Design, synthesis and biological evaluation of Cu(I) and Cu(II) complexes with bis(pyrazolyl)acetates and related bioconjugated ligands

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month/year

Abstract. Due to the success of cisplatin in the treatment of different type of tumors, the development of transition metal-based complexes as drugs have gained a relevant boost. Toxicity and drug resistance phenomena of cisplatin limit its clinical use, so the attention has been focused on other transition metals. Especially copper-based drugs have been investigated on the assumption that an endogenous metal should be less toxic with respect to other metallodrugs and researchers have putted great attention in the study of mechanism of adsorption, distribution, metabolism and excretion of copper-based compounds.

Polydentate nitrogen-containing donor ligands derived from poly(pyrazol-1-yl)methanes bearing organic functional groups on the bridging carbon have recently attracted considerable attention and their coordination chemistry towards main group and transition metals have been extensively studied. I have synthesized two carboxylated heteroscorpionate ligands, $[HC(CO_2H)(pz^{Me2})_2]$ and $[HC(CO_2H)(pz)_2]$ and I have studied their coordination chemistry towards copper(II) acceptors starting from copper chloride or perchlorate and copper(I) acceptors in the presence of phosphane coligands (triphenylphosphine and PTA). The bis(pyrazolyl)acetates ligands have also been functionalized with the potent NMDA receptor antagonist (6,6-diphenyl-1,4-dioxan-2-yl)methanamine, which showed a significant cytotoxic activity on MCF7 human breast cancer cell lines, highly expressing NMDA receptors. Some copper complexes as well as the corresponding uncoordinated ligands were evaluated for their cytotoxic activity towards a panel of several human tumour cell lines.

Key words: copper(I/II) complexes, anticancer drugs, transition metal-based complexes, heteroscorpionate ligands, bioconjugated ligands.

Introduction

In medicinal chemistry organic compounds and natural products were almost the entirety of the drug used. During the past three decades metal complexes have gained a growing interest as pharmaceuticals used as diagnostic agents or as chemotherapeutic drugs [1-7]. The widespread success of cisplatin (cis-diamminedichloroplatinum(II)) in the clinical treatment of various types of neoplasias has placed coordination chemistry of metal-based drugs in the frontline in the fight against cancer in the 20^{th} century [1, 8-10]. The anticancer property of cis-diamminedichloroplatinum(II) was discovered accidentally in 1965 by the American chemist Barnett Rosenberg [11] during an experiment on the influence of electric fields in cellular growth. However, the first who synthesize cisplatin was the Italian chemist Michele Peyrone in 1845 [12], with the historical name of “Peyrone's chloride”; the Nobel Prize Alfred Werner first elucidated the structure only in 1893.

The goodness of cisplatin is the high efficacy in treating a wide range of cancers, especially testicular cancer, for which the overall cure rate exceeds 90% [2]. Unfortunately the cure with cisplatin is limited by the drawbacks, such as the dose limiting effects [13], nephrotoxicity, emetogenesis and neurotoxicity [2] and by inherited or acquired resistance phenomena, only partially overcame by the development of new platinum drugs, such as carboplatin and oxaliplatin [14-17]. The side effects have encouraged an extensive research in this field, and rapidly chemists developed alternative antitumor-active inorganic complexes, based on different metals, with improved pharmacological properties and pointed to different targets [18].

One of the research strategies is focused on the use of biologically active complexes formed by essential ions, based on the assumption that endogenous metals may be less toxic for normal cells with respect to cancer cells. All the biological systems tend to concentrate some elements and to reject others, like a natural selection. The design of copper complexes could be an interesting strategy [19]; the interest in this field has rapidly grown in the last years, as illustrated by the increasing number of publications reported since 2000 (Fig. 1.).

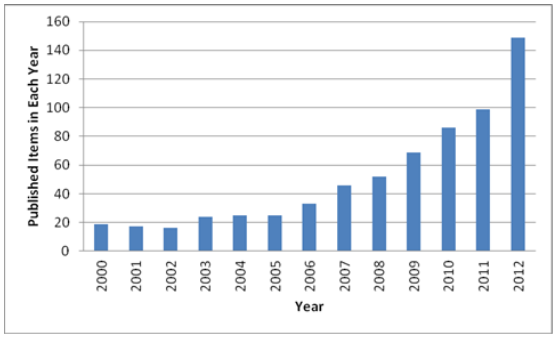

Fig. 1. Number of articles in Web of Science on the topic "copper and anticancer" from 2000 to 2012.

Copper-based complexes showed encouraging perspectives in this field [20-24], even if copper could show toxicity due to its redox activity and its affinity for binding sites that should be occupied by other metals. The basis for the progress of copper complexes is the different response between tumour and normal cells to copper and the change in metabolism of cancer cells. Copper is a micro-nutrient essential for most of the aerobic organisms, employed as a structural and catalytic cofactor, and consequently it is involved in many biological pathways [14, 15, 25]. Take it into account it's reasonable that researchers have put lot of attention on the mechanisms of ADME (absorption [16-18], distribution [20-22], metabolism, and excretion) of copper [23, 24, 26], as well as on its role in the development of cancer and other diseases [27-29].

1. Copper Chemistry

Copper forms a rich variety of coordination complexes with oxidation states Cu(II) and Cu(I), and very few examples of Cu(III) compounds are reported [30]. Coordination chemistry of copper is dominated by Cu(II) derivatives with little, but important examples of Cu(I) compounds. Since copper(I/II) complexes are redox active, frequently labile, and atypical in their preference for distorted coordination geometries, they are much less structurally predictable than other first row transition metal complexes. The variety of accessible arrays allows for a great assortment in the choice of the ligands (from mono- to hexa-dentate chelates), and of the donor atoms (N, O, S and halides) [31]. The redox potential of the physiologically accessible Cu(I)/Cu(II) couple varies dramatically depending upon the ligand environment due to the donor set, geometry, substituents, electronic and steric effects, and chelation[32].

2. Copper complexes as anticancer agents

In review papers about copper complexes as antitumor agents, ligands are generally classified discriminating by the donor atom(s) involved in the bond with the metal in organometallic compounds. Following this rule most of the ligands are classified as S-donor, O-donor, N-donor, P-donor, C-donor, N,O-donor, N-N diimiide, C-donor N-heterocyclic, but also as Shiff bases, polydentate and macrocyclic ones.

Starting from the tris(pyrazolyl)borates, a very useful class of monoanionic, nitrogen-based, auxiliary ligands, some ligands take the name of "heteroscorpionate" because of the shape that they take in the coordination of the metal centre. They readily coordinate usually as face-capping tridentate

ligands to a wide variety of metal ions affording stable metal complexes [33].

Polydentate nitrogen-containing donor ligands derived from poly(pyrazol-1-yl) methanes with [RR'C$(Az)_2$] (Az = azolyl groups; R = H or Az) as general structure and bearing organic functional groups (R') on the bridging carbon have attracted considerable attention and their coordination chemistry towards main group and transition metals have been extensively studied [34-36]. These intriguing heteroscorpionate ligands [33, 37] present different types of functional groups, which successfully broaden the scope of their applications.

