

Synthesis and NMR of RNA with selective isotopic enrichment in the bases

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ABSTRACT

Efficient syntheses of pyrimidine and purine nucleosides and nucleotides with selective ^{13}C enrichment in the base moieties are described. Uridine and cytidine are labeled at position C6 and adenosine and guanosine are labeled at position C8. The selectively labeled nucleosides were converted to nucleoside triphosphates and used with *in vitro* transcription to synthesize labeled RNA. Isotope-edited ^{12}C and ^{13}C sub-spectra of a ω 1-1/2-X-filtered NOESY experiment are demonstrated to be useful for making resonance assignments and for deriving structural information in large (>20 nt) RNA molecules. The labeled RNAs also allow heteronuclear J-couplings and relaxation parameters to be measured without complications from ^{13}C – ^{13}C J-couplings.

INTRODUCTION

NMR studies of RNA structure and dynamics are often hindered by spectral overlap (1). The introduction of methods for the uniform ^{15}N and ^{13}C enrichment of RNA (2–4) has helped resolve overlap problems and has facilitated unambiguous assignments (5,6). Serianni and co-workers have synthesized nucleosides with selective isotopic enrichment in the sugar C1' and C2' positions (7). That work allowed heteronuclear J-couplings to be measured and related to sugar pucker conformation. Complementary methods are described here for selective isotopic enrichment of RNA in the bases.

Nuclear Overhauser enhancements (NOEs) involving base protons are particularly important for determining RNA structure. Spectral overlap of base protons in large RNA molecules, however, often precludes complete assignment of resonances and prevents quantification of cross-peak volumes for distance determination. To resolve this overlap, we decided to specifically ^{13}C enrich the carbons attached to the base protons. This allows isotope-filtered (8) and ^{13}C chemical shift-edited NMR experiments, which were originally developed for studying proteins, to be applied to RNA as well. We report the synthesis and NMR of three RNAs with selective isotopic enrichment of C6 of U and C residues and C8 of G and A residues. Labeling RNA at these positions facilitates NMR assignment. The labels also allow measurement of three bond heteronuclear J-couplings to H1' (7, 9) and ^{13}C relaxation parameters (10).

MATERIALS AND METHODS

General methods

[^{13}C , 99 atom %]Formic acid and [^{13}C , 99 atom %]potassium cyanide were from Cambridge Isotope Laboratories. Uridine phosphorylase (UPase) and purine nucleoside phosphorylase (PNPase) were the generous gifts of the Burroughs Wellcome Co. All other enzymes, urea, Pd-BaSO₄ and trisodium phosphoenolpyruvate (PEP) were from Sigma. Hexamethyldisilazane (HMDS), chlorotrimethylsilane (TMS-Cl) and phosphorylchloride were from Aldrich and were distilled prior to use. 1,2,4-Triazole was from Aldrich and was recrystallized from ethanol. Acetonitrile was from Fisher and was distilled from P₂O₅ and CaH₂. Triethylamine was from Fisher and was distilled from sodium metal. All other chemicals were from Aldrich and were used without further purification.

One-dimensional ^1H , ^{13}C and ^{31}P NMR spectra were acquired on a Bruker AM400 spectrometer with a digital resolution of <0.1 Hz/point. ^1H and ^{13}C data are referenced to internal TMS (for CDCl₃ or D₆-DMSO solutions, δ 0.0 p.p.m.) or TSP (D₂O solutions, δ 0.0 p.p.m.). ^{31}P data are referenced to external TMP (δ 0.0 p.p.m.). ^{13}C spectra were acquired with broadband ^1H decoupling. UV absorbance spectra were recorded in water at pH 7 on a Shimadzu UV160 spectrophotometer. Except for nucleoside monophosphates (NMPs) and nucleoside triphosphates (NTPs), the yields reported were determined from the mass of the products after drying in a vacuum oven overnight at 70°C. For NMPs and NTPs yields from mass measurements are not reliable, because the amount of salt and solvent are unknown. Thus for NMPs and NTPs yields were determined by UV absorbance using the following extinction coefficients ($\text{M}^{-1} \text{cm}^{-1}$): AMP ϵ (259 nm) = 15 400, ATP ϵ (259 nm) = 15 400, CMP ϵ (271 nm) = 9100, CTP ϵ (271 nm) = 9000, GMP ϵ (252 nm) = 13 700, GTP ϵ (253 nm) = 13 700, UMP ϵ (262 nm) = 10 000, UTP ϵ (262 nm) = 10 000 (10). UV absorbance spectra were measured for all compounds and agreed with literature data (11). ^1H , ^{13}C and ^{31}P chemical shifts and J-couplings of synthetic intermediates are given in Tables 1 and 2. ^{13}C chemical shifts are consistent with those reported previously (12). ^{13}C isotopic purity was determined to be 99% for synthetic intermediates by integrating ^1H NMR spectra acquired with one scan. Electron impact mass spectra (EIMS) and fast atom bombardment mass spectra (FABMS) were acquired in positive ion mode with a resolution of 1000–1500 by the Mass Spectrometry and Instrumentation facility at the University of California–Berkeley.

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Table 1. ¹H NMR chemical shift and J-coupling data^a

