High-Yielding Preparation of [3-^{15}N]Cytidine, [4-^{15}NH$_2$]Cytidine, and [3-^{15}N,4-^{15}NH$_2$]Cytidine

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The specific ^{15}N labeling of oligonucleotides has been very useful as a probe in NMR studies aimed at elucidating nucleic acid structures,[1] nucleic acid interactions with proteins or drugs,[2] and local interactions at crucial nitrogen atoms,[3] as well as at providing direct evidence of individual hydrogen bonds in Watson−Crick nucleobase pairs.[4] Two labeling tactics have been reported: (i) introduction of the exocyclic ^{15}N labels in the deprotection step of a modified oligonucleotide[2b,d,5] and (ii) preparation of labeled nucleosides—either chemically[1c,2a,c,3,6] or by microbial fermentation[1d,4,7]—followed by their incorporation into DNA or RNA oligomers. This second approach has stimulated the search for efficient chemical syntheses of ^{15}N-labeled nucleosides. In fact, almost all the nucleosides with ^{15}N in relevant sites have been prepared, either by total synthesis[6c,8] or by chemical transformation of functionalized nucleosides.[9,10]

Selective labeling of the amino group of cytidines has been easily achieved by conversion of uridine to C4-activated derivatives, followed by treatment with $^{15}NH_3$[8e,10c] or with [^{15}N]phthalimide,[10a,b] which was then converted to an amino group. On the other hand, selective labeling of cytidine at N3 has been carried out by total synthesis: Niu obtained isopropylidene-protected [1,3-^{15}N]cytidine in eight steps starting from [$^{15}N_2$]urea and propynoic acid,[8k] while Strazewski and co-workers[8e] prepared [$^{15}N_3$,2-^{17}O]cytidine likewise. Since N3 of cytidine is involved in many hydrogen bond interactions including triple helix formation,[11] practical, alternative routes to [3-^{15}N]cytidine and [3-^{15}N,4-^{15}NH$_2$]cytidine would be welcome. We report herein on such a method, from uridine as the starting material and $^{15}NH_4Cl$ as a cheap label source.

2′,3′,5′-*O*-Triacetyluridine (**1**), obtained in quantitative yield from uridine, was converted to its *N*-nitro derivative (**2**, 96%),[9d] which as shown in Scheme 1 was treated with $^{15}NH_4Cl$ (1.3 equiv), KOH, and Et_3N in CH_3CN-H_2O for 6 days at room temperature, to afford [3-^{15}N]-2′,3′,5′-*O*-triacetyluridine (**1***) as the major compound; since small amounts of deacetylated products are detected, an in situ reacetylation step was quite convenient to improve the yield (76% overall). Despite the fact that protecting groups other than acetyl are also stable under the nitration conditions and treatment with ammonia,[12] **1** is the substrate of choice because of the ease of the protection and deprotection steps. Formation of **1*** relies on a simple ring-opening/ring-closing process, via a reaction intermediate arising from the attack of $^{15}NH_3$ at C4;[9d] the open-chain intermediate cyclizes slowly, with

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(12) For example, by using 5′-*O*-acetyl-2′,3′-*O*-isopropylidene-3-nitrouridine as the substrate and only 1.1 equiv of $^{15}NH_4Cl$, the labeled product was obtained in 75% yield (together with only 9% of deacetylated product).

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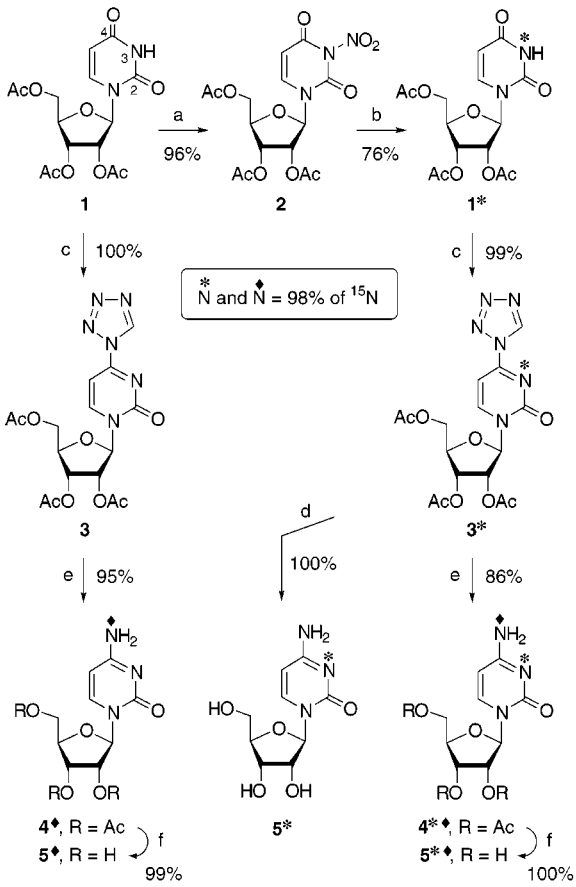
Scheme 1[a]



