Supplementary Materials

Catalysis of a new ribose carbon-insertion reaction by the molybdenum cofactor biosynthetic enzyme MoaA

Angad P. Mehta, Jeremiah W. Hanes, Sameh Abdelwahed, David G. Hilmey, Petra Hänzelmann and Tadhg P. Begley.

Materials:

U-$^{2}H_{10}$-GTP (universally deuterated) and 3',4',5',5'-$^{2}H_{4}$-GTP were purchased from Cambridge Isotopes laboratory. GTP, SAM, NDP kinase were purchased from Sigma-Aldrich.

Over-expression and purification of MoaA:

The MoaA overexpression plasmid was previously described[1]. MoaA was co-expressed in the presence of a plasmid encoding the *suf* operon for in vivo [4Fe-4S] reconstitution *E.coli* BL21 (DE3). An overnight 15 ml culture was grown in LB media in the presence of ampicillin and chloramphenicol. This was then added to 1.5 L of LB media containing ampicillin and chloramphenicol. The cultures were incubated at 37^{0}C with shaking (180 rpm) until the OD_{600} reached 0.45. The culture was then incubated at 4^{0}C without shaking for 1.5 hrs. Then 200 mg of ferrous ammonium sulfate and 200 mg of cysteine were added. This was followed by induction of the culture with 100 µM IPTG. The culture was then incubated at 15^{0}C with shaking (110 rpm) for 14 - 16hrs. The culture was then incubated at 4^{0}C for 3 hrs without shaking. The cells were then harvested and stored in liquid nitrogen overnight before enzyme purification. For enzyme purification, the cell pellets were thawed at room temperature in an anaerobic chamber and suspended in lysis buffer (100 mM Tris-HCl, pH 7.5) in the presence of 2mM DTT, lysozyme (0.2 mg/ml) and benzonase (100 units). This mixture was then cooled in an ice-bath for 2 hrs. The suspension of cells was then sonicated and centrifuged to give the cell-free extract. The enzyme was then purified using standard Ni-NTA chromatography. The column was first incubated with the lysis buffer. The cell-free extract was then passed over the column, which was then washed with 5-6 column volumes of wash buffer (100 mM Tris-HCl, 300 mM NaCl, 20 mM Imidazole, 2mM DTT, pH 7.5). The protein was then eluted using 100 mM Tris-HCl, 300 mM NaCl, 250 mM Imidazole, 2 mM DTT, pH 7.5. The purified enzyme was buffer exchanged into 100 mM Potassium phosphate, 30% glycerol, 2 mM DTT, pH 7.5 using a 10DG column and the purified enzyme was stored in liquid nitrogen.

Over-expression and purification of guanylate kinase:

The guanylate kinase overexpression plasmid[2,3] was transformed into *E.coli* BL21 (DE3). An overnight 15ml culture was grown in LB media in the presence of ampicillin. This was then added to 1.5L of LB media containing ampicillin. The

cultures were incubated at 37^0C with shaking (180 rpm) until the OD_{600} reached 0.55. This was followed by induction of the culture with 100 μM IPTG at 15^0C for 14hrs at 180rpm. The enzyme was purified on a blue sepharose column prepared in wash buffer (15 mM Tris-HCl, 1 mM EDTA, 1 mM DTT). The column was loaded with cell free extract, washed with wash buffer until the OD_{280} was less than 0.02. The enzyme was then eluted with 15 mM Tris-HCl, 1 mM EDTA, 1 mM DTT, 5 mM GMP.

Enzymatic assays:

The enzymatic reaction mixtures contained MoaA (125 μM), GTP (1 mM), SAM (1.5 mM), and sodium dithionite (in excess) and were incubated at room temperature in an anaerobic chamber for 3 hrs. For HPLC and LCMS analysis the enzyme was removed by ultrafiltration using a 10kDa cut-off filter. The samples were then prepared in the anaerobic chamber and sealed before HPLC or LCMS analysis.

For a reaction mixture using a GMP isotopomer as substrate, guanylate kinase (20μM), NDP kinase (20μM), ATP (5 mM), $MgCl_2$ (2 mM), MoaA (125 μM) and GMP (1 mM) were incubated at room temperature for 10 mins. SAM (1.5mM) and sodium dithionite (in excess) were then added and the reactions were run as described above.

LCMS parameters for 5'-deoxyadenosine (5'-dA) and pterin characterization:

LC-ESI-TOF-MS analysis was performed using an Agilent 1260 HPLC system equipped with a binary pump and autosampler and a 1200 diode array detector upstream of a MicroToF-Q II mass spectrometer (Bruker Daltonics, Billerica, MA) using the ESI source in positive ionization mode.

