ORGANIC LETTERS

2007
Vol. 9, No. 16
3057−3060

# Syntheses of (2′)3′-$^{15}N$-Amino-(2′)3′-deoxyguanosine and Determination of Their p$K_a$ Values by $^{15}$N NMR Spectroscopy

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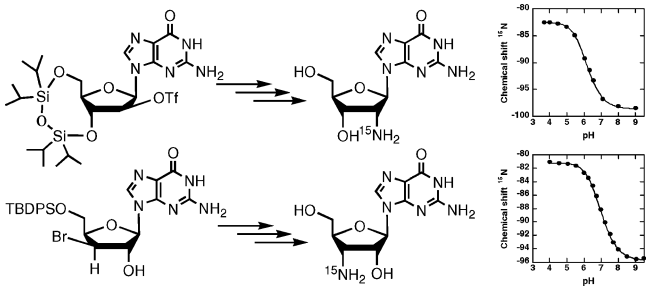
**Received May 15, 2007**

## ABSTRACT




**2′-Amino-2′-deoxyguanosine and 3′-amino-3′-deoxyguanosine are valuable probes for investigating the metal ion interactions at the active site of the group I ribozyme. However, these experiments require a thorough understanding of the protonation state of the amino group at a specific pH. Here, we describe the first syntheses of 2′-$^{15}N$-amino-2′-deoxyadenosine, 2′-$^{15}N$-amino-2′-deoxyguanosine, and 3′-$^{15}N$-amino-3′-deoxyguanosine. The $^{15}$N-enriched nucleus allows convenient and accurate determination of the amine p$K_a$ by $^{15}$N NMR.**

2′-Amino-2′-deoxynucleosides and 3′-amino-3′-deoxynucleosides (herein referred as 2′(3′)-amino-2′(3′)-deoxynucleosides) and oligonucleotides containing them have received much attention in recent years as potential therapeutic agents,[1] chemogenetic agents,[2] and diagnostic and biochemical probes. For example, 3′-amino-3′-deoxyadenosine derivatives inhibit replication of HIV-1 and serve as tailored agonists for activation of an engineered adenosine receptor.[3] As components of antisense oligonucleotides or small interfering RNAs,[4] 2′(3′)-amino-2′(3′)-deoxynucleosides have the potential to improve drug efficacy by imparting resistance to chemical and enzymatic degradation.[5] In addition to their therapeutic potential, the distinct physiochemical properties of 2′(3′)-amino-2′(3′)-deoxynucleosides render them especially powerful tools for exploring RNA structure, function, and dynamics,[6] particularly in defining the roles and environments of specific 2′- or 3′-hydroxyl groups within structured and catalytic RNA molecules.[5,7]

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10.1021/ol071129h CCC: $37.00 
**Published on Web 07/13/2007**

Our interest in 2′(3′)-amino-2′(3′)-deoxyguanosine originates from its value in mechanistic investigations of the group I ribozyme, which efficiently catalyzes nucleotidyl transfer between an oligonucleotide substrate and guanosine.[8] Previous metal ion rescue experiments[9] and crystal structures[10] have implicated metal ions in the catalytic mechanisms of exon-ligation and intron-excision. However, whether three metal ions (Figure 1A) or two metal ions (Figure 1B)

