# Modified DNA Bearing 5-(Methoxycarbonylmethyl)-2′-deoxyuridine: Preparation by PCR with Thermophilic DNA Polymerase and Postsynthetic Derivatization

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*A thymidine analogue bearing a methyl ester at the C5 position was accepted as a substrate by the thermophilic family B DNA polymerases, KOD Dash, Pwo, and Vent(exo−), to form the corresponding PCR product, but not by the thermophilic family A DNA polymerases, Taq, Tth, and T7 thermosequenase. Modified DNA containing this analogue was prepared by PCR on a large scale with KOD Dash DNA polymerase and 5-(methoxycarbonylmethyl)-2′-deoxyuridine 5′-triphosphate as a substrate. The methyl ester of the modified DNA was further allowed to react with tris(2-aminoethyl)amine or histamine by an ester–amide exchange reaction to form the corresponding derivatized DNA bearing a tris(2-aminoethyl)amine or histamine moiety. Hydrolysis of the methyl ester of the modified DNA gave a functionalized DNA bearing an anionic carboxyl group. The derivatized DNA could act as a template for the PCR with KOD Dash DNA polymerase and the natural 2′-deoxythymidine 5′-triphosphate or the modified thymidine analogue as a substrate. The postsynthetic derivatization of the modified DNA may expand the variety of structurally modified DNA produced by PCR.*

**KEYWORDS:**

DNA modification · DNA polymerase · polymerase chain reaction · thymidine analogues

## Introduction

Modified oligonucleotides with a variety of functional groups are an important tool for biological and biochemical studies.[1–3] The C5 position of the pyrimidine ring is an appropriate site for the modification, because it is located in the major groove of double-stranded DNA and does not inhibit A:T base pairing. Previously, we reported the synthesis of thymidine analogues, C5-substituted 2′-deoxyuridines bearing amino linker arms at the C5 position,[4] and their introduction into oligodeoxyribonucleotides.[5]

Usually, modified DNA is prepared chemically by using a DNA synthesizer. However, if a DNA polymerase can accept the modified nucleotide as a substrate and can read through modified residues in the DNA template, modified DNA can be prepared enzymatically by PCR. The resulting modified DNA could be used as a DNA probe, a DNA enzyme, or a DNA aptamer by in vitro selection. Thus, nucleotide analogues that are substrates for DNA and RNA polymerases can be powerful tools for biochemical studies. Some DNA polymerases can use 5′-triphosphates of modified 2′-deoxyuridines with a side chain at the C5 position carrying an (*E*)-propenyl[6–10] or propynyl group.[9–13] The differences in reactivity of C5-substituted 2′-deoxyuridines as substrates may be due to the steric effects of large side-groups and/or to the ionic effects of substituting groups such as cationic amino groups or anionic carboxyl groups. The ability of a modified nucleotide to act as a substrate also depends on the kind of DNA polymerase.[14, 15] Recently, we have shown that KOD Dash DNA polymerase can readily use some new thymidine analogue nucleotides as substrates and can read a template DNA containing the modified thymidine, but no other DNA polymerase, including Taq DNA polymerase, can accept the thymidine analogues as substrates.[14, 16] Among the new thymidine analogues that have an $sp^3$ hybridized carbon at the C5 $\alpha$ position with a substituent group, the analogues bearing a methyl ester, ethylenediamine, or hexamethylenediamine moiety are good substrates for the enzyme,

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and the corresponding modified DNAs can be obtained by PCR with KOD Dash DNA polymerase.[16] However, the analogues bearing a tris(2-aminoethylamine), histamine, or anionic carboxyl group were found to be poor substrates for the enzyme.[14, 16] Little or no corresponding modified DNA can be obtained by PCR with any of these analogues as a substrate.[16]

