

GENE 08146

DNA polymerase fluorescent substrates with reversible 3'-tags

(Nucleotide sequencing; primer extension; gel; genome; termination; modified nucleotides)

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SUMMARY

We have synthesized 3'-substituted-2'-deoxyribonucleotide-5'-triphosphates corresponding to A, T, G and C. The 3' position was esterified by a separate anthranilyc derivative (3'-tag) giving specific fluorescent properties to each nucleotide (nt). These nt acted as substrates with several DNA polymerases leading to chain termination. Upon alkali or enzymatic treatment of the terminated DNA chain, free 3'-hydroxyl groups were recovered and found able to undergo chain extension when incubated with a mixture of dNTPs and a DNA polymerase. Because each tag has different fluorescent properties in itself, i.e., as a free acid, it theoretically is possible, after removal and characterization of the tag, to infer which nt has been inserted. Reiteration of the process can then be used to determine a nt sequence with a non-gel-based method amenable to automation.

INTRODUCTION

DNA sequencing has revolutionized the speed and depth of our understanding of complex molecular biology processes. Presently classical sequencing techniques were introduced around 1977 (Sanger et al., 1977; Maxam and Gilbert, 1977). Dideoxy sequencing (Sanger et al., 1977) has gained wide acceptance and is now the method of choice for determining a nt sequence from a single-stranded (ss) DNA template. During the four enzymatic chain elongations, dideoxy nt are randomly inserted in place of the corresponding deoxy nt. Sequencing reactions generate a complex mixture which is subsequently resolved by polyacrylamide-gel electrophoresis.

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Abbreviations: AMV-RT, avian myeloblastosis virus reverse transcriptase; An, anthranilyl; bp, base pair(s); BSA, bovine serum albumine; kb, kilobase(s) or 1000 bp; nt, nucleotide(s); oligo, oligodeoxyribonucleotide; RTa, reversibly tagged; ss, single strand(ed).

We wanted to reconsider the basic features of the dideoxy sequencing method, in particular the enzymatic reaction itself where most of the complexity of the process is generated and analysed during the following steps of resolution and data acquisition, i.e., gel electrophoresis and sequence reading, respectively. Indeed, we thought that we could take advantage of the molecular recognition performed at the polymerase active site after a single nt incorporation. This would considerably reduce product diversity generated by an extension reaction in the presence of classical deoxy and dideoxy nt. Thus, a canonical sequencing reaction would produce a single adduct in a controlled fashion, allowing identification of the added nt, before iteration of the process, leading to a stepwise, real-time sequence determination. Protection of the 3'-end of the extending DNA molecule would have the desired pr

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As a first step toward such a method, we describe such deoxy nt derivatives and their use by different DNA polymerases. We show using an end-labelled primer and a gel assay that these nt are chain terminators which, once incorporated, can be converted to regular functional

3'-ends. We describe the basic steps of a putative cycle (namely complete incorporation, deprotection and re-incorporation), as a contribution towards a new nt sequencing method that circumvents gel electrophoresis and the use of radio-isotopes.

RESULTS AND DISCUSSION

The aim of this work was to design 3'-modified 2'-deoxynucleotides 5'-triphosphate substrates for DNA polymerases, such that the 3'-moiety would be different for each base G, A, T or C, be easily identified (e.g., fluorescent), and be removed under conditions compatible with DNA stability to restore an unprotected 3'-hydroxyl end.

(a) Synthesis of nt analogs

We synthesized 3'-anthranlyloyl 2'-deoxy-nucleotide-5'-triphosphates derivatives and evaluated them as substrates for several DNA polymerases. For each of the four dATP, dGTP, dTTP and dCTP, the 3'-hydroxyl group was esterified by a distinct anthranilate or fluorescein-based fluorescent residue using the corresponding anhydride or isothiocyanate (Fig. 1). This resulted in different spectrofluorometric properties for each 3'-Reversibly Tagged-dNTP (3'-RTa-dNTP), as a consequence of the respective free acid counterpart (Table I) and allowed discrimination between them thanks to their different fluorimetric absorption or emission spectra. Nuclear magnetic resonance assignments are available upon request.