Compound ^b	H8/H6	H2/H5	H1'	H2'	H3'	H4'	H5'/H5''	Other
1 (DMSO)								9.8, br (N-H) 7.4, br (NH ₂) 3.95 J _{CH} = 10.0 10.0, br (H1 and H3)
2 (DMSO)	7.40 (dd) J _{C6-H6} = 180.6 J _{H5-H6} = 7.6	5.45 (dd) J _{C6-H5} = 4.0 J _{H6-H5} = 7.6						
3 (CDCl ₃)	7.42 (dd) J _{C6-H6} = 180.6 J _{H5-H6} = 8.1	5.62 (dd) J _{C6-H5} = 4.4 J _{H6-H5} = 8.1	6.32 (dd) dd, J ≈ 4.1	5.93 (dd)	5.69 (dd)	4.6–4.9 (o)	4.6–4.9 (o)	9.59 (H3) 7.9–8.1 (<i>o</i> -benzoate) 7.2–7.6 (<i>m</i> - and <i>p</i> -benzoate)
4 (D ₂ O)	7.87 (dd) J _{C6-H6} = 184.1 J _{H5-H6} = 8.1	5.89 (dd) J _{C6-H5} = 4.4 J _{H6-H5} = 8.1	5.92 (dd) J _{C6-H5} = 3.5 J _{H1'-H2'} = 4.5	4.35 (dd)	4.23 (dd)	4.14 (m)	3.7–3.9 (m)	
5 (D ₂ O)	8.12 (dd) J _{C6-H6} = 185.3 J _{H5-H6} = 8.1	6.0 (dd)	6.0 (dd)	4.43 (dd)	4.36 (dd)	4.26 (m)	3.9–4.1 (m)	
6 (D ₂ O)	7.94 (dd) J _{C6-H6} = 184.6 J _{H5-H6} = 8.2	5.98 (dd)	5.99 (dd)	4.2–4.5 (o)	4.2–4.5 (o)	4.2–4.5 (o)	4.2–4.5 (o)	
7 (CDCl ₃)	7.2–8.1 (o) J _{C6-H6} = 4.7 J _{H5-H6} = 7.3	6.72 (dd)	6.24 (dd)	5.69 (dd)	5.62 (dd)	4.5–4.8 (o)	4.5–4.8 (o)	9.00, 7.87 (triazole) 7.2–8.1 (benzoate)
8 (D ₂ O)	7.84 (dd) J _{C6-H6} = 182.9 J _{H5-H6} = 7.6	6.04 (dd) J _{C6-H5} = 4.4 J _{H5-H6} = 7.6	5.90 (dd) J _{C6-H1'} = 3.3 J _{H1'-H2'} = 4.0	4.31 (dd)	4.21 (dd)	4.14 (m)	3.8–4.0 (m)	
9 (D ₂ O)	8.04 (dd) J _{C6-H6} = 183.9 J _{H5-H6} = 7.6	6.12 (dd) J _{C6-H5} = 4.4 J _{H5-H6} = 7.6	6.00 (dd) J _{C6-H1'} = 3.0 J _{H1'-H2'} = 3.9	4.0–4.4 (o)	4.0–4.4 (o)	4.0–4.4 (o)	4.0–4.4 (o)	
10 (D ₂ O)	7.92 (dd) J _{C6-H6} = 183.4 J _{H5-H6} = 7.6	6.13 (dd) J _{C6-H5} = 4.2 J _{H5-H6} = 7.6	6.00 (dd) J _{C6-H1'} = 3.6 J _{H1'-H2'} = 3.9	4.2–4.4 (o)	4.2–4.4 (o)	4.2–4.4 (o)	4.2–4.4 (o)	
11 (DMSO)	8.11 (d) J _{C8-H8} = 209.8	8.13						7.11, br (NH ₂)
12 (D ₂ O)	8.26 (d) J _{C8-H8} = 215.2	8.09	6.01 (dd) J _{C8-H1'} = 3.8 J _{H1'-H2'} = 6.1	4.77 (dd) J _{H2'-H3'} = 6.1 J _{H2'-H3'} = 5.2	4.43 (dd) J _{H3'-H4'} = 3.4	4.30 (m)	3.94, 3.85 (m)	
13 (D ₂ O)	8.59 (d) J _{C8-H8} = 216.3	8.22	6.13 (dd) J _{C8-H1'} = 4.6 J _{H1'-H2'} = 5.7	4.52 (dd)	4.03 (o)	4.38 (m)	4.03 (o)	
14 (D ₂ O)	8.48 (d) J _{C8-H8} = 216.1	8.19	6.12 (dd)	4.56 (dd)	4.42 (m)	4.29 (m)	3.34, 3.19 (m)	
15 (DMSO)	7.69 (d) J _{C8-H8} = 208.0							6.65, br (2-NH ₂) 5.66, br (6-NH ₂)
16 (DMSO)	7.92 (d) J _{C8-H8} = 212.5		5.70 (dd) J _{C8-H1'} = 4.0 J _{H1'-H2'} = 6.3	4.51 (dd) 5.44, br (OH)	4.09 (dd) 5.21, br (OH)	3.91 (m)	3.6 (m, o) 5.64, br (OH)	6.79, br (2-NH ₂) 5.75, br (6-NH ₂)
17 (DMSO)	7.93 (d) J _{C8-H8} = 213.7		5.68 (dd)	4.39 (dd) 5.45, br (OH)	4.09 (dd) 5.20, br (OH)	3.87 (m)	3.64, 3.60 (m) 5.15, br (OH)	10.70, br (H1) 6.48, br (2-NH ₂)
18 (D ₂ O)	8.20 (d) J _{C8-H8} = 216.5		5.93 (dd) J _{C8-H1'} = 4.5 J _{H1'-H2'} = 5.8	4.73 (dd)	4.49 (dd)	4.33 (m)	4.01 (m)	
19 (D ₂ O)	8.14 (d) J _{C8-H8} = 216.5		5.89 (dd)	4.80 (dd)	4.56 (dd)	4.37 (m)	4.27 (m)	

^aChemical shifts in p.p.m. and J-couplings in Hz. Abbreviations: br, broad; d, doublet; dd, doublet of doublets; m, multiplet; o, overlapped.^bDeuterated solvent given in parentheses.

Table 2. ^{13}C and ^{31}P chemical shift and J-coupling data^a

Compound ^b	C8/C6	C2	C1'	C2'	C3'	C4'	C5'	Other	P α	P β	P γ
1	115.3* (C6)	153.2						165.2 (C4) 31.7 (C5, J _{C6-C5} = 62.6)			
2	142.4* (C6)	151.7						164.6 (C4) 100.4 (C5, J _{C6-C5} = 65.8)			
3	139.6* (C6)	150.1	88.1	71.1	73.7	80.5	63.7	162.5 (C4) 103.3 (C5, J _{C6-C5} = 68.4)			
4	166.0, 165.3, 165.2 (benzoate carbonyls), 144.6* (C6)	154.5	92.1	72.2	76.2	86.9	63.5	112.8 (benzoate carbons) 169.0 (C4) 105.0 (C5, J _{C6-C5} = 68.0)			
5	145.0* (C6)	154.8	91.2	72.9	76.8	87.0	66.0	169.0 (C4) 105.5 (C5, J _{C6-C5} = 65.6)	0.6		
6	145.0* (C6)	-	-	-	-	-	-	-	-13.7 (d)	-22.1 (dd)	-8.6 (d)
7	146.3* (C6)		90.0	71.0	74.9	81.0	63.5	165.2 (C4), 96.0 (C5, d)			
8	154.0, 151.7, 151.5, 151.3 (benzoate carbonyls and C2), 144.4* (C6)	160.4	93.1	72.0	76.7	86.5	63.5	129.9, 129.8, 129.6, 129.2, 128.7, 128.5 (benzoate carbons) 168.9 (C4) 98.9 (C5, J _{C6-C5} = 69.3)			
9	144.7* (C6)	160.4	92.2	72.5	77.1	86.1	66.3	169.2 (C4) 99.4 (C5, J _{C6-C5} = 66.3)	-0.4		
10	144.3* (C6)	160.1	92.2	72.1	76.9	85.4	67.7	169.1 (C4) 99.5 (C5, J _{C6-C5} = 68.9)	-13.5 (d)	-22.0 (dd)	-8.5 (d)
11	139.6* (C8)	152.9									
12	143.2* (C8)	155.1	91.1	73.4	76.4	88.5	64.2				
13	143.1* (C8)	155.7	89.7	73.6	77.4	87.6	66.4		-0.5		
14	143.1* (C8)	155.7	89.7	73.1	77.1	86.6	64.0	J _{C4'-P} = 9.1 85.4 J _{C4'-P} = 9.0 J _{C5'-P} = 5.3	-13.7 (d)	-22.0 (dd)	-8.4 (d)
15	136.3* (C8) 148.0 (C6)	160.6						152.9 (C4)			
16	137.0* (C8) 156.6 (C6, J = 6.1)	160.4	87.7	71.2	73.7	86.0	62.2	151.7 (C4, J = 9.9) 113.9 (C5)			
17	136.2* (C8) 157.3 (C6, J = 6.9)	154.1	86.8	70.8	74.1	85.7	61.8	151.8 (C4, J = 9.9) 117.0 (C5)			
18	144.4* (C8) 161.7 (C6)	156.8	89.7	73.4	77.0	87.2	65.5	154.4 (C4) 118.9 (C5)	-0.3		
19	140.5* (C8)	-	-	-	-	-	-	-	-13.9 (d)	-22.0 (dd)	-8.5 (d)

