Supporting Information for

Pseudouridines in rRNA helix 69 play a role in loop stacking interactions

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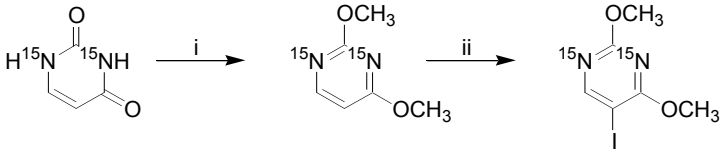
Contents

We describe the approach for labeling pseudouridine and generate fully ^{15}N-enriched, [1,3-^{15}N]-pseudouridine from relatively inexpensive starting materials. Our synthetic strategy involves coupling a pyrimidine precursor, 5-iodo-2,4-dimethoxy-[1,3-^{15}N]-pyrimidine, to a protected D-ribono-1,4-lactone, followed by reduction to generate β-[1,3-^{15}N]-pseudouridine.[1]

[^{15}N]-uracil was generated from relatively inexpensive [^{15}N]-urea according to a literature procedure.[2] [^{15}N]-uracil reacted with phosphorus oxychloride and *N,N*-dimethylaniline.[3] The crude 2,4-dichloro-[1,3-^{15}N]-pyrimidine was treated with sodium methoxide in methanol for 48 h at room temperature to yield **1** in 83% yield. Compound **2** was iodinated under standard conditions with *N*-iodosuccinimide in TFA/TFAA in 92% yield.[3] 5-*O*-*tert*-butyldiphenylsilyl-2,3-*O*-isopropylidene-D-ribono-1,4-lactone **3** was generated in two steps by reacting commercially available D-ribono-1,4-lactone with acetone in H_2SO_4 to generate 2,3-*O*-isopropylidene-D-ribono-1,4-lactone. This product was sufficiently pure without further purification for 5-*O* protection using *tert*-butyldiphenylsiloxylchloride with imidazole to yield compound **3** in 72% yield from **2**.



1 **2**

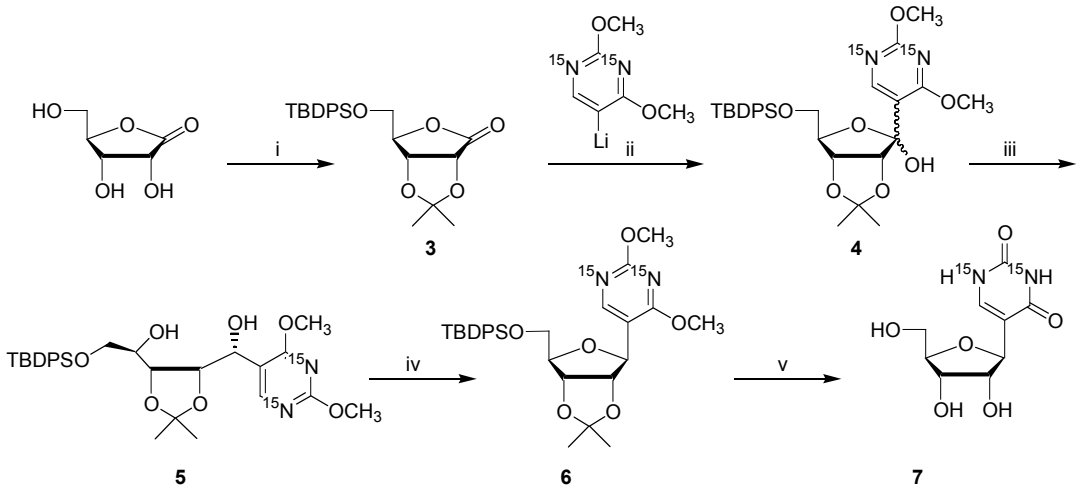
Reagents and Conditions: (i) a) $POCl_3$, *N,N*-dimethylaniline, 100 °C, 3 h; b) $NaOCH_3$, CH_3OH, 2 days, 83%. (ii) *N*-iodosuccinimide, TFA/TFAA, 5 h, 92%.

Lactone **3** was added to a solution of **2** in THF after lithium-halogen exchange with *tert*-butyllithium to afford **4** in 77% yield.[4] Stereoselective reduction of **4** using $ZnCl_2$ and L-selectride gave diol **5** in 90% yield. Compound **5** was treated under Mitsunobu conditions with DIAD and triphenyl phosphine to afford **6** in 75% yield.[1] Methyl groups on the pyrimidine ring were removed by refluxing with NaI and acetic acid for 35 min. Deprotection of the isopropylidene and *tert*-butyldiphenylsilyl groups under acidic conditions (9:1 TFA:H_2O) gave [1,3-^{15}N]-pseudouridine in 96% yield over two steps.[5]

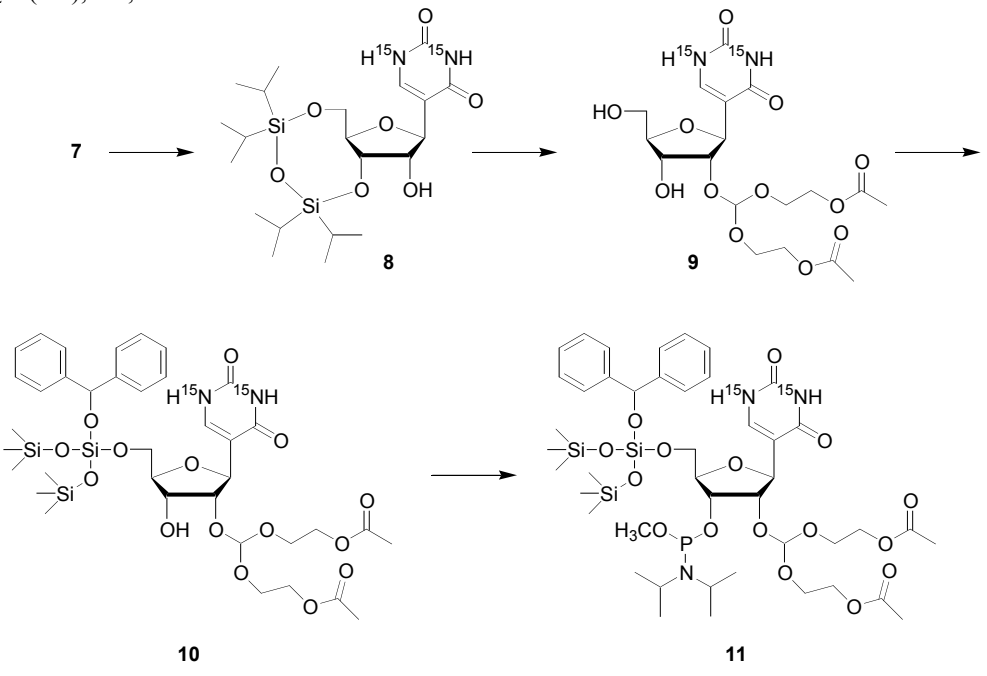
5'-*O*-silyl-2'-*O*-orthoester phosphoramidite chemistry was employed for RNA synthesis.[6,7] For this purpose, a 5'-*O*-BzH-2'-*O*-ACE-[1,3-^{15}N]-pseudouridine phosphoramidite was synthesized. Compound **8** was treated with tris(2-acetoxyethoxy)orthoformate in the presence of pyridinium *p*-toluenesulfonate and 4-(*tert*-butyldimethylsilyloxy)-3-penten-2-one (TBDMS-acac) for 2'-*O*-ACE protection. This reaction requires 55 °C and increasing equivalents of TBDMS-acac and ACE for

[1] S. Hanessian and R. Machaalani, *Tetrahedron Lett.,* **2003**, *44,* 8321.
[2] J. SantaLucia, Jr., L. Shen, Z. Cai, H. Lewis and I. Tinoco, Jr. *Nucleic Acids Res*., **1995**, *23*, 4913.
[3] A. Wada, H. Yasuda, S. Kanatomo, *Synthesis,* **1988**, *10*, 771.
[4] P. J. Grohar and C. S. Chow, *Tetrahedron Lett.,* **1999**, *40*, 2049.
[5] J. J. Chen, J. C. Drach and L. B. Townsend, *J. Org. Chem.,* **2003**, *68*, 4170.
[6] (a) S.A. Scaringe, *Methods Enzymol.* **2000**, *317*, 3. (b) S. A Scaringe,. F. E Wincott and, M.H. Caruthers *J. Am. Chem. Soc*. **1998**, *120*, 11820.
[7] M Meroueh,. P. J. Grohar, J. Qiu,. J. SantaLucia, Jr. S. A. Scaringe and C.S. Chow *Nucleic Acids Res.* **2000**, *28*, 2075.

successful product conversion. The 3',5'-TIPDS group was deprotected using a combination of HF in TMEDA at 0 °C to yield **9** in 65% yield. Compound **9** was reacted with BzHCl and diisopropylamine at 0 °C to afford **10** in 65% yield. Finally phosphoramidite synthesis was completed by the addition of methyl tetraisopropylphosphorodiamidite and *1H*-tetrazole to generate 5'-*O*-BzH-2'-*O*-ACE-[1,3-^{15}N]-pseudouridine phosphoramidite **11** in 52% yield.



Reagents and Conditions: (i) a) acetone, H_2SO_4, 0 °C → rt, 5 h; b) TBDPSCl, imidazole, CH_2Cl_2, 12 h, 85% for two steps. (ii) THF, -72 °C, 2.5 h, 76%. (iii) a) $ZnCl_2$, CH_2Cl_2, -72 °C, 30 min; b) L-selectride, -72 °C → rt, 16 h, 90%. (iv) PPh_3, DIAD, THF, 0 °C → rt, 24 h, 75%. (v) a) NaI, CH_3COOH, 35 min; b) TFA:H_2O (9:1), 1 h, 96%.



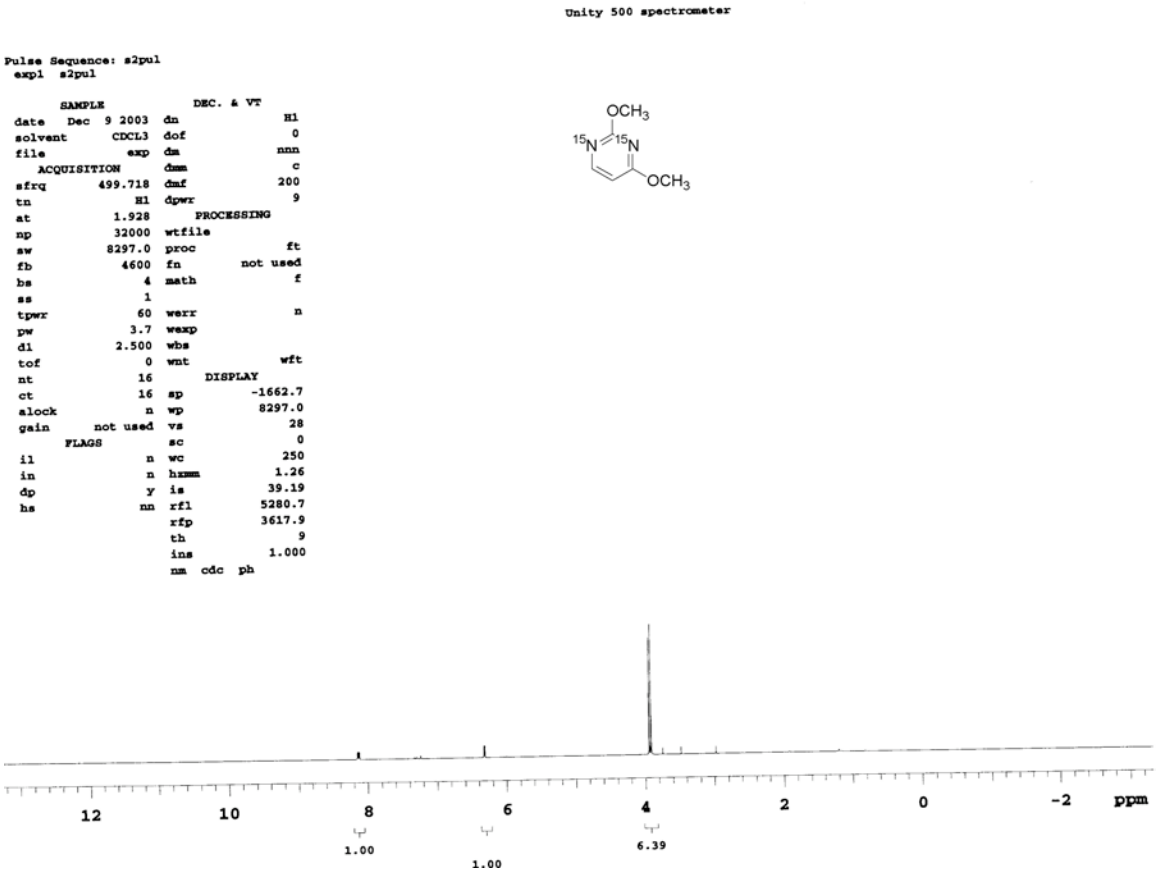
Reagents and Conditions: (i) $TIPDSCl_2$, pyridine, 0 °C → rt, 12 h, 94%. (ii) a) tris(2-acetoxyethyl)orthoformate, pyridinium p-toluenesulfonate, 4-(tert-butyldimethylsilyloxy)-3-penten-2-one, dioxane, 55 °C, 12 h; b) TEMED-HF, CH_3CN, 0 °C, 3 h, 65%. (iii) BzH-Cl, diisopropylamine, CH_2Cl_2, 0 °C, 3 h, 65%. (iv) methyl tetraisopropylphosphorodiamidite, tetrazole, CH_2Cl_2, rt, 12 h, 52%.

Experimental procedures and characterization

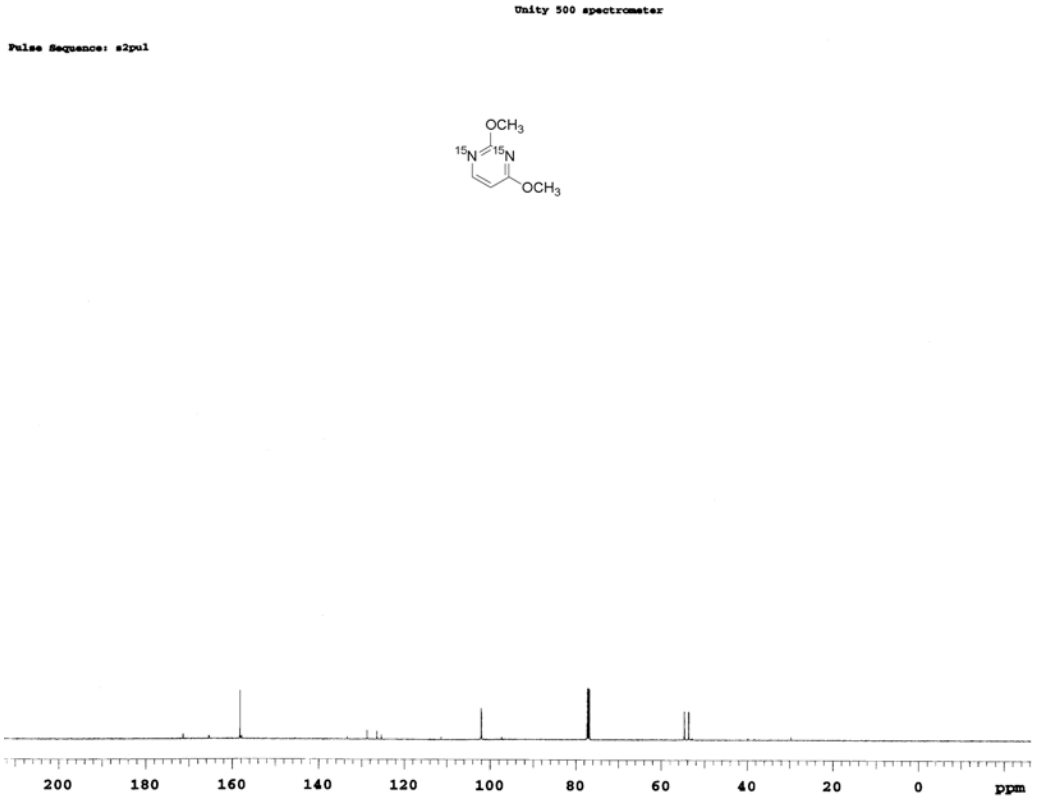
2,4-Dimethoxy-[1,3-^{15}N]-pyrimidine (1)

[1,3-^{15}N]-uracil (1.00 g, 8.77 mmol, 1.0 eq) reacted with 5 mL $POCl_3$ and 3 mL *N,N*-dimethylaniline under argon at 100 °C for 3 h. After TLC showed product conversion, the reaction was quenched with ice and extracted with ether. The organic layer was evaporated to yield a yellow solid. In a separate flask, Na (1.21 g, 52.63 mmol, 6.00 eq) was added to 20 mL of dry methanol at 0 °C. After H_2 effervescence ceased, the yellow solid was added and allowed to stir at room temperature for 2 days. The reaction was quenched with 30% NaOH, extracted with ether and purified on silica gel using 33% ethyl acetate in hexanes to yield an organic liquid **1** in 83% yield (1.03 g). ^{1}H NMR (500 MHz, $CDCl_3$) δ (ppm) 3.91 (s, 3H), 3.94 (s, 3H), 6.32 (d, 1H, J = 5.0 Hz), 8.13 (dd, 1H, J = 5.5, 6.0 Hz); ^{13}C NMR (500 MHz, $CDCl_3$) δ (ppm) 53.62, 53.65, 101.99, 158.19, 165.38 (dd, 1C, J = 9.3 Hz), 171.37 (d, 1C, J = 9.3 Hz); ESI-MS (ES^+) *m/z* calcd for $C_6H_8{}^{15}N_2O_2$ 142.1, found 143.0 (MH^+).

