The differences in the T_2 relaxation rates of the protons in the partially-deuteriated and fully protonated sugar residues in a large oligo-DNA ('NMR-window') gives complementary structural information

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ABSTRACT

Selective incorporation of the stereospecifically deuteriated sugar moieties (>97 atom % ^2H enhancements at $\text{H2}'$, $\text{H2}''$, $\text{H3}'$ and $\text{H5}'/5''$ sites, ~85 atom % ^2H enhancement at $\text{H4}'$ and ~20 atom % ^2H enhancement at $\text{H1}'$) in DNA and RNA by the 'NMR-window' approach has been shown to solve the problem of the resonance overlap [refs. 1, 2 & 3]. Such specific deuterium labelling gives much improved resolution and sensitivity of the residual sugar proton (i.e. $\text{H1}'$ or $\text{H4}'$) vicinal to the deuteriated centers (ref. 3). The T_2 relaxation time of the residual protons also increases considerably in the partially-deuteriated (shown by underline) sugar residues in dinucleotides [$\text{d}(\text{Cp}\underline{\text{G}})$, $\text{d}(\text{Gp}\underline{\text{C}})$, $\text{d}(\text{Ap}\underline{\text{T}})$, $\text{d}(\text{Tp}\underline{\text{A}})$], trinucleotide $\text{r}(\text{A2}'\text{p5}'\text{A2}'\text{p5}'\text{A})$ and 20-mer DNA duplex $^{5'}\text{d}(\underline{\text{C}}^1\text{G}^2\underline{\text{C}}^3\text{G}^4\underline{\text{C}}^5\text{G}^6\underline{\text{C}}^7\text{G}^8\underline{\text{A}}^9\underline{\text{T}}^{10}\underline{\text{T}}^{11}\underline{\text{T}}^{12}\underline{\text{C}}^{13}\underline{\text{G}}^{14}\underline{\text{C}}^{15}\underline{\text{G}}^{16}\underline{\text{C}}^{17}\underline{\text{G}}^{18}\underline{\text{C}}^{19}\underline{\text{G}}^{20})_2^{3'}$. The protons with shorter T_2 can be filtered away using a number of different NMR experiments such as ROESY, MINSY or HAL. The NOE intensity of the cross-peaks in these experiments includes only straight pathway from $\text{H1}'$ to aromatic proton ($i-i$ and $i-i+1$) without any spin-diffusion. The volumes of these NOE cross-peaks could be measured with high accuracy as their intensity is 3 to 4 times larger than the corresponding peaks in the fully protonated residues in the normal NOESY spectra. The structural informations thus obtainable from the residual protons in the partially-deuteriated part of the duplex and the fully protonated part in the 'NMR window' can indeed complement each other.

INTRODUCTION

The determination of the solution structure of large oligo-DNA duplex is generally a complex task because of the undesirable overlap of proton resonances. This complexity even becomes

more serious in the conformational studies with oligo-RNA of comparable molecular weight. The problem of severe spectral overlap makes the assignment of the proton resonances quite ambiguous and this also makes it almost impossible to derive any NOE volumes accurately for the structure determination. The other main problem is the line-broadening that is commonly encountered in the NMR structure determination of even a relatively small oligonucleotide such as a 20-mer DNA duplex³, when the crosspeak between $\text{H1}'$ and $\text{H2}''$ in a DQF-COSY spectrum could not be observed due to the severe overlap of the broad individual lines of the crosspeak.

Our structural studies of partially-deuteriated dinucleotides^{1,2}, trinucleotides^{1,2} and 20-mer DNA duplex³ by NMR spectroscopy, as well as other studies on partially-deuteriated peptides⁴⁻¹¹, suggest that the incorporation of deuterium significantly reduces the linewidths and gives much improved resolution and sensitivity of the proton resonances vicinal to the deuteriated centers. Thus the resonance overcrowding problem in large DNA and RNA may be tackled depending both upon the number and the site(s) of partially deuterium labelled sugar residues incorporated. It is noteworthy that although the uniform ^{15}N and ^{13}C labelling of RNA¹²⁻²⁰ may solve the problem of spectral overlap upto a certain size, but the problems of linewidths as well as the spectral overlap will become acute as the molecular weight increases. The uniform ^{13}C labelling has been shown to increase the linewidth of the α -protons¹⁰ in oligopeptides because of the decrease of T_2 , but the selective deuterium decoupling in $[2-^2\text{H}_R, 2-^{13}\text{C}]$ glycine-labeled *E.coli* thioredoxin indeed causes a 3-fold increase in both ^1H and ^{13}C resolution which result in 9-fold enhancement in the sensitivity²¹.

We have shown that the NMR studies of the partially-deuteriated oligo-RNA^{1,2} and DNA¹⁻³ with our 'NMR-window' approach¹⁻³ reduces the complexity of NOE cross-peaks in all regions except for the aromatic to $\text{H1}'$. We here report on the relaxation properties of residual protons that are vicinal to the deuteriated-carbons in the partially-deuteriated sugars (see Fig. 1)

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and compare them with the fully protonated counterparts in the dinucleotides, trinucleotides and 20-mer DNA (duplex I). We also show how the differences in their T_2 properties can be used to devise various NMR experiments such as ROESY or HAL to filter away the NOEs of the protons with shorter T_2 ; alternatively, it is also possible to make use of a NOESY experiment in which the $H2'/H2''$ resonances in NMR-window part could be saturated using a CW-pulse (MINSY) to visualize only those NOE cross-peaks from the partially-deuterated residues. Clearly, such studies would allow both the construction of new pulse sequences and the formulation of new specific isotope labelling strategy, which should facilitate the investigation of the solution structure of large oligo-DNA and RNA molecules with specific biological functions.

Base = adenine-9-yl; guanine-9-yl;
thymine-1-yl and cytosine-1-yl

Figure 1. A partially-deuterated sugar residue for specific incorporation in the NMR-window¹⁻³ approach (>97 atom % 2H enhancements at $H2'$, $H2''$, $H3'$ and $H5'/5''$ sites, ~85 atom % 2H enhancement at $H4'$ and ~20 atom % 2H enhancement at $H1'$).

