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Synthesis of RNA Nucleotides under Probably Prebiotic Conditions

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Abstract

RNA being composed of multiple covalently linked nucleotides is thought to have been a precursor to life circa 4.3-3.8 billion years ago. Non-enzymatically formed adenosine monophosphate (AMP), more specifically, is a vitally important subtopic of the self-assembly of the first RNA sequence. The goal of this study was to synthesize AMP non-enzymatically under benign conditions that are likely to have existed on early Earth. In this experiment, 3',5'-cAMP was successfully formed using wet-dry cycles at 80°C paired with the minerals zeolite beta, hydroxyapatite, and aerosil 300 in the presence of adenosine, urea, and pyrophosphate. A nuclear magnetic resonance spectrometer was used to characterize the products of each experiment to determine if any adenosine nucleotides were formed. Decreasing the pH and having a higher number of wet-dry cycles results in a higher amount of cAMP formation.

1.0 Introduction

Adenosine nucleotides are vital to all life. Adenosine triphosphate (ATP) especially, is produced in each cell and provides energy to the organism, and is also considered the "universal energy currency"[1]. Nucleotides are extremely important for life as they are the building blocks of nucleic acids like DNA and RNA, energy, and metabolic regulators. In current knowledge, it is not known how many nucleotides are synthesized enzymatically in the body. A series of enzymes are used to synthesize ATP in, more prevalently in eukaryotic organisms. However, there must have been a moment where ATP was initially synthesized non-enzymatically. There are many factors like pH, temperature, phosphate source, and minerals to consider when determining what conditions would be probable for prebiotic, non-enzymatic, phosphorylation of the adenosine nucleoside[1,2].

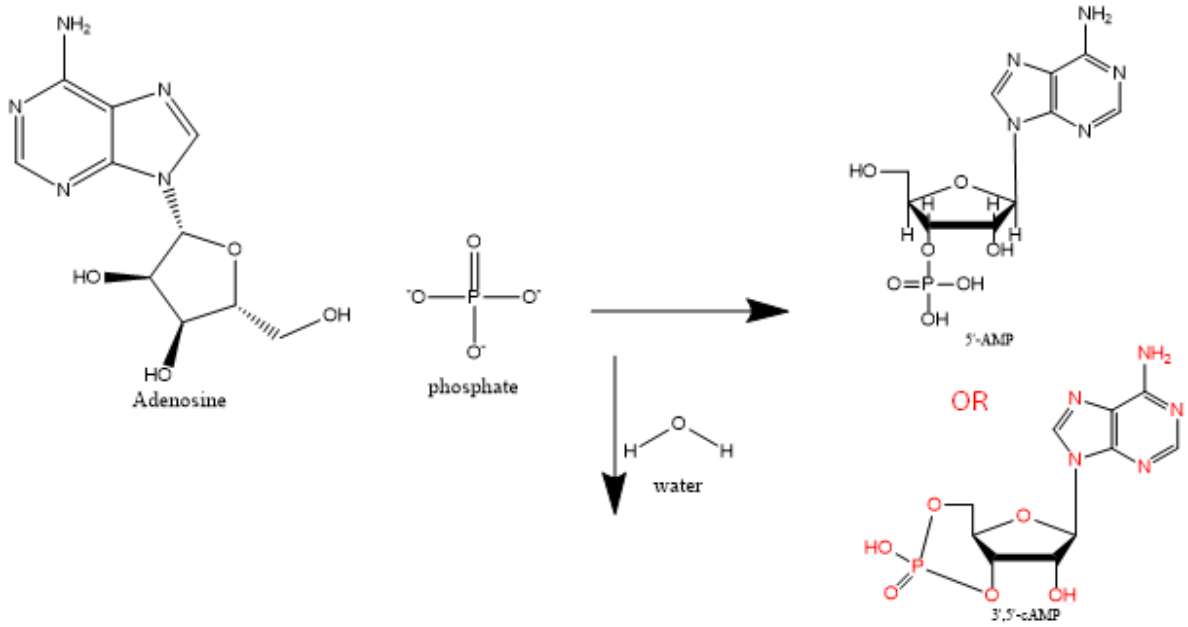

Image 1: The reaction of adenosine and a phosphate, yielding 5'-AMP or 3',5'-cAMP releasing a water molecule. The product depends on the reaction conditions and other molecules present.

The reaction of converting adenosine and an inorganic phosphate to 5'-AMP/3',5'-cyclic adenosine monophosphate (cAMP) is an energetically unfavorable reaction (image 1), meaning it has a positive Gibbs free energy value[3,5]. Knowing this, the reaction of synthesizing an adenosine nucleotide will require a relatively high temperature to succeed, which was done through the wet-dry cycles. This high temperature provided the reaction with sufficient activation energy to complete the reaction. In previous experiments, zeolite (modernite) was used to successfully form adenosine nucleotides (more specifically AMP, ADP, and ATP)[4]. The experiment used wet-dry cycles by adding water along with a 1.5mL potassium phosphate monobasic and ribose, drying the sample at 50°C. The pH of the mentioned sample was 2-3. In this experiment, adenosine with pyrophosphate and urea, multiple different minerals, wet-dry cycles, a variety of

salts, and changes in pH were used to try and replicate the initial synthesis of the first prebiotic adenosine nucleotide.

The most likely prebiotic solvent for these reactions is water, which is what was used in this experiment[2]. Wet-dry cycles were used and involve cycles of adding water, letting the sample totally dry by heating, and repeating. This proved to provide great success not only in this experiment, but in other prominent studies[2,6]. The source of phosphate used in this experiment is sodium pyrophosphate as the pyrophosphate ion as it is stable and it is a "plausible ancestor" of ATP[2]. This is great to measure and compare the chemical shift of different adenosine nucleotides. Finally, urea was used as a condensing/dehydrating agent. The urea absorbed the water produced by the condensation reaction of an inorganic phosphate molecule being attached to the adenosine nucleoside. This is important in this experiment because the phosphorylation of adenosine is thermodynamically unfavorable in an aqueous environment.

Cyclic AMP (cAMP) was successfully synthesized under multiple conditions, confirmed by ^{31}P nuclear magnetic resonance (NMR). ^{31}P NMR is a reliable and easy technique to measure if any adenosine nucleotide was formed. Each standard can be assigned a specific chemical shift in relation to 85% phosphoric acid, providing a reliable analysis for each sample to determine its products. Each sample contained adenosine, sodium pyrophosphate, polymerization salts, and water. Additionally, each sample was put through three wet-dry cycles. The minerals that resulted in cAMP formation were Zeolite beta, hydroxyapatite, and Aerosil 300. As Zeolite beta provided the most pronounced cAMP ^{31}P NMR peak, more experiments were conducted using this mineral (ie. pH change, addition of magnesium sulfate, and the removal of the polymerization salts). The lower pH and increased amount of wet-dry cycles proved to be of significant importance to the formation of cAMP, with possible AMP formation.

2.0 Materials and Methods

2.0.1 Materials:

Urea was obtained from Thermo Fisher Scientific (Germany). Sodium pyrophosphate ($Na_4P_2O_7$) was purchased from Alfa Aesar (Germany). ATP was obtained from Ambeed (Illinois, USA). ADP was bought from Research Products International (Illinois, USA). AMP was purchased from Carbosynth (United Kingdom). cAMP and deuterium oxide (D_2O) were purchased from Thermo Fisher Scientific (Switzerland). Magnesium chloride tetrahydrate ($MgCl_2*4H_2O$) and sodium bicarbonate ($NaHCO_3$) was purchased from VWR (PA, USA). Aerosil 300 silica ($300m^2/g$) was purchased from Evonik (NJ, USA). Adenosine was obtained from Sigma-Aldrich (MO, USA). Magnesium Sulfate hexahydrate ($MgSO_4*6H_2O$) was purchased from Sigma-Aldrich (India). Potassium sulfate (K_2SO_4) was purchased from Thermo Fisher Scientific (Lancashire, UK). 10x polymerization salts were made from 2M NaCl and 0.75M $MgCl_2$, dissolved in MilliQ water, yielding a final concentration of 0.2M NaCl and 0.075M $MgCl_2$. Aluminum oxide (Al_2O_3) was purchased from Sigma-Aldrich (Austria). Calcium silicate (Ca_2SiO_4), hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$). and zinc oxide (ZnO) were obtained from Sigma Aldrich (USA). Zeolite beta (CP814E: surface area: $680m^2/g$, nominal cation form: ammonium) was purchased from Zeolyst International (Kansas, USA). All water used in the experiment was MilliQ water, purified using a Millipore Milli-Q water system. All ^{31}P NMR graphs were plotted using ACD/NMR Processor Academic Edition.

2.1 *Experiment 1: Effects of Salts on Minerals During Drying Down*

This experiment is a replication of Gull et al. 2020, but instead using adenosine as opposed to uridine. The sample size was 6. In each sample, 100mg sodium pyrophosphate,

100mg urea, 500mg adenosine, 100mg of each sample's respective salt and 300mg of each sample's respective mineral were added to a glass tube. 7mL of water was added to each sample and the tubes were vortexed for 1 minute. The pH was measured using 50uL of sample and pH strips. The samples were heated at 80°C for 5 days to completely dry down the sample. After centrifuging at 13,500RPM for 10 minutes, 30mg of sample in 800µL of D_2O, the samples were analyzed using ^{31}P NMR.

Sample	Salt (100mg)	Mineral (300mg)	pH
1	-	-	10.0
2	Magnesium Chloride	-	7.4
3	Magnesium Chloride	Aerosil 300	6.5
4	Sodium Bicarbonate	-	9.0
5	Potassium Sulfate	-	10.0
6	Magnesium Chloride	SPV200 Volclay	8.7

Table 1: A table of the samples used in experiment 1 with their respective salt, mineral, and pH.

2.2 *Experiment 2: Effects of Minerals with 3 Wet-Dry Cycles*

Each sample contained 0.1 mmol(26.6mg) sodium pyrophosphate, 0.1 mmol(26.7mg) adenosine, 1 mmol(60mg) urea, 30µL 10x polymerization salts, 0.1g mineral, and 320µL of water were vortexed in a clear tube for 5 minutes where an either white or brownish gel was observed. The pH was measured using pH strips (table 1). The tubes were placed in an oven at 80°C for 24 hours which allowed for the sample to dry. Twenty-four hours later, 200µL-400µL of water was added to each tube (enough water to dissolve the solid and reform a gel). Each tube was vortexed for around a minute to dissolve all solids and placed back into the oven at 80°C for another 24 hours. This wet-dry cycle was repeated for a total of three cycles (two more times). Fifty milligrams of the final dry solid was dissolved in 800µL D_2O, vortexed for 15 minutes,

centrifuged at 13,500RPM for 10 minutes, and the supernatant was retrieved. The solution was added to an NMR tube (with the internal standard) for analysis.

Sample	Mineral	pH
11	Aerosil 300	8.7
12	Al_2O_3	9.5
13	Calcium silicate	10.0
14	Hydroxyapatite in 10% water	8.7
15	ZnO	8.1
16	Zeolite beta	7.1

Table 2: Along with the reactants above, 0.1g of each mineral was added to each respective sample. The pH's were recorded right before heating.

The pH of each mineral was taken in pure water using pH strips. Twenty to thirty milligrams of each mineral was dissolved in 1mL of water and stirred for 24 hours. The hydroxyapatite was already suspended in water as provided by the manufacturer.

2.3 *Experiment 3: Effects of Wet-Dry Cycles and pH in Presence of Zeolite Beta*

The same amounts of reactants and the same preparation procedure was used as in experiment 3. Sample 16.4 was pH adjusted to 3.5 using 0.5M HCl and sample 16.5 was pH adjusted to 9.0 using 0.5M sodium bicarbonate (table 3). The pH was measured using pH strips (table 3). The wet-dry cycle was repeated for a total of three cycles (samples 16A, 16.1-16.5), two cycles (sample 16B), one cycle (sample 16C), and zero cycles (sample 16D). The samples were again analyzed using ^{31}P NMR, as done in experiment 2.

