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5-Amino-2′-deoxyuridine, a Novel Thymidine Analogue for High-Resolution Footprinting of Protein–DNA Complexes

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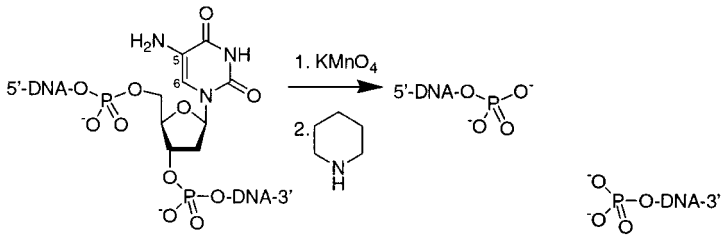
ABSTRACT



5-Amino-2′-deoxyuridine 5′-triphosphate, an analogue of deoxythymidine triphosphate, was synthesized and found to be a substrate of Taq DNA polymerase. The DNA-borne analogue underwent selective chemical reaction with permanganate. The use of 5-amino-dU as an interference probe was validated using the Ada protein/*ada* promoter complex. The performance of 5-amino-dU in interference footprints is similar to that of the previously described analogue 5-hydroxy-dU, but the former is incorporated more readily into DNA during enzymatic polymerization.

Gene expression is regulated by sequence-specific DNA binding proteins. A major goal of the post-genome era is to identify all of these regulatory proteins and map their binding sites in the genome. To facilitate this process, we have developed a biochemical procedure called template-directed interference (TDI) footprinting,[1−4] which enables the determination at single-nucleotide resolution of DNA bases that are contacted by a protein upon formation of a sequence-specific complex. Here we report an improvement in a key chemical component used in TDI footprinting.

In TDI footprinting,[1] analogues of the four naturally occurring 2′-deoxynucleosides are incorporated into DNA enzymatically, via the corresponding 5′-triphosphates. These analogues possess the same base-pairing preferences as their normal counterparts, but bear alterations in structure that (i) interfere with the establishment of intermolecular contacts by a DNA-binding protein and (ii) allow for selective chemical degradation leading to DNA strand cleavage, so as to reveal the positions at which the analogue has been incorporated. During the enzymatic DNA synthesis step, the molar ratio of the analogue triphosphate to its normal counterpart is set to a value that results, on average, in one unit of the analogue being incorporated into each product DNA molecule. The product is therefore a pool of singly substituted DNA molecules that differ in the position of analogue incorporation. This pool is then incubated at equilibrium with the protein of interest, and the DNA molecules that bind the protein are separated from those that do not. The former contain the analogue at positions that are not important for protein binding; the latter contain the analogue at positions that interfere with recognition by the protein. Specific chemical cleavage at the sites of analogue incorporation and gel electrophoretic analysis reveal those positions at which the analogue disrupts protein binding.[5]

We have reported a set of four nucleoside analogue triphosphates (7-deaza-7-nitro dATP, 5-hydroxy dCTP,

