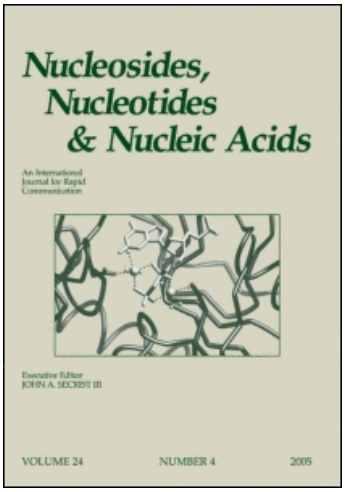

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AMBIGUOUS BASE PAIRING OF 1-(2-DEOXY-β-D-RIBOFURANOSYL) IMIDAZOLE-4-CARBOXAMIDE DURING PCR

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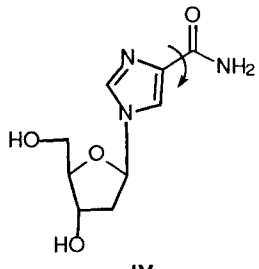
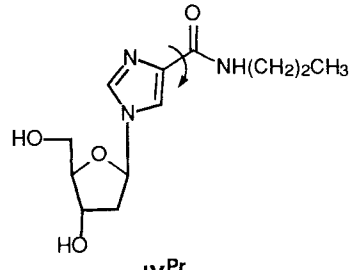
ABSTRACT: The use of 5'-triphosphate of 1-(2-deoxy-β-D-ribofuranosyl)imidazole-4-carboxamide (dYTP) in DNA amplification reaction in place of dATP or dGTP yielded mutations frequencies of $3\text{-}4x10^{-2}$ per base per amplification. A reasonable proportion of transversions (11-15%) was observed in the absence of deletions and insertions.

1-(2-deoxy-β-D-ribofuranosyl)imidazole-4-carboxamide (dY), a simplified deoxypurine analogue resulting from opening the six-membered ring and elimination of C2 and N3, was recently proposed as ambiguous nucleoside. As a result of rotation around the carboxamide and glycosidic bonds, this nucleoside may in principle form hydrogen bonded base pairs with the four canonical bases as well as with itself[1]. The 5'-triphosphate derivative dYTP was found to be substrate for DNA polymerases (Klenow fragment, Sequenase, *Taq* polymerase). In another way the incorporation with the Klenow fragment of the four natural bases and itself opposite Y located in the matrix was observed[2]. These results indicate that in principle dYTP can be incorporated in a variety of conformations and, once part of the template, can be copied ambiguously. It is reported here that dYTP, and its propyl derivative $dY^{Pr}TP$, give rise to ambiguous base pairing during PCR.



dY



dY^{Pr}

The frequency of *Taq* DNA polymerase error generally falls within the range of $2x10^{-3}$ to $<2x10^{-4}$ mutations per nucleotide per amplification[3]. PCR may be rendered highly error prone by using dNTP biases and/or the addition of millimolar concentrations of transition metal cations such as manganese (mutation rate about 10^{-1} per base per amplification)[4,5]. Nucleoside analogues have also been used to decrease the fidelity of PCR. Deoxyinosine may base pair with dA, dG, dC and dT, although it has a clear preference for dC[6]. More recently, dPTP (6-(2-deoxy-β–D-ribofuranosyl)-3,4-dihydro-8H-pyrimido-[4,5-C][1,2] oxazin-7-one triphosphate) and 8-oxo-2'-deoxyguanosine triphosphate have been shown to be efficiently incorporated by *Taq* DNA polymerase[7].

Polymerase chain reaction

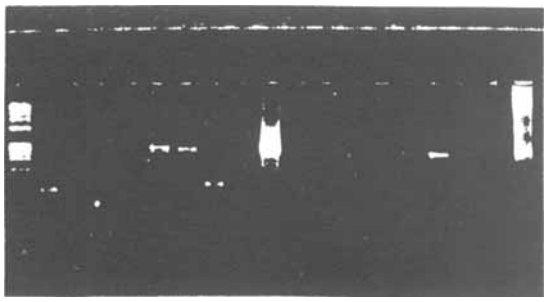
Target DNA for PCR was the gene encoding for the type II dihydrofolate reductase (DHFR), expressed by the *E. coli* plasmid R67. The amplification primers give rise to a PCR product of 240 bp[8]. The title compound dYTP and the N-propyl derivative $dY^{Pr}TP$ were first used to completely substitute for one of the four standard dNTPs (200 μM). No PCR product could be discerned in any reaction (Figure A, samples 1-4 and 10-13). In an attempt to force incorporation of dYTP and $dY^{Pr}TP$, their concentration was increased to 1 mM and the reaction doped with a low concentration of the dNTP for which dYTP or $dY^{Pr}TP$ was substituting. Only when dYTP or $dY^{Pr}TP$ were used as deoxypurine analogues could PCR products be recovered (Figure A, samples 5-8 and 14-17). In the case of dYTP (or $dY^{Pr}TP$) as a dATP analogue, [dATP] could be reduced to 2.5 μM (or 5 μM) while as a dGTP analogue [dGTP] could be reduced to 1 μM (or 1 μM). Below these concentrations no PCR product was recoverable (Figure B).

