Design, synthesis, characterization and biological studies of ruthenium and gold compounds with anticancer properties

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CHAPTER 3

Stability Studies of $[Au(L)Cl_2]Cl{\cdot}xH_2O$ Compounds (where L=azpy, tazpy, 3mazpy, 4mazpy and 3mtazpy and x=1, 2 or 3) in Solution and their Biological Activity. The *In Vitro* Cytotoxic Activity and the Search of an Structure-activity Relationship*

Abstract

Several promising gold compounds have been synthesised and their biological properties have been tested; however, the poor stability (reduction to Au(0)) shown for most of them has hampered further studies. For the therapeutic application of gold compounds, the fine tuning of their chemical reactivity is the most important goal.

Extending chapter 2, now electron-releasing groups substituted in different positions in the pyridine and/or aryl moieties will be introduced in order to investigate the effect imparted on the stability of the gold compound, but also in the cytotoxic activity.

Although stable in the solid state, most of the gold compounds described in the previous chapter show an unexpected reactivity in solution, which is studied in further detail and the findings comprise part of this chapter. The chemical stability of the complex cations, is analyzed by means of nuclear magnetic resonance and electronic spectroscopy. Tricyclic cationic organic derivatives of the original ligands (azpy, tazpy, 4mazpy and 3mtazpy) were produced in solution, then isolated and characterized. The X-ray diffraction studies provide additional support to the structural proposal based on chemical evidence obtained by elemental analysis, NMR, ESI-MS and molar conductivity determinations. To date, no study of the synthesis of these organic charged molecules has been described; in fact they are promising starting materials for the synthesis of more complicated organic structures by themselves.

In the last part of this chapter, 2-(arylazo)pyridine ligands, Au(III) compounds and the organic cyclic cations are investigated as potential cytotoxic and anticancer agents and the *in vitro* cytotoxic activity against cisplatin-sensitive and cisplatin-resistant ovarian carcinoma cell lines, A2780; and cisplatin-sensitive and cisplatin-resistant murine lymphocytic leukaemia cell lines, L1210 is described. The IC_{50} values of the closely related gold(III) compounds were found significant, whereas the 2-(arylazo)pyridine related ligands were found to be less cytotoxic. Significant anticancer activity against the cisplatin resistant cell lines was found for one of the tricyclic salts, ruling out the occurrence of cross-resistance phenomena. The results are discussed in detail and compared and some important observations have been made which are useful in the proposal of a structure-activity relationship.

"Science condemns itself to failure when, yielding to the infatuation of the serious, it aspires to attain being, to contain it, and to possess it; but it finds its truth if it considers itself as a free engagement of thought in the given, aiming, at each discovery, not at fusion with the thing, but at the possibility of new discoveries; what the mind then projects is the concrete accomplishment of its freedom"

Simone de Beauvoir, writer and philosopher (1908-1989)

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3.1 Introduction

The previous chapter described a new series of Au(III) compounds which were synthesised, isolated and fully characterized [1]. In fact the pursued goal of this research project, which deals with a better understanding of the chemical, physical and biological behaviour of these promising Au(III)-2-(arylazo)pyridine compounds had been accomplished only partially. Although stable enough for the isolation and characterization, these gold compounds have to be studied in solution and where possible under physiological conditions; therefore stability studies have been carried out.

It is well known that the unique chemical, spectroscopic and solubility properties in addition to the versatile coordination geometries, imposed by the metal moieties in the coordination compounds, represent useful factors for the potential application in chemotherapy [2, 3]. In fact, these properties could be complementary to the biological properties observed for some ligands (i.e. the organic moiety) [4].

So far, studies towards the design of anticancer metal-based compounds, have been limited mainly to platinum, ruthenium, iron and gold, and some of these metal compounds have been developed till the stage of entering clinical trials [2, 5-9].

Gold was considered until 1972 as a metal with a low reactivity [10]. Some gold(I) compounds were applied in the treatment of rheumatoid arthritis [11-13] and studies of its medical application as anticancer drugs were fully described in literature [12, 14-17]. Opposite to the wide investigation of potential anticancer properties of gold(I) derivatives, the search of potential anticancer gold(III) drugs has been discouraged by the poor stability reported for several Au(III) coordination compounds, in particular under physiological conditions. In fact, the ambiguity relating to the mechanism of biochemical action makes the design of gold complexes difficult, also by the lack of theoretical guidance.

Even though the stability of the gold(III) metal centre may be enhanced by the coordination of ligands with particular properties, such as: a) being strong σ-donor which can stabilize the electrophilic and oxidizing nature of Au(III) ion, b)strong chelating effect to avoid demetalation (multidentate ligands) and c) rigid ligand scaffold to stabilize the four-coordinated Au(III), by raising the kinetic barrier (inner-sphere re-organization energy) for reduction to two coordinate Au(I) [3]); the excess of stabilization may result in a loss of biological activity [18].

Taking into account these facts, some selected gold(III) compounds have been designed by others, showing outstanding cytotoxic activity toward various tumour cell lines with IC_{50} values falling in the range 1–50 μM [18-25].

In order to better understand the mechanism of biological action of this type of metal-based anticancer compounds, or at least to establish some structure activity relationships that could be applied in the design of more effective drugs, it is of paramount importance to identify the molecular components involved in the biochemical interaction.

Although being used since ancient times, gold(III) is recognized only recently for its unique ability to activate carbon-carbon double and triple bonds as soft carbophilic Lewis acid [10, 26-29]. In fact, in the last eight years, an exponential growth of evidence into the benefits of gold as a homogeneous catalyst for the synthesis of particular chemicals has been described [10]. This tendency has been further nourished for the interest in development of resources-saving and safe chemical processes were gold salts can function as green catalysts [30-32].

In this sense gold(III) salts are highly efficient catalyst in the formation of C-C [33, 34], C-O [10, 26, 35], C-N [36, 37], C-S [10, 26, 35] bonds and selective oxidations [38-41]. Moreover, gold(III/I) salts are capable of activating C-H bonds of aromatic and other substrates, opening unprecedented pathways for their functionalization. The activation of non-reactive C-H bonds has been pursued, because it would allow the synthesis of complex molecules from easily available and cheap precursors in fewer steps. By using chiral allenes as substrates, gold catalysts can even be applied in stereo-selective target-oriented synthesis. Even though gold has been used in the human activities since ancient times, it is only known recently that Au(III) salts are able to activate C-H bonds [35, 42] of terminal alkynes, arenes, and β-dicarbonyl compounds by forming nucleophiles that can react with various electrophiles. In fact gold catalyst can play a dual role in these transformations [10]. Moreover, C–H substitution reactions of heterocyclic derivatives are an enduring challenge for organic chemists [35], and results [30] demonstrate that gold catalyst are

effective in synthetic protocols that involve more environmentally benign solvents, room temperature and atmosphere conditions and simple procedures.

Some general facts related to the catalytic activity of gold(III) salts could be enlisted [10] and as all recently developing field, as yet offer more questions than conclusions:

a) Gold is a 'soft' transition metal and thus prefers other soft partners, for example carbon (this might explain the dominance of organic chemistry in this field and the formation of the promising anticancer-active organogold(III) compounds described in the literature [20, 43, 44])
b) Gold shows a small tendency for β-hydride elimination
c) Gold often reacts much faster than other transition metals, which in principle can catalyse the same reactions
d) Organogold intermediates undergo fast proton demetallation
e) Due to the easy reduction and the difficult oxidation of gold, a cross-coupling chemistry seems to be difficult to reach, due to the necessary change of oxidation states
f) In most cases, the mechanism of the reactions, starting with the oxidation state of the catalytically active species are unknown, It is also obvious that often the reactions are possible due to the presence of both Au(I) and Au(III) pre-catalysts.

In addition to the recently discovered chemical reactivity of Au(III) salts, it has been demonstrated that the family of 2-(arylazo)pyridine ligands by itself is susceptible of remarkable and unprecedented metal-mediated chemical reactions. Although in 1993, IUPAC recommended the use of "diazenyl" as an alternative name for the azo function, so for instance, the IUPAC recommended name for azpy would be 2-[(Z)-phenyldiazenyl]pyridine, in the literature, the use of azo is still common, and this name will be used in this thesis.

A considerable number of chemical reports discussing the coordination properties of this family of 2-(arylazo)pyridine ligands with several metals, have appeared in the chemical literature in the past 30 years [45-70]. 2-(arylazo)pyridine ligands under "normal" conditions coordinate through the pyridine and aza nitrogen atoms, forming a stable five-membered chelate ring, acting then as a bidentate ligand [45].

At the same time, unexpected reactions have been reported, like the aromatic ring amination of the pendant aryl ring of coordinated 2-(arylazo)pyridine which does not occur in the free ligand [71, 72]. After several studies, it has been concluded that upon coordination, the aryl C-H bonds at *ortho* and *para* positions are susceptible of amination [73, 74] and that the labilities of co-ligands in the metal complexes play a fundamental role in this process. If a labile metal complex is present (*i.e.* Co^{2+}), the *ortho*-fusion process is favoured. In the absence of any vacant site at the metal centre (inert complex like Rh^{3+}) [75], the amination occurs in the second choice, the *para*-position (figure 3.1). The same kind of amination was reported in Ir(III)-2(arylazo)pyridine complexes [70, 76].

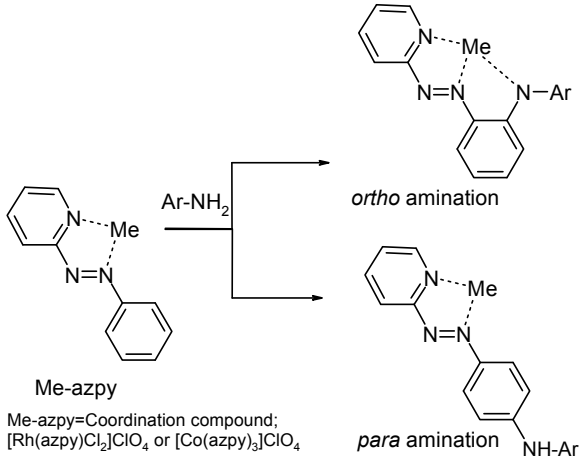

Figure 3.1 Schematic representation of the 2-(phenylazo)pyridine amination process. Upon coordination both, *ortho*- and *para*-C-H bonds of the pendant phenyl group of the ligand are activated. As a result, the amination process can occur at both *ortho* and *para* positions. The selective site of amination can be controlled by a proper selection of the metal compound [71]. The complete coordination compounds structure was omitted for clarity reasons.

Another metal-mediated transformation of 2-(arylazo)pyridines was reported by Banyopadhyay *et al,* [64, 77]. In this case the hydroxylation of the pending ring was observed when in the purification process of the coordination compound, dichlorido{2-(*meta*-tolylazo)pyridine}palladium(II) a crystalline green solid was isolated. The structure of this green compound was determined by X-ray crystallography and is redrawn in figure 3.2, B. Even though the hydroxylation of an aromatic ring is an important process in chemistry and biology, minor efforts have been directed towards mechanistic studies.

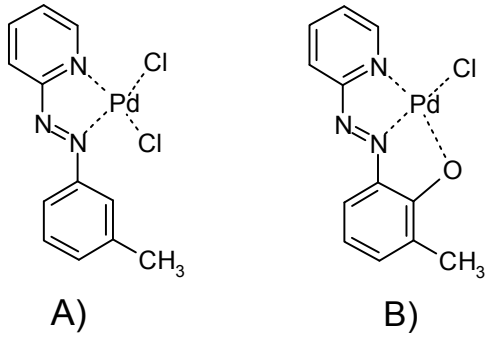

Figure 3.2 Schematic representation of the two products obtained in the synthesis of a palladium compound where the 2-(phenylazo)pyridine metal-mediated hydroxylation is observed [78].

A closely related cobalt-mediated selective activation of a C-H bond in the pendant ring of a 2-(arylazo)pyridine ligand was also reported which develops in turn a facile aromatic hydroxylation in the presence of *m*-chloroperbenzoic acid as hydroxylating agent under ambient conditions [79].

Another unexpected reaction was reported in which azo-splitting of 2-(arylazo)pyridine ligands was observed; in boiling 2-methoxyethanol, K_2ReCl_6 slowly reacts with the ligand affording the corresponding violet crystalline coordination product [78]. The structure of a representative compound has been determined by X-ray crystallography (figure 3.3). The mechanism proposed considers the formation of a metal organo-imido species, which is formed by the reductive cleavage of the azo function. The stoichiometric analysis dictates that complex reactions must be involved, as the ill-defined nature of the other products formed during synthesis hampered any further mechanistic detail.

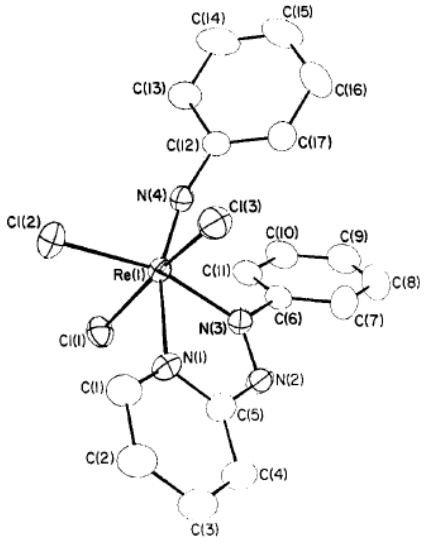

Figure 3.3 ORTEP plot of the molecular structure of a mixed-ligand rhenium compound, with the atom labelling scheme indicated. All atoms are represented by their probability ellipsoids.

