

# Synthesis and Primer Properties of Oligonucleotides Containing 3'-Deoxyψicothymidine Units, Labeled with Fluorescein at the 1'-Position

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Several analogues of the standard M13 sequencing primer that contain up to five 3'-deoxyψicothymidines, or one or two such units labeled with fluorescein at the 1'-position, have been prepared. All these oligonucleotides have been shown to prime the DNA-polymerase-catalyzed synthesis of DNA.

Oligonucleotide conjugates bearing reporter groups have recently found an increasing number of applications as versatile tools in basic research in molecular biology (1, 2), as diagnostic probes (2), and as regulators of gene expression (3, 4). Usually aminoalkyl tethers are employed to introduce reporter groups at the 5'- or 3'-terminus of the oligonucleotide (1-3, 5), at heterocyclic nucleic bases (6, 7), or at internucleosidic phosphodiester bonds (8). All these methods suffer from some shortcomings. 3'- and/or 5'-terminal conjugate groups prevent the enzymatic extension or ligation of the labeled oligonucleotide. Tethers attached to the nucleic bases sometimes weaken the base-pairing (9), and substitution of the phosphodiester bond gives rise to two diastereomers about phosphorus, which are not always easily resolved (10). Recently, several groups have reported on preparation of sugar moiety tethered oligonucleotides that upon hybridization place the reporter group at the minor groove (11-15). This approach leaves all the ionic and tautomeric properties as well as the functional groups of 2'-deoxynucleoside units unchanged and minimizes the steric hindrance for duplex formation. Manoharan *et al.* (11) and Sproat *et al.* (12) attached a linker to the 2'-position of a ribonucleoside unit and used the tether for subsequent labeling of the oligonucleotide. Matsuda *et al.* (13, 14) employed 3'-deoxyψicouridine (1'-(hydroxymethyl)-2'-deoxyuridine) for the same purpose. Both approaches involve a multistep preparation of the phosphoramidite building block, carrying the protected aminoalkyl linker at the sugar moiety, and subsequent use of these monomers in the solid phase DNA synthesis. We have previously reported on derivatization of the 1'-position of 3'-deoxyψicothymidine (1'-(hydroxymethyl)-2'-deoxythymidine) (16) during the course of oligonucleotide synthesis (15). This approach is advantageous, since a normal thymine base is used instead of uracil (13, 14), and the length and reactivity of the linker may be adjusted according to the requirements of the further derivatization without synthesis of new building blocks.

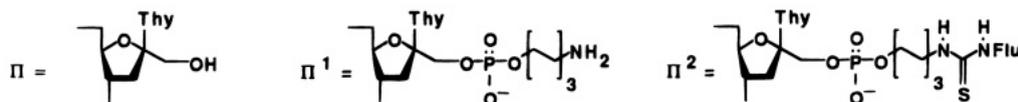
The present report describes the synthesis of oligonucleotides containing up to five 3'-deoxyψicothymidine units Π (oligonucleotides 1-4) or up to two such units labeled with either an aminoalkyl group (Π<sup>1</sup>, oligonucleotides 6, 7) or fluorescein at the 1'-position (Π<sup>2</sup>, oligonucleotides 8, 9). Moreover, their ability to prime DNA-polymerase-catalyzed synthesis of DNA is demonstrated. All the oligonucleotides were analogues of the standard

M13 primer 5, which is widely used in DNA sequencing (17) (Figure 1).

