Effects of the 2-Substituted Adenosine-1,3-diazaphenoxazine 5’-Triphosphate Derivatives on the Single Nucleotide Primer Extension Reaction by DNA Polymerase

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The adenosine triphosphate derivatives of 2-oxo-1,3-diazaphenoxazine (dAdapTP) showed a significant discrimination ability for the template strand including between 8-oxo-2’-deoxyguanosine (8-oxodG) and 2’-deoxyguanosine (dG) by the single nucleotide primer extension reaction using the Klenow Fragment. In this study, we synthesized new dAdapTP derivatives, i.e., 2-amino-dAdapTP, 2-chloro-dAdapTP and 2-iodo-dAdapTP, to investigate the effect on the selectivity and efficiency of incorporation for the primer extension reaction using a variety of DNA polymerases. In contrast to the previously tested dAdapTP, the selectivity and efficiency of the 2-halo-dAdapTP incorporation were dramatically decreased using the Klenow Fragment. Moreover, the efficiency of the 2-amino-dAdapTP incorporation into the T-containing template was almost the same with that of dAdapTP. In the case of the Bsu DNA polymerase, the efficiency of all the dAdapTP derivatives decreased compared to that using the Klenow Fragment. However, the incorporation selectivity of dAdapTP had improved against the oxodG-containing template for all the template sequences including the T-containing template. Moreover, 2-amino-dAdapTP showed a better efficiency than dAdapTP using the Bsu DNA polymerase. The 2-amino group of the adenosine unit may interact with syn-oxodG at the active site of the Bsu DNA polymerase during the single primer extension reaction.

Key words DNA damage; 8-oxo-2’-deoxyguanosine (8-oxodG); enzymatic primer extension; adenosine-1,3-diazaphenoxazine (Adap) triphosphate derivative

Introduction

Genomic DNA is constantly damaged by external or internal stimuli. In particular, the reactive oxygen species (ROS) react at the 8-position of the guanine nucleobase (G) to produce a compound called 8-oxoguanine (oxoG) as the oxidative damage. The 8-oxo-2’-deoxyguanosine (oxodG) can form hydrogen bonding, not only with 2’-deoxycytidine (dC), but also with 2’-deoxyadenosine (dA) in duplex DNA. This property induces the transversion mutation from a GC to a TA base pair during the DNA replication. According to the results of several studies, the amount of oxoG in urine, blood or cell is associated with the various types of diseases such as neurodegenerative diseases, so it becomes a good biomarker. Furthermore, the presence of the oxoG in the DNA sequences may play an important role in biological systems. However, an innovative technique or simple method that can sequence oxodG in DNA does not presently exist. In addition, amplification is necessary due to the low amount of oxoG in the DNA. Thus, in order to identify it, the chemical modification or multistep operation is required. Recently, we developed a novel nucleoside triphosphate, adenosine-1,3-diazaphenoxazine triphosphate (dAdapTP), which showed a discrimination ability for the template strand including between that 8-oxodG and dG using the Klenow Fragment. However, dAdapTP consists of 2’-deoxyadenosine as the basic skeleton, thus it was also incorporated into the primer strands for the thymidine-containing template (T-template). In order to solve this problem, we tested the properties of the 2-substituted adenosine derivatives of the dAdapTP derivatives (Fig. 1) and several commercially-available DNA polymerases.

The syntheses of the 2-chloro-Adap (11) and the 2-iodo-Adap (12) are summarized in Chart 1. These two compounds were synthesized from the 3’-O- and 5’-O-tert-butylidimethylsilyl (TBS) protected 2,4,6-trisopropylbenzenesulfonylethyl nitrite and trimethylsilyl chloride (TMSCl) in dichloromethane at −10°C, and the chlorinated product 5 was obtained in a 43% yield. On the other hand, iodination was carried out using tert-butyl nitrite and trimethylsilyl chloride (TMSCl) in dichloromethane at 5°C, and the chlorinated product 6 was obtained in a 61% yield. A substitution reaction was done using the phenoxazine unit 7 and the corresponding 2-halogenated compounds (5 or 6) in the presence of disopropylethylamine to give the TBS protected compound 8 or 9 in a 68 or 83% yield, respectively. The TBS groups at the 3’- and 5’-hydroxyl group of each compound were removed to produce the 2-chloro-Adap (11) or the 2-iodo-Adap (12) in a good yield. The conventional triphosphate synthesis method is shown in Chart 2. Briefly, these diol compounds (10, 11 and 12) were converted into the 3’-O-Ac compounds via protection and deprotection at the 5’-hydroxyl group with the dimethoxytrityl (DMTr) group (13, 14 and 15). The 5’-hydroxyl group was reacted with phosphorylated reagents. After deprotection under alkaline conditions, the target triphosphate compounds were purified by reverse phase HPLC. Although the isolated or reaction yields were not good because of the remaining corresponding diol material, the 2-amino-dAdapTP (1), 2-chloro-dAdapTP (2) or 2-iodo-dAdapTP (3) were identified by ‘H- and 31P-NMR and high resolution mass spectra measurements.

