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Partially-deuterated Nucleotide Residues in Large DNA Duplex Simplify the Spectral Overlap and Provide both the J-coupling and nOe Informations by the "NMR-window" Approach

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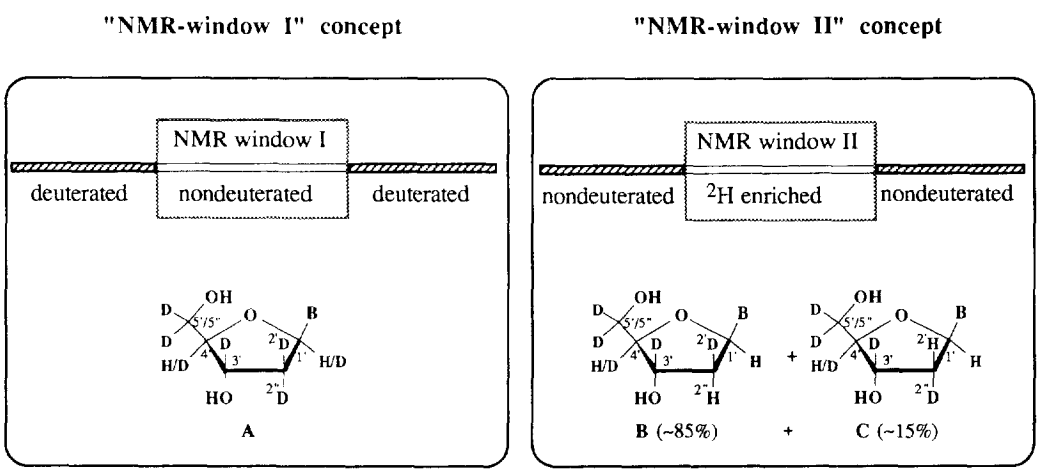
Abstract: *The nucleobase-protected partially-deuterated 5'-O-DMTr-2'#,2''#,3',4'#,5',5''-$^2H_5$-2'-deoxyribonucleoside 3'-phosphoramidite derivatives* ***17a,b*** *-* ***20a,b****, containing C2' isotopomeric mixture of deuterons and protons [~15 atom % ^2H at C2'(R), ~85 atom % ^2H at C2'(S), ; 65 atom % ^2H at C4' (#), >97 atom % ^2H at C3' and C5', i.e. Blocks* ***B*** *and* ***C*** *in Fig. 1], have been site specifically incorporated into a self-complementary 12-mer [d($C^5G^6C^{7*}G^8A^{9*}A^{10}T^{11}T^{12*}C^{13}G^{14*}C^{15}G^{16}$)]$_2$ (**I**) and a 20-mer [d($C^1G^2C^3G^4C^{5*}G^{6*}C^{7*}G^{8*}A^{9*}A^{10*}T^{11*}T^{12*}C^{13*}G^{14*}C^{15*}G^{16*}C^{17}G^{18}C^{19}G^{20}$)]$_2$ (**II**) DNA duplex (N* indicates the partially-deuterated blocks* ***B*** *and* ***C*** *in Fig. 1) by the solid phase synthesis methodology to develop the "NMR window II" concept (for our "NMR window I" concept see refs 5-8). The present "NMR window II" concept simplifies spectral crowding as well as allows the retrieval of both J-coupling and nOe informations from the partially-deuterated nucleotide residues, whereas our older "NMR-window I" concept helped to suppress the unwanted proton resonances by substituting with deuterium but the sensitivity of the proton resonances in the "NMR-window" was poorer in a 20-mer DNA duplex because of the line-broadening. The overall spectral simplification of the spectral crowding in "NMR window II" concept, owing to ≥97% suppression of the proton resonances from C3' and C5', has resulted in an enhancement of the spectral resolution, and thereby following structural information could be obtained in an unambiguous manner: (i) The partial deuteration of C2' along with full suppression of H3' resonance by deuteration creating an C2'-isotopomeric mixture has given us an unprecedented possibility for the extraction of the $^3J_{H1',H2'}$ and $^3J_{H1',H2''}$ coupling constant information easily and unambiguously from DQF-COSY or other double quantum experiments for the 20 base pair long DNA duplex with high accuracy as a consequence of the increased intensity of the crosspeaks because of the elimination of $J_{2'2''}$, $J_{2''3'}$ and $J_{2'3'}$ couplings in these partially-deuterated blocks [i.e. N* residues in duplex (**I**) and (**II**)]. (ii) It is also noteworthy that the T_2 relaxation for the H2'' protons of partially-deuterated residues in deuterated duplex (**I**) has increased by ~1.5 to 2 fold compared to the nondeuterated residues (see Table 1). (iii) Because of the suppression of the proton resonances completely from C3' and C5' as well as owing to the fact that there is only ~15 atom % residual ^1H at C2'(S) in the β-face, we observe only interresidual [$(H2'')_{i,i-1}$ - $(Ar)_i$, $(H1')_{i,i-1}$-$(Ar)_i$] and intraresidual [$(H2''$-$Ar)_i$, $(Ar$-$H1')_i$, $(H1'$-$H2'')_i$, $(H4'$-$H1')_i$, $(H4'$-$H2'')_i$, $(H4'$-$Ar)_i$] nOes using HAL-NOESY experiment, allowing the filtration of all proton resonances belonging to the nondeuterated nucleotides. The comparison of the relative nOe intensities, as judged by comparison of crosspeak to its own diagonal peak at the same mixing time both in deuterated and its non-deuterated counterparts, obtained in HAL-NOESY experiment with that of a standard NOESY experiment, shows that the errors in the nOe volume estimation are quite similar, and thereby allowing the extraction of quantitative interproton distance information in "NMR-window II" concept. (iv) The elimination of proton resonances completely from C3' and C5' and only ~15 atom % residual ^1H at C2' in the β-face has made it possible to eliminate spin-diffusion taking place through the H2'-Ar, H1'-H2', H3'-Ar, H1'-H3', H2'-H3' and H2'-H2'' pathways in the NOESY spectra. (v) The present HAL-NOESY experiment allows an unambiguous extraction of the H1'-H4', H4'-H2'' nOe volumes for large duplex, which are known to be quite sensitive to the sugar conformation.*

Nuclear magnetic resonance (NMR) spectroscopy provides unique information on the variation of the local structure and the dynamics of DNA and RNA under physiological condition[1], which is potentially useful for understanding their biological function. The quality of information that NMR can provide suffers very severely as the molecular weight of DNA or RNA increases. The reasons for this are mainly three-fold: (1) increasing

overlap of proton resonances makes the chemical shift assignments very ambiguous, which also makes J-coupling as well as nOe volume estimation almost impossible, (2) the line-broadening of the individual resonance lines increases because of the decrease of T_2 relaxations whereas T_1 increases due to the slower molecular tumbling of the molecules as they become larger, which also makes it very difficult to extract any J-coupling information; (3) this is furthermore complicated as the uncertainty in the estimation of interproton distance calculation increases because of the spin-diffusion in the NOESY/ROESY experiments. At present, NMR spectroscopy[2] can provide both interproton distance and torsion angle information for only up to 12 - 14-mer DNA duplex [3] or 8 - 12-mer single stranded RNA[4] in an unambiguous manner because of the reasons discussed above. Clearly, the sizes of DNA or RNA molecules that are amenable to NMR study are far from the molecular weights of a biologically functional molecule. It is therefore reasonable to assume that any isotope labelling techniques that remedy the above problems would have a serious impact in the structural studies on DNA or RNA molecules that represent specific biological function.

Our NMR studies on partially-deuterated oligomers have shown,[5-8] that the selective incorporation of specifically deuterated nucleotides of type **A** [>97 atom % ^{2}H at C2', C3', C5'; ~85 atom % ^{2}H at C4'; ~20 atom % ^{2}H at C1'] into DNA or RNA according to the "NMR-window I" approach (Figure 1) can indeed solve the resonance overlap problem. Such deuterium labelling also improved the resolution and sensitivity of the residual sugar protons (*i.e.* H1' & H4')[7,8] in the partially-deuterated nucleotides in a DNA duplex. The observed ~3 fold increase of T_1 and ~10 - 15 fold increase of T_2 relaxation times of the residual H1' and H4' protons in the partially-deuterated residues of type **A** in our "NMR-window I" concept however allowed the determination of their nOe volumes with high accuracy by filtering away the proton signals of the nondeuterated residues (see



B = thymin-1-yl or cytosin-1-yl or adenin-9-yl or guanin-9-yl

Figure 1. The concepts of "NMR-window I" and "NMR-window II" using partially-deuterated building block **A** or the isotopomeric mixture of building blocks **B** + **C**. In "NMR-window I" concept,[5-8] the partially-deuterated building block **A** is introduced (shaded region) in certain parts of DNA through synthetic chemistry to suppress the proton resonances selectively, thereby creating an "NMR-window" of natural protonated part in which the spectral overlap is optimized. The design of "NMR-windows" in an incremental manner with an overlap of one nucleotide enables one to walk on the DNA molecule by a common chemical shift. In "NMR-window II" concept, we have designed and synthesized partially-deuterated isotopomeric mixture of building blocks **B** + **C** and subsequently introduced them specifically in to certain parts of DNA creating an "NMR-window II". In this "NMR-window II" concept, the structural information from the partially-deuterated nucleotide residues can be successfully filtered off from the protonated part by specific NMR experiments (see the text).

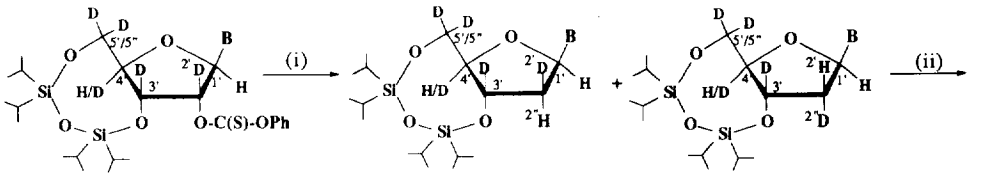
Figure 1) with shorter T_2 using ROESY, MINSY or HAL experiments.[8] On the other hand in NOESY or in DQF-COSY experiments, severe line-broadening of proton resonances from natural nucleotides in the "NMR-window I" region was observed for a 20-mer DNA duplex[7] (*i.e.* a duplex with 40 nucleotides with molecular weight of ~12000) owing to the slow tumbling rate and effective decrease of T_2 relaxations. This line-broadening problem prevented the extraction of the J-coupling constants from the pentose sugar residues of the ^{1}H-NMR visible region in the "NMR-window I" concept (Figure 1), which prompted us to devise further refinement of our deuteration strategy for an improved "NMR-window" concept.

The improved relaxation properties found for the residual H1' and H4' protons during the development of the "NMR window I" concept have led us to argue that a diastereomeric proton at 2' or 2" positions (isotopomer), which would be geminal to a deuterium atom as well as vicinal to C3'-deuteron as in the partially-deuterated isotopomeric 2'-deoxynucleoside blocks **B** + **C** as in Figure 1 would have substantially narrower resonance lines and higher sensitivity than a nucleotide block with 2'/2"-geminal protons. We here show that the incorporation of the isotopomeric mixture of building blocks **B** + **C** as in Figure 1 in the specific region of a DNA duplex ("NMR window II" concept) indeed assists in the extraction of both $^3J_{H1'H2'}$ (*i.e. cis*-coupling) and $^3J_{H1'H2''}$ (*i.e. trans*-coupling) coupling constant information from COSY-type experiments with high accuracy giving a fairly clear picture of the pentose sugar conformation. We also show that, in addition to the J-coupling information, the incorporation of the isotopomeric mixture of building blocks **B** + **C** enables us to extract the crucial interproton distance information such as H1'$_i$-Ar$_i$, H2'/ H2"$_i$-Ar$_i$, H1'-H2"/ H2', H1'-H4' and interresidual Ar-Ar, H2'/H2"$_{i-1}$-Ar$_i$, H1'$_{i-1}$-Ar$_i$ from spin-echo based HAL-NOESY experiments. These benefits of our new "NMR window II" concept have been demonstrated on partially-deuterated Dickerson's 12-mer and a 20-mer DNA duplex.

Results and Discussion

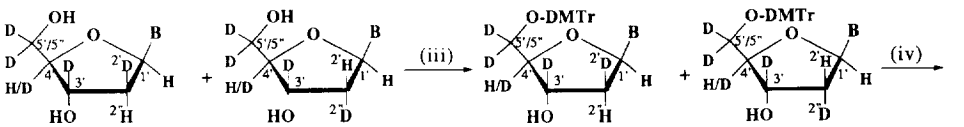
(A) Chemical synthesis of partially-deuterated oligo-DNA. From the various chemical methods[9-16a,b] available for diastereospecific isotope labelling of 2'-deoxynucleosides at C2' (R or S), the free radical reduction of the appropriate 2'-O-phenoxythiocarbonyl derivatives **1** - **4** (Figure 2) by tri-n-butyltin hydride[14] was adopted for the present goal for the synthesis of deutero isotopomeric mixture of building blocks **B** + **C** as in Figure 1. This reaction[14] has the following advantages: (i) it gives higher stereoselectivity for the (2'R)-isomer [*i. e.* proton at the α-face, (type **B** blocks in Figure 1)] over the (2'S)-isomer [*i. e.* proton at the β-face, (type **C** block in Figure 1)]. Heretofore, the data on the preparation of diastereomeric mixture of **B** + **C** type blocks have been available only for adenosine derivatives in ~9:1 ratio[14,17] (ii) since it is used for conversion of precursors **1** - **4** to the corresponding 1'$^{\#}$,2',2",3',4'$^{\#}$,5',5"-$^2H_7$-2'-deoxynucleosides[6] (type **A** in Figure 1, # signifies only partial deuteration[6]), the incorporation of a proton does not require additional chemical manipulation step. Earlier, Ishido *et al.* employed tri-n-butyltin hydride or deuteride[16a,b] reduction under ultrasonification condition to obtain higher distereoselectivity (99:1) for 2'-^{2}H or 2"-^{2}H nucleosides, which are not required for our NMR purpose because we wished to employ easily accessible 3',4',5'/5",2'/2"-$^2H_5$ (C2'/2"-isotopomeric) nucleoside blocks (*i.e.* **B** and **C** type blocks in Fig. 1) for our "NMR-window II" (Fig. 1) concept (see the NMR part below for their usefulness for the conformational studies of large DNA duplex).

The 3',5'-O-(1,1,3,3-tetraisopropyldisiloxan-1,3-diyl(TPDS[18]))-2'-O-phenoxythiocarbonyl(PTC)-2',3',4'$^{\#}$,5',5"-$^2H_5$-ribonucleosides **1** - **4** (Figure 2) were deoxygenated by tri-n-butyltin hydride in the presence of



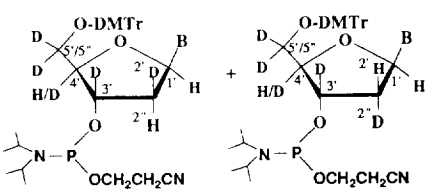
1: B = T
2: B = C^{Bz}
3: B = A^{Bz}
4: B = G^{DPC}_{Ac}

5a: B = T (89%) + **5b**: B = T (11%)
6a: B = C^{Bz} (82%) + **6b**: B = C^{Bz} (18%)
7a: B = A^{Bz} (91%) + **7b**: B = A^{Bz} (9%)
8a: B = G^{DPC}_{Ac} (82%) + **8b**: B = G^{DPC}_{Ac} (18%)



9a: B = T (89%) + **9b**: B = T (11%)
10a: B = C^{Bz} (82%) + **10b**: B = C^{Bz} (18%)
11a: B = A^{Bz} (91%) + **11b**: B = A^{Bz} (9%)
12a: B = G^{DPC}_{Ac} (82%) + **12b**: B = G^{DPC}_{Ac} (18%)

13a: B = T (89%) + **13b**: B = T (11%)
14a: B = C^{Bz} (82%) + **14b**: B = C^{Bz} (18%)
15a: B = A^{Bz} (91%) + **15b**: B = A^{Bz} (9%)
16a: B = G^{DPC}_{Ac} (82%) + **16b**: B = G^{DPC}_{Ac} (18%)



17a: B = T (89%) + **17b**: B = T (11%)
18a: B = C^{Bz} (82%) + **18b**: B = C^{Bz} (18%)
19a: B = A^{Bz} (91%) + **19b**: B = A^{Bz} (9%)
20a: B = G^{DPC}_{Ac} (82%) + **20b**: B = G^{DPC}_{Ac} (18%)

T= thymin-1-yl; $\mathbf{C^{Bz}}$= N^4-benzoylcytosin-1-yl; $\mathbf{A^{Bz}}$= N^6-benzoyladenin-9-yl;
$\mathbf{G^{DPC}_{Ac}}$= N^2-acetyl-O^6-diphenylcarbamoylguanin-9-yl; **DMTr**= 4',4'-dimethoxytrityl.