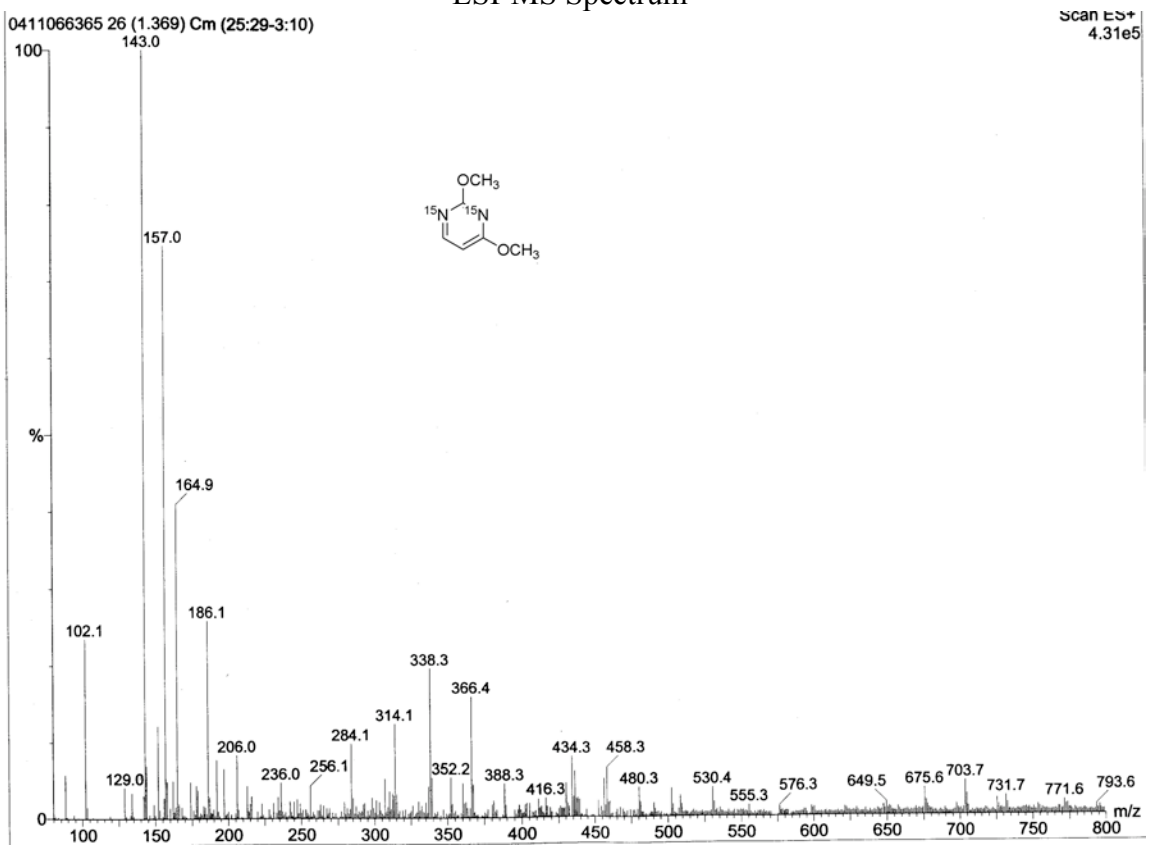
^{1}H NMR Spectrum



^{13}C NMR Spectrum



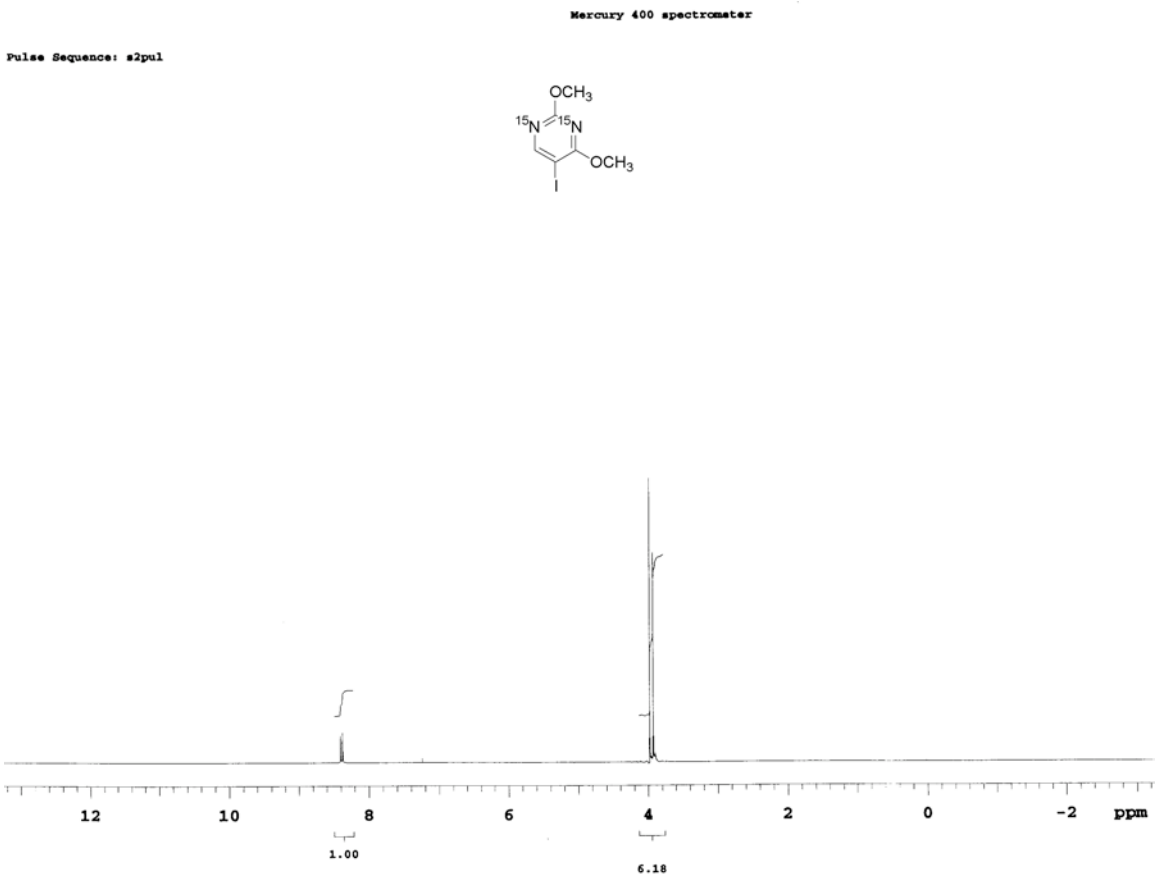
ESI-MS Spectrum



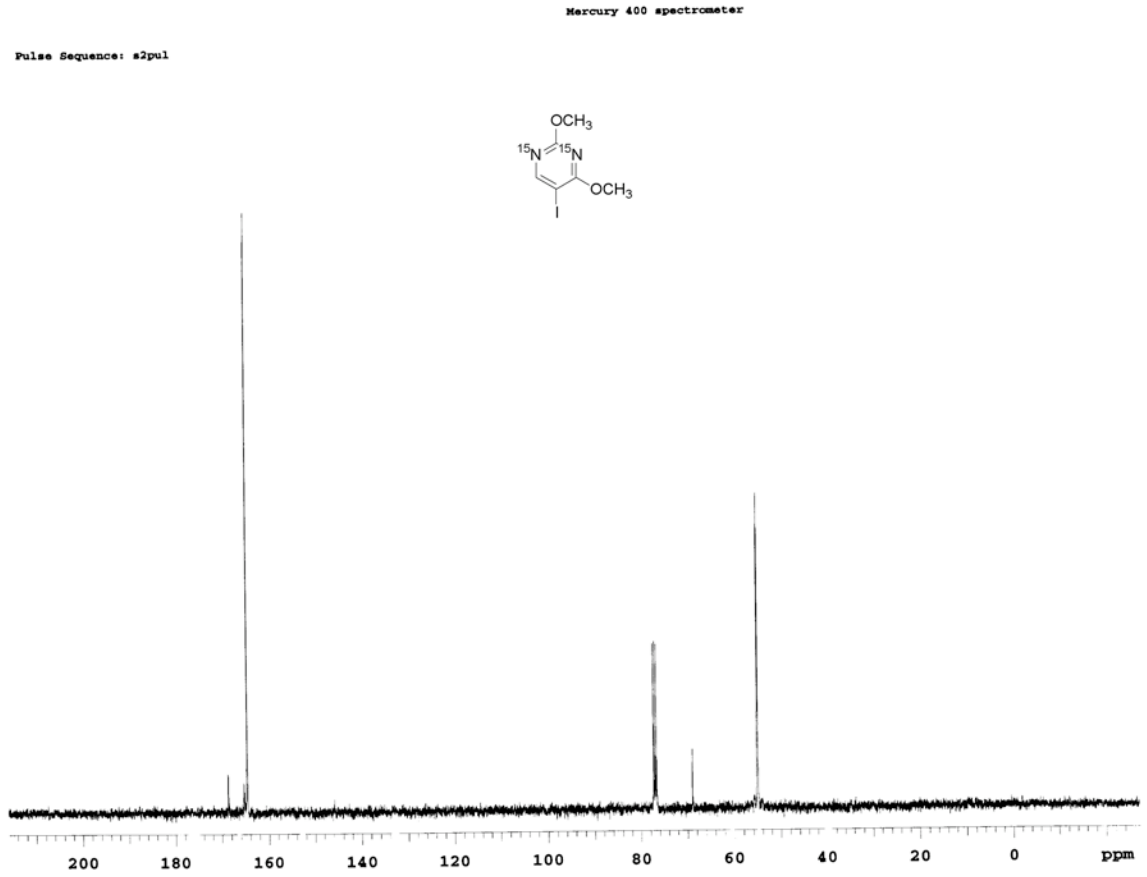
5-Iodo-2,4-dimethoxy-[1,3-^{15}N]-pyrimidine (2)

A solution of trifluoroacetic anhydride (0.7 mL) with trifluoroacetic acid (3.5 mL) was slowly added to compound **1** (0.50 g, 3.52 mmol, 1.0 eq). The clear solution was stirred under argon for several minutes. 1.012 g of *N*-iodosuccinimide (4.49 mmol, 1.3 eq) was added to mixture to afford a dark brown color solution. The reaction was refluxed for ca. 5 hours, after which point, TLC showed proper product conversion. The reaction was quenched by the slow addition of 5% sodium bicarbonate, extracted over chloroform, and washed with sodium thiosulfate. The organic layer was dried and purified on silica gel using a 10 – 20% ethyl acetate gradient in hexanes to yield **2** in 92% yield (0.87 g). ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 3.92 (s, 3H), 3.98 (s, 3H), 8.39 (d, 1H, J = 11.2 Hz); ^{13}C NMR (400 MHz, $CDCl_3$) δ (ppm) 54.97, 55.01, 55.10, 68.87, 164.65, 165.36, 168.67 (d, 1C, J = 7.5 Hz); ESI-MS (ES^+) *m/z* calcd for $C_6H_7I^{15}N_2O_2$ 267.9, found 268.9 (MH^+).

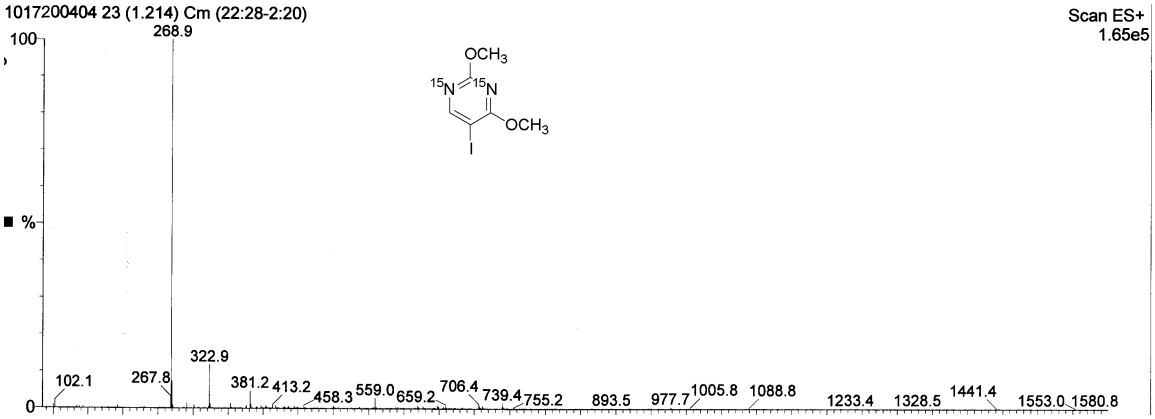
^{1}H NMR Spectrum



^{13}C NMR Spectrum



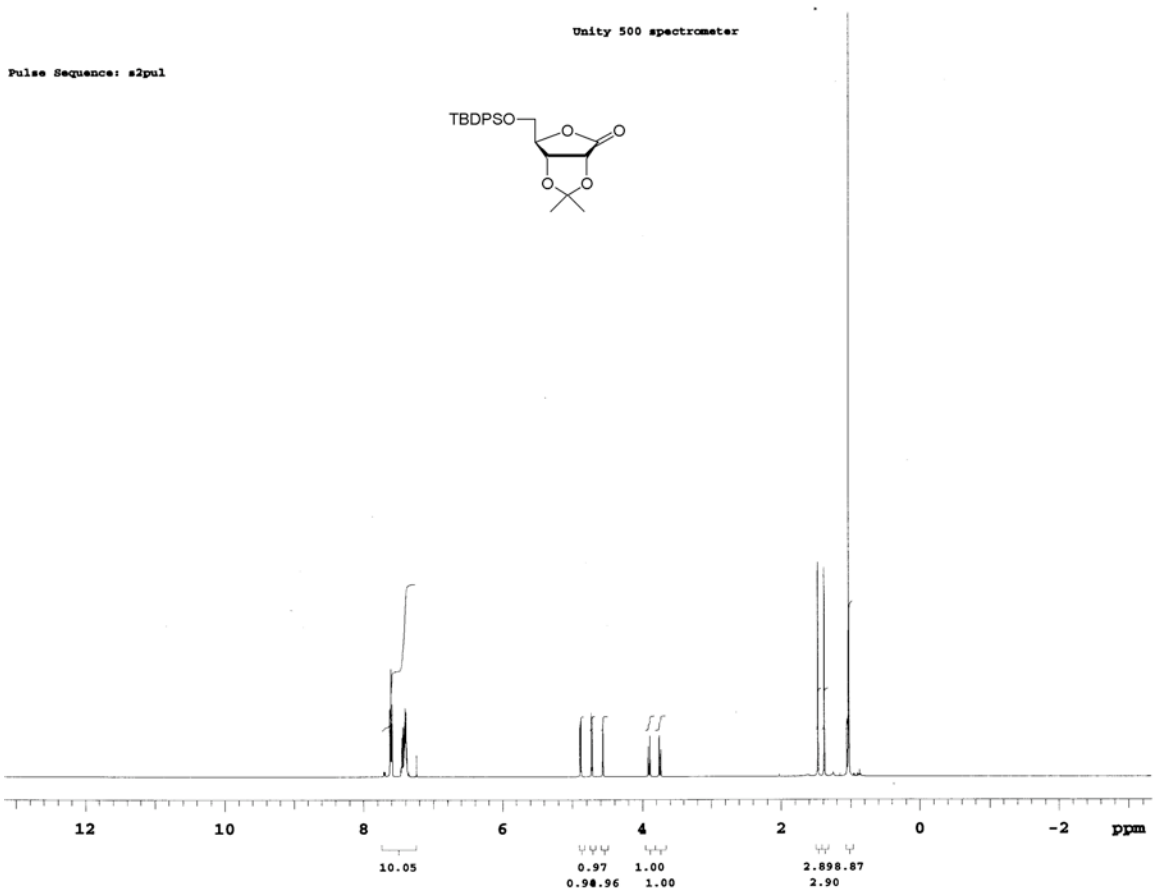
ESI-MS Spectrum



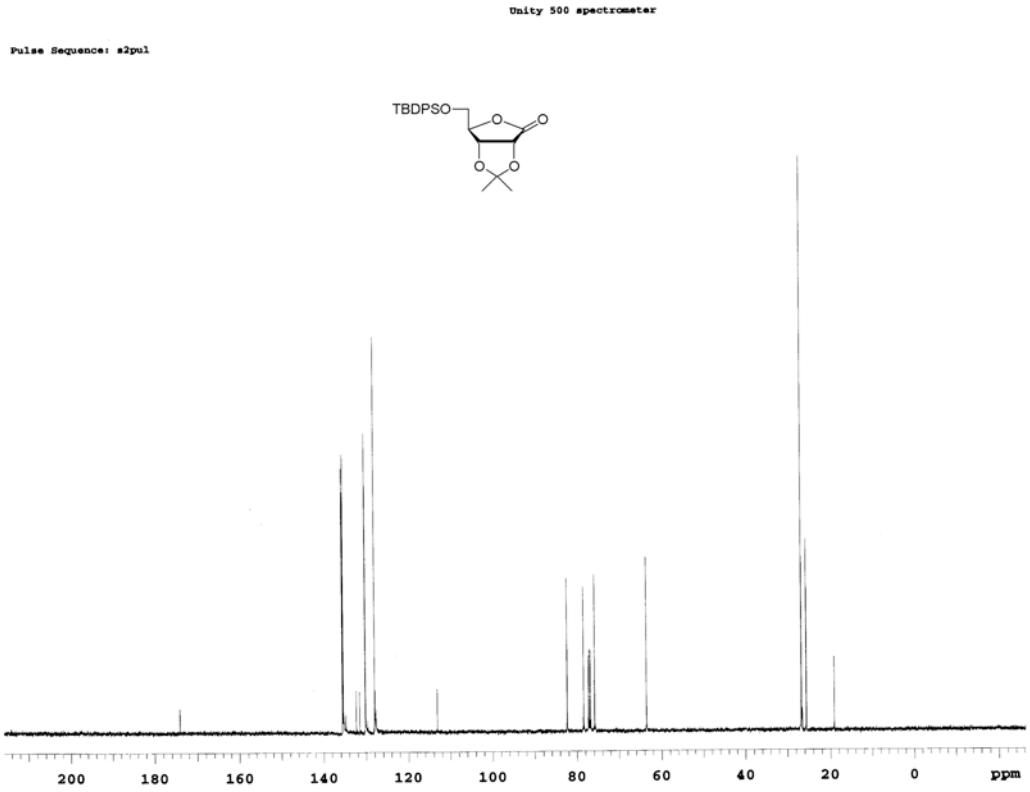
5-*O*-*tert*-Butyldiphenylsilyl-2,3-*O*-isopropylidene-D-ribono-1,4-lactone (3)

D-ribono-1,4-lactone (4.80 g, 32.75 mmol, 1.0 eq) was treated with 100 mL acetone and 1.0 mL H_2SO_4 and stirred at 0 °C for 2 h then at room temperature for 3 h. The reaction was quenched with 5% $NaHCO_3$ and extracted with ethyl acetate to yield 2,3-*O*-isopropylidene-D-ribono-1,4-lactone in 90% yield (5.55 g, 29.51 mmol). This product was dissolved in 30 mL dry dichloromethane followed by imidazole (4.41 g, 64.92 mmol, 2.2 eq) and *tert*-butyldiphenylsilylchloride (8.31 mL, 32.46 mmol, 1.1 eq) was added dropwise slowly to a stirred solution at 0 °C. The reaction was stirred overnight, and quenched with 5% $NaHCO_3$, extracted with ethyl acetate, dried over Na_2SO_4, and purified on silica gel with a 15 – 25% ethyl acetate gradient in hexanes to yield **3** as a white solid (85% yield over two steps, 11.78 g). ^{1}H NMR (500 MHz, $CDCl_3$) δ (ppm) 1.03, 1.05, 1.06 (3s, 9H), 1.38 (s, 3H), 1.47 (s, 3H), 3.74 (dd, 1H, J = 12.0, 1.5 Hz), 3.90 (dd, 1H, J = 11.8, 2.5, 2.0 Hz), 4.56 (t, 1H, J = 2.0 Hz), 4.72 (d, 1H, J = 6.0 Hz), 4.88 (d, 1H, J = 6.0 Hz), 7.38 – 7.47 (m, 6H), 7.59 – 7.62 (m, 4H); ^{13}C NMR (500 MHz, $CDCl_3$) δ (ppm) 19.04, 25.57, 26.52, 26.73, 63.52, 75.80, 78.40, 82.30, 113.13, 127.67, 127.98, 130.16, 131.51, 132.32, 134.76, 135.40, 135.58, 174.08; ESI-MS (ES^+) *m/z* calcd for $C_{24}H_{30}O_5Si$ 426.2, found 449.1 ($M+Na^+$), 465.1 ($M+K^+$), 875.2 ($2M+Na^+$), 891.2 ($2M+K^+$).

