INTRODUCTION OF PHOSPHATE GROUP INTO β -ARBUTIN BY CYCLO-TRIPHOSPHATE

Hideko Maeda^{1*}, Mako Katsushiro¹,
Hiroyuki Nariai², Hirokazu Nakayama¹

(i-hideko@kobepharma-u.ac.jp)

¹Department of Functional Molecular Chemistry, Kobe Pharmaceutical University, 4-19-1 Motoyamakita-machi, Higashinada-ku, Kobe 658-8558 Japan.

²Department of Chemical Science and Engineering, Faculty of Engineering, Kobe University, 1-1 Rokkodai-cho, Nada-ku, Kobe 657-8501 Japan.

Keywords: Phosphorylation; β -Arbutin; *cyclo*-Triphosphate; HPLC; ³¹P NMR

Abstract: Phosphorylation of β -arbutin has been achieved using inorganic *cyclo*-triphosphate (P_{3m}) in aqueous solution. The optimum condition for the phosphorylation of β -arbutin with P_{3m} is β -arbutin : P_{3m} = 0.1 mol/L : 0.5 mol/L, pH 11 and 40°C. 4-Triphospho- β -arbutin was synthesized with the yield of 90%. The reaction mechanism of β -arbutin with P_{3m} was discussed. We have successfully introduced the triphosphate group in one molecule. Phosphorylated β -arbutin is expected that the permeability of β -arbutin into the skin will be increased by introducing a phosphate group.

(Received Jul 7, 2017; Accepted Aug 2, 2017)

INTRODUCTION

β -Arbutin (4-hydroxyphenyl- β -D-glucopyranoside) is found in the leaves of bearberry, blueberry, and pear trees¹. It inhibits melanin synthesis by inhibition of tyrosinase activity^{2,3}. Therefore, β -arbutin is blended to cosmetics as a skin lighting agent⁴. It has also been reported that it has a diuretic action and a urinary tract sterilizing action, and it is regarded as a urinary tract antiseptic⁵. However, the usage is restricted because of its poor water solubility. β -Arbutin is expected to possess higher solubility by the introduction of phosphoryl group.

Duarte et al.⁶ reported that L-ascorbyl-2-phosphate introduced to phosphate group in L-ascorbic acid was non-toxic vitamin C analogue. Kameyama and coworkers indicated that L-ascorbyl-2-phosphate was absorbed percutaneously and retained in the skin⁷. Kobayashi et al. demonstrated that L-ascorbyl-2-phosphate was hydrolyzed and converted to ascorbic acid by acidic phosphatase in lysosomes and alkaline phosphatase in the serum⁸. Then, phosphorylated β -arbutin is expected that the permeability of β -arbutin into the skin will be increased by introducing a phosphate group.

As shown in Fig. 1, sodium *cyclo*-triphosphate, $Na_3P_3O_9$ (P_{3m}) is triphosphate with six-membered ring structure. P_{3m} has been used for phosphorylating agent since Quimby and Flautt⁹ produced amidotriphosphate in 1958. Because of its unique structure, biologically important

compounds having an amino or a hydroxyl group can be phosphorylation by P_{3m} ^{10,11}. Also, the phosphorylation reactions were proceeded in aqueous solution by one-step.

In this study, we examined the phosphorylation reaction of β -arbutin and P_{3m} to synthesize a novel β -arbutin containing phosphate groups.

sodium *cyclo*-triphosphate
(P_{3m})

β -arbutin
(4-hydroxyphenyl- β -
D-glucopyranoside)

FIGURE 1 Structures of sodium *cyclo*-triphosphate (P_{3m}) and β -arbutin

EXPERIMENTAL

Materials and methods

Sodium *cyclo*-triphosphate ($Na_3P_3O_9$, P_{3m}) was purchased from BK Giulini (Ludwigshafen, German). Sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) was purchased from Sigma-Aldrich Chemical Co. (St. Louis, USA). β -Arbutin and other reagents were purchased from Tokyo Kasei Chemicals (Tokyo, Japan) and Wako Pure Chemical Industries, Ltd. (Osaka, Japan), respectively.

