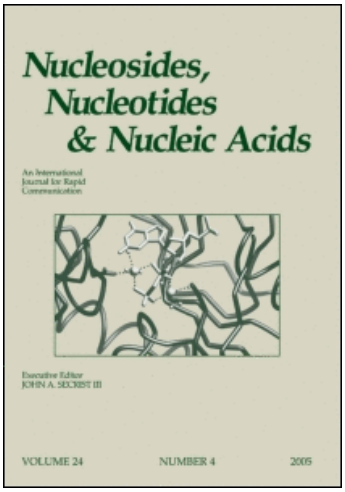

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INTERACTION OF DISTAMYCIN A AND NETROPSIN WITH QUADRUPLEX AND DUPLEX STRUCTURES: A COMPARATIVE ^{1}H-NMR STUDY

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ABSTRACT

Homonuclear NMR techniques have been used to investigate the interactions of the minor groove binding agents distamycin A (Dist-A) and the related drug netropsin (Net) with three quadruplexes characterized by different groove widths: $[d(TGGGGT)]_4$ (Q1), $[d(GGGGTTTTGGGG)]_2$ (Q2), and d(GGGGTTGGGGTGTGGGGTTGGGG) (Q3). Netropsin has been found to be in a fast chemical exchange with all three kinds of quadruplexes, whereas Dist-A interacts tightly with Q1 and, at a less extent, with Q2. In order to determine the degree of selectivity of Dist-A for two- rather than four-stranded DNA, we titrated with Dist-A an equimolar solution of Q1 and the duplex $d(CGCAAATTTGCG)_2$ (D). This comparative ^{1}H-NMR study allowed us to conclude that Dist-A and, consequently, Net possess higher affinity for duplex DNA.

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INTRODUCTION

DNA has been for many years the traditional target for chemotherapeutic intervention in human cancers,[1] and an intense interest continues to exist in the design and synthesis of small molecules that might selectively bind to defined sites in DNA.[2] Targeting sequence-dependent patterns of hydrogen bond donors and acceptors within the major and the minor grooves of duplex DNA, or targeting unique nucleic acid structures represent two different approaches to produce the desired selectivity.

Several nucleic acid ligands, such as the minor groove binding agents distamycin A (Dist-A) and the related drug netropsin (Net) (Fig. 1), have been the subject of a number of investigations, in search of possible correlations between biological activities and binding affinity. Both molecules were found to bind strongly in the minor groove of AT-rich regions of duplex DNA.[3]

In a previous work,[4] we reported the reversible interaction of Dist-A and Net with the parallel stranded $[d(TGGGGT)]_4$. Here, we wish to report the structural characterization of the new quadruplex d(GGGGTTGGGG-TGTGGGGTTGGGG) (Q3), along with the ^{1}H-NMR investigation of the interaction of Dist-A and Net with several tetraplexes characterized by different groove width, along with a comparison of Dist-A affinity for duplex DNA.

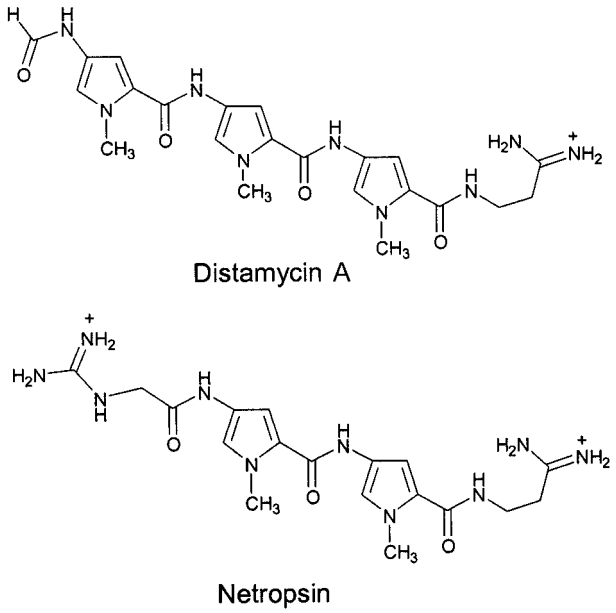

Figure 1. Structures of distamycin A and netropsin.