Finally, it is has been widely described that irradiation of azobenzene in the presence of proton acids [80, 81] or Lewis acids [82, 83], generates a cyclodehydrogenation process, producing benzo[c]cinnoline. In a report based on the previously described reactivity of azo moieties, the photochemical cyclodehydrogenation of 2-(phenylazo)pyridine is available (figure 3.4) [84].

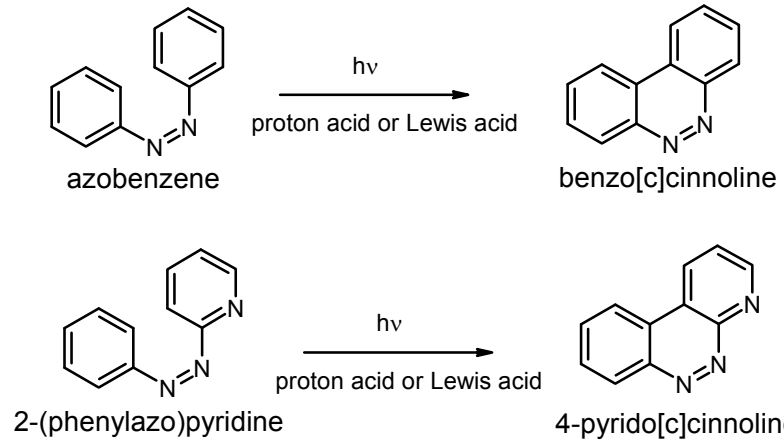

Figure 3.4 Schematic representation of the photochemical cyclodehydrogenation of azobenzene and 2-(phenylazo)pyridine.

Due to their chemical similarity to platinum(II) compounds, it was believed that gold(III) compounds might have DNA as their biological target. However, gold(III) complexes have been reported to interact also with several other cellular components, and recent data indicated mitochondria as a potential target. Nevertheless, so far the mechanism of action of gold(III) compounds is unclear and there is little understanding of how they can elicit their cytostatic activity [17, 19, 25, 85].

In the view of the information just described and in an attempt to establish trends in the biological activity for this family of Au(III) compounds, this chapter comprises detailed chemical stability studies in solution for the coordination compounds described in the previous chapter. As a result of an unexpected reactivity in solution, a series of tricyclic products have now been isolated and characterized by means of several techniques.

The last part of this chapter deals with the cytotoxic activity studies of the new compounds and with a general discussion about some structure-activity relationships observed in the present family of Au(III) compounds.

3.2 Experimental section

3.2.1 Methods and instrumental techniques

Chemicals and solvents (analytical reagent grade) were purchased from Acros, Nova-Biochem and Biosolve and used without further purification, unless otherwise stated. The synthesis of the family of Au(III)-2-(arylazo)pyridine compounds used has been described in detail in the previous chapter. $HAuCl_4 \cdot 3H_2O$ was purchased from Merck. All other reagents were of high purity and used as purchased without any further purification.

Different techniques were employed in the characterization of coordination compounds and tricyclic derivatives synthesized. Elemental analyses were performed with a Perkin Elmer series II CHNS/O 2400 Analyzer. Gold concentration was determined with a VISTA-MPX charged-coupled simultaneous ICP-OES spectrometer (inductively coupled plasma optical emission spectrometer), which was measured in mg/L at 242.794 and 267.594 nm. The experiments were carried out in duplicate. Electrospray mass spectra were recorded on a Finnigan TSQ-quantum instrument using an electrospray ionization technique (ESI-MS). The eluent used was a mixture acetonitrile:water 80:20. The UV-visible (UV-Vis) spectra were recorded using a Varian CARY 50 UV/VIS spectrophotometer operating at room temperature in freshly prepared acetonitrile solutions due to the poor solubility in water. NMR experiments were carried out with a Bruker 300 DPX spectrometer. All spectra were recorded at 21 ºC, unless otherwise indicated. Temperature was kept constant using a variable temperature unit. The software packages XWIN-NMR and XWIN-PLOT were used for edition of the NMR spectra. Tetramethylsilane (TMS) or the deuterated solvent residual peaks were used for calibration. In addition 2D ^{1}H COSY spectra were recorded to confirm the proton assignments. The IR spectra obtained for the products mentioned in this chapter in the 4000-300 cm^{-1} range were recorded as a solid with a Perkin Elmer FT-IR Paragon 1000 spectrophotometer with a single-reflection diamond ATR P/N 10500.

3.2.2 Stability studies

Stability studies were developed in solution and followed by ^{1}H NMR. A solution of the compounds was prepared by dissolving around 1.05 mg of each Au(III) compound in 0.58 mL of deuterated acetone or acetonitrile, with TMS used as the NMR internal standard. The solution in the NMR tube was closed with a proper cap. The number of scans was kept constant in all the determinations.

Several conditions were tested to detect the main factors that could take part in the formation of the tricyclic cations. Factors as light, solvent nature, temperature and atmosphere composition where all considered and analyzed.

The chemical changes in the sample were detected by time-dependent consecutive measurements with ^{1}H NMR. The stability studies were prolonged till no further changes in the spectra were detected. With all the data registered, plots of the chemical shifts against time were used to analyze the decomposition of the Au(III) compounds and formation of the tricyclic organic charged derivatives.

3.2.3. Synthetic procedures

2-(phenylazo)pyridine (*azpy*), 2-(tolylazo)pyridine (t*azpy*), 2-(phenylazo)-3-mehtylpyridine (3m*azpy*), 2-(tolylazo)-3-methylpyridine (3mtazpy), 2-(phenylazo)-4-methylpyridine (4m*azpy*): The ligands were prepared as described in the literature [86] with minor modifications in the purification procedure and the characterization results obtained completely agreed with the reported data.

Dichlorido{2-(phenylazo)pyridine}gold(III) chloride dihydrate, $[Au(azpy)Cl_2]Cl\cdot 2H_2O$ (*Au-azpy*); dichlorido{2-(tolylazo)pyridine}gold(III) chloride dihydrate, $[Au(tazpy)Cl_2]Cl\cdot 2H_2O$ (*Au-tazpy*); dichlorido{2-(phenylazo)-3-methylpyridine} gold(III) chloride trihydrate, $[Au(3mazpy)Cl_2]Cl\cdot 3H_2O$ (*Au-3mazpy*); dichlorido{2-(phenylazo)-4-methylpyridine}gold(III) chloride, $[Au(4mazpy)Cl_2]Cl$ (*Au-4mazpy*) and dichlorido{2-(tolylazo)-3-methylpyridine} gold(III) chloride trihydrate, $[Au(3mtazpy)Cl_2]Cl\cdot 3H_2O$ (*Au-3mtazpy*): The synthetic procedures for all enlisted compounds were fully described in the previous chapter.

Pyrido[2,1-*c*][1,2,4]benzotriazin-11-ium chloride hydrate, $C_{11}H_8N_3Cl\cdot H_2O$ (abbreviated: *pyrium* chloride hydrate): The title compound was synthesized by the following procedure: 0.03g (0.0574mmol) of $[Au(azpy)Cl_2]Cl\cdot 2H_2O$ were solved in 16.57 mL of acetone or acetonitrile. The system was kept under atmospheric conditions at 294 K. Pale yellow needles were obtained after 14 days. The crystals formed after this time were collected by filtration, washed plenty with cold acetone and dried with diethyl ether. Yield: 64.88 % (0.03721 mmol, 8.77 mg). Elemental analysis for $C_{11}H_{10}N_3ClO$: Calculated (%): C, 56.06; N, 17.83 and H, 4.28. Found (%): C, 55.52; N, 17.68 and H, 4.18. ESI-MS: m/z=181.94, $C_{11}H_8N_3^+$, where calculated m/z value is 182.21. ^{1}H NMR (300 MHz, acetone, 21 ºC, s=singlet, d=doublet, t=triplet and m=multiplet): δ=10.6312(d, 1H, H_6'), 9.5012(d, 1H, H_3'), 9.3472(m, 2H, H_{m2}' and H_4'), 9.1410(d, 1H, H_o'), 8.8872(t, 1H, H_5'), 8.6730(t, 1H, H_p') and 8.5156(t, 1H, H_{m1}') ppm.

The same procedure was applied in the synthesis of the following derivatives and the chemical information experimentally obtained is described as follows:

7-methylpyrido[2,1-*c*][1,2,4]benzotriazin-11-ium chloride, (abbreviated: t-*pyrium* chloride): ESI-MS: m/z=195.97, $C_{12}H_{10}N_3^+$, where calculated m/z value is 196.23. ^{1}H NMR (300 MHz, acetonitrile, 21 ºC, s=singlet, d=doublet, t=triplet and m=multiplet): δ=9.927(d, 1H, H_6'), 9.2647(d, 1H, H_3'), 9.0336(m, 1H, H_4'), 8.6625(d, 1H, H_{m2}'), 8.5449(t, 1H, H_5'), 8.3966(t, 1H, H_p'), 8.2050(t, 1H, H_{m1}') and 3.2219(s, 3H, CH_{3o}')ppm.

3-methylpyrido[2,1-*c*][1,2,4]benzotriazin-11-ium chloride, (abbreviated 4m-*pyrium* chloride): ESI-MS: m/z=195.97, $C_{12}H_{10}N_3^+$, where calculated m/z value is 196.23. ^{1}H NMR (300 MHz, acetonitrile, 21 ºC, s=singlet, d=doublet, t=triplet and m=multiplet): δ=9.8191(d, 1H, H_6'), 9.0998(s, 1H, H_3'), 8.9988(d, 1H, H_o'), 8.7902(d, 1H, H_{m2}'), 8.4434(m, 2H, H_5' and H_p'), 8.3223(t, 1H, H_{m1}') and 2.9540(s, 3H, CH_{3py}')ppm.

3,7-methylpyrido[2,1-*c*][1,2,4]benzotriazin-11-ium chloride, (3mt-*pyrium* chloride): ESI-MS: m/z=209.92, $C_{13}H_{12}N_3^+$, where calculated m/z value is 210.26. ^{1}H NMR (300 MHz, acetonitrile, 21 ºC, s=singlet, d=doublet, t=triplet and m=multiplet): δ=9.7958(d, 1H, H_6'), 8.8596(d, 1H, H_4'), 8.6450(d, 1H, H_{m2}'), 8.4049(m, 2H, H_5' and H_p'), 8.1848(t, 1H, H_{m1}'), 3.27082(s, 3H, CH_{3py}') and 3.2237(s, 3H, CH_{3o}')ppm.

3.2.4 X-ray diffraction studies

Light yellow needles from pyrido[2,1-*c*][1,2,4]benzotriazin-11-ium chloride hydrate, $C_{11}H_8N_3Cl{\cdot}H_2O$ (pyrium chloride hydrate), were grown after several days by slow evaporation (14-17 days) of the solvent under atmospheric conditions. The crystal structure of this tricyclic cation, was determined with a Nonius KappaCCD diffractometer equipped with graphite-monochromated Mo Kα radiation (λ=0.71073Å). Reflection data were measured at 150(2) K. The structure was solved with Direct Methods (SHELXS86) [87]. The reduction data was obtained using DENZO [88]. The program used to refine the structure was SHELXL97 [89]. The crystal data are extremely hampered by serious crystal defects as evidenced with synthetic precession images derived from the CCD images. Structure determination was attempted in three space groups ($P2_1/n$, $Pn2_1a$ and Pnma). The same overall structure was arrived at in all three cases, all with signs of disorder. Eventually a disorder model in Pnma was used. Molecular graphics, structure checking and calculations were performed with the PLATON software [90]. All structural drawings and geometrical calculations were prepared using PLATON.

3.2.5 Cytotoxic studies

The human ovarian cell lines, A2780 and A2780R, sensitive and resistant to cisplatin, were derived from untreated patient cells [91]. The cells were grown as monolayers in Dulbecco's modified Eagle's Medium (Gibco, Paisley, Scotland) supplemented with 10% foetal calf serum (Hyclone, Logan, USA), penicillin (100 units/ml: Dufecha, Netherlands) and streptomycin (100 µg/ml: Dufecha, Netherlands). The murine lymphocytic leukaemia cell lines, L1210 and L1210R, sensitive and resistant to cisplatin, respectively, were cultured in Dulbecco's modified Eagle's Medium supplemented with 10% foetal calf serum (Hyclone, Logan, USA), penicillin (100 units/ml: Dufecha, Netherlands) and streptomycin (100 µg/ml: Dufecha, Netherlands). During growth, the cells grew partly in suspension and partly attached to the wall of the flask. The cisplatin-resistant L1210R was obtained by exposure of L1210 cells to cisplatin at the concentration of 10 µM over a period of 3 months and subsequent cloning [92]. For the cell growth assay, cells (2000 cells/100 µl of complete medium/well) were pre-cultured in 96 multi-well plates for 48 h at 37 ºC in a 7% CO_2 containing incubator and subsequently treated with the tested compounds for 72 h. The stock solutions of the compounds in the minimal amount of the corresponding solvent (acetonitrile) were diluted in five subsequent dilutions in order to have final concentrations of 0-200 µM by triplicate. Cisplatin was used as a control and was solved in Millipore water as well. A blank for the solvent was also included in the tests. After 48 or 72 h of incubation time, the surviving cells in cultures treated with the compounds were detected using the MTT method [93, 94]. MTT is a yellow water-soluble tetrazolium salt. The MTT (3-(4,5-dimethylthiazol 2-yl)-2,5-diphenyltetrazolium bromide) assay is a simple non-radioactive colorimetric assay to measure cell cytotoxicity, proliferation or viability. Metabolically active cells are able to convert the dye to a water-insoluble dark blue formazan by reductive cleavage of the tetrazolium ring [95, 96]. MTT in PBS (100 µl at 2.5 mg/ml) was added and the cells were incubated for 2 h. The solution was carefully removed and the remaining crystals were dissolved in 100 µl of DMSO after which the absorbance at 590 nm of each well was determined using a BIO-RAD microplate model 550 reader. The growth inhibition was determined relative to untreated controls. Data were used for construction of response curves and determination of the IC_{50}(concentration of the compound that restricts cell growth to 50% of that compared with the control) values was graphically done by use of GraphPad Prism software, version 3.02, 2000.