Reagents: (i) tri-n-butyltin hydride, AIBN in dry toluene, 76 °C; (ii) 1.0 M TBAF in dry THF, RT; (iii) DMTr-Cl in dry pyridine, RT; (iv) 2-cyanoethyl N,N-diisopropylchlorophosphoramidite, N,N-diisipropylethylamine in dry THF or N,N-diisopropylammonium tetrazolide, (2-cyanoethoxy)bis(N,N-diisopropylamino)phosphine in dry dichloromethane

Figure 2. The synthetic scheme of the partially-deuterated 2'/2" isotopomeric mixture of appropriately protected phosphoramidite building blocks for the solid-phase DNA synthesis.

2,2'-azobis(2-methylpropionitrile) (AIBN) in dry toluene at 75 °C to give an inseparable mixture of isotopomers **5a,b - 8a,b** in 86, 70, 80 and 87 % yields, respectively. The preference for α-face attack by the incoming

hydrogen atom giving predominant (2'R)-isomers has been found to be slightly dependent on the nature of the nucleobase, giving 89, 82, 91 and 82 % of **5a - 8a** as major isotopomers, respectively, as it has been proved by integration of appropriate signals in their ^{1}H-NMR spectra at 500 MHz. After removal of TPDS protection by a treatment with tetrabutylammonium fluoride in dry tetrahydrofuran, the base protected 2'#,2"#,3',4'#,5',5"-$^2H_5$-2'-deoxynucleosides **9a,b - 12a,b** were obtained in 97, 87, 97 and 98 % yields, respectively. These compounds were further transformed to the corresponding 5'-O-DMTr derivatives **13a,b - 16a,b** (80, 89, 83 and 89%, respectively), followed by phosphitylation of the 3'-hydroxyl groups[19-21] to afford the phosphoramidite derivatives **17a,b - 20a,b** in 76, 62, 65 and 66 % yields, respectively.

These specifically deuterated phosphoramidite building blocks in conjunction with the nondeuterated natural counterparts have been subsequently used for the synthesis of the self-complementary Dickerson's DNA dodecamer[22] $[d(C^5G^6C^7*G^8A^9*A^{10}T^{11}T^{12}*C^{13}G^{14}*C^{15}G^{16})]_2$ (**I**) and a 20-mer $[d(C^1G^2C^3G^4C^5*G^6*C^7*G^8*A^9*A^{10}*T^{11}*T^{12}*C^{13}*G^{14}*C^{15}*G^{16}*C^{17}G^{18}C^{19}G^{20})]_2$ (**II**) sequence (N* indicates deuterated blocks **9a,b - 12a,b** according to the "NMR-window II" concept as schematically shown in Figure 1) on an automatic DNA synthesiser using CPG solid support (36 μmol/g loading) in 25 % (453 o.d. units) and 21 % (500 o.d. units) yields, respectively.

The NMR properties of these specifically deuterium labelled duplexes were studied in comparison with their nondeuterated natural counterparts[7,8] $[d(C^5G^6C^7G^8A^9A^{10}T^{11}T^{12}C^{13}G^{14}C^{15}G^{16})]_2$ (**III**) and $[d(C^1G^2C^3G^4C^5G^6C^7G^8A^9A^{10}T^{11}T^{12}C^{13}G^{14}C^{15}G^{16}C^{17}G^{18}C^{19}G^{20})]_2$ (**IV**) and an analogous partially-deuterated 20-mer $[d(\underline{C}^1\underline{G}^2\underline{C}^3\underline{G}^4C^5G^6C^7G^8\ A^9A^{10}T^{11}T^{12}C^{13}G^{14}C^{15}G^{16}\underline{C}^{17}\underline{G}^{18}\underline{C}^{19}\underline{G}^{20})]_2$[7,8] (**V**) according to the "NMR-window I" concept (Figure 1)[7] (specific site of incorporations of deuterated blocks of type **A** are shown by underscore).

(B) NMR spectroscopy. Conformational studies of biologically functional DNA or RNA molecules by NMR spectroscopy is of considerable challenge because as the molecular weight increases the line-broadening increases along with the severe increase of the resonance overlap. The use of three- or four-dimensional triple resonance experiments[23a-e] with *uniform* $^{15}N/^{13}C$ labelled oligo-RNA[23f-m] also suffers considerably because of the above problem, making the extraction of the necessary interproton distance or $^3J_{HH}$ information quite a complex task[23m]. It should be noted that "so far, no structure has been solved using these new techniques"[23m]. Clearly, the use of any of these labelling techniques to extract coupling constant and interproton distance information requires further substantial development in the NMR and isotope labelling methodologies. Since the present enzyme promoted $^{15}N/^{13}C$ isotope labelling technologies cannot be used for oligo-DNA or for non-uniform isotope labelling in general, we[6-8,15] and others[9-14,16,25] have been developing chemical methodologies that will allow non-uniform isotope labelling in both DNA or RNA.

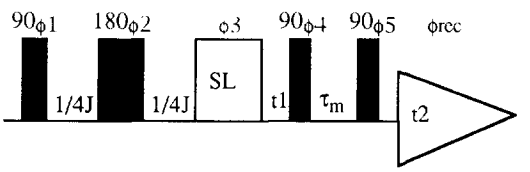


Figure 3. The HAL-NOESY experiment. Phase-cycling: φ1: x, -x; φ2: x; φ3: -y; φ4: 8x, 8-x; φ5: 2x, 2-x, 2y, 2-y; φrec: x, 2-x, x, y, 2-y, y, -x, 2x, -x, -y, 2y, -y. For quadrature detection using TPPI the phases φ1, φ2 and φ3 are incremented by 90° for each experiment. SL is a short high-power spin-lock pulse (500 μsec), τ_m is the mixing time and 1/4J is the delay for the spin-echo.

(1) How does 2'/2",3',4'#,5'/5"-$^{2}H_5$ (C2'/2"-isotopomeric) nucleoside blocks help the extraction of accurate $^{3}J_{1'2'}$ and $^{3}J_{1'2''}$ coupling constants in large DNA duplexes? The assignments of the aromatic and sugar protons for the natural 12-mer (**III**)[3b] and 20-mer (**IV**) DNA duplex[7,8] have been presented earlier. The effect of the chemospecific deuteration at C2', C3', C4' and C5' in the four residues C^{7*}, A^{9*}, T^{12*} and G^{14*} in partially-deuterated 12-mer duplex (**I**), using an isotopomeric mixture of

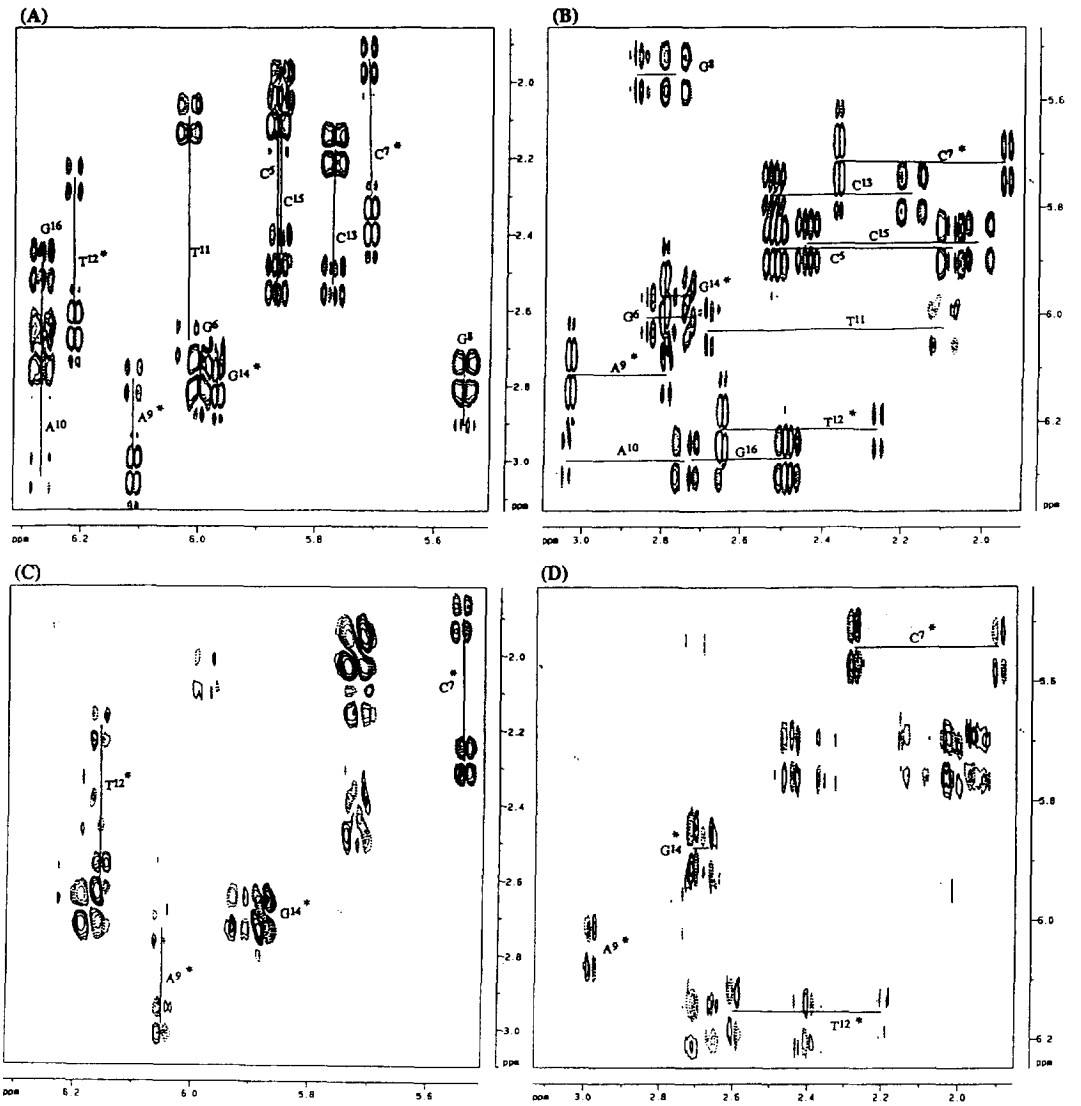


Figure 4. DQF-COSY spectra of the partially deuterated 12-mer (**I**) at 21 °C (Panels A & B) and at 0 °C (Panels C & D) showing H1' to H2'/2" areas of the spectrum. Each nucleotide in 12-mer (**I**) marked by * signifies the partially deuterated isotopomeric nucleotide blocks (originated from phosphoramidite building blocks **17a,b - 20a,b**) Note that despite the slower tumbling rate, the crosspeaks belonging to the partially deuterated residues (C^{7*}, A^{9*}, T^{12*} and G^{14*}) appear clearly even at 0 °C, whereas the crosspeaks belonging to the nondeuterated residues are not clearly visible (Panels C & D) because of the line-broadening [compare the 'up' off-diagonal spectra in the Panel A with C, and the 'down' off-diagonal spectra in the Panel B with D].

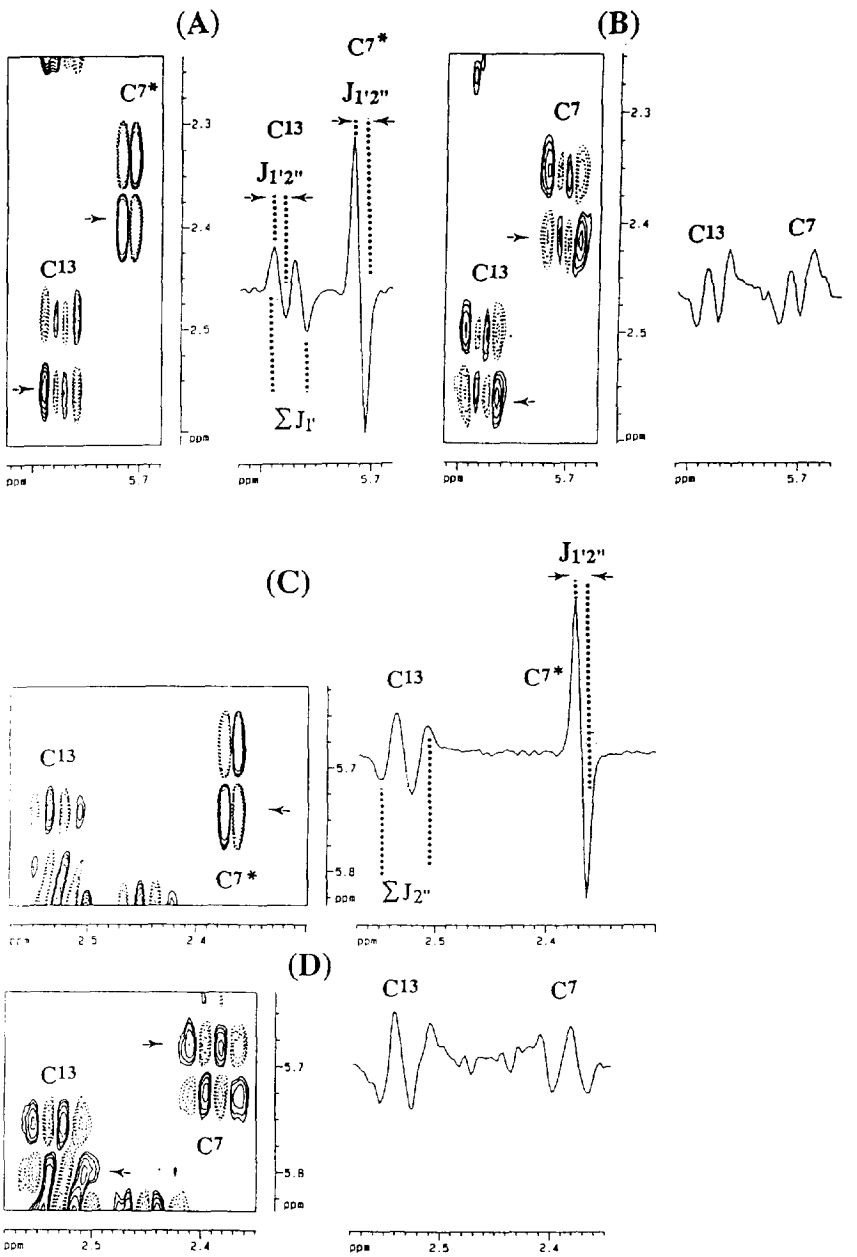


Figure 5. Expanded DQF-COSY spectra (see Fig. 4A) with two crosspeaks belonging to C^{13} and C^{7*} residues of the 12-mer (**I**) (Panels A & C) and natural 12-mer counterpart (**III**) (Panels B & D) showing parts of the spectrum from the 'up' off-diagonal (Panels A & B) and the 'down' off-diagonal (Panels C & D) parts of the spectrum at 21 °C. The arrows near the crosspeaks show the row where the projection has been taken. The comparison of the relative intensity of C^{13} and C^{7*} residues shows the effect of restricted J-coupling network in the partially-deuterated C^{7*} residue compared to the nondeuterated natural C^{13} residue. Note that the 1D projection shown represents the superimposition of two 1D projections through the two crosspeaks.

