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Inorganica Chimica Acta 359 (2006) 2285–2290
Inorganica Chimica Acta
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Note

$[Ru^{III}(medtra)(H_2O)]$ (medtra = *N*-methylethylenediaminetriacetate) complex – A highly efficient NO inhibitor with low toxicity

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Received 7 September 2005; received in revised form 19 December 2005; accepted 29 December 2005
Available online 23 February 2006

Abstract

Stopped-flow kinetic measurements were used to compare the reactivities of $[Ru(medtra)(H_2O)]$ ($medtra^{3-}$ = *N*-methylethylenediaminetriacetate) (**1**) and $[Ru(hedtra)(H_2O)]$ (**2**) ($hedtra^{3-}$ = *N*-hydroxyethylethylenediaminetriacetate) with NO in aqueous solution at 15 °C, pH 7.2 (phosphate buffer). The measured second-order rate constants (3×10^3 and 6×10^4 M^{-1} s^{-1} for **1** and **2**, respectively) are three to four order of magnitudes lower than that for the reaction between $[Ru^{III}(edta)(H_2O)]^-$ (**3**) with NO. However, NO scavenging studies of complexes **1**–**3**, conducted by measuring the difference in nitrite production between treated and untreated murine macrophage cells, revealed that despite being less kinetically reactive toward NO, the $[Ru(medtra)(H_2O)]$ complex exhibited the highest NO scavenging ability and lowest toxicity of compounds **1**–**3**.

Keywords: NO scavenging; Ru^{III}-pac complexes; Kinetics; NO binding; Murine macrophages

1. Introduction

The $[Ru^{III}(pac)(H_2O)]$ complexes (pac = polyaminocarboxylate) by virtue of their ability to bind to a number of biological molecules via a rapid aquo-substitution rate command a reputable position as potential metallodrugs [1]. The *pac* ligands form stable 1:1 (metal:ligand) complexes with ruthenium and function as pentadentate ligands (as represented in Fig. 1) in $[Ru^{III}(pac)(H_2O)]$ complexes [2]. The sixth coordination site of the ruthenium centre is occupied by a water molecule at low pH or by a hydroxide ion at high pH [3–6]. Recent studies show that the Ru^{III}-pac complexes exhibited most of the characteristics of an effective NO scavenger in biological systems [7–14]. Until now, $[Ru(edta)(H_2O)]^-$ ($edta^{4-}$ = ethylenediaminetetraacetate) appeared to be the most promising $[Ru^{III}(pac)(H_2O)]$ NO scavenger [9,11,13].

We have undertaken this study to compare the NO scavenging ability of $Ru(medtra)(H_2O)]$ (**1**) ($medtra^{3-}$ = *N*-methylethylenediaminetriacetate) and $[Ru(hedtra)(H_2O)]$ (**2**) ($hedtra^{3-}$ = *N*-hydroxyethylethylenediaminetriacetate) with that of $[Ru^{III}(edta)(H_2O]^-$ (**3**) ($edta^{4-}$ = ethylenediaminetetraacetate) under biological conditions (pH 7.2). The lability of aquo-substituents on **1** and **2** differs from that of **3** in the following order: $[Ru^{III}(edta)(H_2O]^- \gg [Ru(hedtra)(H_2O)] > [Ru(medtra)(H_2O)]$ [2]. Studies with murine macrophages, described herein, reveal that, despite being least kinetically reactive toward NO, $[Ru(medtra)(H_2O)]$ is the most efficient and least toxic NO scavenger under physiological conditions.

2. Experimental

2.1. Materials

The $K_2[RuCl_5(OH_2)]$ complex used as the precursor complex for the preparation of Ru-pac complexes (**1**–**3**) was prepared by the reported method [15]. The complexes

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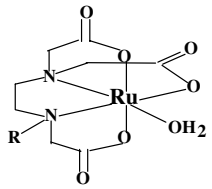
doi:10.1016/j.ica.2005.12.081



1 R = CH_3 ; pac = $medtra^{3-}$ (N-methylethylenediaminetetriacetate)
2 R = CH_2CH_2OH ; pac = $hedtra^{3-}$ (N-hydroxyethylethylenediaminetetriacetate)
3 R = CH_2COO^- ; pac = $edtra^{4-}$ (ethylenediaminetetraacetate)

Fig. 1. Schematic representation of Ru-pac complexes.

