BULLETIN
OF THE
KOREAN CHEMICAL SOCIETY

VOLUME 16, NUMBER 5
MAY 20, 1995

BKCS 16(5) 383-466
ISSN 0253-2964

Communications

Synthesis of Perdeuterated RNAs and Its Applications to NMR Studies

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Received December 5, 1994

Although ribonucleic acid has important biological functions, conformational studies of RNAs at atomic resolution are at a very primitive stage. Only three types of single crystal RNA structures[1~3] are known so far. Solving RNA structures by NMR is more formidable than solving DNA or protein structures of similar size mainly because spectra of RNA sugar protons overlap extensively in a narrow spectral region of 1.5 ppm. Recently, however, structures of a number of small (6-13 nucleotides long) RNA molecules[4~8] were solved at high resolution using backbone torsion angle information from homo- and heteronuclear correlated spectroscopies in addition to distance information from NOESY. Structures of a few, longer (19-31 nucleotides) RNAs[9~14] have also been determined at intermediate resolution.

Most RNA molecules which have biological functions are much larger than current 2-dimensional NMR techniques can handle, which is about 20 nucleotides. This is mainly due to extensive overlapping of sugar proton resonances. The solution to this is to introduce higher than two-dimensional and heteronuclear NMR spectroscopies on labelled samples with ^{13}C, ^{15}N, or ^{2}H. When the molecule is labelled with ^{13}C or with ^{15}N, only the labelled part of the molecule will be seen in the NMR spectrum. On the other hand, when the molecule is labelled with ^{2}H, the substituted part of the molecule will be invisible as far as NMR is concerned, so that the overcrowded spectra will be much simplified. Here, we report the synthesis of a perdeuterated RNA, and show how the spectrum of an RNA enzyme-substrate complex can be simplified by hiding the proton resonances of the perdeuterated part.

The RNA examined is an RNA enzyme of the hammerhead type which cleaves RNAs that base-pair to it (see Fig. 1). This ribozyme is derived from self-cleaving hammerhead RNAs.[15~20] The cleavage occurs under physiological conditions in the presence of Mg^{++}. Symons and coworkers were the first to propose the hammerhead model for the self-cleaving domain[18] in these RNAs (see Figure 1), and it proved to be consistent with artificially designed self-cleaving RNAs.[21~24] The cleavage reaction is a transesterification and results in the production of 2'3'-cyclic phosphate and 5'-hydroxyl termini.[17] The reaction rate increases with increasing pH and $MgCl_2$. Both Mn^{++} and Ca^{++}, but not Zn^{++}, can substitute[25] for Mg^{++}. Cleavages can occur to the 3'-sides of cytidine, adenosine, and uridine, but not guanosine.[22]

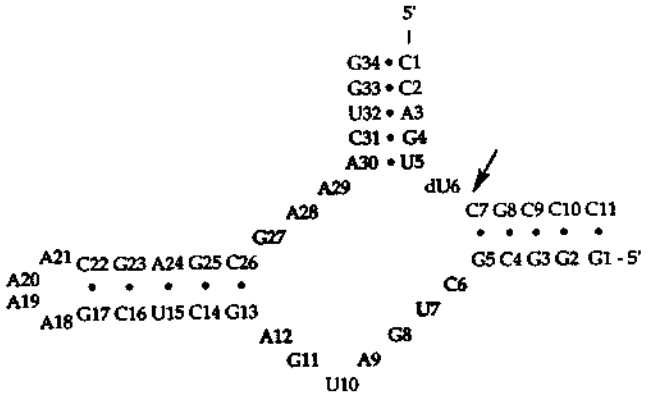

Figure 1. Secondary Structure of Hammerhead RNA: The conserved sequence is shown in box letter. The cleavage site is denoted by an arrow. The longer strand is the ribozyme and the shorter is the modified substrate. The cleavage reaction was blocked by replacing ribo-U to deoxyribo-U at the cleavage site.

