Supplementary Information

Intermediates in the Transformation of Phosphonates to Phosphate by Bacteria

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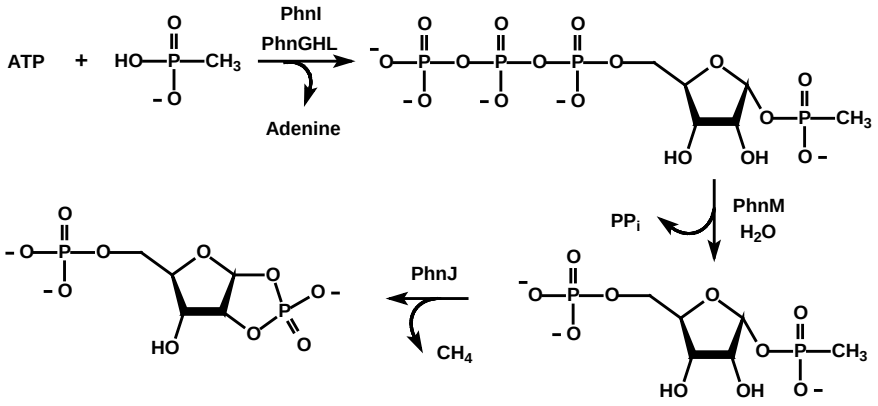


Figure S1: Conversion of methyl phosphonate to ribose-1,2-cyclic phosphate-5-phosphate by the C-P lyase system in *Escherichia coli.*

Supplemental Table S1: Kinetic constants for the reactions catalyzed by PhnI and PhnM

Substrate	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($M^{-1}s^{-1}$)
		PhnI only	
GDP	0.20 ± 0.02	225 ± 37	(1.0 ± 0.1) x 10^3
GTP	5.3 ± 0.2	64 ± 8	(8.3 ± 0.8) x 10^4
ADP	0.065 ± 0.003	204 ±18	(0.32 ± 0.02) x 10^3
ATP	1.40 ± 0.04	95 ± 12	(1.3 ± 0.2) x 10^4
ITP	0.8 ± 0.1	108 ± 12	(7.4 ± 0.6) x 10^3
		PhnG + PhnH + PhnI + PhnL + Factor Xa	
GTP	27 ± 2	80 ± 10	(3.4 ± 0.3) x 10^5
ATP	20 ± 1	56 ± 7	(3.5 ± 0.3) x 10^5
		PhnM	
RPnTP	6.4 ± 0.3	56 ± 12	(1.1 ± 0.2) x 10^5
RTP	6.0 ± 0.2	98 ± 12	(6.1 ± 0.3) x 10^4
RDP	0.08 ± 0.01	200 ± 32	(0.38 ± 0.03) x 10^3

All assays were performed at 30 ^oC at pH 8.5

Product Analysis

Gas Chromatography and GC-MS experiments with PhnJ: The samples for the GC and GC-MS experiments were prepared anaerobically in a glove box. For the GC experiment the concentration of the reconstituted PhnJ was 150 µM. The enzyme was premixed with 2 mM SAM, 1 mM dithionite, 1X Factor Xa buffer, 2 mM α-D-ribose-1-methylphosphonate-5-phosphate in 150 mM HEPES containing 250 mM NaCl, 10 % v/v glycerol at pH 8.5. The reaction volume was 2.3 mL. The reaction was transferred to a NMR tube in the glovebox and the NMR tube was sealed with a self-sealable rubber-cap and further sealed using parafilm to prevent any escape of the contents within the tube. The reaction was initiated by addition of 50 units of Factor Xa using a 1 ml 27G 3/8 allergy syringe (BD). The reactions were allowed to proceed for 6 hours at 12 ^{o}C. 50 µL of the headspace (500 µL) was removed using a gas-tight Hamilton syringe and injected into the GC instrument (Hewlett Packard HP 6890 GC system with FID detector and manual injection) equipped with a 30 m X 0.32 mm I.D. SGE Solgel 1 column used in splitless injection mode, at constant 20 psig He carrier pressure, with a temperature program of 50 ^{o}C/1 min, to 100 ^{o}C @ 10 ^{o}C / min. The same reaction was set up for the GC-MS experiment using a Thermo Electron Corporation DSQ quadrulpole GC-MS instrument with Finnigan Trace GC Ultra chromatograph at 70 E.V. EI ionization. The temperature program and injection mode were the same. The column was a 30 m X 0.25 mm I.D. SGE BP1 column operated at 1 mL/min constant flow He; with the exception that the PhnJ concentration was 160 µM in a final volume of 2.5 mL. 100 µL of the headspace was injected into the GC-MS instrument.

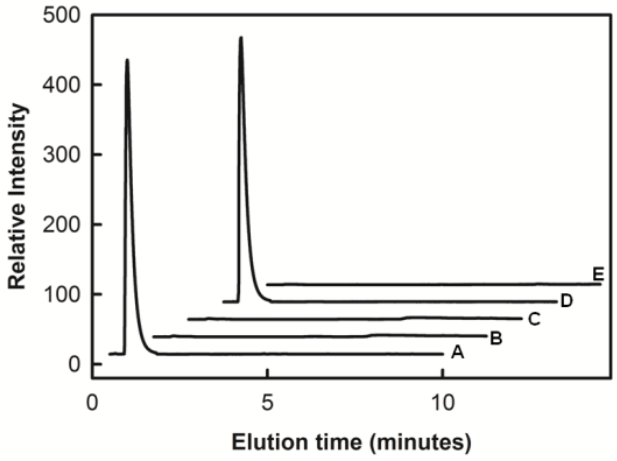


Figure S2: Gas chromatographic analysis for methane formation after incubation of PhnJ (150 µM), SAM (2 mM), dithionite (1 mM), and PRPn (2 mM) in the presence of 50 units Factor-Xa and 1X Factor-Xa buffer in a volume of 2.3 mL with a headspace of ~500 µL. (A) Methane standard (714 ng). (B) Incubation of reaction mixture with deletion of PhnJ. (C) Incubation of reaction mixture with deletion of PRPn. (D) Incubation of entire reaction mixture. (E) Incubation of the reaction mixture with deletion of SAM. (In samples B, C, D and E; 50 µL of the headspace was injected in the GC instrument).

HPLC analysis of the products of SAM: The HPLC analysis for determining the reaction products of SAM with PhnJ was performed on an AKTA Purifier FLPC/HPLC system with a C18 column (Cosmosil 5C18-AR-II 4.6x150 mm Nacalai, USA). All reactions were monitored at 260 nm using a linear gradient of 0 – 50 % buffer B over 10 column volumes over 35 minutes at a flow rate of 1.0 ml/min. Buffer A was 50 mM ammonium acetate pH 5.4 and buffer B was 50 % v/v methanol/water. The loading volume for all the reactions was 250 µL. The C18-column was pre-calibrated with 0.5 mM SAM, 0.5 mM 5'-deoxyadenosine (5DA) and 0.5 mM 5'-deoxy-5'-methylthioadenosine (MTA) for the elution profiles of the standards.

For the reactions involving PhnJ, the concentrations of the components were: PhnJ = 60 µM, SAM = 0.5 mM, dithionite = 1 mM, α-D-ribose-1-methylphosphonate-5-phosphate = 2 mM in 150 mM HEPES, containing 250 mM NaCl, 10 % v/v glycerol and 1X Factor-Xa reaction buffer at pH 8.5. The reaction was initiated by adding 50 units of Factor-Xa to the reaction. The total reaction volume was 1.0 mL. As controls, in one reaction dithionite was eliminated and in another PhnJ was eliminated. All the reactions were kept anaerobic for 3 hours after which the precipitated enzyme was removed by centrifugation. The reaction was filtered through a 30 kDa ultra-filtration membrane and the flow-through was collected. 250 µL of this material was loaded onto the C18-column and the HPLC traces were collected.

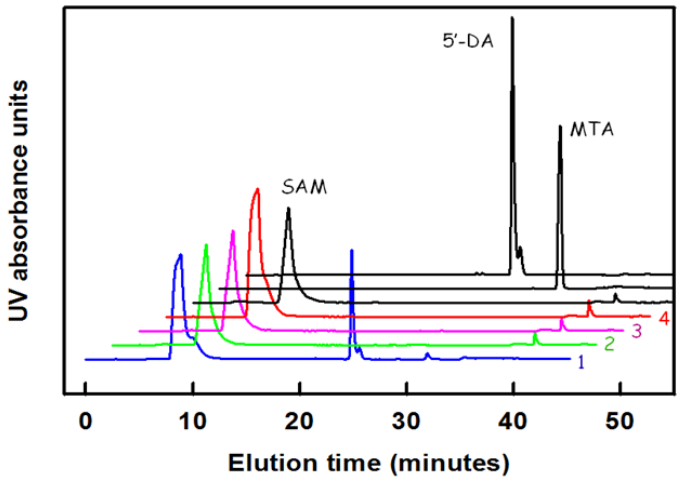


Figure S3: Standards for the detection of the reaction products from S-adenosyl-L-methionine (SAM) by PhnJ in presence of dithionite and Factor-Xa. Standards (black traces): 0.5 mM S-adenosyl-L-methionine (SAM) in 150 mM HEPES, 250 mM NaCl, 10 % v/v glycerol, 50 units Factor Xa and 1X Factor Xa reaction buffer at pH 8.5; 0.5 mM 5'-deoxyadenosine (5'-DA) in 150 mM HEPES, 250 mM NaCl, 10 % v/v glycerol, 50 units Factor Xa and 1X Factor Xa reaction buffer at pH 8.5; 0.5 mM 5'-deoxy-5'-methylthioadenosine (MTA) in 150 mM HEPES, 250 mM NaCl, 10 % v/v glycerol, 50 units Factor Xa and 1X Factor Xa reaction buffer at pH 8.5.

The reaction products of SAM in the reaction catalyzed by PhnJ with PRPn: (1) Reaction products after mixing 60 μM PhnJ, 0.5 mM SAM, 1 mM dithionite, 2 mM α-D-ribose-1-methylphosphonate-5-phosphate in 150 mM HEPES, 250 mM NaCl, 10 % v/v glycerol, 50 units Factor-Xa and 1X Factor-Xa reaction buffer at pH 8.5. (2) Same as 1 except PhnJ was omitted. (3) Same as 1 except that dithionite was omitted. (4) Same as 1 except Factor-Xa omitted. 5'-deoxyadenosine was identified as the major reaction product from SAM during the reaction with PhnJ.

Amino acid analysis of the products of SAM: Amino acid analysis of the reaction products from SAM in the reaction catalyzed by PhnJ was performed in the Protein Chemistry Laboratory, Texas A&M University using the AminoQuant method. The AminoQuant analyzes the samples by pre-column derivatization of the amino acids with o-phthalaldehyde (OPA) and 9-fluoromethyl-chloroformate (FMOC). OPA reacts with primary amino acids and FMOC with secondary amino acids (proline). The derivatized amino acids are separated by reverse phase HPLC and detected by UV absorbance with a diode array detector or by fluorescence using an in-line fluorescence detector.

For the reactions involving PhnJ, the concentrations of the components were: PhnJ = 70 μM, SAM = 2.0 mM, dithionite = 1.0 mM, α-D-ribose-1-methylphosphonate-5-phosphate = 1.0 mM in 150 mM HEPES, containing 250 mM NaCl, 10 % v/v glycerol and 1X Factor Xa reaction buffer at pH 8.5. The reaction was initiated by adding 50 units of Factor Xa to the reaction. The total reaction volume was 1.0 mL. As a control PhnJ was eliminated from the reaction mixture. All the reactions were kept anaerobic for 3 hours after which the precipitated enzyme was removed by centrifugation. The reaction was filtered through a 30 kDa ultra-filtration membrane and the flow through was collected. The control reaction showed less than 0.01 μM L-methionine. In the reaction mixture with PhnJ, 150 μM of L-methionine was detected.

Detection of CH_3OH in PhnJ reconstitution reaction: Alcohol dehydrogenase (ADH) from *Saccharomyces cerevisiae* (Sigma) was used to detect and quantify the amount of methanol (if any produced) from the reaction of α-D-ribose-5-phosphate-1-methylphosphonate [PRPn] with $[4Fe\text{-}4S]^{1+}$ reconstituted PhnJ in the presence of SAM, dithionite and Factor-Xa. A standard calibration curve was generated for the change in absorbance at 340 nm using $[NAD^+]$ = 2 mM, [ADH] = 0.1 mg/mL and varying concentrations of methanol (10 – 100 µM) in 20 mM HEPES buffer pH 7.5 at 30 ^oC. For the PhnJ reaction; [PhnJ] = 150 µM, [SAM] = 2 mM, [dithionite] = 1m M, [PRPn] = 1.5 mM, 50 units of Factor-Xa in 1X Factor-Xa buffer was used in and the enzyme was removed by ultrafiltration through 10 kDa membrane and the unreacted SAM and 5'-deoxyadenosine were removed by treatment with activated charcoal at the end of the reaction. The remaining reaction was incubated with NAD^+ and ADH at pH 7.5 and the changes in spectrum were measured at 340 nm for 5 hours for 30 ^oC. The control used for this test was all the ingredients except PhnJ. The net change in the absorbance corresponded to a methanol concentration < 2 µM. Thus effectively no significant amount of methanol could be in the solution PhnJ reconstitution reaction.

Detection of HCHO in PhnJ reconstitution reaction: The heterocyclic dye Purpald[1,2] (Sigma) was used to detect and quantify the amount of formaldehyde (if any produced) from the reaction of α-D-ribose-5-phosphate-1-methylphosphonate [PRPn] with $[4Fe\text{-}4S]^{1+}$ reconstituted PhnJ in the presence of SAM, dithionite and Factor-Xa. A standard curve was generated by using 100 % formalin (37 % w/v formaldehyde) as the source of formaldehyde and purpald by measuring the change in absorbance at 550 nm. In this experiment the purpald (10 mM) was initially allowed to react with varying formaldehyde concentrations (0 – 15 µM) for 10 minutes and the reaction was quenched with $NaIO_4$ (10 mM) after which the absorbance was measured. For the PhnJ reaction; [PhnJ] = 150 µM, [SAM] = 2 mM, [dithionite] = 1m M, [PRPn] = 1.5 mM, 50 units of Factor-Xa in 1X Factor-Xa buffer. No effort was made to separate any ingredient at the end of the reaction. A reference for this was all the ingredients except PhnJ. Based on the change in absorbanc obtained using purpald, the formaldehyde produced during PhnJ was estimated < 1.5 µM. Thus effectively no significant amount of formaldehyde could be in the solution PhnJ reconstitution reaction.

Cloning and Purification of C-P lyase Proteins

Cloning of phnG from Escherichia coli K12 MG1655: The DNA sequence for *phnG* from *E. coli K12 MG1655* was cloned (gi|16131927). The gene for *phnG* was amplified utilizing the primer pair 5'-AAAAAAGGATCCATGCACGCAGATACCGCGACCCGCCAGCA-3' and 5'-AGAAGAAAGCTTTCATGCGTTGTCTCCGCGAACCATCGTAAAGAAGTCCGA-3' using *Pfu Turbo (Strategene, Agilent). BamHI* and *HindIII* restriction sites were introduced into the forward and the reverse primers, respectively. The PCR product was purified with a PCR cleanup system (Qiagen), digested with *BamHI* and *HindIII*, and ligated into a pET30a(+) vector which was previously digested with *BamHI* and *HindIII*.

Protein Expression and Purification of PhnG: The recombinant plasmid bearing the gene for *phnG* was transformed into *E. coli* BL21 (DE3) competent cells by electroporation. A single colony was grown overnight at 37 ^{o}C in 5 mL of LB medium containing 50 µg/mL kanamycin. Five mL aliquots were used to inoculate 3 L of the same medium. The cell cultures were grown at 37 ^{o}C until the A_{600} reached ~ 0.3 - 0.4. The temperature was reduced to 18 ^{o}C and the culture was induced with 0.5 mM isopropyl-β-thiogalactoside (IPTG) when the A_{600} reached ~ 0.6. The cells were harvested by centrifugation 18 - 21 hours after induction. The cells were resuspended in 50 mM HEPES, 150 mM NaCl and 20 mM imidazole at pH 8.5 (binding buffer), containing 0.1 mg/mL PMSF and lysed by sonication. The soluble proteins were separated from the cell debris by centrifugation at 12000 x g for 15 minutes at 4 ^{o}C. The nucleic acids were removed by dropwise addition of 2% w/v protamine sulfate. After centrifugation, the supernatant solution was applied to a HisTrap column (GE Healthcare 5 mL) previously equilibrated with the binding buffer. The protein was eluted by applying a gradient of 50 mM HEPES, 150 mM NaCl and 1 M imidazole at pH 8.5 (Elution buffer). The fractions

were analyzed for purity by SDS-PAGE. The appropriate fractions were pooled and dialyzed at 4 ^{o}C with 50 mM HEPES, 150 mM NaCl at pH 8.5. The protein was concentrated using a 30 kDa ultra-filtration membrane (Amicon) by centrifugation. Typical yields for PhnG were 15 – 20 mg PhnG / L of LB media (~ 4 gm of cells/L of LB media).