3.3 Results and discussion

3.3.1 Stability studies and characterization of tricyclic derivatives

In the past 40 years several types of gold-based antitumour compounds have been studied and from these studies different mechanisms of biological activity have been proposed to be responsible of the anticancer effect [14, 16, 17, 19, 85, 97]. It has been mentioned in the literature that Au-based compounds can act by mechanisms involving interaction with protein targets mainly by affecting the mitochondrial cell death pathways, or by direct DNA interaction as cisplatin.

Researchers have rationalized trends in activity, based in the indispensable detailed knowledge of the structural and solution chemistry of the complexes. In this thesis, the synthesis, isolation and characterization of a series of Au(III) compounds with different 2-(arylazo)pyridine ligands was accomplished. In order to continue with the elucidation of their structure-activity relationship, their chemical behaviour in solution must be studied in further detail.

Although this family of compounds shows a high stability in the solid state under atmospheric conditions, the corresponding stability studies in solutions were performed and the changes were followed by ^{1}H NMR spectroscopy.

Several factors, probably involved in the chemical transformation of the compounds discussed here were considered; among them, the influence of solvent, temperature, the presence of dioxygen and the influence of light (light sensitivity of Au(III) compounds is well known [98]).

A. Stability studies for Au-azpy

The stability study for Au-azpy will be discussed as follows. In figure 3.5, the spectral changes as a function of time in deuterated acetone are presented. While some resonance peaks gradually appeared, some others disappeared, clearly suggesting that a reaction is taking place. Two important observations need to be stressed: (1) the spectral changes include intense downfield shifts and (2) the integration values for the final product, approximately after 11 days, evidenced the presence of 8 protons, instead of the original 9 protons (hydrogen atoms present in Au-azpy). The studies were also performed in acetonitrile and methanol with similar results (spectra not shown). Keeping the sample under an argon atmosphere, on the other hand, prevented these changes to occur. The same conversion was observed when the sample was kept at 5 ºC in air, but in the dark. Worth mentioning if the fact that, under these conditions, a free azpy ligand solution is not developing this transformation.

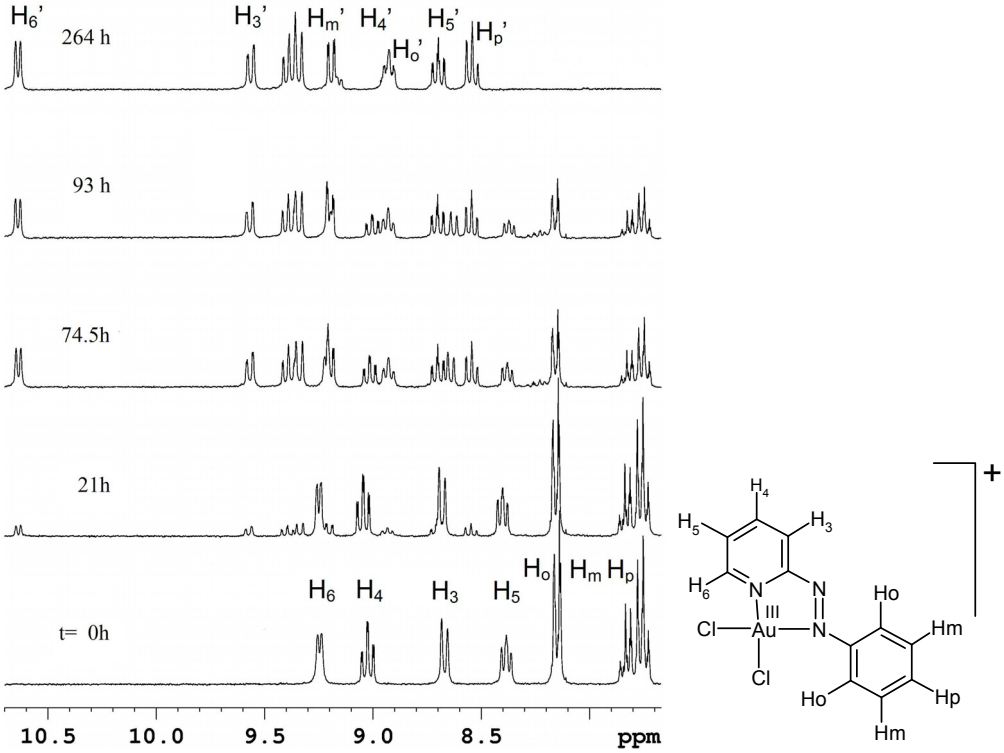

Figure 3.5 Time-dependent ^{1}H NMR study of Au-azpy in deuterated acetone at 21 ºC, using TMS as internal standard. Aromatic region only.

A plot of the integration values as a function of time for some resonance peaks is presented in figure 3.6. Although not studied in detail, the role of the solvent also must be critical here, in allowing the chemical transformation, as is well known in the chemistry of gold(III) coordination compounds [99]. It is also important to stress the fact that the chemical transformation of Au-azpy, in solution (acetone), starts after approximately 21 h, at room temperature.

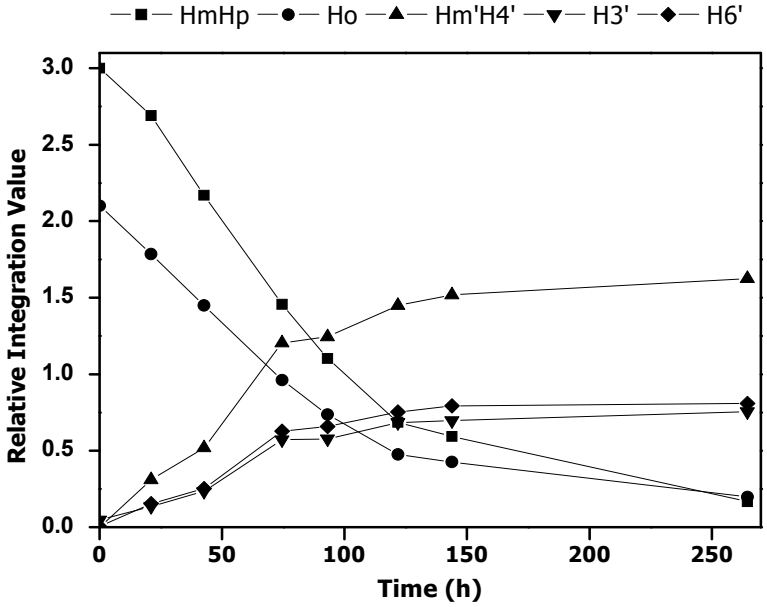

Figure 3.6 Proton resonance peak shifts as a function of time in deuterated acetone solution of Au-azpy at 21 ºC. Shifts for new peaks: H_6', 10.6312; H_3', 9.5012 and H_m'/H_4', 9.3472.

Several hypothetic structures were considered to explain these changes in the spectra, but more information was needed to assure the structure proposal. After the time of the experiment, no metallic gold was deposited so it appears that azpy prevents the reduction of Au(III) to Au(0), even in solution, which in fact renders Au-azpy suitable for further biological studies.

Based on the time taken for the disappearance of the proton signals corresponding to Au-azpy, the synthesis in larger quantities of this by-product was designed (procedure described in the experimental section). The compound, called pyrium chloride hydrate, was successfully isolated as a bright yellow crystalline material, characterized by means of elemental analysis, ^{1}H NMR, ESI-MS and X-ray diffraction studies. In addition, the final solutions obtained from the stability studies of Au-azpy, with 100% of converted product, were analysed by means of ^{1}H NMR and ESI-MS studies and identical results were obtained when comparing the ^{1}H NMR and ESI-MS spectra for the isolated product.

A.1 Pyrido[2,1-*c*][1,2,4]benzotriazin-11-ium chloride hydrate, $C_{11}H_8N_3Cl{\cdot}H_2O$, *pyrium* chloride hydrate

The schematic representation of the new cationic species is shown in figure 3.7.

The synthesis was found to be fully reproducible in the 4 used solvents: acetone, acetonitrile, ethanol and methanol, and all gave the same product. 11 days were required in case of acetone. The yields obtained were above the 60% [1].

The ESI-MS spectrum exhibits the highest mass peak at m/z=181.94, which corresponds to the pyrium cation, $C_{11}H_8N_3^+$ (calculated m/z=182.21). The peak displays the correct isotopomer distribution (figure 3.7).

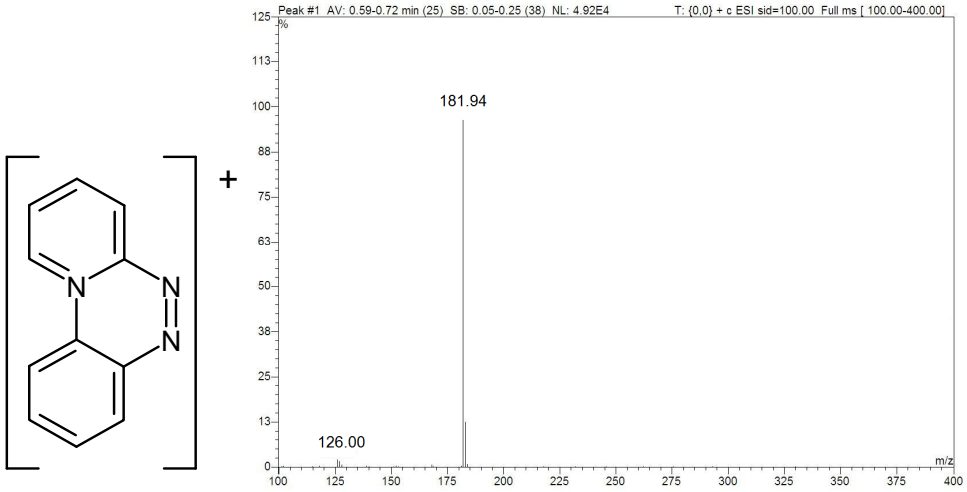

Figure 3.7 Schematic representation of the pyrium cation (left) and ESI-MS positive ion spectrum (m/z in Da).

^{1}H NMR spectrum is in agreement with the structure proposed. Seven different peaks (one pair overlapping) are observed (figure 3.5 after 264 h and figure 3.8). The integration values are in agreement with the molecular formula proposed (8 H atoms). The complete peak assignment was performed by ^{1}H COSY determination (figure 3.8). The presence of a resonance peak assigned to H_3', completely rules out the structure proposed by Pillai *et al.* [84] where photocyclization of azpy produces 4-pyrido[c]cinnoline (figure 3.4). Then it is clear that the crystalline yellow product isolated and the thermodynamically stable product from the Au-azpy solution decomposition, detected after 264 h are the same.

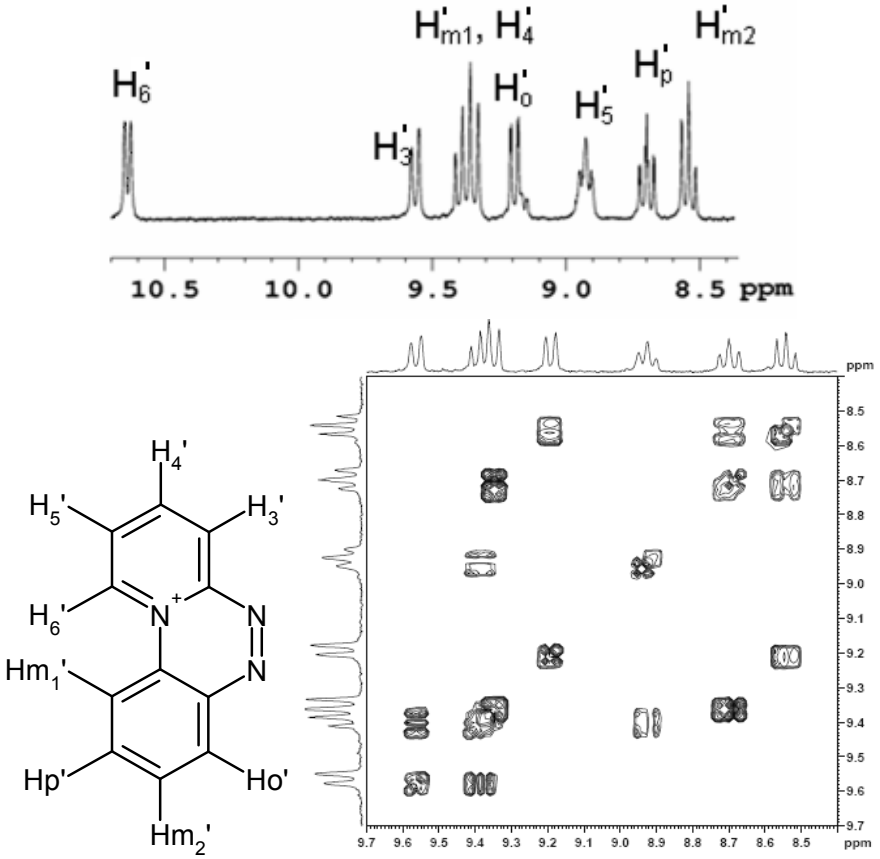

Figure 3.8 1D ^{1}H NMR and 2D ^{1}H COSY spectra of the pyrium cation in deuterated acetone at 21 ºC, using TMS as internal standard. Aromatic region only.

