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# Kinetic and thermodynamic studies of the ligand substitution reactions of the cobalamins

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# CHAPTER 2

## EXPERIMENTAL

### 2.1 Buffers

The buffers used throughout this work are tabulated in Table 2.1. The standard buffers used for calibrating the pH meter were potassium hydrogen phthalate, pH 4.004; potassium dihydrogen phosphate/dipotassium hydrogen phosphate, pH 6.863; borax, pH 9.183; at 25 ºC. (Adjusted from potassium hydrogen phthalate, pH 4.0; potassium dihydrogen phosphate, pH 4.4; dipotassium hydrogen phosphate, pH 9.1; borax, pH 9.1; at 20 ºC).[1]

**Table 2.1** Buffers used.

| Buffer | p$K_a$ | pH Range | Supplier |
|---|---|---|---|
| MES | 6.1 | 5.5–6.7 | Sigma |
| MOPS | 7.2 | 6.5–7.9 | Sigma |
| HEPES | 7.5 | 6.8–8.2 | Sigma |
| Tris/HCl | 8.1 | 7.0–9.0 | BDH |
| CHES | 9.3 | 8.6–10.0 | Sigma |
| CAPS | 10.4 | 9.7–11.1 | Sigma |

### 2.2 pH Measurements

The pH of all solutions were measured with a Metrohm 605 pH meter and a 6.021 00.100 combination glass electrode calibrated against standard buffers – all maintained at the appropriate temperature with a water-circulating bath.

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## 2.3 Data Analysis

All results were analysed with the computer programme FIT[2], which employs a Newton–Raphson procedure.

## 2.4 p$K_a$ Determinations

The p$K_a$ for the proton dissociation of the water ligand was determined spectroscopically for aquacobalamin, aqua-10-chlorocobalamin, aqua-10-nitrosocobalamin, and aquacobalamin in 70% ethanol. Cells with a 2.00 cm pathlength were used in a thermostatted cell holder of a Cary 3E spectrometer. The p$K_a$ determination was monitored at 350 nm for aquacobalamin, 355 and 565 nm for aqua-10-chlorocobalamin, 347 and 350 nm for aqua-10-nitrosocobalamin and at 522 nm for aquacobalamin in a 70% ethanol solution. The concentrations of the solutions were in the range 20–25 μmol $dm^{-3}$ with $I$ = 0.5 M ($NaNO_3$ or $NaClO_4$), 0.01 MOPS, 0.01 M Tris/$HNO_3$ and 0.01 CHES buffers. 10 ml of a solution of the relevant compound at pH 6.0 was placed in a glass cell that had thermostatted water circulating through it to maintain the temperature. A peristaltic pump (Watson Marlow 101U) was then used to transfer 2.5 ml of the solution into the cuvette in the spectrometer where the absorbance value for the relevant pH was obtained. The solution was then transferred back to the glass cell where a drawn-out capillary tube was used to add a small amount of concentrated NaOH solution. This was repeated until a pH of 10.5 was reached for aquacobalamin in both the water and 70% ethanol solutions, a pH of 9.4 for aqua-10-chlorocobalamin and a pH of 12.0 for aqua-10-nitrosocobalamin. The procedure was carried out at four temperatures ranging between 5 and 35 ˚C for each of the p$K_a$ determinations, except that of aquacobalamin in 70% ethanol which was only performed at 25 ˚C. This was due to problems encountered from the slow response time of the electrode in the ethanol solution as well as the deprotonation of ethanol, occurring after a few hours at high concentrations of NaOH, resulting in a colour change of the solution from clear to yellow. The

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experimental data were fitted to an ionisation isotherm (Equation 2.1), which is relevant when only one acid/base equilibrium is present.

$$A_T = A_0\left[\frac{1}{1+\frac{K_a}{[H^+]}}\right] + A_1\left[\frac{1}{1+\frac{[H^+]}{K_a}}\right] \qquad (2.1)$$

In Equation 2.1, $A_T$ is the absorbance at the monitoring wavelength, $K_a$ is the acid dissociation constant, $A_0$ the initial absorbance and $A_1$ the final absorbance. This was performed for each of the temperatures at which data were collected. The thermodynamic parameters, $\Delta H$ and $\Delta S$, were then determined from the slope and intercept, respectively, from plots of ln $K_a$ against $1/T$.

