Pergamon

Tetrahedron 55 (1999) 6603–6622

TETRAHEDRON

The First Example of Sequence-specific Non-uniformly $^{13}C_5$ labelled RNA: Synthesis of the 29mer HIV-1 TAR RNA with ^{13}C Relaxation Window#

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Received 8 February 1999; revised 8 March 1999; accepted 25 March 1999

Abstract: A complete synthetic protocol as well as ^1H- and ^{13}C-NMR data on the monomer building blocks used for the solid-phase synthesis of specifically ^{13}C-labelled (99 atom % ^{13}C) stem (^{27}A and ^{43}G), bulge (^{24}C) and loop (^{31}U) regions of 29mer HIV-1 TAR RNA hairpin starting from the $^{13}C_6$-D-glucose are presented. The complex NMR spectra of ^{13}C-labelled monomer building blocks, due to the interaction of various ^{13}C and ^1H spins, have been assigned. It has been demonstrated by heteronuclear 2D NMR that the non-uniform labelling of the HIV-1 TAR 29mer RNA achieved herein by chemical synthesis provides an optimal opportunity to perform full T_1 and T_2 relaxation measurements (the "NMR Relaxation Window") of each type of sugar-carbons for all four strategically placed ^{13}C-labelled residues in a unique and unprecedented manner because of minimal overlap of ^{13}C resonances compared to uniformly labelled oligo-RNA. © 1999 Elsevier Science Ltd. All rights reserved.

Introduction

One of the major problems of the structure elucidation of functional oligo-RNA by NMR is the severe overlap of the sugar proton resonances,[1] most of which normally appear within a very small stretch of ~1.0 ppm or less centered at ~4.5 ppm. In order to tackle this, Danyluk *et al* introduced the deuteration approach[2a-f] (~90 atom % ^2H incorporation) as early as 1972 by preparing perdeuterated building blocks[2] from RNA digest of blue-green algae grown in D_2O for assignment purposes, which turned out to be inadequate for any high resolution NMR studies because of residual proton signals. In 1986, we introduced stereospecific chemical methodologies to introduce deuterium on the sugar carbons (>97 atom % ^2H incorporation) to suppress specific NMR resonances in nucleosides.[3] Subsequently, we have developed the Uppsala "NMR-window" concept,[4] in which partially-deuterated sugar residues are non-uniformly incorporated into either oligo-DNA[4a-c,e,i] or -RNA[4f] by the solid-phase synthesis protocol or enzymatic means[4g] for simplification of the spectral crowding[4a-c,e-h,j] and coupling patterns,[4e-h,j] increasing NOE intensities,[4c-e,j] probing dynamics by selective T_1 and T_2 measurements,[4k-m] reducing the spin diffusion[4e,j] as well as the line-broadening[4d] associated with ^1H dipolar relaxation. Non-uniform ^2H labelling (with or without ^{13}C enriched blocks)[4l,m] at specific sites in an oligonucleotide molecule ("NMR-window") can be easily achieved today in a desired manner using chemospecifically synthesised phosphoramidite or H-phosphonate derivatives of deuterated nucleoside blocks by solid-phase chemistry.

#*Dedicated to Professor Andrzej B. Legocki on the occasion of his 60th birthday*

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PII: S0040-4020(99)00294-X

The importance of specific non-uniformly deuterium labelled oligo-DNA and -RNA at the sugar has been subsequently recognized in other laboratories: Stereoselectively 2'(R)-deuterated 2'-deoxynucleoside blocks[5] have been used for extraction of the $^{3}J_{H1',H2'}$ and $^{3}J_{H1',H2''}$ coupling constants from COSY-type experiments.[6] Incorporation of isotopomeric 5'(R/S) mixture of ^{2}H-labelled nucleosides[7] facilitated the $^{3}J_{HH}$ determination and unambiguous NOE assignment of the diastereotopic H5'/5" methylene resonances in oligo-DNAs,[8] whereas 5'-$^{2}H/^{13}C$ double-labelled nucleoside incorporated oligo-DNA gave information regarding vicinal ^{1}H-^{31}P coupling constants.[9] Methods for stereoselective 5'-labelling with deuterium have also been developed for 2'-deoxynucleosides.[10] Deuteration of C5/C6 of pyrimidines or 5-methyl of thymine and C8 of purine nucleobases removed unessential crosspeaks in the NOESY spectra of oligo-DNA[11a,b,d] or -RNA.[11c] Sequence specific incorporation of C1'-deuterated nucleosides into an RNA duplex[12] decreased the spectral overcrowding of the aromatic→H1' region in the NOESY spectra. 3',4',5',5"-$^{2}H_4$-Labelled nucleosides[4n,13] were uniformly incorporated into RNA and the effects of this site-specific deuteration on the spectral crowding and relaxation behaviour were studied.[13]

The importance of incorporation of non-uniformly ^{13}C-labelled 2'-deoxyribonucleosides[14] into stretches of oligo-DNA has been well demonstrated for NMR studies in solution: The ^{13}C-labels have been mainly incorporated at C1'/5', but methodologies are also available to introduce the ^{13}C-labels at other sugar carbons.[15] Uniformly ^{13}C-labelled blocks have been selectively incorporated into oligo-DNA to establish the connectivities of nucleobases to their own sugar residues[16] or to extract the $^{3}J_{HH}$ coupling information,[17] whereas site-specific ^{15}N labelling[18] has been used to investigate the structural properties of oligonucleotides. Uniformly or sequence specifically $^{13}C/^{15}N$-labelled DNA oligomers[19] have been prepared by solid-phase chemical synthesis. Uniformly $^{15}N/^{13}C$-labelled oligo-RNA[23] and -DNA[21] molecules have been also prepared by enzymatic synthesis (using NTPs[20] and T7 RNA polymerase[22] or dNTPs[21] and Klenow DNA polymerase), whereas nonuniformly $^{15}N/^{13}C$-labelled oligo-RNA[24] and -DNA[25] molecules have been prepared by ligation of labelled and non-labelled stretches.

The T7 polymerase reaction[22] gives access to uniformly $^{15}N/^{13}C$-labelled RNA,[27,28] which, depending upon the molecular size, leads to spectral overcrowding specially for a large biologically functional oligomers. On the other hand, the non-uniform labelling of RNA by nucleoside type[4g,28] using T7 polymerase reaction[22] chronically suffers from the lack of vital sequential connectivity data. By a combination of the enzymatic transcription and ligation it was possible to synthesise partially ^{13}C-labelled RNA[24] and DNA[25] oligomer, but the low overall yield of this technique calls for substantial improvement. On the contrary, automated solid support synthesis[26] enabled sequence specific incorporation of ^{2}H-[4f,11d,12] or ^{15}N-labelled[18] nucleosides into the oligomer chain, resulting in appreciable resolution and sensitivity enhancement without the loss of connectivity information.

However, this flexibility of the chemical synthesis, to the best of our knowledge, has not yet been exploited for non-uniform ^{13}C-labelling of oligo-RNA. Clearly, development of protocol for such non-uniform ^{13}C-labelling of large biologically important oligo-RNA by solid-phase chemical synthesis will aid in the elucidation of structure, dynamics, and interactions of RNAs such as ribozymes, ribosomal RNAs, transfer RNAs, and genetic control elements. This paper describes the preparation of the ^{13}C-labelled building blocks, their NMR characterization as well as their application in the synthesis of a non-uniformly ^{13}C-labelled 29mer RNA constituting the trans activator regulatory (TAR) element of HIV-1 with bulged hairpin structure, which plays critical role in the regulation of the viral transcription.[27] The 29mer HIV-1 TAR RNA bulged hairpin has been

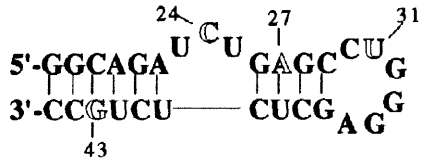
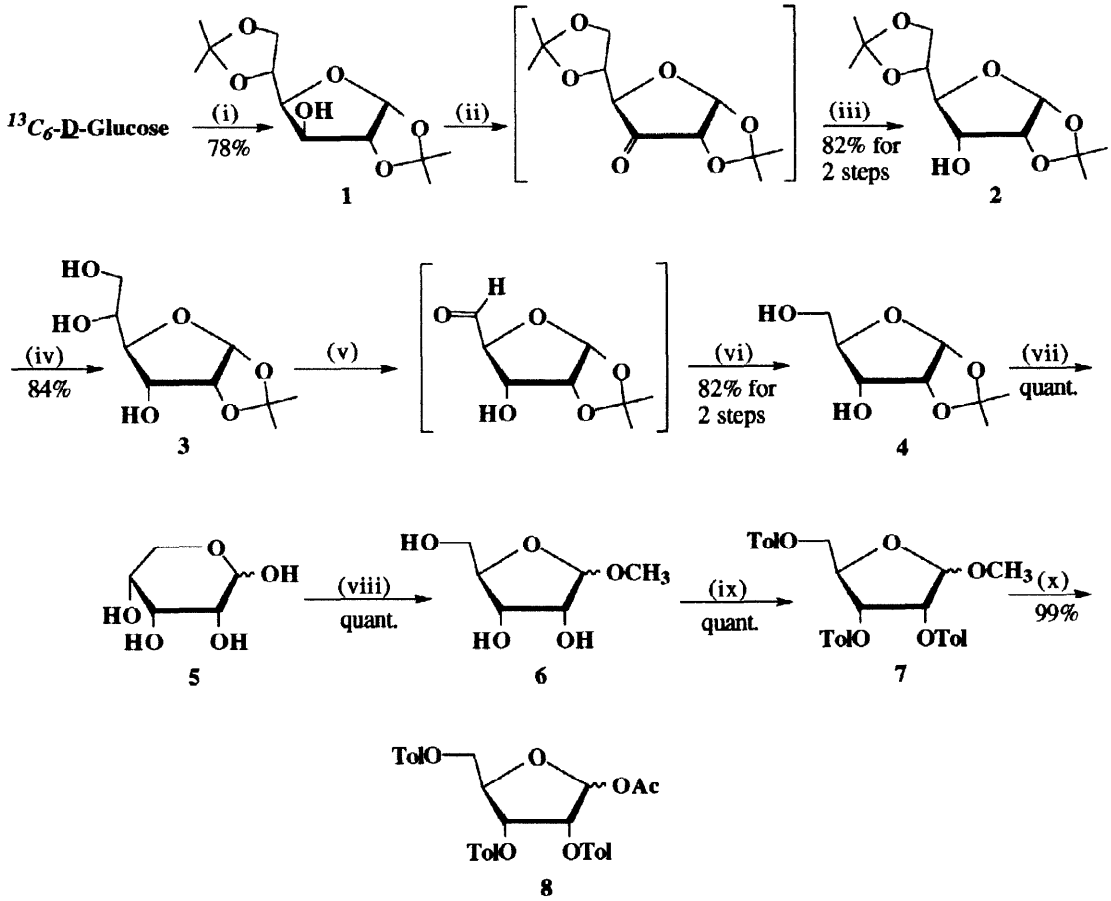


Figure 1. Sequence and secondary structure of the 29mer HIV-1 TAR RNA (open letters and numbering indicate $^{13}C_5$-ribofuranose labelled nucleotides)

labelled with four 1',2',3',4',5'-$^{13}C_5$ ribonucleoside units specifically incorporated into the stem (^{27}A and ^{43}G), bulge (^{24}C) as well as in the loop (^{31}U) (Fig. 1) regions as an illustration of the solid-phase approach compared to the enzymatic non-uniform labelling by residue-type.[28] The structure of this hairpin has been earlier investigated through uniform[28,29] as well as by the residue-type[28] $^{13}C/^{15}N$ labelling, which we deemed will make comparison easier for the evaluation of our ^{13}C relaxation window.