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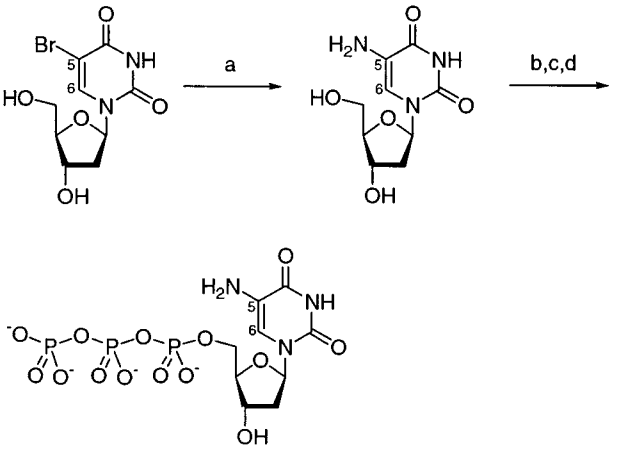
7-deaza-7-nitro dGTP, and 5-hydroxy dUTP) that can be incorporated into DNA via the polymerase chain reaction (PCR).[6] One of these, 5-hydroxy-dU, was less efficient than the others as a substrate for Taq polymerase, resulting in difficulty with amplification of certain sequences. It was therefore desirable to replace 5-hydroxy-dU with a new "T" analogue having improved performance in PCR. To this end, we designed and synthesized a new thymidine (T) analogue, 5-amino-2′-deoxyuridine (5-amino-dU). Like 5-hydroxy-dU, 5-amino-dU is roughly isosteric with T. However, whereas the C4 carbonyl group of T is available for hydrogen bonding to a protein, the C4 carbonyl of the analogue is tied up in an intramolecular hydrogen bonding interaction with the vicinal 5-amino group. Furthermore, the polar 5-amino group of the analogue would be less able to engage in hydrophobic contacts than the 5-methyl group of T. For these reasons, we suspected the analogue would interfere with the establishment of T-specific protein contacts. Finally, we thought that the electron-rich 5,6-enamine moiety of 5-amino-dU might be susceptible to attack by permanganate, a prerequisite for DNA strand cleavage.

5-Amino-dU was prepared by treatment of 5-bromo-dU with liquid ammonia[7] (Scheme 1). We found that the chromophore of 5-amino-dU was completely destroyed by brief exposure to aqueous potassium permanganate and piperidine (data not shown), suggesting the analogue would be selectively cleavable in DNA. Importantly, the nucleoside analogue was found to withstand 30 cycles of thermal cycling in Taq polymerase reaction buffer, as judged by HPLC analysis (data not shown). Encouraged by the results of these tests, we converted 5-amino-dU to the corresponding 5′-triphosphate, using the standard procedure[8] (Scheme 1). The analogue proved to be a relatively poor substrate for chemical phosphorylation, but we could easily purify the desired product by anion-exchange chromatography, so we proceeded without any further optimization of the synthesis.

Scheme 1. Synthesis of 5-Amino-dUTP[a]



[a] Reagents and conditions: (a) liquid NH_3, 55−60 °C, 44 h (65%); (b) $POCl_3$, −10 °C, 3 h; (c) tributylammonium pyrophosphate, 0 °C, 2 min; (d) 1.5 M triethylammonium bicarbonate, pH 7.7, rt, 1 h (overall isolated yield 10% for steps b−d).

To assess the biochemical characteristics of 5-amino-dU, we used the analogue triphosphate in Taq-based PCR amplification of a 209 base pair segment of a plasmid carrying the *E. coli ada* promoter sequence. Unlike 5-hydroxy-dUTP, 5-amino-dUTP supported robust DNA polymerization even under conditions of uniform incorporation, when every T in the sequence is replaced by the analogue (data not shown). To determine the ratio of 5-amino-dUTP/dTTP that affords statistical incorporation of a single 5-amino-dU moiety per DNA strand, we radiolabeled one of the two PCR primers and carried out a series of PCR reactions using different ratios. The PCR products were then treated with 100 μM potassium permanganate followed by 10% aqueous piperidine (a detailed procedure is provided in the Supporting Information). Following evaporation and resuspension in loading dye, the fragments were analyzed

