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Functionalized cationic (η^6-arene)ruthenium(II) complexes for site-specific and covalent anchoring to papain from papaya latex. Synthesis, X-ray structures and reactivity studies

Pierre Haquette [a], Barisa Talbi [a], Sigolène Canaguier [a], Samuel Dagorne [b], Céline Fosse [c], Annie Martel [a], Gérard Jaouen [a], Michèle Salmain [a,*]

[a] Ecole Nationale Supérieure de Chimie de Paris, Laboratoire de chimie et biochimie des complexes moléculaires, UMR CNRS 7576, 11 rue Pierre et Marie Curie, 75231 Paris cedex 05, France
[b] Laboratoire DECOMET, Institut de Chimie, UMR CNRS 7177, Institut Le Bel, 4, rue Blaise Pascal, 67000 Strasbourg, France
[c] Ecole Nationale Supérieure de Chimie de Paris, Laboratoire de synthèse sélective organique et produits naturels, équipe de spectrométrie de masse, UMR CNRS 7573, 11 rue Pierre et Marie Curie, 75231 Paris cedex 05, France

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ABSTRACT

The cationic (η^6-arene)ruthenium complexes **6–9** containing a chloroacetamide or a maleimide functional group on the arene ligand were synthesized and successfully used to introduce ruthenium(II) species to the active site of the cysteine endoproteinase papain in a site-directed and covalent fashion as shown by enzymatic and ESI-MS studies.

We have recently reported the covalent and chemospecific introduction of several transition organometallic complexes including various metals to the enzyme papain from *Carica papaya*. This was achieved by Michael reaction between the sulfhydryl group of the sole free cysteine of this enzyme and organometallic N-substituted maleimides.[1] While our main aim was to provide new heavy metal reagents for protein X-ray crystallography, other groups have reported the covalent anchoring of transition metal complexes to the same protein for preparing artificial metalloenzymes.[2–4] Indeed this concept that relates to the combination of protein hosts and catalytically active metal centres has recently witnessed several successes in the area of enantioselective catalysis.[5–10] In the particular case of papain, the resulting hybrids were catalytically active but the measured enantiomeric excesses were low or even zero.

Nevertheless, we thought it interesting to try to anchor a new series of complexes with potential catalytic activities to papain. For this purpose, we selected the family of (η^6-arene)ruthenium(II) complexes as they display versatile catalytic properties and are tolerant to water and oxygen.[11] Indeed, these complexes with bidentate N,N donor ligands catalyze transfer hydrogenation reactions of ketones and imines.[12] Moreover, dicationic (η^6-arene)ruthenium complexes with bidentate ligands are active in Lewis acid-catalyzed Diels–Alder reactions.[13–17] Most interestingly, some of these complexes are even active catalysts in aqueous medium for the reduction of ketones and imines[18–21] and the isomerization of allylic alcohols.[22,23]

Therefore we designed and synthesized a series of cationic complexes of the general formula $[(\eta^6\text{-arene})Ru(N,N)Cl]^+$ carrying either a chloroacetamide or a maleimide moiety on the arene ligand and studied their reactivity towards papain. These functional groups were selected as they are classical means to specifically target/ block cysteine residues in protein chemistry. One of the artificial metalloprotein constructs was characterized by ESI-MS, which provided good evidence for chemoselective covalent anchoring of the complexes to papain.

The most general method for preparing ruthenium complexes of the $[RuCl_2(\eta^6\text{-arene})]_2$ type is the reaction of 1,4-cyclohexadiene derivatives with ruthenium(III) chloride in alcoholic media. The route used to prepare the complexes described herein, bearing η^6-arene ligands with pendant maleimide or chloracetamide groups, is depicted in Scheme 1.

Cyclohexadiene derivatives with a terminal amino functionality $C_6H_7(CH_2)_nNH_2$ (n = 1 or 2) **1a** and **b** were prepared by Birch reduction of commercially available $C_6H_5(CH_2)_nNH_2$ compounds. Subsequent acylation of **1b** with chloroacetyl chloride afforded the chloroacetamide compound **3** in almost quantitative yield while

* Corresponding author. Tel.: +33 1 44 27 67 32; fax: +33 1 43 26 00 61.
E-mail address: michelesalmain@enscp.fr (M. Salmain).

