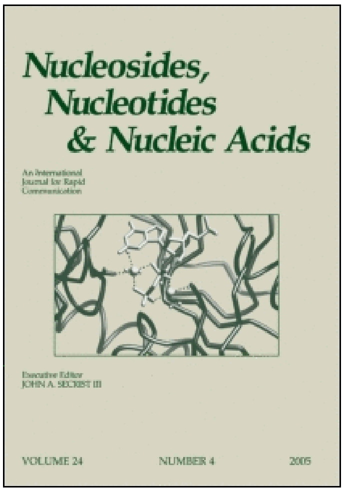

Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

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A. Földesi[a]; S. I. Yamakage[a]; F. P. R. Nilson[a]; T. V. Maltseva[a]; C. Glemarec[a]; J. Chattopadhyaya[a]
[a] Department of Bioorganic Chemistry, Box 581 Biomedical Center, Uppsala University, Uppsala, Sweden

To cite this Article Földesi, A. , Yamakage, S. I. , Nilson, F. P. R. , Maltseva, T. V. , Glemarec, C. and Chattopadhyaya, J.(1997) 'The Application of Uppsala "Nmr-Window" Concept for Conformational Analysis of Biologically Functional Dnas and RNAs by High-Field NMR', Nucleosides, Nucleotides and Nucleic Acids, 16: 5, 517 — 522

To link to this Article: DOI: 10.1080/07328319708002911

URL: http://dx.doi.org/10.1080/07328319708002911

NUCLEOSIDES & NUCLEOTIDES, 16(5&6), 517-522 (1997)

THE APPLICATION OF UPPSALA "NMR-WINDOW" CONCEPT FOR CONFORMATIONAL ANALYSIS OF BIOLOGICALLY FUNCTIONAL DNAS AND RNAS BY HIGH-FIELD NMR

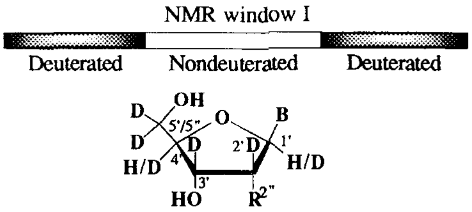
A. Földesi, S.-I. Yamakage, F.P.R. Nilson, T.V. Maltseva,
C. Glemarec and J. Chattopadhyaya*

Department of Bioorganic Chemistry, Box 581, Biomedical Center,
Uppsala University, S-751 23 Uppsala, Sweden

Abstract: The present state-of-the-art of the Uppsala "NMR-window" concept (see refs 1-4, 7-8 & 10-11) developed for the high-field NMR conformational studies on the solution structure of large, biologically functional DNAs and RNAs is summarised. The use of specifically deuterated nucleotide building blocks allows the extraction of the vital J coupling and nOe information to give the NMR-constrained structure refinements. In particular, the improved preparation of C5-deuterio uridine and cytidine blocks (*i.e.* hexadeuterated block) is described for their use in the "NMR-window" concept.

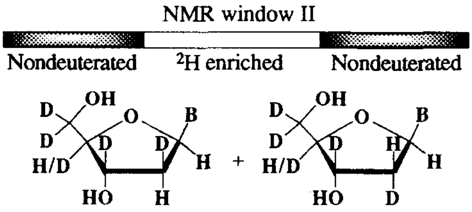
One of the most serious problem encountered in the NMR structure determination of large biologically functional DNA or RNA molecules is the overlapping NMR resonances. In order to overcome this, we have developed non-uniform deuterium labelling technique[1,2] (Uppsala "NMR-window"), which simplifies the NMR spectra and allows the determination of both J couplings and NOE volumes in an unambiguous manner from a small domain of a large molecule (The "NMR-window" concept, see Figs. 1-3)[1-4,7-8,10-11].

