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^{31}P NMR Study on the Reactions of Amino Acids and Sugar Derivatives with Pyrophosphorous Acid as a Possible Prebiotic Phosphonylating Agent

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Abstract

Phosphorus is an essential element in living organisms. Evaluating prebiotic processes that lead to phosphorylated biomolecules is an important step toward understanding the origin of life. Schreibersite ($[Fe,Ni]_3P$) is a meteoritic phosphorus mineral which releases various phosphorus species reactive toward biomolecules. We studied the reactions between biomolecules and pyrophosphorus acid ($H_4P_2O_5$), which is a phosphorous acid derivative released from schreibersite. The reactions between pyrophosphorous acid and molecules having hydroxy groups were carried out under mild alkaline conditions. Notably, some biologically important molecules such as L-serine, L-tyrosine, L-threonine, D-ribose, and D-glyceraldehyde reacted with pyrophosphorous acid to give corresponding phosphonates. These results suggested that if schreibersite and the biomolecules co-existed in the prebiotic earth, they formed the phosphonates which were able to play roles as surrogates or precursors of phosphorylated biomolecules.

Keywords: Prebiotic chemistry | Schreibersite | Pyrophosphorous acid

1. Introduction

Phosphorus is an essential element in living organisms; it appears in the forms of phosphates of nucleotides, the internucleotidic backbones of nucleic acids, phosphorylated amino acids in proteins, phospholipids, phosphorylated sugars, and high-energy intermediates in metabolic systems. Therefore, evaluating prebiotic processes that lead to these phosphorylated biomolecules is an important step toward understanding how precursors of current living systems were generated on the early Earth. Schreibersite ($[Fe,Ni]_3P$),[1] which is a meteoritic phosphorus mineral, is considered to be a source of phosphorus in the primitive environment, and it has been estimated that 10^6–10^{10} kilograms per year of phosphorus fell as schreibersite during the period of late heavy bombardment.[2]

Interestingly, schreibersite in water not only releases phosphoric acid (H_3PO_4) but also lower-oxidation-state phosphorus species, such as phosphorous acid (H_3PO_3), hypophosphoric acid ($H_4P_2O_6$),[3] and pyrophosphorous acid (**1**, $H_4P_2O_5$, Figure 1A) under mild alkaline conditions.[1b] Because the phosphoric acid currently found in nature is mainly present as water-insoluble calcium phosphate (apatite), it is implausible that phosphoric acid of this form was the phosphorylation source on the primitive Earth. On the other hand, the reduced form, namely calcium phosphite, is more soluble in water than calcium phosphate,[4] which suggests that reduced phosphorus species, such as phosphorous acid derivatives, may have played important roles in prebiotic process which lead to the phosphorylated biomolecules. Among reduced phosphorus species, we focused on the reactivities of **1** toward hydroxy groups in this study. We were interested in **1** because this species is generated through the aqueous corrosion of schreibersite, as described above, or by the evaporation of aqueous solutions of sodium phosphonate.[5] In addition, **1** reacts under mild conditions with various chemical species, such as phosphoric acid,[5] the phosphate groups of 5′-mononucleotides,[6,7] and sugar phosphates,[8] to give mixed acid anhydrides. In addition, under mild alkaline conditions, compound **1** reacts with the 2′ or 3′-

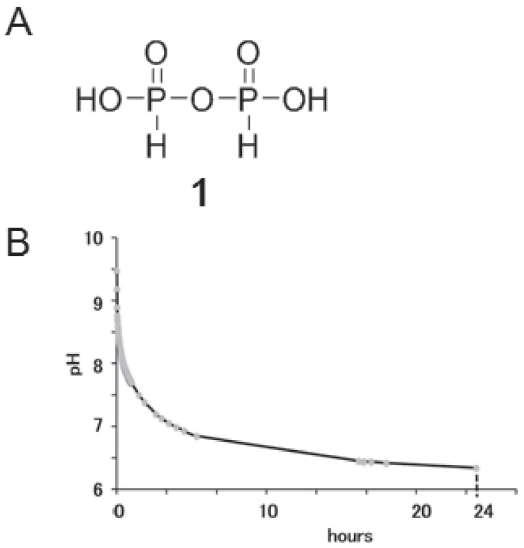


Figure 1. A) The structure of pyrophosphorous acid (**1**). B) Decrease of pH of a 1.0 M aqueous solution of **1**, initially adjusted to pH 9.5, as a function of time.