Mutagenic effects

On the premise that mismatches involving dY or dY^{Pr} would be subject to proof-reading post cloning, 10% of PCR products were chased in a second round of PCR using equimolar dNTPs (200 μM). PCR products were purified and cloned as described[8]. Plating out R67 DHFR on trimethoprim (trim) + ampicillin (ampi) plates yields the functional DHFR variants, while plating on ampicillin alone yields the total collection of variants, functional and defective. The $trim^R/ampi^R$ ratio provides a rapid indication of the efficiency of hypermutagenesis. A series of mutagenesis experiments in which dYTP($dY^{Pr}TP$) at 1 mM replace dATP or dGTP (added at 1-5 μM) revealed that dYTP was mutagenic when used to mimic dATP or dGTP; by contrast $dY^{Pr}TP$ was mutagenic only as a dATP analogue. No evidence of a significantly altered $trim^R/ampi^R$ ratio was found when either product was used to mimic both dATP and dGTP at the same time.

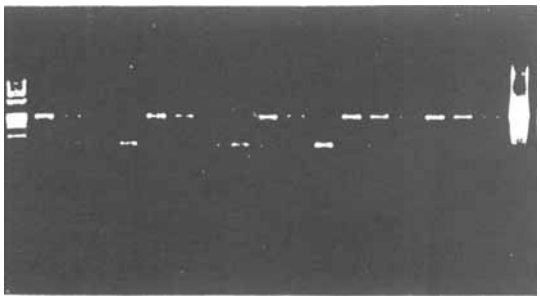
Sequencing data

Clones from mutagenesis generated by dYTP and $dY^{Pr}TP$ were grown up and sequenced. Among the 95 clones sequenced three insertions/deletions were noted (+1, +3 and -3).



Φ 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

(A) The reaction mixtures contained the following deoxytriphosphates: (1) G, C, T and Y^{Pr}; (2) A, C, T and Y^{Pr}; (3) A, G, T and Y^{Pr}; (4) A, G, C and Y^{Pr}; (5) A*, G, C, T and Y^{Pr}; (6) A, G*, C, T and Y^{Pr}; (7) A, G, C*, T and Y^{Pr}; (8) A, G, C, T* and Y^{Pr}; (9) A, G, C, T and Y^{Pr}; samples 10 to 18 contained the same triphosphates except Y in place of Y^{Pr}. All dNTPs concentrations were at 200 μM, except in samples 5-8 and 14-17 in which triphosphate noted with * was at 5 μM.



Φ 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

(B) Samples 1-4 contained Y^{Pr} at 1 mM, G, C and T at 200 μM, and A at 5 μM, 2.5 μM, 1 μM, 0.5 μM, respectively; samples 5-8, same as 1-4 but no Y^{Pr}; samples 9-11 contained Y at 1 mM, G, C and T at 200 μM, and A at 5 μM, 2.5 μM, 1 μM, respectively; samples 12-14 contained Y at 1 mM, A, C and T at 200 μM, and G at 5 μM, 2.5 μM, 1 μM, respectively; samples 12-14, same as 9-11 but no Y; sample 18 contained A, C, G and T at 200 μM.

FIGURE: Incorporation into DNA and extension of dYTP or $dY^{Pr}TP$ by *Taq* DNA polymerase. PCR reaction conditions were 10 mM Tris-HCl pH 8.3, 50 mM KCl, 5 mM $MgCl_2$, 5 ng of plasmid DNA, 100 pmol of each primer and 5U of *Taq* polymerase (Roche-Cetus) in a final volume of 100 μl. Thermal cycling parameters : 95°C for 5 min; then 50x(95°C, 30 sec; 60°C, 30 sec; 72°C 10 min.). Φx174-RF DNA/Hae III digest was noted Φ.

When dYTP and $dY^{Pr}TP$ were used to substitute for dATP, predominantly T→C and A→G transitions were noted with between 10-15% of T→G,A and A→C,T transversions indicating that dYTP and $dY^{Pr}TP$ substrates were accepted essentially in the A-like conformation (Table). When dYTP was used in lieu of dGTP, the mutation spectrum was dominated by C→T and G→A transitions and a few C,G→N transversions. However, the mutation frequency was some 6 fold lower compared to using dYTP as a dATP analogue.

These results suggest that, at least for *Taq* DNA polymerase, dYTP is efficiently incorporated as a dATP analogue. dYTP can substitute for dGTP although at a reduced frequency (~six fold lower). This may stem from the fact that in solution dYTP predominantly adopts an A-like conformation. However, once part of a DNA template rotation about the carboxamide bond allows the base to be copied as G or A.

TABLE

Reaction	clones	total number of mutations	overall mutation frequency[a]	Ti/Tv[b]	resulting substitutions					
					T→C A→G	T→G A→C	T→A A→T	G→A C→T	G→C C→G	G→T C→A
dYTP in place of dATP	32	225	3.0x10-2	192/33	188	10	22	4	1	0
dY^{Pr}TP in place of dATP	34	217	2.6x10-2	193/24	190	7	16	3	1	0
dYTP in place of dGTP	29	37	5.3x10-3	32/5	1	0	1	31	0	4

a, mutation frequency is the number of mutations scored divided by the product of the number of clones sequenced and the target length of the DHFR. b, number of transitions (Ti) and transversions (Tv).

Substitution frequencies of ~$3x10^{-2}$ per base per amplification are comparable to those for a number of other hypermutagenic protocols. The mutation spectrum is, however, richer than either due to the fact that mutation of both DNA strands occurs. However, the infidelity of PCR with dYTP is currently ~2-3 fold less efficient than hypermutagenic PCR with biased dNTP concentrations and Mn^{2+} cations or PCR with the modified base dPTP. Nonetheless dYTP has a number of desirable traits: it produces a reasonable proportion of transversions (11-15%), substitutions were apparently random being free of any hot spots or dinucleotide context, while few frameshifts were noted. Consequently it could be of use in the construction of mutant gene libraries.

The deoxyimidazole-4-carboxamide triphosphates are possibly among the most radical departures so far from the canonical dNTPs used as substrates in PCR.

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