Introduction

Nucleoside triphosphates (NTPs) have very important functions in cells, such as synthetic material for DNA or RNA, as an energy source, or as a transmitter. Therefore, γ-labeled 2'-deoxyoxynucleoside triphosphates (dNTPs) and NTPs are widely used tools in chemical biology, including in DNA sequencing, enzyme inhibition, prodrug systems, and the measurement of enzyme activities and other applications. The application of dNTP to DNA sequencing technology is attracting increasing interest. Hirao et al. previously reported that a PCR reaction via unnatural base pair formation by artificial nucleic acids was successful using γ-amido-dATP. This is an innovative method for promoting unnatural base pair formation by lowering the efficiency by which an enzyme forms natural triphosphates. In the synthesis of γ-amido-dATP, amines are introduced by a treatment with concentrated ammonia solution in water, while maintaining nucleobase selectivity; however, their incorporation efficiency by DNA polymerase was lower than that of dNTP. The results obtained showed the incorporation of these derivatives into the DNA primer while maintaining nucleobase selectivity; however, their incorporation efficiency by DNA polymerase was lower than that of dNTP. This is the first study to demonstrate the successful synthesis of four sets of γ-amido-dNTPs and clarify their properties.

Key words γ-amido-2'-deoxyoxynucleoside triphosphate; one-pot reaction; polymerase reaction

Results and Discussion

The syntheses of γ-amido-modified triphosphates are summarized in Chart 1. Commercially available dNTPs were treated with EDC·HCl in water, and concentrated ammonia solution was then added to the corresponding reaction mixtures. In the case of dATP and 2'-deoxycytidine 5'-triphosphate (dCTP), the starting material completely disappeared and a new peak was observed (Figs. 2A and 2B, respectively). After separation and identification, these compounds were the corresponding γ-amido-modified dATP (1) and γ-amido-modified dCTP (2). In the case of dGTP and thymidine 5'-triphosphate (TTP), the starting triphosphate material also completely disappeared after a 4-hour incubation; however, there were two major new peaks (Figs. 3A and 3B, upper part). After the separation of these peaks, the results of electrospay ionization (ESI)-high...
resolution (HR)-MS measurements indicated that one was the desired \(\gamma\)-amido-modified compound and the other was its EDC adduct, \(\gamma\)-amido-dGTP-EDC (C\(_{18}\)H\(_{33}\)N\(_9\)O\(_{12}\)P\(_3\) (M−H)\(^−\), Calcd. for: 660.1456. Found: 660.1479.) and \(\gamma\)-amido-TTP-EDC (C\(_{18}\)H\(_{34}\)N\(_6\)O\(_{13}\)P\(_3\) (M−H)\(^−\), Calcd. for: 635.1391. Found: 635.1371.). The 31P-NMR spectra of dGTP, \(\gamma\)-amido-dGTP and \(\gamma\)-amido-dGTP-EDC are depicted in Fig. 4. Apparently, by being converted to an amido-modified dGTP, the signal of

(a) EDC·HCl, water at 30 °C; (b) 28% ammonia solution at 30 °C, 31% and 25% in the one-pot reaction (for 1 and 2, respectively, after HPLC purification); (c) 10% NaOH aqueous at 50 °C, 41% and 29% in the one-pot reaction (for 3 and 4, respectively, after HPLC purification).

Chart 1. Syntheses of \(\gamma\)-Amido-Modified Triphosphates (1–4)

Fig. 2. HPLC Charts of the \(\gamma\)-Amidation Reaction of (A) dATP and (B) dCTP

HPLC conditions: column: Nacalai Tesque 5C18-AR-II 4.6 × 250 mm, oven 35 °C, UV monitor 254 nm, Flow rate 1.0 mL/min, Solvent A: 0.1 M TEAA buffer B: CH\(_3\)CN, B concentration 2 to 7%/20 min for dATP or 0 to 2%/20 min for dCTP.