Cloning of *phnH from Escherichia coli K12 MG1655*: The DNA sequence for *phnH* from *E. coli K12 MG1655* was cloned (gi|16131926). The gene for *phnH* was amplified utilizing the primer pair 5'-AAAGAGAATCATATGACCCTGGAAACCGCTTTTATGCTTCCCGTG-3' and 5'-AGAAGAAAGCTTTCAGCACACCTCCACATGAGTGGTTCGCGG-3' using *Pfx Platinum (Invitrogen)*. *NdeI* and *HindIII* restriction sites were introduced into the forward and the reverse primers, respectively. The PCR product was purified with a PCR cleanup system (Promega), digested with *Nde*I and *HindIII*, and ligated into a pET28b(+) vector which was previously digested with *NdeI* and *HindIII*.

Expression and Purification of PhnH: The recombinant plasmid bearing the gene for *phnH* was transformed into *E. coli* BL21 (DE3) competent cells by electroporation. A single colony was grown overnight at 37 ^{o}C in 5 mL of LB medium containing 50 µg/mL kanamycin. Five mL aliquots were used to inoculate 2 L of the same medium. The cell cultures were grown at 37 ^{o}C until the A_{600} reached ~ 0.3 - 0.4. The temperature was reduced to 18 ^{o}C and induced with 0.5 mM IPTG when the A_{600} reached ~ 0.6. The cells were harvested by centrifugation 18 - 21 hours after induction. The cells were resuspended in 50 mM HEPES and 20 mM imidazole at pH 8.0 (binding buffer), containing 0.1 mg/mL PMSF and lysed by sonication. The soluble proteins were separated from the cell debris by centrifugation at 12000 x g for 15 minutes at 4 ^{o}C. Nucleic acids were removed by dropwise addition of 2% w/v protamine sulfate. After centrifugation, the supernatant solution was applied to a HisTrap column (GE Healthcare 5 mL)

previously equilibrated with the binding buffer. The protein was eluted by applying a gradient of 50 mM HEPES and 1 M imidazole at pH 8.0 (elution buffer). The fractions were analyzed for purity by SDS-PAGE. The fractions were pooled and dialyzed at 4 ^{o}C into 50 mM HEPES at pH 8.0. The protein was concentrated using a 30 kDa ultra-filtration membrane (Amicon) by centrifugation. Typical yields for PhnH obtained were 150 – 160 mg PhnH / L of LB media (~ 4 gm of cells/L of LB media).

Cloning of *phnI from Escherichia coli K12 MG1655*: The DNA sequence for *phnI* from *E. coli K12 MG1655* was cloned (gi|16131925). The gene for *phnI* was amplified utilizing the primer pair 5'-AAAAAAGGATCCATGTACGTTGCCGTGAAAGGGGGCGAGAAG-3' and 5'-AGAAGAAAGCTTTTAGCCATGGTTCTGCTCCTGTTGCAGACGTTTGAG-3' using *Pfu Turbo (Strategene, Agilent). BamHI* and *HindIII* restriction sites were introduced into the forward and the reverse primers, respectively. The PCR product was purified with a PCR cleanup system (Qiagen), digested with *BamHI* and *HindIII*, and ligated into a pET28b(+) and pET42a(+) vector which was previously digested with *BamHI* and *HindIII*.

Protein Expression and Purification of PhnH: The recombinant plasmid (pET42a(+)) bearing the gene for *phnI* was transformed into *E. coli* BL21 (DE3) competent cells by electroporation. A single colony was grown overnight at 37 ^{o}C in 5 mL of LB medium containing 50 µg/mL kanamycin. Five mL aliquots were used to inoculate 6 L of the same medium. The cell cultures were grown at 37 ^{o}C the A_{600} reached ~ 0.3 - 0.4. The temperature was reduced to 18 ^{o}C and induced with 0.5 mM IPTG when the A_{600} reached ~ 0.6. The cells were harvested by centrifugation 18 - 21 hours after induction. The cells were resuspended in 100 mM HEPES, 0.5 M NaCl, 10% glycerol (v/v), 1 mM dithiothreitol (DTT) and 1 mM EDTA at pH 8.8 (binding buffer), containing 0.1 mg/mL PMSF,1 µL of protease inhibitor cocktail

(Sigma P8340)/mL and 0.4 mg deoxyribonuclease I (Sigma DN25)/mL. The cells were lysed by sonication. The soluble proteins were separated from the cell debris by centrifugation at 12000 x g for 10 minutes at 4 ^{o}C. After centrifugation, the supernatant solution was applied to a GSTrap column (GE Healthcare 5 mL) previously equilibrated with the binding buffer. The protein was eluted by applying a gradient of 100 mM HEPES, 0.5 M NaCl, 10% glycerol (v/v), 1 mM DTT, 1 mM EDTA and 10 mM reduced glutathione at pH 8.8 (elution buffer). The fractions were analyzed for purity by SDS-PAGE, pooled and concentrated using a 30 kDa ultra-filtration membrane (Amicon) by centrifugation. Typical yields for PhnI obtained were 1 – 1.5 mg PhnI / L of LB media (~ 4 gm of cells/L of LB media).

Cloning of *phnJ from Escherichia coli K12 MG1655*: The DNA sequence for *phnJ* from *E. coli K12 MG1655* was cloned (gi|16131924). The gene for *phnJ* was amplified utilizing the primer pair 5'-AAAAAAGGATCCATGGCTAATCTGAGCGGCTACAACTTTGCCTAC-3' and 5'-AGAAGAAAGCTTTCATTGGTTTTTTGCCTCGCTCTGTTGGCGG-3' using *Pfu Turbo (Strategene, Agilent). BamHI* and *HindIII* restriction sites were introduced into the forward and the reverse primers, respectively. The PCR product was purified with a PCR cleanup system (Qiagen), digested with *BamHI* and *HindIII*, and ligated into a pET28b(+) and pET42a(+) vector which was previously digested with *BamHI* and *HindIII*.

Protein Expression and Purification of PhnJ: The recombinant plasmid (pET42a(+)) bearing the gene for *phnJ* was transformed into *E. coli* BL21 (DE3) competent cells by electroporation. A single colony was grown overnight at 37 ^{o}C in 5 mL of LB medium containing 50 µg/mL kanamycin. Five mL aliquots were used to inoculate 6 L of the same medium. The cell cultures were grown at 37 ^{o}C the A_{600} reached ~ 0.3 - 0.4. The temperature was reduced to 18 ^{o}C and induced with 0.5 mM IPTG when the A_{600} reached ~ 0.6. The cells

were harvested by centrifugation 18 - 20 hours after induction. The cells were resuspended in 100 mM HEPES, 0.5 M NaCl, 10% glycerol (v/v) and 1 mM EDTA at pH 8.8 (binding buffer), containing 0.1 mg/mL PMSF, 1 µL of protease inhibitor cocktail (Sigma P8340)/mL and 0.4 mg deoxyribonuclease I (Sigma DN25)/mL. The cells were lysed by sonication. The soluble proteins were separated from the cell debris by centrifugation at 12000 x g for 10 minutes at 4 ^{o}C. After centrifugation, the supernatant solution was applied to a GSTrap column (GE Healthcare 5 mL) previously equilibrated with the binding buffer. The protein was eluted by applying a gradient of 100 mM HEPES, 0.5 M NaCl, 10% glycerol (v/v), 1 mM EDTA and 10 mM reduced glutathione at pH 8.8 (elution buffer). The fractions were analyzed for purity by SDS-PAGE. The fractions were pooled and concentrated using a 30 kDa ultra-filtration membrane (Amicon) by centrifugation. Typical yields for PhnJ obtained were 2 – 2.5 mg PhnJ / L of LB media (~ 4 gm of cells/L of LB media).

Reconstitution of PhnJ: PhnJ eluted from the GSTrap column (6 – 8 mg from 12 gm cells from 3 L LB media) was taken into the glove box. Argon was bubbled through the PhnJ solution for an hour to remove most of the oxygen from the solution. The PhnJ solution was concentrated using a 30 kDa ultra-filtration membrane (VWR chemicals) to 100 – 150 µM depending on the protein yield. To this concentrated PhnJ solution, 5 mM ferrous sulfate was added dropwise over an hour (50 fold excess). The mixture of PhnJ and Fe^{2+} was allowed to react for 3 hours. 5 mM sodium sulfide was added to the mixture of PhnJ and Fe^{2+} dropwise over an hour (50 fold excess). The mixture was allowed to react for another 3 hours. The insoluble ferrous sulfide was removed from the protein reconstituted with the [4Fe-4S]-cluster by centrifugation. The unreacted iron and sulfide was further separated from the protein by ultra-filtration using a 30 kDa membrane (VWR chemicals). 1 mM sodium dithionite was added to

the resulting protein to reduce the [4Fe-4S]-cluster to the active $[4Fe\text{-}4S]^{+}$ form from the $[4Fe\text{-}4S]^{2+}$ form. PhnJ was further concentrated to 150 – 200 µM (600 – 800 µL) using a 30 kDa ultra-filtration, to separate any residual sodium dithionite. The chemical reconstitution of the [4Fe-4S]-cluster of PhnJ was possible without interference from the N-terminal GST-fusion solubility tag.

Cloning of phnK from Escherichia coli K12: The DNA sequence for *phnK* from *E. coli K12* was cloned (gi|16131923). The gene for *phnK* was amplified utilizing the primer pair 5'-AAAAAAGGATCCATGAATCAACCGTTACTTTCGGTCAATAACCTGACCACCTTTACGCG-3' and 5'-AGAAGAAAGCTTTCAATTCTGCAAAACCGATGACACCAGCAGCTGTGTATACGGA-3' using *Pfu Turbo (Strategene, Agilent). BamHI* and *HindIII* restriction sites were introduced into the forward and the reverse primers, respectively. The PCR product was purified with a PCR cleanup system (Qiagen), digested with *BamHI* and *HindIII*, and ligated into a pET30a(+) and pET42a(+) vector which was previously digested with *BamHI* and *HindIII*.

Protein Expression and Purification of PhnK: The recombinant plasmid (pET42a(+)) bearing the gene for *phnJ* was transformed into *E. coli* BL21 (DE3) competent cells by electroporation. A single colony was grown overnight at 37 ^{o}C in 5 mL of LB medium containing 50 µg/mL kanamycin. Five mL aliquots were used to inoculate 6 L of the same medium. The cell cultures were grown at 37 ^{o}C until the A_{600} reached ~ 0.3 - 0.4. The temperature was reduced to 18 ^{o}C and induced with 0.5 mM IPTG when the A_{600} reached ~ 0.6. The cells were harvested by centrifugation 18 - 21 hours after induction. The cells were resuspended in 100 mM HEPES, 0.5 M NaCl, 10% glycerol (v/v) and 1 mM DTT at pH 8.8 (binding buffer), containing 0.1 mg/mL PMSF and 0.4 mg deoxyribonuclease I (Sigma

DN25)/mL. The cells were lysed by sonication. The soluble proteins were separated from the cell debris by centrifugation at 12000 x g for 10 minutes at 4 ^{o}C. After centrifugation, the supernatant solution was applied to a GSTrap column (GE Healthcare 5 mL) previously equilibrated with the binding buffer. The protein was eluted by applying a gradient of 100 mM HEPES, 0.5 M NaCl, 10% glycerol (v/v), 1 mM DTT and 10 mM reduced glutathione at pH 8.8 (elution buffer). The fractions were analyzed for purity by SDS-PAGE gels. The pure fractions (< 2 % impurities) were pooled and concentrated using a 30 kDa ultra-filtration membrane (Amicon) by centrifugation. Typical yields for PhnK obtained were 1 – 1.5 mg PhnK / L of LB media (~ 4 gm of cells/L of LB media).

Cloning of phnL from Escherichia coli K12: The DNA sequence for *phnL* from *E. coli K12* was cloned (gi|16131922). The gene for *phnL* was amplified utilizing the primer pair 5'-AAAAAAGGATCCATGATTAACGTACAAAACGTCAGTAAAACCTTCATCCTGCACCAGCAA-3' and 5'-AGAAGAAAGCTTTCATGAAGAGGCTCCCATTGGGTGCAGGCGGT-3' using *Pfu Turbo (Strategene, Agilent). BamHI* and *HindIII* restriction sites were introduced into the forward and the reverse primers, respectively. The PCR product was purified with a PCR cleanup system (Qiagen), digested with *BamHI* and *HindIII*, and ligated into a pET30a(+) and pET42a(+) vector which was previously digested with *BamHI* and *HindIII*.

Protein Expression and Purification of PhnL: The recombinant plasmid (pET42a(+)) bearing the gene for *phnL* was transformed into *E. coli* BL21 (DE3) competent cells by electroporation. A single colony was grown overnight at 37 ^{o}C in 5 mL of LB medium containing 50 µg/mL kanamycin. Five mL aliquots were used to inoculate 6 L of the same medium. The cell cultures were grown at 37 ^{o}C the A_{600} reached ~ 0.3 - 0.4. The temperature was reduced to 18 ^{o}C and induced with 0.5 mM IPTG when the A_{600} reached ~ 0.6. The cells

were harvested by centrifugation 18 - 20 hours after induction. The cells were resuspended in 100 mM HEPES, 0.5 M NaCl, 10% glycerol (v/v) and 1 mM DTT at pH 8.5 (Binding buffer), containing 0.1 mg/mL phenylmethylsulfonyl fluoride and 0.4 mg deoxyribonuclease I (Sigma DN25)/mL. The cells were lysed by sonication. The soluble proteins were separated from the cell debris by centrifugation at 12000 x g for 10 minutes at 4 ^{o}C. After centrifugation, the supernatant solution was applied to a GSTrap column (GE Healthcare 5 mL) previously equilibrated with the binding buffer. The protein was eluted by applying a gradient of 100 mM HEPES, 0.5 M NaCl, 10% glycerol (v/v), 1 mM DTT and 10 mM reduced glutathione at pH 8.5 (elution buffer). The fractions were analyzed for purity by SDS-PAGE gels. The fractions were pooled and concentrated using a 30 kDa ultra-filtration membrane (Amicon) by centrifugation. Typical yields for PhnL obtained were 8 – 10 mg PhnL / L of LB media (~ 4 gm of cells/L of LB media).

Cloning of phnM from Escherichia coli K12: The DNA sequence for *phnM* from *E. coli K12* was cloned (gi|16131921). The gene for *phnM* was amplified utilizing the primer pair 5'-GGGACTTCCATATGATTATCAATAACGTTAAGCTGGTGCTGG-3' and 5'-CCGGAATTCTCAGAACACCCTTTTACCCTGACG-3' using *Pfx Platinum (Invitrogen).* *NdeI* and *EcoRI* restriction sites were introduced into the forward and the reverse primers, respectively. The PCR product was purified with a PCR cleanup system (*Promega*), digested with *NdeI* and *EcoRI*, and ligated into a pET30a(+) vector which was previously digested with *NdeI* and *EcoRI*.

Protein Expression and Purification of PhnM: The recombinant plasmid bearing the gene for *phnM* was transformed into *E. coli* BL21 (DE3) competent cells by electroporation. A single colony was grown overnight at 37 ^{o}C in 5 mL of LB medium containing 50 µg/mL kanamycin.