Therefore, we have explored the postsynthetic derivatization of the modified DNA that can be prepared by PCR with the thymidine analogue bearing a methyl ester, because this analogue is a good substrate for KOD Dash DNA polymerase.[16] We have further examined some other DNA polymerases which can accept the thymidine analogue bearing a methyl ester during the PCR, and we found that Pwo and Vent(exo $-$) also produced the corresponding modified DNA. The methyl ester of the resulting modified DNA, produced by PCR on a large scale with KOD Dash DNA polymerase, could further react with a polyamine[17] or hydrolyze to a carboxyl group. The postsynthetic derivatization of the modified DNA bearing a polyamine, histamine, or anionic carboxyl group could form modified DNA with a new functional group, which cannot be obtained directly by PCR and may be useful for the generation of an active DNA enzyme or DNA aptamer by in vitro selection. Here we wish to report the activity of several DNA polymerases for the synthesis of modified DNA, the large-scale synthesis of modified DNA with a methyl ester by PCR, the postsynthetic derivatization of the modified DNA, and the template activity of the resulting DNA after derivatization.

## Results and Discussion

The modified thymidine analogue, 5-(methoxycarbonylmethyl)-2′-deoxyuridine 5′-triphosphate (**1**, Scheme 1), was synthesized according to the method described previously.[14] We studied the ability of the nucleotide **1** to act as a substrate in place of 2′-deoxythymidine 5′-triphosphate (dTTP) for thermostable DNA polymerases under typical PCR conditions with pUC18 plasmid DNA as a template and oligonucleotide A, 5′-GGAAACAGCTATGACCATGATTAC-3′ and oligonucleotide B, 5′-CGACGTTGTAAAACGACGGCCAGT-3′ as primers. Figure 1 shows the results of the PCR with the substrate **1** and several DNA polymerases. All

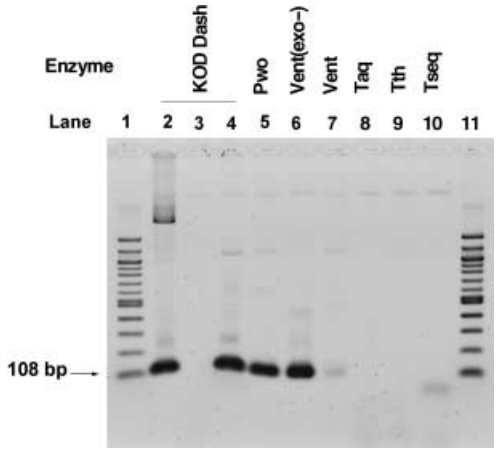
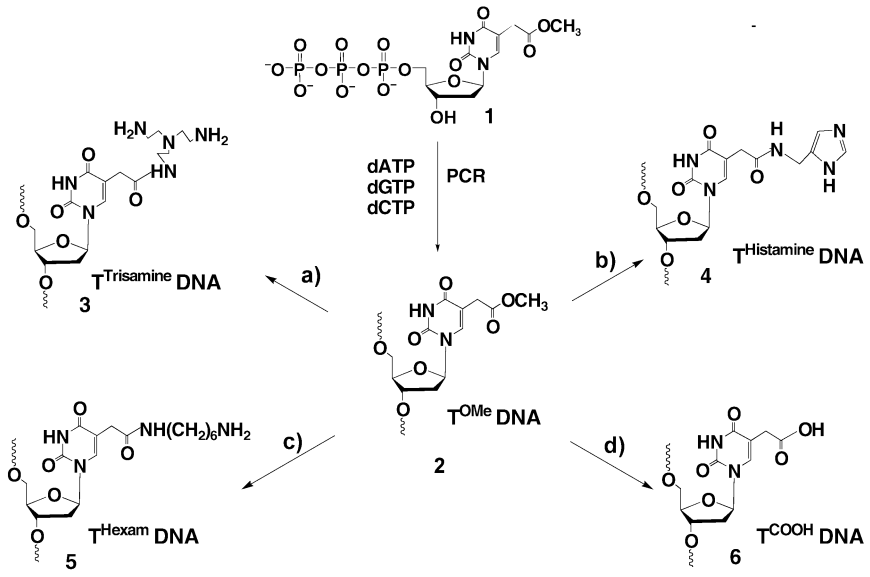