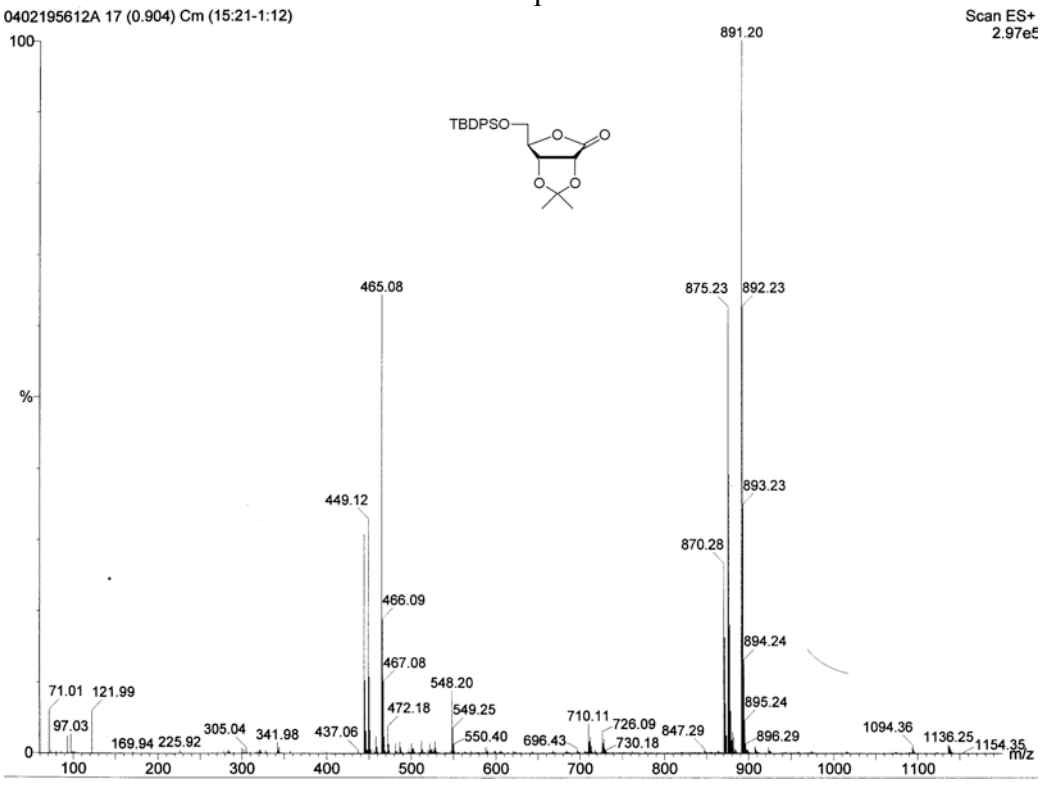
^{1}H NMR Spectrum



^{13}C NMR Spectrum



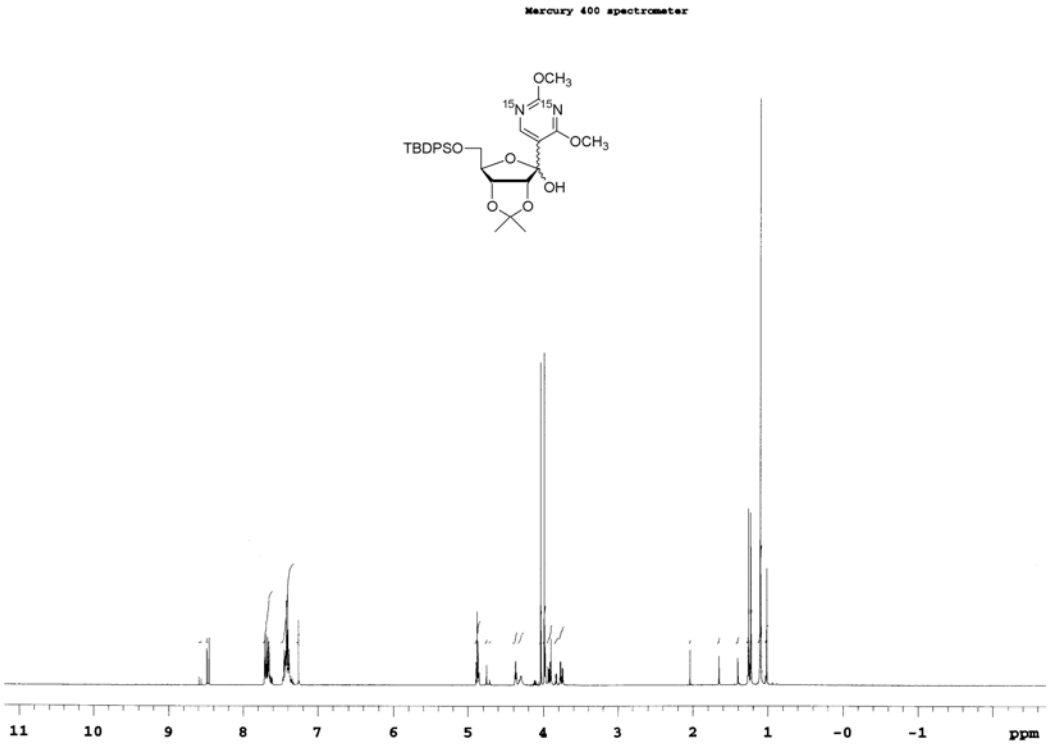
ESI-MS Spectrum



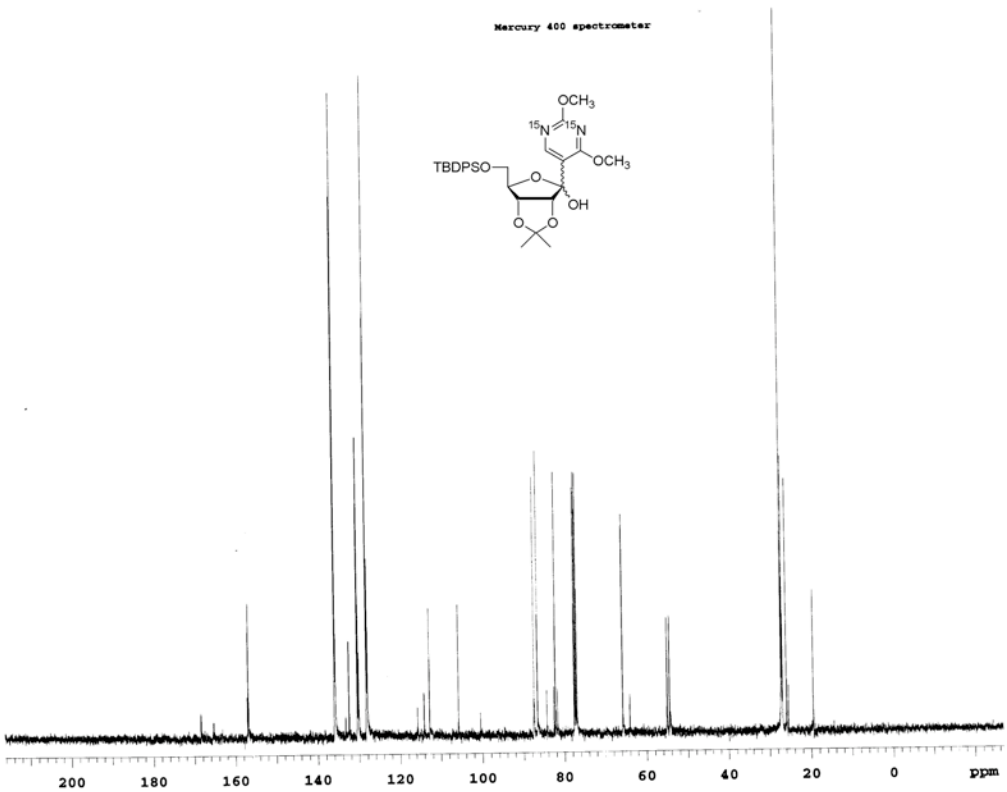
5-[5'-*O*-(*tert*-Butyldiphenylylsilyl)-2',3'-*O*-isopropylidene-D-ribofuranosyl]-[1,3-^{15}N]--2,4-dimethoxypyrimidine (4) (α and β; 7:1)

A 1.7 M solution of *tert*-butyllithium (2.20 mL, 3.74 mmol, 2.0 eq) in pentane was added dropwise to a stirred solution of 5-iodo-2,4-dimethoxy-[1,3-^{15}N]-pyrimidine **2** (0.50 g, 1.87 mmol, 1.0 eq) in anhydrous THF (10 mL) at -72 °C under argon for 30 min. After stirring at room temperature for 5 min, the reaction was cooled to -72 °C for 20 min. Compound **3** (0.88 g, 2.05 mmol, 1.1 eq) in THF (5 mL) at -72 °C was transferred slowly to the reaction by cannula. Stirring was continued at -72 °C for 1.5 h. The reaction was quenched by the addition of water (10 mL) and warmed slowly to room temperature for 30 min. The reaction mixture was extracted with ethyl acetate (3 × 50 mL). The combined organic extracts were dried over Na_2SO_4 and concentrated to afford an orange oil. The product was purified by flash chromatography using a 25 – 40% ethyl acetate gradient in hexanes to give **4** (0.81 g, 76%) as a colorless oil. ^{1}H NMR (400 MHz, $CDCD_3$) δ (ppm) 1.01 (s, 9H), 1.10 (s, 63H), 1.23 (s, 21H), 1.26 (s, 21H), 1.40 (s, 3H), 1.65 (s, 3H), 3.79 (m, 11H), 3.92 (dd, 12H, *J* = 11.2, 4.8 Hz), 3.98 (m, 24H), 4.03 (s, 21H), 4.30 (m, 7H), 4.37 (m, 7H), 4.73 (m, 3H), 4.87 (m, 14H), 7.40 (m, 51H), 7.66 (m, 34H), 8.48 (d, 7H, *J* = 12.0 Hz), 8.58 (d, 1H, *J* = 11.6 Hz); ^{13}C NMR (400 MHz, $CDCD_3$) δ (ppm) 19.43, 25.42, 25.88, 26.86, 27.03, 27.15, 39.88, 54.20, 54.45, 54.49, 55.03, 64.00, 65.73, 81.67, 82.21, 82.59, 84.33, 86.58, 87.47, 100.50, 105.76, 112.79, 114.11, 115.69, 127.97, 128.16, 128.20, 130.05, 130.28, 130.42, 132.43, 132.50, 133.28, 135.83, 136.01, 156.87, 157.10, 157.81, 165.34, 165.45, 168.35, 168.44; ^{15}N NMR (500 MHz, $CDCl_3$) δ (ppm) -142.7, -144.1, -152.6, -153.2; ESI-MS (ES^+) m/z calcd for $C_{30}H_{38}{}^{15}N_2O_7Si$ 568.2, found 569.2 (MH^+), 1137.2 ($2MH^+$).

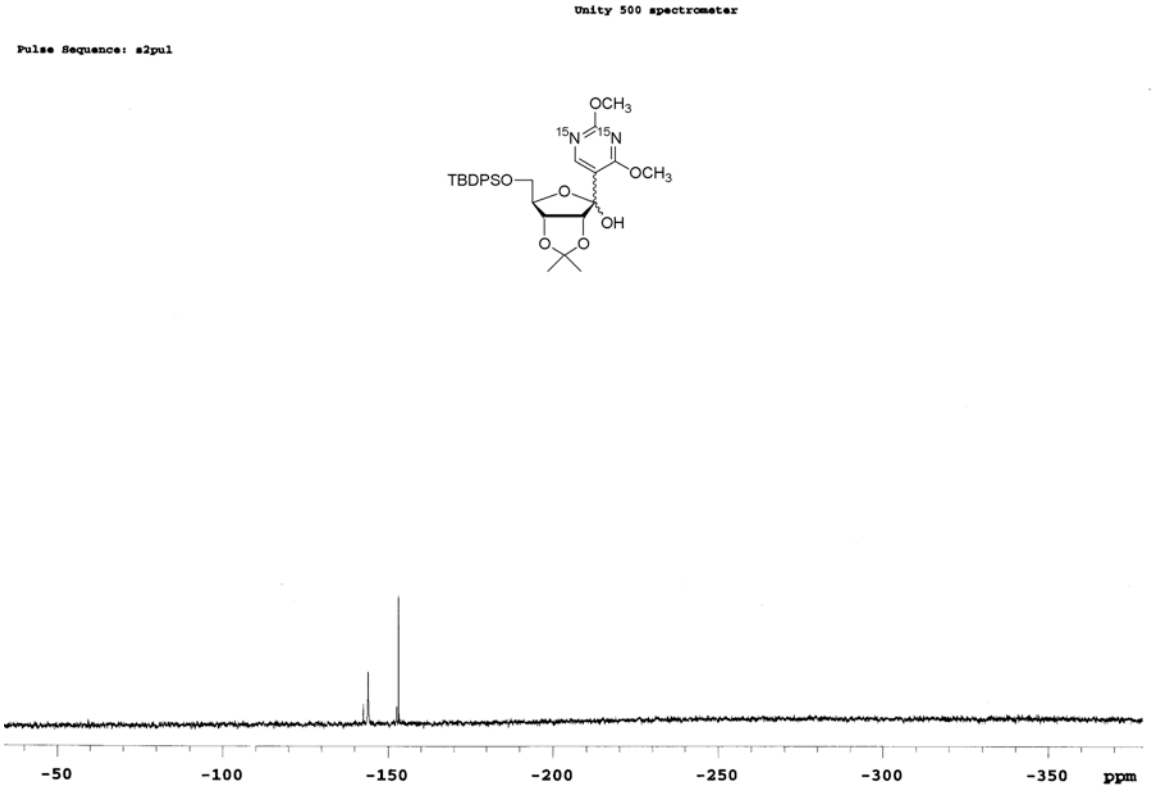
^{1}H NMR Spectrum



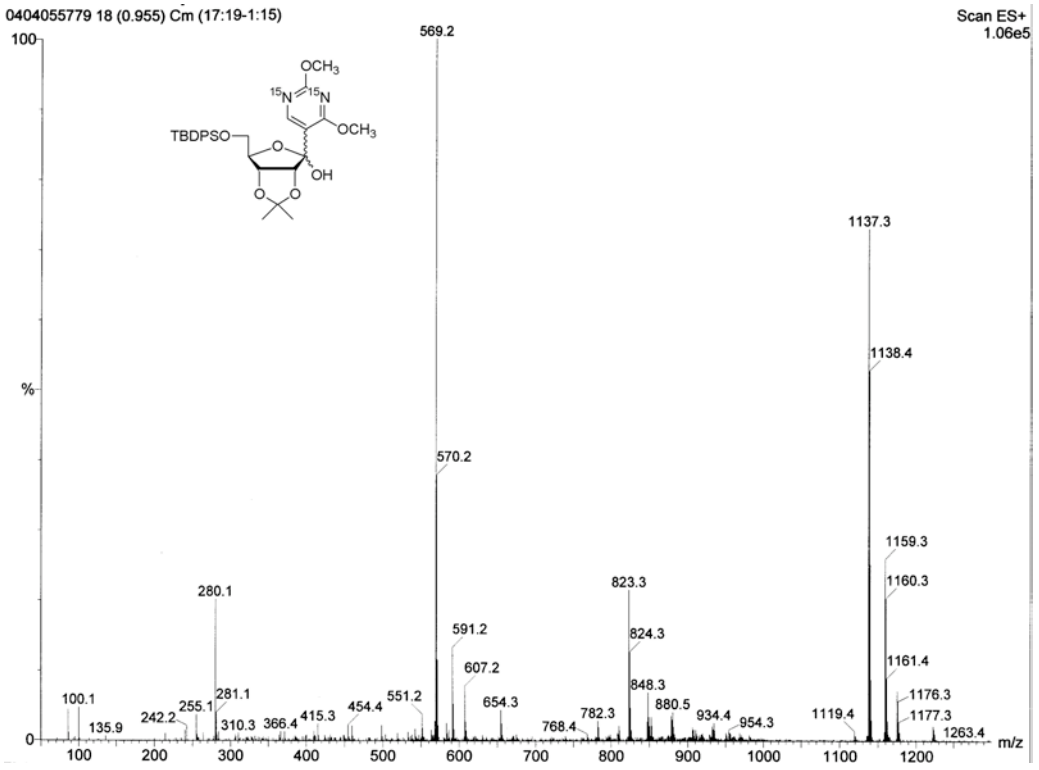
^{13}C NMR Spectrum



^{15}N NMR Spectrum



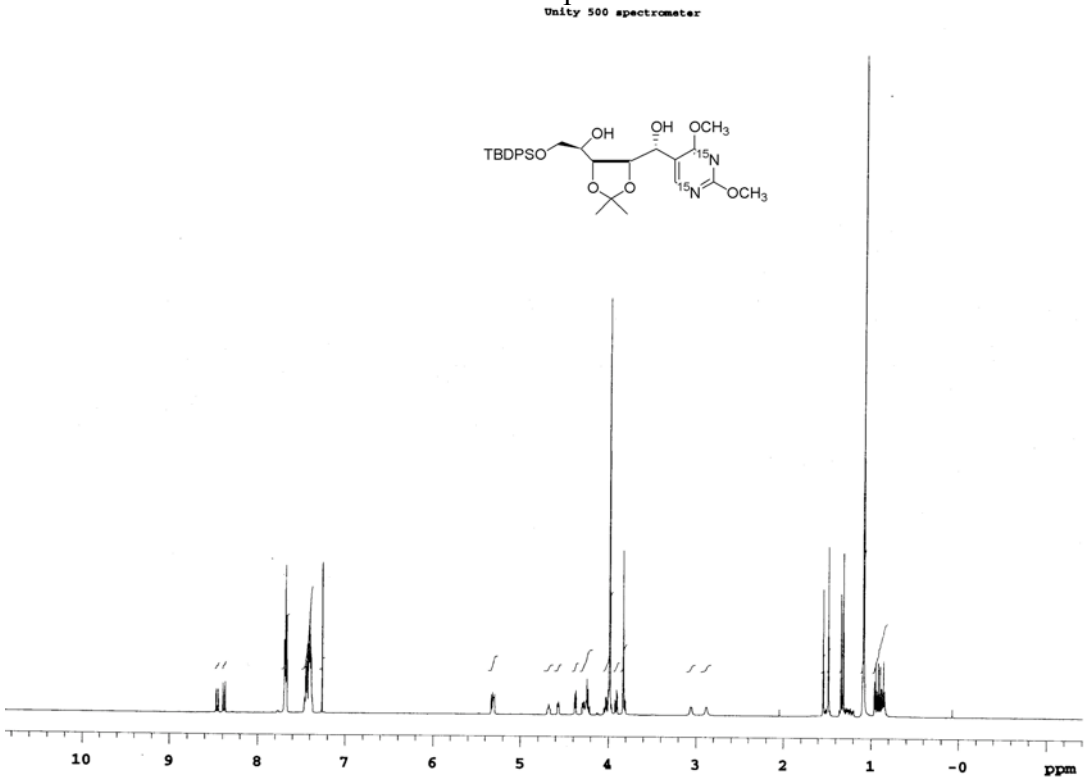
ESI-MS Spectrum



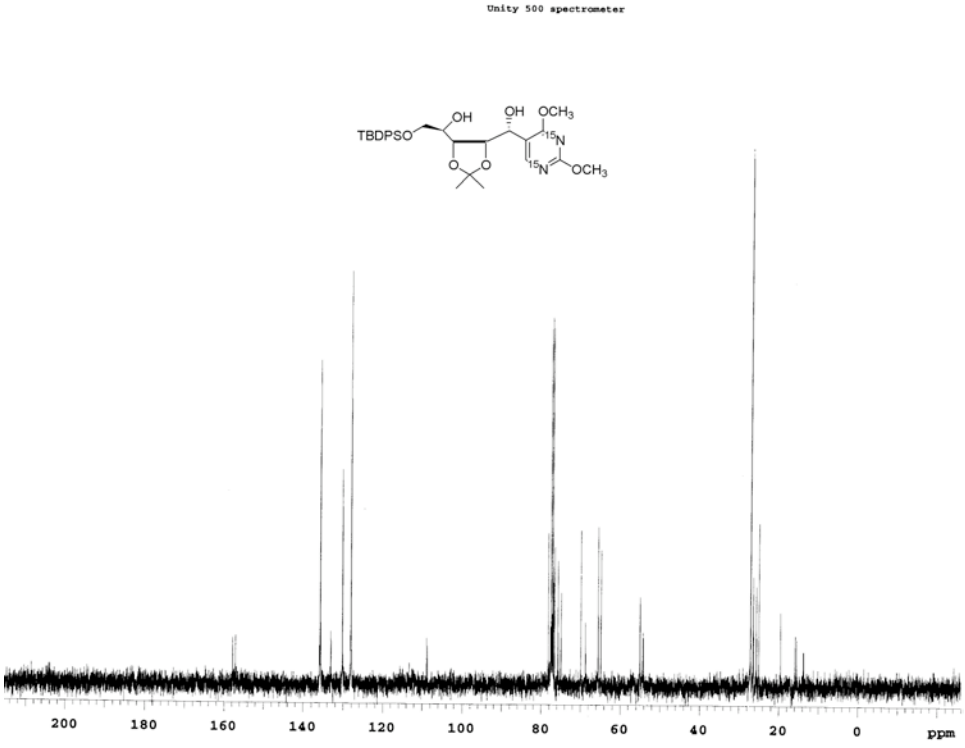
5-[(1*R*,2*S*,3*R*,4*S*)- 5'-*O*-(*tert*-Butyldiphenylylsilyl)-2',3'-*O*-isopropylidene-1',4'-pentandiol]-[1,3-^{15}N]-2,4-dimethoxypyrimidine (5).

A 1.0 M solution of $ZnCl_2$ (1.90 mL, 1.90 mmol, 1.50 eq) in diethyl ether was added dropwise to a solution of **4** (0.720 g, 1.27 mmol, 1.0 eq) in anhydrous DCM (75 mL) at -72 °C under argon. After stirring for 30 min, a 1.0 M solution of L-selectride (4.81 mL, 4.81 mmol, 2.53 eq) in THF was added slowly over 30 min. The reaction was then allowed to warm to room temperature and stirred for 16 h. The reaction was quenched by addition of EtOH (2 mL), water (2 mL), 30% H_2O_2 (2 mL), and 5 N NaOH (2 mL). After workup, the crude product was purified by flash chromatography using a 35 – 50% ethyl acetate gradient in hexanes to give **5** (0.650 g, 90%) as a white foam. ^{1}H NMR (500 MHz, $CDCl_3$) δ (ppm) 1.08 (m, 9H), 1.31 (m, 3H), 1.48 (s, 3H), 2.88 (s, 1H), 3.05 (d, J = 5.5 Hz, 1H), 3.82 (m, 1H), 3.91 (dd, J_1 = 10.0 Hz, J_2 = 3.0 Hz, 1H), 3.99 (m, 6H), 4.25 (m, 2H), 4.37 (dd, J_1 = 5.5 Hz, J_2 = 1.5 Hz, 1H), 5.31(m, 1H), 7.42 (m, 6H), 7.68 (m, 4H), 8.38 (d, J = 11.2 Hz, 1H); ^{13}C NMR (500 MHz, $CDCl_3$) δ (ppm) 19.55, 25.61, 27.03, 27.07, 54.28, 54.98, 64.82, 65.50, 69.89, 75.68, 78.17, 108.86, 128.10, 128.30, 130.14, 130.18, 133.08, 135.74, 135.80, 157.07, 157.77; ^{15}N NMR (500 MHz, $CDCl_3$) δ (ppm) -142.9, -153.4, -153.7; ESI-MS (ES^+) m/z calcd for $C_{30}H_{40}{}^{15}N_2O_7Si$ 570.3, found 571.2 (MH^+), 593.2 ($M+Na^+$).