Sample	Modifications	pH	number of wet-dry cycles
16A	Same as 16	8.1	3

16B	Same as 16, 2 cycles	8.1	2
16C	Same as 16, 1 cycle	8.1	1
16D	Same as 16, 0 cycles	8.1	0
16.1	No zeolite beta	10.0	3
16.2	No polymerization salts	8.7	3
16.3	No polymerization salts, with $MgSO_4*6H_2O$	6.8	3
16.4	Same as 16, pH adjusted to 3.5	3.5	3
16.5	Same as 16, pH adjusted to 9.0	9.0	3

Table 3: Sample 16 was repeated, however with multiple modifications to test the importance of wet-dry cycles, polymerization salts, and pH.

2.4 *Experiment 4: Acid-Base Properties*

To measure the pH of each mineral used, 20mg of each mineral was dissolved in 1mL of water. These samples were allowed to mix for 24 hours and then the pH was measured using pH strips.

2.5 *^{31}P NMR Standards*

To create consistent standards, 0.1mmol of ATP, ADP, AMP, cAMP, and sodium pyrophosphate (pH=3.0, 5.0, 6.5, 6.0, and 10 respectively) were dissolved in 1mL of D_2O. The samples were vortexed and transferred to a 5 cm tall NMR tube. Every sample was run on a Varian 500MHz nuclear magnetic resonance spectrometer with 64 scans, a relaxation delay of 5 seconds, pulse angle of 90 degrees, and an acquisition time of 0.655 seconds. These standards,

and every sample run throughout the experiment contained an internal standard of 85% phosphoric acid (ppm≈0) in a sealed capillary tube.

3.0 Results

3.2.1 Effects of Salts on Minerals During Drying Down

The first experiment provided no significant results. There was no formation of any adenosine nucleotide. This was further investigated in experiment 2 where wet-dry cycles were used instead of drying down each sample. The lack of formation of the adenosine nucleotides in the first experiment suggests that the reaction requires wet-dry cycles. The absence of signals in this experiment is likely due to the different nucleoside (adenosine) and lack of magnesium sulfate used in this experiment compared to the nucleoside (uridine) used in the experiment conducted by Gull et al. 2020.

3.2.2 Effects of Minerals with 3 Wet-Dry Cycles

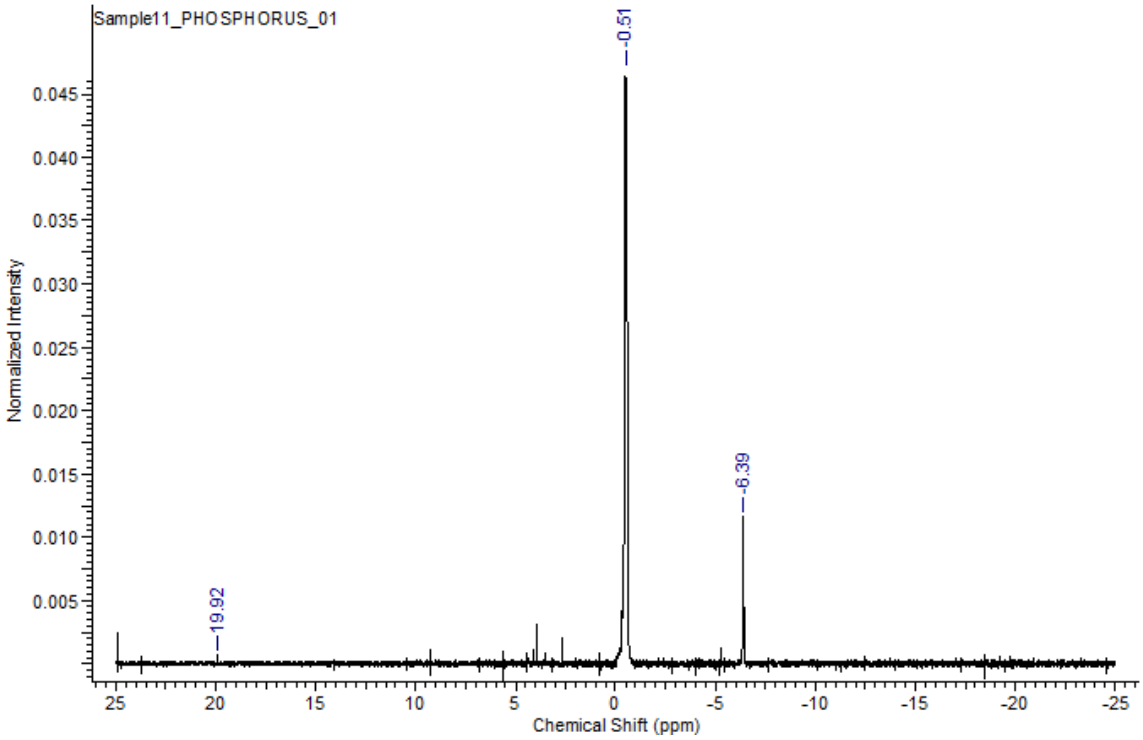

Figure 1: ^{31}P NMR spectrum of sample 11: aerosil 300. This sample resulted in cAMP formation at 19.92ppm (see figure D), but a very low yield hence the small peak. Experimental conditions as in table 2. Note: using an 85% H_3PO_4 internal standard at ~-0.5–0.8ppm.

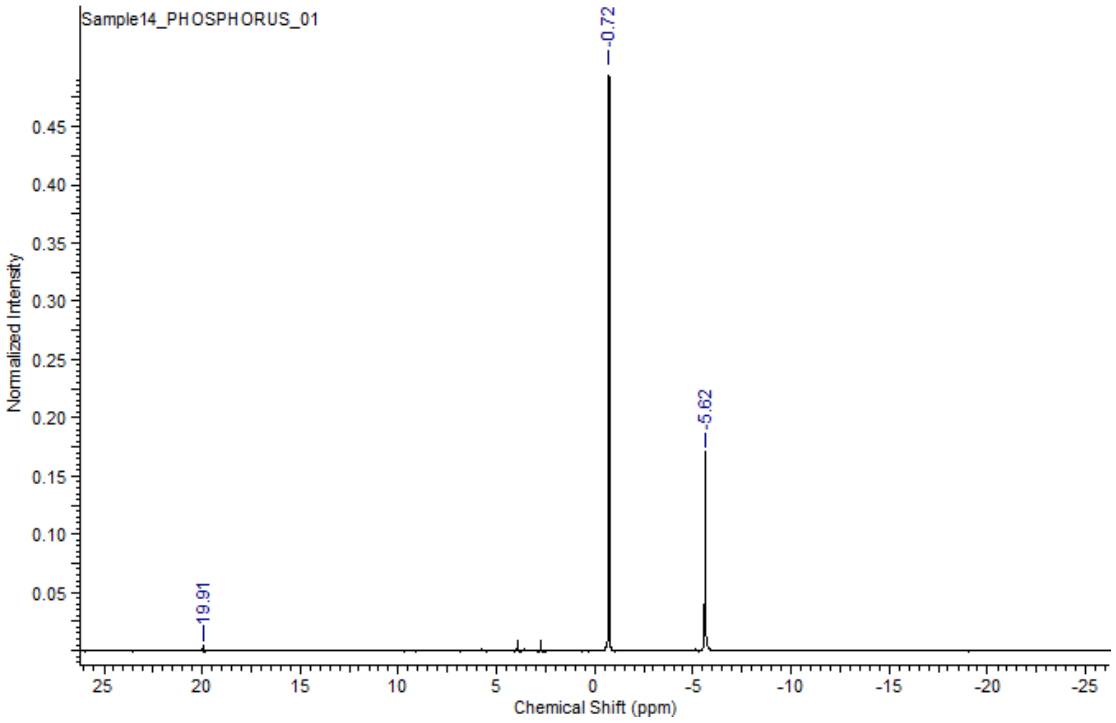

Figure 2: ^{31}P NMR spectrum of sample 14: hydroxyapatite. This sample resulted in cAMP formation at 19.91ppm (see figure D), but a very low yield (hence the small peak). Experimental conditions are located in table 2. Note: using an 85% H_3PO_4 internal standard at ~-0.5–0.8ppm.

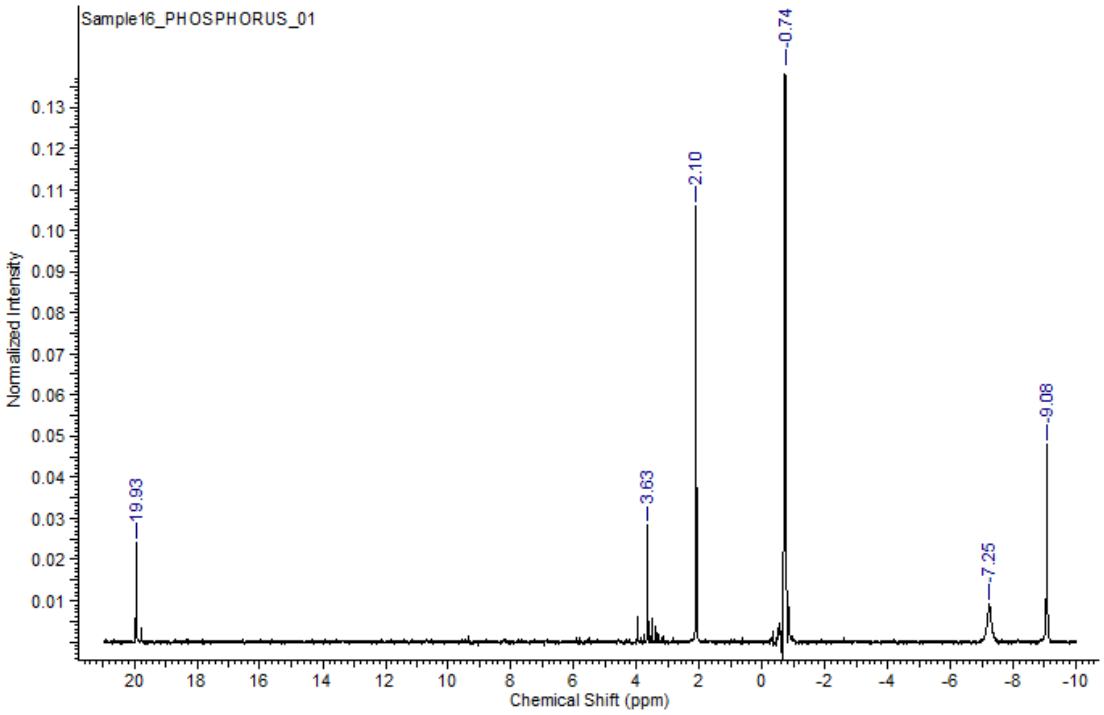

Figure 3: ^{31}P NMR spectrum of sample 16: zeolite beta. This sample resulted in cAMP formation at 19.93ppm (see figure D). Experimental conditions are located in table 1. Note: using an 85% H_3PO_4 internal standard at ~-0.5–0.8ppm.

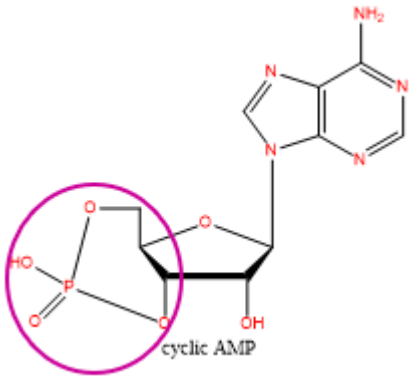

Image 2: Structure of 3'-5' cyclic adenosine monophosphate, the molecule that was produced in both experiments. The phosphate group that was observed in the ^{31}P NMR is circled in purple.

The main purpose of experiment 2 was to determine which mineral would provide the best results using wet-dry cycles. Aerosil 300, hydroxyapatite, and zeolite beta all resulted in cAMP formation, however the peak in figure 3 (zeolite beta), resulted in the largest peak at 19.93ppm. Each of these three minerals are negatively charged, as compared to the more positively charged

ZnO and Al_2O_3. The formation of cAMP can be confirmed as seen in figure D by using a standard of cAMP dissolved in D_2O. The mineral was determined to provide the best results in experiment 2. This experiment also confirmed that the conditions used were adequate for the formation of an adenosine nucleotide. Zeolite beta was further analyzed in experiment 3, testing how the number of wet-dry cycles and pH affect the formation of cAMP.