Five mL aliquots were used to inoculate 6 L of the same medium. The cell cultures were grown at 37 ^{o}C the A_{600} reached ~ 0.3 - 0.4. The temperature was reduced to 18 ^{o}C and induced with 0.5 mM IPTG when the A_{600} reached ~ 0.6 in the presence of 1.0 mM $ZnCl_2$. The cells were harvested by centrifugation 18 - 20 hours after induction. Protein expression was confirmed by SDS-PAGE. The cells were centrifuged and then resuspended in 100 mM HEPES, 1 mM $ZnCl_2$ at pH 8.8 (purification buffer), containing 0.1 mg/mL PMSF and lysed by sonication. The soluble proteins were separated from the cell debris by centrifugation at 12,000 x g for 15 minutes at 4 ^{o}C. The nucleic acids were removed by dropwise addition of 2% w/v protamine sulfate. After centrifugation, solid ammonium sulfate was added to 60% saturation to the supernatant solution. The precipitated protein was dissolved in purification buffer and then applied to a High Load 26/60 Superdex 200 prep grade gel filtration column (GE Healthcare), which was previously equilibrated with the purification buffer. The fractions were pooled and loaded onto a ResourceQ column 6 mL (GE Healthcare) and eluted with a gradient of NaCl (0 – 1M) in 50 mM HEPES, pH 8.8. The fractions were pooled and exchanged into 100 mM HEPES, 1 mM $ZnCl_2$ at pH 8.8. Typical yields for PhnM were 4 - 5 mg / L of LB media (~ 4 gm of cells/L of LB media).

Reaction Kinetics of C-P Lyase Enzymes

Nucleosidase Reaction: To establish the substrate profile for PhnI, a library of nucleosides and nucleotides were incubated with 10 µM PhnI in 100 mM HEPES buffer containing 250 mM NaCl and 10 % v/v glycerol at pH 8.5 for 6 hours. At the end of 6 hours, coupling enzymes capable of deaminating the free base released from the respective substrates were added and the UV-vis spectrum was observed from 240 – 350 nm using a SpectraMax-340 UV-vis spectrometer in a 1.0 mL quartz cuvette. For inosine containing compounds 50 units of xanthine oxidase (Sigma) was added and the spectrum was collected.

After initial spectroscopic screening, 10 µM PhnI was incubated with 2 mM adenine and guanine nucleoside di- and tri-phosphates overnight in the presence of 3 mM Mg^{2+} in 100 mM HEPES buffer containing 250 mM NaCl and 10 % v/v glycerol at pH 8.5 at 30 ^{o}C. The reactions with ATP and GTP were analyzed by HPLC. 0.25 mL of the reaction mixture was loaded onto a ResourceQ column 1 mL (GE Healthcare) pre-equilibrated with 25 mM HEPES pH 8.0. The reaction mixture was eluted with a 0-1 M NaCl gradient over 10 column volumes using an AKTA 10 Basic FPLC system. The ResourceQ column was caliberated with adenine and ATP or guanine and GTP standards prior to loading the reaction to determine the reaction products. In order to establish the products of the enzyme mixture the same reaction was carried out using 10 µM PhnI, 20 µM PhnL, 40 µM PhnG and 40 µM PhnH for 5 mM ATP or GTP in the presence of 7 mM Mg^{2+} under the same conditions.

Data fitting: All kinetic data was fit to equation 1 using non-linear least squares fitting program SigmaPlot 9.0.

$$\upsilon / E_t = k_{cat}[A] / (K_m + [A]) \quad (1)$$

where υ is the initial velocity, [A] = substrate concentration, E_t = total enzyme concentration, k_{cat} = turnover number and K_m = Michaelis constant.

Nucleosidase Kinetics: All kinetic assays were carried out in 50 mM HEPES buffer pH 8.5 at 30 ^oC in the presence of 1.0 mM Mg^{2+} using a SpectraMax-340 UV-vis spectrometer using a 96 well quartz plate. The nucleosidase activity of PhnI with ATP and ADP were measured by coupling the nucleosidase activity to the deamination of the released free base with adenine deaminase (ADE) from *E. coli*[3]. In the assay, the ATP concentrations were varied from 0 – 300 μM and ADP concentrations were varied from 0 – 350 μM. In the assay, the final PhnI concentration was 0.5 μM for kinetic assays with ATP and 1.0 μM for kinetic assays with ADP. The final ADE concentration in the assay was 10.0 μM. To test the kinetics of PhnI in the presence of PhnG, PhnH and PhnL with Factor Xa the following concentrations were used: PhnI = 0.1 μM, PhnG = 0.4 μM, PhnH = 0.4 μM, PhnL = 0.2 μM and Factor Xa = 10 units. In this kinetic assay the methylphosphonate and Mg^{2+} concentrations were constant at 1 and 2 mM respectively. All the kinetics of the ADE coupled assays were measured at 260 nm using a differential extinction coefficient of 4100 $M^{-1}cm^{-1}$ for the decrease in adenine concentration forming hypoxanthine.

The nucleosidase activity of PhnI with GTP and GDP was measured by coupling the nucleosidase activity to the deamination of the released free base guanine by guanine deaminase (GuaD) from *E. coli*[4]. In the assay, GTP concentrations were varied from 0 – 300 μM and GDP concentrations were varied from 0 – 300 μM. In the assay, the final PhnI concentration was 0.1 μM for kinetic assays with GTP and 0.5 μM for kinetic assays with GDP. The final GuaD concentration in the assay was 15.0 μM. To test the kinetics of PhnI in the presence of PhnG, PhnH and PhnL in the presence of Factor Xa the following concentrations were used: PhnI = 0.1

μM, PhnG = 0.4 μM, PhnH = 0.4 μM, PhnL = 0.2 μM and Factor Xa = 10 units. In this kinetic assay the methylphosphonate and Mg^{2+} concentrations were constant at 1 and 2 mM respectively. All of the GuaD coupled assays were measured at 253 nm using a differential extinction coefficient of 3920 $M^{-1}cm^{-1}$ for the decrease in guanine concentration forming xanthine.

The nucleosidase activity of PhnI with ITP and IDP was measured by coupling the nucleosidase activity to the oxidation of the released free base hypoxanthine to uric acid by xanthine oxidase from bovine milk (Sigma). In the assay, ITP concentrations were varied from 0 – 300 μM and IDP concentrations were varied from 0 – 300 μM. In the assay, the final PhnI concentration was 0.5 μM for kinetic assays with ITP and 1.2 μM for kinetic assays with IDP. The final xanthine oxidase was 50 units per assay as described in Sigma catalog. All the kinetics of the xanthine oxidase coupled assays were measured at 293 nm using an extinction coefficient of 12600 $M^{-1}cm^{-1}$ for the increase in uric acid concentration.

Purification of D-ribose-5-triphosphate (RTP) and α-D-ribose-1-methylphosphonate-5-triphosphate (RPnTP): The ResourceQ column (GE Healthcare) was pre-calibrated for the elution profiles of adenine, AMP, ADP and ATP to determine the volumes of elution of each. Baseline separations were obtained for adenine, AMP, ADP and ATP. The binding buffer for these separations included ammonium bicarbonate (100 mM) pH 8.5 and the elution buffer was 1 M ammonium bicarbonate pH 8.5. The gradient was run over 10 column volumes. Since both D-ribose-5-triphosphate (RTP) and α-D-ribose-1-methylphosphonate-5-triphosphate (RPnTP) do not have chromophores to check absorbance, these elution volumes were used as standards to collect fractions.

The enzymatic synthesis of D-ribose-5-triphosphate (RTP) was carried out by incubating 15 µM PhnI with 5 mM ATP and 7 mM Mg^{2+} to a total volume of 1.0 mL for 5 hours in 50 mM HEPES buffer containing 250 mM NaCl and 10 % v/v glycerol at pH 8.5. 0.5 mL of the reaction mixture was loaded onto the ResourceQ column (two times) and the fractions corresponding to the elution volumes of ATP were collected. The solvent was evaporated using a SpeedVac Concentrator (Savant) for about 6 hours. The final yields varied from 1.5 – 2.0 mg. The resulting solid was white in color and was stored at – 20 ^{o}C. D-ribose-5-diphosphate (RDP) was enzymatically synthesized by the reaction of PhnI with ADP. All concentrations were the same except that ADP was used in place of ATP. The purification was also the same except that the fractions eluting around ADP were collected instead of the fractions eluting around ATP from the ResourceQ column. The final yields were also the same.

The enzymatic synthesis of α-D-ribose-1-methylphosphonate-5-triphosphate (RPnTP) was carried out by incubating 10 µM PhnI, 20 µM PhnL, 40 µM PhnG, 40 µM PhnH, 50 units Factor Xa, 1X Factor Xa buffer, 5 mM methylphosphonate, 5 mM ATP and 7 mM Mg^{2+} in 50 mM HEPES buffer containing 250 mM NaCl and 10 % v/v glycerol at pH 8.5 in a total volume of 1.5 mL. 0.5 mL of the reaction mixture was loaded onto the ResourceQ column and all fractions eluting after the elution volumes of ATP were collected. The solvent was evaporated using a SpeedVac Concentrator (Savant). The final yields obtained varied from 1.8 – 2.2 mg. The resulting solid was white in color and was stored at – 20 ^{o}C.

PhnM Kinetics: All PhnM assays were performed using a Pi Colorlock (Gold) phosphate detection colorimetric system from Innova Biosciences according to the manufacturer's instructions. Since all reactions were performed in 50 mM HEPES containing 5 mM Mg^{2+}, 1 mM Zn^{2+} and 5 units of inorganic pyrophosphatase from Baker's yeast (Sigma) at pH 8.5, a

calibration curve was made with this standard assay condition to account for any background phosphate in the assay. All kinetic data was fit to standard Michaelis-Menten kinetics described earlier.

PhnM was shown to cleave the phosphodiester bond of α and β- phosphoryl groups of the triphosphate moiety of the putative substrates releasing pyrophosphate for a triphosphate substrate and phosphate for a diphosphate substrate. For the kinetics with RTP, the RTP concentration in the assay was varied from 0 – 500 µM. The PhnM concentration in the assay was 10 nM . For the kinetics of RPnTP, the concentration of RPnTP was varied from 0 – 750 µM, with a fixed PhnM concentration of 10 nM. For both of these assays, end points for the kinetic assays were collected at 0, 3, 6, 9 and 12 minutes. Inorganic pyrophosphatase was added as a coupling enzyme to the assay to convert the pyrophosphate released from the substrate to phosphate to allow detection by the Pi ColorLock (Gold) kit. For RDP, the concentration of RDP was varied from 0 – 1 mM, with the PhnM concentration being 1.0 µM in the assay.

Purification of α-D-ribose-1-methylphosphonate-5-phosphate: The enzymatic synthesis of α-D-ribose-1-methylphosphonate-5-phosphate was performed by incubating 5 mM RPnTP with 10 µM PhnM, 5 mM Mg^{2+} and 1 mM Zn^{2+} in 50 mM HEPES pH 8.5. The reaction was monitored by quantifying the phosphate produced after adding inorganic pyrophosphatase from Baker's yeast to the reaction over time in conjunction with ^{31}P NMR. The reaction of PhnM with RPnTP was allowed to incubate at 30 ^{o}C for about 5 hours for complete turnover. Zinc supplementation was essential for complete turnover of RPnTP. The reaction was filtered through a 30 kDa ultra-filtration membrane (VWR Chemicals) to remove the enzymes from the mixture. At the end of the filtration the flow through was a mixture of α-D-ribose-1-methylphosphonate-5-phosphate and pyrophosphate containing 5 mM Mg^{2+} and 1 mM Zn^{2+} in

50 mM HEPES pH 8.5. ^{31}P NMR was used to confirm the relative ratios of α-D-ribose-1-methylphosphonate-5-phosphate and pyrophosphate. The α-D-ribose-1-methylphosphonate-5-phosphate purified using this protocol was used as a substrate for all assays with PhnJ.

Enzymatic assays with PhnJ: All enzymatic assays with PhnJ were carried out in a glove box (MBraun Lab Master SP) because of the air sensitivity of the iron-sulfur cluster of PhnJ. All buffers were degassed for oxygen and introduced into the glove box prior to any assay. All assays in the glove box were carried out at 10 – 12 ^{o}C. The buffer used for all assays with PhnJ was 150 mM HEPES, 350 mM NaCl, 10 % v/v glycerol and 1X Factor Xa reaction buffer at pH 6.8. All assays contained fixed concentrations of 2 mM S-adenosyl-L-methionine (SAM) and 1 mM dithionite. The PhnJ concentrations in the assay varied from 100 – 160 μM (6.5 – 10 mg/mL). The substrate, α-D-ribose-1-methylphosphonate-5-phosphate, concentration in the assay varied from 0.5 – 3 mM depending on the nature of the assay. All the reactions were started by the addition of 50 units of Factor Xa. All reactions were allowed to proceed for 3 – 5 hours anaerobically. The reaction volumes were usually 1.0 mL. At the end of the reaction most of the enzyme would have precipitated due to the poor solubility after cleaving the GST-tag. The precipitated enzyme was removed by centrifugation. To the supernatant, 2 mg of activated charcoal (Sigma) was added to remove unreacted SAM and the products of SAM from the reaction for about 30 minutes. 2 mg of activated charcoal was found to be sufficient to remove any SAM and related compounds from control reactions performed earlier in the absence of PhnJ. The activated charcoal was removed by centrifugation and the supernatant was filtered through a 30 kDa ultra-filtration membrane to remove any enzyme that might have remained in solution. The reaction was transferred into a NMR tube anaerobically and the NMR tube was

sealed anaerobically. All the reactions were analyzed by ^{31}P NMR (Varian Inova 300 MHz and 500 MHz) spectroscopy to check for products.

UV-visible spectrum of PhnJ: The chemically reconstituted PhnJ (2 mg/mL ~ 31 µM) was transferred to a 1.0 mL anaerobic cuvette (Precision Cells Inc.) and sealed to prevent air from entering the cuvette. The UV-visible absorbance spectrum was collected in a SpectraMax Plus 384 spectrophotometer from 260 – 700 nm. After the spectrum was collected, PhnJ was treated with 1 mM dithionite anaerobically in the glove box for 1 hour. The excess dithionite was separated from PhnJ using a PD-10 column (GE Healthcare). The PhnJ fractions were collected and concentrated using a 30 kDa ultra-filtration membrane. The reduced form of PhnJ (1.7 mg/mL ~ 27 µM) was again transferred to the 1.0 mL anaerobic cuvette and the spectrum was collected.