building blocks **B** + **C** as in Fig. 1 (the "NMR-window II" concept) is clearly observed in the DQF-COSY spectra through the comparison with the constituent nondeuterated residues at 0 ° or 21 °C or (Fig. 4), and also with the corresponding residues in the natural 12-mer (**III**) (Fig. 5). It is clear from the expanded H1'-H2'/H2" and H2'/H2"-H1' part of the DQF-COSY spectra (Fig. 4) that the crosspeaks for $^3J_{H1',H2'}$ or $^3J_{H1',H2"}$ for the deuterated residues can easily be distinguished as doublets compared to the corresponding complex crosspeaks patterns for the nondeuterated residues in duplex (**I**). Moreover, because of slower tumbling rate at 0 °C, the DQF-COSY spectra (Figs. 4C and 4D) shows an enhanced line-broadening as well as the reduction of the resolution and sensitivity of protons from the nondeuterated parts (compare Figs. 4A and 4C) compared to the protons from the partially-deuterated residues allowing an easy and unambiguous extraction of $^3J_{H1',H2'}$ or $^3J_{H1',H2"}$ coupling constants in the latter. *The projections through the crosspeaks of partially-deuterated residues in deuterated duplex (**I**) compared to the nondeuterated residues (Fig. 5) in the same duplex and in natural 12-mer counterpart (**III**) show that the intensity of the crosspeaks are increased for the deuterated ones because of the*

Table 1. T_1 and T_2 relaxation times for protons from the partially-deuterated 12-mer (**I**) and its fully protonated counterpart (**III**)

proton	12-mer (**I**)		12-mer (**III**)	
	T_1 (s)	T_2 (ms)	T_1 (s)	T_2 (ms)
H8G^6	1.81	93	1.09	36
H8G^{16}	1.71	133	1.09	36
H8G^{14}	2.59	201	1.36	60
H8G^8	2.55	124	1.24	73
H2A^{10}	5.22	-	3.68	237
H2A^9	6.39	291	3.98	139
H6C^5	1.90	-	1.18	86
H6C^{13}	2.39	43	1.64	-
H6C^{15}	2.13	55	1.36	48
H6C^7	2.74	105	1.32	39
H6T^{12}	2.80	76	1.82	56
H6T^{11}	2.64	64	2.05	62
H1'T^{12}	3.17	104	2.10	64
H1'A^9	3.42	47	2.09	-
H1'G^{14}	2.78	169	1.58	-
H1'C^{13}	2.35	88	1.64	-
H1'C^7	3.34	67	1.53	-
H5C^7	2.92	175	1.84	-
H5C^{13}	3.00	115	2.47	69
H2"G^8	-	23	-	-
H2"G^{14}	2.38	57	1.25	21
H2"T^{12}	2.71	65	2.01	-
H2"C^{15}	-	34	-	-
H2"C^7	2.95	42	0.84	-
H2'A^{10}	-	22	-	-
H2'C^{13}	1.92	26	0.93	-
H2'C^{15}	1.61	33	-	-
MeT^{12}	2.36	79	1.36	85
MeT^{11}	2.46	93	1.34	87

elimination of $J_{2'2''}$, $J_{2''3'}$ and $J_{2'3'}$ couplings. It is also noteworthy that the T_2 relaxation for the H2" protons of deuterated residues in deuterated duplex (I) has increased by ~1.5 to 2 fold compared to the nondeuterated residues (Table 1).

The advantages of using our partially-deuterated nucleotide blocks such as **B** and **C** (Fig. 1) are 3-fold:

(1) The linewidths of the H2" protons of the partially deuterated duplex (**I**) and (**II**) at a half-height of the crosspeak resulting from H1-H2" doublet component in the DQF COSY spectrum varied in the range of 4.4-5.5 Hz. The linewidths at half-heights have also been estimated from crosspeaks between H1'-H2" protons in HAL-NOESY experiment. The elimination of the passive coupling and the increase of T_2 of H1' and H2" in the partially deuterated residue, owing to the elimination of the number of relaxation pathways,[16c] decreases the linewidth with an overall increase of the crosspeak intensity. Hence, the incorporation of partially-deuterated nucleotide blocks such as **B** and **C**, shown in Fig. 1, in oligo-DNAs has made it possible to extract the $^3J_{1',2'}$ and $^3J_{1',2''}$ from the partially-deuterated 20-mer DNA duplex (40 nucleotide) (**II**), despite the antiphase cancellation, compared to the natural counterpart (**IV**)(Fig. 6A, D).

(2) Moreover, the simplification of pattern of crosspeak resulting from $^3J_{1',2''}$ doublet causes the increase of the intensity of the crosspeak that, in turn, allows a quantitative determination of $^3J_{1',2''}$ coupling constant through a simulation procedure with higher precision (~0.2 Hz) of the fit to the coupling.[35a] This could be clearly observed upon comparison of the $^3J_{1',2''}$ crosspeaks (up diagonal) of deuteriated residue (Fig. 6A) in duplex (**II**) and the corresponding non-deuterated in duplex (**V**) in Fig. 6B.

(3) Systematic and random errors in the determination of J-coupling such as distortions in the lineshapes and amplitudes caused by experimental artifact[35] could be estimated by the spread of repeated independent measurements, showing the accuracy of the measured coupling constant. In our present work, experimental J-coupling presented in Table 2 have been averaged through six independent data sets, which are obtained from up and down diagonal of two DQF-COSY experiments with and without ^{31}P decoupling and also by one double

Table 2. Vicinal ^1H-^1H spin $J_{1'2''}$ and $J_{1'2'}$ (in Hz) coupling constants for the partially-deuterated 12-mer (**I**) and the natural counterpart (**III**) and the partially-deuterated core 12-mer part in 20-mer (**II**) as determined from DQF-COSY spectra. Note that the errors in proton coupling constants are considerably reduced in the deuterated nucleoside moieties (shown by the shaded region). Note also that the errors shown have been calculated as upper and lower limits of the average values of six independent measurements from up and down diagonal from three independent experiments (see text).

Residue	12-mer (**III**)		12-mer (**I**)		20-mer (**II**)	
	$J_{1'2''}$	$J_{1'2'}$	$J_{1'2''}$	$J_{1'2'}$	$J_{1'2''}$	$J_{1'2'}$
C^5	5.3±0.8	9.0±0.8	5.5±0.8	8.8±0.8	6.0±0.2	9.0±0.2
G^6	4.0±0.8	10.5±0.8	4.5±0.8	10.0±0.8	6.2±0.2	9.7±0.2
C^7	5.3±0.8	9.3±0.8	5.8±0.2	9.0±0.2	6.1±0.2	9.2±0.2
G^8	4.8±0.8	10.5±0.8	4.4±0.8	10.2±0.8	6.0±0.2	10.0±0.2
A^9	6.1±0.8	10.5±0.8	5.5±0.2	10.0±0.2	6.0±0.3	10.2±0.2
A^{10}	5.5±0.8	9.2±0.8	5.7±0.8	9.7±0.8	6.2±0.2	10.2±0.2
T^{11}	6.5±0.8	8.9±0.8	5.0±0.8	9.4±0.8	6.4±0.2	9.2±0.2
T^{12}	6.2±0.8	10.0±0.8	5.8±0.2	9.9±0.2	6.2±0.2	10.2±0.2
C^{13}	5.1±0.8	9.8±0.8	5.0±0.8	9.4±0.8	6.2±0.2	9.8±0.2
G^{14}	4.0±0.8	10.5±0.8	5.2±0.2	10.1±0.2	6.2±0.2	9.9±0.2
C^{15}	5.5±0.8	10.1±0.8	5.3±0.8	9.0±0.8	6.3±0.2	9.5±0.2
G^{16}	5.0±0.8	9.6±0.8	5.5±0.8	8.8±0.8	6.2±0.2	10.2±0.2

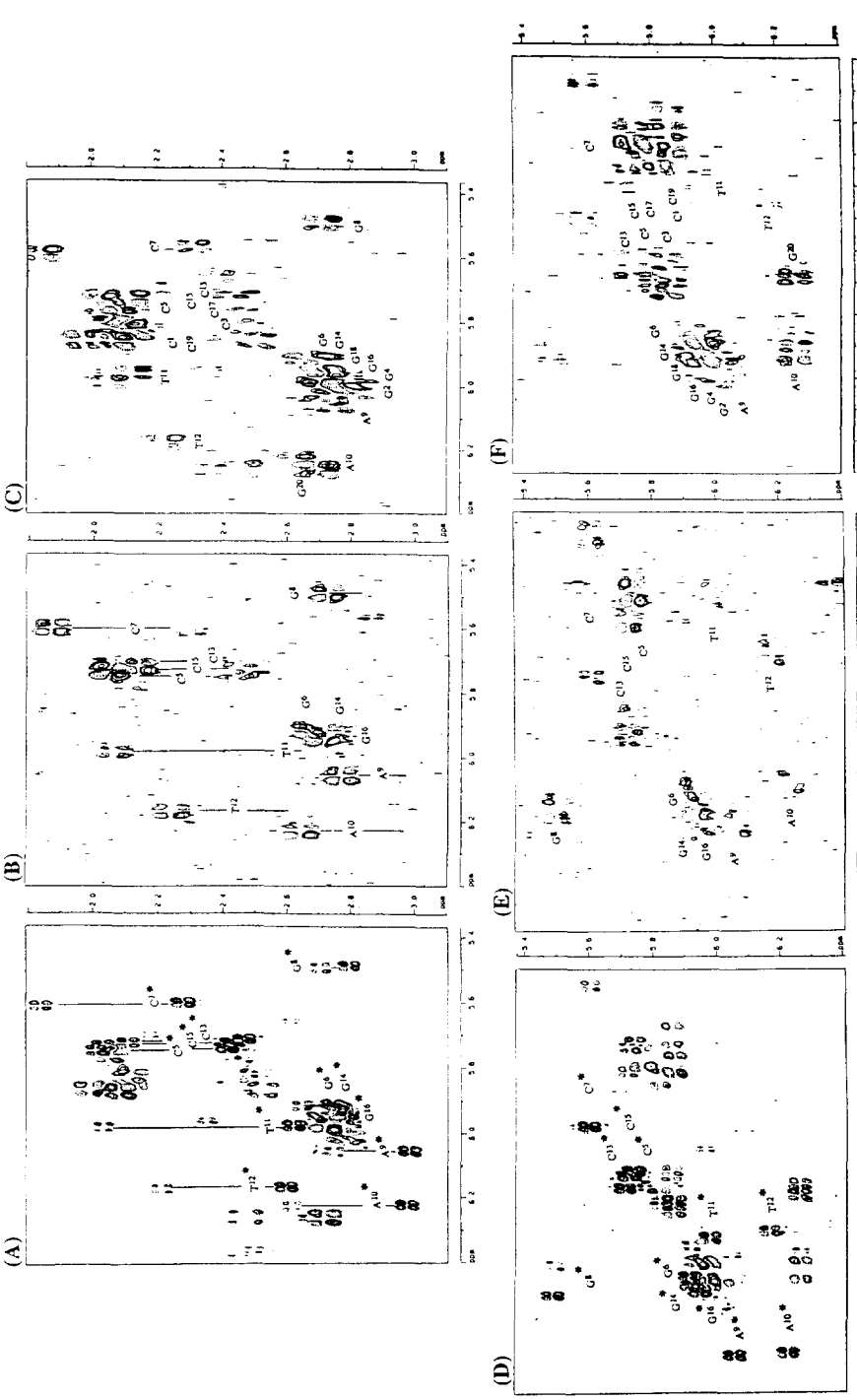


Figure 6. Expanded DQF-COSY spectra in the 'up' off-diagonal (Panels A, B & C) and 'down' off-diagonal (Panels D, E & F) showing the H1' to H2'/2" crosspeak region of the partially-deuterated 20-mer (**II**), prepared with partially-deuterated isotopomeric mononucleotide blocks marked by * (originated from phosphoramidite building blocks **17a,b - 20a,b**) (Panels A & D); the partially-deuterated 20-mer, [d(C^{1}G^{2}C^{3}G^{4}C^{5}G^{6}C^{7}G^{8}A^{9}A^{10}T^{11}T^{12}C^{13}G^{14}C^{15}G^{16}C^{17}G^{18}C^{19}G^{20})]$_2$ (**V**)[7,8], prepared with 1$^{\#}$,2',2",3',4$^{\#}$,5',5"-^{2}H$_7$-2'-deoxynucleosides (underscore indicates partial deuteration using the building block of type A (see ref 6) (Panels B & E) and the corresponding fully protonated natural 20-mer (**IV**) (Panels C & F) at 21 °C.

quantum (DQ) experiment. It may be noted that the digital resolution in these experiments were 1.10 Hz/point in F_2 and 4.39 Hz/point F_1 dimension after the data processing. In order to estimate the J-coupling through crossection of crosspeaks in F2 dimension (as shown in Fig. 5), the back Fourier transformation of this projection has been performed and zero filled again to 16K to obtain the digital resolution 0.27 Hz/point, which has been subsequently used for the simulation of crosspeaks.

Fig. 6 shows a comparative study amongst the various expanded DQF-COSY spectra of various oligo-DNAs: (i) the natural 20-mer (**IV**) (Panels C and F), (ii) the selectively deuterated 20-mer (**V**) (Panels B and E), incorporating the "NMR-window I" concept,[7,8] and (iii) the selectively deuterated analogous 20-mer (**II**) (Panels A and D) in which we have introduced the "NMR-window II" concept. It should be noted that the DQF-COSY spectra shown in all panels (A-F) in Fig. 6 have the same number of scans per experiment thereby showing the sensitivity and line-broadening problem depending upon the nature of deuterium isotope labelling in oligo-DNA. It can easily be seen that in natural 20-mer (**IV**) (Panels C and F) neither quantitative nor qualitative estimation of coupling constants is extractable. Even for the partially-deuterated 20-mer (**V**) (Panels B and E) using our older "NMR-window I" concept, the J-coupling information could not be extracted because of dramatic line-broadening and loss of sensitivity, hence one can see considerable noise in Panels B and E. These problems of line-broadening, low sensitivity and spectral overcrowding, however, have been overcome in the new partially-deuterated 20-mer (**II**) (Panels A and D) using our "NMR-window II" concept: all 12 H1'-H2" and 12 H1'-H2' crosspeaks from the 12 partially-deuterated sugar residues (even though $^3J_{H1',H2"}$ is smaller than $^3J_{H1',H2'}$) are observed with adequate individual resonance line separation (even for G^{16*}, G^{14*}, and G^{6*} residues where the differences in chemical shifts between H2" protons are in the range of 0.05ppm[7]) to accurately estimate the $J_{1'2"}$ coupling constants (Table 2). Noteworthy is the fact that even a ~15 atom% ^1H at the C2' position (due to the minor isotopomer, block **C** (see Figures 1 & 2)) in the partially-deuterated nucleotides is sufficient to observe its H1'-H2' crosspeaks which enables the extraction of $^3J_{H1',H2'}$ coupling constants unambiguously (Panels A and D, Table 2). *This exemplifies the advantages of performing high-field ^1H-NMR measurements with two deuterium isotopomers for the structural analysis of reasonably large DNA duplex in which the major isotopomer* ***5a*** *-* ***8a*** *(~85% of the total mixture) has natural H2" in the α-face and the H2' at the β-face is substituted by ^2H, and the minor isotopmer* ***5b*** *-* ***8b*** *(~15%) in which the H2" in the α-face is substituted by ^2H and the natural H2' is at the β-face.*

It may be noted that Kainosho and coworkers[25a] for the same DNA dodecamer (**III**) containing uniformly ^{13}C-labelled sugar estimated the $^3J_{1'2'}$ and $^3J_{1'2"}$ of the constituent thymidine T^7 residue (by our numbering it is T^{11}) to be 11.3 ± 0.6 and 4.4 ± 0.6 Hz, respectively, from a heteronuclear two-dimensional HCCH-E.COSY experiment whereas we, using our "NMR-window II" technique in combination with a simple 2D DQF-COSY experiment (see above and Table 2 for the accuracy of J-values in our experiments), found that the corresponding coupling constants are 9.2 ± 0.2 Hz and 6.4 ± 0.2 Hz. We presume that their inaccuracy arises mainly because of the line-broadening of the ^{13}C-H2' or ^{13}C-H2" crosspeaks in 2D HCCH-E.COSY experiment.[25a]

