formation; such mechanism includes the formation of a Co_3 cluster intermediate in the four-metal domain that eventually transforms into the final Co_4 cluster.

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Proton NMR Spectra of the Co_4S_{11} Cluster in Metallothioneins: A Theoretical Model

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Abstract: The theory for the electron–nucleus interaction in exchange-coupled dimetallic systems is extended to the case of a four-metal cluster to develop a theoretical model for the ^{1}H NMR spectra of the Co_4S_{11} cluster in Co^{II}_7 reconstituted metallothionein (MT-2) from rabbit liver. A model is obtained that qualitatively rationalizes the isotropic shifts pattern and their temperature dependence in terms of five large and one essentially zero J values for the six exchange coupling constants operative in the four-metal cluster. This finding is consistent with the results from the X-ray and solution structures of the protein. A discussion is presented that shows under what conditions the experimental features are met. Also, the ^{1}H NMR spectra of the already reported Co_4S_{10} cluster, present in the model system $[Co_4(SEt)_{10}]^{2-}$, are discussed.

Metallothioneins (MT) are a class of low molecular weight ($M_w \simeq$ 6–7 kDa), single-chain proteins capable of binding seven bivalent metal ions[1] such as zinc and cadmium.[2] They are bound in two independent clusters of three and four metal ions, respectively.[3-5] The Co^{II}_7 derivative of rabbit liver MT-2 has recently been reported;[6,7] it is proposed that the seven cobalt(II) ions are arranged in the same way as the native metals, i.e., three cobalt(II) ions in the β-domain and four cobalt(II) ions in the α-domain with the cluster structure substantially retained (Figure 1).

It is shown in the preceding paper[7] that the Co^{II}_7 derivative gives a surprisingly well-resolved ^{1}H NMR spectrum characterized by more than 20 isotropically shifted signals spread over a region spanning between 300 and −100 ppm (Figure 2).[6] The signals have been unequivocally assigned to the Co_4 cluster, by comparing the spectra of the Co_7 derivative and the Cd_4Co_3 derivative; the Co_3 cluster in the Cd_4Co_3 derivative does not give sharp isotropically shifted signals.[7] It has been suggested that the Co_3 cluster experiences some fluxional character that broadens the lines beyond detection.[7] Most or all of the isotropically shifted ^{1}H NMR signals of the Co_4 cluster in the Co_7 derivative arise from the β-CH_2 protons of the 11 cysteines involved in metal coordination; some of the less shifted signals, both up and down field, might be assigned to Cys α-CH protons. Two upfield signals (v and w) belong to exchangeable protons. Signals d, e, and g seem to have double intensity.

The cobalt(II) ions in the Co_4 cluster are magnetically coupled with an average magnetic susceptibility per cobalt ion equal to about 50% that of the isolated ion.[7] This result has been evaluated by subtracting the magnetic susceptibility of the Cd_4Co_3 derivative from the total magnetic susceptibility of the Co_7 derivative. The many new levels arising from exchange coupling can account for the decreased susceptibility as well as for the short electron relaxation rates and sharp NMR lines (Figure 2).[7]

^{1}H NMR spectra of paramagnetic species exhibiting such an extraordinary spectral dispersion and resolution contain in principle a wealth of structural information. The isotropic shifts can be contact or dipolar in origin.[8] Since the geometry around each of the metal ions is pseudotetrahedral, it is likely that the shifts are predominantly contact in origin. The contact hyperfine coupling for protons of methylene groups bound to the metal-coordinated sulfurs S are expected to depend on the dihedral angle ϕ between the M–S–C and S–C–H planes[9] according to the following equation:[9-11]

$$A = \beta_0 + B_2 \cos^2 \phi \tag{1}$$

where $B_2 \cos^2 \phi$ is related to the spin density on the proton resulting from hyperconjugation and β_0 is related to spin density resulting from other delocalization mechanisms. For alkylamines β_0 is negligible,[12] whereas no estimates are available for alkyl thiolates. In principle, the spreading of the signals could be accounted for on the basis of the above equation, but the nonequivalence of the four CoS_4 moieties may also contribute heavily to the signal spreading.