^aChemical shifts in p.p.m. and J-couplings in Hz. Asterisks indicate 99% ^{13}C enrichment, all other peak intensities consistent with 1.1% natural abundance. Dashes indicate insufficient signal-to-noise ratio to detect these resonances. Assignments are given in parentheses where appropriate. J-couplings in ^{31}P spectra were observed to be 15.3–15.5 Hz.

^bSolvents are given in Table 1.

Labeled nucleotide syntheses

[6- ^{13}C]Cyanoacetyurea (**1**). **1** was synthesized from 34.6 g (0.37 mol) chloroacetic acid, 19.7 g (0.30 mol) [^{13}C]potassium cyanide (KCN) and 18.6 g (0.31 mol) urea as described (**13**). The yield was 26.0 g (68% from KCN). EIMS m/z 128 (27%), 85 (100%).

[6- ^{13}C]Uracil (**2**). A mixture of 3.4 g 5% Pd-BaSO₄ and 40 ml H₂O in a 500 ml Parr bottle was pre-reduced with H₂. 7.68 g (0.06 mol) of **1** was dissolved in 120 ml boiling acetic acid and 80 ml hot water, added to the Parr bottle and hydrogenated at atmospheric pressure (**14**). The mixture was shaken for 6 h, at which time hydrogen uptake ceased (uptake was 1510 ml). The reaction mixture was heated to 70°C and filtered through glassfiber filter paper (Whatman). The filtrate was evaporated until a white precipitate started to form and stored at 4°C for 2 days. The white product was removed by filtration and dried overnight in a vacuum oven (yield 5.5 g, 81%). EIMS m/z 113 (100%), 70 (54%).

2',3',5'-Tri-*O*-benzoyl[6- ^{13}C]uridine (**3**). **3** was synthesized from 0.56 g (5 mmol) **2** and 2.52 g (5 mmol) 1'-*O*-acetyl-2',3',5'-tri-*O*-benzoyl- β -D-ribofuranose (ATBR) as described (**15,16**). The yield was 2.4 g (86%). FABMS m/z 558 (M+1, 51%), 445.2 (100%). UV (acetonitrile) λ_{min} 211 nm, λ_{max} 230 nm, λ_{sh} ~256 nm.

[6- ^{13}C]Uridine (**4**) (**16**). Freshly prepared ammonia-saturated methanol (100 ml) was added to 2.78 g (5 mmol) **3**. The flask was sealed and allowed to react overnight at 23°C. The oil obtained after rotary evaporation was partitioned between 100 ml water and three portions of 100 ml diethylether. The water layer was concentrated to a solid white glass and crystallized at 23°C from 20 ml hot ethanol. The yield was 1.1 g (90%). FABMS m/z 246 (M+1, 87%), 114 (100%).

[6- ^{13}C]Uridine 5'-monophosphate (**5**) (**17**). 0.75 g (3 mmol) of **4** was dried overnight in a vacuum oven, suspended in 7.5 ml triethylphosphate and stirred for 10 min at 23°C, cooled to 0°C and 0.55 ml (6 mmol) POCl₃ added via syringe. After 6 h the

reaction was quenched while stirring at 0°C by the dropwise addition of 2 M NaOH until pH 4 was achieved, stirring for 1 h and then adjusting to pH 7. This procedure prevented formation of the 5',3'-cyclic phosphate (18). The mixture was partitioned between 20 ml H₂O and four 20 ml portions of diethylether. Two volumes of cold ethanol were added to the water layer and stored overnight at -20°C. The precipitate was pelleted by centrifugation, dissolved in 30 ml water, frozen and lyophilized. The product contains ~95% 5'-UMP, about half an equivalent of sodium phosphate and <5% of 5',2'- and 5',3'-uridine bisphosphate as judged by ³¹P-NMR. The yield was 1.2 g (62%).

[6-¹³C]Uridine 5'-triphosphate (6) (19,20). To 0.653 g (1 mmol) 5 was added 0.655 g (2.8 mmol) PEP, 15 ml distilled water, 0.5 ml 0.05 M ATP, 3 ml 1 M MgCl₂, 3 ml 1 M KCl and 1.5 ml 1 M Tris-HCl, pH 7.6. The pH was adjusted to 7.6 with 1 M NaOH. The solution was degassed with nitrogen for 30 min. To this were added 10 mg DTT, 1500 U myokinase, 240 U pyruvate kinase and 5 mg (5 U) nucleoside monophosphate kinase (NMPK) and the flask capped with a nitrogen-filled balloon. The reaction was stirred at 37°C. After 0.5 h the pH was 9.5 and was adjusted to 7.6 by addition of 1 M HCl. The pH was adjusted again after 1 h and the mixture allowed to stir overnight. ³¹P NMR indicated complete conversion of 5 to 6. Two volumes of cold ethanol were added to the reaction mixture, which was stored overnight at -20°C. The UTP precipitate was pelleted by centrifugation and the supernatant removed. The UTP was redissolved in 25 ml distilled water, frozen and lyophilized. ³¹P NMR indicated that the 5',2'- and 5',3'-uridine bisphosphate contaminants were not converted to the 5'-triphosphate derivatives, which could potentially terminate transcription. The product was used for RNA transcription without further purification. Labeled NTPs yielded the same amount of full-length transcripts as unlabeled NTPs. The yield was 0.65 g (91%).

4-Triazolyl-1-(2',3',5'-tri-O-benzoyl-β-D-ribofuranosyl)-[6-¹³C]2-pyrimidineone (7) (21,22). A mixture of 2.78 g (5 mmol) 3 and 5.18 g (75 mmol) 1,2,4-triazole were dried overnight in a vacuum oven. The solids were dissolved in 50 ml dry acetonitrile and 16 ml dry triethylamine under a nitrogen atmosphere. The mixture was cooled to 0°C and 0.92 ml (10 mmol) POCl₃ were added dropwise and the reaction stirred for 30 min. The mixture was partitioned between 100 ml cold saturated NaHCO₃ and 100 ml chloroform. The chloroform layer was dried over Na₂SO₄ and concentrated to a light yellow syrup.

