Journal of Labelled Compounds and Radiopharmaceuticals—Vol. XXXVIII, No. 7

The First Synthesis of [9,Amino-$^{15}N_2$]Adenine and β-2'-Deoxy-[9,Amino-$^{15}N_2$]Adenosine.

Charles C. Orji and Louis A. Silks III*

NIH Stable Isotopes Resource, Los Alamos National Laboratory, Biochemistry and Spectroscopy Section, CST-4; MS C-345, Los Alamos, NM 87545.

SUMMARY

Summary: β-2'-Deoxy-[9,Amino-$^{15}N_2$]Adenosine has been constructed in 4 steps from commercially available 5-amino-4,6-dichloropyrimidine and $^{15}NH_3$. The reactions have been scaled to provide significant quantities of labeled nucleoside.

Keywords: [9,Amino-$^{15}N_2$]Adenine, β-2'-Deoxy-[9,Amino-$^{15}N_2$]-Adenosine, $^{15}NH_3$.

INTRODUCTION

^{15}N Labeled oligonucleotides have proven to be invaluable probes of nucleic acid structure, drug-binding, and protein-nucleic acid interactions[1]. Many of the single ^{15}N labeled purines and purine-based nucleosides have been constructed (β-2'-deoxy-[1-^{15}N], [amino-^{15}N], [3-^{15}N], [9-^{15}N] and [7-^{15}N]adenosines; β-2'-deoxy-[1-^{15}N], [2-^{15}N], [3-^{15}N], and [7-^{15}N]-guanosines) in sufficient quantity to be useful for chemical construction of oligonucleotides[2]. However, there are very few examples of the preparation of nucleosides which are multiply and specifically labeled with ^{15}N.

We have been interested in the construction of site-specifically ^{15}N labeled 2'-deoxynucleosides. Our first report described a large scale synthesis of β-2'-deoxy[amino-^{15}N]adenosine, which we viewed as a common precursor for a series of multiply labeled purine 2'-deoxynucleosides[3]. Moreover, we have been successful in the first synthesis of β-2'-Deoxy-[9-^{15}N]-adenosine[4]. We now report the first synthesis of doubly ^{15}N-labeled [9,Amino-$^{15}N_2$]adenine and β-2'-Deoxy-[9,Amino-$^{15}N_2$]-adenosine.

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*Author for correspondence. (505-667-0151; e-mail address pete-silks@lanl.gov)]

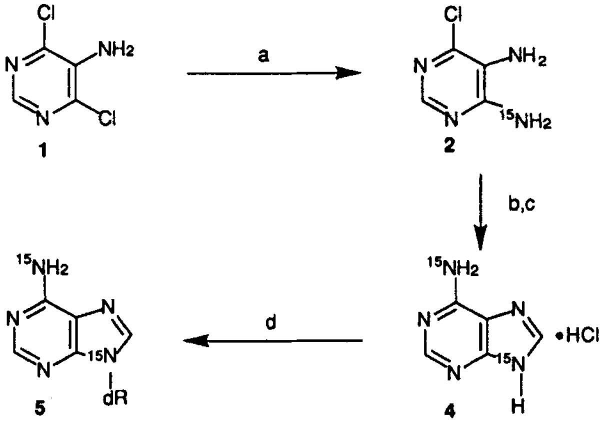
CCC 0362–4803/96/070625–06

Received 30 October 1995
Revised 27 December 1995

RESULTS AND DISCUSSION

To accomplish the synthesis of the title compounds conversion of 5-amino-4,6-dichloropyrimidine to 4-chloro-[5-^{14}N,6-^{15}N]diaminopyrimidine was necessary (Scheme 1). Treatment of **1** with 4 mol equivalents of $^{15}NH_4OH$ at 120-140°C and a pressure of ~100 psi over a period of 7 h gave the monosubstituted product **2** in 95% yield. Recovery of the excess $^{15}NH_3$ was accomplished by simply trapping it with 1 M HCl. Examination of the ^{13}C NMR of **2** revealed four resonances. The C6 resonance, as expected, was split into a doublet ($^1J_{C\text{-}N}$ 20). The isotopic content of **2** was determined by integrating the carbon signals from the C6 singlet (arising from the remaining ^{14}N isotopomer) and the C6 ^{13}C-^{15}N doublet. This process indicated an enrichment greater than 98%, which is in close agreement with the enrichment level of the $^{15}NH_3$. Based on this result, we concluded that dilution of the label at C6 by a Dimroth ring opening/closing process did not take place.[5]