[a] Reagents and conditions: (a) $(CF_3CO)_2O/NH_4NO_3$, CH_2Cl_2, 0 °C; (b) $^{15}NH_4Cl$ (1.3 equiv), KOH (1.1 equiv), Et_3N (1.3 equiv), CH_3CN-H_2O, rt, 6 days, then Ac_2O, pyridine, rt; (c) tetrazole, TsCl, diphenyl phosphate, pyridine, rt, 1.5 days; (d) aq NH_3, 1,4-dioxane, rt, 16 h; (e) $^{15}NH_4Cl$, KOH, Et_3N, CH_3CN-H_2O, rt, 24 h; (f) NH_3, MeOH, rt, 6 h.

loss of $HNNO_2^-$ (nitramide anion, which readily decomposes to give N_2O), to regenerate the six-membered ring.

Activation of C4 of **1** and **1*** as their tetrazolyl derivatives (**3** and **3***, respectively) was achieved quantitatively by the method of Reese and Ubasawa.[13] Conversion of **3*** to [3-^{15}N]-cytidine (**5***) was accomplished quantitatively by ammonolysis in dioxane (73% overall yield from uridine). The proton-decoupled ^{13}C NMR spectrum of **5*** showed the expected doublets for C2 and C4 ($^1J_{CN}$ = 6.7 and 5.5 Hz, respectively), and the proton-coupled ^{15}N NMR spectrum was a singlet at δ −155.1.

Introduction of label at the C4 amino group generally requires a large excess of the isotope-containing precursor,[8e,10c] with one exception, as Kamaike et al.[10a,b] used only 1.5 equiv of [^{15}N]phthalimide. However, our experience indicates that such a labeling can be carried out with nearly stoichiometric amounts of $^{15}NH_4Cl$ in comparable yields, thus avoiding further transformations (cleavage of the phthalyl protecting group). In practice, reaction of $^{15}NH_4Cl/KOH/Et_3N$ with the parent tetrazolyl derivative **3** (1.2 equiv) in CH_3CN-H_2O at room temperature afforded [4-$^{15}NH_2$]-2′,3′,5′-tri-*O*-acetylcytidine (**4♦**) in 95% yield at a multigram scale, using standard glassware and septum equipment. Interestingly, acetate hydrolysis and/or ammonolysis were not observed. A final treatment of **4♦** with an excess of methanolic ammonia furnished quantitatively [4-$^{15}NH_2$]cytidine (**5♦**).

The same straightforward procedure was applied to the synthesis of double-labeled cytidine (**5*♦**). As shown in Scheme 1, tetrazolyl derivative **3*** was converted to **4*♦** in 86% yield by using only 1.0 equiv of $^{15}NH_4Cl$/KOH and 1.1 equiv of Et_3N in CH_3CN-H_2O. Final deprotection with an excess of methanolic ammonia afforded quantitatively the double-labeled cytidine **5*♦**. A singlet at δ −156.4 and a triplet at δ −270.3 ($^1J_{NH}$ = 90 Hz) in the proton-coupled ^{15}N NMR spectrum and the splitting of C2 (dd, $^1J_{CN}$ = 6.7 Hz, $^4J_{CN}$ = 3.7 Hz) and C4 (dd, $^1J_{CN}$ = 19.8 Hz, $^1J_{CN}$ = 5.5 Hz) in the ^{13}C NMR spectrum confirmed the incorporation of the second label.

In summary, starting from uridine and employing only nearly equivalent amounts of $^{15}NH_4Cl$, cytidines labeled either at N3, at the amino group, or at both these nitrogen atoms have been obtained in good-to-excellent yields.

Experimental Section

For general methods, see ref 9d. Coupling constants (*J*) are given in hertz. ^{15}N NMR chemical shifts are referred to external concentrated $H^{15}NO_3$ (negative values upfield). HRMS were registered in the FAB positive mode by the C.A.C.T.I., Universidad de Vigo. $^{15}NH_4Cl$ was purchased from Aldrich (98% of label).