Fig. 3. HPLC Charts of the \(\gamma\)-Amidation Reaction of (A) dGTP and (B) TTP

HPLC conditions: column: Nacalai Tesque 5C18-AR-II 4.6 × 250 mm, oven 35 °C, UV monitor 254 nm, Flow rate 1.0 mL/min, Solvent A: 0.1 M TEAA buffer B: CH\(_3\)CN, B concentration 2 to 7%/20 min.
γ-position was shifted downfield. On the other hand, no change in chemical shift was observed in the EDC adduct product compared with γ-amido-modified compound. This result means that EDC did not connect with phosphate group of the triphosphate. Moreover, the N1 of guanine and the N3 of uracil nucleobases form adducts with EDC under alkali conditions, therefore the predicted structure of EDC adduct of guanine nucleobase is shown in Fig. 4. When 10% NaOH solution in water was added to the reaction mixture and heated to 50 °C, EDC adducts were hydrolyzed to obtain the corresponding γ-amido-modified dGTP (3) and γ-amido-modified TTP (4) in the one-pot reaction (Figs. 3A and 3B, lower part). Using the same procedure, we successfully performed the one-pot synthesis of γ-amido-ATP (5), γ-amido-CTP (6), γ-amido-GTP (7), and γ-amido-uridine 5'-triphosphate (UTP) (8) using the corresponding NTPs as starting materials (Fig. 5).

We then examined the base selectivity of γ-amido-dNTPs in a single nucleotide primer extension reaction using 15-mer FAM-labeled primer DNA, 25-mer template DNA, and DNA polymerase (Fig. 6). We used Klenow fragment as a DNA polymerase, and the gel results obtained are shown in Figs. 6A and 6B. Natural dNTPs were incorporated into complementary nucleobases (Fig. 6A). In the case of γ-amido-dNTPs, the amount of the enzyme had to be increased and the reaction time prolonged; however, the same base selectivity as natural dNTP was observed (Fig. 6B). We then used a different type of DNA polymerase, Bst DNA polymerase, to confirm base selectivity (Figs. 6C, 6D). Although a large amount of the
The enzyme was required for the incorporation of \( \gamma \)-amido-dNTPs, base selectivity was not impaired even when different polymerases were used. We then obtained steady-state kinetic data from a single nucleotide primer extension reaction using the two polymerases, Klenow Fragment and Bst DNA polymerase. The results on steady-state kinetics (\( V_{\text{max}} \) and \( K_M \)) are summarized in Table 1. Regarding Klenow Fragment, the incorporation efficiencies (\( V_{\text{max}}/K_M \)) of \( \gamma \)-amido-dNTPs into complementary nucleobase-containing template DNA were approximately 10-fold lower, and approximately 100-fold lower for dGTP-NH\(_2\). Similarly, for Bst polymerase, the incorporation efficiencies of \( \gamma \)-amido-dNTPs were approximately 10-fold lower (Table 1, entries 9–16). These results indicated large \( K_M \) values for each \( \gamma \)-amido-dNTP, but also that the decreased incorporation efficiency of \( \gamma \)-amido-dNTPs was mainly due to a reduction in \( V_{\text{max}} \) values.

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**Table 1. Steady-State Kinetic Parameters Using Klenow Fragment and Bst DNA Polymerase**