METHODS

Partially-deuterated (shown by underline throughout the text and Tables) 20-mer, $5'd(C^1G^2C^3G^4C^5G^6C^7G^8A^9A^{10}T^{11}T^{12}C^{13}G^{14}C^{15}G^{16}C^{17}G^{18}C^{19}G^{20})_2$ (duplex I), its natural 20-mer counterpart (duplex II) and 12-mer $5'd(C^5G^6C^7G^8A^9A^{10}T^{11}T^{12}C^{13}G^{14}C^{15}G^{16})_2$ (duplex III) were prepared using our literature procedure³. 1H NMR measurements of the duplexes were made in a buffer consisting of 150 mM NaCl, 0.5 mM NaEDTA in 99.98 % D_2O (pH 7.0) at 294 K. The T_1 and T_2 relaxation times have been measured by 1H NMR at 500 MHz for all sugar protons in $d(TpA)^2$, $d(TpA)^2$, $d(ApT)^2$, $d(ApT)^2$, $d(CpG)^1$, $d(CpG)^1$, $d(GpC)^2$, $d(GpC)^2$, $r(A2'p5'A2'p5'A)^1$ and $r(A2'p5'A2'p5'A)^1$ dissolved in 99.98 % D_2O using inversion recovery and the Carr-Purcell-Meibom-Gill (CPMG) pulse sequence²² respectively with a relaxation delay of 10s. Additionally it was possible to measure the T_1 and T_2 relaxation time for some protons in duplex I. Selective T_1 was also measured for the separated resonances of duplex I using the saturation recovery method. Both the intensity and the volume of each resonance were used to calculate the T_1 and T_2 using the T_1/T_2 curve fitting procedure of UXNMR²³. The error for the T_2 values is estimated to be a maximum of 50% for J-coupled and 20% for other protons (i.e. $H1'$ and $H4'$ of partially-deuterated residues and $H2$ and $H8$). For the T_1 values the error is estimated to be approximately 10%. The NOESY²⁴ and ROESY²⁵ spectra were recorded using a sweep width of 9 ppm, with 4096 complex points in F2-dimension and 1024 or 256 experiments of 128 scans

Table 1. Experimentally determined T_2 relaxation times (s).

compound	residue	$H1'$	$H2'$	$H2''$	$H3'$	$H4'$	$H5'$	$H5''$	$H6/H2$	$H5/H8$ /5-Me
$d(CpG)$	C	0.04	0.03	0.02	0.06	†	0.08	0.07	0.18	0.16
$d(CpG)$	C	0.04	0.03	0.03	0.06	0.05	0.05	0.05	0.11	0.11
	ΔT_2	0	0	0.01	0	†	-0.03	-0.02	-0.07	-0.05
$d(CpG)$	G	0.04	0.03	0.03	0.09	0.23	†	†	-	0.19
$d(CpG)$	G	0.96	-	-	-	1.65	-	-	-	0.66
	ΔT_2	0.92	-	-	-	1.42	-	-	-	0.47
$d(GpC)$	G	0.05	0.04	0.04	†	0.06	0.12	0.12	-	0.63
$d(GpC)$	G	0.06	0.06	0.05	†	0.05	0.09	0.11	-	0.15
	ΔT_2	0.01	0.02	0.01	†	-0.01	-0.03	-0.01	-	-0.48
$d(GpC)$	C	0.06	0.07	0.07	0.12	0.12	0.12	0.12	0.05	0.05
$d(GpC)$	C	1.18	-	-	-	2.24	-	-	0.05	0.05
	ΔT_2	1.12	-	-	-	2.12	-	-	0	0
$d(ApT)$	A	0.05	0.05	0.05	†	0.05	0.09	0.11	0.34	0.25
$d(ApT)$	A	0.06	0.06	0.06	†	0.06	0.07	0.08	1.16	0.64
	ΔT_2	0.01	0.01	0.01	†	0.01	-0.02	-0.03	0.82	0.39
$d(ApT)$	T	0.06	0.05	0.05	0.04	□	0.17	†	0.21	0.26
$d(ApT)$	T	1.44	-	-	-	1.88	-	-	0.26	0.38
	ΔT_2	1.38	-	-	-	□	-	-	0.05	0.12
$d(TpA)$	T	0.05	0.03	0.02	0.11	0.23	0.22	0.22	0.34	0.44
$d(TpA)$	T	0.05	0.03	0.03	0.10	0.23	0.21	0.21	0.35	0.46
	ΔT_2	0	0	0.01	-0.01	0	-0.01	-0.01	0.01	0.02
$d(TpA)$	A	0.05	0.05	0.03	†	0.25	0.23	0.23	†	1.28
$d(TpA)$	A	1.49	-	-	-	2.09	-	-	1.79	1.45
	ΔT_2	1.44	-	-	-	1.84	-	-	†	0.17
$r(A2'p5'A2'p5'A)$	Ap	0.08	0.05	-	0.04	†	0.19	0.14	2.38	1.44
$r(A2'p5'A2'p5'A)$	Ap	1.99	-	-	-	1.95	-	-	2.43	1.51
	ΔT_2	1.91	-	-	-	†	-	-	0.04	0.07
$r(A2'p5'A2'p5'A)$	pAp	0.23	†	-	0.04	†	0.09	0.12	2.06	0.84
$r(A2'p5'A2'p5'A)$	pAp	0.25	†	-	0.05	0.06	0.09	0.10	2.10	0.88
	ΔT_2	0.02	†	-	0.01	†	0	-0.02	0.04	0.04
$r(A2'p5'A2'p5'A)$	pA	0.08	0.13	-	0.30	†	†	0.19	2.30	†
$r(A2'p5'A2'p5'A)$	pA	1.54	-	-	-	2.12	-	-	2.52	0.73
	ΔT_2	1.46	-	-	-	†	-	-	0.22	†
Deut 20-mer (I)	1-4, 17-20 [#]	0.11-0.20 [§]	-	-	-	†	-	-	†	†
Deut 20-mer (I)	5-16 [#]	<<0.11 [§]	†	†	†	†	†	†	†	†
20-mer (II)	1-20 [#]	<<0.11 [§]	†	†	†	†	†	†	†	†

†could not be measured, $\Delta T_2 = [T_2 \text{ (in partially-deuterated residue)} - T_2 \text{ (in protonated residue)}]$, underline indicates a partially-deuterated residue shown in Fig. 1; [#]residue number. [§]The correlation time, and therefore the T_2 , of most protons are proportional to the molecular weight, therefore the T_2 decreases nearly linearly with the increase of molecular weight. There is an 11 fold difference in molecular weight between the dimer and the 20-mer, this should imply a T_2 for $H1'$ of a protonated residue of ~5 ms and a T_2 for $H1'$ of a partially-deuterated residue of roughly 140 ms which is in good agreement with the experimental results.