hydroxy groups of inosine[9] and adenosine[6] to give the corresponding 2′ or 3′-*H*-phosphonates. Although the roles of *H*-phosphonates in chemical evolution has not yet been established, it has been hypothesized that dinucleoside *H*-phosphonates were formed by the thermal condensation of nucleosides and nucleoside *H*-phosphonates; these are unstable and were immediately oxidized or thiolated to the stable dinucleoside phosphate or dinucleoside thiophosphate.[10–12] Hence, it is possible that a variety of *H*-phosphonate derivatives were intermediates during the formation of biologically important phosphates. Although the reactions of **1** with some biomolecules bearing hydroxy groups, such as nucleosides, L-DOPA,[13] and catechin,[14] have been clarified, reactions with other molecules, such as amino acids and carbohydrates, which are important in prebiotic chemistry, have not been studied to date.

In this study, we examined the reactions of **1** with a variety of alcohols, including D-ribose, hydroxy-substituted amino acids, and glyceraldehyde. We clarified the phosphonylation chemistry of many types of hydroxy groups, such as 1,2-diols, primary and secondary alcohols, and phenols in the above-mentioned biomolecules under mild alkaline conditions. The results of this study suggest that many types of biomolecules may have been phosphonylated by **1** in the prebiotic environment prior to the appearance of phosphorylating enzymes.

2. Experimental

^{31}P NMR spectra were acquired in D_2O-H_2O (1:5, v/v) on a Varian AS500 or a Bruker BioSpin AVANCE III HD500 spectrometer at 202 MHz. Disodium salt of **1** was prepared following a literature procedure.[15] Reverse-phase HPLC was performed using an XBridge™ Prep C18 (4.6 × 150 mm, 5 µm) column at 30 °C with 30 mM ammonium acetate as the eluent at a flow rate of 3 mL min^{-1}.

The Degradation Rate of Disodium Pyrophosphate Solution under Basic Conditions. Disodium salt of **1** (1.89 g, 10 mmol) was dissolved in 10 mL of distilled water. The initial pH of the mixture was adjusted to 9.5 with 6 M NaOH. The mixture was stirred for 24 h and then analyzed by ^{31}P NMR spectroscopy.

General Procedure for the Reaction of 1 with an Alcohol. The alcohol (R-OH, 1 mmol) and disodium salt of **1** (1.89 g, 10 mmol) were dissolved in 10 mL of distilled water. The mixture was stirred for 3 h during which time the pH of the mixture was maintained at 9 ± 0.5 by the addition of 6 M NaOH. After 3 h, the reaction mixture was acidified to pH 4–5 by the addition of 2 M HCl, and then analyzed by ^{31}P NMR spectroscopy. The ^{31}P NMR chemical shift of phosphorous acid is 4.08 ppm.

Reaction of 1 and Glyceraldehyde. Glyceraldehyde (0.1 mmol) and disodium salt of **1** (0.19 g, 1 mmol) were dissolved in 10 mL of distilled water. The mixture was stirred for 3 h during which time the pH of the mixture was maintained at 9 ± 0.5 by the addition of 6 M NaOH. After 3 h, the reaction mixture was acidified to pH 4–5 by the addition of 2 M HCl, and then analyzed by ^{31}P NMR spectroscopy.

Calculation of the Conversion Yield. The yield (% conversion) was calculated using NMR peak areas as follows. When the initial molar amount of **1** was 10 mmol, the total molar amount of phosphorus atom was 20 mmol. Thus, the molar amount of the product (in mmol) was calculated to be

$$\textit{The molar amount of product} = \frac{\textit{the peak area of the product} \times 20}{\textit{the sum of all peak areas}}$$

Because the initial molar amount of the alcohol was 1 mmol,

$$\%\textit{conversion} = \frac{\textit{the peak area of the product} \times 20\ [\textit{mmol}]}{\textit{the sum of all peak areas}} \times \frac{1}{1\ [\textit{mmol}]}$$

3. Results and Discussion

It has been reported that **1** effectively phosphonylates the 2′ or 3′-hydroxy group of inosine at initial pH values of 9–11.[9] However, phosphorous acid is produced when pyrophosphorous acid is hydrolyzed or alcoholyzed, leading to a gradual decrease in pH, which is problematic. To check its time-dependency, the pH of a 1.0 M aqueous solution of pyrophosphorous acid was measured over a 24 h period after it was initially adjust to a value of 9.5.