Finally, it has been observed that the shifts of a few signals increase with increasing temperature (anti-Curie behavior), whereas for most signals the pattern is normal (Curie behavior). The observation of both Curie and anti-Curie behavior in the same molecule is rather peculiar. In exchange-coupled dimetallic

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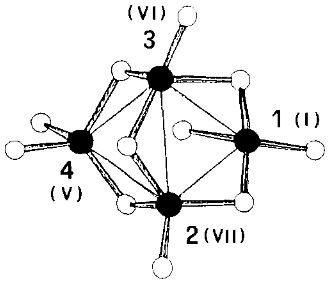

Figure 1. Schematic drawing of the four-metal cluster in MT according to X-ray and solution NMR structures. The filled circles are the cadmium ions. Roman numbers refer to the labeling used in ^{113}Cd NMR studies.

systems with two alike metal ions, either Curie or anti-Curie types of behavior are operative depending on whether the temperature T is larger or smaller than Jk^{-1}, respectively, over the temperature range studied.[13] Only in heterodimers has the coexistence of signals showing both Curie and anti-Curie behavior been observed[14] and theoretically explained.[15,16]

We attempt here to reproduce the essential features of the ^{1}H NMR spectrum of Co$_7$MT by extending a spin Hamiltonian approach that has proven to be successful for rationalizing the contact shifts in dimetallic cluster complexes.[15,16] We take advantage of the available structural information on the native Cd$_5$Zn$_2$ and on Cd$_7$ systems. We show that these structural features can account for the observed temperature dependence of the shifts as well as the observed spreading of the ^{1}H NMR signals.

Theoretical Approach

We consider four $S = {}^3/_2$ states exchange-coupled through the following Hamiltonian:

$$H = J_{12}\mathbf{S}_1\cdot\mathbf{S}_2 + J_{13}\mathbf{S}_1\cdot\mathbf{S}_3 + J_{14}\mathbf{S}_1\cdot\mathbf{S}_4 + J_{23}\mathbf{S}_2\cdot\mathbf{S}_3 + J_{24}\mathbf{S}_2\cdot\mathbf{S}_4 + J_{34}\mathbf{S}_3\cdot\mathbf{S}_4 \quad (2)$$

In this formalism a positive J means antiferromagnetic coupling. The total multiplicity for four coupled S is $(2S + 1)^4$ or 256 when $S = {}^3/_2$. The wave functions of the coupled system are linear combinations of the product spin eigenfunctions of the uncoupled Co(II) $|S_1S_2S_3S_4M_{S1}M_{S2}M_{S3}M_{S4}\rangle$. The matrix elements of the Hamiltonian $\langle S_1S_2S_3S_4M_{S1}M_{S2}M_{S3}M_{S4}|H|S_1S_2S_3S_4M_{S1}M_{S2}M_{S3}M_{S4}\rangle$ can be evaluated as a function of the various J values. The 256 × 256 matrix can be arranged into block form, one block for each group of total M_S values, and diagonalized numerically to give the eigenvalues and eigenfunctions of the coupled system for a given choice of J_{ij} values. The new states can be designated by $|S'M_{S'}\rangle$.

The contact shift originating from the ith metal ion is proportional to the overall expectation values of S_{iz}. The contribution of each S' level to $\langle S_{iz}\rangle$ can be deduced from the coefficients with which an individual cobalt(II) ion is represented in the state, and each S' level can be weighted according to its Boltzmann population.[15] The individual $\langle S_{iz}\rangle$'s divided by the $\langle S_z\rangle$ of the isolated ion reflect the fractional change in the contact shift of a proton when the metal passes from an isolated to an exchange-coupled situation.

From Figure 1, we should note that there is no bridging sulfur between metal 1 and metal 4 in the Cd$_4$ cluster; it is probable that the cobalt(II) derivative retains the same gross cluster structure of the cadmium(II) system. We will start by considering that the Co$_4$ cluster has cubic symmetry with six equal exchange coupling constants, and then we will consider the situation where one of them (relating metal 1 and metal 4) drops to zero. Our aim is to ascertain whether and how strongly the exchange-coupling asymmetry spreads the isotropically shifted signals of the ligand protons and affects the temperature dependence of the ^{1}H NMR signals.

