Lu(III) form 1:2 complexes with ATP, by coordinating with α,β,γ-phosphates of two ATP molecules. The complexes are mixtures of rapidly exchanging diastereomers, and the detailed microscopic structures of the complexes remain to be established by further investigation.

Experimental Section

Materials. The $H_2{}^{17}O$ (51.0 atom % ^{17}O, 38.6 atom % ^{18}O) was obtained from Monsanto. The ^{17}O-depleted water (0.00338 atom % ^{17}O, 0.00135 atom % ^{18}O) was obtained from Yeda Stable Isotopes. The metal oxides Sc_2O_3, La_2O_3, and Lu_2O_3 were of the puratronic grade (99.999%e from Alfa. Unlabeled ATP was obtained from Sigma.

^{17}O-Labeled ATP. The [α-$^{17}O_2$]ATP (38 atom % ^{17}O) and [γ-$^{17}O_3$]ATP (42 atom % ^{17}O) were synthesized by combined chemical and biochemical procedures as described previously.[12,16] The [β-$^{17}O_2$]ATP was synthesized from [β-$^{17}O_3$]ADP (39 atom % ^{17}O) (prepared as described in ref 12) according to the procedure of Wehrli.[31,32] All three labeled samples were newly prepared for this work. The atom % ^{17}O enrichments were determined by the integration method of Tsai et al.[16] on the basis of the quadrupolar effect of ^{17}O in ^{31}P NMR.

Sample Preparations. Stock solutions of $M^{III}Cl_3$ were prepared by dissolving the metal oxides in concentrated HCl upon gentle heating, followed by repetitive rotary evaporation to remove excess HCl. After redissolving in triple-distilled water, the concentration of M(III) was determined by passing the $M^{III}Cl_3$ solution through a cation-exchange column (Dowex 50W-X8, H^+ form, Bio-rad) followed by titrating the released H^+ ions with standardized NaOH. The results were reproducible within ±2% in three independent determinations.

The nucleotides were first converted to sodium salts by passing through a sp-Sephadex C-25 column (Pharmacia). The solution was then passed through a small column of Chelex-100 (Bio-Rad), lyophilized, redissolved in ^{17}O-depleted water, quantified by UV absorption at 259 nm, and used as a stock solution. NMR samples were prepared by mixing proper amounts of $M^{III}Cl_3$ and nucleotide stock solutions (usually in <100-μL quantities) in ^{17}O-depleted water (for ^{17}O NMR), in H_2O/D_2O (3:1 v/v) (for ^{31}P NMR), or in 99.8% D_2O (for ^1H NMR), followed by adjusting to pH 8.0 (direct reading from the pH meter) with NaOH/HCl or NaOD/DCl. In ^{17}O NMR and ^{31}P NMR the experiments were usually begun by taking the spectrum of the free nucleotide as a control (for purity, homogeneity, etc.) followed with successive titration with $M^{III}Cl_3$ (pH was adjusted at each titration). In cases where decomposition of ATP occurred (hydrolysis to ADP and AMP), a new sample was prepared at the later part of the titration. In most cases, <5% decomposition occurred within 3-5 h. One set of experiments usually took 5-8 h. For ^1H NMR, multiple samples of different M(III)/ATP ratios were prepared, lyophilized, dried under vacuum, and redissolved in 99.996% D_2O. Such a process resulted in 10-20% decomposition in the ATP complexes of La(III) and Lu(III).

NMR Methods. ^1H and ^{31}P NMR spectra were obtained from a Bruker WP-200 NMR spectrometer, with deuterium lock, at ambient temperature (30 ± 2 °C). The chemical shifts were referenced to external TSP and 85% H_3PO_4, respectively, with + signal indicating a downfield shift. Homonuclear ^1H-decoupling experiments were performed to aid peak assignments when necessary.

^{17}O NMR spectra were measured on a GE-300 widebore NMR spectrometer. A horizontal, nonspinning probe (10-mm outer diameter, 2-mL sample size) was used for most experiments. The ^{31}P-decoupled ^{17}O NMR experiments were carried out on a spinning horizontal probe (20-mm outer diameter, 4.5-mL sample size). Chemical shifts were referenced to H_2O, with + signal indicating a downfield shift. ^{17}O-depleted water was used in all experiments. In most cases, the T_1 inversion recovery experiment was used to partially suppress the solvent signal on the basis of different relaxation times between the solvent signal and the nucleotide signal.