[6-¹³C]Cytidine (8). Crude 7 was dissolved in 50 ml acetonitrile and 50 ml concentrated ammonia (at 23°C) was added dropwise. The flask was sealed, stirred overnight and concentrated by rotary evaporation. The residue was partitioned between 50 ml water and four 50 ml portions of diethylether. The water layer was concentrated to 20 ml and applied to a 2.5 × 20 cm column containing Dowex 1×2 (200–400 mesh) resin (OH⁻ form) and eluted with distilled water/methanol 7:3 (v/v) (7). The fractions containing cytidine were combined, concentrated by rotary evaporation and crystallized at 23°C from 30 ml hot ethanol. The yield was 1.05 g (86% from 3). FABMS *m/z* 245 (M+1, 55%), 113 (100%).

[6-¹³C]Cytidine 5'-monophosphate (9). 8 was converted to 9 as described for 5, but the reaction was complete in 1 h. The yield was 1.4 g (79%).

[6-¹³C]Cytidine 5'-triphosphate (10). 9 was converted to 10 as described for 6, but 0.589 g (1 mmol) 9 and 0.655 g (2.2 mmol) PEP were used. The yield was 0.91 g (96%).

[8-¹³C]Adenine (11). 4,5,6-Triaminopyrimidine sulfate hydrate (1.246 g, 5.6 mmol) was pulverized, added to a 40 ml vial with a Teflon sealed cap and suspended in 15 ml ethylene glycol. To this was added 0.4 ml (10.4 mmol) [¹³C]formic acid, the vial capped and stirred vigorously overnight at 23°C. The stirred mixture was heated to 90°C (oil bath) for 1 h at which time complete dissolution occurred. The cap was removed and the reaction stirred at 180°C under a nitrogen atmosphere for 2 h to distill off any remaining formic acid and water. Ethylene glycol was removed by rotary evaporation. The oil was dissolved in 25 ml hot water and 1 ml concentrated ammonia. Excess ammonia was removed by rotary evaporation to give a pH of 4–6. After 2 h at 23°C the colored precipitate (unidentified) was removed by filtration. The filtrate was stored overnight at 4°C. The crystalline needles were removed by filtration. The yield was 0.47 g (62%). EIMS *m/z* 136 (100%).

[8-¹³C]Adenosine (12). A mixture of 1.65 g (6.8 mmol) uridine, 0.61 g (4.5 mmol) 11, 25.5 mg KH₂PO₄ and 32.7 mg K₂HPO₄ was dissolved in 75 ml water at 70°C. Upon cooling to 37°C the pH was adjusted to 7.4 with 1 M NaOH and 5000 U PNPase and 2500 U UPase were added. The reaction was stirred at 37°C overnight. Proton NMR indicated reaction completion. The pH was adjusted to 10 with concentrated ammonia. The product was directly loaded on a Dowex 1×2 200–400 (OH⁻ form) column (2.5 × 13 cm) and eluted with 500 ml 30% methanol/water and then 60% methanol/water (7). Fractions containing adenosine were pooled and the solvent removed by rotary evaporation and crystallized at 4°C from 25 ml boiling water. The yield was 1.15 g (95%). FABMS *m/z* 269 (M+1, 100%), 137 (86%).

[¹³C8]Adenosine 5'-monophosphate (13). 12 was converted to 13 as described for 5, but 0.80 g (3 mmol) 12 were suspended in 10.0 ml triethylphosphate and the reaction was complete in 4 h. The yield was 1.5 g (80%).

[¹³C8]Adenosine 5'-triphosphate (14). 13 was converted to 14 as described for 6, except no NMPK was added and 0.627 g (1 mmol) 13 and 0.655 g PEP were used. The yield was 0.7 g (80%).

[8-¹³C]2,6-Diaminopurine (15). 15 was synthesized as described for 11, except 1.33 g (5.6 mmol) 2,4,5,6-tetraaminopyrimidine sulfate and 0.25 ml (6.7 mmol) [¹³C]formic acid were used. The yield was 0.67 g (79%). The product was contaminated with a small amount of ammonium sulfate. EIMS *m/z* 151 (100%). UV λ_{min} 236 nm, 262 nm, λ_{max} 247 nm, 280 nm.

β-D-Ribofuranosyl-[8-¹³C]2,6-diaminopurine (16). The procedure described above for 12 was used, except 0.68 g (4.5 mmol) 15 were used in place of 11. The product was crystallized from ethanol. The yield was 1.15 g (90%). FABMS *m/z* 284 (M+1, 76%), 152 (100%). UV λ_{min} 237 nm, 265 nm, λ_{max} 256 nm, 280 nm.

[8-¹³C]Guanosine (17). A mixture of 2.0 g (7.1 mmol) 16 and 0.174 g K₂HPO₄ was dissolved in 200 ml hot water. The mixture was cooled to 23°C, the pH was adjusted to 7.5 with 1 M HCl and 1000 U adenosine deaminase were added. The mixture turned cloudy after 10 min and precipitate formed after 1 h. The pH was

adjusted to 8 and the mixture reacted overnight. The guanosine was isolated by filtration and dried in a vacuum oven. The yield was 1.8 g (90%). FABMS m/z 285 (M+1, 82%), 153 (100%). UV λ_{\min} 223 nm, λ_{\max} 253 nm, λ_{sh} ~272 nm.

[8- ^{13}C]Guanosine 5'-monophosphate (**18**). **17** was converted to **18** as described for **13**, but with 0.85 g (3 mmol) **17** and the reaction was complete in 4 h. The yield was 1.4 g (80%).

[8- ^{13}C]Guanosine 5'-triphosphate (**19**). **18** was converted to **19** as described for **6**, except 10 U guanylate kinase were added and NMPK was not added and 0.581 g (1 mmol) **18** were used. The yield was 0.52 g (56%).

RNA synthesis

Selectively labeled RNAs were synthesized using T7 RNA polymerase (**23**), the labeled NTPs described above and unlabeled NTPs from Sigma. Since the labeled NTPs contain Mg^{2+} , it was important to re-optimize the added Mg^{2+} concentration so that transcription yields were equivalent to reactions with only unlabeled NTPs. The RNAs were then purified by 20% PAGE, electro-eluted (Schleicher & Schuell), dialyzed against the final buffer for NMR studies, lyophilized several times after addition of 99.9% D_2O and finally dissolved in 99.96% D_2O .

NMR of labeled RNAs

Two-dimensional ω 1-1/2-X-filtered NOESY and 3D HMQC-NOESY spectra were acquired on a Bruker AMX600 spectrometer operating at 600 MHz for proton and 150.9 MHz for carbon, using an inverse 5 mm probe. Data were transferred to a Silicon Graphics computer and processed with FELIX 2.3 (Biosym Tech.). Chemical shifts are referenced to internal TSP for ^1H resonances (0.0 p.p.m.) and external TSP for ^{13}C resonances (0.0 p.p.m.) (**24**).

