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Novel binuclear μ -oxo diruthenium complexes combined with ibuprofen and ketoprofen: Interaction with relevant target biomolecules and anti-allergic potential

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ABSTRACT

This work presents the synthesis and characterization of two novel binuclear ruthenium compounds of general formula $[\text{Ru}_2\text{O}(\text{carb})_2(\text{py})_6](\text{PF}_6)_2$, where py = pyridine and carb are the non-steroidal anti-inflammatory drugs ibuprofen (**1**) and ketoprofen (**2**). Both complexes were characterized by ESI-MS/MS spectrometry. The fragmentation patterns, which confirm the proposed structures, are presented. Besides that, compounds **1** and **2** present the charge transfer transitions within 325–330 nm; and the intra-core transitions around 585 nm, which is the typical spectra profile for $[\text{Ru}_2\text{O}]$ analogues. This suggests the carboxylate bridge has little influence in their electronic structure. The effects of the diruthenium complexes on IgE mediated mast cell activation were evaluated by measuring the enzyme β -hexosaminidase released by mast cells stimulated by antigen. The inhibitory potential of the ketoprofen complex against mast cell stimulation suggests its promising application as a therapeutic agent for treating or preventing IgE-mediated allergic diseases. In addition, *in vitro* metabolism assays had shown that the ibuprofen complex is metabolized by the cytochrome P450 enzymes.

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1. Introduction

Ibuprofen (2-(4-isobutylphenyl)-propionic acid) and ketoprofen (2-(3-benzoylphenyl)-propionic acid) are nonsteroidal anti-inflammatory drugs (NSAIDs), widely used as antipyretic, anti-inflammatory and analgesic agents. In addition, these drugs have provided therapeutic benefits in the prevention of various types of cancer [1–6]. These NSAIDs are also used for treatment of rheumatoid arthritis and osteoarthritis. However, one of the most important side-effects associated with NSAID therapies is gastrointestinal disturbance [1,6–8].

Coordination compounds are used for the treatment of several diseases [9–13] and, particularly, those ones combined with anti-inflammatory drugs have attracted the interest of many researchers [5,6,14,15]. Ruthenium-based drugs show potential biological activity and, due to their reactivity in physiological conditions, they have been used in clinical application against metastatic cells resulting in low systemic toxicity [11]. Moreover, these complexes are versatile regarding

their spectroscopic and electrochemical properties and reactivity toward different classes of ligands [16]. According to literature [6,14], the coordination of NSAID to diruthenium complexes of general formula $[\text{Ru}_2(\text{carb})_4(\text{L})_2]^n$ retained the anti-inflammatory property and decreased ulcerations of the gastrointestinal tract. In this context, the interest on binuclear ruthenium compounds has grown, especially after the discovery that the analogue structure with a μ -oxo and two μ -carboxylate bridges of formula $[\text{M}_2(\mu\text{-O})(\mu\text{-RCOO})]^n$ ($n = 1$ or 2 , $\text{M} = \text{Fe}$, Mn) occur in metalloenzymes [17].

Some of these diruthenium complexes, with different terminal ligands, have been characterized with oxidation numbers $\text{Ru}_2(\text{II,III})$ as well as the mixed-valence forms $\text{Ru}_2(\text{II,III})$ and $\text{Ru}_2(\text{III,IV})$, synthesized by Abe et al. [18]. Such complexes have the general formula $[\text{Ru}_2(\mu\text{-O})(\mu\text{-RCOO})]$, and the coordinated carboxylate groups have various origins, such as CH_3COOH , $\text{CH}_2(\text{CH}_3)\text{COOH}$, PhCOOH , CH_2ClCOOH , CCl_3COOH and CF_3COOH [18–21].

Despite the promising pharmacological potential of these diruthenium complexes, there are few assays regarding the interaction of such complexes with biological relevant targets. One particular and important feature presented in this work is the ability of these new candidates to metallo-drugs to inhibit mast cell degranulation responsible for allergic diseases, which are a major global public health concern [22]. The activation of IgE receptors, $\text{Fc}\epsilon\text{RI}$, on mast cell membrane, is the key event for the initiation and propagation

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of pathophysiological responses involved in allergic conditions [23, 24]. The interaction between antigen and the immune-complex FcεRI-IgE, on mast cell membrane, triggers a signaling of intracellular events leading to degranulation and consequent release of pre-formed allergic mediators in addition to the synthesis and secretion of lipid mediators and cytokines [25]. Thereby, the inhibition of mast cell response, when stimulated by an antigen (allergen), represents an important route for the development of new antiallergic drugs. The current use of anti-inflammatory drugs to treat some allergic disorders [26] has led us to study the potentiality of diruthenium complexes combined with anti-inflammatory drugs to inhibit mast cell activation. This preliminary investigation could, consequently, result in new candidates for anti-allergic drugs.

Other important biological targets are the cytochrome P450 (CYP450) enzymes from rat liver microsomes (RLM), since these enzymes are involved in the biotransformation of compounds of various endogenous and exogenous sources, including drugs. In this context, microsomes are the most popular model to perform *in vitro* metabolism studies and to evaluate the interaction of candidate drugs with the CYP enzymes. This model allows the extrapolation of the results from animals to humans with a certain level of assurance [27].

Considering the relevance of the synthesis of new metallo-drugs and their biological applications, this work presents the synthesis of two novel μ -oxo diruthenium analogues, $[\text{Ru}_2\text{O}(\text{ibu})_2(\text{py})_6](\text{PF}_6)_2$ (**1**) and $[\text{Ru}_2\text{O}(\text{keto})_2(\text{py})_6](\text{PF}_6)_2$ (**2**) (where ibu and keto are the NSAIDs ibuprofen and ketoprofen, respectively) (Fig. 1). The inedited compounds had their structure characterized by means of mass spectrometry and their spectroscopic profile is described by UV–visible experiments. To evaluate the antiallergic potential of compounds **1** and **2** we quantified the release of β -hexosaminidase, accepted as a biological marker for mast cell degranulation [28]. This enzyme is stored in secretory granules into the cytoplasm of the cell and is released when mast cells are immunologically triggered to degranulate. The interaction of compound **1** with the cytochrome P450 (CYP450) enzymes was also accomplished by carrying out an *in vitro* metabolism assay using rat liver microsomes.

2. Experimental

2.1. Synthesis

All reactants for synthetic procedures were purchased from Sigma-Aldrich and used without purifications. The synthesis of compounds $[\text{Ru}_2\text{O}(\text{ibu})_2(\text{py})_6](\text{PF}_6)_2$ and $[\text{Ru}_2\text{O}(\text{keto})_2(\text{py})_6](\text{PF}_6)_2$ followed literature procedures, with slight modifications [20].

2.1.1. $[\text{Ru}_2\text{O}(\text{ibu})_2(\text{py})_6](\text{PF}_6)_2$

1.136 g of ibuprofen (5.501 mmol) was added to a mixture of 135 mL of water and 45 mL of ethanol containing 1.136 g of RuCl_3 (5.477 mmol).

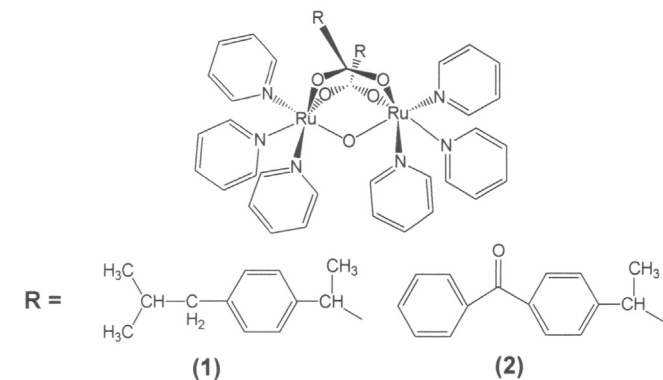


Fig. 1. Pictorial view of the proposed structure for compounds $[\text{Ru}_2\text{O}(\text{ibu})_2(\text{py})_6](\text{PF}_6)_2$ (**1**) and $[\text{Ru}_2\text{O}(\text{keto})_2(\text{py})_6](\text{PF}_6)_2$ (**2**) (ibu and keto are ibuprofen and ketoprofen, respectively).

