PREPARATION OF 1′-C DEUTERATED SYNTHONS FOR RNA SYNTHESIS BY H-PHOSPHONATE METHOD AIMING AT TWO-DIMENSIONAL NMR SECONDARY STRUCTURE STUDIES

Lubos ARNOLD[a], Martina PRESSOVA[a], David SAMAN[a], Martin VOGTHERR[b] and Stefan LIMMER[b]

[a] *Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, 166 10 Prague 6, Czech Republic*

[b] *Laboratorium für Biochemie, Universität Bayreuth, D-95440 Bayreuth, Germany*

Received July 19, 1995
Accepted September 21, 1995

1′-C Deuterated H-phosphonate synthons were prepared via a 12-step procedure starting from [1′-^{2}H]ribose. The procedure included nucleosidation of 1′-*O*-acetyl-2′,3′,5′-*O*-tribenzoyl[1′-^{2}H]ribose with appropriately protected nucleobases and preparation of nucleoside-H-phosphonates by slightly modified described procedures. The automated RNA synthesis of 5′-G*C*U*A*U*UUAU-3′ and 3′-AC*G*A*U*A*AAGU-5′ was performed on a Gene Assembler Plus DNA-synthesizer. These specifically deuterated oligoribonucleotides were subsequently compared with the corresponding non-deuterated sequences using 2D-NMR NOESY spectra. Specific deuterium incorporation resulted in the expected simplification of spectral pattern.

Key words: [1′-^{2}H]Ribose; Nucleoside-H-phosphonates; Oligoribonucleotides, deuterated.

The elucidation of the structure of RNA molecules with molecular weight of ca 8 000 Da and more (about 25 nucleotides) using the standard 2D-NMR method is successful only in rare cases[1–5]. Usually a severe overlap of cross peaks in the spectra prevents an unequivocal assignment (and quantitative peak intensity determination) of proton resonance signals. These difficulties can be overcome by using isotope-labelled nucleotides, especially ^{13}C-ribose- and ^{15}N-base-labelled ones[6–8], and employing three-dimensional NMR methods. This approach is demanding with respect to both the production of the labelled nucleotides and the NMR experiments which require sophisticated NMR equipment and long measuring cycles[9,10].

The alternative approach we used is based on the fact that for the assignment the most important region in the NOESY spectra is that containing NOE contacts between the ribose 1′ and base aromatic (H-6, H-8, and H-2) protons (chemical shifts at about 6.2–5.0 ppm for the 1′ protons and 8.5–6.9 ppm for the aromatic protons). In this "coupling box" a continuous assignment path can be drawn connecting all the nucleotides of one strand which are located in regions with approximately regular helical

geometry. If the 1′ protons are replaced by deuterons, the corresponding interaction is eliminated and hence the cross peak vanishes from the NOESY spectrum. In most of the functionally interesting RNA molecules regions with well-defined secondary structure (helical stems) are combined with regions lacking any pronounced secondary structure. It is obvious that in most cases only the non-helical regions (loops, bulges, pseudo-knots, etc.) without distinct regular base pairing patterns need to be characterized in more detail.

Recently, Chattopadhyaya[11] and co-workers concentrated on non-specific introduction of deuterium into the ribose moiety and on the use of appropriate partly labelled nucleoside synthons for oligonucleotide synthesis. However, the relative ^{2}H isotopic enrichment at the 1′ position reached only ca 20%, which is clearly not sufficient to suppress efficiently the 1′-aromatic cross peaks and consequently to reduce the resonance overlap in this crucially important coupling box.

We decided to use specifically 1′-deuterated ribose to prepare the ribonucleotide H-phosphonate synthons by chemical synthesis.

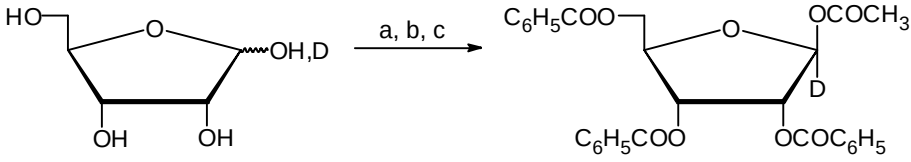
The synthetic procedure leading to 1′-deuterated nucleoside-H-phosphonates (Schemes 1–4), first optimized on non-deuterated compounds, included the following steps (intermediates at all stages were monitored by ^{1}H NMR spectroscopy (Tables I–V) mainly with respect to the presence and content of deuterium in the 1′ position:

1. Stereoselective protection of [1′-^{2}H]ribose (**1**), realized according to the published procedure[11] comprising preparation of 1-*O*-methyl-D-ribofuranose, separation of mixture of stereoisomers by crystallization, benzoylation of the β-isomer leading to 2,3,5-tri-*O*-benzoyl-β-D-ribofuranose and acetylation of the latter yielding 1-*O*-acetyl-2,3,5-tri-*O*-benzoyl-β-D-ribofuranose (**2**) (Scheme 1, Table I).

2. Preparation of appropriately protected nucleobases according to the known procedures: benzoylation[12] of adenine leading to N^6-benzoyladenine, acetylation of guanine followed by reaction with diphenylcarbamoyl chloride[13] leading to N^2-acetyl-O^6-(diphenylcarbamoyl)guanine, trimethylsilylation[14] of uracil leading to O^2,O^4-bis(trimethylsilyl)uracil and acetylation of cytosine followed by trimethylsilylation[14] leading to N^4-acetyl-O^2-(trimethylsilyl)cytosine.