Due to asymmetry in the molecule different peaks are observed. The presence of H_6' at low-field is explained by the strong deshielding effect of the positively charged nitrogen in the pyridine moiety.

Crystals suitable for X-ray diffraction studies were obtained, to confirm the structure proposal of a positively charged tricyclic derivative of azpy, suggested by the other characterization techniques.

A.2. Crystallography

The crystal data suffer from serious crystal defects as evidenced with synthetic precession images derived from the CCD images. Table 3.1 summarizes the crystallographic data.

The results from the X-ray diffraction analysis indicate an organic, aromatic cationic structure (pyrium), in which Cl^- anions function as counterion. A water molecule is also present in the asymmetric unit (figure 3.9). The thermodynamically stable final compound is systematically named pyrido[2,1-*c*][1,2,4]benzotriazin-11-ium chloride hydrate.

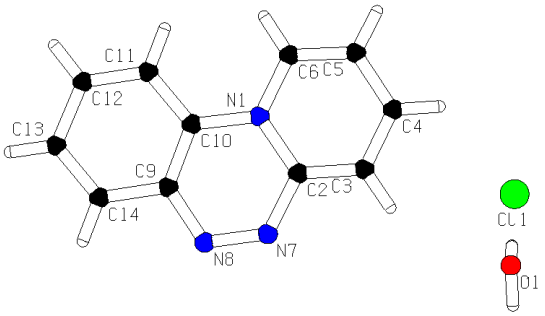

Figure 3.9 X-ray structure of the asymmetric unit of pyrium chloride hydrate and the used atom numbering.

Table 3. 1 Summarized crystallographic data for $C_{11}H_8N_3Cl(H_2O)$, pyrium chloride hydrate.

Property	
Empirical fomula	$C_{11}H_{10}ClN_3O$
Formula weight	235.67
Crystal system	Orthorhombic
Space group	Pnma
a(Å)	5.4591(12)
b(Å)	11.540(3)
c(Å)	16.976(4)
$\alpha=\beta=\gamma$ (º)	90
V(Å^3)	1069.5(4)
Z	4
T(K)	150
D_{calc}(Mgm^{-3})	1.4636(5)
μ(Mo Kα)(mm^{-1})	0.337
F(000)	488.0
Parameters refined	115
R_1[b]	0.095
wR_2[c]	0.213
Crystal dimensions	0.05 x 0.10 x 0.30
Color	Needle, Light yellow

The organic cation is disordered over a crystallographic mirror (figure 3.10). The water molecules form an infinite, cooperative chain of hydrogen bonds, capped at the sides by Cl^- ions (figure 3.11). The packing is additionally stabilized by π-π interactions between the aromatic ring systems.

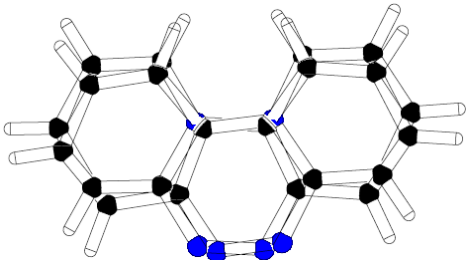

Figure 3.10 View of the disorder over a crystallographic mirror plane, perpendicular to the drawing plane of the pyrium cation.

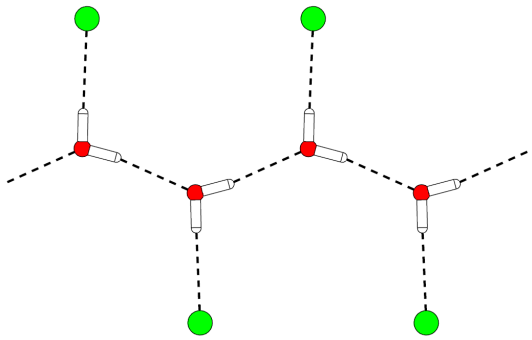

Figure 3.11 View of the infinite cooperative chain of hydrogen bonds between water molecules capped at the sides by Cl^- ions. No hydrogen bonding is observed to the N atoms of the aromatic ring.

This heterocyclic structure is the first pyrido[2,1-*c*][1,2,4]benzotriazin-11-ium core described in literature**.** It is well known that under irradiation, azobenzenes may undergo cyclodehydrogenation, forming benzo[c]cinnoline when proton acids [80, 81] or Lewis acids [82, 83] are present and even more, it was reported for Pillai and Purushothaman [84] the successful photocyclization of azpy. Unfortunately, the final product, 4-pyrido[c]cinnoline (figure 3. 4) was barely characterized.

The analysis of this charged organic structure would suggest the formation of an organometallic gold intermediate, prior to cyclization; however, the time-dependent ^{1}H NMR stability studies do not present any indication of such an intermediate species. The integration values obtained from the ^{1}H NMR spectrum of Au-azpy clearly show the presence of nine protons in agreement with the proposed structure. It is well known that organometallics containing metal-ligand σ-bonds are characterized by long-wavelength LMCT absorptions [98]; however, these are not observed in the corresponding electronic spectra. The ill-defined nature of other products formed during the transformation to the pyrium cation has so far precluded electron accounting and possible elucidation of the reaction stoichiometry and mechanistic proposal. The formation of Au(0) has not been observed and the present chemical evidence is suggesting the presence of Au(III) in solution after complete conversion to the pyrium cation. Although not studied in detail, the role of the solvent must be important in maintaining the oxidative conditions for the stabilization of Au(III). Polar solvents as acetonitrile and methanol render identical results for this system with final cyclization of azpy. Stability studies in water were not developed due to solubility problems.

B. Stability studies for Au-tazpy

The stability study for Au-tazpy will be discussed as follows. The identity of the byproduct detected was established based on the one-dimensional and two-dimensional ^{1}H NMR(spectrum not shown) and ESI-MS determinations.

The spectral changes observed after some time in deuterated acetonitrile are presented in figure 3.12.

As the stability studies for Au-azpy showed, Au-tazpy in solution is also undergoing a chemical transformation. While some resonance peaks gradually appeared some others disappeared. The spectral changes include intense downfield shifts and the integration values for the final product, approximately after 3 days (see the final spectrum -72h- in the series of spectra presented in figure 3.12), evidenced the presence of 10 protons, instead of the original 11 protons (hydrogen atoms present in Au-tazpy). If the evidence obtained for the pyrium cation is considered here, a closely related organic positively charge cation would be proposed as the final product. The schematic representation of this product, the cation called t-pyrium is presented in figure 3.12 along with the numbering scheme used for the ^{1}H NMR peaks assignment of the t-pyrium spectrum (figure 3.12, after 72 h). Due to asymmetry in the final product, 7 different peaks are observed in the aromatic region. The presence of H_6' at low-field is explained by the strong deshielding effect of the positively charged pyridine nitrogen. The low-field shift observed for H_{m2}' would be explained by diamagnetic currents produced for the proximity with H_6'. As in case of the pyrium cation, the production of t-pyrium starts approximately after 24 h. Complete transformation took part in 3 (72 h) days, which is a large difference when considering that 100% conversion to

the pyrium cation took part after 11 days under the same experimental conditions. In this case also metallic gold was observed deposited at the NMR tube walls. The progress of the conversion of Au-tazpy to the t-pyrium cation by ^{1}H NMR does not suggest the presence of other byproducts.

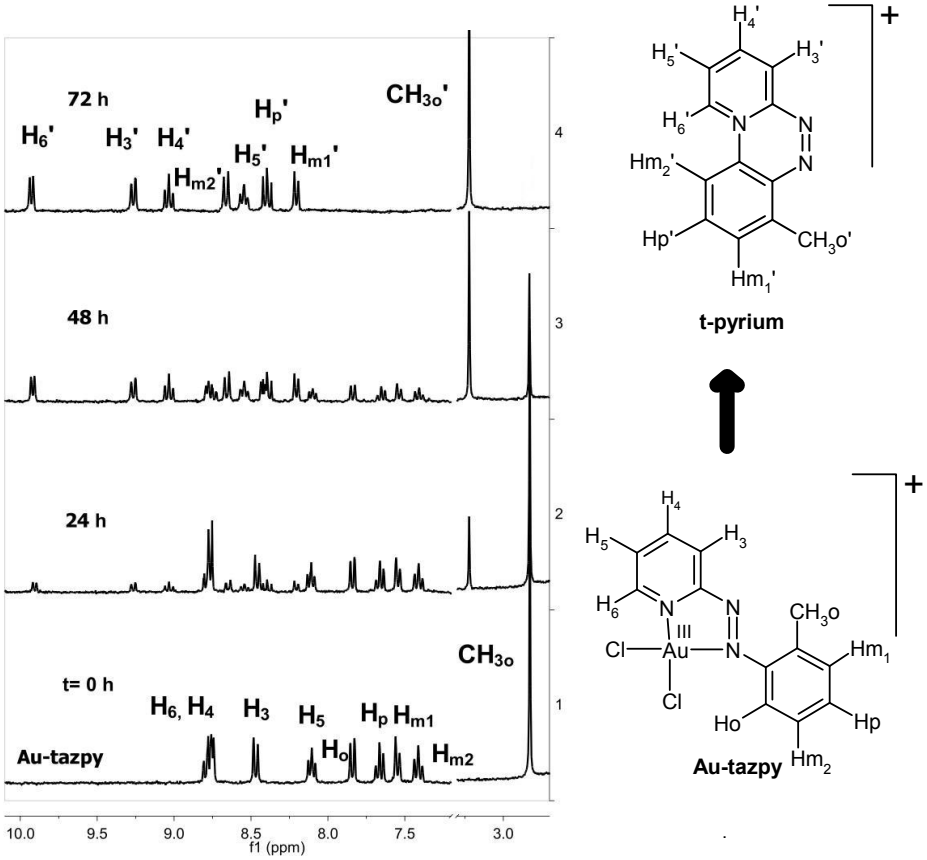

Figure 3.12 Time-dependent ^{1}H NMR study of Au-tazpy in deuterated acetonitrile at 21 ºC, using TMS as internal standard. The numbering scheme corresponding to the peak assignments is displayed in the schematic representation of the new product, the t-pyrium cation and the starting material, Au-tazpy.

The informative fragment ion observed by ESI-MS at 195.93 m/z (calculated m/z=196.23), corresponding to the formula $[C_{12}H_{10}N_3]^+$, where the counterion is lost, supports the structure proposed from the NMR data.

C. Stability studies for Au-4mazpy

The stability studies for Au-4mazpy again showed similar results and the main evidence is shown below. As in the previous case, the identity of the thermodynamically stable product was established based in the one-dimensional ^{1}H NMR, 2D ^{1}H COSY (spectrum not shown) and ESI-MS determinations. The spectral changes that took part after some time in deuterated acetonitrile are presented in figure 3.13.

Several hypothetic structures could be suggested as explanation of the spectral changes. The same kind of effects observed in the stability studies discussed earlier are evident: formation of new peaks and disappearance of some peaks, intense downfield shifts and the integration values in the final product, approximately after 15 days (Figure 3.13, spectrum measured after 312 h), show the presence of 10 protons, instead of the original 11 protons in Au-4mazpy. Then a closely related organic positively charged cation would be proposed as the final product. The schematic representation of this cationic product called 4m-pyrium is presented in figure 3.13 along with the numbering scheme used for the ^{1}H NMR peak assignments (spectrum recorded after 312 h in the figure 3.13). Due to asymmetry in the final product, 7 different peaks are observed in the aromatic region. The presence of H_6' at low-field is explained by the strong deshielding effect of the positive charge at the pyridine nitrogen. The low-field shift observed for H_{m2}' would be explained by diamagnetic currents due to the proximity of H_6'. Another important observation is that more peaks than the peaks corresponding to the starting Au-4mazpy and the 4m-pyrium cation are observed, which could be explained by the presence of intermediate

products. It is possible that the peak appearing after 48 h around 8.5 ppm is evidence for demetallation of the nitrogen in the pyridine ring, as H_6 in the free ligand generates a peak at 8.535 ppm. The first evidence of chemical transformation to the tricyclic cation appears after more than 24 h.

