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ORIGINAL ARTICLE

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Cyclometalated ruthenium(II) complexes as efficient redox mediators in peroxidase catalysis

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Abstract Cyclometalated ruthenium(II) complexes, $[Ru^{II}(C\sim N)(N\sim N)_2]PF_6$ [HC∼N = 2-phenylpyridine (Hphpy) or 2-(4'-tolyl)pyridine; N∼N = 2,2'-bipyridine, 1,10-phenanthroline, or 4,4'-dimethyl-2,2'-bipyridine], are rapidly oxidized by H_2O_2 catalyzed by plant peroxidases to the corresponding Ru^{III} species. The commercial isoenzyme C of horseradish peroxidase (HRP-C) and two recently purified peroxidases from sweet potato (SPP) and royal palm tree (RPTP) have been used. The most favorable conditions for the oxidation have been evaluated by varying the pH, buffer, and H_2O_2 concentrations and the apparent second-order rate constants (k_{app}) have been measured. All the complexes studied are oxidized by HRP-C at similar rates and the rate constants k_{app} are identical to those known for the best substrates of HRP-C (10^6–10^7 M^{-1} s^{-1}). Both cationic (HRP-C) and anionic (SPP and RPTP) peroxidases show similar catalytic efficiency in the oxidation of the Ru^{II} complexes. The mediating capacity of the complexes has been evaluated using the SPP-catalyzed co-oxidation of $[Ru^{II}(phpy)(bpy)_2]PF_6$ and catechol as a poor peroxidase substrate as an example. The rate of enzyme-catalyzed oxidation of catechol increases more than 10,000-fold in the presence of the ruthenium complex. A simple routine for calculating the rate constant k_c for the oxidation of catechol by the Ru^{III} complex generated enzymatically from $[Ru^{II}(phpy)(bpy)_2]^+$ is proposed. It is based on the accepted mechanism of peroxidase catalysis and involves spectrophotometric measurements of the limiting Ru^{II} concentration at different concentrations of catechol. The calculated k_c value of 0.75 M^{-1} s^{-1} shows that the cyclometalated Ru^{II} complexes are efficient mediators in peroxidase catalysis.

Keywords Catechol · Cyclometalated complexes · Plant peroxidases · Redox mediators · Ruthenium complexes

Abbreviations bpy: 2,2'-bipyridine · CTAB: cetyltrimethylammonium bromide · Hphpy: 2-phenylpyridine · HRP-C: isoenzyme C of horseradish peroxidase · Htopy: 2-(4'-tolyl)pyridine · Me_2bpy: 4,4'-dimethyl-2,2'-bipyridine · phen: 1,10-phenanthroline · RPTP: royal palm tree peroxidase · SPP: sweet potato peroxidase

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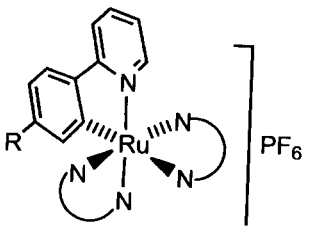
Introduction

Peroxidase (CE 1.11.1.7; donor:hydrogen-peroxide oxidoreductase) is a heme-containing enzyme that catalyzes the oxidation of phenols and aromatic amines (reducing substrates) in the presence of peroxides [1]. The best substrates of peroxidases are ferulic acid, guaiacol, 2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonic acid), *o*-phenylenediamine, *o*-dianizidine, veratryl alcohol, etc. [1, 2]. The peroxidase catalysis obeys a "ping-pong" mechanism [1] according to Eqs. 1, 2, 3:

$$E + H_2O_2 \xrightarrow{k_1} CpdI + H_2O \quad (1)$$



R = H (phpy), Me (topy)

N^N = bpy, phen, 4,4'-Me_2bpy

Fig. 1 Cyclometalated ruthenium(II) complexes used in this work

$$\text{CpdI} + \text{AH}_2 \xrightarrow{k_2} \text{CpdII} + \text{AH}\cdot \quad (2)$$

$$\text{CpdII} + \text{AH}_2 \xrightarrow{k_3} \text{E} + \text{AH}\cdot + \text{H}_2\text{O} \quad (3)$$

Here, E is the ferric enzyme (resting state), Cpd I and Cpd II are Compound I and Compound II, the oxidized intermediates of peroxidase which are by two and one oxidation equivalents above the resting state, respectively, and AH_2 and $AH^{\cdot}$ are an electron donor substrate and the primary radical product of its one-electron oxidation, respectively.