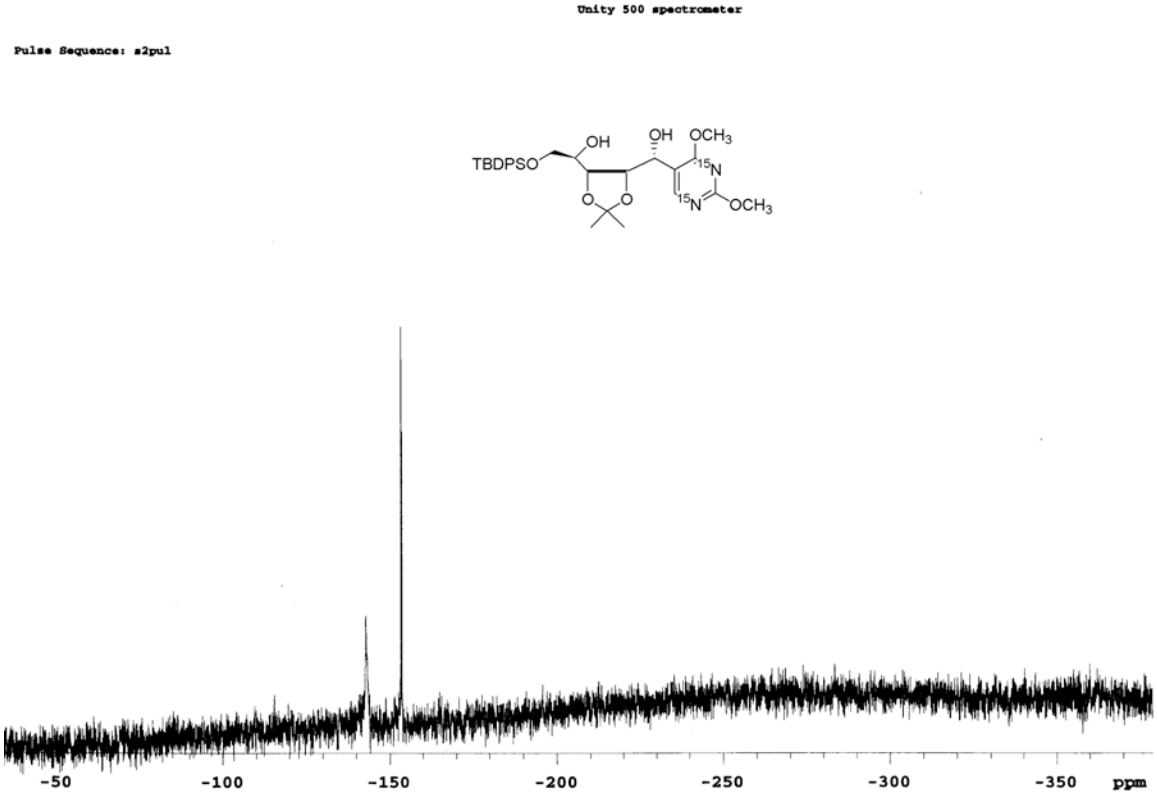
^{1}H NMR Spectrum



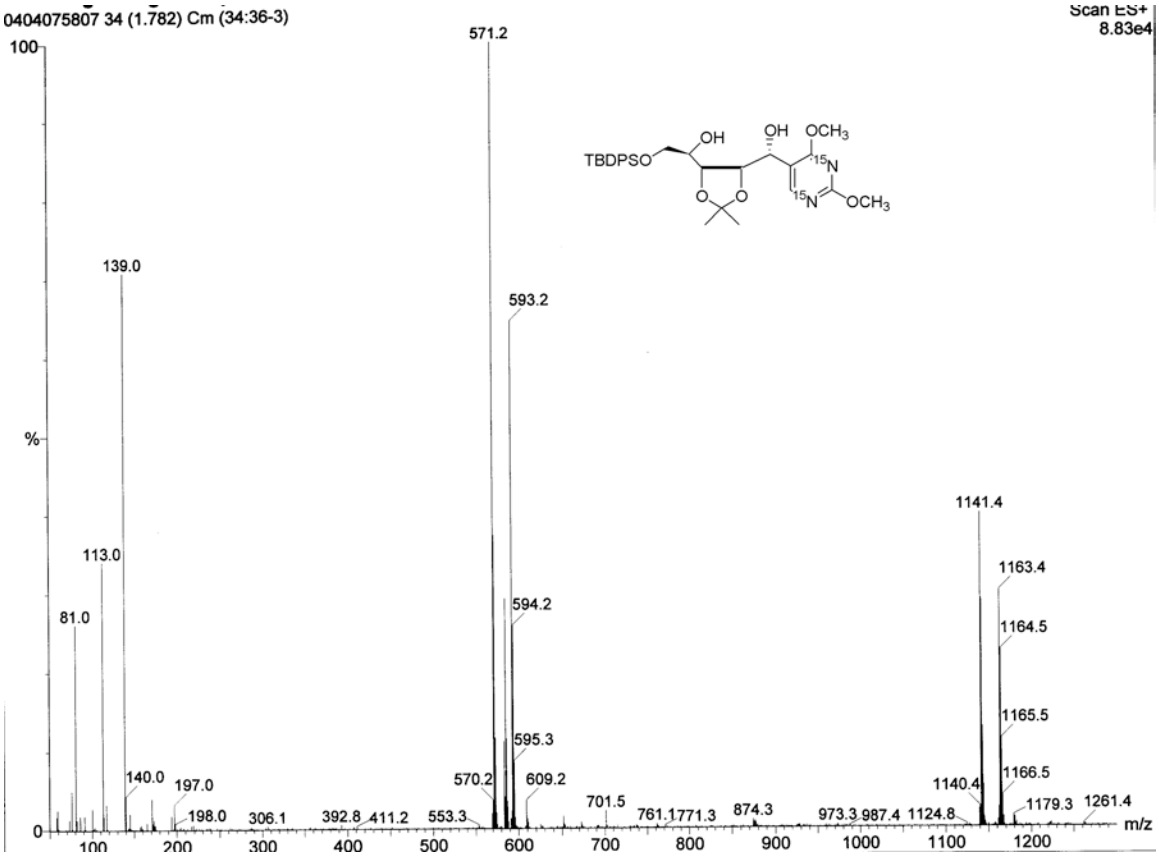
^{13}C NMR Spectrum



^{15}N NMR Spectrum



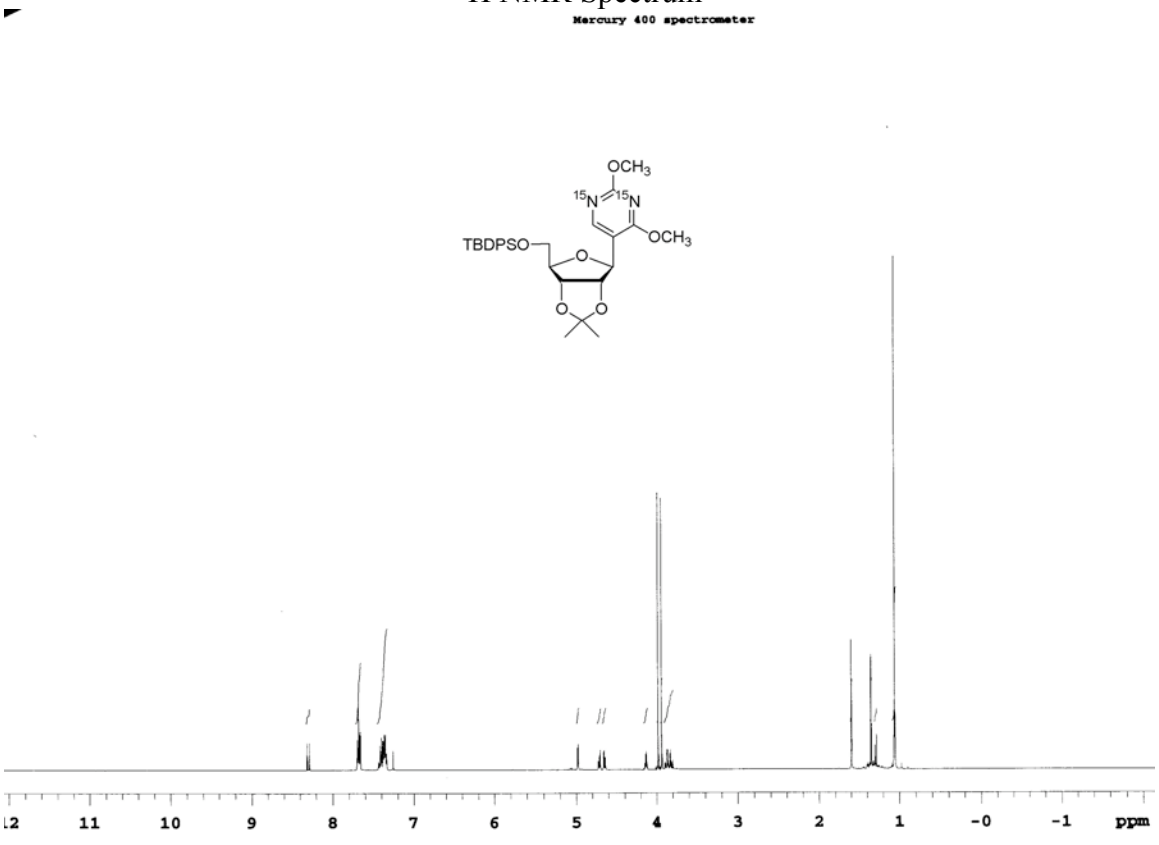
ESI-MS Spectrum



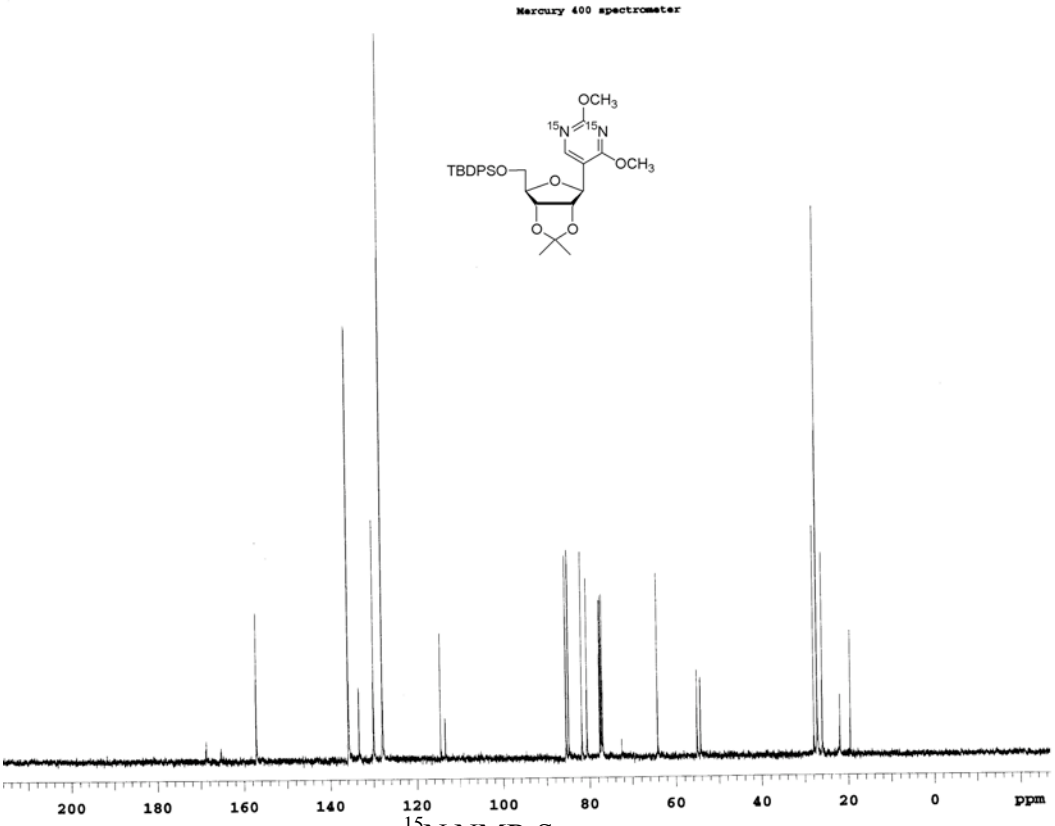
5-[5'-*O*-(*tert*-Butyldiphenylylsilyl)-2',3'-*O*-isopropylidene-β-D-ribofuranosyl]-[1,3-^{15}N]-2,4-dimethoxypyrimidine (6).

Diisopropyl azodicarboxylate (0.370 mL, 1.93 mmole, 2.01 eq) was added to a stirred solution of **5** (0.550 g, 0.964 mmol, 1.0 eq) and triphenylphosphine (0.510 g, 1.93 mmol, 1.05 eq) in anhydrous THF (60 mL) at 0 °C under argon. The reaction was allowed to warm to room temperature slowly and stirred for 24 h. The solvent was removed under reduced pressure. The residue was then purified with flash chromatography using a 20 – 35% ethyl acetate gradient in hexanes to give **6** (0.461 g, 75%) as a colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 1.06 (s, 9H), 1.35 (s, 3H), 1.59 (s, 3H), 3.85 (m, 2H), 3.94 (s, 3H), 3.98 (s, 3H), 4.13 (dd, J_1 = 8.8 Hz, J_2 = 4.0 Hz, 1H), 4.65 (dd, J_1 = 6.4 Hz, J_2 = 4.0 Hz, 1H), 4.72 (dd, J_1 = 7.2 Hz, J_2 = 4.8 Hz, 1H), 4.98 (d, J = 4.0 Hz, 1H), 7.38 (m, 6H), 7.68 (m, 4H), 8.30 (d, J = 11.2 Hz, 1H); ^{13}C NMR (400 MHz, $CDCl_3$) δ (ppm) 19.48, 21.86, 25.91, 27.07, 27.86, 54.28, 54.32, 55.08, 64.19, 80.56, 81.76, 84.86, 85.43, 113.37, 114.50, 127.95, 129.97, 130.01, 133.36, 133.41, 135.85, 135.88, 157.12; ^{15}N NMR (500 MHz, $CDCl_3$) δ (ppm) -142.09, -152.99; HRMS calcd for $C_{26}H_{29}{}^{15}N_2O_6Si$ (M^+–C_4H_9) 495.1736, found 495.1737.