Figure 1B reveals that the pH decreased sharply over the first 2 h, after which it decreased more gently to a value of around 6.5. The ^{31}P NMR spectrum after 24 h is shown in Figure 2. The doublet of triplets observed at −3.92 ppm, (J^1_{PH} = 667 Hz; J^2_{PP} = 7.6 Hz) corresponds to **1**, while the doublet observed at 4.08 ppm (J^1_{PH} = 577 Hz) corresponds to phosphorous acid. The final ratio of phosphorous acid to **1** was determined to be 1:4.8 by peak-area integration, and the ratio did not change afterward. Consequently, in order to study the reactivity of **1** under alkaline conditions, the pH needs to be controlled to avoid decreases in pH. Therefore, the pH of the reaction mixture was maintained at 9.0 ± 0.5 in the experiments described below through the continuous addition of 6 M NaOH.

Under the conditions described above, a variety of compounds bearing hydroxyl groups were reacted with **1** for 3 h, as depicted in Scheme 1, and the products were analyzed by ^{31}P NMR spectroscopy after acidification of the reaction mix-

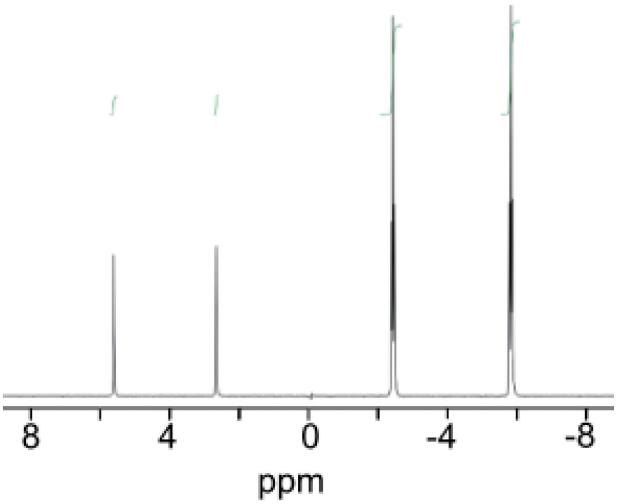
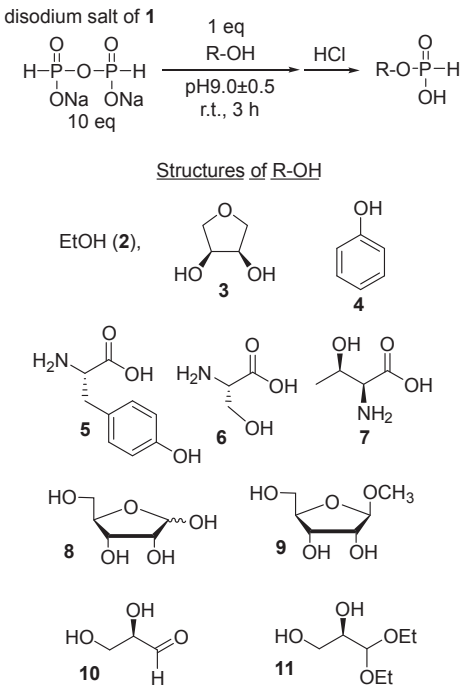


Figure 2. 202 MHz ^{31}P NMR spectrum of a 1.0 M aqueous solution of **1** solution after 24 h. Triplets in the negative region are those of **1**, and the singlets in the positive region are those of phosphorous acid.



Scheme 1. Reactions of **1** with various compounds at pH 9.0 ± 0.5.

ture with 2 M HCl. We studied the reaction of **1** with ethanol (**2**), 1,4-anhydroerythritol (**3**), and phenol (**4**) in order to clarify the reactivities of **1** toward primary alcohol, 1,2-cis-diol, and phenol, respectively. We also studied reactions with L-tyrosine (**5**), L-serine (**6**), and L-threonine (**7**) to clarify the reactivities of amino acids with hydroxy groups in their side chains, while D-ribose (**8**), methyl β-D-riboside (**9**), glyceraldehyde (**10**), and glyceraldehyde diethylacetal (**11**) were reacted in order to determine the reactivities of carbohydrate hydroxy groups and their acetal derivatives.

