# 3′-Fluoro-2′,3′-dideoxyribonucleoside 5′-triphosphates: terminators of DNA synthesis

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It is shown that dNTP(3′F) are terminators of DNA synthesis and may serve as very effective tools for DNA sequencing with *E.coli* DNA polymerase I and AMV reverse transcriptase. The dNTP(3′F) are found to be chain terminator substrates for calf thymus terminal deoxyribonucleotidyl transferase but not for calf thymus DNA polymerase α. The optimal dNTP(3′F) concentration for DNA sequencing by DNA polymerase I is found to be an order of magnitude lower than that of ddNTPs. dNTP(3′F) produce a more clear sequence pattern than do ddNTPs.

*3′-Fluoro-2′,3′-dideoxyribonucleoside 5′-triphosphate Terminator DNA synthesis DNA polymerase Terminal deoxyribonucleotidyl transferase*

## 1. INTRODUCTION

With the aim of finding compounds that may serve as inhibitors of DNA synthesis with different specificity to different DNA polymerases, the dNTP(3′F) analogue of dNTP was studied. The inhibition of DNA synthesis may be due to irreversible incorporation of the nucleotide analogue residues, dNMP(3′F), (likewise ddNTP (I) or dNTP(3′$NH_2$) [2]) at the 3′-terminus of the growing DNA chain resulting in a molecule which has no 3′-hydroxyl group available for chain extension. Application of the compounds for DNA sequencing by the method of Sanger et al. [1] was one of the goals in this work.

We synthesized 4 dNTP(3′F) with thymine, cytosine, adenine and guanine bases and found that all these compounds were powerful terminators of DNA synthesis, catalyzed by *E. coli* DNA polymerase I, AMV reverse transcriptase, and Bollum terminal transferase.

*Abbreviations:* dNTP(3′F), 3′-fluoro-2′,3′-dideoxyribonucleoside 5′-triphosphates with the bases adenine [dATP(3′F)], guanine [dGTP(3′F)], thymine [dTTP-(3′F)] and cytosine [dCTP(3′F)]; dNMP(3′F), 3′-fluoro-2′,3′-dideoxyribonucleoside 5′-monophosphates with the same base nature; dThd(3′F), dCyt(3′F), dAdo(3′F) and dGua(3′F), 3′-fluoro-2′,3′-dideoxynucleoside; ddNTP, 2′,3′-dideoxynucleoside 5′-triphosphates; dNTP(3′$NH_2$), 3′-amino-2′,3′-dideoxynucleoside 5′-triphosphates; rNTP, ribonucleoside 5′-triphosphates

## 2. MATERIALS AND METHODS

The synthesis of dThd(3′F) has been described [3]; the synthesis of dCyd(3′F), dAdo(3′F) and dGuo(3′F) will be published elsewhere. dTMP(3′F), dCMP(3′F) and dAMP(3′C) were synthesized by reaction of the corresponding nucleosides with phosphorus oxychloride in trimethyl phosphate [4] according to [5] and purified by ion-exchange chromatography on Sephadex A-25 ($HCO_3^-$) [5] with yields of 74, 55 and 57%, respectively. dGMP(3′F) was obtained by the action of pyrophosphoryl chloride in ethyl acetate [6] on $N^2$-palmitoyl-dGuo(3′F), subse-

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quently deblocked with concentrated aqueous ammonia and purified by ion-exchange chromatography on Sephadex A-25 ($HCO_3^-$). Elution was effected with a 0.01–0.8 M gradient of TEAB buffer with an 80% yield. dNTP(3'F) were synthesized according to Hoard and Ott [7], isolated in a pure form by chromatography on Sephadex A-25 ($HCO_3^-$) using a linear gradient of TEAB buffer (0.001–0.8 M) as eluent. dNTP(3'F) $Na^+$ salts were obtained as amorphous powders according to [8]. The purity of all synthesized NMP(3'F) and NTP(3'F) was checked by thin-layer chromatography (TLC), UV spectroscopy and degradation to the corresponding fluoronucleosides under the action of alkaline phosphatase.

