DALTON
FULL PAPER

Bromination of phenol red mediated by vanadium(v) peroxo complexes at pH 6.5

Roxana M. Tótaro,[ab] Patricia A. M. Williams,[c] María C. Apella,*[ab] Miguel A. Blesa[de] and Enrique J. Baran[c]

[a] *Centro de Referencia para Lactobacilos (CERELA-CONICET), Chacabuco 145, 4000-San Miguel de Tucumán, Argentina. E-mail: mapella@cerela.org.ar*
[b] *Universidad Nacional de Tucumán, Miguel Lillo 205, 4000-San Miguel de Tucumán, Argentina*
[c] *Centro de Química Inorgánica (CEQUINOR-CONICET,UNLP), Facultad de Ciencias Exactas, UNLP, C. Correo 962, 1900-La Plata, Argentina*
[d] *Unidad de Actividad Química, Comisión Nacional de Energía Atómica, Avenida del Libertador 8250, 1429-Buenos Aires, Argentina*
[e] *Escuela de Posgrado, Universidad Nacional de San Martín, Avenida General Paz y Constituyentes (Iado Provincia), Buenos Aires, Argentina*

Received 17th July 2000, Accepted 9th October 2000
First published as an Advance Article on the web 16th November 2000

The kinetics of bromination of phenol red (HPhR) to yield bromophenol blue (BrPhB) was studied at pH 6.5, in the presence of peroxovanadium(v) species generated by acid decomposition of $[VO(O_2)_2(NH_3)]^-$ and of $[O\{VO(O_2)_2\}_2]^{4-}$. In the concentration ranges 10^{-6}–10^{-7} (HPhR), (1.5–8.0) × 10^{-4} (vanadium complexes) and 0.004–0.12 mol dm^{-3} (bromide), the rate law is $R = k[V]_T\,[Br^-][HS]$, where HS is the substrate undergoing bromination in the rate determining step, with $k = 2.49 \times 10^5$ dm^6 mol^{-2} s^{-1}. Acid treatment of the precursor complexes yields a mixture of $[VO(O_2)L]^n$ complexes, with L = H_2O, $[VO(O_2)(H_2O)]^+$, or O_2^{2-}, $[VO(O_2)_2]^-$. Alkalinization leads to active species that react with bromide to yield a brominated vanadium complex (*e.g.* $[VO(O_2)Br]$), which is postulated to be the active bromination agent. Kinetic data rule out the mediation of hypobromous acid. The results support the idea that five-co-ordinated vanadium species are required in the bromination reaction.

Introduction

Haloperoxidases are enzymes that catalyse the oxidation of a halide (*i.e.* Cl^-, Br^- or I^-) by hydrogen peroxide, or the halide assisted disproportionation of hydrogen peroxide generating dioxygen. They are referred to as chloroperoxidases, bromoperoxidases or iodoperoxidases, depending on the most electronegative halogen they can oxidize.[1–3] The oxidation potentials of the halides are pH-dependent, and acidic conditions are usually required to oxidize the more electronegative halides. These enzymes are involved in the biosynthesis of many halogenated marine natural products, such as terpenes, indoles, phenols and others, which often have important biological and/or pharmacological activities.[1,4,5]