3.2.3 Effects of Wet-Dry Cycles and pH in Presence of Zeolite Beta

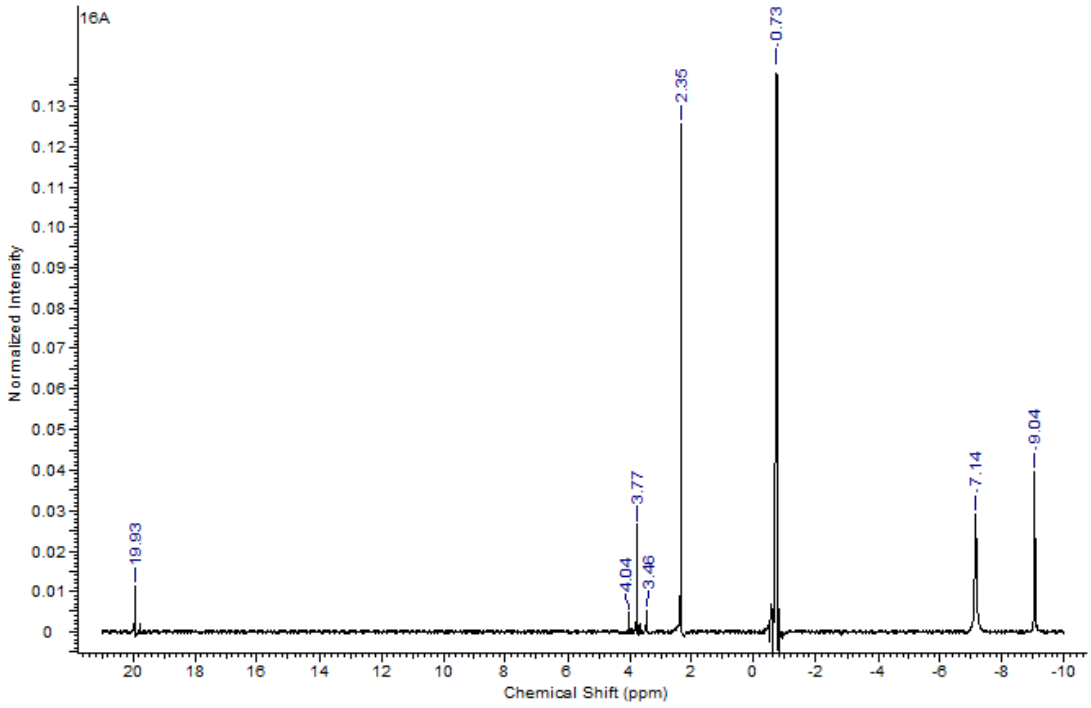

Figure 4: ^{31}P NMR spectrum of sample 16A. This sample resulted in cAMP formation at 19.93ppm. Experimental conditions are located in table 3, this sample went through three wet-dry cycles. Note: using an 85% H_3PO_4 internal standard at ~-0.5–0.8ppm.

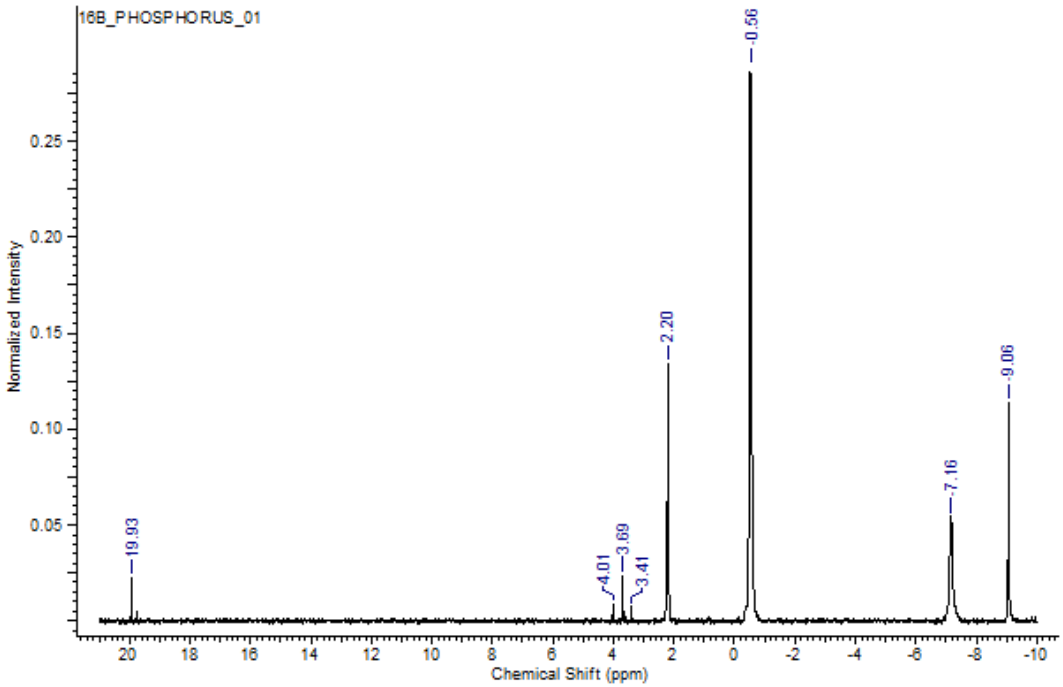

Figure 5: ^{31}P NMR spectrum of sample 16B. This sample resulted in cAMP formation at 19.93ppm. Experimental conditions are located in table 3, this sample went through two wet-dry cycles. Note: using an 85% H_3PO_4 internal standard at ~-0.5–0.8ppm.

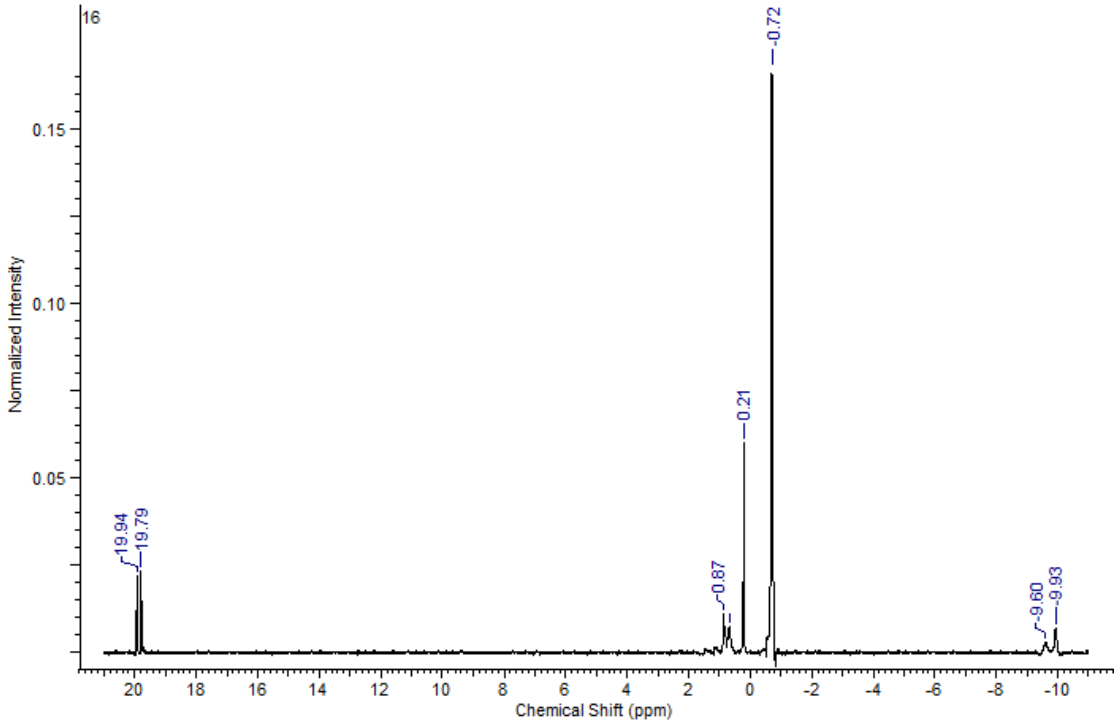

Figure 6: ^{31}P NMR spectrum of sample 16.4. This sample resulted in cAMP and possible AMP formation at 19.93ppm and 0.87ppm. Experimental conditions are located in table 3, this sample

went through three wet-dry cycles. Note: using an 85% H_3PO_4 internal standard at ~-0.5–0.8ppm.

Experiment 2 confirmed that the wet-dry cycles are vital in forming cAMP for these conditions. As seen in figures 4-6, cAMP formed for the low pH conditions, and the higher amount of wet-dry cycles. The adenosine, along with the P_i, have more H^+ ions in solution in acidic conditions allowing the covalent bond between the nucleoside and P_i to form easier. Prebiotic seas were thought to have been slightly acidic due to the increased amount of CO_2 in the atmosphere, further supporting this result[9]. This is the explanation as to why the formation of cAMP seems to be more favorable in acidic environments[10]. In figure 6, a chemical shift at 0.87ppm is present. Comparing it to figure C (supplementary information) where 5'-AMP has a peak at 0.68ppm, this peak may be from adenosine monophosphate[13].

3.2.4 Acid-Base Properties

Mineral	pH
Aerosil 300	4.66
Aluminum oxide	8.62
Calcium silicate	10.75
Hydroxyapatite in 10% water	4.0
Zinc oxide	7.83
Zeolite beta	5.59

Table 4: The pH of each mineral used dissolved in water. Highlighted in blue are the more basic minerals being aluminum oxide, calcium silicate, and zinc oxide. Highlighted in red are the more acidic minerals being aerosil 300, hydroxyapatite, and zeolite beta.

The results of this experiment consist of the minerals used in every other experiment. Three of the minerals are basic in water (aluminum oxide, calcium silicate, and zinc oxide) and three of the minerals are acidic in water (aerosil 300, hydroxyapatite, and zeolite beta). As seen in figures

1-3, cAMP was formed in each of the acidic minerals. However, there was no adenosine nucleotide formation using any of the basic minerals. This acidic mineral can help promote the protonation of the pyrophosphate, thus providing more favorable conditions for a condensation reaction. More data that supports this statement can be found in figure 6. Higher amounts of cAMP formation and possible AMP formation resulted under more acidic conditions.

4.0 Conclusion

Zeolites, hydroxyapatite, and silicates (ie. calcium silicate and Aerosil 300) were important components of the prebiotic planet[11]. Zeolites in particular were present as they have strong active sites and cage-like arrangements that make them likely to host prebiotic synthesis[4]. As previously stated, ancient seas were thought to have been acidic due to the high levels of CO_2 dissolved in the water. The experiments that have been conducted as the lower pH sample using zeolite beta provided the best results, thus supporting the hypothesis. It resulted in the largest formation of cAMP, compared to other minerals such as aerosil 300 and hydroxyapatite. This is due to the larger surface area of the zeolite compounds, as cAMP has a chemical shift at 19.93ppm, as confirmed by the standard run using ^{31}P NMR. The decrease of wet-dry cycles performed in experiment 2 showed the importance of the cycles, resulting in no cAMP formation using 0 cycles. Importantly, the decrease in pH resulted in cAMP and possible AMP formation. The cAMP that was formed in this sample had the largest concentration of any sample. This proves that the lower pH provides more favorable conditions for adenosine nucleotide formation as opposed to the higher pH sample that no significant results were obtained from. Also, the inclusion of the polymerization salts and acidic minerals in experiment 3, along with the 2-3 wet-dry cycles and temperature of 80°C were all vital in cAMP formation as seen in figures K-O where no cAMP was formed. The basic minerals, 0-1 wet-dry cycles, and lack of polymerization

salts showed the necessity of each of these when no cAMP was formed. These experiments confirm that adenosine, a source of phosphate (pyrophosphate in this case), urea, the polymerization salts, and acidic minerals like zeolite beta, hydroxyapatite, and aerosil 300 were all needed reactants to produce cAMP.

