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Synthesis of *N*-functionalized 2,2′-dipyridylamine ligands, complexation to ruthenium (II) and anchoring of complexes to papain from papaya latex

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ABSTRACT

2,2′-Dipyridylamine (dpa) derivatives carrying a thiol-targeted maleimide group located at the end of an alkyl substituent on the central amine were synthesized. Reaction with the organometallic precursors $[(\eta^6\text{-arene})RuCl_2]_2$ (arene = benzene or *p*-cymene) yielded the half-sandwich cationic complexes $[(\eta^6\text{-arene})Ru(dpa)Cl]^+$ where the dipyridylamine derivatives were coordinated as bidentate N,N donor ligands. Enzymatic studies showed that these derivatives were able to inactivate the cysteine endoproteinase papain by *S*-alkylation of the cysteine active site.

1. Introduction

Within the last 2 years, we reported the covalent and chemoselective coupling of several transition organometallic complexes to the cysteine endoproteinase papain from *Carica papaya*. This could be achieved by reacting the sole free sulfhydryl function of papain carried by Cys25 with complexes including a functional chloroacetamide or maleimide group targeted towards thiols [1,2]. Our aim, that was initially to provide protein crystallographers with new heavy metal reagents for structural analysis, shifted towards the preparation of artificial metalloenzymes as papain allows embedding of catalytically active organometallics at a unique and well-defined position [3].

To initiate this research, we selected the family of $(\eta^6$-arene)ruthenium(II) complexes as they display versatile catalytic properties and are tolerant to water and oxygen [4]. For example, dicationic $(\eta^6$-arene)ruthenium(L)(II) complexes where L is a chiral bidentate P,P-, P,N-, N,O- or N,N-ligand are efficient enantioselective catalysts of Diels–Alder reactions thanks to their Lewis-acid character [5]. Most interestingly, $[(\eta^6$-arene)ruthenium(N,N)] compounds are very efficient catalysts for the racemic and asymmetric transfer hydrogenation of ketones and imines in aqueous medium using formate as hydrogen donor [6].

In our previous paper, we reported the synthesis of $[(\eta^6$-arene)ruthenium(N,N)Cl]$^+$ complexes carrying the thiol-targeting function on the arene ligand and studied the reactivity of these compounds towards papain [2]. In this paper, we wish to report the synthesis and reactivity of a novel series of complexes for which the maleimide function is now carried by the bidentate N,N-ligand.

2. Results and discussion

2.1. Synthesis of ligands

To avoid the presence of chiral centers in the final complexes, we chose the symmetrical 2,2′-dipyridylamine core as a scaffold to construct the functionalized ligands and introduced the thiol-reactive maleimide function at the extremity of an alkyl chain substituting the central amine.

Most methods for the preparation of *N*-substituted maleimides involve the reaction of a primary amine with maleic anhydride, followed by dehydration of the intermediate maleamic acid promoted by acid [7]. However, a recently described procedure utilizing a Mitsunobu reaction offers a convenient one-step method starting

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from the alcohol precursor [8]. Furthermore, the starting materials for this method, the dipyridylamino alcohols **1a**,**b** are readily available by Buchwald–Hartwig amination reaction [9] (Scheme 1).

Reaction of 4-amino-1-butanol or 5-amino-1-pentanol with 2 equiv. of 2-bromopyridyne and 2.4 equiv. of sodium-*tert*-butoxide in the presence of 4 mol% *rac*-BINAP and 4 mol% $Pd_2(dba)_3$ in toluene at 70 °C led to the corresponding dipyridylamino alcohols **1a**,**b** in 23–34% yield after purification on silica gel (Scheme 1). Low yields are probably due to the *O*-arylation reaction, which competes with the slower second amination.