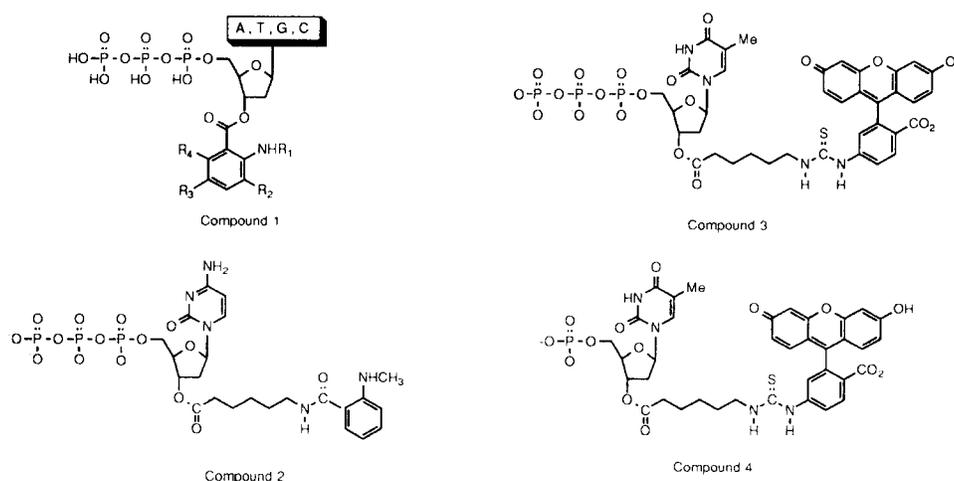


Fig. 1. Chemical structure of 3'-RTa-2'-deoxy-nucleotides. **Methods:** An-dATP (Sarfati et al., 1990) was prepared from dATP and isatoic anhydride essentially by the same procedure as (Hiratsuka, 1982) for the synthesis of An-ATP. *N*-methyl-An-dGTP, 6-methyl-An-dTTP, 3-methyl-An-dCTP and 5-methyl-An-dCTP were synthesized, purified and characterised by the same procedure. 3-Methyl, 5-methyl and 6-methyl-isatoic anhydrides were prepared by the procedure described by Erdmann (1899) for isatoic anhydride. Synthesis of compounds 1, 2, 3 and 4 will be described elsewhere (R.S.S., T. Berthod, C. Guerreiro and B.C., data not shown).

TABLE I

Spectral properties of uncoupled fluorophores^a and their corresponding nt

Anthranlyic derivative ^b				Wavelength (nm)		Coupled to
R1	R2	R3	R4	λ_{max} absorption	λ_{max} emission	
H	H	H	H	315	396.5	dATP
CH ₃	H	H	H	n.d. ^c	416.5	dGTP
H	CH ₃	H	H	312	403	dTTP
H	H	CH ₃	H	317	409	dCTP
H	H	H	CH ₃	289	403	dCTP
Fluorescein				494	523	dTTP

^a **Methods:** Absorption spectra were recorded at 25 °C in a double-beam spectrophotometer in the presence of 50 mM Tris·HCl (pH 8.0). Fluorescence emission and excitation spectra were measured at 25 °C in a LS50B Perkin-Elmer fluorescence spectrophotometer, using a 2-ml cuvette or a 16- μ l liquid chromatography flow cell from Perkin-Elmer. All compounds were excited at their absorption maxima. The slit widths for excitation and emission were 2.5 nm.

^b See Fig. 1 for compound 1 with groups R1–R4.

^c n.d., not determined.

