Supporting Information for

Locked Nucleic Acid (LNA)-Modified Dinucleotide mRNA Cap Analogue: Synthesis, Enzymatic Incorporation, and Utilization

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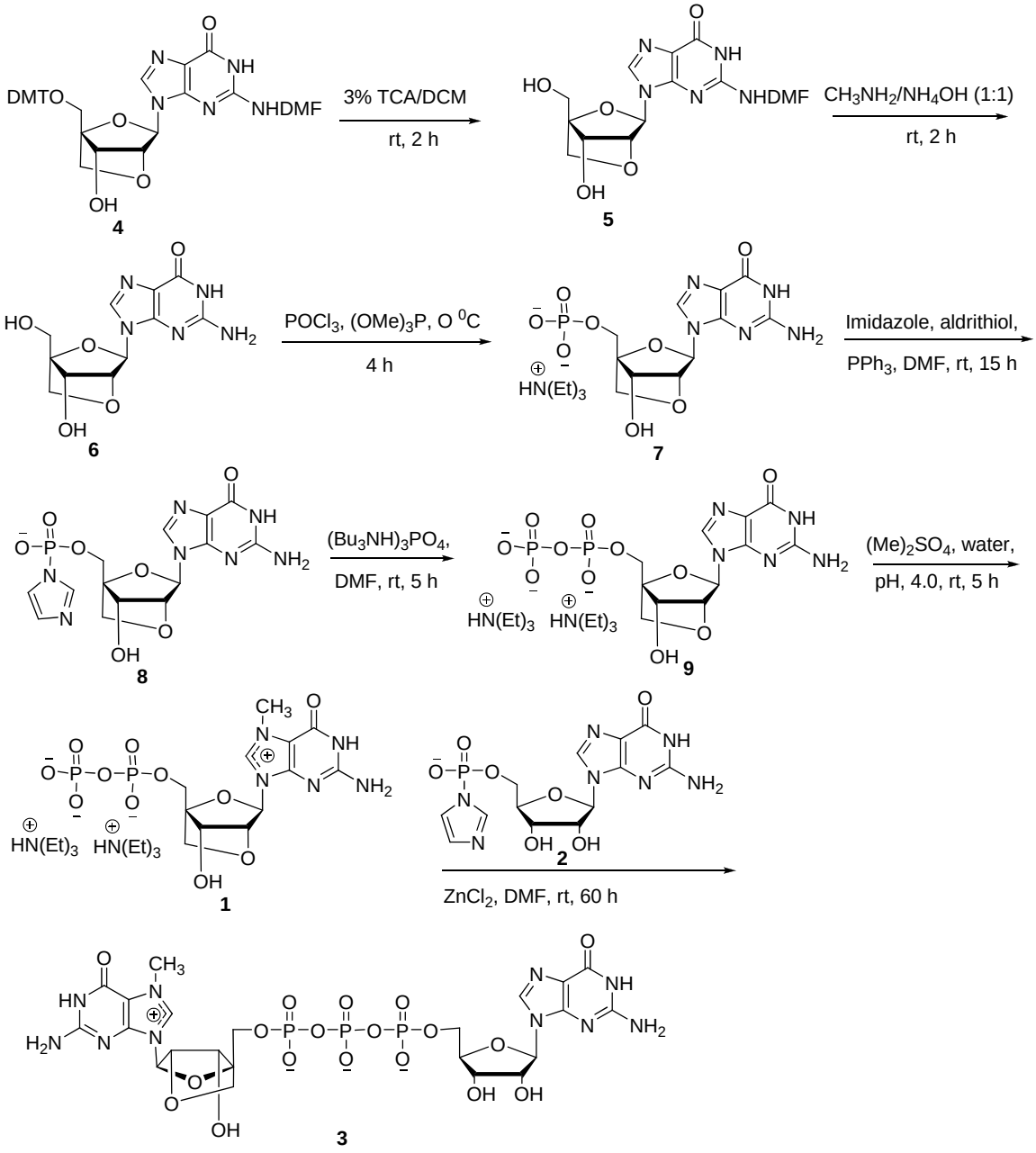
Contents:

1. General Notes and Procedures:

All of the commercial reagents and solvents are used as such without further purification. ^{1}H NMR spectra were recorded in D_2O on a Bruker 400 MHz and ^{31}P NMR were recorded on a Bruker 162 MHz. Chemical shifts are reported in ppm, and signals are described as s (singlet), d (doublet), t(triplet), q (quartet), and m (multiplet). ESI mass was recorded on a Applied Biosystems/Sciex MDX API 150 model and MALDI-TOF was recorded on a Applied Biosystems Voyager DE-PRO model. HPLC was run on a Waters 2996 (Waters Corporation) using anion exchange column. FPLC (fast protein liquid chromatography) was performed on a ÄKTA purifier (Amersham Biosciences, GE Healthcare) using DEAE Sephadex column. The starting material 5'-DMT-N-DMF LNA guanosine was purchased from Exiqon. (Denmark).

2. Synthesis of Novel Cap Analog $m^{7(LNA)}G[5']ppp[5']G$ 3:

The overall synthetic scheme to make **3** is depicted as follows:



Synthesis of DMF-protected LNA Guanosine 5: To a stirred solution of 30 mL 3% trichloroacetic acid in dichloromethane, 5'-DMT-N-DMF LNA guanosine **4** (2.90 g, 4.32 mmol) was added and the reaction mixture was stirred for 2 h at rt. After 2h, the reaction mixture was evaporated under rotavapor. To the resulting orange solid, 25 mL diethyl ether was added and allowed to stir at rt for 30 min. The resulting mixture was filtered and dried under vacuum to get a white colored solid **5** (Yield: 1.55 g, 95%). This crude material was used for next step without further purification.

Synthesis of LNA Guanosine 6: To a stirred solution of 20 mL 1:1 mixture of aqueous 40% methyl amine and 28% ammonium hydroxide, DMF-protected LNA guanosine **5** (1.55 g, 4.09 mmol) was added and the reaction mixture was stirred at rt for 2 h. After 2 h, the reaction mixture was evaporated under rotavapor to get a white colored solid **6** (Yield, 1.15 g, 95%). This crude material was used for next step without further purification.

Synthesis of LNA-GMP 7: To a stirred solution of $POCl_3$ (1.69 g, 11.19 mmol) and $(MeO)_3P$ (15.0 mL) at 0 ^{0}C under argon atmosphere, LNA guanosine **6** (1.10 g, 3.72 mmol) was added and the reaction mixture was stirred for 4 h at 0 ^{0}C. After 4 h, 50.0 mL water was added to the reaction mixture. The resulting reaction mixture was washed with ethyl acetate (2 X 50 mL) to remove phosphorylating agent. The collected aqueous solution was adjusted to pH 1.5 and allowed to stir at 4 ^{0}C for 15 h. After 15 h, the aqueous solution was adjusted to pH 5.5 and loaded on a DEAE Sephadex column The desired product was eluted using a linear gradient of 0-1M TEAB (triethyl ammonium bicarbonate, pH 7.5) and the fractions containing the product were pooled, evaporated and dried in vacuum desiccator over phosphorous pentoxide to give a fine white powder

7 (Yield: 1.47 g, 80%). Data for **7**. ^{1}H NMR (D_2O, 400 MHz) δ 8.01 (s, 1H), 5.91 (s, 1H), 4.63 (s, 1H), 4.58 (s, 1H), 4.16 (m, 3H), 4.03 (d, *J* = 8.4 Hz, 1H), 3.20 (q, *J* = 7.6 Hz, 6H), 1.28 (t, *J* = 7.2 Hz, 9H); ^{31}P NMR (D_2O, 162 MHz) δ -5.40 (s, 1P); MS (m/z): 374 $[M-H]^{+}$.