HPLC analysis was performed on a JASCO

PU-2080i system (JASCO, Tokyo, Japan), using a InertSustain C18 column (250×6.0 mm i.d., 5 μ m, GL Science, Tokyo, Japan) maintained at 40 °C. Isocratic elution using a 20:80 (v/v) methanol/H₂O solution was employed at a flow rate of 0.25 ml/min with UV detection at 254 nm. The amount of sample injected was 100 μ L. The system control, data collection, and data analysis were carried out by JASCO-ChromNAV system (Version 1.18. 03, JASCO, Tokyo, Japan).

³¹P NMR spectra with and without broad band ¹H decoupling and ³¹P-¹H heteronuclear multiple bond correlation spectroscopy (HMBC), and ¹H-¹H correlation spectroscopy (COSY) 2D NMR spectra were obtained with a Bruker Ascend 600 MHz NMR spectrometer (Billerica of Massachusetts, USA) using 85 % H₃PO₄ solution in D₂O as an external standard.

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

RESULTS AND DISCUSSION

Reaction of β -arbutin with P_{3m}

The phosphorylation of β -arbutin with P_{3m} was performed in aqueous solution. Figure 2 shows the HPLC profiles for the reaction solution of β -arbutin (0.1 mol/L) and P_{3m} (0.5 mol/L) incubated at pH 11 at 40 °C.

The peaks at a retention time of about 9 min increased gradually with reaction time, predicting the product of β -arbutin. From the results of NMR and MS data as described later, the peaks at 9 min were found to be 4-triphospho- β -arbutin (product) and the peaks at 11 min were by-product. Although by-product could not be identified only by HPLC, it was found to be diphospho- or monophospho- β -arbutin¹². The other chromatographic peaks at 14 min were assigned to β -arbutin.

FIGURE 2 HPLC profiles of the reaction solution of β -arbutin (0.1 mol/L) with P_{3m} (0.5 mol/L) at pH 11 and 40 °C

The yield of 4-triphospho- β -arbutin increased gradually with reaction time to reach the maximum of 94 % after 17 days. The yield remained constant until 25 d and that of by-product did not increase.

Table 1 summarizes the yield of 4-triphospho- β -arbutin under various reaction conditions. At 25 °C and pH 10, the yield of product was 13, 46, and 57 % in a molar ratio of β -arbutin : P_{3m} = 0.1 mol/L : 0.1 mol/L, 0.1 mol/L : 0.3 mol/L, and 0.1 mol/L : 0.5 mol/L, respectively. The yield of product increased with the increase of initial concentration of P_{3m}. The solubility of P_{3m} is 0.5 mol/L, therefore, a molar ratio of β -arbutin : P_{3m} = 0.1 mol/L : 0.5 mol/L is preferable.

At a molar ratio of β -arbutin : P_{3m} = 0.1 mol/L : 0.5 mol/L and 25 °C, the yield of product was 26 % at pH 8, 35 % at pH 9, 57 % at pH 10, 64 % at pH 11, and 70 % at pH 12. Although the yield of triphospho- β -arbutin improved as the pH increased, those of diphospho- or monophospho- β -arbutin also increased at pH 12. Therefore, pH 11 is preferable.

At a molar ratio of β -arbutin : P_{3m} = 0.1 mol/L : 0.5 mol/L and pH 11, the yield of product was 51 % at 10 °C, 67 % at 25 °C, and 94 % at 40 °C. Although the yield of triphospho- β -arbutin improved as the temperature increased, those of diphospho- or monophospho- β -arbutin also increased at 60 °C. Therefore, preferable temperature is 40 °C.

Consequently, the optimum condition for the phosphorylation of β -arbutin with P_{3m} is β -arbutin : P_{3m} = 0.1 mol/L : 0.5 mol/L, pH 11 and 40 °C.