For the complexes bearing phosphine ligands the distinctive characteristic is the +1 oxidation state of the metal, which is mostly comprised in a four-coordinated tetrahedral environment. Copper coordination sphere is either partially or totally filled by phosphine ligands that efficiently bind the electron-rich d^{10} metal ion. Although Cu(I) is the chemical form generally accepted by the bioinorganic community to describe the active internalization of physiological copper in mammalian cells through copper transporter (Ctr) proteins, still very few studies report on the action of Cu(I) complexes as antitumor agents. This is likely related to the intrinsic difficulty to stabilize copper(I) species, especially in aqueous media. Only the formation of quite robust metal-ligand interactions, as those displayed in the case of copper-phosphine (and copper-NHC) species, prevents hydrolysis and the activation of the redox machinery [38].

Two different coordination spheres have been particularly investigated in the field of copper-phosphine compounds, with the corresponding tetrahedral complexes showing both easy synthesis and appealing biological properties: the homoleptic, mono-cationic $[Cu(P)_4]^+$ arrangement and the mixed-ligand, neutral [Cu(N-N)(P)(X)] assembly, where P represents a monodentate phosphine, N-N an aromatic diimine and X a halide [39].

The work by Berners-Price on 1:2 hydrophilic adducts of copper(I) halides with 1,2-bis(di-2-pyridylphosphino)ethane (P-P) [40] joined the extensive studies performed in the eighties [41, 42] on group 11 metals including lipophilic bis-aryldiphosphines. The lack of selectivity toward tumorigenic and non-tumorigenic cells, and the robustness toward dissociation of these copper adducts made them more promising candidates in the radiopharmaceutical field [43].

3. Mechanistic approaches and proposed biological targets: state of the art

Copper species possess a broader spectrum of activity and a lower toxicity than platinum drugs, and are suggested to be able to overcome inherited and/or acquired resistance to cisplatin. These features are consistent with the hypothesis that copper complexes possess mechanism(s) of action different from platinum drugs that covalently bind to DNA. Moreover, DNA damaging agents, the drugs that promote DNA dysfunction, can induce as drawback gene mutations and chromosomal modification. Hence development of antitumor drugs that act through different pathway than DNA is preferred. Anyway, little information is available on the molecular basis for the mode of action of copper complexes. At present, most investigations still focus on the ability of these complexes, or fragments thereof, to interact with DNA. However, other cellular constituents such as topoisomerases or the proteasome multi-protein complex are emerging as new putative targets [39].

The last studies are confirming that copper-based complexes could be a solution to overcome the problem of toxicity and acquired resistance to platinum drugs, especially for Cu(I) complexes, the chemical form nowadays accepted for internalization of physiological copper in mammalian cells.

3.1. Copper complexes as DNA targeting drugs

Since 1969 copper has been found to possess high DNA binding affinity [44]. Analogously to what has been widely illustrated for cisplatin [45], a crystal structure describing the formation of an adduct between $CuCl_2$ and DNA was published in 1991 [46]. The binding was dependent on copper complex size, electron affinity and geometry of the formed adduct inducing an irreversible modification of the DNA conformational structure.

According to these observations, a high number of copper complexes have been and are still being tested as DNA-targeting metal-based drugs. For

some classes of copper derivatives, the ability to bind DNA has been well established and documented. Copper derivatives have been found capable of non-covalently interact with DNA double helix, rather than forming coordinated covalent adducts with DNA. The non-covalent DNA interactions included intercalative, electrostatic and groove binding (Fig. 2.) of metal complexes, along major or minor DNA groove.

Fig. 2. Schematic representation of intercalative (left) and groove (right) binding.

In most cases, the metal acted as an inorganic modifier of the organic backbone of the bio-active molecule and ligands granted DNA affinity and specificity.

Overall, it has been demonstrated that physico-chemical features, such as the planarity, hydrophobicity and size of the di-imine, the nature of the coligand as well as the coordination geometry of the metal complex, all played important roles in determining the binding/intercalating mode of copper complexes to DNA.

3.2. Copper complexes as Topoisomerase I,II inhibitors

Recent research into the ability of copper complexes to inhibit topoisomerases has served not only to reinforce the significant potential of this class of metal complexes in cancer research but also to expand the array of possible biochemical targets for these molecules. Topoisomerases are essential nuclear enzymes that regulate the overwinding or underwinding of DNA and so they play essential functions in DNA replication and transcription. Topoisomerases create transient nicks (Topo I) or breaks (Topo II) in the double-stranded DNA polymer, allowing DNA to be converted between topological isomers [47].

Currently there is still an increasing interest focusing on the development of new kinds of drugs targeting human topoisomerases and the development of metal complexes as Topo I,II inhibitors fits just in this therapeutic niche. However, unlike that involving DNA, interaction of copper complexes with topoisomerases is a relatively new field of research.

3.3. Copper Complexes as Proteasome Inhibitors

The proteasome is a large multiprotein complex located in both the nucleus and the cytoplasm that selectively modulates and degrades intracellular proteins. The proteasome is part of a major mechanism by which cells regulate the concentration and the stability of particular proteins and decompose unfolded proteins.

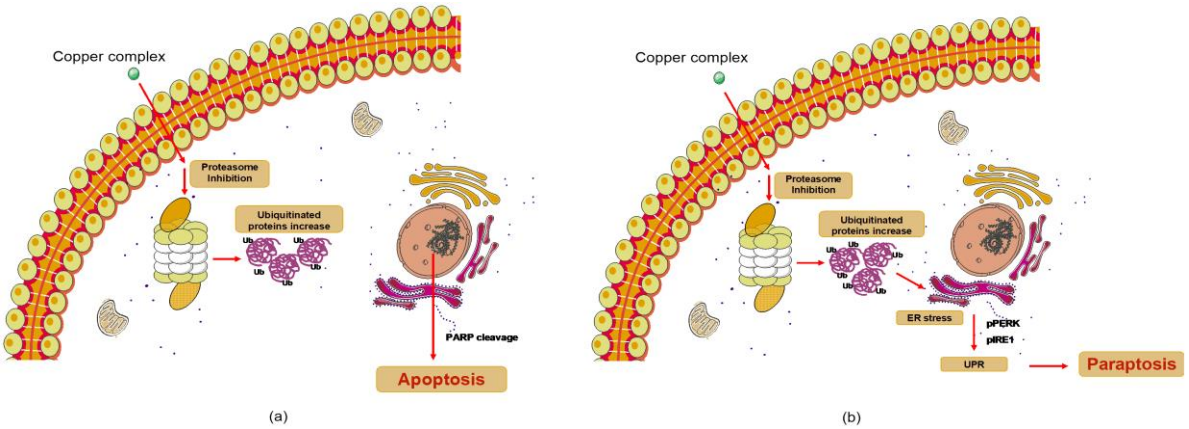

Fig. 3. Schematic diagrams of cellular pathways involved in proteasome inhibition induced by copper compounds. a) Apoptosis triggered by DTC copper(II) complexes; b) paraptosis caused by phosphine copper(I) and thioxotriazole copper(II) complexes.