***Figure 1.*** *Gel electrophoresis assay of the PCR of **1** with several DNA polymerases. Assays were carried out as described in the Experimental Section. Lanes 1 and 11: marker DNA (100 – 2000 bp); Lane 2: natural 108-bp DNA from dTTP with KOD Dash (0.005 U); Lane 3: negative control reaction without dTTP in the presence of KOD Dash (0.05 U); Lane 4: **1** with KOD Dash (0.05 U); Lane 5: **1** with Pwo (0.025 U); Lane 6: **1** with Vent(exo $-$) (0.05 U); Lane 7: **1** with Vent (0.01 U); Lane 8: **1** with Taq (0.05 U); Lane 9: **1** with Tth (0.05 U); Lane 10: **1** with T7 thermosequenase (1 U). All unit quantities were per $\mu$L of final reaction volume.*

DNA polymerases could accept the natural substrate, dTTP, to form a DNA with 108 base pairs in the positive control reactions, but they did not yield DNA in the negative control reactions where no dTTP was used (data in the Supporting Information). We confirmed the previous report that KOD Dash DNA polymerase can accept **1** as a substrate to give a 108-bp DNA product (partially shown as **2**, Scheme 1).[16] The 108-bp DNA containing the primers and the template region has 40 modified thymidines with a single-stretch of 4 successive thymidine residues. Pwo and Vent(exo $-$) DNA polymerases could also accept **1** and form a 108-bp PCR product. On the other hand, Vent, Taq, Tth, and T7 thermosequenase DNA polymerases could not produce the corresponding PCR product. Vent DNA polymerase is likely to accept **1** in the PCR, however, the enzyme's strong exonuclease activity could degrade any DNA formed, which would result in no overall formation of the corresponding product. The DNA polymerases, KOD Dash, Pwo, and Vent(exo $-$), which could accept the modified substrate **1**, all belong to the family B DNA polymerases.




***Scheme 1.*** *Synthesis of the modified DNAs **2** – **6** by PCR and postsynthetic derivatization: a) tris(2-aminoethyl)amine; b) histamine; c) hexamethylenediamine; d) sodium hydroxide. Further details are given in the Experimental Section.*

The DNA polymerases that could not produce the PCR product are classified as family A DNA polymerases, except Vent DNA polymerase which has strong exonuclease activity. Our result that family B DNA polymerases could accept the modified thymidine nucleotide more easily than family A DNA polymerases is in accordance with the result of a recent report by Held and Benner.[15] They have shown that family B DNA polymerases, especially Pwo DNA polymerase, can incorporate a thymidine analogue with 5′-position sulfur functionality to produce the corresponding PCR product.

We prepared the modified DNA **2** by PCR on a large scale with **1** as a substrate (Scheme 1). The modified DNA was obtained in an optical density ($OD_{260nm}$) of 4.5 units after the usual extraction and purification by preparative disk-gel electrophoresis. The modified DNA was converted into the triethylammonium salt from the sodium salt by treatment with a cation-exchange resin so that it would dissolve in *N*,*N*-dimethylformamide (DMF). An ester–amide exchange reaction of the methyl ester of the modified DNA with tris(2-aminoethyl)amine was conducted in DMF at 37 °C to give modified DNA bearing a tris(2-aminoethyl)-amine group at the C5 position of thymidine (partially shown as **3**).[17] Similarly, the modified DNA **2** was treated with histamine or hexamethylenediamine to yield the modified DNAs **4** and **5** with histamine and hexamethylenediamine side chains, respectively. Alkaline hydrolysis of the methyl ester on the modified DNA **2** gave the corresponding modified DNA bearing an anionic carboxyl group at the C5 position (partially shown as **6**).