Reactions of 1 with Ethanol (2), 1,4-Anhydroerythritol (3), and Phenol (4). No product was observed other than

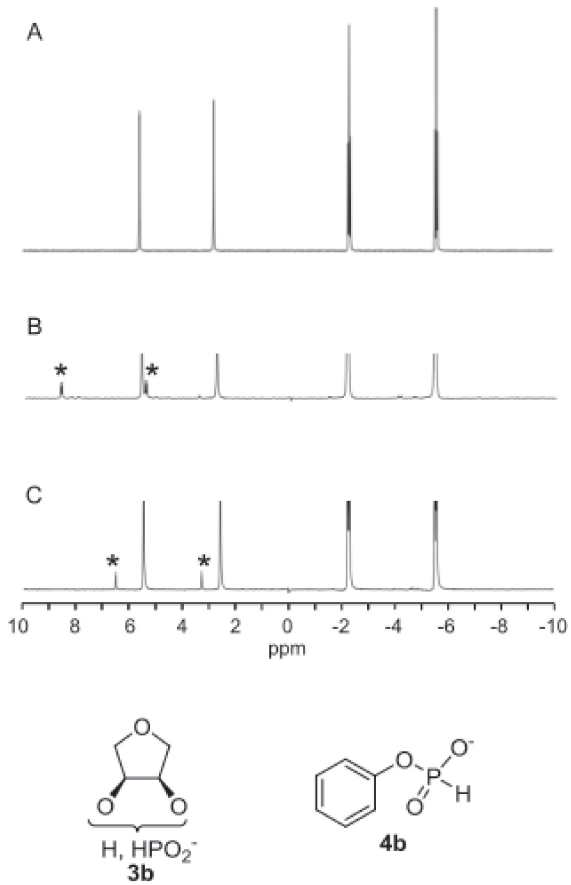
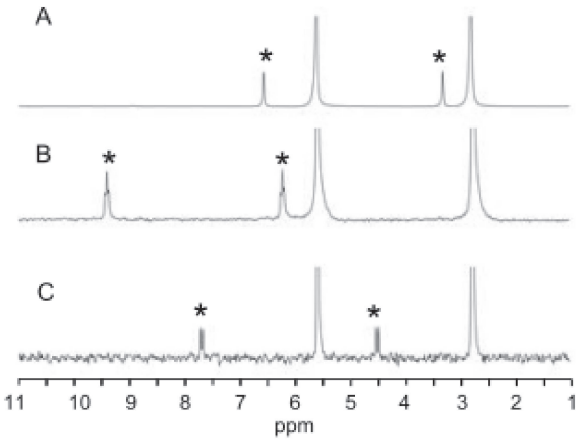


Figure 3. 202 MHz ^{31}P NMR spectra following the reaction of **1** (**1**) with A) ethanol (**2**), B) 1,4-anhydroerythritol (**3**), and C) phenol (**4**). The structures of products **3b** and **4b** are shown. The peak shown by the asterisks in panel B and C are assigned to **3b** and **4b**, respectively.

phosphorous acid when **1** was reacted with ethanol (**2**), whose pK_a is 15.9[16] (Figure 3A); only the doublet of triplets corresponding to **1** and the doublet of phosphorous acid were observed in the ^{31}P NMR spectrum of the reaction mixture. On the other hand, the ^{31}P NMR spectrum of the reaction mixture of the cis-diol **3** (Figure 3B), whose pK_a was lower than that of **1**, and **1** exhibited a doublet of doublets at 7.05 ppm (J^1_{PH} = 646 Hz; J^3_{PH} = 9.5 Hz) that corresponded to a new compound, which is assumed to be **3b**; this compound was formed in 71% conversion as determined by the comparison of the NMR-signal integration as described in the experimental section. Phenol (**4**) produced compound **4b** in 60% conversion; **4b** exhibited a doublet at 4.92 ppm (J^1_{PH} = 654 Hz) (Figure 3C). These results reveal that the NMR conversions were higher for compounds with hydroxy groups of lower pK_a, presumably because **1** reacts with alkoxides rather than alcohols. These reactivities of **3** and **4** are in accordance with previous reports on the reactivities of **1** with nucleosidic 1,2-cis-diols,[6,9] and the phenolic hydroxy groups of L-DOPA[13] and catechin.[14]

L-Tyrosine (**5**), L-serine (**6**), and L-threonine (**7**), which contain hydroxy groups, were reacted under the same reaction conditions, the results of which are displayed in Figure 4. In the case of **5**, the phenolic hydroxy group reacted with **1** to form **5b**, which exhibited a doublet at 4.93 ppm (J^1_{PH} = 658