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We first tested the single nucleotide extension reaction using the fluorescein (FAM)-labeled primer, template DNA (X) (X = oxodG, dG, dA, dC or T), dAdapTP or 2-substituted dAdapTP derivatives (1, 2 and 3) and commercially-available DNA polymerases (Klenow Fragment (exo −), Bsu DNA polymerase, KOD Dash, Bst DNA polymerase, Vent DNA polymerase (exo −) and Taq DNA polymerase). After the single nucleotide extension reaction, the elongated FAM-labeled primer was separated by gel electrophoresis. These gel result pictures are depicted in Fig. 2. Among these polymerases, the elongation products were obviously confirmed using the Klenow Fragment, Bsu DNA polymerase and KOD Dash.

We next obtained the steady-state kinetic data for the single primer extension reaction using the following three polymerases: Klenow Fragment, Bsu DNA polymerase and KOD Dash. The results of the steady-state kinetics (\(V_{\text{max}}\) and \(K_M\)) are summarized in Tables 1, 2 and 3 for the Klenow Fragment, Bsu DNA polymerase and KOD Dash, respectively. In the case of the Klenow Fragment, the incorporation efficiency (\(V_{\text{max}}/K_M\)) of 2-amino-dAdapTP into the oxodG-containing template DNA was reduced while maintaining the incorporation efficiency into the T-containing template (Table 1, entries 1 vs. 6 and 5 vs. 10). The efficiency into the T-containing template of 2-amino-dAdapTP was slightly lower than that of the
natural dATP (Table 1, entry 25). Unfortunately, the selectivity and efficiency of the 2-chloro-dAdapTP and 2-iodo-dAda-
pTP incorporations were dramatically decreased (Table 1, entries 11–20). These results indicated that the 2-substitution of the adenosine unit of dAdapTP reduced the incorporation efficiency but the amino group was expected to interact with the 2-position of the carbonyl group of the thymine nucleobase at the complementary position at the active site of the Klenow Fragment.

The Bsu DNA polymerase showed interesting kinetics results (Table 2). The incorporation efficiency of dAdapTP into the oxodG-containing template was slightly reduced, but the selectivity for the oxodG-containing template was improved (Table 2, entries 1–5). In particular, the incorporation efficiencies for the oxodG-containing template and T-containing template were almost the same (Table 2, entries 1 vs. 5). Interestingly, 2-amino-dAdapTP having an amino group at the 2-position of the adenosine skeleton slightly improved the incorporation efficiency over dATP (Table 2, entries 1 vs. 6 and 5 vs. 10). Interestingly, the efficiency of 2-amino-
dAdapTP for the oxodG-containing template was better than that of dATP, which is believed to be due to multiple hydrogen
Table 1. Steady-State Kinetic Parameter Using Klenow Fragment

| Entry | dNTP  | $X$        | $V_{\text{max}}$ [\% min$^{-1}$] | $K_M$ [\mu M] | $V_{\text{max}}/K_M$ [\% min$^{-1}$ M$^{-1}$] | Relative [%] |
|-------|-------|------------|----------------------------------|---------------|----------------------------------|-------------|
| 1     | dAdapTP | 8-oxodG    | 2.35 (0.37)                     | 0.78 (0.02)   | 3.02 $\times$ 10$^6$                     | 100         |
| 2     | dG     |            | 0.26 (0.01)                     | 3.11 (0.25)   | 0.08 $\times$ 10$^6$                     | 2.75        |
| 3     | dA     |            | 0.99 (0.26)                     | 6.35 (0.34)   | 0.16 $\times$ 10$^6$                     | 5.17        |
| 4     | dC     |            | 0.79 (0.01)                     | 5.40 (0.47)   | 0.15 $\times$ 10$^6$                     | 4.87        |
| 5     | T      |            | 5.23 (0.43)                     | 0.48 (0.14)   | 10.9 $\times$ 10$^6$                    | 360         |
| 6     | 2-Amino-dAdapTP | 8-oxodG    | 1.20 (0.40)                     | 2.60 (1.10)   | 0.46 $\times$ 10$^6$                     | 100         |
| 7     | dG     |            | 0.09 (0.01)                     | 0.64 (0.03)   | 0.14 $\times$ 10$^6$                     | 29.7        |
| 8     | dA     |            | 0.15 (0.02)                     | 1.47 (0.23)   | 0.10 $\times$ 10$^6$                     | 22.2        |
| 9     | dC     |            | 0.15 (0.05)                     | 1.86 (0.92)   | 0.08 $\times$ 10$^6$                     | 18.0        |
| 10    | T      |            | 3.29 (0.03)                     | 0.30 (0.02)   | 10.8 $\times$ 10$^6$                    | 2350        |
| 11    | 2-Chloro-dAdapTP | 8-oxodG    | 0.82 (0.11)                     | 6.17 (1.52)   | 0.13 $\times$ 10$^6$                     | 100         |
| 12    | dG     |            | 0.87 (0.08)                     | 2.31 (0.22)   | 0.38 $\times$ 10$^6$                     | 283         |
| 13    | dA     |            | 0.65 (0.04)                     | 2.30 (0.38)   | 0.28 $\times$ 10$^6$                     | 212         |
| 14    | dC     |            | 0.82 (0.09)                     | 4.23 (0.53)   | 0.19 $\times$ 10$^6$                     | 145         |
| 15    | T      |            | 0.75 (0.10)                     | 5.23 (0.05)   | 0.14 $\times$ 10$^6$                     | 108         |
| 16    | 2-Iodo-dAdapTP | 8-oxodG    | 0.03 (0.01)                     | 1.65 (0.12)   | 0.02 $\times$ 10$^6$                     | 100         |
| 17    | dG     |            | 0.05 (0.02)                     | 0.51 (0.22)   | 0.10 $\times$ 10$^6$                     | 546         |
| 18    | dA     |            | 0.14 (0.02)                     | 1.29 (0.16)   | 0.11 $\times$ 10$^6$                     | 612         |
| 19    | dC     |            | 0.14 (0.03)                     | 2.56 (0.83)   | 0.05 $\times$ 10$^6$                     | 300         |
| 20    | T      |            | 0.10 (0.02)                     | 2.48 (0.51)   | 0.04 $\times$ 10$^6$                     | 211         |
| 21    | dATP$^{(a)}$ | 8-oxodG    | 14.9 (0.97)                     | 4.42 (0.39)   | 3.30 $\times$ 10$^6$                     | 100         |
| 22    | dG     |            | 0.90 (0.06)                     | 3.21 (0.29)   | 0.28 $\times$ 10$^6$                     | 8.44        |
| 23    | dA     |            | 0.85 (0.05)                     | 2.75 (0.17)   | 0.31 $\times$ 10$^6$                     | 9.37        |
| 24    | dC     |            | 0.86 (0.13)                     | 3.83 (0.74)   | 0.23 $\times$ 10$^6$                     | 6.82        |
| 25    | T      |            | 13.8 (1.42)                     | 0.73 (0.06)   | 18.9 $\times$ 10$^6$                    | 572         |