NMR Spectra of Reaction Products

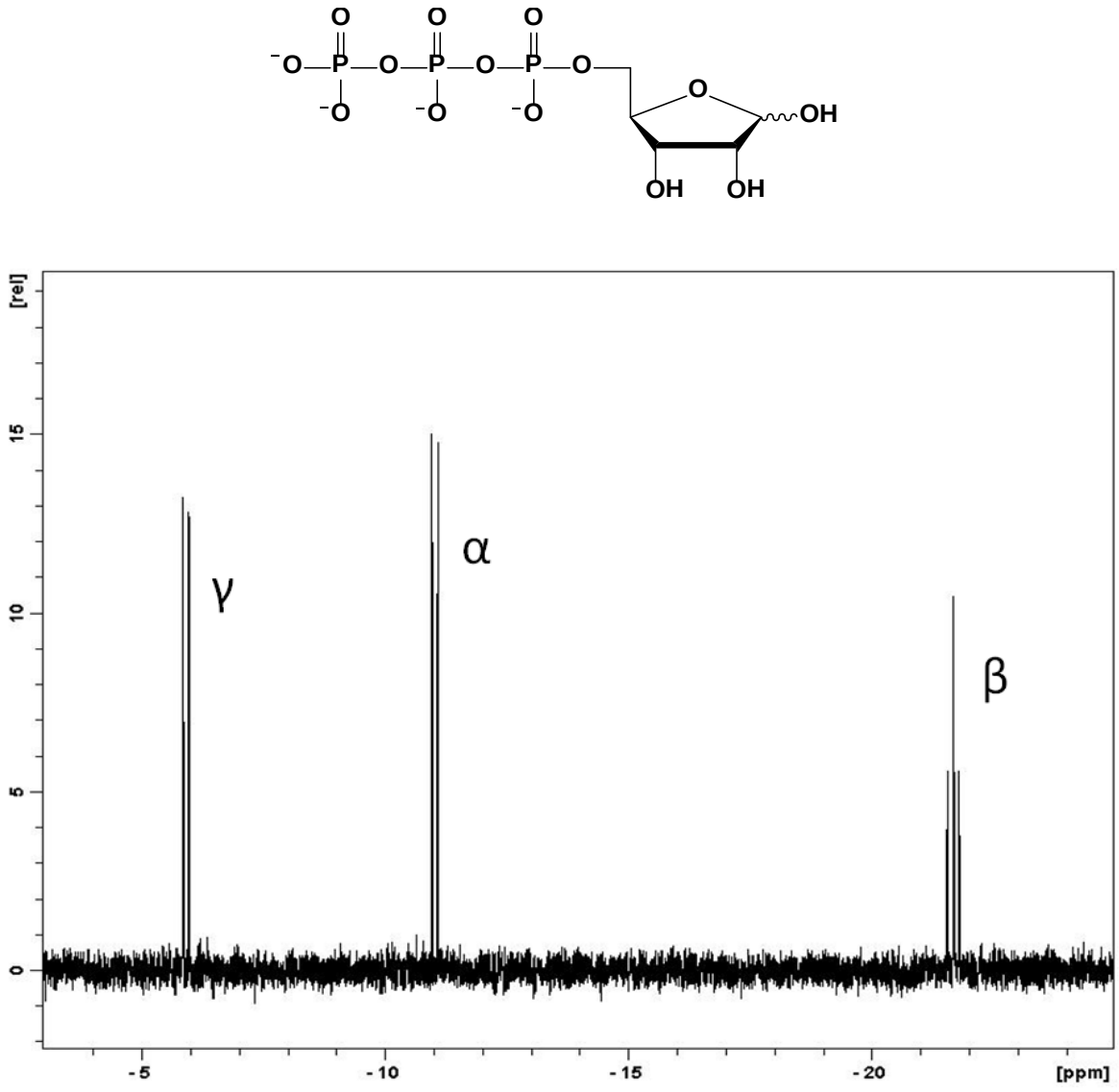


Figure S4: ^{31}P NMR spectrum of D-ribose-5-triphosphate (RTP); the product of the reaction of PhnI (10 µM) with MgATP (5 mM) in 100 mM HEPES buffer pH 9.0. The spectrum shows the tri-phosphate moiety of RTP.

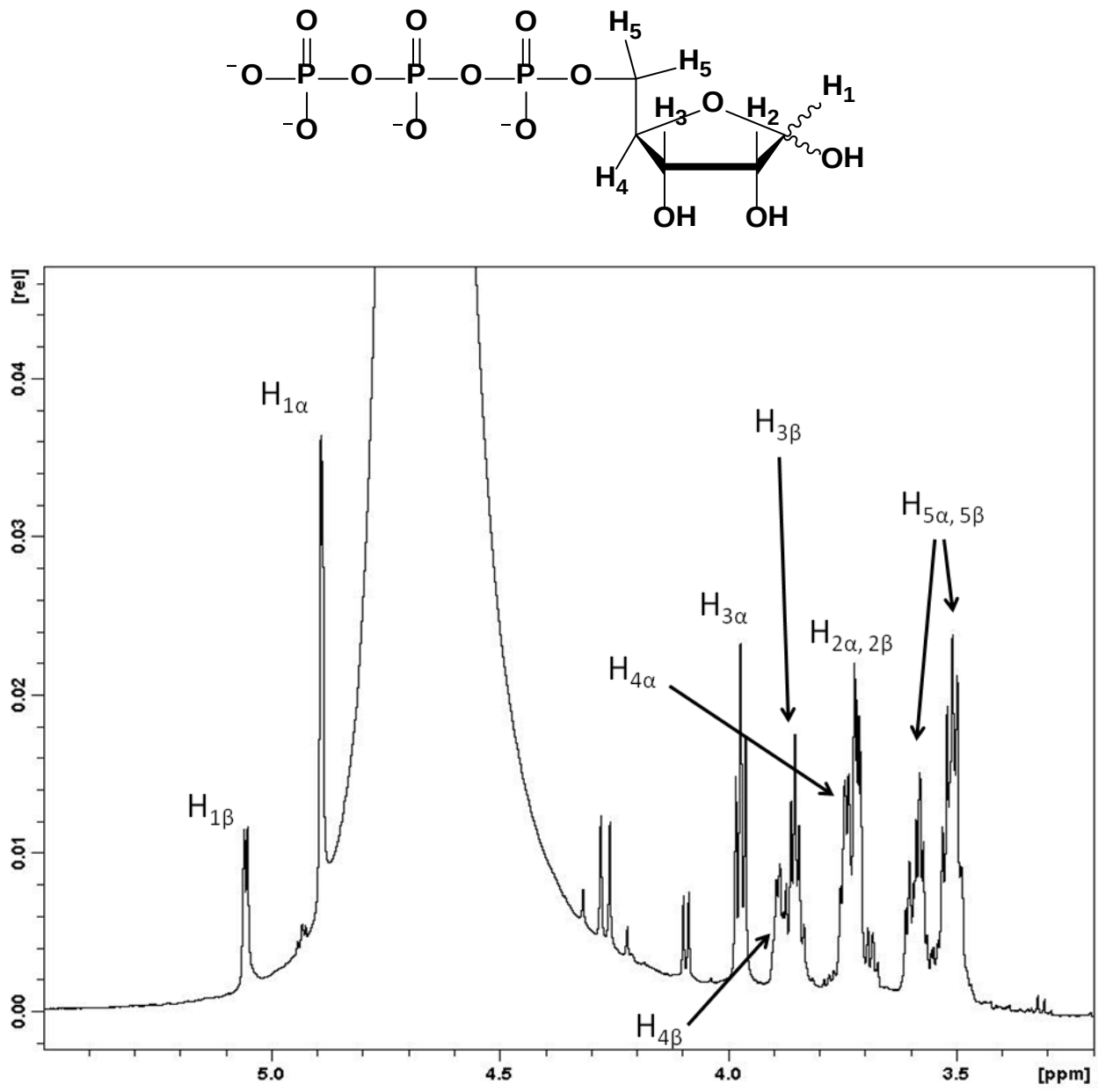


Figure S5: ^{1}H NMR spectra of RTP in 100 mM phosphate buffer pH 8.5, 10% D_2O.

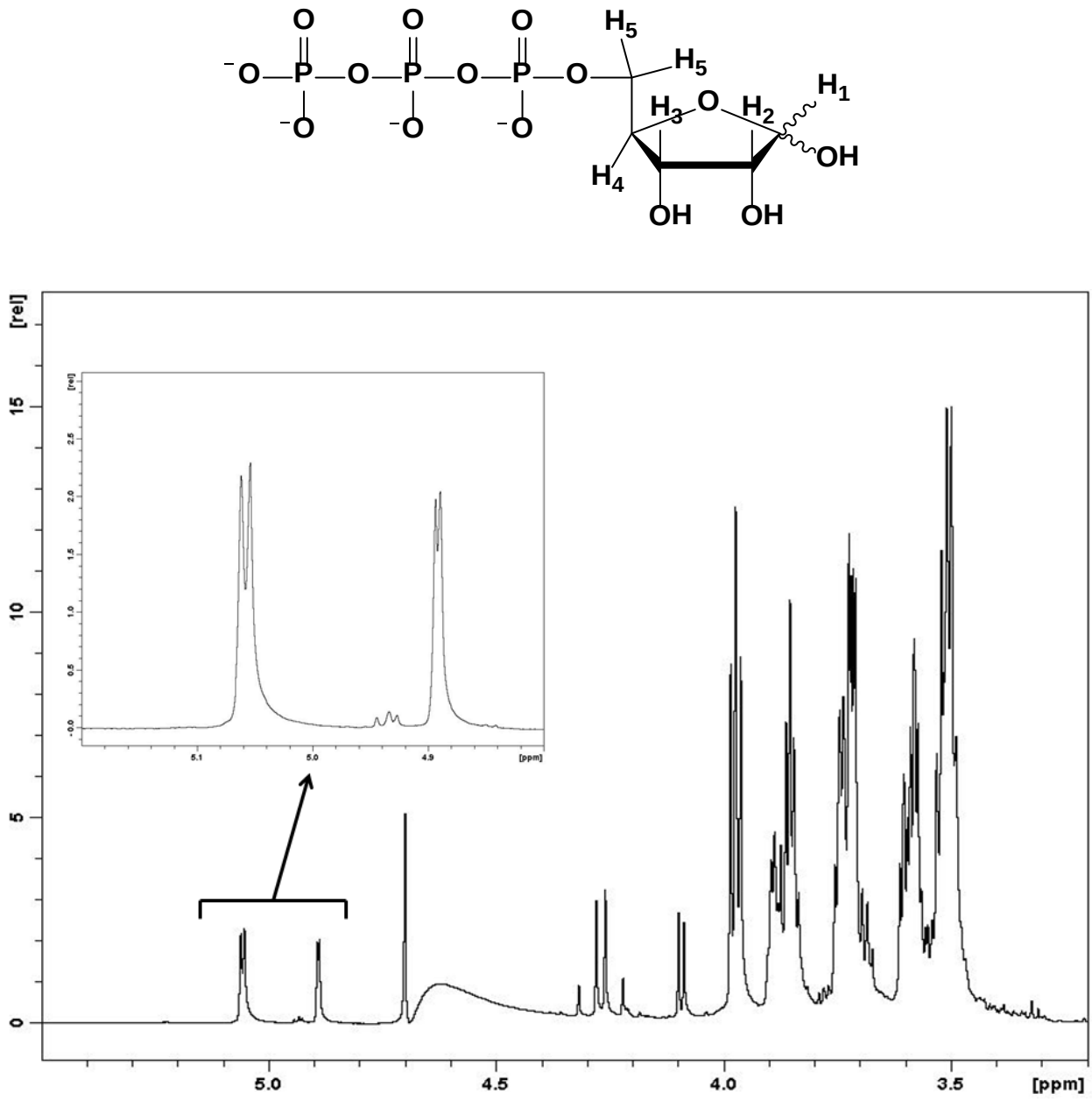


Figure S6: ^{1}H NMR spectrum of RTP in 100 mM phosphate buffer, pH 8.5, 10% D_2O, acquired with WATERGATE solvent suppression on a Bruker Avance III 500 MHz NMR. The inset shows the anomeric protons as doublets (H1-H2 coupling). The coupling constants determined for these were $J_{H1\alpha}$ = 2.4 Hz and $J_{H1\beta}$ = 3.5 Hz.

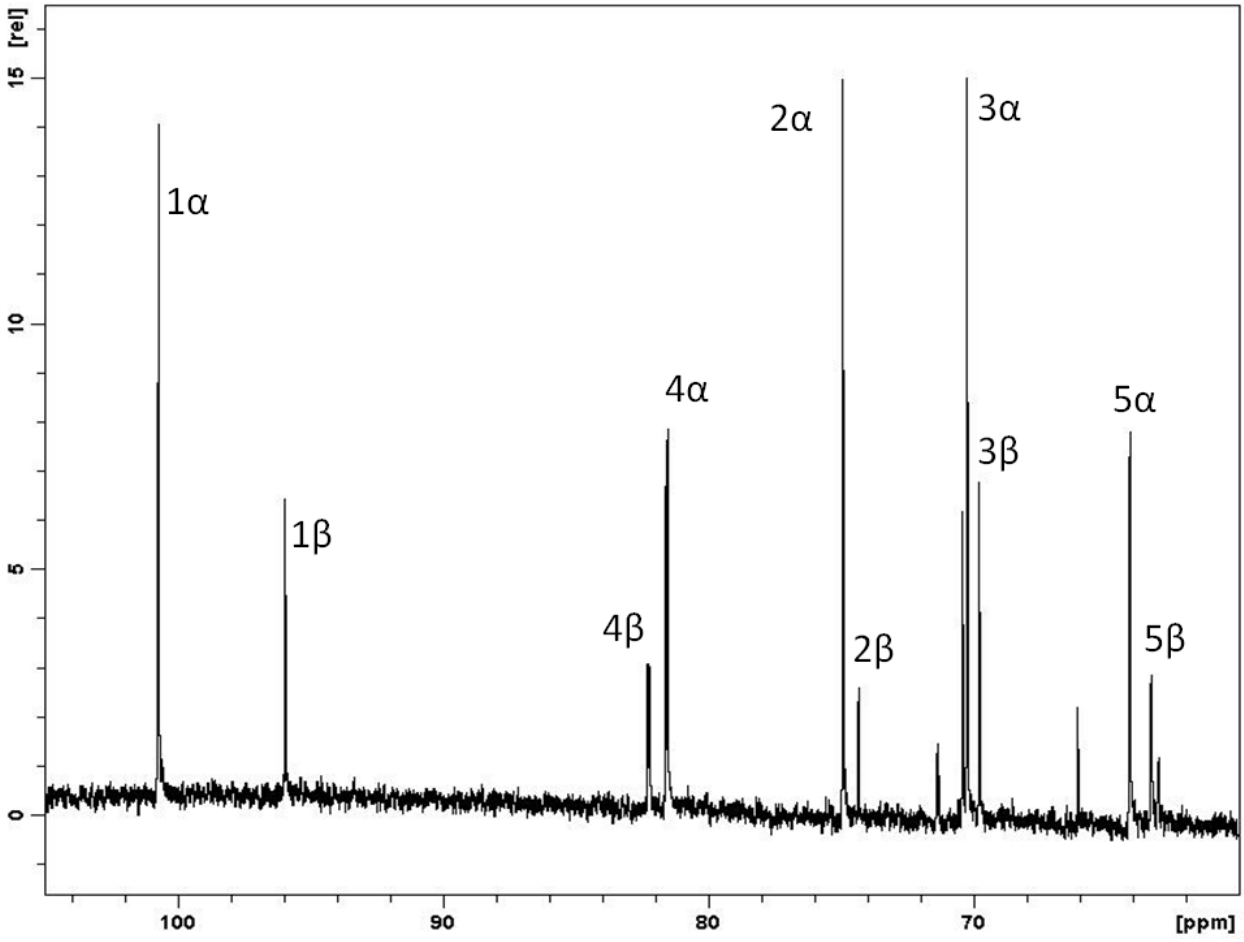
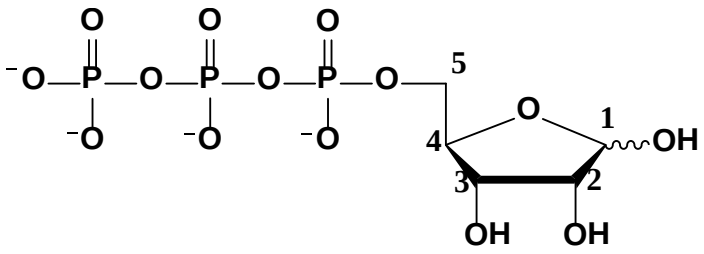


Figure S7: ^{13}C NMR spectrum of RTP in 100 mM phosphate buffer, pH 8.5, 10% D_2O. The numbers represent the numbering of the ribose carbon atoms. Glycerol was an impurity with the RTP during the purification from the reaction buffer.



