PHOSPHORYLATION OF CYTARABINE WITH *CYCLO*-TRIPHOSPHATE IN AQUEOUS SOLUTION

Takuma Kusuhara, Hideko Maeda*, Mitsutomo Tsuhako,
Hirokazu Nakayama

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

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

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Phosphorylation of cytarabine has been achieved using inorganic *cyclo*-triphosphate ($\text{P}_{3\text{m}}$) in aqueous solution. The optimum condition for the phosphorylation of cytarabine with $\text{P}_{3\text{m}}$ is cytarabine : $\text{P}_{3\text{m}}$ = 1 : 10, pH 12 and 25 °C. Cytarabine 5'-triphosphate, cytarabine 3'-triphosphate, and cytarabine 2'-triphosphate were synthesized with the total yield of more than 75 %. The phosphorylated products of cytarabine were stable in neutral and alkaline solution. The reaction mechanism of cytarabine with $\text{P}_{3\text{m}}$ was discussed.

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INTRODUCTION

Synthesis and biological evaluation of nucleotide analogs have been continued for capability of delivering the corresponding nucleotides¹⁻³. 9- β -D-Arabinofuranosyl-cytosine (cytarabine or ara-C) has been applied to many kinds of cancer, but a strong side effect is a serious problem. Cytarabine is expected to possess higher solubility by the introduction of phosphoryl group. So it would be drained outside of the body quickly and then a side effect would be reduced.

Sodium *cyclo*-triphosphate, $\text{Na}_3\text{P}_3\text{O}_9$ ($\text{P}_{3\text{m}}$), is a simple and efficient phosphorylating agent. One of the authors reported the phosphorylation of nucleosides⁴ and nucleotides⁵ by $\text{P}_{3\text{m}}$. The 2'- and 3'-OH groups of the β -D-ribofuranosyl unit on nucleosides and nucleotides were selectively phosphorylated. The main phosphorylated products were 2'- and 3'-monophosphate esters of the nucleosides and nucleotides. However, 2'-deoxyadenosine and 3'-deoxyadenosine could not be phosphorylated at all because of the absence of hydroxyl groups at 2'- or 3'-position of the β -D-ribose. Also, the reactivity of nucleosides and nucleotides strongly depend on the configuration of the hydroxyl groups at C-2' and C-3'. It is important to apply this selective phosphorylation reaction to other related nucleosides and nucleotides.

In this study, the reaction of cytarabine (the hydroxyl groups at C-2' and C-3' are in *trans* position) with $\text{P}_{3\text{m}}$ was examined to develop a selective phosphorylation.

RESULTS AND DISCUSSION

Reaction of cytarabine with $\text{P}_{3\text{m}}$

The phosphorylation of cytarabine with $\text{P}_{3\text{m}}$ was performed in aqueous solution. Figure 1 shows the HPLC profiles for the reaction solution of cytarabine (0.06 M) and $\text{P}_{3\text{m}}$ (0.6 M) incubated at pH 12 at 25 °C. The peak of the product appeared at a retention time of about 14 and 16 min. The total yield of products increased gradually with reaction time to reach the maximum of 75 % after 25 d and was constant until 60 d without hydrolysis of product.

FIGURE 1 HPLC profiles of the reaction solution of cytarabine : $\text{P}_{3\text{m}}$ = 0.06 M : 0.6 M at pH 12 and 25 °C

Table 1 summarizes the total yield of products **1** – **3** under various reaction conditions. Although the HPLC profiles showed two peaks due to the reaction product at the retention time of 14 and 16 min, the

products are three triphosphate esters (**1**, **2**, and **3**) as evidenced by the ^{31}P NMR data.