[3-^{15}N]-2′,3′,5′-Tri-*O*-acetyluridine (1*). $^{15}NH_4Cl$ (209 mg, 3.84 mmol) and KOH (85%, 234 mg, 3.54 mmol) were placed in a 50-mL round-bottomed flask and sealed with a septum. Then, water (7.5 mL), CH_3CN (7.5 mL), Et_3N (535 μL, 3.84 mmol), and a solution of 2′,3′,5′-tri-*O*-acetyl-3-nitrouridine (**2**, 1.227 g, 2.95 mmol)[9d] in CH_3CN (15 mL) were added sequentially, avoiding leakage of ammonia. After 6 days of vigorous stirring, the mixture was saturated with NaCl and extracted with CH_2Cl_2. The aqueous layer was evaporated, and the residue was triturated with CH_2Cl_2−MeOH (9:1). The combined organic layer was dried over anhydrous Na_2SO_4, and the solvent was removed. The residue was dissolved in pyridine (12 mL), and Ac_2O (850 μL, 9.0 mmol) was added and stirred for 3 days. Then, MeOH (2 mL) was added, and the solvent was removed. The residue was purified by column chromatography with ethyl acetate as the eluent to yield [3-^{15}N]-2′,3′,5′-tri-*O*-acetyluridine (**1***, 838 mg, 76%) as colorless crystals: mp 128−129 °C; ^1H (300 MHz, $CDCl_3$) δ 2.10 (s, 3 H), 2.14 (s, 3 H), 2.15 (s, 3 H), 4.34−4.37 (m, 3 H), 5.34−5.36 (m, 2 H), 5.81 (dd, *J* = 8.2, 2.4, 1 H), 6.05 (d, *J* = 4.9, 1 H), 7.42 (d, *J* = 8.2, 1 H), 9.65 (d, *J* = 91.0, 1 H); ^{13}C (75.4 MHz, $CDCl_3$) δ 20.3, 20.4, 20.7, 63.1, 70.1, 72.6, 79.8, 87.4, 103.3 (d, *J* = 7.0), 139.3, 150.2 (d, *J* = 18.3), 162.9 (d, *J* = 9.5), 169.6, 169.6, 170.1; ^{15}N (30.4 MHz, $CDCl_3$) δ −214.9 (dd, *J* = 91.0, 2.4); HRMS, calcd for $C_{15}H_{19}N^{15}NO_9$ $[M + 1]^+$ 372.1061, found 372.1078.

[3-^{15}N]-4-(Tetrazol-1-yl)-1-(2′,3′,5′-tri-*O*-acetyl-β-D-ribofuranosyl)pyrimidin-2(1*H*)-one (3*). Compound **1*** (594 mg, 1.6 mmol), tetrazole (224 mg, 3.2 mmol), diphenyl phosphate (480 mg, 1.92 mmol), and tosyl chloride (610 mg, 3.2 mmol) were dissolved in pyridine (4 mL) and stirred at room temperature for 1.5 days.[10a,13] Afterward, 1 mL of water was added, and the solution was poured into aqueous Na_2CO_3 (saturated) and extracted with CH_2Cl_2. The solvent was dried (Na_2SO_4) and removed by coevaporation with toluene. The residue was purified by column chromatography (CH_2Cl_2−MeOH 95:5) to afford **3*** (670 mg, 99%) as a yellow foam: ^1H (300 MHz, $CDCl_3$) δ 2.10 (s, 3 H), 2.15 (s, 3 H), 2.17 (s, 3 H), 4.39−4.53 (m, 3 H), 5.29 (dd, *J* = 6.4, 5.5, 1 H), 5.50 (dd, *J* = 5.5, 3.7, 1 H), 6.12 (d, *J* = 3.7, 1 H), 7.26 (d, *J* = 7.2, 1 H), 8.40 (d, *J* = 7.2, 1 H), 9.63[14] (s, 1 H); ^{13}C (75.4 MHz, $CDCl_3$) δ 20.3, 20.4, 20.7, 62.4, 69.4, 73.7, 80.2, 90.0, 95.4 (d, *J* = 2.4), 140.7,[14] 147.3, 153.6 (d, *J* = 6.1), 157.5 (d, *J* = 8.2), 169.4, 169.4, 170.0; ^{15}N (30.4 MHz, $CDCl_3$) δ −120.8; HRMS, calcd for $C_{16}H_{19}N_5{}^{15}NO_8$ $[M + 1]^+$ 424.1235, found 424.1233.