$^{(a)}$ Conditions: 1.0 $\mu$M FAM-labelled primer-template duplex, 0.01–0.1 unit/µM Klenow Fragment (exo-), 50 mM NaCl, 10 mM Tris–HCl, 10 mM MgCl$_2$, 1 mM DTT, pH 7.9, 0.1–35 $\mu$M dNTP, incubated at 37°C for 2–10 min in a reaction volume of 10 $\mu$L. Velocity is normalized for the lowest enzyme concentration used. b) ref 17.

In this study, we successfully synthesized the 2-substituted adenosine-1,3-diazaphenoxazine derivatives and evaluated the properties of their triphosphate for the single nucleotide primer extension reaction using DNA polymerase. Based on the value of the steady-state kinetic parameter, we found an interesting DNA polymerase, the Bsu DNA polymerase, concerning the base selectivity between the oxodG-containing template and T-containing template. Furthermore, the amino group substitution at the 2-position of the adenosine unit improved the uptake efficiency, and their selectivity using the Bsu DNA polymerase was better than the results using the Klenow Fragment. These results indicated that the 2-amino-dAdapTP can be successfully incorporated into the active site of the Bsu DNA polymerase.

On the other hand, KOD Dash incorporated the 2-amino-dAdapTP into the primer strand better than dAdapTP for the T-containing template (Table 3, entries 5 vs. 10). The 2-amino group of 2-amino-dAdapTP could interact with the thymine nucleobase during the polymerase reaction of KOD Dash. Interestingly, the incorporation efficiency of the 2-halo-dAdapTP derivatives increased more than that of dAdapTP (Table 3, entries 11–20). These results also indicated that the 2-halo substituted derivative showed the same interaction such as the halogen-oxygen interaction with 2-cabonyl group of thymine or shape fitting in the active site of KOD dash DNA polymerase. However, they had reduced efficiency and selectivity compared to the incorporation of dATP into the template (Table 3, entries 21–25).

Conclusion

In this study, we successfully synthesized the 2-substituted adenosine-1,3-diazaphenoxazine derivatives and evaluated the properties of their triphosphate for the single nucleotide primer extension reaction using DNA polymerase. Based on the value of the steady-state kinetic parameter, we found an interesting DNA polymerase, the Bsu DNA polymerase, concerning the base selectivity between the oxodG-containing template and T-containing template. Furthermore, the amino group substitution at the 2-position of the adenosine unit improved the uptake efficiency, and their selectivity using the Bsu DNA polymerase was better than the results using the Klenow Fragment. These results indicated that the 2-amino-dAdapTP can be successfully incorporated into the active site of the Bsu DNA polymerase to interact with syn-oxodG in the complimentary position. Unfortunately, the halogen substitution at the 2-position of adenosine has a negative effect on the enzymatic incorporation under these conditions. These results encourage us to further modify the dAdapTP derivatives and the functional evaluation of them using various polymerases, which will lead to the development of a new oxodG sequencing technology.