One other way used successfully by us to obtain precise $^3J_{H1',H2'}$ or $^3J_{H1',H2"}$ coupling constants for the ^2H enriched blocks **5a,b** - **8a,b** in the partially-deuterated 12-mer (**I**) and 20-mer (**II**) DNA duplex is the double quantum (DQ) experiment.[26] Using different delays in the echo sequence of this experiment it has been possible to filter away the nondeuterated residues and distinguish the partially-deuterated ones as presented in

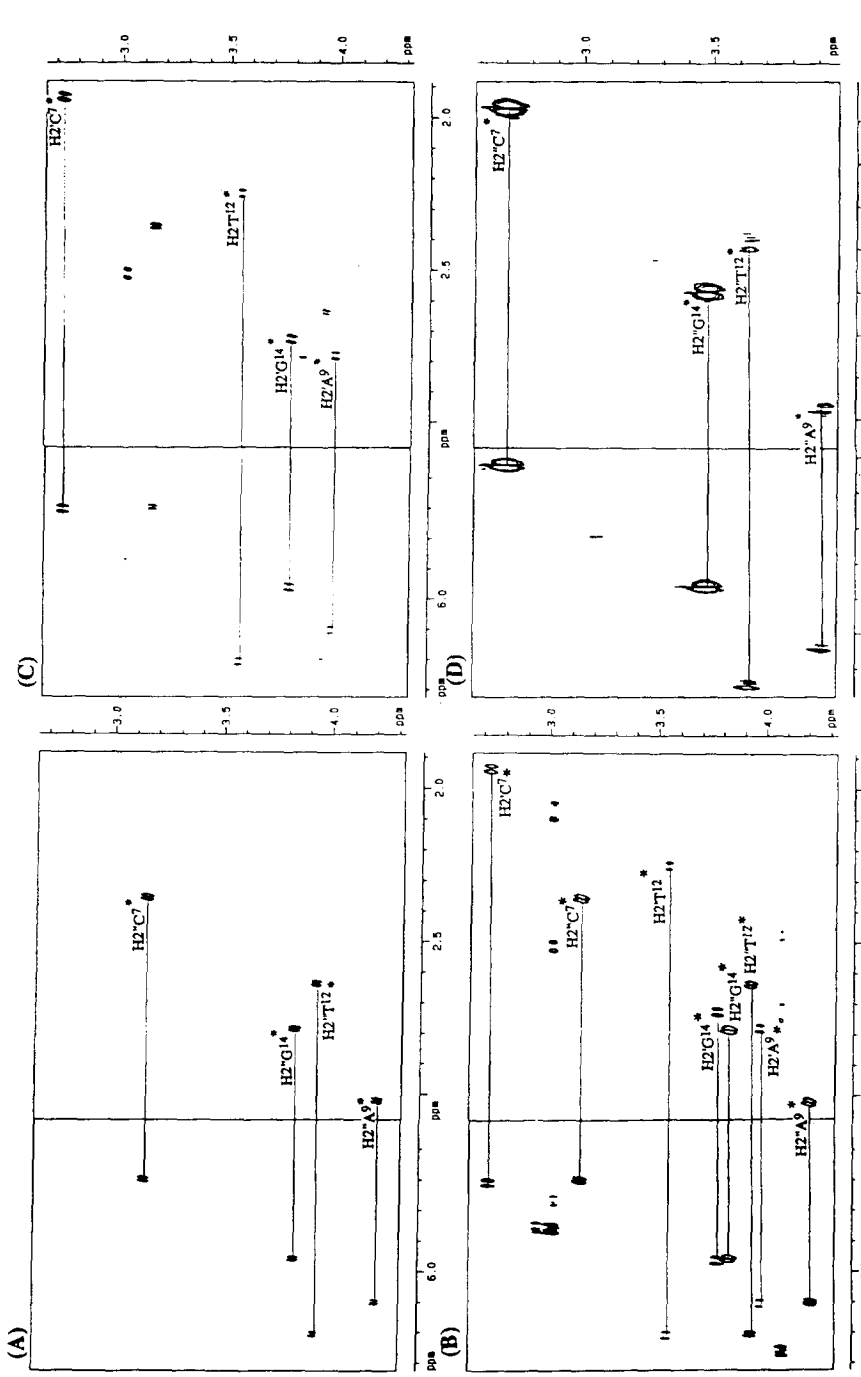


Figure 7. Double Quantum (DQ) spectrum of the partially-deuterated 12-mer (I) at 21°C. (A) shows a DQ spectrum with a delay of 48.1 ms for the spin-echo corresponding to a coupling constant of 5.2 Hz (typical for $^3J_{1'2''}$). Note that only crosspeaks between H1' and H2" of the partially-deuterated residues can be observed using this delay. (B) Shows a DQ spectrum with a delay of 26 ms for the spin-echo corresponding to a coupling constant of 9.6 Hz (typical for $^3J_{1'2'}$). Here all crosspeaks between *H1'/H2"* and *H1'/H2'* of the partially-deuterated residues and some of the others can be observed. (C) shows a DQ spectrum resulting from the subtraction of a DQ spectrum with a delay of 78.1 ms for the spin-echo from a DQ spectrum with a delay of 26 ms for the spin-echo. Here only the crosspeaks between H1' and H2' of the partially-deuterated residues can be observed. (D) DQ spectrum of the 12-mer (I) recorded at 0 °C with a delay of 48.1 ms (same as in experiment A) corresponding to a coupling constant of 5.2 Hz. The crosspeaks between *H1'* and H2" of the partially-deuterated residues can still be observed.

Fig. 7. It is noteworthy that at 0 °C only crosspeaks belonging to the deuterated residues have been clearly observed (Fig. 7D).

(2) The effect of stereoselective deuteration at C2' and C3' in 2'/2",3',4'#,5'/5"-$^{2}H_{5}$ (C2'/2"-isotopomeric) nucleoside blocks on dipole-dipole relaxation in the partially-deuterated DNA duplexes. One of the most important aspect of the structural analyses of biomolecules is to derive their spatial structure using the interproton distance information. Although, the loss of vital interproton distance information obtained from intraresidual $(H1')_i$-$(Ar)_i$, $(H2'/or\ H2'')_i$-$(Ar)_i$, H1'-H2"/orH2', H1'-H4' and interresidual Ar-Ar, $(H2'/H2'')_{i-1}$-$(Ar)_i$, $(H1')_{i-1}$-$(Ar)_i$ nOes would dramatically decrease the confidence of the NMR constrained MD refinement procedure, but, in contradistinction, the loss of interproton distance information from $(H3')_i$-$(Ar)_i$, H1'-H3', $(H4')_i$-$(Ar)_i$, nOes do not have much influence on the final quality of the NMR structure.

The aim of the present study is to test the reliability of nOe information from the isotopomeric mixture, generated from blocks such as **B** and **C** (Fig. 1) in the partially-deuterated "NMR-window II" part, for the extraction of the interproton distances. The main question here to address is if it is acceptable to use numerous deutero isotopomeric mixture for the estimation of quantitative proton-proton distances from the point of view of their relative proton relaxation properties. Although the number of isotopomeric DNAs in a sample under our measurement condition is equal to 2^n (where n is the number of deuterated nucleoside moieties in a oligo-DNA), the contribution to the volume of the each *intraresidual nOe crosspeak* in a NOESY type experiment arises from either the major (*i.e.* 2'S) or the minor (*i.e.* 2'R) isotopomer of a particular nucleoside moiety, whereas the corresponding *diagonal peak* for H1' contains information from both isotopomers (owing to the fact that there is no isotope-induced H1' chemical shift difference[5]), the ratio of which could be however accurately estimated *a priori* at the monomeric level from the H2'/H2" integration for further use for calculating $(a_{ij})/(a_{ii})$ in eqn. 1.

It should also be addressed how the intraresidual relaxation pathways influence the interresidual relaxation pathways in eqn. 1 such as $[H(i)_{n-1}$-$H(j)_n)]$ since there is no guarantee of common relaxation pathways between two neighbouring residues from one isotopomeric oligo-DNA to the next. To evaluate this, we have examined the ratio of the intensity of the crosspeak (a_{ij}) and intensity of the corresponding diagonal peak (a_{ii}) at the same mixing time for the partially-deuterated 12-mer (**I**).

$$\frac{a_{ij}}{a_{ii}} \approx -\sigma_{ij}\tau_m + \frac{1}{2}\sum_{k\neq i,j}\sigma_{ik}\sigma_{kj}\tau_m^2 \qquad \text{eqn (1)}$$

The ratio of $(a_{ij})/(a_{ii})$ at short mixing times represent the value of the cross-relaxation rate (σ_{ij}) between observed spins i and j[28] without influence of leakage relaxation (eqn. 1). The comparison of the build-up curves of this ratio (a_{ij}/a_{ii}) derived from the interresidual crosspeaks $(H2'')_{i-1}$-$(Ar)_i$ and $(H1')_{i-1}$-$(Ar)_i$ in both the major isotopomer of partially-deuterated and the nondeuterated sugar residue in 12-mer duplexes (**I**) and (**III**), respectively, depending upon the mixing time up to 250 ms, shows that there is no difference in the range of their experimental errors (see Figs. 8B and 9B) [Note that in (**I**) and (**III**), the diagonal peaks and crosspeaks that have been taken for determination of $(a_{ij})/(a_{ii})$ ratio are well separated from each other, and therefore the error of estimation of their ratios is very low]. This means that the intraresidual relaxation pathways have no significant influence on the interresidual relaxation pathways (*i.e.* the 2nd term in eqn. 1), hence the nOes derived from the isotopomeric mixture are reliable for proton-proton distance estimation using the complete relaxed matrix approach.[27]

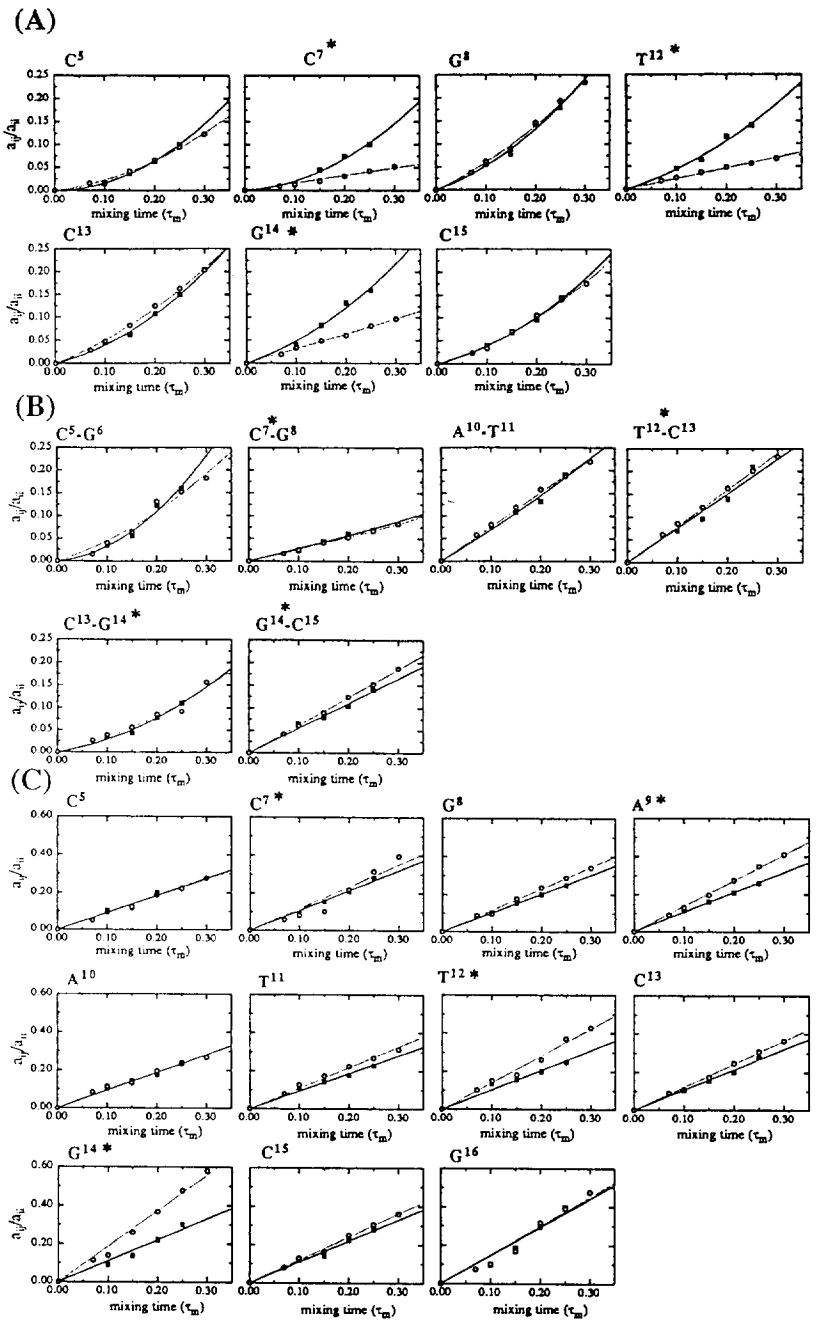


Figure 8. The nOe build-up curves of the completely uncrowded crosspeaks at various mixing times for the partially-deuterated 12-mer duplex (**I**) (dashed line), and the natural counterpart duplex (**III**) (solid line) showing the dependence of the normalised volume of the crosspeaks (a_{ij}) to their own diagonal peaks (a_{ii}) at the same mixing time: (A) H2"$_i$-H8/H6$_i$, (B) H2"$_i$-H8/H6$_{i+1}$, (C) H1'-H2". Each Panel shows the curves for the crosspeaks which are labelled by corresponding symbol as in the text. The comparison shows that the contribution due to spin-diffusion in H2"$_i$-H8/H6$_i$ crosspeak is much reduced in the partially-deuterated residues due to the elimination of H2'-H2" pathway.

The futher advantage of the incorporation of the ^2H at C3' as in blocks **B** and **C** (Fig. 1) is the elimination of intraresidual spin-diffusion allowing the extraction of accurate intraresidual distances $(H1')_i$-$(H4')_i$ and $(H2'')_i$-$(H4')_i$ which are very sensitive to the sugar conformation.[1c] Clearly, this spin-diffusion problem will be completely uncontrollable if one used either natural nucleosides or even partially-deuterated (2'R)-[2'-^2H] /or (2'S)-[2'-^2H]) nucleoside blocks.[16c]

It has been pointed out above that in case of deuterated blocks **5** - **8**, a few relaxation pathways such as H2'-Ar, H1'-H2', H3'-Ar, H1'- H3', H2'-H3' and H2'-H2" for the residual sugar and nucleobase protons have been eliminated. The result of these eliminations can easily be detected qualitatively upon inspection of the mixing time (τ_m) dependant build-up curve (Figs. 8 and 9), which is defined as the ratio of the intensity of the crosspeaks (a_{ij}) and intensity of the corresponding diagonal peak (a_{ii}) at the same mixing time (a_{ij}/a_{ii}))[28] (eqn. (1)).