Some substrates are effectively oxidized by peroxidases, whereas the reactivity of others is low. Mediators may, however, increase the rate of oxidation of poor substrates. Mediators are compounds which are oxidized by enzymes first (Eq. 4) and only then, using their oxidative potential, react with substrate molecules to form product(s) (Eq. 5):

$$\text{CpdI/II} + \text{M}_{red} \rightarrow \text{E} + \text{M}_{ox} \quad (4)$$

$$\text{M}_{ox} + \text{AH}_2 \rightarrow \text{M}_{red} + \text{AH}\cdot \quad (5)$$

The oxidized mediator (M_{ox}) is converted to the reduced form (M_{red}) and participates further in catalysis. Thus, a mediator plays a role of an electron shuttle between the active site of the enzyme and a target molecule.

Mediators are widely used in amperometric biosensors [3, 4, 5], where they shuttle electrons between the enzyme active sites and electrodes, and are promising in organic and polymer chemistry [6, 7]. Therefore, the synthesis and thorough characterization of novel redox mediators are of great current interest [8, 9]. Recently, it has been shown that cyclometalated complexes of ruthenium(II) with diimine ligands (Fig. 1) are highly efficient in the mediated electron transfer involving both glucose oxidase/D-glucose and HRP/hydrogen peroxide [10]. In the present work, we report on the mediating properties of the cyclometalated ruthenium(II) complexes containing a σ Ru–C bond and show their high efficacy as mediators for plant peroxidases.

Materials and methods

Materials

Isoenzyme C of HRP (RZ 2.9) was purchased from Sigma. Peroxidases from sweet potato tubers (SPP) (RZ 3.4) and royal palm tree leaves (RPTP) (RZ 2.8) were purified as described previously [11, 12]. H_2O_2 and catechol were obtained from Merck. The ruthenium(II) complexes ($[Ru(phpy)(bpy)_2]PF_6$, $[Ru(phpy)(phen)_2]PF_6$, $[Ru(phpy)(Me_2bpy)_2]PF_6$, $[Ru(topy)(bpy)_2]PF_6$, and $[Ru(topy)(phen)_2]PF_6$) were synthesized and characterized as described elsewhere [10].

Oxidation of ruthenium(II) complexes

The kinetics of peroxidase-catalyzed oxidation of Ru^{II} complexes by H_2O_2 was determined spectrophotometrically as follows. An enzyme solution (10 µL, 3.8×10^{-7} M) was added to 2 mL of 10–100 mM citrate-phosphate buffer (pH 2.7–7.7) containing 0.01–0.1 mM of ruthenium(II) complex, 1 mM CTAB, and 0.25–8.0 mM H_2O_2, and the absorbance change was then measured at 25 °C. Wavelengths and extinction coefficients used for the ruthenium(II) complexes studied are listed in Table 1. The optimal conditions for SPP catalysis with the highest second-order rate constants (k_{app}) were determined by varying the concentrations of H_2O_2 and substrates. The H_2O_2 concentration was determined by monitoring A_{240} using $\epsilon = 43.6\ M^{-1}\ cm^{-1}$ [13]. The required dilutions of H_2O_2 were made daily.

Oxidation of catechol

The kinetics of the SPP-catalyzed oxidation of catechol by hydrogen peroxide in the presence and in the absence of $[Ru^{II}(phpy)(bpy)_2]PF_6$ was studied as follows. A solution of SPP (10 µL, 3.8×10^{-7} M) was added to 2 mL of 10 mM citrate-phosphate buffer (pH 4.5) containing 5 mM catechol, ruthenium(II) complex (0 or 0.1 mM), 1 mM CTAB, and 0.82 mM H_2O_2 and the mixture was incubated at 25 °C. At different time intervals, aliquots were withdrawn and mixed quickly with methanol (2:3 by volume) to

Table 1 Spectral properties and apparent second-order rate constants for the oxidation of ruthenium(II) complexes by H_2O_2 in the presence of plant peroxidases[a]