Experimental

General

The $^1$H-NMR (400 MHz, 500 MHz), $^{13}$C-NMR (125 MHz) and $^{31}$P-NMR (202 MHz) spectra were recorded by Varian UNITY-400 and Bruker Ascend-500 spectrometers. The high-resolution electrospray ionization (HR-ESI)-MS were recorded by a Bruker microTOF II. The FAM labelled primer and template DNAs were purchased from Gene Design, Inc., or Genenet Co., Ltd., Japan.

$^{3,5'}$-Bis-O-tert-butylmethylsilyl-2'-deoxy-6-O-[(2,4,6-trisopropylphenyl)sulfonyl]-2-chloro-guanosine (5) Under
Under an argon atmosphere, to a solution of tert-butyl nitrite (310 µL, 2.62 mmol) in dry CH₂Cl₂ (13 mL) was added the solution of 4 (1.0 g, 1.31 mmol) in dry CH₂Cl₂ (13 mL) and TMSCl (330 µL, 2.61 mmol) at −10°C. After stirring for 90 min at the same temperature, the reaction was quenched by a saturated NaHCO₃ solution. The organic layer was washed with water and a saturated NaCl solution, then dried over Na₂SO₄. The solvent was removed under reduced pressure, then the residue was purified by silica gel column chromatography (kanto 60N, Hexane/EtOAc = 10/1) to obtain a yellow foam (445 mg, 0.56 mmol, 43%). ¹H-NMR (400 MHz, CDCl₃) δ: 8.31 (1H, s), 7.20 (2H, s), 6.39 (1H, t, J = 6.4 Hz), 4.61–4.58 (1H, m), 4.33–4.26 (2H, m), 3.99 (1H, dt, J = 6.7, 3.4 Hz), 3.86 (1H, dd, J = 11.3, 4.0 Hz), 3.74 (1H, dd, J = 11.3, 3.1 Hz), 2.94–2.87 (1H, m), 2.60–2.54 (1H, m), 2.45–2.39 (1H, m), 1.27–1.24 (18H, m), 0.89 (9H, s), 0.88 (9H, s), 0.08 (6H, s), 0.06 (3H, s), 0.05 (3H, s); ¹³C-NMR (125 MHz, CDCl₃) δ: 159.7, 154.8, 154.6, 152.2, 146.9, 131.0, 124.0, 122.0, 88.4, 85.1, 71.9, 62.8, 41.6, 34.5, 30.0, 26.1, 25.9, 24.8, 24.7, 23.7, 23.6, 18.6, 18.2, −4.5, −4.7, −5.3, −5.4; IR (neat, cm⁻¹) 2956.2, 1601.6, 1565.2, 1391.3, 1256.2; HRMS (ESI-time-of flight (TOF)) Caled for C₃₇H₆₁IN₄O₆SSi₂Na [M + Na⁺]: 803.3431, 805.3405. Found: 803.3445, 805.3405.

** Entry | dNTP | X | Vmax [% min⁻¹] | KM [µM] | Vmax/KM [% min⁻¹ M⁻¹] | Relative [%]
---|---|---|---|---|---|---
1 | dAdapTP | 8-oxodG | 1.37 (0.21) | 1.42 (0.22) | 0.97 × 10⁶ | 100
2 | dG | 0.20 (0.02) | 3.30 (0.15) | 0.06 × 10⁶ | 6.26
3 | dA | 0.19 (0.02) | 3.15 (0.63) | 0.06 × 10⁶ | 6.12
4 | dC | 0.14 (0.01) | 2.85 (0.68) | 0.05 × 10⁶ | 4.91
5 | T | 2.12 (0.49) | 2.13 (0.71) | 0.99 × 10⁶ | 103
6 | 2-Amino-dAdapTP | 8-oxodG | 2.02 (0.98) | 1.52 (0.87) | 1.33 × 10⁶ | 100
7 | dG | 0.20 (0.04) | 3.20 (1.54) | 0.06 × 10⁶ | 4.74
8 | dA | 0.28 (0.06) | 3.47 (0.88) | 0.08 × 10⁶ | 6.08
9 | dC | 0.17 (0.02) | 2.55 (0.52) | 0.03 × 10⁶ | 5.17
10 | T | 3.14 (0.52) | 1.72 (0.84) | 1.82 × 10⁶ | 137
11 | 2-Chloro-dAdapTP | 8-oxodG | 0.16 (0.01) | 1.24 (0.29) | 0.13 × 10⁶ | 100
12 | dG | 0.12 (0.04) | 1.72 (0.72) | 0.07 × 10⁶ | 50.3
13 | dA | 0.21 (0.07) | 1.14 (0.07) | 0.19 × 10⁶ | 140
14 | dC | 0.21 (0.01) | 1.14 (0.07) | 0.19 × 10⁶ | 140
15 | T | 0.55 (0.06) | 2.02 (0.49) | 0.27 × 10⁶ | 206
16 | 2-Iodo-dAdapTP | 8-oxodG | __³) | __³) | __³) | __³)
17 | dG | __³) | __³) | __³) | __³)
18 | dA | __³) | __³) | __³) | __³)
19 | dC | __³) | __³) | __³) | __³)
20 | T | __³) | __³) | __³) | __³)
21 | dATP | 8-oxodG | 2.10 (0.92) | 3.65 (0.42) | 0.58 × 10⁶ | 100
22 | dG | 0.30 (0.03) | 4.36 (0.45) | 0.07 × 10⁶ | 11.9
23 | dA | 0.09 (0.01) | 0.73 (0.21) | 0.12 × 10⁶ | 21.3
24 | dC | 0.10 (0.01) | 2.28 (0.27) | 0.04 × 10⁶ | 7.71
25 | T | 8.19 (0.94) | 0.62 (0.16) | 13.1 × 10⁶ | 2278