## 2.5 Kinetics of the Ligand Substitution Reactions of Aquacobalamin

### 2.5.1 Stopped-flow Spectrometry

Hydroxocobalamin (stored at –20°C; >99% pure, HPLC) was purchased from Roussel. NaOCN (Fluka), $NaN_3$ (Riedel-de-Haën), KSeCN (Sigma), $Na_2S_2O_3$, NaSCN and $NaNO_2$ (Saarchem, South Africa) were of the highest purity available and used as received. Water was purified using a Millipore RO unit and further purified through a Millipore MilliQ system (18 MΩ cm). Preliminary spectroscopic investigations were carried out on a Cary 3E UV-vis spectrometer. All reactions were studied under pseudo-first-order conditions with aquacobalamin concentrations between 50 and 80 μM, buffered with 0.1 M CHES at pH 9.00. The total ionic strength was adjusted to 2.2 M with $NaNO_3$. No spectroscopic evidence was found for the coordination of $NO_3^-$ to aquacobalamin. Ligand solutions were also buffered with CHES and adjusted with $NaNO_3$ in order to obtain the same pH and ionic strength values as that of the aquacobalamin solutions. The ligand concentrations ranged from 0.02–0.2 M for the ligands $SCN^-$, $N_3^-$, $SeCN^-$ and $S_2O_3^{2-}$; 0.04–0.4 M for $NO_2^-$; and 0.2–1 M for $OCN^-$. The kinetics of the reactions was monitored at 375 nm (the absorbance due to $NO_3^-$ is

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negligible). Equal volumes (0.1 $cm^3$) of the two solutions were mixed in a Hi-Tech SF-51 stopped-flow spectrometer (cell pathlength 1.00 cm) interfaced through a DAS-50 A/D board with a personal computer. The layout of the apparatus can be seen in Figure 2.1.



**Figure 2.1** Stopped-flow spectrometer used to collect all the kinetic data in this work.

Pseudo-first-order rate constants, $k_{obs}$, were obtained by curve-fitting the absorbance versus time traces to an equation of the form $A_1\exp(-k_{obs}t) + A_2$, with $A_1$, $A_2$ and $k_{obs}$ as variable parameters, using a non-linear least-squares programme that employs a Newton–Raphson procedure. An example of such a trace for the $OCN^-$ ligand is shown in Figure 2.2.

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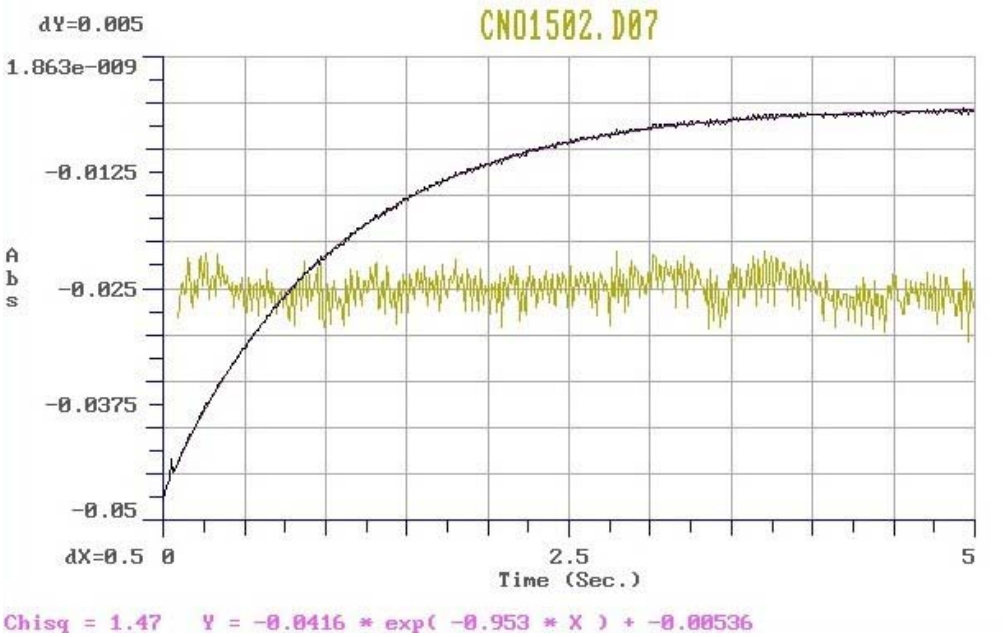



**Figure 2.2** An example of a trace recorded for the $OCN^-$ ligand with the fit obtained using a non-linear least-squares programme that employs a Newton–Raphson procedure. The yellow trace represents a plot of the residuals.