$K[Ru^{III}(hedta)Cl]$, $K[Ru^{III}(hedtra)Cl]$ and $K[Ru^{III}(medtra)Cl]$ were prepared by treating $K_2[RuCl_5(H_2O)]$ with the respective ligands as described in the literature [16]. The complexes were characterized by comparing the spectral (UV–Vis and IR) and micro-analysis (C,H,N) data obtained for complexes **1**–**3** with previously reported data [3–6]. All the $K[Ru^{III}(pac)Cl]$ complexes are rapidly aquated when dissolved in water to form their corresponding aquo-species [3–6]. Table 1 summarizes acid-dissociation constants along with spectral and electrochemical data for $[Ru^{III}(pac)(H_2O)]$ complexes.

The NO solution was prepared by dropwise addition of an aqueous solution of H_2SO_4 (2 M) to $NaNO_2$ (solid) under $N_{2(g)}$ in a Kipps apparatus as described in the literature [13]. The gas produced was purged sequentially through NaOH solution (1 M), cold distilled water, and finally a degassed phosphate buffer solution saturated with NO (final concentration estimated [17,18] was $\sim 1.5 \times 10^{-3}$M at 25 °C). All other chemicals used were of A.R. grade. Doubly distilled H_2O was used throughout the experiments.

2.2. Instrumentation

The electronic absorption spectra were measured with a Lambda 35 (Perkin–Elmer) UV–Vis spectrophotometer. The cell compartment was equipped with a PTP-1 Peltier System for temperature control of the test solution. All kinetic measurements were carried out with a SF-61SX2 (HI-TECH) stopped-flow spectrophotometer coupled with an on-line data analyzer (KinetAsyst3). The solution temperature during kinetic experiments was maintained within ±0.1 °C using a circulating water bath (JEIO TECH RW-1025G). The substitution reaction was followed by monitoring changes in absorbance at 300 nm. Rate constant data were measured under pseudo-first order conditions of excess (10–50-fold) substituting ligands. The pH of the solutions was measured with a Mettler Toledo MA 235 pH/Ion analyzer. A phosphate buffer was used to maintain the pH of the solution for the kinetic studies. All the spectral and kinetic measurements were performed under anaerobic conditions. Dilution of a stock solution of NO (1.5×10^{-3}M) was carried out using degassed phosphate buffer solution. The experimentally observed rate constant data (k_{obs}) represented as an average of several kinetic runs (at least five to six) are reproducible within ±4%.

Table 1
Spectral, electrochemical and acid-dissociation constant data for $[Ru^{III}(pac)(H_2O)]$ complexes

Complex	λ_{max} (nm) (ε_{max}/M^{-1} cm^{-1}) in water	$E_{1/2}$ (V vs. NHE)	pK_1	pK_2
$[Ru^{III}(medtra)(H_2O)]$	290 (2400 ± 30) 380 (920 ± 60)	−0.10		6.3
$[Ru^{III}(hedtra)(H_2O)]$	285 (1950 ± 20) 350 (850 ± 20)	−0.07		4.9
$[Ru^{III}(hedta)(H_2O)]$	280 (2800 ± 50) 350 (680 ± 30)	−0.04	2.4	7.6

2.3. Murine macrophage assay for NO scavenging by Ru-pac complex

Swiss albino mice (av. wt. 25–30 g) were used. All animals were housed under standard conditions of temperature (25 ± 5 °C). A 12 h light/dark cycle was maintained and animals were provided with standard pellet diet and water ad libitum. To elicit activated peritoneal macrophages, Swiss albino mice were injected i.p. with starch (2% in autoclaved phosphate buffered saline (PBS), 0.02 M, pH 7.2, 2 ml/animal). After 48 h, the peritoneal cells were lavaged with chilled, sterile PBS (10 ml) and centrifuged at 2000 rpm for 10 min at 4 °C. The resultant cell pellet was washed in PBS and finally re-suspended in complete RPMI-1640 medium supplemented with 10% heat inactivated fetal calf serum (HIFCS). Macrophage viability (>95%) was confirmed by Trypan Blue exclusion.