### 2.1. *DNA synthesis*

DNA synthesis by DNA polymerase from *E. coli* and $\alpha$ calf thymus was according to [2]: DNA pBRS2950 was cut by *Eco*RI restriction endonuclease at the unique site. The DNA ends were partially hydrolyzed with *E. coli Eco*III exonuclease for 10–15 min. The lengths of the hydrolyzed ends were 80–120 nucleotides. The hydrolyzed ends were then partially (20–40 nucleotides) restored with DNA polymerase I and [$\alpha$-$^{32}$P]NTPs. The enzymes were destroyed by heating, and dNTPs removed by gel filtration on a Sephadex G-100 column. The $^{32}$P-labelled DNA was used with DNA polymerases I or $\alpha$ for DNA chain elongation with all 4 unlabelled dNTP and one of the dNTP(3'F).

Reaction conditions for DNA polymerase I were: 50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 0.1 mM EDTA, 10 mg/ml BSA, 0.25 $\mu$M dNTP (each of), 50 $\mu$M dNTP(3'F), 10 units DNA polymerase I, 0.5 $\mu$g $^{32}$P-labelled pBRS2950 DNA; total volume 20 $\mu$l, incubation time 30 s at 37°C. For DNA polymerase $\alpha$: 50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 0.1 mM EDTA, 10 $\mu$g/ml BSA, 10 $\mu$M dNTP (each of), 500 $\mu$M dNTP(3'F), 4 units DNA polymerase $\alpha$, 0.5 $\mu$g $^{32}$P-labelled pBRS2950 DNA; total volume 20 $\mu$l, incubation time 30 min at 37°C.

Then the DNA was cut with the *Hin*dIII restriction endonuclease (Institute of Applied Enzymology, Vilnus) into two parts 40–130 bp and 3400 bp long and analyzed by 20% polyacrylamide gel electrophoresis under denaturing conditions [2].

### 2.2. *Reaction conditions for terminal deoxyribonucleotidyl transferase (TdT)*

These conditions were: 0.1 M potassium

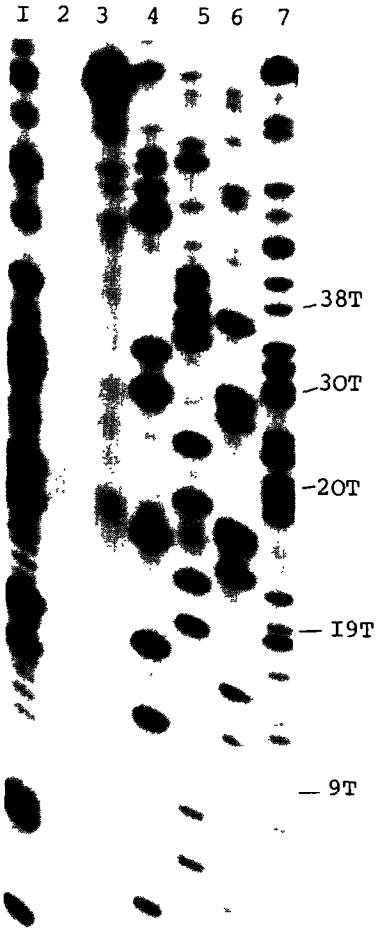


Fig.1. Electropherogram of pBRS2950 DNA elongated in the presence of dNTP(3'F). (1) Initial [$^{32}$P]DNA. (2) DNA hydrolyzed by DNA polymerase I in the absence of dNTPs. (3) DNA synthesized for 30 s in the presence of 0.25 $\mu$M natural dNTPs without inhibitor. (4–7) The original DNA elongated in the presence of dNTPs and dNTP(3'F), (4) dATP(3'F), (5) dGTP(3'F), (6) dCTP(3'F), (7) dTTP(3'F). The DNA sequence is: ACCGTGTTTA CTTCATGTTG TCAACCGTTT TGTTTCACCT TATGTGCGTG.

cacodylate (pH 6.8), 0.1 M $CH_3COOK$, 5 mM $MgCl_2$, 0.5 mM $CoCl_2$, 200 $\mu$M dNTP (or an analog), 1 $\mu$M 5'-[$^{32}$P]oligodeoxyribonucleotide of 12 nucleotides in length, 0.1 units/$\mu$l TdT, total volume 10 $\mu$l, reaction time 1 h at 37°C. Reaction was initiated by enzyme addition and terminated with 10 mM EDTA. Reaction products were analyzed by 30% polyacrylamide gel electrophoresis under denaturing conditions. One unit was the enzyme amount sufficient to incorporate 1 nmol [$\alpha$-$^{32}$P]dCTP into oligonucleotide under the above-described conditions for 1 h. The enzyme was purified from calf thymus. The specific activity of the enzyme was 20 units/$\mu$g.