The ubiquitin proteasome-dependent degradation system is essential for many cellular functions, including processes of primary importance for carcinogenesis such as proliferation, apoptosis, angiogenesis and metastasis formation [48].

It has been shown that cancer cells are more sensitive to proteasome inhibition than normal cells. Thus, targeting the ubiquitin-proteasome pathway has emerged as a favourable anticancer strategy [49, 50] and currently the development of proteasome inhibitors as novel anti-cancer agents is under intensive investigation.

Copper-mediated proteasome-inhibitory activity could be enhanced by the choice of appropriate bidentate ligands but blocked by stronger copper polydentate chelators such as EDTA. This feature denoted that substitution-inert copper complexes, in which copper was totally sequestered by the ligand framework, could not act as proteasome inhibitors because the metal was likely incapable to interact with cell substrates at molecular level [20].

The first case of copper complexes inhibiting proteasome function has been reported by Dou and co-workers [51].

4. *In vivo* antitumor studies

Although many different *in vitro* assays, both cell-based and molecular-target driven, have been used to identify lead compounds, the most common step following *in vitro* assays is efficacy assessments in animal tumour models. Actually, there are series of reports in literature describing *in vitro* cytotoxic activity investigations as no predictive assays of *in vivo* antitumor efficacy. Animal models have several advantages over *in vitro* cell cultures as tumours develop vasculature and interact with the stroma; therefore, they allow evaluation of toxicity and provide pharmacokinetic data of the agent. The typical development plan for a cancer agent also requires studies on preclinical models in which critically important measures of antitumor effectiveness (i.e. the increase in the life time and/or tumour growth delay in tumour bearing mice) can be monitored according to the standard protocol of the experimental evaluation of antitumor drugs (National Cancer Institute or NCI, USA).

Despite huge interest in the development of copper based compounds that are poorly toxic and highly active as antitumor drugs, nowadays there is still a paucity of studies investigating the *in vivo* antitumor activity of copper(I,II) complexes. Actually, although several classes of copper(I,II) complexes have been proposed as very promising cytotoxic agents, for very few of them a remarkable *in vivo* activity has been demonstrated so far.

5. Heteroscorpionate ligands: poly(pyrazolyl) acetate and their applications

Bis(pyrazol-1-yl)carboxylic acids belong to a versatile class of N,N,O-donor ligands, which are useful in the field of enzyme modelling. They are particularly interesting, due to the k^3-N,N,O coordination behaviour, in the field of model complexes, regarding zinc and iron enzymes with a 2-histidine-1-carboxylate metal bonding topic [52-55].

Burzlaff *et al.* have identified the heteroscorpionate ligand bis(3,5-dimethylpyrazol-1-yl)acetic acid as suitable as model for the active sites of the facial 2-histidine-1-carboxylate triad in iron and zinc containing enzymes [39].

Otero and coworkers first synthesized in 1999 a bis(pyrazol-1-yl)acetate ligand; they studied the coordination ability of bis(3,5-dimethylpyrazol-1-yl)acetate ligands towards group IV and group V metals. The synthesis of the ligand was performed starting from 3,5-dimethylpyrazole to achieve (3,5-dimethylpyrazol-1-yl)methane by reaction with CH_2Cl_2. The deprotonation with Bu^nLi at the bridging CH_2 in presence of CO_2 gives the lithium bis(3,5-dimethylpyrazol-1-yl)acetate salt.

In addition to this multistep synthesis Burzlaff *et al.* have designed a synthesis starting from commercially available dibromo- or dichloro-acetic acid and 2 equivalents of 3,5-dimethylpyrazole, with an excess of potassium hydroxide and potassium carbonate, using tetra-n-butylammonium bromide as phase-transfer catalyst. After acidification and extraction with diethyl ether the synthesis affords bis(3,5-dimethylpyrazol-1-yl)acetic acid with reasonable yield. This procedure works as well using pyrazole.

Due to their characteristics and the catalytic activity shown in different reactions (like olefin polymerization, olefin oxidation, hydrogen/ deuterium exchange and metathesis reaction),

several studies have been carried out on transition metal complexes with bis(pyrazol-1-yl)acetate ligands.

6. NMDA receptors

NMDA receptors are cation channels with high calcium permeability involved in many aspects of the biology of higher organisms. The opening of the NMDA receptor associated cation channel is controlled by various ligands interacting with different binding sites at the receptor.

This last binding site is located within the cation channel and compounds interacting with the PCP site behave as non-competitive NMDA receptor antagonists by inhibiting the Ca^{2+}-ions influx through the cation channel blockade [56].

Recent findings suggest a complex relationship between NMDA and σ functions. Sigma receptors were initially classified as opioid receptor subtypes [57], and subsequently it was postulated that they were identical to the PCP binding site at the NMDA receptor channel [58]. Further studies demonstrated that they were distinct from both opioid receptors and PCP/NMDA receptor complexes.

both σ receptor subtypes are overexpressed in many human and rodent tumour cell lines [59]. Because of their widespread expression in many human tissues and their involvement in several pathophysiological processes, σ receptors have proved to be highly attractive pharmacological targets for the potential treatment of various pathologies, including neuropathic pain, depression, cocaine abuse, epilepsy, psychosis, as well as Alzheimer's and Parkinson's diseases [60]. Moreover, σ_1 antagonists and σ_2 agonists may be useful as anticancer agents and radio-labelled ligands as selective tumour imaging agents [61].

On this topic 1,4-dioxane nucleus (Fig.4.a), differently and properly substituted in 2 and 5 or 6 positions, has already proved to be a suitable scaffold for building ligands selectively targeting different receptors [62].

On the basis of these considerations the bis(pyrazolyl)acetate ligands have been functionalized through amidic bond with (±)-(6,6-Diphenyl-1,4-dioxan-2-yl)methanamine (Fig. 4.b) to give bioconjugated ligands to be coordinated to copper.

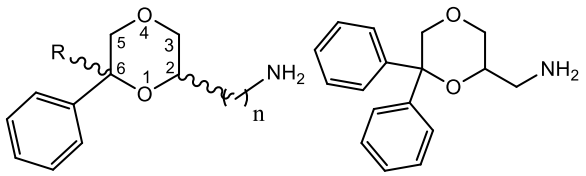

Fig. 4. Structure of: a) 1,4- dioxane scaffold; b) (±)-(6,6-Diphenyl-1,4-dioxan-2-yl)methanamine.

