

## Formation of purine-purine mispairs by *Sulfolobus solfataricus* DNA polymerase IV

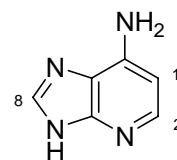
Lindsey DeCarlo, A.S. Prakasha Gowda, Zucui Suo, and Thomas E. Spratt

### SYNTHESIS.

The majority of reagents and solvents were from Aldrich Chemical Co. (Milwaukee, WI). 2'-Deoxy-7-deazaadenosine (7-deaza-dA) and 2'-deoxy-7-deazaguanosine (7-deaza-dG) were purchased from Berry and Associates (Dexter, MI). The triphosphates were prepared by the Ludwig procedure (1) with modifications as described (2). The phosphoramidites for 7-deaza-dA and 7-deaza-dG were purchased from Glen Research (Sterling, VA). NMR spectra were determined with a Bruker Avance II 500 MHz spectrometer. The chemical shifts are referenced to TMS unless indicated.  $\delta$  is in ppm, and  $J$  is in Hz

*1-Deazaadenine*. 7-Nitroimidazo[4,5-*b*]pyridine-1-oxide (3,4) (1.0 g, 5.5 mmol) was dissolved in 10 mL H<sub>2</sub>O and cooled in an ice bath. Approximately 6 equivalents (60 mL) of an ice-cold 10% (w/v) solution of TiCl<sub>3</sub> in 20-30% HCl was added dropwise. The reaction was monitored spectrophotometrically by observing the decrease of a peak at 345 nm and an increase in a peak at 308 nm. The pH was brought to 9 by the addition of saturated Na<sub>2</sub>CO<sub>3</sub>. The precipitate was filtered and the filtrate evaporated. The product was isolated by titrating the solid with acetone.

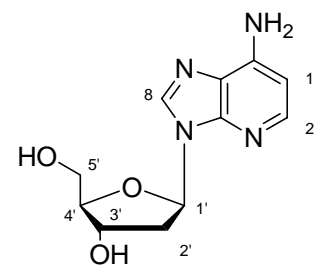
<sup>1</sup>H NMR DMSO-d<sub>6</sub>. 8.05(1H, s, 8-CH), 7.82(1H, d, 2-CH), 6.34(1H, d, 1-CH), 6.25(2H, br-s, NH<sub>2</sub>)



*1-Deaza-2'-deoxyadenosine*. 2'-Deoxythymidine (2 g, 8.2 mmol) and 1-deazaadenine (0.5 g, 3.7 mmol) were slowly stirred in 500 mL 10 mM sodium phosphate (pH 7.0) with 500 units purine nucleoside phosphorylase and 500 units thymidine phosphorylase. The reaction was monitored by HPLC with a 4.6 × 250 mm Prodigy ODS(3) reverse-phase column. The gradient was 0 to 20% acetonitrile over 20 min in 100 mM Et<sub>3</sub>N-HOAc (pH 7.0) at 1 mL/min. The elution order was thymine (7 min), 1-deazaadenine (8.3 min), 2'-deoxythymidine (11.5 min), and 1-deaza-2'-deoxyadenosine (14 min). After the reaction reached >95% completion the solvent

was evaporated to approximately 25 mL. The precipitate, which was predominately 2'-deoxythymidine, was removed by filtration and the filtrate was applied to a 2.6 × 100 cm Sephadex G-15 column which was equilibrated with H<sub>2</sub>O. The product eluted after approximately 1.4 L. Ten milliliter fractions were collected and the elution of the reactants and products monitored by silica gel TLC developed with 1-butanol/acetic acid/H<sub>2</sub>O (8/1/1); 2'-deoxythymidine R<sub>f</sub> = 0.53, thymine R<sub>f</sub> = 0.50, 1-deaza-2'-deoxyadenosine R<sub>f</sub> = 0.28, and 1-deazaadenine R<sub>f</sub> = 0.23. The appropriate fractions were collected and evaporated.

<sup>1</sup>H NMR (DMSO-d<sub>6</sub>) δ 8.26(1H, s, 8-CH), 7.78(1H,d, 2-CH), 6.44(2H, br-s, NH<sub>2</sub>), 6.34 (2H, m, 1-CH and 1'-CH), 5.76 (1H, d/d, 5'-OH), 5.27(1H, d, 3'-OH), 4.41(1H, m, 4'-CH), 3.91(1H, m, 3'-CH), 3.63 (1H, d/t, 5'-CH), 3.53(1H, m, 5'-CH) 2.78(1H, d/d/d, 2'-CH), 2.20, (1H, d/d/d, 2'-CH).