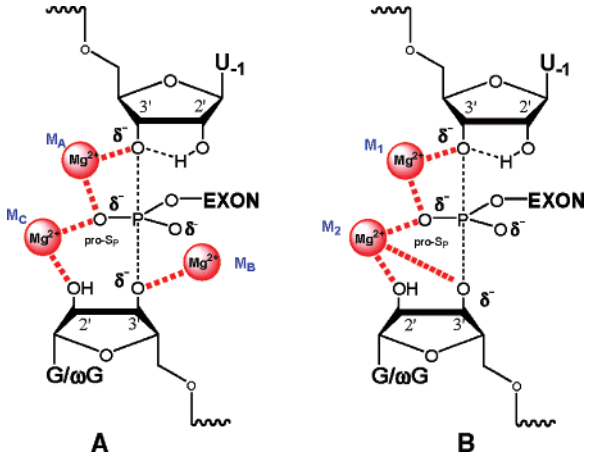


**Figure 1.** Models of the transition state for both step 1 and step 2 of the group I intron RNA splicing reaction with (A) three catalytic metals and (B) two catalytic metals. In both models, there is a metal ion, $M_A$ or $M_1$, which coordinates with the 3′-hydroxyl group of the $U_{-1}$ residue. However, in the three-metal ion model, two distinct metal ions, $M_B$ and $M_C$, coordinate with the 3′- and 2′-hydroxyl groups of guanosine, respectively, whereas in the two-metal ion model, $M_2$ coordinates with both the 3′- and 2′-hydroxyl groups.[10]

contribute to transition-state interactions remains unclear. The crystal structure of the *Azoarcus* intron suggests that the 3′-oxygen of the guanosine cofactor interacts with $M_2$ in the ground state (Figure 1B). The guanosine analogue, 3′-amino-3′-deoxyguanosine, provides a probe to test this interaction via metal ion rescue experiments in the same manner that studies using 2′-amino-2′-deoxyguanosine have established the interaction between $M_C$ and the guanosine 2′-hydroxyl group as shown in Figure 1A.[11]

Defining the interactions between 3′-amino-3′-deoxyguanosine and the ribozyme requires knowledge of the amine p$K_a$, as protonation may compete with metal ion coordinations and influence binding of the guanosine analogue to the ribozyme. Eckstein et al. have used $^{13}$C NMR to determine the ribofuranosyl amine p$K_a$ of 2′-amino-2′-deoxycytidine in a dinucleotide.[12] The corresponding p$K_a$ value for 2′-amino-2′-deoxyguanosine has not been determined by NMR but was inferred from biochemical experiments.[11] We wanted to use $^{15}$N NMR to determine the p$K_a$ values of 2′(3′)-amino-2′(3′)-deoxyguanosine, which requires the installation of a $^{15}$N-enriched amino group at the 2′(3′)-α position of guanosine. The low natural abundance of $^{15}$N (0.36%) allows facile chemical shift assignment of $^{15}$N-enriched (98%) nuclei, which yield one singlet in the $^{15}$N NMR spectrum. Moreover, the ionization state of the amine influences the $^{15}$N chemical shift, allowing a sensitive and convenient way to determine the p$K_a$ of the amino group.[13] Herein, we describe the first syntheses of 2′-$^{15}$*N*-amino-2′-deoxyadenosine, 2′-$^{15}$*N*-amino-2′-deoxyguanosine, and 3′-$^{15}$*N*-amino-3′-deoxyguanosine and determination of their p$K_a$ values using $^{15}$N NMR.

To synthesize 2′(3′)-$^{15}$*N*-amino-2′(3′)-deoxyguanosine, we considered strategies that introduce the $^{15}$N atom into the *C*-2′- or *C*-3′ position of guanosine using the commercially available $^{15}$*N*-labeled reagents. Some of these include $^{15}NH_3$, $PhCH_2{}^{15}NH_2$, and phthalimide-$^{15}$N potassium salt. Considering the nucleophilicity of these $^{15}$N-labeled reagents, the most straightforward approach would involve $S_N2$ displacement reactions with the 2′- or 3′-β-triflate derivative of guanosine. Iodide or bromide has been reported to displace the 2′-β-triflate from the 3′,5′-O-disilyl protected guanosine derivative (**1b**) to give the corresponding 2′-α-halo-2′-deoxyguanosine derivatives.[14] We recently reported that *t*-butylthiol[15] reacts with **1b** smoothly to generate a 2′-*t*-butylthio-2′-deoxyguanosine derivative. Direct $S_N2$ displacement reaction of **1b** with amines, however, has not been reported.

