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A NEW CLASS OF 3'-MODIFIED NUCLEOTIDES AS CHAIN-TERMINATORS FOR DNA-SEQUENCING

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Abstract: We describe the use of 3'-modified thymidine nucleotides as potent chain terminators in Sanger's DNA sequencing reactions by application of T7 DNA polymerase and Mn^{2+}-ions. These nucleotides contain fluorescein which is linked directly or by mono-, di- and tetraglycine units to the 3'-position of a 3'-amino-2'-3'-dideoxyribose.

Introduction

During the last 6 years numerous investigations in non-radioactive Sanger sequencing methods have been done. Therefore base attached nucleotide-dye conjugates were synthesized[1,2]. These base-labeled nucleotides are responsible for an increased error rate during DNA-sequencing compared to the commercially available dideoxyterminators (ddNTP).

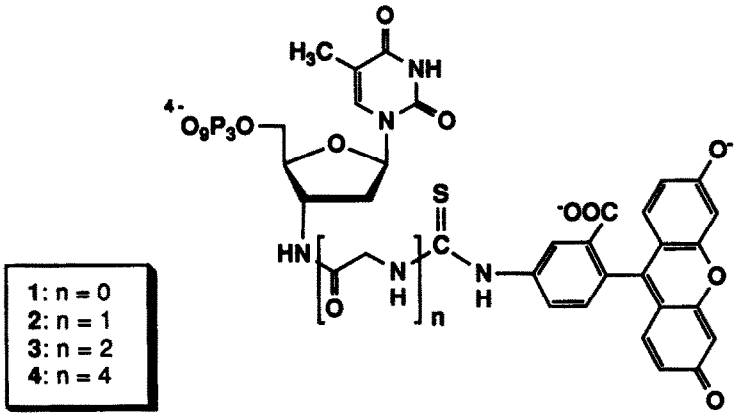

Figure 1. Structures (charged) of 3'-modified nucleotides tested as chain terminators in DNA-sequencing.

This is due to sterical hindrance of the base modified terminators which are not well accepted by commonly used DNA polymerases. As it is known from the DNA polymerase inhibitor azidothymidine (AZT)[3] a 3'-modification is well accepted during the polymerisation reaction. In view of this, our aim was to examine the influence of a dye attached to the 3'-position of a nucleotide during polymerase reaction. Therefore we synthesized compounds **1-4** (Figure 1). Starting from 3'-amino-2'-3'-dideoxythymidine, fluoresceine isothiocyanate (FITC) was coupled directly (**1**)[4], via mono- (**2**), di- (**3**) and tetraglycine (**4**) units. The fluorescence labeling of the glycine units was easily performed on the 2-chlorotrityl resin[5] by use of FITC under basic conditions. With these compounds we were able to study the sterical influence of the dye residue to the substrate specificity of T7 DNA polymerase.

Results and Discussion

The performance of compounds **1-4** was tested by sequence analysis of the bacteriophage vector m13mp18 using the universal primer and T7 DNA polymerase. Subsequently the polymerisation products were separated on an A.L.F. DNA-sequencer: DNA-fragments which are produced by the enzymatic chain extension reaction are separated by polyacrylamide gel electrophoresis, detected and identified when they migrate through the laser beam of the fluorescence detection system. Fluorescent signals were amplified by addition of a fluorescent universal primer since different fluorescence intensities for nucleotides **1-4** were measured (Figure 2). Figure 2 shows the relation of fluorescence emission F (λ_{ex} = 488 nm; λ_{em} = 520 nm) to absorbance A (λ_{abs} = 265 nm) simultaneously detected during reversed phase HPLC analysis. For compound **1** we obtained the smallest fluorescence intensity. For compounds **2-4** intensity increases in proportion to the length of the glycine-linker. As described by Tabor and Richardson[6] incorporation of dideoxynucleotides by T7 DNA polymerase is more efficient when Mn^{2+} rather than Mg^{2+} is used as catalyst.

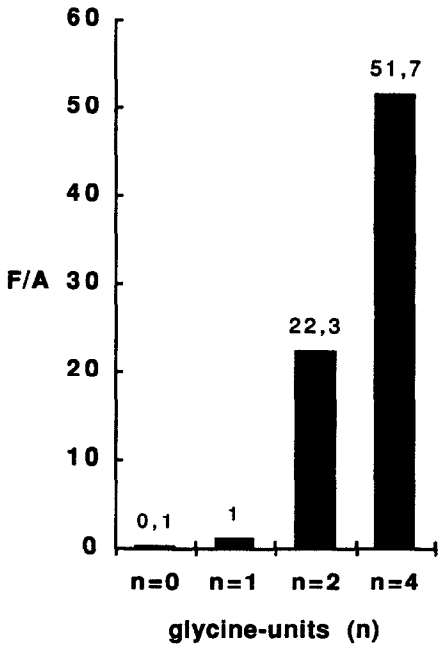

Figure 2. Quotient of fluorescence emission F (λ_{ex} = 488 nm; λ_{em} = 520 nm) and absorbance A (λ_{abs} = 265 nm) of compounds **1-4**.