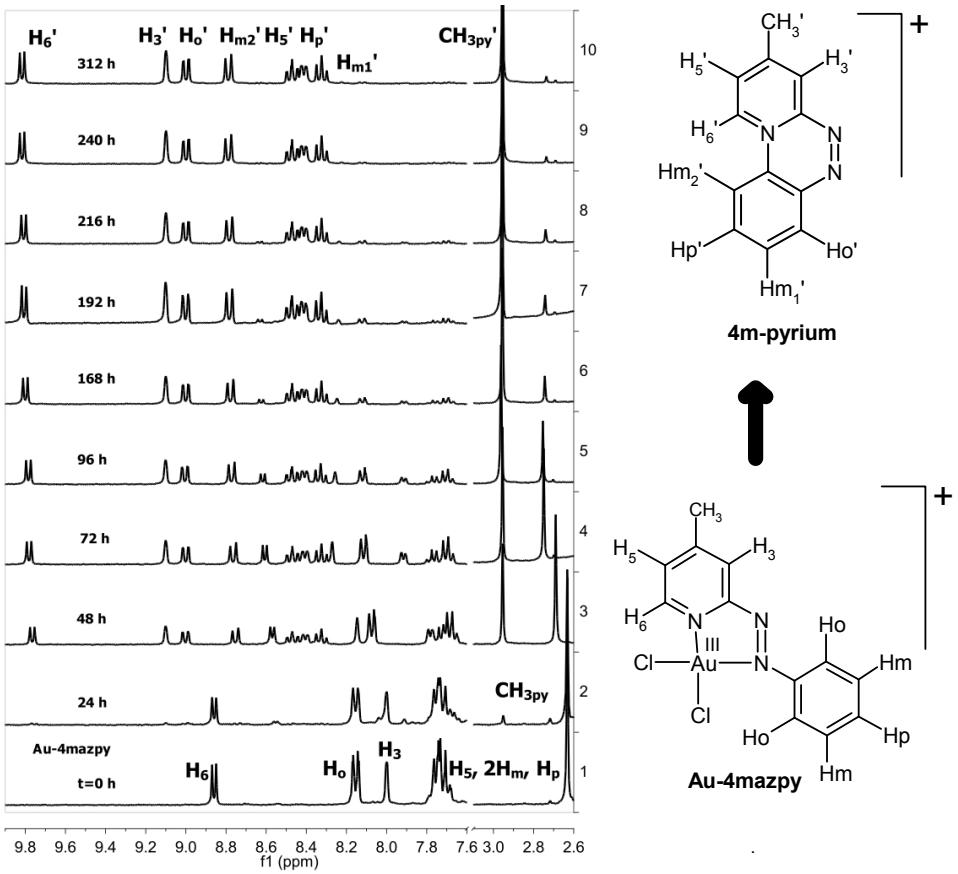

Figure 3.13 Time-dependent ^{1}H NMR study of Au-4mazpy in deuterated acetonitrile at 21 ºC, using TMS as internal standard. The numbering scheme corresponding to the peak assignments is described in the schematic representation of the new product, the 4m-pyrium cation and the starting material, Au-4mazpy.

The fragment ion observed by ESI-MS at 195.93 m/z (calculated m/z=196.23) corresponds to the formula $[C_{12}H_{10}N_3]^+$, where the counterion is lost, giving support to the structure proposed based in the NMR data. Complete transformation took part in 15 days comparable to the time that 100% conversion to pyrium was needed. No metallic gold was observed deposited in the NMR tube walls.

D. Stability studies for Au-3mtmazpy

The stability study of Au-3mtazpy is discussed as follows. The chemical characterization of the thermodynamically favoured product was established with the help of one-dimensional and two-dimensional ^{1}H NMR and ESI-MS determinations.

The spectral changes observed after some time in deuterated acetonitrile are presented in figure 3.14. As in the other stability studies for the Au(III)-2-(arylazo)pyridine compounds, previously described in this thesis (chapter 2), Au-3mtazpy in solution, undergoes a chemical transformation. It is clear that some resonance peaks gradually appeared, while some others disappeared. Again, the spectral changes include intense downfield shifts and the integration values for the final thermodynamically favoured product, after 45 days (1080 h), correspond to 12 hydrogen atoms, instead of the original 13 (hydrogen atoms present in Au-3mtazpy). So, also in this case, a closely related organic positively charge cation would be proposed as the final product.

The schematic representation of this cationic product called 3mt-pyrium is shown in figure 3.14 along with the numbering scheme used for the ^{1}H NMR peak assignments (last spectrum after 1080 h), which were confirmed by 2D ^{1}H COSY experiments (data not shown). Due to asymmetry in the final product, 5 different peaks (integration value=6H) are observed in the

aromatic region, corresponding to the 6 aromatic hydrogen atoms present in the structure. The presence of H_6' at low-field is explained by the strong deshielding effect of the positively charged pyridine nitrogen. The low-field shift observed for H_{m2}' could be generated by diamagnetic currents produce for the proximity with H_6'. It is important to mention here that even though the reaction was followed for months, a remaining byproduct (approximately in 34%), closely related to the 3mt-pyrium cation could be detected in the solution (figure 3.15). This byproduct is not the original free ligand (in *trans* configuration), because the signals at the high field region, assigned to the hydrogen atoms in the methyl moieties, are shifted considerably and both peaks (CH_{3o} and CH_{3py}) present magnetically equivalent environments, which is not the case in the free ligand.

Without further information it is difficult to make a structural proposal, but it is possible that the structure could be the free ligand, but converted to the *cis* configuration. This conversion is strongly suggested by the appearance of small peaks after 24h in the reaction system. For instance, the peak around 8.46 ppm, a typical broad doublet, due to the magnetic influence of the nitrogen in pyridine in close proximity, suggests that a hydrogen atom *ortho* to this pyridine-nitrogen is responsible of this resonance peak. The upfield shift is explained as a result of magnetic interactions with the vicinal aromatic ring in *cis* orientation. Peaks at higher field are normally observed in *cis*-azo compounds than the ones in *trans* conformation [100]. Supporting this observation are the resonance peaks (two really close singlets) around 2.77 ppm, which must belong to the methyl moieties (one in the pyridine ring and the other in the phenyl ring), which are almost magnetically equivalent. This could be explained just considering that the methyl moieties are in a *cis* configuration in the ligand. In fact, the methyl moieties in the tricyclic product generate resonance peaks almost magnetically equivalent, but downfield shifted due to the positive charge in the pyridine nitrogen. The changes in Au-3mtazpy in solution start before the first 24 hours.

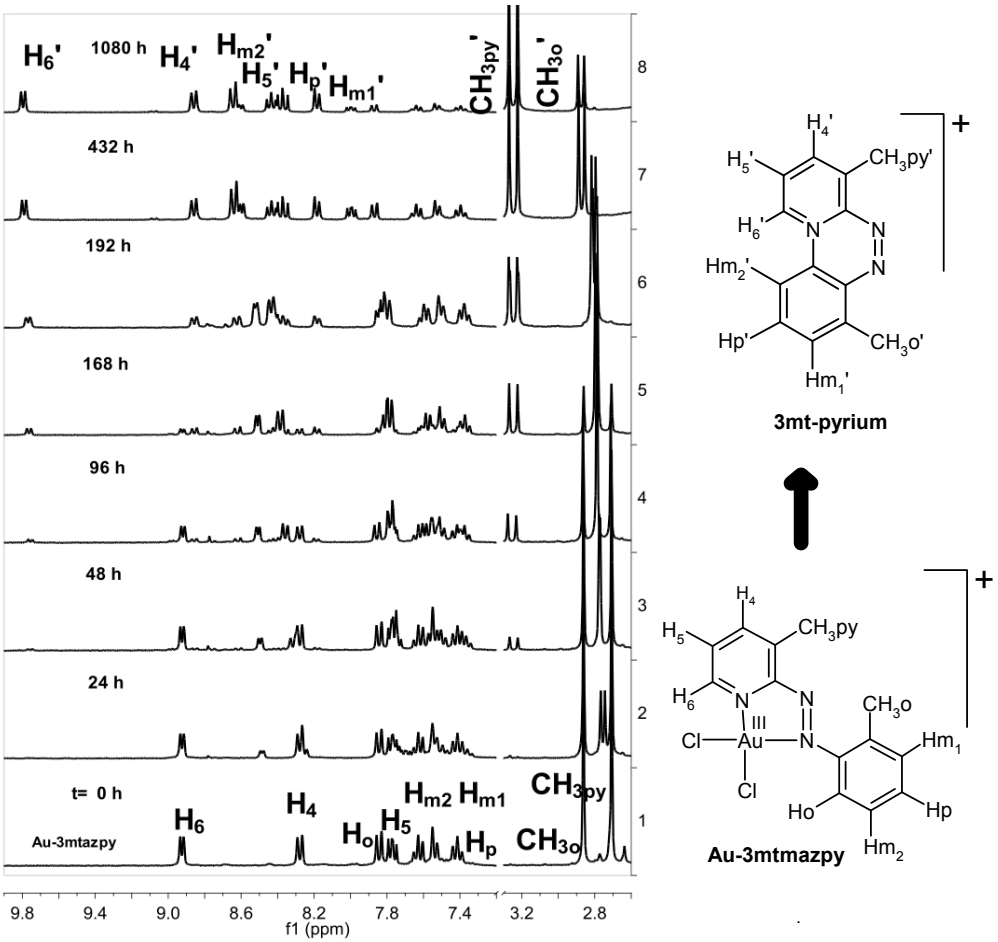

Figure 3.14 Time-dependent ^{1}H NMR study of Au-3mtazpy in deuterated acetonitrile at 21 ºC, using TMS as internal standard. The numbering scheme corresponding to the peak assignments is displayed in the schematic representation of the new product, the 3mt-pyrium cation and the starting material, Au-3mtazpy.

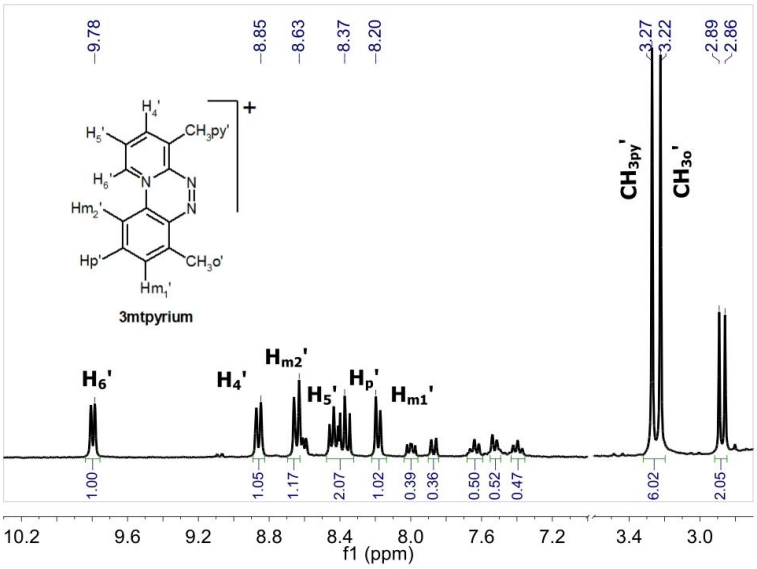

Figure 3.15 1D ^{1}H NMR spectrum of the 3mt-pyrium cation in deuterated acetonitrile at 21 ºC, using TMS as internal standard. This is the stable spectrum obtained after several days. The numbering corresponding to the peak assignments is displayed in the schematic representation of the cationic 3mt-pyrium.

The informative fragment ion observed by ESI-MS at 209.95 m/z (calculated m/z=210.26), corresponding to the formula $[C_{13}H_{12}N_3]^+$, where the counterion is lost, gives support to the structure proposed based on NMR data. Metallic gold was observed deposited at the NMR tube walls.

E. Stability studies for Au-3mazpy

The stability study for the Au-3mazpy system shows a completely different picture by not only producing trace amounts of the tricyclic cation but also a new organic derivative is produced. The spectral changes observed after some time in deuterated acetonitrile are presented in figure 3.16.

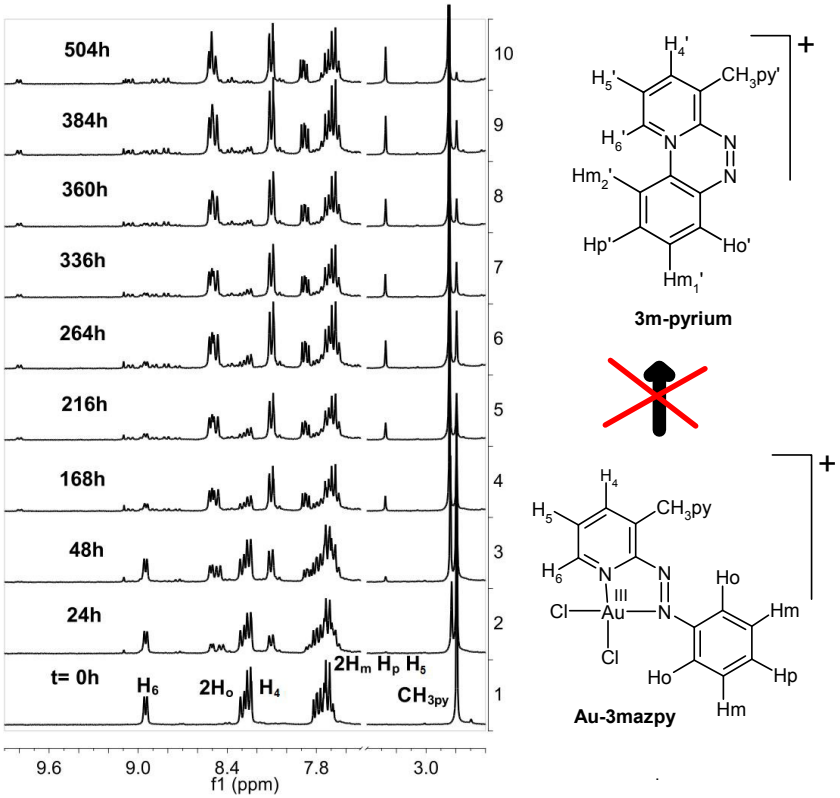

Figure 3.16 Time-dependent ^{1}H NMR study of Au-3mazpy in deuterated acetonitrile at 21 ºC, using TMS as internal standard. The numbering scheme corresponding to the peak assignments is displayed in the schematic representation of Au-3mazpy.