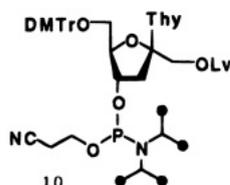
We have described previously the preparation of building block 10 (Figure 2) derived from 3'-deoxyψicothymidine and demonstrated its efficiency in the oligonucleotide condensation (15). However, since the introduction of several modified units may bring additional sterical hindrance in the growing oligonucleotide chain, and hence decrease the coupling efficiency, the ABI 392 DNA synthesizer was programmed to use a longer coupling step (180 s) when the oligonucleotide chain was elongated with 10. The coupling yield of each ψicothymidine unit proved to be about 98%, as determined by a trityl assay. After the chain assembly was completed, oligonucleotides 1-4 were deblocked in the conventional manner. Modified oligomers 6 and 7 were obtained as follows. The reaction columns were removed from the synthesizer upon completion of the chain elongation (DMTr-On synthesis) (18), 1'-O-levulinyl groups were cleaved (15), and the reaction columns were reinstalled to the synthesizer. The aminoalkyl phosphate groups were introduced using a commercial Aminolink-2 and a prolonged coupling step (180 s) as part of the standard protocol (18). For the preparation of 6 one coupling was sufficient. To obtain 7, two couplings, separated by acetonitrile wash and argon flush, were applied. Upon iodine oxidation, 6 and 7 were deblocked in a usual manner. All oligonucleotides prepared were isolated by successive anion exchange and reversed phase HPLC and finally desalted by gel filtration (15). The HPLC analysis of the oligonucleotides 1-4, 6, and 7, digested with a mixture of phosphodiesterases I and II in the presence of alkaline phosphatase (15), verified the presence of expected nucleosides in the correct ratio. Oligonucleotides 6 and 7 were finally reacted with FITC using two different methods. In method A the labeling was performed in a sodium carbonate buffer at pH 10.3 under standard conditions (13). The isolated yield of monolabeled oligonucleotide 8 was repeatedly 50-60% and that of the double labeled 9 30-40%. In method B acetylated long chain (alkylamino)-CPG was used as a carrier of oligonucleotides. Compounds 6 and 7 (2-3 OD) were dried in the presence of the carrier (2-3 mg), and the labeling was performed with a 3% solution of FITC in a mixture of pyridine-N,N-diisopropylethylamine-water (8:1:1, 100 μL). After the reaction was completed (12 h, rt), the carriers and the adsorbed oligonucleotides were washed with dioxane (1.5 mL), dioxane-pyridine (19:1, 5 × 1.5 mL), and finally with ether (1.5 mL). Labeled oligonucleotides were then dissolved in water, purified

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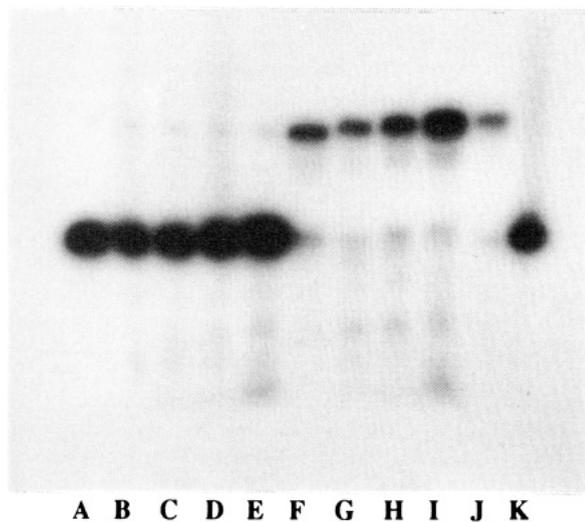
- |   |   |
|---|---|
| 1 | 5'-GpTpTpTpTpCpCpCpApCpΠpCpApCpGpApC-3'                             |
| 2 | 5'-GpTpΠpTpΠpCpCpCpApCpTpCpApCpGpApC-3'                             |
| 3 | 5'-GpTpΠpTpΠpCpCpCpApCpΠpCpApCpGpApC-3'                             |
| 4 | 5'-GpΠpΠpΠpΠpCpCpCpApCpΠpCpApCpGpApC-3'                             |
| 5 | 5'-GpTpTpTpTpCpCpCpApCpTpCpApCpGpApC-3'                             |
| 6 | 5'-GpTpTpTpTpCpCpCpApCpΠ <sup>1</sup> pCpApCpGpApC-3'               |
| 7 | 5'-GpTpΠ <sup>1</sup> pTpΠ <sup>1</sup> pCpCpCpApCpTpCpApCpGpApC-3' |
| 8 | 5'-GpTpTpTpTpCpCpCpApCpΠ <sup>2</sup> pCpApCpGpApC-3'               |
| 9 | 5'-GpTpΠ <sup>2</sup> pTpΠ <sup>2</sup> pCpCpCpApCpTpCpApCpGpApC-3' |



Flu = Fluorescein

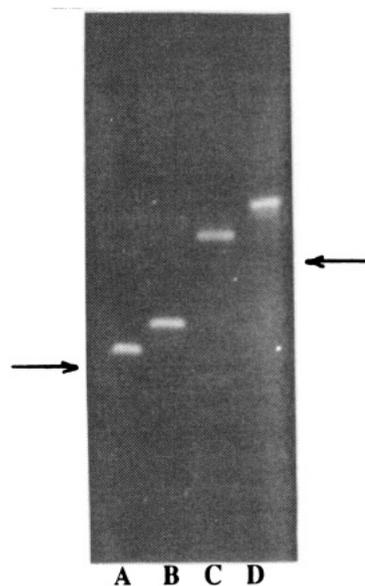
**Figure 1.** Structures of oligonucleotides 1–9.