This mixture was allowed to warm to 70 °C for 10 min, with color change from brown to reddish brown. After heating, 7 mL of pyridine (87 mmol) was added to this solution and then, it was allowed to react under reflux for 1 h, leading to a green solution. After the reaction medium reached room temperature, 4.540 g of NH_4PF_6 (27 mmol) was added. The reaction medium was allowed to stand for 2 days at room temperature. The solid isolated by filtration was purified in a neutral alumina adsorption column and the fraction of interest (purified product) was eluted with mobile phase 8 dichloromethane/2 acetonitrile (v/v). $\eta = 20.6\%$. Elemental Analysis, Exp. (calc): %C 47.91 (48.2); %H 4.61 (4.6); %N 5.98 (6.0). $\nu_{\text{asCOO}^-} = 1448 \text{ cm}^{-1}$ and $\nu_{\text{sCOO}^-} = 1403 \text{ cm}^{-1}$; $\Delta\nu = 43 \text{ cm}^{-1}$ (bridging coordination). ^1H NMR: 0.81 ppm (m, 6H); 0.87 ppm (d, 3H); 1.74 ppm (m, 1H); 2.36 ppm (d, 2H); 3.17 ppm (m, 1H); 6.45 ppm (d, 2H); 6.82 ppm (d, 2H); 7.2–7.0 ppm (m, 4H); 7.65–7.38 ppm (m, 6H); 8.2 ppm (m, 2H); 8.38 ppm (t, 1H); 9.5 ppm (t, 2H).

2.1.2. $[\text{Ru}_2\text{O}(\text{keto})_2(\text{py})_6](\text{PF}_6)_2$

1.065 g of ketoprofen (4.188 mmol) was added to a mixture of 105 mL of water and 35 mL of ethanol containing 0.8784 g of RuCl_3 (4.234 mmol). This mixture was allowed to warm to 70 °C for 30 min, with color change from brown to reddish brown. After heating, 7 mL of pyridine (87 mmol) was added to this solution and then, it was allowed to react under reflux for 1 h, leading to a green solution. After the reaction medium reached room temperature, 3.490 g of NH_4PF_6 (21 mmol) was added. The reaction medium was allowed to stand for 2 days at room temperature. The solid isolated by filtration was purified in a neutral alumina adsorption column and the fraction of interest (purified product) was eluted with mobile phase 8 dichloromethane/2 acetonitrile (v/v). $\eta = 12\%$. Elemental Analysis, Exp. (calc): %C 49.75 (50.0); %H 3.78 (3.8); %N 5.56 (5.6). $\nu_{\text{asCOO}^-} = 1449 \text{ cm}^{-1}$ and $\nu_{\text{sCOO}^-} = 1404 \text{ cm}^{-1}$; $\Delta\nu = 45 \text{ cm}^{-1}$ (bridging coordination). ^1H NMR: 0.91 ppm (m, 3H); 3.34 ppm (m, 1H); 6.84 ppm (d, 1H); 6.99 ppm (d, 1H); 7.22–7.05 ppm (m, 4H); 7.74–7.38 ppm (m, 13H); 8.38–8.07 ppm (m, 4H); 9.1 ppm (t, 1H).

Caution: due to the *trans*-effect exerted by the μ -oxo bridge, both compounds might present reactivity in coordinating solvents if in solution for long periods.

3. Measurements

3.1. Characterization

Mass spectra (ESI/MS and ESI-MS/MS) were obtained in an electron spray Mass Spectrometer Q-TOF II-Micromass UK, under the following experimental conditions: infusion pump 250 $\mu\text{L/h}$; sample concentration: 200 $\mu\text{g/mL}$ (dissolved in methanol); cone voltage: 30 V; mass range: 150–1200. All the analyses were performed in positive detection mode. Electronic spectra were recorded on a UV–visible-NIR spectrophotometer – Model U-3501 HITACHI, ranging from 200 nm to 800 nm, using a quartz cuvette of 1 cm optical path and acetonitrile solutions. Infrared spectra were obtained from samples dispersed in KBr pellets in the region 400–4000 cm^{-1} with resolution of 4 cm^{-1} in an IR spectrophotometer Shimadzu Prestige 21. ^1H NMR spectra were recorded on a Bruker DRX500, 500 MHz spectrometer, from acetonitrile $1 \times 10^{-2} \text{ mol} \times \text{L}^{-1}$ solutions.

3.2. Interaction with CYP enzymes present in rat liver microsomes

3.2.1. Reagents

Due to the known reactivity of $[\text{Ru}_2\text{O}(\text{carb})_2(\text{py})_6](\text{PF}_6)_2$ complexes in solution [29], a standard stock solution of the $[\text{Ru}_2\text{O}(\text{carb})_2(\text{py})_6](\text{PF}_6)_2$ complexes under investigation was not prepared. Daily, a Ru-complex solution at a concentration of 1 mg/mL in acetonitrile was prepared. Sodium chloride and sodium dihydrogen phosphate were obtained from Merck. Sodium hydroxide and potassium chloride were obtained from Nuclear.

Glycerol and Tris (hydroxymethyl)aminomethane were obtained from J.T. Baker, ethylenediaminetetraacetic acid (EDTA) from Carlo Erba. NADP^+ , glucose-6-phosphate and glucose-6-phosphate dehydrogenase were obtained from Sigma-Aldrich.

3.2.2. HPLC conditions

To perform the *in vitro* metabolism study, the authors used a High Performance Liquid Chromatography from Shimadzu (Kyoto, Japan) comprising a LC-20AT solvent pump unit, a DGU-20A₃ online degasser, a CBM-20A system controller and a SPD-M20A diode array detector operating from 190 to 800 nm. Injections were performed manually through a 20 μL loop employing a Rheodyne injector (Model 7725i). Data were collected using a LC solution software 1.25 also from Shimadzu. The analyses of the $[\text{Ru}_2\text{O}(\text{ibu})_2(\text{py})_6](\text{PF}_6)_2$ were performed on a silica analytical column acquired from Shimadzu (Shim-pack® CLC-SIL, 250 mm x 4.0 mm, 5 μm particle size). The mobile phase used was a mixture of acetonitrile:methanol (9.5:0.5, v/v) at a flow rate of 0.5 mL min⁻¹.

3.2.3. Animals and microsomal preparation

Male Wistar rats weighting 180–220 g were obtained from the College of Pharmaceutical Sciences of Ribeirão Preto – University of São Paulo (Ethical Approval no 11.1.1047.53.4). Animals were fed in a normal condition and acclimatized at 12 h light/dark cycle. The animals were sacrificed by decapitation and the livers were removed and placed in ice-cold 0.05 mol/L Tris–HCl buffer (pH 7.4), containing 0.15 mol/L KCl. The livers were minced with scissors and washed three times with Tris–HCl buffer. After that, the slices were homogenized with a MA 181 potter equipment (Marconi, Brazil). The homogenate was centrifuged in a HIMAC CF 15D2 centrifuge (Hitachi, Japan) at 10,000 $\times g$ for 15 min at 4 °C and the resulting supernatant was ultracentrifuged employing an XL-70 Beckman ultracentrifuge (Beckman, USA) at 100,000 $\times g$ for 60 min at 4 °C in order to obtain the microsomal pellet. The obtained pellet was resuspended in HEPES–HCl buffer (pH 7.4; 0.05 mol/L), containing 20% glycerol and 0.001 mol/L EDTA and stored at –170 °C until use. The protein concentration was determined by the biuret method using a BCA Kit (Labtest, Brazil).

3.2.4. Microsomal incubation conditions

Incubations ($n = 3$) were performed with the reconstituted rat liver microsomes in 10 mL amber tubes, using a shaking water bath at 37 °C. The incubation mixture consisted of a cofactor solution, microsomal preparation (rat liver microsomes), phosphate buffer 0.250 mol/L pH 7.4 and the Ru-complex (25 μL , 50 $\mu\text{g}/\text{mL}$) in a total volume of 1.0 mL. The cofactor solution consisted of NADP^+ (0.25 mM), glucose-6-phosphate (5 mM) and glucose-6-phosphate dehydrogenase (0.5 units) in Tris–HCl buffer (Tris–HCl 0.05 mol/L–KCl 0.15 mol/L, pH 7.4). After 5 min pre-warmed at 37 °C, the metabolic reaction was initiated by the addition of the rat microsomal preparation and it was carried out for 90 min. After this period, the reaction was terminated by the addition of cold acetonitrile (500 μL). This mixture was agitated for 30 s in a mixer and then centrifuged for 8 min at 2860 $\times g$ (Hitachi CF16RXII, Himac, Japan). The supernatant was collected (100 μL), transferred to another tube and evaporated using a gentle flow of compressed air. The residue was solubilized in 100 μL of mobile phase and injected into the chromatography system. Control incubations ($n = 3$) were performed in the absence of cofactor solution and in the absence of microsomal preparation. The difference between ‘with’ and ‘without’ NADP^+ was considered as CYP450-mediated metabolism.

3.3. Antiallergic potential

3.3.1. Mast cell culture

RBL-2H3 (Rat Basophilic Leukemia) mast cells [30] were maintained in monolayer culture in minimum essential medium with L-glutamine and Eagle's salt, supplemented with 20% fetal bovine serum (Atlanta

Biological, Atlanta, USA) and 50 $\mu\text{g}/\text{mL}$ gentamicin sulfate (Invitrogen Corp., Carlsbad, USA). The cells were used from 3 to 5 days after passage, and released from culture flasks by treatment with 0.5% trypsin–EDTA for 5 min at 37 °C, centrifuged at 1000 $\times g$ for 5 min, (centrifuge Sorvall Legend, model Mach 1.6 R. Thermo Fisher Scientific, Waltham, USA) and resuspended at 1×10^6 cell/mL. Reagents used were from Gibco (Carlsbad, USA), except when indicated.