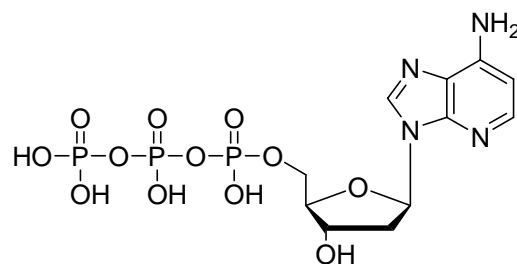


<sup>1</sup>H NMR 80% DMSO-d<sub>6</sub> 20% D<sub>2</sub>O. 8.24(1H, s, 8-CH), 7.80(1H,d, J=5.50 Hz, 2-CH), 6.44(1H, d, J=5.50 Hz, 1-CH), 6.34 (1H, d/d, J= 2.75, 1.25 Hz, 1'-CH), 4.40(1H, m, 4'-CH), 3.92(1H, m, 3'-CH), 3.62 (1H, d/d, J=12.2, 3.3 Hz, 5'-CH), 3.54(1H, d/d, J=12.5, 3.5 Hz, 5'-CH) 2.69(1H, m, 2'-CH), 2.25, (1H, d/d/d, 2'-CH).

MS *m/z* (relative intensity) 251 (M+1, 100), 133(57)

*1-Deaza-2'-deoxyadenosine 5'-triphosphate* was prepared from 1-deaza-2'-deoxyadenosine by the Ludwig procedure (1) with modifications as described (2). The triphosphate was purified by ion exchange chromatography with a 0.1 M to 1 M Et<sub>3</sub>N-HCO<sub>3</sub> (pH 7.5) gradient at 1 mL/min using a Mono Q HR 10/10 column (Amersham Biosciences). The triphosphate was characterized by broad-band decoupled <sup>31</sup>P-NMR. Three phosphate signals were detected, very similar to that found with a dATP sample.

<sup>1</sup>H NMR D<sub>2</sub>O. 8.29(1H, s, 8-CH), 7.80(1H,d, 2-CH), 6.44 (1H, d, 1-CH), 6.25 (1H, m, 1-CH) 4.40(1H, m, 4'-CH), 3.84(1H, m, 3'-CH), 3.65 (1H, m, 5'-CH), 3.55(1H, m, 5'-CH),

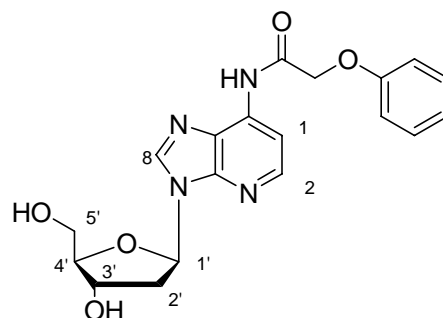


2.88(1H, m, 2'-CH), 2.29, (1H, m, 2'-CH)

<sup>31</sup>P NMR D<sub>2</sub>O/H<sub>2</sub>O (1/1) (external Na<sub>2</sub>PO<sub>4</sub>) 43.8 (s), 39.2 (s), and 28.1 (s).

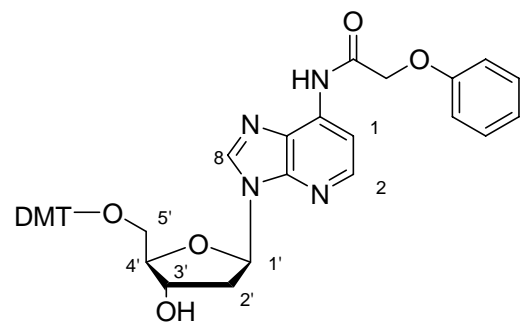
*1-Deaza-2'-deoxy-N<sup>2</sup>-phenoxyacetyladenosine*. 1-Deaza-2'-deoxyadenosine (0.51 g, 2.0 mmol), dried by repeated anhydrous pyridine evaporations, was suspended in 20 mL anhydrous pyridine and cooled in an ice bath. Freshly distilled chlorotrimethylsilane (1.03 mL, 8 mmol) was added dropwise. After stirring for 60 min in the ice bath, phenoxyacetyl chloride (375 μL, 1.3 mmol) was added dropwise and the solution was stirred overnight as the temperature rose to room temperature. The reaction mixture was cooled on ice and then mixed with 10 mL ice cold H<sub>2</sub>O. After 10 min, 10 mL of cold concentrated NH<sub>4</sub>OH was added. The solution was evaporated and the product was purified by silica gel chromatography by elution with initially 5% CH<sub>3</sub>OH/CH<sub>2</sub>Cl<sub>2</sub>, followed by 15% CH<sub>3</sub>OH/CH<sub>2</sub>Cl<sub>2</sub>. The yield was 0.78 g (100%).

<sup>1</sup>H NMR DMSO-d<sub>6</sub>. 8.8 (1H, br-s, NH), 8.27 (1H, s, 8-CH), 7.78(1H,d, 2-CH), 7.3 (2H, d, phenyl), 7.1(3H, m, phenyl), 6.55(1H, d, 1-CH) 6.34 (1H, m, 1'-CH), 5.7 (1H, d/d, 5'-OH), 5.37(1H, d, 3'-OH), 4.6 (2H, s, CH<sub>2</sub>) 4.41(1H, m, 4'-CH), 3.9(1H, m, 3'-CH), 3.6 (1H, m, 5'-CH), 3.51(1H, m, 5'-CH) 2.72(1H, m, 2'-CH), 2.20, (1H, d/d/d, 2'-CH). HRMS (EI): Exact mass calcd for C<sub>19</sub> H<sub>20</sub> O<sub>5</sub> N<sub>4</sub> 384.1434 . Found 384.1429.