in F1-dimension. The relaxation delay was 2s. The spectra were then zero-filled to 2K by 2K or 2K by 1K real data points before applying a 2 Hz line-broadening factor in F2 and F1-dimension. Baseline correction was performed in both dimensions. The mixing times used were 30, 50, 70, 100, 150, 180 and 200 ms. A NOESY experiment was also performed in which the H2'/H2'' resonances were saturated using a CW-pulse (MINSY)²⁶. The other parameters were as mentioned above using 256 experiments of 64 scans. The mixing time was 150 ms. A third NOESY experiment was performed in which the last pulse was replaced by (90° - 1/4J - 180° - 1/4J - SL) (HAL)²⁷. The other parameters were as mentioned above using 256 experiments of 64 scans. The mixing time was 150 ms. A NOESY experiment using a combination of the MINSY and HAL sequences was also performed using the parameters above. σ and τ_c were calculated for different crosspeaks in both the NOESY and ROESY spectra and are presented in Table 4.

RESULTS

The results in Tables 1 and 2 show that there is a substantial increase of T_1 and T_2 of the residual protons (i.e. H1' and H4') in partially-deuteriated residues (Fig. 1). It is most clearly seen in the dimers where the T_2 of the H1' and H4' increases more than 20 times in the partially-deuteriated sugar moieties compared to the natural counterparts. A larger T_2 for the H1' in the partially-deuteriated residues was also observed compared to that of H1' in the fully protonated residues in the 20-mer (duplex I). The T_1 values, on the other hand, increase only by 2 to 3 fold. The T_2 of the H8 and H2 of adenosine, the H6 and 5-Me protons of thymidine and the H6 and H5 protons of cytidine seems

to be marginally affected (<30%) or unaffected by the partial deuteration of their respective sugar residues at the dimer level. We however do not see a comfortable trend in the T_2 properties of H2 and H8 of adenosine in d(ApT) and H8 of guanosine when their natural dinucleotides are compared with deuteriated counterparts (Table 1). The change in the T_2 of aromatic protons is however much less than the increase of T_2 for H1' and H4' in the partially-deuteriated sugar residues, which has a considerable impact on the resolution and intensity of NOE crosspeaks involving these protons (*vide infra*).

Table 3 shows T_1 (by inversion and saturation recovery experiments) and T_2 measured for some protons of the partially-deuteriated 20-mer (duplex I). The table shows that there is no difference in the non-selective T_1 between the aromatic protons in the deuteriated and the non-deuteriated part of the molecule. However, comparing the selective T_1 there is an approximately 3 fold increase in the relaxation time of the aromatic protons of the partially-deuteriated residues. This should be due to the reduced number of relaxation pathways available for these protons as only the H1' of their own sugar residue is still protonated. Since the ^1H to ^2H dipolar interactions are only approximately 6% of that of ^1H to ^1H at the same distance they should in most cases be negligible. The non-selective T_1 of the H1 of the partially-deuteriated residues shows approximately 2 times increase, whereas the selective T_1 shows a 3 times increase.

The difference in the T_2 relaxation time can be however used to obtain different types of information from NOESY and ROESY spectra as the dipole-dipole relaxation process in these type of experiments are dependent on T_1 and T_2 respectively. A ROESY spectrum of the partially-deuteriated 20-mer (duplex I) at a mixing time of 150 ms showed only crosspeaks between

Table 2. Experimentally determined T_1 relaxation times (s).

compound	residue	H1'	H2'	H2''	H3'	H4'	H5'	H5''	H6/H2	H5/H8 /5-Me
d(CpG)	C	1.59	0.49	0.44	1.13	†	0.49	0.46	1.26	2.67
d(CpG)	C	1.75	0.56	0.51	1.22	1.57	0.54	0.54	1.39	2.72
	ΔT_1	0.16	0.07	0.07	0.09	†	0.05	0.08	0.13	0.05
d(CpG)	G	1.62	0.49	0.56	1.36	1.27	†	†	-	0.92
d(CpG)	G	4.22	-	-	-	4.50	-	-	-	2.72
	ΔT_1	2.60	-	-	-	3.23	-	-	-	1.80
d(GpC)	G	1.50	0.47	0.47	†	1.40	0.52	0.51	-	1.78
d(GpC)	G	1.49	0.42	0.44	†	1.45	0.48	0.46	-	0.69
	ΔT_1	-0.01	-0.05	-0.03	†	0.05	-0.04	-0.05	-	-1.09
d(GpC)	C	1.76	0.58	0.63	1.34	1.14	0.52	0.53	1.15	2.41
d(GpC)	C	4.05	-	-	-	4.32	-	-	1.71	2.11
	ΔT_1	2.29	-	-	-	3.18	-	-	0.56	-0.30
d(ApT)	A	1.22	0.40	0.40	†	1.16	0.44	0.44	1.62	0.97
d(ApT)	A	1.51	0.43	0.43	†	1.34	0.48	0.46	5.05	1.85
	ΔT_1	0.29	0.03	0.03	†	0.18	0.04	0.02	3.43	0.88
d(ApT)	T	1.55	0.51	0.51	1.21	†	0.41	†	1.04	0.85
d(ApT)	T	4.32	-	-	-	3.67	-	-	1.89	0.89
	ΔT_1	2.77	-	-	-	†	-	-	0.85	0.04
d(TpA)	T	1.52	0.40	0.44	1.14	1.40	0.46	0.45	1.21	0.98
d(TpA)	T	1.66	0.40	0.48	1.12	1.47	0.47	0.45	1.21	0.98
	ΔT_1	0.14	0	0.04	-0.02	0.07	0.01	0	0	0
d(TpA)	A	1.76	0.49	0.55	†	1.28	†	†	5.41	1.51
d(TpA)	A	4.78	-	-	-	4.44	-	-	5.73	3.56
	ΔT_1	3.02	-	-	-	3.16	-	-	0.32	2.05
r(A2p5'A2p5'A)	Ap	2.31	1.69	-	†	†	0.46	0.45	5.35	1.63
r(A2p5'A2p5'A)	Ap	3.52	-	-	-	5.30	-	-	6.58	3.16
	ΔT_1	1.21	-	-	-	†	-	-	1.23	1.53
r(A2p5'A2p5'A)	pAp	2.23	†	-	†	†	0.43	0.43	5.06	1.64
r(A2p5'A2p5'A)	pAp	2.78	†	-	1.38	1.25	0.47	0.45	6.43	3.32
	ΔT_1	0.55	†	-	†	†	0.04	0.02	1.37	1.68
r(A2p5'A2p5'A)	pA	3.04	1.21	-	1.18	1.17	†	†	6.16	1.40
r(A2p5'A2p5'A)	pA	5.71	-	-	-	5.43	-	-	7.57	1.60
	ΔT_1	2.67	-	-	-	4.26	-	-	1.41	0.20
Deut 20-mer (I)	1-4, 17-20 [#]	2.62 - 4.95	-	-	-	†	-	-	†	†
Deut 20-mer (I)	5-16 [#]	†	†	†	†	†	†	†	†	†
20-mer (II)	1-20 [#]	†	†	†	†	†	†	†	†	†