Results and Discussion

One of the major problems in the chemical synthesis of 99 atom% ^{13}C enriched sugar or nucleoside derivatives is that it is relatively *difficult to monitor the progress of a reaction or purity of a product* during the

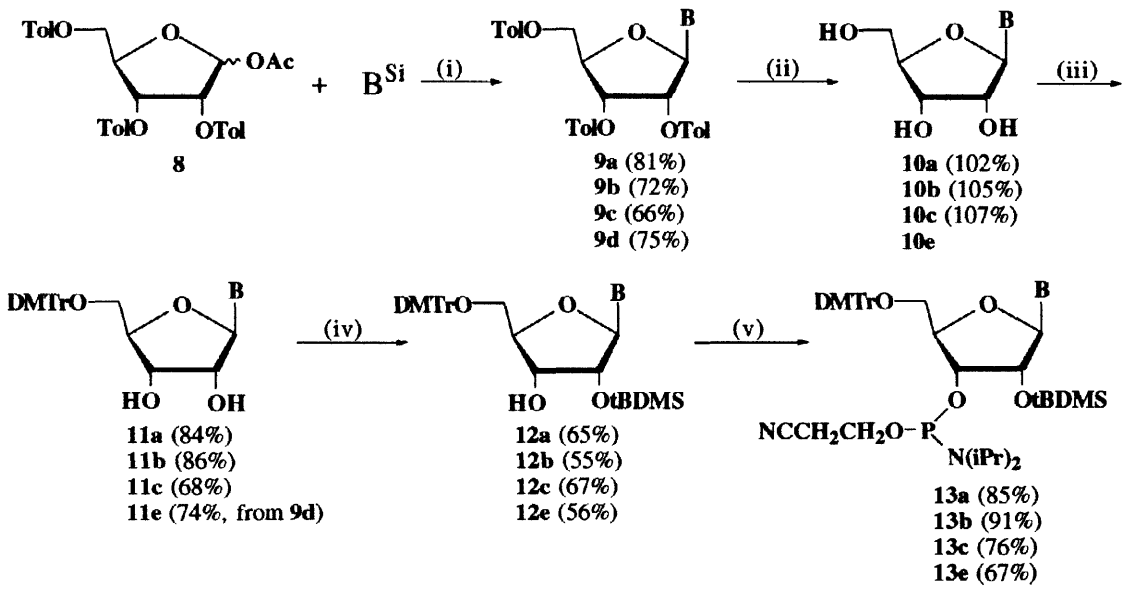


Scheme 1. Synthesis of 1-*O*-acetyl-2,3,5-tri-*O*-toluoyl-α,β-D-ribofuranose-$^{13}C_5$ (**8**). Reaction conditions: i. acetone, $ZnCl_2$, H_3PO_4; ii. PDC, acetic anhydride, methylene chloride; iii. $NaBH_4$, ethanol; iv. 80% acetic acid, r. t.; v. $NaIO_4$, ethanol-water, r. t.; vi. $NaBH_4$, ethanol, r. t.; vii. 60% acetic acid, 80 °C; viii. methanol, H_2SO_4, 4 °C; ix. toluoyl chloride, pyridine; x. acetic acid, acetic anhydride, H_2SO_4, methylene chloride, 0 °C.

synthesis of the monomeric block in a standard NMR spectrometer of a synthetic lab because of the complex unresolved spectrum originating from the one- and two-bond ^{1}H-^{13}C couplings. Of course, the easiest way to circumvent this problem is broadband ^{13}C decoupling (*i.e.* X-decoupling). Similarly, monitoring the product purity by standard 1D ^{13}C-NMR becomes a complex issue because of ^{13}C-^{13}C as well as ^{1}H-^{13}C couplings, which can only be circumvented by selective ^{13}C decoupling or in an HSQC experiment, which can uniquely assign the resonances, but the resolution of the experiment can not guarantee the purity of the product. Hence, a detailed spectral characterization of all four 99 atom % ^{13}C enriched phosphoramidite blocks including their intermediates starting from commercially available $^{13}C_6$-D-glucose[30] has been performed including the synthesis and isolation of non-uniformly ^{13}C-labelled 29mer HIV-1 TAR RNA as well its NMR characterization.

$^{13}C_6$-D-Glucose (99 atom% ^{13}C) was converted into 1,2:5,6-di-*O*-isopropylidene-α-D-glucoforanose **1**[30a,b,31,32c] (78%). Compound **1** was transformed[32] into *allo*-diastereomer **2**[30a,32c] (in 82% yield) by oxidation/reduction reaction sequence. Selective hydrolysis of 5,6-*O*-isopropylidene group[4i] gave **3**[30a,32b] (84%), which was oxidized[33] and then reduced to D-ribose derivative **4**[30a,b,32b] (81%) with $NaBH_4$. Acidic hydrolysis[4i] of the remaining isopropylidene group gave D-ribose **5**,[47,48] which was converted into its α/β-methyl furanosides **6**[4a] in a quantitative manner. The mixture of α and β-methyl furanosides **6** was converted to 1-*O*-acetyl-2,3,5-tri-*O*-(4-toluoyl)-α/β-D-ribofuranose (**8**)[4a] (~3:10 α/β mixture in 42% yield from $^{13}C_6$-D-glucose).

Condensation of **8** (Scheme 2) under Vorbrüggen's condition[34] gave protected nucleosides **9a-c**[4a] in 66-81% yields. Protected guanosine **9d**[4a] was prepared by condensation of N^2-acetyl-O^6-diphenylcarbamoyl-guanine[35] and protected sugar **8**, which gave small amount of N^7 isomer. In the case of synthesis of protected uridine **9c**, the temperature of condensation plays a crucial role. In temperatures above 45 °C, considerable amount of N^1,N^3-diribosyl uracil was formed (up to 50% at 70 °C), which is an undesired side product, con-



Scheme 2. Synthesis of ribonucleoside-$^{13}C_5$ phosphoramidites. B^{Si}: silylated nucleobase B: a. N^6-benzolyladenin-9-yl; b. N^4-benzoylcytidin-1-yl; c. uracil-1-yl; d. N^2-acetyl-O^6-diphenylcarbamoylguanin-9-yl; e. N^2-isobutyrylguanin-9-yl. Reaction conditions: i. $TfOSi(CH_3)_3$, 1,2-dichloroethane or toluene, 45-80 °C; ii. 2N NaOH-ethanol-pyridine 6 min. (a-c), or methanolic ammonia 2 days (d), r. t., then isobutyric anhydride, TMS-Cl, pyridine (e); iii. DMTr-Cl, pyridine; iv. $AgNO_3$, tBDMS-Cl, THF-pyridine; v. 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite, DIPEA, THF, r. t.

suming valuable labelled ribose derivative, and lowering the yield of desired labelled uridine.

Deprotection of the *O*-acyl groups from **9a-c** was achieved with aqueous 2*N* NaOH-ethanol-pyridine.[4a] In case of guanosine **9d**, complete deprotection (with methanolic ammonia hydrolysis) and re-protection (transient protection technique[36]) by isobutyryl group at N^2-site was necessary, since separation of the 2'-*O*- and 3'-*O*-*t*BDMS regioisomers did not work in our hands with N^2-acetyl-O^6-diphenylcarbamoyl group on guanosine. In all cases deacylated nucleosides **10a-c,e**[4b,30a,50] were not purified, but subjected to dimethoxytritylation as crude mixtures, giving high yields of 5'-*O*-dimethoxytrityl derivatives **11a-c,e**.[30a,52] Introducing *t*-butyldimethylsilyl as 2'-OH blocking group[26] presents the problem of maximising the yield of the desired 2'-*O*-*t*BDMS isomer **12a-c,e**.[30a,37,42] To achieve this, we applied the silver nitrate procedure[37] for silylation, which

Table IA. ^{1}H-NMR chemical shifts[a] (δ) of different ^{13}C-labelled sugar derivatives (in $CDCl_3$)

Comp.	H1	H2	H3	H4	H5	H5'	H6	H6'
1	6.24; 5.63 *5.93 (5.94)*	4.79; 4.24 *4.51 (4.35)*	4.59; 4.07 *4.33 (4.33)*	4.32; 3.81 *4.07 (4.07)*	4.60; 4.08 *4.34 (4.36)*	-	4.41; 3.91 *4.16 (4.17)*	4.24; 3.75 *3.99 (3.98)*
2	6.11; 5.50 *5.80 (5.82)*	4.88; 4.31 *4.60 (4.62)*	4.31; 3.82 *4.07 (4.06)*	4.06; 3.58 *3.82 (3.82)*	4.55; 4.06 *4.31 (4.31)*	-	4.31; 3.82 *4.07 (4.04)*	4.31; 3.82 *4.07 (4.04)*
3	6.07; 5.46 *5.75 (5.81)*	4.88; 4.32 *4.60 (4.64)*	4.33; 3.89 *4.10(4.14)*	4.12; 3.62 *3.87 (3.88)*	4.32; 3.76 *4.04 (4.04)*	-	4.12; 3.62 *3.87 (3.85)*	4.03; 3.53 *3.78 (3.78)*
4	6.07; 5.44 *5.76 (5.71)*	4.88; 4.33 *4.61 (4.65)*	4.16; 3.68 *3.92 (4.05)*	4.12; 3.64 *3.88 (3.88)*	4.06; 3.54 *3.80 (3.83)*	3.81; 3.32 *3.57 (3.52)*	-	-
5	*	*	*	*	*	*	-	-
6 α [β]	5.25; 4.61 *4.95 (4.94)* [5.15; 4.51 *4.83 (4.84)*]	**	**	**	**	**	-	-
7	5.90; 5.40 *5.65 (5.65)*	5.37; 4.84 *5.11 (5.13)*	6.09; 5.59 *5.84 (5.84)*	4.96; 4.47 *4.72 (4.72)*	4.96; 4.47 *4.72 (4.71)*	4.74; 4.25 *4.50 (4.49)*	-	-
8	6.71; 6.11 *6.41 (6.41)*	6.01; 5.48 *5.75;(5.76)*	6.11; 5.62 *5.87 (5.87)*	5.01; 4.50 *4.76 (4.77)*	4.98; 4.49 *4.74 (4.73)*	4.73; 4.24 *4.49 (4.48)*	-	-

*Due to ^{1}H - ^{13}C coupling and anomeric/pyranose/furanose equilibrium proton signals appear as diffuse multiplets
**Mixture of anomers - severe signals overlap
[a] observed values, *average, (^{12}C value)*

gives improved ratio of the 2'-isomer. If considerable amount of 2',3'-bis-*O*-*t*BDMS product was formed (in case of G and A), it was desilylated with TBAF to the starting material and the silylation reaction was repeated. If there were only traces of bis-silylated isomer present, the undesired 3'-*O*-*t*BDMS isomer, after separation, was isomerised[38,39] in methanol - triethylamine or 10% aqueous pyridine to the mixture of 2'-*O*-*t*BDMS and 3'-*O*-*t*BDMS derivatives, which were separated by column chromatography. It has been found that the 5'-*O*-DMTr

Table IB. One bond ^{1}H - ^{13}C coupling constants (in Hz, obtained from 1D ^{1}H-NMR spectra, Δδ = 1 Hz) for the various ^{13}C-labelled sugars

Comp.	H1	H2	H3	H4	H5	H5'	H6	H6'
1	186	164	154	oc	154	-	149	oc
2	182	oc	oc	143	148	-	oc	oc
3	186	164	oc	oc	oc	-	oc	oc
4	185	163	oc	oc	143	143	-	-
6	174	oc	oc	oc	oc	oc	-	-
7	162	oc	oc	oc	oc	148	-	-
8b	182	162	149	148	146	147	-	-