(b) Incorporation of 3'-RTa-dNTPs

Since these nt analogs did not contain a 3'-hydroxyl group, their incorporation into an elongating DNA strand resulted in chain termination. This point was assessed using the solid-phase assay described in Methods in the legend to Fig. 3. A primed ss oligo (Fig. 2, substrate 31-G,A,T,C) was incubated with the complementary 3'-RTa-dNTP adjacent to the primer and various DNA polymerases, and the resulting product was



Fig. 2. Diagram of the system used to measure enzymatic insertion of 3'-modified nt. The synthetic primer is either biotinylated in 5' (substrate 31-G,A,T,C) or ³²P-end-labelled (substrate 26-C) and annealed to a complementary template.

assayed for free 3'-hydroxyl groups available for further extension with a chase containing a radio-labelled deoxy nt (Fig. 3A). Typically, this assay indicated that some incorporation of the 3'-modified nt might have occurred, as higher counts were always found in the control (unblocked) relative to the various enzyme/substrates tested. Several DNA polymerases could have extended the primer with a 3'-modified deoxy nt to some extent, the Sequenase and the M-MuLV reverse transcriptase being respectively the most and the least efficient under the experimental conditions tested here. Unmodified T7 DNA polymerase, *Taq* polymerase and Klenow fragment of DNA polymerase I were also able to use such substrates (data not shown).

However, the observed blocking was not complete under some of these conditions (Fig. 3A), and such assays did not tell us what may have happened at the molecular level.

Consequently, AMV-reverse transcriptase, *Taq* DNA polymerase and modified T7 DNA polymerase were selected for further studies using a ³²P-end-labelled primer extension assay followed by denaturing polyacrylamide-gel electrophoresis. Fig. 3B shows the result of such an assay in conjunction with its corresponding solid-phase assay. Modified T7 DNA polymerase was able to incorporate very rapidly 3'-RTa-dGTP in front of its cognate base up to a plateau value dependent on the concentration of the modified nt, and comparison of Fig. 3A and B showed that the solid-phase assay correlated well with the primer extension assay when relative band intensities were determined upon densitometric analysis (data not shown). The equilibrium was not exclusively dependent on nt concentration, since addition of more enzyme was able to displace this equilibrium towards further incorporation. The plateau value did not come from a rapid inactivation of the enzyme, since a chase of classical deoxy-nt lead to rapid extension of the remaining 21-mer up to 31-mer (data not shown). In Fig. 3B, concentrations of 3'-RTa-dGTP above 1 mM were able to displace this plateau value to nearly 100% incorporation in less than 1 min. Although 3'-RTa-dNTPs did act as chain terminators, they did not compete out significantly ddNTPs in classical Sanger dideoxy sequencing reactions even with

a fivefold molar excess relative to ddNTPs. For example, addition of 40 μ M of 3'-RT-dCTP to a M13 sequencing ddC termination mix made of 80 μ M of each dGTP, dATP, dCTP and dTTP, 8 μ M ddCTP and 50 mM NaCl did not lead to significant shortening of sequencing products upon examination of polyacrylamide gel autoradiograms obtained with commercial T7 sequencing kits. Taken with the fact that high concentrations of 3'-RTa-dNTPs were needed to reach high incorporation levels, this may indicate that these modified nt are not very efficient chain terminators, a result awaiting precise determination of their K_m , V_{max} and k_{cat} (work in progress). The DNA polymerases used here had different kinetic behaviors ranging from very slow (e.g., AMV-RT, data not shown), to moderate (e.g., *Taq* DNA polymerase), to instantaneous incorporation (e.g., Sequenase) up to the plateau value. For example, one can compare the Sequenase enzyme in Fig. 3B with the *Taq* DNA polymerase in Fig. 5A. We do not know if this kinetic behavior is related to the processivity of these polymerases. No products smaller in size than the primer were detected, except when DNA polymerases having a 3' to 5' exonuclease activity were used, a finding which indicates that such activity should not be present when one expects a single addition product.