Experimental section

All reagents were purchased from Aldrich and used without further purification. All solvents were dried, degassed and distilled prior to use. Elemental analyses (C,H,N,S) were performed in-house with a Fisons Instruments 1108 CHNS-O Elemental Analyser. Melting points were taken on an SMP3 Stuart Scientific Instrument. IR spectra were recorded from 4000 to 100 cm^{-1} with a Perkin-Elmer SPECTRUM ONE System FT-IR instrument. IR annotations used: br = broad, m = medium, s = strong, sh = shoulder, w = weak. ^{1}H and ^{31}P NMR spectra were recorded on an Oxford-400 Varian spectrometer (400.4 MHz for ^{1}H and 162.1 MHz for ^{31}P). Chemical shifts, in ppm, for ^{1}H NMR spectra are relative to internal Me_4Si. ^{31}P NMR chemical shifts were referenced to a 85% H_3PO_4 standard. The ^{31}P NMR spectroscopic data were accumulated with ^{1}H decoupling. NMR annotations used: AB q = AB quartet, br = broad, s br = broad singlet, d = doublet, dd = doublet of doublets, m = multiplet, s = singlet, v br = very broad. Electrospray ionization mass spectra (ESIMS) were obtained in positive-(ESI(+)MS) or negative-ion (ESI(-)MS) mode on a Series 1100 MSD detector HP spectrometer, using a methanol or acetonitrile mobile phase. The compounds were added to reagent grade methanol to give solutions of approximate concentration 0.1 mM. These solutions were injected (1 μL) into the spectrometer via a HPLC HP 1090 Series II fitted with an autosampler. The pump delivered the solutions to the mass spectrometer source at a flow rate of 300 μL min^{-1}, and nitrogen was employed both as a drying and nebulising gas. Capillary voltages were typically 4000 V and 3500 V for the

positive- and negative-ion mode, respectively. Confirmation of all major species in this ESI-MS study was aided by comparison of the observed and predicted isotope distribution patterns, the latter calculated using the IsoPro 3.0 computer program.

1. Synthesis of the ligands

Synthesis of $[HC(CO_2H)(pz)_2]$ (LH, 1)

Dichloroacetic acid (4.191 g, 32.500 mmol) was dissolved in THF (100 mL), then KOH (7.294 g, 130.000 mmol) and K_2CO_3 (17.967 g, 130.000 mmol) were added and stirred for 5'. Pyrazole (pz, 4.424 g, 65.000 mmol) and tetra-n-butylammonium bromide (TBAB, 0.638 g, 1.980 mmol), serving as phase-transfer catalyst, were later added. The reaction mixture was heated under reflux for 12 h. The solvent was evaporated at reduced pressure, the residue dissolved in water (100 mL) and acidified to pH 7 with concentrated HCl. The solution was extracted with diethyl ether (2 x 100 mL). The water phase was further acidified with HCl concentrated to pH 1.5 and stirred for 2 h. A white precipitate was formed that was filtered off, washed by diethyl ether, and dried under reduced pressure to give ligand LH (1) in 48% yield.

Synthesis of $[HC(CO_2H)(pz^{Me2})_2]$ (L^2H, 2)

Dichloroacetic acid (4.191 g, 32.500 mmol) was dissolved in tetrahydrofuran (THF) (100 mL), then KOH (7.294 g, 130.000 mmol) and K_2CO_3 (17.967 g, 130.000 mmol) were added and the solution was stirred for 5'. 3,5-dimethylpyrazole (pz^{Me2}) (6.248 g, 65.000 mmol) and TBAB (0.638 g, 1.980 mmol), serving as phase-transfer catalyst, were later added. The reaction mixture was heated under reflux for 12 h. The solvent was evaporated at reduced pressure, the residue dissolved in water (100 mL) and acidified to pH 7 with concentrated HCl. The solution was extracted with diethyl ether (2 x 100 mL). The water phase was further acidified with HCl concentrated to pH 1.5 and stirred for 2 h. A white foam was formed. The residue was separated by filtration, washed by diethyl ether and dried under reduced pressure to give ligand L^2H (2) in 41% yield.

Synthesis of (±)-(6,6-Diphenyl-1,4-dioxan-2-yl)methanamine (NMDA, 3)

The syntheses of NMDA and the related bioconjugated ligands L^{NMDA} and L^{2NMDA} were performed in collaboration with Dr. Fabio Del Bello of the School of Pharmacy of the University of Camerino.

(a) 2,2-Diphenyloxirane.

Sodium hydride (60% dispersion in mineral oil, 2.195 g, 54.880 mmol) was added to a solution of benzophenone (5.000 g, 27.440 mmol) and trimethylsulfonium iodide (11.199 g, 54.880 mmol) in dimethylsulfoxide (DMSO, 30 mL) at room temperature and the reaction mixture was stirred overnight at room temperature. The mixture was poured into water and extracted with diethyl ether (Et_2O). The organic layer was concentrated under reduced pressure to give the title compound as a white solid in 89% yield. 2-(Allyloxy)-1,1-diphenylethanol.

(b) 2-(Allyloxy)-1,1-diphenylethanol.

2,2-Diphenyloxirane (a, 6.300 g, 32.104 mmol) was added dropwise to a stirred solution of freshly cut sodium (0.220 g, 9.569 mmol) in allyl alcohol (22 mL) at room temperature. After 1 h at room temperature, the reaction mixture was refluxed for 20 h. Most of the unreacted allyl alcohol was then separated by distillation at atmospheric pressure. After cooling to room temperature, 6N H_2SO_4 (0.6 mL) was added to the residual solution to neutralize the sodium alloxide, and solvent removal was continued to afford a residual oil, which was purified by column chromatography, eluting with cyclohexane/ethyl acetate (10:0.05) to give a solid in 85% yield.

(c) 2-(Oxiran-2-ylmethoxy)-1,1-diphenylethanol.

m-Chloroperbenzoic acid (50%) (11.600 g, 33.610 mmol) was added to a solution of b (3.000 g, 11.796 mmol) in CH_2Cl_2 (120 mL). After 20 h at room temperature under stirring the reaction mixture was washed with 10% Na_2SO_3, 5% Na_2CO_3, and H_2O. Removal of dried solvents afforded a solid in 90% yield. Mp. 83-84°C.

(d) (6,6-Diphenyl-1,4-dioxan-2-yl)methanol.

A solution of c (29.400 g, 108.760 mmol) and (1S)-(+)-10-camphorsulfonic acid (3.300 g, 14.206 mmol) in CH_2Cl_2 (500 mL) was refluxed for 8 h.

The reaction mixture was then washed with $NaHCO_3$ saturated solution and dried over Na_2SO_4. Removal of the solvent gave a residue, which was purified by column chromatography eluting with cyclohexane/ethyl acetate (9:1) to afford a solid in 60% yield.

(e) (6,6-Diphenyl-1,4-dioxan-2-yl)methyl 4-Methyl benzenesulfonate.

Tosyl chloride (0.400 g, 2.098 mmol) was added to a stirred solution of d (0.400 g, 1.480 mmol) in pyridine (5 mL) at 0°C for 30 min. After 3 h at 0°C, the mixture was left for 20 h at 4°C in the freezer. Then it was poured into ice and concentrated HCl (5 mL) and extracted with $CHCl_3$. The organic layers were washed with 2N HCl (15 mL), $NaHCO_3$ saturated solution (15 mL), and H_2O (15 mL) and then dried over Na_2SO_4. The evaporation of the solvent afforded e as a solid in 69% yield.

(f) 6-(Azidomethyl)-2,2-diphenyl-1,4-dioxane.