[oc]peak overlapping makes estimation of coupling constants impossible

group of guanosine derivative **11e** is considerably more labile than any other 5'-*O*-DMTr protected nucleosides **11a-c**, hence we had to be specially careful in handling this. It should be noted that if 5'-*O*-DMTr is inadvertently lost before silylationreaction, *t*BDMS group is introduced both into 5'-*O*- and into 2'-*O*- (or 3'-*O*-) positions. The resulted derivatives co-migrate with their 5'-*O*-DMTr counterpart and are practically impossible to separate by column chromatography. One should have in mind that in automated oligonucletide synthesis such 5'-*O*-silyated units would give rise to "capped" sequences, interrupting chain growth at those sites. As even 1% drop in coupling efficiency has a considerably deteriorating effect on the oligoribonucleotide synthesis, even such small contamination would have profound effect on the overall yield of the precious ^{13}C-labelled oligo-RNA. For this reason, all chromatographic operations with 5'-*O*-dimethoxytrilylated guanosine derivatives were performed with solvents containing small amounts of pyridine (0.1 - 0.3%). Pure 5'-*O*-DMTr-2'-*O*-*t*BDMS nucleosides **12a-c,e** were phosphitylated by isomerisation-free procedure[40,41] with 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite to yield the desired building blocks **13a-c,e**[49] for oligonucleotide synthesis. Yields of phosphitylation were in the range of 80% for A, C and U derivatives **12a-c**, or close to 70% for G derivative **12e**. In the latter case, the side product (^{31}P-NMR signal around 14 ppm) was contaminating the guanosine phosphoramidite. The side product could not be removed with the rest of the impurities during routine silica gel chromatography; only the second reverse-phase chromatography (see experimental section) was necessary to remove this.

During the synthesis, the identity and purity of the intermediates were ascertained by ^1H-NMR, which was complex owing to ~99 atom % ^{13}C isotope enrichment. The ^1H-^{13}C spin-spin couplings and the line broadening in the NMR spectra of the ^{13}C-labelled derivatives made the analysis of spectra quite cumbersome and very ambiguous. The identity of the intermediates however can be accurately assessed by calculating middle-points of the J-coupled multiplicities of the sugar protons and comparing them with the spectra of the non-labelled counter-

Table IIA. ^1H-NMR chemical shifts (δ) of different ^{13}C-labelled nucleoside derivatives (in $CDCl_3$)

Comp.	Si-*t*Bu	$SiCH_3$	H1'	H2'	H3'	H4'	H5'	H5''
9a[a]	-	-	6.82; 6.19 *6.55; (6.51)*	6.67; 6.09 *6.37; (6.33)*	6.51; 5.93 *6.22; (6.23)*	5.18; 4.62 *4.83; (4.81)*	5.11; 4.55 *4.90; (4.90)*	4.95; 4.40 *4.68; (4.67)*
9b[a]	-	-	6.83; 6.19 *6.51; (6.52)*	6.08; 5.51 *5.84; (5.80)*	6.19; 5.59 *5.89; (5.87)*	5.04; 4.48 *4.76; (4.75)*	5.11; 4.56 *4.84; (4.85)*	4.95; 4.41 *4.68; (4.67)*
9c[a]	-	-	6.59; 5.98 *6.30; (6.35)*	6,07; 5,50 *5.66; (5.72)*	5.96; 5.35 *5.89; (5.85)*	4.91; 4.34 *4.62; (4.67)*	5.02; 4.46 *4.74; (4.72)*	4.85; 4.26 *4.55; (4.62)*
9d[a]	-	-	6.64; 6.03 *6.34; (6.35)*	6.51; 5.92 *6.30; (6.23)*	6.51; 5.92 *6.30; (6.22)*	5.08; 4.54 *4.86; (4.83)*	5.08; 4.54 *4.86; (4.88)*	4.94; 4.42 *4.68; (4.70)*
11a[a]	-	-	6.39; 5.77 *6.08; (6.07)*	5.19; 4.67 *4.93; (4.90)*	4.77; 4.20 *4.48; (4.47)*	4.67; 4.13 *4.40; (4.41)*	3.75; 3.18 *3.46; (3.46)*	3.60; 3.05 *3.33; (3.31)*
11b[a]	-	-	6.21; 5.56 *5.89; (5.90)*	4.70; 4.15 *4.42; (4.42)*	4.70; 4.15 *4.42; (4.42)*	4,70; 4.15 *4.42; (4.42)*	3,69; 3.16 *3.42; (3.44)*	3.69; 3.16 *3.42; (3.44)*
11c[a]	-	-	6.20; 5.56 *5.88; (5.95)*	4.70; 4.14 *4.42; (4.45)*	4.62; 4.06 *4.34; (4.35)*	4.46; 3.91 *4.19; (4.18)*	3.76; 3.24 *3.50; (3.50)*	3.76; 3.24 *3.50;(3.50)*
11e[a]	-	-	6.16; 5.53 *5.84; (5.81)*	5.53; 4.97 *5.28; (5.30)*	4.84; 4.28 *4.56; (4.57)*	4.57; 4.01 *4.29; (4.27)*	3.70; 3.19 *3.44; (3.49)*	3.41; 2.83 *3.12; (3.09)*
12a[b]	0.85	-0.001; -0.147 *6.11; (6.11)*	6.42; 5.80 *5.02; (5.03)*	5.30; 4.75 *4.37; (4.37)*	4.65; 4.09 *4.28; (4.29)*	4.57; 4.01 *3.55; (3.55)*	3.84; 3.26 *3.42; (3.40)*	3.62; 3.16
12b[b]	0.96	0.32; 0.22 *5.95; (5.95)*	6.27; 5.62 *4.32; (4.32)*	4.61; 4.03 *4.40; (4.39)*	4.68; 4.12 *4.12; (4.11)*	4.38; 3.87 *3.61; (3.61)*	3.91; 3.30 *3.51; (3.54)*	3.77; 3.25
12c[b]	0.93	0.18; 0.16 *5.95; (5.96)*	6.27; 5.63 *4.35; (4.36)*	4.63; 4.07 *4.35; (4.35)*	4.63; 4.07 *4.07; (4.11)*	4.38; 3.76 *3.50; (3.53)*	3.77; 3.24 *3.50; (3.48)*	3.77; 3.24
12e[b]	0.83	0.018 -0.190 *5.72; (5.72)*	6.03; 5.41 *5.30; (5.33)*	5.56; 5.03 *4.37; (4.34)*	4.68; 4.05 *4.22; (4.23)*	4.49; 3.94 *3.55; (3.60)*	3.80; 3.30 *3.00; (3.03)*	3.26; 2.74

[a] obs. values, *average, (^{12}C. value)* [b] obs. values, *average, (lit. value)*[42]

Table IIB. One bond ^{1}H - ^{13}C coupling constants (in Hz) for different ^{13}C-labelled nucleosides (values of accuracy of 1Hz are calculated from 1 D ^{1}H-NMR spectra with peaks clearly separated).

Comp.	H1'	H2'	H3'	H4'	H5'	H5"
9a	166	157	158	151	153	148
9b	172	156	162	149	150	148
9c	167	oc	*163*	oc	151	154
9d	166	*158*	*158*	*148*	*148*	147
11a	167	oc	154	146	oc	oc
11b	173	*147*	*147*	*147*	*144*	*144*
11c	172	150	151	148	*145*	*145*
11e	171	151	152	151	oc	155
12a	166	149	151	150	oc	*144*
12b	176	156	149	oc	oc	oc
12c	173	*151*	*151*	oc	*146*	*146*
12e	165	144	151	150	oc	142

[oc]peak overlapping makes estimation of coupling constants impossible
italic mean values for two or more peaks are very close, exact figure difficult to estimate

parts[42] (Tables I & II), but the purities could not be ascertained simply by looking at the sugar-proton resonances. The purity however can be estimated by looking at the resonances of the aromatic protons of the aglycone or the resonances arising from the protecting groups. In contrast, the ^{13}C-NMR spectra exhibited not only dramatically high sensitivity, but revealed also ^{13}C-^{13}C coupling patterns (Table III-V), which are not observable for the compounds with natural ^{13}C abundance. The Tables IA & IIA contain chemical shift data, which correspond closely to the values for the non-labelled compounds, whereas Tables III-V show ^{13}C-^{13}C coupling constants, non-observable without ^{13}C isotope enrichment. In the case of ^{1}H spectra line broadening is so severe that interpretation of the spectra becomes difficult. Equilibrium mixture of D-ribose **5** anomeric/py-

Table III. ^{13}C NMR chemical shifts (δ), multiplicities and one bond ^{13}C-^{13}C coupling constants (Hz) of various ^{13}C-labelled sugar derivatives (in $CDCl_3$)

Comp.	C1	C2	C3	C4	C5	C6
1	105.3, *d*, (33.2)	85.1, *dd*, (33.2, 43.8)	75.1, *dd*, (43.8, 37.5)	81.2, *dd*, (37.5, 48.4)	73.4, *dd*, (34.3, 48.4)	67.6, *d*, (34.3)
2	104.0, *d*, (32.2)	80.4 – 78.5 (*m*)	75.7, *dd*, (33.7, 43.8)	80.4 – 78.5 (*m*)	72.5, *dd*, (39.3, 39.3)	66.4, *d*, (33.8)
3	104.4, *d*, (34.8)	80.8 – 79.7 (*m*)	70.7, *dd*, (40.3, 40.5)	80.8 – 79.7 (*m*)	71.7, *dd*, (43.4, 41.3)	62.8, *d*, (41.3)
4	104.4, *d*, (34.6)	81.1 – 79.4 (*m*)	70.7 *dd*, (40.3, 40.4)	81.1 – 79.4 (*m*)	60.4, *d*, (42.9)	-
5*	101.1, *d*, (46.2) Fβ 96.4, *d*, (40.7) Fα 93.9, *d*, (46.2)# Pβ 93.6, *d* (43.6) Pα	75.2, *dd*, Fβ 71.2, *dd*, Pβ 70.3 Pα	70.8 Fβ 70.3 Fα 69.1, *t*, Pβ 69.3 Pα	82.6, *t*, Fβ 83.2, *t*, Fα 67.3, *t*, Pβ 67.5, *t* Pα	62.6, *d*, Fβ 61.5, *d*, Fα 63.1, *d*, Pβ 63.1, *d* Pα	
6 β, [α] anomer	108.7, *d*, (47.1), [102.8, *d*, (40.8)]	75.4, *dd*, (36.1, 47.1)	71.1, *dd*, (36.1, 39.8)	84.5, *dd*, (39.8, 39.8)	64.7, *d*, (39.8) [62.7, *d*, (43.9)]	
7 β, [α] anomer	106.5, *d*, (47.0), [102.0, *d*, (43.2)]	75.4, *dd*, (38.8; 47.1)	72.3, *dd*, (38.7, 40.1)	79.2, *dd*, (39.8,43.8)	64.7, *d* (43.8)	-
8 β, [α] anomer	98.5, *d*, (44.4), [94.4, *d*, (44.3)]	74.8, *dd*, (44.6, 38.5)	71.2, *dd*, (38.5, 40.8)	80.1, *dd*, (40.8, 41.8)	63.6, *d*, (41.8) [63.8, *d*, (40.2)]	-

*only anomeric carbon data for the four isomers could be extracted from the overlapping signals
# predominant isomer
F = furanose; P = pyranose

Table IV. ^{13}C NMR chemical shifts (δ) and ^{13}C-^{13}C coupling constants (Hz) of various ^{13}C-labelled nucleoside derivatives

