Phosphorylation of hydroxyproline (Hyp) has been achieved using inorganic trisodium-cyclo-triphosphate (P$_{3m}$) in aqueous solution. In the reaction of Hyp (1.5 M) and P$_{3m}$ (0.5 M), at pH 11, and 10 °C, N-triphosphoryl hydroxyproline (P$_{1}$-(N)-Hyp) was synthesized. An intramolecular attack of carboxyl group on a phosphorus atom of P$_{1}$-(N)-Hyp yields cyclic phosphate derivative (P$_{1}$-(N,O)-Hyp). Hydrolysis of cyclic phosphate derivative occurs simultaneously to form N-phosphoryl hydroxyproline (P$_{1}$-Hyp). Here, we were able to show that Hyp reacts with P$_{3m}$ to form the intermediates (P$_{1}$-(N)-Hyp and P$_{1}$-(N,O)-Hyp) and give the final product (P$_{1}$-(N)-Hyp). The optimum condition for P$_{1}$-(N)-Hyp was Hyp : P$_{3m}$ = 2(1.0 M) : 1(0.5 M), pH 13, and 25 °C. P$_{1}$-(N)-Hyp was synthesized by dissolving Hyp and P$_{3m}$ in H$_{2}$O under the optimum conditions for 7 days, and isolated by adding 2-propanol to the reaction solution. The moisture retention of P$_{1}$-(N)-Hyp and Hyp alone were compared by measuring amount of water. The moisture retention of the P$_{1}$-(N)-Hyp can be increased more effectively than that of Hyp alone.

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INTRODUCTION

4-Hydroxy pyrrolidine-2-carboxylic acid (Hydroxyproline : Hyp) is a natural amino acid and contained in collagen and elastin. It was promoting proliferation of epidermal cells, and moisturizing the stratum corneum. Since Hyp is rarely found in proteins other than collagen, measurement of Hyp is used as a marker for quantifying collagen or gelatin content.

Vitamin C (ascorbic acid), which is generally known as one type of nutrient, plays an important role in the skin. Because it is an unstable in aqueous solution, various derivatives have been synthesized. Kameyama and coworkers indicated that L-ascorbyl-2-phosphate was absorbed percutaneously and retained in the skin than vitamin C alone. Then, Duarte et al. reported that L-ascorbyl-2-phosphate was non-toxic vitamin C analogue and indicated that the phosphate derivative of vitamin C is superior to ordinary vitamin C for permeability to skin. Therefore, introduction of a phosphate group into Hyp is expected to enhance penetration of Hyp into the skin.

We succeeded in the phosphorylation of amino acids, aminoalcohols, carbohydrates and dendrimers using inorganic phosphorylating agents such as trisodium-cyclo-triphosphate (P$_{3m}$). Phosphorylation with this agent can be achieved by a one-pot reaction in aqueous solution at 25 °C without side reactions.

FIGURE 1 Structure of hydroxyproline (Hyp) and trisodium-cyclo-triphosphonate (P$_{3m}$).

In the present work, we studied the phosphorylation of Hyp with P$_{3m}$ in aqueous solution in order to synthesize phosphate derivatives of Hyp, and then investigated the moisture retaining for improvement of the phosphate derivative of Hyp.
**Materials and methods**

**Materials**

Trisodium cyclo-triphosphate, Na₃P₂O₇ (P₃m) was purchased from BK Giulini GmbH (Ludwigshafen am Rhein, Germany). Hydroxyproline and sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) were purchased from Wako Chemical Industries, Ltd. (Osaka, Japan), and Sigma (St. Louis, USA). Unless otherwise stated, guaranteed grade reagents from Wako Chemical Industries, Ltd. (Osaka, Japan), were used.

**NMR measurements**

$^{1}$H- and $^{1}$H-$^{31}$P correlation spectroscopy (COSY) NMR spectra were measured with a Bruker Avance III 600 MHz spectrometer (Billericia of Massachusetts, USA). Samples were dissolved in D₂O (99.9%) with DSS as an external standard. $^{31}$P-NMR spectra with and without broad band $^{1}$H-decoupling and $^{1}$H-$^{31}$P heteronuclear multiple bond correlation spectroscopy (HMBC) spectra were obtained with a Bruker Avance III 600 MHz spectrometer. As an external standard, 85% H₃PO₄ was used.