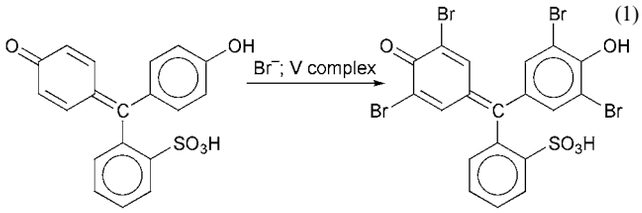
Some of the haloperoxidases contain V in the active site. Vanadium bromoperoxidases (V-BrPO) have been isolated from numerous brown and green algae and also from a terrestrial lichen.[2] They are all acidic glycoproteins with very similar amino acid composition, molecular weight, charge and vanadium content.[3] Preliminary structural studies suggest close similarities with the chloroperoxidase isolated from the fungus *Curvularia inaequalis* (particularly in the active site region), the structure of which has recently been determined.[3] This chloroperoxidase contains trigonal-bipyramidal vanadate, ligated by azide (probably substituted for an OH^- group of the native enzyme[6] during crystallization), three non-protein oxygen atoms and the N-imidazole atom from an histidine residue.[6,7] Vanadium bonding to the protein is strengthened by multiple hydrogen bonding between the vanadate oxygen atoms and positively charged protein residues.[6] It can be removed by dialysis against EDTA in citrate buffer and vanadate can reactivate the enzyme. Treatment of the apoenzyme with phosphate generates phosphatase activity, suggesting evolutionary relationships between peroxidases and phosphatases.[8]

Available mechanistic studies suggest that V-BrPO forms a complex with hydrogen peroxide, which then oxidizes bromide to form an otherwise unknown intermediate that brominates the organic substrate;[2,5] apparently, the redox state of the metal does not change during the catalytic cycle. Hydrogen peroxide can also disproportionate indirectly to H_2O and O_2 in the presence of halides, and can inhibit or inactivate V-BrPO under certain conditions.[5] Thus, the presence of H_2O_2 is crucial to generate the brominating intermediate.[9]

Although the exact nature of the peroxidic intermediate is not known, a large number of model systems mimicking enzymatic activity have been described; these systems are based on the interaction of vanadium centers with peroxo groups.[3,5,8,9]

A number of peroxovanadium(v) complexes carry out a variety of oxidation reactions, including alkene epoxidation and hydroxylation, aromatic alkane and alcohol oxidation, sulfide and halide oxidation, *etc*.[10] Other simple peroxovanadium(v) complexes are also able to brominate organic substrates in acidic or neutral media.[11,12] This biomimetic activity has been related to the simple structure of the active vanadium site in V-BrPO. It has been found that *cis*-VO_2^+ is catalytically active in acidic media, whilst vanadate ($H_2VO_4^-$ + HVO_4^{2-}) is inactive at pH values close to 7. It has also been suggested that activity requires the presence of the dimeric peroxidic species $[\{VO(O_2)\}_2O_2]$.[5]

In the present paper we report results of the study of phenol red (HPhR) bromination to yield bromophenol blue, eqn. (1), by species arising from monomeric and dimeric



(1)

oxodiperoxovanadium(v) complexes, $[VO(O_2)_2(NH_3)]^-$ and $[O\{VO(O_2)_2\}_2]^{4-}$, respectively.

Experimental

All chemicals were reagent grade used as supplied. Doubly distilled water was employed in all experiments.

Preparation of the vanadium complexes

The ammonium salts of the two complexes, $NH_4[VO(O_2)_2(NH_3)]$ and $[NH_4]_4[O\{VO(O_2)_2\}_2]$, were prepared using well known procedures.[13,14]

Kinetic experiments

These were performed by monitoring the changes in absorbance at 595 nm, corresponding to a maximum of bromophenol blue (BrPhB), on a Shimadzu UV-300 spectrophotometer provided with a thermostatted stopped-flow accessory. Measurements were done at 25 °C, pH 6.5 (phosphate buffer) and ionic strength 1.0 mol dm^{-3} (NaCl). Two types of experiments were performed: (a) the reagents were dissolved in a medium of pH 6.5, and the reaction was started by mixing a solution containing vanadium complex and bromide with a solution of phenol red; (b) the vanadium complexes were dissolved in a strongly acidic solution (pH 1), and stored for five minutes prior to mixing with a bromide and phenol red containing solution of pH 6.5. Owing to the employed concentrations, the pH of the reaction mixture was again 6.5. Incubation times larger than 5 min led to the same results, and our experimental conditions precluded the possibility to explore shorter times. The concentrations of the reagents were varied in the ranges 10^{-6}–10^{-7} (phenol red), $(1.5–4.0) \times 10^{-4}$ (vanadium complexes) and 0.00375–0.12 mol dm^{-3} (bromide). The main source of error in the kinetic measurements was the deviations of bromide and vanadium concentrations from the nominal values. Duplicate kinetic runs established an upper limit of 8% for this error. Errors associated with deviations from linear behaviour during data treatment were found to be negligible.