In this case, it is possible to identify the formation of the tricyclic derivative in the spectra, at low-field, as this is the behaviour observed in the other cases presented here. The yield anyway is considerably small (integration values indicates less than 10%) (figure 3.17).

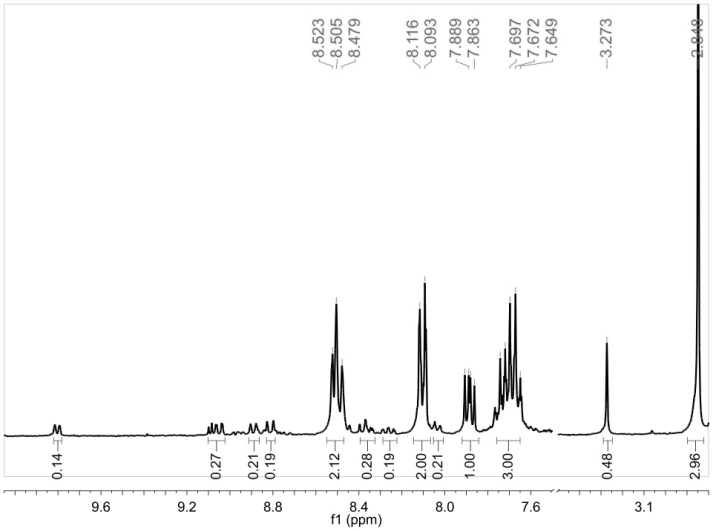

Figure 3.17 1D ^{1}H NMR spectrum of the final reaction solution at the end of the corresponding stability studies (504 h) in deuterated acetonitrile at 21 ºC, using TMS as internal standard.

Clearly the chemical reactivity is different and it must be directly related to the presence of a methyl moiety in the pyridine ring. The stable product obtained presents a spectrum with a pattern of peaks more similar to the free ligand, but is not identical as seen from figure 3.18. Anyway, this favoured product contains 8 aromatic hydrogen atoms in its structure as deduced from the integration values.

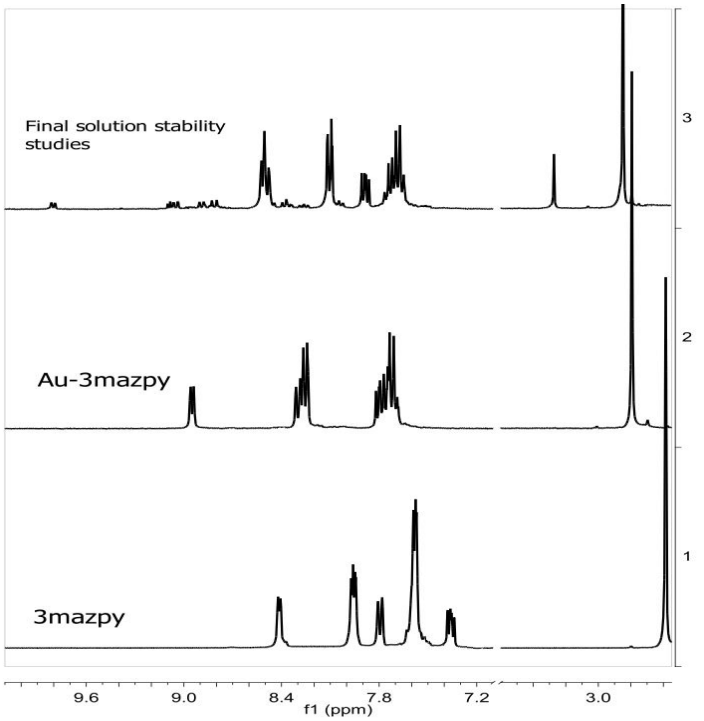

Figure 3.18 ^{1}H NMR spectra of 3mazpy(1), Au-3mazpy (2) and final stable product observed in the stability studies(3). All spectra were recorded in deuterated acetonitrile at 21 ºC, using TMS as internal standard.

The presence of some tricyclic compound is also evident from the peak in ESI-MS at 195.93 m/z, corresponding to the formula $[C_{12}H_{10}N_3]^+$, as well as the peak that corresponds to the ligand by itself, but no other byproduct could be identified.

The first evidence of production of the tricyclic product is observed just after approximately 48 h (figure 3.16). No metallic gold was deposited at the NMR tube walls in this case.

It is quite clear that the five studied gold(III) compounds develop different kinetic stabilities in solution, despite the subtle differences in the chemical structure of all them. The differential stability of these compounds, monitored over a convenient period of time, is best described by the half-life values. These were calculated from the changes in the integration values of different peaks from the ^{1}H NMR spectra as a function of time and then a kinetic exponential dissociation model (one phase) was applied. The results are listed in table 3.2. From the stability studies, it is evident, that more than one chemical step is involved in the entire cyclization process. The peaks selected for the calculation of half-life data, where those peaks showing a clear degradation process (disappearance of the resonance peak) with time and where no other byproduct peaks could interfere with the integration values and final calculations. Au-3mazpy and Au-tazpy were found to be the most- and least-stable compounds, respectively, within the range of half-life values (range=55 h).

Table 3.2 Half-life data calculated for the Au(III) compounds in acetonitrile by means of an exponential dissociation model (one phase) and based on the integration values of selected ^{1}H NMR peaks obtained from stability studies.

Compound	t ½ (h)
Au-tazpy	44.6
Au-4mazpy	56.3
Au-azpy	68.0
Au-3mtazpy	81.7
Au-3mazpy	99.5*

*In this case the tricyclic cationic derivative was not the main product

The use of different 2-(arylazo)pyridine derivatives in the synthesis of Au(III) compounds was undertaken realising that the presence of at least two chelating nitrogen donors could lower the reduction potential of the metal centre and thereby stabilize the Au(III) compounds. In fact, several reasonably stable Au(III) compounds with pyridine-containing ligands have been described in the literature. As presented above and clearly supported by the half life data, Au(III)-2-(arylazo)pyridine derivatives are stable enough for at least 24 h.

Photocyclodehydrogenation has been observed in stilbenes [101], diphenylamines [102] and Schiff-bases [103] under neutral conditions [83, 104]. As a difference, the same procedure for azobenzene occurs just in highly acidic solutions or in presence of Lewis acids by a photochemical disproportionation mechanism [81, 83, 105, 106]. Quantum yield data for the photochemical cyclization of azobenzene in sulphuric acid of different normalities have shown clearly, that the conjugated acid of azobenzene, and not the free base, is the species which undergoes ring-closure (figure 3.19) [105]. The initial process apparently involves *cis*↔*trans* isomerisation and cyclization of the *cis*-isomer being accompanied by loss of hydrogen. The fate of hydrogen is unknown up till now, but it may be noted that the presence of atmospheric oxygen or an oxidizing agent, seems to be essential for such a cyclization [105].

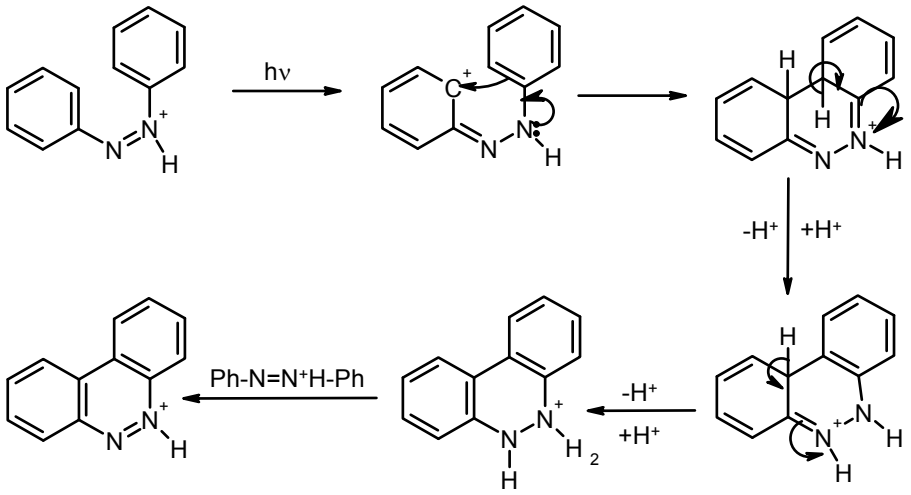

Figure 3.19 Schematic representation of the mechanistic proposal for the photochemical cyclodehydrogenation of azobenzenes in acidic conditions based on [105].

As mentioned in the introduction, azpy was described as having the same photocyclization process as azobenzene, producing 4-pyrido[c]cinnoline (figure 3.4), although poor chemical information was reported [84].

Even though it is not the main goal of this thesis to getting into detail of this unexpected reactivity, the mechanism of cyclization that is taking part in the Au(III)-2-(arylazo)pyridine compounds could be proposed, based on the chemical evidence obtained from the stability studies, and also based in the information obtained from the literature.

First of all, it is suggested that the presence of gold(III) coordinated to the ligand is an indispensable requirement in these conditions. From the stability studies, there is strong evidence that suggest that more than one step is involved in the entire cyclization process. The coordination of gold(III) maintains the *trans* configuration of the ligand (figure 3.20, structure A). As a preliminary activation step, it is believed that a semi *cis*-configuration is generated and probably stabilized through hydrogen bond interactions (figure 3.20, structure B). This activation step could be supported by the observation of small low-field shifts, for most of the resonance peaks in the ^{1}H NMR spectra after a few hours in solution, which could probe a spatial reorganization (figure 3.13 and 3.14). These diamagnetic shifts could be also explained through the generation of inductive effects from electronegative atoms (chloride or nitrogen), or even steric repulsions that results from conformation changes. The gold(III) compounds generate a partially positive charge through the five-membered coordination ring (figure 3.20, structure B). Then, because of this partial positive charge, the stereochemical conformation, and the mesomeric effect, a stabilising π-polarization is generated where the *ortho* and *para* positions in the phenyl ring become electron deficient (figure 3.20, structure C).

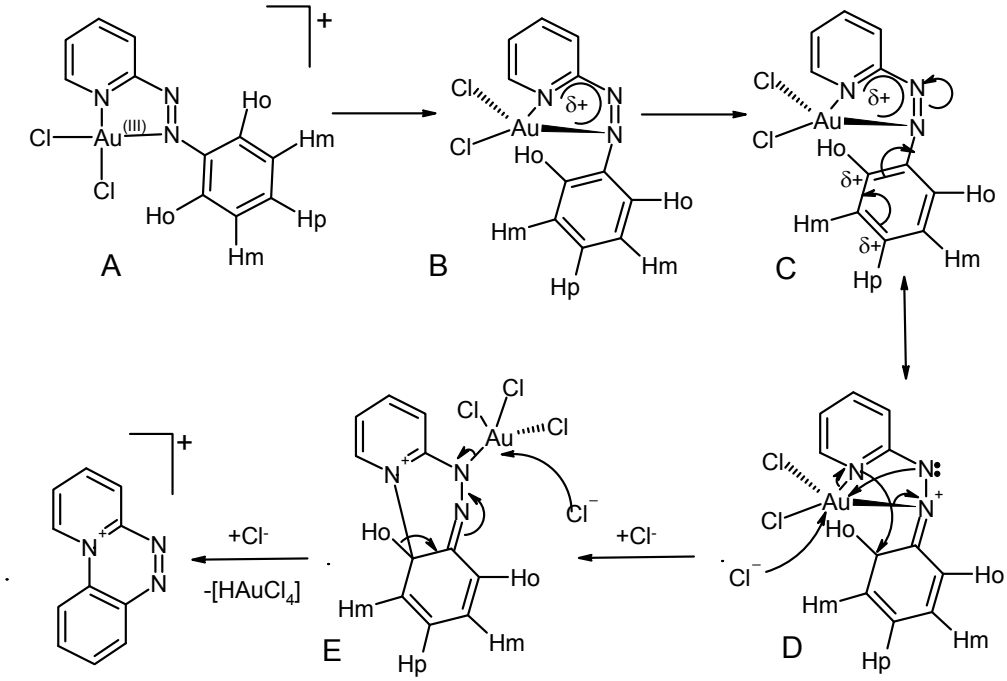

Figure 3.20 Schematic representation of the mechanistic proposal for the formation of the pyrium cation. The same mechanistic proposal could be applied in the generation of the t-pyrium, 3m-pyrium, 3mt-pyrium and 4m-pyrium cations.

At the same time, the ambidentate nature of the azo group, which is the result of having two nitrogen atoms with identical donor properties [107], equally able to participate in coordination, becomes differentiated through the nucleophilic "concerted" attack of the pyridine nitrogen to the electrophilic *ortho* position in the aryl ring. Then, the nitrogen from the azo group directly attached to the pyridine ring develops a negative charge, highly efficient in coordinating gold(III), so that migration of the Au(III) centre is induced. The feasibility of these structural changes is mainly based on the observation of just a few intermediates, the stability of the final products and the extra stabilization attributed in part to the gain in resonance energy (figure 3.20, structure D). The participation of chloride anions is suggested, because the starting material contains them as counterion and it could also explain, at least in part, the stabilization of the anion, $[AuCl_4]^-$, with Au(III), as no metallic gold is observed in some cases. Finally this intramolecular re-

arrangement produces the tricyclic cationic compound from which the driving force could be attributed in part to the gain in resonance energy (figure 3.20, structure E and the final tricyclic cation).