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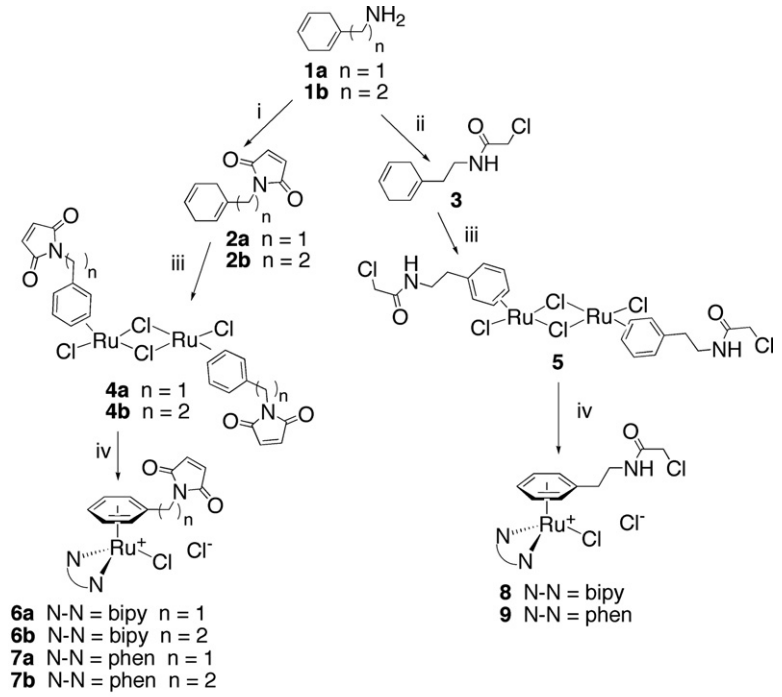

Scheme 1. Preparation of (η^6-arene)ruthenium(II) complexes **6**–**9** with a pendant maleimide or chloroacetamide function.

reaction of **1a** and **1b** with maleic anhydride followed by cyclization promoted by sodium acetate in acetic anhydride led to the corresponding maleimide compounds **2a** and **2b** in moderate yield. Reduction of ruthenium(III) chloride in refluxing ethanol in the presence of a stoichiometric amount of **2a** and **2b** or **3** afforded orange solids of the dimeric η^6-arene complexes **4a**, **4b** and **5**, respectively, in good yields. These complexes are soluble in methanol and DMSO but are converted in the latter solvent to the corresponding [$RuCl_2(\eta^6$-arene)(DMSO)] complex within a few days. The X-ray structure of the neutral complex $RuCl_2[\eta^6$-$(C_6H_5(CH_2)_2NHC(O)CH_2Cl)$(DMSO)] **10** is depicted in Figure 1.

Complex **10** adopts a typical piano-stool geometry with a pseudo-tetrahedral arrangement of the arene, the chloride ligands and the S atom of the coordinated DMSO ligand around the ruthenium centre. Bond lengths and bond angles were comparable to the closely related 1,4,9,10-tetrahydroanthracene ruthenium complex [(η^6-$C_{14}H_{14}$)$RuCl_2$(DMSO)][24] and the *p*-cymene ruthenium complex [(η^6-$C_{10}H_{14}$)$RuCl_2$(DMSO)].[25]

When treated with the N,N donor ligands 2,2'-bispyridyl or 1,10-phenanthroline, the dimeric complexes **4a, b** and **5** were converted to the monocationic complexes **6–9** containing N,N-chelate. Complexes **6–9** are soluble in methanol and water. In the latter solvent, the monocationic chloro complexes are in equilibrium with the dicationic aqua complexes resulting from the abstraction of the chloride ligand. The X-ray structure of complex **8** carrying a pendant chloroacetamide group is depicted in Figure 2.