Results and Discussion

The experimental temperature dependence of the isotropically shifted ^{1}H NMR signals of the Co$_7$ system, in the temperature range 278–323 K, is shown in Figure 3. The ^{1}H NMR spectra have been recorded at 200 MHz at 5 K intervals. It can be noted that while most of the signals follow a Curie-like behavior, there are six signals (k, m, p, q, r, and s) that exhibit a more or less anti-Curie behavior.

To calibrate our theoretical model against experimental facts, we first consider the symmetrical situation in which the four cobalt(II) ions are at the vertices of a regular tetrahedron with six equal J magnetic coupling constants. By taking all the six J values in the Hamiltonian (2) equal one another and a value of 50 cm^{-1}, which is consistent with the experimental magnetic susceptibility (vide infra), the energy levels of Figure 4A are obtained. The corresponding expectation values $\langle S_{iz}\rangle$ for each of the four metal ions are then evaluated[15,16] by

$$\langle S_{iz}\rangle = \frac{\sum_{S'M_{S'}} \exp\left[-\frac{E_{S'M_{S'}}}{kT}\right]\langle S'M_{S'}|S_{iz}|S'M_{S'}\rangle}{\sum_{S'M_{S'}} \exp\left[-\frac{E_{S'M_{S'}}}{kT}\right]} \quad (3)$$

where the sum is over all the energy levels for the coupled system. The $\langle S_{iz}\rangle$ values are shown in Figure 5A as a function of $1/T$. It can be noted that all four $\langle S_{iz}\rangle$ values are equal and that they decrease with increasing temperature (Curie behavior), although less than for isolated metal ions.

The contact shift for a proton sensing a single metal ion is given by[8,9]

$$\left[\frac{\Delta\nu}{\nu_0}\right]^{\text{con}} = -\frac{2\pi}{h\gamma_N B_0}A\langle S_z\rangle \quad (4)$$

where A is the hyperfine coupling constant, γ_N is the nuclear magnetogyric ratio, and B_0 is the external magnetic field. The kth proton of a terminal S–CH$_2$ bound to metal i experiences a contact shift proportional to $A_{ik}\langle S_{iz}\rangle$, where A_{ik} is the Fermi hyperfine coupling constant between metal i and proton k; on the other hand the kth proton of the S–CH$_2$ group bridging metal i and metal j experiences a contact shift proportional to $(A_{ik}\langle S_{iz}\rangle + A_{jk}\langle S_{jz}\rangle)$.

To obtain an estimate of the isotropic shifts, we proceed with our calculations by taking a value of A corresponding to a room-temperature downfield shift of 250 ppm in a hypothetical isolated cobalt(II) complex. This value is consistent with that experienced by the Cys β-CH$_2$ protons of cobalt(II) liver alcohol dehydrogenase in which a single cobalt(II) ion is bound to two cysteines. The four signals of the Cys β-CH$_2$ protons fall between 200 and 300 ppm.[17] Holm has reported[18] a value of 190 ppm for the β-CH$_2$ protons of the [Co(SEt)$_4$]$^{2-}$ complex in which the CH$_2$ protons are made equivalent by rapid rotation; the maximum shift calculated from eq 1 is 380 ppm if $\beta_0 = 0$. We will take all A values equal, although they may differ because of the dihedral angle and because, for bridging cysteines, the metal–sulfur bonds may be weaker. Using an A value corresponding to 250 ppm and asking that all six J's are equal to 50 cm^{-1}, we find that the calculated shift values at 300 K are 120 ppm for the terminal methylenes and 240 ppm for the bridging methylenes (Figure 5C). Of course, their temperature dependence follows the behavior of the $\langle S_{iz}\rangle$ values of the cobalt(II) ions to which they are attached. Since the $\langle S_{iz}\rangle$ values are all equal and of Curie type, all the isotropic shifts increase with decreasing temperature, even if the