Synthesis of ImLNA-GMP 8: To a stirred solution of LNA-GMP TEA salt **7** (1.40 g, 2.95 mmol) in 20 mL dry DMF, imidazole (0.96 g, 14.77 mmol), triphenyl phosphine (1.54 g, 5.88 mmol), aldrithiol (1.30 g, 5.90 mmol) and triethylamine (0.30 g, 2.97 mmol) were added. The reaction mixture was stirred under argon atmosphere at rt for 15 h. To a solution of sodium perchlorate (1.50 g) in 100 mL acetone in a centrifuge tube at 0 ^{0}C, the above reaction mixture was added slowly for 5 minutes. The resulting mixture was centrifuged and the supernatant liquid was removed. The solid was ground with a new portion of acetone (100 mL), cooled, and centrifuged again. This process was repeated for two more times, and the resulting solid was dried in a vacuum desiccator over P_2O_5 to give a white powder **8** (Yield: 1.14 g, 87%).

Synthesis of LNA-GDP 9: To a stirred solution of ImLNA-GMP **8** (1.10 g, 2.46 mmol) and zinc chloride (0.67 g, 4.92 mmol) in 10.0 mL dry DMF, 15 mL of 1M tris(tributylammonium) phosphate in DMF was added under argon atmosphere. The reaction mixture was stirred at rt for 5 h. After 5 h, the reaction mixture was diluted with 50.0 mL of water. The resulting reaction mixture was washed with ethyl acetate (2 X 50 mL) to remove phosphorylating agent. The collected aqueous solution was adjusted to pH 5.5 and loaded on a DEAE Sephadex column. The desired product was eluted using a linear gradient of 0-1M TEAB and the fractions containing the product were pooled, evaporated and dried in vacuum desiccator over phosphorous pentoxide to give a fine

white powder **9** (Yield 1.12 g, 70%). Data for **9**. ^{1}H NMR (D_2O, 400 MHz) δ 8.00 (s, 1H), 5.95 (s, 1H), 4.63 (s, 2H), 4.37 (m, 2H), 4.14 (d, *J* = 8.4 Hz, 1H), 4.04 (d, *J* = 8.4 Hz, 1H), 3.20 (q, *J* = 7.2 Hz, 12H), 1.28 (t, *J* = 7.6 Hz, 18H); ^{31}P NMR (D_2O, 162 MHz) δ -8.94 (d, *J* = 20.9 Hz, 1P), -9.99 (d, *J* = 21.3 Hz, 1P); MS (m/z): 454 $[M-H]^+$.

Synthesis of $m^{7(LNA)}$GDP 1: To a stirred solution of LNA-GDP **9** (0.90 g, 1.37 mmol) in 20.0 mL of water, acetic acid was added slowly to adjust the pH of the solution to 4.0. To this mixture, dimethyl sulfate (2.0 mL) was added drop wise over a period of 30 min. and the reaction mixture was allowed to stir at rt for 5 h. As the methylation proceeds, the pH drops down to around 2.0 and the pH was readjusted back to 4.0 using 1M NaOH solution. After 5 h, the reaction mixture was extracted with ethyl acetate (3 X 50 mL) to remove unreacted excess dimethyl sulfate. The collected aqueous solution was adjusted to pH 5.5 and loaded on a DEAE Sephadex column. The desired product was eluted using a linear gradient of 0-1M TEAB and the fractions containing the product were pooled, evaporated and dried in vacuum desiccator over phosphorous pentoxide to give a fine white powder **1** (Yield 0.70 g, 76%). Data for **1**. ^{1}H NMR (D_2O, 400 MHz) δ 6.05 (s, 1H), 4.73 (s, 1H), 4.55 (s, 1H), 4.42 (m, 1H), 4.32 (m, 1H), 4.13 (s, 3H), 4.11 (d, *J* = 6.0 Hz, 1H), 4.00 (d, *J* = 8.8 Hz, 1H), 3.20 (q, *J* = 7.2 Hz, 12H), 1.28 (t, *J* = 7.2 Hz, 18H); ^{31}P NMR (D_2O, 162 MHz) δ -6.18 (d, *J* = 23.0 Hz, 1P), -9.56 (d, *J* = 22.8 Hz, 1P); MS (m/z): 468 $[M-H]^+$.

Synthesis of $m^{7(LNA)}$G[5']ppp[5']G 3: To a stirred solution of $m^{7(LNA)}$GDP **1** (0.17 g, 0.25 mmol) and ImGMP **2** (0.10 g, 0.23 mmol) in 10.0 mL dry DMF, zinc chloride (0.13 g, 0.96 mmol) was added under argon atmosphere and the reaction mixture was stirred at rt for 60 h. After 60 h, the reaction mixture was added to a solution of EDTA disodium

(0.71 g, 1.91 mmol) in 100.0 mL of water at 0 ^{0}C. The resulting aqueous solution was adjusted to pH 5.5 and loaded on a DEAE Sephadex column. The desired product was eluted using a linear gradient of 0-1M TEAB and the fractions containing the product were pooled, evaporated and concentrated to 10.0 mL TEA salt of **3**. The resulting 10.0 mL was passed through a Strata-X-AW column and washed with 10.0 mL water followed by 10.0 mL MeOH. Then, the desired compound was eluted with 15.0 mL of $NH_4OH/MeOH/H_2O$ (2/25/73) and the collected solution was evaporated and dried to give a fine white powder **3**. (Yield: 0.13 g, 65%) Data for **3**. ^{1}H NMR (D_2O, 400 MHz) δ 8.02 (s, 1H), 5.77 (d, *J* = 6.4 Hz, 1H), 5.71 (s, 1H), 4.65 (m, 1H), 4.58 (s, 1H), 4.46 (m, 3H), 4.37 (m, 1H), 4.31 (s, 1H), 4.28 (m, 2H), 4.12 (d, *J* = 8.4 Hz, 1H), 4.04 (s, 3H), 4.03 (d, *J* = 6.8 Hz, 1H); ^{31}P NMR (D_2O, 162 MHz) δ -10.12 (d, *J* = 19.9 Hz, 1P), -10.42 (d, *J* = 19.8 Hz, 1P), -22.03 (t, *J* = 19.9 Hz, 1P); MS (m/z): 815 $[M]^{+}$.

3. Gel shift (capping efficiency) and HPLC assay:

The capping efficiency of $m^{7(LNA)}$G[5']ppp[5']G **3** was compared with the standard cap m^{7}G[5']ppp[5']G **4**. The pTri β actin template was used in an *in vitro* transcription reaction omitting pyrimidine nucleotides, resulting in the termination of transcription after the first 7 coded nucleotides. Syntheses of the capped and uncapped oligoribonucleotides performed by using the MAXIscript kit (Ambion, Inc.), following manufacturer's protocol. Typically, 20 µl of the transcription reactions contained the following final concentrations of components: linearized pTri β actin vector template, 25 ng/µl (0.5 µg total); ATP, 2 mM; GTP, 0.4 mM; standard mCAP **4** or modified cap **3**, 1.6 mM each in separate reaction; reaction buffer, 1X; T7 RNA polymerase, 50 units/µl; and (α-^{32}P) ATP, 800 (Ci/mmol). In the control reaction, no cap analog was added. The

transcription reactions were incubated at 37 ^{o}C for 2 h, after which the reaction mixtures (10 μl) were then applied to a 20% dPAGE gel. Radiation in the gel bands of interest were quantified by a phosphorimager (GE Healthcare). In order to determine the orientation of the incorporated cap analog, the HPLC assay was developed. For this purpose, the above transcription reaction was scaled up 3X and the use of (α-^{32}P) ATP was eliminated from the reaction mixture. The remaining plasmid pTri β actin was hydrolyzed by adding 6 μl of turbo DNase to the reaction mixture, and further incubated at 37 ^{o}C for 15 min. The resulting crude reaction mixture was used as such for HPLC analysis using anion exchange column. The general structure of standard capped mRNA is depicted in Figure 1.