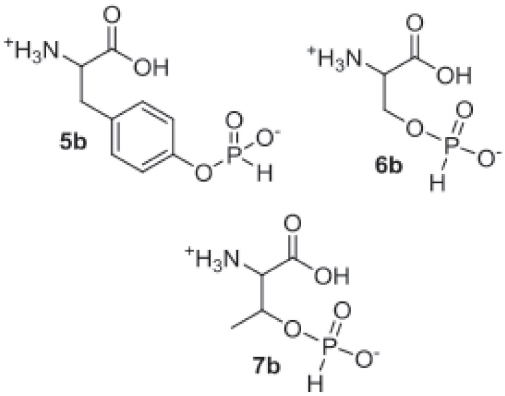


Figure 4. 202 MHz ^{31}P NMR spectra following the reactions of A) L-tyrosine (**5**), B) L-serine (**6**), and C) L-threonine (**7**) with **1** for 3 h. The peak shown by the asterisks in panel A, B and C are assigned to **5b**, **6b**, and **7b**, respectively. The structures of products **5b**, **6b**, and **7b** are shown.

Hz) in its ^{31}P NMR spectrum (Figure 4A); the yield of this phosphonate was calculated to be 61%. In the case of **6** (Figure 4B), a doublet of triplets ($J^1_{PH} = 646$ Hz; $J^3_{PH} = 5.9$ Hz) was observed; the splitting pattern of this peak suggested the presence of a C<u>H</u>$_2$-O-P-<u>H</u> unit bearing one and two protons on the phosphorus atom and the carbon atom β to the phosphorus, respectively; the yield was determined to be 86%. In the case of **7** (Figure 4C), the observed doublet of doublets ($J^1_{PH} = 646$ Hz; $J^3_{PH} = 9.5$ Hz) suggested the presence of a C<u>H</u>-O-P-<u>H</u> unit, and the reaction conversion was 14%.

Since **5** contains a phenolic hydroxy group, the formation of **5b** was expected on the basis of the reactivity of **4**, as shown in Figure 3. However, the formation of **6b** and **7b**, which are phosphonates of the aliphatic alcohols present in **6** and **7**, respectively, was unexpected, since **2** was unreactive (Figure 3A). Considering that the pK_a of the hydroxy group of serine has been estimated to be greater than 15,[17] the formation of the corresponding alkoxide at pH 9 is implausible. The acetate ion has been reported to accelerated the phosphonylation of ADP by **1**;[7] hence, the carboxylate ion of serine may also accelerate this reaction in an intramolecular or intermolecular manner.

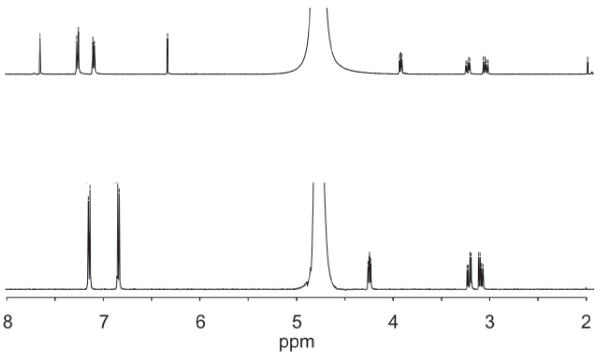


Figure 5. 500 MHz ^{1}H NMR spectra. Upper panel: the isolated *O*-phosphonylated L-tyrosine **5b**; lower panel: L-tyrosine (**5**).

The low yield of **5** compared to that of **4** is ascribable to the low solubility of **5**, which is about 0.01 M at pH 9.5.[18] In addition, the lower yield of **7** compared to **6** is possibly due to greater steric hindrance associated with the secondary hydroxy group in **7** compared to that of the primary hydroxy group in **6**.

In the case of **5**, product **5b** was isolated from the reaction mixture by reverse-phase HPLC and its structure was confirmed by ^{1}H NMR spectroscopy (Figure 5, upper panel). The ^{1}H NMR spectrum of **5b** exhibited a doublet at 6.99 ppm ($J^1_{HP} = 657$ Hz) for the proton directly attached to the phosphorus atom, in addition to signals that correspond to the L-tyrosine skeleton.

On the other hand, although products **6b** and **7b** could not be isolated, their structures were also confirmed by ^{1}H NMR spectroscopy of the crude reaction mixtures, as shown in Figures S1 and S2, respectively.