At a pH 12, 25 °C, and a molar ratio of cytarabine : $\text{P}_{3\text{m}}$ = 1 : 20 (0.03 M : 0.6 M), 1 : 10 (0.06 M : 0.6 M), 1 : 5 (0.12 M : 0.6 M), or 1 : 2 (0.3 M : 0.6 M), the total yield of products **1** - **3** were 73, 75, 67, and 60 %, respectively. One of us already reported that the reaction of adenosine⁴ and $\text{P}_{3\text{m}}$. The molar ratio for the phosphorylation of adenosine with $\text{P}_{3\text{m}}$ is adenosine : $\text{P}_{3\text{m}}$ = 0.05 M : 0.5 M, that is, $\text{P}_{3\text{m}}$ excess. Therefore, a molar ratio of cytarabine : $\text{P}_{3\text{m}}$ = 1 : 10 is preferable. At a molar ratio of 1 : 10 and 25 °C, the total yield of products **1** - **3** were 66 % at pH 12.5, 75 % at pH 12, and 13 % at pH 10. It was thought that the reaction is easier than at pH 12 rather than pH 10 because proton of OH group of arabinofuranose unit is easy to dissociate. Therefore, pH 12 is suitable pH. At a molar ratio of 1 : 10 and pH 12, the total yield of products **1** - **3** were 75 % at 25 °C, 64 % at 40 °C, and 20 % at 70 °C. The yield at 25 °C remained constant after 56 d without hydrolysis of triphosphate derivative. However, the yield at 40 °C increased with the reaction time, reaching 64 % after 19 d, and then decreased gradually. Therefore, 25 °C is preferable temperature. Consequently, the optimum condition for the phosphorylation of cytarabine with $\text{P}_{3\text{m}}$ is cytarabine : $\text{P}_{3\text{m}}$ = 1 : 10, pH 12 and 25 °C.

TABLE 1 Total yield of products **1** - **3**

Conc. (M)		Reaction conditions			Total Yield (%)
Cytarabine	$\text{P}_{3\text{m}}$	pH	Temp. (°C)	Time (d)	
0.03	0.6	12	25	15	73
0.06	0.6	10	25	33	13
		12	25	25	75
		12.5	25	14	66
		12	40	19	64
		12	70	8	20
0.12	0.6	12	25	17	67
0.30	0.6	12	25	15	60

To identify the reaction product in the phosphorylation of cytarabine with $\text{P}_{3\text{m}}$, ^{31}P and ^1H NMR spectra were measured. As shown in Fig. 2, the ^{31}P NMR spectrum with ^1H -nondecoupling of reaction solution of cytarabine with $\text{P}_{3\text{m}}$ indicated that the products are three triphosphate esters (**1**, **2**, and **3**), although the HPLC profiles showed two peaks due to the reaction product. The ^1H non-decoupling spectrum had a doublet of triplet at -9.6 ppm, a doublet of doublet at -10.6, and a doublet of doublet at -11.5 ppm. Their peaks became doublet on ^1H decoupling, which is characteristic for P_α . The doublets at -4.57, -4.62, and -4.74 ppm were assigned to the end phosphorus atom (P_γ). The other doublet of doublet around -20.0 ppm is the characteristic of the middle phosphorus atom (P_β) of triphosphate. A previous work indicated that the

phosphorylation product of aminoalcohols⁶ or carbohydrates⁷⁻¹³ with $\text{P}_{3\text{m}}$ is triphosphate esters or aminoalcohols or carbohydrates with a $-\text{O}-\text{P}_\alpha-\text{O}-$ bond. These products show a characteristic P_α signal around -10 ppm in their ^{31}P NMR spectra. In order to assign the site of phosphorylation, ^{31}P - ^1H heteronuclear multiple bond correlation spectroscopy (HMBC) 2D-NMR spectrum was measured.

Figure 3 shows the ^{31}P - ^1H HMBC 2D-NMR spectrum of products **1** - **3**. The peaks at -9.6, -4.74, and -20.0 ppm of the ^{31}P NMR spectrum were assigned to product **1**. A correlation of P_α at -9.6 ppm of product **1** and ^1H signal at 4.15 and 4.18 ppm. The multiplets at 4.15 and 4.18 ppm could be assigned to $\text{H}_\text{A}-5'$ and $\text{H}_\text{B}-5'$ of product **1**. The down-field shift from 3.73 and 3.81 ppm due to cytarabine itself to 4.15 and 4.18 ppm indicates the phosphorylation of cytarabine with $\text{P}_{3\text{m}}$. This assignment was confirmed by ^1H - ^1H COSY NMR spectrum. The $^3J_{\text{P}_\alpha\text{H}5'}$ value was 5.4 Hz, which is obtained from the ^{31}P NMR data. Therefore product **1** was verified to be cytarabine 5'-triphosphate.