The dipyridylamino alcohols were converted into the corresponding *N*-alkylmaleimides **2a**,**b** by a Mitsunobu reaction with maleimide in the presence of 1 equiv. of PPh_3 and 1 equiv. of DEAD in THF at −60 °C for 15 min and then at RT for 40 h in about 60% yield after purification by chromatography on silica gel (Scheme 1). While cyclization by intramolecular Mitsunobu reaction leading to the substitution of hydroxyl group with one pyridyl ring was observed from the dipyridylamino alcohol with a 3-carbon spacer (unpublished results), no such side reaction occurred with longer chains.

2.2. Complexation by Ru(II)

Surprisingly, the only example of complexation of a ligand presenting the 2,2′-dipyridylamine motif by arene ruthenium species involved tri-2-pyridylamine. In this case, bidentate coordination was shown to occur by two of the three pyridyl substituents [10].

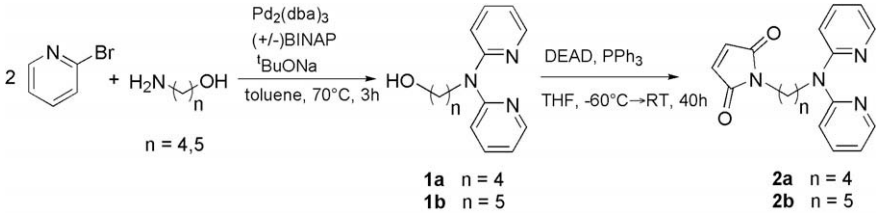
Reaction of $[Ru(p\text{-cymene})Cl_2]_2$ with 2 equiv. of *N*-substituted dipyridylamine **2a**,**b** in CH_2Cl_2 at RT for 16 h led to the corresponding mononuclear cationic complexes **3a**,**b** in quantitative yield. In the same way, reaction of $[Ru(benzene)Cl_2]_2$ with compound **2b** led to complex **4** (Scheme 2). Complexes **3a**,**b** and **4** are air-stable orange solids, soluble in polar solvents (water and methanol).

In the ^1H NMR spectra of compounds **3a**,**b** and **4**, the presence of a doublet of doublets at *ca.* 8.8 ppm (two protons) attributed to the CH groups adjacent to the pyridyl nitrogen, indicated the symmetrical chelation of the ligand to Ru by the pyridyl nitrogen atoms.

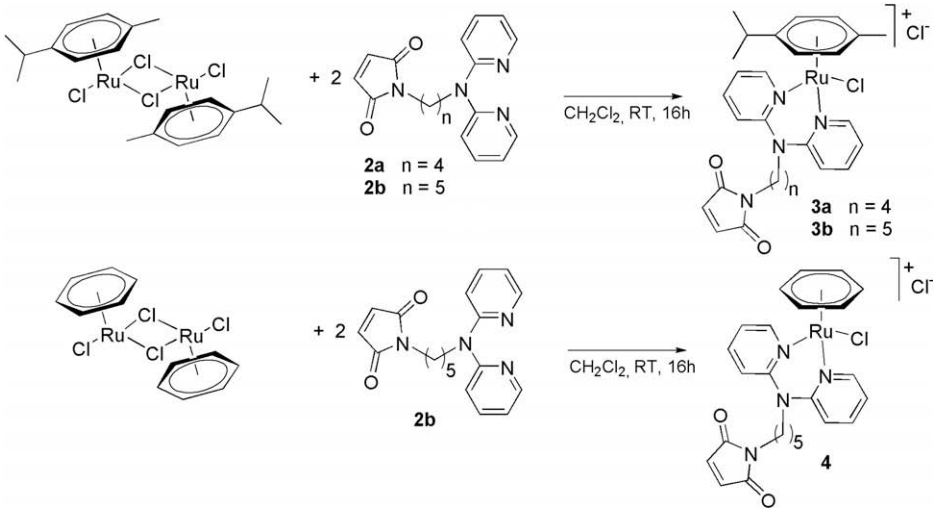
2.3. Reactivity studies with papain

In the past few years, several half-sandwich ruthenium complexes have been reported as enzyme inhibitors. In these complexes, the metal either suitably organized the ligands in the three-dimensional space so that an inhibitory activity was conferred. This is the case for the kinases reversible inhibitors derived from indolocarbazoles [11]. Alternatively, the metal brought a special reactivity. This is the case for glutathione *S*-transferase irreversible inhibitors [12] and p-glycoprotein inhibitors [13]. We have also reported the ability of several half-sandwich ruthenium to irreversibly inhibit the enzyme papain, this property being brought by the presence of a suitable function on the arene ligand [2].