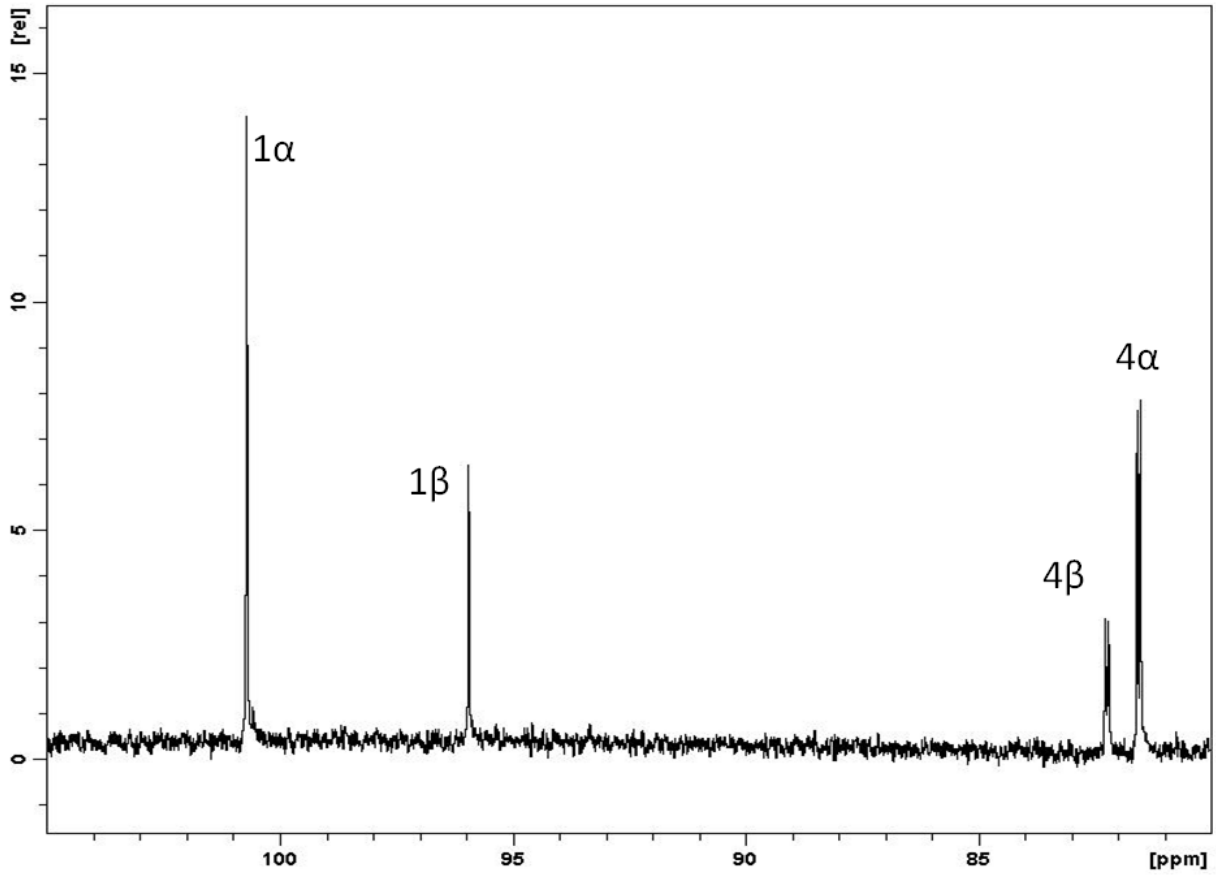


Figure S8: ^{13}C NMR spectrum of RTP in 100 mM phosphate buffer, pH 8.5, 10% D_2O showing C-1 and C-4 of the ribose moiety. The C-1 is a singlet (lack of phosphorus coupling at C-1), whereas the C-4 is a doublet from the coupling of the phosphorus at C-5.

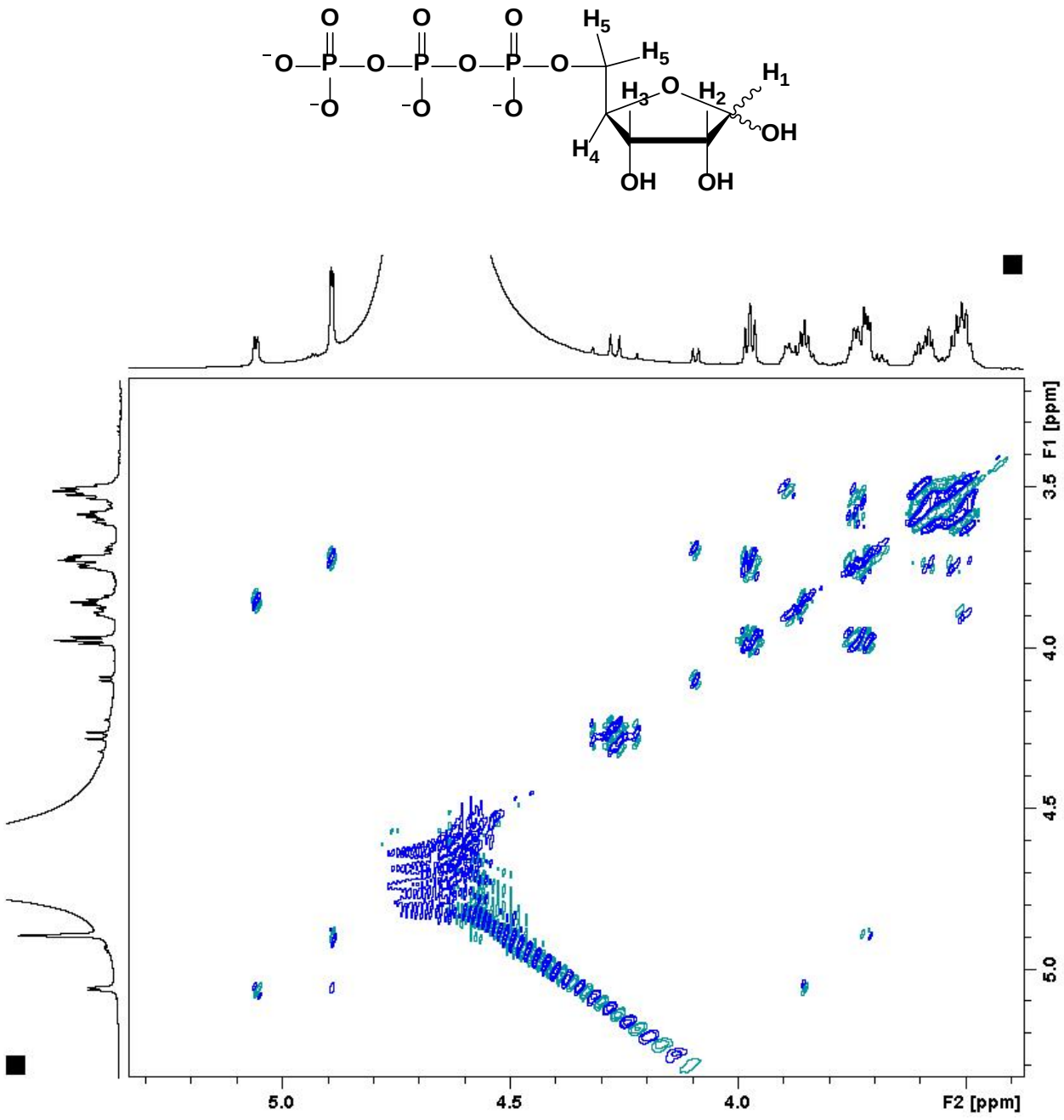


Figure S9: COSY (^{1}H-^{1}H) NMR spectrum of RTP in 100 mM phosphate buffer at pH 8.5 on a Bruker Avance III 500 MHz NMR.

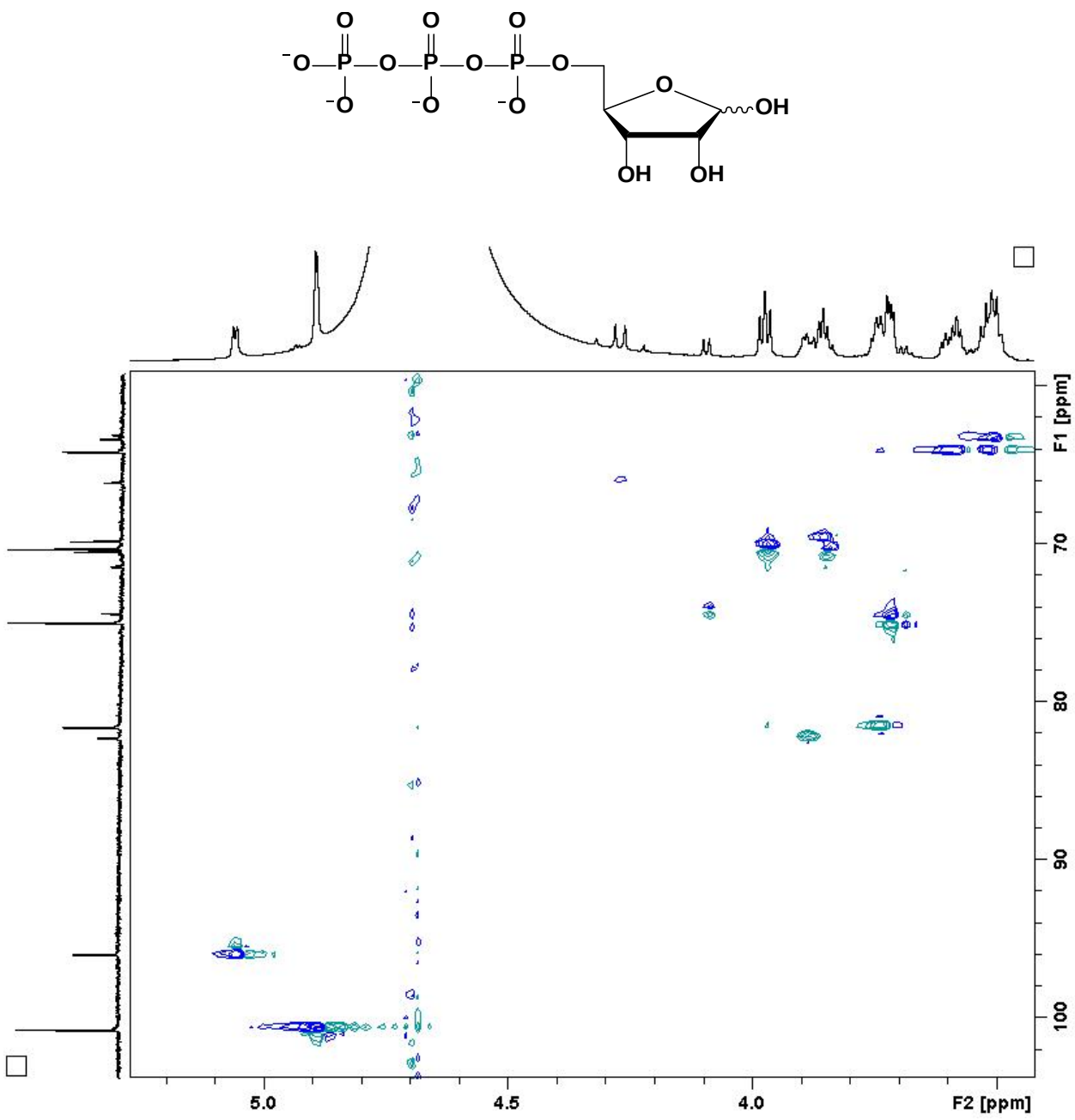


Figure S10: HSQC (^{1}H-^{13}C) NMR spectrum of RTP in 100 mM phosphate buffer at pH 8.5 on a Bruker Avance III 500 MHz NMR.

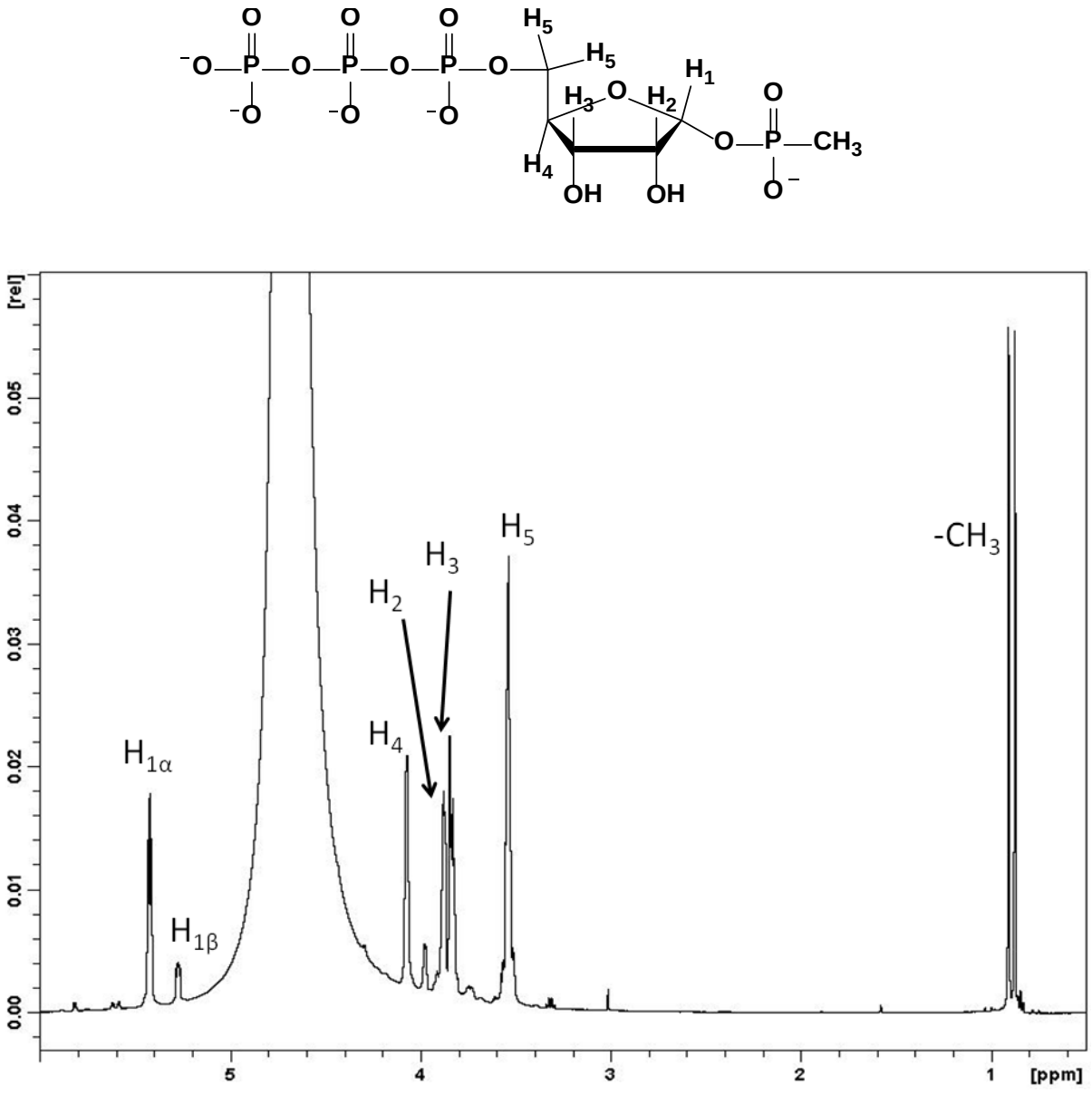
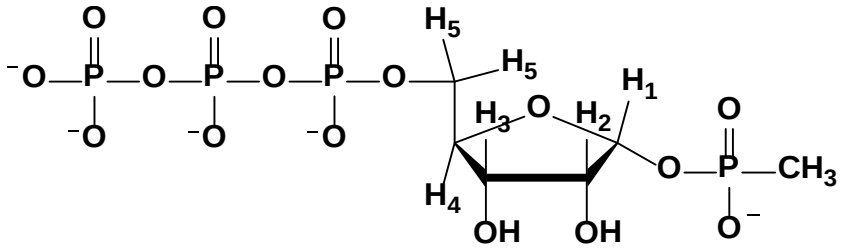


Figure S11: ^{1}H NMR spectrum of α-D-ribose-1-methylphosphonate-5-triphosphate (RPnTP) in 100 mM phosphate buffer, pH 8.5 on a Bruker Avance III 500 MHz NMR. A small amount of the β-anomer is also formed in the reaction.