LC conditions:

A – 20mM *N,N*-dimethylhexylamine, 10mM ammonium acetate, pH 6.4
B – 75% Methanol, 25% Water
Column – Agilent Poroshell 120, EC-C18, 2.7μm, 3x10mm

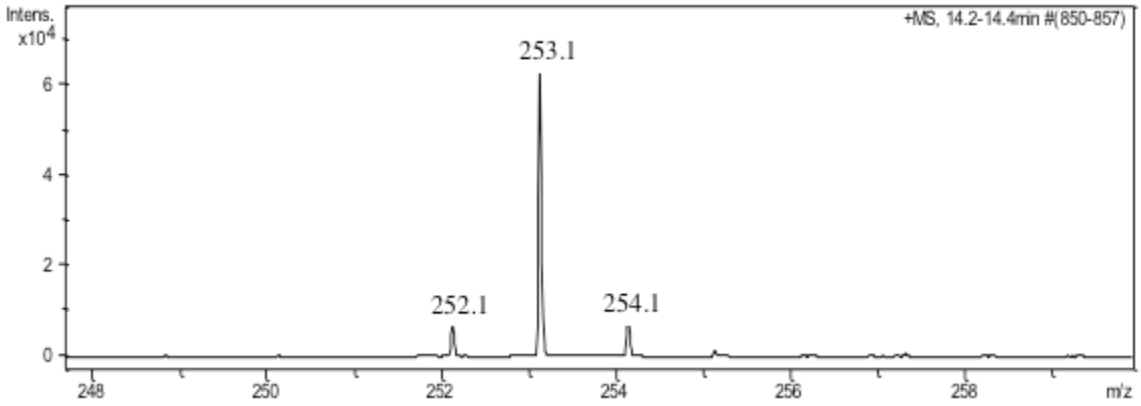
LC method:

0 min – 100% A, 5 mins – 100% A, 15 mins - 60% A 40% B, 27 mins – 10% A 90% B, 36min – 100% A

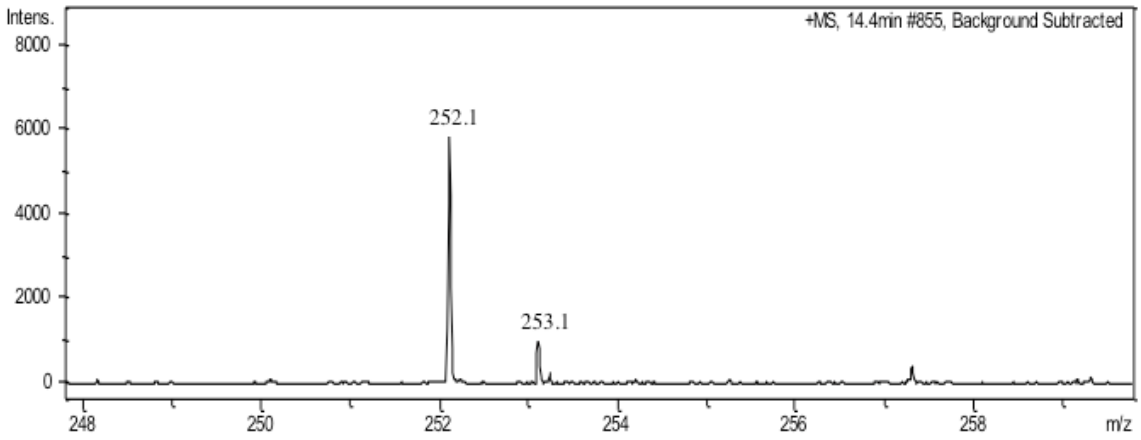
MS parameters:

Capillary, -4500 V; Capillary offset, -500 V; Nebulizer gas, 3.0 bar; Dry gas, 10.0 L/min; Dry gas temperature, 200°C; Funnel 1 RF, 200.0 Vpp; Funnel 2 RF, 250.0 Vpp; ISCID, 0.0 eV; Hexapole RF, 150 Vpp; Quadrapole, Ion energy, 5.0 eV; Low mass, 80 *m/z*; Collision cell, collision energy, 10.0 eV; Collision RF, 150.0 Vpp, Transfer time, 100.0 μs; Prepulse storage, 5.0 μs. Data was processed with DataAnalysis ver. 4.0 SP4 (Bruker Daltonics, Billerica, MA).

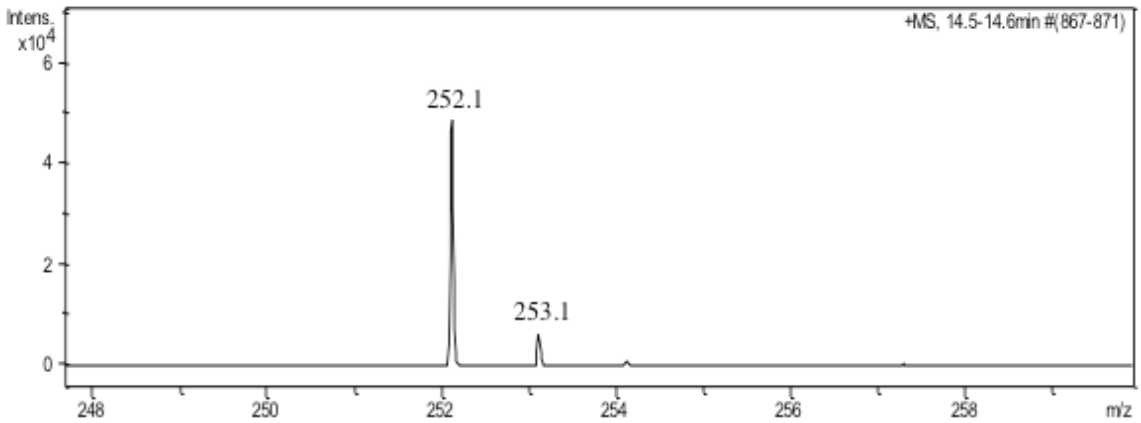
1. MoaA assays with 3',4',5',5'-$^{2}H_4$-GTP:



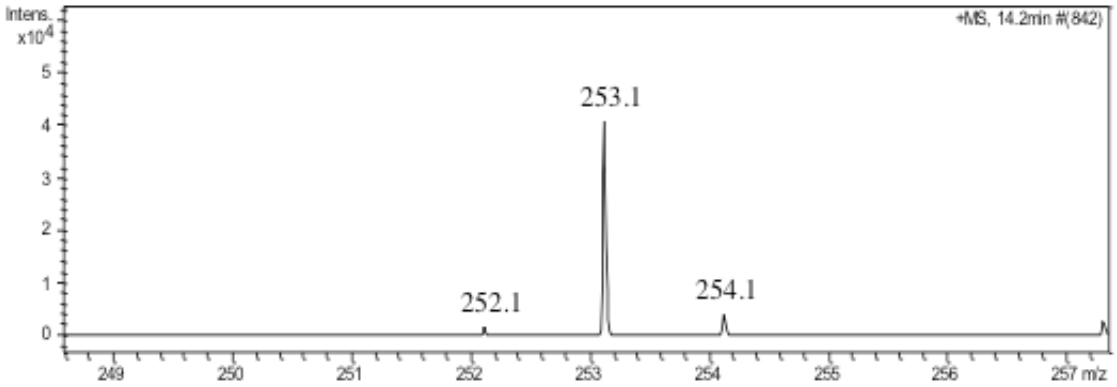
2. MoaA assays with 8'-$^{2}H_4$-GTP:



3. MoaA assays with 2'-$^{2}H_4$-GTP:



4. MoaA assays with U-$^{2}H_{10}$-GTP (universally deuteriated):



5. MoaA assays with 3'-$^{2}H_{4}$-GTP

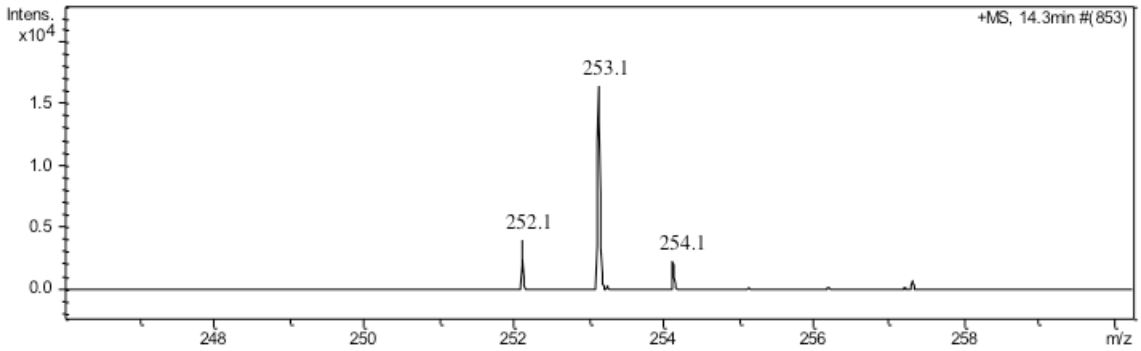


Figure S1: Mass spectrometry data for 5'-dA formation with GTP isotopomers – (see table in Figure 2E).

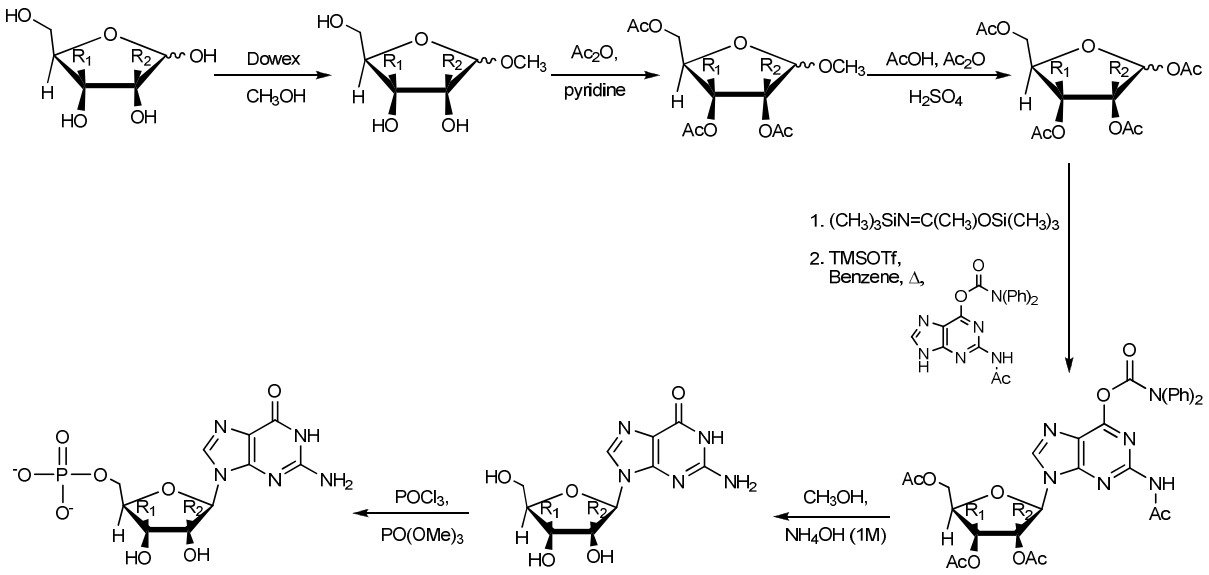
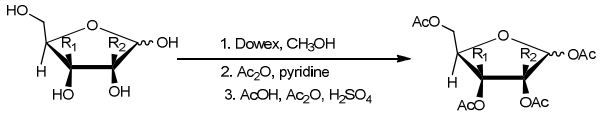


Figure S2: Synthesis of Guanosine monophosphate (GMP) isotopomers - 2'-^{2}H-GMP and 3'-^{2}H-GMP[4,5,6].