Spectral characterization

Visible spectra were recorded at room temperature on a diode-array Hewlett-Packard 8452-A spectrophotometer.

Results and discussion

Direct reaction at pH 6.5

No bromination reaction is observed with either vanadium complex. Neither the monomeric species, nor the dimeric one, is able to generate oxidized bromine species. The species $[VO(O_2)_2(NH_3)]^-$ and $[O\{VO(O_2)_2\}_2]^{4-}$ are kinetically stable at this pH value and cannot interact with Br^-.

Reaction at pH 6.5 after incubation of the vanadium solution at pH 1

In this case the reaction proceeds smoothly. Under our experimental conditions, the reaction is first order in the concentration of each of the reagents (organic substrate, bromide and vanadium complex). The first order dependency on the organic substrate is demonstrated by the linearity of plots of ln $[(A - A_\infty)/(A_0 - A_\infty)]$ *vs.* time (see Fig. 1), where A, A_∞ and A_0 are the absorbances at 595 nm, measured at time t, $t \longrightarrow \infty$, and $t = 0$, respectively (at $t = 0$ the absorbance is already appreciable). This first order dependence holds for the following molar concentration ranges: phenol red (initial) $< 10^{-6}$, $0.00375 < [Br^-] < 0.12$, and $1.5 < [V] \times 10^4 < 8$.

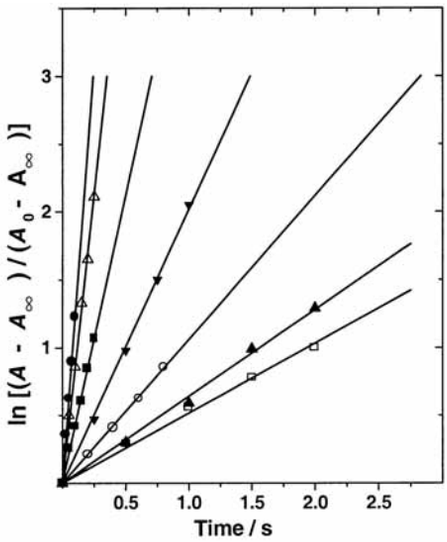


Fig. 1 Typical first order plot, ln $[(A - A_\infty)/(A_0 - A_\infty)]$ *vs.* time. The lines correspond to various Br^- concentrations: (□) 3.75×10^{-3}; (▲) 5.0×10^{-3}; (○) 7.5×10^{-3}; (▼) 1.50×10^{-2}; (■) 3.00×10^{-2}; (△) 6.00×10^{-2}; (●) 9.00×10^{-2} mol dm^{-3} at pH 6.5 and 25.0 °C. $[O\{VO(O_2)_2\}_2^{4-}] = 3.0 \times 10^{-4}$ mol dm^{-3}; $\lambda = 595$ nm. Similar behaviour is found at other $[O\{VO(O_2)_2\}_2^{4-}]$ values, and when $[VO(O_2)_2(NH_3)]^-$ is substituted for $[O\{VO(O_2)_2\}_2]^{4-}$.