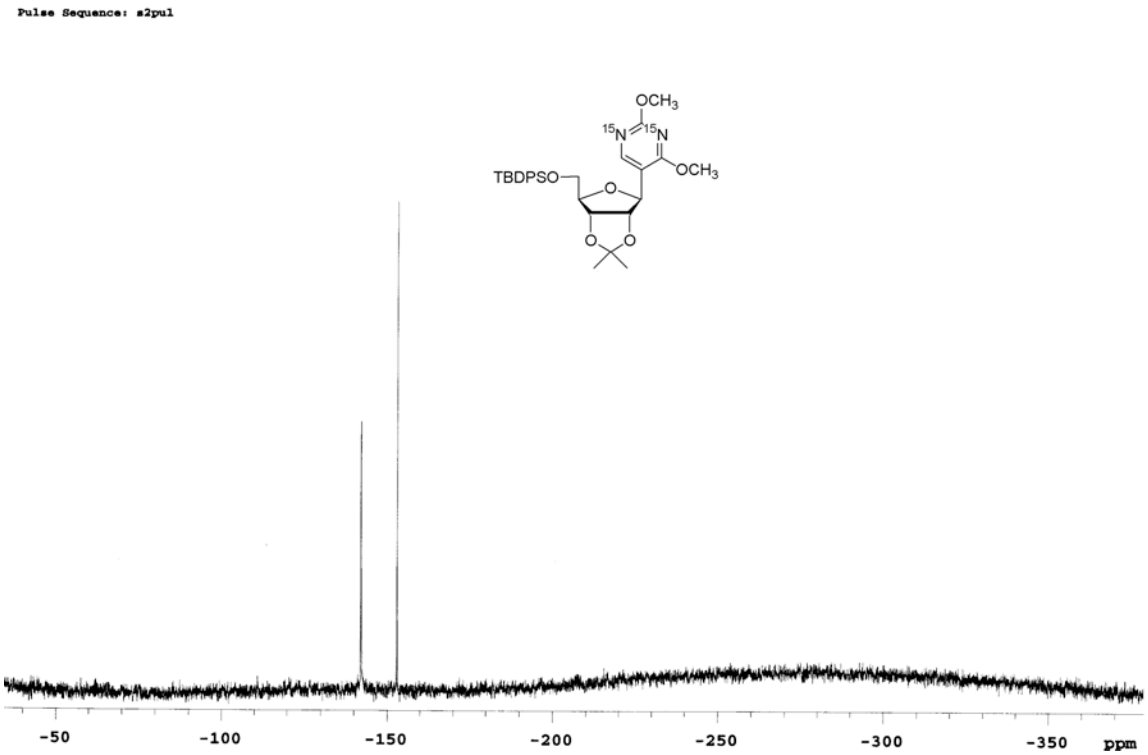
^{1}H NMR Spectrum



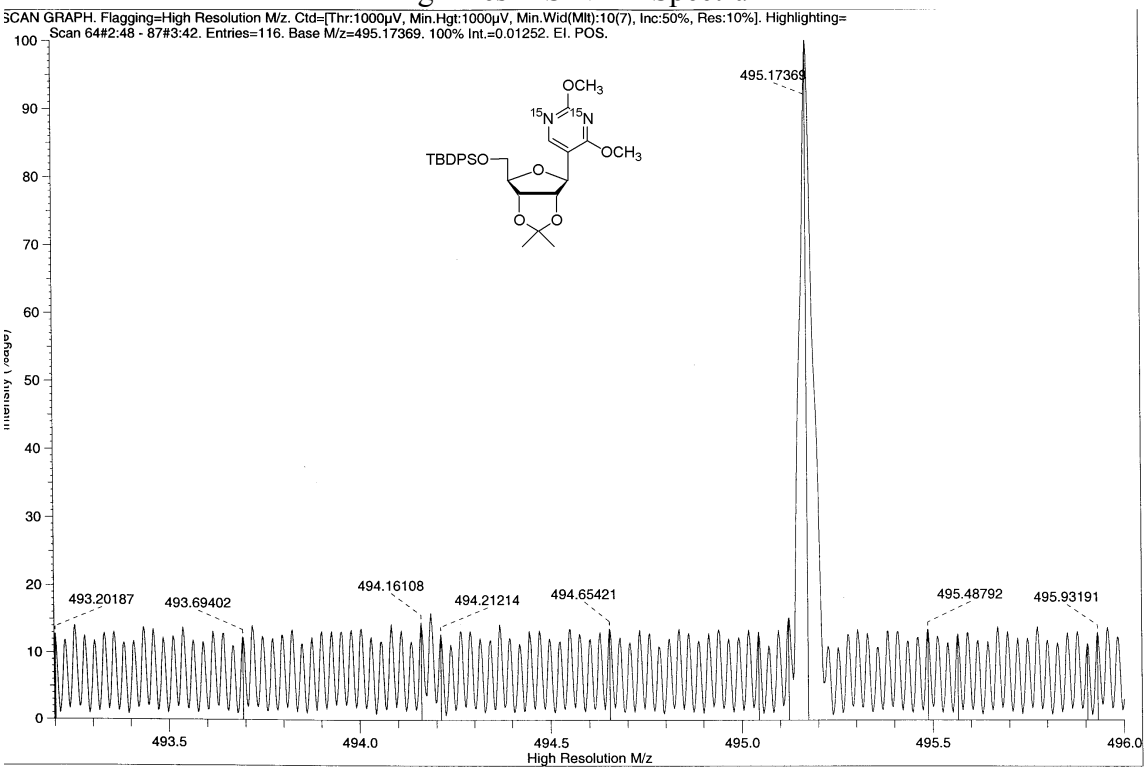
^{13}C NMR Spectrum



^{15}N NMR Spectrum



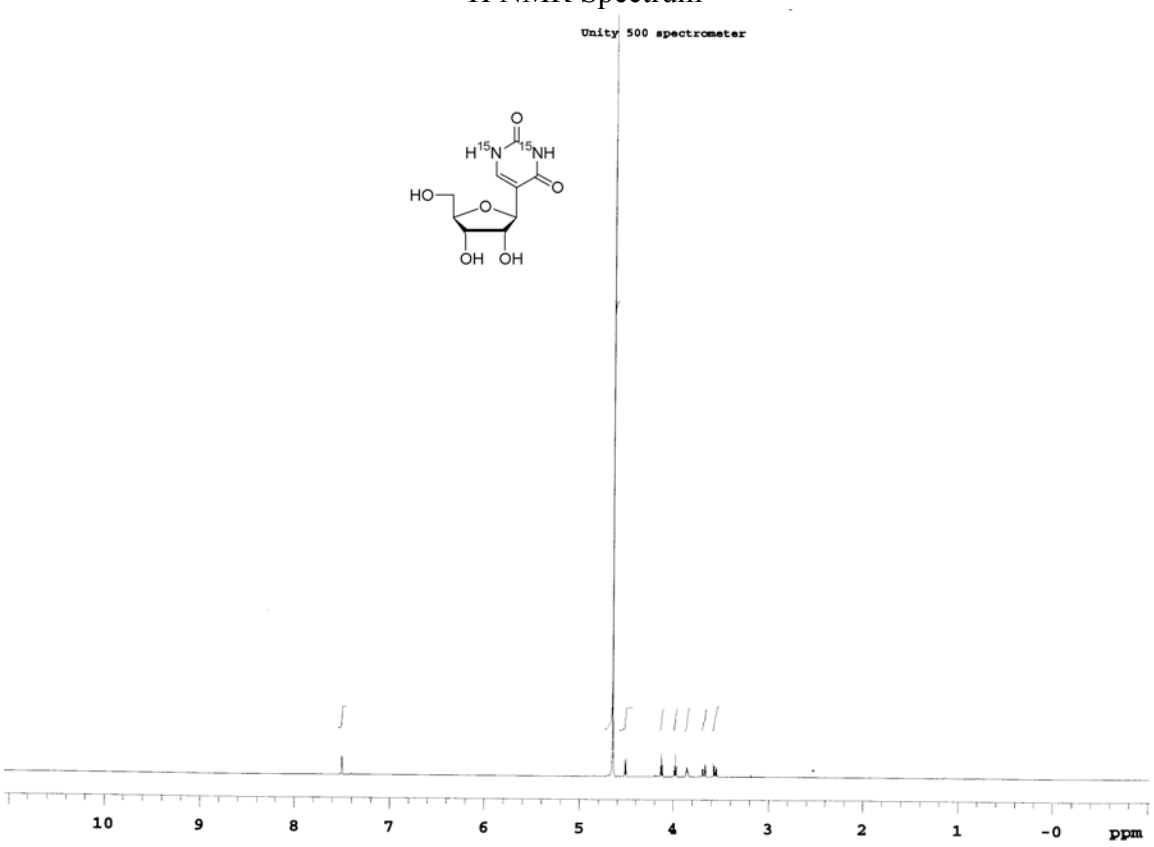
High-Res MS NMR Spectrum



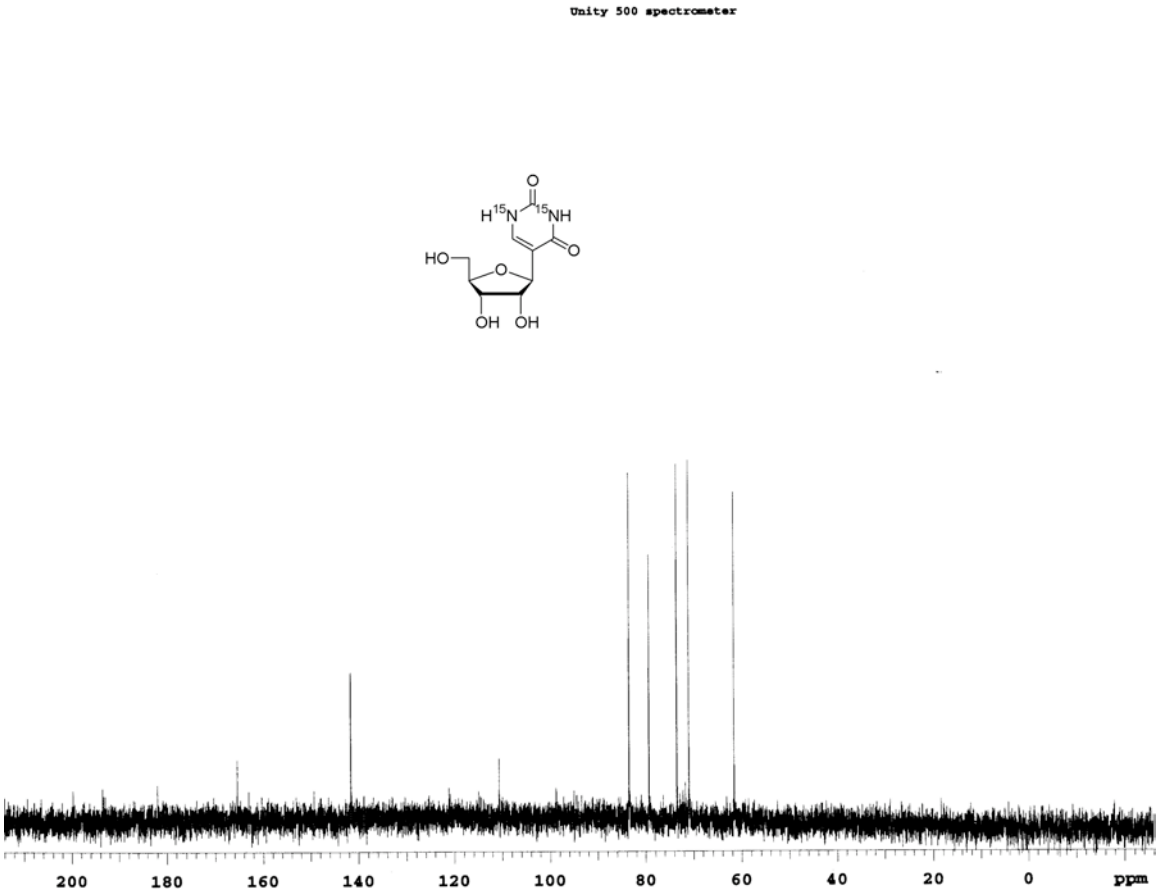
5-(β-D-Ribofuranosyl)uracil ([1,3-^{15}N]-Pseudouridine) (7)

To a solution of **6** (0.35 g, 0.63 mmol, 1.0 eq) in glacial acetic acid (15 mL) was added sodium iodide (0.38 g, 2.53 mmol, 4.0 eq) and heated to reflux for 35 min. The reaction mixture was poured onto ice and the resulting aqueous layer extracted with chloroform (3 × 30 mL). The combined organic extracts were washed with saturated solutions of $Na_2S_2O_3$ and $NaHCO_3$ for several times and dried over Na_2SO_4. The solvent was evaporated under reduced pressure to afford a light-yellow oil. A white solution was obtained after TFA/H_2O (9/1, 10 mL) added to this light-yellow oil. Stirring was continued for 1 h. TFA and water were evaporated under reduced pressure in hot water bath. The product was washed with chloroform for several times to give **7** (0.15 g, 96%) as a light-yellow powder. mp 211–212 °C; ^{1}H NMR (500 MHz, D_2O) δ (ppm) 3.56 (dd, J_1 = 13.0 Hz, J_2 = 5.0 Hz, 1H), 3.68 (dd, J_1 = 12.0 Hz, J_2 = 3.0 Hz, 1H), 3.85 (m, 1H), 3.98 (t, J = 5.5 Hz, 1H), 4.13 (t, J = 5.5 Hz, 1H), 4.51 (d, J = 5.5 Hz, 1H), 7.50 (d, J = 3.0 Hz, 1H); ^{13}C NMR (500 MHz, D_2O) δ (ppm) 61.60, 70.92,73.45, 79.27, 83.46, 110.59, 141.54, 141.63, 165.36; ^{15}N NMR (500 MHz, D_2O) δ (ppm) -209.13, -234.91; ESI-MS (ES^+) m/z calcd for $C_9H_{12}{}^{15}N_2O_6$ 246.1, found 247.0 (MH^+), 269.0 ($M+Na^+$).

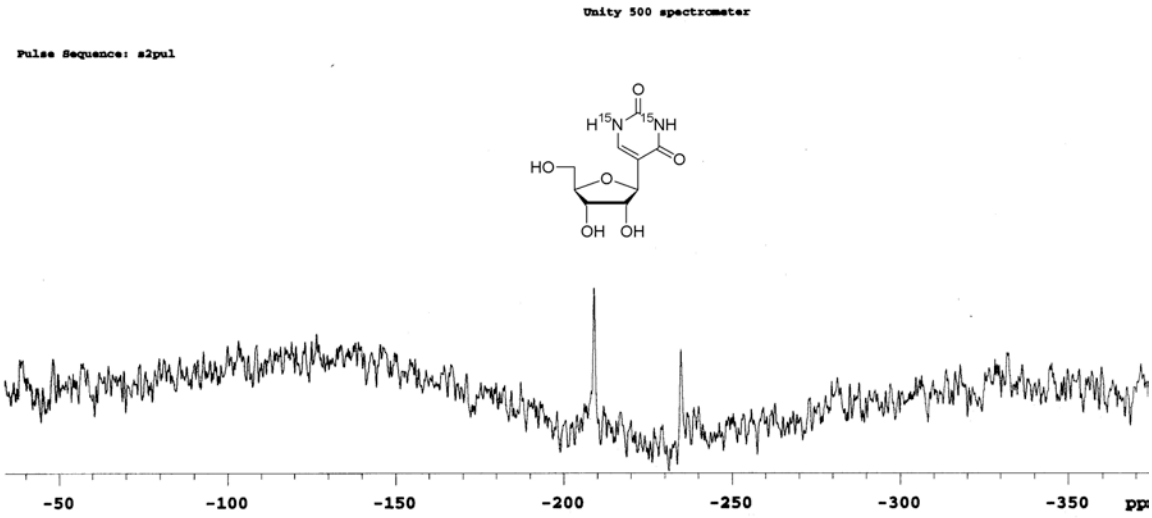
^{1}H NMR Spectrum



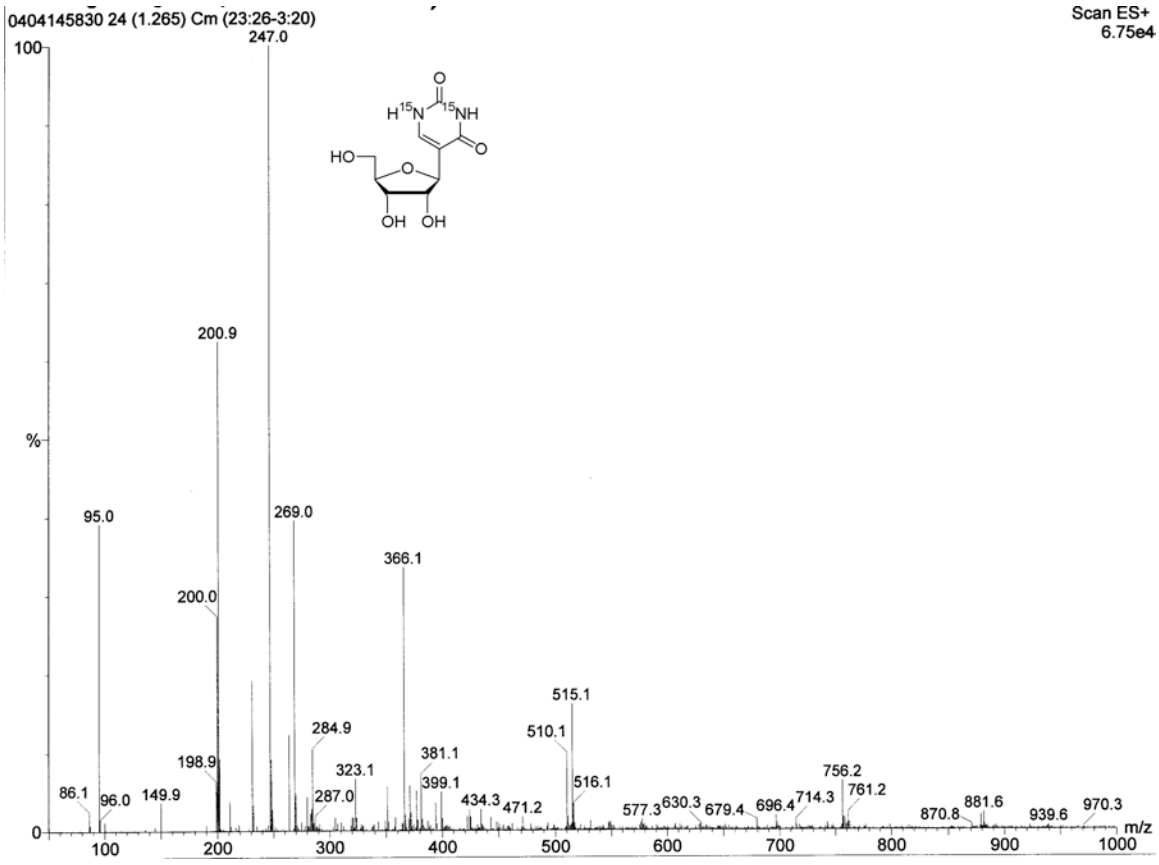
^{13}C NMR Spectrum



^{15}N NMR Spectrum



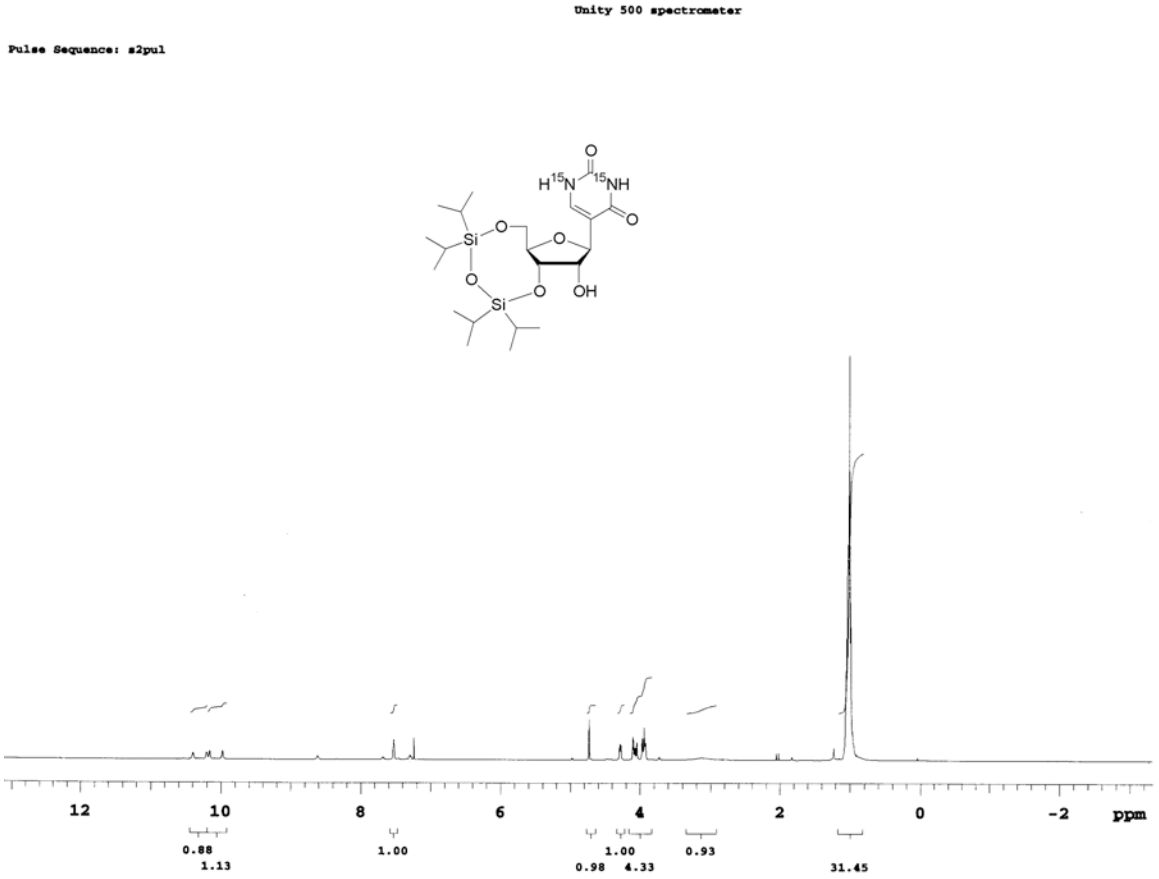
ESI-MS Spectrum



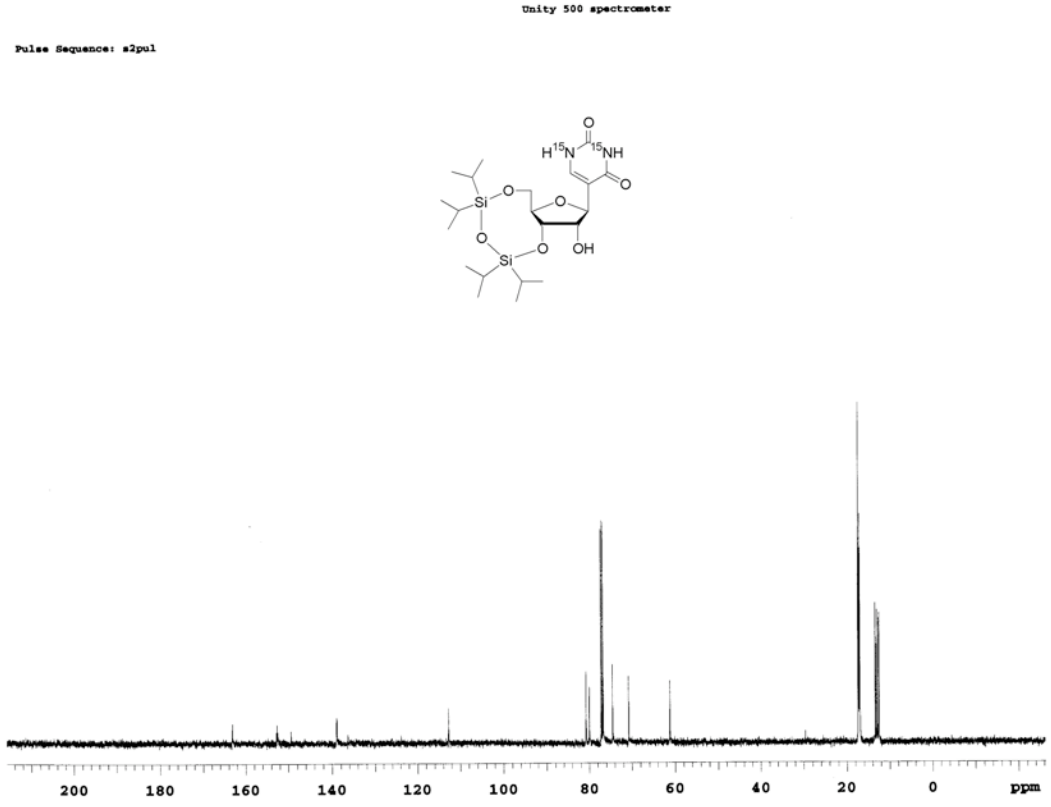
3',5'-*O*-(1,1,3,3-Tetraisopropyl-1,3-disiloxanediyl)-[1,3-^{15}N]-pseudouridine (8)

To a round-bottom flask, **7** (0.12 g, 0.47 mmol, 1.0 eq) was azeotroped with benzene for 2 h. This compound was dissolved in 10 mL of distilled pyridine at 0 °C. After 20 min, 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane (0.16 mL, 0.51 mmol, 1.1 eq) was added dropwise and left to stir at 0 °C for 1 h and overnight at room temperature. The following day, the reaction was dried under reduced pressure, and the product was purified on silica gel using 50% ethyl acetate in hexanes to isolate **8** as a white crystalline solid in 94% yield (0.22 g). ^{1}H NMR (500 MHz, $CDCl_3$) δ (ppm) 1.01 – 1.08 (m, 28H), 3.13 (s, 1H), 3.92 – 4.00 (m, 2H), 4.05 – 4.10 (m, 2H), 4.28 (dd, 1H, J = 5.8, 2.0 Hz), 4.73 (s, 1H), 7.53 (s, 1H), 10.07 (d, 1H, J = 91 Hz), 10.30 (d, 1H, J = 97.5 Hz); ^{13}C NMR (500 MHz, $CDCl_3$) δ (ppm) 12.52, 12.65, 13.01, 13.34, 16.90, 16.99, 17.01, 17.11, 17.26, 17.30, 17.39, 61.18, 70.74, 74.52, 79.99, 80.81, 112.72 (J = 3.4 Hz) 138.91 (J = 6.7 Hz), 152.63, 163.15; ^{15}N NMR (500 MHz, $CDCl_3$) δ (ppm) -214.61, -245.66; ESI-MS (ES^+) *m/z* calcd for $C_{21}H_{38}{}^{15}N_2O_7Si_2$ 488.2, found 511.2 ($M+Na^+$), 527.1 ($M+K^+$).