TABLE 1 Yields of 4-triphospho- β -arbutin

Reaction conditions					
Conc. (mol/L)		pH	Temp. (°C)	Time (d)	Yield (%)
β -arbutin	P _{3m}				
0.1	0.1	10	25	27	13
0.1	0.3	10	25	13	46
0.1	0.5	10	25	23	57
0.1	0.5	12	25	9	70
		11	25	10	64
		10	25	13	57
		9	25	24	35
		8	25	22	26
0.1	0.5	11	10	23	51
		11	25	17	67
		11	40	17	94

The procedure for the isolation of 4-triphospho- β -arbutin

Product (4-triphospho- β -arbutin) was synthesized by dissolving β -arbutin (1.3613 g, 0.5 mol/L) and P_{3m} (1.5294 g, 0.5 mol/L) in H₂O (20 mL) at 40 °C, and adjusting the solution to pH 11 by adding 6 M NaOH solution. After seven days, the yield of the 4-triphospho- β -arbutin due to the peak at 9 min by HPLC was 62 %. Then the reaction solution was

adjusted to pH 7. Although the phosphorylated β -arbutin undergo hydrolysis to β -arbutin and triphosphate with the passage of time at 25 °C and high pH, it is stable at pH 7.

The separation of product was accomplished by anion-exchange chromatography on a 2×80 cm column filled with Dowex 1-X2 resin (100–200 mesh, Cl⁻ form). Elution was carried out with distilled water until no further β -arbutin appeared. Then elution was carried out with aqueous 0.3 mol/L KCl. Fractions of 50 mL were collected during the elution process, and those fractions obtained during the volume range of 400 to 450 mL (during which the product was obtained) were analyzed via HPLC. Then, each fraction was concentrated via freeze-drying. For the purpose of desalting, an aqueous solution of the concentrate was passed over a PD-10 column (GE Healthcare UK Ltd, Little Chalfont, England). Each 0.5 mL fraction was measured by HPLC, and the fractionated solutions containing only 4-triphospho- β -arbutin was freeze-dried.

An aqueous solution of the concentration of 0.5 mg/mL was measured by HPLC. 4-Triphospho- β -arbutin was 90 % in purity. The ESI-MS spectrum of aliquot solution showed the molecular ion for the deprotonated 4-triphospho- β -arbutin.

4-Triphospho- β -arbutin : ¹H NMR (D₂O) δ : 7.1695 (2H, m, H3, H5), 7.0415 (2H, m, H2, H6), 5.0085 (1H, d, $J_{H1',H2'} = 7.8$ Hz, H-1'), 3.4695 (1H, dd, $J_{H1',H2'} = 7.8$ Hz, $J_{H2',H3'} = 9.6$ Hz, H-2'), 3.527 (1H, dd, $J_{H2',H3'} = 9.6$ Hz, $J_{H3',H4'} = 9.0$ Hz, H-3'), 3.417 (1H, dd, $J_{H3',H4'} = 9.0$ Hz, $J_{H4',H5'} = 9.6$ Hz, H-4'), 3.533 (1H, m, $J_{H4',H5'} = 9.6$ Hz, $J_{H5',H6'} = 1.8$ Hz, $J_{H5',H6'} = 5.4$ Hz, H-5'), 3.843 (1H, dd, $J_{H5',H6'} = 1.8$ Hz, $J_{H6',H6''} = 12.6$ Hz, H-6'), 3.676 (1H, dd, $J_{H5',H6''} = 5.4$ Hz, $J_{H6',H6''} = 12.6$ Hz, H-6''). ³¹P NMR (D₂O) δ : -15.03 (1P, d, $J_{P_{\alpha},P_{\beta}} = 19.2$ Hz, P _{α}), -21.85 (1P, dd, $J_{P_{\alpha},P_{\beta}} = 19.2$ Hz, $J_{P_{\beta},P_{\gamma}} = 19.7$ Hz, P _{β}), -6.32 (1P, d, $J_{P_{\beta},P_{\gamma}} = 19.7$ Hz, P _{γ}).

ESI-MS m/z : 510.98859 Calcd [C₁₂H₁₉O₁₆P₃]⁻ Found : 510.98222.

Assignment of 4-triphospho- β -arbutin

To identify the 4-triphospho- β -arbutin in the phosphorylation of β -arbutin with P_{3m}, the isolation procedure as described above was achieved. As shown in Fig. 3(c), the ³¹P NMR spectrum with ¹H decoupling showed a doublet at -6.32 ppm (P _{γ}), a doublet at -15.03 ppm (P _{α}), and a doublet of doublets at -21.85 ppm (P _{β}). These peaks are characteristic for triphosphate esters^{13,14}. The doublet at -15.03 ppm did not change with ¹H non-decoupling in Fig. 3 (b). It indicates that phosphorylation occurs in places not affected by protons¹⁵. That is, it was presumed that it occurred not on β -D-glucose moiety

but on phenolic OH group of β -arbutin. A singlet at 2.19 ppm is assigned to monophosphate (P₁).