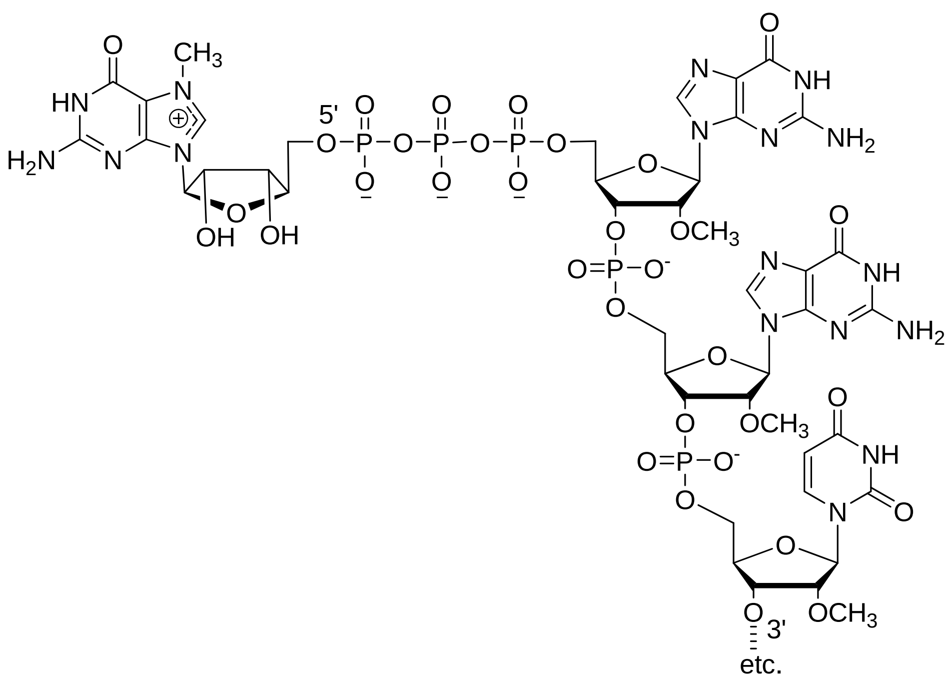


Figure 1. The chemical structure of the 5' terminus of a 'capped' mRNA with standard cap **4**.

4. *In vitro* Transcription of the Luciferase mRNA Poly(A):

T7 RNA polymerase transcription was performed by using the MEGAscript kit (Ambion, Inc.). All transcription reactions were performed in a 20 µl final volume at the following final concentrations of components: linearized AmbLuc poly(A) DNA, (1.0 µg total); 1X reaction buffer; ATP, UTP, and CTP, 7.5 mM each; and 50 units/µl of T7 RNA polymerase. Additionally, GTP was present at 7.5 mM in the no-cap control; and in the reactions with cap analog included GTP was present at 1.5 mM while the cap analog (standard mCAP **4** or modified cap **3**) was present at 6.0 mM. The transcription reactions were incubated at 37 ^{o}C for 2 h. In order to hydrolyze the remaining plasmid DNA, 1 µl of turbo DNase was added to the reaction mixture, and further incubated at 37 ^{o}C for 15 min. Purifications of the RNA from these transcription reactions were done by using the MEGAclear™ Kit (Ambion, Inc.) as per manufacturer's protocol. The transcription yield of modified cap **3** was comparable with the standard cap **4**. (Figure 2)

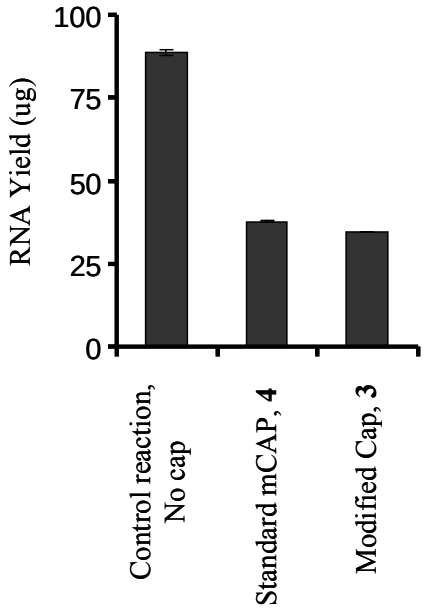


Figure 2. Yield of T7 RNA polymerase transcription reaction with standard **4** and LNA-modified cap **3** analogs

5. Assay of Intracellular Stability of Luciferase mRNA:

In vitro transcribed, 5′-capped RNAs from standard mCAP **4**, mixed population of 5′-capped and uncapped mRNA poly (A) from modified cap **3**, and uncapped control luciferase mRNAs from the above transcriptions were transfected into HeLa cells by electroporation. Cells were centrifuged at 300g for 5 min, and resuspended in siPORTER electroporation buffer. 75,000 cells in a total volume of 75 μL were placed in 1 mm pathlength cuvettes, 1 μg of mRNA was added, and electroporation performed with Bio-Rad Genepulser at 400 V/250 mF. Following discharge, cells were incubated in cuvettes at 37 °C for 10 min, then transferred to plates with prewarmed complete medium (75,000 cells per well of 24 well plate), and placed at 37 °C in a 5% CO_2 humidified atmosphere. Cells were harvested at various time intervals post-electroporation (4 h - 10 h) and processed similar to described in Kore et al., 2008 except trypsinization step was added.[1] Trypsinization is the most efficient procedure that allows gentle removal of cells from the plates without releasing RNA bound to the wells, and also removes RNA that are tightly associated with cell surface components. Overall, addition of this step guarantees that only RNA that is truly inside cells will be left. Briefly, cells were washed with 1 mL of PBS 3 times by rotating the plate to ensure coverage of the cells followed by treatment with 100 μL 0.05% trypsin for 3 minutes at 37ºC, then inactivated by adding 1 mL of complete growth medium (DMEM + 10% FBS). Media was swirled around and pipetted up and down to dislodge cell clumps. Cells were transferred to 1.5 mL eppendorf tubes, pelleted at 800 g for 3 minutes, and cell pellets were washed 2 times with 1 mL of PBS by flicking the tube to disrupt the pellet. Pellets were then lysed with 1.25 mL of the TaqMan® MicroRNA Cells-to-CT™ lysis buffer (Applied Biosystems P/N 4391848).

Lysates were mixed by inverting the tubes 5 times and then incubated at room temperature for 8 minutes. Lysis reactions were stopped by adding 125 µL Stop Solution followed by inverting the tubes to mix and incubated at room temperature for another 2 minutes. Total RNA was then isolated with TaqMan® Gene Expression Cells-to-CT™ (Applied Biosystems), and levels of fLuc mRNA were determined by real-time PCR (7900HT Fast Real-Time PCR System, Applied Biosystems). Luciferase mRNA remaining at each time point was converted to a percent of the RNA present at earliest time point (4h), and the results were plotted as log10([RNA]) versus time.

6. Luciferase (Transfection) Assay:

HeLa cells (60,000/well in 24 well-plates) were seeded at least 12 h before transfection in growth medium without antibiotics. A complex of 5′-capped RNA was prepared by mixing 600 ng (2 µl) of RNA, 2.5 µl of TFX-20 (Promega), and 300 µl of serum-free DMEM in polystyrene tubes and incubated for 15 min at room temperature. After the incubation, media from the pre-plated HeLa cells was removed and 200 µl of the complex was added to each well. The plates were incubated for 1 h at 37 °C, and then 1 ml of pre-warmed media with serum was added. The transfected plates were incubated at 37 °C. Cells were harvested and lysed at 5, 10, 15, 20, 25, 30, and 40 h post-transfection. The cells were harvested and lysed by removing the media and adding 100 µl of 1X passive lysis buffer (Promega). The plate was mixed carefully to disrupt the cells and 10 µl of cell lysates from each transfections was mixed with 100 µl of luciferase substrate (Promega) and measured immediately on a luminometer (POLARstar OPTIMA, BMG Labtech) in 96-well plates. The fold induction of luciferase protein data was normalized to the control reaction, i.e. the no cap, mRNA poly(A) transfection results.