† could not be measured, $\Delta T_1 = [T_1(\text{in partially-deuteriated residue}) - T_1(\text{in protonated residue})]$, underline indicates a partially-deuteriated residue, in which the level of deuteration is H1': $\approx 20\%$, H2', H2'', H3', H5', H5'': $> 97\%$ and H4': $\approx 85\%$ ^{1,2}, # : residue number.

Table 3. Different T_1 and T_2 relaxation times of some of the protons in the partially-deuteriated 20-mer duplex (I).

Type of proton	$T_{1,ns}$ (s) [§]	$T_{1,s}$ (s) [§]	$T_{2,ns}$ (s) [£]	T_{2p} (s) [¥]
H8A ⁹ +H8A ¹⁰	2.166	0.261	#	0.060
H6C ¹	1.863	0.704	#	0.130
H6T ¹¹	1.706	0.138	#	#
H8G ²	1.369	0.719	#	0.100
H1'C ¹ +H1'C ¹⁹	3.456	0.992	0.161	0.177
H1'T ¹²	1.792	0.226	#	0.038
H6C ⁷	1.482	0.231	#	0.090
H2A ¹⁰	3.393	0.772	0.159	#
H2A ⁹	3.294	0.614	0.120	#
H1'G ²⁰	3.220	0.538	0.157	0.148
H1'G ²	3.376	0.514	0.108	0.113
H1'G ⁴	2.606	0.438	#	#
H1'G ¹⁸	3.409	0.474	0.114	#
H1'C ³	4.699	0.718	0.201	0.185
H1'C ¹⁷	3.059	0.405	0.132	0.140
MeT ¹²	2.281	0.455	#	#
H2"A ⁹ +H2"A ¹⁰	#	0.149	#	0.033
H5C ¹³	#	0.199	#	0.090
H8G ²⁰	1.362	0.719	#	0.105

proton does not exist, or is masked by other protons, $T_{1,ns}$ is nonselective and is obtained by inversion recovery experiment, $T_{1,s}$ is selective and is obtained by saturation recovery experiment, $T_{2,ns}$ is nonselective and is obtained by CPMG pulse-sequence, T_{2p} is obtained from ROESY experiment using buildup curves of the diagonal peaks at different mixing times.

aromatic and H1' protons of the partially-deuteriated residues and between H6 and H5 protons of all residues (Fig. 3B). On the other hand, a NOESY spectrum of the partially-deuteriated 20-mer recorded under identical conditions showed all possible crosspeaks from both the partially-deuteriated and the protonated parts (Fig. 3A).

Table 4 shows the σ values calculated from different NOESY and ROESY spectra. The σ for the H6-H5 crosspeaks from all cytidine residues are the same in both the partially-deuteriated and the non-deuteriated residues of the 20-mer (duplex I), which are comparable to the natural 20-mer (duplex II) and the 12-mer (duplex III). The reason is that the correlation time perpendicular to the helix axis^{28,29} does not change upon increasing the size of the molecule from a 12-mer to a 20-mer duplex. The σ for H6-H5C have also been calculated from experiments with different relaxation delays (2 and 7 s) but no difference was observed.

One of the most important questions in the determination of interproton distances is the effect of spin-diffusion. The σ of the H6/H8-H1' crosspeaks have been calculated both from the build-up rates and from individual NOESY experiments at 150 and 200 ms where spin-diffusion could be expected to start to play a role. The NOESY and ROESY data in Table 4 show there is no significant difference in σ at 150 or 200 ms compared to the σ from build-up curves (from 30, 50, 70, 100, 150, 180 and 200 ms) for the H6/H8-H1' crosspeaks of the partially-deuteriated sugars in duplex (I). But for the protonated sugars, the σ from the 150 or 200 ms NOESY spectra is usually larger than the σ from the build-up curve. A comparison of the σ values of the partially-deuteriated residues in the 20-mer (duplex I) with those of the corresponding residues in the duplex II shows that the σ

Figure 2. The pulse sequences used for a (A) normal NOESY²⁴, (B) normal ROESY²⁵, (C) MINSY²⁶ experiment in which the H2'/H2'' region is saturated during the mixing time, (D) HAL²⁷ which is a NOESY experiment followed by an echo and a short spinlock, and (E) a combined MINSY-HAL experiment.

values increase for the latter due to the contribution from the spin-diffusion. A similar observation can be also made for the σ values for the H6/H8_i-H1'_{i-1} crosspeaks. These observations show that a reduction in the number of relaxation pathways for the H1' by selective substitution of vicinal protons by deuterons (i.e. H2'/2'' → D2'/2'') is a very powerful tool in obtaining NOE volumes that can be used for the determination of correct aromatic-H1' distances.