### 2.3. *Reaction conditions for AMV reverse transcriptase*

These conditions were 30 mM Tris-HCl (pH 8.4), 6 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 50 mM KCl, 0.1 mM EDTA, 0.01 $\mu$M [$^{32}$P]oligonucleotide primer, 0.01 $\mu$M single-stranded MI3mp8 template and 0.2 $\mu$g pBR322 DNA carrier, 10 $\mu$M dNTP (of each). Reaction was initiated by the addition of enzyme to 3.5 units/20 $\mu$l concentration and terminated after 3 min by adding 1 mM of the dNTP(3'F). After 30 min at 42°C, 10 mM EDTA was added and the resulting mixtures were analyzed via polyacrylamide gel electrophoresis under denaturing conditions.

## 3. RESULTS AND DISCUSSION

Fig.1 shows the 'termination' pattern of the [$^{32}$P]DNA elongated in the presence of dNTP(3'F). The sequencing pattern with a minimum of misleading bands is typical. The optimal concentration of dNTP(3'F) for the reading of 50–100 long nucleotides in the gel was found to be 50 $\mu$M, the dNTP concentration being 0.25 $\mu$M. For a longer DNA one needs a lower concentration

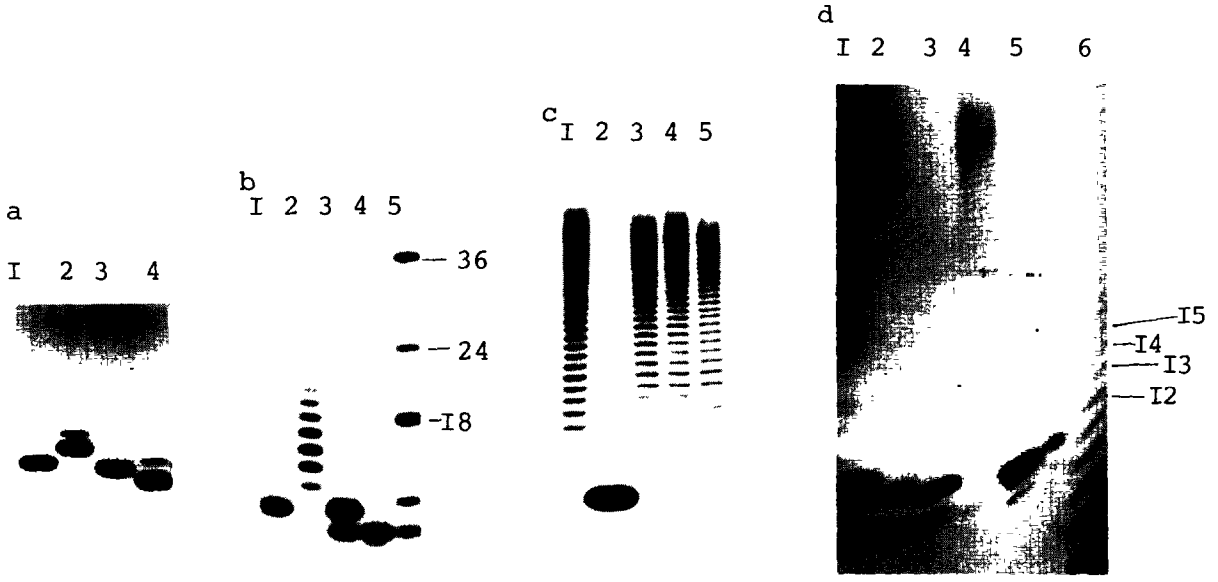


Fig.2. Elongation of [$^{32}$P]oligodeoxynucleotide by TdT in the presence of analogs as described in the text. (a) 1, dATP(3'F); 2, rCTP; 3, ddCTP; 4, without nucleotides. Reaction conditions are described in the text. (b) The same as (a) but: 1, rUTP; 2, dTTP; 3, dTTP(3'F); 4, without nucleotides; incubation time 0.5 h; 5, length marker oligonucleotides. (c) the same as (b) but simultaneously with 20 mM inhibitor the 200 mM dTTP was added. The experiment imitates the second step of the experiment displayed in (d). Tracks: 1, dATP(3'F); 2, original oligonucleotide; 3, dTTP(3'F); 4, ddTTP; 5, without nucleotides. (d) The [$^{32}$P]oligonucleotide was incubated with TdT as described in (a) with: 1, rCTP; 2, dTTP; 3, dATP(3'F); 4, without nucleotides and the probes were diluted 10-fold with fresh incubation mixture lacking oligonucleotide and inhibitor but containing 20 mM dTTP and further incubated for 0.5 h at 37°C; track 5, initial [$^{32}$P]oligonucleotide. Track 6, [$^{32}$P]poly(dT). Numbers denote the length of oligonucleotide in electropherograms.

of dNTP(3'F) or a higher concentration of dNTP (not shown).