It should be noted that the NMR spectra described above were acquired after the reaction mixture was acidified by the addition of 2 M HCl. In the absence of acidification, the spectra exhibited peaks corresponding to phosphonamidate-type products that contain P-N bonds, resulting in complicated spectra (data not shown). However, the P-N bonds were unstable, even at pH 7 or below, hence these compounds were not observed in the above-mentioned NMR spectra.

Reactions of 1 with D-Ribose and Glyceraldehyde. D-Ribose was reacted with **1** and the ^{31}P NMR spectrum of the reaction mixture is shown in Figure 6A; this spectrum is clearly very complex and signals corresponding to at least fifteen species, which could not be unambiguously assigned, were observed. However, the splitting patterns of the observed signals revealed the positions of the phosphonylated hydroxy groups. For example peaks 2 and 3 (Figure 6A) are doublets and most likely correspond to phosphorus atoms introduced at secondary hydroxy groups, such as positions 2 or 3 of D-ribofuranose, or 2, 3, or 4 of D-ribopyranose. Peak 15 is a doublet of doublets, suggesting that it belongs to a phosphorus atom introduced at the primary hydroxy group of a 5-position. Hence, we conclude that both the secondary and primary hydroxy groups of D-ribose were phosphonylated by **1**. The presence of many signals in the NMR spectrum is ascribable to products that exist as mixtures of isomers. For example, D-ribose exists in both D-furanose and D-pyranose forms. In addition, the presence of α and β anomeric forms further complicates the spectrum of the

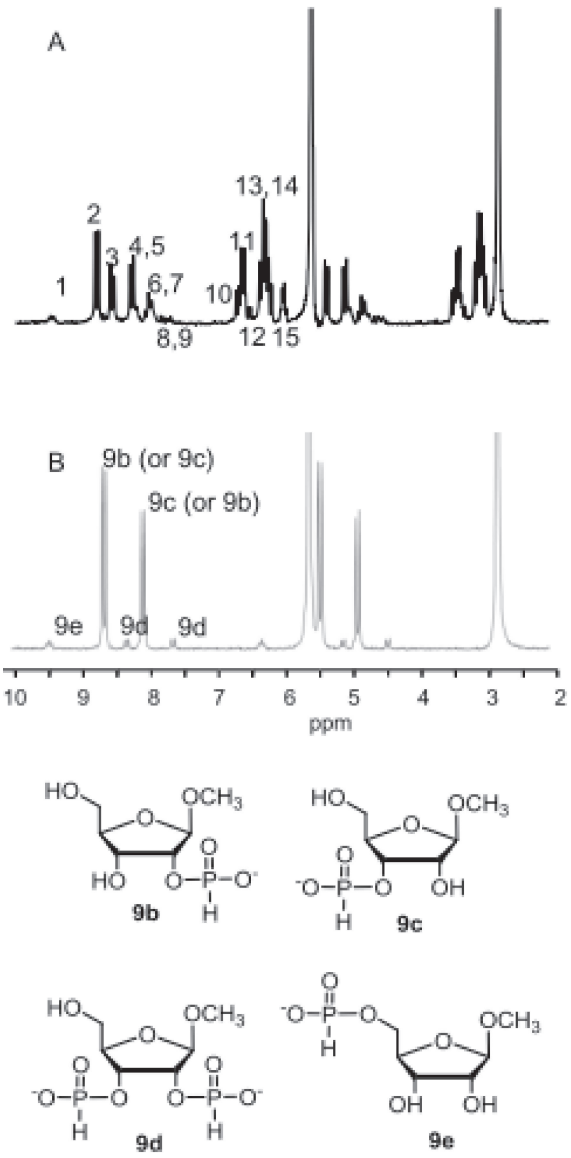


Figure 6. ^{31}P NMR spectra and expected products of the reaction mixtures of **1** and A) D-ribose (**8**) and B) methyl D-ribofuranoside (**9**).