In the future, I would attempt to form ADP or ATP using the same reaction, but with many more wet-dry cycles. The pH being lowered more could assist in confirmed AMP formation. Also, more experiments would be conducted using hydroxyapatite as this was another likely source of phosphate that may have been present in prebiotic ATP formation[9]. The role of minerals in non-enzymatic polymerization of RNA have been further enhanced by salts like $MgCl_2$ has been shown in past studies[12]. Formation of 3',5'-cyclic guanosine monophosphate in Morasch et al. 2014 resulted in long strands of RNA, meaning that the formation of cAMP in our experiments may also be a step that led to RNA non enzymatic, prebiotic, synthesis[10]. The previous studies add to this body of work, emphasizing the importance of minerals in prebiotic nucleotide and RNA oligomerization.

5.0 References

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6.0 Appendix:

Supplementary Information:

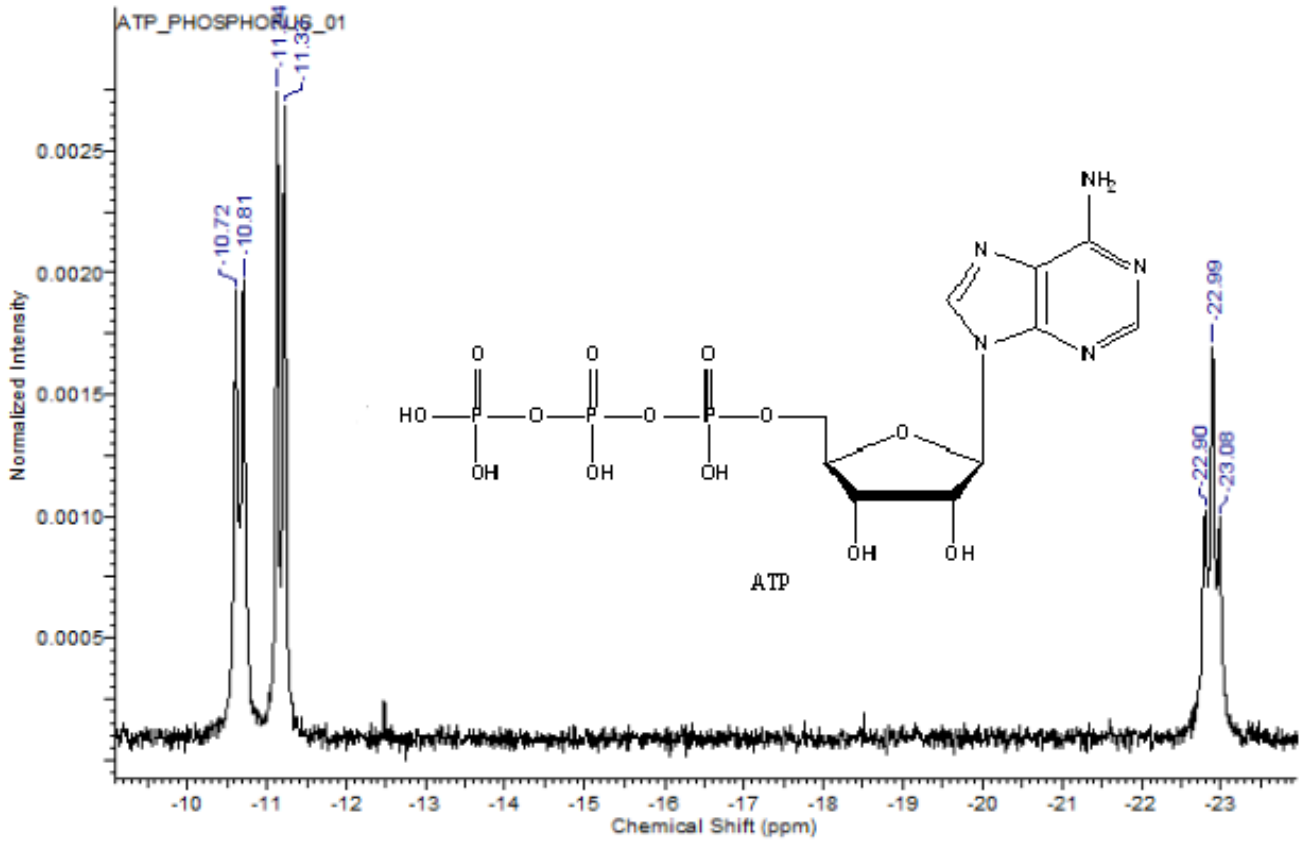

Figure A: ^{31}P NMR spectrum of the 5'-ATP standard, along with the structure of 5'-ATP. These chemical shifts will be used as comparison in the experiment to determine if any ATP is synthesized. Note: using an 85% H_3PO_4 internal standard at ~-0.5–0.8ppm.

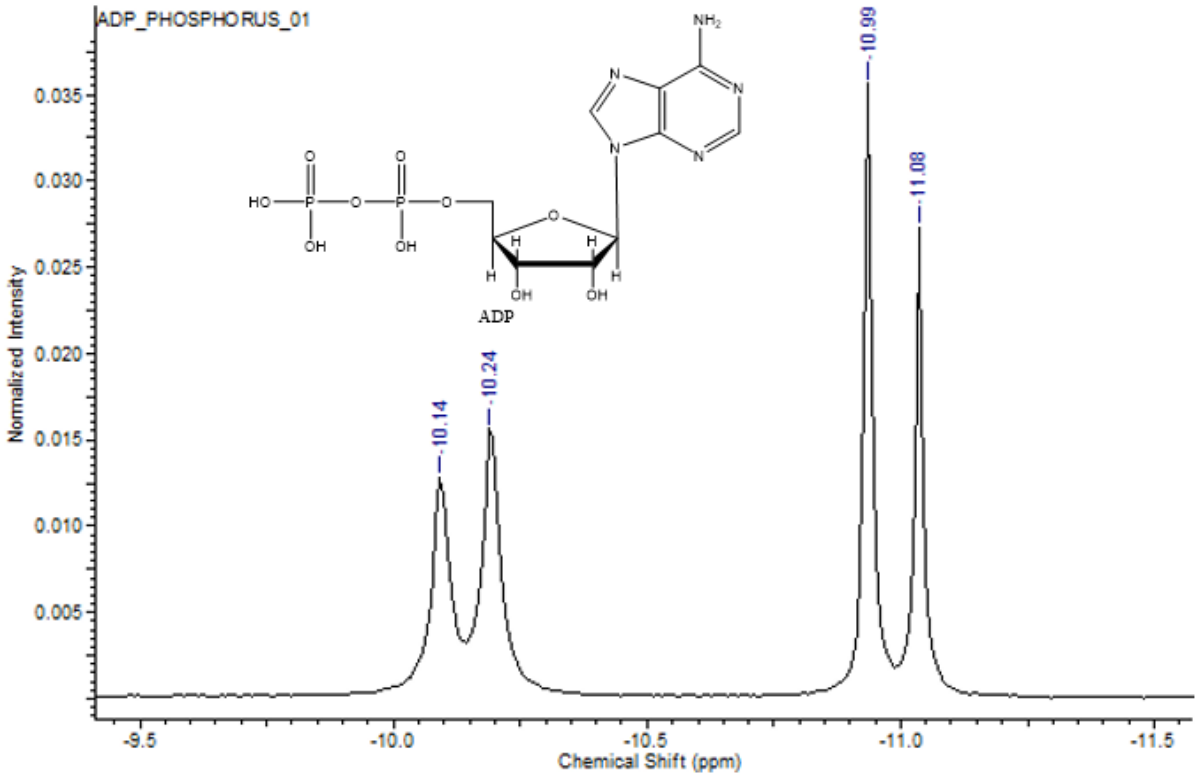

Figure B: ^{31}P NMR spectrum of the 5'-ADP standard, along with the structure of 5'-ADP. These chemical shifts will be used as comparison in the experiment to determine if any ATP is synthesized. Note: using an 85% H_3PO_4 internal standard at ~-0.5–0.8ppm.

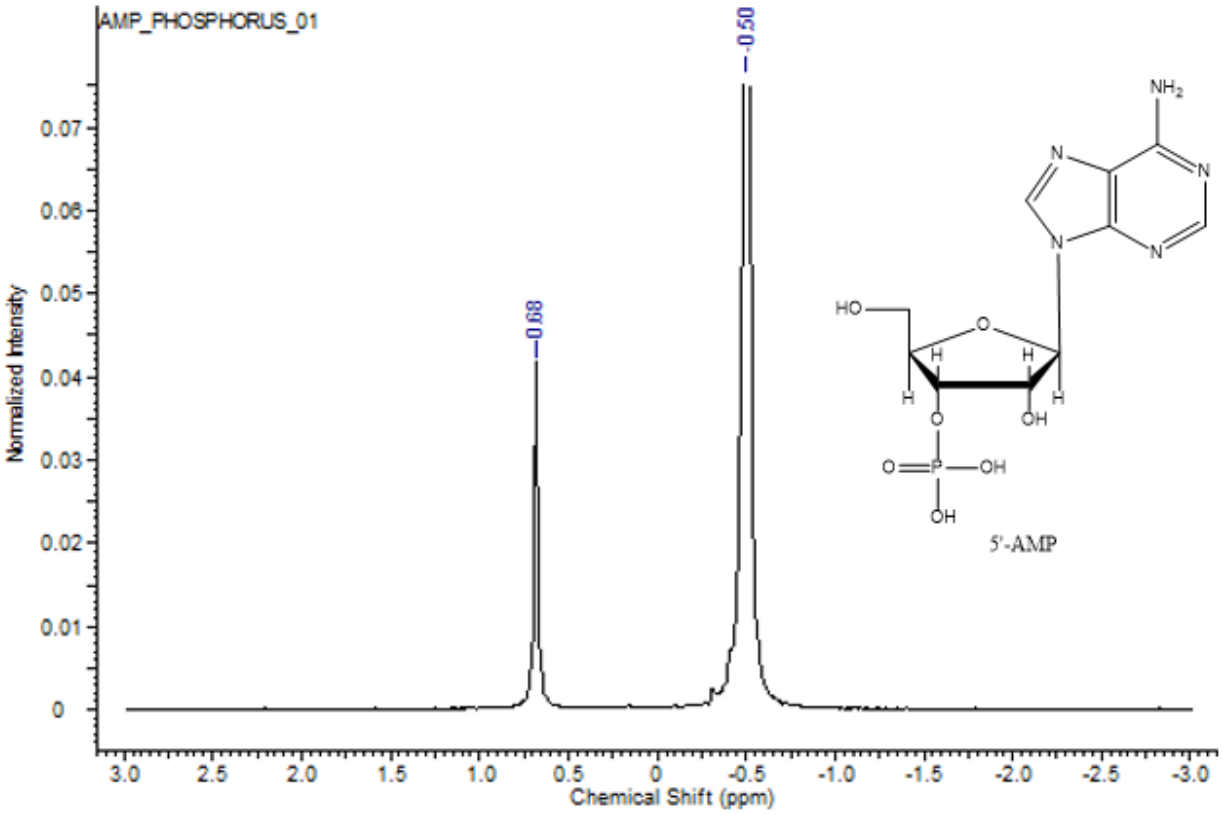

Figure C: ^{31}P NMR spectrum of the 5'-AMP standard, along with the structure of 5'-AMP. These chemical shifts will be used as comparison in the experiment to determine if any ATP is synthesized. Note: using an 85% H_3PO_4 internal standard at ~-0.5–0.8ppm.