| Entry | polymerase | dNTP | X | \( V_{\text{max}} \) (\% min\(^{-1}\)) | \( K_M \) (\( \mu \)M) | Efficiency | Ratio |
|-------|------------|------|---|------------------|-----------------|-----------|-------|
| 1     | Klenow Fragment exo\(^{-}\) | dGTP | dC | 9.71 (0.22)       | 0.72 (0.20)     | 14.3 \times 10^6 (4.21) | 143   |
| 2     | dGTP-NH\(_2\) | 0.59 (0.11) | 6.02 (0.26) | 0.10 \times 10^6 (0.02) | 10.8 |
| 3     | dATP | T | 0.51 (0.20) | 0.19 (0.22) | 3.57 \times 10^6 (1.87) | 10.8 |
| 4     | dATP-NH\(_2\) | 0.10 (0.02) | 0.36 (0.11) | 0.33 \times 10^6 (0.17) | 12.6 |
| 5     | dCTP | dG | 0.56 (0.14) | 0.21 (0.04) | 2.64 \times 10^6 (0.50) | 10.9 |
| 6     | dCTP-NH\(_2\) | 0.27 (0.09) | 1.31 (0.09) | 0.21 \times 10^6 (0.07) | 12.6 |
| 7     | TTP | dA | 0.87 (0.13) | 0.76 (0.22) | 1.16 \times 10^6 (0.18) | 14.5 |
| 8     | TTP-NH\(_2\) | 0.06 (0.01) | 0.27 (0.11) | 0.08 \times 10^6 (0.0) | 12.6 |
| 9     | Bst DNA polymerase 3.0 | dGTP | dC | 16.3 (5.43) | 0.58 (0.52) | 10.4 \times 10^6 (0.63) | 12.6 |
| 10    | dGTP-NH\(_2\) | 2.23 (0.95) | 2.34 (1.08) | 0.95 \times 10^6 (0.05) | 12.6 |
| 11    | dATP | T | 15.1 (2.26) | 1.72 (0.06) | 8.74 \times 10^6 (1.26) | 8.5 |
| 12    | dATP-NH\(_2\) | 1.87 (0.79) | 1.61 (0.94) | 1.28 \times 10^6 (0.36) | 12.6 |
| 13    | dCTP | dG | 14.7 (4.26) | 1.34 (0.28) | 11.1 \times 10^6 (3.20) | 8.5 |
| 14    | dCTP-NH\(_2\) | 1.38 (0.37) | 1.05 (0.16) | 1.30 \times 10^6 (0.16) | 12.6 |
| 15    | TTP | dA | 16.4 (2.46) | 1.45 (0.45) | 11.6 \times 10^6 (3.53) | 5.4 |
| 16    | TTP-NH\(_2\) | 1.64 (0.63) | 0.78 (0.31) | 2.13 \times 10^6 (0.70) | 12.6 |

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\( a \) Conditions: 1.0\( \mu \)M FAM-labeled primer-template duplex, 0.001–0.1 unit/\( \mu \)L Klenow Fragment (exo\(^{-}\)), 50 mM NaCl, 10 mM MgCl\(_2\), 1 mM dithiothreitol (DTT) pH 7.9, 1–12\( \mu \)M dNTPs, incubated at 37°C for 0.5–10 min, or 0.01–0.1 unit/\( \mu \)L Bst 3.0 DNA polymerase, 20 mM Tris–HCl, 10 mM (NH\(_4\))\(_2\)SO\(_4\), 150 mM KCl, 2 mM MgSO\(_4\), 0.1% Tween 20, pH 8.8, 2–12\( \mu \)M dNTPs, incubated at 37°C for 1 min in a reaction volume of 10\( \mu \)L. Standard deviations are given in parentheses (three independent experiments). Velocity is normalized for the lowest enzyme concentration used. \( b \) Efficiency = \( V_{\text{max}}/K_M \). \( c \) Ratio = Efficiency\(_{\text{dNTP}}$/Efficiency\(_{\text{dNTP-NH\(_2\)}}$.
HR-ESI-MS measurements. Purity and structure of which were elucidated by NMR and HR-ESI-MS measurements.