**HPLC measurements**

HPLC was carried out using a JASCO PU-980 HPLC (Tokyo, Japan) coupled with a JASCO PU-2080i flow injection system to detect phosphate by the post-column reaction. A column (150 x 6.0 mm I.D.) packed with the polystyrene-based anion-exchanger (TSK gel, SAX, 5 μm, TOSOH, Japan) was used for the analysis of phosphate. The column temperature was maintained at 40 °C. A convex gradient technique using 0.2 and 0.45 M potassium chloride solutions was employed for the analysis of phosphate. Detection of phosphorus was carried out by spectrophotometry of a phosphorus-molybdenum heteropoly blue complex at 830 nm. The amount of sample injected was 100 μL. The flow rate of the eluent was 1.0 mL min⁻¹. The system control, data collection, and data analysis were carried by JASCO-ChromNAV system (Version 1.18.03, JASCO, Tokyo, Japan).

**MS measurements**

Electrospray ionization mass spectrometry (ESI-MS) was performed using a Thermo Scientific Exactive™ mass spectrometer (Thermo Fisher Scientific, Commonwealth of Massachusetts, USA). The mass spectrometer was operated in the negative-ion mode.

**Determination of moisture retention**

The determination of moisture content was measured with the water content actually applied to the skin by Moisture Analyzer SK-III (Beauty garage, Tokyo, Japan).

First of all, cotton included with Milli-Q water, a concentration of 1.0% Hyp aqueous solution, and 1.0% P₁(N)-Hyp. Five subjects were tested on the forearm for 5 - 60 min and the water content was measured.

**RESULTS AND DISCUSSION**

**Reaction of hydroxyproline with P₃m**

Phosphorylation was carried out according to the previously described method. Figure 2 shows HPLC analysis served as a tool for evaluating the yields of products by their peak areas.

![Figure 2](image_url)  
**FIGURE 2** HPLC profiles for the reaction solution of Hyp (1.0 M) and P₃m (0.5 M) at pH 13 and 25 °C

Two peaks attributed to the phosphorylated products appeared at retention times of 10 min (product 2) and 25 min (product 1) after 1 d incubation. The peak area of product 1 decreased gradually, whereas that of product 2 gradually increased with the passage of reaction time.

Other chromatographic peaks were assigned to monophosphate: P₁, diphosphate: P₂, triphosphate: P₃, and trisodium-cyclo-triphosphate: P₃m on the basis of the retention times for the authentic samples. From the results of $^{31}$P NMR spectra, product 2 was found to be a monophosphate derivative of Hyp and product 1 was a triphosphate derivative of Hyp.

![Figure 3](image_url)  
**FIGURE 3** Changes of the amounts of the products in the reaction solution of Hyp (1.0 M) and P₃m (0.5 M) at pH 13 and 25 °C
Figure 3 shows changes of the amounts of the products in the reaction of Hyp (1.0 M) and P_{3m} (0.5 M) at pH 13 and 25 °C. The yield of product 1 increased with the passage of reaction time, reaching about 71% after 1 d, then it decreased gradually. This decrease is due to the hydrolysis of product 1 to form product 2. The yields of product 2 increased with reaction time, reaching a maximum of 63% (after 17 d).

The reaction of Hyp with P_{3m} may be explained by the following mechanism (Scheme 1). P_{3m} are easily attacked by nucleophilic reagents such as ammonia, amino acid, and alcohol. In the present study, the lone electron pair on the imino group nucleophilically attacks a phosphorus atom of P_{3m} to give the product 1. An intramolecular attack of carboxyl group on a phosphorus atom of 1 yields cyclic phosphate derivative (P_{1-(N,O)}-Hyp). Hydrolysis of this intermediate occurs simultaneously to form the product 2. Here, we were able to show that Hyp react with P_{3m} to form the intermediates 1 and P_{1-(N,O)}-Hyp, final product 2. Therefore, we concluded that imino group reacted selectively and 4-OH did not react. So we studied for optimum reaction conditions to produce stable monophosphate derivative of Hyp (product 2, P_{1-(N)}-Hyp).