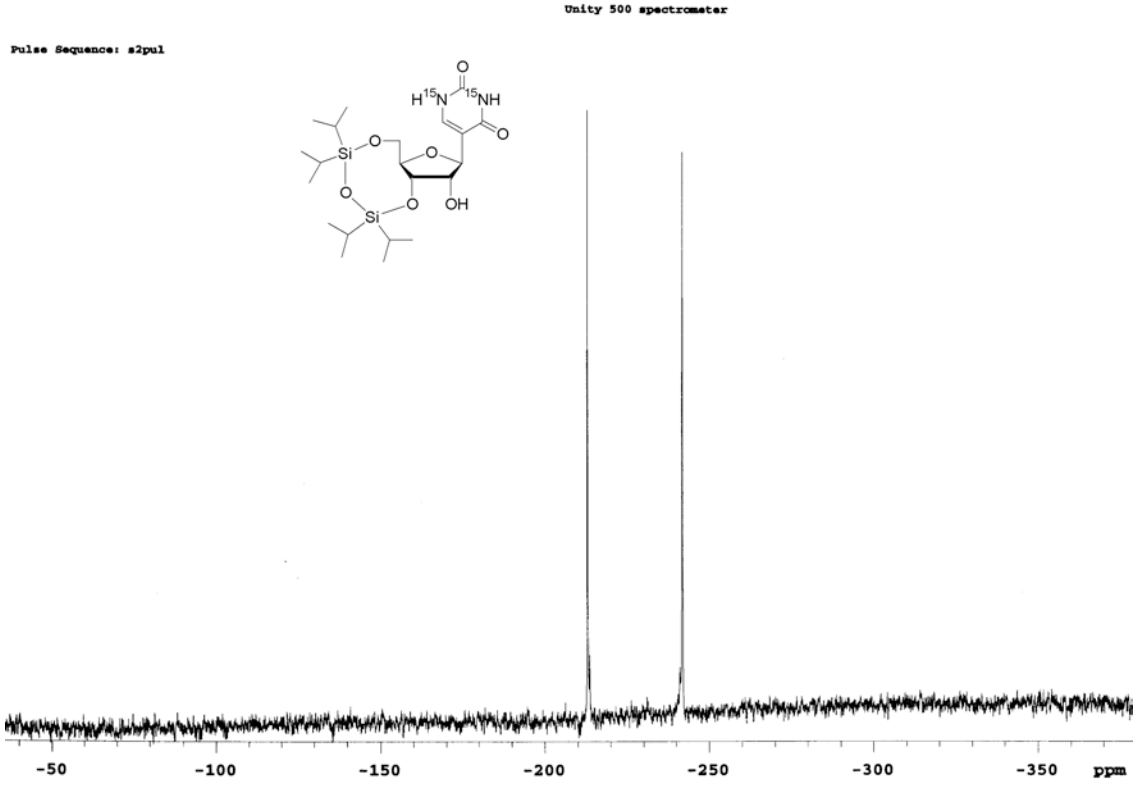
^{1}H NMR Spectrum



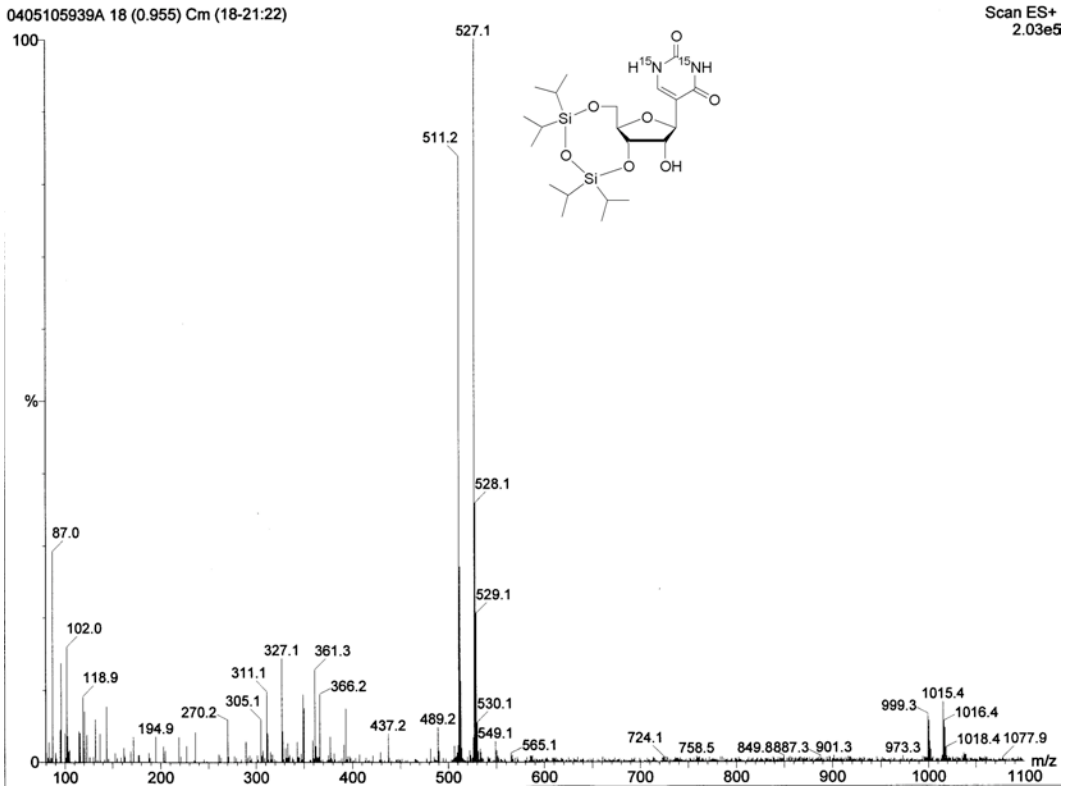
^{13}C NMR Spectrum



^{15}N NMR Spectrum



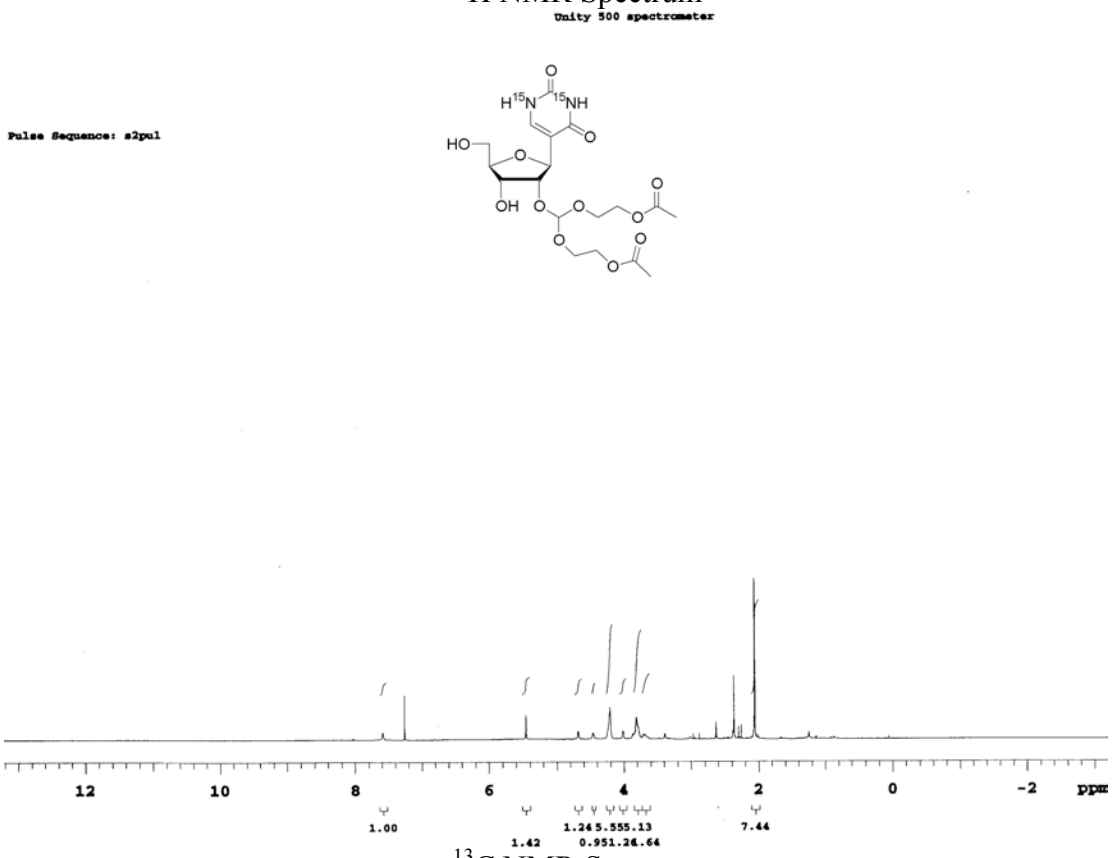
ESI-MS Spectrum



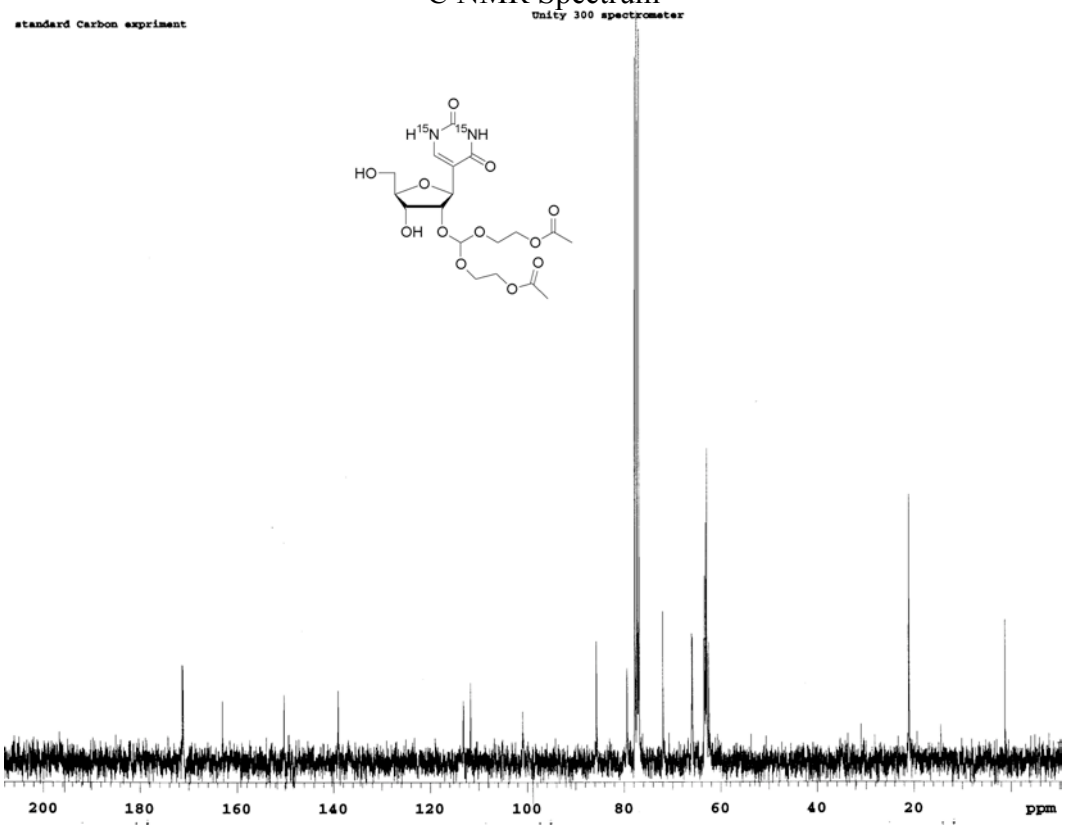
2'-*O*-[Bis(2-acetoxyethoxy)methyl]-[1,3-^{15}N]-pseudouridine (9)

Compound **8** (0.22 g, 0.44 mmol) and a sample of 3',5'-*O*-(1,1,3,3-tetraisopropyl-1,3-disiloxanediyl)-pseudouridine (0.22 g, 0.44 mmol) were dissolved in 5 mL of anhydrous 1,4-dioxane to give a solution of 50% ^{15}N-enriched compound (0.430 g, 0.882 mmol, 1.0 eq). Tris(2-acetoxyethoxy)orthoformate (1.65 g, 5.11 mmol, 5.8 eq) and pyridinium *p*-toluenesulfonate (0.04 g, 0.8 mmol, 0.4 eq) were then added to give a clear solution. The reaction was stirred for 1 h at room temperature, and then 4-(*tert*-butyldimethylsilyloxy)-3-penten-2-one (0.91 g, 3.35 mmol, 3.8 eq) was added. The solution was stirred and heated to 55 °C with a condenser overnight. The reaction mixture was monitored by TLC and showed efficient product conversion. *N,N,N′,N′*-Tetramethylethylenediamine (TMEDA; 0.11 g, 0.93 mmol, 1.1 eq) was added and stirred for 15 min at room temperature. The crude mixture was evaporated and the intermediate was partially purified on silica gel to yield a partially purified intermediate. The intermediate was dissolved in 1.78 mL of CH_3CN, and cooled to 0 °C to give solution A. To a separate flask, solution B was prepared at 0 °C by the addition of CH_3CN (1.78 mL), TMEDA (0.66 mL), and 48% aq. HF (0.10 mL). Solution B was stirred for 10 min at 0 °C. Solution B was transferred dropwise to solution A by cannula. The reaction was stirred at 0 °C for 3 h, and TLC showed proper product conversion. The solvent was evaporated, and the crude product was purified by flash chromatography on silica gel with 1% TMEDA in a 33-88% EtOAc in hexanes gradient to give **9** as a pale yellow oil in 65% yield (0.26 g, 0.57 mmol). ^{1}H NMR (500 MHz, $CDCl_3$) δ (ppm) 2.038 (s, 3H), 2.043 (s, 3H), 3.66 – 3.83 (m, 6H), 3.85 (s, 1H), 4.00 (m, 1H), 4.19 – 4.21 (m, 5H), 4.44 (m, 1H), 4.67 (d, 1H, J = 4.5), 5.44 (s, 1H), 7.57 (s, 1H); ^{13}C NMR (300 MHz, $CDCl_3$) δ (ppm) 21.02, 62.47, 62.85, 63.04, 63.28, 63.41, 65.83, 65.96, 71.96, 77.71, 79.34, 85.65, 111.63, 113.15, 138.98, 150.17, 162.94, 171.25; ^{15}N NMR (500 MHz, $CDCl_3$) δ (ppm) -213.02, -241.93; ESI-MS (ES^+) *m/z* calcd for $C_{20}H_{26}{}^{15}N_2O_{12}$ 464.1 and $C_{20}H_{26}N_2O_{12}$ 462.1, found 485.0 ($M+Na^+$), 487.0 ($M(^{15}N)+Na^+$).

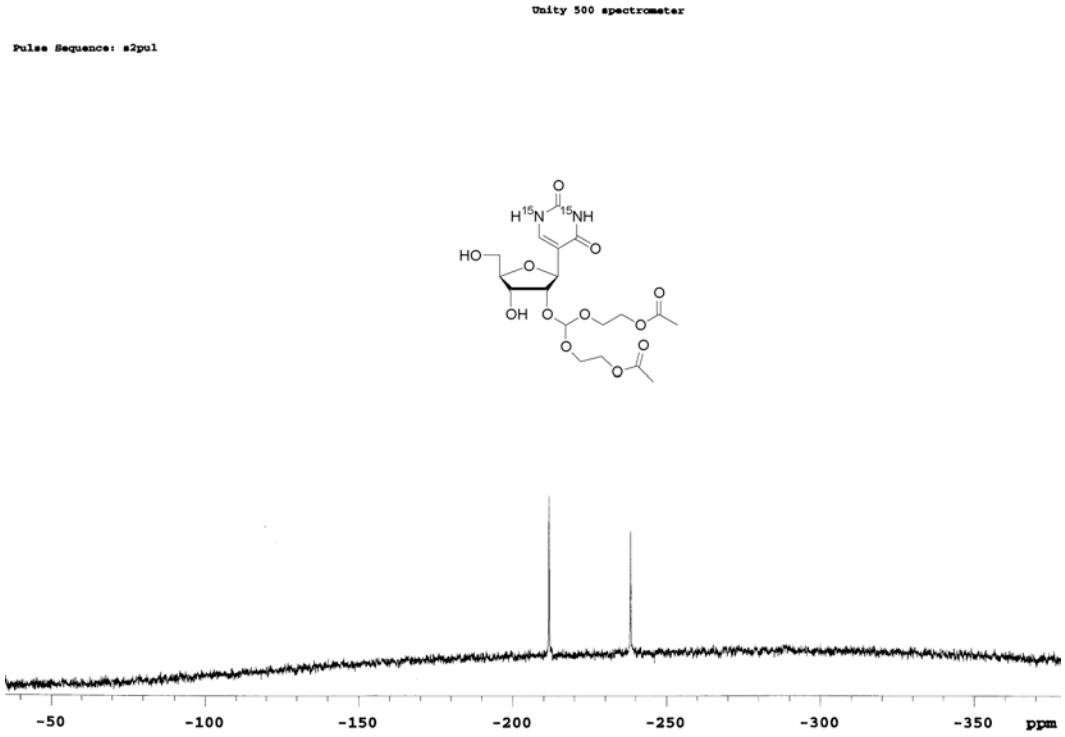
^{1}H NMR Spectrum



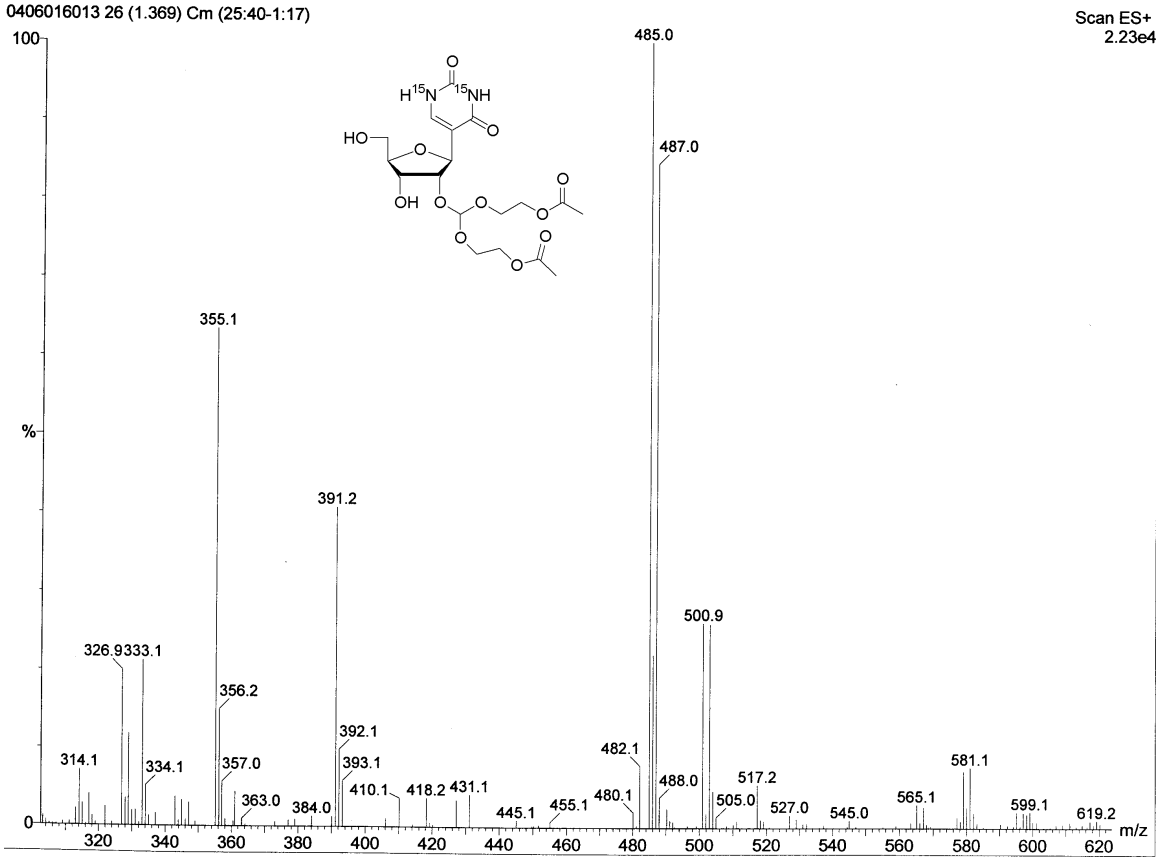
^{13}C NMR Spectrum



^{15}N NMR Spectrum



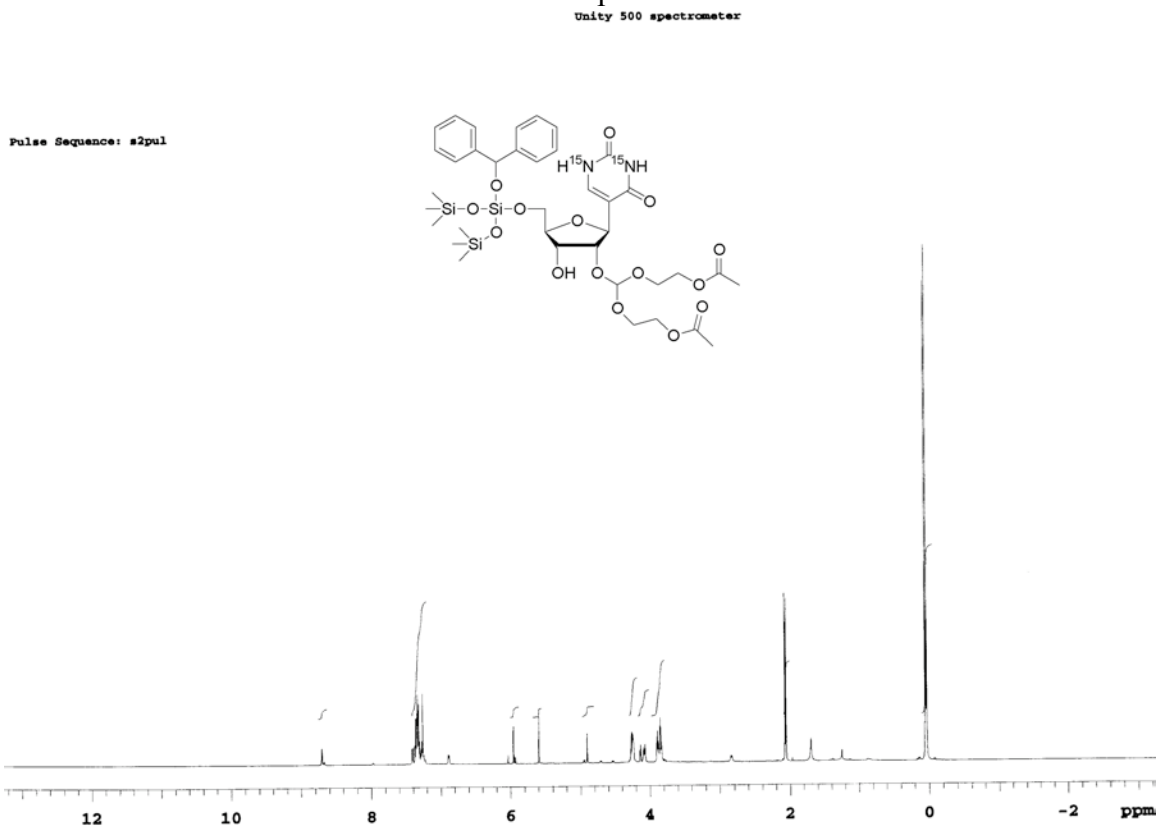
ESI-MS Spectrum



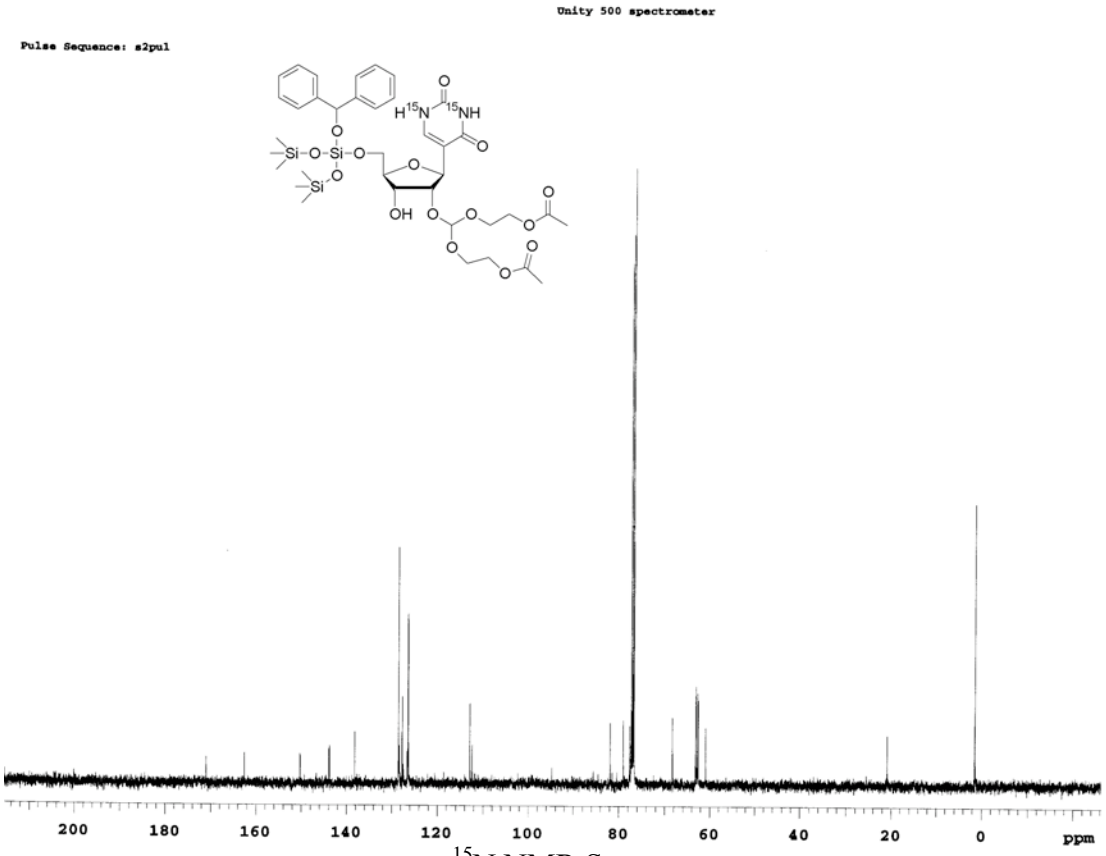
5'-*O*-[Benzhydryloxybis(trimethylsilyloxy]-2'-*O*-[bis(2-acetoxyethoxy)methyl]-[1,3-^{15}N]-pseudouridine (10)