Scheme 1

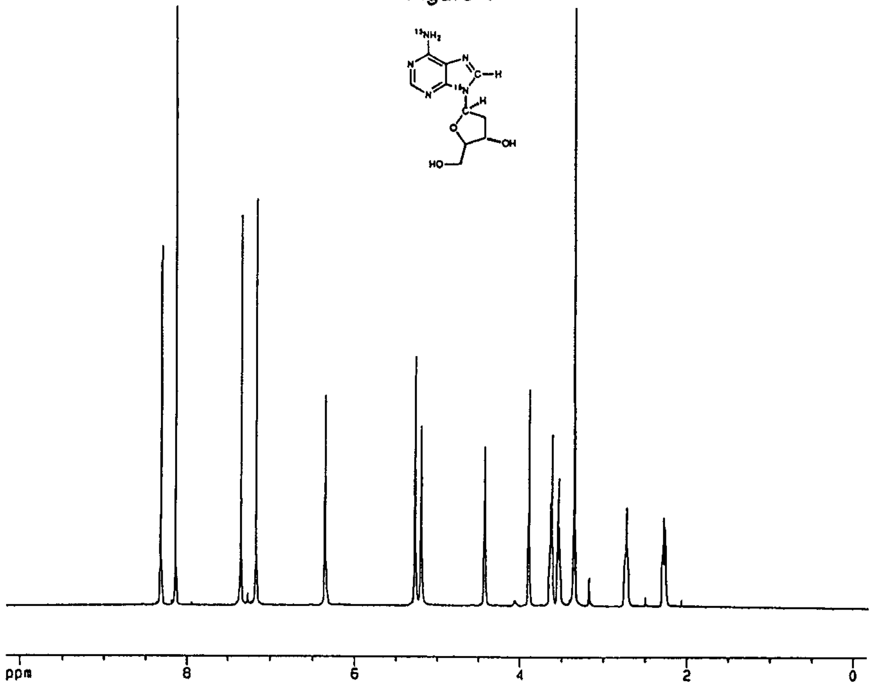


dR = β-2-deoxyribose

Scheme 1 *Reagents:* (a) 26% $^{15}NH_4OH$, 120 °C (97%); (b) diethoxymethylacetate (DEMA) (86-90%); (c) $^{15}NH_4OH$ (95%) followed by 1 M HCl; (d) thymidine, thymidine phosphorylase, nucleoside phosphorylase (81% for both c and d).

The annulation reaction of **2** can be performed in neat DEMA at 100 °C for 3.5 h. Purification gave a 90% yield of the desired product **3**. The addition of $^{15}NH_4OH$ to **3** was carried out in a stainless steel Parr 50 mL reaction vessel that was fitted with a pressure gauge and an internal thermocouple. The reaction was monitored for completion using thin layer chromatography (methanol/methylene chloride; 30% v/v). Upon completion the reaction vessel was cooled and connected to a solution of 1.0 M HCl. The pressure was released and the excess ammonia was then trapped with 1 M HCl. The pressure was released and the excess ammonia was then trapped with 1 M HCl (78% recovery[6]). The resulting solution was concentrated *in vacuo* to give a yellow solid. Acidification of the crude adenine with 1.0 M HCl allowed for the isolation of the salt from water/methanol mixtures as a yellow precipitate. Leonard and coworkers[7] have investigated the propensity of adenine to undergo a Dimroth type of rearrangement under autoclaving conditions to give mixtures of [amino-^{15}N] and [1-^{15}N] adenine in varying yields. Their results suggest that these conditions promoted some pyrimidine ring opening between N1 and C2 and reclosure to either N1 or N6 (amino group). These authors report that after 48 h (120°C), 24% scrambling was apparent (i.e., 12 % of the ^{15}N label appeared at N1). In addition, for the rearranged material, they report that H2 possessed a coupling constant of J = 16 Hz. Based on Leonard's report we have identified, upon extended reaction time and temperatures greater than 150 °C, what appeared to be a small amount (~2-5%) of the rearranged product (by ^{1}H NMR). Therefore, to suppress this rearrangement, we recommend that reaction times always be less than 12 h. The crude **4** was carried through the next reaction. Enzymatic β-ribosylation (effected with thymidine phosphorylase, nucleoside phosphorylase, and thymidine) over a period of four days, followed by purification, gives the β-2'-deoxy-[9,amino-^{15}N$_2$]adenosine **5** in 81% yield for two steps. The ^{1}H NMR spectrum of **5** is illustrated in Figure 1 (3.36 ppm is water). It clearly shows the presence of ^{15}N at positions N9 and NH$_2$ *via* the $^2J_{N9\text{-}H8}$ (8.33 ppm) and $^1J_{N\text{-}H}$ amino couplings (7.26 ppm).