*1-Deaza-2'-deoxy-5'-O-(dimethoxytrityl)-N<sup>2</sup>-phenoxyacetyladenosine*. 1-Deaza-2'-deoxy-N<sup>2</sup>-phenoxyacetyladenosine (0.78 g, 2 mmol), dried by repeated anhydrous pyridine evaporations, was suspended in 20 mL anhydrous pyridine and cooled in an ice bath. 4,4'-Dimethoxytrityl chloride (1.0 g, 3 mmol) was added and the reaction was removed from the ice bath and stirred for 5 h. Methanol (10 mL) was added and the solvent evaporated. The product was purified by silica gel chromatography by elution with 1-5% CH<sub>3</sub>OH/CH<sub>2</sub>Cl<sub>2</sub> in a 75% yield.

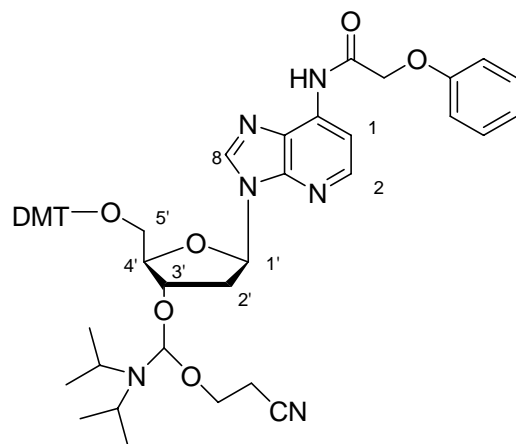
<sup>1</sup>H NMR CDCl<sub>3</sub>. 8.7 (1H, br-s, NH), 8.07 (1H, s, 8-CH), 7.78(1H,d, 2-CH), 7.3 (10H, d, phenyl and trityl), 7.1(4H, m, phenyl and trityl), 6.75 (4H, d, trityl), 6.55(1H, d, 1-CH), 6.34 (1H, m, 1'-CH), 4.65(2H, s, CH<sub>2</sub>) 4.41(1H, m, 4'-CH), 3.91(1H, m, 3'-CH), 3.75 (6H, s, trityl-OCH<sub>3</sub>), 3.69 (1H, d/t, 5'-CH), 3.63(1H, m, 5'-CH)



2.72(1H, m, 2'-CH), 2.25, (1H, m, 2'-CH). HRMS (EI): Exact mass calcd for C<sub>40</sub> H<sub>38</sub> O<sub>7</sub> N<sub>4</sub> [Na]<sup>+</sup>,709.74209. Found 709.74213.

*1-Deaza-2'-deoxy-5'-O-(dimethoxytrityl)-N<sup>2</sup>-phenoxyacetyladenosine 3'-[(2-cyanoethyl)-(N,N-diisopropyl)]phosphoramidite.* 1-Deaza-2'-deoxy-5'-O-(dimethoxytrityl)-N<sup>2</sup>-phenoxyacetyladenosine (0.2 g, 310 μmol) dissolved in 2 mL anhydrous acetonitrile with 80 μL diisopropylethylamine (460, 310 μmol). 2-Cyanoethyl N,N-diisopropylchlorophosphoramidite (77 μL, 340 μmol) was added and the reaction was stirred for 30-60 min. Methylene chloride (10 mL) was added and the solution was washed with water (10 mL) three times. The solution was dried with Na<sub>2</sub>SO<sub>4</sub> and evaporated to yield 200 mg of an oil. This was used without further purification for the oligodeoxynucleotide synthesis. For analysis the diastereomers of the phosphoramidite were separated by flash chromatography using 2% MeOH/0.1% Et<sub>3</sub>N/CH<sub>2</sub>Cl<sub>2</sub>. The <sup>1</sup>H NMR spectra for the upper band is shown in Figure S1 and that for the lower band is in Figure S2.

The upper band. <sup>1</sup>H NMR CDCl<sub>3</sub>. 9.58 (1H, br-s, NH), 8.32 (1H, d, J=6.0 Hz, 1 or 2), 8.27 (1H,d,J=5.4 Hz, 1 or 2), 8.20(1H, s, 8), 7.44(2H,m, Ar), 7.39(2H,m, Ar), 7.32(4H,m, Ar), 7.28(2H, m, Ar), 7.23(1H,m, Ar), 7.11(3H,m, Ar), 6.81(4H,m, Ar), 6.60(1H, t, 1'), 4.81(1H,m,3'), 4.74(2H,s,COCH<sub>2</sub>O), 4.34(1H,m,4'), 3.81(6H,s,CH<sub>3</sub>O), 3.71(2H,m,OCH<sub>2</sub>), 3.65(2H,m, NCH), 3.48(1H,d/d, 5'), 3.35(1H,d/d, 5'), 2.96(1H,m,2'), 2.64(1H,m,2'), 2.50(2H,t,CH<sub>2</sub>CN),



1.21(12H,m,CH<sub>3</sub>). HRMS (EI): Exact mass calcd for C<sub>49</sub> H<sub>55</sub> O<sub>8</sub> N<sub>6</sub> P [Na]<sup>+</sup>, 909.3711. Found 909.3671.