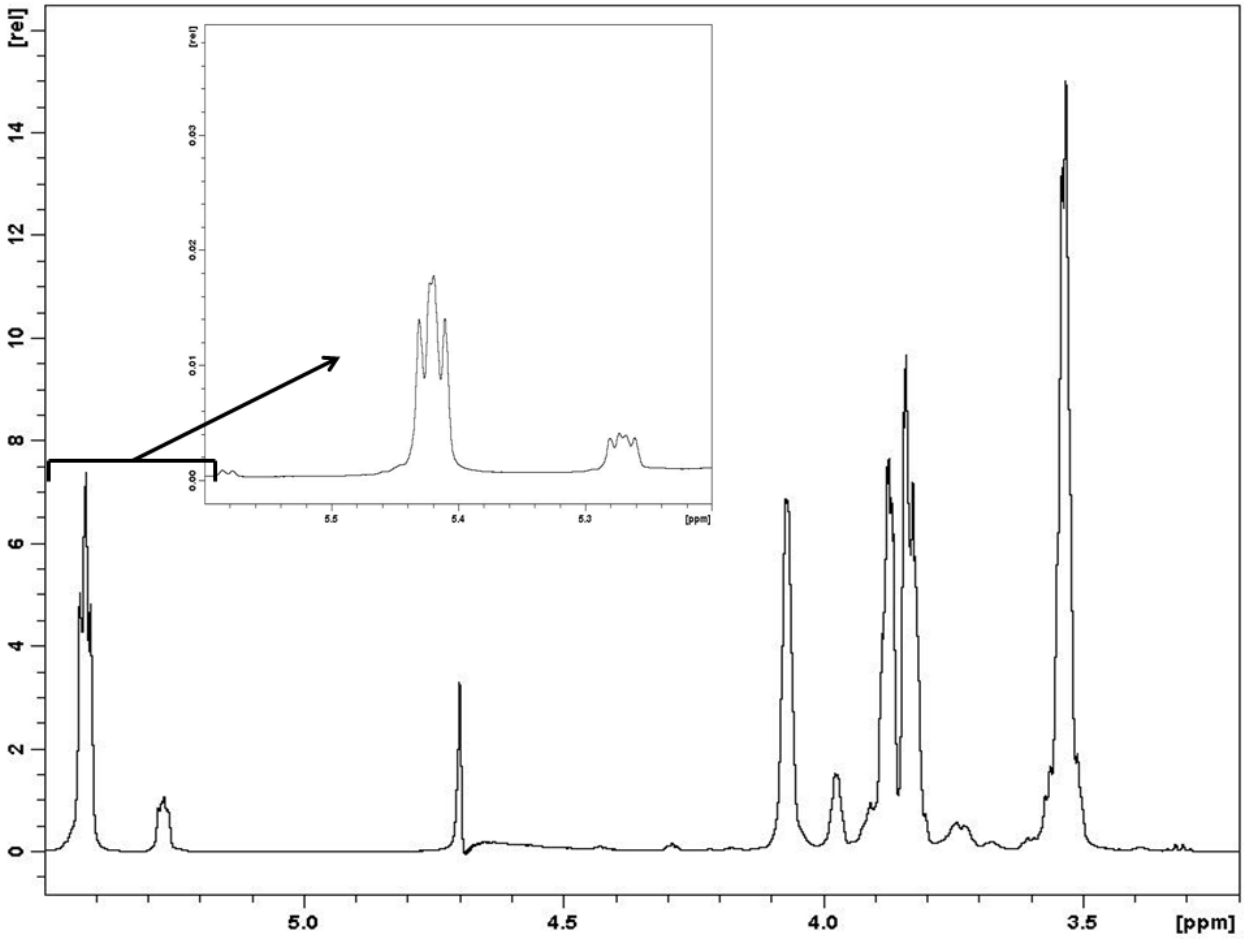


Figure S12: ^{1}H NMR spectrum of the ribose moiety of α-D-ribose-1-methylphosphonate-5-triphosphate (RPnTP) in 100 mM phosphate buffer, pH 8.5 using WATERGATE solvent suppression on a Bruker Avance III 500 MHz NMR. The inset shows the anomeric protons split as a quartet (doublet of doublets) resulting from the phosphorus of the methylphosphonate attached to C-1. The coupling constants for the major product: $H_{1\alpha}$ are $J_{(H1-H2)}$ = 4.3 Hz and $J_{(H1-P1)}$ = 6.1 Hz.

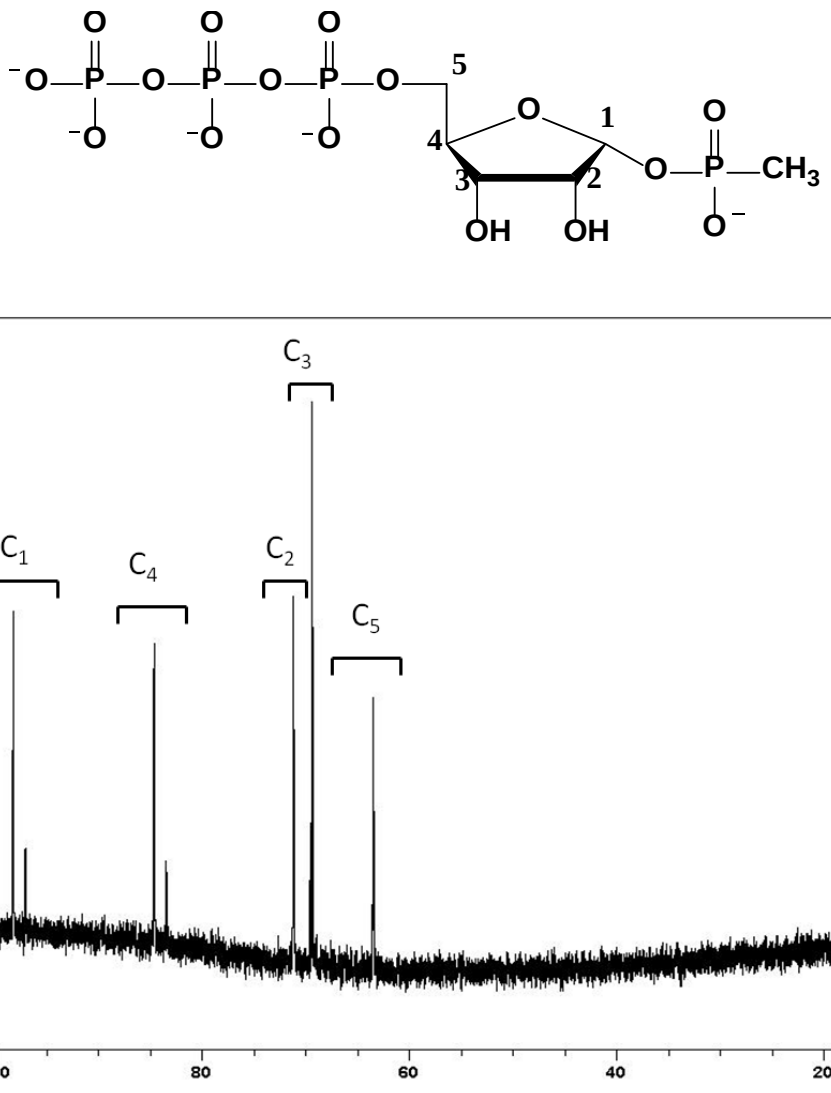


Figure S13: ^{13}C NMR spectrum of RPnTP in 100 mM phosphate buffer, pH 8.5 on a Bruker Avance III 500 MHz NMR. The carbon atoms of RPnTP are labeled in the ^{13}C NMR spectrum. There are two anomers observed in the ^{13}C NMR spectrum with the α-anomer being the major enzymatic product. The six carbon atoms of the molecule are observed in this spectrum with the methyl group of the 1-methylphosphonate upfield from the ribose carbon atoms and split as doublet from the strong coupling to the phosphorus.

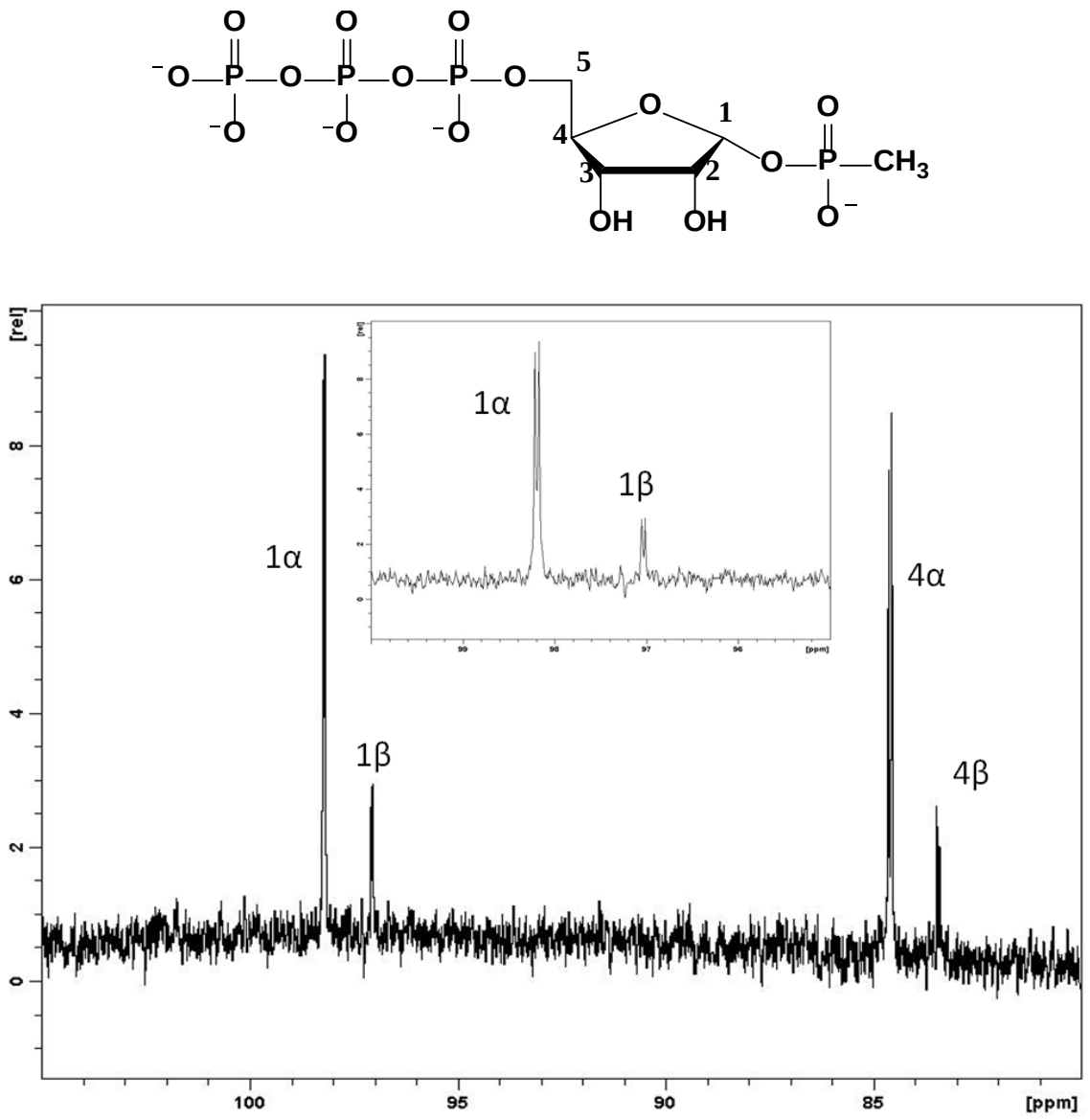


Figure S14: ^{13}C NMR spectrum of RPnTP in 100 mM phosphate buffer at pH 8.5 on a Bruker Avance III 500 MHz NMR showing C-1 and C-4 of the ribose moiety. Both C-1 and C-4 are doublets for both anomers. The inset shows the splitting of the C-1 anomers from the 1-methylphosphonate adduct to form RPnTP.

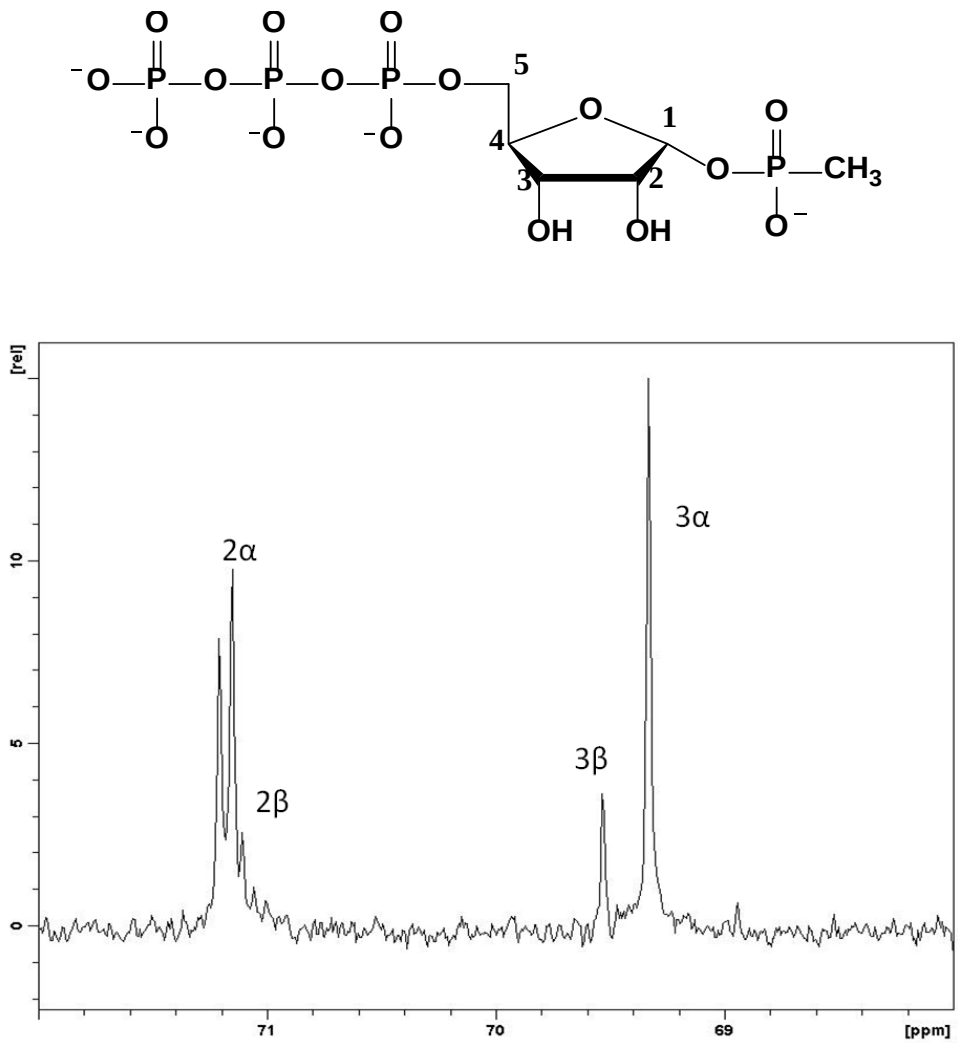


Figure S15: ^{13}C NMR spectrum of RPnTP in 100 mM phosphate buffer pH 8.5 on a Bruker Avance III 500 MHz NMR showing C-2 and C-3 of the ribose moiety. The C-2 of the ribose moiety is a doublet and C-3 of the ribose moiety is a singlet.