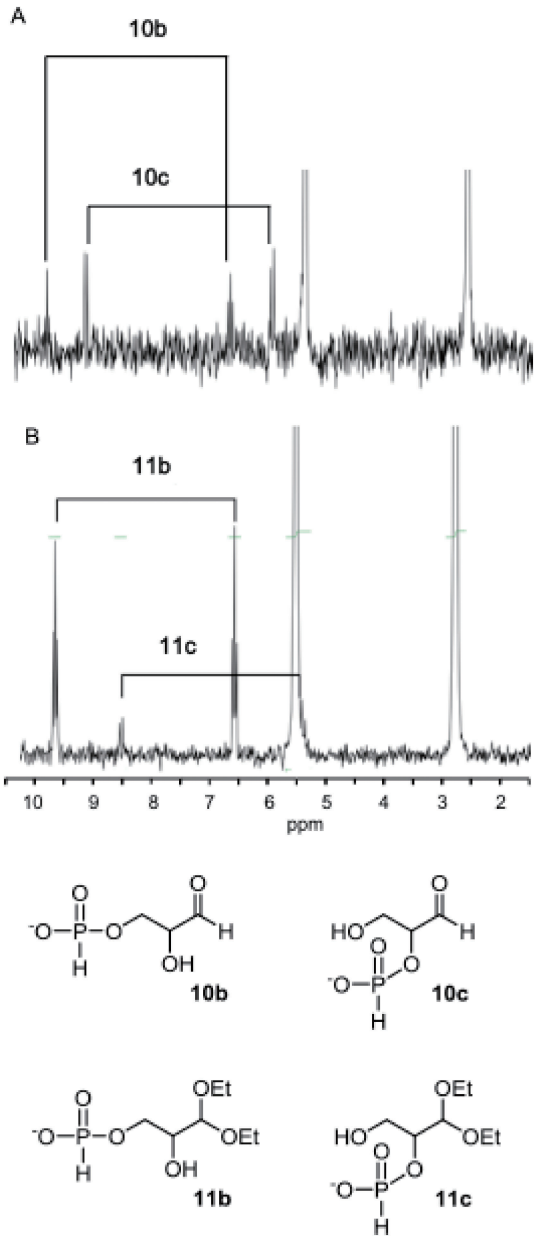


Figure 7. ^{31}P NMR spectra of the reaction mixture following the reaction of **1** with A) glyceraldehyde (**10**) and B) glyceraldehyde diethylacetal (**11**).

product mixture. Moreover, although their proportions may be small, ring-opened forms may also be present in the reaction mixture.

To simplify the reaction mixture, we reacted methyl β-D-ribofuranoside (**9**) with **1**; the ^{31}P NMR spectrum of this reaction system is shown in Figure 6B. Two different doublets of doublets, both with $J^3_{PH} = 10.1$ Hz and $J^1_{PH} = 649$ Hz, correspond to the 2- and 3-phosphonylated ribofuranosides **9b** and **9c**. In addition, two different doublets of doublets ($J^3_{PH} = 10.1$ Hz; $J^1_{PH} = 650$ Hz) and a doublet of triplets ($J^3_{PH} = 6.9$ Hz; $J^1_{PH} = 640$ Hz) were observed; these peaks are assigned to the diphosphonate **9d** and 5′-phosphonate **9e**, respectively. These results indicate that, despite the low reactivity of the primary hydroxy group, the rather acidic cis-diol as well as the primary alcohol reacted with **1**.

The reaction of glyceraldehyde (**10**) with **1** was studied next. It is known that glyceraldehyde is dimeric in highly concentrated solutions;[19] therefore, we reacted 10 mM of glyceraldehyde with 100 mM **1**. The ^{31}P NMR spectrum of the reaction mixture is displayed in Figure 7A. A doublet of doublets ($J^3_{PH} = 11.0$ Hz; $J^1_{PH} = 649$ Hz) was observed at 7.68 ppm. In addition, a doublet of triplets ($J^3_{PH} = 7.7$ Hz; $J^1_{PH} = 639$ Hz) was observed at 8.38 ppm. The doublet of triplets observed in the ^{31}P NMR spectrum is suggestive of the presence of a C<u>H</u>$_2$-O-P-<u>H</u> component in **10b**, resulting from the phosphonylation of the primary hydroxy group. Similarly, the doublet of doublet suggests that **10c** contains a C<u>H</u>-O-P-<u>H</u> unit that arises from the phosphonylation of the secondary hydroxy group. Based on these observations, we determined that **1** reacted with the primary hydroxy group in 4.4% conversion, and with the secondary hydroxy group in 5.8%. The reactions of the primary and

secondary hydroxy groups were also confirmed by the reaction of glyceraldehyde diethyl acetal, which is a model compound. As shown in Figure 7B, the doublet of doublets ($J^3_{PH} = 11.5$ Hz; $J^1_{PH} = 648$ Hz at 6.99 ppm) is assigned to the secondary phosphonate (**11c**), while the doublet of triplets ($J^3_{PH} = 7.2$ Hz; $J^1_{PH} = 638$ Hz at 8.17 ppm) is assigned to the primary phosphonate (**11b**).