Figure 1



^{1}H NMR spectrum of β-2'-deoxy-[9,amino-^{15}N$_2$]adenosine

CONCLUSION

This four-step process constitutes an efficient synthesis of the previously unreported β-2'-deoxy-[9,amino-$^{15}N_2$]adenosine. The process uses an economical source of ^{15}N, does not require any protection or deprotection steps, and the excess isotope used in the reaction can be recovered as its HCl salt[6]. Moreover, these multiply site-specifically labeled nucleic acids are essential for the investigation of both the structure and function of nucleic acid biomolecular complexes by nuclear magnetic resonance spectroscopy experiments. We are currently exploring the feasibility of applying this route to the synthesis of β-2'-deoxy [8-^{13}C; 9,amino-$^{15}N_2$]adenosine and these results will be reported in due course.

Chemicals--$^{15}NH_3$ (99.2% ^{15}N) was prepared at Los Alamos National Laboratory. 5-Amino-4,6-dichloropyrimidine was purchased either from Aldrich Chemical Co. or Fluka. Thymidine, thymidine phosphorylase, and purine nucleoside phophorylase were purchased from Sigma Chemical Co. Compound **2** has been synthesized and characterized.[4]

NMR Methods--The ^{1}H, ^{13}C, and ^{15}N NMR spectra were recorded as DMSO-d6 or D_2O solutions on Bruker AM-200, AC-250, WM-300, or AMX-500 NMR spectrometers. ^{1}H chemical shifts are expressed in parts per million with respect to tetramethylsilane at 0.0 ppm; ^{13}C chemical shifts are referenced with respect to internal $CDCl_3$ (d = 77.0 ppm with respect to tetramethylsilane at 0.0 ppm), DMSO (39.5 ppm), CD_3OD (49.0 ppm), or D_2O (external reference doped with methanol); ^{15}N NMR chemical shifts are referenced with respect to 2.5 M solution of potassium [^{15}N]nitrate.

[9,Amino-$^{15}N_2$]adenine•HCl (4)--To 6-chloro[9-^{15}N]purine (0.540 g, 3.47 mmol) in a 50 mL Parr stainless steel reactor was added $^{15}NH_3$ solution [0.960 g of a 26% aqueous solution (0.250 g, 13.87 mmol), 4 mol equivalent] and ethanol (3.5 mL) and the reaction was heated to 140 °C for 7 h. The warm reaction mixture (to ensure complete transfer of unreacted $^{15}NH_3$) was vented into cold 1M HCl solution contained in two flasks in tandem. The crude reaction mixture was acidified with 2M HCl. Silica gel was added and the solvent was removed *in vacuo* using a rotary evaporator equipped with a clean, liquid nitrogen-cooled trap. The dry, sample-coated silica gel was applied to a silica gel column and eluted with $CH_3OH:NH_4OH:CH_2Cl_2$ (25:5:70; v/v). Removal of solvent *in vacuo* gave 0.68 g of the title compound as the hydrate. This material was used in the ribosylation reaction without further purification. Recovery of $^{15}NH_4Cl$ was performed in the following manner. The silica gel column (previously purged with N_2) was eluted with water and the eluent was added to the combined 1M HCl solutions. Also, the rotary evaporator trap was washed with 1M HCl solution and the wash solution was added to this mixture. After removal of solvent *in vacuo*, the recovered crude $^{15}NH_4Cl$ was crystallized from H_2O/ethanol to give 0.470 g