1,2,3,5-Tetra-O-acetylribofuranoside[4].

Dowex 8 × 50W, 200–400, H^+ cation-exchange resin (300 mg) was washed with anhydrous methanol and then stirred for 4.5 h at room temperature with ribose (260 mg, 1.72 mmol) in anhydrous methanol (6 mL). Reaction was monitored by TLC (CHCl3: MeOH, 85:15) for completion. The resin was removed by filtration and washed with anhydrous methanol. The combined filtrate and washings were evaporated to give 1-O-methyl- ribofuranoside which was dissolved in anhydrous pyridine (5 mL) and cold in ice bath then treated with acetic anhydride (0.5 mL, 564 mg, 5.48 mmol) and stirred at room temperature for 4 h. The reaction mixture was cooled (ice-bath), diluted with water (25 mL) and the resultant aqueous solution was extracted with chloroform (4 × 25 mL). The combined organic extracts were washed with water (4 × 50 mL), dried and then co-evaporated with toluene to give 1-O-methyl-2,3,5-tri-O-acetyl-ribofuranoside which was subjected to next reaction without further purification. A cooled (ice-bath) solution of 1-O-methyl-2,3,5-tri-O-acetyl-ribofuranoside (403 mg, 1.39 mmol) and acetic anhydride (1.4 mL, 1.56 g, 15.16 mmol) in glacial acetic acid (10 mL) was treated drop-wise with conc. sulfuric acid (0.2mL) and then stirred at room temperature for 3 h. The reaction mixture was cooled (ice-bath) and ice (10 g) was added with continued stirring. When the ice had melted, the resultant aqueous solution was extracted with chloroform (4 × 25 mL). The combined organic extracts were washed successively with water (2 × 50 mL), a saturated aqueous solution of sodium bicarbonate (2 × 50 mL) and water

(50 mL), then dried and evaporated to give 1,2,3,5-tetra-O-acetyl-ribofuranoside (355 mg, 80%) as a virtually colorless oil which solidified after standing at room temperature.

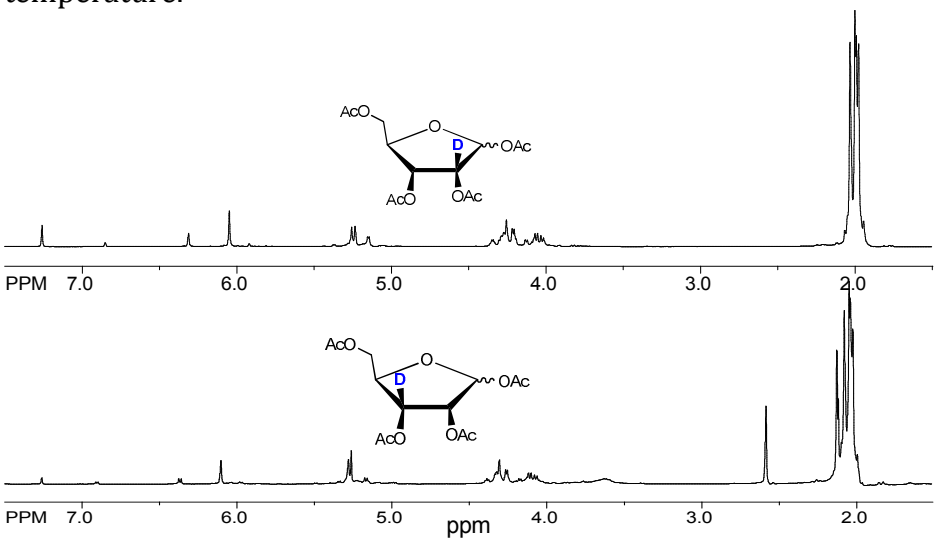
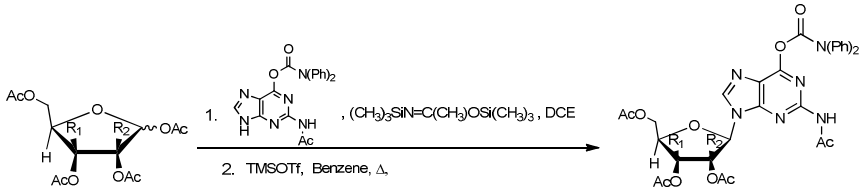


Figure S3: ^{1}H-NMR ($CDCl_3$) of 2'-^{2}H & 3'-^{2}H of 1,2,3,5-Tetra-O-acetyl-ribofuranoside

[2-^{2}H] Tetraacetyl ribofuranose:^{1}H NMR ($CDCl_3$) δ 6.41 (d, 0.3, H1α), 6.15 (s, 0.7, H1β), 5.3 (d, 0.7, H3β), 5.2 (d, 0.3, H3 α), 4.1–4.5 (m, 3, H4, H5, H5') 2.10 (s, 3, CH_3CO), 2.07 (s, 3, CH_3CO), 2.06 (s, 3, CH_3CO), 2.04 (s, 3, CH_3CO).