3. Nucleosidation starting with 2,3,5-tri-*O*-benzoyl-1-*O*-acetyl[1-^{2}H]ribose and subsequent hydrolysis with saturated methanolic ammonia (Scheme 2, Table II); the condensation[14] with N^6-benzoyladenine gave [1′-^{2}H]adenosine (**3**), condensation[15] with N^4-acetyl-O^2-(trimethylsilyl)cytosine [1′-^{2}H]cytidine (**4**), condensation[16] with N^2-acetyl-O^6-(diphenylcarbamoyl)guanine gave [1′-^{2}H]guanosine (**5**), condensation[15] with O^2,O^4-bis(trimethylsilyl)uracil afforded [1′-^{2}H]uridine (**6**).

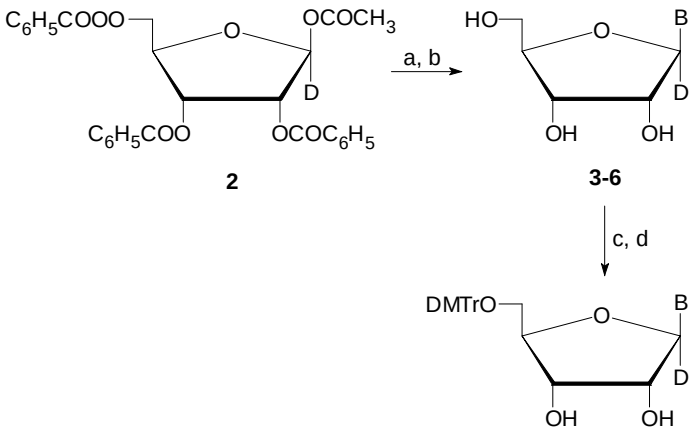
4. Step-by-step protection[17–20] comprising preparation of 5′-*O*-DMTr-*N*-protected nucleosides **7–10**, silylation leading to mixture of 2′ (**11–14**) and 3′ (**15–18**) isomers, and their chromatographic separation (Scheme 3, Tables III and IV).



1 **2**

a) MeOH, H_2SO_4; b) benzoylchloride, pyridine; c) CH_3COOH, $(CH_3CO)_2O$, H_2SO_4

SCHEME 1



2 **3-6** **7-10**

	B
3	adenine-9-yl
4	cytosine-1-yl
5	guanine-9-yl
6	uracil-1-yl

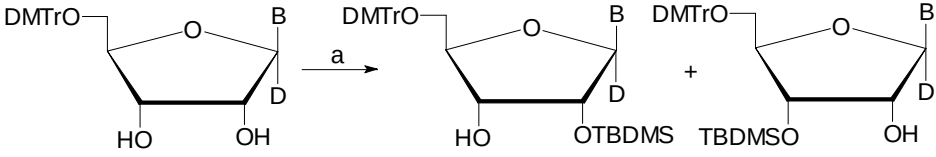
	B
7	N^6-dimethylaminomethyleneadenine-9-yl
8	N^4-benzoylcytosine-1-yl
9	N^2-dimethylaminomethyleneguanine-9-yl
10	uracil-1-yl

a) N^6-benzoyladenine, CH_3CN, $SnCl_4$; or N^4-acetyl-O^2-(trimethylsilyl)cytosine, $(CH_2Cl)_2$, $SnCl_4$; or N^2-acetyl-O^6-(diphenylcarbamoyl)guanine, N,O-bis(trimethylsilyl)acetamide),$(CH_2Cl)_2$, toluene, $CF_3SO_3Si(CH_3)_3$; or O^2,O^4-di(trimethylsilyl)uracil, $(CH_2Cl)_2$, $SnCl_4$;

b) NH_4OH, MeOH
c) in case of **3** and **5** *N,N*-dimethylformamide dimethylacetal;
in case of **4** benzanhydride, methanol, ethanol

d) 4,4'-dimethoxytriphenylmethyl (DMTr-Cl) chloride, pyridine

SCHEME 2

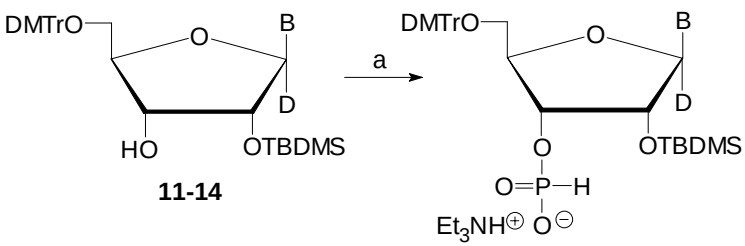


7-10 **11-14** **15-18**

a) *tert*-butyldimethylsilyl chloride (TBDMS-Cl), imidazole, pyridine

	B
11, 15	N^6-dimethylaminomethyleneadenine-9-yl
12, 16	N^4-benzoylcytosine-1-yl
13, 17	N^2-dimethylaminomethyleneguanine-9-yl
14, 18	uracil-1-yl

SCHEME 3



11-14 **19-22**

a) PCl_3, imidazole, Et_3N, CH_2Cl_2

	B
19	N^6-dimethylaminomethyleneadenine-9-yl
20	N^4-benzoylcytosine-1-yl
21	N^2-dimethylaminomethyleneguanine-9-yl
22	uracil-1-yl