Ruthenium(II) complex	$E^{o\prime}$ (mV vs. SCE)	λ (nm)	ϵ ($M^{-1}\ cm^{-1}$)	$k_{app}\times10^{-7}$ ($M^{-1}\ s^{-1}$)		
				HRP	SPP	RPTP
$[Ru(phpy)(bpy)_2]PF_6$	280	548	9400	4.3 ± 0.1	0.56 ± 0.07	1.8 ± 0.1
$[Ru(phpy)(phen)_2]PF_6$	280	480	12500	2.4 ± 0.1	0.63 ± 0.07	1.5 ± 0.1
$[Ru(phpy)(Me_2bpy)_2]PF_6$	185	549	8500	2.7 ± 0.2	0.72 ± 0.08	1.8 ± 0.1
$[Ru(topy)(bpy)_2]PF_6$	230	550	7100	1.6 ± 0.1	0.68 ± 0.06	2.3 ± 0.1
$[Ru(topy)(phen)_2]PF_6$	265	480	12800	1.2 ± 0.08	0.24 ± 0.08	1.7 ± 0.15

[a]Experimental conditions: 10 mM citrate-phosphate buffer (pH 4.5), 1 mM CTAB, $[Ru^{II}] = 0.1$ mM, [peroxidase] = 1.5×10^{-10} M, peroxide concentration for HRP, SPP, and RTPT was 3.3, 0.82, and 0.12 mM, respectively; 25 °C

quench the enzymatic reaction. The amount of catechol in the samples was measured by HPLC using a Vydac C18 (4×250 mm) column and a C18 (HP) precolumn. Catechol was detected at 279 nm. A MeOH/H_2O/MeCOOH = 15:85:1 mixture was used as eluent. The elution rate was 1 mL/min.

Quantification of the oxidation of catechol by $[Ru^{III}(phpy)(bpy)_2]^{2+}$

The sweet potato peroxidase-catalyzed co-oxidation of $[Ru^{II}(phpy)(bpy)_2]PF_6$ and catechol by H_2O_2 was carried out as follows. The complex $[Ru(phpy)(bpy)_2]PF_6$ (0.1 mM) and catechol (0–0.5 mM) were dissolved in 10 mM citrate-phosphate buffer (pH 4.5) containing 1 mM CTAB. The reaction was initiated by addition of SPP (10 µL, 3.8×10^{-7} M) and then H_2O_2 (10 µL, 18 mM) to 2 mL of the substrate solution. A decrease in the concentration of the Ru^{II} complex was monitored by measuring the optical density at 548 nm and 25 °C. The final concentration of $[Ru^{III}(phpy)(bpy)_2]^{2+}$ was calculated as the difference between the initial and the final concentrations of $[Ru^{II}(phpy)(bpy)_2]PF_6$.

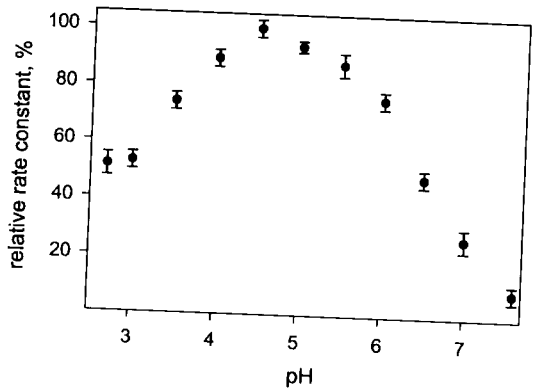


Fig. 2 Effect of pH on horseradish peroxidase activity measured with $[Ru^{II}(phpy)(bpy)_2]PF_6$. Conditions: $[HRP\text{-}C] = 1.5\times10^{-10}$ M; $[H_2O_2] = 0.35$ mM; $[Ru^{II}] = 0.1$ mM; 10 mM citrate-phosphate and phosphate buffers containing 1 mM CTAB

Results and discussion

Optimization of the conditions of the enzymatic reaction

Complexes of ruthenium(II) with diimine and acido ligands have been shown to react readily with oxidoreductases [14, 15, 16]. This is why ruthenium compounds are used as mediators in amperometric biosensors [3, 4, 5]. Recently, a novel synthetic approach to cyclometalated ruthenium(II) complexes containing a σ metal–carbon bond has been introduced [10]. It allows for the synthesis of a variety of complexes with redox potentials in the range 185–280 mV versus SCE, which show significant reactivity toward glucose oxidase and HRP due to a rigid ligand shell imposed by a cyclometalated ligand.