For this reason we used Mn^{2+} catalysis in the sequencing reactions. All four modified nucleotides are well accepted by the enzyme and show equivalent termination quality compared with ddTTP terminated reactions. Best results were obtained with Mn^{2+} as catalyst and concentrations of 150 µM for **1** and 200 µM for **2**, **3** and **4**. The concentration of dNTP's was 1000 µM in all experiments. Under these conditions we were able to detect

T-bases up to 400 bp. Figure 3 serves as an example of a sequencing experiment using compound **4** (lower curve) and ddTTP with a concentration of 15 μM (upper curve) as chain terminators.

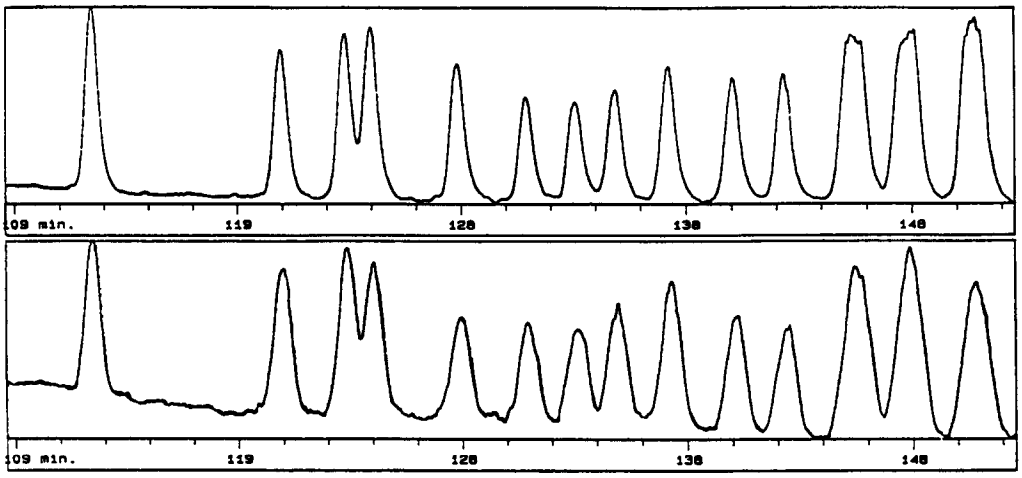

Figure 3. Raw data output of a sequencing experiment using T7 DNA polymerase and compound **4** (lower curve) and ddTTP (upper curve) as chain terminators analysed on an A.L.F. DNA-sequencer.

This output demonstrates the possibility of getting a high resolution T-lane sequence with the newly developed 3'-modified chain-terminators. These results indicate that the length of the linker shows insignificant difference in substrate specificity under these conditions and therefore it should be possible in future to use 3'-modified nucleotides for non-radioactive DNA sequencing methods. Another advantage to existing optical DNA sequencing methods is chain-termination by modification. This means that the modification in the furanose moiety induces the termination of the polymerisation reaction while containing the fluorescence marker. Our observation of insignificant difference in substrate specificity with regard to 3'-modified nucleosides is consistent with the suggestion of Tabor and Richardson[6] that the furanose moiety in the Mn^{2+}-nucleotide complex shows a conformation in which bulky substituents at the 3'-position are pushed away from the active site of the enzyme[7]. After having finished this publication we learned that Hovinen et al. have shown 3'-modified nucleotides as substrates for Tet/z-polymerase[8].

Experimental Section

Synthesis of compounds ***2-4***

The labeling of the glycines was performed on resin bound glycines by adding 3 eq FITC in N, N-dimethylformamide/dichloromethane and 1,5 eq diisopropylethylamine. After two days reaction at room temperature the labeled glycines were cleaved from the resin[5]. Coupling of the labeled glycines with 3'-amino-2'-3'-dideoxy-5'-triphosphate-thymidine was performed according to the method of Bannwarth et al.[9].

Synthesis of compound 1

The labeling reaction leading to compound **1** was performed in solution instead of using solid phase techniques as described above for compounds **2-4**.

Sequencing reactions with modified chain terminators (1-4)

The reaction vial for the annealing reaction with T7 DNA polymerase contains 1 µg of m13mp18 ss-DNA (5 µl), 2 µl fluorescein labeled universal primer (1 pmol, Pharmacia LKB), 2 µl Mn-buffer 1 (311 mM Tris-HCl, pH 7.5), 2 µl Mn-buffer 2 (177 mM dithiothreitol (DTT)) and 2 µl Mn-buffer 3 (62 mM $MnCl_2$, 460 mM sodiumisocitrate). The mixture is heated to 70°C and then cooled to 25°C in 40 min. To the annealed template-primer-complex 2 µl of diluted T7 DNA polymerase (4 units, Pharmacia) were added. Meanwhile 3 µl termination mix (T-mix 1: includes 150 µM **1**; T-mix 2 : includes 200 µM **2**; T-mix 3: includes 200 µM **3**; T-mix 4: includes 200 µM **4**; each of the mixes also include: 1 mM dATP, 1 mM dGTP, 1 mM dCTP, 1 mM dTTP, 50 mM NaCl and 40 mM Tris HCl, pH 7.5) were pipetted into four vials and warmed for at least one minute at 37°C. Immediately 3.8 µl of the annealing mix were pipetted into each of the prewarmed termination mixes. After incubation for 10 minutes at 37°C, 4 µl of stop solution (deionized formamide, blue dextrane) were added to each reaction. The reaction solutions were heated for 2 min at 90°C and loaded (6 µl) into the appropriate wells of a 6 % denaturing sequencing gel.

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