3.3.2. β -Hexosaminidase release assay

IgE-mediated degranulation of mast cells was performed as described by Naal and others [30]. RBL-2H3 cells were initially sensitized with a fivefold molar excess of anti-DNP IgE (purified as previously described [31]), over Fc ϵ RI and plated onto 96-well plate at 5×10^5 cell/mL for 24 h, at 37 °C and 5% CO_2 atmosphere. Next, cells were washed twice with 200 μL Tyrode's buffer (135 mM NaCl, 5 mM KCl, 1.8 mM CaCl_2 , 1 mM MgCl_2 , 5.6 mM glucose, 20 mM HEPES, and 1 mg/mL bovine serum albumin (BSA) at pH 7.4) and pretreated, for 20 min, with different concentrations of diruthenium complexes (1–200 μM , from a DMSO stock solution), diluted in Tyrode's buffer. Cell degranulation was then stimulated by addition of 100 μL of 0.1 $\mu\text{g}/\text{mL}$ DNP–BSA (BSA conjugate with an average of 15 DNP groups), prepared as previously described [32], diluted in Tyrode's buffer. Samples were then incubated for 1 h at 37 °C, after which cells were placed on ice to stop degranulation. Cell degranulation was measured by determining the amount of released β -hexosaminidase. In this way, 25 μL of cell supernatant and 100 μL of 1.2 mM β -hexosaminidase substrate (4-methylumbelliferyl-N-acetyl- β -D-glucosaminide, MUG, Sigma-Aldrich), in 0.05 M sodium acetate buffer (pH 4.4), were mixed in separated 96-well plate and incubated for 30 min, at 37 °C. The enzyme–substrate reaction was stopped by addition of 175 μL of 0.1 M carbonate buffer, pH 10. Controls without antigen were used to measure spontaneous release. Total β -hexosaminidase release was obtained by lysing cells with 0.1% Triton-X 100 before removing the supernatant. Released β -hexosaminidase was quantified by measuring the fluorescence intensity of the product of β -hexosaminidase-mediated cleavage of MUG, methylumbelliferone, in a microplate reader (BioTEK, Winooski, USA) using 360 nm excitation and 450 nm emission filters. The percentage of stimulated β -hexosaminidase-release was calculated according to Eq. (1). Values of IC_{50} (concentration of diruthenium complexes necessary to inhibit the degranulation of β -hexosaminidase from cells stimulated with antigen by 50%) were determined graphically. The inhibitory effect of diruthenium complexes on β -hexosaminidase release was compared to ketotifen fumarate. Unless otherwise specified, reagents were from Mallinckrodt Baker Inc., Phillipsburg, USA.

$$\% \beta\text{-Hexosaminidase} = \left(\frac{S-N}{T-N} \right) \times 100$$

N: Normal, fluorescence from vehicle (Tyrode's buffer); S: Sample, fluorescence from (+) DNP–BSA, (+) or (–) Ru-complex samples; T: Total, fluorescence from Triton X-100-lysed cell samples.

3.3.3. β -Hexosaminidase activity assay

RBL-2H3 cell suspension, at 5×10^5 cell/mL, was sonicated during 20 min for cell lysis and release of β -hexosaminidase enzyme. After centrifugation at 1000 $\times g$ for 5 min, 45 μL of supernatant was incubated with 5 μL of diruthenium complex sample solutions (1–300 μM) and 50 μL of β -hexosaminidase substrate (MUG) solution (final concentration 1.2 mM), for 60 min. The enzyme–substrate reaction was stopped by addition of 200 μL of stop solution (0.1 M carbonate buffer, pH 10.0). β -Hexosaminidase activity was determined from fluorescence measurements of hydrolyzed MUG, as described above. Samples not treated with metallo-complexes were taken as 100% of β -hexosaminidase activity.

3.3.4. Cell viability assay

RBL-2H3 cells were seeded onto 96-well microplates at 1.5×10^5 cell/mL and incubated overnight at 37 °C. After the washing step with PBS buffer, cells were treated with different concentrations of the diruthenium complexes (1–200 μ M), diluted in culture medium, following an incubation step for 2 h at 37 °C. Next, samples were treated with 5 μ L of a 10 mg/mL resazurin stock solution (Sigma-Aldrich) for 4 incubation periods. Cell viability was accessed by the fluorescence of resorufin, a fluorescent product resulting from reduction of resazurin by viable cells (excitation in 530 nm and emission in 590 nm). Fluorescence achieved from non-treated control samples was considered as 100% of cell viability.

3.3.5. Statistical analysis

Results were expressed as mean $\pm$ SD. Statistical significance was assessed by one-way ANOVA followed by Dunnett's *posthoc* pairwise comparisons. Differences were considered statistically significant at * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$

4. Results and discussion

4.1. Structural characterization — ESI mass spectrometry

The structure of binuclear ruthenium complexes of general formula $[\text{Ru}_2\text{O}(\text{RCOO})_2(\text{L})_6](\text{PF}_6)_2$ typically has been characterized by means of X-ray and NMR measurements [33–35]. Although mass spectrometry has been used to study these complexes, to our knowledge none of the previous works have explored the use of fragmentation patterns obtained from ESI-MS/MS measurements (electron spray ionization mass spectrometry) to this end. Therefore, in the absence of suitable crystals to perform X-ray analysis and also considering the originality of the ESI-MS use for the structural characterization of $[\text{Ru}_2\text{O}]$ complexes, we report the data below.

Fig. 2 displays the correspondence between the isotopic pattern observed for the molecular-ion of both $[\text{Ru}_2\text{O}(\text{ibu})_2(\text{py})_6](\text{PF}_6)_2$ and $[\text{Ru}_2\text{O}(\text{keto})_2(\text{py})_6](\text{PF}_6)_2$ complexes and the theoretical prediction.

Table 1 depicts the fragments observed in the ESI MS/MS spectra (collision induced dissociation, CID) of the two complexes.

As one can see in Fig. 2, the molecular ions $[\mathbf{1} - \text{PF}_6]^+$ and $[\mathbf{2} - \text{PF}_6]^+$ are detected as a characteristic cluster of isotopologue ions, with the most abundant being that of m/z 1249 and m/z 1345, respectively. The isotopic multiplicity is mostly characteristic of the presence of two Ru atoms, which is a multiple isotope element (^{104}Ru (18.7%), ^{102}Ru (31.6%), ^{101}Ru (17.0%), ^{100}Ru (12.6%), ^{99}Ru (12.7%), ^{98}Ru (1.88%) and ^{96}Ru (5.52%)). Both gaseous ions were then selected and dissociated via 20 eV collisions with argon (spectra available as SI). Until a certain point, compounds **1** and **2** show a gas-phase behavior nearly identical, therefore in this first moment, it will be commented only on the ESI-MS/MS results for **1**, keeping in mind that the discussion also holds for complex **2**.

Collision induced dissociation (CID) is observed to some extent for compound **1**, affording mainly +1 fragments originated from successive pyridine losses and formation of gaseous phase adducts with one methanol molecule. Thus, the observed fragment ion at m/z 1044 is ascribed to the loss of three pyridines and the association of one methanol molecule, from the molecular ion at m/z 1249. The gaseous ion at m/z 1044 further fragments with two successive losses of one pyridine molecule, yielding the fragments at m/z 965 and m/z 886, respectively (Table 1). Interestingly, in this fragmentation path, loss of methanol at the expense of pyridine molecules was not observed, suggesting the importance of the adduct formation with solvent in stabilizing the gaseous ions.

Finally, a fragment ion is observed at m/z 727, which was assigned to the loss of four pyridines and one ibuprofen from the molecular ion $[\mathbf{1} - \text{PF}_6]^+$, with charge maintenance. This ion is less abundant than the others that originated in the successive pyridine losses suggesting, as expected, that pyridine is more loosely bound to the $[\text{Ru}_2\text{O}]$ unit than the bridging ibuprofen. Therefore, the fragmentation path centered on carboxylate losses is less prone than the one centered on pyridine losses.