Therefore, the ability of the three dipyridylamine ruthenium(II) **3a**,**b** and **4** together with the dipyridylamine ligand **2b** to inhibit papain was tested on the affinity-purified enzyme. This protein, belonging to the cysteine endoproteinases family, catalyzes the hydrolysis of peptide bonds in peptides and proteins [14]. Chemical modification of its active site cysteine residue (Cys25) leads to enzyme inactivation [15]. This provides a convenient means for assessing the reaction of the complexes with papain and may even furnish data on the kinetics of the reaction. In a typical experiment, the enzyme was mixed with the complex or the ligand in large excess so that pseudo-first-order conditions were applied. The enzymatic activity of the mixture was periodically assayed on the substrate PFLNa that yields, in the presence of active enzyme, *p*-nitroaniline. The formation of this yellow compound can easily



Scheme 1.



Scheme 2.

be monitored colorimetrically (λ_{max} = 412 nm) [16]. As expected, gradual loss of enzymatic activity was observed in the presence of all the compounds tested and the enzyme became fully inactivated after a certain time in all cases. Semi-logarithmic plots of initial PFLNa hydrolysis rate versus time were linear and gave the pseudo-first-order rate constants of inactivation k_{obs}. For an easier comparison with published data on related maleimide inhibitors, we calculated the $k_{obs}/[I]$ ratios (I is the inactivator) which is equivalent to the second-order rate constant k_2 (Table 1).

It appears that all the compounds tested were found to inactivate papain in a time-dependent fashion but the rate by which inactivation occurred clearly depended on the structure of the compound. In the 5-carbon chain series, complexation by the cationic (*p*-cymene)RuCl entity had only a slight enhancing effect on the inactivation rate, with respect to that of the ligand alone, whereas complexation by the cationic (benzene)RuCl had an enhancing effect by a factor of 3. All the compounds tested reacted with papain faster than the model compound *N*-pentylmaleimide. The enhancing effect varied from ca. 2 to 6.

For compound **3a**, the pseudo-first-order rate constant experiment was repeated with several concentrations of complex in the range of 10–100 μM. In these experimental conditions, the pseudo-first-order rate constant showed a linear relationship with respect to the concentration of **3a** (Fig. 1) without any observed saturation effect which would account for a preliminary binding step of the complex to the enzyme before thiol alkylation occurs. Thus, the reaction of papain with **3a** appeared as a simple bimolecular process in the tested reaction conditions with a second-order rate constant of inactivation k_2 of 4200 ± 260 M^{-1} min^{-1} (Table 1). Quite unexpectedly, this complex **3a** inactivated papain at a very high rate, which was almost twice higher than that of the 5-carbon chain analog **3b**. This finding was very surprising since, in the organic series (alkyl substituted maleimides), the rate of inactivation tends to decrease with the chain length [17]. On the whole, this compound was the fastest organometallic inactivator of papain ever tested. With respect to *N*-butylmaleimide, the organometallic entity $[(p\text{-cymene})Ru(N,N)Cl]^+$ enhanced the inactivation rate of papain by a factor of 17.

No doubt the protein environment around the sulfhydryl function had a marked influence on its reactivity with the maleimides since no effect was noticed on the reaction rate of *N*-alkylmaleimides of various chain lengths with the small thiol glutathione [18]. It is known that the catalytic site of papain contains several hydrophobic residues, that is Tyr61, Tyr67, Trp69, Phe207, Val133 and Val157. The latter two aminoacids form the S2 subsite, which is responsible for the specificity of papain for peptide substrates and inhibitors containing a phenylalanine residue at the P2 position [19]. It may be inferred that, because of the presence of aromatic substituents, favorable electronic interactions (π–π or π–cation) between papain and the maleimides tested may occur, which would account for the fast inactivation rates. However, steric parameters may also be involved in the interaction between papain and the complexes (see results for **3b** and **4** that only differ by the aromatic ligand substituents).