Production of Perdeuterated Nucleotides. Perdeuterated RNA source was a generous gift from Dr. P.B. Moore of Yale University. The mass production of perdeuterated RNAs was achieved[26] by growing *E. coli* in 100% D_2O and by purifying rRNAs. About 40,000 ODU (optical density unit at 260 nm) ($\approx$1.3 g) of rRNAs were dialyzed extensively against a buffer containing 30 mM NaOAc, pH 5.3 and 10 μM $ZnSO_4$. The RNA concentration was adjusted to 20 ODU/mL with the same buffer, which made a reaction vol-

ume of 2 liter. About 0.2 g Nuclease P1 (from Pharmacia), which hydrolyzes RNA and DNA to nucleoside 5'-monophosphates,[27] was added to the mixture and was incubated at 37 ℃ overnight. The absorbance had increased to 30 ODU/mL by the next morning due to hyperchromic effect. The reaction mixture was incubated for additional 12 hours. No further increase in absorbance was noted. The solvent was removed by rotary evaporation and the nuclease by chromatography using a G-25 Sephadex column. About 55,000 ODU of perdeuterated 5'-NMPs were obtained from this preparation.

The extent of perdeuteration of the NMPs was checked by proton NMR spectroscopy. No notable peak except the HDO solvent peak was observed (data not shown).

Conversion from 5'-NMP to 5'-NTP. The perdeuterated nucleoside 5'-monophosphates were converted to nucleoside 5'-triphosphates (dNTP) enzymatically using the method of Whitesides and coworkers.[28,29] NMPs were converted to NDPs using commercially available adenylate kinase (AdK), guanylate kinase (GK), and nucleosidemonophosphate kinase (NMPK). AdK phosphorylates AMP,[28] CMP,[28] and, less effectively, UMP.[29] GK phosphorylates GMP, and NMPK all the four NMPs. These enzymes use ATP as phosphoryl donor. A single enzyme, pyruvate kinase (PK) converts all NDPs to NTPs using phosphoenolpyruvate (PEP) as its phosphate source.

About 17,000 ODU perdeuterated NMPs, 0.5 g $MgCl_2$ and 0.2 g KCl were dissolved in 30 mL Tris-HCl buffer, pH 7.6. The mixture was degassed by purging N_2 gas for 30 min. Then 0.3 g PEP, 10 μL 2-mercaptoethanol and 30 mg ATP were added. A catalytic amount of ATP was required to start the reaction. The pH of the mixture was readjusted to 7.6. Finally, 1.5 kU PK and 4 U NMPK (both from Sigma) were added. Positive N_2 gas pressure was applied during the reaction and the pH was kept between 7.5 and 7.7 using a peristaltic pump driven by a pH controller. Concentrated HCl was added to keep the volume small. About 2 mL concentrated HCl was consumed for the first 1 hour. After 2 hours, there was no further increase in pH. To make the reaction go to completion, 8 U GK, 5 kU AdK, and additional 1 kU PK and 0.2 g PEP (all from Sigma) were added. When these were added, the pH increased slightly but not much (from 7.60 to 7.66). The pH kept constant for the next 3 hours. The reaction was stopped after 5 hours, and the perdeuterated NTPs were precipitated by adding 60 mL cold ethanol and maintaining the reaction mixture at −20 ℃ for 3 hours.

The dNTP synthesis reaction was checked by making RNAs from the product (see below), and by poly(ethylene) imine cellulose thin layer chromatography.[33,34] The reaction was about 90% complete from the TLC assay. Since the total amount of the nucleotides after the ethanol precipitation was 15,600 ODU, the yield of the final product of this reaction was $15{,}600 \times 0.9/17{,}000 = 80\%$.