FIGURE 2 ^{31}P NMR spectra of reaction solution of cytarabine : $\text{P}_{3\text{m}}$ = 0.06 M : 0.6 M at pH 12, 25 °C, and 25 d

FIGURE 3 ^{31}P - ^1H 2D-NMR spectrum of products **1** - **3** cytarabine : $\text{P}_{3\text{m}}$ = 0.06 M : 0.6 M, pH 12, 25 °C, 25 d

The other peaks at -10.6, -4.62, and -20.0 ppm of the ^{31}P NMR spectrum were assigned to product **2**. A correlation of P_α at -10.6 ppm of product **2** and ^1H signal at 4.53 ppm is shown in Fig. 3. The doublet

of doublets of doublets at 4.53 ppm was assigned to H-3' based on ^1H - ^1H COSY NMR spectrum. The down-field shift from 4.01 ppm due to cytarabine itself to 4.53 ppm indicates the phosphorylation of cytarabine with $\text{P}_{3\text{m}}$. Therefore product **2** was verified to be cytarabine 3'-triphosphate.

The other peaks at -11.5, -4.57, and -20.0 ppm of the ^{31}P NMR spectra were assigned to product **3**. A correlation of P_α at -11.5 ppm of product **3** and ^1H signal at 4.67 ppm is shown in Fig. 3. The multiplet at 4.67 ppm was assigned to H-2' based on ^1H - ^1H COSY NMR spectrum. The down-field shift from 4.29 ppm due to cytarabine itself to 4.67 ppm indicates the phosphorylation of cytarabine with $\text{P}_{3\text{m}}$. Therefore product **3** was verified to be cytarabine 2'-triphosphate.

Reaction mechanism of cytarabine with $\text{P}_{3\text{m}}$

The reaction of cytarabine with $\text{P}_{3\text{m}}$ may be explained by the following mechanism. At pH 12, $\text{P}_{3\text{m}}$ is easily attacked by nucleophilic reagents such as amines⁶, amino acids¹⁴ and cyclodextrins¹³. In the present study, the lone electron pair on the hydroxyl group of cytarabine nucleophilically attacks a phosphorus atom of $\text{P}_{3\text{m}}$ to cleave its six-membered ring (Scheme 1). The 2'-OH, 3'-OH, or 5'-OH of β -D-arabinofuranose unit reacts with $\text{P}_{3\text{m}}$ to form cytarabine 5'-triphosphate (**1**), cytarabine 3'-triphosphate (**2**), and cytarabine 2'-triphosphate (**3**). Previous works showed that the hydroxyl groups at C-5' of nucleosides and nucleotides could not react with $\text{P}_{3\text{m}}$, and C-2' and C-3' of them which are in *cis* position react with $\text{P}_{3\text{m}}$ to form monophosphate esters^{4,5}. On the other hand, cytarabine, in which the hydroxyl groups at C-2' and C-3' are in *trans* position reacts with $\text{P}_{3\text{m}}$ to form triphosphate esters (**1**, **2**, and **3**).

SCHEME 1 Reaction mechanism of cytarabine with $\text{P}_{3\text{m}}$

Stability of phosphorylated cytarabine

Figure 4 shows the changes of the amount of phosphorylated products at pH 2, 7, and 12. The products were synthesized under the reaction condition of pH 12, 25 °C, and a molar ratio of cytarabine : $\text{P}_{3\text{m}}$ = 0.06 M : 0.6 M for 25 d. At pH 2, phosphorylated products were decomposed gradually and decreased to 38 % after 50 days. On the other hand, phosphorylated products were stable at pH 12 for 30 days, but after 30 d they were decomposed gradually. However, it was stable at pH 7 for 56 days. Therefore, phosphorylated products of cytarabine were stable in neutral and alkaline solution.