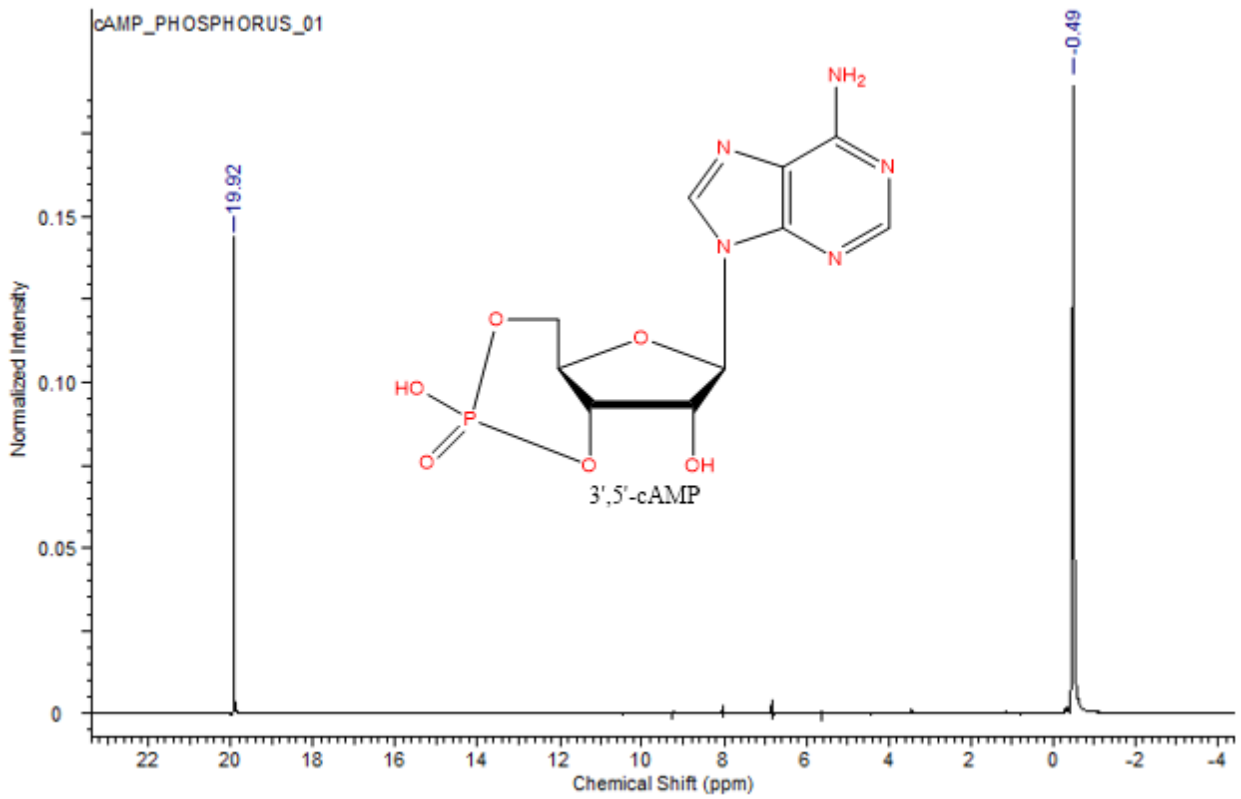

Figure D: ^{31}P NMR spectrum of the cAMP standard at 19.92ppm. These chemical shifts will be used as comparison in the experiment to determine if any cAMP is synthesized. Note: using an 85% H_3PO_4 internal standard at ~-0.5–0.8ppm.

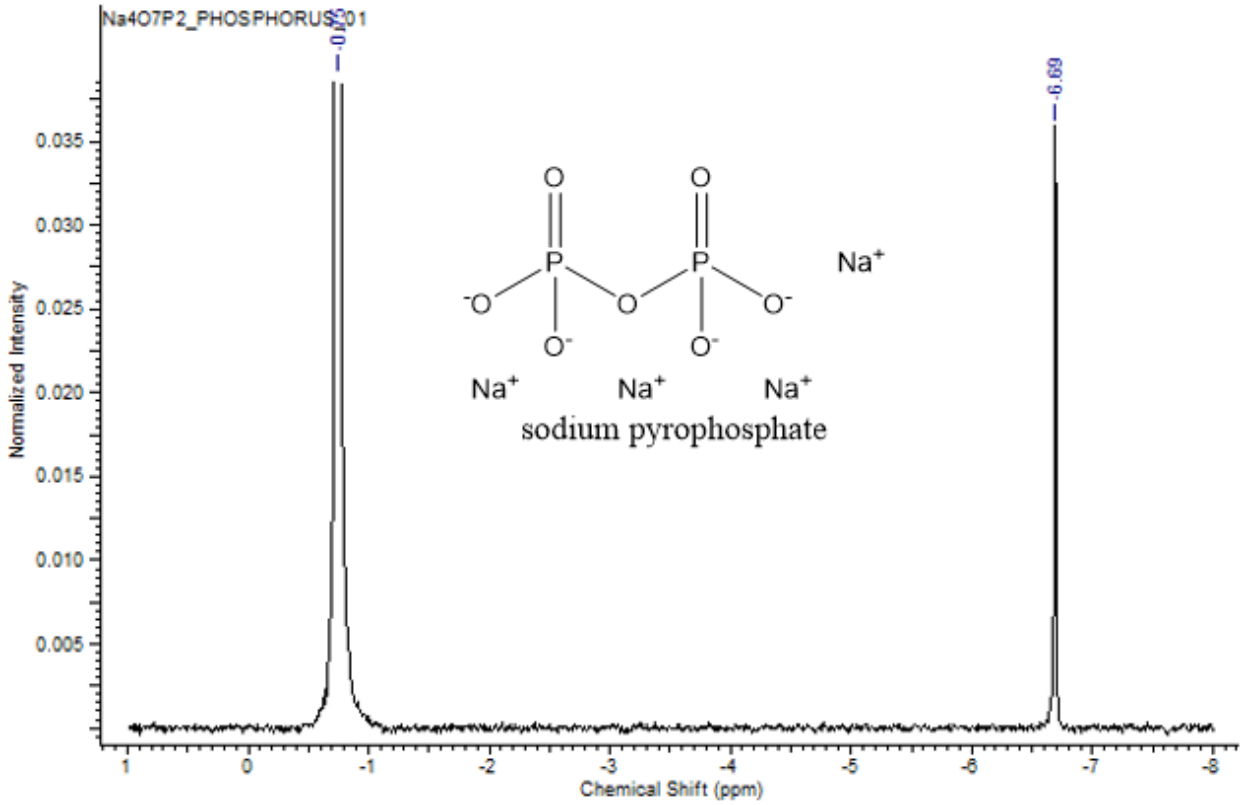

Figure E: ^{31}P NMR spectrum of the [sodium] pyrophosphate standard at -6.69ppm, along with the structure of sodium pyrophosphate. These chemical shifts will be used as comparison in the experiment to determine if there is any pyrophosphate remaining (unreacted). Note: using an 85% H_3PO_4 internal standard at ~-0.5–0.8ppm.

Sample	PPM obtained in this study	Reference	PPM reported by reference	Referenced Standard
ATP*	-11.24, -11.33, -22.90, -23.08, -22.99, -10.72, -10.81	Walker et al. (2019)	-10.1, -18.57, -4.9	85% H_3PO_4
ADP*	-10.99, -11.08, -10.14, -10.24	Walker et al. (2019)	-9.5, -5.6	85% H_3PO_4
AMP*	0.68	Lian et al. (2016)	0.9	Na_2HPO_4
cAMP*	19.92	Gull et al. (2020)	19-20	85% H_3PO_4(external standard)/spiked using 5'-UMP
$Na_4P_2O_7$*	-6.69	Gull et al. (2020)	-8	85% H_3PO_4(external standard)/spiked using 5'-UMP

Table A: Summary of ^{31}P NMR chemical shifts of standards, along with reported values from other experiments. *blue=alpha phosphate, red=beta phosphate, and green=gamma phosphate

Sample	Mineral (100mg)	Important Modifications	Nucleotide Synthesis?	pH	wet/dry cycles
11	Aerosil 300	Type of mineral	Yes, cAMP	8.7	3
12	Al_2O_3	Type of mineral	No	9.5	3
13	Calcium silicate	Type of mineral	No	10.0	3
14	Hydroxyapatie in 10% water	Type of mineral	Yes, cAMP	8.7	3
15	ZnO	Type of mineral	No	8.1	3
16	Zeolite Beta	Type of	Yes, cAMP	8.1	3

		mineral			
16A	Zeolite Beta	Number of wet/dry cycles	Yes, cAMP	8.1	3
16B	Zeolite Beta	Number of wet/dry cycles	Yes, cAMP	8.1	2
16C	Zeolite Beta	Number of wet/dry cycles	No	8.1	1
16D	Zeolite Beta	Number of wet/dry cycles	No	8.1	0
16.1	No mineral	No mineral	No	10.0	3
16.2	Zeolite Beta	No polymerization salts	No	8.7	3
16.3	Zeolite Beta	No polymerization salts, with $MgSO_4*6H_2O$	No	6.8	3
16.4	Zeolite Beta	pH adjusted to 3.5	Yes, cAMP & possible AMP	3.5	3
16.5	Zeolite Beta	pH adjusted to 9.0	No	9.0	3

Table B: A table of each experiment with important information about each sample. Each sample (respectively) contained 0.1mmol(26.6mg) sodium pyrophosphate, 0.1mmol(26.7mg) adenosine, and 1mmol(60mg) urea. All samples that underwent a wet/dry cycle were heated at 80°C for 24 hours. Then 200µL of water was added to each sample, vortexed for ~10 minutes, and placed back in the oven. Note: blue backgrounds are samples of the first experiment, while yellow backgrounds are samples of the second experiment.

Experiment	$Na_4P_2O_7$	Adenosine	Urea	$NaHCO_3$	$MgCl_2$	K_2SO_4	Mineral	pH	T	Vol (H_2O)
1	*	*	*					10	80 °C	7 mL
2	*	*	*		*			7.4		
3	*	*	*		*		Aerosil 300	6.5-7		
4	*	*	*	*				9		
5	*	*	*			*		10		
6	*	*	*		*		Volclay	8.7		

Table C: An in-depth of the experimental conditions of experiment 1.

Sample	$Na_4P_2O_7$	Adenosine	Urea	Polymerization salts	Aerosil 300	Aluminium oxide	Calcium silicate	Hydroxyapatite	Zinc oxide	Zeolite Beta	pH	T	Inital water added (µL)	Water added each cycle (µL)
11	*	*	*	*	*						8.7	80 °C	620	400
12	*	*	*	*		*					9.5		320	200
13	*	*	*	*			*				10		120	200
14	*	*	*	*				*			8.7		120	200
15	*	*	*	*					*		8.1		320	200
16	*	*	*	*						*	7.1		320	200

Table D: An in-depth of the experimental conditions of experiment 2.

Sample	Na4P2O7	Adenosine	Urea	Polymerization salts	Zeolite Beta	$MgSO_4$	pH	T	Inital water added (µL)	Water added each cycle (µL)	Number of wet-dry cycles
16 A	*	*	*	*	*		8.1	80 °C	320	200	3
16B	*	*	*	*	*		8.1		320	200	2
16C	*	*	*	*	*		8.1		320	200	1
16D	*	*	*	*	*		8.1		320	0	0
16.1	*	*	*	*			10.0		320	200	3
16.2	*	*	*		*		8.7		320	200	3
16.3	*	*	*		*	*	6.8		320	200	3
16.4	*	*	*	*	*		3.5		320	200	3
16.5	*	*	*	*	*		9.0		320	200	3

Table E: An in-depth of the experimental conditions of experiment 3.