SCHEME 4

5. Phosphitylation of the 2′-*O*-silylated intermediates **11–14** according to the published procedure[21] leading to the following nucleoside triethylammonium H-phosphonates (Scheme 4, Table V), or 2′-*O*-(*tert*-butyldimethylsilyl)-5′-*O*-(4,4′-dimethoxytrityl)[1′-^{2}H]-N^6-dimethylaminomethyleneadenosine-3′-H-phosphonate (**19**), or 2′-*O*-(*tert*-butyldimethylsilyl)-5′-*O*-(4,4′-dimethoxytrityl)[1′-^{2}H]-N^4-benzoylcytidine-3′-H-phosphonate (**20**), or 2′-*O*-(*tert*-butyldimethylsilyl)-5′-*O*-(4,4′-dimethoxytrityl)[1′-^{2}H]-N^2-dimethylaminomethyleneguanosine-3′-H-phosphonate (**21**), or 2′-*O*-(*tert*-butyldimethylsilyl)-5′-*O*-(4,4′-dimethoxytrityl)[1′-^{2}H]uridine-3′-H-phosphonate (**22**).

Automated synthesis of RNA fragments was performed on an automatic synthesizer according to a modified synthesizer program[22] with coupling time 3 min on the 1.5 μmol scale. The four following oligoribonucleotides were synthesized: non-deuterated Rz-1 and Rz-2, and deuterated Rz-1 and Rz-2.

Non-deuterated

		11	12	13	14	15	16	17	18	19	
Rz-1	5′-	G	C	U	A	U	U	U	A	U	-3′
Rz-2	3′-	A	C	G	A	U	A	A	G	U	-5′
		10	9	8	7	6	5	4	3	2	1

Deuterated

			11	12	13	14	15	16	17	18	19	
Rz-1	5′-		G*	C*	U*	A*	U*	U	U	A	U	-3′
Rz-2	3′-	A	C*	G*	A*	U*	A*	A	A	G	U	-5′
		10	9	8	7	6	5	4	3	2	1	

EXPERIMENTAL

The nucleobases were purchased from Fluka, D-[1-^{2}H]ribose (isotopic enrichment >98%) was from Omicron Chemicals (South Bend, IN, U.S.A.). Pyridine (dry), acetonitrile (distilled from P_2O_5 and CaH_2), dichloromethane (dry), *N*,*N*-dimethylformamide dimethylacetal, 4,4′-dimethoxytriphenylmethyl chloride, *tert*-butyldimethylchlorosilane, imidazole (puriss.), triethylamine (puriss.), 1-adamantanecarbonyl chloride, tetrabutylammonium fluoride and dichloroacetic acid were Fluka products and were used without further purification. Phosphorus trichloride (2 M solution in dichloromethane) was purchased from Aldrich. DEAE-Sephacel and Sephadex G-15 were obtained from Pharmacia. TLC was carried out on Silufol UV_{254} (Kavalier) using the following eluents: S1, ether–acetone 95 : 5; S2, chloroform–methanol 7 : 3; S3, ether–acetone 9 : 1; S4, 2-propanol–ammonia–water 7 : 1 : 2; S5, chloroform–methanol 8 : 2; S6, chloroform–methanol–triethylamine 95 : 4.5 : 0.5; S7, toluene–ethyl acetate 2 : 1; S8, chloroform–methanol 9 : 1. The automated RNA synthesis was performed on a Gene Assembler Plus DNA-synthesizer (Pharmacia). Ultraviolet spectra were measured on a UV-VIS

spectrometer Unicam 8625. ^{1}H NMR spectra of intermediates and H-phosphonate synthons were measured on a Varian UNITY-500 (499.8 MHz) spectrometer in hexadeuteriodimethyl sulfoxide (99.8 at.% D, Aldrich); for standardization the central line of the non-deuterated solvent (δ 2.50 ppm) was used. 2D-NMR spectra of oligonucleotides were measured on a Bruker DRX 500 instrument equipped with an Aspect X-32 computer. 500 MHz-NOESY spectra in D_2O buffer solution were recorded at 16 °C using standard procedures with mixing times of 300 ms. The spectra were acquired with 4 096 complex data points in the domain t_2 and 512 complex data points in the domain t_1 and a recycle delay of 1.5 s employing zero-filling to 1 024 data points in the F1 frequency dimension in the Fourier-transformed spectra (no zero filling in F2).

For the 2D-measurements, RNA (8–13 mg) was dissolved in 0.5 ml D_2O buffer solution containing 0.1 mol l^{-1} NaCl, 10 mmol l^{-1} sodium phosphate, pH 6.5. The resonances were referenced to internal 2,2-dimethyl-2-silapentane-5-sulfonate. RNA annealing was achieved by heating the samples at 80 °C for five minutes and subsequent cooling to room temperature.

1-*O*-Acetyl-2,3,5-tri-*O*-benzoyl[1-^{2}H]ribose (**2**)

The title compound was prepared according to the published procedures[11] starting from [1-^{2}H]ribose (15 g, 0.1 mol); yield 20.5 g (41%) after recrystallization from 1-propanol.

TABLE I
Spectral ^{1}H NMR parameters (δ, ppm; *J*, Hz) of starting ribose **1** and compound **2**

Parameter	Compound		
	1(a)	**1**(b)	**2**[a]
δ(H-2)	3.37 dd	3.21 dd	5.73 d
δ(H-3)	3.72 dt	3.71 dt	5.86 dd
δ(H-4)	3.49 dt	3.54 dt	4.84 dt
δ(H-5a)	3.28 dd	3.44 dd	4.50 dd
δ(H-5b)	3.61 dd	3.59 dd	4.72 dd
δ(1-OH)	6.04 s	6.30 s	–
δ(2-OH)	4.76 d	4.73 d	–
δ(3-OH)	5.02 d	4.62 d	–
J(2,3)	2.7	2.9	4.9
J(3,4)	3.9	3.3	7.1
J(4,5a)	3.9	6.6	4.2
J(4,5b)	7.8	3.3	3.4
J(5a,5b)	11.2	10.7	12.2
J(2,OH	7.1	6.8	–
J(3,OH)	5.6	5.1	–

[a] Other proton signals: 1.93 s ($OCOCH_3$); 7.52–8.03 m (OBz).