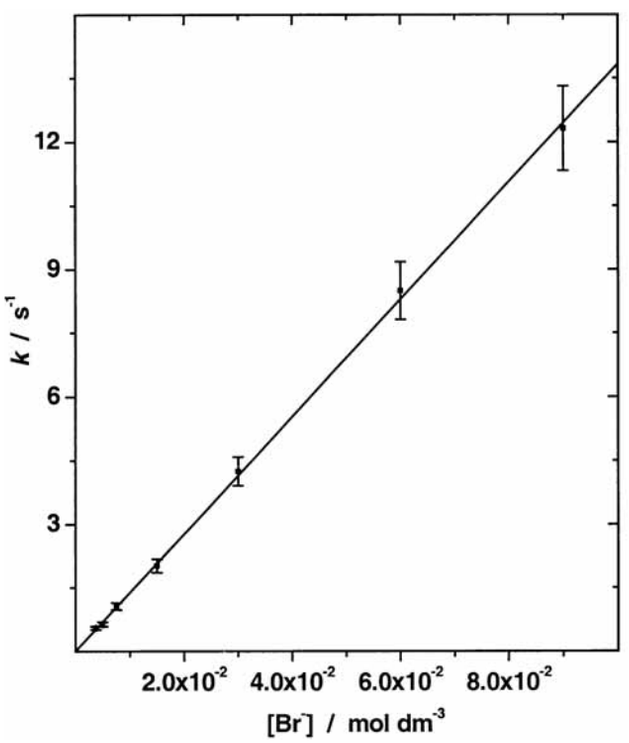


Fig. 2 Dependence of the pseudo first order rate constants derived from Fig. 1 on $[Br^-]$; $[V^V] = 6.0 \times 10^{-4}$ mol dm^{-3}. The straight line yields $R = 0.9997$. Similar results are obtained when $[Br^-]$ is varied at constant [V], and when $[VO(O_2)_2(NH_3)]^-$ is substituted for $[O\{VO(O_2)_2\}_2]^{4-}$.

The rate constants derived from the pseudo-first order plots on the concentrations vary linearly with $[Br^-]$ at constant vanadium concentration, and *vice versa*. Fig. 2 shows an example. From these results, the rate law (2) is derived, where

Table 1 Pseudo second order rate constants, in experiments performed by keeping constant either $[Br^-]$ or $[V^V]$

Series	Vanadium complex	10^4 $[V^V]$/ mol dm^{-3}	10^3 $[Br^-]$/ mol dm^{-3}	10^{-2} Pseudo second order rate constant[a]/ dm^3 mol^{-1} s^{-1}	R[a] (correlation factor)
1	$[VO(O_2)_2(NH_3)]^-$	1.5–4.0	7.5	34.2 ± 2.7	0.9945
2	$[VO(O_2)_2(NH_3)]^-$	3.0	3.75–120	0.82 ± 0.06	0.9992
3	$[O\{VO(O_2)_2\}_2]^{4-}$	3.0–8.0	3.75	9.19 ± 0.74	0.9985
4	$[O\{VO(O_2)_2\}_2]^{4-}$	6.0	3.75–90	1.38 ± 0.11	0.9997

[a] From the slope of the line in Fig. 2, or in other equivalent figures, not shown. The relatively high errors in the rate constants reflect the uncertainties in the reagent concentrations.

$$R = k[V]_T[Br^-][HS] \quad (2)$$

HS is the substrate undergoing bromination in the rate determining step (see below).

The main product of the bromination of phenol red is the tetrabromo-derivative (bromophenol), see eqn. (1); four successive bromination reactions are involved. The actual rate determining step in the course of the successive four bromination reactions is not known, and cannot be derived from our measurements. Several reaction mechanisms can be envisaged that lead to first order kinetics; these differ in the identification of the most sluggish bromination step and hence of the nature of HS (*e.g.* phenol red or any of its partially brominated derivatives). Although in principle it is expected that the fourth bromination is the slowest reaction, k may in principle be linked with the reactivity of any of several species. This ambiguity is however irrelevant for our purposes, insofar as the substrate is the same in all experiments.