(A) **The "NMR-window I" concept**[1,2] (Fig. 1) allowed the assignment of the resonances for the nondeuterated sugar protons and allowed the determination of the $^{3}J_{HH}$ coupling constants using the building blocks of type **A** or **B** (Fig. 1) (>97 atom % ^{2}H at C2', 2", C3' and C5', ~85 atom % ^{2}H at C4' and ~20 atom % ^{2}H at C1') in short oligo-DNAs and RNAs[1,2]. Further comparative studies[3,4] on the self-complementary DNA dodecamer $[d(C^{5}G^{6}C^{7}G^{8}A^{9}A^{10}T^{11}T^{12}C^{13}G^{14}C^{15}G^{16})]_2$ and a 20mer $[d(C^{1*}G^{2*}C^{3*}G^{4*}C^{5}G^{6}C^{7}G^{8}A^{9}A^{10}T^{11}T^{12}C^{13}G^{14}C^{15}G^{16}C^{17*}G^{18*}C^{19*}G^{20*})]_2$ (N* indicates deuterated blocks type A) DNA duplexes showed[3] that the reduced resonance overlap in NOESY type spectra indeed helps in the full assignment of the chemical shifts as well as in the determination of the NOE volumes in an accurate manner. It has also been found[4] that a proton vicinal to a deuteron has a ~2-3 fold increase of T_1 and ~20 fold increase of



Building block of type **A**: R = D
Building block of type **B**: R = OH

Fig. 1

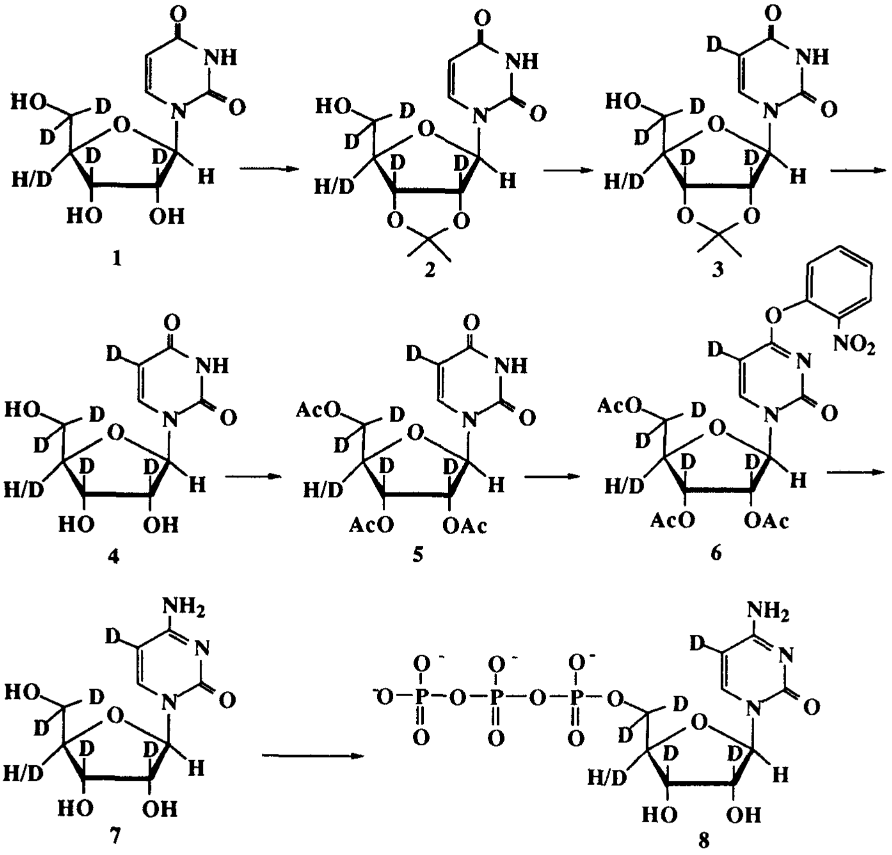


Isotopomer **C** (~85%) + Isotopomer **D** (~15%)