Overall, compound **2** presents more extensive fragmentations than **1**, following two distinct pathways. The same fragmentation path of

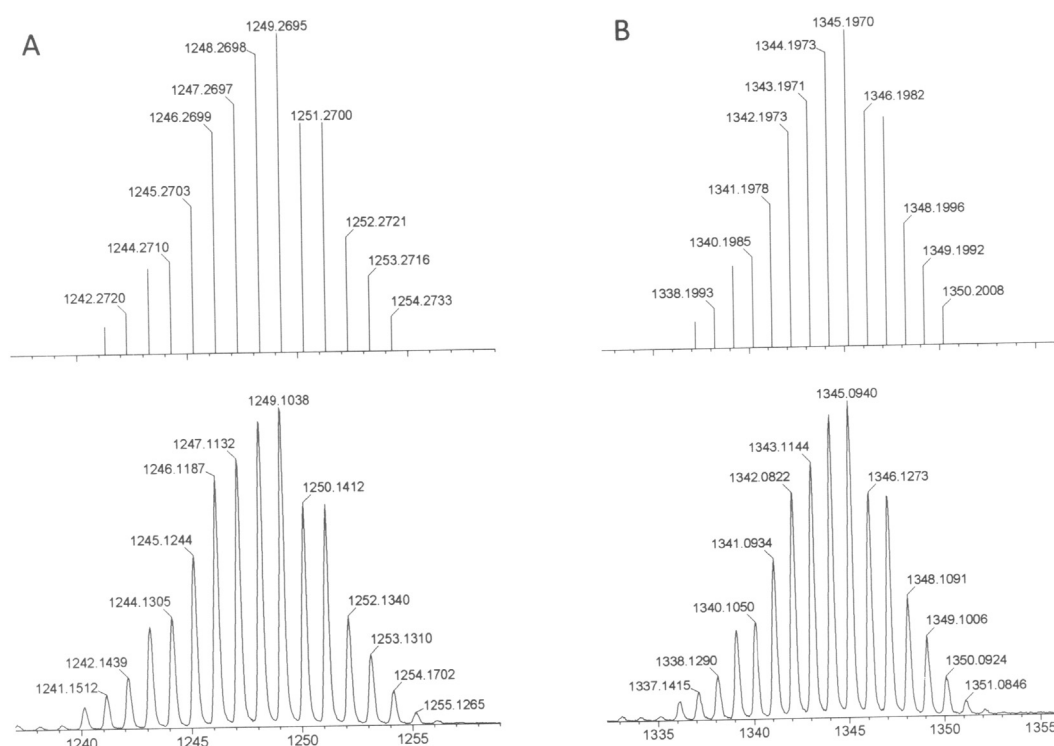


Fig. 2. ESI MS of (A) $[\text{Ru}_2\text{O}(\text{ibu})_2(\text{py})_6](\text{PF}_6)_2$ and (B) $[\text{Ru}_2\text{O}(\text{keto})_2(\text{py})_6](\text{PF}_6)_2$ recorded from methanolic solutions (bottom) and the theoretical prediction for the isotopic pattern (top).

Table 1Gaseous phase fragments observed for compounds **1** and **2** in the ESI MS/MS spectra.

[Ru ₂ O(ibu) ₂ (py) ₆](PF ₆) ₂ (1)		[Ru ₂ O(keto) ₂ (py) ₆](PF ₆) ₂ (2)	
[1 – PF ₆] ⁺	<i>m/z</i> 1249	[2 – PF ₆] ⁺	<i>m/z</i> 1345
[1 – PF ₆ – 3py + CH ₃ OH] ⁺	<i>m/z</i> 1044	[2 – PF ₆ – 3py + CH ₃ OH] ⁺	<i>m/z</i> 1140
[1 – PF ₆ – 4py + CH ₃ OH] ⁺	<i>m/z</i> 965	[2 – PF ₆ – 4py + CH ₃ OH] ⁺	<i>m/z</i> 1061
[1 – PF ₆ – 5py + CH ₃ OH] ⁺	<i>m/z</i> 886	[2 – PF ₆ – 5py + CH ₃ OH] ⁺	<i>m/z</i> 981
		[2 – PF ₆ – 6py + CH ₃ OH] ⁺	<i>m/z</i> 902
[1 – PF ₆ – 4py – 1 ibu] ⁺	<i>m/z</i> 727		
		[2 – PF ₆ – 1keto – (C ₁₅ H ₁₃ O)] ⁺	<i>m/z</i> 883
		[2 – PF ₆ – 1py – 1keto – (C ₁₅ H ₁₃ O)] ⁺	<i>m/z</i> 804
		[2 – PF ₆ – 2py – 1keto – (C ₁₅ H ₁₃ O)] ⁺	<i>m/z</i> 725

pyridine losses and methanol adduct formation is observed. However, **2** displays one last fragmentation process, losing the sixth pyridine ligand originating the singly charged ion at *m/z* 902.

Although complex **2** also loses one carboxylate molecule, apparently it follows a different path from that observed for **1**, since it initially does it without loss of pyridine and it displays a cleavage on the remaining ketoprofen ligand, losing the fragment C₁₅H₁₃O, generating the ion at *m/z* 883. This ion further fragments, performing two successive losses of one pyridine molecule, yielding the fragments at *m/z* 804 and *m/z* 725, respectively (Table 1).

The fragmentation schemes as well as the ESI-MS/MS spectra of compounds **1** and **2** are available as the Supporting information.

4.2. Spectroscopy

Typically, the electronic spectra of the [Ru₂O] complexes display strong bands in the range between 300 nm–400 nm and 550 nm–600 nm, assigned to metal-to-ligand charge transfer and intra-core transitions, respectively. This last one fundamentally has metallic character and had been rationalized in terms of a set of metal- $d\pi$ - π - π interactions [21,25]. The characteristic bands of this class of complexes can be seen in Fig. 3 for compounds [Ru₂O(ibu)₂(py)₆](PF₆)₂ and [Ru₂O(keto)₂(py)₆](PF₆)₂.

Both compounds present the charge transfer transitions at 326 nm (14,500 mol × L^{−1} × cm^{−1}) and at 328 nm (14,985 mol × L^{−1} × cm^{−1}); and the intra-core transitions at 586 nm (7275 mol × L^{−1} × cm^{−1} and 5960 mol × L^{−1} × cm^{−1}) for [Ru₂O(ibu)₂(py)₆](PF₆)₂ and [Ru₂O(keto)₂(py)₆](PF₆)₂, respectively. Inspection of Fig. 3 shows that the typical spectra profile of this class of compounds is maintained. These observations confirm that the compounds investigated in this work actually present the [Ru₂O] core

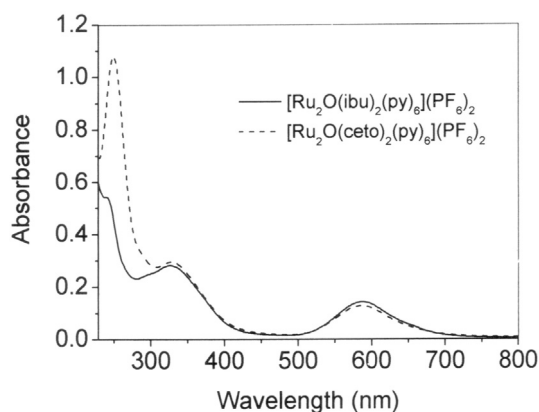


Fig. 3. Electronic spectra of compounds [Ru₂O(ibu)₂(py)₆](PF₆)₂ and [Ru₂O(keto)₂(py)₆](PF₆)₂ obtained in acetonitrile solution (concentration 2 × 10^{−5} mol/L).

structure and also suggest that the carboxylate bridge has very little influence in their electronic structure.

4.3. Anti-allergic potential

Degranulation of mast cells stimulated by allergens is considered a key event in allergic diseases [23,24]. Consequently, one of the strategies to find new anti-allergic candidates is to monitor the capacity of new molecules to inhibit mast cell activation and degranulation. In this perspective, rat basophilic leukemia (RBL-2H3) cells are largely used as a convenient model to study mast cell degranulation [28, 36–38] since these cells are mucosal mast cells with similar functions to primary mast cells and normal basophils [39]. Additionally, these cells are robust and grow up very well in different surfaces.

Pretreatment of IgE-sensitized RBL cells with [Ru₂O(keto)₂(py)₆](PF₆)₂ (compound **2**), followed by cell stimulation with allergen (DNP-BSA), showed a strong concentration-dependent inhibition of β -hexosaminidase release with a IC₅₀ equal to 18 ± 1 μ M (Fig. 4A). It is important to emphasize that the inhibitory activity of compound **2** was considerably more potent than that exhibited by ketotifen fumarate (IC₅₀ = 154.7 μ M), determined from previous work [37]. Ketotifen is a mast cell stabilizer commonly used as a positive control in mast cell degranulation assays [40,41]. Interestingly, contrary to that observed for the Ru-ketoprofen complex, cell treatment with ketoprofen alone did not inhibit degranulation of mast cells stimulated by the antigen as expected (Fig. 4A).