7. Molecular Modeling:

Molecular modeling of cap analogs were carried out to understand whether electrostatic interaction of LNA-modified cap **3** analog with eIF4E protein is more favorable compared to standard cap analog **4**. The crystal structure of eIF4E-m^{7}GpppA-4E-BP1 peptide[2,3] was used as a starting point to build the coordinates of two eIF4E protein complexes each containing either standard cap **4** or LNA-modifed cap **3** analog. Both complexes were built and optimized with Accelrys Discovery Studio Software V2.02 software (trial version) using CHARMm force field.[4] The adenosine residue was mutated to guanosine and +1 charge was introduced on the N^7-nitrogen of guanosine residue. In the case of LNA modified cap **3** containing complex, a methylene bridge between 2′-O and 4′-C of the N^7 guanosine residue of the cap analog was also introduced. Both the structures were subjected to energy minimization using CHARMm force field with a convergence threshold of 0.01 kcal/mol Å. The stability of each complex was obtained by calculating the interaction energies (a sum of both van der Waals and electrostatic energies) of individual complex with implicit distance-dependent dielectric model. The structural characteristics of both the minimized structures were similar to the crystal structure of eIF4E-m^{7}GpppA- 4E-BP1 peptide that the phosphate groups of the cap forms "molecular anchor" by interacting with the amino acids of eIF4E protein. However, it appears from the molecular modeling study that additional one more amino acid (Arg112) also participates in molecular anchoring step during the formation of LNA modified cap-**3** eIF4E complex (Figure 3).

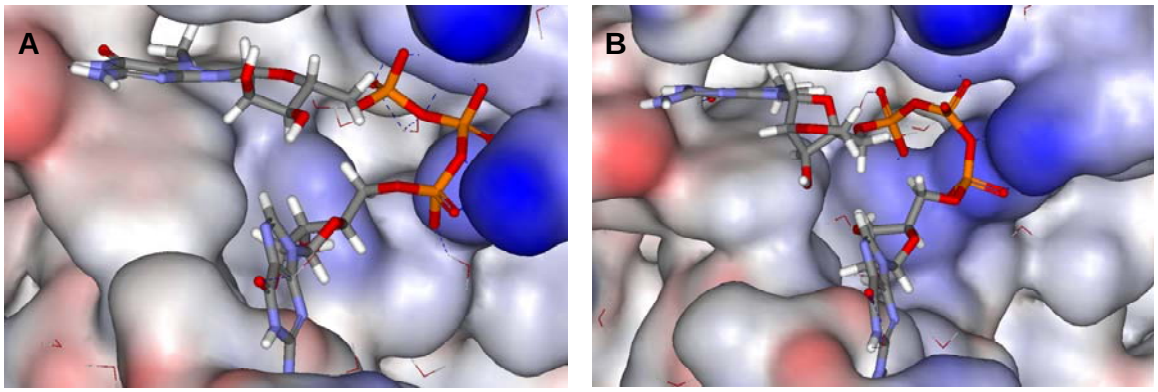


Figure 3. (A) Energy minimized structure of standard cap **4**-eIF4E complex. (B) LNA-modified cap **3**-eIF4E complex.

The ball-and-stick model of the resulting complex is shown in Figure 4.

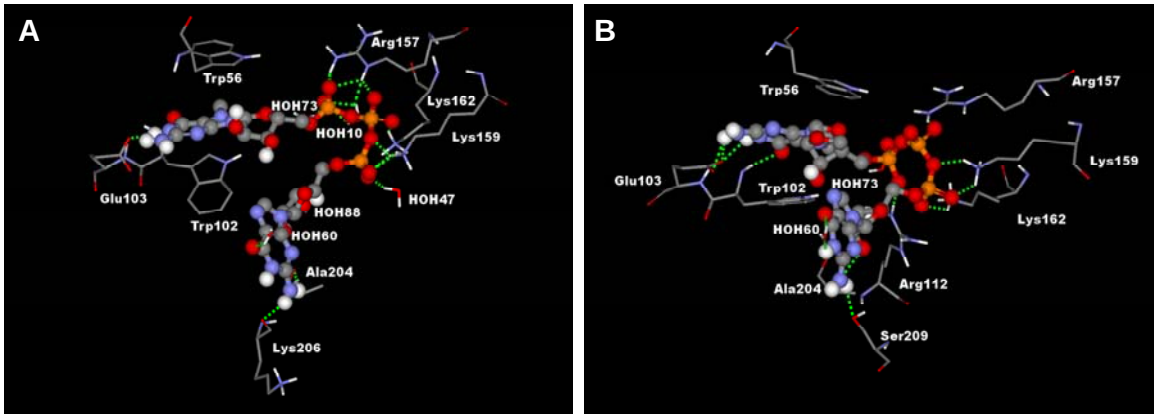


Figure 4. (A) Standard cap-eIF4E complex. (B) $m^{7(LNA)}G[5']ppp[5']$ **3**-eIF4E complex.

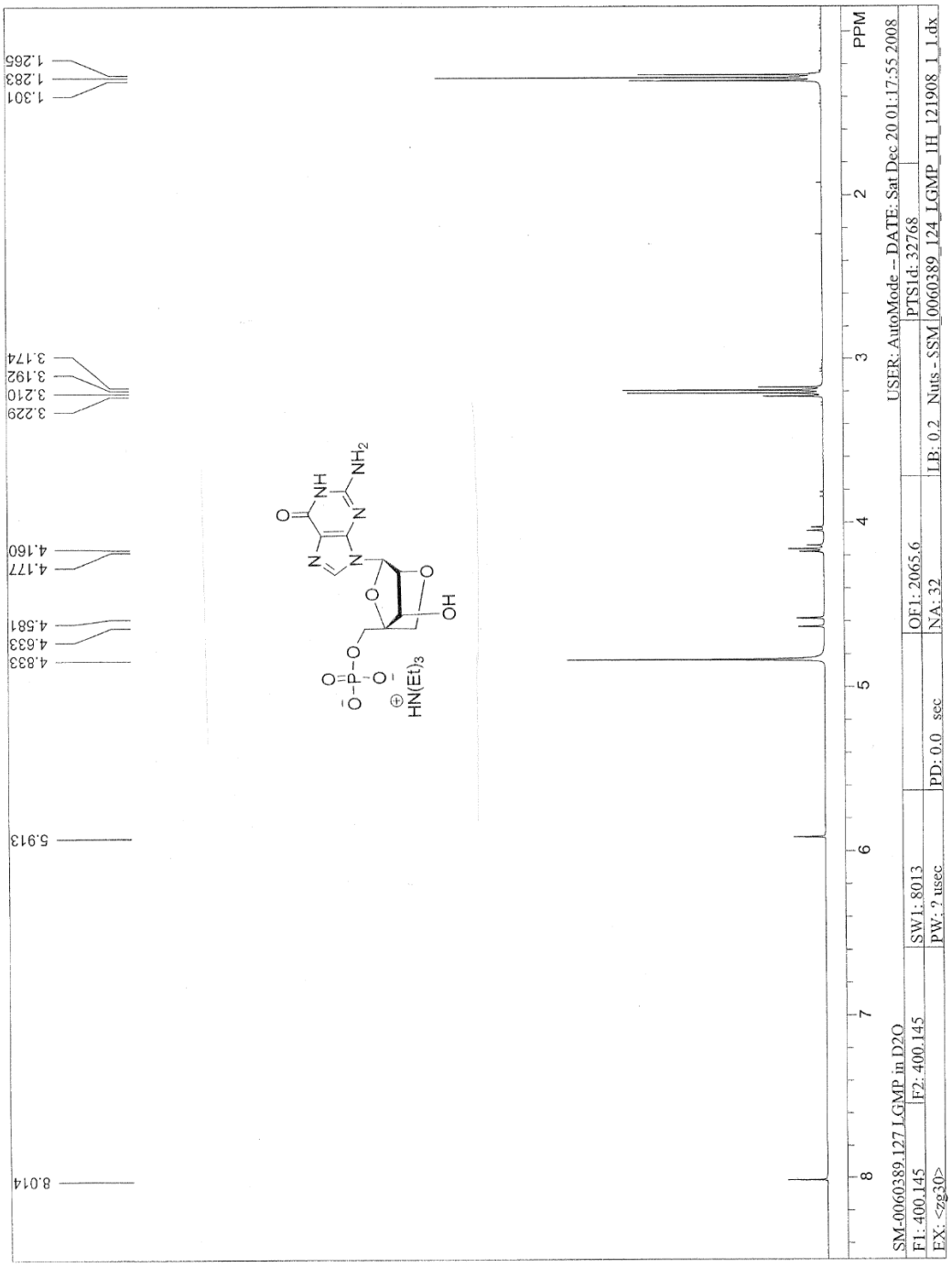
The modeling results show that LNA modified cap **3**-eIF4E complex is more stable by 47.283 Kcal/mol compared to standard cap-eIF4E complex (Table 1). Both the energy-minimized complexes were stable during standard dynamics cascade simulation. During the simulation the structure was heated to 2ps followed by equilibration for 1ps and production for 2ps. At each stage, 1fs time step was used.