A NOESY experiment (Fig. 2A) in which the H2'/H2'' protons are saturated during the mixing time (MINSY)²⁶ (Fig. 2C) have been suggested as a means to reduce the number of relaxation pathways (H1'-H2' and H1'-H2''). In a MINSY experiment with duplex I, we expected to observe most of the aromatic-H1' crosspeak belonging to both the deuteriated and non-deuteriated residues. We also expected an enhancement of the NOE intensities of the aromatic-H1' crosspeak of the residues belonging to the 'H NMR-window' because of the reduction of the number of relaxation pathways in the same manner as those from the partially-deuteriated residues. To our surprise, this MINSY experiment ($\tau_m = 150$ ms) showed only crosspeaks arising from the partially-deuteriated residues (Fig. 3C). The crosspeaks that are found in this MINSY experiment (Fig. 3C) are identical to those found in the ROESY experiment (Fig. 3B). The observation of these aromatic-H1' crosspeaks from the partially-deuteriated residues confirmed our earlier assignments³ and helped to calculate accurately the intra and interresidual NOEs (data not shown). It is noteworthy that σ calculated from the MINSY experiment (Fig. 3C) are in good agreement with σ from NOESY (Fig. 3A) and ROESY experiments (Fig. 3B) (Table 4, $2\sigma^{noe} = \sigma^{roe}$). Both MINSY and ROESY experiments gave the same kind of information, but as T_1 is much longer than T_2 we have been able to use longer mixing times in the MINSY experiment which resulted in more intense crosspeaks and more correct NOE volumes for distance calculations, and therefore it should be preferable to ROESY experiments. The correlation time of H1' of a partially-deuteriated sugar in partially-deuteriated 20-mer duplex (I) has been calculated from the ratio of $T_{1,ns}/T_{1,s}$ ^{30,31} and was found to be 5.75 ± 0.65 ns, which is close in value to the one found for the H5-H6 vector (Table 4). It is consistent with the influence of anisotropic rotation and internal motions on NOE intensities^{28,29}. The H5-H6 of cytidine and the H1'-H6/H8 vectors are nearly perpendicular to the axis of the helix and consequently their correlation time is the same as τ_s ^{28,29} (the correlation for the rotation along the short axis). Using the ratio for a 20-mer of τ_L/τ_s of approximately 2.5^{28,29}, the τ_L (the correlation for rotation along the long axis) should be equal to

Table 4. Dipole-dipole relaxation rates calculated for different crosspeaks of duplexes I, and II and III.

Type of crosspeak	Deuteriated 20-mer (I)										20-mer (II)	12-mer (III)
	σ^{noe} from NOESY			σ^{noe} from build up curve		σ^{roe} from ROESY		σ^{noe} MINSY	σ^{noe} HAL	σ^{noe} from NOESY		
	$\tau_m=0.20s$, RD=2s	$\tau_m=0.15s$, RD=2s	$\tau_m=0.20s$, RD=7s	NOESY	ROESY	$\tau_m=0.10s$, RD=2s	$\tau_m=0.15s$, RD=2s	$\tau_m=0.20s$, RD=2s	$\tau_m=0.15s$, RD=2s	$\tau_m=0.020s$, RD=2s	$\tau_m=0.20s$, RD=2s	
H6C ¹ -H5C ¹	1.20	2.03	1.38	0.97±0.06	1.91±0.29	2.42	1.95	1.11	#	1.42	£	
H6C ³ -H5C ³	1.29	1.28	1.58	1.16±0.11	2.00±0.20	1.81	1.79	1.02	#	1.45	£	
H6C ⁵ -H5C ⁵	¥	¥	¥	¥	¥	¥	¥	#	#	¥	1.55	
H6C ⁷ -H5C ⁷	1.31	1.50	1.48	0.95±0.11	1.81±0.20	1.59	1.12	1.00	#	1.20	1.02	
H6C ¹³ -H5C ¹³	1.22	1.07	1.09	0.92±0.07	1.87±0.17	1.44	0.81	0.82	#	1.05	1.21	
H6C ¹⁵ -H5C ¹⁵	¥	¥	¥	¥	¥	¥	¥	#	#	¥	1.17	
H6C ¹⁹ -H5C ¹⁹	1.31	1.00	1.44	0.68±0.07	1.82±0.19	1.68	1.81	0.84	#	1.31	£	
	$\sigma_{\text{noe}}=1.24\pm0.29^*$, $\tau_c=5.41\pm1.15$					$\sigma_{\text{roe}}=1.88\pm0.07^{**}$				$\sigma_{\text{noe}}=1.29\pm0.16^*$	$\sigma_{\text{noe}}=1.24\pm0.22^*$	
H6C ¹ -H1'C ¹	0.26	0.38	0.34	0.15±0.02	0.34±0.03	0.66	0.48	0.24	0.27	0.33	£	
H8G ² -H1'G ²	0.10	0.09	0.14	0.08±0.001	0.34±0.06	0.10	0.09	0.08	0.12	0.39	£	
H6C ³ -H1'C ³	0.14	0.17	0.21	0.11±0.10	0.36±0.09	0.19	0.20	0.18	0.20	0.60	£	
H6C ⁷ -H1'C ⁷	0.21	0.21	0.25	0.12±0.01	#	#	#	#	#	0.33	0.15	
H8G ⁸ -H1'G ⁸	¥	0.12	0.18	0.10±0.02	#	#	#	#	#	0.36	0.21	
H8A ¹⁰ -H1'A ¹⁰	0.32	0.28	0.28	0.11±0.02	#	#	#	#	#	0.52	0.19	
H6T ¹¹ -H1'T ¹¹	0.46	0.38	0.58	0.10±0.01	#	#	#	#	#	0.52	0.24	
H6T ¹² -H1'T ¹²	0.19	0.13	0.19	0.17±0.02	#	#	#	#	#	0.34	0.28	
H6C ¹³ -H1'C ¹³	¥	0.46	0.59	0.11±0.02	#	#	#	#	#	0.61	¥	
H6C ¹⁷ -H1'C ¹⁷	¥	¥	¥	¥	¥	0.13	0.26	0.12	0.18	¥	£	
H6G ¹⁸ -H1'G ¹⁸	¥	¥	¥	¥	¥	0.24	0.22	0.10	0.09	¥	£	
H6C ¹⁹ -H1'C ¹⁹	0.12	0.17	0.16	0.11±0.01	0.35±0.05	0.25	0.25	0.11	0.17	0.58	£	
H8G ²⁰ -H1'G ²⁰	0.16	0.16	0.16	0.01±0.001	0.36±0.05	0.11	0.08	0.12	¥	0.25	£	
	$\sigma_{\text{noe}}=0.11\pm0.04^{**}$					$\sigma_{\text{roe}}=0.35\pm0.01^{**}$				$\sigma_{\text{noe}}=0.44\pm0.12^*$	$\sigma_{\text{noe}}=0.21\pm0.04^*$	
H1'C ¹ -H8G ²	0.13	0.09	0.11	0.08±0.01	0.30±0.05	0.16	0.19	0.07	0.11	0.26	£	
H1'G ² -H6C ³	¥	¥	¥	¥	0.34±0.03	0.27	0.34	0.26	0.29	¥	£	
H1'C ³ -H8G ⁴	0.20	0.16	0.23	0.11±0.01	0.34±0.04	0.21	0.26	0.14	0.23	0.53	£	
H1'G ⁶ -H6C ⁷	0.49	0.48	0.35	0.15±0.01	#	#	#	#	#	0.38	0.23	
H1'C ⁷ -H8G ⁸	0.17	0.14	0.13	0.08±0.02	#	#	#	#	#	¥	0.12	
H1'G ⁸ -H8A ⁹	0.29	0.46	0.26	0.19±0.01	#	#	#	#	#	0.41	0.35	
H1'A ¹⁰ -H6T ¹¹	0.33	0.30	0.27	0.17±0.01	#	#	#	#	#	0.32	0.36	
H1'T ¹² -H6C ¹³	0.24	0.27	0.21	0.17±0.01	#	#	#	#	#	0.41	0.26	
H1'G ¹⁶ -H6C ¹⁷	¥	¥	¥	¥	0.35±0.03	0.19	0.18	0.15	0.20	¥	£	
H1'C ¹⁷ -H8G ¹⁸	¥	¥	¥	¥	0.34±0.04	0.17	0.27	0.13	0.18	¥	£	
H1'G ¹⁸ -H6C ¹⁹	¥	¥	¥	¥	0.34±0.05	0.48	0.59	0.16	0.32	¥	£	
H1'C ¹⁹ -H8G ²⁰	0.12	0.06	0.09	0.10±0.01	0.30±0.05	0.18	0.12	0.08	0.11	0.19	£	
	$\sigma_{\text{noe}}=0.13\pm0.04^{**}$					$\sigma_{\text{roe}}=0.33\pm0.02^{**}$				$\sigma_{\text{noe}}=0.36\pm0.10^*$	$\sigma_{\text{noe}}=0.26\pm0.09^*$	