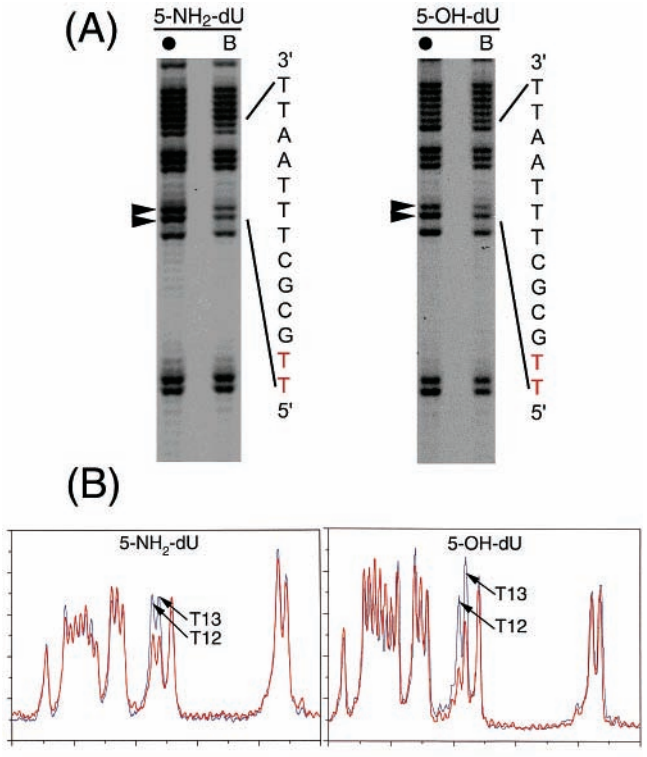


Figure 1. A comparison of TDI footprints generated by 5-amino-dU with those generated by 5-hydroxy-dU. (A) Phosphorimage renderings of TDI footprints of both analogues made by the Ada protein on the *ada* promoter. The sequence is typed next to the image, and the T residues showing interference (T12 and T13) are in red. The lane denoted by "•" represents the pattern of the cleaved starting DNA pool, and the lane labeled B corresponds to cleaved protein-bound DNA. (B) Densitometric analysis: blue, cleaved starting DNA pool; red, cleaved protein-bound DNA.

(5) A scheme depicting the overall procedure for TDI footprinting, accompanied by a detailed experimental procedure, is available in the Supporting Information.

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on 6% polyacrylamide sequencing gel. By visual inspection of the gel (Supporting Information), we empirically determined that 40 mol % of 5-amino-dUTP relative to the total T nucleoside triphosphates (dTTP plus 5-amino-dUTP) afforded PCR products containing approximately one analogue per strand. The gel analysis further indicated that 5-amino-dU faithfully base-pairs with A during PCR, as cleavage was observed only at T positions in the sequence. The background cleavage of unmodified DNA at T positions was negligible under these conditions (Supporting Information).

To evaluate the interference characteristics of the new analogue, we incubated the statistically modified 209 base-pair DNA product with the Ada protein, isolated the protein-bound DNA fraction, cleaved it, and analyzed the products on a 6% DNA sequencing gel. For purposes of comparison, we carried out a parallel interference experiment with 5-hydroxy-dU. Comparison of the pattern of bands for the initial 5-amino-dU-containing DNA pool (Figure 1A, left-hand panel, dot) with that of the protein-bound fraction (B lane) reveals interference at two positions, T12 and T13; this can be seen more clearly in the densitometric traces in Figure 1B. We previously reported significant interference by 5-hydroxy-dU at these same positions,[6] a result corroborated here (Figure 1, right-hand panel). The high-resolution NMR structure of the sequence-specific Ada protein/DNA complex[9] reveals a hydrophobic interaction between the π-face of Phe114 and the methyl-bearing edges of both T12 and T13; 5-amino-dU, like 5-hydroxy-dU, clearly interferes with this interaction.

Here we have introduced a new T analogue for use in TDI footprinting of protein−DNA interactions. This analogue performs similarly to the previously reported one, 5-hydroxy-dU, in footprints of the Ada protein on its promoter sequence, but the newer analogue shows improved behavior in PCR amplification, especially under demanding conditions. We therefore recommend the use of 5-amino-dU as the T analogue of choice in future TDI footprinting experiments. We also note that 5-amino-dU could be useful for the preparation of randomly labeled probes for DNA microarray analysis.[10]

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Supporting Information Available: Detailed experimental procedures, characterization of 5-amino-dUTP, and 4 figures describing (1) an overall scheme for TDI footprinting, (2) the base-pairing properties of 5-amino-dU and 5-amino-dUTP/dTTP ratio determination, (3) cleavage of T vs 5-amino-dU residues in DNA, and (4) native polyacrylamide gel electrophoresis separation of Ada protein-bound DNA. This material is available free of charge via the Internet at http://pubs.acs.org.

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