Table 1: Interaction energy of the standard cap **4**-eIF4E and LNA-modified cap **3**-eIF4E complexes.

Molecule Name	**Interaction Energy (kcal/mol)**	**VDW Interaction Energy (kcal/mol)**	**Electrostatic Interaction Energy (kcal/mol)**
Standard cap **4**-eIF4E complex	-358.52	-31.14	-327.39
LNA-modified cap **3** -eIF4E complex	-311.24	-26.37	-284.87
Energy difference between standard cap **4**-complex and LNA-modified cap **3**-complex	-47.28	-4.76	-42.52

References:

1. Kore, A. R.; Shanmugasundaram, M.; Vlassov, A. V. *Bioorg, Med. Chem. Lett*. **2008**, *18*, 4828-4832.
2. Tomoo, K.; Matsushita, Y.; Fujisaki, H.; Abiko, F.; Shen, X.; Taniguchi, T.; Miyagawa, H.; Kitamura, K.; Miura, K.; Ishida, T. *Biochim. Biophys. Acta* **2005,** *1753*, 191-208.
3. The PDB file was downloaded from protein data bank website, http://www.rcsb.org/pdb/cgi/explore.cgi?pdbId=1wkw.
4. Accelrys Software Inc., *Discovery Studio Modeling Environment, Release 2.01*, San Diego: Accelrys Software Inc., 2008.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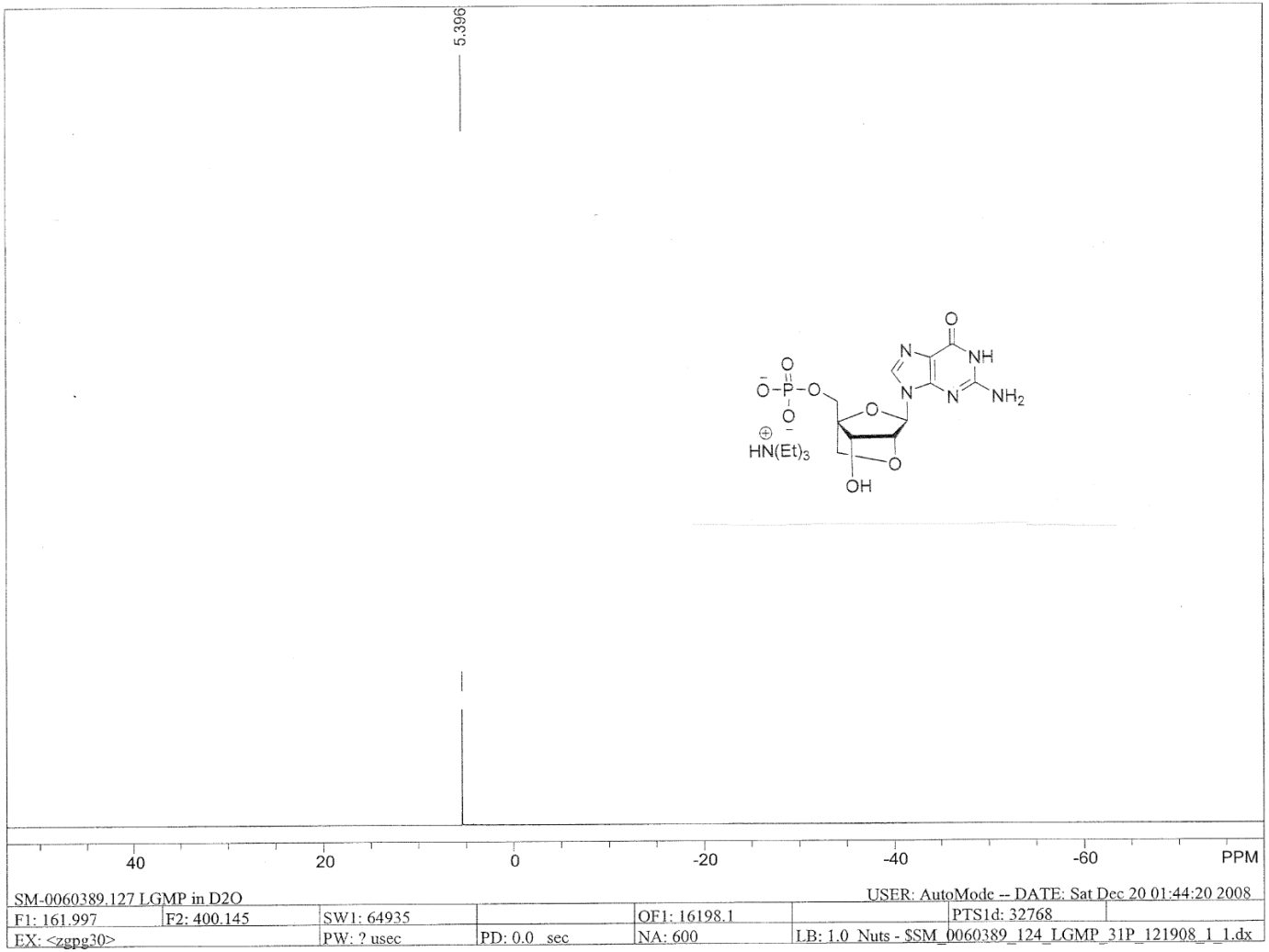