µM FAM-labelled primer-template duplex, 0.01–0.1 unit/µL Bsu DNA polymerase, 50 mM NaCl, 10 mM Tris–HCl, 10 mM MgCl₂, 1 mM DTT, pH 7.9, 0.1–35 µM dNTP, incubated at 37°C for 2–10 min in a reaction volume of 10 µL. Velocity is normalized for the lowest enzyme concentration used. b) Single nucleotide incorporation reaction did not occur, therefore, the parameters were not determined.

**General Procedure of Coupling Reaction with Phenoza- line Unit** Under an argon atmosphere, a reaction mixture of 5 or 6 (1.0 eq.) and DIPEA (1.5 eq.) in 1-propanol (0.1 M) was refluxed at 100°C for 5 h. The reaction was quenched by a saturated aqueous NaHCO₃ solution. The 1-propanol was removed under reduced pressure. The res-
Table 3. Steady-State Kinetic Parameter Using KOD Dash

| Entry | dNTP | X     | $V_{\text{max}}$ [% min$^{-1}$] | $K_M$ [µM] | $V_{\text{max}}/K_M$ [% min$^{-1}$ M$^{-1}$] | Relative [%] |
|-------|------|-------|-------------------------------|------------|--------------------------------------------|-------------|
| 1     | dAdApTP | 8-oxoG | 0.06 (0.01) | 10.5 (4.12) | 0.53 × 10$^4$ | 100 |
| 2     | dG    | _b_   | _b_   | _b_ | _b_ | _b_ |
| 3     | dA    | _b_   | _b_   | _b_ | _b_ | _b_ |
| 4     | dC    | _b_   | _b_   | _b_ | _b_ | _b_ |
| 5     | T     | 4.85 (0.18) | 13.9 (1.00) | 34.9 × 10$^4$ | 6551 |
| 6     | 2-Amino-dAdApTP | 8-oxoG | 0.05 (0.01) | 8.65 (1.70) | 0.63 × 10$^4$ | 100 |
| 7     | dG    | _b_   | _b_   | _b_ | _b_ | _b_ |
| 8     | dA    | _b_   | _b_   | _b_ | _b_ | _b_ |
| 9     | dC    | _b_   | _b_   | _b_ | _b_ | _b_ |
| 10    | T     | 3.94 (0.11) | 5.89 (0.19) | 66.8 × 10$^4$ | 10600 |
| 11    | 2-Chloro-dAdApTP | 8-oxoG | 0.57 (0.20) | 12.9 (6.00) | 4.42 × 10$^4$ | 100 |
| 12    | dG    | _b_   | _b_   | _b_ | _b_ | _b_ |
| 13    | dA    | _b_   | _b_   | _b_ | _b_ | _b_ |
| 14    | dC    | _b_   | _b_   | _b_ | _b_ | _b_ |
| 15    | T     | 4.66 (0.30) | 15.6 (1.51) | 29.9 × 10$^4$ | 677 |
| 16    | 2-Lodo-dAdApTP | 8-oxoG | 0.27 (0.05) | 3.36 (1.00) | 8.07 × 10$^4$ | 100 |
| 17    | dG    | _b_   | _b_   | _b_ | _b_ | _b_ |
| 18    | dA    | _b_   | _b_   | _b_ | _b_ | _b_ |
| 19    | dC    | _b_   | _b_   | _b_ | _b_ | _b_ |
| 20    | T     | 1.24 (0.13) | 0.75 (0.07) | 164 × 10$^4$ | 2031 |
| 21    | dATP  | 8-oxoG | 0.12 (0.92) | 1.17 (0.40) | 10.0 × 10$^4$ | 100 |
| 22    | dG    | _b_   | _b_   | _b_ | _b_ | _b_ |
| 23    | dA    | _b_   | _b_   | _b_ | _b_ | _b_ |
| 24    | dC    | _b_   | _b_   | _b_ | _b_ | _b_ |
| 25    | T     | 1.26 (2.65) | 1.71 (0.22) | 7356 × 10$^4$ | 73450 |

a) Conditions: 1.0µM FAM-labelled primer-template duplex, 0.01-0.1 unit/µL KOD Dash, 20mM Tris·HCl, 8mM MgCl$_2$, 7.5mM DTT, 2.5µg BSA, pH 7.5, 0.1-3.5µM dNTP, incubated at 37°C for 5-50min in a reaction volume of 10µL. Velocity is normalized for the lowest enzyme concentration used. b) Single nucleotide incorporation reaction did not occur, therefore, the parameters were not determined.

idue was purified by silica gel column chromatography (kanto 60N, Hexane/EtOAc = 1/1 to 1/5) to give the corresponding coupling compounds 8 or 9.