NaN_3 (0.300 g, 4.615 mmol) was added to a solution of e (0.960 g, 2.261 mmol) in dimethyl-formamide (DMF, 5 mL). The mixture was stirred at 100°C for 3 h and then poured into H_2O and extracted with Et_2O. The organic layer was washed with brine, dried over Na_2SO_4 and concentrated in vacuo to give 0.85 g of azide f, which was employed in the next step without further purification.

(NMDA, 3) (±)-(6,6-Diphenyl-1,4-dioxan-2-yl) methanamine.

A solution of f (0.250 g, 0.846 mmol) in Et_2O (10 mL) was added dropwise, at 0°C, to a suspension of $LiAlH_4$ (0.360 g, 9.486 mmol) in Et_2O (10 mL). The mixture was stirred at room temperature for 4 h. The reaction was quenched with saturated Na_2SO_4 solution and filtered. After evaporation of the filtrate, the residue was purified by column chromatography eluting with $CHCl_3$/MeOH (95:5) to obtain an oil in 79% yield.

Synthesis of L^{NMDA} (4)

Carbonyldiimidazole (CDI, 0.302 g, 1.860 mmol) was added to a solution of LH (1) (0.357 g, 1.860 mmol) in THF. The reaction mixture was stirred at reflux for 2 h. Then it was cooled to 0°C, NMDA was added (0.501 g, 1.860 mmol) and the solution was stirred at room temperature for 3 h. Then it was dried under reduced pressure, and the oil formed dissolved in $CHCl_3$ and washed with $NaHCO_3$ saturated solution and HCl 2N. The $CHCl_3$ phase was dried with Na_2SO_4, filtered and dried under reduced pressure to give a solid, which was purified by column chromatography, eluting first with cyclohexane, and then with cyclohexane/ethyl acetate (5:5), obtained in 47% yield.

Synthesis of L^{2NMDA} (5)

Carbonyldiimidazole (CDI, 0.180 g, 1.110 mmol) was added to a solution of L^2H (2) (0.275 g, 1.110 mmol) in THF. The reaction mixture was stirred for 2 h. Then it was cooled to 0°C, NMDA was added (0.299 g, 1.110 mmol) and the solution was stirred at room temperature (r.t.) for 3 h. Then it was dried under reduced pressure, and the oil formed dissolved in $CHCl_3$ and washed by $NaHCO_3$ saturated solution and HCl 2N. The $CHCl_3$ phase was dried with Na_2SO_4, filtered and dried under reduced pressure to give a solid, which was purified by column chromatography, eluting first with cyclohexane/ethyl acetate (EtOAc) (7:3), and then with cyclohexane/EtOAc (5:5), obtained in 54% yield.

2. Synthesis of the complexes

Synthesis of $[(L^{OMe})CuCl_2]$ (6)

To a methanol suspension (50 mL) of LH (1, 0.192 g, 1.000 mmol) $CuCl_2 \cdot 2H_2O$ was added (0.170 g, 1.000 mmol). The reaction mixture was stirred at room temperature for 3 h to obtain a green precipitate, which was filtered off and dried at reduced pressure to give a light green complex $[(L^{OMe})CuCl_2]$ (6) in 81% yield.

Synthesis of $[(L^{2OMe})CuCl_2]$ (7)

To a methanol solution (50 mL) of L^2H (2, 0.248 g, 1.000 mmol) $CuCl_2 \cdot 2H_2O$ was added (0.170 g, 1.000 mmol). The reaction mixture was stirred at room temperature for 3 h, filtered, and dried at reduced pressure to give the green complex $[(L^{2OMe})CuCl_2]$ (7) in 90% yield.

By dissolving the crude complex 7 in methanol solution and by slow evaporation of the solution, single blue crystals suitable for X-ray diffraction analysis were obtained.

Synthesis of $[(L^{NMDA})CuCl_2]$ (8)

To a methanol suspension (25 mL) of L^{NMDA} (4, 0.015 g, 0.338 mmol) $CuCl_2 \cdot 2H_2O$ was added (0.006

g, 0.338 mmol). The reaction mixture was stirred at room temperature for 12 h to obtain a light blue precipitate, which was filtered off and dried at reduced pressure to give the cyan complex $[(L^{NMDA})CuCl_2]$ (8) in 61% yield.

Synthesis of $[(L^{2NMDA})CuCl_2]\cdot H_2O$ (9)

To a methanol solution (25 mL) of L^{2NMDA} (5, 0.015 g, 0.300 mmol) $CuCl_2\cdot 2H_2O$ (0.005 g, 0.300 mmol) was added. The reaction mixture was stirred at room temperature for 12 h to obtain a light blue solution, which was evaporated at reduced pressure to give the brown complex $[(L^{2NMDA})CuCl_2]\cdot H_2O$ (9) in 65% yield.

Synthesis of $[(LH)Cu(PPh_3)_2]PF_6$ (10)

To an acetonitrile suspension (50 mL) of triphenylphosphine (PPh_3, 0.524 g, 2.000 mmol), $Cu(MeCN)_4PF_6$ was added (0.373 g, 1.000 mmol). The reaction mixture was stirred at room temperature for 3 h, then LH (1) was added (0.192 g, 1.000 mmol) and the suspension stirred overnight. The reaction mixture was filtered, and dried at reduced pressure; the solid was washed by Et_2O to remove the excess of PPh_3 and dried at reduced pressure to give the white complex $[(LH)Cu(PPh_3)_2]PF_6$ (14) in 40% yield

Synthesis of $[(L^2H)Cu(PPh_3)_2]PF_6$ (11)

To an acetonitrile suspension (50 mL) of PPh_3 (0.524 g, 2.000 mmol), $Cu(MeCN)_4PF_6$ was added (0.373 g, 1.000 mmol). The reaction mixture was stirred at room temperature for 3 h, then L^2H (2) was added (0.248 g, 1.000 mmol) and the suspension stirred overnight. The reaction mixture was filtered and dried at reduced pressure; the solid was washed by Et_2O to remove the excess of PPh_3 and dried at reduced pressure to give complex $[(L^2H)Cu(PPh_3)_2]PF_6$ (15) in 62% yield.

Synthesis of $[(LH)Cu(PTA)_2]PF_6$ (12)

To an acetonitrile suspension (50 mL) of PTA (0.236 g, 1.500 mmol), $Cu(MeCN)_4PF_6$ was added (0.280 g, 0.750 mmol). The reaction mixture was stirred at room temperature for 3 h, then a methanol suspension of LH (1) was added (0.144 g, 0.750 mmol) and the suspension was stirred overnight. The reaction mixture was filtered and dried at reduced pressure to give complex $[(LH)Cu(PTA)_2]PF_6\cdot MeCN$ (12) in 63% yield.

Synthesis of $[(L^2H)Cu(PTA)_2]PF_6$ (13)

To an acetonitrile suspension (50 mL) of PTA (0.236 g, 1.500 mmol), $Cu(MeCN)_4PF_6$ was added (0.280 g, 0.750 mmol). The reaction mixture was stirred at room temperature for 3 h, then a methanol suspension of L^2H (2) was added (0.186 g, 0.750 mmol) and the suspension stirred overnight. The reaction mixture was filtered and dried at reduced pressure to give complex $[(L^2H)Cu(PTA)_2]PF_6$ (13) in 50% yield.