Nucleoside derivatives	C1'	C2'	C3'	C4'	C5'
9a	86.6, *d*, (43.6)	73.6, *dd*, (37.5, 43.6)	71.2 dd, (37.5, 38.9)	80.9 dd, (38.9, 43.1)	63.2, d, (43.1)
9b	89.0, d, (42.7)	74.7, dd, (42.7, 40.2)	71.0, dd, (40.2, 39.5)	80.9, dd, (39.5, 43.1)	63.5, d, (43.1)
9c	87.6, d, (43.7)	73.6, dd, (43.7, 40.5)	71.1, dd, (40.5, 39.5)	80.8, dd, (39.5, 43.1)	63.6, d, (43.1)
9d	87.0, d, (43.6)	74.1, dd, (43.6, 39.5)	71.4, dd, (39.5, 37.1)	80.9, dd, (37.1, 43.7)	63.6, d, (43.7)
11a	90.4, d, (41.7)	75.6,dd, (41.6, 37.6)	72.2, dd, (37.6, 37.2)	85.8, dd, (37.1, 43.1)	63.5, d, (43.2)
11b	93.6, d, (40.0)	77.1, dd, (40.0, 37.4)	71.8, dd, (37.4, 39.9)	86.0, dd, (39.9, 42.6)	62.9, d, (42.6)
11c	90.7, d, (41.1)	75.6, dd, (41.1, 39.4)	69.9, dd, (39.4, 37.9)	84.0, dd, (37.9, 43.1)	62.0, d, (43.1)
11e	90.1, d, (43.5)	72.6, dd, (43.5, 39.6)	71.3, dd, (39.6, 38.1)	85.2, dd, (38.0, 43.6)	63.7, d, (43.7)
12a	88.5, d, (43.1)	75.8, dd, (43.1, 37.4)	71.6, dd, (37.4, 36.9)	84.3, dd, (36.9, 43.1)	63.3, d, (43.1)
12b	90.8, d, (42.0)	76.7, dd, (42.0, 37.9)	69.2, dd, (37.9, 39.7)	83.3, dd, (39.7, 43.3)	61.5, d, (43.3)
12c	88.8, d, (43.1)	76.4, dd, (43.1, 36.9)	70.5, dd, (36.9, 41.0)	83.6, dd, (41.0, 43.1)	62.4, d, (43.1)
12e	90.5, d, (43.6)	74.2, dd, (43.6, 39.4)	72.3, dd, (39.4, 39.9)	86.1, dd, (39.9, 43.6)	63.2, d, (43.6)

ranose/furanose forms gives a set of signals which is impossible to be assigned without 2D techniques.

The coupling between neighbouring carbon atoms in the synthesised molecules opens the possibility of gathering wealth of information on the magnetic resonance phenomena, non-observable in normal abundance molecules. The values extracted from monomer units can be the starting point in analysis of oligomers. Measurements of these effects are under way and will be published elsewhere.

The phosphoramidites **13a-c,e** were used for the synthesis of the 29mer HIV-1 TAR RNA sequence (Fig. 1). During the synthesis we have attempted to recover amidites, which are lost according to routine synthetic protocol. In the course of the routine automated synthesis, large excess (approx. 10 eq.) of amidites are used to ensure maximum yield of the coupling reaction and are consequently directed to waste. The cost of the lost amidites, although considerable, is accepted in the synthesis of non modified or non labelled oligomers. The ^{13}C-labelled units are incomparably more expensive than regular ones and efforts to recover or recycle at least part of them are well justified. This problem had already drawn some attention[43,44] and there are reports on procedures of recovery of amidites. The amidite solution leaving the reaction column contains large excess of activating agent, usually tetrazole, and is prone to reaction with nucleophiles, such as hydroxyl compounds. Even traces of water convert the activated amidite to H-phosphonate diester. In the Beaucage's procedure,[43] the activated amidite solution was quenched with excess of diisopropylamine under rigorously anhydrous conditions. This allowed to convert activated amidite back to its stable form. Although it was successful in the case of deoxyribonucleoside amidites, which were recovered with the yield of ca. 55%, in the case of ribonucleotides

Table V. Carbon and phosphorus chemical shifts (δ) and $^{13}C - ^{13}C$ coupling constants (Hz) of ^{13}C-labelled nucleoside phosphoramidites **13a-c,e** (in $CDCl_3$ – DABCO)

Phosphor-amidites	C1'	C2'	C3'	C4'	C5'	31P
13a	88.6, d, (42.0) 88.4, d, (43.1)	75.8 - 71.7 (m)	75.8 - 71.7; (m)	84.9 - 83.4 (m)	63.3, d, (43.1) 63.2, d, (42.6)	151.7, 149.8, 149.68
13b	91.0, d, (41.0) 90.7, d (39.5)	76.6 - 74.9 (m)	72.4 - 69.4; (m)	82.6 - 81.1 (m)	61.9, d, (43.1) 61.3, d, (44.1)	150.8, 150.6, 149.9, 149.8
13c	89.0, d, (40.0) 88.4, d, (44.1)	76.5 - 74.7 (m)	72.8 - 71.2; (m)	83.4 - 82.2 (m)	62.6, d, (42.6) 62.1, d, (42.6)	150.8 , 150.3, 150.1
13e	88.8, d, (44.1) 86.6, d, (43.8)	77.2 - 76.0 (m)	74.1 - 71.5; (m)	85.1 - 83.9 (m)	63.4, d, (42.6) 63.2, d, (42.8)	151.5, 149:5, 149.4

this technique did not promise much success due to greater steric hindrance caused by bulky 2'-*O*-*t*BDMS group, and technical difficulties in collection of the amidites under anhydrous conditions.

The other approach utilises the hydrolysis product *i.e.* H-phosphonate diester. It can be further hydrolysed back to the nucleoside, which subsequently can be phosphitylated to the amidite. This route, although indirect, is more feasible in the case of 2'-*O*-tBDMS ribonucleosides. Brill[44] applied fluoride ion for hydrolysis of P-O bond. This reagent, applied in conjunction with the use of 2'-*O*-acid labile protection, can not be used when *O*-alkylsilyl blocking groups are present, as it would cause desilylation of nucleoside. We found that after removal of tetrazole by extraction with water, the remaining H-phosphonate diester can be hydrolysed to the nucleoside with excess of DIPEA. Very little amount of phosphonate monoester is formed under these conditions.

The 29mer RNA was synthesized on an eight port ABI 392 DNA/RNA synthesizer using standard 1 μmol scale RNA program from ABI (see experimental part for the details). Base-protecting groups were removed in methanolic ammonia[45] at 55 °C for 17 h and 2'-*O*-silyl deprotection was achieved with triethylamine trihydrofluoride.[46] The crude RNA was precipitated with ethanol and purified by PAGE. Pure oligoribonucleotide was extensively dialysed against sterilized water using Centricon-3 concentrators,[20a] precipitated from 70% ethanol and sodium exchanged using Dowex 50WX (Na^+ form). The electrophoretogram of the crude and purified RNA is shown in Fig. 2.

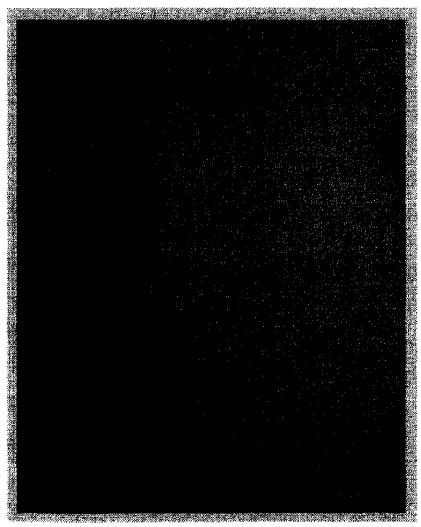


Figure 2. The crude (lane *a*) and PAGE purified (lane *b*) 29mer RNA analyzed by denaturing (20% polyacrylamide, 7 M urea) gel electrophoresis. Lane *c*: xylene cyanol and bromophenol blue tracking dyes.

NMR results

The structural integrity of 29mer RNA as well as the location of four ^{13}C-labelled nucleotide moieties were proven by detailed 2D-NMR studies, which also showed an optimal dispersion of sugar ^{13}C resonances in these four residues, thereby allowing to perform specific ^{13}C relaxation studies (will be reported elsewhere). In the Fig. 3, the expanded regions of Ar-H1' crosspeaks in the DQF-COSY (Panel A) and conventional NOESY (Panel B) spectra of specifically $^{13}C_5$-ribose labelled (*i.e.* ^{24}C, ^{27}A, ^{31}U and ^{43}G residues) 29mer TAR RNA are compared. All 15 crosspeaks of H5-H6 at natural abundance as expected according to the sequence of 29mer TAR RNA (Fig. 4) has been observed (Panel A), consistent with the target sequence shown in Fig. 1. The cytosine and uridine (labelled by capital C and U in Panel A) residues could be easily discriminated by differences in their H5-H6 coupling constants (7.6 and 8.8 Hz, respectively[1]). The same crosspeak could be identified in NOESY spectra as shown in boxes (Panel B). The occurrence of a single set of resonances from the $^{13}C_5$-ribose labelled 29mer TAR RNA proves that it has a specific single and unique structure as earlier published in literature.[28,29]

In the Fig. 4, HSQC-CT spectra of specifically $^{13}C_5$-ribose labelled 29mer TAR RNA are presented. All five regions for ^1H-^{13}C crosspeaks, [$^1H(1')$-$^{13}C(1')$, $^1H(2')$-$^{13}C(2')$, $^1H(3')$-$^{13}C(3')$, $^1H(4')$-$^{13}C(4')$, $^1H(5')$-$^{13}C(5')$], corresponding to four $^{13}C_5$-ribose labelled residues in 29mer TAR RNA are clearly observed. Even at