2.4. Measurement of nitric oxide (NO) production in macrophages

Macrophages obtained by peritoneal lavage, as described above, were seeded (2×10^5 cells/200 μl/ well) in RPMI-1640 (Phenol red free) medium supplemented with 10% HIFCS in 96 well tissue culture plates and incubated at 37 °C, 5% CO_2 for 3–4 h. Ru-pac complexes (**1**–**3**) were co-incubated with lipopolysaccharide (LPS, 10 μg/ml) for an additional 48 h at 37 °C, 5% CO_2. At the end of 48 h, 100 μl of the supernatant was withdrawn from each well and nitrite accumulation as an indicator of NO production was measured using the Griess reagent [8]. Briefly, 100 μl of Griess reagent (1:1 of naphthyl ethylene diamine dihydrochloride (NED), 0.1% in water) and sulfanilamide (1% in 5% orthophosphoric acid) was added to 100 μl of supernatant and incubated in dark at room temperature (25–30 °C for 10 min). Absorbances at 550 nm were measured in a microplate ELISA reader (BioRad model 680). A standard curve using sodium nitrite (0–100 μM) was used to calculate the concentrations of nitrite. To demonstrate specificity, macrophages were incubated in the presence

of *N*-monomethyl arginine (L-NMMA, 0.1 mM), an established inhibitor of NO production. A standard curve was generated using sodium nitrite. The % inhibition of NO production by the Ru^{III}-pac complexes was calculated as follows: $([NaNO_2]_{\text{control macrophages}} - [NaNO_2]_{\text{test macrophages}})/[NaNO_2]_{\text{control macrophages}} \times 100$.

2.5. Cytotoxicity assay

To determine viability of macrophages in the presence of the compounds, macrophages were seeded (2×10^5 cells/200 μl/well) in RPMI-1640 phenol red free medium supplemented with 10% HIFCS in 96-well tissue culture plates and incubated at 37 °C, 5% CO_2 for 3–4 h. The Ru-pac complexes (**1**–**3**) along with LPS (10 μg/ml) were incubated at 37 °C, 5% CO_2 for 48 h. At the end of exposure of the compounds (**1**–**3**), the MTS assay was performed. MTS {3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt, 2 mg/ml} was prepared in PBS and stored in the dark at −20 °C. A stock solution of PMS (phenazine methosulfate, 0.92 mg/ml in PBS) was similarly prepared. After MTS and PMS were mixed in a 5:1 ratio, each well was treated with MTS/PMS mixture (20 μl). The plates were then incubated