The lower band. (1H, s, 8-CH), 7.78(1H,d, 2-CH), 7.3 (10H, d, phenyl and trityl), 7.1(4H, m, phenyl and trityl), 6.75 (4H, d, trityl) 6.55(1H, d, 1-CH) 6.34 (1H, m, 1'-CH), 4.65(2H, s, CH<sub>2</sub>) 4.41(1H, m, 4'-CH), 3.91(1H, m, 3'-CH), 3.75 (6H, s, trityl-OCH<sub>3</sub>), 3.69 (1H, d/t, 5'-CH), 3.63(1H, m, 5'-CH) 2.72(1H, m, 2'-CH), 2.25, (1H, m, 2'-CH). HRMS (EI): Exact mass calcd for C<sub>49</sub> H<sub>55</sub> O<sub>8</sub> N<sub>6</sub> P [Na]<sup>+</sup>, 909.3711. Found 909.37396.

### **Oligodeoxynucleotide synthesis.**

The oligodeoxynucleotides containing 1-deaza-dA, 7-deaza-dA, and 7-deaza-dG were synthesized by the phosphoramidite method on an Expedite 8098 DNA synthesizer with standard protocols. The oligodeoxynucleotides were purified by PAGE followed by reverse-phase HPLC. They were judged > 95% pure by PAGE in which the oligonucleotides, which were 5'-labeled with <sup>32</sup>P-phosphate, appeared as single bands.

*Enzyme hydrolysis* Oligodeoxynucleotide (0.1 A260 unit) was incubated in 50 mM Tris-HCl (pH 8.5), 15 mM MgCl<sub>2</sub> at 37°C with snake venom phosphodiesterase (1 unit) (United States Biochemical, Cleveland OH) for 1 h followed by bacterial alkaline phosphatase (10 units) (Sigma) for 1 h. Analysis of the base compositions of the oligodeoxynucleotides was performed using a 4.6 x 250 mm Luna 5μ C18(2) column (Phenomenex, Torrance, CA) with 100 mM sodium phosphate (pH 4.8) as buffer A and methanol as B. Separation of the nucleosides was effected with a linear gradient from 5 to 50% B over 45 min. The retention times (min) were as follows: dC (11.4), dG (22.5), dT (25.9), 7-deaza-dG (30.8), dA (33.0), 1-deaza-dA (39), and 7-deaza-dA (45 min).