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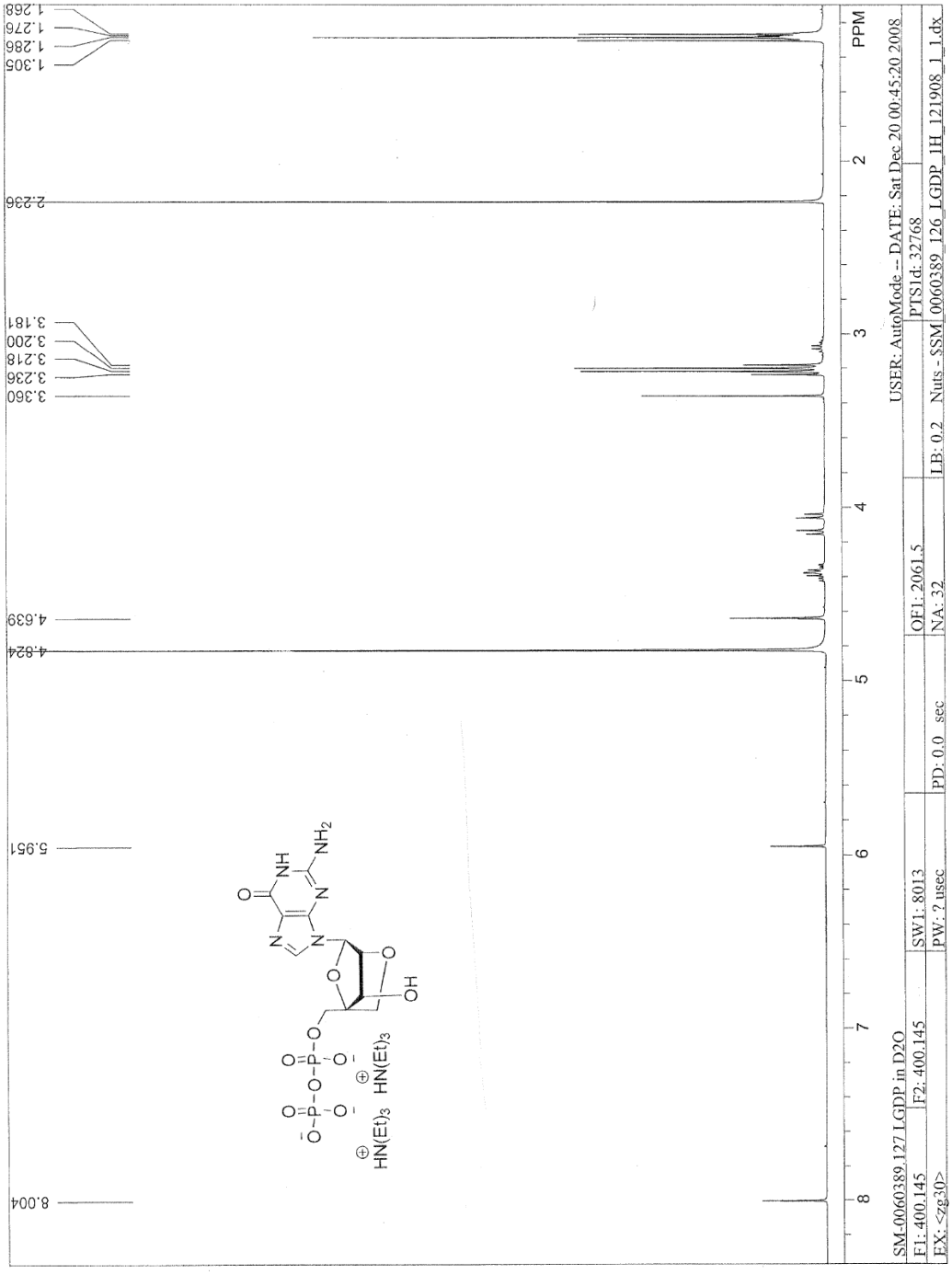
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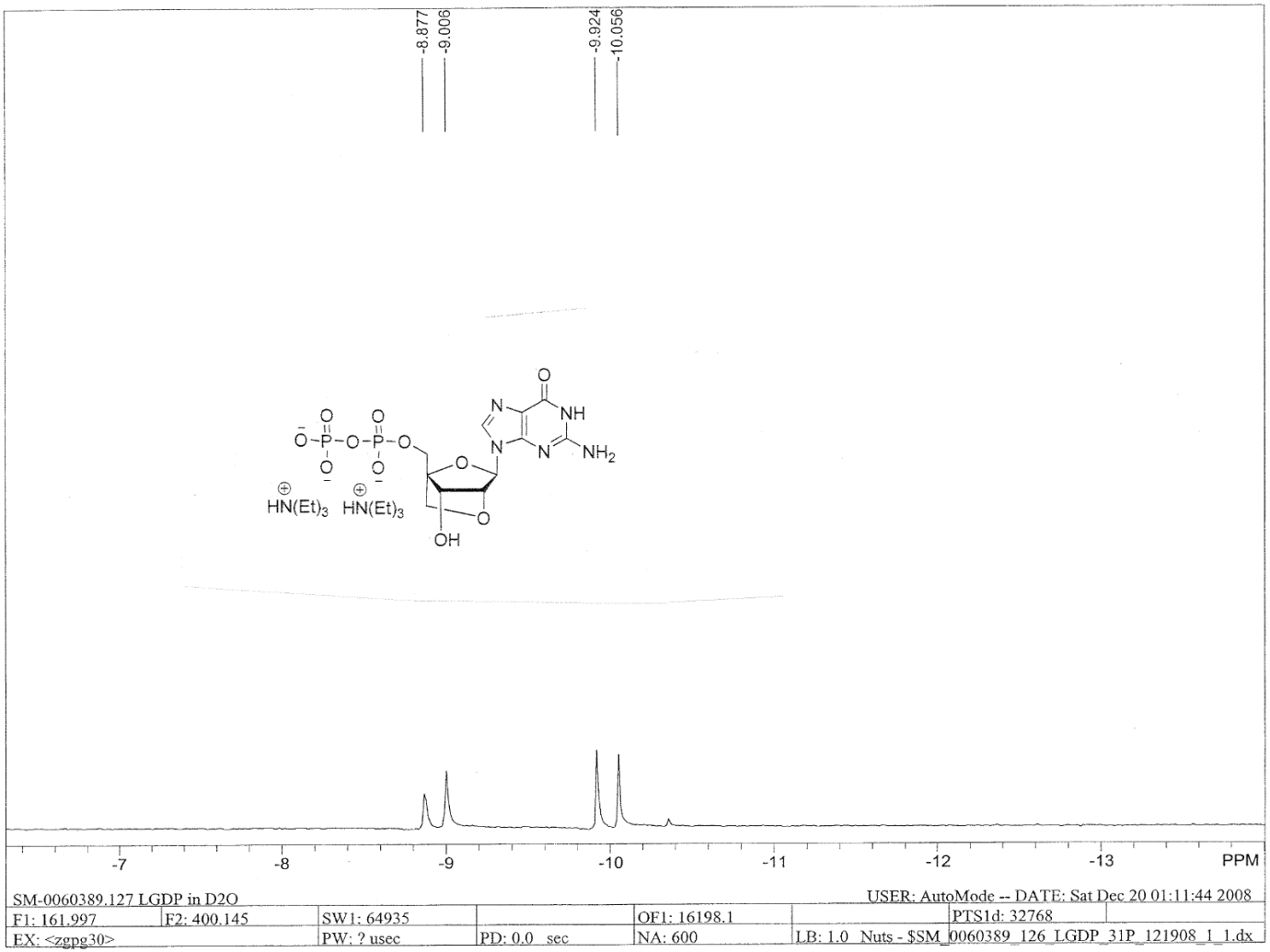
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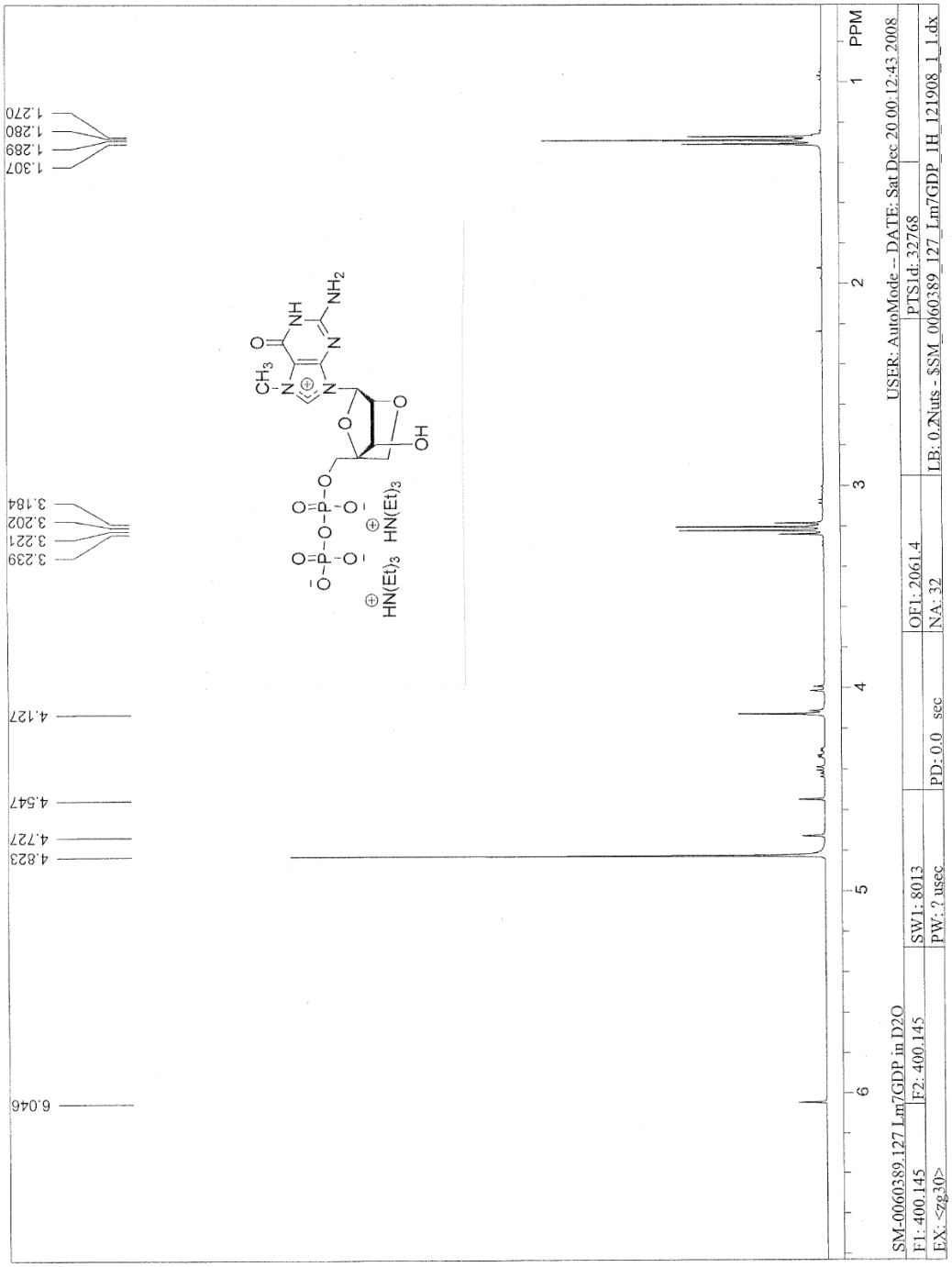
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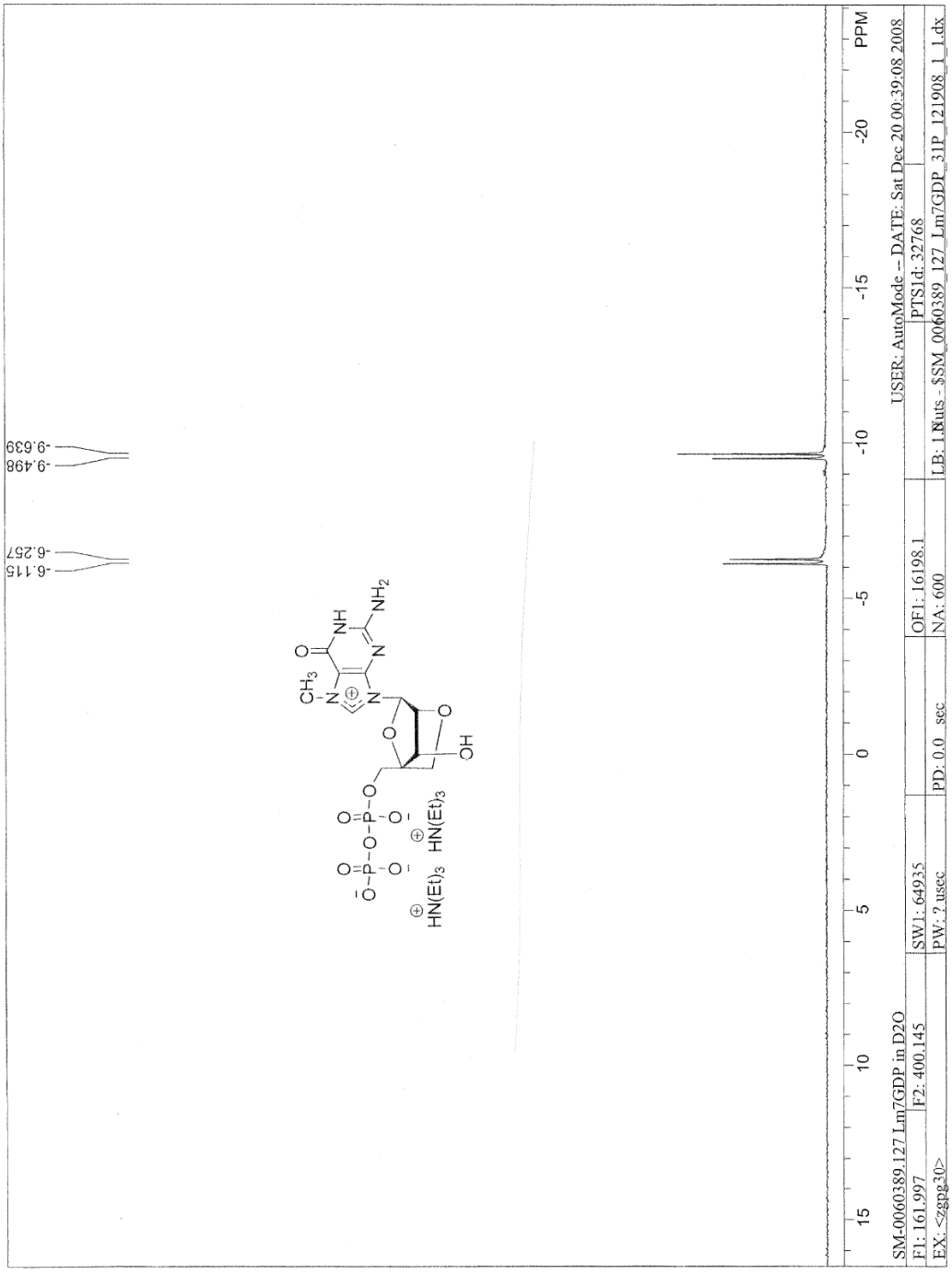