Table 1
Rate constants for maleimide inactivation of papain[a].

Compound	[*I*] (μM)	$k_{obs}/[I]$ (M^{-1} min^{-1})	k_2 (M^{-1} min^{-1})
3a	10–100		4200 ± 260
N-Butylmaleimide	40–200		260[b]
4	100	2600	
3b	100	980	
2b	500	860	
N-Pentylmaleimide	70–300		410[b]

[a] Conditions: r.t., H_2O/DMSO 95:5, [papain] = 2.1 μM.
[b] Lit. [17]; conditions: 25 °C, 0.15 M potassium phosphate, pH 6.8, with 0.5% EtOH, [papain] = 0.093 μM.

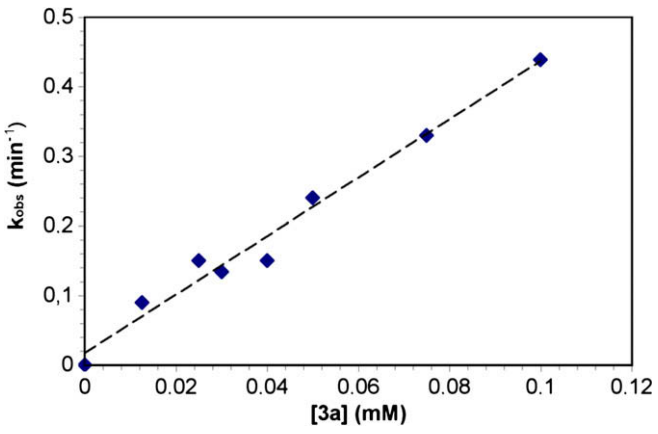


Fig. 1.

3. Conclusions

2,2′-Dipyridylamine derivatives carrying a thiol-targeted maleimide group positioned at the end of an alkyl chain substituting the central amine were synthesized and characterized by usual spectroscopic methods. Reaction with the organometallic precursors $[(\eta^6\text{-arene})RuCl_2]_2$ (arene = benzene or *p*-cymene) yielded the half-sandwich cationic complexes $[(\eta^6\text{-arene})Ru(dpa)Cl]^+$ where the dipyridylamine derivatives dpa were coordinated as bidentate N,N donor ligands. Enzymatic studies showed that these derivatives were able to inactivate the cysteine endoproteinase papain by *S*-alkylation of the active site cysteine yielding to artificial metalloproteins. Studies delineating their potential activity as Lewis-acid catalysts or in ketone reduction by hydrogen transfer are currently under way. This new anchoring approach should extend the range of metal catalysts to be associated to papain as catalytically active dipyridylamine complexes of Cu [9f,g], Ni [20], Pd [21], Zn [22] and Mg [20] have already been reported.

4. Experimental

4.1. General

Solvents were dried and distilled by standard procedures and all reactions and manipulations were performed under an inert atmosphere of argon or nitrogen using standard Schlenk and vacuum-line techniques. Flash chromatography was performed on silica gel Merck 60 (40–63 μm). NMR spectra were recorded on 300 and 400 MHz Bruker spectrometers. High resolution mass spectra were recorded on a MStation JMS 700 (Jeol). $[Ru(p\text{-cymene})(Cl)_2]_2$ and $[Ru(benzene)(Cl)_2]_2$ were prepared according to Ref. [23].

Papain from papaya latex (EC 3.4.22.2) was obtained as a suspension in acetate buffer (Sigma P3125) and further purified by affinity chromatography using the procedure described in Ref. [24]. Under these conditions, between 2.5 and 3.4 mg of fully active enzyme was typically recovered from 10 mg of starting material. Purified papain was stored as a 0.25 mg/mL aqueous solution at −20 °C. L-Pyroglutamyl-L-phenylalanyl-L-leucine-*p*-nitroanilide (PFLNa) was purchased from Bachem. A 1 mM stock solution was prepared in DMSO. Enzymatic assays were performed in 100 mM phosphate, 300 mM KCl, 0.1 mM EDTA, pH 6.5, containing 10% DMSO.