By comparison with Figure 3(a) and 3(b), it found that diphosphate (P₂), triphosphate (P₃), and P_{3m} in the reaction solution were separated after isolation. The purity of product was determined to be 94 % from the integration of ³¹P NMR signals. The remaining 6 % is the P₁ and by-product.

FIGURE 3 ³¹P NMR spectra of reaction solution (a) and isolation product (b and c)

(a) ¹H non-decoupling spectrum of the reaction solution, (b) ¹H non-decoupling spectrum of the isolation product, (c) ¹H decoupling spectrum of the isolation product

In order to confirm the site of phosphorylation, ³¹P-¹H heteronuclear multiple bond correlation spectroscopy (HMBC) 2D NMR spectrum was measured. Two correlations of P _{α} at -15.03 ppm of product and ¹H signal at 7.17 and 7.04 ppm were observed. The multiplets at 7.17 and 7.04 ppm could be assigned to (H-3 and H-5) and (H-2 and H-6) of the product. The down-field shift from 6.83 and 6.47 ppm due to β -arbutin itself to 7.17 and 7.04 ppm indicates the phosphorylation of β -arbutin with P_{3m}¹⁶. This assignment was confirmed by ¹H-¹H COSY NMR spectrum.

Furthermore, since there were no peaks correspond to β -arbutin in the ¹H NMR data, it could be seen that the raw material was removed by isolation.

FIGURE 4 ^{31}P - ^1H HMBC 2D-NMR spectrum of isolation product

Figure 5 shows ^1H - ^1H COSY NMR spectra of isolation product. All protons on a β -D-glucopyranosyl unit will have a correlation with all other protons on the same β -D-glucopyranosyl unit. Thus, the peak at 5.01, 3.84, 3.68, 3.53, 3.53, 3.42, and 3.47 ppm could be assigned to H-1', H-6', H-6'', H-3', H-5', H-2' and H-4' proton of β -D-glucopyranosyl unit, respectively. The chemical shifts of all protons on a β -D-glucopyranosyl unit did not change before and after phosphorylation. This suggests that β -D-glucopyranosyl of β -arbutin have not been phosphorylated. Therefore, we concluded that 4-OH on phenyl group reacted selectively in spite of having five hydroxyl groups.

FIGURE 5 ^1H - ^1H COSY NMR spectra of isolation product

In order to confirm, electrospray ionization mass spectrometry (ESI-MS) was performed. The ESI-MS spectrum shows the molecular ion peak due to triphospho- β -arbutin ($m/z = 510.98859$).

Reaction mechanism of β -arbutin with P_{3m}

The reaction of β -arbutin with P_{3m} may be explained by the following mechanism. P_{3m} with six-membered structure consists phosphorus and oxygen atom. Since the electronegativity of oxygen atom is higher than phosphorus atom, the electron pair is attracted to the oxygen atom and the electron density of phosphorus atom becomes smaller and becomes $\text{P}^{\delta+}$. At pH 12, $\text{P}^{\delta+}$ is easily attacked by nucleophilic reagents such as amino acid¹⁷ and alcohol¹⁸.

In the present study, the lone electron pair on the hydroxyphenyl group of β -arbutin nucleophilically attacks a phosphorus atom of P_{3m} to give 4-triphospho- β -arbutin. The hydroxylphenyl group of β -arbutin was phosphorylated selectively. Why did not it respond to hydroxyl groups of D-glucopyranosyl unit? The value of $\text{p}K_1$ of D-glucose¹⁹ is 12.28, whereas that of hydroxyphenyl group²⁰ is 9.0. Therefore, hydroxylphenyl group of β -arbutin was phosphorylated selectively.