Synthesis of $[(L^{NMDA})Cu(PTA)_2]PF_6$ (14)

To an acetonitrile suspension (50 mL) of PTA (0.094 g, 0.600 mmol) $Cu(MeCN)_4PF_6$ was added (0.112 g, 0.300 mmol). The reaction mixture was stirred at room temperature for 3 h, then a methanol suspension of L^{NMDA} (4) was added (0.133 g, 0.300 mmol) and the suspension stirred overnight. The reaction mixture was filtered, and dried at reduced pressure to give complex $[(L^{NMDA})Cu(PTA)_2]PF_6$ (14) in 62% yield.

Synthesis of $[(L^{2NMDA})Cu(PTA)_2]PF_6$ (15)

To an acetonitrile suspension (50 mL) of PTA (0.094 g, 0.600 mmol) $Cu(MeCN)_4PF_6$ was added (0.112 g, 0.300 mmol). The reaction mixture was stirred at room temperature for 3 h, then a methanol suspension of L^{2NMDA} (5) was added (0.150 g, 0.300 mmol) and the suspension stirred overnight. The reaction mixture was filtered, and dried at reduced pressure to give complex $[(L^{2NMDA})Cu(PTA)_2]PF_6$ (15) in 59% yield.

Synthesis of $[(LH)Cu(L)ClO_4]$ (16)

To a methanol suspension (25 mL) of LH (1) (0.384 g, 2.000 mmol) a water solution (25 mL) of $Cu(ClO_4)2\cdot 6H_2O$ was added (0.371g, 1.000 mmol). The suspension was stirred at room temperature for 3 h. The precipitate was filtered off and dried at reduced pressure to give the blue complex $[(LH)Cu(L)ClO_4]$ (16) in 69% yield Mp. 223-224°C. By dissolving the crude complex 16 in DMSO solution and by slow evaporation of the solution, single blue crystals suitable for X-ray diffraction analysis (Table 3.1. and Table 3.2.) were obtained. The X-ray diffraction study revealed that the

structure of the crystals corresponds to the $[(L)_2Cu]$. The same crystals were obtained by dissolving the crude complex 16 in DMSO/CH_3OH (2:1) solution and by slow evaporation of the solution.

Results and discussion

1. Ligands

Starting from dichloroacetic acid and pyrazole (pz) for LH (**1**), 3,5-dimethylpyrazole (pz^{Me2}) for L^2H (**2**), with an excess of potassium carbonate and potassium hydroxide in THF solvent, with a small quantity of TBAB as phase-transfer catalyst, I have synthesized, in a reasonable yield bis(pyrazol-1-yl)acetic-acid $[HC(CO_2H)(pz)_2]$ (**1**) and bis(3,5-dimethylpyrazol-1-yl)acetic-acid $[HC(CO_2H)(pz^{Me2})_2]$ (**2**), following the one-step synthesis design by Burzlaff *et al.* (Fig. 5.). LH is soluble in acetone, methanol, THF and DMSO, while L^2H is soluble in water, methanol, ethanol, DMSO ethyl acetate and THF. The infrared spectra carried out on solid samples of **1** and **2** showed all the expected bands for the ligands: weak absorptions in the range 2927-3177 cm^{-1} due to the pyrazole rings, broad peaks at 2454 and 2429 cm^{-1} for **1** and **2** respectively, attributable to the stretching of the OH of the carboxylic groups.

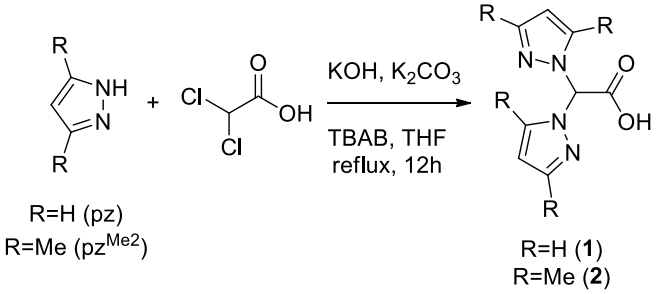

Fig. 5. Reaction scheme of the synthesis of **1** and **2**

The asymmetric stretchings of C=O of **1** and **2** are detected respectively at 1720 and 1740 cm^{-1} as strong peaks.

The ^1H NMR spectra of **1** and **2** showed a single set of resonances for pyrazole rings, indicating that the pyrazoles are equivalent. The signals at δ 7.41 and 6.91 ppm, for **1** and **2** respectively, are attributable to the bridging carbons and are diagnostic for the effective substitution of the chlorides on the acetic acid with the pyrazoles. The molecular structures of the **1** and **2** are confirmed by the ESIMS study by the presence in the negative-ions spectra of the molecular peaks of the $[L]^-$ and $[L^2]^-$ species. The synthesis of NMDA (**3**) was performed in 7 steps (Fig. 6.).

To a solution of LH (**1**) and L^2H (**2**) respectively, CDI was added to promote amide bond formation between ligands and NMDA (**3**), later added at 0°C. After separation and purification by column chromatography L^{NMDA} (**4**) and L^{2NMDA} (**5**) were formed in a reasonable yield and purity. The ligand **4** is soluble in acetonitrile, chloroform and DMSO, while **5** is soluble in methanol, acetonitrile, chloroform and DMSO. The infrared spectra carried out on solid samples of **4** and **5** showed all the expected bands for the ligands: in particular, weak absorptions due to the CH stretchings have been observed in the range 2860-3157 cm^{-1}, while peaks attributable to the amide stretchings are present at 3287 cm^{-1} and 3423 cm^{-1}, respectively for L^{NMDA} and L^{2NMDA}. The asymmetric stretchings of the C=O groups are detected as strong peaks at 1681 and 1702 cm^{-1}, respectively, in the typical range for the amide groups.

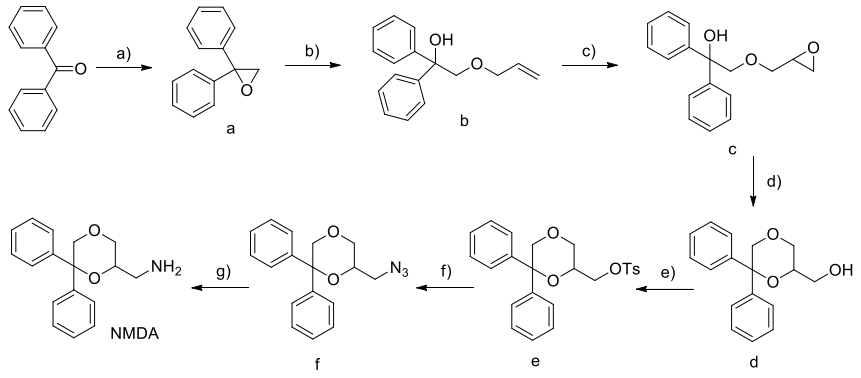

Fig. 6. Reaction scheme of the synthesis of **3**: (a) NaH, $(CH_3)_3SI$, DMSO (b) Na, CH_2=$CHCH_2OH$; (c) *m*-CPBA, CH_2Cl_2; (d) (1*S*)-(+)-CSA, CH_2Cl_2; (e) *p*-TsCl, pyridine; (f) NaN_3, DMF; (g) $LiAlH_4$, Et_2O.