RESULTS AND DISCUSSION

Quadruplexes can be formed by a wide array of topologies and strand stoichiometries.[5] For the present study three different quadruplexes have been designed. In particular, we prepared $[d(TGGGGT)]_4$ (Q1), $[d(GGGGTTTTGGGG)]_2$ (Q2) and d(GGGGTTGGGGTGTGGGGTTGGGG) (Q3).

All oligonucleotides were synthesized using solid phase β-cyanoethyl phosphoramidite chemistry, cleaved from the support and purified by standard procedures.

Q1 NMR sample was prepared at a concentration of 1.3 mM (0.5 mL, 90% H_2O/ 10% D_2O), having 10 mM potassium phosphate, 70 mM KCl, 0.2 mM EDTA (pH 7.0). The structure of the four stranded hexamer $[d(TGGGGT)]_4$ has been already extensively studied by NMR and x-ray crystallography.[6] It has a fourfold symmetry with all strands parallel to each other, which afford four grooves of identical medium width, and all nucleosides in an anti conformation.

The NMR sample of Q2 was prepared at a concentration of 1.5 mM (0.5 mL, 90% H_2O/ 10% D_2O), having 10 mM sodium phosphate, 70 mM NaCl, 0.2 mM EDTA (pH 7.0), since the usage of potassium buffer prevented us to obtain a well defined species in solution. This tetraplex has been already characterized by NMR.[7] The whole of NMR data are consistent with the structure reported in the literature. It possesses four G-tetrads with guanosines that adopt the *syn-syn-anti-anti* conformations, which results in a quadruplex of alternative wide, medium, narrow, medium width grooves between strands.

Q3, to the best of our knowledge, has never been described before. Its NMR sample was prepared at a concentration of 1.0 mM (0.5 mL, 90% H_2O/ 10% D_2O), having 10 mM potassium phosphate, 70 mM KCl, 0.2 mM EDTA (pH 7.0). Its structural characterization began with obtaining the ^{1}H-NMR spectrum (Fig. 2A). This was recorded using pulsed-field gradient WATERGATE[8] for H_2O suppression. The one-dimensional proton spectrum shows that a single, well-defined species, is plainly observable in solution, since there are 23 signals from seventeen G-H8 and six T-H6 protons in the aromatic region. Furthermore, the presence of 16 imino peaks (Fig. 2A) resonating at 11–12 ppm indicates the formation of a G-quadruplex structure, consisting of four G-tetrads.

The exchange rates of the imino protons with water were determined by partially drying a sample in water and reconstituting the sample in D_2O. Periodic examination of the imino proton spectrum shows that they exchange very slowly, in agreement to what observed for other quadruplex structures.[9]

The proton spectrum of the quadruplex has been partially assigned on the basis of NOESY and TOCSY data obtained at 500 MHz (Table 1).

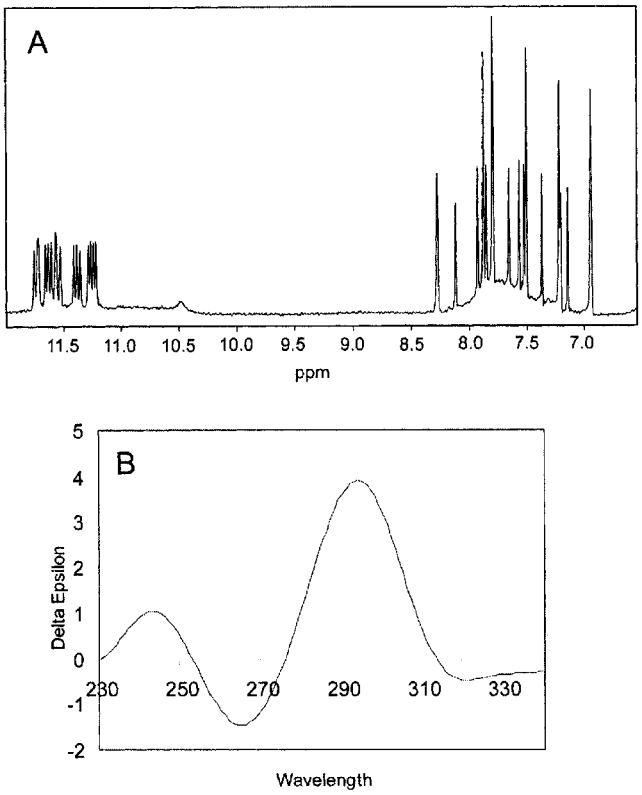

Figure 2. (A) Selected region of the NMR proton spectrum of Q3 (500 MHz, T = 300 K); (B) CD spectra of Q3.