3',5'-Bis-O-tert-butyldimethylsilyl-2'-deoxy-6-N-[[1,3-di-aza-3-methyl-2-oxo-phenoxazine-9-yloxy]ethyl]-2-chloro-adenosine (8) Yellow foam (260mg, 0.34mmol, 68%). 1H-NMR (500MHz, CDCl$_3$) δ = 8.72 (1H, br), 8.23 (1H, s), 6.78 (1H, t, $J$ = 8.2Hz), 6.66 (1H, d, $J$ = 8.1Hz), 6.43 (1H, d, $J$ = 8.0Hz), 6.34 (1H, t, $J$ = 6.7Hz), 6.31 (1H, s), 4.63–4.61 (1H, m), 4.18 (2H, br), 4.02–3.99 (1H, m), 3.84 (2H, br), 3.83 (1H, dd, $J$ = 10.9, 5.9Hz), 3.74 (1H, dd, $J$ = 11.0, 4.2Hz), 3.18 (3H, s), 2.82–2.77 (1H, m), 2.43 (1H, ddd, $J$ = 13.1, 5.9, 3.2Hz), 0.92 (9H, s), 0.87 (9H, s), 0.11 (6H, s), 0.06 (3H, s), 0.05 (3H, s); 13C-NMR (125MHz, CDCl$_3$) δ = 155.1, 154.8, 151.9, 151.1, 149.4, 145.1, 139.9, 129.3, 124.4, 123.5, 118.4, 110.4, 88.3, 85.2, 77.4, 72.6, 63.2, 41.8, 40.1, 35.9, 29.8, 29.4, 26.1, 26.0, 18.5, 18.2, −4.5, −4.6, −5.3, −5.4; IR (neat, cm$^{-1}$) 2928.3, 1630.4, 1473.2, 1260.8; HRMS (ESI-TOF) Calcd for C$_{35}$H$_{51}$O$_{6}$Si$_{2}$Na [M+Na]$^+$: 879.2870. Found: 879.2879.

General Procedure of Deprotection Reaction of TBS Group Under an argon atmosphere, Et$_3$N·3HF (0.19mL, 1.18mmol) and triethylamine (0.18mL, 1.29mmol) were added to a solution of compound 8 or 9 (0.30mmol) in dry pyridine (3.0mL). After stirring for 20h, the solvent was removed under reduced pressure. The residue was purified by amino-silica gel column chromatography (CHCl$_3$/MeOH = 30/1) to obtain the corresponding diol products.

2'-Deoxy-6-N-[[1,3-di-aza-3-methyl-2-oxo-phenoxazine-9-yloxy]ethyl]-2-chloro-adenosine (2-Chloro-Adap) (11) Pale yellow powder (150mg, 0.28mmol, 93%). 1H-NMR (500MHz, dimethyl sulfoxide (DMSO)-d$_6$) δ = 9.94 (1H, br), 8.88 (1H, brs), 8.40 (1H, s), 7.49 (1H, br), 6.75 (1H, t, $J$ = 8.2Hz), 6.61 (1H, d, $J$ = 7.8Hz), 6.41 (1H, d, $J$ = 8.2Hz), 6.27 (1H, t, $J$ = 6.6Hz), 5.32 (1H, d, $J$ = 4.2Hz), 4.95 (1H, t, $J$ = 5.5Hz), 4.39 (1H, br), 4.05 (2H, br), 3.86 (3H, m), 3.61–3.57 (1H, m), 3.53–3.49 (1H, m), 3.19 (3H, s), 2.64 (1H, qui, $J$ = 6.6Hz), 2.31–2.29 (1H, m); 13C-NMR (125MHz,
DMSO-d$_6$) $\delta$: 154.8, 154.6, 154.0, 153.2, 149.4, 146.0, 142.2, 139.9, 129.2, 125.9, 122.9, 118.6, 115.8, 107.9, 106.8, 88.0, 83.6, 70.7, 67.3, 61.6, 40.1, 39.6, 36.7; IR (neat, cm$^{-1}$) 3481.7, 2928.0, 1670.1, 1630.0, 1512.5, 1473.0, 1317.8; HRMS (ESI-TOF) Calculated for C$_2$H$_2$Cl$_3$N$_7$O$_6$Na [M + Na$^{+}$]: 856.1321, 567.1294. Found: 565.1324, 567.1294.