N^6-Benzoyladenine

N^6-Benzoyladenine was prepared according to the published procedure[12] starting from adenine (75 g, 0.55 mol); yield 94.5 g (60%) after recrystallization from ethanol, m.p. 242–242.5 °C.

N^4-Acetyl-*O*^{2}-(trimethylsilyl)cytosine

N^4-Acetyl-*O*^{2}-(trimethylsilyl)cytosine was prepared according to the published procedure[14] starting from *N*^{4}-acetylcytosine (7.65 g, 50 mmol); yield 11.25 g (100%) of the crude product.

TABLE II
Spectral ^{1}H NMR parameters (δ, ppm; *J*, Hz) of 1′-deuterated nucleosides

Parameter	Compound			
	3[a]	**4**[b]	**5**[c]	**6**[d]
δ(H-2)	4.60 dd	3.93 dd	4.39 dd	4.02 bt
δ(H-3)	4.14 dt	3.92 dt[e]	4.08 dt	3.96 dt
δ(H-4)	3.96 q	3.80 dt[e]	3.86 dt	3.83 bq
δ(H-5a)	3.55 ddd	3.53 ddd	3.52 ddd	3.54 ddd
δ(H-5b)	3.67 ddd	3.64 ddd	3.61 ddd	3.61 ddd
δ(2-OH)	5.43 d	5.26 d	5.39 d	5.38 d
δ(3-OH)	5.18 d	5.03 t	5.12 d	5.09 d
δ(5-OH)	5.41 dd	4.96 t	5.04 t	5.12 t
δ(NH_2)	7.34 bs	7.15 bs	7.93 bs	–
δ(NH)	–	–	10.63 bs	11.19 bs
J(2,3)	5.1	5.2[e]	4.9	4.6
J(3,4)	3.2	4.7[e]	3.5	3.6
J(4,5a)	3.7	3.4	4.1	3.8
J(4,5b)	3.9	3.2	4.1	3.8
J(5a,5b)	12.2	12.2	12.1	12.2
J(2,OH)	6.6	5.1	6.1	5.6
J(3,OH)	4.6	5.3	4.6	4.9
J(5a,OH)	7.1	5.3	5.8	5.4
J(5b,OH)	4.4	5.3	5.4	5.4

Other proton signals: [a] H-2, 8.34 s; H-8, 8.13 s. [b] H-5, 5.69 d (7.5); H-6, 7.83 d (7.5). [c] H-8, 7.93 s. [d] H-5, 5.63 bd (8.0); H-6, 7.86 d (8.0). [e] Chemical shifts and coupling constants determined by means of simulative-iterative calculations.

N^2-Acetyl-O^6-(diphenylcarbamoyl)guanine

N^2-Acetyl-O^6-(diphenylcarbamoyl)guanine was prepared according to the published procedures[13] starting from guanine (60.44 g, 0.40 mol); yield 35.53 g (21%) after recrystallization from ethanol.

O^2,O^4-Bis(trimethylsilyl)uracil

O^2,O^4-Bis(trimethylsilyl)uracil was prepared according to the published procedure[14] starting from uracil (5.6 g, 50 mmol); yield 12.27 g (96%), b.p. 107–109 °C/13 Pa.

[1′-^{2}H]Adenosine (**3**)

N^6-Benzoyladenine was condensed with 1-*O*-acetyl-2,3,5-tri-*O*-benzoyl[1-^{2}H]ribose by the known method[14]. The obtained N^6-benzoyl-2′,3′,5′-tri-*O*-benzoyl[1′-^{2}H]adenosine (R_F 0.53, S1) deprotected by treatment with saturated methanolic ammonia for 24 h to give the title compound (R_F 0.46, S2).

TABLE III
Spectral ^{1}H NMR parameters (δ, ppm; *J*, Hz) of fully protected 1′-deuterated nucleoside-2′ isomers

Parameter	Compound			
	11[a]	**12**[b]	**13**[c]	**14**[d]
δ(H-2)	4.87 d	4.17 d	4.57 d	4.21 d
δ(H-3)	4.24 ddd	4.21 ddd	4.18 dt	4.07 q
δ(H-4)	4.10 q	4.07 ddd	4.05 dt	3.99 ddd
δ(H-5a)	3.24 dd	3.35 dd	3.23 dd	3.24 dd
δ(H-5b)	3.28 dd	3.39 dd	3.30 dd	3.31 dd
δ(3-OH)	5.15 d	5.16 d	5.09 d	5.13 d
δ(NH)	–	11.25 bs	11.33 s	11.38 s
δ(CH_3-Si)	–0.06 s	0.10 s	–0.05 s	0.05 s
	–0.17 s	0.13 s	0.01 s	0.06 s
δ((CH_3)$_3$C-Si)	0.73 s	0.89 s	0.80 s	0.85 s
J(2,3)	5.1	4.4	5.2	5.1
J(3,4)	4.3	8.1	5.4	5.6
J(4,5a)	5.1	2.1	2.9	2.6
J(4,5b)	4.2	3.9	5.5	4.1
J(5a,5b)	10.7	10.8	10.5	10.8
J(3,OH)	6.1	6.4	5.9	6.1