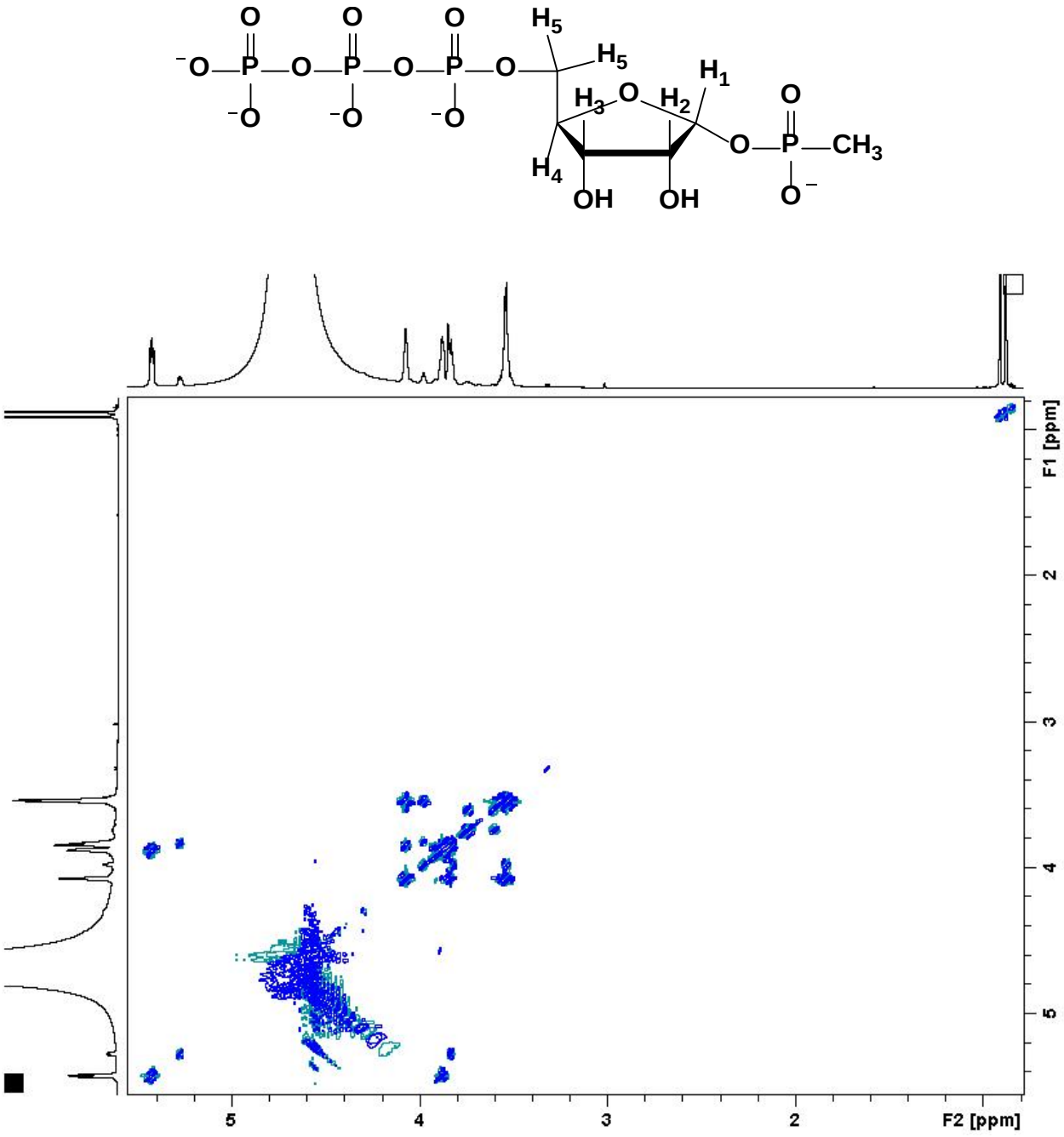


Figure S16: COSY (^{1}H-^{1}H) NMR spectrum of RPnTP in 100 mM phosphate buffer at pH 8.5 on a Bruker Avance III 500 MHz NMR.

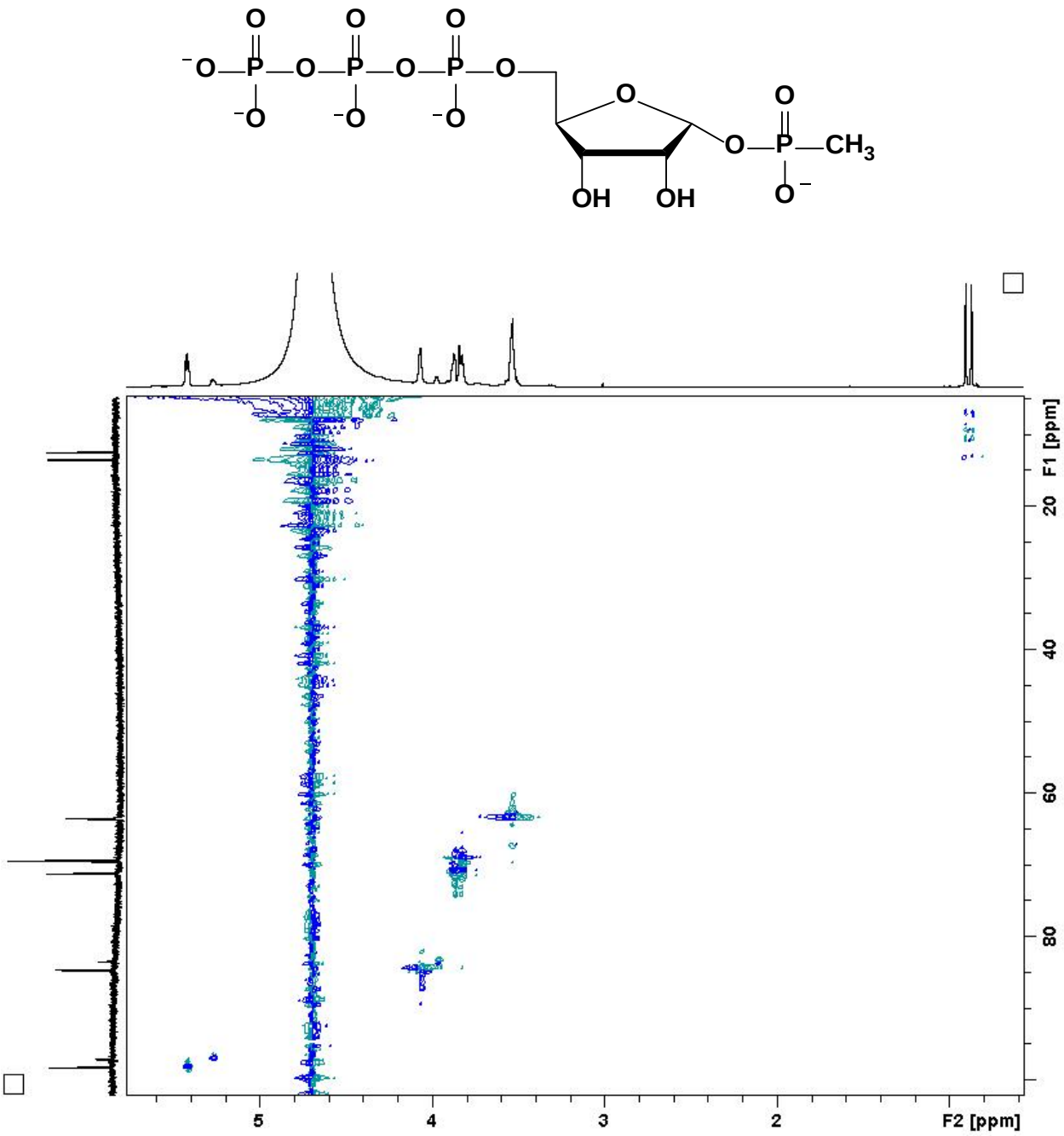


Figure S17: HSQC (^{1}H-^{13}C) NMR spectrum of RPnTP in 100 mM phosphate buffer at pH 8.5 on a Bruker Avance III 500 MHz NMR.

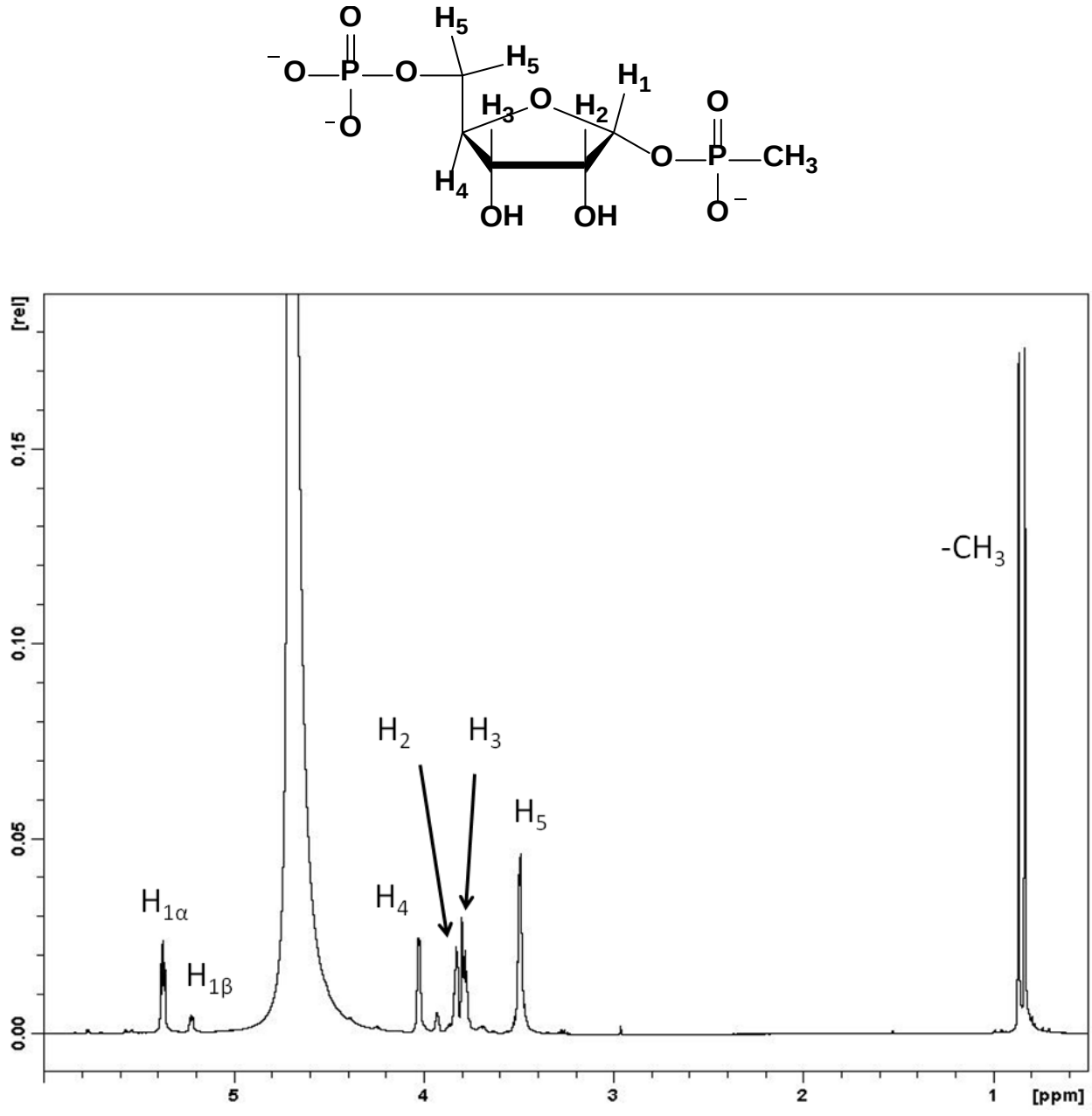


Figure S18: ^{1}H NMR spectrum of α-D-ribose-1-methylphosphonate-5-phosphate (RPnP) in 100 mM phosphate buffer pH 8.5 on a Bruker Avance III 500 MHz NMR.A small amount of the β-anomer is also formed in the reaction.

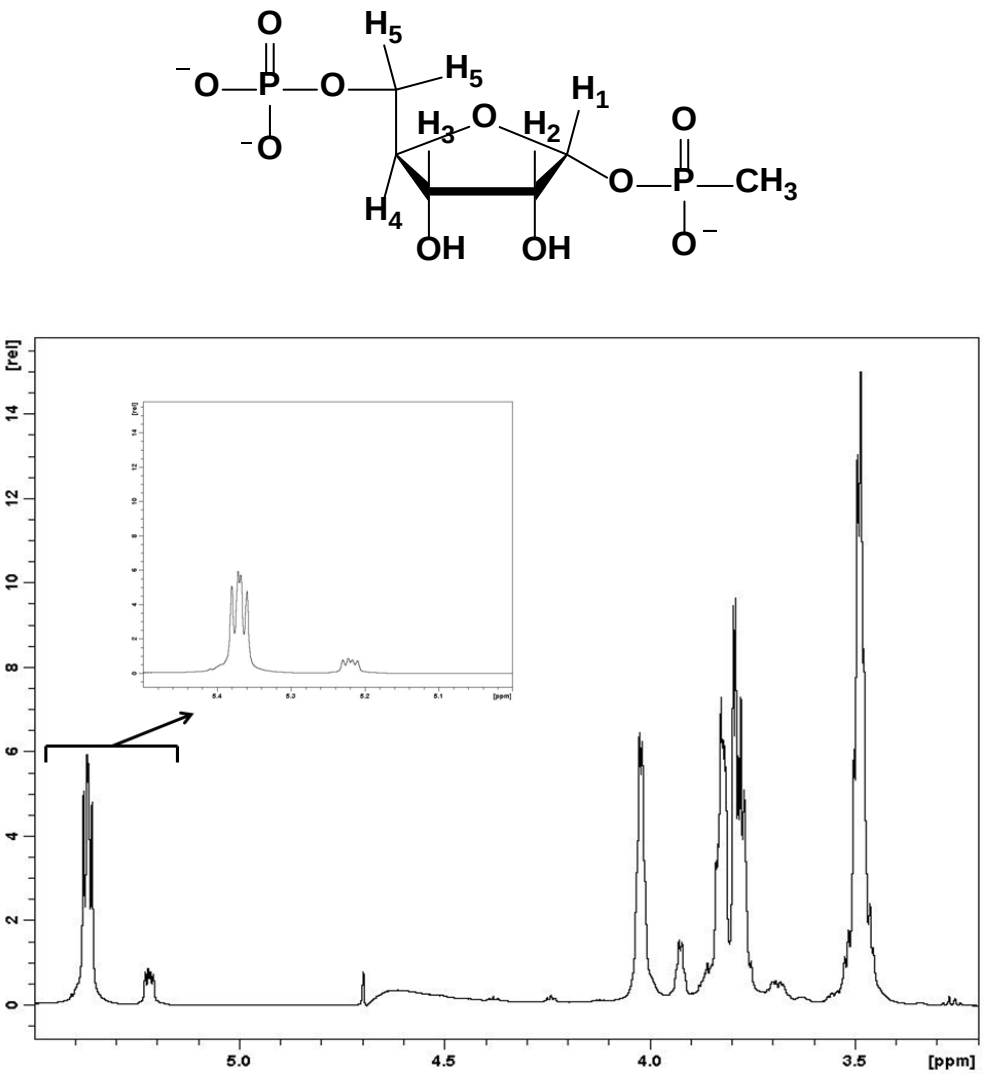


Figure S19: ^{1}H NMR spectrum of the ribose moiety of the α-D-ribose-1-methylphosphonate-5-phosphate (RPnP) in 100 mM phosphate buffer pH 8.5 using WATERGATE solvent suppression on a Bruker Avance III 500 MHz NMR. The inset shows the anomeric protons split as a quartet (doublet of doublets) resulting from the phosphorus of the methylphosphonate attached to C-1. The coupling constants for the major product are $H_{1\alpha}$ are $J_{(H1\text{-}H2)}$ = 4.2 Hz and $J_{(H1\text{-}P1)}$ = 6.3 Hz.

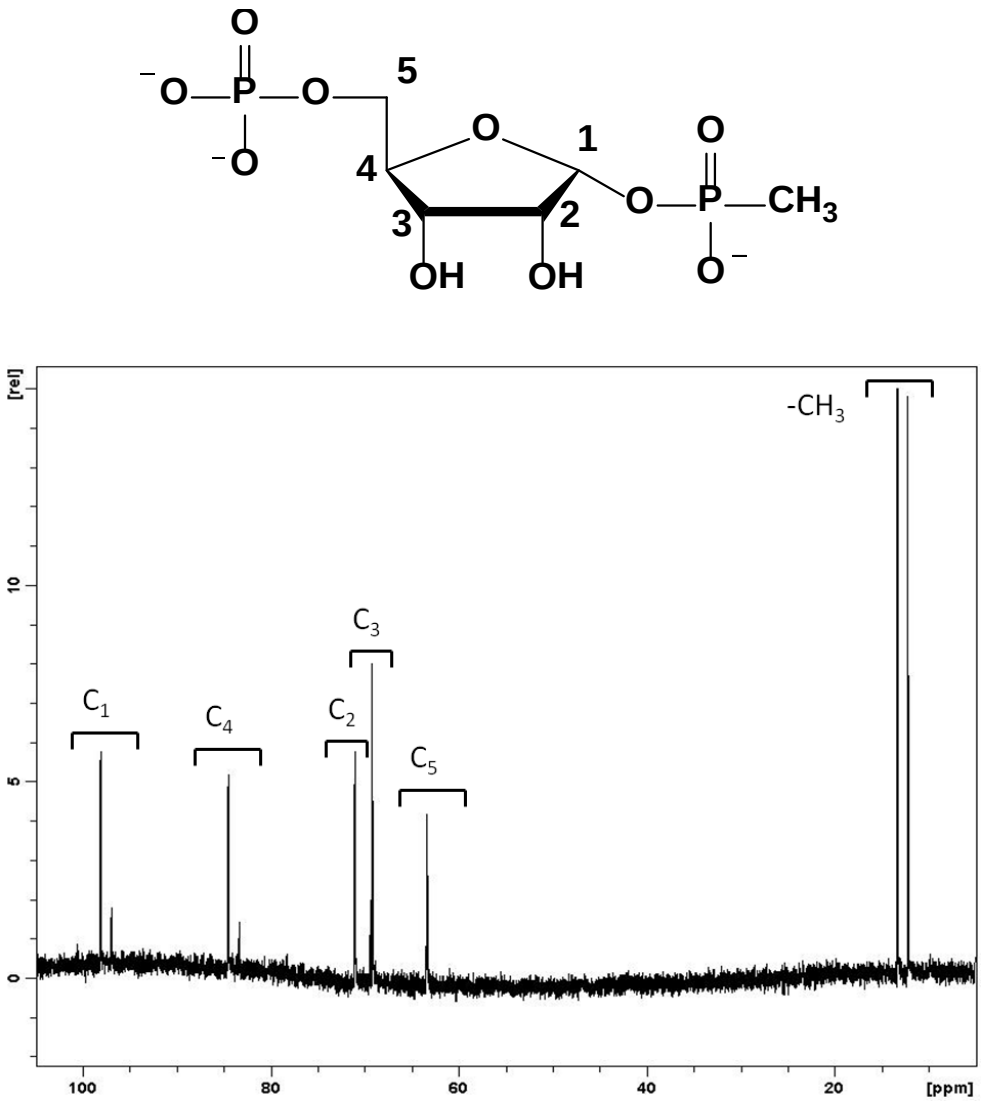


Figure S20: ^{13}C NMR spectrum of RPnP in 100 mM phosphate buffer pH 8.5 on a Bruker Avance III 500 MHz NMR. The carbon atoms of the RPnP are identified in the ^{13}C NMR spectrum. The two anomers are clearly seen in the ^{13}C NMR spectrum with the α-anomer being the major product of the reaction of PhnM with RPnTP in the presence of Zn^{2+}.

