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Bioorganic & Medicinal Chemistry Letters 17 (2007) 776–779
Bioorganic & Medicinal Chemistry Letters

Chemico-enzymatic synthesis of a new fluorescent-labeled DNA by PCR with a thymidine nucleotide analogue bearing an acridone derivative

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Received 11 July 2006; revised 23 October 2006; accepted 25 October 2006
Available online 28 October 2006

Abstract—Triphosphate of a thymidine analogue bearing acridone was prepared and incorporated as a substrate for PCR using KOD Dash DNA polymerase forming a new fluorescent-labeled DNA that is useful as a DNA probe.

DNAs tagged with a fluorescent label are important tools for biological and biochemical studies and for diagnostic use as a DNA probe. Various methods have been reported for the synthesis of fluorescent-tagged DNA.[1–3] DNA with high density of labels could increase detection sensitivity. Polymerase chain reaction is suitable for simultaneous labeling and amplification of the DNA with a fluorophore, if a fluorescent-labeled nucleotide can be a substrate for DNA polymerase during PCR. Taq and KOD Dash DNA polymerases can accept some 5′-triphosphates of 2′-deoxyuridine derivatives bearing fluorescein or cyanine dyes via an amino linker arm at the C5 position yielding the corresponding fluorescent-labeled DNA during PCR, although co-existence of natural substrate TTP is required for the reaction.[3–7] Complete displacement of the natural substrate TTP with the fluorescent-tagged 2′-deoxyuridine derivatives resulted in loss of the formation of the fluorescent-tagged PCR product. Although fluorescein and cyanine dyes are widely used as a fluorescent-labeling agent for various biomolecules such as DNA or protein, these molecules have disadvantages, such as sensitivity to photobleaching and fluorescent quenching in acidic and basic conditions. Moreover, they are large bulky molecules with negative or positive charges, which results in loss of the substrate activity of the labeled nucleotide in PCR.

The acridone moiety is highly fluorescent and stable against photo-degradation, oxidation, and heat.[8–10] Further, acridone is a rather small molecule. Several types of acridone derivatives have been prepared and used as a fluorescent label for peptides,[11] antibody,[12,13] and amino acids.[14] However, to our knowledge, no report has appeared on the conjugate of DNA and an acridone derivative as a fluorescent DNA probe. Previously, we reported that triphosphate of a thymidine analogue bearing a methylene group at the C5 α-position with an amino-linker arm could be accepted by KOD Dash DNA polymerase as a substrate in PCR.[15] The triphosphate of the thymidine analogue with an amino-linker was further reacted with an acridone derivative yielding a new thymidine analogue tagged with acridone. The thymidine analogue was found to be a good substrate for KOD Dash DNA polymerase without co-existence of a natural substrate. Here, we report the preparation of the acridone-tagged thymidine analogue and its enzymatic incorporation into the DNA by PCR using KOD Dash DNA polymerase, along with the fluorescent properties of the resulting DNA.

We first undertook preparation of 2-acridone acetic acid (**3**) to introduce the acridone moiety to the modified thymidine or DNA bearing an amino-linker via an amide-bond formation. Compound **3** was prepared as a possible anti-inflammatory agent by Taraporewala and Kaufman.[16] We prepared **3** by modification of their method as shown in Scheme 1. Ullmann–Goldberg condensation of *o*-bromo benzoic acid with methyl

Keywords: Acridone; Fluorescent-labeled DNA; PCR; DNA probe.
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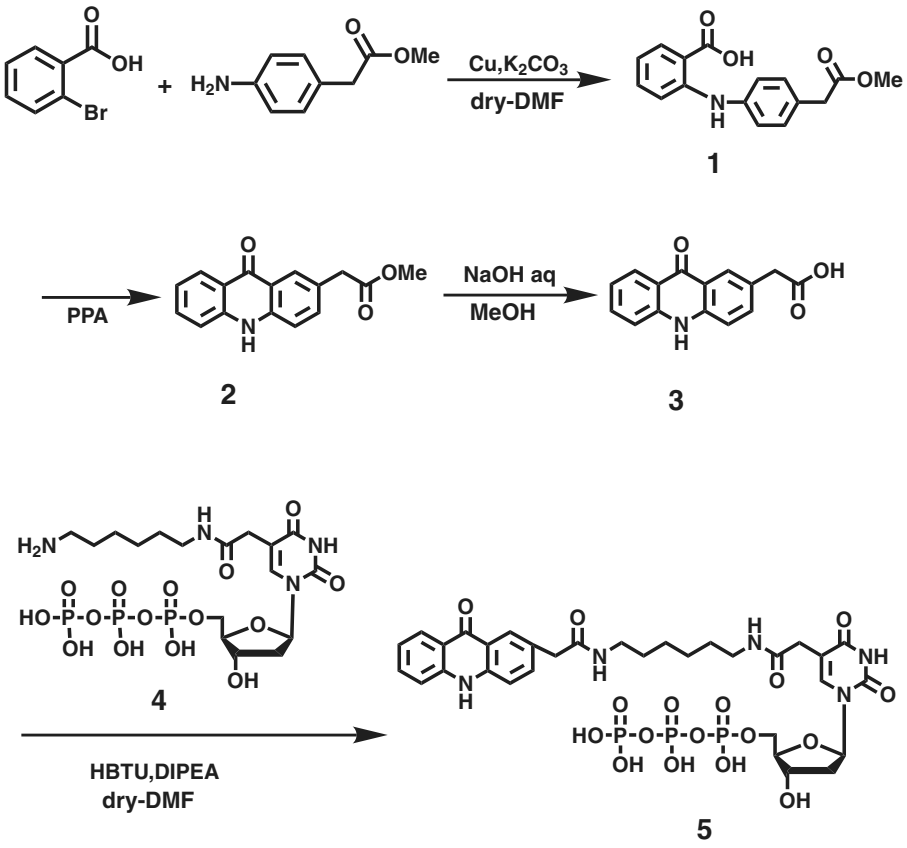
doi:10.1016/j.bmcl.2006.10.072