Compound **8** crystallizes in the triclinic space group $P\bar{1}$. The asymmetric unit comprises two molecules of the cationic complex and two chloride counterions. These counterions are hydrogen-bonded to the NH amide of neighbouring molecules (distance = 2.217 Å). Complex **8** adopts a piano-stool, pseudo-tetrahedral coordination geometry at the ruthenium atom. The chloroacetamide chain is moved above the plane of the arene ligand. The distance between the centroid and the ruthenium atom is 1.69 Å, very close to related monocationic structures with phenanthroline and bipyridyl ligands. The distances between Ru and the 6 carbons of the arene ligand range from 2.153 to 2.246 Å, the longest bond being measured between Ru and the substituted aromatic carbon C16. The interplanar angle between the arene and the bipyridyl planes is 47.94°, which is significantly smaller than that measured for [$Ru(\eta^6$-$C_6H_5(CH_2)_3OH)$(bipy)Cl]BF_4 (58.5°),[26] [$Ru(\eta^6$-$C_6H_5CH_2COOH)$(phen)Cl]OTf (58.4°)[27] and [$Ru(\eta^6$-$C_6H_6)$-(phen)Cl]Cl (62.7°).[18] The cation displays an eclipsed conformation for its substituent methylene carbon atom C17 with respect to the

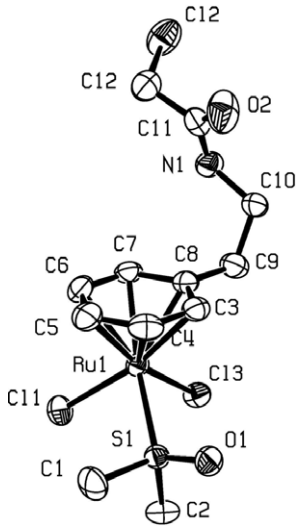

Figure 1. Molecular structure of complex **10** calculated at 50% probability level. Selected bond distances (Å) and bond angles (°): Ru(1)–Cl(1), 2.4026(7), Ru(1)–Cl(3), 2.4125(7), Ru(1)–S(1), 2.3400(6), Ru(1)–C(3), 2.197(3), Ru(1)–C(4), 2.158(3), Ru(1)–C(5), 2.194(3), Ru(1)–C(6), 2.212(3), Ru(1)–C(7), 2.222(2), Ru(1)–C(8), 2.217(3), Cl(1)–Ru(1)Cl(3), 88.26(3), Cl(1)–Ru(1)–S(1), 86.05(2), Cl(3)–Ru(1)–S(1), 85.96(2).

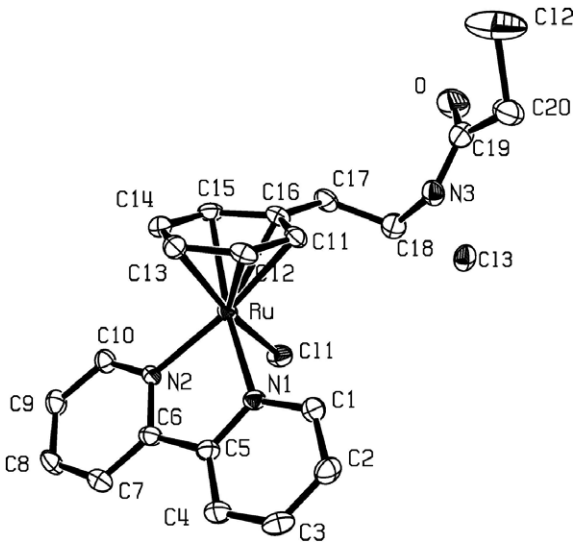

Figure 2. Molecular structure of complex **8** calculated at 50% probability level. Selected bond distances (Å) and bond angles (°): Ru–Cl(1), 2.3978(7), Ru–N(1), 2.091(2), Ru–N(2), 2.083(2), Ru–C(11), 2.210(3), Ru–C(12), 2.153(3), Ru–C(13), 2.192(3), Ru–C(14), 2.196(3), Ru–C(15), 2.213(2), Ru–C(16), 2.246(2), Cl(1)–Ru–N(1), 86.67(6), Cl(1)–Ru–N(2), 85.60(6), N(1)–Ru–N(2), 76.84(8).

Cl ligand as previously observed in the case of [Ru(η^6-C_6H_5–$(CH_2)_3OH$)(bipy)Cl]BF_4.