Thy = thymine-1-yl; DMTr = 4,4'-dimethoxytrityl; Lv = levulinyl.

**Figure 2.** Structure of the building block 10.**Figure 3.** Autoradiogram of 20% PAGE: lines A, K, <sup>32</sup>P-labeled commercial 17nt standard M13 sequencing primer 5; lines B–E, <sup>32</sup>P-labeled 1–4; line F, enzymatic elongation of <sup>32</sup>P-labeled commercial standard M13 sequencing primer 5 on 27nt matrix; lines G–J, enzymatic elongation of <sup>32</sup>P-labeled 1–4 on 27nt matrix.

by a successive anion exchange and reversed-phase HPLC, and finally desalted. The isolated yield of **8** was 80–90% and that of double labeled **9** was 70–80% repeatedly. The UV–vis absorption spectra of the labeled **8** and **9** exhibited the characteristic fluorescein absorption at 490 nm and oligonucleotide absorption at 260 nm, the ratio A (490 nm)/A (260 nm) observed with **9** being approximately 2-fold compared to that of **8**.

Matsuda *et al.* (13, 14) have shown that oligonucleotides containing a 1'-derivatized 3'-deoxypsycouridine unit hybridize with complementary DNA strands. The aim of this work was to test the ability of the oligonucleotides 1–4, as well as that of the FITC-labeled **8** and **9**,

**Figure 4.** Photograph of 20% PAGE under the long wavelength UV: lines A, B, **8** and **9**; lines C, D, enzymatic elongation of **8** and **9** on 27nt matrix. The arrow on the left indicates the position of <sup>32</sup>P-labeled commercial 17nt standard M13 sequencing primer 5; the arrow on the right indicates the position of enzymatically elongated <sup>32</sup>P-labeled commercial standard M13 sequencing primer 5 on 27nt matrix.

to prime the DNA–polymerase-catalyzed reaction and to compare their priming ability to that of commercial standard M13 sequencing primer 5. 5'-<sup>32</sup>P-Labeled (19) derivatives 1–4 and FITC-labeled **8** and **9** were annealed to the synthetic 27-nt. complementary strand, and the polymerase reaction, employing Sequenase version 2.0, was performed.<sup>1</sup> The products were analyzed with PAGE. Figure 3 shows the autoradiography of PAGE,

<sup>1</sup> The elongation reaction mixtures contained the following: 1.25 pmol of <sup>32</sup>P-labeled 1–5, annealed to an equal amount of synthetic matrix 5'-GTTTTACAACGTCGTGACTGGGAAAAC-3'; 1–2 units of Sequenase version 2.0; 0.06 M 1,4-dithio-D,L-threitol; 75 μM each 2'-deoxynucleoside 5'-triphosphate, 40 mM Tris–HCl pH 7.5; 20 mM MgCl<sub>2</sub>; 50 mM NaCl. The chain elongation reaction of FITC-labeled primers contained 60 pmol of **8** and **9** and an equal amount of synthetic matrix. After 10 min at 37 °C reactions were stopped by 10 times dilution with "stop-solution" (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylenecyanol). Products of the chain elongation reactions were analyzed with 20% PAGE.

referring to the experiment with primers 1–5. Figure 4 demonstrates the photograph of PAGE taken under the long wavelength UV and referring to the DNA–polymerase reaction with luminescent primers 8 and 9. It is clearly seen that oligonucleotides containing up to five 3'-deoxyisocytosine units are all able to serve as efficient primers in the DNA–polymerase synthesis of DNA, analogously to the commercial M13 sequencing primer 5. The attachment of one or two fluorescein molecules *via* (aminoalkyl)phospho-linker to the 1'-position of 3'-deoxyisocytosine still does not prevent the oligonucleotide analogues to hybridize with the complementary DNA and prime the enzymatic reaction.

In summary, the introduction of 1'-modified nucleosides and the subsequent tethering may easily be performed on an automated DNA synthesizer. Neither several 3'-deoxyisocytosine units nor their derivatives tethered with bulky substituents at O-1' abolish the ability of modified oligonucleotide to hybridize the complementary DNA strand and prime the polymerase reaction. The data presented demonstrate an alternative approach for the labeling of DNA with reporter groups.

#### ACKNOWLEDGMENT

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