Other proton signals: [a] H-2, 8.34 s; H-8, 8.36 s; =C**H**, 8.90 s; N(CH_3)$_2$, 3.13 s, 3.19 s; DMT, 3.73 s, 6.83–6.85 m, 7.17–7.42 m. [b] H-5, 7.99 dd (1.7, 7.4); H-6, 8.39 d (7.4); DMT, 3.76 s, 6.90–6.94 m, 7.24–7.42 m, 7.50–7.54 m, 7.60–7.64 m. [c] H-8, 7.91 s; =C**H**, 8.46 s; N(CH_3)$_2$, 3.03 s, 3.06 s; DMT, 3.73 s, 6.83–6.87 m, 7.23–7.40 m. [d] H-5, 5.30 dd (2.0, 8.0); H-6, 7.75 d (8.0); DMT, 3.74 s, 6.88–6.92 m, 7.12–7.38 m.

[1′-^{2}H]Cytidine (**4**)

N^4-Acetyl-O^2-(trimethylsilyl)cytosine was condensed with 1-*O*-acetyl-2,3,5-tri-*O*-benzoyl[1-^{2}H]ribose by the method described by Hrebabecky[15]. The obtained N^4-acetyl-2′,3′,5′-tri-*O*-benzoyl[1′-^{2}H]-cytidine (R_F 0.15, S1) was deprotected by treatment with saturated methanolic ammonia for 24 h to give the title compound (R_F 0.13, S2).

[1′-^{2}H]Guanosine (**5**)

N^2-Acetyl-O^6-(diphenylcarbamoyl)guanine was condensed with 1-*O*-acetyl-2,3,5-tri-*O*-benzoyl[1-^{2}H]-ribose by the method described by Jenny et. al.[16]. The obtained N^2-acetyl-2′,3′,5′-tri-*O*-benzoyl-O^6-(diphenylcarbamoyl)[1′-^{2}H]guanosine (R_F 0.60, S3) was deprotected by treatment with saturated methanolic ammonia for 24 h to give the title compound (R_F 0.46, S4).

TABLE IV
Spectral ^{1}H NMR parameters (δ, ppm; *J*, Hz) of fully protected 1′-deuterated nucleoside-3′ isomers

Parameter	Compound			
	15[a]	**16**[b]	**17**[c]	**18**[d]
δ(H-2)	4.88 dd	4.07 t	4.57 dd	4.10 t
δ(H-3)	4.52 t	4.28 dd	4.31 t	4.15 t
δ(H-4)	4.03 q	4.05 dt	3.94 dt	3.91 dt
δ(H-5a)	3.11 dd	3.22 dd	3.15 dd	3.16 dd
δ(H-5b)	3.35 dd	3.51 dd	3.28 dd	3.34 dd
δ(2-OH)	5.36 d	5.48 d	5.36 d	5.34 d
δ(NH)	–	11.26 bs	11.33 bs	11.36 bs
δ(CH_3-Si)	0.04 s	–0.07 s	–0.01 s	–0.03 s
	0.08 s	0.02 s	0.05 s	0.02 s
δ((CH_3)$_3$C-Si)	0.84 s	0.74 s	0.80 s	0.77 s
J(2,3)	5.6	4.6	5.1	5.6
J(3,4)	4.4	7.8	5.1	5.4
J(4,5a)	4.8	3.4	5.3	4.4
J(4,5b)	4.3	2.5	3.8	3.2
J(5a,5b)	10.5	10.9	10.5	10.7
J(2,OH)	6.4	5.6	6.1	5.9

Other proton signals: [a] H-2, 8.35 s; H-8, 8.40 s; =C**H**, 8.90 s; N(CH_3)$_2$, 3.12 s, 3.19 s; DMT, 3.72 s, 6.80–6.84 m, 7.17–7.35 m. [b] H-5, 7.99 dd (1.2, 7.3); H-6, 8.48 d (7.3); DMT, 3.75 s, 6.89–6.93 m, 7.15–7.41 m, 7.49–7.53 m, 7.60–7.64 m. [c] H-8, 7.96 s; =C**H**, 8.44 s; N(CH_3)$_2$, 3.03 s, 3.07 s; DMT, 3.72 s, 6.81–6.85 m, 7.19–7.35 m. [d] H-5, 5.37 bd (8.0); H-6, 7.77 d (8.0); DMT, 3.73 s, 6.87–6.91 m, 7.19–7.38 m.

[1′-^{2}H]Uridine (**6**)

O^2,O^4-Bis(trimethylsilyl)uracil (2.86 ml, 10.5 mmol) was condensed with 1-*O*-acetyl-2,3,5-tri-*O*-benzoyl[1-^{2}H]ribose (4.77 g, 9.5 mmol) by the method described by Hrebabecky[15]. The obtained 2′,3′,5′-tri-*O*-benzoyl[1′-^{2}H]uridine (R_F 0.66, S1) was deprotected by treatment with saturated methanolic ammonia for 24 h to give the title compound (R_F 0.15, S5).

2′-*O*-(*tert*-Butyldimethylsilyl)-5′-*O*-(4,4′-dimethoxytrityl)-N^6-dimethylaminomethylene[1′-^{2}H]-adenosine (**11**)

Step-by-step protection of the nucleoside was performed according to the published procedure[17] starting from [1′-^{2}H]adenosine (2.67 g, 10 mmol); the yield 1.19 g (16%) of the title compound as a chromatographically pure solid foam (R_F 0.55, S6).