Scheme 1. Synthetic procedure of acridone acetic acid **3** and acridone-tagged thymidine analogue **5**.

p-aminophenyl acetate in dry DMF in the presence of potassium carbonate and copper powder catalyst[17] gave *N*-phenylanthranilic acid derivative (**1**) in 65% yield. Cyclodehydration of **1** was carried out with polyphosphoric acid at 85 °C for 1 h. Usual workup of the reaction mixture and chromatography on silica gel using 5% methanol/dichloromethane as an eluent gave **2** as a white solid in 61% yield. The methyl ester of **2** was hydrolyzed with sodium hydroxide in aqueous methanol to yield **3** in 66% yield. Its structure was confirmed by NMR and ESI-mass spectra and spectroscopic properties are listed in the Note.[18] Compound **3** displayed high fluorescence intensity in aqueous solution, quantum yield of **3** was 0.894 when excited at 365 nm. 5′-Triphosphate of 5-aminohexylcarbamoylmethyl-2′-deoxyuridine (**4**) was prepared by the procedure described previously.[15,19] The acridone derivative **3** (0.8 mg, 3 μmol) was coupled with **4** (10 ODU_{260}, 1.0 μmol) in the presence of HBTU, diisopropylethylamine, and tributylamine in dry DMF for 1 h at room temperature. The purified acridone-tagged thymidine analogue **5** was obtained in 5% yield (1.0 ODU_{260}) after purification by HPLC on an ODS-silica gel column, and confirmed by an ESI-mass spectrum.[18]

We examined the incorporation of the acridone-labeled thymidine triphosphate **5** in place of TTP during PCR using KOD Dash DNA polymerases. pUC18 plasmid DNA, and DNA A, 5′-GGAAACAGCTATGACCAT GATTAC-3′, and DNA B, 5′-CGACGTTGTAAAAC

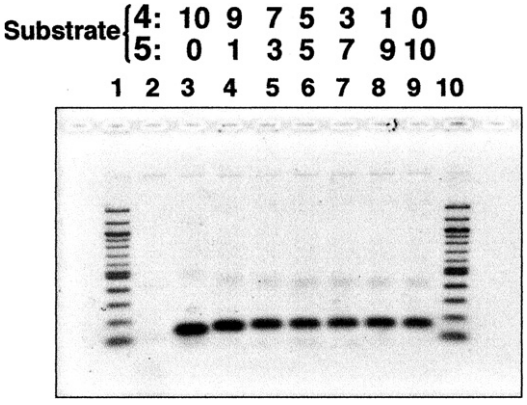


Figure 1. PCR assays of the acridone-tagged TTP analogue using KOD Dash DNA polymerase. The mixture (20 μl) for PCR contained 0.5 ng/μl DNA template (pUC18, 2686 bp), 0.4 μM of each primer, modified dNTP mix (0.2 mM of each nucleotide), and 0.5 U/10 μl of DNA polymerase in the buffer supplied by the maker for the DNA polymerase reaction. PCR assays were carried out at 94 °C/1 min, 30 cycles of 94 °C/30 s, 52 °C/30 s, 74 °C/1 min, and 74 °C/5 min. The reaction mixture was quenched by addition of formamide-dye solution and PCR products were separated by 2% agarose gel electrophoresis. The gel was visualized after staining with ethidium bromide. lanes 1 and 10: marker DNA (100–1200 bp); lane 2: negative control without TTP; lane 3: positive control **4**; lane 4: **4** + **5** (9:1); lane 5: **4** + **5** (7:3); lane 6: **4** + **5** (1:1); lane 7: **4** + **5** (3:7); lane 8: **4** + **5** (1:9); and lane 9: **5**. All reaction mixtures contained dNTP (dATP+dCTP+dGTP) in addition to TTP analogues.