Solution A was made by the addition of **9** (0.24 g, 0.52 mmol, 1.0 eq) in 2.9 mL of dry CH_2Cl_2 and 0.11 mL of distilled diisopropylamine to yield a clear yellow solution. The solution was cooled to 0 °C. To another flask, solution B was made by adding diisopropylamine (0.26 mL, 1.23 mmol) dropwise to benzhydryloxybis(trimethylsiloxy)silyl chloride (1.30 g, 3.08 mmol, 5.9 eq) in 1.23 mL of dry CH_2Cl_2 at 0 °C. 0.35 mL of solution B (0.5 eq) was added to solution A dropwise, and every 10 min, 0.25 eq aliquots were added. The reaction was stirred for 3 h at 0 °C and product formation monitored by TLC. Upon completion, the reaction was quenched with 5% $NaHCO_3$ and extracted with CH_2Cl_2. The organic layer was washed with brine, dried over Na_2SO_4, concentrated, and purified on silica gel with 33-50% EtOAc in hexanes to give **10** as a clear oil in 65% yield (0.29 g). ^{1}H NMR (500 MHz, $CDCl_3$) δ (ppm) 0.04 (s, 9H), 0.06 (s, 9H), 2.06 (s, 6H), 2.07 (s, 6H), 3.83–3.90 (m, 4H), 4.07–4.14 (m, 2H), 4.24–4.28 (m, 4 H), 4.91 (s, 1H), 5.60 (s, 1H), 5.96 (s, 1H), 7.25–7.37 (m, 8H), 7.41 (d, 1H, J = 5.0 Hz), 8.70 (s, 1H); ^{13}C NMR (500 MHz, $CDCl_3$) δ (ppm) 1.43, 20.85, 60.90, 62.60, 62.71, 63.08, 63.16, 68.28, 77.60, 78.96, 81.83, 112.37, 112.88, 126.33, 126.44, 126.57, 127.66, 127.71, 128.31, 128.36, 128.49, 138.16, 143.77, 143.89, 150.28, 162.54, 170.99; ^{15}N NMR (500 MHz, $CDCl_3$) δ (ppm) -213.66, -243.49; ESI-MS (ES^+) *m/z* calculated for $C_{37}H_{54}{}^{15}N_2O_{15}Si_3$ 852.3 and $C_{37}H_{54}N_2O_{15}Si_3$ 850.3, found 889.2 ($M+K^+$), 891.1 ($M(^{15}N)+K^+$).

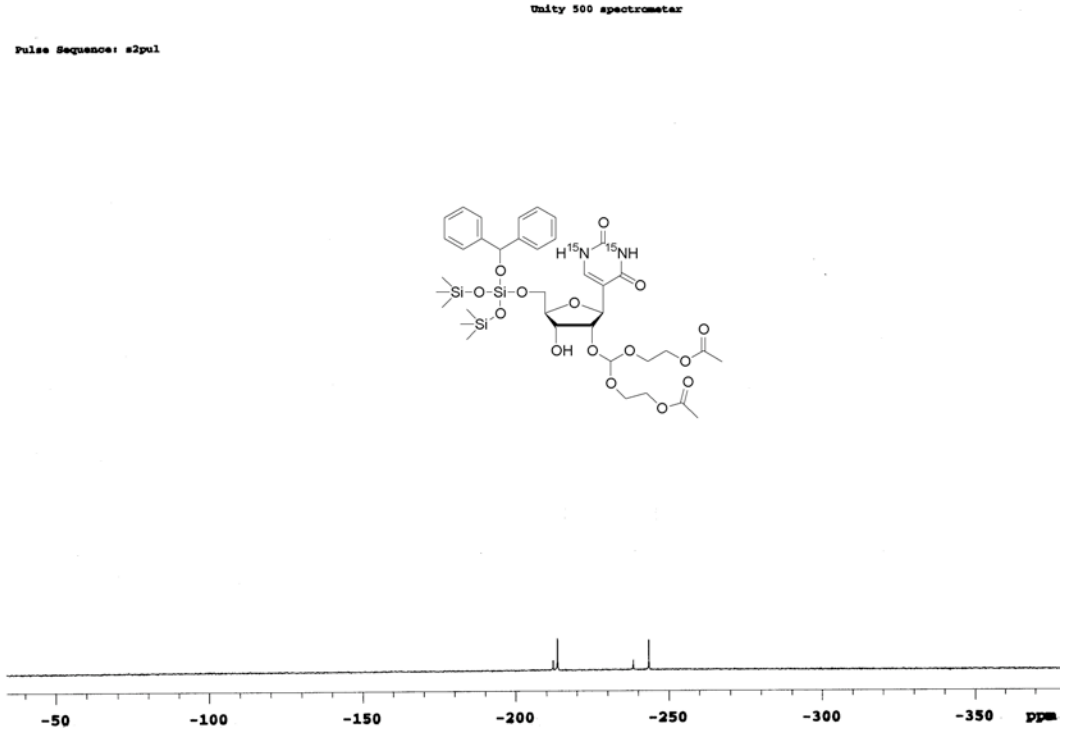
^{1}H NMR Spectrum



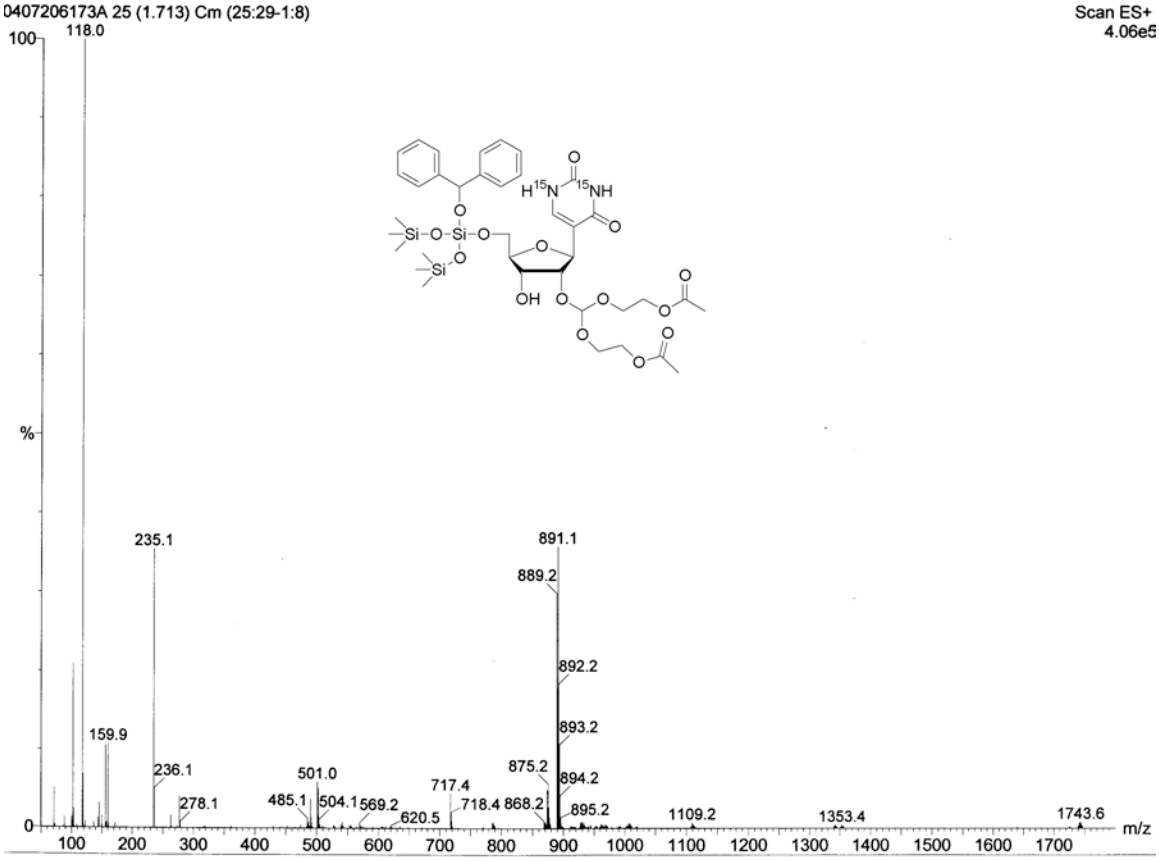
^{13}C NMR Spectrum



^{15}N NMR Spectrum



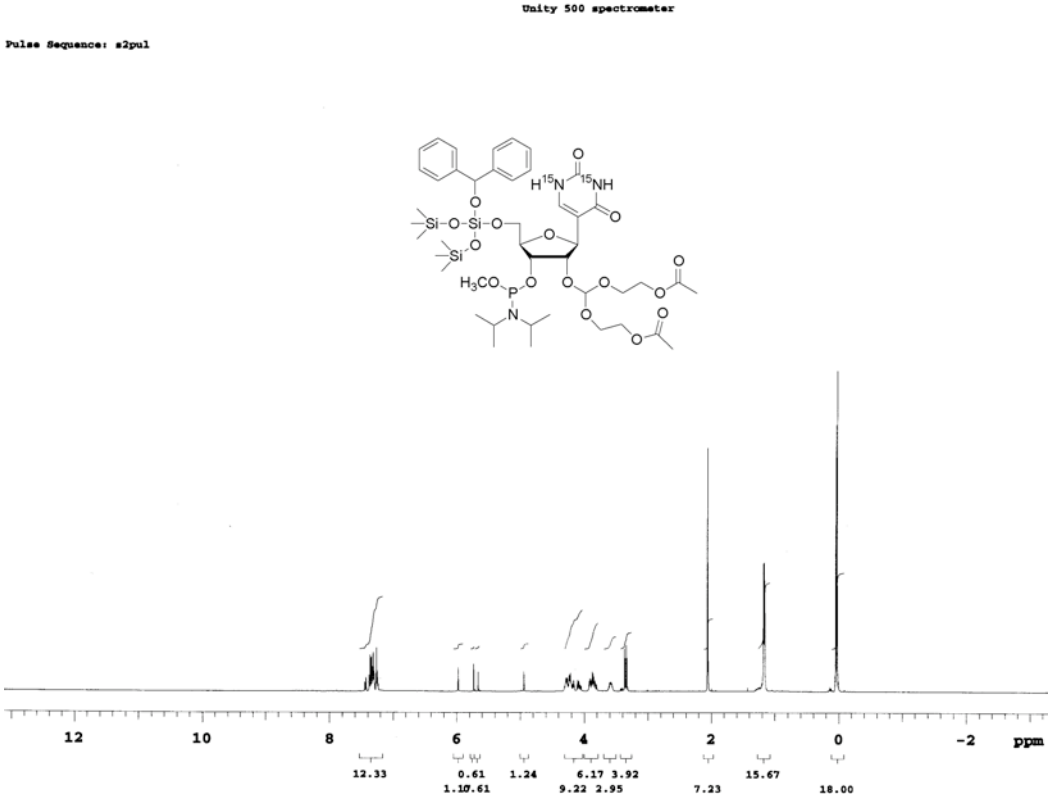
ESI-MS Spectrum



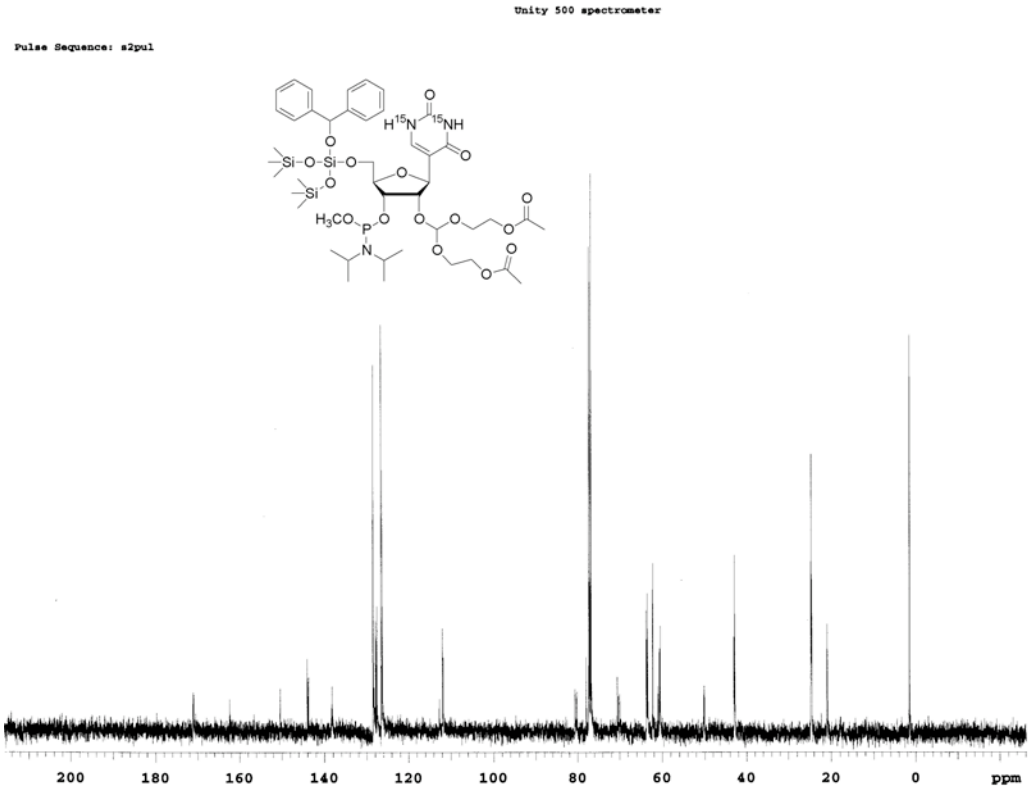
5'-*O*-[Benzhydryloxybis(trimethylsilyloxy)silyl]-2'-*O*-[bis(2-acetoxyethoxy)methyl]-[1,3-^{15}N]-pseudouridine-3'-(methyl-*N*,*N*-diisopropyl)phosphoramidite (11)

Compound **10** (0.17 g, 0.20 mmol, 1.0 eq) was dissolved in 2.2 mL of CH_2Cl_2. To this stirring solution, 1*H*-tetrazole (0.03 g, 0.20 mmol, 1.0 eq), and methyl tetraisopropyl phosphorodiamidite (0.16 mL, 0.54 mmol, 2.8 eq) was added and the reaction stirred overnight. The next day, TLC showed proper product conversion, and the reaction was quenched with 5% $NaHCO_3$ and extracted with CH_2Cl_2. The organic layer was dried over Na_2SO_4, and concentrated. The product was purified on silica gel with 10% TEA in a 25 – 33% acetone in hexanes gradient to afford **11** as a colorless oil (0.10 g, 0.10 mmol) in 52% yield. ^{1}H NMR (500 MHz, $CDCl_3$) δ (ppm) (mixture of diastereomers) 0.03–0.05 (3s, 18H), 1.162, 1.167, 1.175, 1.180 (4s, 12H), 2.05, 2.06 (2s, 6H), 3.34, 3.36 (dd, 3H, *J* = 5.0 Hz), 3.55–3.61 (m, 2H), 3.80–3.93 (m, 5H), 4.05–4.31 (m, 8H), 4.94 (s, 1H), 5.66 (s, 1H), 5.73 (s, 1H), 5.98 (s, 1H), 7.24–7.37 (m, 10H), 7.43 (d, 1H, *J* = 1.5 Hz), 7.44 (d, 1H, *J* = 1.0 Hz); ^{13}C NMR (500 MHz, $CDCl_3$) δ (ppm) (mixture of diastereomers) 1.46, 20.87, 20.91, 24.52, 24.57, 24.68, 24.74, 42.81, 42.90, 43.00, 50.06, 60.46, 60.58, 60.98, 62.20, 63.39, 63.47, 63.61, 63.69, 70.05, 70.39, 70.54, 77.35, 77.90, 80.16, 80.61, 111.75, 111.89, 112.69, 126.25, 126.30, 126.52, 127.55, 127.73, 127.80, 128.42, 128.47, 128.51, 138.20, 143.685, 143.76, 143.97, 150.36, 162.29, 170.98; ^{15}N NMR (500 MHz, $CDCl_3$) δ (ppm) (mixture of diastereomers) -211.83, -238.36; ^{31}P NMR (400 MHz, $CDCl_3$) δ (ppm) (mixture of diastereomers) -71.37, -70.14; ESI-MS (ES^+) *m/z* calculated for $C_{44}H_{70}N^{15}N_2O_{16}PSi_3$ 1013.4 and $C_{44}H_{70}N_3O_{16}PSi_3$ 1011.4, found 1050.3 ($M+K^+$), 1052.3 ($M(^{15}N)+K^+$); Anal. calcd for 50% $C_{44}H_{70}N^{15}N_2O_{16}PSi_3$ and 50% $C_{44}H_{70}N_2{}^{15}NO_{16}PSi_3$: C, 52.16; H, 6.96; N, 4.25. Found C, 51.99; H, 6.95; N, 4.20.