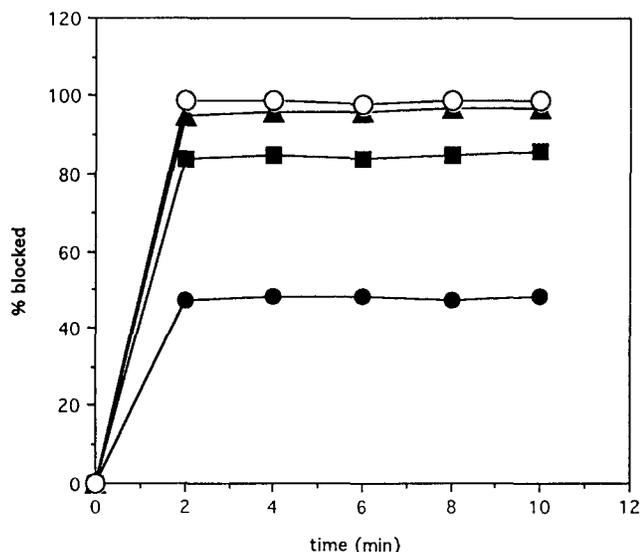
These results show that, despite a relatively bulky 3'-group, these modified nt are still accepted by the enzyme. However, the nature of the 3'-substitute played a key role in the incorporation level, as shown in Fig. 4.

A spacer arm was esterified in the 3' position of dTTP and dCTP, allowing facile coupling of *N*-methyl-anthranilic and fluorescein derivatives (compounds 2 and 3), respectively (R.S.S., T. Berthod, C. Guerreiro and B.C., unpublished results). This led to very slow and incomplete incorporation for the 3'-fluorescein derivative of dTTP, no matter which enzyme was used, but had a dramatic effect for the *N*-methyl-anthranilic derivative (Fig. 4A and not shown). Indeed, 500 μ M of this 3'-substituted nt were sufficient to drive the incorporation reaction close to completion with modified T7 DNA polymerase. To our surprise, two band products in equilibrium with one another were obtained. The fact that this was again independent of the enzyme used (Sequenase, AMV-RT or *Taq* DNA polymerase) suggested that these two products were probably conformers or differed only by a net electric charge under the electrophoretic conditions used here, but this awaits further characterization of the addition products.

(c) The tags can be chemically or enzymatically removed

Chemical or enzymatic removal of such a tag is shown in Fig. 5. Panel A shows incorporation of 3'-RTa-dGMP in front of its cognate dC base (template 26-C of Fig. 2),