SCHEME 1 Reaction of β -arbutin with P_{3m}

Conclusion

In the reaction of β -arbutin with P_{3m} , 4-triphospho- β -arbutin was synthesized in the yield of 90 %. The purity of 4-triphospho- β -arbutin was determined to be 94% from the integration of ^{31}P NMR signals. We have successfully introduced the triphosphate group in one molecule. This result suggests that phosphorylated β -arbutin is expected that the permeability of β -arbutin into the skin will be increased by introducing a phosphate group.

ACKNOWLEDGEMENTS

The authors thank associate professor C. Tode of Kobe Pharmaceutical University for the measurements of ^{31}P , ^{31}P - ^1H HMBC, ^1H - ^1H COSY, ^1H - ^1H TOCSY 2D NMR spectra. This work was partially supported by the Promotion and Mutual Aid Corporation for Private School of Japan.

REFERENCES

1. *The Merck Index 12th ed.* Edited by S. Budavari (Merck and Co., Whitehouse Station, N.J., 1996), p. 816.
2. M. Nakajima, I. Shinoda, Y. Fukuwatari, H. Hayasawa, *Pigment Cell Res.*, **11**, 12 (1998).
3. Y.-J. Lim, E.H. Lee, T.H. Kang, S.K. Ha, M.S. Oh, S.M. Kim, T.-J. Yoon, C. Kang, J.-H. Park, S.Y. Kim, *Arch. Pharm. Res.*, **32**, 367 (2009).
4. S. Pavez, M. Kang, H.-S. Chung, C. Cho, M.-C. Hong, M.-K. Shin, H. Bae, *Phytotherapy Res.*, **20**, 921 (2006).
5. G. Schindler, U. Patzak, B. Brinkhaus, A. von Nieciecki, J. Wittig, N. Kraehmer, I. Gloeckl, M. Veit, *J. Clin. Pharmacol.*, **42**, 920 (2002).
6. T.L. Duarte, I.E. Almeida, in *Handbook of diet, nutrition and skin*. Edited by V.R. Preedy (Springer, Berlin, 2012), pp. 115-127.
7. K. Kameyama, C. Sakai, S. Kondoh, K. Yonemoto, S. Nishiyama, M. Tagawa, T. Murata, T. Ohnuma, J. Quigley, A. Dorsky, D. Bucks, K. Blanock, *J. Am. Acad. Dermatol.*, **34**, 29 (1996).
8. S. Kobayashi, M. Takehana, M. Kanke, S. Itoh, E. Ogata, *Photochem. Photobiol.*, **67**, 669 (1998).
9. O.T. Quimby, T.J. Flautt, *Z. Anorg. Allg. Chem.*, **296**, 220 (1958).
10. H. Maeda, T. Kusuhara, M. Tsuhako, H. Nakayama, *Chem. Pharm. Bull.*, **59** (12), 1447 (2011).
11. H. Maeda, H. Kanazawa, M. Komatsu, H. Nariai, H. Nakayama, *Phosphorus Res. Bull.*, **30**, 1 (2015).
12. H. Inoue, M. Watanabe, H. Nakayama, M. Tsuhako, *Chem. Pharm. Bull.*, **48**, 802 (2000).
13. H.J. Vogel, in *Phosphorous-31 NMR Principles and Applications*. Edited by D.G. Gorenstein (Academic Press, New York, 1984), p.114.
14. M. Cohn, T.R. Hughes Jr., *J. Biol. Chem.*, **235**, 3250 (1960).
15. H. Inoue, Y. Baba, H. Miyajima, M. Tsuhako, *Chem. Pharm. Bull.*, **40**, 3127 (1992).
16. H. Inoue, N. Tone, H. Nakayama, M. Tsuhako, *Chem. Pharm. Bull.*, **50**, 1453 (2002).
17. J. Rabinowitz, *Helv. Chim. Acta*, **52**, 2663 (1969).
18. W. Feldmann, *Chem. Ber.*, **100**, 3850 (1967).
19. J.A. Dean, in *Lange's Handbook of Chemistry 15th ed.* Edited by J.A. Dean (McGraw-Hill, New York, 1999), 8.48.
20. *The Merck Index 12th ed.* Edited by S. Budavari (Merck and Co., Whitehouse Station, N.J., 1996), p. 7390.