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Uncovering Remote Nuclear-Spin Connectivities by Relayed Zero and Double Quantum Coherence

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Abstract: A novel method is described which allows identification of spin-spin coupling networks in complex spin systems. The method is a two-dimensional time-domain experiment in which double (and zero) quantum states are excited and allowed to evolve for a period t_1 after which signals from these excited states are converted into transverse magnetization. This magnetization is then relayed to remote spins and subsequently recorded in the detection period t_2. The method is applied to assign the proton spectrum of the arginine residue in a vasopressin analogue.

NMR has been shown to be a powerful tool for structure determination of complex molecules such as polypeptides and small proteins in solution.[1] This structural information is obtained from two-dimensional nuclear Overhauser effect (2DNOE) experiments where through-space connectivities between remote nuclear spins are detected.[2] In order to make use of these through-space connectivities the resonance assignments of the spins in the molecule must first be made. The inability to make specific resonance assignments in complex molecules is often a major limitation in how much information can be extracted from the NMR experiment. Thus, procedures for simplifying resonance assignments are of prime importance for studies of complex

† Present address: Rutgers University, Department of Chemistry, Busch Campus, Piscataway, NJ 08954. A novel method is described which allows identification of spin-spin coupling networks in complex spin systems. The method is a two-dimensional time-domain experiment in which double (and zero) quantum states are excited and allowed to evolve for a period t_1 after which signals from these excited states are converted into transverse magnetization. This magnetization is then relayed to remote spins and subsequently recorded in the detection period t_2. The method is applied to assign the proton spectrum of the arginine residue in a vasopressin analogue.

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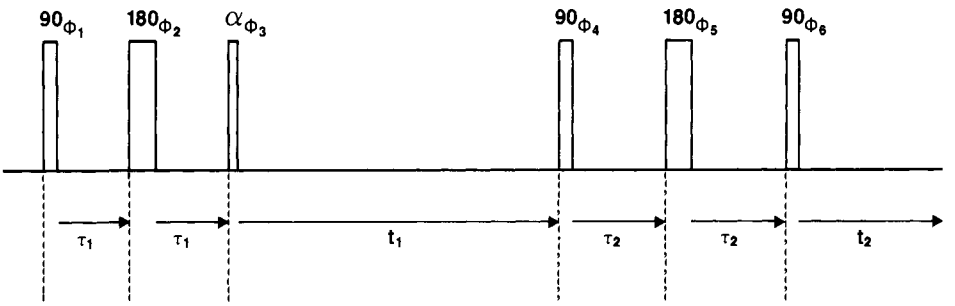

Figure 1. Pulse sequence used in zero and double quantum relay experiments: The phase sequence is listed in Table I. The flip angle α should not be larger than 45° at which optimal sensitivity is achieved. The τ_1 and τ_2 interals should be related to the spin–spin coupling constants, generally $\tau_1 = \tau_2 = 1/(8J)$. The phases ϕ_j of the radio frequency pulses are listed in Table I.

molecules. In this paper we present a novel procedure that allows identification of families of resonances belonging to the same network of coupled spins.[3]

Let us consider two spins, a and d, which are linked together by two intermediates b and c such as a–b–c–d. If b and c are in a crowded region of a spectrum as often encountered with α and β protons in polypeptides, then it becomes difficult to prove that a and d belong to the same group of spins with standard NMR methods. Several procedures have recently been introduced that try to uncover remote connectivites of spins. In one approach, one-dimensional spectra are autocorrelated by relayed polarization transfer[4-7] or by isotropic mixing.[8] Alternatively, indirect detection of multiple quantum coherence[9-10] provides information about remote connectivites. Both methods have their limitations. In multiple quantum experiments, the analysis of spectra is complicated by the occurrence of extra peaks which have various origins such as higher order combination transitions and transitions between magnetically equivalent spins. In this respect, the former method[4-7] of correlating single quantum spectra is more straightforward, but problems may arise if the resonances of nuclei are in crowded regions of the spectrum, also single quantum relay spectra do not distinguish between direct and remote connectivites.