As previously mentioned, antigen-mast cell activation triggers a signaling cascade of chemical reactions that leads to the secretion of chemical mediators responsible for the allergic inflammation symptoms. These chemical mediators are divided into three classes of bioactive molecules: lipid mediators (prostaglandins, leukotrienes), cytokines (IL-4, TNF- α) and cytoplasmic granule contents (histamine, β -hexosaminidase, serotonin) [42]. In general, the non-steroidal anti-inflammatory drugs (NSAIDs) such as ketoprofen and ibuprofen, inhibit the two isoforms of the cyclooxygenase, named as COX-1 and COX-2, that catalyze the early stage in the generation of prostaglandin, molecules involved in allergic inflammation [43]. COX-1 is constitutively expressed in many types of cells while COX-2 is induced mainly in inflammatory process [44,45]. The production of prostaglandins in antigen-activated mast cells, including RBL, is catalyzed by COX-2 via arachidonic acid cascade [43]. Therefore, we expected that ketoprofen and ibuprofen alone could also have a positive effect in inhibiting the release of β -hexosaminidase through another pathway of the cell signaling cascade, which did not occur (see Fig. 4A and B for ketoprofen and ibuprofen, respectively).

Interestingly, the coordination of the ketoprofen with the μ -oxo-diruthenium core led to a new molecule able to access the mast cells and inhibiting β -hexosaminidase release. To certify if the inhibitory activity of compound **2** was mainly ascribed to its ability to decrease the mast cell degranulation, it was investigated whether its action was due inhibition of the enzyme activity or the inhibition of the β -hexosaminidase release, the latter being the expected action for a

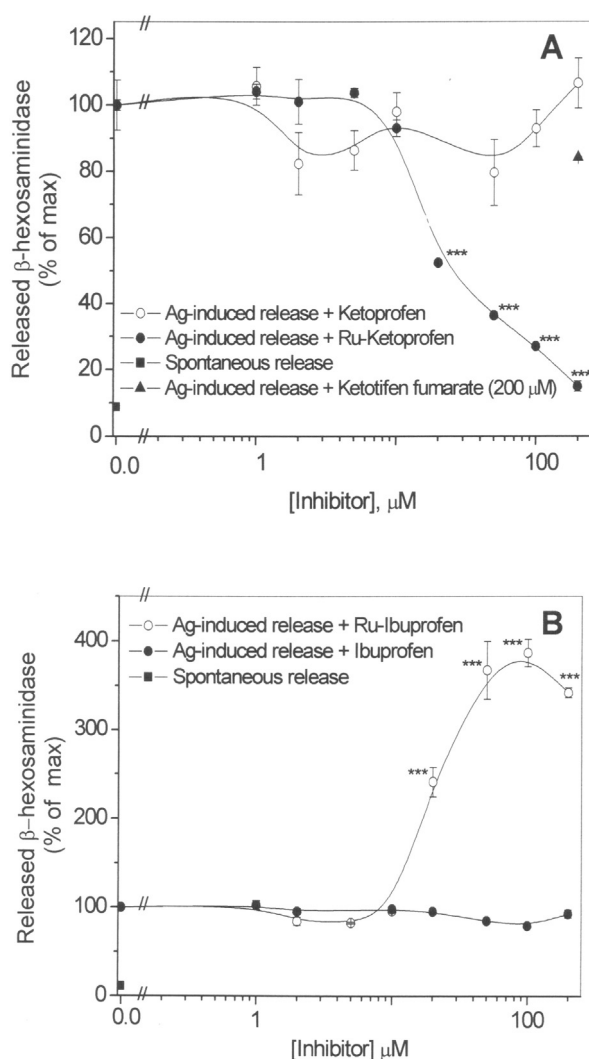


Fig. 4. $[\text{Ru}_2\text{O}(\text{keto})_2(\text{py})_6](\text{PF}_6)_2$ (compound **2**) inhibits FcεRI-mediated RBL mast cells degranulation with higher potency than ketotifen fumarate (A) while $[\text{Ru}_2\text{O}(\text{ibu})_2(\text{py})_6](\text{PF}_6)_2$ (compound **1**) causes β -hexosaminidase release (B). Anti-DNP-IgE-sensitized RBL cells were treated with the complexes $[\text{Ru}_2\text{O}(\text{keto})_2(\text{py})_6](\text{PF}_6)_2$ (0–200 μM), $[\text{Ru}_2\text{O}(\text{ibu})_2(\text{py})_6](\text{PF}_6)_2$ (0–200 μM) or ketotifen fumarate (200 μM) for 20 min, and stimulated with antigen (Ag) followed by 1 h incubation. Cell supernatant was treated with 1.2 mM MUG and incubated for another 30 min. β -Hexosaminidase release was quantified as indicated in the materials and method section. Ketotifen fumarate, in the concentration of 200 μM (in B), was used as positive control. Data were expressed as mean $\pm$ SD of three independent experiments. One-way ANOVA, followed by Dunnett's test, indicates statistical significance of $***p < 0.001$ in comparison to antigen-control sample.

bioactive compound. Fig. 5 shows that the highest concentrations of compound **2** moderately inhibit β -hexosaminidase activity since 200 μM of compound **2** causes 37% inhibition of enzyme while the same concentration causes 85% inhibition of degranulation (Fig. 5). Therefore, the results proved that the reduction in the percentage of released β -hexosaminidase enzyme is primarily assigned to the ability of compound **2** to inhibit mast cell degranulation.

Incubation of RBL-2H3 cells with compound **2** for 2 h caused a small decrease in cell viability only at the highest concentrations, proving that its inhibitory activity against mast cell degranulation is not caused by a cytotoxic effect (Fig. 5). As shown in Fig. 5 only a minor reduction in cell viability (about 20%) was observed after cell treatment with the highest drug concentration (200 μM). These results are consistent with a primary inhibitory effect of compound **2** on reducing antigen-stimulated mast

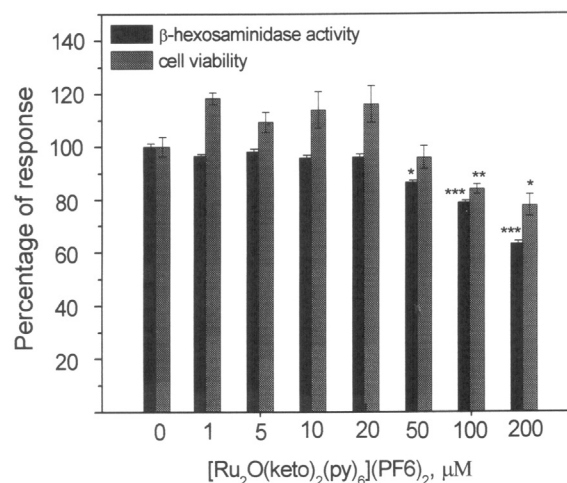


Fig. 5. Compound **2** causes a small decrease in both β -hexosaminidase activity and cell viability. β -hexosaminidase activity was quantified through fluorescence measurements of methyl-umbelliferone after cell supernatant was incubated with compound **2** (1–200 μM) and 1.2 mM MUG solution for 1 h at 37 $^\circ\text{C}$. Cell viability was evaluated by means of fluorescence measurement of resorufin, a compound resulting from the reduction of resazurin by viable cells. One representative experiment of three, for each bioassay, is shown. One way ANOVA followed by Dunnett's test, indicates statistical significance of $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$, in comparison to untreated control samples.

cell degranulation, and point out this new molecule as a promising candidate to explore the mechanisms of action in allergen-stimulated mast cells for future application as an antiallergic drug. It is important to emphasize that compound **2** does not stimulate mast cell degranulation, which suggests that this compound does not present allergenic potential, a required property for its future pharmacological applications as an antiallergic drug or others.

In contrast to the results obtained for compound **2**, the interaction with compound **1** caused the release of β -hexosaminidase as shown in Fig. 4B. This behavior could be caused by cell stimulation (allergenic property) or cell death (by necrosis). However, the first possibility was discarded since compound **1** has shown cytotoxic effect to RBL cells, when incubated with the cells for 20 min (time of the bioassay)

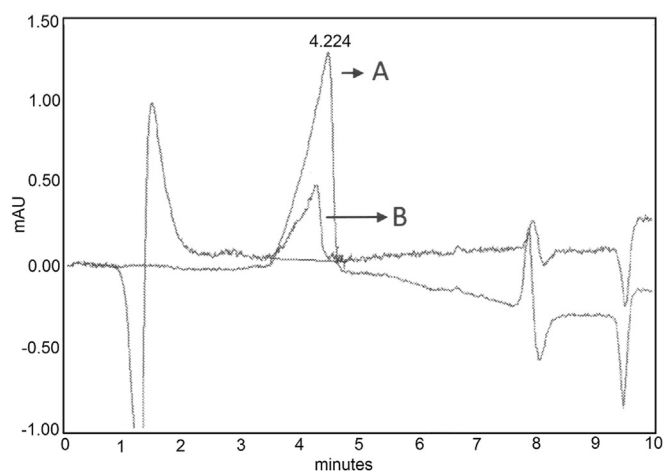


Fig. 6. Interaction study of $[\text{Ru}_2\text{O}(\text{ibu})_2(\text{py})_6](\text{PF}_6)_2$ complex with CYP450 from rats. (A) Chromatogram regarding the control sample without addition of cofactors; (B) chromatogram obtained for the *in vitro* metabolism study. The peak with the highest intensity (retention time = 4.2 min) is the $[\text{Ru}_2\text{O}(\text{ibu})_2(\text{py})_6](\text{PF}_6)_2$ complex before interacting with CYP450 enzymes; and the peak with lower intensity is the $[\text{Ru}_2\text{O}(\text{ibu})_2(\text{py})_6](\text{PF}_6)_2$ complex after interacting with CYP450. Chromatograms conditions: silica column and acetonitrile: methanol (9.5:0.5 v/v) as mobile phase. Flow rate: 0.50 mL/min. Detection at 586 nm. Injection volume: 20 μL . Sample concentration: 0.025 mg/mL.

or 2 h (Fig. S5 B and C, [Supplementary material](#)). The cytotoxic effect of compound **1** makes its use impracticable as an antiallergic drug, but does not rule out the application of it in other types of diseases such as cancer, for example, as observed for other diruthenium–ibuprofen compound [46].