$^{2}$-Deoxy-6-N-[(1,3-diaza-3-methyl-2-oxophenoxazine-9-yloxy)-ethyl]-2-ido-adenosine (12) (2-Ido-AdapTP) (12) Pale yellow powder (140 mg, 0.22 mmol, 73%). $^{1}$H-NMR (500 MHz, DMSO-d$_6$) $\delta$: 9.93 (1H, br), 8.72 (1H, brs), 8.32 (1H, s), 7.51 (1H, br), 6.77 (1H, t, $J$ = 8.0 Hz), 6.64 (1H, d, $J$ = 8.0 Hz), 6.42 (1H, d, $J$ = 8.0 Hz), 6.26 (1H, t, $J$ = 6.5 Hz), 5.32 (1H, d, $J$ = 4.1 Hz), 4.92 (1H, t, $J$ = 5.4 Hz), 4.38 (1H, br), 4.04 (2H, br), 3.85 (3H, m), 3.61–3.56 (1H, br), 3.52–3.48 (1H, m), 3.19 (3H, s), 2.62 (1H, qui, $J$ = 6.6 Hz), 2.29–2.26 (1H, m); $^{13}$C-NMR (125 MHz, DMSO-d$_6$) $\delta$: 154.1, 154.0, 153.8, 148.9, 146.0, 142.2, 139.2, 129.1, 125.9, 122.9, 119.8, 107.9, 106.8, 88.0, 83.5, 70.7, 67.3, 61.6, 48.6, 39.6, 36.6; IR (neat, cm$^{-1}$) 3257.9, 2916.7, 2354.2, 1696.2, 1627.5, 1559.3, 1505.9, 1476.2, 1280.6; HRMS (ESI-TOF) Calculated for C$_{32}$H$_{23}$Cl$_3$N$_7$O$_6$Na [M + Na$^{+}$]: 867.0677. Found: 857.0663.

**General Procedure of Synthesis of 3'-O-Acetyl Compound**

Under an argon atmosphere, DMTrCl (135 mg, 0.40 mmol) was added to a solution of compound 10,11 or 12 (0.20 mmol) in dry pyridine (1.5 mL). After stirring for 90 min, acetic anhydride (56.7 µL, 0.60 mmol) was added to the reaction mixture. After stirring for 1 h, the solvent was removed under reduced pressure, then the residue was dissolved in 3% trichloroacetic acid in CH$_2$Cl$_2$ (10 mL). After stirring for 1 h, the solvent was removed under reduced pressure, then the residue was purified by silica gel column chromatography (CH$_2$Cl$_2$/MeOH = 100/1 to 50/1) to obtain the corresponding 3'-O-acetyl compound.

3'-O-Acetyl-2'-deoxy-6-N-[(1,3-diaza-3-methyl-2-oxophenoxazine-9-yloxy)-ethyl]-2-phenoxyacetylamino-adenosine (13) (2-Chloro-dAdapTP) (1) A solution was obtained as a colorless solution (15 mg, 0.05 mmol) in 1,4-dioxane (0.4 mL) added to the solution of 13, 14 or 15 (0.05 mmol) in pyridine/1,4-dioxane (50/50, 0.5 mL), then the mixture was stirred for 30 min at room temperature. The reaction mixture was treated with a 0.38 M solution of tributylammonium pyrophosphate in DMF (0.25 mL, 0.10 mmol) and tributylamine (57 µL, 0.38 M solution of tributylammonium pyrophosphate in DMF) (19.2 mg, 0.10 mmol) in 1,4-dioxane (0.4 mL) was added to the reaction mixture. After stirring for 5 min, which was treated with a 5% NaHSO$_3$ solution (2 mL) for 30 min, the solvent was evaporated under reduced pressure, then the residue was dissolved in 28% ammonium hydroxide (2 mL) for 10 min. The reaction mixture was then treated with 1% iodine in pyridine–water (98/2, 2.0 mL) for 30 min. After stirring was treated with a 5% NaH$_2$PO$_4$ solution (1.3 mL) for 30 min. The solvent was evaporated under reduced pressure, then the residue was dissolved in 28% ammonium solution (15 mL). After stirring for 12 h, the solvent was removed under reduced pressure. The residue was washed with 75% ethanol in water containing 0.07 M NaCl (1.3 mL), and the precipitate was dissolved in water and purified by HPLC (HPLC conditions: Column (Shiseido CAPCELL PAK C18-MG), Buffer (A: 20 mM TEAA, B: CH$_3$CN, B conc. 10 to 50%/20 min linear gradient), Flow rate (1.0 mL/min), UV-detector (254 nm), 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, whose purity and structure were determined by NMR and HR-ESI-MS measurements. Their concentrations were determined by NMR measurements with dATP at a known concentration as the internal standard.