Reaction of **7b** with 1 mole equiv of cysteine ethyl ester was monitored by ^{1}H NMR in D_2O. Immediate disappearance of the singlet at 6.8 ppm assigned to the vinylic protons of the maleimide double bond was observed on the spectrum of the mixture, indicating that reaction was fast and quantitative. Conversely, no change of ^{1}H NMR spectrum of the mixture of **9** and cysteine ethyl ester in D_2O was observed within 2 days, indicating that no reaction occurred between these compounds. Instead, formation of the corresponding dicationic aqua complex was shown to occur.

Reactivity studies were then pursued with papain. This protein is a member of the cysteine endoproteinases family and catalyzes the hydrolysis of peptide bonds in peptides and proteins.[28] Chemical modification of its active site cysteine (Cys25) is known to induce the loss of its catalytic activity.[29] Therefore, if alkylation of the sulfhydryl group of Cys25 occurs, no more peptidase activity should be measured for the enzyme treated by the complexes. The kinetics of inactivation may be monitored indirectly by periodically assaying the catalytic activity of the enzyme-complex mixtures. In a typical experiment, papain (3.5 μM) was incubated with complexes **6**–**9** (100 μM, that is, pseudo-first order conditions) in a DMSO/H_2O 5:95 mixture. The hydrolytic activity of the mixtures was periodically assayed on the chromogenic substrate PFLNa.[30] Semi-logarithmic plots of initial hydrolysis rates versus time were linear and gave the pseudo-first order rate constants of inactivation k_{obs} (Table 1).

It appears that the kinetics of the inactivation process was weakly dependent on the structure of the complex but markedly dependent on the nature of the tethered reactive function. In the malemide series (complexes **6** and **7**), the presence of the phenanthroline ligand had an enhancing effect on the rate of inactivation of papain when the spacer arm between the maleimide ring and the complex was long. For the short chain complexes, the effect was opposite. For the phenanthroline derivatives, shortening of the spacer arm decreased the rate of inactivation by one third. This finding is most certainly related to steric hindrance effects. The presence of the bulky [(η^6-arene)Ru(N–N)Cl]$^+$ entity at close proximity of the reactive maleimide group may slow down the rate of reaction with the thiol of Cys25. No such effect was noticed in the bipy series. Interestingly, all the new maleimide derivatives inactivated papain much faster than NEM. Such a behaviour was previously noticed with our former series of organometallic maleimides and was attributed to the hydrophobic character of the complexes.[1]

Table 1
Irreversible inactivation of papain by complexes **6**–**9**

Compound	k_{obs} (min^{-1})
6a	0.112 ± 0.009
6b	0.090 ± 0.004
7a	0.100 ± 0.004
7b	0.159 ± 0.001
NEM	0.036[a]
8	0.035 ± 0.001
9	0.049 ± 0.003
$FcCOCH_2Cl$	0.028[b]
TPCK[c]	0.121 ± 0.005

Pseudo-first order rate constants k_{obs} measured for [complex] = 100 μM.
[a] *N*-Ethylmaleimide; lit.[33]
[b] Lit.[32]
[c] *N*-*p*-Tosyl L-phenylalanine chloromethyl ketone.

Both chloroacetamides **8** and **9** were also able to inactivate papain in a time-dependent fashion, although no reaction had previously been noticed with cysteine ethyl ester in D_2O (see above). The higher reactivity of papain's active site cysteine towards electrophiles with respect to cysteine and glutathione has been abundantly discussed.[31] It is related to the unusually low pK_a of the thiol of Cys25 (3–4) and the existence of an ion pair with the imidazole side chain of His159 under physiological conditions. The rates of inactivation with **8** and **9** were ca. three times slower that those measured with the maleimide complexes. The phenanthroline complex **9** reacted slightly faster that the bispyridine derivative **8** as in the maleimide series with the longer spacer arm. The pseudo-first order rate constants were in the same order of magnitude as that reported for ferrocenyl chloromethyl ketone.[32] The k_{obs}/[complex] values, respectively, equal to 5.8 and 8.2 M^{-1} s^{-1} for **8** and **9** were only two to three times smaller than that measured for the prototype irreversible inhibitor TPCK which was equal to 20.2 M^{-1} s^{-1} under the same experimental conditions.