After the derivatization of the modified DNA **2** (in portions of 0.5 $OD_{260nm}$ units) and purification by ethanol precipitation, we obtained the modified DNAs **3**–**6** in 0.28, 0.36, 0.38, and 0.48 $OD_{260nm}$ units, respectively. Figure 2 shows the results of the

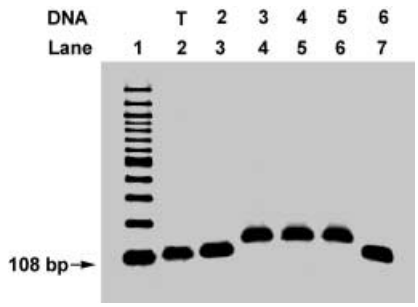



***Figure 2.*** *Gel electrophoresis assay of the modified DNAs obtained by post-synthetic derivatization. Lane 1: marker DNA; Lane 2: natural 108-bp DNA produced by PCR; Lane 3: modified DNA **2** bearing the methyl ester modification; Lane 4: modified DNA **3** bearing the tris(2-aminoethyl)amine side chain; Lane 5: modified DNA **4** bearing the histamine moiety; Lane 6: modified DNA **5** bearing the hexamenthylenediamine modification; Lane 7: modified DNA **6** bearing the carboxyl group.*

gel electrophoresis of the modified DNAs obtained by the postsynthetic derivatization. The modified DNAs **3**–**5**, with tris(2-aminoethyl)amine, histamine, and hexamethylenediamine side chains, respectively, showed lower mobility shift on the electrophoresis gel due to the mass increase and charge associated with the modified thymidine. The modified DNAs were further subjected to enzyme digestion by using nuclease P1 and, subsequently, alkaline phosphatase to confirm the conversion of the modified thymidine residue. A typical example of the enzyme digestion results for modified DNA **3** as analyzed by HPLC is shown in Figure 3. Peaks corresponding to the natural

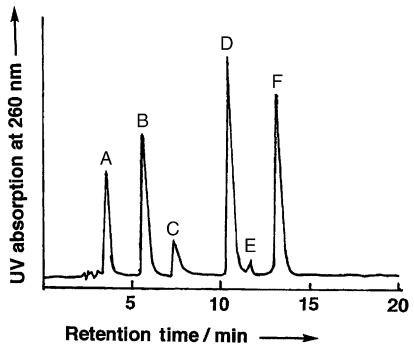



***Figure 3.*** *HPLC profile of the enzyme-digested modified DNA **3**. Peak assignment: A: $dT^{Trisamine}$; B: dC; C: $dT^{COOH}$; D: dG; E: dT; F: dA.*

nucleosides A, G, C, and T, along with the modified thymidine residue derived from the postsynthetic derivatization, were generated by the enzyme digestion. The latter compound was identified by coinjection of an authentic sample prepared from 5-(methoxycarbonylmethyl)-2′-deoxyuridine with tris(2-aminoethyl)amine. Natural thymidine is derived from the primers for the PCR. The HPLC trace also showed formation of some 5-(carboxylmethyl)-2′-deoxyuridine which was identified by coinjection with an authentic sample. This indicates that the methyl ester in the modified DNA **2** was hydrolyzed to the carboxyl group in nearly 30% yield during the PCR and/or the post synthetic derivatization. The nucleoside composition estimated by HPLC analysis roughly corresponded to that expected for the modified DNA.

We examined the template activity of the modified DNAs obtained by postsynthetic derivatization. Small amounts of the modified DNA (0.8 ng) and dTTP or modified thymidine nucleotide **1** were used as a template and a substrate, respectively, for the PCR with KOD Dash DNA polymerase. All the modified DNAs had template activity and the enzyme accepted both natural dTTP and **1** to form the corresponding 108-bp DNA (Figure 4). The DNA prepared by PCR with dTTP in the presence of the modified DNA templates **3** and **6** had the same base sequence as that of the original pUC18 DNA template (data in the Supporting Information). The results indicate that the enzyme read through the modified thymidine in the DNA template, accepted the complementary substrate, and continued the chain elongation until the end of the template to form the corresponding 108-bp DNA without mutation.