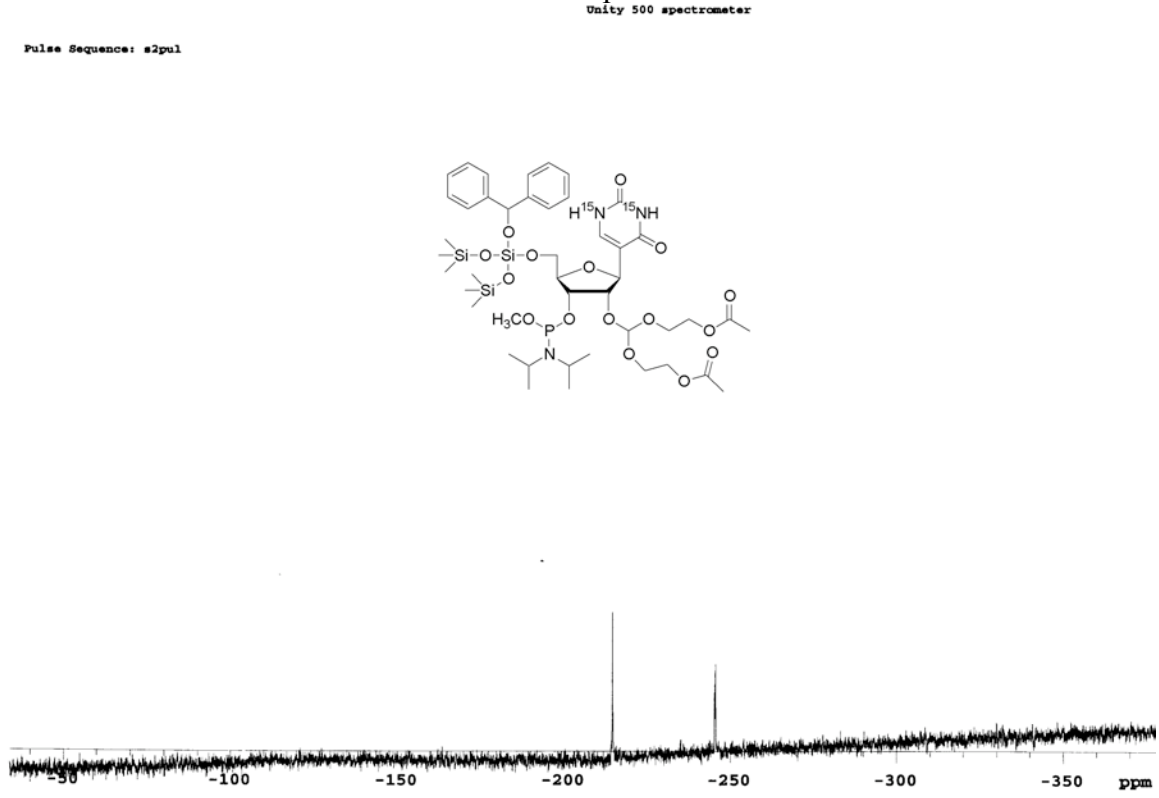
^{1}H NMR Spectrum



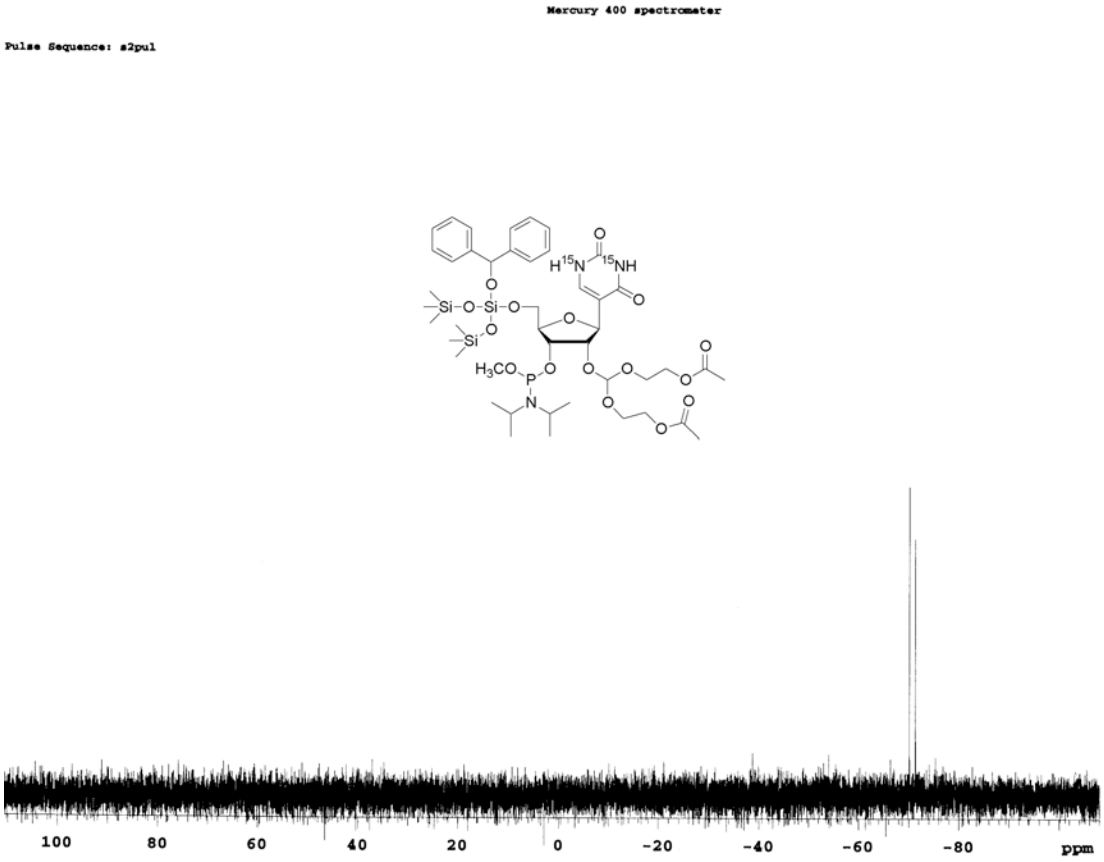
^{13}C NMR Spectrum



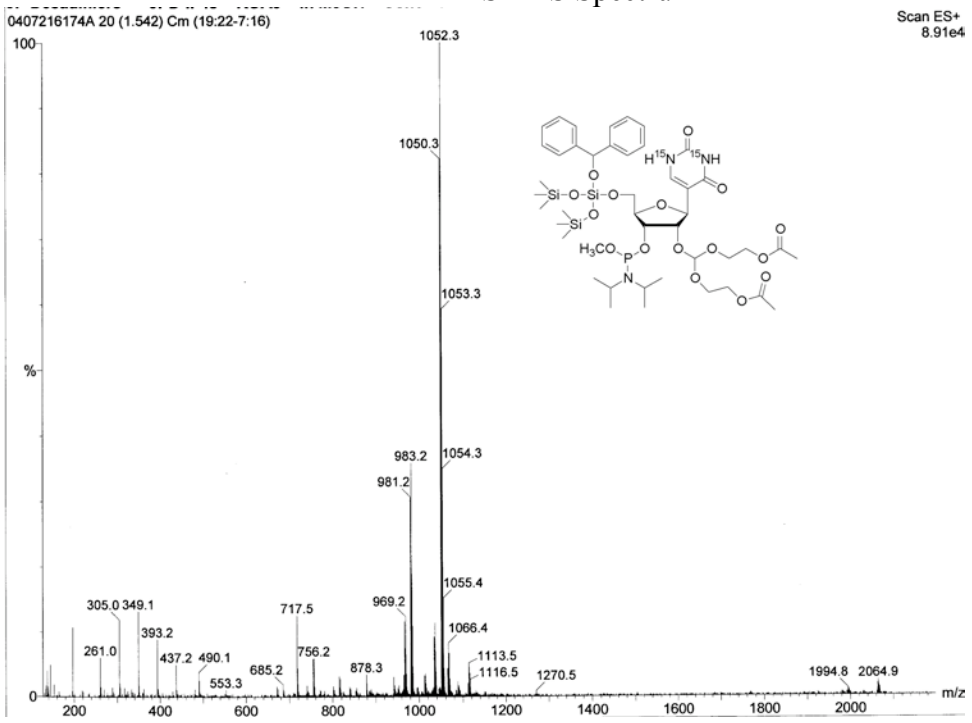
^{15}N NMR Spectrum



^{31}P NMR Spectrum



ESI-MS Spectrum



ElementalAnalysis

Analysis	Theory	% Found	
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H	6.96	6.95	
N	4.25	4.20	
P	3.06	*	

M.P./B.P. oil

Hygroscopic: Yes Explosive. No

Molecular Formula: $C_{44}H_{70}N_2NO_{16}PSi_3$

To Be Dried: No X
Yes ☐ at °C

ANALYZE for: CHN, P ☐ Single ☐ Duplicate

m/m Lab Established in 1966
MIDWEST MICROLAB, LLC
7212 N. SHADELAND AVE. INDIANAPOLIS, IN 46250
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Synthesis, Deprotection, and Purification of Modified RNA Oligonucleotides

RNAs were synthesized on 1.0 μmol scales using polystyrene supports as described previously. The sequences of the three RNAs are as follows, with the numbering based on the full-length *E. coli* 23S rRNA: 5'-$G_{1906}GCCG^{15}N\Psi_{1911}AAC\Psi_{1915}A\Psi_{1917}AACGGUC_{1924}$-3' (referred to as $^{15}N\Psi\Psi\Psi$; the names of the RNAs correspond to the nucleotides at positions 1911, 1915, and 1917, respectively), and 5'-$G_{1906}GCCG\Psi_{1911}AAC^{15}N\Psi_{1915}A\Psi_{1917}AACGGUC_{1924}$-3' ($\Psi^{15}N\Psi\Psi$), and 5'-$G_{1906}GCCG\Psi_{1911}AAC^{15}N\Psi_{1915}A\Psi_{1917}AACGGUC_{1924}$-3' ($\Psi\Psi^{15}N\Psi$), where $^{15}N\Psi$ refers to the a [1,3-^{15}N]pseudouridine insertion site. The crude RNAs were deprotected using 100 mM NaOAc buffer (pH 3.8) and heating the solution at 60 °C for 30 min. The RNAs were purified on a Xterra MS C18 column (2.5 μm, 10 x 50 mm, Waters, MA) using HPLC. The eluent was 0.1 M TEAA buffer, pH 7.0 with a 7 to 11 % linear gradient of acetonitrile over 17 minutes at a flow rate of 4.5 mL/min. Following HPLC purification, the RNAs were ethanol precipitated with NaOAc, and dialyzed for 3 days against RNase-free, double-deionized water.

RNA concentrations were calculated using Beer's law and single-stranded extinction coefficient (ε) was calculated to be 188,860 cm^{-1} M^{-1} for each RNA. The extinction coefficient for uridine (1.0×10^4 cm^{-1} M^{-1} at pH 7.0)[8] was used for pseudouridine because the nearest-neighbor extinction coefficient is unknown.

For NMR sample preparation, the RNA was dissolved in 200 μL 90% H_2O and 10% D_2O containing a buffer which contained NMR buffer (30 mM NaCl, 10 mM sodium phosphate and 0.5 mM Na_2EDTA, at pH 6.5). This solution was heated to 90 °C for 2 minutes, and slowly cooled to room temperature for proper hairpin annealing. The solution was added to an NMR Shigemi tube, and residual RNA left in the centrifuge tube was washed with 80 μL, and added to NMR Shigemi tube to yield a final volume of 280 μL. The RNA concentrations for the RNAs were determined to be in the range from 0.5 – 1.0 mM.

[8] Richards, E. G. In *Handbook of Biochemistry Molecular Biology: Nucleic Acids*; Fasman, G. D., Ed.; CRC Press: Cleveland, OH, 1975, 596 – 599.

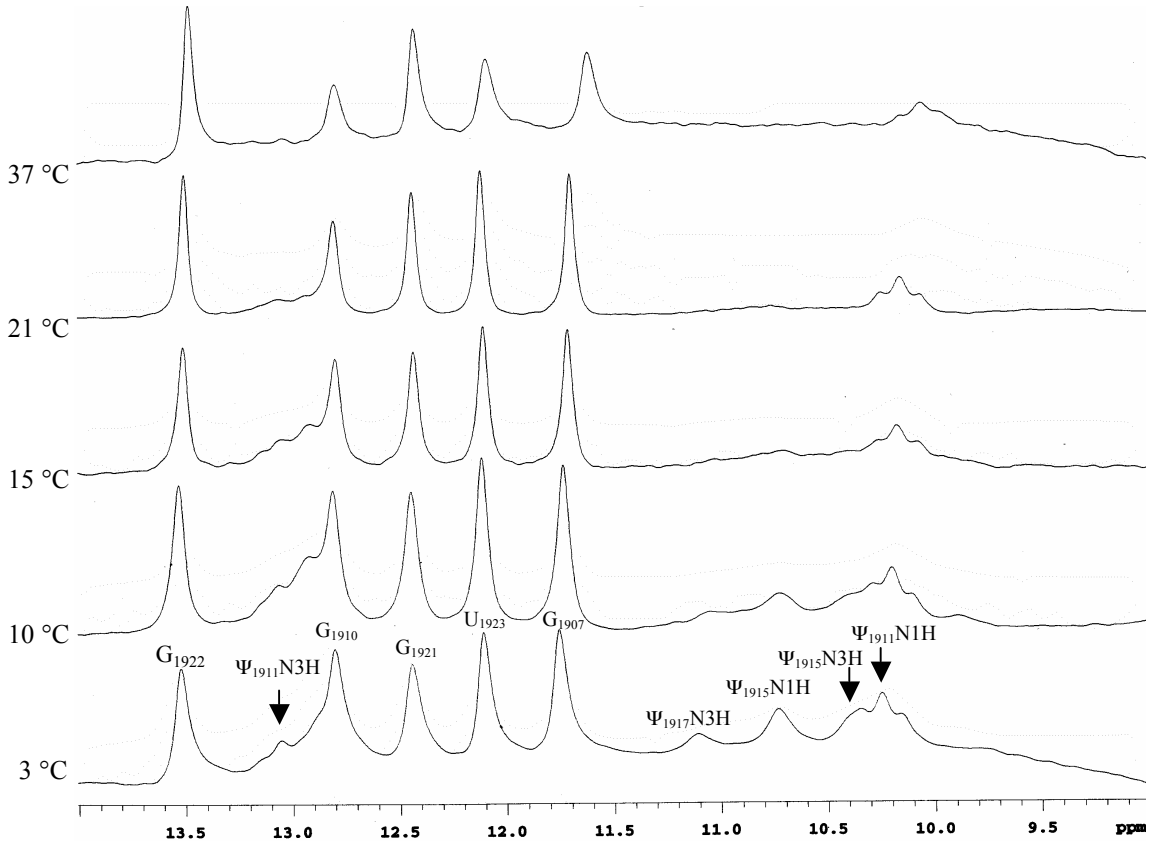
Temperature-dependent imino ^{1}H NMR spectra

^{1}H NMR spectra for Ψ^{15}NΨΨ, Ψ^{15}NΨΨ, and ΨΨ^{15}NΨ were obtained on a Varian Unity 500 MHz spectrometer at 3, 10, 15, 21 and 37. Table 1 illustrates the chemical shifts of the imino region (9 – 15 ppm) at 3 °C.

Table S1: Imino chemical shift resonances of ^{15}NΨΨΨ, Ψ^{15}NΨΨ, and ΨΨ^{15}NΨ.

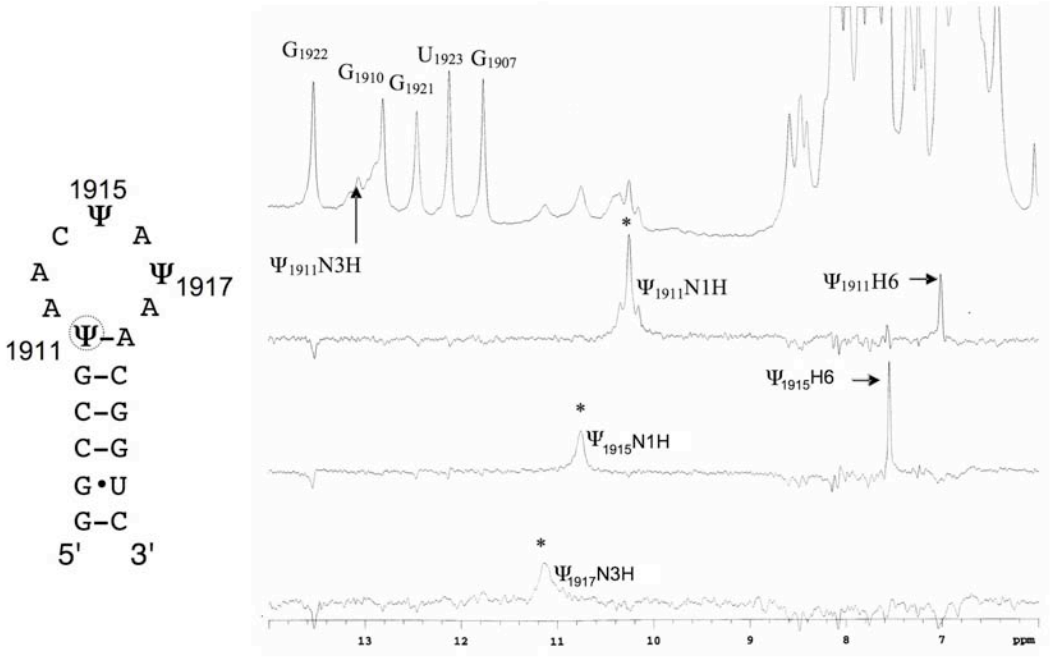
	^{15}NΨΨΨ	Ψ^{15}NΨΨ	ΨΨ^{15}NΨ
G1922	13.5	13.5	13.5
Ψ1911N3H	13.1	13.1	13.1
G1910	12.8	12.8	12.8
G1921	12.4	12.5	12.4
U1923	12.1	12.1	12.1
G1907	11.8	11.8	11.8
Ψ1917N3H	11.1	11.1	11.2
Ψ1915N1H	10.7	10.8	10.7
Ψ1915N3H	10.4	10.4	overlap with 10.3
Ψ1911N3H	10.3	10.3	10.3

Temperature-dependent spectra of ^{15}NΨΨΨ are shown below.



1D imino NOE difference spectroscopy of ^{15}NΨΨΨ

1D imino NOE difference spectra of ^{15}NΨΨΨ were obtained at 3 °C on a Unity Varian 500 MHz spectrometer. Asterisks indicate irradiated protons and arrows show NOEs.



2D ^{15}N HMQC of Ψ^{15}NΨΨ

A 2D ^{15}N HMQC spectrum of Ψ^{15}NΨΨ was obtained at 3 °C on a Bruker 700 MHz spectrometer. Ψ_{1915}N1 was assigned to 135 ppm, based on its correlation with the triplet at 10.7 ppm. The triplet at 10.7 was assigned to Ψ_{1915}N1H because of a strong NOE at 7.4 ppm (Ψ_{1915}H6). Ψ_{1915}N3 was assigned to 159 ppm, because it correlates to a broad proton peak at 10.4 ppm (Ψ_{1915}N3H).

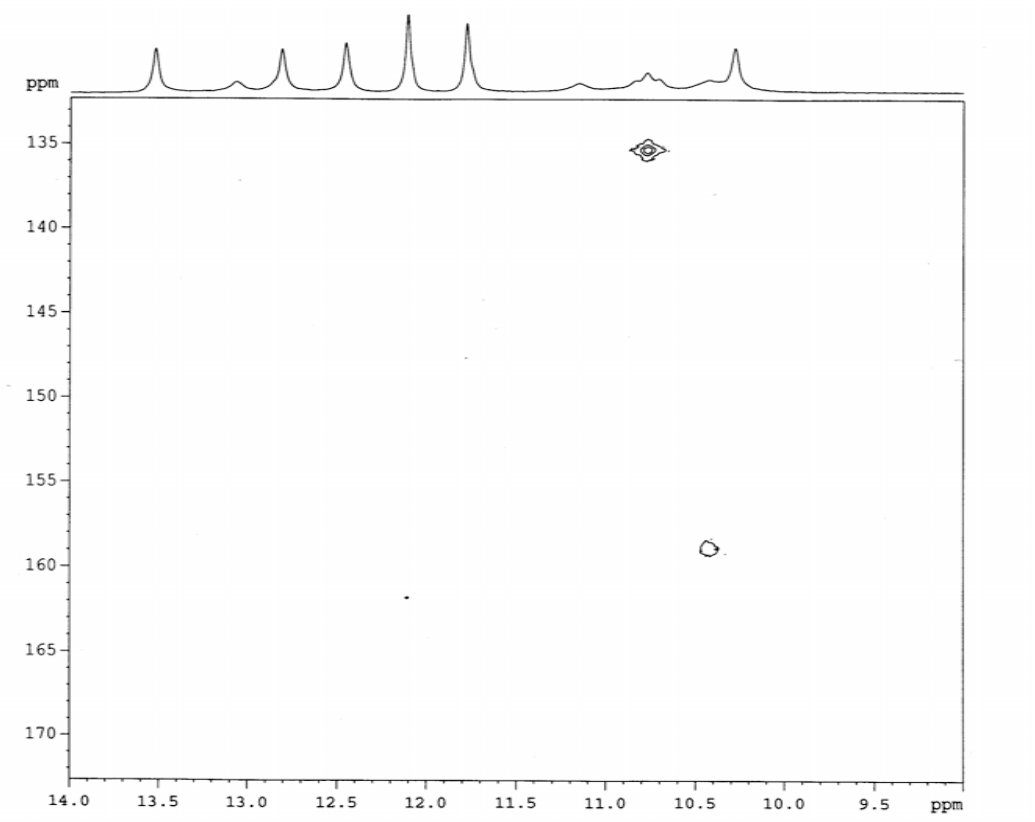


Table S2: Key NOEs involving pseudouridines observed in ΨΨΨ H69 RNA (5'-GGCCGΨAACΨAΨAACGGUC-3') at 37 °C in 99.96% D_2O 0.1 M NaCl, 10 mM sodium phosphate, pH 7 buffer. The results are consistent with strong stacking of C1914 with Ψ1915 and tri-nucleotide stacking of A1916, Ψ1917, and A1918.

Protons	NOE intensity[a]
Ψ1915H6-C1914H6	weak
Ψ1915H6-C1914H1'	weak
Ψ1915H6-C1914H2'	medium
Ψ1915H6-C1914H3'	strong
Ψ1915H6-A1916H8	very weak
Ψ1915H1'-A1916H8	weak
Ψ1915H2'-A1916H8	weak
Ψ1915H6-A1916H2	medium
Ψ1917H6-A1916H8	weak
Ψ1917H6-A1918H8	medium
Ψ1917H6-A1916H2	weak
Ψ1917H6-A1916H1'	weak
Ψ1917H1'-A1916H1'	weak
Ψ1917H6-A1916H2'	strong
Ψ1917H1'-A1916H2	weak
Ψ1917H2'-A1916H2	weak
Ψ1917H3'-A1916H2	weak
Ψ1917H1'-A1918H8	weak
Ψ1917H2'-A1918H8	medium
Ψ1917H3'-A1918H8	strong
Ψ1917H1'-A1918H1'	weak
Ψ1917H2'-A1918H1'	weak

[a] NOE intensities are listed as strong (2-3 Å), medium (2-4 Å) and weak (3-5 Å), and very weak (3-6 Å) distance restraints.