A completely satisfactory explanation of the effects of substituents on the reactivity is not yet possible on the basis of the proposed mechanism of cyclization, although a more critical examination could be made if the ill-defined byproducts present in solution were better known.

The faster the reaction proceeds, the more difficult it is to observe the changes in the spectrum as observed in the case of Au-tazpy.

Based on this mechanism proposal, it is clear that most probably, the reduced production of the tricyclic compound, in case of Au-3mazpy, is due to steric factors as coordination of Au(III) to the azo nitrogen will be space demanding. Also of consideration is the fact that electron-releasing groups, as the methyl moieties at position 3 (instead of H_3) will induce a reduction in the partial positive charge generated in the coordination ring, so the resonance effect could be reduced as well. The presence of a methyl group in the aryl ring could help in providing a better mesomeric effect, but it could be also the case that instead of a nucleophilic attack by the pyridine nitrogen, a Au(III) attack could take part and then an organometallic structure must be formed, as in the case of palladium, producing, the tricyclic product [35] shown in figure 3.21. In fact, Huttel and Vicente, reported independently the formation of organometallic gold(III) compounds with azobenzene derivatives [108, 109].

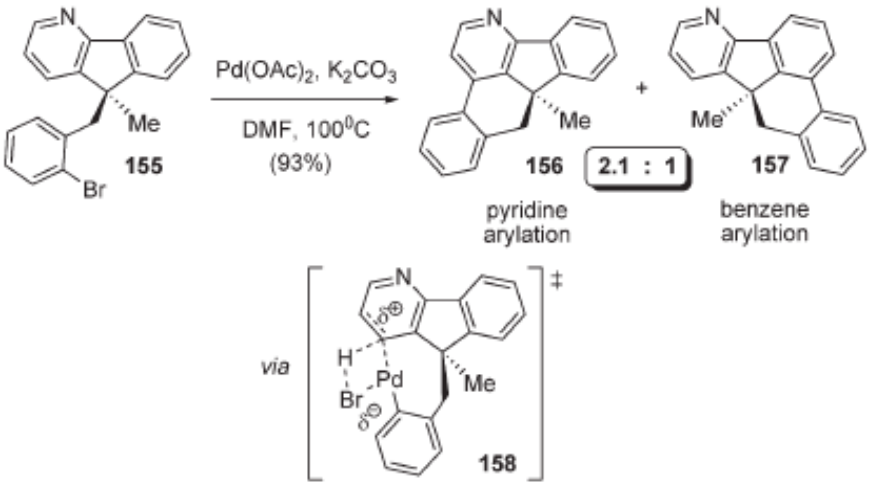

Figure 3.20 Schematic representation of the Pd-catalyzed arylation of pyridine [35].

The nucleophilic substitution in aryl rings is believed to occur anyway as a result of the effect on the aryl ring in azpy upon coordination, as the ring becomes electrophilic. The production of metallic gold in case of Au-tazpy and Au-3mtazpy could be related to side-reactions once the tricyclic structure is formed. This is not further discussed.

The nucleophilic attack produced in Au-4mazpy is completed in less time than azpy, probably because of the electron-releasing effect of the methyl group in the *para* position of the pyridine ring, which could increase the nucleophilic nature of the pyridine nitrogen. This increased nucleophilic effect could also produce an extra stabilization of the transient species suggested to be produced in the mechanistic proposal.

From some of the solutions used in the stability studies that were kept at the freezer, crystals of Au-4mazpyCl_3 were observed after some days. A good quality crystal was isolated from the solution and the diffraction studies performed and detailed results are presented in table 3.3 and figure 3.22. Table 3.4 includes selected bond distances and angles for Au-4mazpyCl_3.

It is interesting to note that the key structural features of Au-4mazpyCl_3 are quite similar to those observed in previously reported Au(III) compounds [18, 110] with pyridyl-based ligands and halogens. Specifically, bond lengths for Au-Cl are described around 2.25 Å, while the present observed values vary between 2.25-2.28 Å in Au-4mazpyCl_3. The smallest one being for Au-Cl2 which is *trans* to the pyridine nitrogen. In addition, the bond distance for Au-N has been described in literature around 2.04 Å, as is the distance observed in Au-4mazpyCl_3. Cl-Au-Cl angles were quite comparable with literature data as well. The plane of the ligand is perpendicular to the plane

described by the chlorides coordinated to Au(III). This conformation offers smaller steric repulsions with ring protons. Worthy to mention is the torsion angle of 10.3° between the pyridine ring and the phenyl ring in the ligand coordinated to Au(III). This assures also less steric effect and possibly provides intramolecular hydrogen bond extra stabilization around the azo moiety.

Table 3.3 Summarized crystallographic data for $C_{12}H_{11}AuCl_3N_3$, Au-4mazpyCl$_3$.

Property	
Empirical formula	$C_{12}H_{11}AuCl_3N_3$
Formula weight	500.56
Crystal system	Triclinic
Space group	P-1
a(Å)	9.3925(4)
b(Å)	9.8683(5)
c(Å)	10.0532(6)
α (º)	115.121(1)
β (º)	96.151(2)
γ (º)	113.660(2)
V(Å^3)	728.21(7)
Z	2
T(K)	150(2)
D_{calc}(Mgm^{-3})	2.283(2)
μ(Mo Kα)(mm^{-1})	10.639
F(000)	468.0
Parameters refined	173
R_1[a]	0.0138(3126)
wR_2[b]	0.0299(3343)
GOF	1.03
$\Delta\rho_{max,}$ e Å^{-3}	0.50
$\Delta\rho_{min,}$ e Å^{-3}	-0.62
Crystal dimensions	0.02 x 0.09 x 0.15
Colour	Needle, yellow

[a] $R1 = \Sigma||F_o| - |F_c||/\Sigma|F_o|$. [b] $wR2 = \{\Sigma[w(F_o^2 - F_c^2)^2]/\Sigma[w(F_o^2)^2]\}^{1/2}$

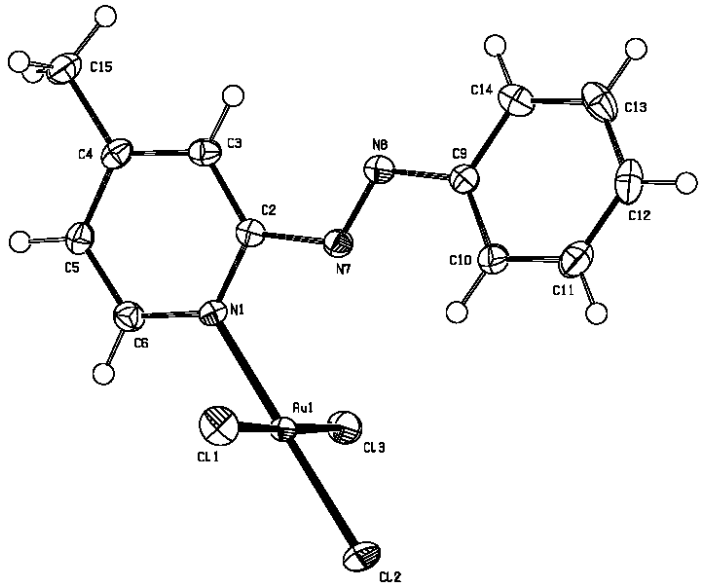

Figure 3.22 Molecular structure of the asymmetric unit of Au-4mazpyCl$_3$ and the used atom numbering.

Most probably, the isolation of this crystalline product was the result of the extra stabilization from the methyl moiety at *para* position in the pyridine ring, but also due to the reduced temperature, so the delicate equilibrium between cyclization and coordination of Au(III) can perhaps be controlled.

Table 3.4 Selected geometric parameters (Å, °) for Au-4mazpyCl_3.

Distances (Å)			
C2-C3	1.378 (4)	C10-C11	1.376 (4)
C3-C4	1.391 (4)	N7-N8	1.252 (3)
C2-N1	1.339 (3)	Au-N1	2.042 (3)
C2-N7	1.422 (4)	Au-Cl1	2.273 (9)
C9-N8	1.422(4)	Au-Cl2	2.255 (8)
C9-C10	1.391 (4)	Au-Cl3	2.281 (8)
C3-H···N8	2.525	C10-H···N7	2.468
C14-H···N8	2.513	Cl2a···H-C14	2.727
Angles (°)			
Cl1-Au1-Cl2	91.25 (3)	C2-N7-N8-C9	176.1 (2)
Cl3-Au1-N1	89.64 (6)	C10-C9-N8-N7	-10.3 (4)
Au1-N1-C6	121.14 (17)	N1-C2-N7-N8	172.7(2)
N1-C2-N7	112.0 (2)	C3-C2-N7-N8	-9.6 (4)

It was also attempted to observe transient species in solution of the other gold derivatives at reduced temperature, but so far with negative results. The addition of a counterion to stabilize the structures was tried and positively charged ligands were crystallized and diffraction studies performed in case of 3mazpy and 3mtazpy. This observation could suggest that, Au(III) coordination is partially hampered by steric factors as the ligand prefers to be protonated at the pyridine ring instead of forming the coordination compounds. The crystal properties are fully described in appendix A of this thesis and the X-ray structures are depicted in figure 3.23 and 3.24.

The results discussed earlier in this chapter, clearly describe a kinetic-stability tendency, depending on the ligand coordinated to the trivalent gold ion. This observation will provide an effective tool for tuning the reactivity of this family of compounds in solution. It is believed that incorporation of electron-releasing and electron-withdrawing groups at the pyridine and/or aryl rings will control the chemical properties of this family of Au(III) compounds.

It is important to stress the fact that heterocyclic rings are present as fundamental components in all living systems, and play an important role in the homeostatic mechanism. Researchers all over the world are working to discover and optimize new reaction procedures for the construction of heterocycles. Now a facile route for the synthesis of a new family of heterocycles was described and a considerable number of possible applications could result from these positively charged tricyclic compounds.

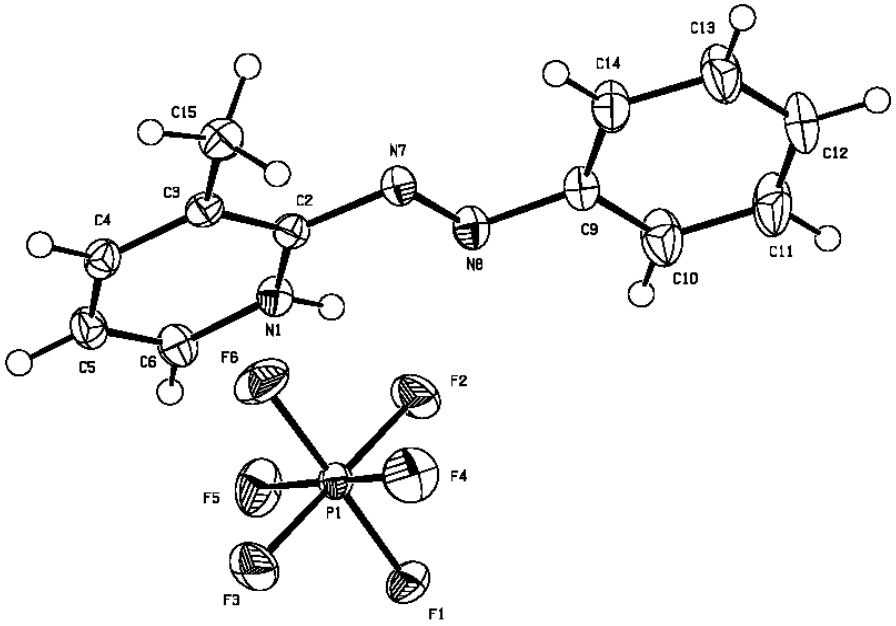

Figure 3.23 X-ray structure of the asymmetric unit of 3mazpyH with the hexafluoridophosphate anion and used atomic numbering.

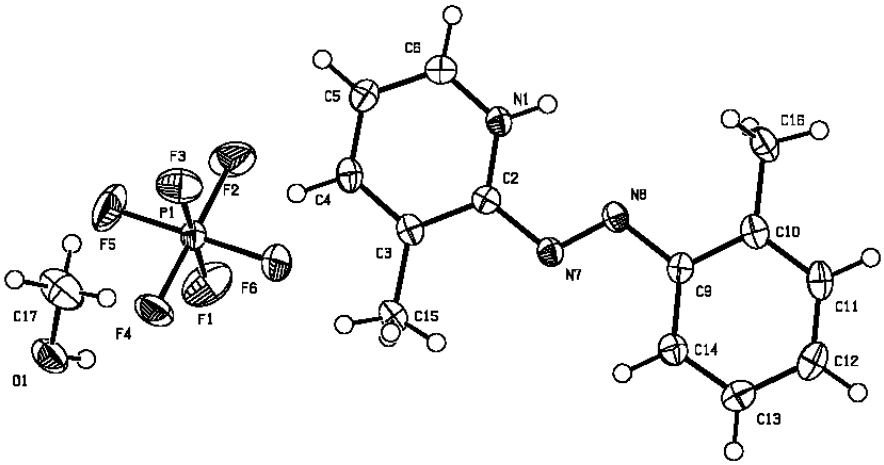

Figure 3.24 X-ray structure of the asymmetric unit of 3mtazpyH with the hexafluoridophosphate anion and used atom numbering.

3.3.2 In vitro cytotoxic activity of 2-(arylazo)pyridine analogues, Au(III)-2-(arylazo)pyridine compounds and the pyrium cation

The purity of the compounds described in this report has been estimated by elemental analysis, IR spectroscopy, ^{1}H NMR spectrometry, chromatography and equivalent conductance and after completing the characterization, also the cytotoxic properties were determined.