Synthesis of Perdeuterated Hammerhead Ribozyme. The perdeuterated Hammerhead ribozyme was synthesized[30] using T7 RNA polymerase and a synthetic DNA template. The length of the ribozyme is 34 nucleotides long and its sequence is shown in Fig. 1. The transcription reaction condition was optimized by adjusting dNTP concentration, DNA template concentration, amount of T7 RNA polymerase, and incubation time. The following condition was used for the transcription reaction; 40 mM tris, pH 8.1, 1 mM spermidine, 0.01%(v/v) Triton X-100, 8% polyethylene glycol (MW 8000), 36 mM $MgCl_2$, 20 mM dNTP, 1.2 μM DNA template, 100 U/μL T7 RNA polymerase. The mixture was incubated at 37 ℃ for 2 hours. The RNA transcript was phenol extracted, ethanol precipitated, and purified using 20% polyacrylamide gel electrophoresis (denaturing condition). About 336 ODU (0.97 μmole) perdeuterated ribozyme was made from three 10 mL transcription reactions, resulting in the yield of 11 ODU/mL reaction. The biological activity of the ribozyme was confirmed by the cleavage reaction of a substrate (see Fig. 2).

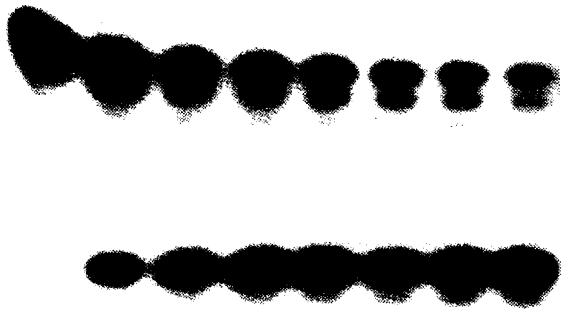

Figure 2. Cleavage of RNA by Perdeuterated Ribozyme: Sequence of the RNA substrate is shown in Fig. 1 except that the nucleotide at the cleavage site is not modified so that it can be cleavable by the ribozyme. The substrate was ^{32}P-labelled at the 5'-end was incubated with the perdeuterated ribozyme in a buffer containing 50 mM sodium cacodylate, pH 6.5 and 20 mM magnesium chloride at 37 ℃. The incubation times are 0 min. for lane 1, 2 min. for 2, 5 min. for 3, 8 min. for 4, 13 min. for 5, 20 min. for 6, 30 min. for 7, and 1 hour for 8. The ratio of the ribozyme to the substrate is about 1.

Synthesis of Protonated RNA Substrate. A protonated RNA substrate analogue (see Fig. 1) was synthesized chemically by the Yale University School of Medicine Protein and Nucleic Acid Chemistry Facility on an Applied Biosystems 380B synthesizer. The substrate was not cleavable since the nucleotide at the cleavage site was substituted for deoxyribonucleotide (rU→dU). Cleavable substrate was also made to check the activity of the synthesized ribozyme (Fig. 2). The synthesized molecules were purified by HPLC on a Nucleogen-DEAE 60-7 ion-exchange column.

Confirmation of the Synthesized Molecules by NMR Spectroscopy. The ribozyme and the non-cleavable substrate were mixed together with 1 : 1 molar ratio. The concentrations of the RNAs were calculated from the absorbances at 260 nm and the extinction coefficients calculated from nearest-neighbor approximation.[31] The combined complex was dialyzed against 5 mM cacodylate, pH 6.5, 100 mM NaCl, and 1 mM EDTA. The RNA concentration of the NMR sample was 2 mM.

1-D NMR spectrum was taken in H_2O using 1-1 spin-echo water suppression pulse sequence.[32] Fig. 3(a) shows imino proton resonances of the ribozyme-substrate complex at 22.4 ℃. The resonances between 14.1 ppm and 11.9 ppm are from Watson-Crick base-paired imino protons, and those between 11.6 ppm and 9.6 ppm are from G·A base-paired imino protons (G11 and G27) or non-hydrogen-bonded imino pro-