¥: could not be calculated, #: crosspeak does not appear in spectra, £: protons do not exist. The σ values were calculated using the following formula: $\sigma = (a_{ij}/a_{ii} + a_{ji}/a_{jj})/2\tau_m$, for ROESY spectra offset correction of both crosspeaks and diagonal peaks were performed. σ values marked with * are the averages of all the values available in this table, those marked ** are the averages using only the data from build-up. Note that $2\sigma^{\text{noe}} = \sigma^{\text{roe}}$. τ_c calculated using $\tau_c = (10\sigma^6)/((\mu/4\pi)^2\hbar^2\gamma^4)$. The average τ_c for the H5-H6 crosspeaks for protonated 20-mer and 12-mer is 5.58 ± 0.63 and 5.41 ± 8.87 respectively.

8.64 ns for 20-mer duplexes I and II. The vector H1'(i)–H6/H8(i+1) is nearly parallel to the helix axis and it should be determined by $\tau_L^{28,29}$. The average distance for the H1'(i)–H6/H8(i+1) vector of duplex I, calculated using the average σ and the value of τ_L , is 3.9 Å.

Figs. 4A–D show experimental and theoretical build-up curves for different types of diagonal and crosspeaks for both NOESY and ROESY spectra. For the NOESY spectra the relaxation rate RL (for all symbols and nomenclature see ref. 32–34) for the diagonal peaks have been varied between 1 and 5 s^{−1} (same region as observed from the selective T₁ measurements). For the experimental crosspeaks three different types of dipole–dipole relaxation, i.e. between H6/H8–H1', H6–H5 and H2'–H2'', have been plotted in Figs. 4A and 4C. The data for the experimental NOESY spectrum (Fig. 4A) and the theoretical NOE volume calculations^{32–34} (Fig. 4B) show that there are no difference in the NOE volumes between crosspeaks arising from fully protonated and partially-deuteriated residues. A completely different situation is however observed from the data of the experimental ROESY spectrum (Fig. 4C) and the theoretical ROE volume calculations³⁵ (Fig. 4D). The RL obtained from the experimental data is varied between 7 and 10 s^{−1} for all protons (i.e. H1' and H5 and H6) of the partially-deuteriated residues and H5/H6 of fully-protonated residues (Fig. 4C), which fit with their observed T₂ values (Table 3). The RL of sugar protons of

the fully protonated residues were found to be more than 30 s^{−1} (Fig. 4C). The data shows that for a mixing time of 100 ms the intensity of the diagonal peak should have decreased by ≈ 50% for the partially-deuteriated residues, whereas only ≈ 3% of the diagonal peaks would be left from the fully protonated residues (Fig. 4D). A similar situation is also observed for the crosspeaks: Amongst the crosspeaks of the aromatic-H1' protons in the mixing time between 100 and 150 ms, only crosspeaks from the partially-deuteriated residues could reach a detectable maximum intensity (Figs. 4C & 4D). It is noteworthy that the crosspeaks between H5 and H6 from partially-deuteriated residues and those from the fully protonated residues in duplex (I) behave in a slightly different manner in the ROESY spectra (Fig. 4C) whereas they showed similar behaviour in the NOESY spectra (Fig. 4A). As pointed out above (Table 4), the σ for H5 and H6 for both deuteriated and protonated residues are the same. It means that the difference observed in these crosspeaks in ROESY is due to different RL. The difference in intensity between the H5–H6 crosspeaks from the partially-deuteriated and fully protonated residues increase with the increasing mixing time (Fig. 4C). For example, in 200 ms mixing time the H5–H6 crosspeaks from the fully protonated residues disappear completely. Note that this difference in the NOE volumes decreases at the shorter mixing times (i.e. 30–50 ms) (Fig. 4C). It is unfortunate that T₂ data could not be obtained for any H5 or H6 protons of the duplex