A mediator should be a good substrate of an enzyme. Therefore, the kinetics of peroxidase-catalyzed oxidation of the cyclometalated ruthenium(II) complexes shown in Fig. 1 has been studied (Table 1). Three plant peroxidases, i.e. commercially available HRP-C and two anionic peroxidases purified from the peel of sweet potato tubers [11] and the leaves of royal palm tree [12], have been used. All measurements were carried out in the presence of CTAB detergent for increasing the solubility of the ruthenium complexes, which are marginally soluble in water. CTAB stabilizes the solutions of the complexes in 10 mM citrate-phosphate buffer (pH 4.5) and no precipitation occurs.

It is well documented that the favorable conditions for peroxidase catalysis are determined by the chemical nature of the substrates [12, 17, 18]. Therefore, the pH optimum of the peroxidase-catalyzed oxidation of each complex by H_2O_2 was determined. It was around 4.5 for all complexes studied (Fig. 2), independent of the peroxidase used (SPP, RPTP, or HRP-C). The buffer concentration affected also the efficiency of peroxidase catalysis. In particular, the rate constant for

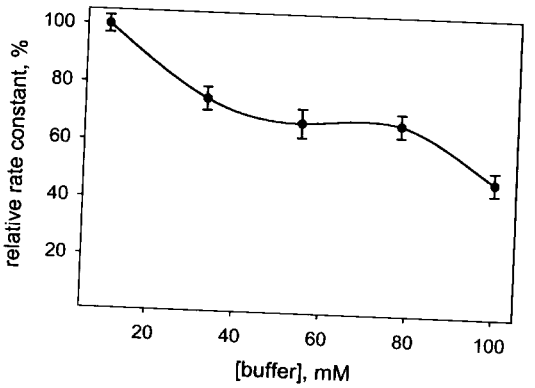


Fig. 3 Effect of citrate-phosphate buffer concentration on horseradish peroxidase activity measured with $[Ru^{II}(phpy)(bpy)_2]PF_6$. Conditions: $[HRP\text{-}C] = 1.5\times10^{-10}$ M; $[H_2O_2] = 0.35$ mM; $[Ru^{II}] = 0.1$ mM; 10–100 mM buffer (pH 4.5) containing 1 mM CTAB

HRP-C-catalyzed oxidation of ruthenium(II) complexes decreases gradually on increasing the citrate-phosphate buffer concentration (Fig. 3). The same effect has been observed for the anionic SPP and RPTP enzymes. Therefore, all further measurements have been carried out in the 10 mM buffer. A similar effect of the buffer concentration was recently reported for other substrates of SPP and RPTP such as guaiacol, 2,2'-azinobis (3-ethylbenzthiazoline-6-sulfonic acid), *o*-phenylenediamine, *o*-dianisidine, and ferulic acid [11, 12].

Hydrogen peroxide is a suicide substrate, because peroxidases are inactivated at higher H_2O_2 concentrations [19, 20, 21]. The mechanism of peroxidase inactivation by H_2O_2 has been described in detail previously [22, 23, 24]. Hence, for each ruthenium(II) complex the concentration of H_2O_2 that ensures the maximal catalytic activity has been determined. It should be noted that the optimal $[H_2O_2]$ did not depend on the nature of the complexes. This was, however, not true for all peroxidases used. The optimal concentration of H_2O_2 for

RPTP was 0.12 mM, whereas the maximal activity of SPP and HRP-C has been measured at 0.82 and 3.3 mM H_2O_2, respectively.

Reactivity of cyclometalated Ru^{II} complexes toward peroxidases

The efficacy of the oxidation of cyclometalated ruthenium(II) complexes by plant peroxidases has been determined under the most favorable conditions. The corresponding second-order rate constants (k_{app}) have been calculated using Eq. 6, since the reaction rate is directly proportional to the concentrations of the enzymes and the complexes at concentrations ranges of 0.1–3 nM and 0.01–0.1 mM, respectively:

$$k_{app} = \frac{\text{rate}}{[S]_0[E]_0} \quad (6)$$

where $[S]_0$ and $[E]_0$ are total concentrations of the ruthenium complex and peroxidase, respectively.

The second-order rate constants k_{app} measured under the steady state correspond to k_3 (Eq. 3) [25]. It is important to note that since Cpd II is normally rate-limiting and the steady-state reaction exhibits second-order kinetics, then reduction of Cpd II also follows overall second-order kinetics.