From our experience the sequencing pattern of dNTP(3'F) analogs is clearer when compared with those of ddNTP and dNTP(3'$NH_2$).

The results obtained with AMV reverse transcriptase show a clear termination pattern with all 4 dNTP(3'F) analogs (not shown) with efficiency similar to dNTP(3'$NH_2$), araNTP(3'$NH_2$) and some other dNTP analogs [9].

Similar to ddNTP and in contrast to dNTP(3'$NH_2$), the dNTP(3'F)s were unable (not shown) to terminate DNA polymerase $\alpha$ of calf thymus. In addition, the data of Schroeder and Jantschak [10] indicate that DNA polymerase from *M. luteus* and reverse transcriptase of Raucher leukemia virus are able to utilize dTTP(3'F) as substrates whereas DNA polymerase of T4 phage cannot. These data taken together show that different DNA polymerases can be selectively inhibited with different terminating nucleotides.

Fig.2 shows that dNTP(3'F) can be incorporated into the 3'-end of oligonucleotide by TdT. The enzyme can readily incorporate dNMP(3'F) residues at the 3'-end of a primer, thus preventing further elongation of the primer in the presence of dNTP. Incorporation of the dNMP(3'F) is irreversible within the time scale of the experiment. The relative rate of incorporation of synonymic dNTP(3'F) and dNTP was found to be 20% at equal concentrations (200 $\mu$M). The high efficiency of dNTP(3'F) incorporation together with the inability of the analogs to inhibit DNA polymerase $\alpha$ may permit one to use the analogs for the study of the function of TdT. The ability of the corresponding nucleotides to be phosphorylated and incorporated into the pool of dNTP has been demonstrated for crude extract of carcinoma cells [11].

## 4. CONCLUSIONS

(i) The data described above show that dNTP(3'F) might be used for DNA sequencing.

(ii) Specific incorporation of the nucleotide containing the F atom at the 3'-end of DNA presents new possibilities for investigation of protein-nucleic acid interaction using NMR, for instance, to study the interaction of DNA polymerase with the correctly or incorrectly terminated primer.

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## REFERENCES

[1] Sanger, F., Neiden, S. and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463–5467.
[2] Chidgeavadze, Z.G., Beabealashvilli, R.Sh., Atrazhev, A.M., Kukhanova, M.K., Azhayev, A.V. and Krayevsky, A.A. (1984) Nucleic Acids Res. 12, 1671–1686.
[3] Kowollik, G., Etzold, G., Janta-Lipinski, M., Von Gaertner, K. and Langen, P. (1973) J. Pract. Chem. 315, 895–900.
[4] Yoshikawa, M., Kato, T. and Takenshi, T. (1979) Bull. Chem. Soc. Jap. 42, 3505–3508.
[5] Mikhailopulo, I.A. and Cramer, F. (1982) FEBS Lett. 144, 109–111.
[6] Epstein, D.A., March, Y.V., Schryver, B.B., Larsen, M.A., Barnett, J.W., Werheiden, J.P.H. and Prisbe, E.J. (1982) J. Biol. Chem. 257, 13390–13397.
[7] Hoard, D.E. and Ott, D.G. (1965) J. Am. Chem. Soc. 87, 1785–1788.
[8] Moffatt, J.G. (1964) Can. J. Chem. 42, 599–604.
[9] Beabealashvilli, R.Sh., Scamrov, A.V., Kutateladze, T.V., Mazo, A.M., Krayevsky, A.A. and Kukhanova, M.K. (1985) Nucleic Acids Res., submitted.
[10] Schroeder, C. and Jantschak, J. (1980) Allg. Mikrobiol. 20, 657–662.
[11] Langen, P., Kowollik, G., Etzold, G., Vennerm, H. and Reinert, H. (1972) Acta Biol. Med. Germ. 20, 483–494.