[3-2H] Tetraacetyl ribofuranose: ^{1}H NMR ($CDCl_3$) δ 6.41 (d, 0.3, H1 α), 6.15 (s, 0.7, H1β), 5.2–5.3 (m, 1, H2), 4.1–4.5 (m, 3, H4, H5, H5'), 2.10 (s, 3, CH_3CO), 2.08 (s, 3, CH_3CO), 2.06 (s, 3, CH_3CO), 2.04 (s, 3, CH_3CO).

General Procedure for the Synthesis of 2-Acetylamino-2' ,3' ,5' –tri-O-acetyl-6-O-(N,N-diphenylcarbamoy)-guanosine[5].



2-Acetylamino-6-O-diphenylcarbamoylguanine (220 mg, 0.63 mmol) was suspended in 1,2-dichloroethane (6.5 ml) under nitrogen and treated with N,O-bis-(trimethylsilyl)-acetamide (0.32 ml). The mixture was heated to 80°C and stirred for 15 min when some of the suspension dissolved. The solvents were removed in *vacuo*. The residue was suspended in dry benzene (3 ml) under nitrogen and fresh trimethylsilyltrifluoromethanesulfonate (150 uL) was added.
1,2,3,5-Tetra-O-acetyl-D-ribofuranose (200 mg, 0.62 mmol) dissolved dry benzene (3 ml) was added to the mixture which was heated to 80°C for 1 h. The solution was diluted with EtOAc, washed with water and brine and dried ($MgSO_4$). The solvent was removed in *vacuo* to yield a yellow foam, which was absorbed onto silica gel

and purified by column chromatography on silica gel using Hexanes : Ethyl acetate (1:1) as eluent.

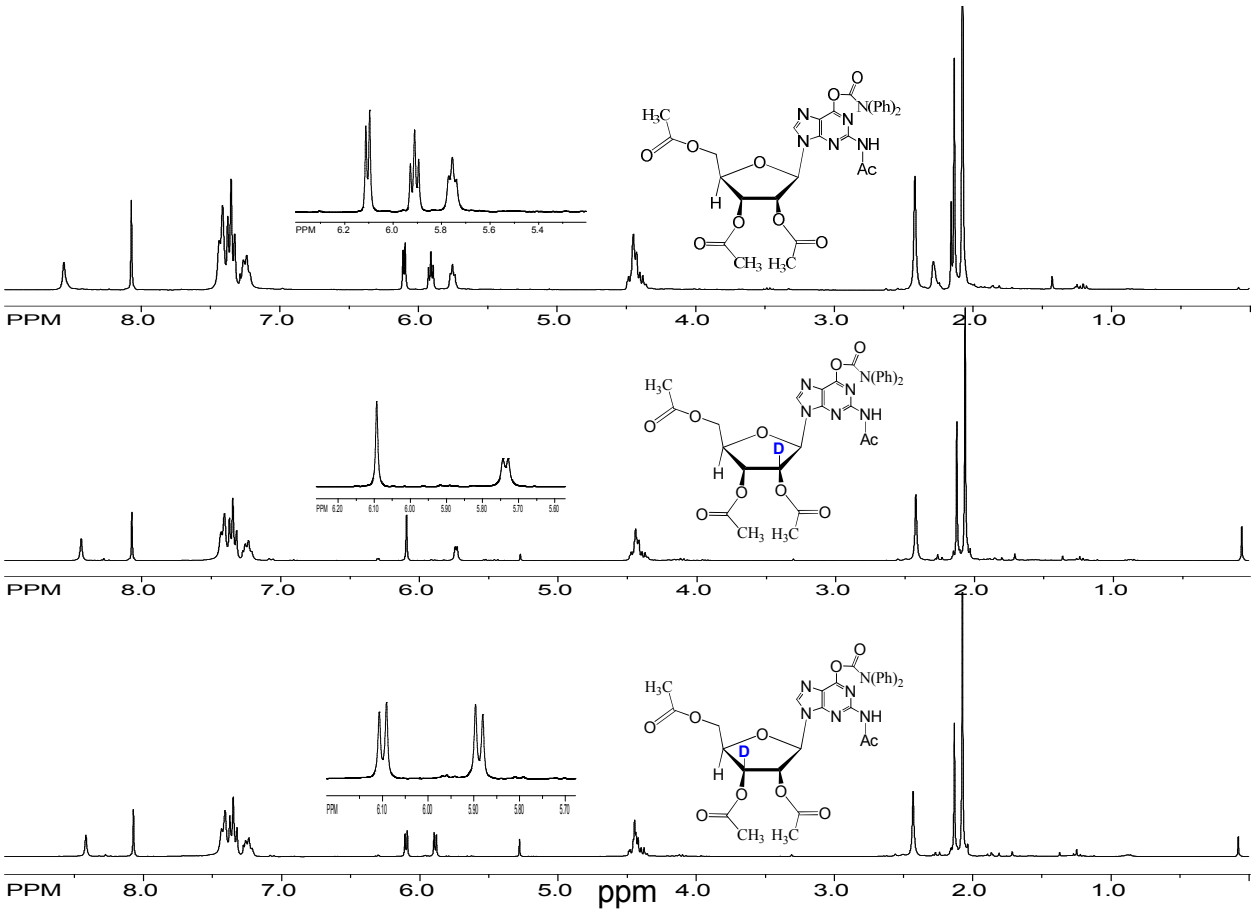
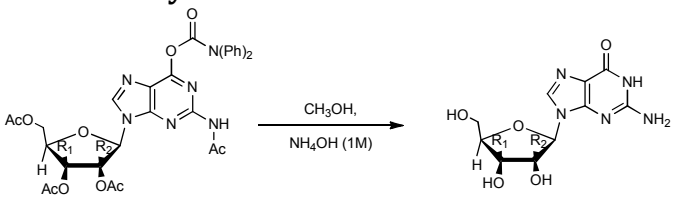