![Scheme 1 Reaction mechanism of Hyp with P_{3m}](image)

**Optimum reaction conditions to produce N-phosphoryl hydroxyproline (product 2, P_{1-(N)}-Hyp)**

Table 1 summarizes the yield of product 2 (P_{1-(N)}-Hyp) obtained in the reaction of Hyp with P_{3m} under various conditions. The optimum condition for obtaining the P_{1-(N)}-Hyp are as follows: Hyp : P_{3m} = 2 : 1 (1.0 M : 0.5 M), pH 13, and 25 °C.

| Conc. (M) | Temp. (°C) | pH | Time (d) | Yield (%) |
|-----------|------------|----|----------|-----------|
| Hyp       | P_{3m}     |    |          |           |
| 0.5       | 0.5        | 25 | 11       | 4         | 6         |
|           |            |    | 12       | 17        | 20        |
|           |            |    | 13       | 9         | 27        |
| 1.0       | 0.5        | 10 | 13       | 21        | 27        |
|           |            |    | 25       | 13        | 63        |
|           |            |    | 40       | 13        | 18        |
| 1.5       | 0.5        | 25 | 13       | 16        | 61        |

**Isolation of N-phosphoryl hydroxyproline (P_{1-(N)}-Hyp)**

N-phosphoryl hydroxyproline (P_{1-(N)}-Hyp) was synthesized by dissolving Hyp (3.113 g, 1.0 mol/L) and P_{3m} (1.5294 g, 0.5 mol/L) in H_{2}O (20 mL) at 25 °C, and adjusting the solution to pH 13 by adding 6 M NaOH solution. After seven days, the yield of the P_{1-(N)}-Hyp due to the peak at 10 min by HPLC was 62%.

When 10 mL 2-propanol was added to 20 mL of the reaction solution, diphosphate (P_{2}) and P_{3m} were precipitated. While the supernatant by adding 10 mL of 2-propanol was cooled at 4 °C overnight, colloidal precipitation appeared. Then, it was separated into the supernatant and colloidal precipitation with a separating funnel, and the upper layer was evaporated and the sirupy solid with yellowish white was obtained.

An aqueous solution of the concentration of 1 mg/mL was measured by HPLC. P_{1-(N)}-Hyp was 97% in purity. The Electrospray ionization mass spectrometry (ESI-MS) spectrum of aliquot solution showed the molecular ion for the deprotonated.

**Table 1 Yields of P_{1-(N)}-Hyp**

| Conc. (M) | Temp. (°C) | pH | Time (d) | Yield (%) |
|-----------|------------|----|----------|-----------|
| Hyp       | P_{3m}     |    |          |           |
| 0.5       | 0.5        | 25 | 11       | 4         | 6         |
|           |            |    | 12       | 17        | 20        |
|           |            |    | 13       | 9         | 27        |
| 1.0       | 0.5        | 10 | 13       | 21        | 27        |
|           |            |    | 25       | 13        | 63        |
|           |            |    | 40       | 13        | 18        |
| 1.5       | 0.5        | 25 | 13       | 16        | 61        |

**N-phosphoryl hydroxyproline (product 2, P_{1-(N)}-Hyp)**

P_{1-(N)}-Hyp : 1H NMR (D_{2}O) δ 4.286 (1H, dddd, J_{H3,H4} = 2.5 Hz, J_{H3,H4} = 6.0 Hz, J_{H4,H5} = 5.3 Hz, J_{H4,H5} = 1.0 Hz, H-4), 3.524 (1H, dd, J_{H2,H3} = 7.8 Hz, J_{H2,H3} = 9.3 Hz, H2), 3.127 (1H, dd, J_{H4,H5} = 5.3 Hz, J_{H4,H5} = 12.3 Hz, H5), 2.55 (1H, ddd, J_{H4,H5} = 10.5 Hz, J_{H5,H5',p} = 3.0 Hz, H5'), 1.939 (1H, dddd, J_{H2,H3} = 7.5 Hz, J_{H2,H3} = 2.5 Hz, J_{H3,H3'} = 13.5 Hz, J_{H3,H3'} = 1.0 Hz, H-3), 1.746 (1H, ddd, J_{H2,H3} = 9.0 Hz, J_{H3,H3'} = 6.0 Hz, J_{H3,H3'} = 13.5 Hz, H-3'). 

^{31}P NMR (D_{2}O) δ 11.74 (1P, m, J_{H5,p} = 3.0 Hz).

ESI-MS m/z : 210.025 Calcd [C_{3}H_{3}NO_{4}P] Found : 210.017.
Assignment of N-phosphoryl hydroxyproline (P₁-(N)-Hyp)  
To identify the N-phosphoryl hydroxyproline (P₁-(N)-Hyp), $^{31}$P and $^1$H NMR spectra were measured. Figure 4 shows the $^{31}$P NMR spectra for the isolation product.