After lyophilization of the fraction, the residue was increased. Therefore, this study provides important information for further biological application. This might be attributed also less likely to associate with magnesium ion by chemical modification of the triphosphate. 3)


g-γ-Amido-dGTP (3) and γ-Amido-TTP (4)

A mixture of a solution of dGTP or TTP (100 mM, 350 µL, 35 µmol) and a solution of EDC·HCl (400 mM, 4.9 mL, 1.96 mmol, 56 equiv) was incubated at 30 °C for 30 min. An ammonium solution (28% in water, 5 µL, 73 µmol, 2.1 equiv.) was added to the mixture, which was incubated at 30 °C for 4 h. Aqueous sodium hydroxide (10% in water, 420 µL) was added to the mixture, which was incubated at 50 °C for 7 h. The reaction mixture was neutralized with hydrochloric acid (10% in water) and purified by HPLC (HPLC conditions: Column (Shiseido CAPCELL PAK C18-MG), Buffer (A: 0.1 M TEAA, B: CH3CN, B conc. 2 to 7%/20 min for dATP or 0 to 2%/20 min for dCTP, linear gradient), flow rate (1.0 mL/min), UV detector (254 nm), and column oven (35 °C)). After lyophilization of the fraction, the residue was dissolved in deionized water. The resulting solution was treated with Dowex Resins (Na+ form) to convert the counter cation to a sodium ion, the purity and structure of which were elucidated by NMR and HR-ESI-MS measurements.

Fig. 7. Gel Result of a Primer Extension Reaction Using Four γ-Amido-dNTPs

Conditions: 1.0 μM FAM-labeled primer (15 mer), 1.0 μM template DNA (dG) (25 mer), polymerase (Klenow Fragment (exo −)) in the corresponding reaction buffer, 10 μM each of γ-amido-dNTP, reaction in 10 μL at 37 °C for 1 h and a 15% denatured polyacrylamide gel.

Conclusion

In the present study, we successfully synthesized γ-amido-modified dNTPs (1, 2, 3, and 4) and NTPs (5, 6, 7, and 8) in a one-pot reaction. The reaction conditions employed are the first example to obtain pure compounds using corresponding nucleoside triphosphates as the starting materials in an easy and simple method. The results of the single nucleotide extension reaction confirmed that base selectivity did not change and incorporation efficiency by the enzyme decreased as compare with the properties of corresponding non-modified dNTPs. The present results also revealed that the primer extension reaction proceeded when the amount of the enzyme was increased. Therefore, this study provides important information for further biological application.

Experimental

General 1H-NMR (500 MHz) and 31P-NMR (202 MHz) spectra were recorded using the Bruker Ascend-500 spectrometer. High-resolution mass spectra (HR-ESI-MS) were recorded by Bruker micrOTOF II. The FAM-labeled primer and template DNAs were purchased from Gene Design Inc. or Genenet Co., Ltd., Japan.

Synthesis of γ-Amido-dATP (1) and γ-Amido-dCTP (2)

A mixture of a solution of dATP or dCTP (100 mM, 350 µL, 35 µmol) and a solution of EDC·HCl (400 mM, 4.9 mL, 1.96 mmol, 56 equiv) was incubated at 30 °C for 30 min. An ammonium solution (28% in water, 5 µL, 73 µmol, 2.1 equiv.) was added to the mixture, which was incubated at 30 °C for 4 h. The reaction mixture was diluted with water (5.3 mL) and purified by HPLC (HPLC conditions: Column (Shiseido CAPCELL PAK C18-MG), Buffer (A: 0.1 M TEAA, B: CH3CN, B conc. 2 to 7%/20 min for dATP or 0 to 2%/20 min for dCTP, linear gradient), flow rate (1.0 mL/min), UV detector (254 nm), and column oven (35 °C)). After lyophilization of the fraction, the residue was dissolved in deionized water. The resulting solution was treated with Dowex Resins (Na+ form) to convert the counter cation to a sodium ion, the purity and structure of which were elucidated by NMR and HR-ESI-MS measurements.
γ-Amido-UTP (8)

$^{31}$P-NMR (162 MHz, D$_2$O) δ: −0.83, −11.28, −22.30, (HRMS (ESI-TOF) C$_9$H$_{15}$N$_3$O$_{14}$P$_3$ (M-H)$^-$, Calcd. for: 481.9761. Found: 481.9786).