At least six half-lives were monitored and fitted for each ligand concentration and these recorded traces were averaged to obtain the final $k_{obs}$ value. A water-circulating bath was used to maintain the temperature (± 0.1 $^{o}$C) of the system and each ligand was studied at at least four temperatures. Since hydroxocobalamin is inert to substitution,[3,4] values of $k_{obs}$ were converted into a pH-independent value, $k$, using Equation 2.2,

$$k = k_{obs}(1+10^{pH-pK_{Co}}) \qquad (2.2)$$

where $K_{Co}$ is the acid dissociation constant for coordinated $H_2O$ in aquacobalamin, corrected for its temperature variation, p$K_{Co}$ 8.09 (25 $^{o}$C), $I$ = 0.5 M, $NaNO_3$; $\Delta H$ = 36.0 ± 1.9 kJ mol$^{-1}$, $\Delta S$ = –34 ± 6 J K$^{-1}$ mol$^{-1}$ (see Chapter 3). Since the p$K_L$ of the ligands studied were in the region of 4.7 and below, they did not have to be included in the calculation as they occur unprotonated and will coordinate to aquacobalamin at the operating pH value.

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### 2.5.2 Molecular Orbital Calculations

Molecular orbital calculations were performed using HYPERCHEM V. 4.5.[5] The species were initially geometry-optimised by semi-empirical molecular orbital calculations using the PM3 model.[6,7] This was followed by full geometry optimisation using an *ab initio* method at the Hartree–Fock level of theory with the STO-6G* minimal basis set,[8] the Raffenetti integral format,[9] a self-consistent field (SCF) convergence limit of $10^{-5}$ kcal $mol^{-1}$, and an energy convergence criterion of 0.01 kcal $mol^{-1}$ for the root-mean-square gradient. More sophisticated basis sets such as split-valence or polarised basis sets could not be used because of the lack of parameters for Se.

## 2.6 Solvent Effects

Pyridine (Merck) solutions in 70% ethanol in the concentration range 0.2–2.0 M were buffered with MOPS (0.1 M) in order to maintain a pH of 7.00. Aquacobalamin solutions (50–80 μM) were also made up in 70% ethanol and buffered with MOPS (0.1 M). Stopped-flow kinetics were performed with the aquacobalamin and pyridine solutions in the same manner as described in Section 2.3.1 at four temperatures (5.0, 15.0, 25.0 and 35.0 ºC).

## 2.7 Kinetics of the Ligand Substitution Reactions of Iodocobalamin

Aquacobalamin solutions (*ca*. 50 uM) were prepared with NaI (Saarchem) in order to form the compound iodocobalamin. Sufficient NaI was added to aquacobalamin to ensure >97% complex formation (log *K* for coordination of $I^-$ by aquacobalamin = 1.5[10]). Ligand solutions of imidazole (Merck), $N_3^-$ and $S_2O_3^{2-}$ were prepared in the concentration ranges 0.2–2.0 M for $N_3^-$ and imidazole, and 0.2–0.7 M for $S_2O_3^{2-}$. All solutions were buffered at pH 7.00 with MOPS (0.1 M) and the ionic strength was maintained at 2.2 M with $NaClO_4$. The kinetics were studied as previously described in Section 2.3.1 at a wavelength of 560 nm. The ligand substitution reactions of aquacobalamin with imidazole and

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$S_2O_3^{2-}$ were studied at the temperatures 10.0, 15.0, 20.0 and 25.0 ºC and with $N_3^-$ at 10.0, 15.0, 25.0 and 30.0 ºC.