TABLE V
Spectral ^{1}H NMR parameters (δ, ppm; *J*, Hz) of nucleoside-H-phosphonate synthons

Parameter	Compound			
	19[a]	**20**[b]	**21**[c]	**22**[d]
δ(H-2)	5.04 d	4.31 d	4.77 d	4.31 d
δ(H-3)	4.72 ddd	4.63 ddd	4.79 dt	4.59 dt
δ(H-4)	4.31 dt	4.20 dt	4.18 ddd	4.15 dt
δ(H-5a)	3.32 m	3.39 m	3.23 dd	3.27 dd
δ(H-5b)	3.32 m	3.39 m	3.32 dd	3.35 dd
δ(NH)	–	11.24 bs	11.26 bs	11.37 bd
δ(CH_3-Si)	–0.07 s	0.11 s	–0.09 s	0.05 s
	–0.25 s	0.14 s	0.01 s	0.08 s
δ((CH_3)$_3$C)	0.67 s	0.86 s	0.77 s	0.83 s
J(2,3)	4.9	4.2	5.1	4.9
J(3,4)	2.7	7.5	4.4	4.2
J(4,5a)	4.4	2.9	2.6	2.7
J(4,5b)	4.4	2.9	5.9	4.2
J(5a,5b)	–[e]	–[e]	10.4	10.7
J(H-3,^{31}P)	10.3	10.1	9.8	10.4

Other proton signals: [a] H-2, 8.29 s; H-8, 8.33 s; =C**H**, 8.90 s; N(CH_3)$_2$, 3.13 s, 3.19 s; DMT, 3.73 s, 6.81–6.84 m, 7.19–7.34 m. [b] H-5, 7.06 d (7.6); H-6, 8.37 d (7.6); DMT, 3.75 s, 6.89–6.93 m, 7.19–7.44 m, 7.49–7.53 m, 7.60–7.64 m. [c] H-8, 7.90 s; =C**H**, 8.58 s; N(CH_3)$_2$, 3.03 s, 3.07 s; DMT, 3.73 s, 6.80–6.84 m, 7.19–7.38 m. [d] H-5, 5.25 dd (1.8, 8.1); H-6, 7.71 d (8.1); DMT, 3.74 s, 6.87–6.91 m, 7.19–7.34 m. [e] Value not determined.

N^4-Benzoyl-2′-*O*-(*tert*-butyldimethylsilyl)-5′-*O*-(4,4′-dimethoxytrityl)[1′-^{2}H]cytidine (**12**)

Step-by-step protection of the nucleoside was performed according to the published procedures[18–20] starting from [1′-^{2}H]cytidine (2.43 g, 10 mmol); yield 3.2 g (42%) of the title compound as a chromatographically pure solid foam (R_F 0.29, S7).

2′-*O*-(*tert*-Butyldimethylsilyl)-5′-*O*-(4,4′-dimethoxytrityl)-N^2-dimethylaminomethylene[1′-^{2}H]-guanosine (**13**)

Step-by-step protection of the nucleoside was performed according to the published procedure[17] starting from [1′-^{2}H]guanosine (2.83 g, 10 mmol); yield 1.35 g (18%) of the title compound as a chromatographically pure solid foam (R_F 0.26, S6).

2′-*O*-(*tert*-Butyldimethylsilyl)-5′-*O*-(4,4′-dimethoxytrityl)[1′-^{2}H]uridine (**14**)

Step-by-step protection of the nucleoside was performed according to the published procedure[18,19] starting from [1′-^{2}H]uridine (2.44 g, 10 mmol); yield 1.65 g (25%) of the title compound as a chromatographically pure solid foam (R_F 0.32, S7).

Nucleoside-H-phosphonates

Phosphitylation of all appropriately protected nucleosides was carried out by the slightly modified Froehler method[17,21] with 85–95% yield of triethylammonium H-phosphonates:

2′-O-(tert-Butyldimethylsilyl)-5′-O-(4,4′-dimethoxytrityl)-N^6-dimethylaminomethylene[1′-^{2}H]adenosine-3′-H-phosphonate (**19**) (96%), R_F 0.20, S8.

N^4-Benzoyl-2′-O-(tert-butyldimethylsilyl)-5′-O-(4,4′-dimethoxytrityl)[1′-^{2}H]cytidine-3′-H-phosphonate (**20**) (92%), R_F 0.25, S8.

2′-O-(tert-Butyldimethylsilyl)-5′-O-(4,4′-dimethoxytrityl)-N^2-dimethylaminomethylene[1′-^{2}H]guanosine-3′-H-phosphonate (**21**) (85%), R_F 0.20, S8.

2′-O-(tert-Butyldimethylsilyl)-5′-O-(4,4′-dimethoxytrityl)[1′-^{2}H]uridine-3′-H-phosphonate (**22**) (86%), R_F 0.25, S8.