Figure S4: ^{1}H-NMR ($CDCl_3$) of 2'-^{2}H & 3'-^{2}H of 2-Acetylamino-2' ,3' ,5' –tri-O-acetyl-6-O-(N,N-diphenylcarbamoy)-guanosine.

General Procedure for the Synthesis of Guanosine[5]:



2-Acetylamino-2',3',5'-tri-O-acetyl-6-O-(N,N-diphenylcarbamoyl)-guanine (180 mg, 0.28 mmo1) was dissolved in methanol (3 ml). 1N aqueous ammonium hydroxide was added until turbidity. After stirring overnight at 4^{o}C, the solvents were removed in *vacuo* and the residue was recrystallised from water as an off-white solid.

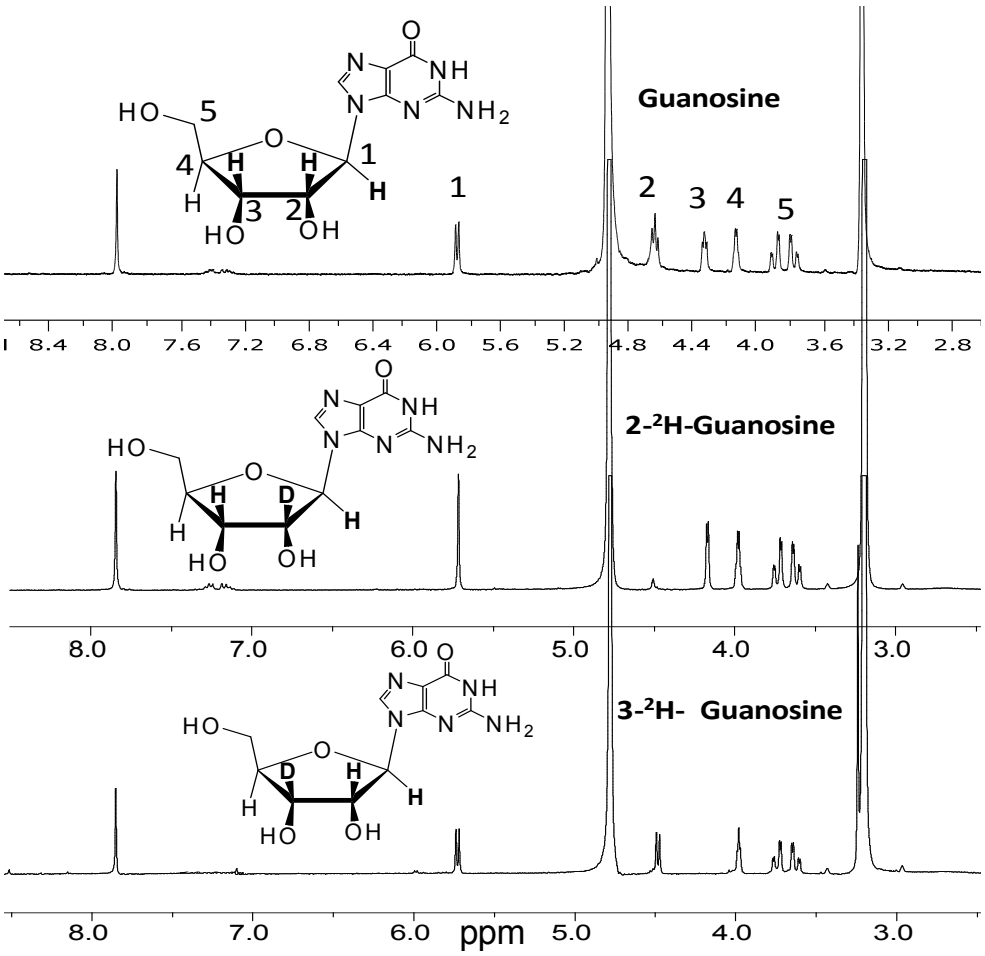
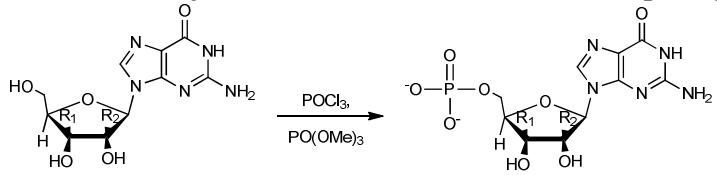


Figure S5: ^{1}H-NMR (MeOD) of Guanosine, 2-^{2}H- Guanosine & 3-^{2}H- Guanosine

General Procedure for the Synthesis of Guanosine-5'-Monophosphate [6].