The singlets at 10.36 and 11.74 ppm were monophosphate derivatives of Hyp. A previous work indicated that glycine reacted with $P_{30}$ to give N-phosphoryl glycine (P₁-(N)-Gly), 5-oxo-1,3,2-oxazaphospholidin-2-olate 2-oxide (P₁-(N,O)-Gly), which was a five-membered cyclic anhydride, N-(N-phosphonomethyl)glycine (P₁-(N)-Gly), and N-triphosphoryl glycine (P₁-(N)-Gly). In the $^1$H decoupled- $^{31}$P NMR spectrum, three singlets at 13.6, 9.2, and 8.9 ppm could be assigned to (P₁-(N,O)-Gly), (P₁-(N)-Gly), and (P₁-(N)-GlyGly), respectively.

Five-membered cyclic phosphate was assigned to a singlet at about 20 ppm on the basis of experimental and theoretical work. Therefore, the singlets at 11.74 and 10.36 ppm is assigned to the P₁-(N)-Hyp and N-(N-phosphonomethyl)hydroxyproline (P₁-(N)-HyprHyp), respectively.

The $^1$H-$^{31}$P 2D HMBC spectrum showed the correlations between P at 11.74 ppm and the $^1$H signal at 3.13 ppm and P at 10.36 ppm and the $^1$H signal at 3.13 ppm. The signal at 3.13 ppm was assigned to H-5 of product based on the $^1$H-$^1$H COSY spectrum. This shows that Hyp reacts with $P_{30}$ to form both P₁-(N)-Hyp (product 2) and N-(N-phosphonomethyl)hydroxyproline (P₁-(N)-HypHyp).

In order to confirm, ESI-MS was performed. The ESI-MS spectrum showed the small peak due to P₁-(N)-HypHyp (m/z = 323.065) in addition to the molecular ion peak due to P₁-(N)-Hyp (m/z = 210.017).

It was thought that a small amount of dimeric monophosphate derivative was also included in the peak at retention time of 10 min of HPLC. Although we could not separate the P₁-(N)-Hyp and P₁-(N)-HyprHyp, the main product was found to be P₁-(N)-Hyp.

Improvement of moisture retaining property of P₁-(N)-Hyp  
The moisture retention of the phosphorylation product obtained in this work was evaluated. Figure 5 shows that the water content in five subjects is compared before application and 10 minutes after application. The water content increased in all cases compared to before the experiment. It was found that the water content of the Milli-Q water and the Hyp aqueous solution after 10 min was about 31 and 47%, whereas that of the P₁-(N)-Hyp aqueous solution was high as 63%.

![Image](image_url)

FIGURE 5 Comparison of the skin moisture content after 10 min. Mean of five determinations ± standard error.

Figure 6 indicates that the changes amount of the water content in five subjects applied to for 5-60 min. The water content increased in all cases, especially when P₁-(N)-Hyp was used, was about 70% after 15 minutes and constant after 60 min. Therefore, it was shown that the moisture retention of Hyp improves by introducing a phosphate group into Hyp.

![Image](image_url)

FIGURE 6 Changes of the skin moisture content (%) after 5-60 min. Mean of five determinations ± standard error.  
- • : Milli-Q water, ■ : 1.0 % Hyp aqueous solution, and ▲ : 1.0 % P₁-(N)-Hyp
CONCLUSION

The reaction of Hyp with $P_{3m}$ gave $N$-triphosphoryl hydroxyproline ($P_3$-(N)-Hyp, product 1), cyclic phosphate derivative ($P_3$-(N,O)-Hyp), $N$-phosphoryl hydroxyproline ($P_3$-(N)-Hyp, product 2), and $N$-(N-phosphonohydroxyprolyl) hydroxyproline ($P_3$-(N)-Hyp). At a molar ratio of Hyp : $P_{3m} = 2$ (1.0 M) : 1 (0.5 M), pH 13, and 25 ºC, the main product was found to be $P_3$-(N)-Hyp.

The moisture retention of the $P_3$-(N)-Hyp can be increased more effectively than that of Hyp alone. These results suggest that the synthesis of novel anionic derivative of Hyp would be expected to lead to increased application of Hyp phosphate.

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