The NOESY data demonstrates that eight G residues are in a *syn* conformation since the G-H8/H1′ intraresidue NOEs of these residues are very strong. This is in a perfect agreement with a quadruplex structure formed by antiparallel strands.[7,9] Moreover, CD spectrum of Q3 is typical of folded quadruplexes involving deoxy-guanosine alternating between *syn* and *anti* conformations about the glycosidic bond[10] in exhibiting a maximum at 293 nm and a minimum at 265 nm (Fig. 2B).

A thorough analysis of all NMR data, allowed us to conclude that the folding pattern is very similar to that observed for the thrombin-binding aptamer (TBA),[9] with four G-tetrads formed from guanosines of alternating *anti-syn-anti-syn* conformation, and each *syn*-guanosine donating hydrogen bonds to an adjacent *anti*-guanosine, and accepting hydrogen bonds from the other adjacent guanosine. Each tetrad stacks upon the other, so that consecutive G's in each DNA strand adopt an alternating *5′-3′-syn-anti*-relationship. This kind of quadruplex results in a rectangular G-tetrad core with grooves of alternating wide-narrow-wide-narrow widths.

Table 1. Non–exchangeable Proton Chemical Shifts for Q3 in 10 mM KH_2PO_4, 70 mM KCl, 0.2 mM EDTA (pH 7.0, T = 300 K)

Base (5′–3′)	H8/H6	H1′	H2′/H2″	H3′	H4′	H5′/H5″	H2/Me
G1	7.14	5.89	2.72–3.05	5.05		4.18	
G2	8.12	6.20	2.36–3.17	5.08	4.43	3.96	
G3	7.21	5.89	2.76–3.13	5.11		4.19	
G4	8.26	5.93	2.35–3.02	5.13	4.44	4.24	
T5	7.81	6.14	2.11–2.52	4.85		4.25	1.93
T6	6.93	5.91	1.97–2.61	4.84			0.71
G7	7.53	6.16	3.08–3.44	4.86	4.48		
G8	7.85	6.15	2.50–2.80	4.90		4.26	
G9	7.56	6.15	3.06–3.70	4.87	4.38	4.18	
G10	7.79	6.18	2.39–2.74	5.02			
T11	7.93	6.49	2.48–2.59	4.85	4.43	4.22	1.97
G12	7.37	5.53	1.85–2.12	4.83		3.96	
T13	6.94	5.52	1.88–2.42	4.56	3.84	3.47	1.70
G14	7.57	6.16	3.09–3.70				
G15	7.80	6.16	2.40–2.74	5.02	4.38		
G16	7.21	5.89	2.76–3.13	5.11		4.21	
G17	8.27	5.93	2.35–3.02	5.13	4.45	4.23	
T18	7.81	6.14	2.11–2.52	4.85		4.25	1.93
T19	6.93	5.91	1.97–2.61	4.85			0.71
G20	7.51	6.17	3.10–3.45		4.34		
G21	7.87	6.16	2.50–2.80	5.06		4.27	
G22	7.57	6.15	3.06–3.70			4.18	
G23	7.79	6.18	2.39–2.74	5.02			

Thus, there are alternative strand orientations that result in distinct distribution of glycosidic conformations in the G-tetrads. One consequence of this variation in glycosidic conformations is the distinct nature of the grooves that are formed between adjacent DNA strands (Fig. 3). All three kinds of grooves (narrow, medium and wide) described above differ significantly in size, electrostatic potential, hydrogen-bonding characteristics, steric effects, and hydration.

In principle, it is possible for Dist-A and Net to recognize different grooves. Therefore, we thought it necessary to investigate the binding mode of these molecules with each quadruplex described above.