## 2.8 Formation Constants of Aquacobalamin and Aqua-10-chlorocobalamin

The formation constants were determined for both aquacobalamin and aqua-10-chlorocobalamin with the anionic ligands: $NO_2^-$, $SCN^-$, $S_2O_3^{2-}$, $SeCN^-$, $OCN^-$ and $N_3^-$, and the neutral ligands: imidazole, pyridine and methylamine (Aldrich). Professor Kenneth L. Brown and Dr Xiang Zou of the University of Ohio supplied the aqua-10-chlorocobalamin and the synthesis can be found in Ref. 11. A series of solutions were prepared by addition of an aliquot of a stock solution of the ligand to the cobalt corrin (either aquacobalamin [*ca*. 50 μM] or aqua-10-chlorocobalamin [*ca*. 30 μM]). The solutions were buffered at the appropriate pH (this was dependent on the pH of the ligand so that maximum complex formation could be obtained i.e. the ligand needed to occur as an anion in solution) and an ionic strength of 2.2 M was maintained with $NaClO_4$. The volumes of the mixtures of the cobalt corrin with the ligand were adjusted to 5.00 $cm^3$ with water. The solutions were then covered with foil to prevent light degradation and allowed to equilibrate overnight for approximately 15 hours in a water-circulating bath. It was found that this allowed sufficient time to reach equilibrium, with no further spectroscopic changes being noticed. Absorbance measurements were then taken for each of the solutions and plotted using either Equation 2.3 or 2.4 (see Chapter 8) to determine the equilibrium constants.

$$A_T = \frac{A_0 + A_1 K[L]}{1 + K[L]} \tag{2.3}$$

$$\frac{A - A_0}{A_1 - A} = K\left([L]_T - \frac{A_0 - A}{A_0 - A_1}[M]_T\right) \tag{2.4}$$

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### 2.8.1 Molecular Orbital Calculations

Semi-empirical molecular orbital calculations were performed on the crystal structure of aquacobalamin[12] and aqua-10-chlorocobalamin[11] using HYPERCHEM[13] with the ZINDO/1 model[14,15] and the RHF method with a SCF convergence limit of $10^{-5}$ kcal $mol^{-1}$.

## 2.9 Ligand Substitution Reactions of Aqua-10-chlorocobalamin

The ligand substitution kinetics of aqua-10-chlorocobalamin were investigated with the ligands pyridine and $N_3^-$. Solutions of aqua-10-chlorocobalamin (*ca*. 50 M) were buffered with $NaClO_4$ (2.2 M) and adjusted to pH 7.0 with MOPS for the reactions with pyridine and pH 9.0, with CHES, for the reactions with $N_3^-$. Pyridine solutions were prepared in the range 0.05–1.6 M and buffered at pH 7.0 while the $N_3^-$ solutions were in the range 0.2–1.8 M and buffered at pH 9.0. The kinetics was monitored at a wavelength of 375 nm at the temperatures 10.0, 15.0, 20.0 and 25.0 ºC for pyridine and 5.0, 10.1, 15.0 and 24.9 ºC for $N_3^-$. The kinetic experiments were carried out in the same manner as that described in Section 2.3.1.

## 2.10 Synthesis of Aqua-10-nitrosocobalamin

This synthesis has been modified from that reported by Wagner[16] for obtaining cyano-10-nitrosocobalamin from cyanocobalamin. In a typical experiment, hydroxocobalamin (0.109 g) was added to glacial acetic acid (40 ml) in a round bottom flask and stirred under nitrogen. Dried NaCl (0.6239 g) was added to a separate round bottom flask to which nitrosylsulfuricacid (1.2587 g) (Aldrich) was added. The brown nitrosyl chloride gas that formed was carried over into the solution of hydroxocobalamin and glacial acetic acid in the nitrogen stream and bubbled through for four hours. The reaction was followed by HPLC and a maximum of 40% conversion to aqua-10-nitrosocobalamin was obtained. Prolonged exposure to the NOCl gas resulted in decomposition of the product.

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Water was then added to the solution and the mixture was desalted on XAD (using a 33 × 2 cm column) and eluted with 10% acetonitrile. Further purification was performed by HPLC. The solvents used were 0.025 M phosphate buffer (dihydrogen phosphate/dipotassium hydrogen phosphate), at pH 6.863, HPLC grade acetonitrile and deionised water. The gradient elution programme is shown in Table 2.2 and a typical HPLC chromatogram in Figure 2.3. Finally, lypholisation produced an orange-red solid.