The values for k derived from the different sets of experiments shown in Table 1 are, respectively (4.55 ± 0.36; 2.72 ± 0.22; 2.45 ± 0.20 and 2.31 ± 0.19) × 10^5 dm^6 mol^{-2} s^{-1}. Except for the first value, good agreement is obtained from the different sets of experiments, and it may be assumed that a single rate constant $k = (2.49 \pm 0.22) \times 10^5$ dm^6 mol^{-2} s^{-1} suffices to describe both systems. The origin of the discrepancy in the first series of experiments is not clear, although it might be related to a large systematic error in the bromide concentration. In agreement, the common experimental point in Series 1 and 2 deviates most from the straight line corresponding to Series 2. Series 1 shows also a somewhat more important scatter of the data (see R values in Table 1), and is also composed of a low number of individual data points (four). Consequently, we have disregarded Series 1 results in our analysis.

A possible mechanism implies the participation of a common intermediate, generated by the attack of acid on the vanadium complexes. Acidification of $[VO(O_2)_2(NH_3)]^-$ or $[O\{VO(O_2)_2\}_2]^{4-}$ solutions originates red species; Fig. 3 shows the absorption spectrum of $[VO(O_2)_2(NH_3)]^-$ after exposure to acid of pH 1; a band centred around 450 nm with $\varepsilon = 215$ dm^3 mol^{-1} cm^{-1} is observed. Similar treatment of $[O\{VO(O_2)_2\}_2]^{4-}$ yields the same spectrum, albeit with a somewhat higher molar absorptivity ($\varepsilon = 270$ dm^3 mol^{-1} cm^{-1}). This band is characteristic of a charge transfer transition in $[VO(O_2)L]^n$ complexes,[15–17] and we assume that in both cases a mixture of the species with L = H_2O, $[VO(O_2)(H_2O)]^+$, and O_2^{2-}, $[VO(O_2)_2]^-$, is formed.[5] The actual number of coordinated water molecules in $[VO(O_2)]^+$ is not known; the species is probably five-co-ordinated.[18,19] The release of NH_3 from $[VO(O_2)_2(NH_3)]^-$ is quantitative at pH 1 and negligible at pH > 2 (eqn. (3)). Hydrogen peroxide is also released to generate the monoperoxo complex (eqn. (4)).

$$[VO(O_2)_2(NH_3)]^- + H^+ \longrightarrow [VO(O_2)_2]^- + NH_4^+ \quad (3)$$

$$[VO(O_2)_2(NH_3)]^- + 3H^+ \longrightarrow [VO(O_2)]^+ + H_2O_2 + NH_4^+ \quad (4)$$

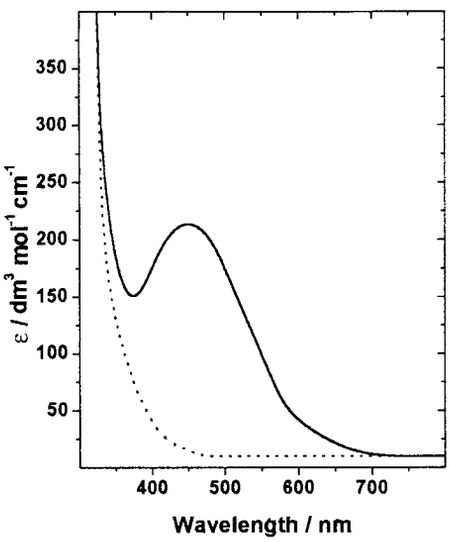


Fig. 3 Visible absorption spectrum of the species formed from $[VO(O_2)_2(NH_3)]^-$ at pH 1 (full line). For comparison, the spectrum at pH 5 is also shown (dashed line).