Fig. 2

T_2. This increase in T_2 of the residual protons in partially-deuterated residues of the specifically labelled oligomers allowed to filter away protons with shorter T_2 using a number of different NMR experiments (ROESY, MINSY, HAL)[4]. It has also emerged from these studies[3,4] that the C5 position of pyrimidine residues should be deuterated in order to remove the strong H5-H6 cross-peaks in both DQF-COSY and NOESY type experiments and interresidual [aromatic $H5_{(i+1)}$→aromatic $H\text{-}8/H6_{(i)}$] as well as [aromatic $H5_{(i)}$→$(H2'/H2'')_{(i-1)}$] cross-peaks in NOESY type experiments. This has been accomplished by subjecting the d_5-uridine derivative (**2**) to NaOD catalysed exchange reaction[5] in a mixture of DMF:D_2O (3:1, v/v) (Scheme 1) at 75 °C to give deuterium incorporation >95 atom% as evidenced from the comparison of integral values belonging to the residual H5 and nondeuterated H6 and H-1' signals in the ^{1}H-NMR spectrum. The d_6-uridine (**4**) obtained was subsequently transformed[6] to d_6-cytidine (**5**) through the displacement of an O^4-(2-nitrophenyl) leaving group of the fully protected d_6-uridine derivative (**6**) with liquid ammonia in dry THF getting both C5 deuterated nucleosides from a common precursor.

(B) Structural studies of large biologically functional DNAs by NMR: A new NMR-window concept has been developed[7] (Fig. 2) in which the partially deuterium labelled isotopomeric mixture of blocks of type **C** and **D** (Fig. 2) (~15 atom % ^{2}H at C2'(R̲), ~85 atom % ^{2}H at C2'(S̲), 65 atom % ^{2}H at C4' (#), >97 atom % ^{2}H at C3' and C5') provides the structural information. High field ^{1}H-NMR studies on the non-uniformly deuterium labelled self-complementary DNA dodecamer $[d(C^5G^6C^{7*}G^8A^{9*}A^{10}T^{11}T^{12*}C^{13}G^{14*}C^{15}G^{16})]_2$ and a 20mer $[d(C^1G^2C^3G^4C^{5*}G^{6*}C^{7*}G^{8*}A^{9*}A^{10*}T^{11*}T^{12*}C^{13*}G^{14*}C^{15*}G^{16*}C^{17}G^{18}C^{19}G^{20})]_2$ sequence (N* indicates deuterated blocks type **C** + **D** in Fig, 2) showed that (i) the partial deuteration allows extraction of the $^3J_{H1',H2'}$ and $^3J_{H1',H2''}$ coupling constants from DQF-COSY experiments, (ii) the T_2 relaxation for the H2" of partially-deuterated residues has



Scheme 1

increased by ~1.5 to 2 fold compared to the nondeuterated ones, (iii) almost all important nOe volumes can also be obtained in a HAL-NOESY experiment, which effectively filters away signals having ${}^3J_{H1',H2'}$ couplings *i.e.* the ones belonging to nondeuterated nucleotides, (iv) the spin-diffusion in NOESY experiments has been reduced and (v) the HAL-NOESY experiment gives the H1'-H4', H4'-H2" nOe volumes qualitatively which are useful markers of the sugar conformations.

(C) Structural studies on large, biologically functional RNAs by NMR: Our NMR studies[8] on a chemically synthesized biologically important 21mer RNA hairpin[9] and its deuterium labelled analogue, constructed using "NMR-window I" concept as in Fig. 1 using block type **B**, (shown in Fig 3A, "outline font" denotes deuterated blocks) with additional C5 deuteration at pyrimidines in the stem region showed a significant spectral simplification for the loop region allowing an unambiguous NMR assignment