The results obtained here showed that all Ru^{II} complexes are oxidized by the same peroxidase with similar k_{app} and, therefore, the nature of ligands did not affect much the oxidation rate of the ruthenium complexes. Their redox potentials are too close for revealing any correlation with k_{app} (Table 1). It should be mentioned that a poor correlation for substituted ferrocene was observed when their redox potentials were varied in the range of 450 mV [26]. The data in Table 1 show that HRP-C and RPTP are slightly more active than SPP. The k_{app} values obtained for HRP-C and RPTP equal ca. 10^7 M^{-1} s^{-1}. Similar k_{app} (or k_3) values have been previously reported for the plant and fungal peroxidase-catalyzed oxidation of ferulic acid and 2,2'-azinobis (3-ethylbenzthiazoline-6-sulfonic acid), which are known to be the best peroxidase substrates [12, 17, 27]. Thus, all five studied organometallic ruthenium complexes are considered to refer to very reactive substrates for cationic and anionic plant peroxidases.

Mediating capability of $[Ru^{II}(phpy)(bpy)_2]PF_6$

Since all the ruthenium complexes demonstrate similar reactivity, the mediating efficiency has been evaluated for $[Ru^{II}(phpy)(bpy)_2]PF_6$ with catechol as a poor substrate of SPP [11]. The kinetics of the SPP-catalyzed oxidation of catechol by H_2O_2 was measured using HPLC and has been run under the most favorable conditions for the oxidation of Ru^{II} complexes. A pseudo-first-order rate constant for the enzymatic oxidation of catechol in the absence of $[Ru^{II}(phpy)(bpy)_2]PF_6$ (k_{c-m}) equals 2×10^{-7} s^{-1}. This confirms that catechol is a very poor substrate for SPP. The first-order rate constant in the presence of the ruthenium complex (k_{c+m}) equals 9×10^{-3} s^{-1}, i.e. it is by more than four orders of magnitude higher than that in the absence of the mediator (k_{c-m}). This comparison indicates that the cyclometalated ruthenium(II) complexes with a σ Ru–C bond are both good substrates and efficient mediators for the oxidation of organic compounds by plant peroxidases.

Determination of the second-order rate constant for the oxidation of catechol by enzymatically generated ruthenium(III) species

The kinetics of SPP-catalyzed oxidation of $[Ru^{II}(phpy)(bpy)_2]PF_6$ in the absence of catechol is accompanied by a rapid and significant decrease in absorbance at 548 nm due to the $Ru^{II} \rightarrow Ru^{III}$ transition. When the reaction is complete, the optical density remains unchanged for a long period of time (Fig. 4). The ruthenium(II) complex is also converted into ruthenium(III) in the presence of catechol, but the oxidation is incomplete. It is also seen that the optical density increases gradually after a rapid decrease due to the accumulation of colored products of the catechol oxidation. Remarkably, the degree of the $Ru^{II/III}$ conversion depends systematically on the catechol concentration in the reaction medium and there is an inverse dependence between concentrations of catechol and the ruthenium(III) complex generated (Fig. 4). As will be shown below, this reaction can be used for the quantitative estimation of the second-order rate constant for the oxidation of a poor peroxidase substrate by a mediator.

Our strategy, which is applicable to "poor" peroxidase substrates, is actually based on the mechanism of peroxidase catalysis given by Eqs. 1, 2, 3. The rate-limiting step in the peroxidase catalysis under the steady state is the reaction between Cpd II and a substrate

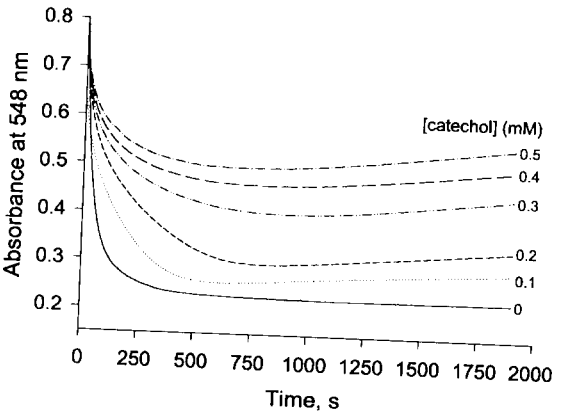


Fig. 4 Kinetics of enzymatic co-oxidation of $[Ru^{II}(phpy)(bpy)_2]PF_6$ and catechol by hydrogen peroxide in the presence of sweet potato peroxidase. Conditions: $[SPP] = 6.7\times10^{-9}$ M; $[H_2O_2] = 0.82$ mM; $[Ru^{II}] = 0.1$ mM; 10 mM citrate-phosphate buffer (pH 4.5) containing 1 mM CTAB