In the case of $[O\{VO(O_2)_2\}_2]^{4-}$ irreversible breakage of the oxo-bridge takes place, alongside release of hydrogen peroxide in the case of formation of the monoperoxo complex (eqns. (5) and (6)).

$$[O\{VO(O_2)_2\}_2]^{4-} + 2\ H^+ \longrightarrow 2\ [VO(O_2)_2]^- + H_2O \quad (5)$$

$$[O\{VO(O_2)_2\}_2]^{4-} + 6\ H^+ \longrightarrow 2\ [VO(O_2)]^+ + 2\ H_2O_2 + H_2O \quad (6)$$

Alkalinization of the resulting solution leads to unknown vanadium complexes. It has been proposed that the active species for halide oxidation is the μ-peroxo dimer, $[\{VO(O_2)\}_2O_2]$.[5,20] In our systems free oxidized bromine species are not observed; only phenol red bromination to yield bromophenol blue is detected. The kinetic data at pH 6.5 are compatible with equilibrated complexation of a vanadium complex by bromide, followed by irreversible bromination of phenol red, eqns. (7) and (8). For simplicity we shall assume

$$Br^- + [VO(O_2)]^+ \rightleftharpoons [VO(O_2)Br] \quad ; K_7 = k_7/k_{-7} \quad (7)$$

$$H^+ + [VO(O_2)Br] + HS \longrightarrow VO_2^+ + BrPhB + H_2O\ ;\ k_8 \quad (8)$$

that the vanadium complex is $[VO(O_2)]^+$, although other species, such as the mentioned dimer, cannot be excluded (see below). The third order rate constant was calculated on the assumption that the dimer breaks down to yield two monomers. The kinetic results preclude simultaneous occurrence of both monomers and dimers, since the shift in the dimerization equilibrium with increasing vanadium concentration would

otherwise result in a more complex kinetic behaviour. As mentioned before, the product of reaction (8) may in fact be a precursor to bromophenol blue, that evolves rapidly to this species in the reaction medium. For $K_7 \ll 1$ and $k_{-7} \gg k_8$ $[H^+][HS]$ the derived kinetic law is given by eqn. (9), that agrees

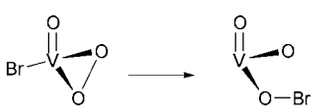
$$d[BrPhB]/dt = k'_8 (k_7/k_{-7}) [HS][Br^-][V] \quad (9)$$

with the empirical expression (2), with $k = k'_8 (k_7/k_{-7})$. The rate constant $k'_8 = k_8 f[H^+]$ is an undefined function of acidity; our experiments, at constant pH, do not give information about the participation of H^+ in the activated state.

This mechanism suggests that phenol red bromination is mediated by halide co-ordination, forming $[VO(O_2)Br]$. Our data rule out the possible formation of hypobromous acid, either directly or from this complex in the slow step of the reaction; such a scheme would require a zero order in the concentration of organic substrate. In other cases, the latter behaviour is observed;[21] a possible origin of the discrepancy is the low HPhR concentration used by us. At higher concentrations the condition $k_8 [H^+][HS] \gg k_{-7}$ may be valid, leading to the kinetic law (10).

$$d[BrPhB]/dt = k_7[Br^-][V] \quad (10)$$

The intermediate $[VO(O_2)Br]$ has been postulated by other authors,[5,22] who suggest that bromination is mediated by a previous internal reorganization, leading to the formation of co-ordinated hypobromite, eqn. (11). Assuming that the active



(11)

species is $[VO(O_2)]^+$, our results agree with this possibility, and also suggest that the initial site of attack of bromide on $[VO(O_2)]^+$ must be the vanadium atom. This conclusion is borne out by the need to generate previously $[VO(O_2)]^+$ from either $[VO(O_2)_2(NH_3)]^-$ or $[O\{VO(O_2)_2\}_2]^{4-}$ in strongly acid media (pH ≈ 1); six-co-ordinated V atoms, as in the precursor complexes, are not active.