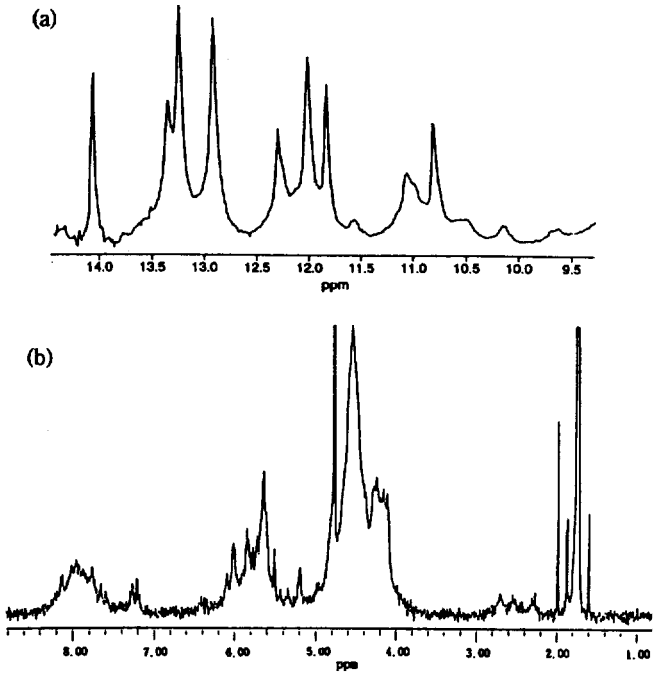

Figure 3. Proton NMR Spectra of Hammerhead RNA: The buffer condition was 5 mM sodium cacodylate, pH 6.5, 1 mM EDTA and 100 mM sodium chloride, and the RNA concentration was about 1 mM. The imino proton spectrum in H_2O(a) and the non-exchangeable proton spectrum in D_2O(b) were taken at 22.4 ℃ from 500 MHz GE Omega Spectrometer. The low signal to noise of spectrum (b) is due to small number of scans (8 scans).

tons which are protected from the exchange with solvent protons. The presence and chemical shifts of these resonances prove that the ribozyme and the substrate are base-paired as shown in Fig. 1. Fig. 3(b) shows non-exchangeable proton resonance spectrum of the complex in D_2O. The spectrum is simplified as much as one quarter; the peaks are from the substrate (11-mer) since all the protons of the ribozyme (34-mer) are substituted for deuterium.

In conclusion, the whole Hammerhead RNA was too large to be investigated by current techniques of NMR. To restrict the size of the system to be studied by NMR, the ribozyme part of the Hammerhead RNA was synthesized from perdeuterated NTPs which do not show proton NMR signals. The substrated part was synthesized chemically from regular protonated reagents. The yield of the conversion reaction (dNMP to dNTP) was 80%, and that of the transcription reaction was 11 ODU/mL reaction. The existence of the imino proton resonances confirmed correct base-pairing between the ribozyme and the substrate of the Hammerhead RNA. The simplified proton NMR spectrum of the Hammerhead RNA proves the usefulness of perdeuteration in the NMR study of RNAs larger than 30 nucleotides.

Acknowledgment. This project has started in Dr. Peter B. Moore's laboratory of Yale University. We thank Dr. Moore for providing us perdeuterated rRNAs. This work was supported by a grant (1994) from the Ministry of Science and Technology (MOST), the Republic of Korea.

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Photocycloaddition Reaction of N¹-(4-Methyl-3-pentenyl)-uracil and -thymine

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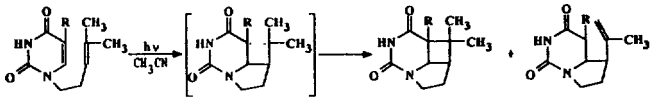
Received December 19, 1994

Intramolecular [2+2] photocycloadditions of cyclic α,β-enone tethered to suitable alkenes afford the construction of carbocyclic and heterocyclic ring systems with high regio- and/or stereoselectivity.[1] When combined with the subsequent cleavage of the cyclobutane ring of the photoproducts by various chemical means, the overall transformation constitutes an annelative two-carbon ring or carbon-hetero ring expansion of original enones. This process is now the most widely used photochemical reaction in synthetic organic chemistry.[2] In conjunction with ongoing studies in our laboratories on the synthesis of heterocyclic compounds containing diaza group in analogy with previously reported routes,[3] we now report the intramolecular equivalent of this process using N¹-(4-methyl-3-pentenyl)-uracil (**1a**) and -thymine (**1b**).