The proposed method offers a way around these problems by combining double (or zero) quantum excitation with a single quantum relay of magnetization in the mixing period. In this experiment one can clearly establish the connectivity pathway between remote nuclear spins via multiple quantum transitions of intermediate nuclei. The principle of this technique is illustrated by the proton double and zero quantum relay spectra of the *n*-butyl group in *n*-butyl acetate. The usefulness of this experiment for resonance assignments in more complex molecules is shown by making the assignment of the arginine residue in a vasopressin analogue.

Experimental Section

NMR. All NMR experiments were performed on a JEOL GX-500 spectrometer operating at 500 MHz for protons. All data processing was done on a VAX 11/780 computer with software developed by Dr. D. Hare. *n*-Butyl acetate was dissolved in acetone-d_6. The zero and double quantum relay spectra in Figure 2 were obtained by storing even and odd number scans according to Table I into two different files. Subsequent addition of these two files generated the zero quantum signal (Figure 2A) whereas their diifference gave rise to the double quantum signal (Figure 2B). The vasopressin analogue was dissolved in Me_2SO-d_6 containing 4% trifluoroacetic acid. The experiments were performed at 60 °C. To obtain the spectrum in Figure 3, a data matrix of 512 points in t_1 and 2048 points in t_2 was recorded. After the Fourier transformation in t_2 the t_1 FID's were zero filled to 1024 points. Sine bell apodization was applied in t_2, and cosine apodization in t_1. Both intervals τ_1 and τ_2 were 15 ms in all experiments.

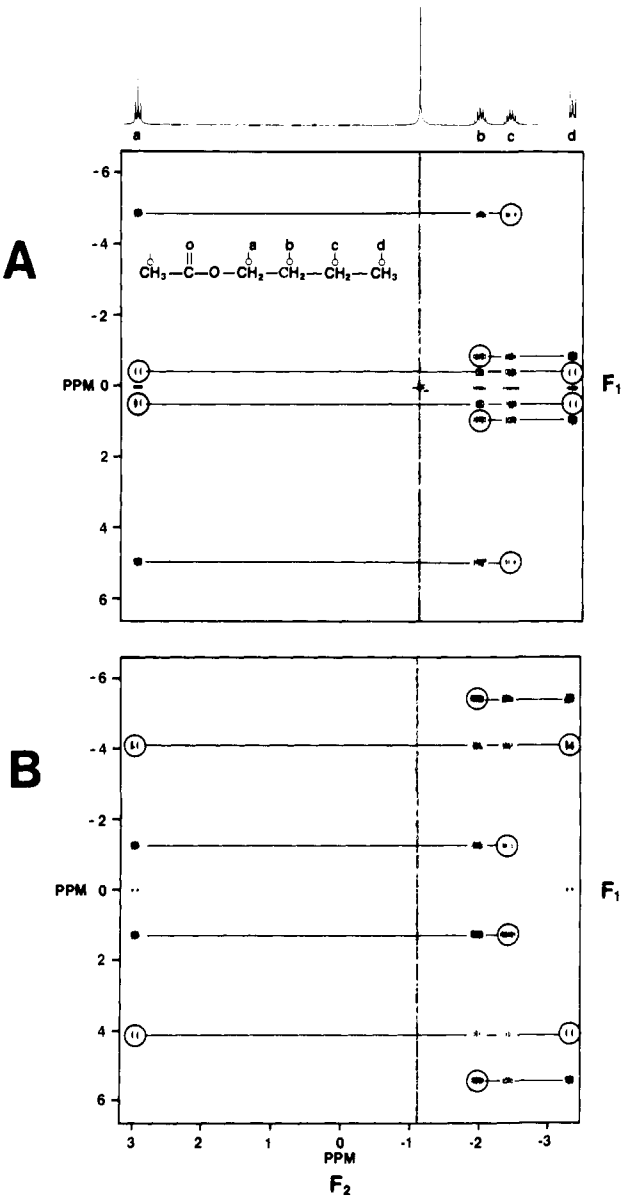

Figure 2. The zero (A) and double quantum spectra (B) of *n*-butyl acetate are depicted. They have been obtained with the pulse sequence depicted in Figure 1 and Table I. The data matrices consist of 512 points in each frequency domain. The flip angle α was 45°, τ_1 = 16.5 ms, and τ_2 = 14 ms. Relayed peaks are circled. Chemical shift in both dimensions was referenced to the carrier frequency set to 0 ppm.