Figure 4. Experimental (panels A and C) and theoretical (panels B and D) buildup curves for the NOESY (panels A and B) and ROESY (panels C and D) spectra. For theoretical estimation³²⁻³⁴ of the buildup curves, the approximation of two-spin system has been used, where the NOE/ or ROE cross (a_{ij}) and diagonal (a_{ii}) peaks volume at mixing time τ_m are: $a_{ii}(\tau_m)/a_{ii}(0) = 0.5a_{ii}(\tau_m = 0)(1 \pm e^{-Rc_{ij}\tau_m}) - RL\tau_m$, where Rc_{ij} is the cross relaxation rate and RL is the leakage rate constant³²⁻³⁴ and are equal to $Rc = [(q_i - q_j)^2 + 4\sigma_{ij}\sigma_{ji}]^{1/2}$ and $RL = [1/2(q_i + q_j) - 1/2Rc]$. $\sigma_{ij} = \sigma_{ji}$ and $q_i = q_j$ are cross-relaxation rates³²⁻³⁴. The Y-coordinates are the value of $(\ln[a_{ii}/a_{io}] + 1)$ diagonal peaks (left side) and $(\ln[a_{ij}/a_{io}] + 1)$ (right side) crosspeaks; the X-coordinates are the mixing time (τ_m) (s). In panels A and C the experimental diagonal volumes are connected by a solid line and crosspeaks by dashed (H5-H6), dotted (H1'-H8/H6) or dashed-dotted (H2'-H8/H6) lines. The diagonal and crosspeak data belonging to the same residue are marked by the same symbol as denoted under the panels. In panels B and C the theoretical data have been calculated for Rc of 0.4, 2.0 and 15.0 for the NOESY spectra (panel B) and 0.8, 4.0 and 30.0 for the ROESY spectra (panel D). For RL of 3.0 and 5.0 for the NOESY spectra (panel B) and 7.0, 10.0 and 30.0 for the ROESY spectra (panel D). Rc values have been calculated using the program MORASS^{ref} for the B-DNA structure of the 20-mer (duplex II) for H5-H6 of cytosine and the interresidual distances: $(H1'-H8/H6)_i$ and $(H2'-H8/H6)_i$ [the correlation time used is the same as the experimental one: 5.5 ns (Table 4)]. RL value have been used as average for the corresponding group of protons (H5, H6, H8, H1', H2'), the values for RL and Rc are plotted near theoretical curves in panels B and D. Theoretical diagonal and cross peak curves belonging to the same proton are represented by the same type of line: thin-solid line for $(H1'-H8/H6)_i$ cross peak and $H1'$ proton diagonal peaks; dashed and dashed-dotted lines for $(H5-H6)$ cross-peaks for the partially deuterated and non-deuterated residue, respectively; thick-solid lines for $(H2'-H8/H6)_i$ cross peak and $H2'$ diagonal peaks.

Figure 3. Contour plots of the aromatic to anomeric regions in NOESY (panel A, see the pulse sequence in Fig. 2A), ROESY (panel B, see the pulse sequence in Fig. 2B), MINSY (panel C, see the pulse sequence in Fig. 2C), HAL (panel D, see the pulse sequence in Fig. 2D), MINSY-HAL (panel E, see the pulse sequence in Fig. 2E) experiments with duplex I at 294 K at a mixing time of 150 ms. The panel F shows a spectrum resulting from the subtraction of the MINSY from NOESY. The crosspeaks marked A-G are the H6-H5 crosspeaks: A of C¹, B of C¹³, C of C³, D of C¹⁹, E of C¹⁷, F of C⁵ and C¹⁵ and G of C⁷. Crosspeaks i-iii of 3C are crosspeaks from the protonated part of the 20-mer (duplex I), appearing due to incomplete saturation of the H2'/H2'' region of the spectrum. In figs. 3B-E the connectivity of the two separate ends of the molecule are marked with dashed and solid lines. In 3A and 3E the connectivities are marked with solid lines. Note that in panel F the H5-H6 crosspeaks: B, C, E-G can not be used for volume calculations.

The concept of NMR-window

NOE and dihedral angle informations are obtainable through NMR-window(s) A or/and B and the informations obtained are complementary. While NMR-window B is completely protonated, NMR-window A is specifically chemically labeled by ^2H in a non-uniform manner leaving some chosen observable sugar proton(s). The remarkable difference in the relaxation properties of the sugar protons, depending upon the nature of vicinal isotope (^2H or ^{13}C), enable specific NMR experiments to perform, and allow the extraction of specific NMR informations. Note that the creation of such non-uniformly labeled NMR-windows is only possible by regio and stereocontrolled chemical synthesis and would help to tackle the solution structure of large DNA or RNA by NMR.

Figure 5. The ^1H -NMR window concept for the determination of local structure of large biologically functional oligo-DNA or RNA

(I), but from the extrapolation of the diagonal peak volume to zero mixing time using ROESY data, we estimated the R_L for H5 and H6 protons of the fully protonated residues and partially-deuteriated residues to be $10 \pm 2 \text{ s}^{-1}$ and $7 \pm 2 \text{ s}^{-1}$, respectively (Table 3). The theoretical³⁵ curves at these particular R_L show (Fig. 4D) that the difference in volumes of H5 and H6 crosspeaks from the deuteriated residues and the corresponding natural counterpart in duplex (I) at 150 ms mixing time should be at least twice but the experimental data show at least a 3-fold difference (Fig. 4C), the reason for this difference in R_L is however not clear to us. The above data show that in ROESY spectra of $\tau_m > 100 \text{ ms}$ only crosspeaks from aromatic-H1' from the partially-deuteriated residues and H5-H6 from all residues appear; the intensities and volumes of these H5-H6 crosspeaks however show clearly observable differences depending on whether they belong to the partially-deuteriated or fully protonated residues (Fig. 2B). These data suggest that the subtraction of a ROESY spectrum from a normal NOESY spectrum indeed eliminates all NOE crosspeaks resulting from H1' and aromatic in the partially-deuteriated sugars. A systematic analysis of the subtraction of NOE volumes (data not shown) has indicated that the error introduced into the crosspeak volume calculations of the resulting subtracted NOESY spectrum is not more than 30% which is well within the error limit for NOE volume calculations³⁶. The spinlock in the ROESY experiment serves in this case as a filter to separate the sugar protons that relax rapidly from those that relax slowly due to the absence or presence of deuterium at the vicinal carbon(s). There are however two main disadvantages of the ROESY experiment: (1) there is a need for offset correction of the diagonal and crosspeaks, and (2) there is also a need to use a narrow range of mixing times (i.e. 100 to 150 ms for duplex I) because of still relatively short T_2 relaxation time for the proton vicinal to deuterium.

In the HAL experiment (Fig. 2D) which consists of a normal NOESY followed by a Hahn-echo and a short spinlock²⁷ the protons which have a very short T_2 could also be filtered away from protons having longer T_2 . We have performed this experiment and the result is very similar (Fig. 3D) to that of the ROESY experiment (3B), but the former has the additional advantage of eliminating the splitting due to J-coupling of H5 and H6 cytidine protons which helps in the calculation of H5-H6 crosspeak volume and it may also help in the extraction of the

other NOE crosspeaks that may overlap in this H5-H6 region. However, this elimination of J-coupling also makes it impossible to subtract the HAL spectrum from the NOESY spectrum.