4.4. *In vitro* metabolism

Fig. 6 presents the result of the interaction of $[\text{Ru}_2\text{O}(\text{ibu})_2(\text{py})_6](\text{PF}_6)_2$ with CYP450 enzymes present in rat liver microsomes, RLM. These are subcellular fractions that consist of hepatocyte endoplasmic reticulum vesicles. Usually, they are employed for the evaluation of phase I metabolism, since they contain many cytochrome P450 (CYP450) metabolizing enzymes. The types of reactions performed by CYP450 could be of reduction and hydrolysis, but are mainly of oxidative nature. This system probably represents one of the most widely used *in vitro* systems for investigating the metabolic profile of a drug candidate [47,48].

The chromatogram depicted in Fig. 4 shows a decrease in approximately 50% of the amount of complex **1** in the microsomal medium, after incubation with rat liver microsomes. This result suggests that compound **1** may suffer an oxidative process in its metabolism by the cytochrome P450 enzymes of a mammalian species. The interaction with CYP450 enzymes is the first step to eliminate a drug from the body aiming its excretion. Therefore, the $[\text{Ru}_2\text{O}(\text{ibu})_2(\text{py})_6](\text{PF}_6)_2$ complex probably will be modified, *i.e.* metabolized, before being excreted. However, the modification in its structure or the determination of any metabolites is virtually impossible to determine under the employed conditions, mainly due to the low concentration of the complex used in the assay. The concentration should be low to favor the solubility of the complex in the microsomal medium and to prevent the inhibition of the complex under the CYP450 enzymes.

5. Conclusion

This work presented the synthesis of two novel binuclear ruthenium complexes, $[\text{Ru}_2\text{O}(\text{ibu})_2(\text{py})_6](\text{PF}_6)_2$ and $[\text{Ru}_2\text{O}(\text{keto})_2(\text{py})_6](\text{PF}_6)_2$, which combine in the same structure the $[\text{Ru}_2\text{O}]$ core, which is a biomimetic analogue of the hemerythrin active center, and the nonsteroidal anti-inflammatory drugs ibuprofen and ketoprofen. Both complexes were characterized by ESI-MS/MS spectrometry and UV–visible spectroscopy. The results are fully consistent with the proposed structures and, apparently, the combination with the NSAIDs does not disturb the electronic behavior of the metal center.

Complex $[\text{Ru}_2\text{O}(\text{keto})_2(\text{py})_6](\text{PF}_6)_2$ was identified as a promising leading compound for the development of novel anti-allergic drugs, considering its potentiality to inhibit the release of allergic mediators from antigen-stimulated mast cells. Investigation about the molecular mechanism responsible for the antiallergic potential of $[\text{Ru}_2\text{O}(\text{keto})_2(\text{py})_6](\text{PF}_6)_2$ is part of the forthcoming work that could endorse the therapeutical applicability of this new ruthenium complex. The fact that the compound $[\text{Ru}_2\text{O}(\text{keto})_2(\text{py})_6](\text{PF}_6)_2$ does not stimulate the degranulation of mast cells is also very positive and suggests that this molecule shows no allergenic potential and can be safely used for other pharmacological applications besides antiallergic. In contrast, complex $[\text{Ru}_2\text{O}(\text{ibu})_2(\text{py})_6](\text{PF}_6)_2$ stimulates the release of β -hexosaminidase, due to its high cytotoxicity for the RBL-2H3 cell line, which makes its use impracticable as anti-allergy, but does not rule out other biological applications.

Besides that, the preliminary essay of the *in vitro* metabolism of the $[\text{Ru}_2\text{O}(\text{ibu})_2(\text{py})_6](\text{PF}_6)_2$ complex, showed its interaction with CYP450 enzymes by the reduction of complex peak in the chromatogram due to the microsomal metabolism carried out by rat liver microsomes. This observation suggests a favorable biological path for the metabolization of this class of compounds.

Overall, both results presented in this work – antiallergic potential and *in vitro* metabolism – have positive implications for the intended use of these compounds as potential metallo-drugs.

Abbreviations

py	pyridine
carb	carboxylate
ibu	ibuprofen
keto	ketoprofen
ESI	electron spray ionization
MS	mass spectrometry
CID	collision induced dissociation
Q-TOF	time of flight quadrupole
NSAID	no-steroidal anti-inflammatory drug
CYP450	cytochrome P450
RLM	rat liver microsomes
UV	ultra-violet
NIR	near infra-red
EDTA	ethylenediaminetetraacetic acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
RBL	rat basophilic leukemia
MUG	methylumbelliferyl-N-acetyl- β -D-glucosaminide
COX	cyclooxygenase

Acknowledgments

The authors thank Drs. Barbara Baird and David Holowka (Cornell University) for the kind gifts DNP-BSA and anti-DNP IgE. The authors also thank the Brazilian agencies Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) (2012/23245-6), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) (470176/2011-3) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for financial support.

Appendix A. Supplementary data

The ESI MS/MS spectra for compounds **1** and **2**, a HPLC chromatogram for each complex, as well as the scheme for their fragmentation patterns are available as supplementary material. The result of the cytotoxicity assay for both compounds is also available.

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Supplementary Material

Novel binuclear «-oxo diruthenium complexes combined with ibuprofen and ketoprofen: interaction with relevant target biomolecules and anti-allergic potential

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Electron Spray Ionization Mass Spectrometry – ESI MS/MS

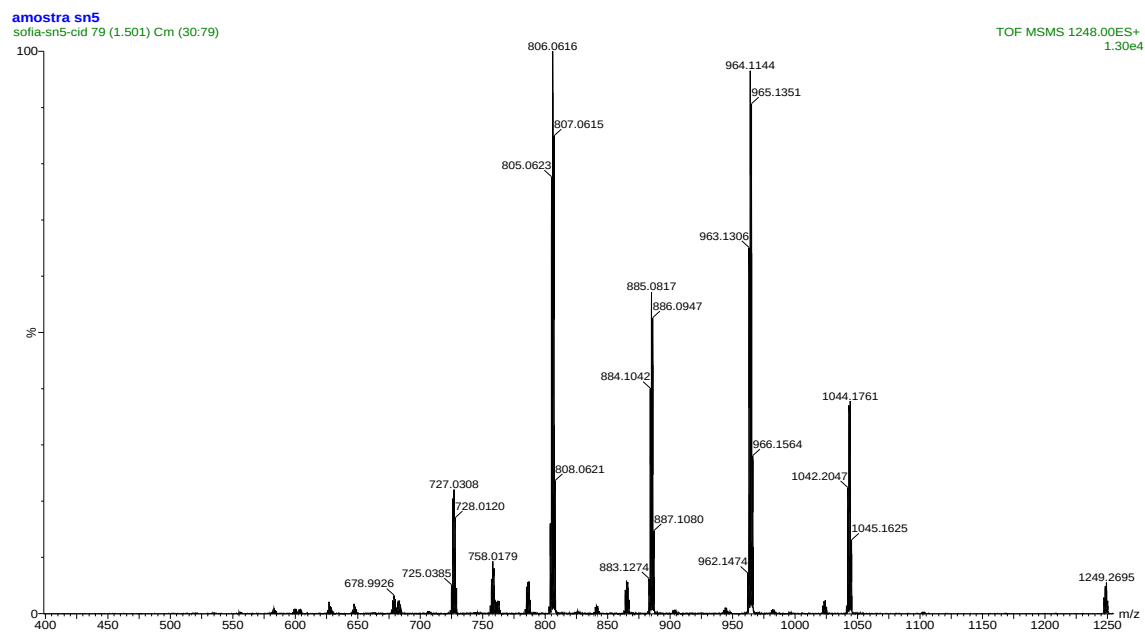


Figure S1. ESI MS/MS spectrum of complex 1, $[\text{Ru}_2\text{O}(\text{ibu})_2(\text{py})_6](\text{PF}_6)_2$. Recorded from methanolic solution, positive acquisition mode.