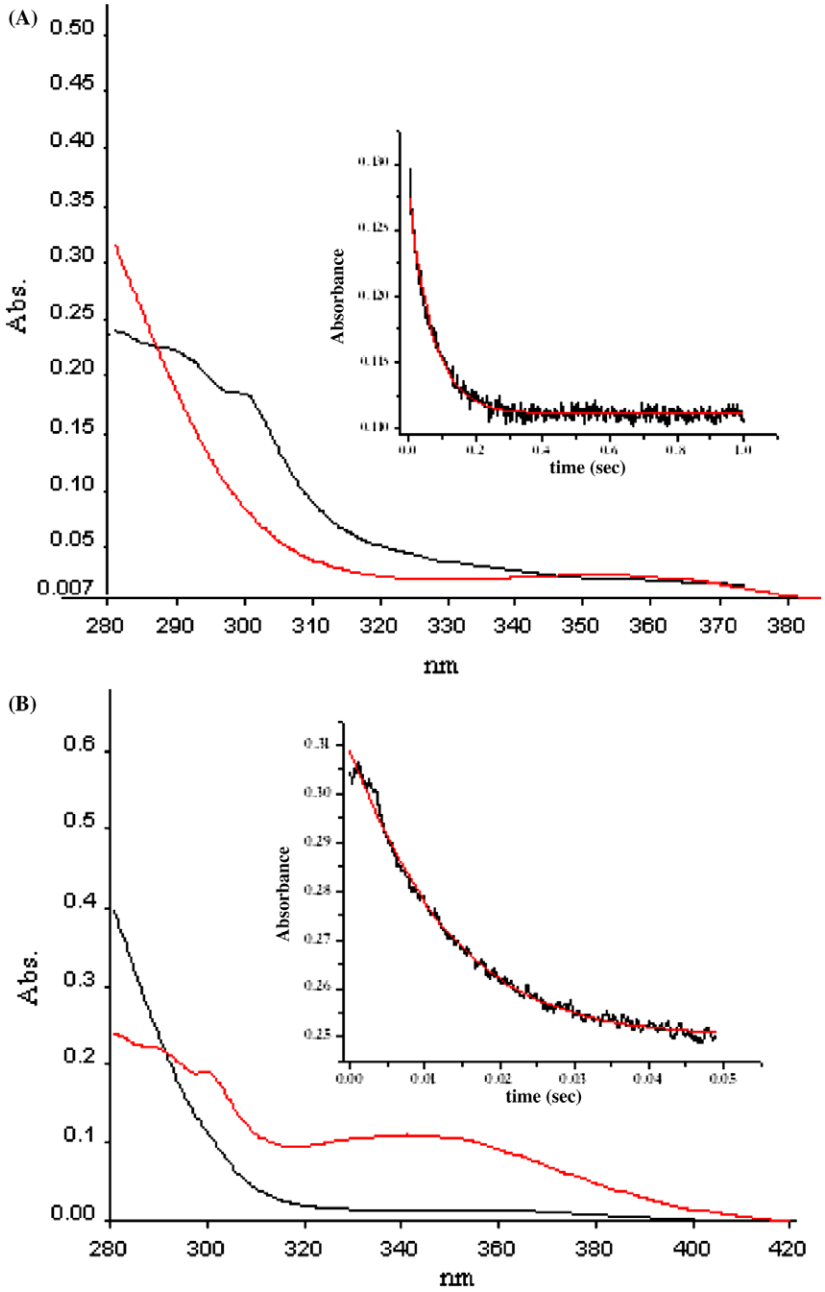


Fig. 2. Spectral changes associated with the reaction of NO with (A) **1** and (B). Inset: Kinetic traces for the reaction of NO with (A) **1** and (B) **2** at 15 °C, pH 7.2 (phosphate buffer), $[Ru^{III}] = 2 \times 10^{-5}$ M, $[NO] = 1.5 \times 10^{-3}$ M.

at 37 °C for 3 h and absorbances measured at 490 nm using a microplate ELISA reader (BioRad model 680). The % viability was calculated as follows: ($^{490}A_{\text{treated macrophages}}$/$^{490}A_{\text{control}}$) × 100, where $^{490}A_{\text{treated macrophages}}$ and $^{490}A_{\text{control}}$ are the specific absorbance of treated macrophages and control macrophages, respectively, at 490 nm. The specific absorbance was calculated by subtracting absorbance of the medium from the total absorbance.

3. Results and discussion

3.1. Kinetics of the interaction of Ru^{III}-pac complexes with NO

The representative example of the spectral changes that occurred upon addition of NO dissolved in degassed phosphate buffer to an aqueous solution of **1** and **2** is shown in the inset of Fig. 2. The kinetics of the interaction of $[Ru^{III}(edta)(H_2O)]^-$ (**3**) with NO in phosphate buffer was reported earlier [9]. Similar, but comparatively much slower kinetic behavior was exhibited by complexes **1** and **2** while reacting with NO under the specified conditions. Kinetic traces (decay at 300 nm) typical of the reaction of **1** and **2** with NO shown in Fig. 2 are single exponential in nature. The values of the observed rate constant (k_{obs}) obtained by performing single exponential analysis of the kinetic traces increased linearly with [NO] with a negligible intercept (Fig. 3), indicating that neither a reverse aquation of the substituted product nor parallel reaction contributes significantly. Considering that $[Ru^{III}(pac)(OH)]^-$ would be the predominating species at pH 7.2 [3–6], the kinetic behavior of the present system can be rationalized in terms of the following reaction (1):