Materials. The deuterated solvents were obtained from Merck (Isotopes). The peptide was synthesized by Dr. W. F. Huffman and co-

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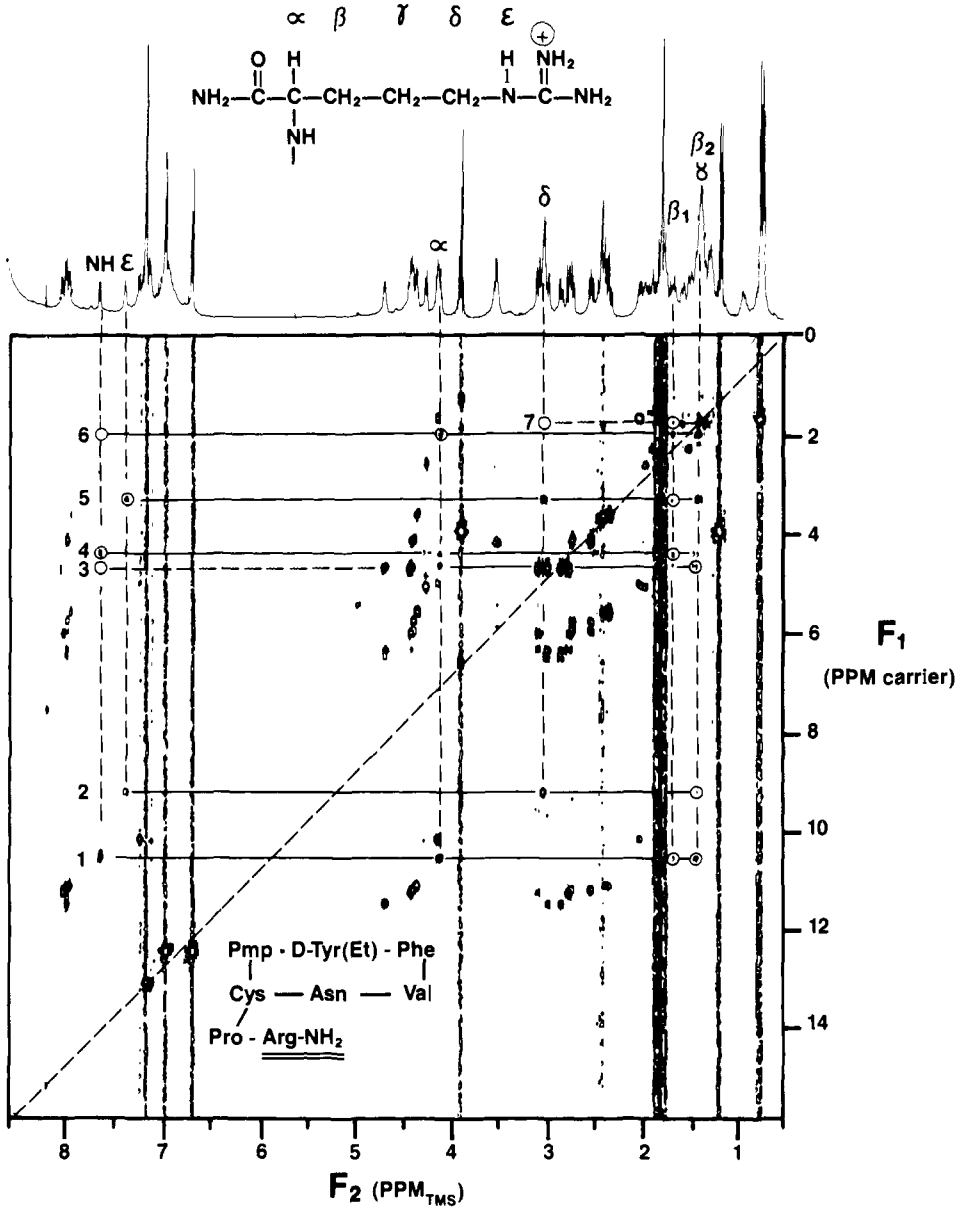