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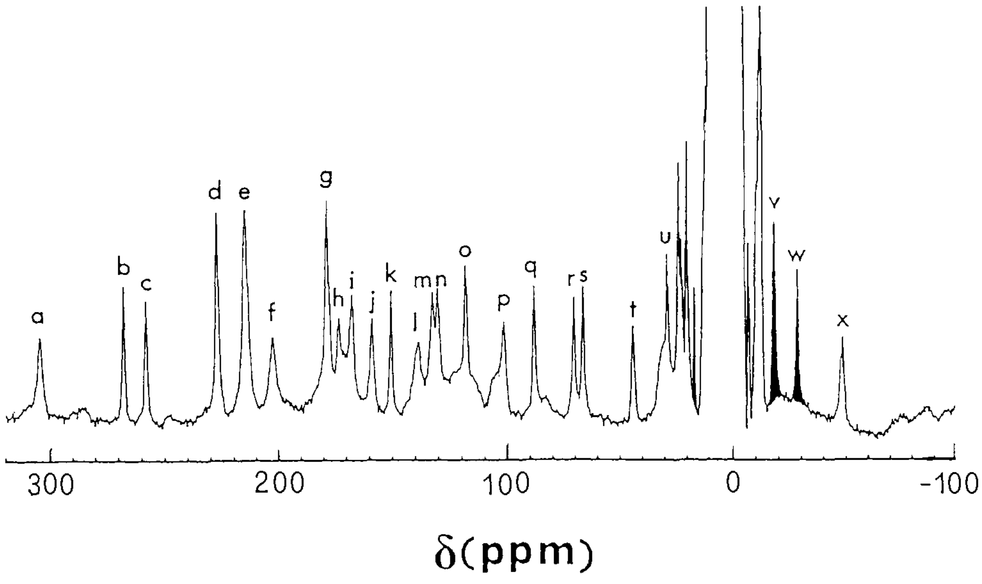


Figure 2. The 298 K 90-MHz ^{1}H NMR spectrum of Co_7MT, pH 8.5, Tris HCl 0.05 M. The filled signals disappear in D_2O.

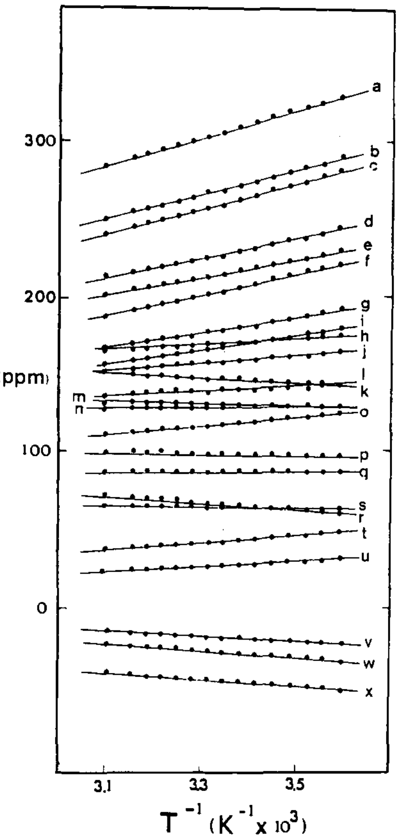


Figure 3. Temperature dependence of the chemical shifts of the isotropically shifted signals in Co_7MT in the temperature range 278–323 K (conditions 200 MHz, pH 8.5).

slope is markedly smaller than for a mononuclear system. This prediction as a result is different from the experimental pattern observed for some of the signals for the Co_4 system.

The magnetic susceptibility value can be obtained through the following equation:[13]

$$\chi = \mu_0 \frac{\mu_B^2}{3kT} \frac{\sum g_i^2(2S_i + 1)S_i(S_i + 1)\exp(-E_i^\circ/kT)}{\sum(2S_i + 1)\exp(-E_i^\circ/kT)} \quad (5)$$

With a choice of J of 50 cm^{-1} the magnetic susceptibility at room temperature is 55% of that expected from the sum of four isolated ions (spin-only values with $g = 2.0023$), consistent with the experimental data.[7] Although the experimental magnetic susceptibility matches well with the calculated value, it is clear that the temperature dependence of the signals cannot be accounted for on the basis of this model.