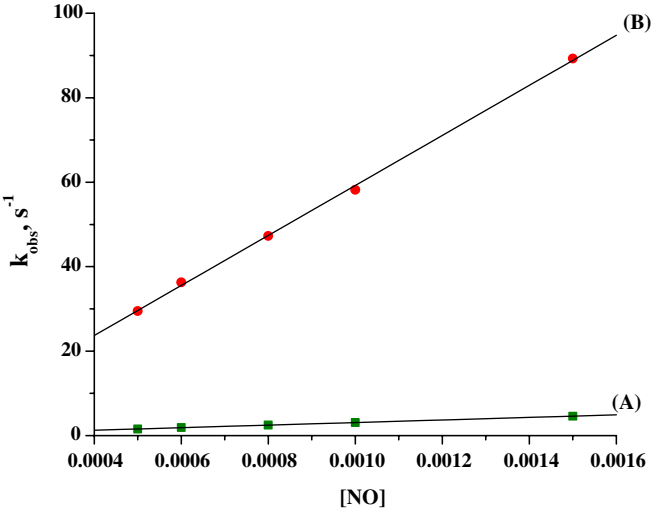


Fig. 3. Plots of k_{obs} vs. [NO] for the reaction of NO with (A) **1** and (B) **2** at 15 °C, pH 7.2 (phosphate buffer), $[Ru^{III}] = 2 \times 10^{-5}$ M, $[NO] = 1.5 \times 10^{-3}$ M.

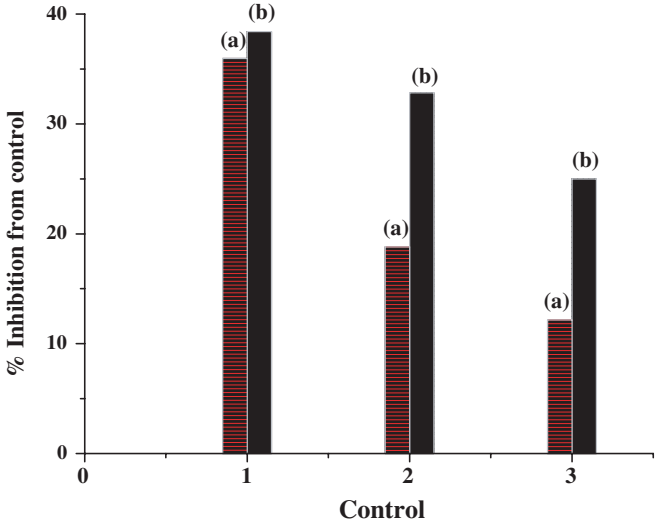


Fig. 4. Inhibition of NO production in murine peritoneal macrophages by Ru^{III}-pac complexes (**1**–**3**, 100 μM) in absence of (a) or presence (b) of LPS (10 μg/ml).

Table 2
Value of second-order rate constant (k) for the formation of $[Ru^{III}(pac)(Nu)]$ in the reaction of $[Ru^{III}(pac)(H_2O)]$ complexes with some selected nucleophiles at 25 °C

Ru^{III}-pac complex	k (M^{-1} s^{-1})					
	Thiourea	Thiocyanate	Azide	Pyridine	Cysteine	Glutathione
$[Ru^{III}(medtra)(H_2O)]$	2.34[a]	0.28[a]	NR	NR	0.3[b]	0.37[b]
$[Ru^{III}(hedtra)(H_2O)]$	22.6[c]	6.8[c]	18.5[c]	18[d]	2.59[e]	1.97[e]
$[Ru^{III}(edta)(H_2O)]^-$	2970[f]	270[g]	1885[f]	6300[g]	170[i]	260[i]
$[Ru^{III}(medtra)(OH)]^-$	0.15[a]	0.04[a]	NR	NR	0.35	0.41
$[Ru^{III}(hedtra)(OH)]^-$	2.8[c]	1.1[c]	2.6[c]	NR	0.6[e]	0.8[e]
$[Ru^{III}(edta)(OH)]^{2-}$	NR	32[f]	75[e]	NR	425[h]	520[h]

[a] Ref. [6].
[b] Unpublished data.
[c] Ref. [5].
[d] Ref. [19].
[e] Ref. [20].
[f] Ref. [4].
[g] Ref. [3].
[h] Ref. [20].
[i] At pH 7.2, Ref. [21]; NR, not reported.