A suspension of Guanosine (50 mg, 0.177 mmol) in TEP, trimethylphosphate, (0.6 mL) was heated at 50°C for 15 min and then cooled to 0°C. Water (1.59 uL, 0.0885 mmol) was added followed by $POCl_3$ (33uL, 0.354 mmol) at 0°C, and the entire mixture was further stirred at 0°C for 1.5 h. The mixture was poured into ice-water (1 mL) and stirred for another hour before it neutralized by ammonium bicarbonate solution. The reaction mixture was lyophilized to give white product which then purified by Prep HPLC using a SPLC-18DB column (10 x 250 mm, 5 µ, Supelco) at a flow rate of 2 mL/min using an Agilent 100 HPLC system with quaternary pump and manual injector.

HPLC conditions for purification of GMP isotopomers:

Agilent 1260 HPLC was used with a fraction collector for the purification of both 5'-dA and compound Z. LC-18-T column (3.0 x 150 mm, 3 µm, Supelco) was used for the purification.
Chromatography conditions were as follows:
A: water
B: 5mM Ammonium formate pH 6.6
C: Methanol

0 min – 100%B, 7 mins- 10%A 90%B, 12 mins – 25%A 60%B 15%C, 17 min – 25%A 10%B 65%C, 19 mins – 100% B, 30 mins – 100%A

8-^{2}H- GMP was synthesized by H/D exchange with D_2O. GMP was heated at 80^{0}C in D_2O and the completion of the reaction was monitered by NMR.

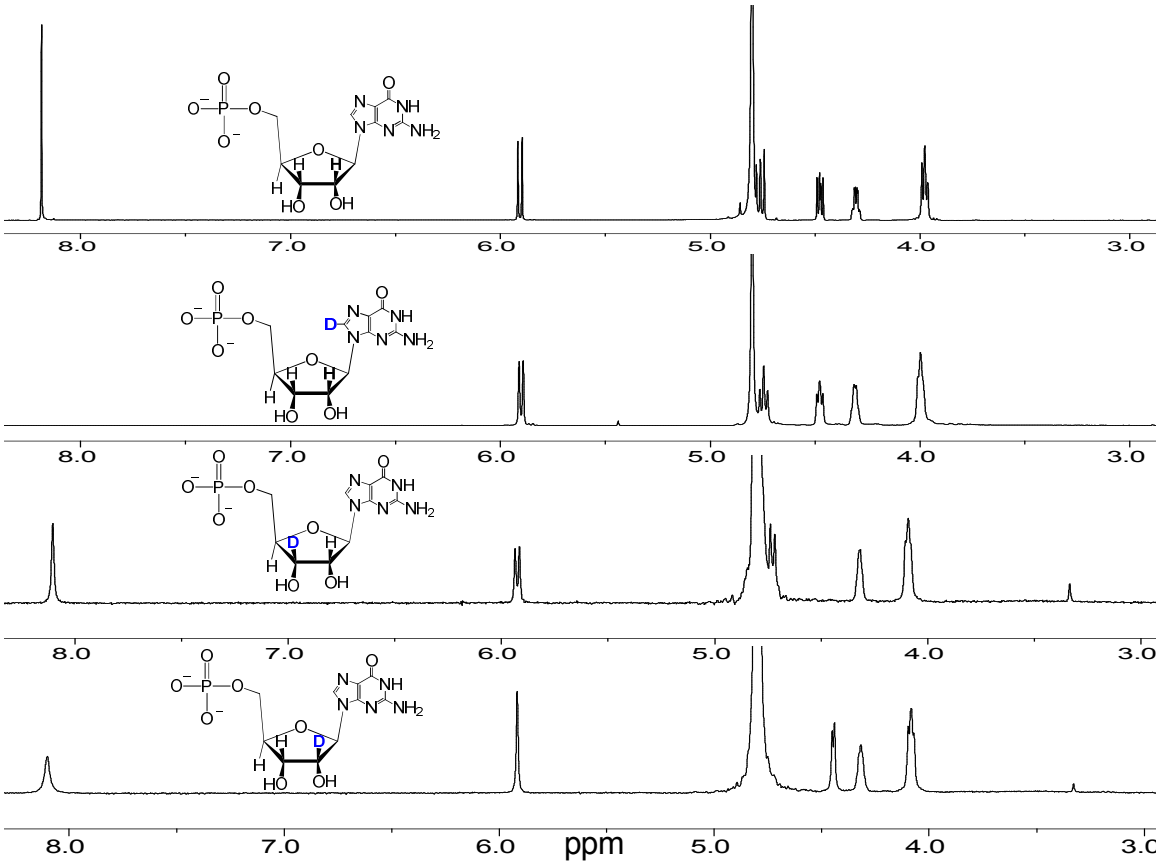


Figure S7: ^{1}H-NMR (D_2O) of GMP, 8-^{2}H- GMP, 3-^{2}H- GMP & 2-^{2}H- GMP.