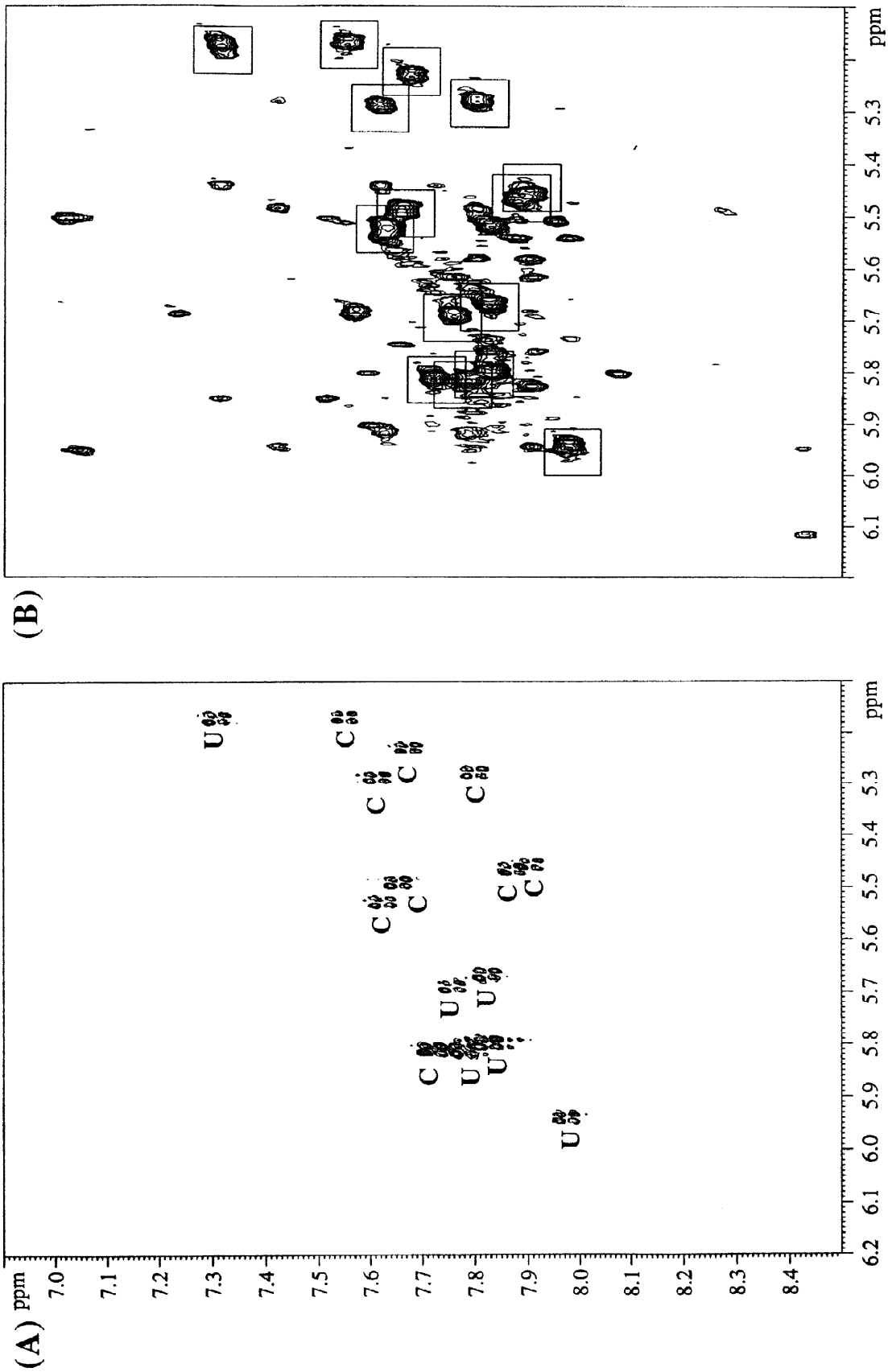


Figure 3. The expanded regions of H5-H6 crosspeaks in the DQF-COSY spectrum (Panel A) and corresponding region in the conventional NOESY (Panel B) spectrum of the selectively ^{13}C-ribose labelled (*i.e.* residues ^{24}C, ^{27}A, ^{31}U and ^{43}G) 29mer TAR RNA are compared. In Panel A, 15 H5-H6 crosspeaks for cytosine (labelled by capital C) and uridine (labelled by capital U) are found as expected, consistent with the TAR RNA sequence shown in Fig. 1. The corresponding crosspeaks in the NOESY spectrum (Panel B) are surrounded in boxes. Note that the chemical shifts of the aromatic pyrimidine protons are quite comparable to those found in the literature for a similar RNA sequence.[28]

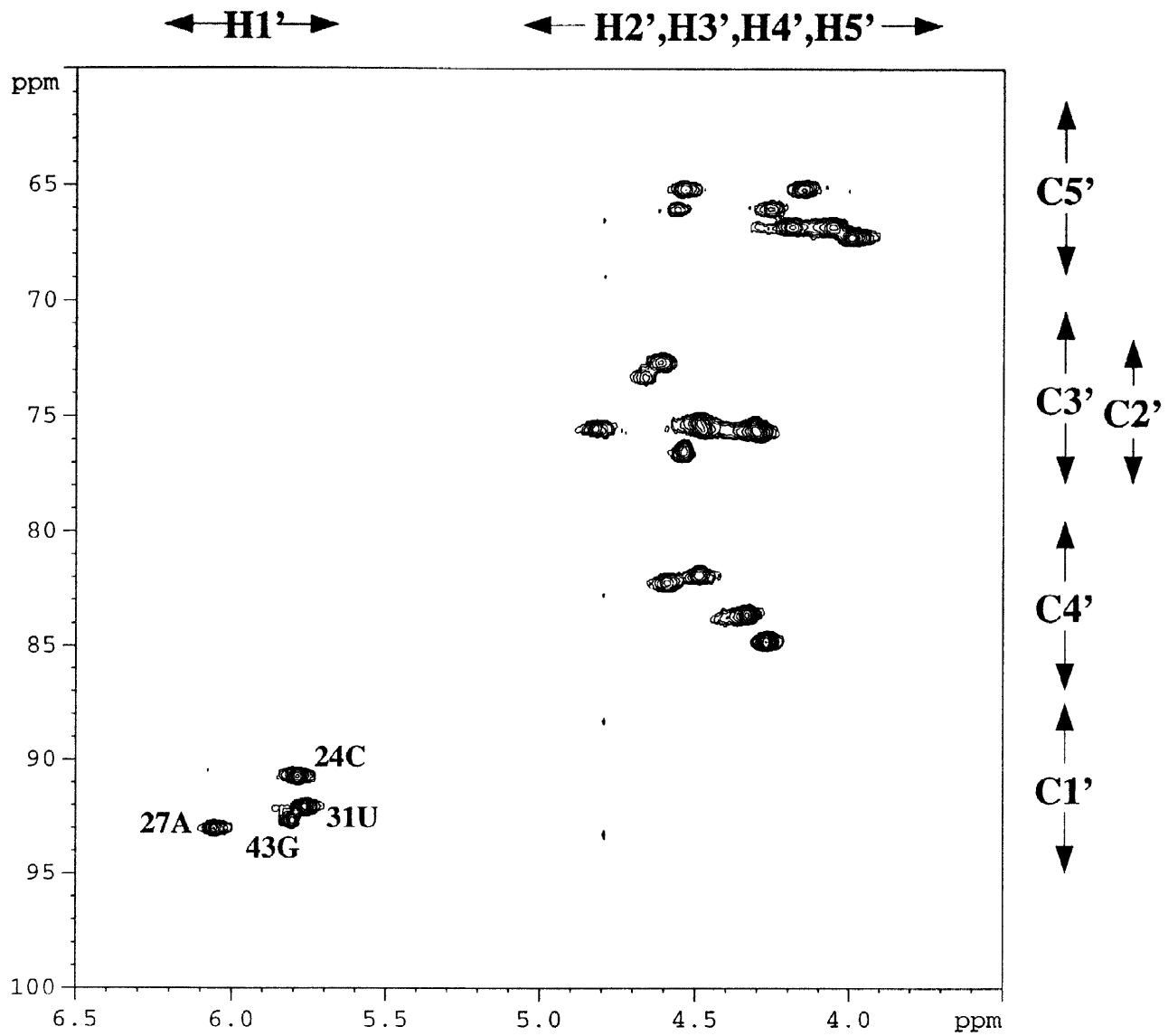


Figure 4. HSQC-CT spectra of specifically $^{13}C_5$-ribose labelled (*i.e.* ^{24}C, ^{27}A, ^{31}U and ^{43}G residues) 29mer TAR RNA are shown. On the right (δ ^{13}C) and top (δ ^{1}H) of the spectra, the chemical shifts of different types of sugar-carbon or -proton regions are defined by arrows. For five carbon-proton regions, four sets of ^{1}H-^{13}C crosspeaks corresponding to four different specifically labelled residues are clearly observed.

concentration of 0.23 mM, the experiment has been performed with 32 number of scans and was accomplished during 5 h, which gave an excellent opportunity to perform the relaxation studies of these selectively labelled residues in RNA, thereby overcoming the problem of overcrowding of chemical shifts in ^{1}H-^{13}C experiments usually encountered routinely with the uniformly ^{13}C labelled RNA (compare our HSQC-CT spectra in Fig. 4 with the HSQC spectra [Fig. 6 in ref. 30e] of uniformly labelled 30mer HIV-2 TAR RNA[30e]). Clearly, non-uniform specific labelling as in our 29mer RNA achieved by chemical synthesis allowed us to perform full T_1 and T_2 relaxation studies (the "NMR relaxation window") of each type of sugar-carbons for all four strategically placed ^{13}C-labelled residues in a unique and unprecedented manner.

In the [^{1}H,^{1}H]-NOESY with X(ω_2)-half-filter experiment presented in Fig. 5 (Panel A), the Ar_i-$H1'_i$-Ar_{i+1} crosspeaks belonging to ^{24}C, ^{27}A, ^{31}U and ^{43}G residues are clearly observed, but the corresponding crosspeaks

for all other non-labelled residues are suppressed. The assignments for H1' and H8 of ^{27}A and ^{43}G residues have been made (Panels A and B) in accordance with the assignment presented in literature[29c] for ^{28}C(H8) and ^{20}A (H2) resonances. The discrimination between two other sets of H1' and H6 chemical shifts of pyrimidine nucleotides has been accomplished basing on the J-coupling of H5-H6 of U and C residues. For ^{43}G and ^{31}U residues, these crosspeaks could be only identified in [^{1}H,^{1}H]-NOESY with X(ω_2)-half-filtering experiment (Panel A) but not in conventional NOESY experiments because of their severe overlap with H5-H6 crosspeaks (Panel B). Thus, information obtained in the X(ω_2)-half-filter [^{1}H,^{1}H]-NOESY experiment and the conventional NOESY experiment are complementary, which allowed us to confirm the presence and location of all four ^{13}C-labelled nucleotide residues in the 29mer TAR RNA in addition to the strategy used for stepwise addition of various phosphoramidite blocks in the solid-phase RNA synthesis.

Experimental

All reagents were from Aldrich and, unless otherwise stated, were used without further purification. The $^{13}C_6$-D-glucose (99% isotopic purity) was from Martek Biosciences Corp (USA). Thin layer chromatography (TLC) was performed on Merck glass backed silica gel plates cat no 5769 or Whatman C18 reverse-phase plates, cat no 4803-600. Solvent systems used were: 2-propanol-conc. ammonia-water 7:1:2, v/v/v (System A), methylene chloride-methanol 19:1 (System B), methylene chloride-methanol 9:1, v/v (System C), hexane-ethyl acetate 7:3, v/v (System D), methylene chloride-ethyl acetate 3:2, v/v (System E), acetone-water 4:1, v/v (System F). Methylene chloride, ethyl acetate, cyclohexane, acetone, methanol and ethanol were distilled prior to use. Toluene and pyridine were refluxed over CaH_2 for 6 h, distilled with exclusion of moisture and stored over molecular sieves (4 Å). THF was refluxed over CaH_2 under nitrogen and distilled immediately before use with rigorous exclusion of moisture.
NMR spectra of the monomeric units were recorded with a Jeol GX 270 spectrometer operating at 270.2 MHz for ^{1}H, 67.9 MHz for ^{13}C and 109.4 MHz for ^{31}P. Chemical shifts are reported in ppm relative to TMS (internal standard) for ^{1}H and ^{13}C and H_3PO_4 (external) for ^{31}P spectra.
The characteristic ^{1}H, ^{13}C and ^{31}P data for compounds **1-13** are collected and shown in Tables I-V.