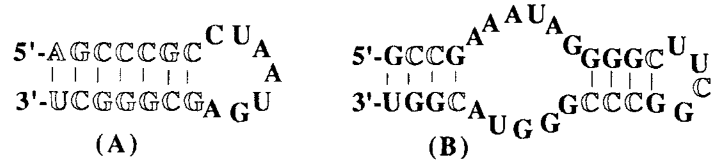


Fig. 3

the non-exchangeable aromatic and sugar protons. By the use of the non-uniformly deuterium labelled 21mer, it was possible to obtain an average 19.9 intra- and internucleotidyl NOE constraints[10] for the seven loop nucleotides together with the dihedral angle constraints for their sugar residues. In a variation of our "NMR-window I" concept[11], cytidine-d_6 5'-triphosphate block (**8**) has been prepared[12] and used in T7 RNA polymerase transcription reaction with good efficiency in order to incorporate specific deuterium labelling for the simplification of the NMR spectra of a biologically functional 31mer RNA subunit of *E. coli* RNase P RNA[13] (Fig. 3B, "outline font" denotes these deuterated C residues). This incorporation of specifically deuterium labelled nucleosides (>95 atom % ^{2}H at C5, >97 atom % ^{2}H at C2', C3' and C5', 65 atom % ^{2}H at C4') in the deuterated analogue of 31mer RNA has increased the number of resonances assigned from 134 (in the fully natural 31mer RNA) to 175 out of total possible 235 resonances giving an average of 22.8 distance constraints/nucleotide in the stem regions resulting in a more accurate NMR structure[11].

Experimental section

Materials and Methods. Deuterium labelled 2',3',4'#,5',5"-$^2H_5$-β-D-uridine (**1**) was prepared by our published procedure[1,2]. 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. DMF was stirred with P_2O_5 overnight followed by distillation under nitrogen. The distilled DMF was further dried sequentially with and stored over molecular sieves (3 Å). DEAE-Sephadex A-25 was purchased from Pharmacia. ^{1}H- and ^{31}P-NMR spectra were recorded on a Jeol FX 90 Q NMR maschine at 90 and 36 MHz, respectively, using TMS (0.0 ppm) or acetonitrile (for D_2O solutions, set at 2.0 ppm) as internal standards for ^{1}H or 85 % phosphoric acid (0.0 ppm) as external standard for ^{31}P spectra. Chemical shifts are reported in ppm (δ scale).

2',3'-O-Isopropylidene-2',3',4'#,5',5"-$^2H_5$-uridine (2). To a stirred suspension of 2',3',4'#,5',5"-$^2H_5$-uridine (1.25 g, 5 mmol) in dry acetone (10 ml), toluenesulfonic acid (95 mg, 0.5 mmol) was added followed by dropwise addition of triethyl ortoformate (1.6 ml, 10 mmol) over a period of 15 min ar RT. After 1.5 h stirring, sodium bicarbonate was added. After an additional 15 min, the reaction mixture was filtered, evaporated and the residue was crystallysed from methanol/diethylether to give compound **2** (1.35 g, 93 %). ^{1}H-NMR (D_2O): 7.71 (d, $J_{H6,H5}$ = 8.1 Hz, 1H) H-6; 5.80 (s, 1H) H-1'; 5.79 (d, 1H) H-5; 4.29 (s, 0.35H) H-4'#; 1.53 & 1.33 (2xs, 6H) methyls of isopropylidene.

2',3'-O-Isopropylidene-2',3',4'#,5',5",5-$^2H_6$-uridine (3). Compound **2** (1.35 g, 4.7 mmol), lyophilised from D_2O (5 ml), was dissolved in a mixture of dry DMF (9 ml) and 10% NaOD in D_2O (3 ml) and heated at 75 °C for 25 h. Then the reaction mixture was allowed to cool to RT and it was neutralised upon addition of Dowex 50 WX (H^+ form), filtered and washed with ethanol. The solvent was removed and the residue crystallised as for compound **2** to afford **3** (1.32 g, 98 %). ^{1}H-NMR (D_2O): 7.61 (s, 1H) H-6; 5.75 (s, 1H) H-1'; 4.29 (s, 0.35H) H-4'#; 1.58 & 1.36 (2xs, 6H) methyls of isopropylidene.

2',3',4'#,5',5",5-$^2H_6$-Uridine (4). Treatment of compound **3** (1.32 g, 4.55 mmol) with 80% aq. acetic acid at RT for 3 days followed by evaporation and a treatment with methanolic ammonia for 1.5 days at RT yielded compound **4** (1.14 g, quantitative). ^{1}H-NMR (D_2O): 7.19 (s, 1H) H-6; 5.21 (s, 1H) H-1'; 3.42 (s, 0.35H) H-4'#.