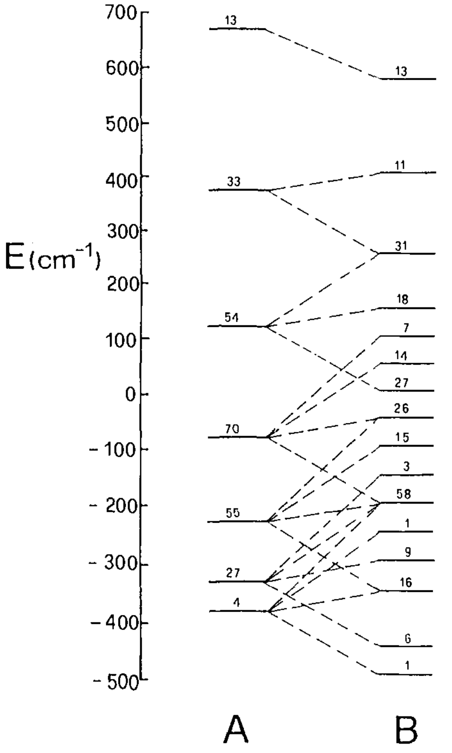


Figure 4. Predicted patterns of the energy levels in the four-metal cluster according to two different models of magnetic coupling: (A) all J's = 50 cm^{-1}; (B) J_{14} = 0, other J's = 50 cm^{-1}.

Holm et al. had previously reported on the ^{1}H NMR properties of a tetranuclear $[Co_4(SEt)_{10}]^{2-}$ complex.[18] The methylene protons of ethane thiolate give rise to two signals centered at 117 and 105 ppm downfield and have been assigned to bridging and terminal protons, respectively. Within the framework of our treatment here, these values are consistent with J being about 45 cm^{-1} and A being 190 ppm for terminal protons, as observed for $[Co(SEt)_4]^{2-}$,[18] and 106 ppm for bridging protons.

The range of the J values that would fit the 50% reduced magnetic susceptibility of the Co_4MT system is rather narrow. As long as the J's are equal, the temperature dependence is of Curie type or anti-Curie type for all the signals as in the homodimers. Under the condition of 50% reduced magnetic suscep-

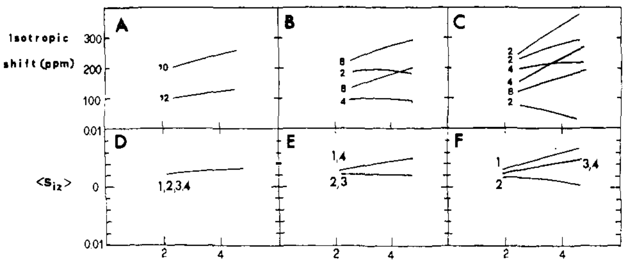


Figure 5. Calculated $\langle S_{iz}\rangle$ (lower) and isotropic shift values (upper) for β-CH_2 protons of metal-bound cysteines in the Co_4 cluster according to the considered models of magnetic coupling: (A and D) all J's = 50 cm^{-1}; (B and E) J_{14} = 0, other J's = 50 cm^{-1} (Figure 1); (C and F) J_{14} = J_{13} = 0, other J's = 50 cm^{-1} (structure IB). Numbers in the lower figures refer to the cobalt(II) ions according to the labeling reported in Figure 1; in the upper figures they refer to the number of protons that experience the same isotropic shift.

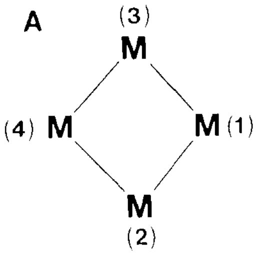
tibility the temperature dependence around room temperature is of Curie type as shown in Figure 5A,D. At this point we would like to underline that the absolute values of the calculated isotropic shifts are not so important; the hyperfine coupling between protons and metal ions cannot be the same for all the 22 protons because of different ϕ angles even if, in first approximation, equal metal–sulfur distances are assumed.

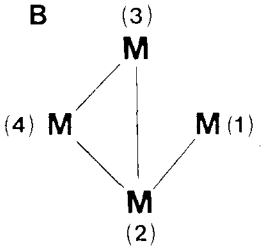
If we now take into consideration that there is no cysteine bridging metal 1 and metal 4 in both the X-ray[3] and the solution NMR[4,5] structures, we may consider a second situation in which J_{1-4} = 0, all other J's retaining their previous values. The new energy level diagram is depicted in Figure 4B; the temperature dependence of $\langle S_{iz}\rangle$ for each metal ion and the predicted shifts for the β-methylene protons, again using a single A value of 250 ppm, are respectively shown in Figure 5, parts B and E, respectively. We observe that a larger spreading of the calculated signals is obtained. Furthermore, six signals are predicted to display a slight anti-Curie behavior. We recall again that in the experimental spectrum there are indeed six signals showing an anti-Curie behavior. The model also predicts a further differentiation of the other proton signals in two classes of eight protons each, with different temperature dependence slopes. A minor variation of J slightly affects the slope of the anti-Curie signals, although their characterization remains unequivocal. The calculated magnetic susceptibility for this situation is very similar to that obtained previously and thus is still consistent with the experiment.