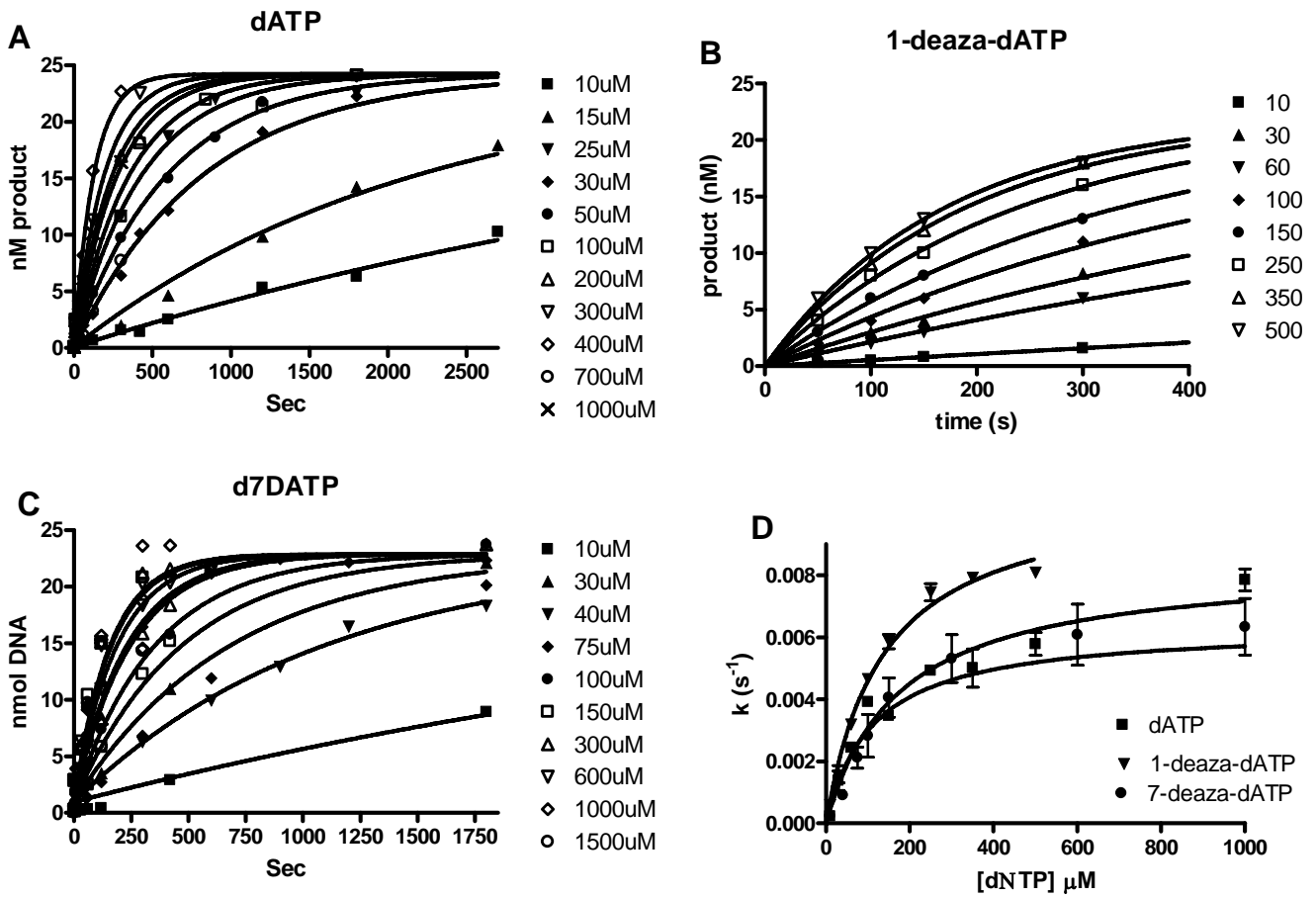


Figure S3. Dpo4-catalyzed incorporation of dNTPs opposite dA in 50 mM Tris-HCl (pH 7.8), 5 mM MgCl<sub>2</sub>, 30 mM NaCl, 50  $\mu$ M EDTA, 2.5 mM DTT, 50  $\mu$ g /mL BSA. The concentration of Dpo4 was 100 nM while the DNA duplex concentration was 25 nM. Panels A-C show the time courses for the incorporation of (A) dATP, (B) 1-deaza-dATP, and (C) 7-deaza-dATP. The concentrations of each dNTP are showed in each panel ( $\mu$ M). D. Plot of  $k$  versus [dNTP]. Each point is the mean of three independent experiments in which the error bars show the standard deviation.