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[3-^{15}N]Cytidine (5*). Aqueous ammonia (32%, 1 mL) was added to a solution of **3*** (98 mg, 0.23 mmol) in 1,4-dioxane (1 mL). The mixture is stirred for 16 h at room temperature, and the solvent was removed. The residue was heated in a vacuum oven at 100 °C for 2 h, to remove acetamide and tetrazole, to afford **5*** (57 mg, quantitative yield): mp 216 °C (dec); ^{1}H (300 MHz, DMSO-d_6) δ 3.53 (dd, J = 12.2, 3.4, 1 H), 3.64 (dd, J = 12.2, 3.3, 1 H), 3.80 (m, 1 H), 3.90−3.96 (m, 2 H), 4.94 (d, J = 5.1, 1 H), 5.01 (t, J = 5.2, 1 H), 5.24 (d, J = 5.1, 1 H), 5.70 (d, J = 7.3, 1 H), 5.76 (d, J = 3.7, 1 H), 7.11 (s, 1 H), 7.16 (s, 1 H), 7.83 (d, J = 7.3, 1 H); ^{13}C (75.4 MHz, DMSO-d_6) δ 60.8, 69.6, 74.2, 84.2, 89.4, 94.0 (br), 141.7, 155.6 (d, J = 6.7), 165.7 (d, J = 5.5); ^{15}N (30.4 MHz, DMSO-d_6) δ −155.1; HRMS, calcd for $C_9H_{14}N_2{}^{15}NO_5$ $[M + 1]^+$ 245.0904, found 245.0916.

[4-^{15}NH$_2$]-2′,3′,5′-Tri-*O*-acetylcytidine (4♦). $^{15}NH_4Cl$ (272 mg, 5.0 mmol) and KOH (85%, 330 mg, 5.0 mmol) were placed in a 50-mL round flask and sealed with a septum. Water (10 mL), CH_3CN (10 mL), Et_3N (770 μL, 5.5 mmol), and a solution of 4-(tetrazol-1-yl)-1-(2′,3′,5′-tri-*O*-acetyl-β-D-ribofuranosyl)pyrimidin-2(1*H*)-one[10a,13] (**3**, 2.619 g, 6.2 mmol) in CH_3CN (20 mL) were then added sequentially with syringe equipment, avoiding leakage of ammonia. After vigorous stirring for 24 h, the solvent was removed, and the residue was purified by column chromatography (CH_2Cl_2−MeOH 9:1) to obtain **4♦** (1.756 g, 95%) as a white foam: ^{1}H (300 MHz, $CDCl_3$) δ 2.09 (s, 3 H), 2.10 (s, 3 H), 2.12 (s, 3 H), 4.28−4.41 (m, 2 H), 5.40 (dd, J = 5.8, 5.5, 1 H), 5.47 (dd, J = 5.8, 4.0, 1 H), 5.90 (d, J = 4.0, 1 H), 5.99 (d, J = 7.6, 1 H), 6.71 (br d, J = 89, 1 H), 7.39 (d, J = 7.6, 1 H), 8.27 (br d, J = 89, 1 H); ^{13}C (75.4 MHz, $CDCl_3$) δ 20.5, 20.5, 20.7, 63.0, 70.0, 73.4, 79.1, 90.0, 96.1 (br), 141.0, 155.6 (d, J = 3.7), 166.1 (d, J = 20.7), 169.6, 169.7, 170.4; ^{15}N (30.4 MHz, $CDCl_3$) δ −274.9 (t, J = 89); HRMS, calcd for $C_{15}H_{20}N_2{}^{15}NO_8$ $[M + 1]^+$ 371.1221, found 371.1238.

[4-^{15}NH$_2$]Cytidine (5♦). Saturated ammonia in MeOH (23 mL) was added to a solution of **4♦** (1.736 g, 4.69 mmol) in MeOH (23 mL). Stirring for 6 h at room temperature and solvent removal in a vacuum afforded a foam that by coevaporation twice with MeOH and once with CH_2Cl_2−MeOH, followed by purification by heating in a vacuum oven at 100 °C for 2 h to remove acetamide, gave **5♦** (1.140 g, 99% yield) as a white powder: mp 216 °C (dec); ^{1}H (300 MHz, DMSO-d_6) δ 3.53 (dd, J = 12.2, 3.4, 1 H), 3.64 (dd, J = 12.2, 3.3, 1 H), 3.80 (m, 1 H), 3.90−3.96 (m, 2 H), 4.94 (d, J = 5.1, 1 H), 5.01 (t, J = 5.2, 1 H), 5.24 (d, J = 5.1, 1 H), 5.68 (d, J = 7.4, 1 H), 5.74 (d, J = 3.6, 1 H), 7.06 (d, J = 89.0, 1 H), 7.12 (d, J = 89.0, 1 H), 7.82 (d, J = 7.4, 1 H); ^{13}C (75.4 MHz, DMSO-d_6) δ 60.8, 69.6, 74.2, 84.3, 89.4, 94.2 (d, J = 4.0), 141.9, 155.4 (d, J = 3.7), 165.5 (d, J = 19.8); ^{15}N (30.4 MHz, DMSO-d_6) δ −271.8 (t, J = 89.0); HRMS, calcd for $C_9H_{14}N_2{}^{15}NO_5$ $[M + 1]^+$ 245.0904, found 245.0916.