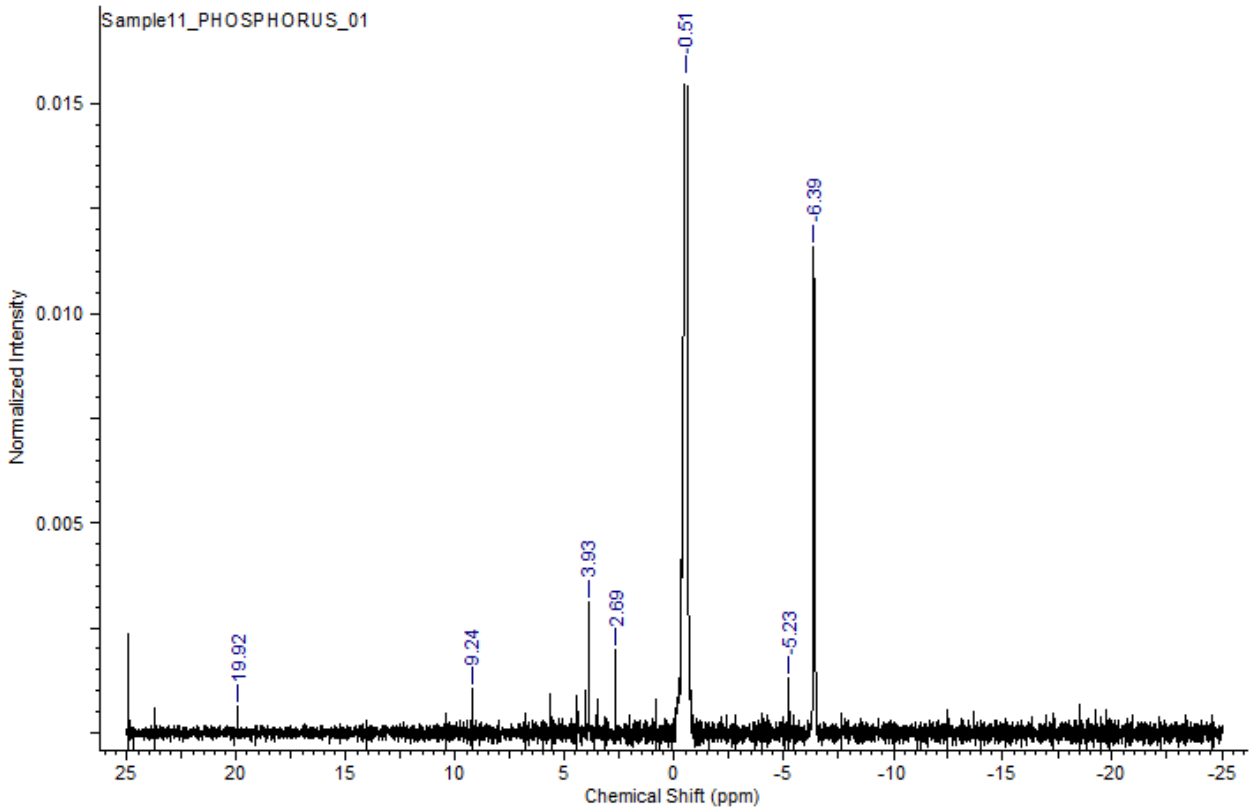

Figure F: ^{31}P NMR spectrum of sample 11. Note: using an 85% H_3PO_4 internal standard at ~-0.5–0.8ppm.

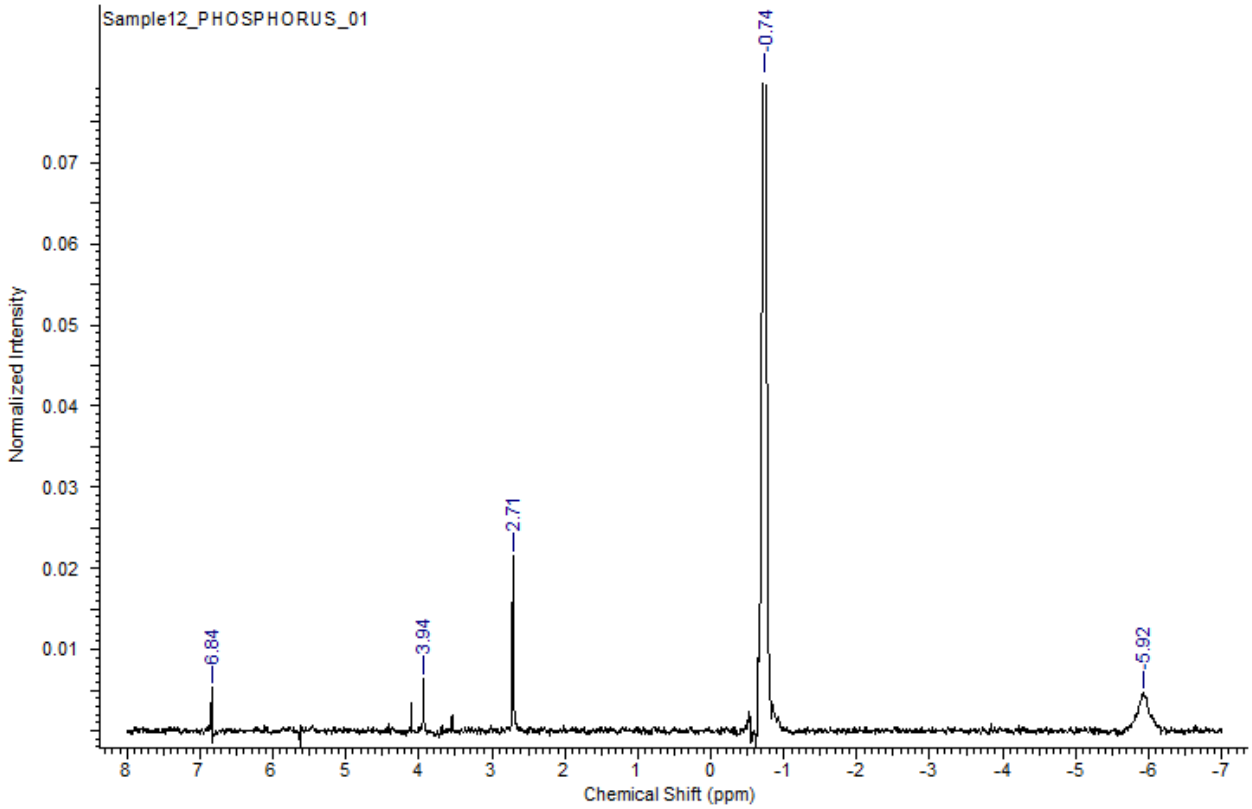

Figure G: ^{31}P NMR spectrum of sample 12. Note: using an 85% H_3PO_4 internal standard at ~-0.5–0.8ppm.

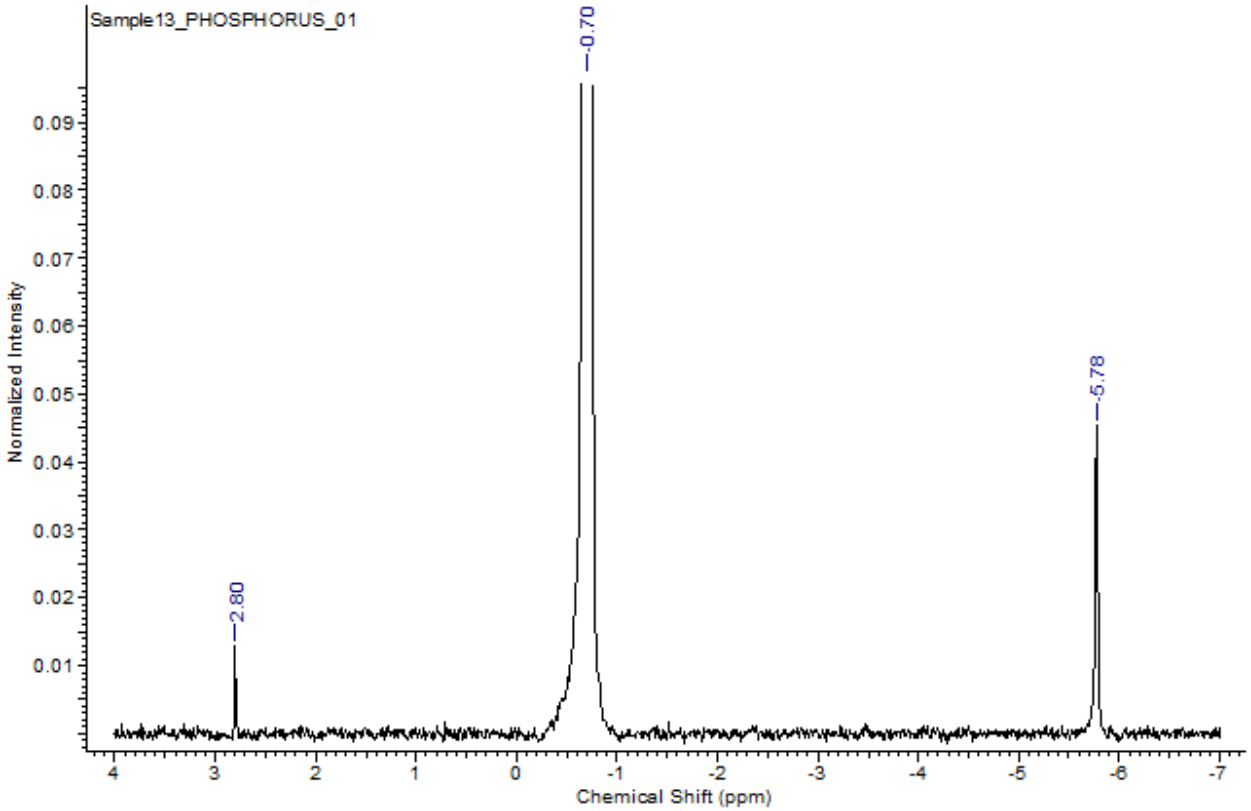

Figure H: ^{31}P NMR spectrum of sample 13. Note: using an 85% H_3PO_4 internal standard at ~-0.5–0.8ppm.

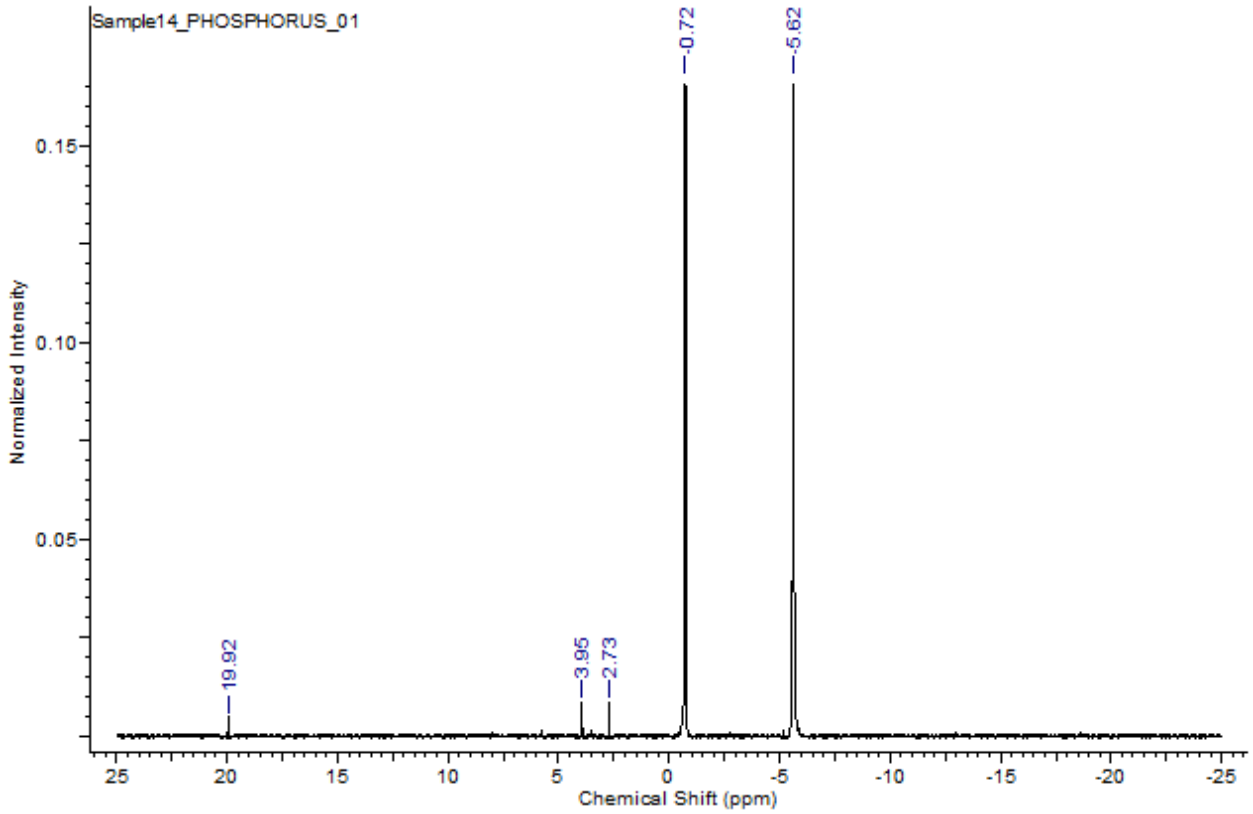

Figure I: ^{31}P NMR spectrum of sample 14. Note: using an 85% H_3PO_4 internal standard at ~-0.5–0.8ppm.