Figure 3. This figure shows the double quantum relay spectrum of the cyclic octapeptide whose formula is indicated on the lower part of the figure. (PMP stands for β-mercapto-β,β-cyclopentamethylenenpropionic acid.) There are 1024 points in the F_1 and 2048 points in the F_2 dimension. Again α was set to 45° and $\tau_1 = \tau_2 = 15$ ms. Arginine peaks belonging to the same double quantum transition are connected by horizontal bars with the relay peaks marked by circles. The arginine double quantum transitions are numbered as follows: 1, indicating the HN, H_α- transition; 2, the H_δ, H_ϵ-; 3, the H_α, $H_{\beta 1}$-; 4, the H_α, $H_{\beta 2}$-; 5, the H_γ, H_δ-; 6, the $H_{\beta 1}$, $H_{\beta 2}$- and $H_{\beta 1}$, H_γ-; and 7, the $H_{\beta 2}$, H_γ- transitions. The chemical shift in F_1 was referenced to the carrier frequency set to 0 ppm whereas in F_2 Me_4Si was set to 0 ppm.

workers at Smith Kline + French Laboratories.

Results and Discussion

The pulse sequence used for the double (and zero) quantum relay experiment is shown in Figure 1 with the phase cycling given in Table I. The zero quantum, and double quantum, relay spectra of *n*-butyl acetate are shown in Figure 2, parts A and B, respectively. Connectivities between spins are indicated by horizontal bars in Figure 2. It is also possible to distinguish remote and direct connectivities in these spectra.

Zero (double) quantum transitions are only excited between directly coupled spins. Therefore, directly coupled protons have common zero (double) quantum peaks which appear in the vertical F_1 dimension in Figure 2, at the difference (sum) of the resonance offsets of the two coupled protons. On the other hand, the circled peaks in Figure 2 have an offset from the origin in F_1 which cannot be generated by differences (sums) of the single quantum frequencies, which appear along the horizontal F_2 domain. They are therefore readily recognized as relayed peaks which reveal remote connectivites. In double quantum spectra, the assignment is further facilitated by the fact that resonances of directly coupled protons always give rise to pairs of peaks along the F_2 axis, centered around the diagonal ($2F_1$, F_2) of the spectrum as depicted in Figure 3.

The excitation sequence[11,12] (90_x–τ_1–180_y–τ_1–45_y) as shown in Figure 1 generates both zero and double quantum coherence between coupled protons, e.g., b and c in Figure 2, which then evolve during the evolution time t_1. The first pulse after t_1 converts these coherences back into transverse magnetization of the participating spins b and c. In the following mixing period $2\tau_2$ this magnetization is partially relayed to neighboring spins a and d in a coupling network such as a–b–c–d. In the resulting two-dimensional spectrum, the zero (double) quantum resonance (b–c) in F_1 appears on all four single quantum resonances a, b, c, and d in F_2 as seen in Figure 2. Therefore, the remote connectivity between a and d can be visualized by a horizontal bar along F_2. This relay of zero (double) quantum coherence does not require any long-range couplings between remote spins. All relayed zero

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Table I. Phase Table for the Pulse Sequence in Figure 1[a]

							ϕ receiver	
no.	ϕ_1	ϕ_2	ϕ_3	ϕ_4	ϕ_5	ϕ_6	zero quantum observation	double quantum observation
1	x	y	y	y	$-x$	x	x	x
2	x	y	y	$-x$	$-y$	y	y	$-y$
3	x	y	y	$-y$	x	$-x$	$-x$	$-x$
4	x	y	y	x	y	$-y$	$-y$	y
5	x	$-y$	y	y	$-x$	x	x	x
6	x	$-y$	y	$-x$	$-y$	y	y	$-y$
7	x	$-y$	y	$-y$	x	$-x$	$-x$	$-x$
8	x	$-y$	y	x	y	$-y$	$-y$	y
9	x	y	$-y$	y	$-x$	x	$-x$	$-x$
10	x	y	$-y$	$-x$	$-y$	y	$-y$	y
11	x	y	$-y$	$-y$	x	$-x$	x	x
12	x	y	$-y$	x	y	$-y$	y	$-y$
13	x	$-y$	$-y$	y	$-x$	x	$-x$	$-x$
14	x	$-y$	$-y$	$-x$	$-y$	y	$-y$	y
15	x	$-y$	$-y$	$-y$	x	$-x$	x	x
16	x	$-y$	$-y$	x	y	$-y$	y	$-y$

[a] Storage of even and odd numbered FID in different files allows the computation of both the zero quantum and the double quantum relay spectra.