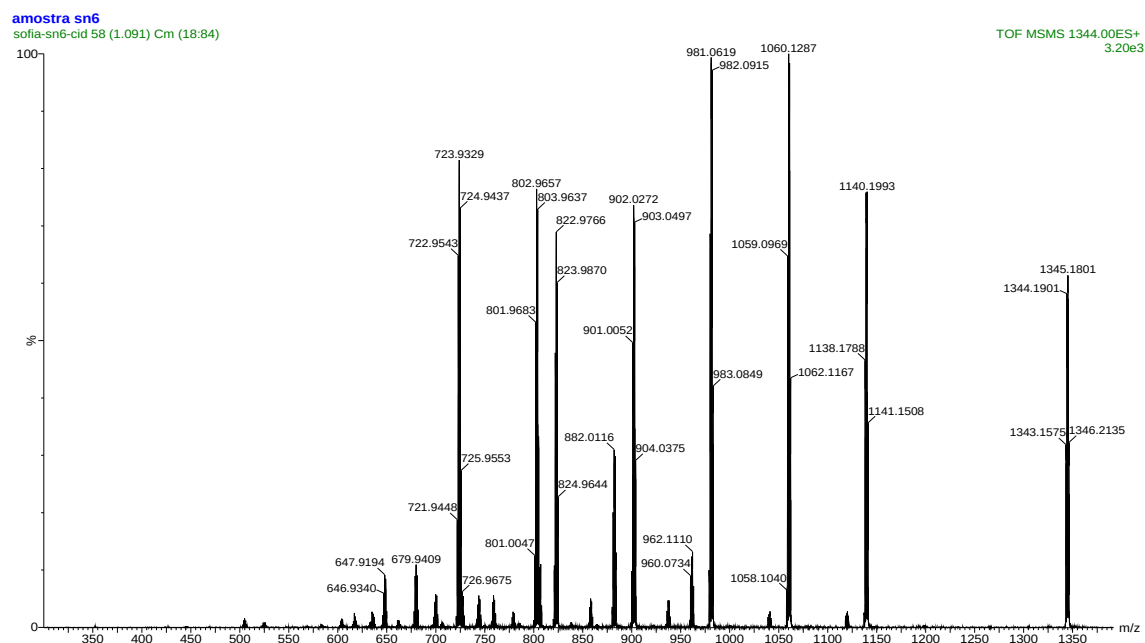


Figure S2. ESI MS/MS spectrum of complex 2, $[\text{Ru}_2\text{O}(\text{keto})_2(\text{py})_6](\text{PF}_6)_2$. Recorded from methanolic solution, positive acquisition mode.

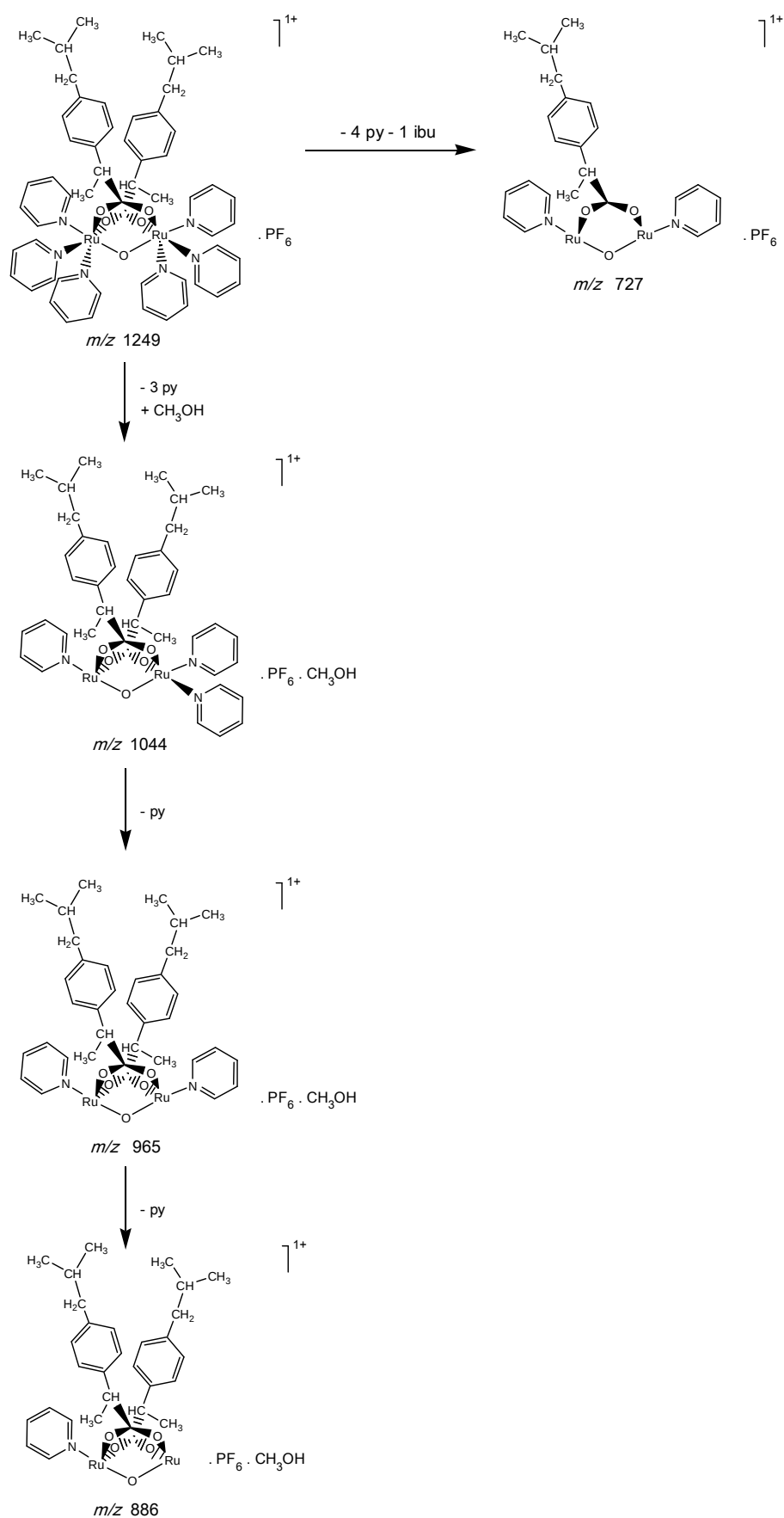


Figure S3. Dissociation pattern of compound $[\text{Ru}_2\text{O}(\text{ibu})_2(\text{py})_6](\text{PF}_6)_2$