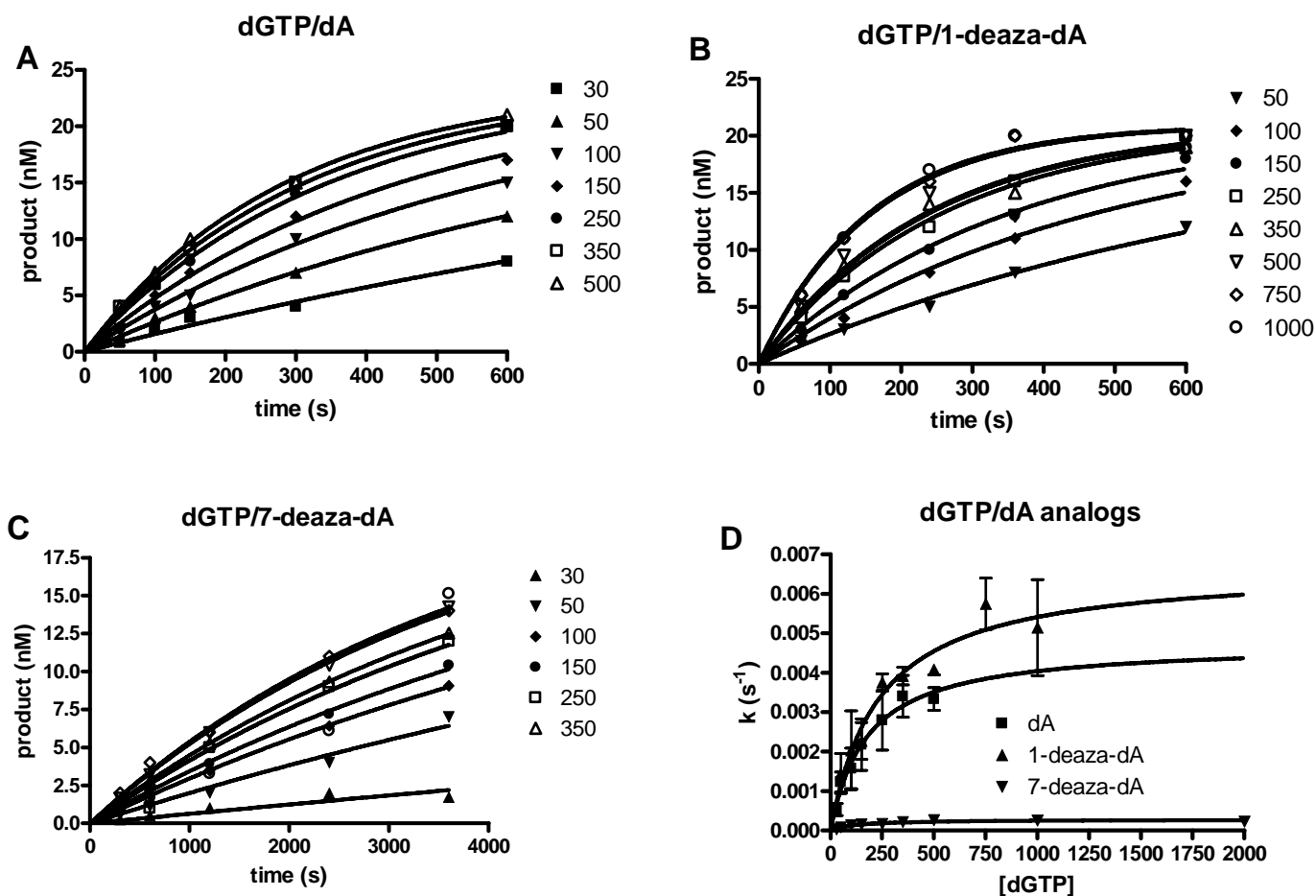


Figure S4. Dpo4-catalyzed incorporation of dGTPs opposite adenine, 1-deazaadenine and 7-deazaadenine in 50 mM Tris-HCl (pH 7.8), 5 mM MgCl<sub>2</sub>, 30 mM NaCl, 50 μM EDTA, 2.5 mM DTT, 50 μg/mL BSA. The concentration of Dpo4 was 100 nM while the DNA duplex concentration was 25 nM. Panels A-C show the time courses for the incorporation of dGTP opposite (A) dA (B) 1-deaza-dA, and (C) 7-deaza-dA. The concentrations of each dNTP are showed in each panel (μM). D. Plot of  $k$  versus [dGTP] for the incorporation opposite dA (■), 1-deaza-dA (▲), and 7-deaza-dA (▼). Each point is the mean of three independent experiments in which the error bars show the standard deviation.

1. Ludwig,J. (1981) A new route to nucleoside 5'-triphosphates. *Acta Biochim. et Biophys. Acad. Sci. Hung.*, *16*, 131-133.
2. Meyer,A.S., Blandino,M., and Spratt,T.E. (2004) E. coli DNA polymerase I (Klenow fragment) uses a hydrogen bonding fork from Arg668 to the primer terminus and incoming deoxynucleotide triphosphate to catalyze DNA replication. *J. Biol. Chem.*, *279*, 33043-33046.
3. Antonini,I., Cristalli,G., Franchetti,P., Grifantini,M., Martelli,S., and Petrelli,F. (1984) Deaza analogues of adenosine as inhibitors of blood platelet aggregation. *J. Pharm. Sci.*, *73*, 366-369.
4. Cristalli,G., Franchetti,P., Grifantini,M., Vittori,S., Bordoni,T., and Geroni,C. (1987) Improved synthesis and antitumor activity of 1-deazaadenosine. *J. Med. Chem.*, *30*, 1686-1688.