FIGURE 4 Stability of phosphorylated cytarabine synthesized under the condition of cytarabine : $\text{P}_{3\text{m}}$ = 0.06 M : 0.6 M, pH 12, 25 °C, and 25 d

■ : pH 2, ■ : pH 7, ■ : pH 12

Conclusion

In the reactions of cytarabine with $\text{P}_{3\text{m}}$, cytarabine 5'-triphosphate (**1**), cytarabine 3'-triphosphate (**2**), and cytarabine 2'-triphosphate (**3**) were synthesized in the total yield of 75 %. These results suggest that the synthesis of novel anionic molecules of cytarabine containing phosphate groups is a promising area for the application to mononucleotide prodrug.

EXPERIMENTAL

Materials and methods

cyclo-Triphosphate, $\text{Na}_3\text{P}_3\text{O}_9$ ($\text{P}_{3\text{m}}$), was prepared according to the previous paper¹⁵. Sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) was purchased from Sigma-Aldrich Chemical Co. (St. Louis, USA). Cytarabine and other reagents were purchased from Wako Chemicals (Osaka, Japan).

^{31}P NMR spectra with and without broad band ^1H decoupling and ^{31}P - ^1H 2D HMBC spectra were obtained with a Varian INOVA-500 spectrometer using 85 % H_3PO_4 as an external standard.

HPLC analysis was carried out with a JASCO HPLC system consisting of a PU-2080i pump, a co-2060 Plus column oven, a V-530 UV-visible detector (JASCO, Japan). A column (150 × 6.0 mm I.D.) packed with the polystyrene-based

anion-exchanger (TSK gel, SAX, 5 μ m, TOSOH, Japan) was used. The column temperature was maintained at 40 °C. An isocratic elution technique using 0.33 M potassium chloride solution was employed. The flow rate of the eluent was 1.0 mL min⁻¹. The UV absorbance of the effluent was monitored continuously at 271 nm. The system control, data collection, and data analysis were carried by JASCO-BORWIN system.

The semipreparative HPLC was carried out with a JASCO HPLC system consisting of a PU-2080i pump, a co-2060 Plus column oven, a Ubest-30 UV-visible detector (JASCO, Japan). An ODS-3 column (250 $\times$ 10.0 mm I.D., 5 μ m, GL Science, Japan) was used and the column temperature was maintained at 40 °C. The amount of sample injection was 1 mL. An isocratic elution technique using 4 % methanol-0.002 M ammonium acetic acid solution was employed. The flow rate of the eluent was 4.0 mL min⁻¹. The UV absorbance of the effluent was monitored continuously at 271 nm. The system control, data collection, and data analysis were carried by JASCO-ChromNAV system.

The procedure for the syntheses of products 1 - 3

The reaction of cytarabine (0.06 M, 10 mL) with P_{3m} (0.6 M, 10 mL) was carried out at pH 12 by adding 6 M sodium hydroxide aqueous solution at 25 °C. After 25 d, the reaction solution was subjected to semipreparative HPLC to yield products 1 - 3. It was collected about 6 min from about 3 min at retention time. 20 times injection produced about 200 mL preparative solution. Then, the solution was concentrated to 10 mL. For the purpose of desalting, an aqueous solution of the concentrate was passed over an InartSep C18 column (GL Science, Japan). Each 2.4 mL fraction was measured by HPLC, and the fractionated solution containing only products 1 - 3 were dried in vacuo.