In opposition to the behaviour of the two investigated starting complexes, vanadium bromoperoxidases do not require any previous transformation to develop catalytic activity under physiological conditions. Seemingly, both the possibility of stabilization of the pyramidal $[VO(O_2)L]^n$ moiety (L = bidentate chelate) and the operation of conditions that define k_{13} $[H^+][HS] \gg k_{-12}$, are involved, eqns. (12) and (13).

$$[VO(O_2)L]^n + Br^- \rightleftharpoons [VO(O_2)L(Br)]^{(n-1)};\ K_{12} = k_{12}/k_{-12} \quad (12)$$

$$H^+ + [VO(O_2)L(Br)]^{(n-1)} + HS \longrightarrow [VO_2L]^n + BrS + H_2O;\ k_{13} \quad (13)$$

Reactions (12) and (13) are analogous to (7) and (8). In the case of bromoperoxidases the catalytic cycle is completed with reaction (14); it is well known that hydrogen peroxide regenerates the catalytic activity of vanadium(V) peroxidases.[23]

$$[VO_2L]^n + H_2O_2 \longrightarrow [VO(O_2)L]^n + H_2O \quad (14)$$

Acknowledgements

This work was supported by the University of Tucumán, CONICET, CIC-PBA and ANPCT (PICT 00119). R. M. T. thanks CONICET for a fellowship. M. C. A., M. A. B. and E. J. B. are members of the Research Career from CONICET. P. A. M. W. is a member of Research Career from CIC-PBA.

References

1 S. L. Neidelman and J. Geigert, in *Biohalogenation. Principles, Basic Roles and Applications*, Ellis Horwood Ltd., New York, 1986.
2 A. Butler, in *Bioinorganic Catalysis*, ed. J. Reedijk, Marcel Dekker, New York, 1993, pp. 425–445.
3 A. Butler and A. H. Baldwin, *Struct. Bonding* (*Berlin*), 1997, **89**, 109.
4 A. Butler and J. V. Walker, *Chem. Rev*., 1993, **93**, 1937.
5 A. Butler, *Coord. Chem. Rev.*, 1999, **187**, 17.
6 A. Messerschmidt and R. Wever, *Proc. Natl. Acad. Sci. U.S.A.*, 1996, **93**, 392.
7 A. Messerschmidt, *Struct. Bonding* (*Berlin*), 1998, **90**, 37.
8 D. Rehder, *Coord. Chem. Rev.*, 1999, **182**, 297.
9 V. Conte, O. Bortolini, M. Carraro and S. Moro, *J. Inorg. Biochem.*, 2000, **80**, 41.
10 A. Butler, M. J. Clague and G. E. Meister, *Chem. Rev.*, 1994, **94**, 625.
11 V. Conte, F. Di Furia and S. Moro, *Tetrahedron Lett.*, 1994, **35**, 7429.
12 R. I. de la Rosa, M. J. Clague and A. Butler, *J. Am. Chem. Soc.*, 1992, **114**, 760.
13 C. A. Strydon and D. de Wall, *J. Therm. Anal.*, 1992, **38**, 943.
14 R. Stomberg, S. Olson and I. B. Svenson, *Acta Chem. Scand.*, *Ser. A*, 1984, **38**, 653.
15 M. Orhanovic and R. G. Wilkins, *J. Am. Chem. Soc.*, 1967, **89**, 278.
16 C. Djordjevic, S. Craig and E. Sinn, *Inorg. Chem.*, 1985, **24**, 1281.
17 C. Djordjevic, B. Puryear, N. Vuletic, C. Abelt and S. Sheffield, *Inorg. Chem.*, 1988, **27**, 2926.
18 O. Howard and J. Hunt, *J. Chem. Soc.*, *Dalton Trans.*, 1979, 1388.
19 J. Jaswal and A. Tracey, *Inorg. Chem.*, 1991, **30**, 3722.
20 M. J. Clague and A. Butler, *J. Am. Chem. Soc.*, 1995, **117**, 3475.
21 G. Colpas, B. Hamstra, J. Kampf and V. Pecoraro, *J. Am. Chem. Soc.*, 1996, **118**, 3469.
22 M. J. Clague, N. L. Keder and A. Butler, *Inorg. Chem.*, 1993, **32**, 4754.
23 M. G. M. Tromp, G. Olafsson, B. E. Krenn and R. Wever, *Biochim. Biophys. Acta*, 1990, **1040**, 192.