4. Conclusion

Many phosphate derivatives are expected to be candidate prebiotic phosphorylating agents. For example, trimetaphosphate has been reported to phosphorylate nucleosides and nucleotides,[20] and amino acids to form short peptides[20c,21a] and N-[21b] or O-phosphorylated serines.[21a] In addition, recent studies on nitrogenous phosphorus species, such as diamidophosphate and amidotriphosphate, revealed that they react with a variety of biomolecules,[20c,22] such as glycolaldehyde, ribose, glyceraldehyde, and amino acids, to give the corresponding phosphate derivatives. On the other hand, in this study we investigated the reactions of pyrophosphorous acid (**1**) as a potential prebiotic phosphonylating agent, which resulted in the production of a variety of *H*-phosphonates that are the reduced analogs of phosphates under milder conditions which are required for the phosphorylation by the phosphate derivatives. Our study revealed for the first time that **1** phosphonylates the hydroxy groups of amino-acid side chains; the products formed, namely **5b**, **6b**, and **7b**, are *H*-phosphonate-type analogs of phosphorylated amino acids. The formation of amino acids **5**–**7** under prebiotic and extraterrestrial conditions, have been reported.[23] Hence, if these amino acids and pyrophosphorous acid coexisted under prebiotic conditions, they may have reacted to form the phosphonates. These O-phosphonylated amino acids may also have modified the properties of prebiotically formed peptides and enhanced their functionalities.

In addition to amino acids, we showed that **1** reacts with some carbohydrates, namely D-ribose and glyceraldehyde; while the hydroxy groups of the 1,2-diol components of D-ribose (**8**) and methyl D-riboside (**9**) were the most reactive, position 5 also reacted to give the corresponding phosphonates. Moreover, the reaction with glyceraldehyde gave glyceraldehyde-3-phosphonate (**10b**), which is an analog of glyceraldehyde-3-phosphate, a prebiotic glycolysis intermediate.[24] The same reaction also gave glyceraldehyde-2-phosphonate (**10c**); upon carbonyl-group oxidation **10c** would give glyceric acid 2-phosphonate, the analog of glyceric acid 2-phosphate, which is also a glycolysis intermediate and a precursor of pyruvate.

The roles of these biomolecule-derived *H*-phosphonates in prebiotic chemistry have not yet been clarified. One possibility was that the *H*-phosphonate derivatives possessed some functions as phosphate analogs without being oxidized to phosphates. Another possibility is that they were oxidized to the corresponding phosphates. Organic-synthesis methods[25] for the oxidation of monoalkyl *H*-phosphonates have been reported, however oxidations in aqueous media are unknown. Several phosphorous acid (H_3PO_3) oxidants, such as $K_2S_2O_8$,[26] I_2,[27] Br_2,[28] Pd^{2+},[29] and dissolved oxygen with UV irradiation,[30,31] have been reported, but they have not been applied to the oxidation of monoalkyl *H*-phosphonates. However, our preliminary experiments suggested that the reaction of $K_2S_2O_8$ or Fenton's reagent with ethyl *H*-phosphonate gave ethyl phosphate in yields of 72% and 6%, respectively (Figure S3). These two reagents commonly generate hydroxyl radicals; hence, we proposed that hydroxyl radicals react with ethyl *H*-phosphonate, and the generated monoalkyl phosphoryl radical is oxidized to ethyl phosphate. The involvement of hydroxyl radicals in the origin of life has recently been proposed,[32] and the involvement of phosphoryl radicals has also been suggested.[2,33] Hence, the *H*-phosphonate monoesters generated in reactions with **1** may become oxidized or converted into more complex molecules through the phosphoryl radical pathway, and such reactions possibly played important roles in the prebiotic generation of phosphorylated biomolecules. Such reactions should be studied further in order to clarify the roles of **1** and monoalkyl *H*-phosphonates on the prebiotic earth.

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Supporting Information

^{1}H-NMR spectra of **6b**, **7b**, and ^{31}P-NMR spectra of the oxidation of ethyl phosphonate. This material is available on https://doi.org/10.1246/bcsj.20180392.

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