Enzymatic assays:

The enzymatic reaction mixtures contained MoaA (125 μM), MoaC (50 μM), GTP (1 mM), SAM (1.5 mM), $MgCl_2$ (100 μM) and sodium dithionite (in excess) and were incubated at room temperature in an anaerobic chamber for 6 hrs. For HPLC and LCMS analysis, the enzyme was removed by ultrafiltration using a 10kDa cut-off filter. The samples were then oxidized by KI/I_2 as described previously[6], purified by HPLC and the fluorescent compound with a retention time of 11 minutes was analyzed by NMR.

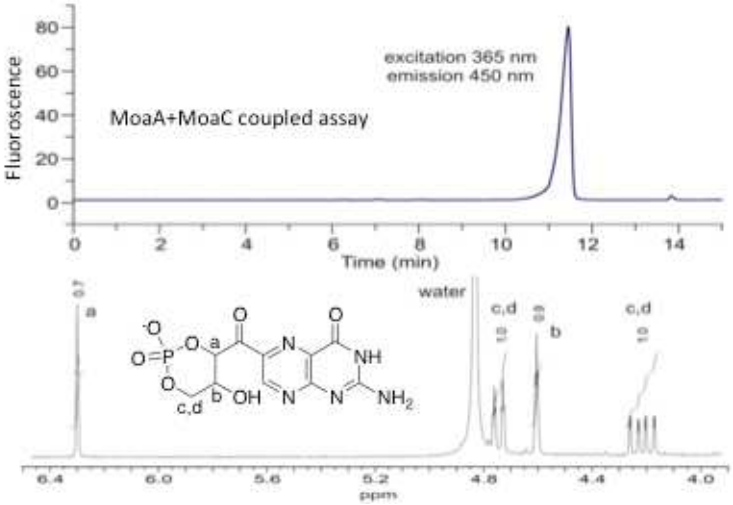


Figure S8: NMR characterization of the oxidized derivative of MoaA and MoaC product (4).

HPLC conditions for purification of compound 4:

Agilent 1260 HPLC was used with a fraction collector for the purification of both 5'-dA and compound 4. LC-18-T column (3.0 x 150 mm, 3 μm, Supelco) was used for the purification.

Chromatography conditions were as follows:

A: water

B: 5 mM Ammonium formate pH 6.6

C: Methanol

0 min – 100%B, 4 mins- 10%A 90%B, 9 mins – 25%A 60%B 15%C, 14 min – 25%A 10%B 65%C, 17 mins – 100% B, 24 mins – 100%A

HPLC conditions for purification of 5'-deoxyadenosine (5'-dA):
Agilent 1260 HPLC was used with a fraction collector for the purification of both 5'-dA and compound Z. LC-18-T column (3.0 x 150 mm, 3 µm, Supelco) was used for the purification.
Chromatography conditions were as follows:
A: water
B: 5mM Ammonium formate pH 6.6
C: Methanol

0 min – 100%B, 4 mins- 10%A 90%B, 9 mins – 25%A 60%B 15%C, 14 min – 25%A 10%B 65%C, 17 mins – 100% B, 24 mins – 100%A

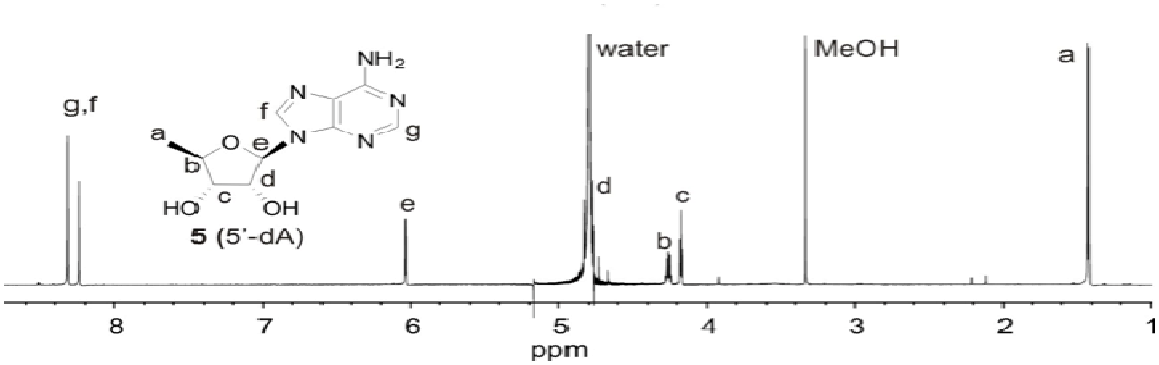


Figure S9: NMR characterization of 5'-dA

References:

1. Hänzelmann, P., Schindelin, H. Proceedings of the National Academy of Sciences,101 (35), August 2004, (MoaA over-expression)
2. Berger, A., Schiltz, E., Schulz, G.E., Eur. J. Biochem. 184,433-443 (1989)
3. Konard, M. J. Biol. Chem. 267 (36), 1992, 25652-55
4. Baldwin, Alexander D. Baldwin, James D. Young, Maurice P. Gallagher, Peter J. F. enderson and Richard B. Herbert. Org. Biomol. Chem., 2005, 3, 462-470
5. Baker, S.J., Young, D.W. Journal of Labelled Compounds and Radiopharmaceuticals. Volume 43, Issue 10, September 2000, pages 1023–1032 (labeled GTP)
6. Ikemoto, T., Haze, A., Hatano, H., Kitamoto, Y., Ishida, M., Nara, K. Chemical & pharmaceutical bulletin 43(2), 210-215, 1995 (Phosphorylation)