**Table 2.2** HPLC elution programme used throughout this work.

| **Time /min** | **% Phosphate Buffer** | **% $CH_3CN$** | **Flow Rate / ml $min^{-1}$** |
|---|---|---|---|
| 0 | 95 | 5 | 2.0 |
| 1 | 95 | 5 | 2.0 |
| 8 | 65 | 35 | 2.0 |
| 13 | 65 | 35 | 2.0 |
| 14 | 95 | 5 | 2.0 |

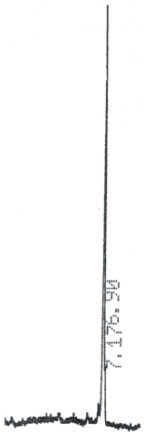



**Figure 2.3** HPLC chromatogram of aqua-10-nitrosocobalamin (retention time 7.17 min) using the gradient elution programme reported in Table 2.2.

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### 2.10.1 NMR Spectroscopy

NMR spectroscopic data for aqua-10-nitrosocobalamin were collected and interpreted by Professor Kenneth L. Brown and Dr Xiang Zou at Ohio University, USA. NMR spectra were obtained on a Varian Unity Inova 500 MHz NMR spectrometer equipped with pulsed field gradients on samples in 95% $H_2O$–5% $D_2O$. TOCSY and ROESY spectra were collected with Watergate solvent suppression and heteronuclear (HMQC and HMBC) experiments were also carried out. The data from the $^{1}H$ and $^{13}C$ NMR assignments, along with the spectra can be found in Appendix 1.

### 2.10.2 FAB-Mass Spectrometry

The FAB-mass spectrometry was carried out at the Nabraska Center for Mass Spectrometry, University of Nebraska. The molecular ion peak $(MH)^+$ occurs at 1374.6 and $(MNa)^+$ occurs at 1396.6, thus giving a molecular weight of 1373.6. The molecular weight of OH-10-nitrosocobalamin is expected to be 1373.8. The spectra can be seen in Appendix 2.

### 2.10.3 Crystals of Aqua-10-nitrosocobalamin

Crystals were grown in a 50% aqueous acetonitrile solution and obtained by slow evaporation of the solution (Figure 2.4). This did not produce diffraction quality crystals and hence an attempt was made to grow them by the hanging drop method of vapour diffusion. This procedure was followed from work carried out by Randaccio *et al.*[17,18] A 2 µl solution of aqua-10-nitrosocobalamin (25 mg/ml) was mixed with 2 µl of a precipitant solution. This solution contained LiCl (3 M) and PEG4000 (10%). This same precipitant solution was then placed in the container below the hanging drop mixture containing aqua-10-nitrosocobalamin.

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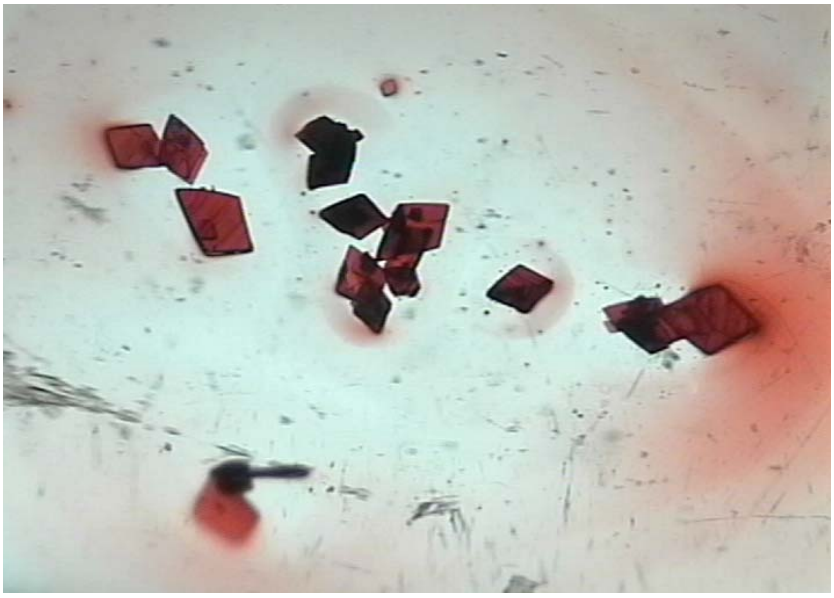


**Figure 2.4** Crystals of aqua-10-nitrosocobalamin grown from a 50% acetonitrile/water solution and obtained by slow evaporation of the solution.