(double) quantum peaks are circled in Figure 2. Zero and double quantum transitions are closely related since they are two photon processes[13] which in our case induce correlated motions of spin pairs. Therefore, the spectrum in Figure 2B provides the same information as the zero quantum relay spectrum in Figure 2A, with the only difference being that the peaks appear at the double quantum frequencies in F_1 (e.g., b + c) thus creating a different peak pattern. This may be an important advantage in analyzing complex spectra because overlapping peaks in a zero quantum spectrum may be resolved in a double quantum spectrum or vice versa.

To address the question of sensitivity, we will compare our double quantum excitation sequence (first three pulses in Figure 1) with the standard sequence 90_x–τ_1–180_y–τ_1–90_x in terms of multiple quantum coherence generated in a pair of protons. For $\tau_1 = 1/4J$ the standard sequence yields the spin density operator formulated in the product operator formalism[14] $\sigma = C/2i(I_1^+I_2^+ - I_1^-I_2^-) = C\{2QT\}_y$, where $C = N_s\hbar\gamma/kT$. Our sequence yields $\sigma = \sin^2(45°)\, C/2(I_1^+I_2^+ + I_1^-I_2^- + I_1^+I_2^- + I_1^-I_2^+) = C/2[\{2QT\}_x + \{ZQT\}_x]$. Apparently, in the proposed method the intensity of the double quantum coherence is reduced by a factor of 2, but there is a gain of zero quantum signal. Hence, there is no overall loss in sensitivity if both zero and double quantum spectra are calculated from the same data set.

It is also important to note that both the zero quantum and the double quantum relay spectrum can be computed from the same data set.[15] For instance, even and odd numbered FID's according to the phase list in Table I can be stored in different memory blocks of the computer. When the receiver phase has been set to accumulate zero quantum signals, then a subsequent addition of these two memory blocks generates the zero quantum signal, whereas a subtraction yields the double quantum signal. Therefore, a single experiment as depicted in Figure 1 and Table I generates both zero quantum and double quantum relay spectra.

A crucial aspect of this experiment is the multiple quantum excitation sequence[13] 90_x–τ_1–180_y–τ_1–α_y shown in Figure 1. This sequence does not excite double quantum coherence between magnetically equivalent spins, also higher order combination transitions in which four or more spins participate in a double or zero quantum transition are considerably reduced. The absence of these extra transitions greatly simplifies the interpretation of zero and double quantum relay spectra. Excitation and mixing intervals τ_1 and τ_2 are adjusted to the coupling constants involved. We found $\tau_{1,2} = 1/8J$ to be a good choice where J is the average proton–proton coupling constant. In the presence of a large dispersion of J values one might repeat the experiment with different τ_1, τ_2 values.

To illustrate the potential of the double quantum relay experiment we attempted to assign the proton spectrum of the arginine residue in an analogue of the cyclic peptide arginine vasopressin. Figure 3 depicts the double quantum relay spectrum of this peptide. The arginine double quantum frequencies are indicated by horizontal bars. Relayed double quantum peaks are circled. The carrier frequency was set at the right end of the spectrum. Nonrelayed double quantum peaks are centered around the axis ($2F_1$, F_2).

The two β-protons are nonequivalent with one β-resonance overlapping the γ-resonance. This is apparent since the NH–α–transition (No. 1 in Figure 3) is relayed to β_1 and β_2 and the ϵ–δ-transition (No. 2) is relayed to the γ-resonance which overlaps with β_2. The δ–γ transition (No. 5) shows a relay peak to ϵ and to β_1. The β_2–γ double quantum transition (No. 7) has a weak relay peak to δ (which is not seen in this plot but can be observed at lower contour levels).

Strongly coupled spins such as the phenyl protons in the lower left of Figure 3 or the β_2, γ protons in the arginine residue give rise to double quantum peaks on the diagonal ($2F_1$,F_2) and are therefore clear evidence of strong coupling. The spectrum in Figure 3 demonstrates that connectivities can also be established via groups of strongly coupled spins (β_2, γ protons).

Conclusion

We have shown that the experiments discussed here allow identification of connectivity networks in complex spin systems by relaying double and zero quantum coherence of coupled spins onto their neighbors. Both the double and zero quantum relay spectra can be obtained from the same data set with appropriate data collection.

Acknowledgment. The authors thank Dr. Dennis Hare for providing them with his excellent 2D NMR software. Recently, we learned that a similar but less versatile method has been developed independently by Macura et al.[16]

Registry No. Vasopressin analogue, 90332-82-4; arginine, 74-79-3.

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