The above problems of the ROESY experiment has been however bypassed by performing Redfield's NOESY (MINSY) experiment with saturation of H2'/H2'' protons of the residues belonging to the NMR-window during the mixing time²⁸. The subtraction of a MINSY spectrum from a NOESY spectrum results in a near perfect picture (Fig. 3F) of the crosspeaks of the core fully protonated residues of duplex I.

DISCUSSION

The maximum size of DNA molecule that is amenable to full assignment and measurement of all NOE crosspeaks is most probably limited to a symmetrical duplex of 24 residues. The first reason is the large proton overlap in the spectra of oligo-DNA and, the second reason is owing to the line-broadening caused by the short relaxation time. These inherent deficiencies of NMR methods are not simply curable by stronger magnetic field or multidimensional NMR. The conformational states of such small DNA molecules that are amenable to NMR structural study may not significantly represent the biological structure and function that are associated with the larger ones. We have recently established the 'NMR-window'¹⁻³ approach and have advocated the use of partially-deuteriated nucleotide residues ($> 97 \text{ atom } \% ^2\text{H}$ enhancements at H2', H2'', H3' and H5'/5'' sites, $\sim 85 \text{ atom } \% ^2\text{H}$ enhancement at H4' and $\sim 20 \text{ atom } \% ^2\text{H}$ enhancement at H1') which are to be introduced in a DNA by synthetic means in a specific manner to simplify the overlap of proton resonances, allowing the examination of a specific non-deuteriated region in a large DNA duplex. During this investigation³ we observed for the first time that the presence of deuterium had a profound influence on the relaxation properties of the neighbouring residual protons (i.e. H1' and H4'). A comparison of the H8/H6 to H1' NOE crosspeaks between $5'\text{d}(\text{C}^1\text{G}^2\text{C}^3\text{G}^4\text{C}^5\text{G}^6\text{C}^7\text{G}^8\text{A}^9 \text{ A}^{10}\text{T}^{11}\text{T}^{12}\text{C}^{13}\text{G}^{14}\text{C}^{15}\text{G}^{16}\text{C}^{17}\text{G}^{18}\text{C}^{19}\text{G}^{20})_23'$ and its partially-deuteriated counterpart $5'\text{d}(\text{C}^1\text{G}^2\text{C}^3\text{G}^4\text{C}^5\text{G}^6\text{C}^7\text{G}^8\text{A}^9\text{A}^{10}\text{T}^{11}\text{T}^{12}\text{C}^{13}\text{G}^{14}\text{C}^{15}\text{G}^{16}\text{C}^{17}\text{G}^{18}\text{C}^{19}\text{G}^{20})_23'$ shows that the linewidths are considerably decreased for the crosspeaks belonging to the partially-deuteriated residues in the latter. There are three possible reasons for this behaviour: (1) The T_2

relaxation times of the H1' protons are increased due to the presence of deuterium in the H2', H2'' and H3' positions. (2) The absence of H1'-H2'/H2'' coupling constants in the partially-deuteriated residues will enhance the intensity of the H8/H6 to H1' crosspeaks. (3) There are a reduced number of relaxation pathways available in the partially-deuteriated residues. Some of these reasons have been however attributed to the effect of deuteriation on the linewidths and calculation of NOESY intensities in proteins^{37,38}. In oligonucleotides it has earlier been shown by us¹ that the T₁ relaxation times of H1' in partially-deuteriated residues are increased in d(GpC) (3.9s) compared to d(GpC) (2s) and in r(A2'p5'A2'p5'A) (3s and 4s respectively) compared to r(A2'p5'A2'p5'A) (2.1s and 2.5s respectively)¹. The increase in T₁ should not influence the linewidths but the present work shows that both the T₁ and the T₂ relaxation times change simultaneously. Our result shows for the first time that there is considerably more increase in the T₂ relaxation time of sugar protons in partially-deuteriated residues in all oligonucleotides investigated in this work (Tables 1–3). The protons with shorter T₂ can be filtered away using a number of different experiments which should include a refocussing period for quickly relaxable protons, for example a ROESY (Fig. 2B) or HAL (Fig. 2D) experiment, whereas a NOESY experiment in which the H2'/H2'' resonances in the 'NMR-window' part were saturated using a CW-pulse (MINSY) could be successfully used (Fig. 2D) to exhibit the NOE crosspeaks only from the partially-deuteriated residues similar to a ROESY experiment. The intensity of the crosspeaks in these experiments includes only straight pathway from H1' to aromatic (i–i and i–i+1) without spin-diffusion. The volumes of these peaks could be measured with high accuracy as their intensities are 3 to 4 times larger than the corresponding peaks for the protonated residues in normal NOESY spectra.

This means that the informations obtainable from the deuteriated part of the duplex and the protonated part in the NMR window are indeed complementary. This should allow the specific incorporation of our partially-deuteriated residues anywhere in a large DNA duplex and extract structural informations from this part by taking into account of the difference in T₂ relaxation properties between the partially-deuteriated residues and the fully protonated ones (Fig. 5).

In summary, this paper along with other studies in the conformational studies of proteins^{4–9,39–41} has clearly shown that the selective deuteriation labelling solves the spectral overlap problem and also gives much improved resolution and sensitivity of the proton vicinal to the deuteriated centers. It is likely that a rational design and synthesis of partially-deuteriated biologically functional DNA or RNA would allow the extraction of both NOE and some dihedral angle information. Using appropriately chosen deuteriation sites in the sugar moiety or a combination of such differently deuterium labelled isotopomers in a large DNA or RNA molecule one should be able to specifically define the sets (usually pairs are desired) of interacting nuclei for NOE and for the dihedral angle information. Such careful engineering of DNA or RNA molecule with deuterium will effectively eliminate spin-spin coupling and relaxation effects from nuclei outside of these sets (Fig. 5) under a given measurement condition. It is also possible to selectively create a 'NMR window' in which one employs ¹³C enriched sugar moieties along with parts of the DNA molecule that are partially-deuteriated. All these demands of selective and yet non-uniformly isotopically enriched large DNA or RNA or peptides^{4–9,39–41} clearly requires regio and stereospecific chemical operations that only synthetic chemistry can offer.

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