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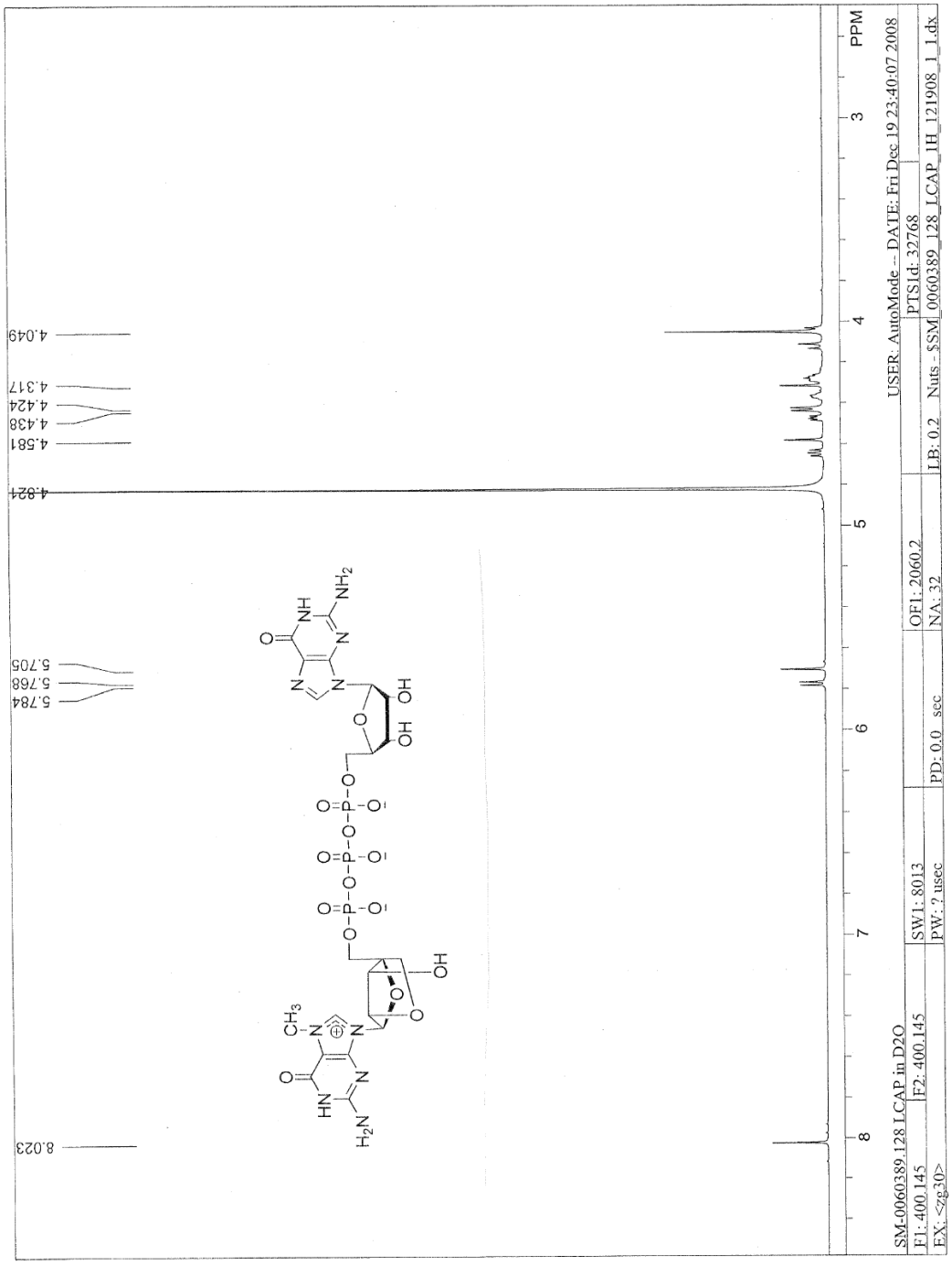
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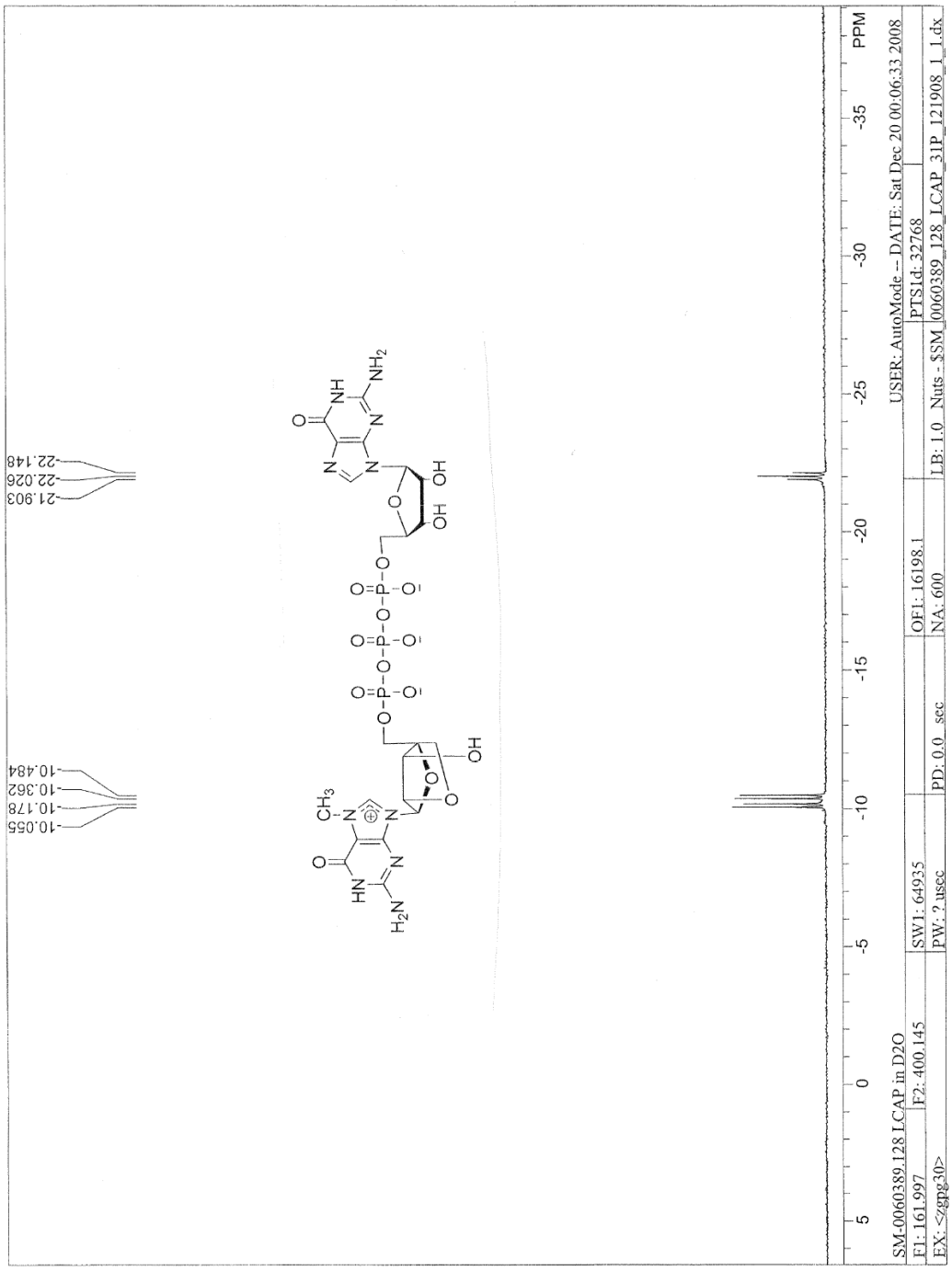
4.824
4.581
4.438
4.424
4.317
4.049
PPM
SM-0060389_128 LCAP in D2O
F1: 400.145
F2: 400.145
EX: <zg30>
SW1: 8013
PW: ? usec
PD: 0.0 sec
OF1: 2060.2
NA: 32
USER: AutoMode -- DATE: Fri Dec 19 23:40:07 2008
PTS1d: 32768
LB: 0.2 Nuts - $SM_0060389_128_LCAP_1H_121908_1_1.dx


-22.148
-22.026
-21.903
-10.484
-10.362
-10.178
-10.055
5
0
-5
-10
-15
-20
-25
-30
-35
PPM
SM-0060389.128 LCAP in D2O
USER: AutoMode -- DATE: Sat Dec 20 00:06:33 2008
F1: 161.997
F2: 400.145
SW1: 64935
OF1: 16198.1
PTS1d: 32768
EX: <zgpg30>
PW: ? usec
PD: 0.0 sec
NA: 600
LB: 1.0 Nuts - $SM_0060389_128_LCAP_31P_121908_1_1.dx