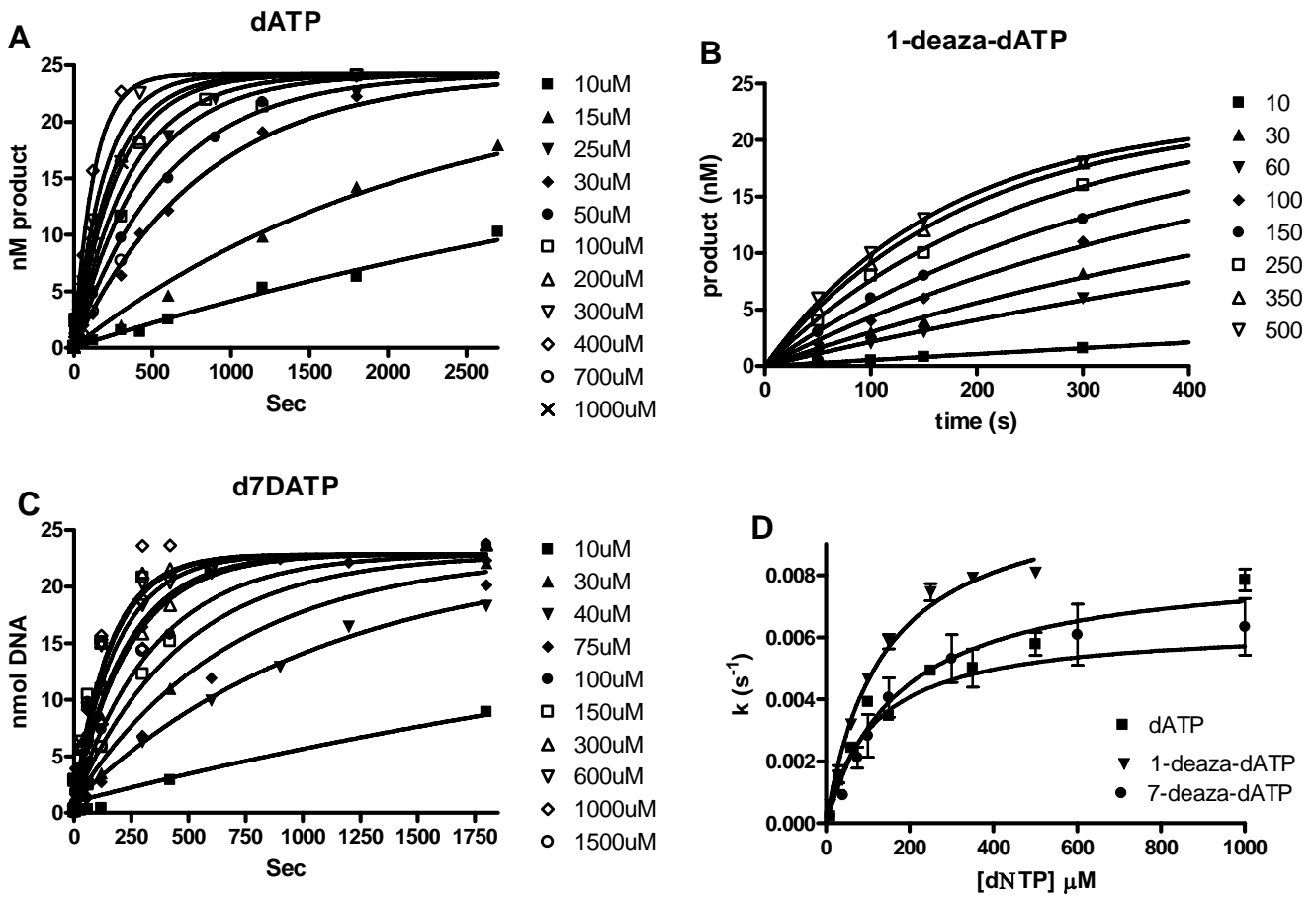


Figure S3. Dpo4-catalyzed incorporation of dNTPs opposite dA in 50 mM Tris-HCl (pH 7.8), 5 mM  $MgCl_2$ , 30 mM NaCl, 50  $\mu$ M EDTA, 2.5 mM DTT, 50  $\mu$ g /mL BSA. The concentration of Dpo4 was 100 nM while the DNA duplex concentration was 25 nM. Panels A-C show the time courses for the incorporation of (A) dATP, (B) 1-deaza-dATP, and (C) 7-deaza-dATP. The concentrations of each dNTP are showed in each panel ( $\mu$ M). D. Plot of  $k$  versus [dNTP]. Each point is the mean of three independent experiments in which the error bars show the standard deviation.