A



B

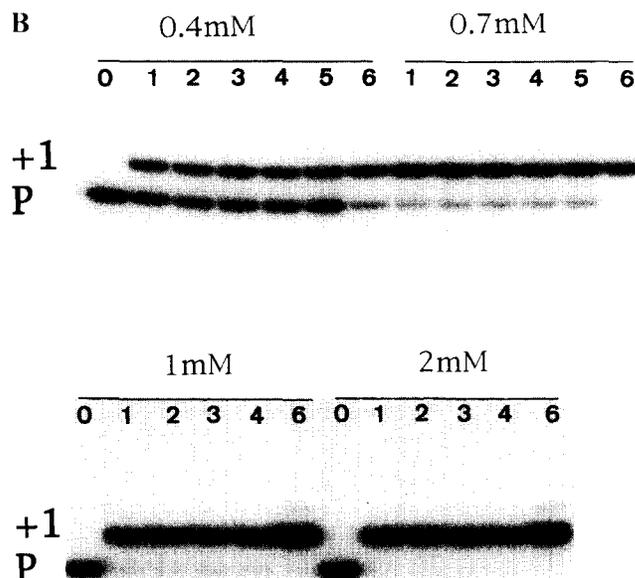


Fig. 3. Incorporation assay into DNA. (A) Incorporation of 3'-RTa-dNTPs with a solid-phase assay. **Methods:** Approx. 2 pmol of 5'-biotinylated 21-mer (5'-Bio-ATACTTTAAGGATATGTATCC) were bound to M-280 Dynabeads as described by the manufacturer and hybridized to an excess (50 pmol) of a complementary oligo presenting a 5' tail (Fig. 2). Annealing was for 1 h at room temperature in the presence of 1 M NaCl/5 mM Tris-HCl pH 7.5/0.5 mM EDTA. After removal of the unbound oligo, washed beads were suspended in 50 mM Tris-HCl pH 8.0/10 mM MgCl₂/10 mM dithiothreitol/100 µg BSA per ml and incubated in the presence of one 3'-RTa-dNTP at various concentrations and a DNA polymerase at 37 °C. The reaction was terminated with 20 mM EDTA/0.01% Triton X-100, the beads washed and their concentration determined under the microscope with a hemacytometer before being assayed for free 3'-hydroxyl group with radiolabel incorporation as follows: beads carrying the hybridized oligos were incubated in the same buffer as for 3'-RTa-dNTPs supplemented with a mix of dNTPs containing [α -³⁵S]dATP and AMV-RT at 37 °C. The beads were washed until radioactive counts reached background level in the supernatants and the concentration of the beads was determined in an aliquot as above. The beads were then dispersed in scintillation counting cocktail (Aquasafe 300, Zinsser Analytic). The amount of radiolabel was estimated relative to the unblocked control in pre-set channels of the corresponding isotopes in a scintillation counter. Concentration of 3'-RTa-dGTP: (■) 0.4 mM; (●) 0.7 mM; (▲) 1 mM; (○) 2 mM. (B) End-labelled primer extension and gel assay. Simple standing start reactions were performed exactly as described (Boosalis et al., 1987) using the primer:template 26-C of Fig. 2 and 5 units of modified T7 DNA polymerase. Incubation was for 0 min (lane 0), 2 min (lane 1), 4 min (lane 2), 6 min (lane 3), 8 min (lane 4), 10 min (lane 5). After 10 min, 5 units were added and the incubation extended to 20 min (lane 6). P: primer (21-mer). The reaction products were subjected to electrophoresis through a 15% denaturing polyacrylamide gel, which was subsequently autoradiographed.

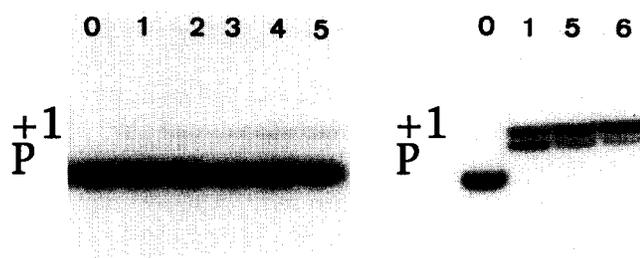


Fig. 4. Incorporation of compound 2 (2 mM, left panel) and compound 3 (0.5 mM, right panel) into primer:template 31-A and 31-G of Fig. 2, respectively, using 5 units of modified T7 DNA polymerase. Incubation times are 0, 1, 2, 3, 4, 5 and 10 min for lanes 0, 1, 2, 3, 4, 5 and 6, respectively. P, primer (21-mer). The reaction products were subjected to electrophoresis through a 15% denaturing polyacrylamide gel, which was subsequently autoradiographed.

saponification with 0.1 M NaOH, neutralization, re-annealing and re-incorporation of ddAMP using *Taq* DNA polymerase. In both incorporations, a single-nt adduct is detected, at the expected level, indicating the

easy deprotection of the 3'-end of the growing DNA chain (lane 6), to give a functional 3'-hydroxyl end. Omission of the alkaline treatment did not allow a second primer extension (lane 5).

Fig. 5B shows a thin-layer chromatogram of the time-course of compound 4 reacted with proteinase K. After 2 h, complete removal of the tag was obtained, and absence of a ninhydrin positive spot indicated that the ester linkage was indeed the cleaved bond.

(d) The chemical nature of the 3' bond is important for a convenient re-incorporation

Of particular importance was the nature of the chemical bond between the ribosyl moiety and the anthranlyloyl substituents. Ethers or esters are both expected to restore a hydroxyl group upon deprotection. We reasoned that ether bonds would be hard to cleave under mild conditions compatible with DNA chemical stability, whereas chemical deprotection using alkali has the present disad-

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