4.2. Synthetic procedures

*4.2.1. Synthesis of compounds **1a**,**b***

4.2.1.1. General procedure. 2-Bromopyridine (15 mmol, 1.43 mL), aminoalcohol (15 mmol), $Pd_2(dba)_3$ (0.15 mmol, 137 mg, 8%mol Pd), BINAP (0.3 mmol, 187 mg), and *t*-BuONa (18 mmol, 1.73 g) in toluene (75 mL) were stirred at 70 °C for 3 h. The suspension was filtered and the solvent was evaporated to give a red oil. The product was purified by chromatography on silica gel (eluent:ethyl acetate/petroleum ether 8:2).

*4.2.1.2. 4-(Dipyridin-2-ylamino)butan-1-ol (**1a**).* From 4-aminobutanol. Light yellow oil. Yield 23%.

^{1}H NMR ($CDCl_3$, 300 MHz) δ = 8.32 (ddd, 2H, *J* = 5.1, 2.1, 1.0 Hz), 7.51 (ddd, 2H, *J* = 8.5, 7.1, 2.1 Hz), 7.09 (dt, 2H, *J* = 8.5, 1.0 Hz), 6.85 (ddd, 2H, *J* = 7.2, 4.9, 0.9 Hz), 4.27–4.22 (m, 2H), 3.75 (t, 2H, *J* = 6.0 Hz), 1.82 (quintet, 2H, *J* = 7.2 Hz), 1.63 (quintet, 2H, *J* = 6.6 Hz); ^{13}C NMR ($CDCl_3$, 75 MHz) δ = 157.2, 148.2, 137.3, 117.0, 114.8, 62.6, 47.3, 28.6, 24.7; HRMS calcd for $C_{14}H_{18}N_3O$ (M+H) 244.1450, found 244.1448.

*4.2.1.3. 5-(Dipyridin-2-ylamino)pentan-1-ol (**1b**).* From 5-aminopropanol. Light yellow oil. Yield 34%.

^{1}H NMR ($CDCl_3$, 300 MHz) δ = 8.32 (ddd, 2H, *J* = 4.9, 1.9, 0.8 Hz), 7.50 (ddd, 2H, *J* = 8.5, 7.4, 2.1 Hz), 7.06 (d, 2H, *J* = 8.3 Hz), 6.84 (ddd, 2H, *J* = 7.2, 4.9, 0.8 Hz), 4.21–4.16 (m, 2H), 3.62 (t, 2H, *J* = 6.3 Hz), 1.95 (s large, 1H), 1.73 (quintet, 2H, *J* = 7.7 Hz), 1.61 (quintet, 2H, *J* = 6.6 Hz), 1.49–1.39 (m, 2H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ = 157.5, 148.3, 137.2, 116.9, 114.8, 62.7, 48.1, 32.4, 27.7, 23.0; HRMS calcd for $C_{15}H_{20}N_3O$ (M+H) 258.1606, found 258.1604.

*4.2.2. Synthesis of compounds (**2a**,**b**)*

4.2.2.1. General procedure. To a solution of PPh_3 (1.6 mmol, 420 mg) in THF (10 ml) at −60 °C, DEAD (1.6 mmol, 252 μL) was added dropwise. The mixture was stirred at −60 °C for 15 min. Compound **1a** or **1b** (1.6 mmol) was added and the solution was stirred at −60 °C for 15 min. Maleimide (3.2 mmol, 310 mg) was added. The solution was allowed to warm up to room temperature and was then stirred for 40 h. The solvent was evaporated to give a yellow–orange oil. After purification of the product by chromatography on silica gel (eluent:petroleum ether/ethyl acetate 3:2), excess diethylhydrazine-1,2-dicarboxylate was eliminated by crystallization in toluene.