## 2.11 Synthesis of Aqua-10-aminocobalamin

Wagner[16] synthesised cyano-10-aminocobalamin by the reduction of cyano-10-nitrosocobalamin with $NaBH_4$ and observed a blue colour change. This reduction was thus applied to aqua-10-nitrosocobalamin. $NaBH_4$ ($1.3 \times 10^{-1}$ mg) was added to aqua-10-nitrosocobalamin ($1.0 \times 10^{-1}$ mg) in methanol (15 ml) and the solution turned blue after stirring for a few minutes. HCl (0.1 M, 2 ml) was then added and the methanol was removed in vacuo. The aqua-10-nitrosocobalamin was completely converted into the amino derivative (confirmed by HPLC, Figure 2.5). Unfortunately, further purification could not be carried out due to the instability of the compound (see Chapter 11).

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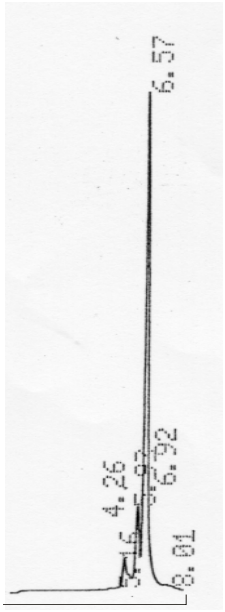



**Figure 2.5** HPLC chromatogram of aqua-10-aminocobalamin (retention time 6.57 min) using the gradient elution programme reported in Table 2.2.

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**REFERENCES FOR CHAPTER 2**


1. Merck Catalogue, Chemical Reagents, 2000.
2. G. Brink, FIT, a computer programme for non-linear least-squares fitting using the Marquard algorithm, University of the Witwatersrand, Johannesburg, South Africa, 1992.
3. H. M. Marques, K. L. Brown, and D. W. Jacobsen, *The Journal of Biological Chemistry*, 1988, **263**, 12378.
4. H. M. Marques, E. L. J. Breet and F. F. Prinsloo, *Journal of the Chemical Society, Dalton Transactions*, 1991, 2941.
5. HYPERCHEM, Molecular Visualization and Simulation, v. 4.5, Hypercube Inc., Waterloo, Ontario, 1995.
6. J. J. P. Stewart, *Journal of Computational Chemistry,* 1989, **10**, 209.
7. J. J. P. Stewart, *Journal of Computational Chemistry,* 1989, **10**, 221.
8. J. B. Collins, P. V. Schleyer, J. S. Binkley and J. A. Pople, *Journal of Chemical Physics*, 1976, **64**, 5142.
9. R. C. Raffenetti, *Chemistry and Physics Letters,* 1973, **20**, 335.
10. J. M. Pratt and R. G. Thorp, *Advances in Inorganic Chemistry and Radiochemistry*, 1969, **12**, 375.
11. K. L. Brown, S. Cheng, X Zou, J. D. Zubkowski, E. J. Valente, L. Knapton and H. M. Marques, *Inorganic Chemistry*, 1997, 36, 3666.
12. C. Kratky, G. Färber, K. Gruber, K. Wilson, Z. Dauter, H. Nolting, R. Konrat and B. Kräutler, *Journal of the American Chemical Society*, 1995, **117**, 4654.
13. HYPERCHEM v. 7.03 *Computational Chemistry Programs*, Hypercube, Inc., Gainesville, Florida, 2002.
14. A. D. Bacon and M. C. Zerner, *Theoretica Chimica Acta*, 1979, **53**, 21.
15. W. P. Anderson, W. D. Edwards and M. C. Zerner, *Inorganic Chemistry*, 1986, **25**, 2728.
16. F. Wagner, *Proceedings of the Royal Chemical Society, Series A*, 1965, **288**, 344.

17. L. Randaccio, S. Geremia, G. Nardin, M. Slouf and I. Srnova, *Inorganic Chemistry*, 1999, **38**, 4087.
18. L. Randaccio, M. Furlan, S. Geremia, M. Slouf, I. Srnova and D. Toffoli, *Inorganic Chemistry*, 2000, **39**, 3403.