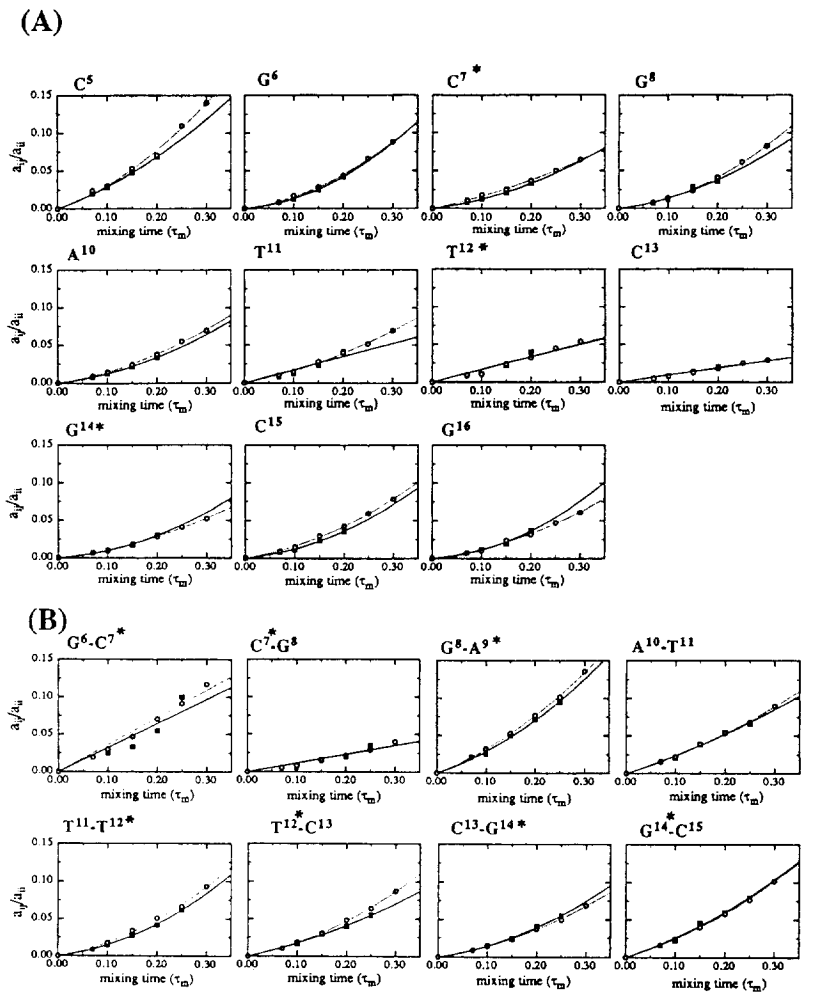


Figure 9. The nOe build-up curves of the completely uncrowded crosspeaks at various mixing times for the partially-deuterated 12-mer duplex (**I**) (dashed line), and the natural counterpart duplex (**III**) (solid line) showing the dependence of the normalised volume of the crosspeaks (a_{ij}) to their own diagonal peaks (a_{ii}) at the same mixing time: (A) $H1'_i$-H8/H6$_i$ and (B) $H1'_i$-H8/H6$_{i+1}$. Each Panel shows the curves for the crosspeaks labelled by corresponding symbol according to the text.

The build-up curves for the crosspeaks $(H2''\text{-}Ar)_i$, which are known to be affected by spin-diffusion through the H2'-Ar and H2'-H2" pathways in the NOESY spectra are presented in Fig. 8A for both the partially-deuterated and nondeuterated sugar residues in 12-mer duplex (**I**) as well as for the natural counterpart in duplex (**III**). A comparison of the build-up curves for the deuterated moieties C^{7*}, T^{12*}, and G^{14*} in duplex (**I**) with the counterparts in the nondeuterated duplex (**III**) shows (data presented only for the noncrowded crosspeaks) that the volumes from nOe experiment are less susceptible to spin-diffusion in the deuterated residues at $\tau_m > 100$ ms than in the natural counterpart. A comparison of interresidual $[(H2'')_{i-1}$ - $(Ar)_i$ (Fig. 8B), $(H1')_{i-1}$-$(Ar)_i$ (Fig. 9B)] and intraresidual $[(H1'\text{-}Ar)_i$ (Fig. 9A)] crosspeaks shows that there is no difference up to 250 ms in the crosspeak intensity between deuterated and nondeuterated residues suggesting (i) a negligible contribution of diffusional pathway through the H2'-H2" bond in the relative intensity of these crosspeaks, and (ii) the magnitude of contributions of H1'-H2" diffusion pathway to the direct dipole-dipole interresidual $(H2'')_{i-1}$ - $(Ar)_i$, $(H1')_{i-1}$-$(Ar)_i$ and intraresidual $(H1'\text{-}Ar)_i$ relaxation pathways for building block **B** and the natural sugar are similar. Earlier, a comparison of the σ relaxation rate of $(H1'\text{-}Ar)_i$ for the 1'#,2',2",3',4'#,5',5"-$^2H_7$-2'-deoxynucleotide residues[6] (*i.e.* Block **A** in Fig. 1) in partially-deuterated 20-mer (**V**)[7,8] with the nondeuterated natural 20-mer (**IV**) shows however that the σ values increase in the latter due to the contribution from spin-diffusion from all coupling pathways. Our present study with the partially-deuterated 12-mer (**I**) compared with natural 12-mer (**III**) shows that the normalised intensities (a_{ij}/a_{ii})) of H1'-H2" crosspeaks are slightly higher for the partially-deuterated residues compared to the natural ones. This increase could also be the result of the elimination of the extra H2'-H2" relaxation pathway. Interestingly, the error for the volume of crosspeaks for the deuterated residues are smaller because of higher intentensity of nOe crosspeaks and absence of splitting by $J_{1'2'}$ and $J_{2'2''}$ couplings compared to those of the nondeuterated residues.

In our efforts to obtain interproton distance information in the "NMR-window II" approach with the partially-deuterated nucleoside incorporated DNA duplex, we wished to select an appropriate nOe experiment to distinguish the partially-deuterated from the natural counterpart. This led us to consider various pulse sequences in different NOESY experiments[29,36a-f] that allow a change in the processes responsible for producing crosspeaks. Most of the approaches reported in the literature[29,36a-f] have been introduced only for the assignment purposes. Crosspeaks in the resulting 2D spectra appear through a two-step pathway linking the participating signals: For the pulse sequence in Fig. 3, the pathway is a through-coupling step in the preparation period followed by evolution and an nOe step. Note that the resulting spectrum is not symmetrical about the diagonal since the pathway $I \xrightarrow{J} I \xrightarrow{t_1} I(F_1) \xrightarrow{nOe} X(F_2)$ does not imply the existence of the reverse pathway $X \xrightarrow{J} X \xrightarrow{t_1} X(F_1) \xrightarrow{nOe} I(F_2)$.[36d,e] In heteronuclear experiments, Otting and Wüthrich[37] were the first to include high-power spin-lock purging pulses at the end of the INEPT transfer to improve the elimination of undesired coherences coming from protons not coupled to heterospins. Kyogoku et al[29] used a similar approach in a homonuclear experiment in which they put together Hahn-echo and spin-lock pulse-building blocks (HAL experiment) to filter off the unwanted magnetization due to the dephasing of the coupling term through B_1 inhomogeneity. In addition, the SQC-NOESY experiments,[36c,f] in which Hahn-echo and spin-lock pulse-building blocks in conjunction with polarization transfer through ^{13}C or ^{15}N, have been used to evaluate interproton distances provided the desirable protons have connectivity with the heteronucleus. This heteronuclear SQC-NOESY[36c] experiment is not symmetric. Individual cross peaks are expected to reflect the same specific attenuation factors as their 'diagonal' counterpart (at the same F1 frequency), responsible for the relaxation of the

parent transverse and heteronuclear antiphase coherences during the INEPT transfers and t1.[36c] Therefore the volume ratio must be calculated using connectivities with the same F1 frequency.

It has been pointed out[36c] that compared to the other qualitative methods (build-up rate ratio, crosspeak ratio) the approach to evaluate the distances through SQC-NOESY experiments[36c] has two advantages: (1) It was independent of the difference in leakage relaxation of the reference and unknown proton pairs, although

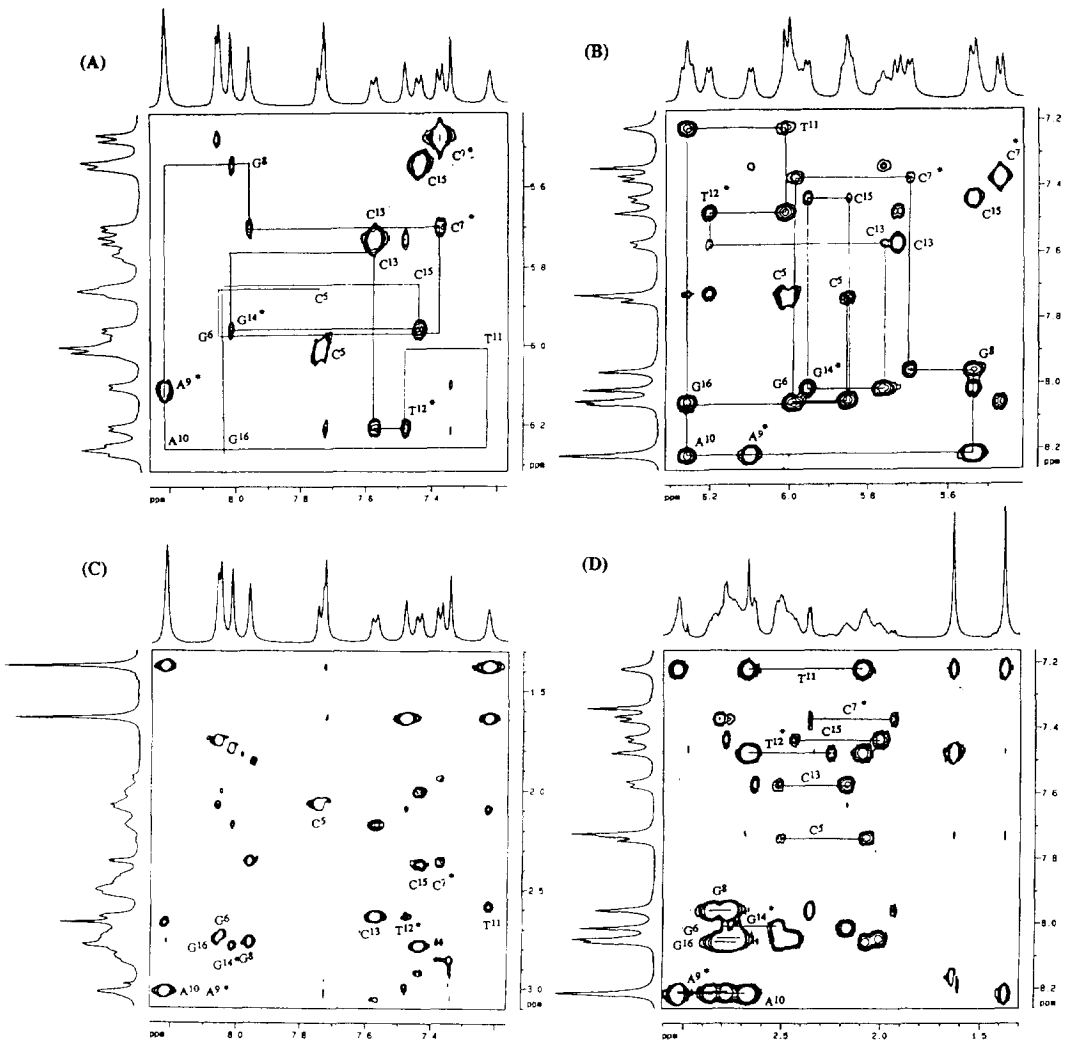


Figure 10. HAL-NOESY spectrum of the partially-deuterated 12-mer (I) using a delay of 28.7 ms ($^3J_{1'2'}$ = 8.7 Hz) for the spin-echo at mixing time t_m = 200ms. In Panels (A) and (B), only the aromatic to H1' parts of the spectrum are shown. In Panels (C) and (D) aromatic to H2', H2" and CH_3 are shown. Below the diagonal (Panels B & D), the spectra are identical to a normal NOESY. Above the diagonal (Panels A & C), there are however some differences in the spectra: In Panel (A), only the H1' of the partially deuterated residues (C^{7*}, A^{9*}, T^{12*} and G^{14*}) show crosspeak with its own aromatic proton or the one belonging to the next residue. Additionally, the crosspeaks belonging to H6 to H5 and the H6/H8 to H5 of the next residue are also visible. In Panel C, only $H2"_i$ - $H6/H8_i$ crosspeaks labelled by the symbol of residue, whereas $H2"_i$ - $H6/H8_{i+1}$ crosspeaks can be found lying in the same column.

they are expected to be the same. (2) It can be applied outside the linear build-up region when a pairwise interaction model can be assumed. In this work, the mixing time in the experiment was varied from 50-100 ms in order to avoid spin-diffusion. The main limitation however has been the overlap of diagonal peaks.

The above plethora of literature data stimulated us to choose a homonuclear experiment in which the same

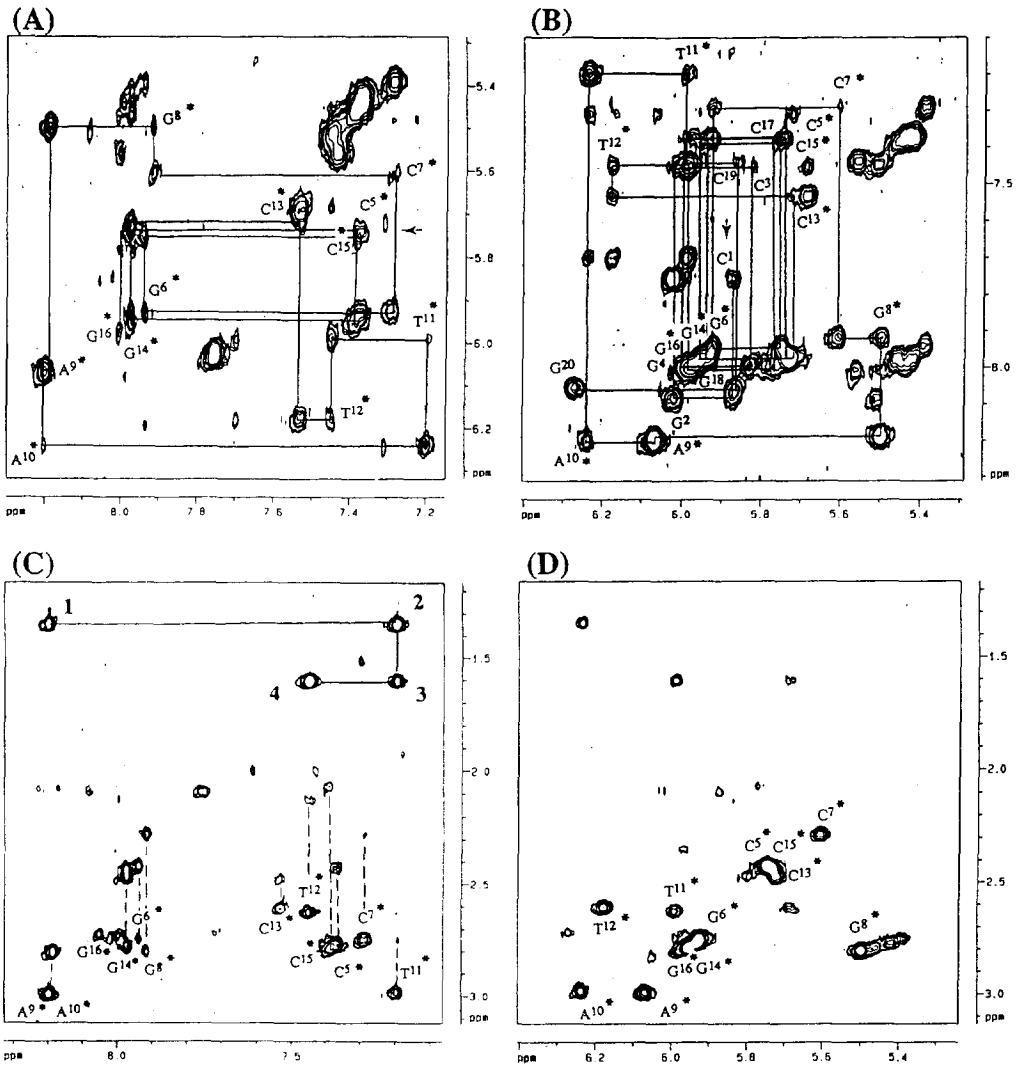


Figure 11. HAL-NOESY spectrum of the partially-deuterated 20-mer (**II**) using a delay of 28.7 ms ($^3J_{1'2'}$ = 8.7 Hz) for the spin-echo at mixing time t_m = 200 ms. In Panels (A) and (B), only the aromatic to H1' parts of spectrum are shown. The 'down' off-diagonal spectrum of aromatic to H1' region (Panel B) is identical with the normal NOESY spectrum. The 'up' off-diagonal spectrum of aromatic to H1' region however shows (Panel A) only the crosspeaks from the partially deuterated central 12 residues of the 20-mer duplex (**II**) (they are connected by the solid line and the starting direction has been shown by an arrow). Additionally, the crosspeaks for H6 to H5 and the H6/H8 to H5 of the (n-1) residue are also visible. Panel (C) shows only the crosspeaks of the partially deuterated central 12 residues: $H2''_i$ - $H6/H8_i$ labelled by the residue number and connected by dashed line with $H2''_i$ - $H6/H8_{i+1}$. Panel (D) shows only $H2''_i$ - $H1'_i$ of the partially deuterated central 12 residues.