Automated Synthesis of RNA Fragments

The H-phosphonates were dissolved in pyridine–acetonitrile (1 : 1) to a concentration of 0.1 mol l^{-1} each, 1-adamantanecarbonyl chloride was dissolved in the same mixture to a concentration of 0.4 mol l^{-1}. The automated oligoribonucleotide synthesis was performed according to a modified synthesizer protocol[22] with coupling time 3 min on 1.5 μmol scale. Cleavage of the crude oligoribonucleotide from the CPG-support and deprotection were carried out by the successive treatment with saturated methanolic ammonia (24 h) and 1 M TBAF in DMF (24 h) at room temperature. Work-up of the deprotection mixtures was carried out on a DEAE-Sephacel column (1 × 2 cm) using 0.01 and 1 M LiCl as eluents, and followed by desalting on G-15 Sephadex column (2 × 40 cm). Two separate syntheses of each fragment afforded 150 OU for non-deuterated Rz-1, 140 OU for non-deuterated Rz-2, 149 OU for deuterated Rz-1 and 145 OU for deuterated Rz-2.

RESULTS AND DISCUSSION

The described method of chemical synthesis provided [1′-^{2}H]-protected nucleoside-H-phosphonate synthons with isotopic enrichment >98% in the position 1′. This was con-

firmed by ^{1}H NMR spectra of compounds **2–22** (Tables I–V), where no residual signal of 1′-^{1}H proton was observed.

The effect of 1′-ribose deuteration on the NOESY spectra of the duplex formed by the oligoribonucleotide strands Rz-1 and Rz-2 is demonstrated in Fig. 1 where a part of the 1′-ribose-base aromatic proton cross peak region (frequently referred to as "finger-print region") is displayed.

The corresponding spectral sections for the non-deuterated (*a*) and the partly deuterated samples (*b*) are compared there. Since unpurified, crude products have been used for the NMR studies, a few cross peaks, due to impurities (in particular truncated frag-

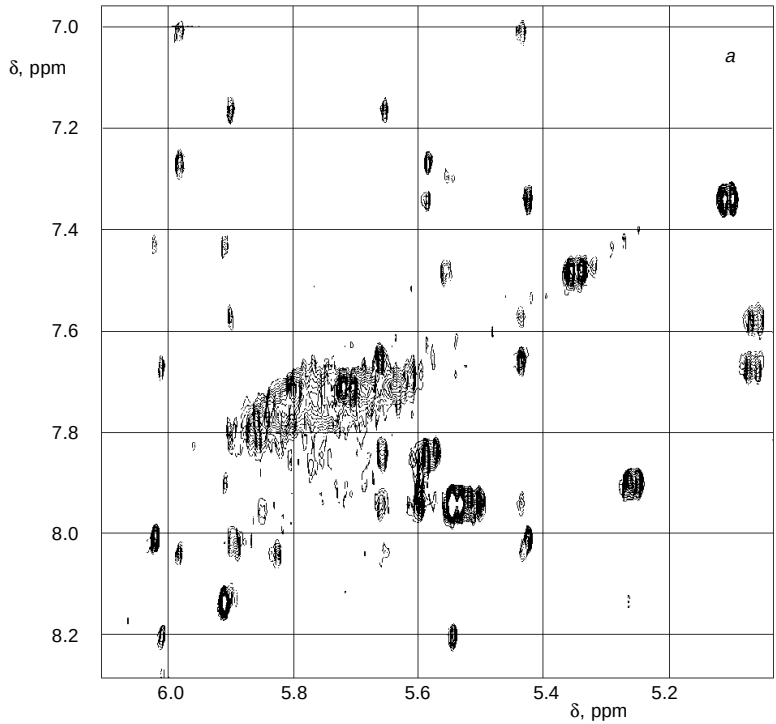


FIG. 1

Part of the NOESY spectrum (mixing time 300 ms, 4K data points in frequency domain F2, 1K data points in domain F1, relaxation delay 1.5 s) of the duplex formed by the oligoribonucleotide strands Rz-1 and Rz-2 (see text), containing the cross peaks resulting from 1′-ribose-base aromatic proton interactions. *a* Spectrum of the non-deuterated sample. The assignment path for the Rz-1 strand is indicated by solid lines. For numbering see text. *b* The same part of the NOESY spectrum as in *a* for the partially deuterated sample (nucleotides 5–9 in Rz-2 and nucleotides 11–15 in Rz-1). The missing cross peaks due to NOE contacts between the nucleotides of the Rz-1 strand with reference to *a* are marked

ments) are also seen. Nevertheless, it is immediately evident that the number of cross peaks in the spectrum of the partially deuterated sample is considerably reduced as it was expected. The missing cross peaks are due to NOE interactions involving 1′-ribose protons of (specifically deuterated) residues 5–9, and 11–15 (numbering according to the sequence scheme, vide supra), whereas all cross peaks resulting from interactions between nucleotides 1–4, and 16–19 are still present (cf. Fig. 1*b*). Moreover, the strong cross peaks originating from the pyrimidine (H-5)–(H-6) interactions – both for the non-deuterated and the partially deuterated residues – are found in the spectrum. They can be removed, too, by deuteration of the pyrimidine C-5 position which is intended in a further step of this work.