To demonstrate that papain inactivation really resulted from the alkylation of the sulfhydryl group of Cys25, the following experiment was performed. Freshly affinity-purified papain was allowed to react with an excess of **8** until no more enzymatic activity was detected for the mixture. After extensive dialysis to remove excess reagent, the solution was concentrated and analyzed by ESI-MS. The resulting spectrum (Fig. 3) displayed several peaks ranging from m/z 997 to 1494.9, corresponding to different charge states of the protein. The charge state of each peak was first calculated, assuming that neighbouring peaks differ by one positive charge. Then the mass was manually determined for each peak and averaged to yield 23 894 ± 2 Da. It was significantly higher than the theoretical mass of the conjugate resulting from the alkylation of papain's sulfhydryl group (23 882 Da) but very close within experimental error to the mass of the adduct where the chloride ligand is replaced by a formato ligand (23 892 Da). This substitution is likely to occur within the experimental conditions used for the mass analysis (high concentration of formic acid at high temperature). Indeed, the ESI-MS analysis of complex **8** performed in the same conditions showed that this transformation did occur.

In conclusion, several (η^6-arene)ruthenium(II) complexes carrying a maleimide or a chloroacetamide function on the arene ligand were synthesized for the purpose of covalently anchoring procatalytic species into the active site of the cysteine endoproteinase papain. Reactivity studies of the complexes with model cysteine derivatives in water showed that S-alkylation took place only with the maleimide derivatives. Nonetheless, both the maleimide and

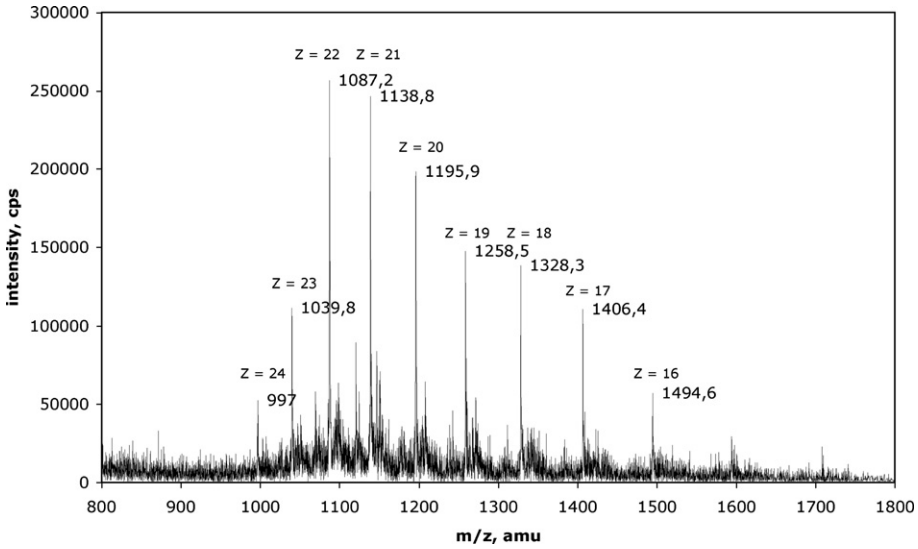

Figure 3. ESI-MS of adduct of papain with complex **8**.

chloroacetamide complexes were shown to behave as irreversible inhibitors of the enzyme, as expected if alkylation of the sulfydryl group of Cys25 occurs. The rates of inactivation were shown to depend on the organometallic entity to some extent with the maleimide derivatives reacting three times faster than the corresponding chloroacetamide derivatives. Preliminary experiments showed that one of the new complexes was not only able to catalyze a transfer hydrogenation reaction in aqueous medium but also accelerated a Diels–Alder reaction in water. Next work will be devoted to study the catalytic properties of the complexes once embedded within papain.

Acknowledgements

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Supplementary data

Experimental synthetic procedures and characterizations of ligands and complexes, enzymatic assay protocols; crystal data and data collection parameters for **8** and **10**. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tetlet.2008.05.043.

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