Table 3
Effect of Ru^{III}-pac complexes on NO production in macrophages[a]

Ru^{III}-pac complexes	LPS (−ve)			LPS (+ve)		
	$NaNO_2$ (μM)[c]	% Inhibition[d]	% Viability[e]	$NaNO_2$ (μM)[c]	% Inhibition[d]	% Viability[e]
Control	7.65		100	8.1		100
1[b]	4.9	35.95	90.45	5.3	39.57	100
2[b]	6.21	18.82	76.86	5.44	32.84	68.07
3[b]	6.72	12.16	59.95	6.35	21.60	59.58

[a] See experimental for details.
[b] $[Ru^{III}] = 100$ μM.
[c] A standard curve using sodium nitrite (0–100 μM) was used to calculate concentrations of nitrite.
[d] % Inhibition with Ru^{III}-pac complexes was calculated as described in Section 2.
[e] % Viability of macrophages treated with Ru^{III}-pac complexes was measured as described in Section 2.

$$[Ru^{III}(pac)(OH)] + NO \xrightarrow{k} \underset{(pac = medtra^{3-};\ hedtra^{3-})}{[Ru^{III}(pac)(NO)]} + OH^- \quad (1)$$

for which the following rate expressions (Eq. (2)) is derived.

$$k_{obs} = k_f[NO] \quad (2)$$

The values of the second-order rate constant (k) for the reaction of NO (15 °C and pH 7.2) with **1** and **2** are 3×10^3 and 6×10^4 M^{-1} s^{-1}, respectively. It had been reported earlier [9,12,13] that the rate of the aquo-substitution of $[Ru^{III}(edta)(H_2O/OH)]^{-/2-}$ with NO is very fast ($1.95–3.29 \times 10^7$ M^{-1} s^{-1} at 7.3 °C) in the studied pH range 6.5–8.0. Comparison of the rate constant values (k) obtained in the present studies with that reported for the reaction of Ru-edta complex with NO [9,12,13] revealed the following reactivity order towards NO binding: **3** ≫ **2** > **1**. The $[Ru^{III}(edta)(H_2O/OH)]^{-/2-}$ complex binds with NO at a much higher rate than **1** and **2**, whereas **2** exhibited a higher reactivity than **1**. Table 2 summarizes the previously reported data for the reaction between **1** and **3** and some selected nucleophiles. The data in Table 2 substantiate our results and underscore the significant difference in the rate constants for the aqua complexes as compared to the hydroxo complexes. Within the series of aqua/hydroxo complexes shown in Table 2, the rate constants substantially decrease along the series edta ≫ hedtra > medtra (see Fig. 4).

3.2. NO scavenging activity of $[Ru^{III}(pac)(H_2O)]^-$ complexes

The results (Table 3) of the studies revealed that the addition of **1**–**3** (100 μM) to macrophages reduced the NO_2^- levels in the system both in the presence and absence of LPS, indicating that all three Ru-pac complexes (**1**–**3**) are capable of scavenging NO in biological systems. In the present investigation, the NO scavenging efficiency of **3** was comparable to that reported earlier [13]. The most significant findings were that both complexes **1** and **2**, despite being less reactive than **3** towards NO binding in aqueous solution, lowered the nitrite levels more efficiently than **3**. Although due to its remarkable lability, the $[Ru^{III}(edta)(H_2O)]^-$ complex (**3**) rapidly binds with NO [9,12,13], it also binds sulfur containing bio-molecules at a much faster rate than **1** and **2** (Table 2). The affinity that **3** exhibits for sulfur containing biomolecules may explain its increased cytotoxicity. In contrast, the slower reactivity toward cellular thio-molecules displayed by **1** and **2** may explain why **2** is less toxic than **3** and, most importantly, why **1** exhibits no toxicity at all. While evaluating complexes for use as potential NO scavengers it is important to consider the potential scavenger's affinity for thio-containing molecules to ensure that the decrease in NO production is not due to enhanced cytotoxicity.

4. Conclusion

Results of kinetic studies revealed that **1** and **2** react with NO in a similar manner as reported for the corresponding 'edta' complex (**3**), but at much slower rates. The NO scavenging ability of **1** and **2** has been demonstrated. In both (LPS treated and non-treated) cases, NO scavenging efficiency of **1** and **2** is appreciably higher than that reported for **3**. It may be envisaged that the lower rate of deactivation through binding of cellular thio-molecules could be one of the reasons. Further, the lower efficiency observed in NO scavenging for **3** could be associated with its deactivation by sulfur containing compounds, since **3** binds sulfur containing compounds more readily than does **1** or **2**.

Acknowledgements

We thank Dr. G.P. Sinha, Director of this institute for his encouragement with respect to this work. D.C. gratefully acknowledges financial support from the DST, Govt. of India (Grant SP/S1/F35/99). A.M. is thankful to CSIR for RA.

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