The reduction of the number of cross peaks in the fingerprint region (as well as the 1′–2′region) can facilitate the resonance assignment considerably, in particular for distinctly greater RNA molecules (>20 nucleotides) with extended helical stem regions. Usually, these stems need not to be analyzed extensively since they do not display severe deviations from regular helical geometry (at least if they contain only regular

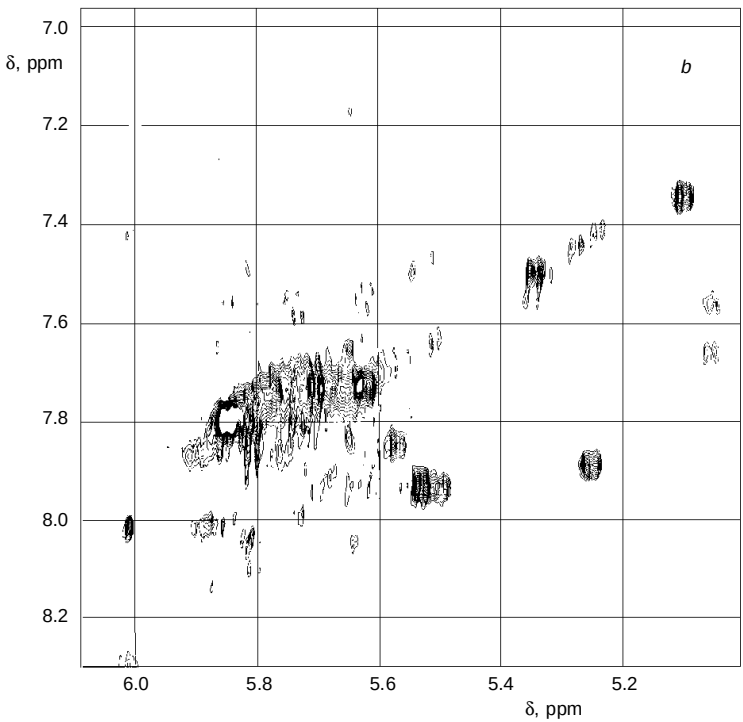


FIG. 1
(*Continued*)

Watson–Crick base pairs). Rather, regions with non-regular arrangement of the nucleotides, as, e.g., in loops, bulges, pseudoknots etc., need to be structurally characterized in greater detail. To this end, in the first step of an NMR structure determination a safe assignment of the resonances of non-exchangeable protons has to be achieved. As mentioned already before, especially for RNA molecules with more than 20 nucleotides the cross peak overlap in the NOESY spectrum region being most important for the resonance assignment can be substantially diminished making use of the above-described deuterium labelling. Accordingly, the resonance assignment can be simplified, in particular, if additionally the number and/or position of the partially deuterated residues is varied along the sequence region of major interest.

Of course, the synthetic procedure presented in this work can also be used to produce specifically ^{13}C-labelled synthons starting from correspondingly ^{13}C-labelled ribose. This would be interesting in view of a detailed structural and conformational characterization of certain particularly important sites in a given RNA sequence by means of multidimensional heteronuclear NMR spectroscopy.

This work was supported by the Volkswagen-Stiftung and the Deutsche Forschungsgemeinschaft, Projekt Sp 243/6-1.

REFERENCES

1. Szewczak A. A., Moore P. B., Chan Y.-L., Wool I. G.: Proc. Natl. Acad. Sci. U.S.A. *90*, 9581 (1993).
2. Puglisi J. D., Wyatt J. R., Tinoco I., jr.: J. Mol. Biol. *214*, 437 (1990).
3. Chastain M., Tinoco I., jr.: Biochemistry *31*, 12733 (1992).
4. Borer P. N., Lin Y., Wang S., Roggenbuck M. W., Gott J. M., Uhlenbeck O. C., Pelczer I.: Biochemistry *34*, 6488 (1995).
5. Moore P. B.: Acc. Chem. Res. *28*, 251 (1995).
6. Batey R. T., Inada M., Kujawinski E., Puglisi J. D., Williamson J. R.: Nucleic Acids Res. *20*, 4515 (1992).
7. Nikonowicz E. A., Sirr A., Legautt P., Jucker F., Baer L. M., Pardi A.: Nucleic Acids Res. *20*, 4507 (1992).
8. Michnicka M. J., Harper J. W., King G. C.: Biochemistry *32*, 395 (1993).
9. Nikonowicz E. A., Pardi A: J. Mol. Biol. *232*, 1141 (1993).
10. Allain F. H.-T., Varani G.: J. Mol. Biol. *250*, 333 (1995).
11. Foldesi A., Nilson F. P. R., Glemarec C., Gioeli C., Chattopadhyaya J.: Tetrahedron *48*, 9033 (1992).
12. Ness N. K. in: *Synthetic Procedures in Nucleic Acid Chemistry* (W. W. Zorbach and R. S. Tipson, Eds), Vol. 1, p. 183. Interscience, New York 1968.
13. Zou R., Robins M. J. : Can. J. Chem. *65*, 1436 (1987).
14. Hrebabecky H., Dockal J., Holy A.: Collect. Czech. Chem. Commun. *59*, 1408 (1994).
15. Hrebabecky H., Holy A.: Carbohydrate Res. *216*, 179 (1991).
16. Jenny T. F., Benner S. A.: Tetrahedron Lett. *33*, 6619 (1992).

17. Arnold L., Smrt J., Zajicek J., Ott G., Schiesswohl M., Sprinzl M.: Collect. Czech. Chem. Commun. *56*, 1948 (1991).
18. Schaller H., Weinmann G., Lerch B., Khorana H. G.: J. Am. Chem. Soc. *85*, 3821 (1963).
19. Usman N., Ogilvie K. K., Jiang M.-J., Cedergren R. J.: J. Am. Chem. Soc. *109*, 7845 (1987).
20. Otter B. A., Fox J. J. in: Ref.[12], Vol. 1, p. 285.
21. Froehler B. C., Ng P. G., Matteucci M. D.: Nucleic Acids Res. *14*, 5399 (1986).
22. Ott G., Arnold L., Smrt J., Sobkowski M., Limmer S., Hofmann H-P., Sprinzl M.: Nucleosides Nucleotides *13*, 1069 (1993).