'pulse-building' block consisting of spin-echo period $\Delta-\pi-\Delta$ followed by a spin-lock pulse, as in hetero[36c,f] or homonuclear experiment,[29] before evoluation and the mixing period, was used to obtain the final non-symmetrical NOESY spectrum with similar attenuation of the diagonal and crosspeaks at the same F_1 frequency by $\cos(\pi J2\Delta)$[29,36c] (Fig. 3) (notated as F_1 isotope-filtered 2D nOe experiments[36b]). In this study, F_1 versus F_2 isotope-filtered 2D nOe experiments were chosen since Bax and Weiss[38] have already shown that such an experiment has the advantage of higher digital resolution, which in our case allowed the Ar-H1' to be resolved with $^3J_{1'2''}$ coupling. In principle, both the HAL-NOESY (Fig. 3) and HAL experiments[29] could be combined and treated in the same way as the original NOESY experiment where the volume of both 'up' and 'down' off-diagonal crosspeaks are averaged (see experimental section) to be used for the distance information.

In the case of $J_{1'2'} \sim 8.7$ Hz, the delay (Δ) of 28.7 ms completely eliminated the observable magnetisation from H1' and H2' protons of the nondeuterated residues such that during the t1 period only magnetisation of the isolated or two-spin systems not involved in $J_{1'2'}$ coupling are evolved. In the HAL-NOESY experiment (Fig. 3), the diagonal peaks with the $J_{H1'\text{-}H2'}$ coupling disappear, but the other diagonal peaks from the H8, H2'' for the deuterated residues either remain as isolated-spin system or reduce in intensity as $\cos(\pi J_{ij}\Delta)$ term[29] in case of two spin system, $J_{1'2''}$ or J_{H5H6}, upon application of the HAL pulse sequence. The final spectrum of partially-

Table 3. Comparison of normalised (to its own diagonal at the same mixing time) volumes of a NOESY spectrum and a HAL-NOESY spectrum of the partially-deuterated 12-mer (I): $[d(C^5G^6C^{7*}G^8A^{9*}A^{10}T^{11*}T^{12}C^{13}G^{14*}C^{15}G^{16})]_2$ (* denotes partially-deuterated residues). In the HAL-NOESY spectrum all crosspeaks have only been normalised along the F2 axis. Normalisation along the F1 axis can give completely wrong results in HAL-NOESY spectra. The data given are the average from both the upper and the lower crosspeaks were it is possible, otherwise only the value for the uncrowded crosspeak is given.

crosspeak	NOESY	HAL	crosspeak	NOESY	HAL	crosspeak	NOESY	HAL
(H6-H1') C^5	0.071	a	(H1'-H4') C^7	0.037	0.023	(H1'-H2'') T^{11}	0.096	c
(H8-H1') G^6	0.041	0.052	(H1'-H4') G^8	0.061	c	(H1'-H2'') T^{12}	0.224	0.261
(H6-H1') C^7	0.037	0.035	(H1'-H4') A^9	0.031	0.058	(H1'-H2'') C^{13}	0.106	c
(H8-H1') G^8	0.041	0.043	(H1'-H4') A^{10}	0.065	c	(H1'-H2'') G^{14}	0.218	0.268
(H8-H1') A^{10}	0.038	0.045	(H1'-H4') T^{11}	0.062	c	(H1'-H2'') C^{15}	0.107	c
(H6-H1') T^{11}	0.041	0.070	(H1'-H4') T^{12}	0.058	0.055	(H1'-H2'') G^{16}	0.207	c
(H6-H1') T^{12}	0.032	0.034	(H1'-H4') C^{13}	0.049	c	(H6-H2'') C^7	0.035	0.014
(H6-H1') C^{13}	0.017	0.020	(H1'-H4') G^{14}	0.035	0.051	(H8-H2'') G^8	0.151	0.198
(H8-H1') G^{14}	0.026	0.028	(H1'-H4') C^{15}	0.036	c	(H8-H2'') C^{15}	0.118	0.076
(H6-H1') C^{15}	0.043	0.035	(H1'-H4') G^{16}	0.052	c	$H8G^6$-H2'C^5	0.052	0.087
(H8-H1') G^{16}	0.031	0.027	(H6-H2') C^7	0.063	0.047	$H6C^7$-H2'G^6	0.054	b
$H8G^6$-H1'C^5	0.017	b	(H8-H2') G^8	0.140	0.363	$H8G^8$-H2'C^7	0.014	0.014
$H6C^7$-H1'G^6	0.071	0.045	(H8-H2') A^{10}	0.193	b	$H8A^9$-H2'G^8	0.092	b
$H8G^8$-H1'C^7	0.026	0.028	(H6-H2') T^{11}	0.258	0.366	$H6T^{12}$-H2'T^{11}	0.102	0.145
$H8A^9$-H1'G^8	0.077	0.080	(H6-H2') T^{12}	0.047	0.030	$H6C^{13}$-H2'T^{12}	0.208	0.174
$H6T^{11}$-H1'A^{10}	0.053	0.083	(H6-H2') C^{13}	0.265	0.357	$H8G^{14}$-H2'C^{13}	0.056	0.073
$H6T^{12}$-H1'T^{11}	0.051	0.065	(H6-H2') C^{15}	0.312	0.197	$H8G^{16}$-H2'C^{15}	0.057	0.065
$H6C^{13}$-H1'T^{12}	0.048	0.057	(H1'-H2'') C^5	0.098	c	$H6C^7$-H2''G^6	0.087	0.054
$H8G^{14}$-H1'C^{13}	0.037	0.043	(H1'-H2'') G^6	0.166	c	$H8G^8$-H2''C^7	0.077	0.059
$H6C^{15}$-H1'G^{14}	0.058	0.050	(H1'-H2'') C^7	0.178	0.216	$H6T^{11}$-H2''A^{10}	0.253	0.293
$H8G^{16}$-H1'C^{15}	0.023	b	(H1'-H2'') G^8	0.179	c	$H6C^{13}$-H2''T^{12}	0.145	0.177
(H1'-H4') C^5	0.047	c	(H1'-H2'') A^9	0.262	0.278	$H6C^{15}$-H2''G^{14}	0.180	0.099
(H1'-H4') G^6	0.067	c	(H1'-H2'') A^{11}	0.119	c	$H8G^{16}$-H2''C^{15}	0.066	0.073

a: it could not be normalised because its own diagonal peak along the F2 axis is crowded, b: crowded crosspeak on the lower half of the spectra and no crosspeak exists on the upper half, c:does not exist since it does not belong to a deuterated residue.

deuterated 12-mer (**I**) is not symmetrical (Figs. 10): the H1'/H2"-Ar part of the spectrum, which is the 'down' off-diagonal shows all crosspeaks from both deuterated and nondeuterated residues (Figs. 10B and D), whereas the 'up' off-diagonal part of the same spectrum shows the crosspeaks belonging only to the partially-deuterated residues (Fig. 10A). A normalisation of the intensity of the crosspeaks to their own diagonal peaks in the F_2 dimension at the same mixing time (Table 3) allows cancellation of the $\cos(\pi J_{ij} \Delta)$ term because both the diagonal and the corresponding crosspeaks depend on this term in the same manner. Table 3 clearly shows that the volumes of the crosspeaks obtainable from either HAL-NOESY or NOESY experiments are quite comparable within the experimental error. The value of R-factor between the NOESY and HAL-NOESY volumes defined as $\frac{\sum |I_{NOESY} - I_{HAL-NOESY}|}{\sum I_{NOESY}}$ is 0.325 (< 8% deviation in interproton distances). It is important that this R-factor is the same as those used in any structural refinement procedure.[27] *This led us to conclude that volumes from our HAL-NOESY experiment can be used as constraints with the same level of confidence as those obtained from normal NOESY spectra for full matrix relaxation analyses using MARDIGRAS[27a] or MORASS[27b] in the MD refinement procedures.*

We have furthermore expanded this work to our partially-deuterated 20-mer duplex (**II**) showing basically the same result as we obtained for 12-mer duplex (**I**). Although we used only 32 scans for each of 512 experiments for both 12-mer (**I**) and 20-mer (**II**) duplexes, but the results are indeed comparable. Panel A in Fig. 9 shows the expanded region of Ar-H1' 'up' off-diagonal crosspeaks of partially-deuterated 20-mer (**II**) which belong to only deuterated residues, whereas panel B shows 'down' off-diagonal peaks of the same spectrum where one observes all Ar-H1' crosspeaks for all 20 sugar residues in 20-mer duplex (**II**). This finding clearly shows the usefulness of the partially-deuterated blocks **B** + **C** in Figure 1 in the creation of "NMR-window II".

The main limitation of this experiment is that the intensities of the signals of the H1' and H2" protons are reduced because $J_{1'2"}$ coupled spin system is affected by the spin-echo, thereby giving a slightly reduced sensitivity. The second limitation is the crowded aromatic diagonal peak region (including H5 of cytosine residue), which will make it difficult for larger DNA duplex to use the diagonal aromatic and H1' region for normalisation decreasing the confidence of the data. This problem is now being tackled in our lab using nucleotide residues containing both deuterated sugar and nucleobases.[30]

(3) The proton-proton distances between H1'-H4', H4'-H2" are sensitive to the sugar conformational changes and hence are useful probes for the determination of sugar conformation. The higher sensitivity of the proton-proton distances H1'-H4', H4'-H2" to the sugar conformation is well known.[1c,24] In N-type conformation ($P \leq 30°$) the distance between H4'-H2" is ~2.3 - 2.4 Å and it is ~ 3.8 - 4.0 Å in the S-type conformation ($140° \leq P \leq 180°$), whereas the H1'-H4' distance for both S and N-type conformations are similar ~2.8 - 3.4 Å but quite sensitive to the small variation of the sugar conformation. These data complement the $^3J_{H1'H2'}$ and/or $^3J_{H1'H2"}$ coupling constants information.[31]

As it has been mentioned above, the HAL-NOESY spectrum is not symmetrical. Fig. 12 shows the expanded HAL-NOESY spectrum of 12-mer duplex (**I**). Panel B shows that the 'up' off-diagonal H1' to H4' region is quite crowded because all 12 crosspeaks of this type from 12 sugar moieties exist here, whereas in Panel A which is the 'down' off-diagonal region of the same HAL-NOESY spectrum only the crosspeaks belonging to the H1' resonances of deuterated residues can be observed [H1' to H4' and some of the $(H1')_{i-1}$ to

$(H5')_i$ crosspeaks]. In Panels A and B of Fig. 13 we show the symmetrical NOESY spectrum of the same duplex (**I**), which are equally crowded. Since the ^2H enrichment at the C4' is ~65%, and therefore the residual ^1H is only ~35% in blocks **5a,b - 8a,b**, hence the H1' to H4' crosspeaks are quite weak. As a result, the extraction of relative intensities of nOe crosspeaks (a_{ij}/a_{jj}) has been possible in a quantitative manner from the

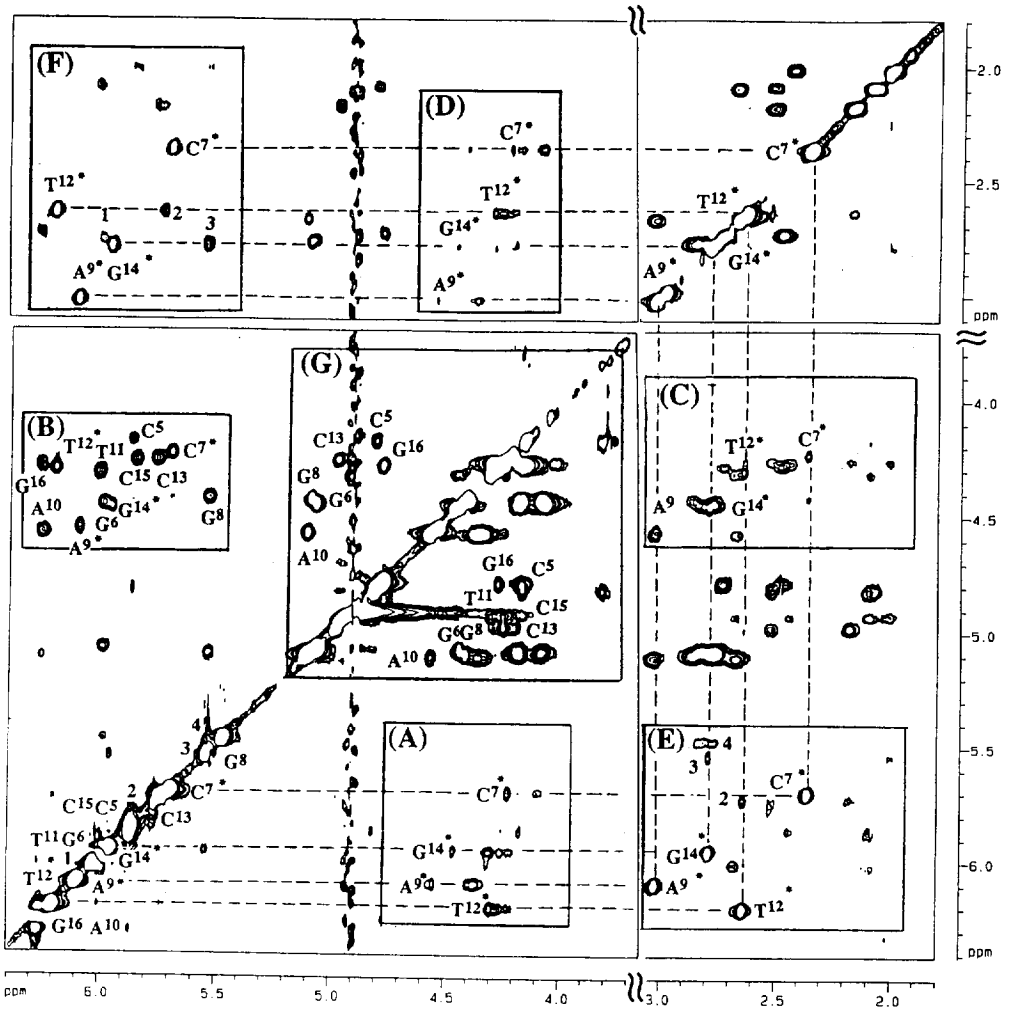


Figure 12. Expanded HAL-NOESY spectrum between 6.4 - 3.7 ppm and 3.1 - 1.8 ppm of the partially-deuterated 12-mer (**I**) using a delay of 28.7 ms for spin-echo at mixing time t_m = 150 ms with 128 scans. In Panel A and B, the H1'-H4'/5'/5" regions of crosspeaks are shown. In Panel B, the H1-H4' crosspeaks for all residues are observed (they are labelled by corresponding symbol and number of residue) but in Panel A only those belonging to the partially deuterated residues (C^{7*}, A^{9*}, T^{12*} and G^{14*}) are observed. In Panels C and D the H4'-H2'/H2" and H5'/H5"-H2'/H2" crosspeaks are presented. In Panel D, the crosspeaks belonging to the partially deuterated residues are observed (H4'-H2" crosspeaks are labelled), but in Panel C the crosspeaks from all sugar residues could be observed (the only crosspeaks belonging to the deuterated residue are labelled). In Panels E and F, the H1'-H2'/H2" regions are presented. The H1'-H2" crosspeaks are observed only for the partially deuterated residues as they are labelled. The crosspeaks between $H5_i$-$H2"_{i-1}$ have been observed and are labelled in the above panels by arabic numbers in the same way as for the corresponding diagonal peaks $H5_i$: (1) C^5(H5), (2) C^{13}(H5), (3) C^{15}(H5), (4) C^7(H5). In Panel G, the H3', H4', H5', H5" region is presented. The crosspeaks belonging to nondeuterated residues are observed and labelled. The diagonal peaks belonging to H5' and H5" protons are eliminated through the echo filter.