*4.2.2.2. 1-[4-(Dipyridin-2-ylamino)butyl]-1H-pyrrole-2,5-dione (**2a**).* From compound **1a**. Light yellow oil. Yield 60%.

^{1}H NMR ($CDCl_3$, 300 MHz) δ = 8.33 (ddd, 2H, *J* = 4.9, 1.9, 0.8 Hz), 7.50 (ddd, 2H, *J* = 8.5, 7.4, 2.1 Hz), 7.06 (dt, 2H, *J* = 8.3, 0.9 Hz), 6.84 (ddd, 2H, *J* = 7.1, 4.9, 0.9 Hz), 6.65 (s, 2H), 4.20 (t, 2H, *J* = 7.0 Hz), 3.53 (t, 2H, *J* = 6.9 Hz), 1.72–1.62 (m, 4H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ = 170.8, 157.3, 148.4, 137.1, 134.0, 116.9, 114.7, 47.5, 37.7, 26.0, 25.5; HRMS calcd for $C_{18}H_{19}N_4O_2$ (M+H) 323.1508, found 323.1513.

*4.2.2.3. 1-[5-(Dipyridin-2-ylamino)pentyl]-1H-pyrrole-2,5-dione (**2b**).* From compound **1b**. Light yellow oil. Yield 56%.

^{1}H NMR ($CDCl_3$, 300 MHz) δ = 8.32 (ddd, 2H, *J* = 4.9, 1.9, 0.8 Hz), 7.49 (ddd, 2H, *J* = 8.5, 7.3, 2.1 Hz), 7.06 (d, 2H, *J* = 8.3 Hz), 6.83 (ddd, 2H, *J* = 7.1, 4.9, 0.9 Hz), 6.65 (s, 2H), 4.18–4.13 (m, 2H), 3.48 (t, 2H, *J* = 7.2 Hz), 1.79 (s large, 1H), 1.71 (quintet, 2H, *J* = 7.5 Hz), 1.60 (quintet, 2H, *J* = 7.5 Hz), 1.38–1.26 (m, 2H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ = 170.8, 157.4, 148.3, 137.1, 134.0, 116.9, 114.6, 47.9, 37.9, 28.3, 27.8, 24.2; HRMS calcd for $C_{19}H_{21}N_4O_2$ (M+H) 337.1665, found 337.1661.

*4.2.3. Synthesis of complexes **3a**,**b** and **4***

4.2.3.1. General procedure. Ligand **2a**,**b** (0.28 mmol) and arene-Ru dimer (0.14 mmol) in 5 mL of dry CH_2Cl_2 were stirred under argon for 16 h. The solvent was evaporated, the solid residue was washed with diethylether (3 × 5 mL) and dried under vacuum.

*4.2.3.2. Ru{1-[4-(dipyridin-2-ylamino)butyl]-1H-pyrrole-2,5-dione)}-(p-cymene)(Cl) (**3a**).* From compound **2a** and [Ru(*p*-cymene)Cl_2]$_2$. Orange–brown powder. Quantitative yield.

^{1}H NMR (CD_3OD, 400 MHz) δ = 8.74 (dd, 2H, *J* = 5.7, 1.5 Hz), 8.03 (ddd, 2H, *J* = 8.5, 7.4, 1.9 Hz), 7.49 (d, 2H, *J* = 8.1 Hz), 7.33 (m, 2 H), 6.83 (s, 2H), 5.81 (d, 2H, *J* = 6.4 Hz), 5.58 (d, 2H, *J* = 6.2 Hz), 4.10–4.15 (m, 2H), 3.71–3.74 (m, 2H), 2.75 (hept, 1H, *J* = 7.0 Hz), 1.91 (s, 3H), 1.77–1.90 (4 m, 4H), 1.25 (dd, 6H, *J* = 6.8, 2.3 Hz); ^{13}C NMR (CD_3OD, 100 MHz) δ = 172.8, 158.4, 155.8, 142.4, 135.5, 122.3, 117.3, 106.9, 102.0, 86.8, 85.5, 50.8, 37.9, 32.0, 27.2, 26.2, 22.6,18.2; HRMS calcd for $C_{28}H_{32}N_4O_2Ru$ 593.1257, found 593.12518.