[3-^{15}N,4-^{15}NH$_2$]-2′,3′,5′-Tri-*O*-acetylcytidine (4*♦). $^{15}NH_4Cl$ (55 mg, 1.0 mmol) and KOH (85%, 66 mg, 1.0 mmol) were placed in a 10-mL round flask and sealed with a septum. Water (2 mL), CH_3CN (2 mL), Et_3N (153 μL, 1.1 mmol), and a solution of **3*** (423 mg, 1.0 mmol) in CH_3CN (4 mL) were then added sequentially with syringe equipment, avoiding leakage of ammonia. After vigorous stirring for 24 h, the solvent was removed, and the residue was purified by column chromatography (CH_2Cl_2−MeOH, 9:1) to obtain **4*♦** (320 mg, 86%) as a white foam: ^{1}H (300 MHz, $CDCl_3$) δ 2.09 (s, 3 H), 2.10 (s, 3 H), 2.12 (s, 3 H), 4.28−4.40 (m, 2 H), 5.40 (t, J = 5.8, 1 H), 5.47 (dd, J = 5.8, 4.0, 1 H), 5.90 (d, J = 4.0, 1 H), 5.99 (d, J = 7.6, 1 H), 6.71 (br d, J = 88.0, 1 H), 7.39 (d, J = 7.6, 1 H), 8.27 (br d, J = 88, 1 H); ^{13}C (75.4 MHz, $CDCl_3$) δ 20.5, 20.5, 20.7, 63.0, 69.9, 73.4, 79.1, 90.0, 96.1 (br), 141.0, 155.6 (dd, J = 7.6, 3.7), 166.1 (dd, J = 20.5, 6.1), 169.6, 169.7, 170.4; ^{15}N (30.4 MHz, $CDCl_3$) δ −165.5, −274.9 (t, J = 88); HRMS, calcd for $C_{15}H_{20}N^{15}N_2O_8$ $[M + 1]^+$ 372.1191, found 372.1209.

[3-^{15}N,4-^{15}NH$_2$]Cytidine (5*♦). Saturated ammonia in MeOH (1 mL) was added to a solution of **4*♦** (108 mg, 0.29 mmol) in MeOH (1 mL). After stirring for 6 h at room temperature, solvent removal and heating in a vacuum oven at 100 °C for 2 h to remove acetamide gave **5*♦** (71 mg, 100% yield) as a white solid: mp 216 °C (dec); ^{1}H (300 MHz, DMSO-d_6) δ 3.53 (dd, J = 12.2, 3.4, 1 H), 3.64 (dd, J = 12.2, 3.3, 1 H), 3.80 (m, 1 H), 3.90−3.96 (m, 2 H), 4.94 (d, J = 5.1, 1 H), 5.01 (t, J = 5.2, 1 H), 5.24 (d, J = 5.1, 1 H), 5.72 (d, J = 7.3, 1 H), 5.78 (d, J = 3.6, 1 H), 7.17 (d, J = 90.0, 1 H), 7.21 (d, J = 90.0, 1 H), 7.84 (d, J = 7.3, 1 H); ^{13}C (75.4 MHz, DMSO-d_6) δ 60.8, 69.6, 74.2, 84.3, 89.4, 94.2 (br), 141.8, 155.5 (dd, J = 6.7, 3.7), 165.7 (dd, J = 19.8, 5.5); ^{15}N (30.4 MHz, DMSO-d_6): δ −156.4, −272.3 (t, J = 90.0); HRMS, calcd for $C_9H_{14}N^{15}N_2O_5$ $[M + 1]^+$ 246.0874, found 246.0886.

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(14) These ^{1}H and ^{13}C chemical shifts, confirmed by 2D NMR to belong to H5 and C5 of the tetrazole ring, indicate that **3*** is the 1-tetrazolyl derivative (rather than the 2-tetrazolyl isomer), as established by comparison with the spectra of 1-phenyl- and 2-phenyltetrazole (Könnecke, A.; Lippmann, E.; Kleinpeter, E. *Tetrahedron* **1977**, *33*, 1399−1403 and references therein).