2-Amino-dAdapTP (1) A solution was obtained as a colorless solution (1.0 µmol, 2%). $^{1}$H-NMR (500 MHz, D$_2$O) $\delta$: 7.99 (1H, brs), 7.24 (1H, s), 6.84 (1H, dd, $J$ = 8.6, 7.3 Hz), 6.77 (1H, d, $J$ = 8.5 Hz), 6.37 (1H, d, $J$ = 7.9 Hz), 6.21 (1H, dd, $J$ = 7.2, 6.5 Hz), 4.51 (2H, br), 4.27 (1H, br), 4.26–4.22 (1H, m), 4.18–4.15 (1H, m), 4.00 (2H, br), 3.37 (3H, s), 2.75–2.70 (1H, m), 2.54–2.49 (1H, m); 31P-NMR (125 MHz, CH$_2$Cl$_2$) $\delta$: 7.09, -11.5, -23.2; HRMS (ESI-TOF) Calculated for C$_{32}$H$_{27}$N$_9$O$_{15}$P$_3$ [M + Na$^{+}$]: 762.0834. Found: 762.0832.

2-Chloro-dAdapTP (2) A solution was obtained as a colorless solution (15 µmol, 30%). $^{1}$H-NMR (400 MHz, D$_2$O) $\delta$: 8.44 (1H, s), 7.07 (1H, s), 6.92 (1H, t, $J$ = 8.2 Hz), 6.70 (1H, d, $J$ = 8.2 Hz), 6.48 (1H, br), 6.43 (1H, d, $J$ = 8.2 Hz), 4.45 (2H, br), 4.37 (1H, br), 4.33–4.29 (1H, m), 4.24–4.21 (1H, m), 3.74 (2H, br), 3.26 (3H, s), 2.78 (1H, br), 2.72 (1H, br); 31P-NMR
(202 MHz, D₂O) δ: −6.6, −11.2, −21.9; HRMS (ESI-TOF) Calcd for C₃₂H₅₂ClN₀₈O₁₅P₃ [M⁺H]⁺: 781.0335, 783.0309. Found: 781.0349, 783.0336.

2-ido-dAdapTP (3) A solution was obtained as a colorless solution (0.5 μmol, 1%). ¹H-NMR (500 MHz, D₂O) δ: 8.29 (1H, brs), 7.22 (1H, s), 6.85 (1H, t, J = 8.3 Hz), 6.76 (1H, d, J = 8.3 Hz), 6.37 (1H, d, J = 7.9 Hz), 4.51 (2H, br), 4.31 (1H, br), 4.29–4.25 (1H, m), 4.22–4.19 (1H, m), 3.93 (1H, br), 3.79 (1H, br), 3.44 (3H, s), 2.74 (1H, br), 2.63 (1H, br). ³¹P-NMR (202 MHz, D₂O) δ: −6.4, −10.8, −20.7; HRMS (ESI-TOF) Calcd for C₃₂H₅₂I₀₈P₃ [M⁺H]⁺: 872.9691. Found: 872.9704.

Single Nucleotide Primer Extension Reaction The mixture of the template DNA (X) (final conc. 1.0 μM, 25 mer, 5’-CGACAGTTA X GGTAGGTTATGGC; X = 8-oxoEtG, dG, dA, dC or T) and primer (final conc. 1.0 μM, 15 mer of FAM-labeled primer, 5’-FAM-CGCATAACCCTTTACC in the corresponding buffer (Klenow Fragment (exo−)) and Bsu DNA polymerase: 10 mM Tris–HCl, 50 mM NaCl, 10 mM MgCl₂ and 1 mM DTT, pH 8.8, New England Biolabs Japan, Inc.) was annealed at 90°C for 5 min. The corresponding dAdapTP derivatives (final concentration of 50 μM) in the same buffer (Klenow Fragment (exo−)) and Bsu DNA polymerase: 10 mM Tris–HCl, 50 mM KCl, 1.5 mM MgCl₂, pH 8.3, New England Biolabs Japan, Inc.) was annealed at 90°C for 5 min. The corresponding dAdapTP derivatives (final concentration of 50 μM in 10 μL of reaction volume), and DNA polymerase (1.0 unit) were added, and the mixture (10 μL) was incubated at 37°C for 10 min. The reaction was quenched with loading buffer and analyzed by 15% denaturing polyacrylamide gel electrophoresis. The bands were visualized using a fluorescence imager (LAS4000).

Steady-State Kinetics Study The mixture of the template DNA (X) (X = oxoEtG, dG, dA, dC or T) (1.0 μM) and 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 dAdapTP derivatives solution (0.2–70 μM) in the same buffer at 37°C. The enzyme concentrations (0.01–0.1 unit/mL) and reaction times (2–50 min) were adjusted in the different dAdapTP derivative reactions to achieve a 1–20% incorporation, then the reactions were quenched with loading buffer and analyzed by 15% denaturing polyacrylamide gel electrophoresis. The bands were visualized and quantified using a fluorescence imager (LAS4000). The relative velocity v was calculated from the ratio of the extended product (Iₓ) to the remaining primer (Iₓ) as follows: v = Iₓ/Iₓₚ, where t represents the reaction time, which was normalized to the lowest enzyme concentration used. The apparent Vₘₐₓ and Kₘ values were obtained from the Hanes–Woolf plots using the data points of at least five deoxyribonucleotide triphosphate (dNTP) concentrations. The average values were obtained in three different independent experiments, and in parentheses showing the standard deviations.

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Conflict of Interest The authors declare no conflict of interest.

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