HAL-NOESY experiment only for the 12-mer duplex (I) (see Table 3), which also shows their similarity with those of the corresponding NOESY data (Table 3) *(vide infra)*.

Panel D (Fig. 12) shows the 'up' off-diagonal region belonging to H2", H4' crosspeaks arising only from deuterated residues, whereas Panel C should show all crosspeaks of this type from all sugar residues. But some crosspeaks in Panel C are too weak to be observed because of the long distances between the H2", H4' protons, suggesting that those H4'-H2" crosspeaks that are not observable most probably arise from S-type sugar conformation (P ≈180°). It is likely that they would be observable if we could only engineer higher residual atom % ^{1}H at C4'.

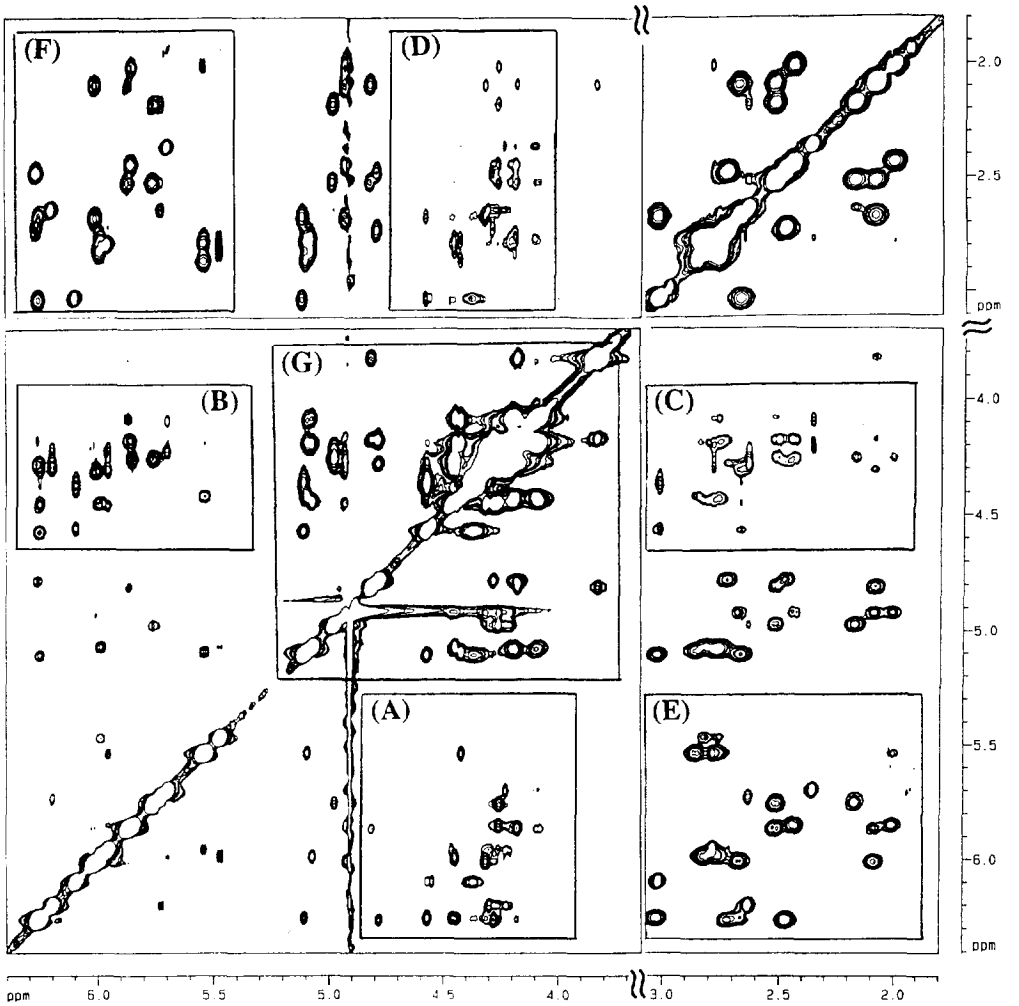


Figure 13. Expanded NOESY spectrum of the partially-deuterated 12-mer (I) between 6.4 - 3.7 ppm and 3.1 - 1.8 ppm at mixing time t_m = 150 ms with 32 scans. The spectrum is symmetrical to the diagonal. In the Panels A - G, the following spectral regions are presented: (A, B) H1'-H4'/H5'/H5"; (C, D) H4'-H2'/H2" and H5'/H5"-H2'/H2"; (E, F) H1'-H2'/H2"; (G) H3'/H4'/H5'/H5".

It is noteworthy that although in Panel G the diagonal should have consisted of the residual H3', H4', H5' and H5" signals but the signals arising from H5'/5" protons are completely eliminated through the echo filter (delay 28.7 ms), and hence only H3' and H4' diagonal peaks are observed. As a result, a significant simplification is achieved in the 'up' off-diagonal part of the spectrum as shown in Panel B, where only crosspeaks belonging to H1' and H4' protons can be observed, whereas in Panel G only crosspeaks belonging to H3', H4' protons of the nondeuterated residues are present (Figure 12). We are now planning a lower enrichment at C4' with ^2H for observing the H1'-H4' and H4'-H2" nOe crosspeaks using the "NMR-window II" concept for larger DNA duplex than have been possible up to now.

Conclusions

The incorporation of new selectively deuterated isotopomeric building blocks **5a,b** - **8a,b** in DNA duplex has four distinct advantages over the existing methods: (i) It allows extraction of the $^3J_{H1',H2'}$ and $^3J_{H1',H2"}$ coupling constant information easily and unambiguously for even a 20 base-pair DNA duplex (*i.e.* 40 nucleotide residues) with a molecular weight of ~12.000 with high accuracy. (ii) The interresidual [$(H2")_{i-1}$ - $(Ar)_i$, $(H1')_{i-1}$-$(Ar)_i$] and intraresidual, [$(H2"\text{-}Ar)_i$, $(Ar\text{-}H1')_i$, $(H1'\text{-}H2")_i$, $(H4'\text{-}H1')_i$, $(H4'\text{-}H2")_i$, $(H4'\text{-}Ar)_i$] nOe volumes can be easily obtained using the HAL-NOESY experiment by filtering away signals belonging to the nondeuterated nucleotides. (iii) The spin-diffusion taking place through the H2'-Ar, H1'-H2', H3'-Ar, H1'-H3', H2'-H3' and H2'-H2" pathways in the NOESY spectra can be successfully eliminated.

MD calculations on duplexes (**I**) and (**II**) using the above NMR data as constraints are in progress. Further work is also in progress in this lab on the use of partially-deuterated blocks, which are fully-deuterated at C3' with deuterio isotopomeric mixture both at C5'(S + R) and C2'(S + R) and fully protonated centers at C1' and C4', for the conformational analysis of large DNA duplex by NMR spectroscopy.

Experimental Section

Materials and Methods. The deuterium labelled 2',3',4'#,5',5"-$^2H_5$-β-D-ribonucleoside derivatives **1 - 4** were prepared by our published procedure[5]. 2,2'-Azobis(2-methylpropionitrile) (AIBN) was purchased from Merck. Tetra-n-butylammonium fluoride was purchased from Fluka. Tri-n-butyltin hydride and 1H-tetrazole were purchased from Aldrich. 1,3-Dichloro-1,1,3,3-tetraisopropyldisiloxane[18], *N,N*-diisopropylammonium tetrazolide[19], (2-cyanoethoxy)bis(diisopropylamino) phosphine[21] and 2-cyanoethyl *N,N*-diisopropyl-chlorophosphoramidite[21] were prepared using literature procedures. Pyridine and toluene were distilled after being refluxed over calcium hydride for 3 - 4 h, dichloromethane, dichloroethene and acetonitrile were stirred with phosphorus pentoxide overnight followed by distillation under nitrogen. The distilled acetonitrile was stored over molecular sieves (3 Å) overnight.

Analytical TLC was carried out using Merck pre-coated Kieselgel 60 F_{254} glass backed plates. Kieselgel G60 from Merck was used for short column chromatographic separations. DEAE-Sephadex G-25 super fine, DNA grade (Pharmacia, Sweden) was used for gel filtrations.

^1H-NMR spectra were recorded on a Jeol FX 90 Q, JNM GX 270 or Bruker AMX-500 spectrometer at 90, 270 MHz or 500 MHz, respectively, using TMS (0.0 ppm) or acetonitrile for D_2O solutions (2.0 ppm) as internal standards. ^{31}P-NMR spectra were recorded at 36 MHz in the same solvent as for ^1H-NMR spectra using 85 % phosphoric acid (0.0 ppm) as external standard. Chemical shifts are reported in ppm (δ scale).

NMR spectroscopy of oligo-DNAs. All spectra were recorded in D_2O at 21°C or at 0°C with a sweep width of 9 ppm. In F_2 dimension 4K complex data points were used while in F_1 dimension either 256 or 512

experiments of 32 scans each. Before Fourier transformation each spectrum was zero-filled twice in the F_1 dimension and a 2 Hz line-broadening function was applied in the F_2-dimension and a $\pi/2$ shifted sinesquare window in F_1 dimension. The final resolution were either 2K by 1K or 2K by 2K real data points. For the HAL-NOESY experiment, the pulse sequence scheme and phase cycle of which is presented in Fig. 3., a delay of 28.7 ms was used for the spin-echo (corresponding to a $^3J_{1'2'} = 8.7$ Hz coupling). Mixing times $\tau_m = 30$, 50, 70, 100, 150, 200, 250 and 300 ms in NOESY and τ_m=100, 150, 200 ms in HAL-NOESY experiments have been used. The volume of the crosspeaks in NOESY and HAL-NOESY spectra at these mixing times have been calculated by the program AURELIA[32]. The double quantum spectra (DQ)[26] were recorded using 512 experiments of 32 scans of 8K complex data points. The spectra were zero-filled twice in the F_1 dimension and a 2 Hz line-broadening function and a $\pi/2$ shifted sinesquare window were applied in the F_1 and F_2 dimensions, respectively. After Fourier transformation the final resolution were 4K by 2K real data points. The different delays used were 26, 48.1 and 78.1 ms corresponding to coupling constants of 9.6, 5.2 and 3.2 Hz, respectively. All DQF-COSY[33] spectra were recorded at both 21 °C and 0 °C. 256 experiments of 32 scans and 4K complex data points were used. The spectra were then processed using $\pi/4$ shifted sinesquare windows in both dimensions and zero-filling in F_1 dimension to yield a 4K by 1K real data points matrix. The T_1 values were measured at 21 °C using inversion recovery with a relaxation delay of 20 s. T_2 was measured by using the Carr-Purcell-Meibom-Gill (CPMG)[34] sequence at 21 °C, also with a relaxation delay of 20 s.

(A) General procedure for the preparation of 2'-O-phenoxythiocarbonyl derivatives of nucleosides.

3',5'-O-(TPDS)-2'#,2"#,3',4'#,5',5"-$^2H_5$-thymidine (5a+b). After coevaporation with dry toluene, the phenoxythiocarbonyl ester **1** (1.96 g, 3.05 mmol) was dissolved in the same solvent (25 ml), AIBN (98 mg, 0.61 mmol, 0.2 mol eq.) and tri-n-butyltin hydride (1.24 ml, 4.58 mmol, 1.5 mol eq.) were added. After degassing by bubbling nitrogen through the solution (20 min.), the reaction mixture was heated at 75 °C under nitrogen atmosphere for 3.5 h. Volatile materials were evaporated on a rotavapor and the residual oil was subjected to column chromatography to afford isotopomeric mixture of **5a+b** (1.29 g, 86 %). ^{1}H-NMR ($CDCl_3$, 500 MHz): 8.52 (br. s., 1H) N-H; 7.43 (d, $J_{5CH3,6}$= 1.1 Hz, 1H) H-6; 6.07 (m, 1H) H-1'; 3.75 (s, 0.35H) H-4'#; 2.47 (d, $J_{H1',H2"}$= 7.6 Hz) H/D-2"# (89:11 ratio); 2.23 (d, $J_{H1',H2'}$= 1.9 Hz) H/D-2'# (11:89 ratio); 1.93 (d, 3H) 5-CH_3; 1.1-1.0 (m, 24H) methyls of TPDS.

3',5'-O-(TPDS)-2'#,2"#,3',4'#,5',5"-$^2H_5$-N^4-benzoyl-2'-deoxycytidine (6a+b). Procedure for preparation of **5a+b** was used for 14 h to convert compound **2** (1.62 g, 2.22 mmol) to isotopomeric mixture of **6a+b** (0.9 g, 70%). ^{1}H-NMR ($CDCl_3$, 500 MHz): 8.93 (br. s., 1H) N-H; 8.33 (d, $J_{H5,H6}$= 7.6 Hz, 1H) H-6; 8.0 - 7.4 (m, 6H) benzoyl + H-5; 6.09 (m, 1H) H-1'; 3.83 (s, 0.35H) H-4'#; 2.59 (d, $J_{H1',H2"}$= 7.1 Hz) H/D-2"# (82:18 ratio); 2.35 (d, $J_{H1',H2'}$= 1.0 Hz) H/D-2'# (18:82 ratio); 1.1 - 1.0 (m, 24H) methyls of TPDS.

3',5'-O-(TPDS)-2'#,2"#,3',4'#,5',5"-$^2H_5$-N^6-benzoyl-2'-deoxyadenosine (7a+b) Treatment of compound **3** (1.6 g, 2.12 mmol) according to the procedure for preparation of **5a+b** was used to give isotopomeric mixture of **7a+b** (1.02 g; 80%). ^{1}H-NMR ($CDCl_3$, 500 MHz): 9.10 (br. s., 1H) N-H; 8.78 (s, 1H) H-8; 8.23 (s, 1H) H-2; 8.1 - 7.4 (m, 5H) benzoyl; 6.36 (m, 1H) H-1'; 3.92 (s, 0.35H) H-4'#; 2.76 (d, $J_{H1',H2'}$= 2.0 Hz) H/D-2'# (9:91 ratio); 2.68 (d, $J_{H1',H2"}$= 7.6 Hz) H/D-2"# (91:9 ratio); 1.1 - 1.0 (m, 24H) methyls of TPDS.

3',5'-O-(TPDS)-2'#,2"#,3',4'#,5',5"-$^2H_5$-N^2-acetyl-O^6-diphenylcarbamoyl-2'-deoxyguanosine (8a+b). Compound **4** (1.32 g, 1.46 mmol) was treated according to the procedure for preparation of **5a+b** to afford an isotopomeric mixture of **8a+b** (0.95 g, 87%). ^{1}H-NMR ($CDCl_3$, 500 MHz): 8.17 (s, 1H) H-8; 7.96 (br. s., 1H) N-H; 7.5 - 7.2 (m, 10H) DPC; 6.27 (m, 1H) H-1'; 3.88 (s, 0.35H) H-4'#; 2.63 (d, $J_{H1',H2'}$= 2.5 Hz) H/D-2'# (18:82 ratio); 2.61 (d, $J_{H1',H2"}$= 7.2 Hz) H/D-2"# (82:18 ratio); 2.55 (s, 3H) Ac; 1.1 - 1.0 (m, 24H) methyls of TPDS.

(B) General procedure for the deprotection of the 3',5'-O-(TPDS) group to prepare the isotopomeric mixture of base-protected 2'-deoxynucleosides.

2'#,2"#,3',4'#,5',5"-$^2H_5$-Thymidine (9a+b). Compounds **5a+b** (1.17 g, 2.39 mmol) were dissolved in dry tetrahydrofurane (24 ml) and 1.0 M TBAF solution in dry THF (2.4 ml, 1.0 mol. eq.) was added. After stirring for 5 min, volatile materials were evaporated and the residue was purified on silica gel

column to get the isotopomeric mixture of **9a+b** (0.57 g, 97 %). ^{1}H-NMR (D_2O, 270 MHz): 7.57 (d, $J_{5CH3,6}$= 1.2 Hz, 1H) H-6; 6.22 (d, $J_{H1',H2''}$= 6.6 Hz, 1H) H-1'; 3.94 (s, 0.35H) H-4'#; 2.29 (d) H/D-2''# (89:11 ratio); 2.26 (s) H/D-2'# (11:89 ratio); 1.83 (d, 3H) 5-CH_3.