We first tried to introduce the 2′-amino group directly by treating **1b** with ammonia but obtained no desired product under various conditions.[16] The 2′-β-triflate derivatives of adenosine, cytosine and uridine react with phthalimide to afford the corresponding 2′-α-phthalimido analogues.[17] However, we observed no reaction for guanosine analogue **1b** under similar conditions. Based on the previous work showing that the 2′-β-triflate derivative of adenosine (**1a**) reacts with methylamine and dimethylamine,[18] we attempted

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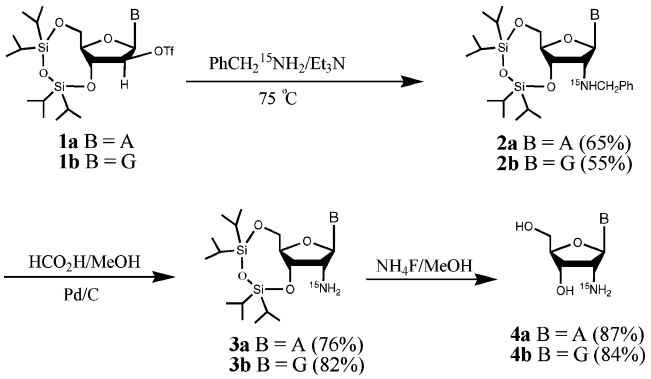
to use $PhCH_2{}^{15}NH_2$ for the synthesis of 2′-$^{15}N$-benzylamino-2′-deoxyadenosine and 2′-$^{15}N$-benzylamino-2′-deoxyguanosine, anticipating that the benzyl group could be subsequently removed by catalytic transfer hydrogenation[19] (Scheme 1).

**Scheme 1.** Syntheses of 2′-$^{15}N$-Amino-2′-deoxypurine Nucleosides

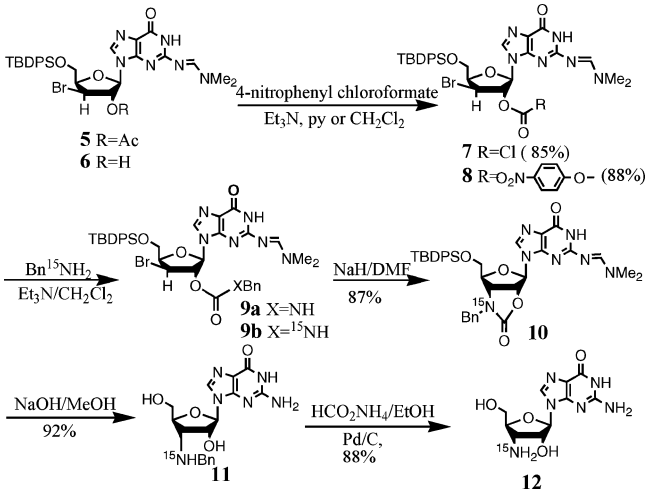



Reaction of **1a**[20] with $^{15}N$-labeled benzylamine in THF in the presence of triethylamine[21] at 75 °C for 2 days gave **2a** in 65% yield. Hydrogenolysis with formic acid and Pd/C in methanol for 6 h removed the benzyl protecting group to afford **3a** in 76% yield. Removal of the 3′,5′-silyl protecting group with TBAF generated product **4a**. It was purified by reversed-phase column chromatography but the *tetra*-butylammonium salt could not be removed completely (as indicated by $^1$H NMR). To circumvent this problem, we treated **3a** with ammonium fluoride ($NH_4F$) in methanol[22] and obtained **4a** in 87% yield, which showed no contamination of $NH_4F$ (as revealed by the absence of a $^{19}$F NMR signal) after purification by reversed-phase chromatography.

We synthesized 2′-$^{15}N$-amino-2′-deoxyguanosine (**4b**) using the same approach. Although **1b** behaved differently from **1a** in its reaction with phthalimide, we found that **1b** reacted smoothly with $^{15}N$-benzylamine in DMF[23] at 75 °C to generate **2b** in 55% yield. Direct displacement of the 2′-$\beta$-triflate from guanosine by an amine to generate the corresponding 2′-α-amino-2′-deoxyguanosine analogue has not been reported previously. We expect that other primary amines may react similarly. Removal of benzyl (82%) and silyl protecting groups (84%) in the same manner as described for the adenosine derivative afforded 2′-$^{15}N$-amino-2′-deoxyguanosine **4b** (Scheme 1).