2',3',5'-O-Acetyl-2',3',4'#,5',5",5-$^2H_6$-uridine (5). Compound **4** (280 mg, 1.1 mmol), was acetylated upon treatment with acetic anhydride (0.95 ml, 10 mmol) in dry pyridine at RT. Sodium bicarbonate work up afforded compound **5** (380 mg, 91 %). ^{1}H-NMR ($CDCl_3$): 8.76 (br. s., 1H) NH; 7.39 (s, 1H) H-6; 6.03 (s, 1H) H-1'; 4.35 (s, 0.35H) H-4'#; 2.15, 2.14 & 2.11 (3xs, 9H) methyls of acetyls.

2',3',5'-O-Acetyl-O^4-(2-nitrophenyl)-2',3',4'#,5',5",5-$^2H_6$-uridine (6). To nucleoside **5** (365 mg, 0.97 mmol) in dry dichloromethane (10 ml) dry triethylamine (1.36 ml), mesitylenesulfonyl chloride (0.64 g) and dimethylaminopyridine (30 mg) were added and the mixture was stirred for 50 min. 2-Nitrophenol (633 mg) and DABCO (22 mg) were added and stirring was maintained for 2 h. Normal work up and column chromatography gave compound **6** (400 mg, 83 %). ^{1}H-NMR ($CDCl_3$): 8.2 - 7.2 (m, 4H) 2-nitrophenyl; 7.92 (s, 1H) H-6; 6.09 (s, 1H) H-1'; 4.40 (s, 0.35H) H-4'#; 2.17 & 2.10 (2xs, 9H) methyls of acetyls.

2',3',4'#,5',5",5-$^2H_6$-Cytidine (7). Compound **6** (400 mg, 0.8 mmol) was dissolved in dry THF (~15 ml) and liquid ammonia (~20 ml) was added. The reaction mixture was sealed in a steel bomb for 4 days. After evaporation of volatile matters, further treatment with aq. ammonia for 5 h and work up afforded compound **7** (180 mg, 90%). ^{1}H-NMR (D_2O): 7.89 (s, 1H) H-6; 5.94 (s, 1H) H-1'; 4.16 (s, 0.35H) H-4'#.

2',3',4'#,5',5",5-$^2H_6$-Cytidine 5'-triphosphate (8). To a suspension of compound **7** (50 mg, ~0.2 mmol) in dry trimethyl phosphate (1 ml), phosphorus oxychloride (41 µl) was added at 0 °C followed by stirring for 3 h. To 0.5 M tri-n-butylammonium pyrophosphate in dry DMF (2.4 ml) , tri-n-butylamine (0.25 ml) was added at 0 °C and the resulting solution was transferred into the reaction mixture. After 1 min stirring, 1M TEAB (~20 ml) was added and stirring was maintained for an additional 3 h in ice bath then the reaction mixture was kept in a refrigerator overnight. After evaporation of the volatile matters separation on a DEAE-Sephadex A-25 ion exchange column (HCO_3^- form) with a linear gradient of 0→0.25 M, 0.25→0.5 M TEAB (500 ml each) afforded CTP derivative **7** (1206 A_{260} o.d. units, 81%) which was passed through a Dowex 50 WX (Na^+ form) column to form the sodium salt. ^{1}H-NMR (D_2O): 7.94 (s, 1H) H-6; 5.95 (s, 1H) H-1'; 4.22 (d, $J_{H4',P\alpha}$=2.93 Hz, 0.35H) H-4'#. ^{31}P-NMR (D_2O): -10.19 (d, $J_{P\alpha,P\beta}$= 19.5 Hz, P_α); -11.28 (d, $J_{P\gamma,P\beta}$= 19.5 Hz, P_γ); -22.98 (t, P_β).

Acknowledgements

Authors thank Swedish Board for Technical Development (NUTEK), Swedish Natural Science Research Council (NFR) and Swedish Research Council for Engineering

Sciences (TFR) for generous financial support. Authors also thank Wallenbergstiftelsen, University of Uppsala and Forskningsrådsnämnden (FRN) for financial supports for purchasing the 500 and 600 MHz NMR spectrometers.

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