[1,2,3,4,5,6-$^{13}C_6$]-1,2:5,6-Di-*O*-isopropylidene-α-D-glucofuranose (1). [1,2,3,4,5,6-$^{13}C_6$]-D-Glucose (10 g, 53.7 mmol) was suspended in dry acetone (70 mL), anhydrous $ZnCl_2$ (8 g, 58.7 mmol) was added, followed by 85% H_3PO_4 (0.3 mL). The mixture was stirred at room temperature under nitrogen for 30 h. Unreacted sugar was filtered and thoroughly washed with dry acetone. The filtrate was neutralised with 50% NaOH and evaporated. The resulting oil was partitioned between methylene chloride and water. The aqueous phase was extracted with methylene chloride once more and the combined extracts were dried ($MgSO_4$) and evaporated. Crystallisation of the residue from hexane gave product as colourless needles (mp. 110-111 °C[32c]). Unreacted glucose (1.85 g, 9.9 mmol) was processed in the same way ($ZnCl_2$, 1.6 g, 12 mmol; H_3PO_4, 0.06 mL; acetone 13 mL). The mother liquors from crystallisation from both experiments were evaporated (4.4 g of oil) and treated with 70% acetic acid at 100 °C for 3 h and evaporated. The residue was rendered free of acetic acid by evaporation of water (2x) and toluene (3x). After careful drying in vacuo, 2.64 g (14.2 mmol) of glucose was recovered, which was converted into the title compound ($ZnCl_2$, 2.38 g, 16.7 mmol; H_3PO_4, 0.09 mL; acetone 50 mL).Combined yield of the desired compound **1**[30a,b,31,32c]: 11.15 g (41.9 mmol, 78%). R_f: 0.39 (System B).
[1,2,3,4,5,6-$^{13}C_6$]-1,2:5,6-Di-*O*-isopropylidene-α-D-allofuranose (2). Pyridinium dichromate (15.75 g, 41.92 mmol) was suspended in dry methylene chloride (110 mL), acetic anhydride (12.6 mL, 13.6 g, 125.75 mmol) was added. To this solution, **1** (11.15 g, 41.92 mmol) in dry methylene chloride (40 mL) was added, and the mixture was refluxed under dry condition for 3 h. After cooling, ethyl acetate (300 mL) was added and the resulted slurry was transferred on top of a silica gel column packed in ethyl acetate. The solution was filtered through the column with the aid of pressure and the column was washed with ethyl acetate (550 mL) until the product was eluted completely. The solvent was evaporated and the residue was rendered free of acetic anhydride and pyridine by evaporation of dry toluene (3x). The residue (10.52 g) was dissolved in ethanol (100 mL), cooled in ice, sodium borohydride (0.84 g, 22.21 mmol) was added and the solution was stirred overnight, then poured into water (400 mL) and extracted with methylene chloride (3 x 100 mL). Combined extracts were dried ($MgSO_4$) and evaporated, leaving an oil, which crystallised into solid mass (sufficiently pure for the next step). Combined yield of **2**[30a] from all experiments: 9.18 g (34.5 mmol, 82%). Analytical sample was recrystal-

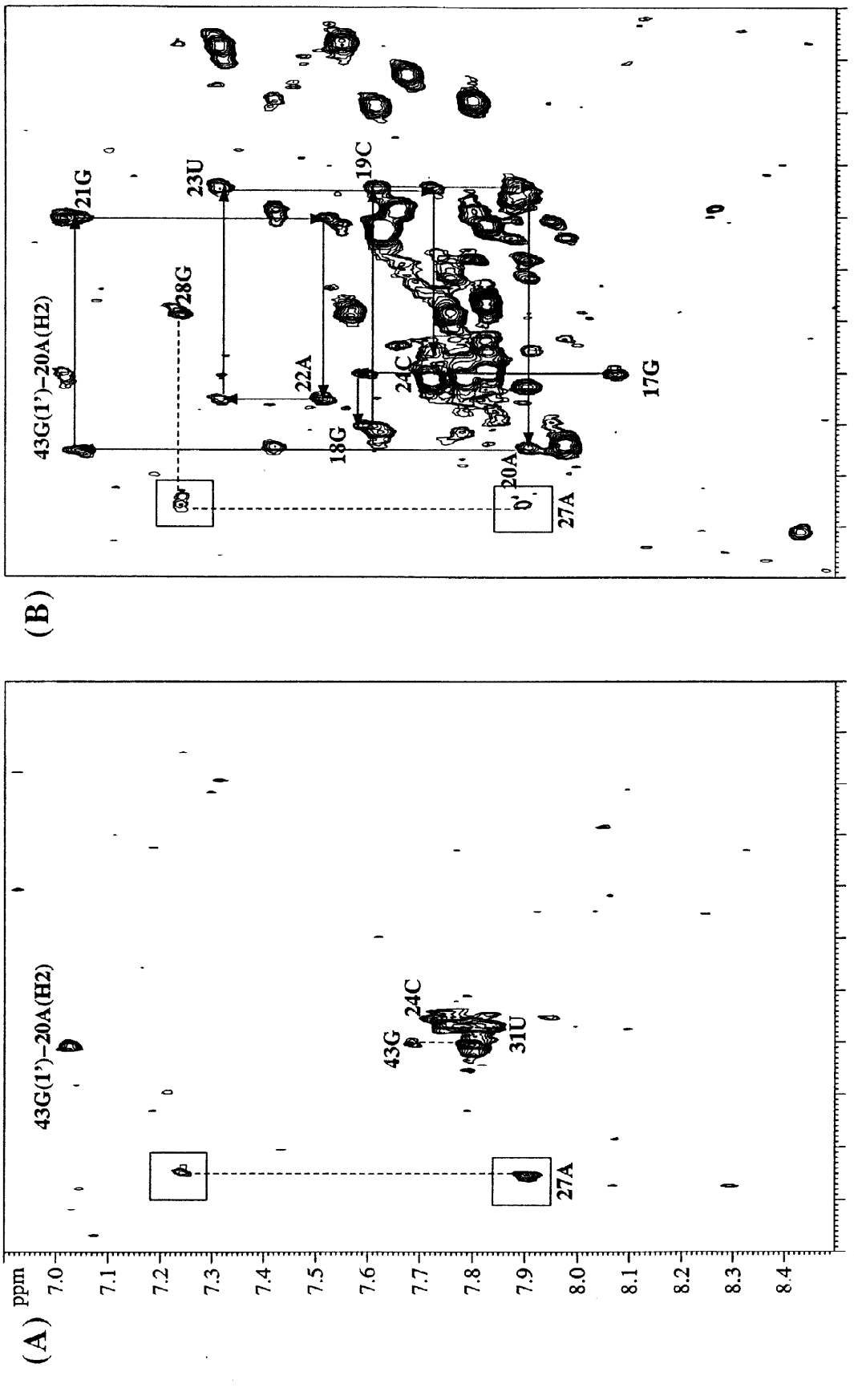


Figure 5. The expanded regions of Ar-H1' of [^{1}H,^{1}H]-NOESY with X(ω_2)-half-filter (Panel A) and the corresponding region in the NOESY spectrum with decoupling of ^{13}C in F1 and F2 dimensions (Panel B) of specifically $^{13}C_5$-ribose labelled (*i.e.* ^{24}C, ^{27}A, ^{31}U and ^{43}G residues) 29mer TAR RNA are compared. In Panel A, the 4 pairs of Ar$_i$-H1'$_i$-Ar$_{i+1}$ crosspeaks are observed as expected for specifically labelled ^{24}C, ^{27}A, ^{31}U, ^{43}G residues. Two corresponding crosspeaks in the NOESY spectrum (Panel B) surrounded by boxes for residue ^{27}A are indicated. In Panel B, the sequential connectivity starting from the 5'-terminal ^{17}G upto ^{24}C is shown by arrows according to the assignments found in the literature.[28]

lised from hexane (mp. 76-78 °C[32c]). R_f: 0.39 (System B).

[1,2,3,4,5,6-$^{13}C_6$]-1,2-*O*-Isopropylidene-α-D-allofuranose (3). Compound **2** (9.18 g, 34.5 mmol) was dissolved in 80% aqueous acetic acid (25 mL) and left at room temperature for 20 h. Solvent was evaporated and the residue crystallised from ethyl acetate, giving the product **3**[30a] as white crystals (mp. 125-127 °C[32b]). Yield 6.5 g (28.7 mmol, 84%). R_f: 0.42 (System C).

[1,2,3,4,5-$^{13}C_5$]-1,2-*O*-Isopropylidene-α-D-ribofuranose (4). Sodium metaperiodate (6.45 g, 30.3 mmol) was dissolved in water (63 mL) and was added to the solution of **3** (6.5 g, 28.7 mmol) in ethanol (63 mL) with stirring. Thick slurry formed and the stirring was maintained for 30 min. The precipitate was filtered off and washed with ethanol. Ethylene glycol (0.1 mL) was added to the combined filtrates and solvents were evaporated. Ethanol was added to the residue, the precipitate filtered off and the procedure repeated, until no precipitate formed. Residue was dissolved in ethanol (200 mL), sodium borohydride (820 mg, 21.7 mmol) was added and solution was stirred overnight. After filtering off the precipitate, the solvent was evaporated, residue dissolved in water and passed through an ion exchange column (Amberlyst A-21, OH^- form) and eluted with water. The alkaline eluate was neutralised with 2N H_2SO_4 and evaporated. To the residue methanol was added and precipitated salts were filtered off. After repeating this procedure, the title compound **4**[30a,b,32b] was obtained as colourless oil (4.6 g, 23.5 mmol, 82%). R_f: 0.26 (System B).

[1,2,3,4,5-$^{13}C_5$]-D-Ribose (5). Compound **4** (4.6 g, 23.5 mmol) was dissolved in 60% aqueous acetic acid and stirred at 80 °C overnight. Solvent was evaporated and the resulting sugar **5**[47,48] was rendered free of water and acetic acid by repeated coevaporation with dry toluene. Yield 3.6 g (yellow syrup, quant.).

[1,2,3,4,5-$^{13}C_5$]-Methyl α/β-D-ribofuranoside (6). Conc. H_2SO_4 (0.3 mL) dissolved in ice-cold methanol (3 mL) was added to a ice-cooled solution of the ribose **5** in dry methanol (100 mL). The reaction mixture was left at 4 °C for 24 h. It was neutralised by passing through an ion exchange column (Amberlyst A-21, OH^- form, pre-washed with methanol) and eluting with methanol (450 mL). Combined eluates were evaporated, leaving product **6**[4a] as yellow syrup. R_f: 0.33 (System C). Yield 3.97 g (quant.).

[1,2,3,4,5-$^{13}C_5$]-1-*O*-Methyl-2,3,5-tri-*O*-(4-toluoyl)-α/β-D-ribofuranoside (7). Methyl riboside **6** (3.97 g, 23.4 mmol) was rendered dry by repeated evaporation of dry pyridine, then it was dissolved in dry pyridine (75 mL), cooled in ice and 4-toluoyl chloride (11.45 ml, 13.38 g, 86.6 mmol) was added with stirring. Mixture was stirred for 30 min at 0 °C, then at room temperature overnight. It was poured in ice-cold solution of sat. $NaHCO_3$ and stirred for 2 h. Viscous oil precipitated and was extracted with methylene chloride. Extracts were dried ($MgSO_4$) and evaporated, leaving a thick syrup of **7**[4a,b] (12.3 g, 23.4 mmol, sufficiently pure for the next step). R_f: 0.52 (System D).

[1,2,3,4,5-$^{13}C_5$]-1-*O*-Acetyl-2,3,5-tri-*O*-(4-toluoyl)-α/β-D-ribofuranoside (8). Compound **7** (12.3 g, 23.4 mmol) was dissolved in dry methylene chloride (90 mL) and cooled in ice, and was added to a ice-cooled solution of acetic anhydride (14 mL, 16.37 g, 151 mmol), acetic acid (11.5 mL, 12.08g, 200 mmol) and conc. H_2SO_4 (0.1 mL). After stirring for 15 min, reaction mixture was poured carefully into cold aq $NaHCO_3$ solution. After neutralisation of acids, the product was extracted with ethyl ether (3 x 100 mL). Extracts were dried ($MgSO_4$) and evaporated. The resulting oil was crystallised from methanol, giving the β-anomer of the product **8**[4a] as colourless crystals (mp.127-128 °C) (6.5 g), R_f: 0.43 (System D). Evaporation of mother liquors gave essentially pure mixture of anomers, which can be used for condensation with silylated nucleobases (5.8 g). Combined yield 12.3 g (22.3 mmol, 42% from D-glucose).