Encouraged by the successful introduction of the 2′-$^{15}N$-benzylamino group into adenosine and guanosine, we reasoned that the 3′-$\beta$-triflate derivative of guanosine would react similarly with $^{15}N$-benzylamine to give the corresponding 3′-$^{15}N$-benzylamino-3′-deoxyguanosine derivative. However, we had difficulty in preparing the 3′-$\beta$-triflate of guanosine due to poor selectivity during protection of the 2′- and 5′-hydroxyl groups.[24] As an alternative, we prepared the corresponding 3′-$\beta$-bromo-3′-deoxyguanosine derivative (**5**, Scheme 2) from 5′-*O*-TBDPS-guanosine.[25] However, we found that treatment of **5** with benzylamine formed the 2′,3′-epoxide derivative exclusively. Probably, benzylamine first removes the 2′-acetyl group, releasing the 2′-hydroxyl group, which then attacks the *C*-3′ from the α-face.

**Scheme 2.** Synthesis of 3′-$^{15}N$-Amino-3′-deoxyguanosine




Subsequent attempts to synthesize 3′-$^{15}N$-amino-3′-deoxyguanosine were carried out by modifying Zhang's recent synthesis of 3′-amino-3′-deoxyguanosine.[25] The key steps of their synthesis include the intramolecular $S_N2$ reaction of **9a** (Scheme 2), followed by ring opening under basic conditions to introduce the 3′-benzylamino group. The intermediate **9a** was prepared by treating 3′-bromoguanosine derivative **6** with benzyl isocyanate. Unfortunately, $^{15}N$-benzyl isocyanate is not commercially available and preparing it from expensive $^{15}N$-benzylamine seemed impractical. To use $^{15}N$-benzylamine directly, we adopted a two-step strategy to introduce a $^{15}N$-labeled benzylaminocarbonyl group at the 2′-α-*C* position of **6**. Reaction of **6** with 4-nitrophenyl chloroformate[26] first gave an intermediate, which then reacted with $^{15}N$-benzylamine to give the 2′-*O*-$^{15}N$-benzylaminocarbonylguanosine analogue. We found that the reaction of **6** with 4-nitrophenyl chloroformate gave different major products depending upon the solvent. In dichloromethane, the reaction gave chloroformate **7** as the major product, while in pyridine, carbonate **8** formed as the major product (Scheme 2). Both **7** and **8** reacted readily with $^{15}N$-benzylamine in dichloromethane in the presence of triethylamine to give the key

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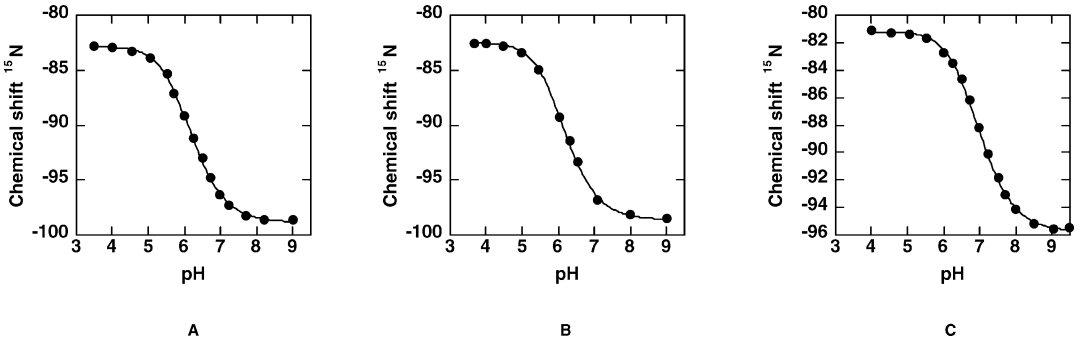


**Figure 2.** Determination of aminonucleoside p$K_a$ values by following $^{15}$N NMR chemical shifts versus pH. (A) 2′-$^{15}$*N*-amino-2′-deoxyadenosine, p$K_a$ = 6.2; (B) 2′-$^{15}$*N*-amino-2′-deoxyguanosine, p$K_a$ = 6.2; (C) 3′-$^{15}$*N*-amino-3′-deoxyguanosine, p$K_a$ = 7.0. Data were fit according to eq 1.

intermediate **9b**. Cyclization with NaH in DMF gave intermediate **10** in 87% yield.[27] Removal of the protecting groups[25] gave 3′-$^{15}$*N*-amino-3′-deoxyguanosine **12**.