The cytotoxicity of $HAuCl_4$, all 2-(arylazo)pyridine ligands, Au(III)-2-(arylazo)pyridine compounds and pyrium chloride was determined using the following human ovarian carcinoma cell lines, A2780, sensitive and A2780R resistant to cisplatin and murine leukaemia cell line L1210, sensitive and resistant to cisplatin. This was planned to asses a possible lack of cross-resistance with cisplatin. Cisplatin, the azopyridine ligands and $HAuCl_4$ were included as reference compounds and have been evaluated under the same conditions. Cross resistance profiles were evaluated by means of the resistance factor (RF), which is defined as the ratio between IC_{50} values calculated for a cisplatin-resistant cell line and the sensitive parental cell line, respectively ($RF=IC_{50resistant}/IC_{50sensitive}$). An RF of <2 was considered to denote non-cross resistance [111]. The data have been summarized in tables 3.5 (72 h) and 3.6 (48 h).

A first approach of the cytotoxic activity was obtained for azpy, Au-azpy and the isolated pyrium chloride hydrate after 72 h of incubation. It was observed that the compounds tested in the non-resistant A2780 cell line show a lower cytotoxic effect than cisplatin, but all compounds present IC_{50} values within the micromolar range, which is generally considered to be a promising cytotoxic activity. The Au(III) compounds ($HAuCl_4$ and Au-azpy) show significant cytotoxic activity, with IC_{50} values ranging from 10-62 μM. Similar values have been reported for other Au(III)-pyridine containing compounds [19, 112]. The $AuCl_4^-$ ion is also quite cytotoxic and clearly a minimum degree of cross-resistance is observed in both solid tumours and leukaemia cells. Au-azpy shows a high cytotoxic activity in the human solid tumours, but lower toxicity in leukaemia cells. In addition some degree of cross-resistance is observed. This evidence proves that Au-azpy is able to travel inside the cells. Important cytotoxic effects were also observed by azpy in the tested cell lines.

In particular it should be pointed out that the pyrium cation has a high cytotoxic activity. In fact pyrium chloride shows a cytotoxic activity within the same range as found for cisplatin in the sensitive line and it is even significantly more cytotoxic than cisplatin in the resistant cell lines, where it is at least 2 times more cytotoxic than cisplatin; this implies that the tricyclic product does overcome cisplatin resistance. For A2780R, studies indicate that the mechanism of resistance to cisplatin is multifactorial involving increased DNA repair, decreased drug uptake and elevated glutathione levels and some evidence underline the charge as an important factor involved in antitumor activity. Anyway more studies with different cell lines and *in vitro* studies with biologically relevant fluids should be undertaken. The murine models have been of some value in identifying agents effective in the treatment of human leukaemia and lymphomas. Remarkably Au-azpy, $HAuCl_4$ and pyrium chloride hydrate retain a high cytotoxicity towards the cisplatin resistant cell line. The cytotoxic activity of the free ligand does not diminish the fact that the presence of Au(III)

increased substantially the cytotoxic properties and even non-cross resistance effect. The potent cytotoxic effect registered by Au-azpy could be partially due to the presence of the pyrium cation, as most probably this organic charged tricyclic compound is being formed during the performance of the test.

Table 3.5 *In vitro* cytotoxicity assay of selected compounds incubated during 72 h[a].

	IC_{50}, μM					
Cell line	A2780	A2780R	RF	L1210/0	L1210/2	RF
Compound						
$HAuCl_4$	19.2	15.9	0.82	18.5	18.2	0.98
Azpy	23.1	26.4	1.14	22.6	45.6	2.01
Au-azpy	10.5	12.6	1.20	56.7	62.5	1.10
Pyrium chloride hydrate	9.2	5.4	0.59	17.0	10.6	0.62
Cisplatin	2.42	9.9	4.06	2.08	19.9	9.6

[a]The cytotoxic effect of other 2-(arylazo)derivatives and Au(III) compounds was not included in these tests due to the prolonged incubation time.

These first tests helped to assess the cytotoxic effect of Au-azpy, azpy and pyrium chloride hydrate. The half-life data obtained for the other compounds suggested that a reduction in the incubation time could provide more accurate cytotoxic information of each compound, so it was decided to perform the cytotoxic studies at 48 h (A2780, A2780R), and the results for all the Au compounds and free ligands are included in the following table (table 3.6).

Analysing the results obtained for the free ligands, notably, the methyl substitution in the pyridine ring in azpy resulted in a higher anticancer activity for 4mazpy, 3mtazpy and especially for 3mazpy. On the contrary, the methyl substitution in the *ortho* position of the aryl ring in azpy produces a small reduction in the anticancer activity, as can be observed in tazpy and 3mtazpy. Possibly, this effect could be related to enhanced lipophilic-properties which facilitate transport into the cells. But also could be hypothesized as a result of the ability of these ligands to induce intracellular metal-deficiency states or even the formation of metal complexes that themselves are tumour growth inhibitors, like in case of terpyridine and related derivatives [113]. The anticancer activity of 4mazpy, 3mazpy and 3mtazpy is quite remarkable by itself. 3mtazpy shows the best resistance factor.

The markedly different cytotoxic activity of the free ligands is an important observation that could not be analysed if controls of the free ligands would not have been included in the biological tests. When studying coordination compounds, it should be stressed the need of performing biological tests even for the free ligands. This protocol requirement has often been omitted in regular bases, in the biological studies, published in the specialized literature.

Table 3.6 *In vitro* cytotoxicity assay of selected compounds incubated during 48 h.

	IC_{50}, μM		
Cell line	A2780	A2780R	RF
Compound			
Cisplatin	2.8	14.9	5.3
$HAuCl_4$	52.2	97.9	1.9
Au-tazpy	22.7	30.7	1.3
Au-4mazpy	36.1	46.9	1.3
Au-azpy	41.0	80.1	1.9
Au-3mtazpy	28.5	26.5	0.9
Au-3mazpy	11.1	11.1	1
tazpy	143.1	145.9	1.0
4mazpy	39.0	42.5	1.1
azpy	126.2	167.9	1.3
3mtazpy	27.9	17.9	0.6
3mazpy	22.7	22.8	1.0

The marked differences in the cytotoxic activity of these Au(III) compounds containing different heterocyclic ligands suggest that the cytotoxic activity of these compounds is determined by several factors: a) the identity and stability of the Au(III) compounds, b) the nature of the

substituents on the heterocyclic and non heterocyclic aromatic rings and c) the byproducts formed (closely related to the incubation times).

All Au(III) compounds exhibited potent *in vitro* anticancer activities toward the cancer cell line A2780, sensitive and resistant to cisplatin with micromolar IC_{50} values. Notably, Au-3mazpy exhibits the highest cytotoxicity toward A2780 and A2780R cells and also overcome cisplatin-resistance (RF=1). This biological response could stand partially, on the stability, as Au-3mazpy was found to be the more stable compound among all the Au(III) compounds, but also in the high toxicity of the ligand by itself.

In fact all the Au-based compounds present IC_{50} values less than 50 μM, with exception of $HAuCl_4$ and Au-azpy in A2780R. From the results after 72 h of incubation, the activity of Au-azpy, azpy and $HAuCl_4$, appear to increase as the incubation time increases. The higher activity of Au-azpy (after 72 h) in particular, could be associated at the higher levels of the pyrium cation (IC_{50}=9.2 and 5.4 in A2780 and A2780R, respectively) instead of the effect of the gold compound by itself.

Interestingly, although the IC_{50} values of free azpy and tazpy (table 3.6) are higher than 100 μM, once coordinated to Au(III), they display IC_{50} values low enough and comparable to the other compounds with more toxic free ligands. This evidence could suggest that the biological effect is not mediated by the ligand alone following complex disruption. In fact, although 3mazpy is quite active by itself, the anticancer effect is enhanced twice once coordinated to gold.

Due to the poor stability of most of the Au(III) compounds, it is strongly recommended to perform the cytotoxic tests at different incubation times.

With the information obtained, it appears that the presence of an electron-releasing group in the pyridine ring, at the *meta* position, will increase the stability of the Au(III) compounds and also the biological activity. Anyway, the observed IC_{50} values for all the Au(III) compounds studied in this chapter, suggest that changes in the extended aromatic array do not substantially increase their cytotoxicity. The effect of electron-withdrawing agents might be a next step in the study.

Gold compounds possess a variety of therapeutic potentials ranging from attenuation of inflammation, suppression of autoimmunity, and promising antitumor activity. It was believed that platinum and gold compounds would target DNA, as both show similar chemical properties. Extensive evidence in the literature indicates, by the contrary, that Au(I) and Au(III) compounds display a different mechanism of action than cisplatin. Gold compounds can interact with several cell components and in particular mitochondria appear as potential targets. Alternative molecular targets for these gold compounds are biologically important thiol-containing molecules, such as cathepsin B, or thioredoxin reductase, but the mechanism of action of Au(I) and Au(III) compounds is still unclear.

The Au(III)-2-(arylazo)pyridine compounds show promising anticancer activity, in particular, two of them (Au-3mazpy and 3mtazpy) exhibit rather encouraging cytotoxic properties, being also able to overcome resistance to cisplatin. Au-3mazpy was found even more toxic than cisplatin in the resistant cell line.

A detailed discussion of structure-activity relationships would be premature. Further work is required to produce a target-specific drug with a suitable pharmacological activity and toxicity profile.

3.4 Concluding remarks

Several authors have repeatedly demonstrated that Au(III) compounds have a great potential as antitumour agents. The major disadvantage recalls in the high reactivity of Au(III) by itself. It has been challenging to design and synthesize physiologically stable Au(III) compounds, specially due to the lack of knowledge in Au(III) chemistry.

In an attempt to provide more chemical evidence, in this chapter, the stability in solution, for the Au(III)-2-(arylazo)pyridine compounds, was studied in detail through ^1H NMR studies, which proved to be a useful tool. The results clearly describe a kinetic-stability tendency, depending on the ligand coordinated to the trivalent gold ion. Half-life values were calculated based on an exponential dissociation model (one phase) from the integration values of selected ^1H NMR peaks from the stability studies. The chemistry of 2-(arylazo)pyridine systems has been under study for the past 30 years, but their interaction with gold(III) is described here for the first time.

The chemical reactivity of most of the Au(III)-2-(arylazo)pyridine compounds, in acetonitrile solution, proved to produced a new family of positively charged aromatic tricyclic compounds. A mechanistic proposal for this unexpected reactivity is described in detail and partially demonstrated through the chemical evidence obtained; however, further mechanistic studies are needed as well as more experimental evidence. A full characterization, including X-ray diffraction studies for the pyrium cation, one of the tricyclic compounds formed, is described in this chapter and ^{1}H NMR and ESI-MS have been shown to be powerful tools for the characterization of the other tricyclic derivatives. No studies on the synthesis of these organic-charged molecules have been reported yet; so the organic compounds obtained represent promising starting materials for the synthesis of more elaborated organic structures. Worthy mentioning is the high cytotoxic activity developed by the pyrium cation in the *in vitro* studies.

The stability studies also showed that the Au(III)-2-(arylazo)pyridine compounds are reasonably stable in solution, during the 48-72 h *in vitro* cytotoxic tests. All Au(III) compounds and free ligands were found to behave as potential cytotoxic and anticancer agents against cisplatin-sensitive and cisplatin-resistant ovarian carcinoma cell lines, A2780; and cisplatin-sensitive and cisplatin-resistant murine lymphocytic leukaemia cell lines, L1210 .

The free ligands show quite remarkable cytotoxic activity against the cell lines tested and introduction of a methyl moiety in the pyridine ring in azpy, increases the cytotoxicity around five times. Further studies are required to outline a clearer structure-activity relationship.

The IC_{50} values of the closely related Au(III) compounds were determined showing a moderate-high cytotoxicity and the results were discussed and compared and some important details were found useful in the proposal of a structure-activity relationship.

In general terms, it was shown that the antitumor activity of this series of Au(III) compounds appears to be a function of a number of factors, such as the nature of the ligand, the Au(III) compound structure, the stability of this compound an even the byproducts formed in solution, as well as the incubation times. The observed IC_{50} values suggest that changes in the ligands coordinated to Au(III) appear to increase the cytotoxicity of the metal base compound, but in a moderate way. Since reduced cytotoxicity was observed with $HAuCl_4$, or some of the ligands alone, it could concluded that, the Au(III) compounds play an important role in the antitumor activity of this series. All the Au(III)-2-(arylazo)pyridine compounds could overcome cisplatin-resistance. Au-3mazpy showed the highest cytotoxic activity ($IC_{50=}$11 μM) and also the highest stability in solution.

Even though an improvement in the knowledge of the synthesis, reactivity in solution and cytotoxicity of the compounds described in this thesis is observed, many questions have arisen. The current results may form an important ground for future *in vitro* and *in vivo* studies and are important for the future design of antitumor Au(III)-2-(arylazo)pyridine compounds, but it is necessary to develop the stability studies under closely similar physiological conditions where the reactivity of the Au(III) could be tested in the presence of biological reducing agents as glutathione or ascorbic acid. It looks also transcendental to study the interaction of these compounds with biological important targets as DNA, proteins and enzymes. The cellular uptake of this set of compounds must be studied in detail as well. Finally more dramatic modifications in the ligands would reveal important chemical information that will be translated into a proposal of structure-activity relationships

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