The ω 1-1/2-X-filtered NOESY pulse sequence (**8**) was used with additional phase cycling of the first proton pulse to reduce TPPI diamond artifacts (**2, 25**). The ^1H and ^{13}C carriers were set to 4.7 and 139.7 p.p.m. The spectral width was 5000 Hz in both the t1 and t2 dimensions. The initial t1 increment was set to minimize baseline distortion and simplify phasing (**26**). The residual HDO resonance was saturated during the ~3.0 s recycle delay. Quadrature detection was achieved in t1 and t2 by TPPI (**27**) with ~350 and 2048 points. Two dummy scans were performed before each t1 increment to ensure a steady state (**8**). Spectra were acquired with NOESY mixing times of 150 (Fig. 2) and 250 ms (Fig. 3). The 1/2J delays were set to 2.4–2.7 ms. Each t1 increment was performed twice with 32 scans each and stored in different data locations, once with and once without the ^{13}C 180° pulse. The total acquisition time was 22 h. The sum of these experiments produced the sub-spectrum of NOEs from ^{12}C bound protons and the difference produced the sub-spectrum of NOEs from ^{13}C bound protons. The data were multiplied by a 30° phase-shifted sine bell skewed by a factor of 0.7 in t2 and 45° phase-shifted skewed sine bell in t1, zero filled to give a 1024 × 1024 real data matrix and Fourier transformed.

Three-dimensional HMQC-NOESY (**28**) was acquired with the ^1H carrier set to 4.7 p.p.m. for HDO pre-saturation during the 2.0 s recycle delay and then changed to 7.5 p.p.m. for all pulses except the last proton 90° pulse, which was at 4.7 p.p.m. The ^{13}C carrier was set to 139.7 p.p.m. Spectral widths of 1500, 1200 and 5000 Hz were used in the t1, t2 and t3 dimensions, with initial

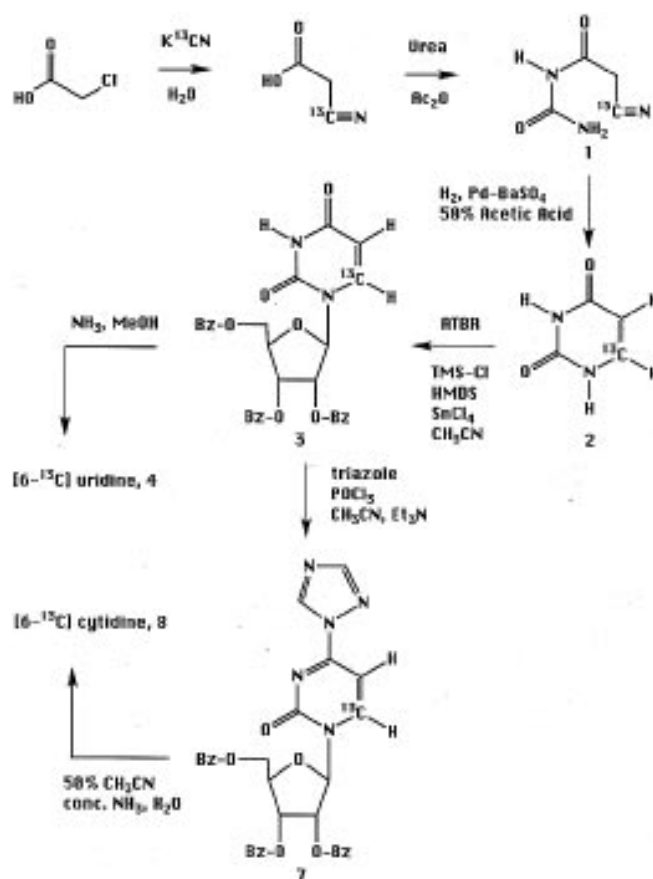


Figure 1. Labeled pyrimidine nucleoside synthesis.

delays as described (**26**). Quadrature detection was achieved in all dimensions by TPPI with 32, 64 and 1024 real points in t1, t2 and t3 respectively. Thirty two scans were acquired for each increment for an acquisition time of 44 h. The FIDs were zero filled to give a final matrix of 64 × 128 × 1024 real points. The data were processed with a 90° phase-shifted sine bell and 2 Hz exponential line broadening in each dimension and Fourier transformed.

RESULTS AND DISCUSSION

Synthesis

A convergent strategy (Fig. 1) for isotopic enrichment of uridine and cytidine was adopted. Since uracil is much easier to synthesize than cytosine (**29**), this strategy decreased the number of steps and improved yields for cytidine. Particular attention was given to improving yields and simplifying literature procedures. The labeled base and nucleoside syntheses described here can be coupled with the various isotopomeric riboses (**30**) to give virtually any desired nucleoside labeling strategy.

Four previously published methods for isotopic enrichment of uracil are: (i) condensation of malic acid and urea (**31**); (ii) condensation of propionic acid and urea with polyphosphoric acid (**32**); (iii) condensation of cyanate and β -alanine (**15**); (iv) preparation (**13**) and cyclization (**33**) of cyanoacetylurea. Methods (i) and (ii) each proceed in one step with 55% and 93% yields respectively. These methods are useful only for C2, N1 and N3 isotopic enrichment, because isotopically enriched malic acid

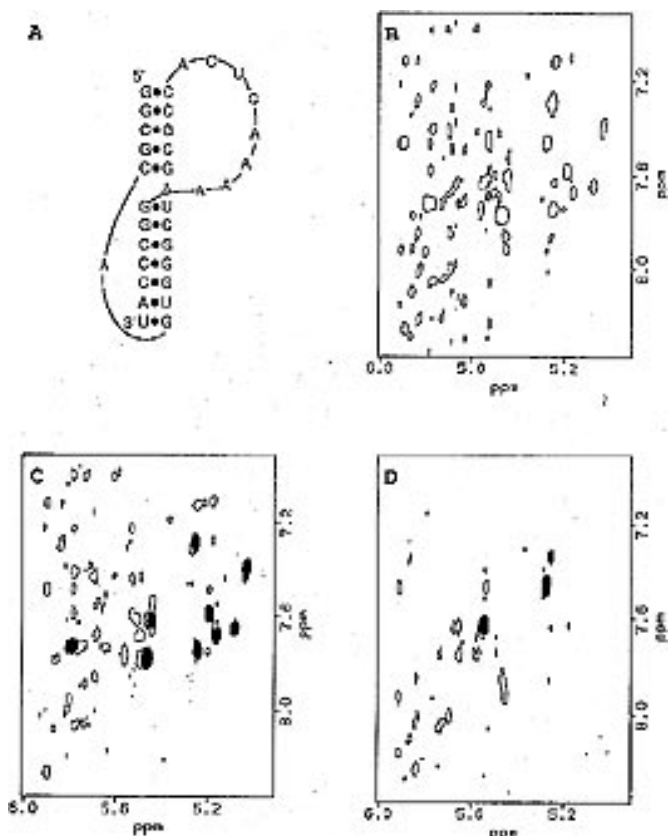


Figure 2. The base H6/H8/H2 to H1/H5 regions of NOESY spectra. (A) Pseudoknot from the *gag-pro* frameshift site of MMTV. (B) NOESY spectrum of an unlabeled sample. (C and D) ω 1-1/2-X-filtered NOESY with the A and U residues labeled at positions C8 and C6 respectively. (C) The sub-spectrum of NOEs from ^{12}C bound protons (from G and C residues along with A-H2 resonances). (D) The sub-spectrum of NOEs from ^{13}C bound protons (from A and U residues). Cross-peaks from pyrimidine H5-H6 NOEs are shaded in (C) and (D). This labeling strategy allows resonances to be identified by residue type.