**Single Nucleotide Primer Extension Reaction** The mixture of template DNA (X) (final conc. 1.0 µM, 25’ CGACAGTTA X GGTAGGTTATGCG; X = dG, dA, dC or T) and the primer (final conc. 1.0 µM, 15 mer of the FAM-labeled primer, 5’-FAM-CGCATAACCCCATACC) in the corresponding buffer (Klenow Fragment (exo I)) (10 mM Tris–HCl, 50 mM NaCl, 10 mM MgCl$_2$, and 1 mM DTT) was added to the mixture at 37°C in a reaction volume of 10 µL. After a 1-hour incubation, the reaction was quenched with loading buffer and analyzed by 15% denaturing polyacrylamide gel electrophoresis. Bands were visualized using a fluorescence imager (LAS4000).

**Steady-State Kinetics Study** A mixture of template DNA (X) (X = dG, dA, dC or T) (1.0 µM) and the FAM-labeled primer (1.0 µM) in the corresponding buffer was annealed at 90°C for 5 min, and the corresponding polymerase was added to the mixture at 37°C. The reaction was initiated by the addition of an identical volume of the corresponding dNTP derivative solution (1–12 µM) in the same buffer at 37°C. Enzyme concentrations (0.001–0.1 unit/µL) were added, and the mixture (10 µL) was incubated at 37°C for 1–5 min. The reaction was quenched with loading buffer and analyzed by 15% denaturing polyacrylamide gel electrophoresis. Bands were visualized using a fluorescence imager (LAS4000).

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**Conflict of Interest** The authors declare no conflict of interest.

**References**

1) Blackburn M., Gait M., Loakes D., Williams D., “Nucleic acids in chemistry and biology,” 3rd ed., The Royal Society of Chemistry, Cambridge, U.K., 2006.

2) Hirao I., Kimoto M., Mitsui T., Fujisawa T., Kawai R., Sato A., Harada Y., Yokoyama S., Nat. Methods, 3, 729–735 (2006).

3) Thavoncharoensub N., Maruyama K., Heh C. H., Leong K. H., Shi H., Shigematsu Y., Sasaki S., Taniguchi Y., Nucleotides and Nucleic Acids, 38, 578–589 (2019).

4) Camarasa M.-J., ChemMedChem, 13, 1885–1889 (2018).

5) Hardt N., Hacker S. M., Marx A., Org. Biomol. Chem., 11, 8298–8305 (2013).

6) Stumber M., Herrmann C., Wohlgenuth S., Kalbitzer H. R., Jahn W., Geyer M., Eur. J. Biochem., 269, 3270–3278 (2002).

7) Hacker S. M., Mex M., Marx A., J. Org. Chem., 77, 10450–10454 (2012).

8) Masuda S., Tomohiro T., Hatanaka Y., Bioorg. Med. Chem. Lett., 21, 2252–2254 (2011).

9) Grachev M. A., Dobrikov M. I., Kaorre V. D., Pressman E. K., Roschke V. V., Shishkin G. V., FEBS Lett., 162, 266–269 (1983).

10) Wray J., Jahn W., FEBS Lett., 518, 97–100 (2002).

11) Espinasse A., Wen X., Goodpaster J. D., Carlson E. E., ACS Chem. Biol., 15, 1252–1260 (2020).

12) Koch H.-G., Jensen-Tretzer C., Jessen H. J., J. Org. Chem., 85, 14496–14506 (2020).

13) Singh J., Ripp A., Haas T. M., Qiu D., Keller M., Wender P. A., Siegel J. S., Baldridge K. K., Jessen H. J., J. Am. Chem. Soc., 141, 15013–15017 (2019).

14) Gibbard C., Bhowmik S., Kariki M., Kim E.-K., Krishnamurthy R., Nat. Chem., 10, 212–217 (2018).

15) Serdjukow S., Kink F., Steigenberger B., Tomás-Gamasa M., Carell T., Chem. Commun., 50, 1861–1863 (2014).

16) Mitchell D. III, Renda A. J., Douds C. A., Babitzke P., Assmann S. M., Bevilacqua P. C., RN1, 25, 147–157 (2019).