The ^{1}H NMR spectra of L^{NMDA} (**4**) and L^{2NMDA} (**5**) showed all the expected signals for the bioconjugated ligands. Interestingly, a double set of resonances appears for the pyrazole rings, indicating that the pyrazoles are not equivalents.

To confirm the stoichiometry the elemental analysis gives a positive matching between the calculated and the measured values for C, H and N**.**

2. Complexes

The copper complexes $[(L^{OMe})CuCl_2]$ (**6**, light green) and $[(L^{2OMe})CuCl_2]$ (**7**, green) have been prepared from the reaction of $CuCl_2{\cdot}2H_2O$ with LH (**1**) and L^2H (**2**) respectively, in methanol solution at room temperature. The compounds are soluble in water and DMSO, and **7** is also soluble in methanol.

Interestingly, based on the spectroscopic analysis we observed the metal-catalysed esterification of the carboxylic group with methanol used as solvent in the synthesis. The infrared spectra carried out on the solid samples for **6** and **7** showed the absence of the OH stretchings of the carboxylic groups (present at 2450 and 2460 cm^{-1} in the free ligands, respectively), and the appearance of strong absorptions of C=O at 1748 and 1757 cm^{-1}, respectively, in a range typical for non-coordinating esters groups. The formation of the complexes **6** and **7** are confirmed by the presence in the positive-ions spectra of the peaks at *m/z* 304 and 360 respectively attributable to the $[(L^{OMe})CuCl]^+$ and $[(L^{2OMe})CuCl]^+$ species.

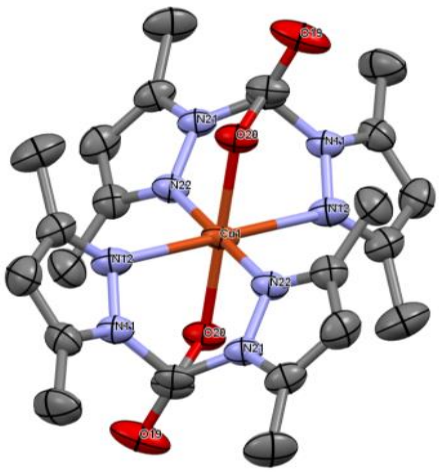

Fig. 7. Crystal structure of $[(L^2)_2Cu]$.

By dissolving the crude complex $[(L^{2OMe})CuCl_2]$ (**7**) in methanol solution and by slow evaporation of the solution, single blue crystals suitable for X-ray diffraction analysis were obtained and the analysis gives the molecular structure (Fig. 7.) in which the metal centre is hexacoordinated by two anionic ligands in the typical "scorpionate" fashion.

The copper complexes $[(L^{NMDA})CuCl_2]$ (**8**) and $[(L^{2NMDA})CuCl_2]{\cdot}H_2O$ (**9**), cyan and brown solids, have been prepared from the reaction of $CuCl_2{\cdot}2H_2O$ with L^{NMDA} (**4**) and L^{2NMDA} (**5**) respectively, in methanol solution for **8** and in methanol suspension for **9**, at room temperature. The compounds are soluble in DMSO, and **9** is also soluble in methanol, ethanol and chloroform. The infrared spectra carried out on solid samples **8** and **9** showed all the expected bands for the complexes reported. The strong absorption at 1667 and 1678 cm^{-1} for **9** and **8** respectively, without a significant variation with respect to the absorptions detectable in the free ligands, indicate that the carbonyl groups are not involved in the coordination of the metal. The copper centre results in a tetracoordinated environment with the ligand chelating in a bidentate fashion, and the other two positions occupied by the chlorides. The ESIMS study showed in the positive ion spectrum of **9** a major peak, at *m/z* 531, attributable to the $[(L^{2NMDA})_2Cu]^{++}$ species, confirming the complex formation, while in the spectrum of **8** a peak at *m/z* 984 is attributable to the $[(L^{NMDA})_2CuCl]^+$ species. The blue copper complex $[(LH)Cu(L)ClO_4]$ (**16**) has been prepared from the reaction of $CuClO_4{\cdot}6H_2O$ with LH (**1**) in a water/methanol suspension at room temperature. The compound is water soluble.

By dissolving the crude complex **16** in DMSO solution and by slow evaporation of the solution, single blue crystals suitable for X-ray diffraction analysis were obtained and the analysis results in the molecular structure (Fig. 8.) in which the metal centre is hexacoordinated by two anionic ligands in the typical "scorpionate" fashion.

The copper(I) complexes $[(LH)Cu(PPh_3)_2]PF_6$ (**10**) and $[(L^2H)Cu(PPh_3)_2]PF_6$ (**11**) have been prepared from the reaction of triphenylphosphine and $Cu(MeCN)_4PF_6$, in the presence of the ligands **1** and **2**, respectively. The mixture was filtered and dried at reduced pressure, and the residues washed by diethyl ether to obtain the complexes as white solids.

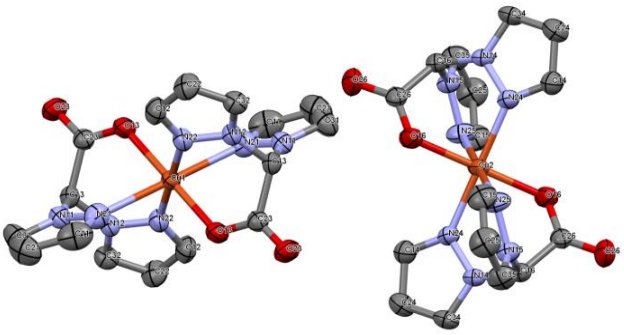

Fig. 8. Crystal structure of $[(L)_2Cu]$.

The compounds are soluble in methanol, chloroform and DMSO, and complex **11** is also soluble in acetone. The acetonitrile solution stabilizes the copper in the oxidation state +1 and with this solvent the esterification was not observed. The ^{1}H NMR spectra of **10** and **11**, recorded in DMSO and $CDCl_3$ solution, respectively, showed a single set of resonances for the pz rings, indicating that the pyrazoles are equivalent. The triphenilphoshine coligands showed a characteristic series of resonances in the aromatic region at δ 7.21-7.61 ppm, with an integration of the peaks, with respect to the ligand resonances, that confirms the 1:2 stoichiometric ratio between the ligand and the PPh_3. The infrared spectra carried out on the solid samples of **10** and **11** showed all the expected bands for the heteroscorpionate ligands and triphenilphoshine coligands. The r.t. $^{31}P\{^1H\}$ NMR spectrum of $[(LH)Cu(PPh_3)_2]PF_6$ (**10)** in DMSO solution, gave a singlet centred at δ -3.10 ppm, along with the characteristic septet centred at about δ -143.10 ppm due to the PF_6 counteranion. The $^{31}P\{^1H\}$ NMR spectrum of $[(L^2H)Cu(PPh_3)_2]PF_6$ (**11**), recorded at room temperature in $CDCl_3$ solution, gave a singlet centred at δ -2.85 ppm, along with a minor peak at δ 8.03 ppm; the characteristic septet due to the PF_6 counteranion is detectable at δ-143.31 ppm.