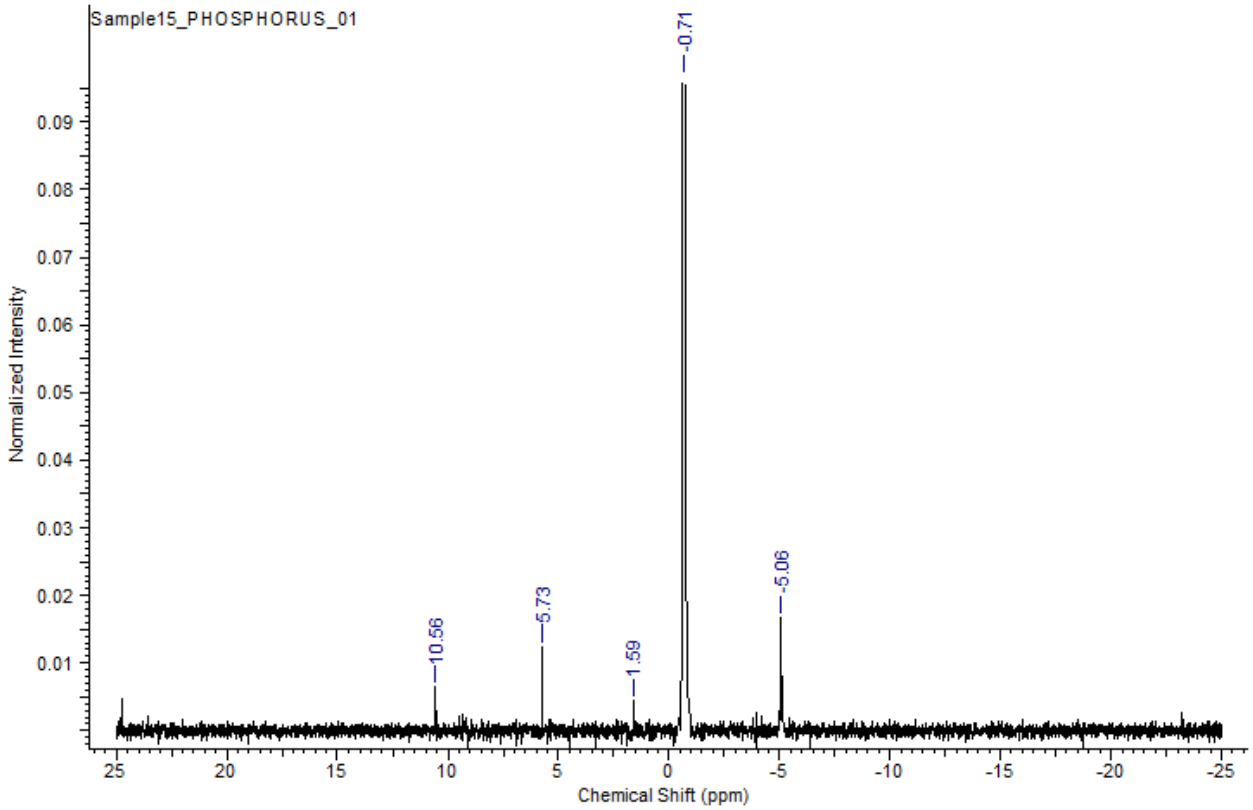

Figure J: ^{31}P NMR spectrum of sample 15. Note: using an 85% H_3PO_4 internal standard at ~-0.5–0.8ppm.

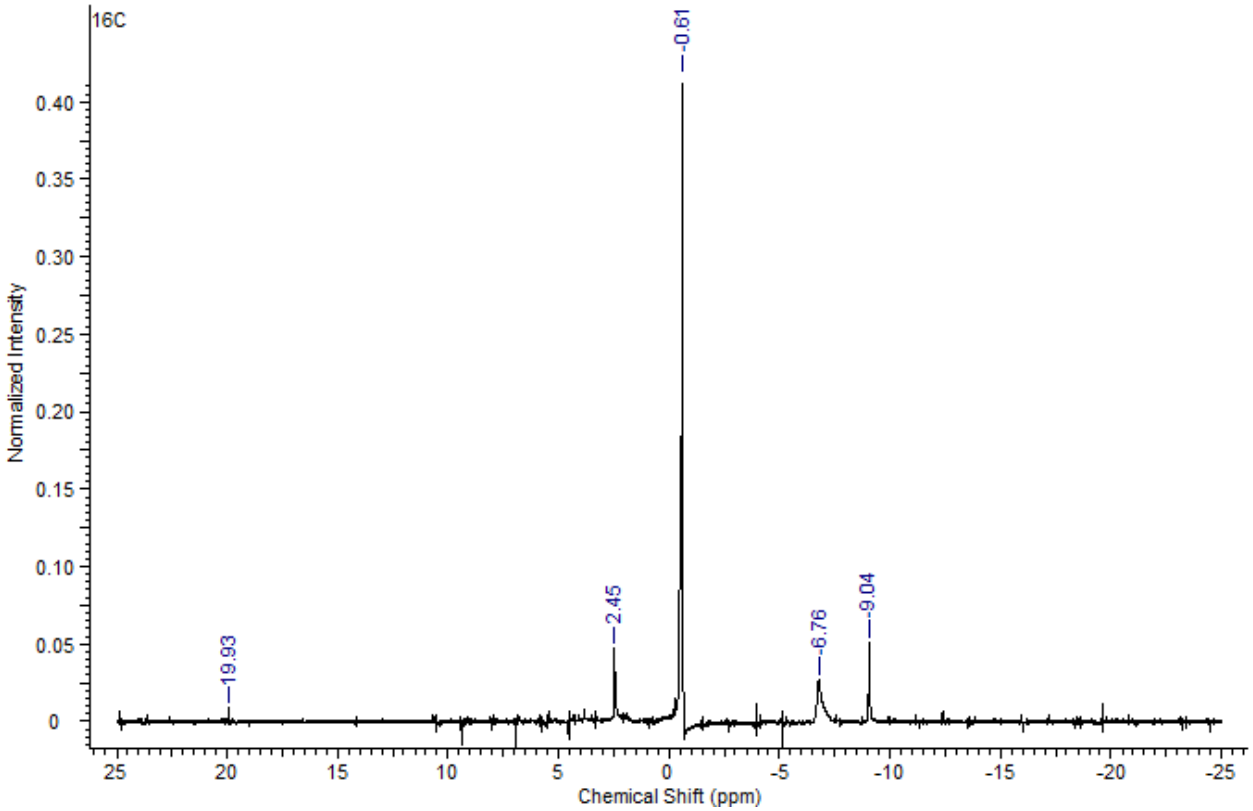

Figure K: ^{31}P NMR spectrum of sample 16C. Note: using an 85% H_3PO_4 internal standard at ~-0.5–0.8ppm.

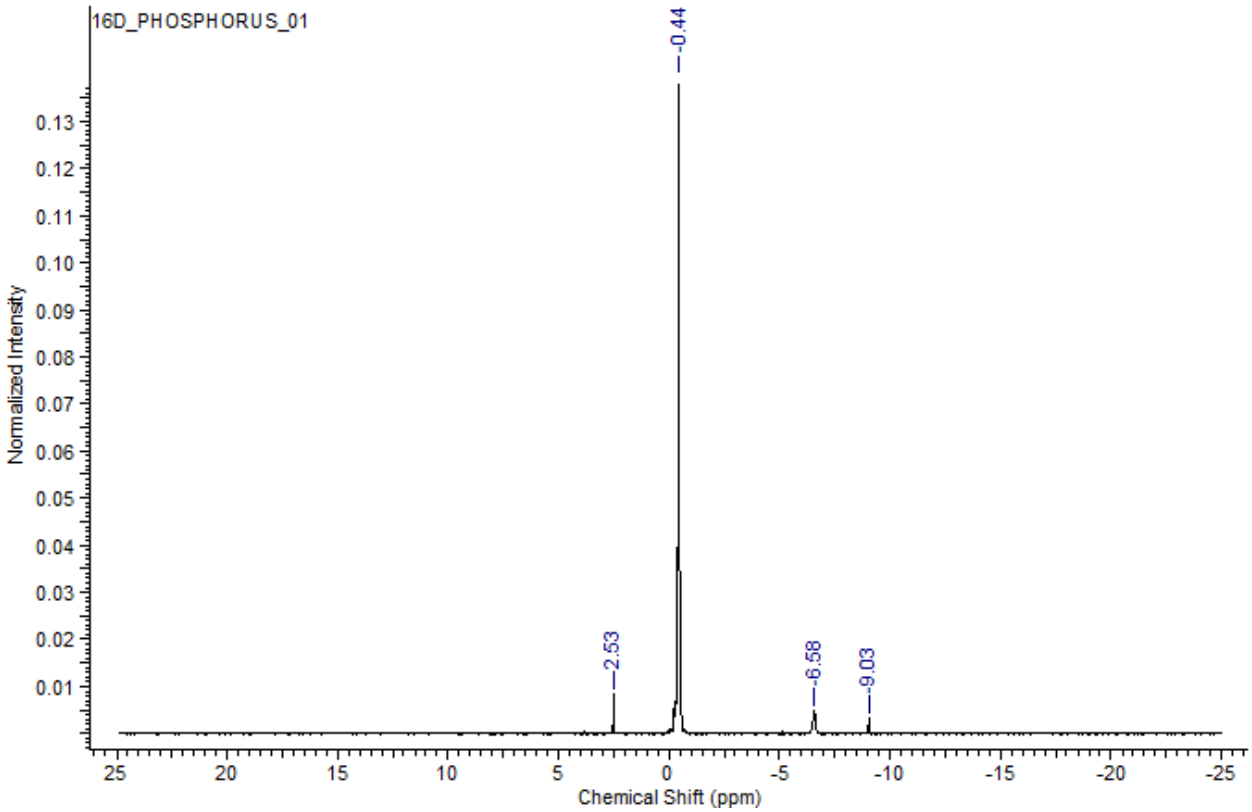

Figure L: ^{31}P NMR spectrum of sample 16D. Note: using an 85% H_3PO_4 internal standard at ~-0.5–0.8ppm.