and propynoic acid are not readily available. Method (iii) is reported to proceed with high yield (~80%), but requires six synthetic steps. Preparation of cyanoacetylurea from chloroacetic acid, cyanide and urea is straightforward with yields between 60 and 78% (13). Several methods for cyclizing cyanoacetylurea are available, but yields are 20–50%, with substantial side products (33). The yield (~80%) and purity (~99%) of uracil were improved using Pd-BaSO₄ instead of PtO₂ to catalyze the hydrogenation. The starting materials (potassium cyanide, chloroacetic acid, urea and hydrogen gas) are commercially available with ^2H , ^{13}C and ^{15}N enrichment; thus regiospecific and uniform enrichment of uracil is possible. We note that for ^{15}N enrichment of N1 or N3 the present method is not regiospecific (15).

2',3',5'-Tri-*O*-benzoyluridine is synthesized from uracil and ATBR using acetonitrile as the solvent and SnCl₄ as catalyst (15,16). We also attempted to use dichloroethane as the solvent and trimethylsilyltriflate as catalyst (7), but this resulted in substantial N3 ribosylation (~25%) and N1,N3 bisribosylation (~10%), as found by others (16). Both [6- ^{13}C]uridine and [6- ^{13}C]cytidine are synthesized from intermediate [6- ^{13}C]2',3',5'-tribenzoyl-uridine. Treatment with ammonia-

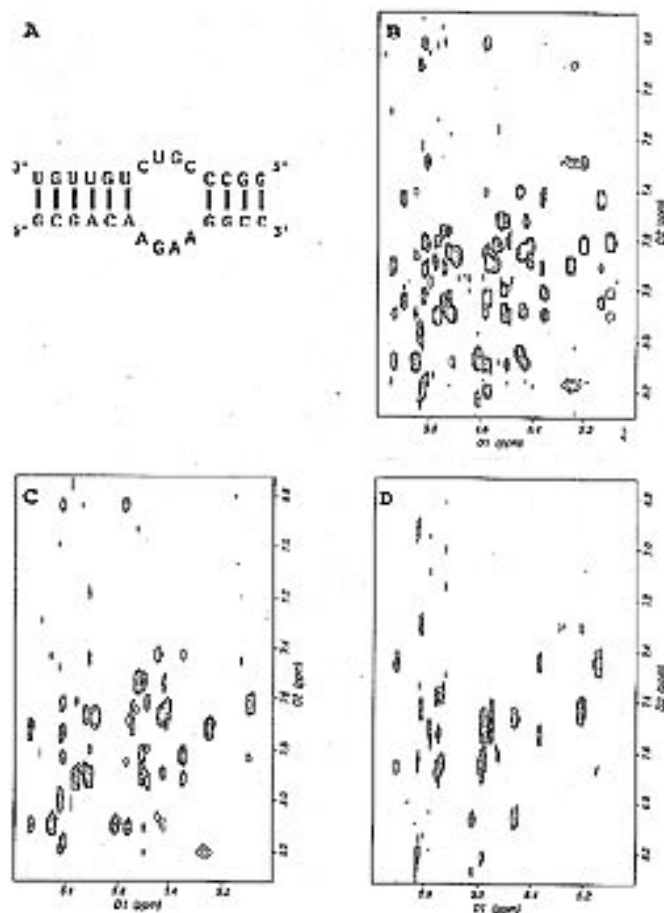


Figure 3. The base H6/H8/H2 to H1/H5 regions of NOESY spectra. (A) Internal loop from a hairpin ribozyme derived from the minus strand of tobacco clearing spot virus satellite RNA. (B) NOESY spectrum of an unlabeled sample. (C and D) ω 1-1/2-X-filtered NOESY with the lower strand labeled at position C8 for A and G residues and position C6 of C residues. No residues of the upper strand were labeled. (C) The sub-spectrum of NOEs from ^{12}C bound protons (upper strand). (D) The sub-spectrum of NOEs from ^{13}C bound protons (lower strand). This labeling strategy allows separate NOE walks along each strand.

saturated methanol removes the benzoyl groups to give uridine. Treatment with triazole in the presence of phosphorylchloride gives a triazolyl intermediate (21,22; Fig. 1), which is converted to cytidine by treatment with aqueous ammonia/acetonitrile. The triazolyl intermediate may also be used to synthesize alkylated pyrimidine nucleosides (34).

[8- ^{13}C]Adenine and [8- ^{13}C]2,6-diaminopurine were synthesized from [^{13}C]formic acid and the appropriate pyrimidine dissolved in ethylene glycol with a procedure adapted from that used to synthesize guanine (35). After completion of this work Dr Peter Silks (Stable Isotope Resource, Los Alamos National Laboratory, personal communication) informed us that performing the reaction in two steps, first by reacting triaminopyrimidine with formic acid in water for 2 h at 70°C and then removing the solvent and heating the solid at 180°C for 2 h over a stream of dry argon results in clean synthesis of adenine.

[8- ^{13}C]Adenosine was synthesized using coupled UPase and PNPase (36). Uridine is the ribose source and is in slight excess (1.5 equivalents) to ensure complete adenosine synthesis. Other purine nucleosides can also be synthesized by this route by simply

changing the input purine. Guanosine, however, was not efficiently synthesized, because guanine is insoluble. 2,6-Diaminopurine, however, is soluble and allows synthesis of diaminopurine riboside. Diaminopurine riboside is converted in high yield to guanosine by treatment with adenosine deaminase (37).

Adenosine and diaminopurine riboside can also be synthesized chemically. Adenine or diaminopurine are benzoylated with 70–80% yield by reacting with benzoic anhydride (38,39). Adenosine is synthesized by condensing *N*-benzoyladenine with ATBR in dichloroethane with trimethylsilyltriflate as catalyst (82% yield) (7). Alternatively, adenosine can be synthesized with ~80% yield by reacting unprotected adenine with ATBR in acetonitrile with SnCl₄ as catalyst (40). Most of the conventional methods for condensing purines with ATBR result in low yields for diaminopurine riboside. The use of 2,6-bisbenzamidopurine with HgCl₂ as catalyst has been reported to work well however (39).