The second theoretical model is thus quite successful. Indeed, we have been able to predict both Curie and anti-Curie behavior in a homometallic cluster by simply reducing the symmetry of the Hamiltonian. The model would predict that $\langle S_z\rangle$ of metals 2 and 3 has an anti-Curie temperature dependence and $\langle S_z\rangle$ of metals 1 and 4 has a Curie temperature dependence. This holds in every homometallic cluster: the metals with a larger number of exchange interactions have anti-Curie behavior. Analogously, in heterodimetallic systems, the ion with smaller S shows anti-Curie behavior. In the present case, the anti-Curie signals k, m, p, q, r, and s should belong to the two terminal CH_2's of metals 2 and 3 and to the CH_2 of the bridging cysteine. Preliminary ^1H NOE experiments indeed confirm that each pair of signals k and r, m and p, and q and s belong to a single cysteine. The eight proton signals showing the more pronounced Curie behavior should belong to the four terminal cysteines of metals 1 and 4 and the other eight signals with less pronounced Curie behavior should belong to the four cysteines bridging "Curie" and "anti-Curie" metals.

We may further note that signals k and r have a more pronounced anti-Curie behavior, m and p have a smaller slope, and q and s are almost flat. This cannot be predicted by a model in which metals 2 and 3 are equivalent. In a model in which the two anti-Curie metal ions are inequivalent, signals k and r would belong to the terminal Cys of the more anti-Curie, q and s to the terminal Cys of the less anti-Curie, and m and p to the bridging Cys. The inequivalence between M(2) and M(3) may result from a variety of situations. The five NMR ^{113}Cd–^{113}Cd J coupling constants in Cd_4MT are 48, 38, 33, 33, and 32 Hz, indicating that indeed there are five similar but not equivalent magnetic interactions.[19]

It should be noted that if we put two J values equal to zero, we should distinguish between a symmetric and an asymmetric situation. The first (structure A) is obtained by eliminating





magnetic interactions between two different pairs of metals; the second (structure B) by eliminating the magnetic interactions of the same metal with two of its three neighbors. In the symmetric case we never expect coexistence of Curie and anti-Curie behavior of the various $\langle S_{iz}\rangle$. In the asymmetric case one should expect anti-Curie for the only metal retaining three bridges. This should give rise to only two signals showing marked anti-Curie behavior (Figure 5C,F). This case should therefore be regarded as a possible limit case of the inequivalence of the two anti-Curie metal ions in the five-J model.

We should keep in mind, however, that we cannot hope to obtain a full understanding of such a complicated spectrum. The analysis would be considered successful if a model predicts the temperature dependence of the isotropic shifts within the structural framework available from X-ray and solution investigations of the analogous cadmium(II) derivative. The actual shifts will depend on the Co–S(terminal) and Co–S–Co distances, which will not all be equal. As a consequence, the five J values, which we have set to 50 cm^{-1}, will assume a range of values for the various exchange interactions.

Finally, it should be taken into consideration that the shift of a proton of a cysteine bridging two metals A and B is given by

$$A_A\langle S_{A_z}\rangle + A_B\langle S_{B_z}\rangle \tag{6}$$

where the A's are given by eq 1. In the case of one $\langle S_z\rangle$ being Curie and the other anti-Curie, the ϕ angles of eq 1 determine which of the two contributions, and to what extent, is prevailing.

In summary we have provided a theoretical approach that in principle is capable of predicting the isotropic shifts of protons of ligands in metal clusters as long as the exchange coupling is a relatively small perturbation of the whole system and the structural details are known. A full interpretation of the isotropic shifts of the protons of cysteines involved in the Co_4 cluster is not the goal of these studies and will not be attempted due to the complexity of the system. Nevertheless the exercise has been quite instructive. In particular, the temperature dependence of the signals is reproduced by assuming that only one of the six exchange coupling constants is zero; this is consistent with the structural details of the known MT derivatives.

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