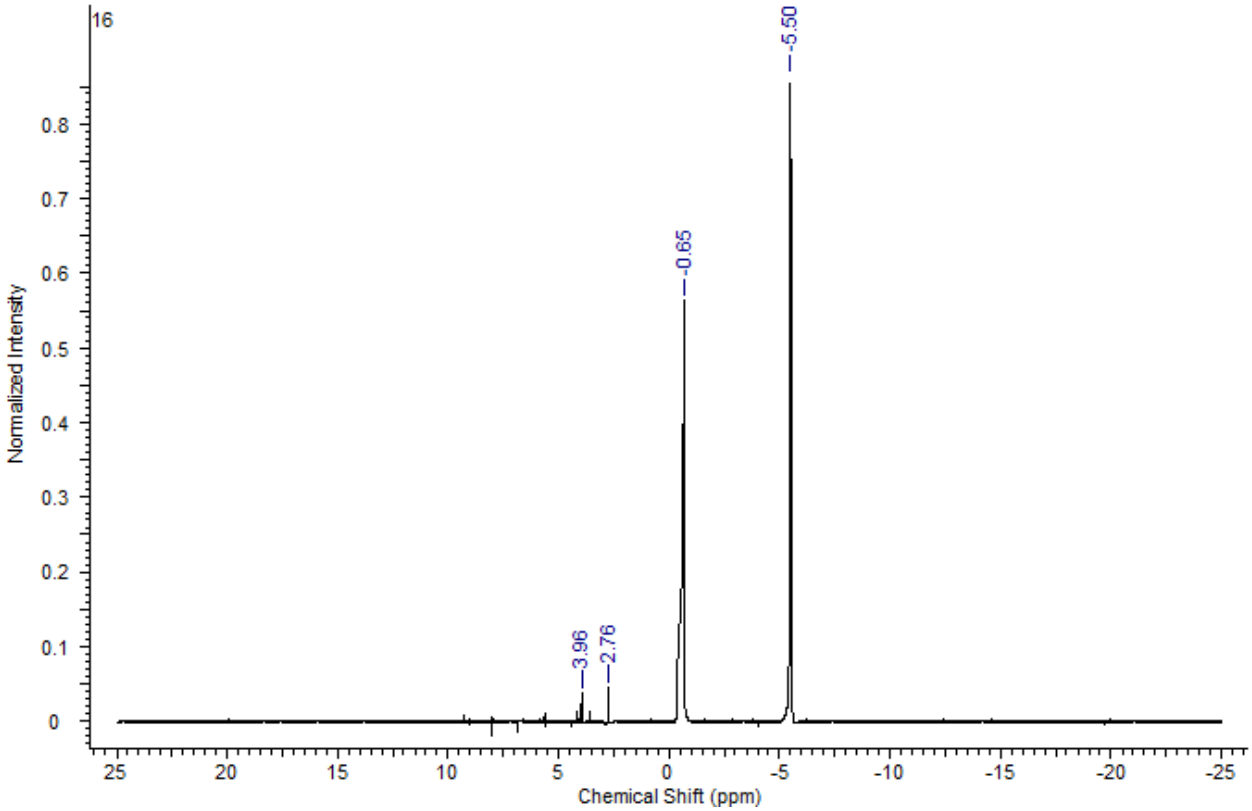

Figure M: ^{31}P NMR spectrum of sample 16.1. Note: using an 85% H_3PO_4 internal standard at ~-0.5–0.8ppm.

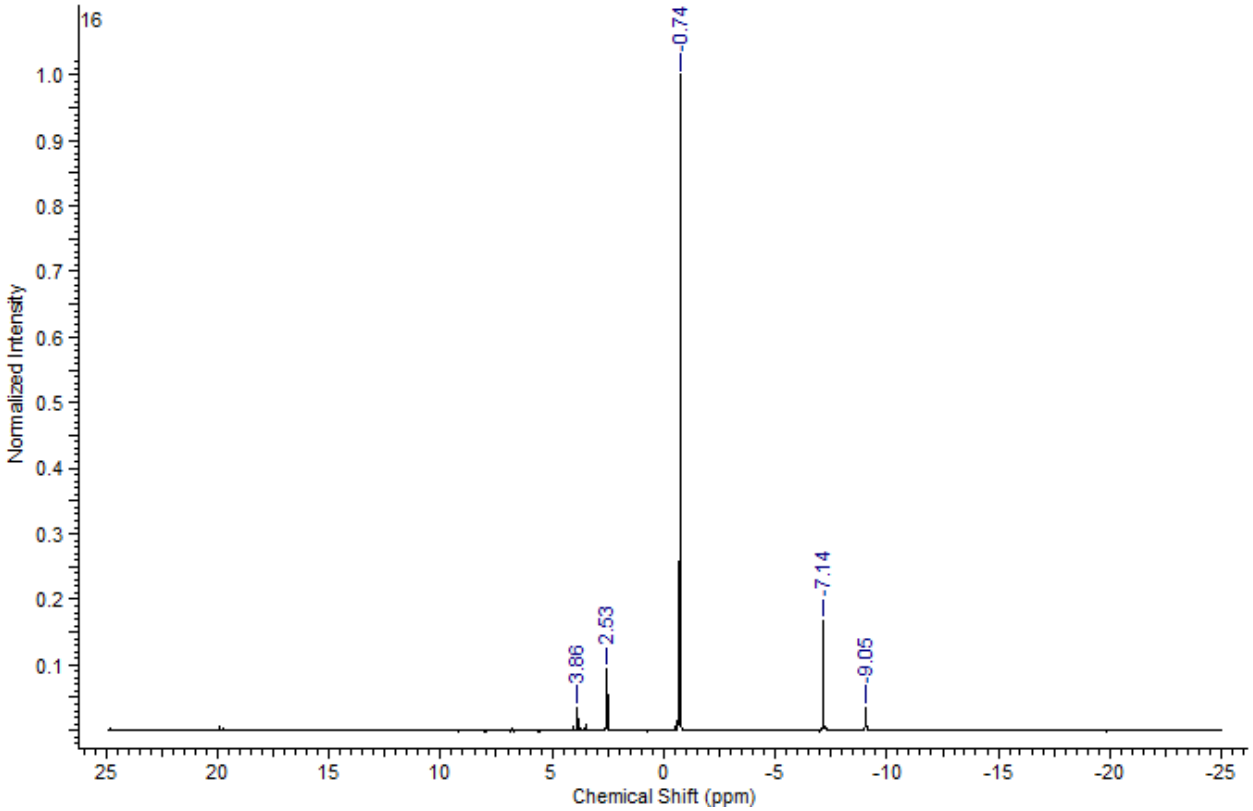

Figure N: ^{31}P NMR spectrum of sample 16.2. Note: using an 85% H_3PO_4 internal standard at ~-0.5–0.8ppm.

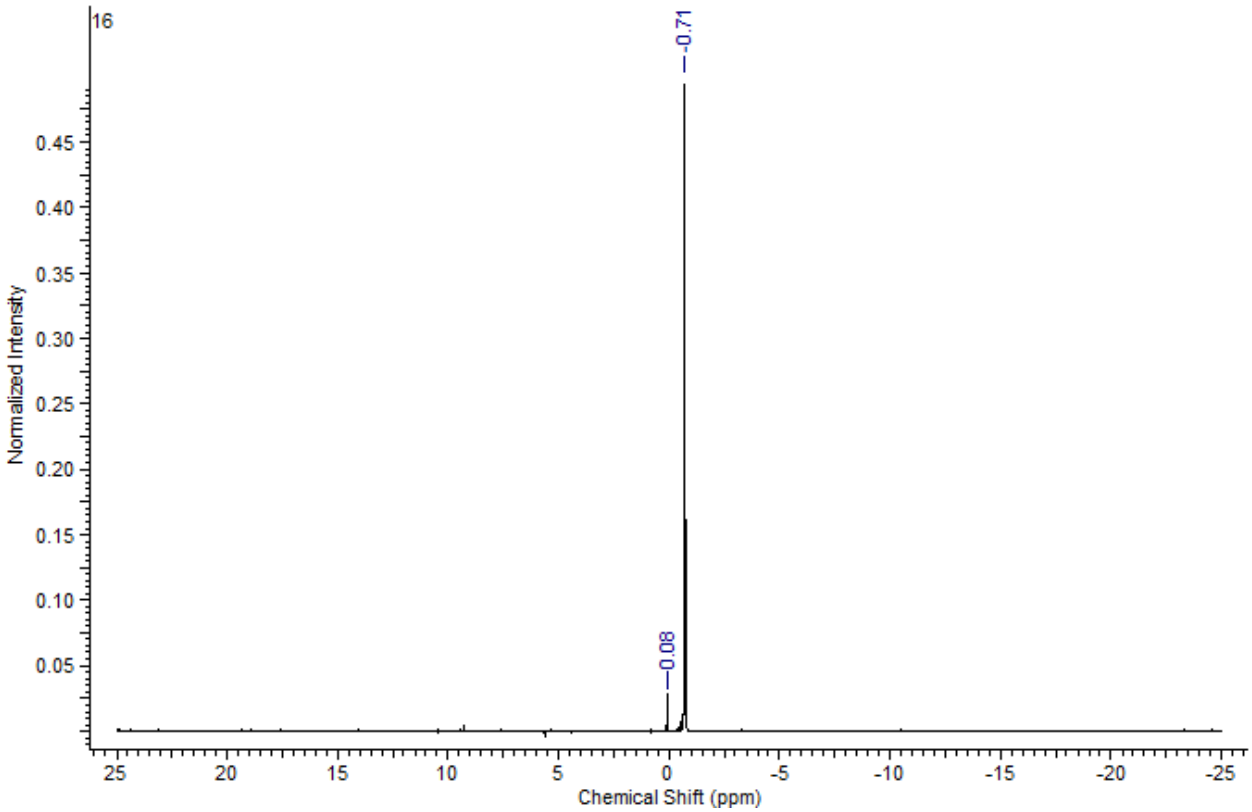

Figure O: ^{31}P NMR spectrum of sample 16.3. Note: using an 85% H_3PO_4 internal standard at ~-0.5–0.8ppm.

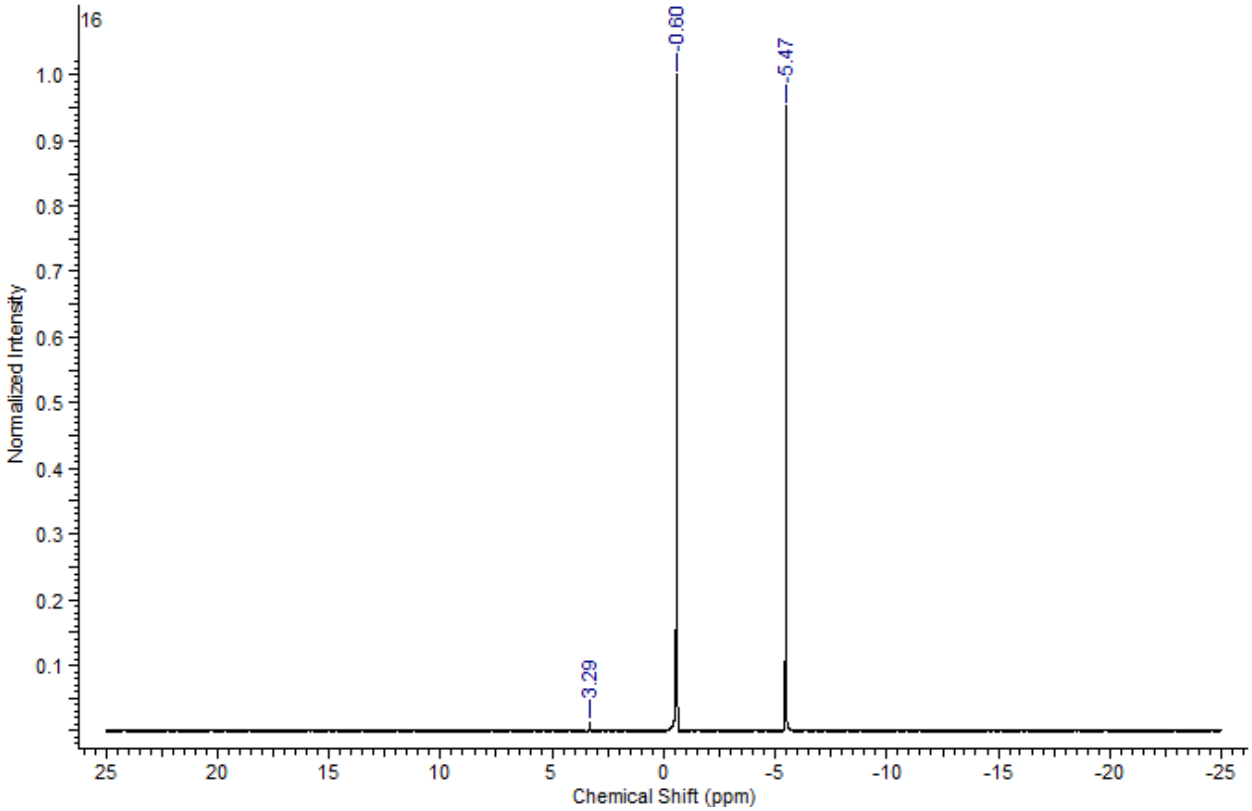

Figure P: ^{31}P NMR spectrum of sample 16.5. Note: using an 85% H_3PO_4 internal standard at ~-0.5–0.8ppm.