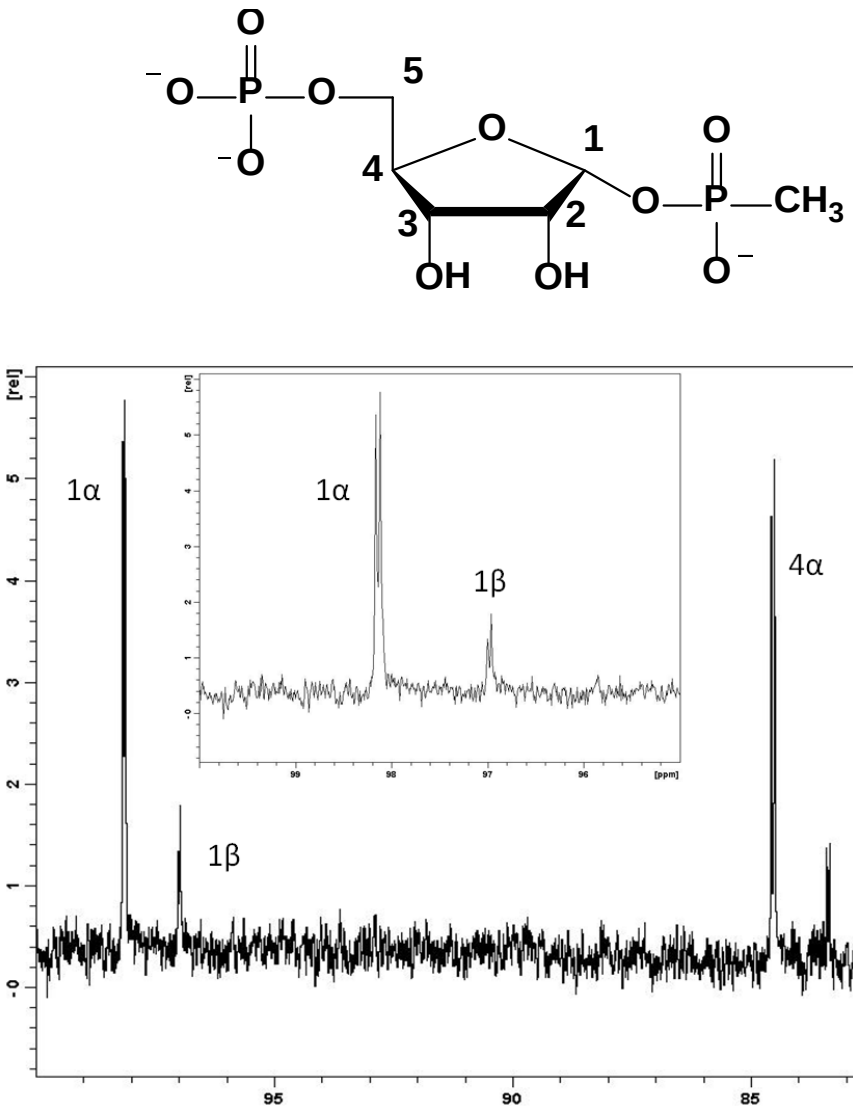


Figure S21: ^{13}C NMR spectrum of RPnP in 100 mM phosphate buffer at pH 8.5 on a Bruker Avance III 500 MHz NMR showing C-1 and C-4 of the ribose moiety. The inset shows the splitting of C-1 by the phosphorus of the phosphonate moiety.

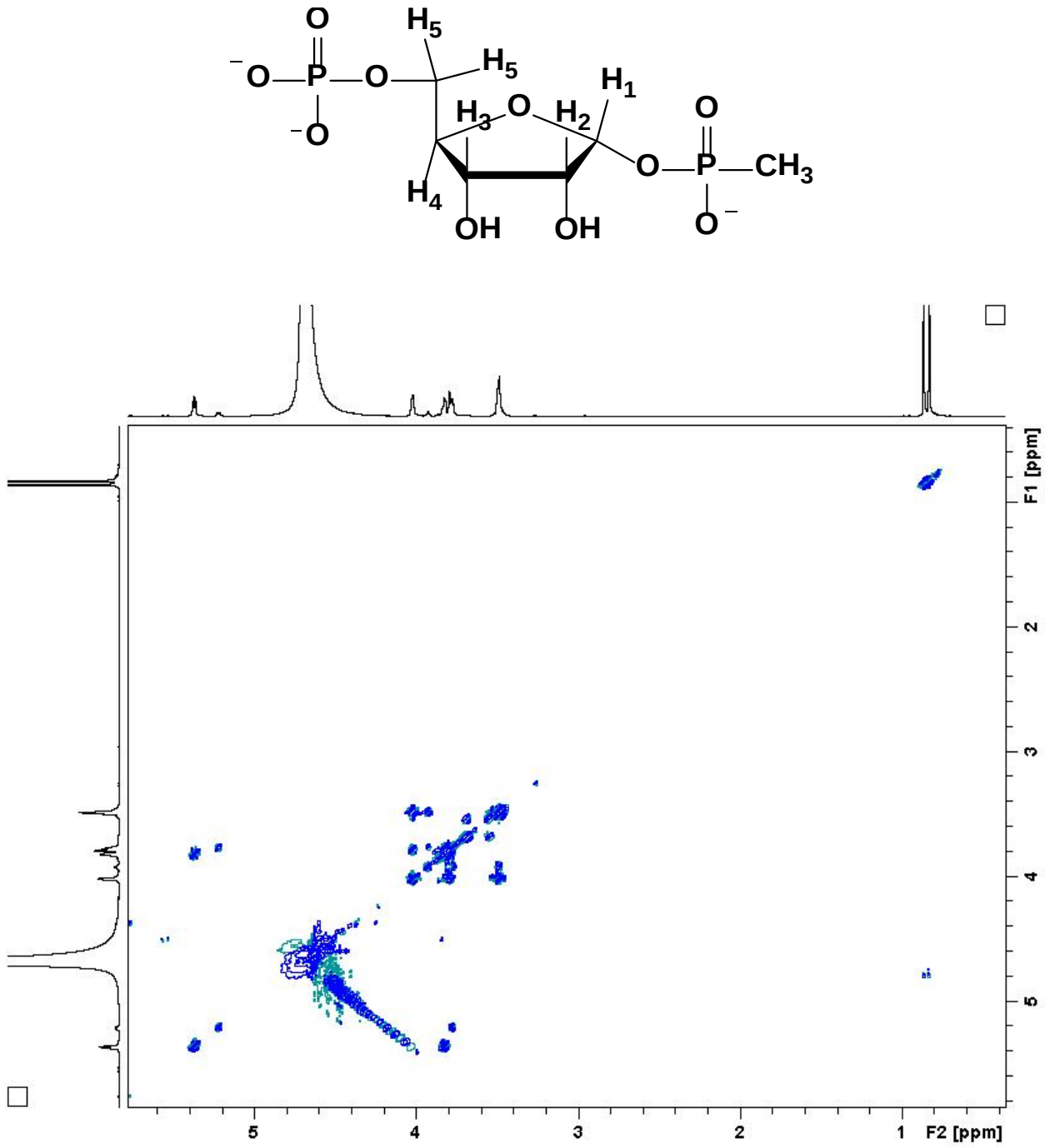


Figure S22: COSY (^{1}H-^{1}H) NMR spectrum of RPnP in 100 mM phosphate buffer at pH 8.5 on a Bruker Avance III 500 MHz NMR.

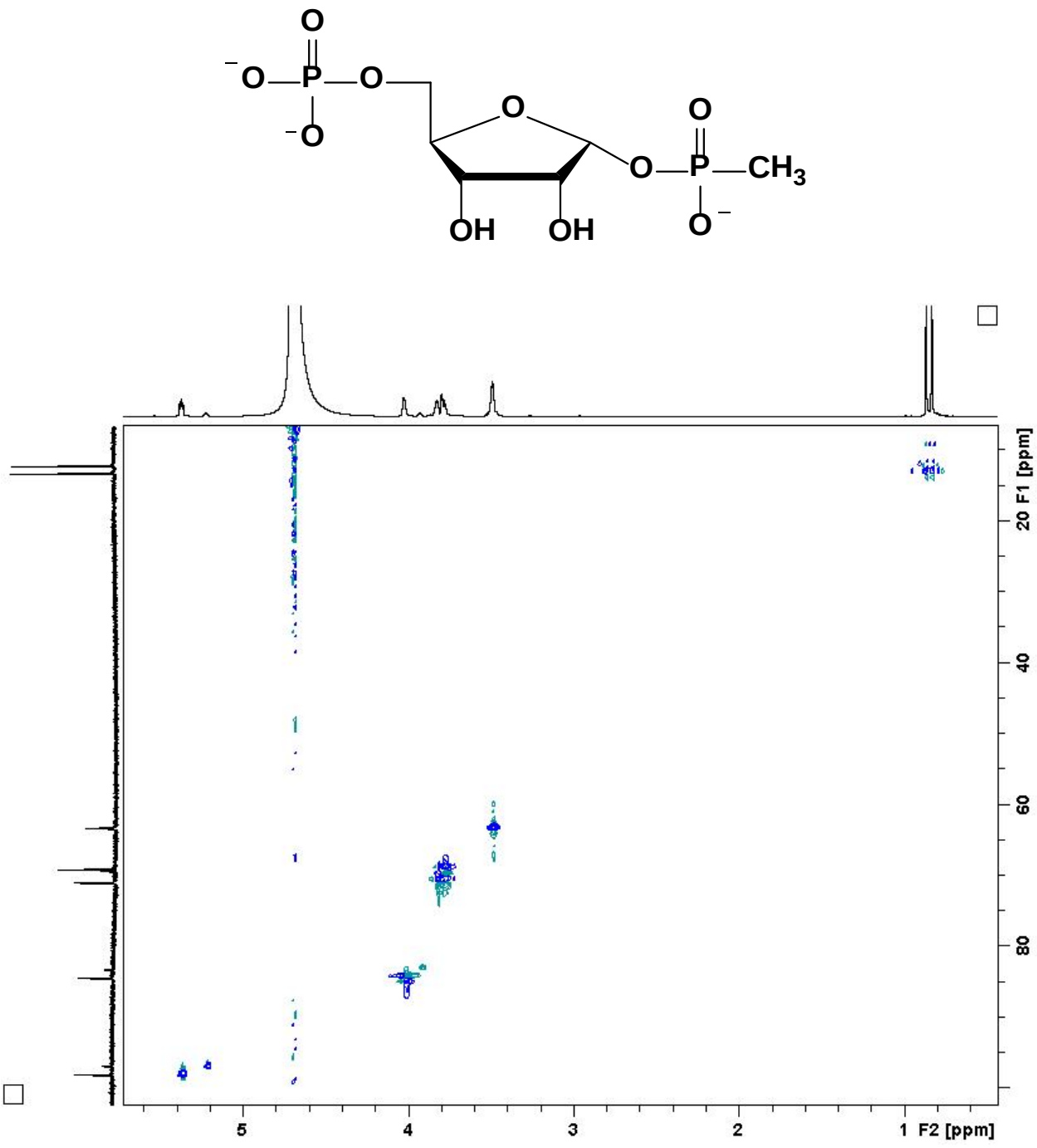


Figure S23: HSQC (^{1}H-^{13}C) NMR spectrum of RPnP in 100 mM phosphate buffer at pH 8.5 on a Bruker Avance III 500 MHz NMR.

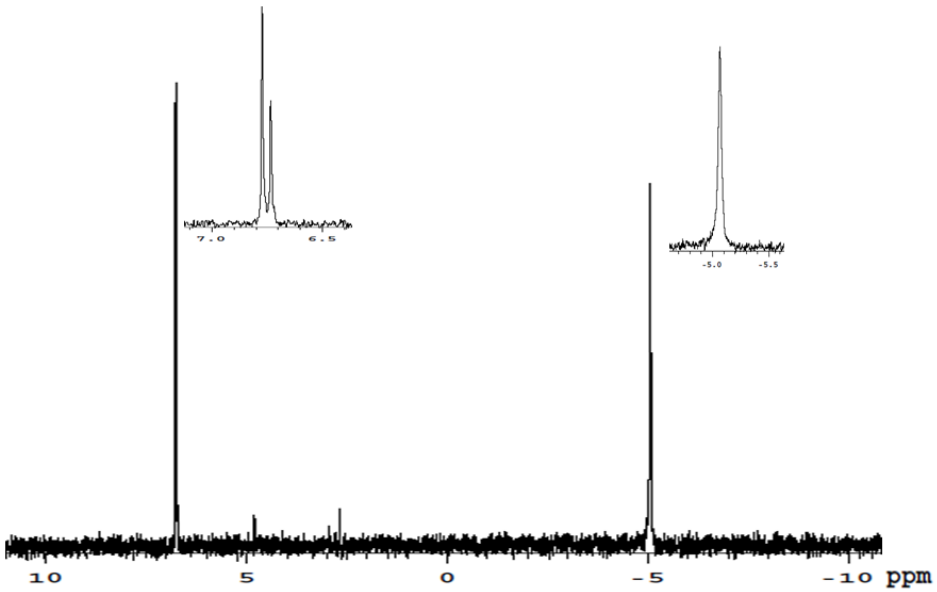


Figure S24: ^{31}P NMR spectrum of the reaction catalyzed by PhnM (10 µM) with 1 mM RTP in the presence of 2 mM Mg^{2+} and 1 mM Zn^{2+} in 50 mM HEPES buffer pH 8.5 containing 35 % H_2O^{18}. The phosphoryl group of ribose-5-phosphate is at ~ 6.8 ppm and the pyrophosphate is at -5.0 ppm. There are two resonances at ~6.8 ppm due to the partial O^{18}-labelling of the phosphate. There is no extra resonance for the pyrophosphate and thus the attack of water on the triphosphate moiety of RTP in on the α–phosphoryl group.

References:

1. Quesenberry, M. S. & Lee, Y. C. A rapid formaldehyde assay using purpald reagent: application under periodation conditions. *Anal. Biochem.* **234,** 50-55 (1995).
2. Avigad, G. A simple spectrophotometric determination of formaldehyde and other aldehydes: application to periodate-oxidized glycol systems. *Anal. Biochem.* **134,** 499-504 (1983).
3. Kamat, S. S. *et al.* Catalytic mechanism and three-dimensional structure of adenine deaminase. *Biochemistry.* **50,** 1917-1927 (2011).
4. Hall, R. S. *et al.* The hunt for 8-oxoguanine deaminase. *J. Am. Chem. Soc.* **132,** 1762-1763 (2010).