The water soluble phosphine PTA was used in the reaction with $Cu(MeCN)_4PF_6$ and the ligands **1** and **2** to synthesize the Cu(I) complexes $[(LH)Cu(PTA)_2]PF_6$ (**12**) and $[(L^2H)Cu(PTA)_2]PF_6$ (**13**). The white compounds as expected are water soluble, and also soluble in MeCN and DMSO. The ^{1}H NMR spectra of **12** and **13**, recorded in DMSO solution, showed a single set of resonances for the pz rings, indicating that the pyrazoles are equivalent. The PTA coligands showed a characteristic series of resonances in the region at δ 4.10-4.67 ppm, with an integration of the peaks, with respect to the ligand resonances, that confirms the 1:2 stoichiometric ratio between the ligand and the PTA. The $^{31}P\{^1H\}$ NMR spectra of $[(LH)Cu(PTA)_2]PF_6$ (**12**) and $[(L^2H)Cu(PTA)_2]PF_6$ (**13**), recorded at room temperature in D_2O solution, gave broad singlets centred at δ -85.54 ppm and at δ -85.87 ppm respectively, along with the characteristic septet centred at about δ-143.95 ppm and d -144.03 ppm due to the PF_6 counteranion, with the J(H-H) = 12.4 Hz, in accordance with the literature for analogues phosphine complexes [63]. The signals at δ -85.54 ppm and at d -85.87 ppm are downfield shifted with respect to the signal exhibited by the uncoordinated PTA ligand (δ -97.70 ppm in D_2O solution) confirming the formation and the stoichiometry of the complexes.

In analogy with the reactions for the formation of the complexes **12** and **13** two synthesis were performed with the functionalized ligands L^{NMDA} (**4**) and L^{2NMDA} (**5**) obtaining the two complexes $[(L^{NMDA})Cu(PTA)_2]PF_6$ (**14**) and $[(L^{2NMDA})Cu\ (PTA)_2]PF_6$ (**15**). The coligand PTA was used in the reaction with $Cu(MeCN)_4PF_6$ and the ligands **4** and **5** (Fig. 9.). The white compounds are soluble in MeCN and DMSO.

The infrared spectra carried out on the solid samples of **14** and **15** showed all the expected bands for the bioconjugated ligands and PTA coligands: the absorptions due to the C=O stretchings are detectable at 1696 and 1698 cm^{-1}, respectively, with no significant variations with respect to the same absorptions detectable in the spectra of the free ligands; the broad absorption at about 2925 cm^{-1} are due to the CH stretchings.

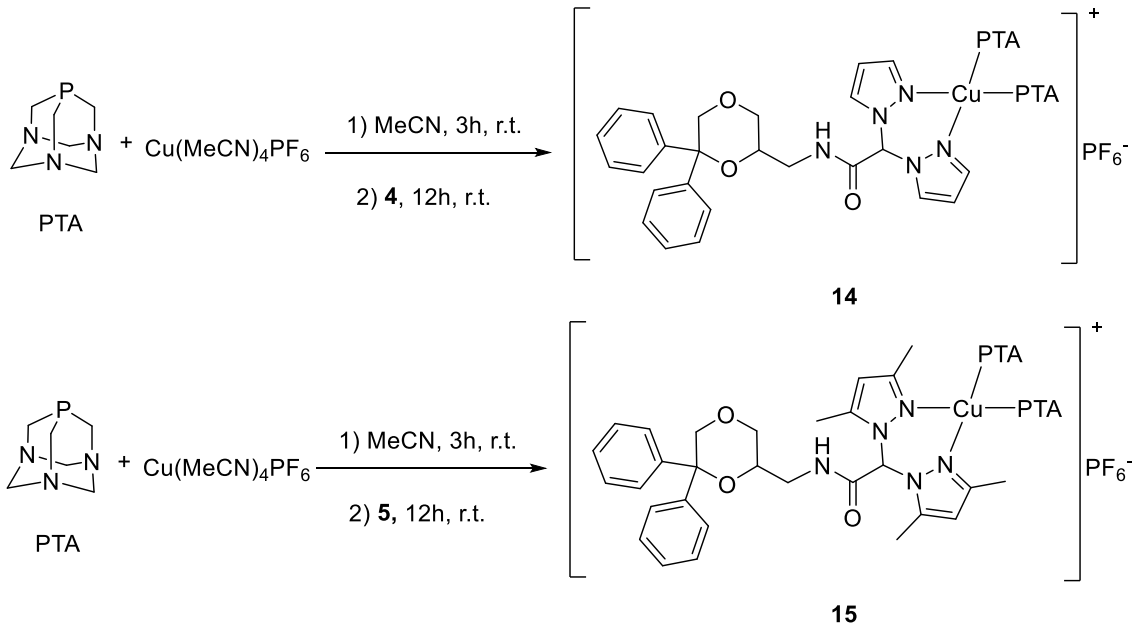

Fig. 9. Reaction scheme of the syntheses of **14** and **15**.

The ^{1}H NMR spectra of **14** and **15**, recorded in DMSO solution, showed all the signals attributable to the ligands **4** and **5** and the PTA coligands with similar patterns and small shifts observed upon complex formation.

The ^{31}P{^{1}H} NMR spectra of **14** and **15**, recorded at room temperature in CD_3CN solution, gave broad singlets centred at d -91.63 and -89.53 ppm, respectively, along with the characteristic septet centred at about d -143.52 ppm due to the PF_6 counteranion, in agreement with the coordination of two phosphines around the metal

The ESIMS study was conducted by dissolving compounds **14** and **15** in acetonitrile and recording the spectra in positive- and negative-ion mode. The formation of the complexes **14** and **15** is confirmed by the presence in the positive-ion spectra of the peaks at *m/z* 663 and 719, attributable to the $[(L^{NMDA})CuPTA]^+$ and $[(L^{2NMDA})CuPTA]^+$ species, respectively. In the negative ion spectra the $[PF_6]^-$ ion is observed as the major peak for both the complexes at *m/z* 145.

Conclusions

The side effects of platinum-based antitumor agents move the research on biologically active complexes formed by essential ions. Copper is one of these elements that possess a rich variety of coordination possibilities, two oxidation states available and different targets to avoid pharmaco-resistance.

Bis(pyrazolyl)acetate ligands were synthesized for the aim of the research, and related Cu(I) and Cu (II) complexes were characterized. To enhance the selectivity of the potential drugs and move on a targeted cancer therapy, bioconjugated ligands using the potent NMDA receptor antagonist (6,6-diphenyl-1,4-dioxan-2-yl)methanamine were design and synthesized with the corresponding copper complexes.

Biological evaluation of the most promising complexes is in progress, and the studies on this field have a great potential still to be explored.

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