(78%). ^{1}H (NaOD/D$_2$O) δ 8.06 (d, $^{2}J_{1H\text{-}15N}$ = 12, 1H), 8.22 (s, 1H); ^{13}C (NaOD/D$_2$O) δ 120.96 (s), 150.31 (d), 153.43 (s), 155.00 (d, J 18.36), 160.30 (d, J 4.8). ^{15}N (NaOD/D$_2$O) δ -149.57 (d, $^{2}J_{1H\text{-}15N}$ = 12, N9), -307.45 (s, amino).

β-2'-Deoxy-[9,amino-^{15}N$_2$]adenosine (5)--To a 250 mL round bottom flask was added the crude [9,amino-^{15}N$_2$]adenine hydrochloride hydrate (0.680 g), thymidine (3.32 g, 13.7 mmol), 20 mM KH$_2$PO$_4$ (46 mL). The mixture was stirred for 5 min and the pH adjusted to 7.4 with 1 M KOH. Thymidine phosphorylase (44 units) and purine nucleoside phosphorylase (79 units) were added and the reaction mixture was stirred at 41-44 °C for 4 days. We feel that the progress of the reaction should be monitored using reverse phase HPLC for completion (Beckman Ultrasphere Ion Pair C18 column 4.5 mm x 15 cm; buffer was 83.3 mM triethyl ammonium bicarbonate, 4% methanol, and pH = 7. The following gradient was used: 0-1 min 100% buffer, 1-15 min 0-15% acetonitrile, 15-25 min 15-30% acetonitrile. The [9,amino-^{15}N$_2$]adenine possessed a retention time of 7 min and the β-2'-deoxy-[9,amino-^{15}N$_2$]adenosine possessed a retention time of 10 min). The reaction mixture was evaporated to give a residue which was triturated several times with methanol. The methanol soluble portion of the product was separated by flash column chromatography [MeOH:CH$_2$Cl$_2$ (20:80; v/v)]. Removal of the solvents from the combined column fractions *in vacuo* gave 0.710 g (81% overall yield based on 6-chloro-[9-^{15}N]purine) of the title compound. ^{1}H (DMSO-d6) δ 2.25-2.30 (m, 1H, H2'), 2.70-2.76 (m, 1H, H2''), 3.52- 3.56 (m, 1H, H5), 3.62-3.66 (m, 1H, H5), 3.90 (q, J 2.3, 1H, H3'), 4.41-4.44 (m, 1H, H4'), 5.20 (t, J = 5.5, 1H, OH5), 5.28 (d, J = 3.7, OH3), 6.35 (t, J = 7.3 Hz, 1H, H1'), 7.26 (d, J = 90.2, 1H, ^{15}NH$_2$), 8.14 (s, 1H, H2), 8.33 (d, J = 8.2, 1H, H8); ^{13}C (DMSO-d6) δ 39.6 (C2'), 61.8 (C5'), 70.9 (C3'), 83.9 (d, J = 10.8, C1'), 87.9 (C4'), 119.2 (dd, ^{2}J = 2.6, 8.4 C5), 139.5 (d, J = 10.3, C4), 148.8 (d, J = 19.4, C8), 152.3 (s, C2), 156.0 (d, J = 20.5, C6); ^{15}N (DMSO-d6) δ -201.0 (d, J = 7.8, N9), -292.1 (d, J = 90.1, amino). IR ν (cm^{-1}) 3297, 3109, 1635, 1599, 1575, 1204, 1150, 1094, 1056. Analysis for $C_{10}H_{13}{}^{14}N_3{}^{15}N_2O_3{\cdot}H_2O$: Calcd. C, 44.28; H, 5.57; N, 26.55. Found C, 44.40; H, 5.50; N, 26.24.

Acknowledgment. This work was supported by the National Stable Isotopes Resource, NIH Division of Research Resources (RR 02231) and the Los Alamos National Laboratory Directed Research And Development funding (LDRD XL2G).

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