At this point all four natural nucleosides are selectively enriched and the decision as to how to couple the nucleosides into a polynucleotide needs to be made. The two best methods for preparing RNA are automated chemical synthesis (41) and T7 RNA polymerase-catalyzed transcription from a DNA template (23). The chemical method allows for maximum flexibility in the labeling strategy by simply varying the phosphoramidites used. However, a substantial portion of the labeled monomer is lost in the synthetic process. For the synthesis of RNAs with >20 bases T7 RNA polymerase-catalyzed transcription is more efficient than chemical synthesis. Since we are most interested in applications of ¹³C labeling to large RNAs, we decided to convert the labeled nucleosides into NTPs for use in transcription. One disadvantage of RNA synthesis by transcription is that all nucleotides of a given type (i.e. A, C, G or U) must be either labeled or unlabeled. The 5'-end of RNA can be selectively labeled by priming the transcription with labeled GMP (23).

The method of Yoshikawa *et al.* was used for the 5'-selective phosphorylation of nucleosides (17). Trimethylphosphate and triethylphosphate give essentially the same ratios of 5'-monophosphorylated and 5',3'- or 5',2'-bisphosphorylated products. Triethylphosphate, however, is easily removed by ether extraction, while removal of trimethylphosphate requires ion exchange chromatography. Complete removal of the solvent is critical for obtaining high yields of NTPs in the next step. NMPs were converted to NTPs with the enzymatic procedure described (2,19).

NMR of ¹³C-labeled RNA

Selective and uniform isotope enrichment strategies are complementary. Uniform enrichment allows all nuclei to be monitored simultaneously and allows for powerful one bond transfer experiments (e.g. H-C-C-H TOCSY) (6). Disadvantages of uniform enrichment are that ¹³C-¹³C J-couplings complicate resonance line shape, which decreases spectral resolution and sensitivity and makes measurement of ¹H-¹H, ¹H-¹³C and ¹³C-³¹P couplings more difficult. Uniform enrichment also makes the interpretation of relaxation parameters in terms of molecular motion more difficult (10). Constant time experiments, which remove ¹³C-¹³C J evolution, suffer from the disadvantage of lower sensitivity, due to relaxation during the long evolution period. For the selective enrichment described here no ¹³C-¹³C J-couplings are present.

Spectral overlap of base protons in large RNA molecules often hinders complete assignment of resonances and prevents quantification of cross-peak volumes for distance determinations. One way to reduce spectral overlap is to selectively deuterate some residues and then observe the remaining resonances. This approach has been used with success by several workers (1,42). One problem with this approach is that the spectral information from the deuterated resonances is lost, necessitating the synthesis of another sample with these residues undeuterated. Further, different samples are often prepared with slightly different oligomer or salt concentrations, resulting in slight changes in resonance chemical shifts and ambiguities in assignments (1).

An alternative, which affords the equivalent information as two deuteration experiments, uses selective labeling with ¹³C in conjunction with 1/2-X-filtered NOESY experiments (8). Information from both ¹²C and ¹³C bound protons is retained in these experiments. Simplified ¹²C and ¹³C sub-spectra are obtained by either adding or subtracting the signals from two pulse sequences which differ only by the presence or absence of a ¹³C 180° pulse. Since the two sub-spectra are derived from the same data, chemical shifts from the two spectra can be rigorously compared.

It is worthwhile to consider various selective labeling strategies. The goal is to maximize the information obtainable while minimizing the number of samples prepared and NMR experiments performed. The synthesis of RNA by transcription requires that all nucleotides of a given type must be either labeled or unlabeled. Nonetheless, several labeling strategies are still possible. For example, one, two, three or all four nucleotides can be labeled. In multi-strand complexes of RNA one strand at a time might be labeled. Some factors which should be considered in choosing among these possibilities include: (i) resolving resonances from residues which are a critical part of the molecule being studied; (ii) maximizing the total number of resonances which can be resolved by synthesizing a single labeled sample; (iii) removing the overlap of strong H5-H6 cross-peaks with other critical cross-peaks. For the assignment of very broad resonances labeling the attached carbon would allow for sensitive detection of the proton resonance using HMQC. Another consideration is heteronuclear relaxation, which broadens the signals of protons bound to ¹³C. This suggests it might be best not to label residues with weak NOEs; this effect is minimized by performing 1/2-X-filtration in ω1 instead of ω2 (8).

Applications

One generally useful strategy is to label one purine and one pyrimidine so that only AC, AU, GC or GU resonances are in the ¹³C-edited sub-spectrum and the remaining resonances are in the ¹²C-edited sub-spectrum. NOEs from adenine H2 protons are also in the ¹²C-edited sub-spectrum, but are easily distinguished from purine H8 protons by their usual lack of NOEs to ribose sugar protons and by the unique C2 carbon chemical shift (observed in natural abundance HMQC experiments;43). In each sub-spectrum, pyrimidines are distinguished from purines by the presence or absence of a strong H5-H6 cross-peak. *Syn* residues can also give a strong cross-peak, but are usually easily distinguished by their lack of NOEs from the base proton to sugar protons and by the absence of the strong cross-peak in DQF-COSY. Thus this strategy allows resonances to be identified by residue type (Fig. 2). Alternatively, C and U residues can be

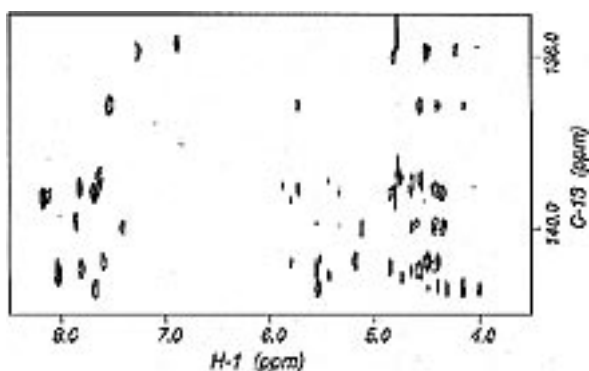


Figure 4. 2D HMQC-NOESY spectrum of the hairpin ribozyme internal loop. This spectrum spreads out NOE information according to the protons attached ^{13}C shift in the first dimension, unlike NOESY which uses two proton frequency axes.

distinguished by the C5 chemical shift (43) and A and G residues can be distinguished in uniformly ^{13}C -enriched RNA by H-C-C-H TOCSY (44).