[1',2',3',4',5'-$^{13}C_5$]-2',3',5'-tri-*O*-(4-Toluoyl)-N^6-benzoyladenosine (9a). N^6-Benzoyladenine (1.53 g, 6.41 mmol, 1.42 eq.) was rendered dry by evaporation of anhydrous toluene (2x), then suspended in 1,1,1,3,3,3-hexamethyldisilazane (38 ml), followed by addition of chlorotrimethylsilane (3.8 ml). The mixture was heated with stirring at 120 °C for 2 h under nitrogen. The clear solution was evaporated and then dry toluene was evaporated (3x) to remove traces of 1,1,1,3,3,3-hexamethyldisilazane. Compound **8** (2.5 g, 4.52 mmol, 1 eq.) was dried *in vacuo* for 3 h, then dissolved in dry 1,2-dichloroethane (40 mL) and added to the oily silylated N^6-benzoyladenine followed by addition of trimethylsilyl triflate (1.5 mL). The solution was stirred under nitrogen at 80 °C for 4 h. After cooling, the solution was poured into sat. $NaHCO_3$, methylene chloride (100 mL) was added, the aqueous phase was separated and extracted two more times with methylene chloride. Combined organic phases were washed with water, dried ($MgSO_4$) and evaporated to semisolid mass. Short column chromatography (methylene chloride - methanol 0 to 3%) yielded the title compound **9a**[4a] (2.67 g, 3.68 mmol, 81%). R_f: 0.45 (System B).

[1',2',3',4',5'-$^{13}C_5$]-2',3',5'-tri-*O*-(4-Toluoyl)-N^4-benzoylcytidine (9b). N^4-Benzoylcytosine (1.98 g, 9.23 mmol, 1.46 eq.), was condensed with sugar **8** (3.46 g, 6.28 mmol, 1 eq.), as described for compound **9a**, except for the temperature of the condensation reaction (65 °C). The crude product **9b** was crystallised from ethanol (mp. 197-198 °C). R_f: 0.59 (System B). Yield of **9b**[4a] (3.23 g, 4.57 mmol, 72%).

[1',2',3',4',5'-$^{13}C_5$]-2',3',5'-tri-*O*-(4-Toluoyl)-uridine (9c). Uracil (0.70 g, 6.28 mmol, 1.5 eq), was condensed with sugar **8** (2.29 g, 4.15 mmol), as described for the compound **9a**, except for the temperature (45 °C for 6 h). R_f: 0.33 (System B). Yield of **9c**[4a] (1.65 g, 2.73 mmol, 66%).

[1',2',3',4',5'-$^{13}C_5$]-2',3',5'-tri-*O*-(4-Toluoyl)-N^2-acetyl-O^6-diphenylcarbamoylguanosine (9d). N^2-acetyl-O^6-diphenylcarbamoylguanine (3.57 g, 9.19 mmol, 1.5 eq.) was condensed with sugar **8** (3.36 g, 6.09 mmol) in toluene at 65 °C. R_f: 0.40 (System B). Yield of **9d**[4a] (4.01 g, 4.56 mmol, 75%).

[1',2',3',4',5'-$^{13}C_5$]-N^6-Benzoyladenosine (10a). Compound **9a** (2.64 g, 3.61 mmol) was dissolved in ethanol - pyridine 1:1 (v/v, 22 mL) and the mixture of 2N NaOH and ethanol (15 + 15 mL) was added. After 6 min stirring at room temperature, Dowex 50 (H^+) was added to neutralise the base. The resin was filtered and washed with ethanol (50 mL) and pyridine (50 mL). Combined filtrates were evaporated and dried by evaporation of toluene. The remaining solid was triturated with ethyl ether (50 mL) and filtered. Solid was washed with ethyl ether once again and after drying, the crude product **10a**[4b,30a,b] was obtained (1.39 g, 3.69 mmol, 102%). It was used directly for the next step.

[1',2',3',4',5'-$^{13}C_5$]-N^4-Benzoylcytidine (10b). Compound **9b** (3.23 g, 4.57 mmol) was deprotected as described for **9a**, giving crude product **10b**[4b,30a] (1.70 g, 4.83 mmol, 105%), which was used directly for the next step.

[1',2',3',4',5'-$^{13}C_5$]-Uridine (10c). Compound **9c** (1.65 g, 2.73 mmol) was deprotected as described for **9a**, except that after evaporation of volatile matters, the remaining solid was partitioned between methylene chloride and water (30 +30 mL), the water phase was separated, extracted with methylene chloride (2x) and evaporated to a gum of **10c**[4b,30a] (730 mg, 2.92 mmol, 107 %), which was used directly for the next step.

[1',2',3',4',5'-$^{13}C_5$]-N^2-Isobutyrylguanosine (10e). Compound **9d** (4.01 g, 4.56 mmol) was dissolved in methanolic ammonia (23% w/v, 200 mL) and left at room temperature for 48 h. The flask was opened and left under the hood for several hours to allow ammonia gas to escape and during this time clear solution turned into a thick suspension. The slurry was carefully evaporated (finally at 0.2 torr), the remaining solid was triturated with ethyl ether (50 ml) and filtered. The procedure was repeated 2x, leaving a white powder (1.8 g, 6.16 mmol, 135%), which was found to be homogeneous on TLC, R_f: 0.48 (System A). This was used for the next step without purification. The compound was carefully dried (105 °C, 0.2 torr, 12 h), then dry pyridine (25 mL) was added and the reaction mixture was cooled in ice bath. Trimethylchlorosilane (7 mL, 6.02 g, 55.4 mmol) was added and the resulted suspension was stirred for 3 h. With cooling in ice bath, isobutyric anhydride (1 ml, 0.95 g, 6 mmol) was added and stirring continued overnight (the bath reached room temperature during this time).The flask was cooled in ice again, and water (10 ml) was slowly added. After 15 min. stirring, conc. ammonia (5mL) was added, stirring continued for 20 min and solvents were evaporated. The resulting solid mass was suspended in pyridine (30 mL), filtered and washed with pyridine (2x). Combined filtrates were evaporated to a semi-solid, which was partitioned between water (30 mL) and methylene chloride (40 mL). Water phase was separated and extracted with methylene chloride (2x). Organic extracts were back extracted with water (2x15 mL). Combined water solutions were evaporated, co-evaporated with dry pyridine (4x), leaving the crude isobutyrylated guanosine **10e**[50] (2 g). R_f: 0.23 (System C).

[1',2',3',4',5'-$^{13}C_5$]-5'-*O*-DMTr-N^6-benzoyladenosine (11a). Compound **10a** (1.39 g, 3.69 mmol) was rendered dry by evaporation of dry pyridine (3 x 10 mL), redissolved in pyridine (20 mL) and dimethoxytrityl chloride (1.54 g, 4.54 mmol) was added. The solution was left overnight, pyridine was evaporated, residue freed from pyridine by evaporation of toluene. It was dissolved in methylene chloride (50 mL), washed with sat. $NaHCO_3$, water, and dried ($MgSO_4$). After evaporation of the solvent, the remaining oil was subjected to short column chromatography (methanol in methylene chloride from 0 to 5%) giving the product **11a**[30a] (2.11 g, 3.11 mmol, 84%). R_f: 0.40 (System C).

[1',2',3',4',5'-$^{13}C_5$]-5'-*O*-DMTr-N^4-benzoylcytidine (11b). Compound **10b** (1.7 g of crude, corresponding to 4.57 mmol) was dimethoxytritylated as described for **10a**, giving the title compound **11b**[30a] (2.59 g, 3.96 mmol, 86%) after chromatography (methylene chloride - methanol 0 to 3%). R_f: 0.44 (system C).

[1',2',3',4',5'-$^{13}C_5$]-5'-*O*-DMTr-uridine (11c). Compound **10c** (730 mg, 2.92 mmol) was dimethoxy-tritylated as described for **10a**. After chromatography (methylene chloride - methanol 1 to 5%), the title compound **11c**[30a] was obtained (1.03 g, 1.87 mmol, 68%). R_f: 0.40 (System C).

[1',2',3',4',5'-$^{13}C_5$]-5'-*O*-DMTr-N^2-isobutyrylguanosine (11e). Crude **10e** was dissolved in pyridine (35 mL), dimethoxytrityl chloride (2 g, 5.90 mmol) was added and the solution was left for 24 h. Pyridine was evaporated, the residue was dissolved in methylene chloride and washed first with sat. $NaHCO_3$ followed by water. The organic phase was dried ($MgSO_4$), filtered and evaporated. Chromatography (methylene chloride - methanol from 1 to 5%) gave the title compound **11e**[51] (2.2 g, 3.33 mmol, 74% from **9d**). R_f: 0.46 (System C).

[1',2',3',4',5'-$^{13}C_5$]-5'-*O*-DMTr-2'-*O*-*t*-butyldimethylsilyl-N^6-benzoyladenosine (12a). Compound **11a** (2.05 g, 3.02 mmol) was dissolved in dry THF (23 mL), dry pyridine (0.55 mL) was added, followed by silver nitrate (0.83 g, 4.9 mmol). This mixture was stirred for 15 min, then *t*-butyldimethylchlorosilane (0.79 g. 5.25 mmol) was added. The reaction mixture was stirred in the dark for 5 h, when TLC showed only traces of the starting nucleoside. Solution was filtered through a Celite bed, which was washed with methylene chloride (100 mL) and the filtrates were evaporated. The residue, after dissolving in methylene chloride, was washed with sat $NaHCO_3$, water and dried with $MgSO_4$. Solvent was evaporated and the remaining oil after short column chromatography (cyclohexane-ethyl acetate from 15% to 60%) gave the title compound **12a**[30a,37,42] (1.28 g, 1.61 mmol) along with 3'-*O*-silylated nucleoside (R_f : 0.46 (System E), 0.8 g, 1 mmol) and 2',3'-*O*-bis-silylated nucleoside (R_f: 0.93 (System E), 0.16 g, 0.17 mmol). Side products were desilylated (TBAF/THF, 5 mmol; 20 min. room temp.) and starting material was recovered by chromatography (0.67 g, 0.99 mmol).Repeated synthesis gave additional crop of 2'-*O*-silyl isomer **12a**. R_f: 0.67 (System E). Combined yield of **12a**: (1.56 g, 1.97 mmol, 65%).

[1',2',3',4',5'-$^{13}C_5$]-5'-*O*-DMTr-2'-*O*-*t*-butyldimethylsilyl-N^4-benzoylcytidine (12b). Compound **11b** (2.55 g, 3.89 mmol), THF (30 mL), pyridine (0.7mL), silver nitrate (1.08 g, 6.36 mmol) and *t*-butyldimethylchlorosilane (1.03 g, 6.84 mmol) gave after chromatography (methylene chloride-ethyl acetate from 2 to 18%) the title compound **12b**[30a,37,42] (R_f: 0.64 (System E) 1.67 g, 2.17 mmol, 55%), along with 3'-*O*-silylated (R_f: 0.24 (System E), 0.37 g, 0.48 mmol) and 2',3'-*O*-bis-silylated (R_f: 0.85 (System E) 0.12 g, 0.13 mmol) derivatives.

[1',2',3',4',5'-$^{13}C_5$]-5'-*O*-DMTr-2'-*O*-*t*-butyldimethylsilyl-uridine (12c). Compound **11c** (0.92 g, 1.67 mmol), THF (13 mL), pyridine (0.3 mL), silver nitrate (0.45 g, 2.67 mmol) and *t*-butyldimethylchlorosilane (0.43 g, 2.87 mmol) were reacted as for **11a** and after chromatography (methylene chloride - ethyl acetate from 0 to 20% of the latter), equilibration of the 3'-*O*-silylated isomer (R_f: 0.48 (System E)) and separation, gave the desired compound **12c**[30a,37,42] (R_f: 0.70 (System E), 0.75 g, 1.13 mmol, 67%).