2'#,2''#,3',4'#,5',5''-$^2H_5$-N^4-Benzoyl-2'-deoxycytidine (10a+b) When compounds **6a+b** (0.97 g, 1.67 mmol) were treated according to procedure for preparation of **9a+b**, the isotopomeric mixture of **10a+b** (0.49 g, 87%) was obtained. ^{1}H-NMR (DMSO-d_6): 8.49 (d, $J_{H5,H6}$= 7.3 Hz, 1H) H-6; 8.2 - 7.5 (m, 5H) benzoyl; 7.43 (d, 1H) H-5; 6.26 (d, $J_{H1',H2''}$= 6.1 Hz, 1H) H-1'; 5.34 (s, 1H) OH; 5.03 (s, 1H) OH; 3.98 (s, 0.35H) H-4'#; 2.42 (d) H/D-2''# (82:18 ratio); 2.14 (s) H/D-2'# (18:82 ratio).

2'#,2''#,3',4'#,5',5''-$^2H_5$-N^6-Benzoyl-2'-deoxyadenosine (11a+b). Compounds **7a+b** (0.92g, 1.5 mmol) was subjected to the treatment described for **9a+b** to give the isotopomeric mixture of **11a+b** (0.52 g, 96%). ^{1}H-NMR ($CDCl_3$/DMSO-d_6): 9.55 (br. s., 1H) N-H; 8.77 (s, 1H) H-8; 8.20 (s, 1H) H-2; 8.1 - 7.4 (m, 5H) benzoyl; 6.45 (d, $J_{H1',H2''}$= 5.6 Hz, 1H) H-1'; 5.64 (s, 1H) OH; 4.59 (s, 1H) OH; 4.21 (s, 0.35H) H-4'#; 2.39 (d) H/D-2''#.

2'#,2''#,3',4'#,5',5''-$^2H_5$-N^2-Acetyl-O^6-diphenylcarbamoyl-2'-deoxyguanosine (12a+b). Upon treatment of compounds **8a+b** (0.89 g, 1.19 mmol) with TBAF as described for **9a+b**, the isotopomeric mixture of **12a+b** (0.60 g, 98%) was obtained. ^{1}H-NMR ($CDCl_3$): 8.64 (br. s., 1H) N-H; 8.13 (s, 1H) H-8; 7.5 - 7.2 (m, 10H) DPC; 6.24 (d, $J_{H1',H2''}$= 6.4 Hz, 1H) H-1'; 4.00 (s, 0.35H) H-4'#; 2.74 (m) H/D-2'# (18:82 ratio); 2.34 (s, 3H) N^2-acetyl; 2.29 (d) H/D-2''# (82:18 ratio).

(C) General procedure for the protection of the 5'-hydroxyl group of base-protected 2'-deoxynucleosides.

5'-O-DMTr-2'#,2''#,3',4'#,5',5''-$^2H_5$-thymidine (13a+b). Compounds **9a+b** (0.56 g, 2.27 mmol) were rendered anhydrous by repeated coevaporation with dry pyridine (3 x 10 ml), then they were treated with DMTr-Cl (1 g, 2.95 mmol) in the same solvent (11 ml) for 2 h at room temperature. The reaction mixture was poured into saturated sodium bicarbonate solution and extracted with dichloromethane. After drying over magnesium sulfate, the organic phase was evaporated, coevaporated with toluene and the residual foam was chromatographed to obtain the isotopomeric mixture of **13a+b** (1.0 g, 80 %) as a foam. ^{1}H-NMR ($CDCl_3$ + DABCO): 7.60 (br. s., 1H) H-6; 7.4 - 6.8 (m, 13H) DMTr; 6.43 (d, $J_{H1',H2''}$= 6.1 Hz, 1H) H-1'; 4.04 (s, 0.35H) H-4'#; 3.78 (s, 6H) 2xOCH_3; 2.39 (d) H/D-2''# (89:11 ratio); 2.26 (s) H/D-2'# (11:89 ratio); 1.46 (d, 3H) 5-CH_3.

5'-O-DMTr-2'#,2''#,3',4'#,5',5''-$^2H_5$-N^4-benzoyl-2'-deoxycytidine (14a+b) Upon subjecting compounds **10a+b** (0.49 g, 1.46 mmol) to a treatment described for the preparation of **13a+b**, the isotopomeric mixture of **14a+b** (0.86 g, 89%) was obtained. ^{1}H-NMR ($CDCl_3$ + DABCO): 8.33 (d, $J_{H5,H6}$= 7.6 Hz, 1H) H-6; 8.0 - 6.8 (m, 19H) DMTr + benzoyl + H-5; 6.30 (d, $J_{H1',H2''}$= 6.3 Hz, 1H) H-1'; 4.13 (s, 0.35H) H-4'#; 3.80 (s, 6H) 2xOCH_3; 2.72 (d) H/D-2''# (82:18 ratio); 2.28 (m) H/D-2'# (18:82 ratio).

5'-O-DMTr-2'#,2''#,3',4'#,5',5''-$^2H_5$-N^6-benzoyl-2'-deoxyadenosine (15a+b). Compounds **11a+b** (0.52 g, 1.44 mmol) were treated according to procedure described for **13a+b** to afford the isotopomeric mixture of **15a+b** (0.79 g, 83%). ^{1}H-NMR ($CDCl_3$ + DABCO): 9.20 (br. s., 1H) N-H; 8.72 (s, 1H) H-8; 8.15 (s, 1H) H-2; 8.1 - 6.7 (m, 18H) DMTr + benzoyl; 6.48 (d, $J_{H1',H2''}$= 6.4 Hz, 1H) H-1'; 4.15 (s, 0.35H) H-4'#; 3.76 (s, 6H) 2xOCH_3; 2.54 (d) H/D-2''# (91:9 ratio); 2.38 (m) H/D-2'# (9:91 ratio).

5'-O-DMTr-2'#,2''#,3',4'#,5',5''-$^2H_5$-N^2-acetyl-O^6-diphenylcarbamoyl-2'-deoxy-guanosine (16a+b). Compounds **12a+b** (0.60 g, 1.17 mmol) were converted into the isotopomeric mixture of **16a+b** (0.80 g, 85%) according to procedure described for **13a+b**. ^{1}H-NMR ($CDCl_3$ + DABCO): 8.16 (br. s., 1H) N-H; 8.09 (s, 1H) H-8; 7.5 - 6.7 (m, 23H) DMTr + DPC; 6.44 (d, $J_{H1',H2''}$= 6.4 Hz, 1H) H-1'; 4.14 (s, 0.35H) H-4'#; 3.73 (s, 6H) 2xOCH_3; 2.50 (d) H/D-2''#; 2.38 (s, 3H) N^2-Ac.

(D) General procedures for the phosphytilation 5'-O-DMTr-base-protected-2'-deoxy-nucleosides.

Method I: 5'-O-DMTr-2'#,2''#,3',4'#,5',5''-$^2H_5$-thymidine 3'-(2-(cyanoethyl)-*N*,*N*-diisopropylamino) phosphoramidite (17a+b). Compounds **13a+b** (0.99 g, 1.8 mmol) were dissolved in dry tetrahydrofuran (9.1 ml) and *N*,*N*-diisopropylethylamine (0.67 ml, 3.9 mmol) was added under nitrogen atmosphere. 2-Cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite (0.57 ml, 2.6 mmol) was added to this solution by dropwise addition. The reaction mixture was stirred for 90 min at ambient temperature. After 90 min, dry ethanol (0.4 ml) was added to the reaction mixture and stirred for 20 min. Finally the reaction mixture

was extracted with ethylacetate and washed with brine three times. The organic phase was dried over $MgSO_4$ and evaporated. The residue was subjected to short column chromatography to give the isotopomeric mixture of phosphoramidites **17a+b** (1.05 g, 76 %) as a foam. ^{1}H-NMR ($CDCl_3$ + DABCO): 8.55 (m, 1H) N-H; 7.65 & 7.60 (2xbr. s., 1H) H-6; 7.6 - 6.8 (m, 13H) DMTr; 6.37 (2xd, $J_{H1',H2"}$= 5.62 Hz, 1H) H-1'; 4.16 (2xs, 0.35H) H-4'#; 3.79 (s, 6H) 2xOCH_3; 3.67 - 3.52 (m, 4H) -CH_2CH_2 CN and 2x$(CH_3)_2CH$; 2.64 (m, 2.89H)CH_2CH_2CN and H/D-2"#; 1.42 (s, 3H) 5-CH_3; 1.20-1.01 (m, 12H) $(CH_3)_2$ CH-x2. ^{31}P-NMR: 148.35, 148.78.

5'-O-DMTr-2'#,2"#,3',4'#,5',5"-$^2H_5$-N^4-benzoyl-2'-deoxycytidine 3'-(2-(cyanoethyl)-N,N-diisopropylamino) phosphoramidite (18a+b). Compounds **14a+b** (0.83 g, 1.3 mmol) were converted into the isotopomeric mixture of **18a+b** (0.68 g, 62%) according to Method I. ^{1}H-NMR ($CDCl_3$ + DABCO): 8.38 (dx2, $J_{5,6}$= 7.8 Hz, 1H) H-6; 8.0 - 7.4 (m, 19H) benzoyl, H-5 and DMTr; 6.28 (dx2, $J_{H1',H2"}$= 6.11 Hz, 1H) H-1'; 4.23 (s, 0.35H) H-4'#; 3.81 (s, 6H) 2xOCH_3; 3.67 - 3.52 (m, 4H) CH_2CH_2 CN, 2x$(CH_3)_2CH$; 2.76 - 2.38 (m, 2.82H) CH_2 CH_2CN and H/D-2"#; 1.30 - 1.05 (m, 12H) 2x$(CH_3)_2$ CH. ^{31}P-NMR: 149.24, 148.66.

Method II: 5'-O-DMTr-2'#,2"#,3',4'#,5',5"-$^2H_5$-N^6-benzoyl-2'-deoxyadenosine 3'-(2-(cyanoethyl)-N,N-diisopropylamino) phosphoramidite (19a+b). The mixture of compounds **15a+b** (0.77 g, 1.16 mmol) was dissolved in dry dichloromethane (11 ml). To this solution stirred under nitrogen atmosphere, N,N-diisopropylammonium tetrazolide (97 mg, 0.55 mmol) was added followed by (2-cyanoethoxy)bis(N,N-diisipropylamino)phosphine (0.49 ml, 1.65 mmol) and stirring was maintained overnight. The reaction mixture was diluted with dichloromethane and extracted with saturated sodium bicarbonate. After two additional extraction with brine, the organic phase was dried over $MgSO_4$ and evaporated to a foam. Short column chromatography followed by precipitation from n-hexane afforded the isotopomeric mixture of compounds **19a+b** (0.65 g, 65%). ^{1}H-NMR ($CDCl_3$ + DABCO): 9.1 (br. s., 1H) N-H; 8.74 (s, 1H) H-8; 8.22 & 8.19 (2xs, 1H) H-2; 8.1 - 6.7 (m, 18H) DMTr + benzoyl; 6.51 (d, $J_{H1',H2"}$= 6.1 Hz, 1H) H-1'; 4.31 (s, 0.35H) H-4'#; 4.2 - 3.2 (m, 2H) CH_2 CH_2CN; 3.77 (s, 6H) 2xOCH_3; 2.7 - 2.3 (m, 4H) CH_2CH_2 CN, 2x$(CH_3)_2CH$, H-2"#, H-2'#; 1.3 - 1.0 (m, 12H) 2x$(CH_3)_2$CH. ^{31}P-NMR: 149.49, 148.84.

5'-O-DMTr-2'#,2"#,3',4'#,5',5"-$^2H_5$-N^2-acetyl-O^6-diphenylcarbamoyl-2'-deoxyguanosine 3'-(2-(cyanoethyl)-N,N-diisopropylamino) phosphoramidite (20a+b). Compounds **16a+b** (0.80 g, 0.99 mmol) were treated as described in Method II to obtain compound **20a+b** as a mixture of isotopomers (0.65 g, 66 %). ^{1}H-NMR ($CDCl_3$ + DABCO): 8.13 & 8.11 (2xs, 1H) H-8; 7.96 & 7.89 (2xbr. s., 1H) N-H; 7.5 - 6.7 (m, 23H) DMTr + phenyls; 6.38 (d, $J_{H1',H2"}$= 5.9 Hz, 1H) H-1'; 4.29 & 4.27 (2xs, 0.35H) H-4'#; 3.9 - 3.3 (m, 2H) CH_2 CH_2CN; 3.73 (s, 6H) 2xOCH_3; 2.7 - 2.3 (m, 6H) CH_2CH_2 CN, 2x$(CH_3)_2CH$, H-2"#, H-2'#, N^2-Ac; 1.3 - 1.0 (m, 12H) 2x$(CH_3)_2$CH. ^{31}P-NMR: 149.06, 148.75.

DNA Synthesis and Purification. The deuterium labelled 12-mer (**I**) and 20-mer (**II**) were prepared by the solid phase phosphoramidite method on a Pharmacia LKB Gene Assembler Special automatic synthesiser of eight ports. The standard programs for 1.3 μmol scale synthesis from Pharmacia were modified in a way to have 2 min. coupling time. After deprotection in 32% aqueous ammonia solution for 7 days at room temperature, the solvent was evaporated. The residue was redissolved in water and extracted (3x) with dichloromethane and finally with diethylether. Purification of the oligomers having palindromic sequences was carried out by preparative ion exchange HPLC (Gilson system consisting of Model 305 &306 Pumps, 811C Dynamic Mixer and 118 UV Detector) on a Millipore Protein Pak^{TM} Q 8HR 1000Å 8μm column (10x100 mm) under denaturating condition (pH = 12) with a linear gradient of 45% → 60% buffer B (1 M NaCl in 0.01 M NaOH) in buffer A (0.01 M NaOH) over a period of 30 min with a loading of 50 o.d. units of crude DNA per injection. Appropriate peaks were collected, pooled and desalted using a Sephadex G 25 gel filtration column. Finally, the purified samples [453 o.d. units, 25 % for duplex (**I**) and 500 od units, 21% for duplex (**II**)] were lyophilised together with the appropriate buffer used for NMR spectroscopy from D_2O (99.9 % D atom). The syntheses of the corresponding counterpart duplexe 12-mer (**III**) and 20-mer (**IV**) with natural building blocks as well as 20-mer (**V**) partially-deuterated according to the "NMR-window I" concept have been published elsewhere[7,8]. Since the core parts of 20-mers (**II**) and (**IV**) have the same sequence as dodecamer (**I**) and (**III**), the numbering of residues in the dodecamers (**I**) and (**III**) are the same as the corresponding numbering of 12 core residues in the 20mer (**II**) and (**IV**).

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deutero isotopomeric mixture of **B** + **C** type building blocks (Fig. 1) (see the NMR part); (iii) For NMR experiments, the presence of H3' proton in 2'(S) or/and (R) deuterio-2'-deoxynucleosides causes a J-coupling with H2' creating serious complications from the passive $^3J_{2'3'}$ coupling in the down off-diagonal part of the spectrum (see the NMR part for more detailed discussions), and this would make the extraction of $^3J_{2'3'}$ coupling constant very inaccurate as in the nondeuterated natural counterpart. This we successfully circumvent by using C3'-deuterated **B** + **C** type building blocks (Fig. 1). (iv) Furthermore, the presence of H3' in 2'(S) or/and (R) deuterio-2'-deoxynucleosides, in contrast to our **B** + **C** type building blocks, as in Fig. 1, would have considerable spin-diffusion through the additional H2'(2")-H3' pathway as well as will cause line-broadening effects particularly in NOESY-type experiments and also partially in DQF-COSY-type experiments decreasing the accuracy of distance and dihedral angle information.

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