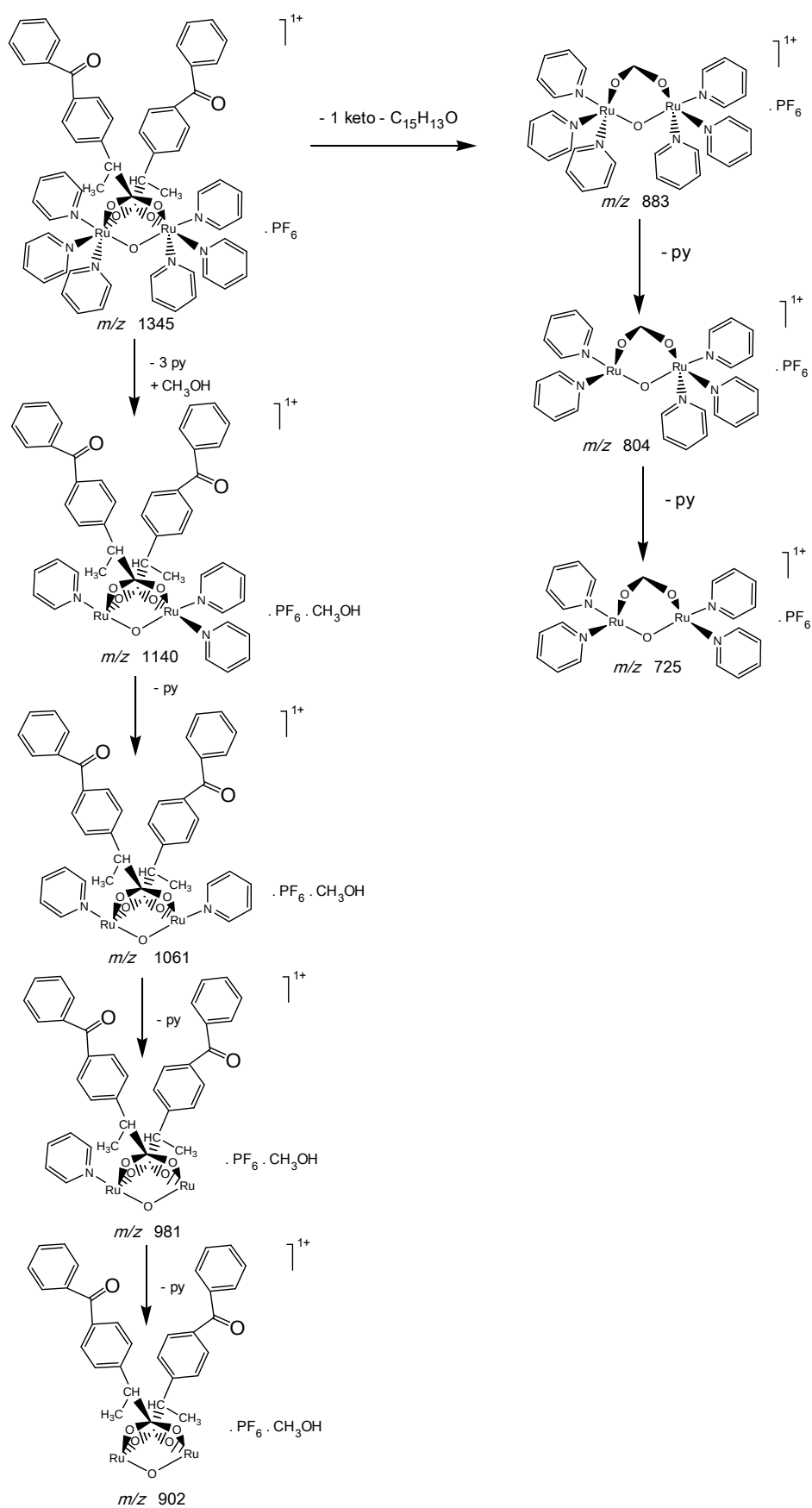


Figure S4. Dissociation pattern of compound $[\text{Ru}_2\text{O}(\text{keto})_2(\text{py})_6](\text{PF}_6)_2$

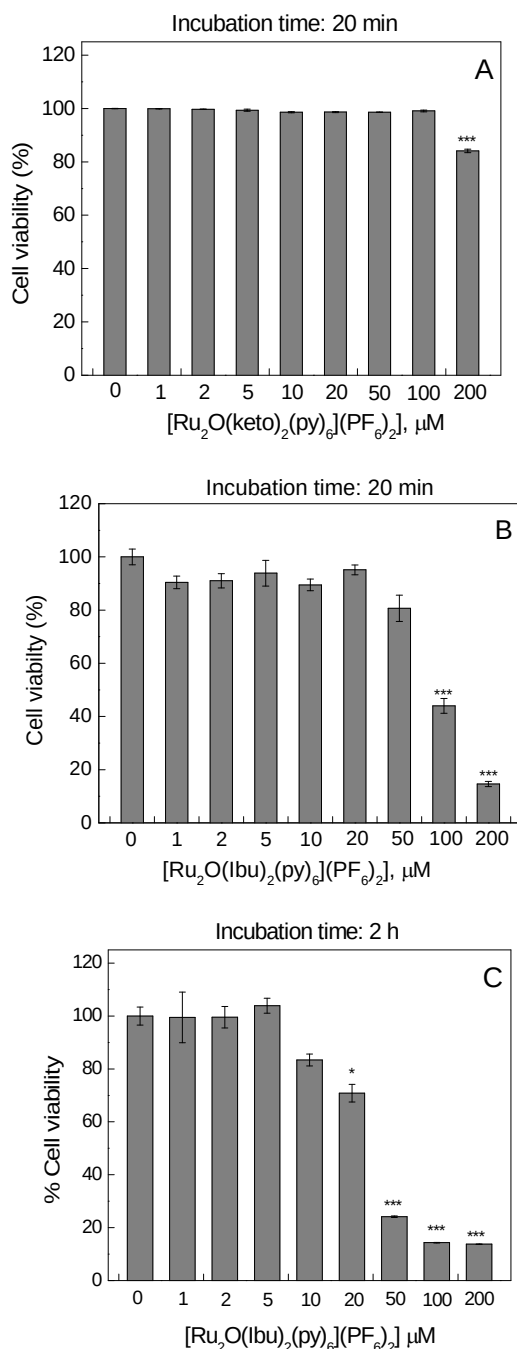


Figure S5. Compound **2** [Ru₂O(keto)₂(py)₆](PF₆)₂] causes a small decrease in cell viability (**A**) during the time of 20 minutes, required for the β-hexosaminidase release assay. However the compound **1** [Ru₂O(Ibu)₂(py)₆](PF₆)₂] showed cytotoxicity in higher concentrations, such as 100 and 200 μM, during 20 minutes incubation (**B**) and higher toxicity for a prolonged time of incubation (2 h) (**C**). Cell viability was evaluated by means of fluorescence measurement of resorufin, a compound resulting from the reduction of resazurin by viable cells. One representative experiment of three, for each bioassay, is shown. One way ANOVA followed by Dunnett's test, indicates statistical significance of **p*<0.05 and ****p*<0.001, in comparison to untreated control samples.

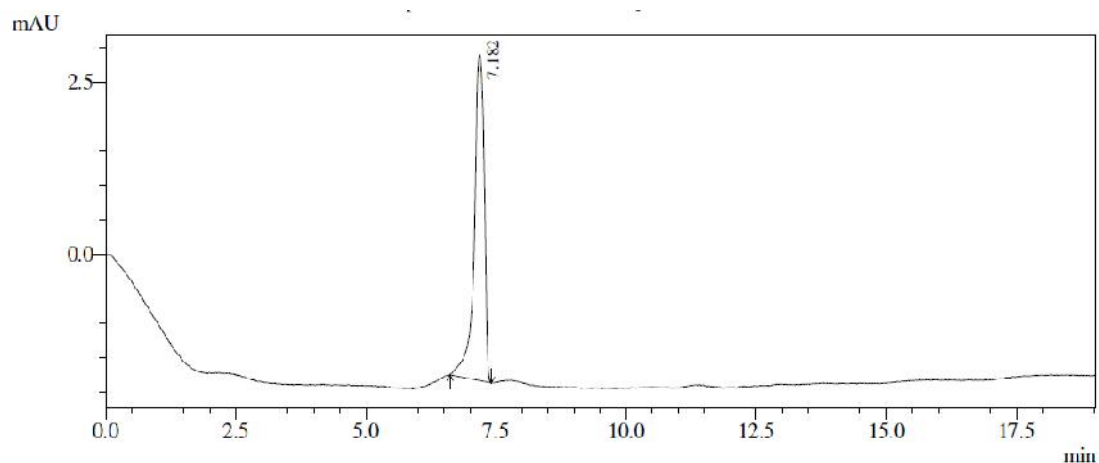


Figure S6. $[\text{Ru}_2\text{O}(\text{ibu})_2(\text{py})_6](\text{PF}_6)_2$ chromatogram performed on a silica analytical column acquired from Shimadzu (Shim-pack[®] CLC-SIL, 4.0 mm $\times$ 250 mm, 5 μm particle size). The mobile phase used was a mixture of acetonitrile : methanol (9.5:0.5, v/v) at a flow rate of 0.5 mL min⁻¹.

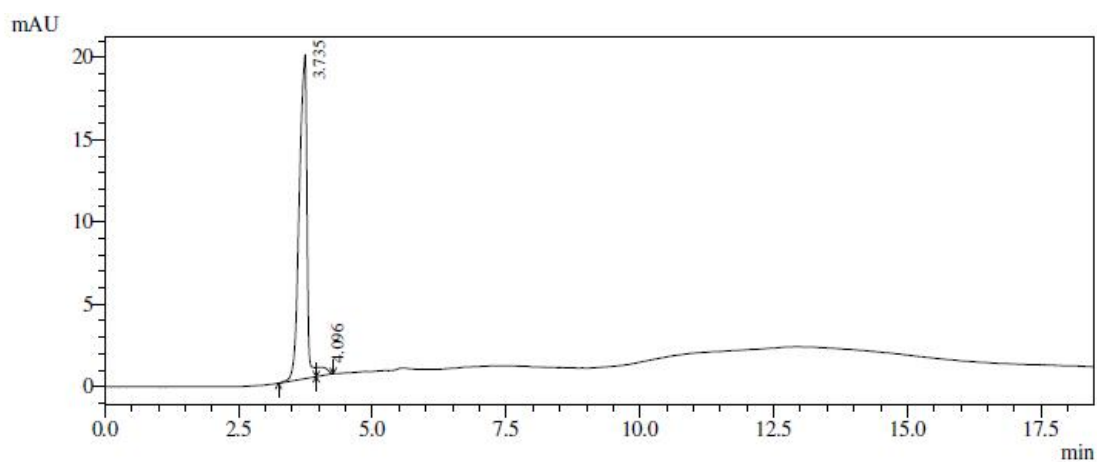


Figure S7. $[\text{Ru}_2\text{O}(\text{keto})_2(\text{py})_6](\text{PF}_6)_2$ chromatogram performed on a silica analytical column acquired from Shimadzu (Shim-pack[®] CLC-SIL, 4.0 mm $\times$ 250 mm, 5 μm particle size). The mobile phase used was a mixture of acetonitrile : ethanol (9.5:0.5, v/v) at a flow rate of 0.5 mL min⁻¹.