The modified DNA bearing the tris(2-aminoethyl)amine, histamine, or carboxyl groups can hardly be obtained directly by PCR with modified thymidine analogues bearing these substituent groups, because even KOD Dash DNA polymerase cannot incorporate these thymidine analogues as substrates. The modified DNA **2** can be prepared easily by PCR with KOD Dash DNA polymerase and the thymidine nucleotide analogue **1**.

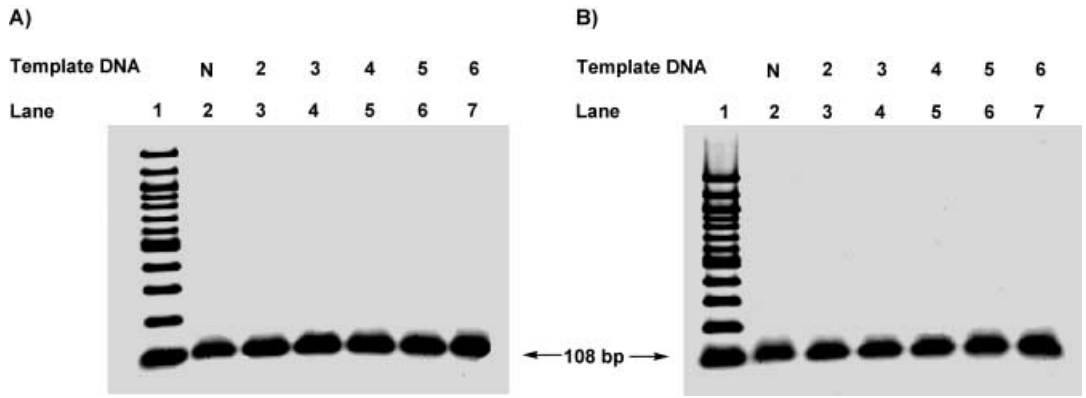



***Figure 4.*** *Gel electrophoresis assay of the PCR with the modified DNA as a template. A) PCR with the natural substrates (dATP, dGTP, dCTP, dTTP). B) PCR with the modified thymidine **1** as a substrate (dATP, dGTP, dCTP, **1**). Lane 1: marker DNA; Lane 2: natural 108-bp DNA as the template; Lane 3: modified DNA **2** as the template; Lane 4: modified DNA **3** as the template; Lane 5: modified DNA **4** as the template; Lane 6: modified DNA **5** as the template; Lane 7: modified DNA **6** as the template.*

The present study demonstrates that postsynthetic derivatization of the modified DNA **2** could expand the range of accessible modified DNAs bearing functional groups such as tris(2-aminoethyl)amine, histamine, or an anionic carboxyl moiety. These functional groups in the modified DNA could improve the catalytic or binding ability of a DNA enzyme or a DNA aptamer, respectively, which may be obtained by a combination of PCR, postsynthetic derivatization, and in vitro selection.

## Experimental Section

**Materials**: 2′-Deoxynucleoside 5′-triphosphates (dNTPs), dATP, dGTP, dCTP, and dTTP, were purchased from Seikagaku Kogyo. Alkaline phosphatase was from Worthington Biochemicals, and nuclease P1 was from Yamasa Co. KOD Dash, Taq, and Tth DNA polymerases were obtained from Toyobo Co. Vent and Vent(exo $-$) DNA polymerases were from New England Biolabs. T7 thermosequenase was purchased from Amersham Bioscience. The 5′-triphosphate of 5-(methoxycarbonylmethyl)-2′-deoxyuridine (**1**) was prepared as described previously.[14] Other C5-substituted deoxyuridine derivatives were prepared from 5-(methoxycarbonylmethyl)-2′-deoxyuridine.[5] pUC18 plasmid DNA was from Toyobo Co. The oligonucleotides used as primers, DNA A, 5′-GGAAACAGCTATGACCATGATTAC-3′ and DNA B, 5′-CGACGTTGTAAAACGACGGCCAGT-3′, were from Sawaday Technology Co. All other chemicals were reagent grade and were used without further purification.