The results of the titration of Q1 with Dist-A and Net have been already reported.[4] Below 2:1 ligand:tetraplex stoichiometry, Dist-A, in a dimeric form, binds each groove of the tetraplex to form short-lived complexes on the NMR time scale. At higher drug:DNA ratios, a second Dist-A dimer tightly and specifically binds the tetraplex, to give a 4:1 complex, in slow exchange with the 2:1 complex. The dyad symmetry of the 4:1 complex is consistent with a model comprising two Dist-A dimers simultaneously spanning, in fast

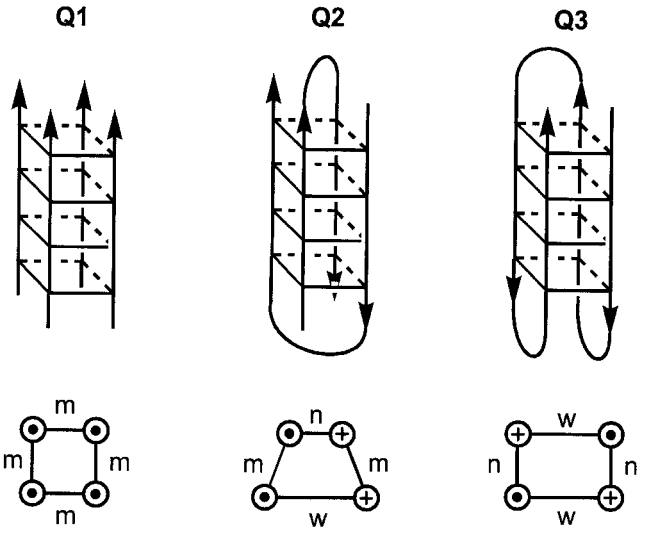

Figure 3. Schematic illustration of $[d(TGGGGT)]_4$ (Q1), $[d(GGGGTTTTGGGG)]_2$ (Q2) and d(GGGGTTGGGGTGTGGGGTTGGGG) (Q3) quadruplex structures. Strand direction along each edge of the quadruplex is indicated by arrows. Top views are reported below each structure. Types of grooves are indicated as narrow (n), medium (m) and wide (w). Strand polarity is indicated by the arrow tails (crosses) and heads (points) in circles.

reorientation, two opposite grooves of the tetraplex (Fig. 4). Notably, a fully saturated complex with all grooves occupied by drug molecules was not observed. Binding of dimer Dist-A may, most likely, expand the binding groove (as observed with duplex DNA),[3] and simultaneously reduce the size of the two adjacent grooves, thus preventing a further interaction with other Dist-A molecules. On the other hand, addition of Net to $[d(TGGGGT)]_4$ provides a complex where the ligand is in fast exchange on the NMR time scale with its binding sites on the tetraplex. Netropsin complexes the tetraplex with one drug molecule bound per groove.

The titration profile of Q3 with Dist-A is quite different from that observed for Q1 with the same molecule. Particularly, after the first addition of the drug, changes in the chemical shift of DNA proton resonances are observable, whereas a new set of broad signals appear between 6 and 7 ppm, which have been assigned to the Dist-A protons. The increase of Dist-A concentration up to 4 mol equiv causes drug resonances to gradually grow in intensity and change in chemical shift, along with a progressive drift of DNA signals. At ligand:DNA ratio of 4:1 the titration is virtually completed. The absence of any doubling of the NMR signals during the titration indicates that the ligand is in fast exchange on the chemical shift time scale with its binding sites on the oligonucleotide. This fast exchange behavior could not be changed to a slow or intermediate regime by altering the temperature of the system.

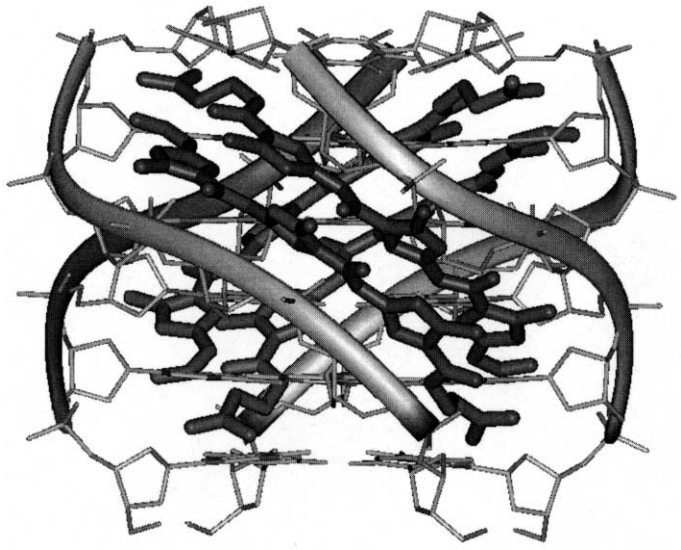

Figure 4. 3D model of the proposed binding mode of Dist-A to parallel-stranded tetraplex Q1.