To determine the p$K_a$ values by $^{15}$N NMR, we dissolved each $^{15}$*N*-labeled nucleoside in deionized water and combined the solutions with an equal volume of buffer.[28] We kept the final concentration of nucleoside in each sample (17 mM) well below the buffer concentration (250 mM).[12] We acquired $^{15}$N NMR spectra using 90% formamide in DMSO-$d_6$ as the reference. In all cases, $^{15}$N chemical shifts moved upfield with increasing pH as shown in Figure 2. The chemical shifts were plotted against pH and fit to eq 1, which was derived from the Henderson−Hasselbalch equation,[29]

$$\delta = \delta_{acid} + \delta_{base}10^{(pH-pK_a)}/[1 + 10^{(pH-pK_a)}] \qquad (1)$$

where $\delta$ is the $^{15}$N chemical shift and $\delta_{acid}$ and $\delta_{base}$ represent the chemical shift values at the low and high extremes of pH, respectively. The influence of pH on the measured $^{15}$N chemical shifts in Figure 2 follows the above equation, where the p$K_a$ value corresponds to the midpoint of the curve. In all cases, the fits gave very good correlation coefficients ($R$ > 0.999). Our data indicate that both 2′-$^{15}$*N*-amino-2′-deoxyadenosine and 2′-$^{15}$*N*-amino-2′-deoxyguanosine ionize with p$K_a$ = 6.2 (Figure 2A,B), identical to that obtained for 2′-amino-2′-deoxycytidine by $^{13}$C NMR. These observations suggest that the nucleobase exerts little influence on the p$K_a$ of the 2′-amino group, as observed for the 2′-hydroxyl group p$K_a$ in natural RNA.[30] The regioisomer 3′-$^{15}$*N*-amino-3′-deoxyguanosine ionizes with p$K_a$ = 7.0 (Figure 2C), higher than its 2′-counterpart.

In conclusion, we have efficiently synthesized 2′-$^{15}$*N*-amino-2′-deoxyadenosine and 2′-$^{15}$*N*-amino-2′-deoxyguanosine by $S_N2$ displacement of the 2′-$\beta$-triflate analogues of adenosine and guanosine with $^{15}$*N*-benzylamine. We prepared 3′-$^{15}$*N*-amino-3′-deoxy-guanosine using a two-step strategy to introduce an $^{15}$*N*-labeled benzylaminocarbonyl group at the 2′-α-*C* position by first treating **6** with 4-nitrophenyl chloroformate followed by treatment with $^{15}$*N*-benzylamine. We determined the p$K_a$ values for the ribofuranosyl amines in these nucleoside analogues by monitoring the chemical shifts of their $^{15}$N NMR spectra as a function of pH. The p$K_a$ value for 3′-amino-3′-deoxyguanosine allows us to calculate the relative concentrations of the ionized and neutral species at specific pH values. This fundamental physiochemical information will help to define the interaction of 3′-amino-3′-deoxyguanosine with the group I intron.

**Acknowledgment.** Q.D. is a Research Assistant Professor at the University of Chicago, J.L. is a Research Associate, and J.A.P. is an Investigator of the Howard Hughes Medical Institute. We thank the following members of the Piccirilli laboratory for critical comments on the manuscript: J. Ye, N-S. Li, R. Fong, J. Frederiksen, T. Novak, R. Sengupta, K. Sundaram, and S. Koo.

**Supporting Information Available:** Full experimental and analytical data and the $^1$H and $^{13}$C NMR spectra for compounds **2a**,**b**, **3a**,**b**, **4a**,**b**, and **7**−**12**. This material is available free of charge via the Internet at http://pubs.acs.org.

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(27) In the report from Zhang et al.,[25] the 5′-TBDPS silyl protecting group was also removed in this step. However, under the same reaction conditions, the protecting group survived in our experiments.

(28) The following buffers were used: sodium acetate, pH 4.5−5.50; NaMES, pH 5.75−6.50; NaMOPS, pH 6.75−7.50; NaEPPS, pH 7.75−8.50; NaCHES, pH 9.0−9.5.

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