**Analytical methods and gel electrophoresis**: UV spectra were recorded on a Hitachi 3200 spectrometer. HPLC on an ODS silica gel column (4 $\times$ 250 mm) was carried out with a linear-gradient elution (2 to 30% acetonitrile in 50 mM triethylammonium acetate (pH 7.0)) over 30 min at a flow rate of 1.0 mL min$^{-1}$. Gel electrophoresis was carried out using 2% agarose gel at 100 V for 40 min and was visualized by staining with ethidium bromide. Preparative disk-gel electrophoresis was done on a Nihon-Eido NA-1800 apparatus with a 2% agarose gel column (21 $\times$ 50 mm) at 25 V.

**PCR with dTTP or modified nucleotide 1 and KOD Dash DNA polymerase**: The mixture (20 μL) for the small-scale PCR contained the DNA template (pUC18 2686 bp; 0.5 ng μL$^{-1}$), the primers (0.2 μM of each), natural dNTPs or modified dNTP mix (0.2 mM of each nucleotide), and DNA polymerase (0.005 or 0.05 U μL$^{-1}$, respectively) in the buffer supplied by the maker for the DNA polymerase reaction. Different quantities of enzyme were used for PCR assays with the other DNA polymerases (see the legend of Figure 1). PCR assays were carried out at 94 °C for 1 min, 30 cycles of 94 °C for 30 s/50 °C for 30 s/74 °C for 1 min, and 74 °C for 5 min. The reaction mixture was quenched by addition of formamide dye solution and the PCR products were analyzed by 2% agarose gel electrophoresis.

**Large-scale synthesis and purification of modified DNA obtained from PCR with modified nucleotide 1**: The reaction mixture (10 mL), containing the DNA template (5 μg), the primers (0.2 μM of each), modified dNTP mix (0.2 mM of each nucleotide), and DNA polymerase (500 U) in the buffer supplied by the maker for the DNA polymerase reaction, was divided into 100 portions (0.1 mL each). Each portion was placed in a 200-μL tube and was set on a PCR thermal cycler. The PCR was carried out under the same conditions as described above for the small-scale synthesis. All PCR products were collected together and redivided into 20 portions (each 0.5 mL), and each portion was placed in a 1.5-mL eppendorf tube. The mixture in each tube was treated twice with PCI solution (0.5 mL; phenol saturated with tris(hydroxymethyl)aminomethane (Tris)/HCl (pH 8.0):chloroform:isoamyl alcohol (25:24:1)) and then twice with CIA solution (0.5 mL; chloroform:isoamyl alcohol (24:1)) to remove the enzymes. The organic phase was removed between each treatment. The aqueous phase was concentrated to 0.1 mL by rotary evaporation. The two 0.1 mL solutions were combined to place enough DNA in one tube for easy precipitation, and ethanol (0.5 mL) and 3 M NaOAc (20 μL; pH 5.2) were added. After shaking, the mixture was kept in a freezer at $-$20 °C for 20 min and then centrifuged at 4 °C and 10000 rpm for 30 min. Ethanol was removed from the tube and 70% ethanol (0.8 mL) was added. The tube was shaken, kept in a freezer at $-$20 °C for 20 min, and centrifuged as before, then ethanol was removed from the tube. The precipitated DNA was dissolved in distilled water (0.1 mL), and all 10 DNA solutions were combined and dried by rotary evaporation. The DNA was redissolved in distilled water (0.3 mL) and purified by a preparative disk-gel electrophoresis. Fractions (each 0.5 mL) eluted from the gel were collected and the appropriate fractions containing modified DNA showing UV absorbance at 260 nm were collected. The modified DNA was checked by agarose gel electrophoresis. The modified DNA bearing 5-(methoxycarbonylmethyl)-2′-deoxyuridine was obtained in 4.5 OD units at 260 nm.