A comprehensive analysis of NOESY spectra of the final complex shows that the structure of the tetraplex remains similar to the original conformation upon binding of distamycin A, as indicated by the presence of all the intranucleotide and internucleotide connectivities of the free tetraplex.

As for Dist-A, titration of Q3 with Net lead only to small changes in chemical shift of DNA proton resonances, along with the appearance of the drug proton signals. No intermolecular NOEs can be observed in the 2D NOESY experiments for both complexes.

Further, addition of Dist-A to Q2 causes gradual and severe line broadening of DNA and ligand resonances. This prevented us from studying the complex by 2D NMR experiments.

As observed for all quadruplex structures considered so far, addition of Net to Q2 lead to the formation of short-lived complexes on the NMR time scale. Progressive addition of Net causes the ligand resonances to grow in intensity and both the DNA and the ligand resonances to gradually change in chemical shift. Surprisingly, the resonances of the residues of the entire crossover loop (T5-T6-T7-T8) and the resonances of the residues adjacent to this (G3-G4 and G9-G10) are particularly affected upon the binding of Net. This suggests that Net mostly interacts with that part of the quadruplex structure. NOESY experiments at 300 K (500 MHz) performed at different mixing times shows only a single ligand:DNA contact between Net-H3 proton at 6.64 ppm and the broad aromatic DNA proton G4-H8 at 7.95.

In summary, Dist-A shows high affinity towards Q1. This quadruplex possesses four grooves of identical medium width, comparable to minor groove width of duplex DNA. Q3 is characterized, instead, by two wide and

two narrow grooves. The titration data clearly indicate that the ligand/DNA complex is in fast chemical exchange with respect to chemical shift on the NMR time scale. On the other hand, the crossover quadruplex Q2 possesses two grooves of medium width very similar to those found in Q1, along with other two grooves of wide and narrow width, respectively. The severe line broadening observed throughout the titration indicate a clear interaction with the target molecule. Typically, line broadening can be explained assuming that binding of the ligand to the oligonucleotide is on a comparable NMR time scale.

Based on what observed for Q1 and Q3, we believe that Dist-A interacts mostly with grooves of medium width. Netropsin showed a very low affinity with all DNA quadruplex structures studied in this investigation.

Distamycin A and netropsin are well-known duplex binders, but nothing is known about their degree of selectivity for four- rather than two-stranded DNA. In order to qualitatively clarify this point, we prepared and titrated with Dist-A an equimolar solution of the quadruplex Q1 and duplex $d(CGCAAATTTGCG)_2$ (D). The former represents, to the best of our knowledge, the quadruplex for which Dist-A possesses the highest degree of affinity, and the latter is a very well studied distamycin duplex DNA target.[3] Two separate annealing were preformed for Q1 and D. Thus, the two oligomers were gathered and analyzed by ^{1}H-NMR ($T = 295$ K, 500 MHz). All resonances in the proton spectrum of the mixture are consistent with the presence of the duplex and the quadruplex.[3,6]

A selected region of the spectra obtained at several points of the titration of the mixture is presented in Fig. 5. After the initial addition of drug the spectra clearly show that the signals belonging to D are particularly affected upon addition of Dist-A, whereas quadruplex resonances do not show any significant change. In particular, at ratio of drug-DNA less than 1:1, we observe the appearance of many new resonances. In fact, as consequence of slow exchange on the NMR time scale of Dist-A with the duplex, all signals belonging to both the free dodecamer and the complex are observed. The spectra are further complicated by a progressive broadening of the signals of the duplex. Nearly at 2:1:1 ligand:tetraplex:duplex stoichiometry, that is when all duplex molecules are saturated by the drug, further addition of Dist-A causes a progressive drift of quadruplex DNA signals, along with a further broadening of the duplex resonances. The titration continues, as expected, with the tight interaction of a second Dist-A dimer with the quadruplex, along with the consequent complication of the spectra as described above.