N¹-alkenylpyrimidines (**1a, 1b**) were prepared by alkylation of uracil and thymine, respectively, with 5-bromo-2-methyl-2-pentene in the presence of potassium carbonate.[3a] Direct and/or Pyrex-filtered irradiation of **1** with a 200W medium pressure Hg lamp in CH_3CN at room temperature gave on distillation of the solvent and seperation by tlc the intramolecular adduct **2**.[4]



1 4 2 3

a. R=H b. R=CH3 a. R=H b. R=CH3 a. R=H b. R=CH3

The structural assignment rests on ^1H NMR spectral data. The alternative cross adduct[2a,2b] is ruled out from consideration by careful examination of the distinguishing pattern of ^1H NMR spectrum of **2** as observed in the case of 1,3-diazatricyclo[5.2.1.0^{5,10}]decan-2,4-dione.[3a]

In contrast to the previous study with N¹-(3-butenyl)-thymine, irradiation of **1** for 10 hr afforded not only **2** but also **3** in the ratio of 1 : 8. It appears that **3** results either from a hydrogen shift in the intermediate biradical **4** or from Type II cleavage of initially formed **2**. We could also reconfirm that extensive photolysis of **2** (CH_3CN/Pyrex) leads to **3** by Type II cleavage of the bond in the cyclobutane ring initiated by a hydrogen abstraction of carbonyl group as shown in **5**. Thus, irradiation of **2** in CH_3CN with 200W

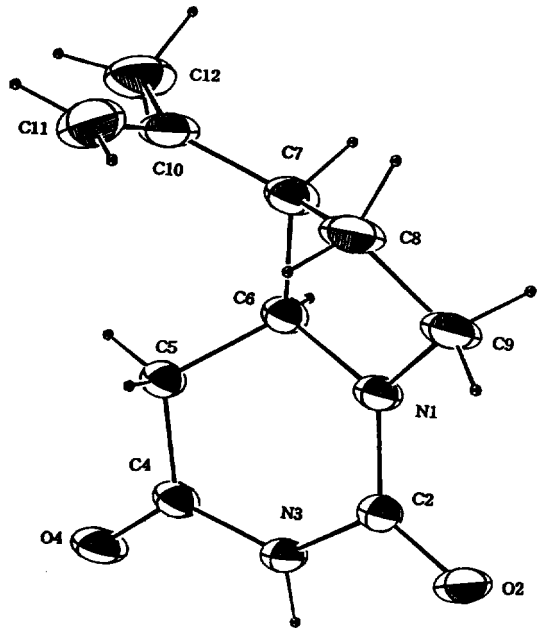


Figure 1. A Molecular Structure of 7(1-methylethenyl)-1,3-diazabicyclo[4.3.0]nonan-2,4-dione with atom labeling.

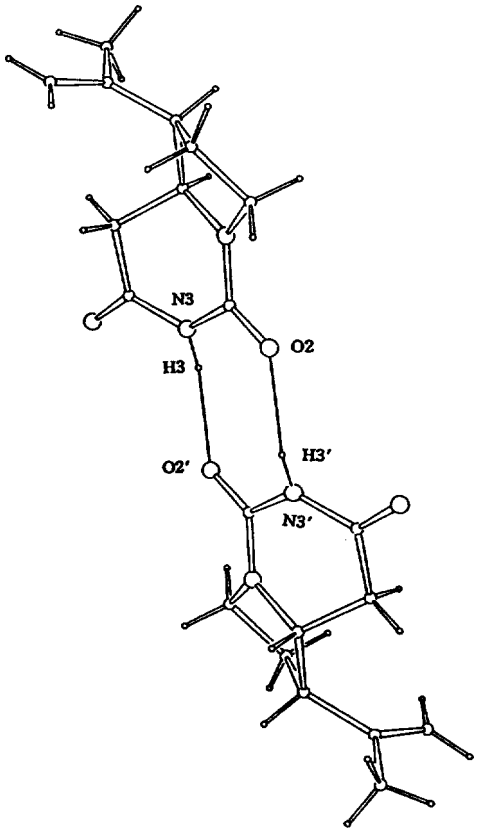


Figure 2. Hydrogen bonding interaction (thin line) between two molecules (d(N3-O2')=2.858 (2) Å, ∠(N3-H3–O2')=159.83(5)°).