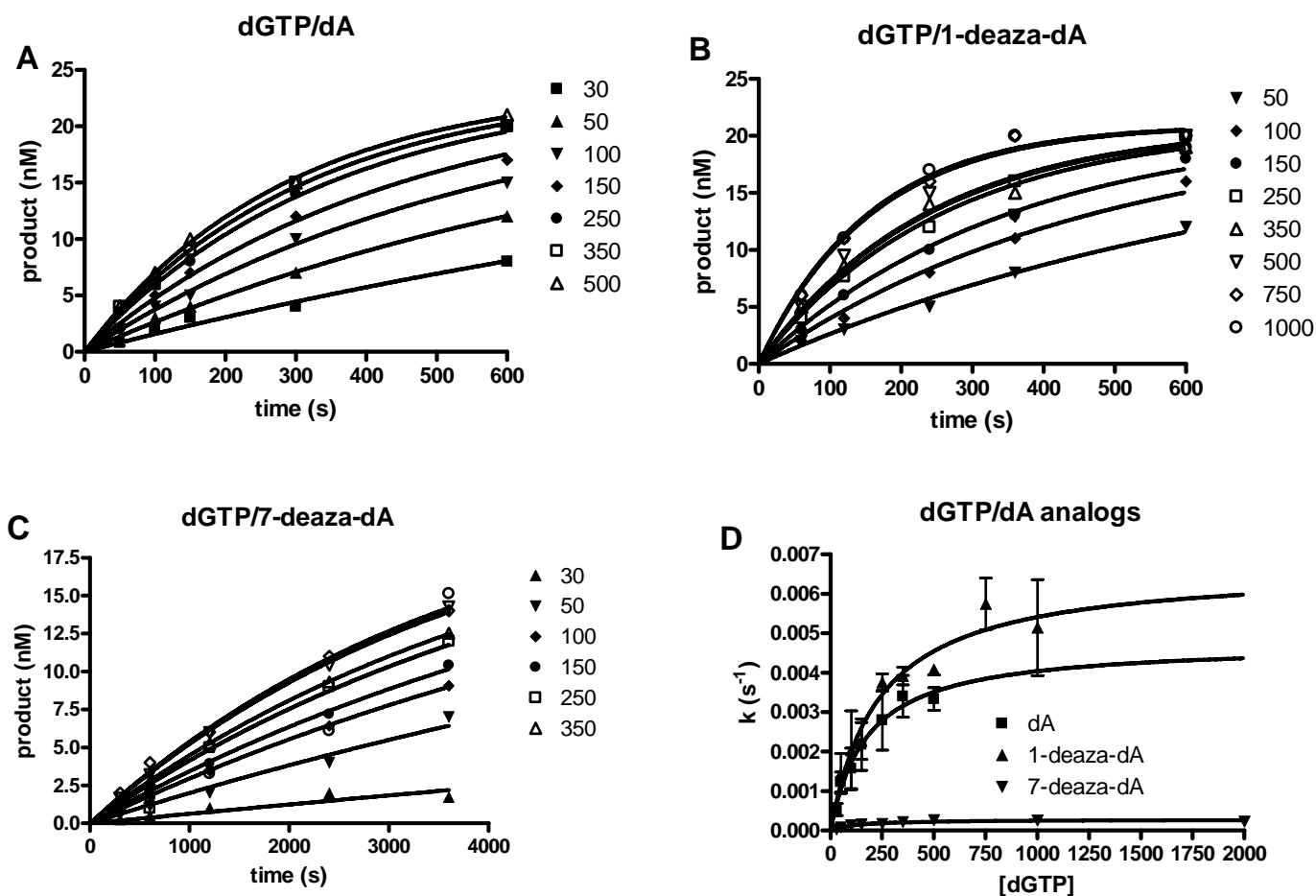


Figure S4. Dpo4-catalyzed incorporation of dGTPs opposite adenine, 1-deazaadenine and 7-deazaadenine in 50 mM Tris-HCl (pH 7.8), 5 mM  $\text{MgCl}_2$ , 30 mM NaCl, 50  $\mu\text{M}$  EDTA, 2.5 mM DTT, 50  $\mu\text{g}/\text{mL}$  BSA. The concentration of Dpo4 was 100 nM while the DNA duplex concentration was 25 nM. Panels A-C show the time courses for the incorporation of dGTP opposite (A) dA (B) 1-deaza-dA, and (C) 7-deaza-dA. The concentrations of each dNTP are showed in each panel ( $\mu\text{M}$ ). D. Plot of  $k$  versus [dGTP] for the incorporation opposite dA (■), 1-deaza-dA (▲), and 7-deaza-dA (▼). Each point is the mean of three independent experiments in which the error bars show the standard deviation.

1. Ludwig,J. (1981) A new route to nucleoside 5'-triphosphates. *Acta Biochim. et Biophys. Acad. Sci. Hung.*, *16*, 131-133.
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Figure S1

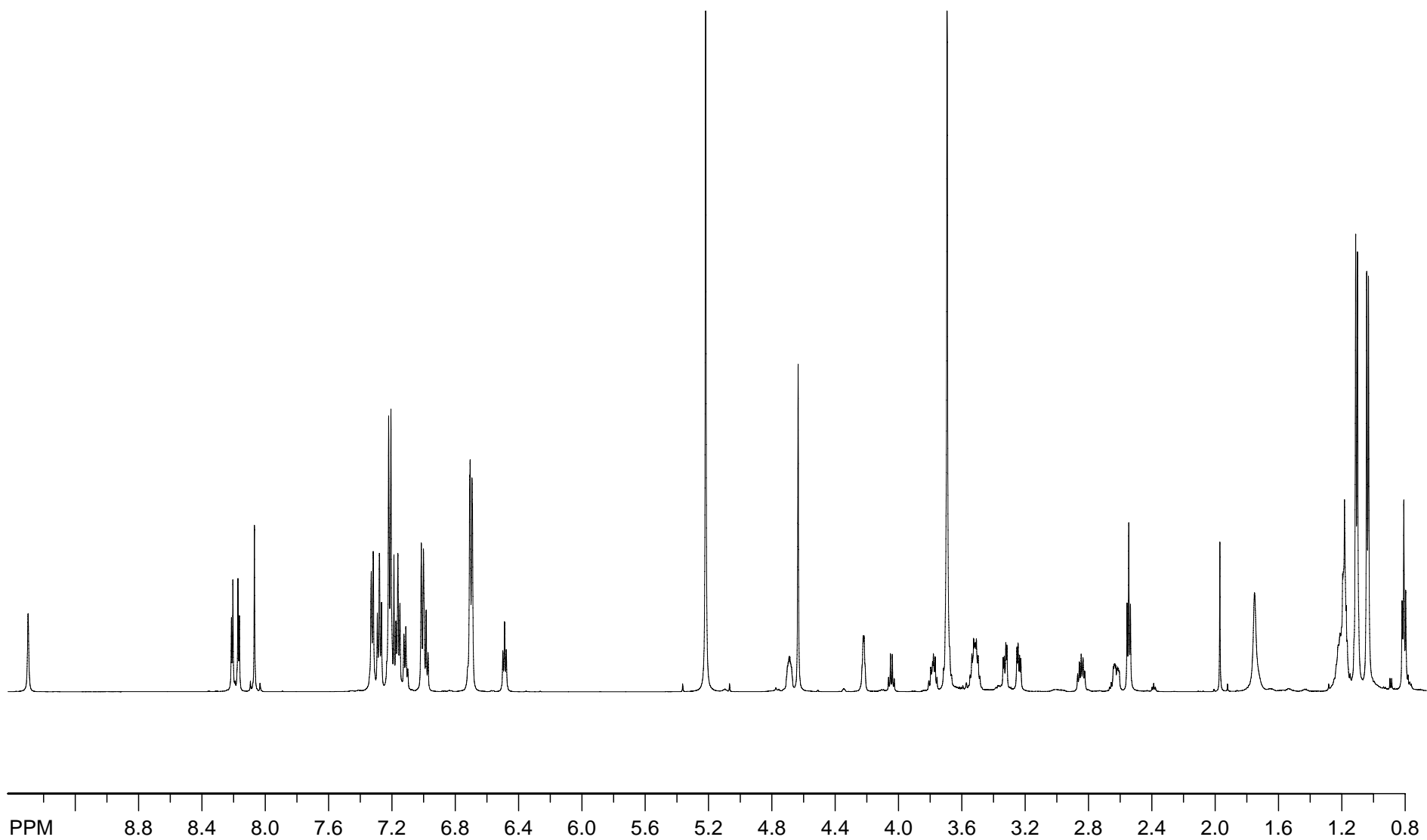


Figure S2