This ^{1}H-NMR study, allowed us to preliminarily conclude that Dist-A, and consequently Net, possess higher affinity for duplex than quadruplex DNA. Nevertheless, this investigation provides more insight into the interaction of minor groove binders with DNA quadruplex structures, suggesting that, in principle, they are able to discriminate among their various

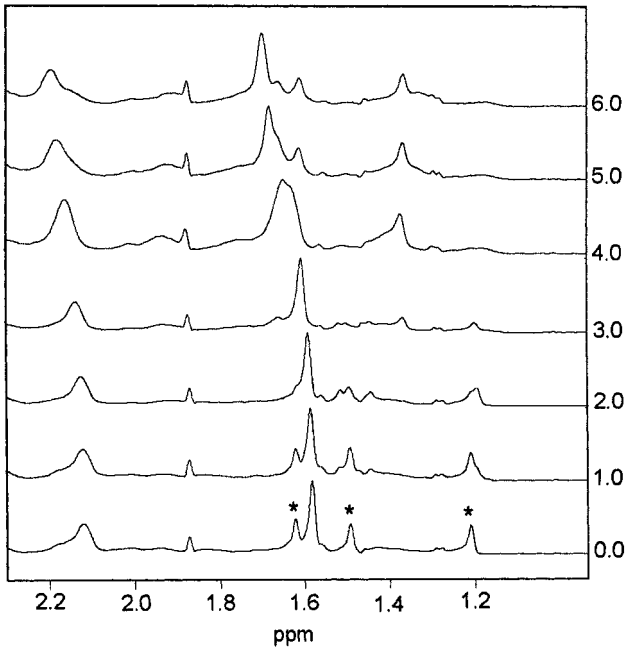

Figure 5. Methyl regions of NMR proton spectra acquired at several points in a titration of the equimolar solution of $[d(TGGGGT)]_4$ (Q1) and $d(CGCAAATTTGCG)_2$(D). ^1H NMR spectra were recorded using pulsed-field gradient WATERGATE[8] for H_2O suppression. A time domain deconvolution was used to further reduce the H_2O signal. The drug/DNA mole ratios are shown along the side of the spectra. The duplex resonances are indicated by asterisks.

topologies, characterized by different groove widths, as indicated by the behavior of Dist-A. Unfortunately, the low affinity of Net for all three quadruplex structures studied here, prevented us from performing a comparative study for this molecule as well. In order to clarify this point an investigation with other minor groove binders will be undertaken in our laboratories.

MATERIALS AND METHODS

The oligonucleotides were synthesized on a Millipore Cyclon Plus DNA synthesizer, using solid phase β-cyanoethyl phosphoramidite chemistry, cleaved from the support and purified by standard procedures. HPLC purifications were carried out with a Waters 515 Pump equipped with a UV detector a computerized controller. Q1 and Q3 NMR samples were prepared at a concentration of 1.3 and 1.0 mM quadruplex concentration respectively, in 0.5 mL (H_2O/D_2O 9:1) buffer solution having 10 mM potassium phosphate, 70 mM KCl, 0.2 mM EDTA, pH 7.0. Q2 was prepared at a concentration of 1.5 mM (H_2O/D_2O 9:1), having 10 mM sodium phosphate,

70 mM NaCl, 0.2 mM EDTA, pH 7.0. For D_2O experiments, the H_2O was replaced with D_2O by drying down the sample, lyophilization and redissolution in D_2O alone. NMR spectra were recorded with a Bruker AMX 500 spectrometer. 1D proton spectra of samples in H_2O were recorded using pulsed-field gradient WATERGATE[8] for H_2O suppression. Phase sensitive NOESY spectra[11] were recorded with mixing times of 50, 100, 200 ms ($T = 300$ K). Pulsed-field gradient WATERGATE was used for NOESY spectra in H_2O. TOCSY spectra[12] with mixing times of 120 ms were recorded with D_2O solutions. NOESY and TOCSY were recorded using TPPI[13] procedure for quadrature detection. In all 2D experiments the time domain data consisted of 2048 complex points in t_2 and 400–512 fids in t_1 dimension. The relaxation delay was kept at 1.2 s for all experiments. The NMR data were processed on a SGI O2 workstation using FELIX 98 software (Byosym, San Diego, CA).

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