*4.2.3.3. Ru{1-[5-(dipyridin-2-ylamino)pentyl]-1H-pyrrole-2,5-dione)}-(p-cymene)(Cl) (**3b**).* From compound **2b** and [Ru(*p*-cymene)Cl_2]$_2$. Orange–brown powder. Quantitative yield.

^{1}H NMR (CD_3OD, 400 MHz) δ = 8.78 (dd, 2H, *J* = 5.9, 1.6 Hz), 8.07 (ddd, 2H, *J* = 8.6, 7.4, 2.0 Hz), 7.53 (d, 2H, *J* = 8.2 Hz), 7.36 (ddd, 2H, *J* = 7.0, 5.8, 0.8 Hz), 6.87 (s, 2H), 5.86 (d, 2H, *J* = 6.2 Hz), 5.65 (d, 2H, *J* = 6.2 Hz), 4.11–4.07 (m, 2H), 3.60 (t, 2H, *J* = 6.8 Hz), 2.83 (hept, 1H, *J* = 6.6 Hz), 2.04 (s, 3H), 1.92–1.85 (m, 2H), 1.76 (quintet, 2H, *J* = 6.6 Hz), 1.54 (quintet, 2H, *J* = 7.4 Hz) 1.28 (d, 6H, *J* = 7.0 Hz); ^{13}C NMR (CD_3OD, 100 MHz) δ = 172.7, 158.4, 155.8, 142.3, 135.4, 122.2, 117.4, 107.1, 101.6, 86.7, 85.6, 51.3, 37.8, 32.0, 28.9, 27.9, 24.7, 22.6, 18.2; HRMS calcd for $C_{29}H_{34}ClN_4O_2Ru$ 607.1407, found 607.1414.

*4.2.3.4. Ru{1-[5-(dipyridin-2-ylamino)pentyl]-1H-pyrrole-2,5-dione)}-(benzene)(Cl) (**4**).* From compound **2b** and [Ru(benzene)Cl]$_2$. Orange–brown powder. Quantitative yield.

^{1}H NMR (CD_3OD, 400 MHz) δ = 8.88 (dd, 2H, *J* = 5.9, 1.6 Hz), 8.06 (ddd, 2H, *J* = 8.6, 7.4, 2.0 Hz), 7.52 (d, 2H, *J* = 8.2 Hz), 7.33 (ddd, 2H, *J* = 7.0, 5.9, 1.2 Hz), 6.86 (s, 2H), 5.94 (s, 6H), 4.12–4.07 (m, 2H), 3.59 (t, 2H, *J* = 6.6 Hz), 1.92–1.82 (m, 2H), 1.72 (quintet, 2H, *J* = 6.6 Hz), 1.51 (quintet, 2H, *J* = 7.4 Hz); ^{13}C NMR (CD_3OD, 100 MHz) δ = 172.7, 158.4, 156.3, 142.4, 135.4, 122.0, 117.4, 88.1, 51.4, 37.9, 28.9, 28.0, 24.7; HRMS calcd for $C_{25}H_{26}ClN_4O_2Ru$ 551.0788, found 551.0790.

4.3. Papain inactivation studies

Papain (2.1 μM) and compound to be tested (concentration ranging from 10 to 500 μM) were incubated at room temperature in water/DMSO 95:5 mixture (final volume 100 μL). At defined time intervals, an aliquot (15 μL) was dispensed in duplicate into wells of a 96-well microtiter plate (Greiner) and the substrate (135 μL; 0.111 mM in assay buffer/DMSO 8:1) was added to the wells. The $OD_{415\ nm}$ was immediately monitored for 2 min with a microtiter plate reader (Biorad model 680).

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