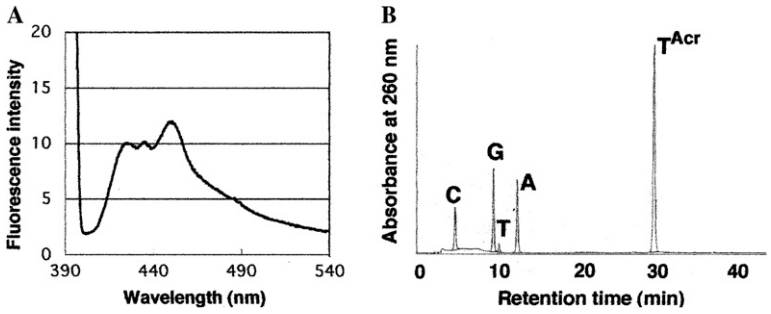


Figure 2. (A) Fluorescence spectrum of the acridone-tagged DNA produced by PCR. Fluorescence spectra were taken with excitation at 388 nm. (B) HPLC profiles of the digested products of the acridone-tagged DNA by nuclease P1 and alkaline phosphatase. HPLC condition: Wakosil 5C18 (4×25 cm); linear gradient elution of acetonitrile (2.1–37.1% in 35 min) in 50 mM triethylammonium acetate (pH 7.2) at flow rate of 1.0 ml/min. HPLC was monitored by UV absorption at 260 nm. Only peak of T^{acr} was observed when HPLC was monitored by fluorescence at 450 nm with excitation at 390 nm (data not shown).

GACGGCCAGT-3′, were used as a template and primers, respectively, for the PCR assay. PCR was carried out according to the procedure shown in the legend of Figure 1. The PCR products were analyzed by 2% agarose gel electrophoresis and visualized with ethidium bromide. PCR with the thymidine derivative **4** demonstrated formation of the 108 base-pair DNA product in accordance with the previous report (Fig. 1, lane 3).[15] Combined use of **4** and **5** in 9:1, 7:3, 5:5, 3:7, and 1:9 molar ratio as a substrate yielded the full-length PCR product, as expected (lanes 4–8). Complete displacement of **4** with **5** also gave the corresponding 108 base-pair DNA with acridone as a fluorescent label (lane 9). Successful PCR with this template and primers requires the incorporation of 40 thymidine analogues with a single stretch of four successive thymidine residues. Thus, multi-labeling of DNA with acridone could be accomplished by using the substrate **5** and KOD Dash DNA polymerase. It is reported that no fluorescent-labeled DNA could be obtained by PCR without combined use of natural TTP and a fluorescent-labeled thymidine analogue, when cyanine dyes or fluorescence was used as a labeling agent.[3–7] A bulky fluorescent molecule in these thymidine analogues suppresses the successive incorporation of the modified substrate.

We prepared the modified DNA by PCR in large scale, and the resulting acridone-tagged DNA was characterized by fluorescence spectrum and nuclease digestion. In brief, 10 portions of 0.1 ml reaction mixture containing 1 nM DNA template, 1 μM of each primer, modified dNTP mix (0.2 mM of each nucleotide), and 5 U of DNA polymerase in the buffer supplied by the maker for the DNA polymerase reaction were put in reaction tubes and set on a PCR thermal cycler. The PCR was carried out under the same conditions as described above. All PCR products were collected, passed through a spin column (cut-off, 10 k), and separated by 2% agarose gel. The modified DNA on the gel was cut out, extracted with TBE buffer, and passed through a spin column for desalting. The modified DNA (0.70 $OD_{260\ nm}$, 0.18 nmol) was obtained in a pure form. The molar absorption coefficient of the modified DNA was estimated from the sum of those of the DNA and acridone.

Fluorescence spectrum of the resulting modified DNA is shown in Figure 2A, which showed the incorporation of acridone moiety. Figure 2B shows a HPLC profile of digested products of the modified DNA by nuclease P1 and alkaline phosphatase. The acridone-tagged thymidine was confirmed by comparing the retention time with that of the authentic sample synthesized by coupling of **3** and the corresponding thymidine analogue, 5-aminohexylcarbamoylmethyl-2′-deoxyuridine.[20] The composition of the normal nucleosides and acridone-tagged thymidine nearly corresponded to the desired composition of the 108 mer DNA ($A/G/C/T/T^{Acr}$ = 49:59:59:9:40).

In conclusion, we prepared 5′-triphosphate of new acridone-tagged thymidine. PCR using this modified nucleotide with KOD Dash DNA polymerase provides a new entry for the synthesis of a DNA probe with a high density of fluorescence, and complements the previous method of the DNA probe synthesis, because acridone has strong fluorescence intensity and is stable against photo-degradation.

Acknowledgments

This work was supported in party by Grant-in-Aid for Scientific Research from the Japan Society for Promotion of Science and by PRESTO from the Japan Science and Technology Agency.

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18. Compound **3**; ESI-Mass (*m*/*z*, negative mode): 252.2. Calcd for $[M-H]^-$ 252.1. ^1H NMR (δ, ppm): 3.79 (s, 2H), 7.29 (t, 1H), 7.50–7.54 (m, 2H), 7.68–7,75 (m, 2H), 8.26 (d, 1H), 8.36 (m, 1H). Absorption: λ_{max} 388 nm (ε 5500), 407 nm (ε 4900). Fluorescence: λ_{max} 423 nm, 448 nm (excitation 388 nm). Compound **5**; ESI-Mass (*m*/*z*, negative mode): 858.2. Calcd for $[M-H]^-$ 858.2.
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