Cytarabine 5'-triphosphate (product 1) : ¹H NMR (D₂O) δ : 6.13 (1H, d, $J_{H1',H2'}$ = 5.0 Hz, H-1'), 4.33 (1H, dd, $J_{H1',H2'}$ = 5.0 Hz, $J_{H2',H3'}$ = 5.5 Hz, H-2'), 4.19 (1H, dd, $J_{H2',H3'}$ = 5.5 Hz, $J_{H3',H4'}$ = 5.5 Hz, H-3'), 4.00 (1H, m, H-4'), 4.15 (1H, m, H_A-5'), 4.18 (1H, m, H_B-5'), 7.79 (1H, d, $J_{H4,H5}$ = 7.5 Hz, H-4), 6.00 (1H, d, $J_{H4,H5}$ = 7.5 Hz, H-5). ³¹P NMR (D₂O) δ : -9.6 (1P, dt, $J_{P_{\alpha},P_{\beta}}$ = 19.0 Hz, $J_{P_{\alpha},H_A5'}$ = 5.4 Hz, $J_{P_{\alpha},H_B5'}$ = 5.4 Hz, P _{α}), -20.0 (1P, $J_{P_{\alpha},P_{\beta}}$ = 19.0 Hz, $J_{P_{\beta},P_{\gamma}}$ = 19.5 Hz, P _{β}), -4.74 (1P, d, $J_{P_{\beta},P_{\gamma}}$ = 19.5 Hz, P _{γ}).

Cytarabine 3'-triphosphate (product 2) : ¹H NMR (D₂O) δ : 6.11 (1H, d, $J_{H1',H2'}$ = 4.5 Hz, H-1'), 4.49 (1H, dd, $J_{H1',H2'}$ = 4.5 Hz, $J_{H2',H3'}$ = 2.5 Hz, H-2'), 4.53 (1H, ddd, $J_{H2',H3'}$ = 2.5 Hz, $J_{H3',H4'}$ = 4.0 Hz, $J_{P_{\alpha},H3'}$ = 9.0 Hz, H-3'), 4.15 (1H, m, H-4'), 3.77 (1H, dd, $J_{H4',H_A5'}$ = 4.0 Hz, $J_{H_A5',H_B5'}$ = 12.5 Hz, H_A-5'), 3.85 (1H, dd, $J_{H4',H_B5'}$ = 6.5 Hz, $J_{H_A5',H_B5'}$ = 12.5 Hz, H_B-5'), 7.70 (1H, d, $J_{H4,H5}$ = 7.5 Hz, H-4), 6.08 (1H, d,

$J_{H4,H5}$ = 7.5 Hz, H-5). ³¹P NMR (D₂O) δ : -10.6 (1P, dd, $J_{P_{\alpha},P_{\beta}}$ = 18.0 Hz, $J_{P_{\alpha},H3'}$ = 9.0 Hz, P _{α}), -20.0 (1P, $J_{P_{\alpha},P_{\beta}}$ = 18.0 Hz, $J_{P_{\beta},P_{\gamma}}$ = 18.5 Hz, P _{β}), -4.62 (1P, d, $J_{P_{\beta},P_{\gamma}}$ = 18.5 Hz, P _{γ}).

Cytarabine 2'-triphosphate (product 3) : ¹H NMR (D₂O) δ : 6.24 (1H, d, $J_{H1',H2'}$ = 3.0 Hz, H-1'), 4.67 (1H, m, H-2'), 4.39 (1H, m, H-3'), 3.95 (1H, m, H-4'), 3.74 (1H, m, H_A-5'), 3.80 (1H, m, H_B-5'), 7.61 (1H, d, $J_{H4,H5}$ = 7.5 Hz, H-4), 5.93 (1H, d, $J_{H4,H5}$ = 7.5 Hz, H-5). ³¹P NMR (D₂O) δ : -11.5 (1P, dd, $J_{P_{\alpha},P_{\beta}}$ = 19.0 Hz, $J_{P_{\alpha},H2'}$ = 10.3 Hz, P _{α}), -20.0 (1P, $J_{P_{\alpha},P_{\beta}}$ = 19.0 Hz, $J_{P_{\beta},P_{\gamma}}$ = 19.5 Hz, P _{β}), -4.57 (1P, d, $J_{P_{\beta},P_{\gamma}}$ = 19.5 Hz, P _{γ}).

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