[1',2',3',4',5'-$^{13}C_5$]-5'-*O*-DMTr-2'-*O*-*t*-butyldimethylsilyl-N^2-isobutyrylguanosine (12e). Compound **11e** (2.15 g, 3.25 mmol), THF (25 ml), pyridine (0.6 ml), silver nitrate (0.89 g, 5.27 mmol) and *t*-butyldimethylchlorosilane (0.83 g, 5.53 mmol) were reacted as for **11a** and after chromatography (mixture of chloroform, containing 0.1% pyridine, and ethyl acetate from 5% to 30% of the latter) gave the desired compound **12e** (R_f: 0.45 (System E), 0.7 g, 0.9 mmol), 2',3'-*O*-bis-silylated (R_f: 0.62 (System E) 0.3 g, 0.33 mmol), 3'-*O*-silylated isomer (R_f : 0.30 (System E), 0.43 g, 0.55 mmol) and unreacted **11e** (0.45 g, 0.68 mmol). Bis-silylated isomer was treated with TBAF/THF (3 mmol) and the starting material **11e** was recovered. It was combined with unreacted portion (together 0.8 g, 1.2 mmol) and silylated again. The 3'-*O*-silyl isomer was equilibrated in methanol - 3% triethylamine for 1 h, giving approx. 1:1 mixture of 2'- and 3'-*O*-silyl derivatives. After repeated chromatography and equilibrations, the combined yield of the desired **12e**[42] was: (1.41 g, 1.82 mmol, 56%).

[1',2',3',4',5'-$^{13}C_5$]-5'-*O*-DMTr-2'-*O*-*t*-butyldimethylsilyl-N^6-benzoyladenosine 3'-*O*-(2-cyanoethyl *N*,*N*-diisopropylphosphoramidite) (13a). Protected adenosine **12a** (751 mg, 0.95 mmol) was placed in a 10 ml flask closed with rubber septum with hypodermic needle inserted. Flask with starting material was dried in vacuum desiccator at 0.2 torr for 3 h. Dry argon gas was introduced into the desiccator and dry THF (4.5 mL) was added to the flask through the septum, followed by *N*,*N*-diisopropylethylamine (DIPEA, 0.825 mL). 2-Cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite (0.443 mL, 0.470 g, 1.98 mmol) was added dropwise to the solution. The reaction mixture was stirred for 3 h at room temperature. After that time dry methanol (0.5 mL) was added and reaction mixture was stirred for 15 min. Triethylamine (1 mL) was added and the solution was diluted with ethyl acetate (30 mL) and washed with brine three times. The organic phase was dried ($MgSO_4$) and evaporated to a foam, which was chromatographed on silica gel (cyclohexane- 3% triethylamine, gradient from 30% to 70% of ethyl acetate). Pure nucleoside phosphoramidite **13a**[49] was dissolved in dry benzene-0.5% triethylamine (6 mL/mmol), frozen and freeze-dried, leaving a hard foam (0.806 g, 0.81 mmol, 85%). R_f: 0.38 (RP-18, System F).

[1',2',3',4',5'-$^{13}C_5$]-5'-*O*-DMTr-2'-*O*-*t*-butyldimethylsilyl-N^4-benzoylcytidine 3'-*O*-(2-cyanoethyl *N*,*N*-diisopropylphosphoramidite) (13b). The cytidine phosphoramidite was obtained as described for **13a**. From **12b** (0.800 g, 1.04 mmol), phosphoramidite **13b**[49] (0.922 g, 0.95 mmol, 91%) was obtained after chromatography (silica gel, cyclohexane-3% triethylamine with gradient of ethyl acetate from 20% to 50%). R_f: 0.42 (RP-18, System F).

[1',2',3',4',5'-$^{13}C_5$]-5'-*O*-DMTr-2'-*O*-*t*-butyldimethylsilyl-uridine 3'-*O*-(2-cyanoethyl-*N*,*N*-diisopropylphosphoramidite) (13c). The uridine phosphoramidite was obtained as described for **13a**. From 0.407 g (0.62 mmol) of **12c**, after chromatography (silica gel, cyclohexane-3% triethylamine with gradient

of ethyl acetate from 20% to 45%), 0.408 g (0.47 mmol, 76%) of uridine phosphoramidite **13c**[49] was obtained. R_f: 0.47 (RP-18, System F).

[1',2',3',4',5'-$^{13}C_5$]-5'-*O*-DMTr-2'-*O*-*t*-butyldimethylsilyl-N^2-isobutyrylguanosine 3'-*O*-(2-cyanoethyl N,N-diisopropylphosphoramidite) (13e). The guanosine phosphoramidite was obtained as described for **13a**. From 0.423 g (0.55 mmol) of **12e** after chromatography (silica gel, cyclohexane-3% triethylamine, gradient of methylene chloride from 20% to 50%, then silanized silica gel, water-3% triethylamine, gradient of acetone from 30% to 60%), 0.370 g (0.38 mmol, 67%) of phosphoramidite **13e**[49] was obtained. R_f: 0.58 (RP-18, System F).

General procedure for the recovery of excess of ribonucleosides (Model experiment)

The effluents from the reaction column containing tetrazole-activated phosphoramidites were collected in a vial, containing moist acetonitrile. After collection of the amidite-containing fractions, the effluent was directed back to the waste port of the synthesizer. After completion of the synthesis, contents of the four vials, containing four amidite hydrolysates were concentrated to near dryness and partitioned between water and methylene chloride. After repeated extraction with water, the organic phase was evaporated to a foam, which consisted of practically pure H-phosphonate diesters and could be stored without decomposition. The hydrolysis of collected crude diesters was accomplished by dissolving them in acetonitrile and adding ca 5 eq. of DIPEA. The course of hydrolysis was monitored with TLC. After disappearance of the diester (16 h, r. t.), the mixture was evaporated, and the remaining foam was purified by short column chromatography. Yields of the nucleosides **12a, 12b, 12c** were in the range of 40 – 45% (**12e** was obtained with the yield of 15%).

Oligo-RNA synthesis and purification

The 29mer RNA was synthesized on an eight port ABI 392 DNA/RNA synthesiser. Four natural and four ^{13}C-labelled phosphoramidites were dissolved in dry acetonitrile to 0.11 M concentration. Standard 1 μmol scale RNA program from ABI was used for the synthesis which provided an average stepwise coupling efficiency of 97%. All 12 x 1 μmol scale syntheses were deprotected separately using the following standard procedure: (i) Base-protecting groups were removed in methanolic ammonia at 55 °C for 17 h and the volatile matters were removed in vacuo in a rotavapor; (ii) 2'-*O*-silyl deprotection was performed on the residue with neat triethylamine trihydrofluoride (700 μL of the reagent per 1 μmol of the crude RNA) for 24 h at r.t. followed by quenching with 100 μL of 3 M sodium acetate, dilution with 5 mL of ethanol, chilling at -30 °C overnight, centrifuging at cold (~4°C) at 12000 r.p.m., followed by a single washing of the precipitated RNA with 100 μL of cold 70% ethanol; (iii) fully deprotected crude RNA (approximately 110 A_{260} o.d. units per one 1 μmol scale RNA synthesis) was purified by denaturing (20% polyacrylamide, 7 M urea) gel electrophoresis with standard TBE buffer (50 mM tris-borate, 1 mM EDTA, pH 8). Appropriate bands were excised, crushed, and the product was extracted with 0.3 M sodium acetate (5 mL) overnight. Extraction was repeated to ensure a complete sample recovery until no UV absobing material is observable on the gel. Extracts were evaporated in vacuo in a rotavapor, and the purity was confirmed by PAGE (20% polyacrylamide, 7 M urea).

Combined extracts were dissolved in 4 mL of sterilised water, placed into two Centricon-3 concentrators (Amicon Inc.) and extensively dialysed against sterilised water (6 x 2 mL each). Concentrated salt free solutions of the pure oligo-RNA were combined and lyophilised. The RNA was precipitated three times from 70% ethanol (1.5 mL) at -50 °C (ethanol-dry ice) overnight and lyophilised. The resulting sample, containing 110 o.d. of pure RNA (Fig. 2), was dissolved in water, sodium exchanged using Dowex 50WX (Na^+ form, freshly generated by washing with 1.0 M NaOH, followed by washing with sterile water till pH 6.0 - 6.5, sterile water was used as the eluent with a UV monitor) and then lyophilised.

NMR experiments with the 29mer TAR RNA.

Specifically ^{13}C-labelled 29mer TAR RNA (Fig. 1) was dissolved in D_2O (99.98%) to a final concentration of ~0.23 mM (14 o.d. in 0.2 mL of Shigemi tube) with pH 7.0 and experiments were conducted at 20 °C.

The NMR experiments were carried out on a Bruker DRX 600 spectrometer at 14.1 T operating at 150.90 MHz for ^{13}C equipped with a Bruker digital lock and QXI-probehead and the switching ^2H lock - ^2H pulse device. All ^1H pulses were applied with a 26.3 kHz field and ^1H broadband decoupling was accomplished with WALTZ16 sequence using a 2500 Hz rf field. All ^{13}C pulses were applied with a 20 kHz field and ^{13}C decoupling was performed using GARP with a 3.85 kHz field strength.

DQF-COSY spectra were acquired with spectral width of 6009.6 Hz, acquiring 4096 complex points in t_2 and 512 in t_1, with 64 scans per FID, a relaxation delay of 2.0 s.

Conventional NOESY spectra were acquired with spectral width of 6009.6 Hz, acquiring 4096 complex points in t_2 and 512 in t_1, with 128 scans per FID, a relaxation delay of 2.0 s, and a mixing time of 300 ms.

The experimental schemes of [^1H,^1H]-NOESY with X(ω_2)-half-filter and heteronuclear (^{13}C) broadband decoupling in ω_1 and ω_2 dimension has been used as described in literature[52], with τ = 3.4 ms. Otherwise, the

condition of the experiment was the same as for the conventional NOESY experiment. The data for NOESY type experiments were multiplied with a square-cosine window function in F1 dimension and cosine window function in F2 dimension before Fourier transformation and a baseline correction were applied. Carriers are positioned at 4.8 ppm for ^{1}H in all experiments and 80 ppm for ^{13}C in area of $^{13}C(1')$.

HSQC-CT spectra were acquired with a constant time interval of 26.6 ms to avoid the ^{13}C-^{13}C splitting in F1 dimension with spectral widths of 6009.6 and 9054.0 Hz for proton and carbon dimensions, respectively; 2048 and 256 complex points were acquired for t_2 and t_1, respectively, with 32 scans per FID. Carriers are positioned at 4.8 ppm for ^{1}H in all experiments and 80 ppm for ^{13}C in area of $^{13}C(1')$. A relaxation delay of 2 s was employed.

To avoid the spinning artefacts, all spectra were measured on non-spinning samples.

Acknowledgements

JM and EZ have equally contributed to the project. This work was supported by grants from the State Committee for Scientific Research, Republic of Poland (3T09A11412) to RWA, from the Swedish Board for Technical Development (NUTEK) to JC, from the Swedish Natural Science Research Council (K-KU 12067-300 to AF & K-AA/Ku04626-321 to JC) and from the Swedish Research Council for Engineering Sciences (TFR) to JC. Authors thank Wallenbergsstiftelsen, University of Uppsala and Forskningsrådsnämnden (FRN) for their financial supports for the purchase of the 500 and 600 MHz NMR spectrometers. Authors also thank Dr. Zofia Gdaniec for her help in some of the NMR experiments and Danuta Wieckowska and Grazyna Dominiak for their excellent technical assistance.

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