Figure 2A shows the secondary structure of a 34 nt pseudoknot derived from the *gag-pro* region of the mRNA of mouse mammary tumor virus (MMTV) (45). Initial assignment of some proton resonances was achieved by analyzing NOESY spectra of unlabeled samples. However, the G+C-rich stems of the pseudoknot resulted in severe proton spectral overlap. To reduce spectral overlap we labeled C8 of the adenosines and C6 of the uridines and used the 1/2-X-filtered NOESY experiment. The ^{13}C -edited sub-spectrum of the labeled pseudoknot contains NOEs from ^{13}C bound H8 and H6 protons of A and U residues (Fig. 2D) to other protons. The ^{12}C -edited sub-spectrum of this sample (Fig. 2C) has NOEs from the H8 and H6 protons of G and C residues (along with adenine H2 protons) to other protons. Figure 2B shows the base to H1' region of the NOESY spectrum of an unlabeled sample. Effectively, spectrum 2B is the sum of 2C and 2D. The synthesis of a pseudoknot sample with only cytidine residues labeled at C6 was also useful for deducing cytidine assignments (data not shown). These experiments confirmed assignments for the stems and established H8/H6, H5 and H1' resonance assignments of most other residues. Out of four U residues in the pseudoknot sequence only two of them have strong H5-H6 NOEs and corresponding H6-H1' NOEs in either Figure 2D or B. Weak or absent cross-peaks from residues U8 and U13 are attributed to broad linewidths of their proton resonances. Comparison of 1/2-X-filtered-NOESY spectra with the NOESY spectrum of the unlabeled pseudoknot facilitated sequential assignment of H2' protons. Spectral overlap in the sugar proton region made resonance assignment difficult beyond H2' protons. The structure of the MMTV pseudoknot is presented elsewhere (46).

Another strategy is to label all residues of one strand of a multi-stranded RNA complex. Labeling one strand allows for separate sequential NOE walks for the labeled and unlabeled strands in the ^{13}C -edited and ^{12}C -edited sub-spectra of the ω 1-1/2-X-filtered NOESY (Fig. 3). The double-stranded internal loop shown in Figure 3A is derived from the hairpin ribozyme found in the minus strand of tobacco ring spot virus satellite RNA (47). Figure 3B shows the base to H1' region of the NOESY spectrum of an unlabeled sample. The NOESY spectrum for the 28 nt internal loop is very crowded in some regions. The aromatic

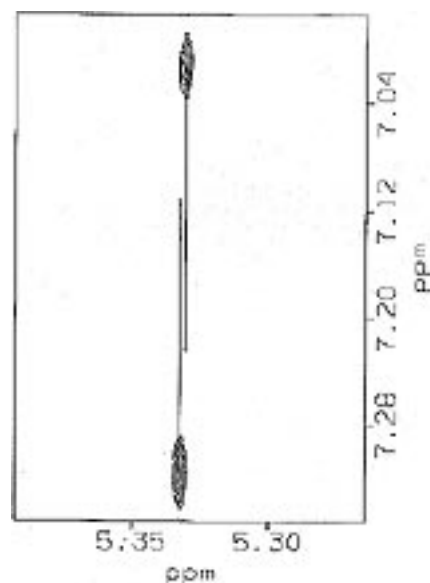


Figure 5. The intra-residue H6-H1' cross-peak of residue C4 of (GGACUC-GUCC)₂ with all cytidine residues labeled at position C6 of the normal NOESY spectrum (without decoupling in either dimension). Note the ECOSY-type cross-peak fine structure reveals the large one bond coupling $J_{\text{H6-C6}} = 184$ Hz in ω 1 and the small three bond coupling $J_{\text{C6-H1'}} = 1.2$ Hz in ω 2.

protons of seven pyrimidines and one purine have resonances between 7.60 and 7.69 p.p.m. The large H5-H6 cross-peaks from pyrimidines have made this spectral area particularly crowded; the labeling experiment described below showed that the H1'-H8 cross-peak from G8 is completely buried underneath the H5-H6 cross-peak from U28. Due to the severe spectral overlap, only two out of the above eight resonances were assigned. In addition, four H1' protons resonate between 5.81 and 5.82 p.p.m. Therefore, the H1' to H8/H6 NOE connectivities could only extend from G1 to A7 on the bottom strand and from G15 to G17 and G20 to U28 of the top strand. Only two out of the five H2 aromatic protons from adenines were definitively assigned.

To overcome the spectral overlap problem we selectively ^{13}C -labeled the lower strand of the internal loop at C8 of each purine and C6 of each pyrimidine. Figure 3C and D shows the ^{12}C -edited and the ^{13}C -edited sub-spectra of the ω 1-1/2-X-filtered NOESY experiment of the lower strand-labeled sample. Separate sequential walks along each strand can be performed in each sub-spectrum. This allowed unambiguous assignment of base and H1' resonances of all residues in both strands. NOE cross-peaks from base protons to sugar H2', H3', H4', H5' and H5'' protons, which are critical for structure determination, are also more resolved in both the ^{12}C -edited and ^{13}C -edited sub-spectra than in the NOESY of the unlabeled sample (data not shown).

An alternative to isotope-filtered experiments is to take advantage of the chemical shift of ^{13}C resonances using 2D HMQC-NOESY (48) and 3D HMQC-NOESY (28). The 2D HMQC-NOESY spreads out NOE information according to ^{13}C shift in the first dimension, unlike NOESY which uses two proton frequency axes. Figure 4 shows the 2D HMQC-NOESY spectrum of the hairpin ribozyme internal loop. This spectrum allows for auto-correlation peaks to be resolved which would normally lie on the diagonal of a NOESY spectrum. NOEs that overlap by

coincidence in the NOESY spectrum may be resolved in this spectrum and vice versa. This spectrum contains the same information as the ^{13}C -edited sub-spectrum of the $\omega 1$ -1/2-X-filtered NOESY (Fig. 3D), only the shifts in $\omega 1$ are from the attached ^{13}C of the base proton involved in the NOE instead of the base proton chemical shift. The 2D HMQC-NOESY spectrum shown in Figure 4 is actually the first t2 increment of the 3D HMQC-NOESY. The spectrum was acquired in 41 min. The 3D HMQC-NOESY further resolves overlap and was useful for confirming many of the assignments derived from the 1/2-X-filtered experiments, particularly in the sugar region (data not shown).

An added bonus for ^{13}C labeled molecules is the possibility of obtaining new structural information, for example J-couplings involving ^{13}C . In non-decoupled NMR experiments ECOSY-type cross-peak fine structure is observed which allows the measurement of small heteronuclear couplings (2,49). Figure 5 shows the C4 intra-residue H6-H1' NOESY cross-peak (without decoupling in either dimension) of (GGACUCGUCC)₂, where the cytidine residues are labeled at position C6. ECOSY-type cross-peak fine structure reveals the large one bond coupling $J_{\text{H6-C6}} = 184$ Hz in $\omega 1$ and the small three bond coupling $J_{\text{C6-H1'}} = 1.2$ Hz in $\omega 2$. In principle, three bond J-couplings from C8/C6 to H1' and C2' could be used to determine the glycosidic torsion angle (2,7,9,50).

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