**Postsynthetic derivatization of the modified DNA containing 5-(methoxycarbonylmethyl)-2′-deoxyuridine**: The modified DNA (4.5 OD units at 260 nm) was passed through a cation-exchange resin

column (Dowex 50 WX-8 ($HN^+Et_3$ form), 100 – 200 mesh, 5 × 70 mm) to exchange the counter cation of the modified DNA from $Na^+$ to $HN^+Et_3$. This is to make the DNA soluble in DMF, as is necessary for the derivatization reaction. After passing through the column, the solution of DNA in the $HN^+Et_3$ form was collected, divided into five portions, placed in five Eppendorf tubes, and dried by rotary evaporation. Each tube contained 0.5 $OD_{260nm}$ units of the modified DNA. The following ester – amide exchange reaction was used for the derivatization. The modified DNA (0.5 $OD_{260nm}$ units) was treated with 50% tris(2-aminoethyl)amine in DMF (0.1 mL), 50% hexamethylenediamine in DMF (0.1 mL), or 10% histamine in DMF (0.1 mL) at 37 °C or 50 °C overnight. Ethanol (0.5 mL) and 3 M NaOAc (20 μL; pH 5.2) were added to each reaction mixture and the solutions were kept in a freezer at −20 °C for 20 min. The mixture in the tube was centrifuged at 4 °C and 10000 rpm for 20 min, and the solvent was removed from the tube. Ethanol precipitation was conducted again with 70% ethanol (0.5 mL) and the precipitated modified DNA was dried by roatry evaporation. We measured the $OD_{260nm}$ of the resulting modified DNA.

The DNA bearing an anionic carboxyl group was obtained by hydrolysis of the methyl ester of the modified DNA **2**. The modified DNA was treated with 0.1 M NaOH (0.1 mL) at 37 °C overnight. Extraction of the DNA bearing an anionic carboxyl group was performed in essentially the same manner as described above, except additional glycogen (1 μL; 20 μg μL$^{-1}$) was added to the DNA mixture to improve the recovery of the DNA by ethanol precipitation. After extraction, the modified DNAs were checked by 2% agarose gel electrophoresis and enzyme digestion.

**Enzyme digestion of the modified DNAs**: The modified DNAs (0.15 $OD_{260nm}$ units) were treated overnight with nuclease P1 (5 μg) in 100 mM NaOAc buffer (20 μL; pH 5.3) at 37 °C. Subsequently, alkaline phosphatase (1 U) and 50 mM Tris/HCl (24 μL; pH 9.0) containing 10 mM $MgCl_2$ were added to the mixture. The whole solution was incubated at 37 °C overnight. The reaction mixture was analyzed by reverse-phase HPLC on a Wakosil 5C-18 column. Identification of the modified nucleoside obtained by the enzyme digestion was done by the coinjection of an authentic sample prepared by treatment of 5-(methoxycarbonylmethyl)-2′-deoxyuridine with tris(2-aminoethylamine), hexamethylenediamine, histamine, or lithium hydroxide in methanol.

**PCR with the modified DNAs obtained by derivatization as templates**: The mixture (20 μL) for the PCR with the derivatized DNA templates contained the DNA (0.04 ng μL$^{-1}$), the primers (0.2 μM of each), natural dNTPs or modified dNTP mix (0.2 mM of each nucleotide), and the DNA polymerase (0.05 or 0.25 U per 10 μL, respectively) in the buffer supplied by the maker for the DNA polymerase reaction. PCR assays were carried out at 94 °C for 1 min, 30 cycles of 94 °C for 30 s/50 °C for 30 s/74 °C for 1 min, and 74 °C for 5 min. The reaction products were separated by 2% agarose gel electrophoresis and were visualized by ethidium bromide staining.

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