molecule (Eq. 3) [1]. Since catechol (C_{red}) is a "poor" substrate for SPP, our assumption is that Cpd II reacts only with the ruthenium(II) complex and the product of this reaction, viz. Ru^{III}, oxidizes catechol via Eq. 7:

$$Ru^{III} + C_{red} \xrightarrow{k_c} C_{ox} + Ru^{II} \quad (7)$$

An expression for the concentration of ruthenium(III) is then given by Eq. 8:

$$d[Ru^{III}]/dt = k_3[CpdII][Ru^{II}] - k_c[Ru^{III}][C_{red}] \quad (8)$$

Since the rate $= k_{app}[E]_0[Ru^{II}]_0 = k_3[Cpd\ II][Ru^{II}]_0$, it follows that:

$$d[Ru^{III}]/dt = k_{app}[E]_0[Ru^{II}] - k_c[Ru^{III}][C_{red}] \quad (9)$$

If $[C_{red}] \approx [C_{red}]_0$, integration of Eq. 9 using the boundary conditions $[Ru^{III}] = 0$ when $t = 0$ leads to Eq. 10:

$$[Ru^{III}] = \frac{k[Ru^{II}]_0}{k + k_c[C_{red}]_0}\left(1 - e^{-(k + k_c[C_{red}]_0)t}\right) \quad (10)$$

where $k = k_{app}\ [E]_0$. The limiting value of the Ru^{III} complex concentration when $t \to \infty$ is given by Eq. 11:

$$[Ru^{III}]_{lim} = \frac{k[Ru^{II}]_0}{k + k_c[C_{red}]_0} \quad (11)$$

which after rearrangement gives:

$$\frac{[Ru^{II}]_0}{[Ru^{III}]_{lim}} = 1 + \frac{k_c[C_{red}]_0}{k} = 1 + \frac{k_c[C_{red}]_0}{k_{app}[E]_0} \quad (12)$$

Hence, the ratio $[Ru^{II}]_0/[Ru^{III}]_{lim}$ plotted versus $[C_{red}]_0$ should give a straight line, the slope and intercept of which equal $k_c/(k_{app}[E]_0)$ and 1, respectively. The value of k_{app} must be determined in an independent experiment in the absence of catechol. The dependence of $[Ru^{II}]_0/[Ru^{III}]_{lim}$ versus $[C_{red}]_0$ is shown in Fig. 5. The rate constant k_{app} for the oxidation of $[Ru^{II}(phpy)(bpy)_2]PF_6$ by SPP equals $5.6 \times 10^6\ M^{-1}\ s^{-1}$ (Table 1).

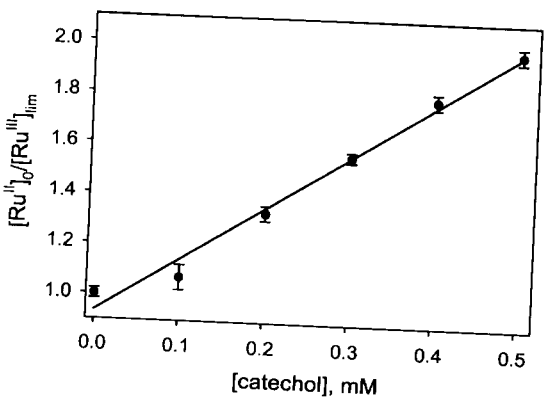


Fig. 5 A plot for determination of the second-order rate constant (k_c) for the reaction between catechol and $[Ru^{III}(phpy)(bpy)_2]^{2+}$ based on Eq. 12

Therefore, the calculated second-order rate constant k_c for the interaction between the Ru^{III} complex and catechol (Eq. 7) equals $0.75 \pm 0.04\ M^{-1}\ s^{-1}$. The presented routine allowed us to quantitatively evaluate the mediating ability of $[Ru^{II}(phpy)(bpy)_2]PF_6$ and might be useful in a search of new mediators of oxidoreductases.

Conclusion

We have demonstrated that the cyclometalated ruthenium(II) complexes containing a σ Ru–C bond are efficient mediators for plant peroxidases and we have developed a simple procedure for estimating the mediating ability of chemical compounds using visible spectroscopy. The second-order rate constant for the oxidation of poor peroxidase substrates by mediators can easily be determined.

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