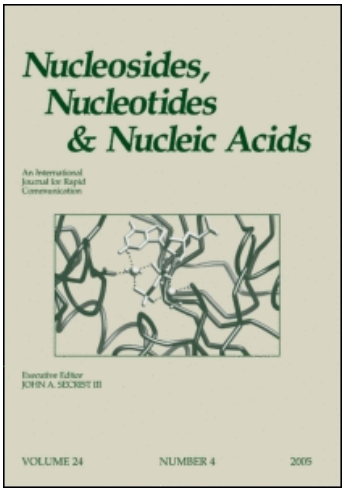

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SYNTHESIS AND STUDY OF THE FLUORESCEIN CONJUGATE OF THE NUCLEOTIDE dPTP

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ABSTRACT

The synthesis of a fluorescein conjugate on the non-natural base P is described. The ability of the newly synthesised fluorescein - dPTP and of a fluorescein-11-dUTP to compete with the natural nucleotide TTP was also studied. Overall the efficiency of labelling a nucleic acid with a fluorescein moiety was found to be approximately equal.

The bicyclic nucleobase analogue 6H,8H-3-4dihydropyrimido[4,5-c]-[1,2]-oxazin-7-one (P) is a mimic of both cytosine and thymidine, since it can form stable hydrogen bonded pairs with either guanine or adenine. This property arises because the base analogue is able to exist in both the imino and amino tautomer. The corresponding deoxyribonucleoside of P (dP) has been made and incorporated into oligonucleotides via chemical synthesis as its phosphoramidite derivative (1,2) and via enzymatic incorporation as its triphosphate derivative (3) (dPTP). In the latter case dPTP was shown to be an excellent substrate for the Taq polymerase with incorporation in place of TTP, at only a fourfold lower efficiency, although substitution for dCTP gave much reduced efficiency (3). This highlighted the potential for the dP to be used in probes and primers where the base analogue can be placed into positions of degeneracy to reduce the number of probes and primers required. The synthesis of functionalised derivative of dPTP would allow the introduction of a fluorescent label such as fluorescien via enzymatic means.

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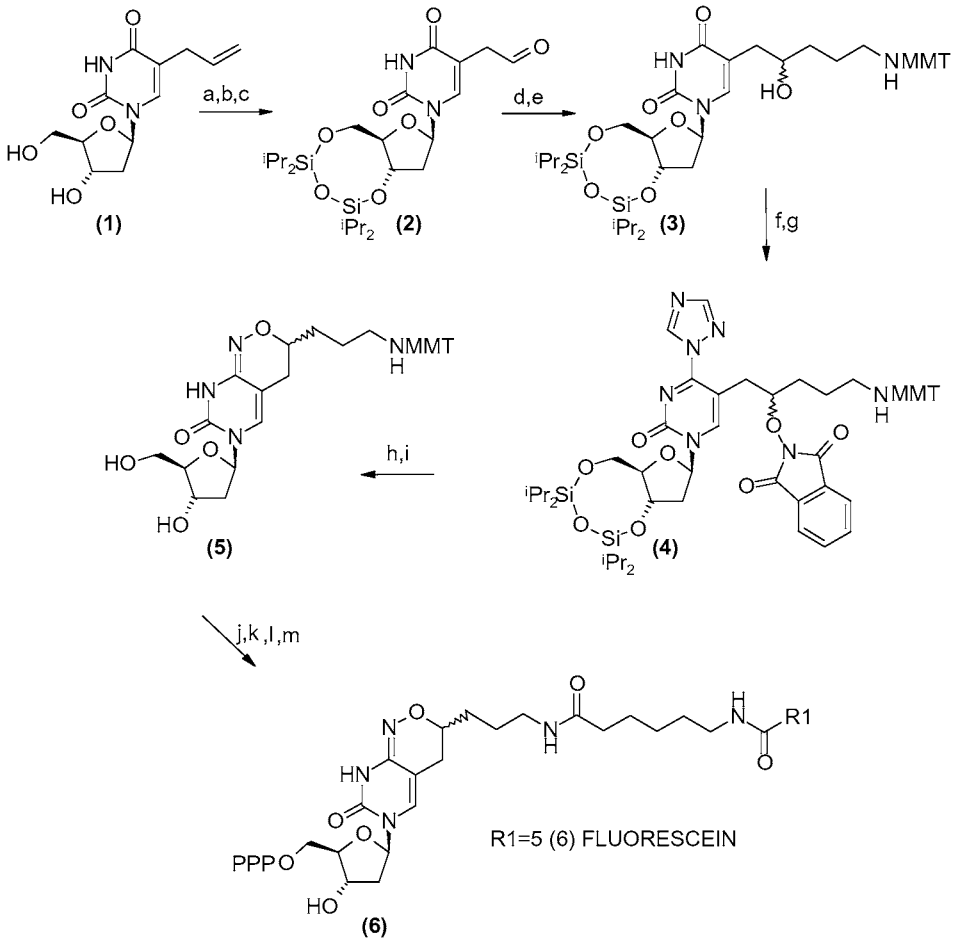

Figure 1. **Reagents** a) $TIPDSCl_2$, py, N_2, 0°C, 68% b) $K_2OsO_4 2H_2O$, N-methylmorpholino-N-oxide, acetone, 72% c) $NalO_4$, THF, H_2O, 93% d) 2,2,5,5-tetramethyl-1-aza-2,5-disilacyclopentane-1-propyl magnesium bromide, Et_2O, THF, N_2, not isolated then e) MMTCl, Et_3N, DCM, 61% the two steps f) N-hydroxyphthalimide, Ph_3P, DEAD, THF, N_2g) 1,2,4, triazole, $POCl_3$, Et_3N, MeCN 55% over the two steps h) NH_3, dioxane i) Bu_4NF, THF 68% over two steps j) AcOH, MeCN, H_2O not isolated then k) NHS of N-(trifluoroacetyl)caproic acid, Et_3N, DMF, 60% over two steps l) $POCl_3$, $(MeO)_3PO$, $(EtO)_3PO$ then $Bu_3NH\ H_3P_2O_7$, DMF, 40% m) NH_3(aq) then NHS ester of 5-(6) carboxyl fluorescein, DMF, Na_2CO_3 buffer pH 8.5.

A multi-step synthetic route, as shown in Figure 1, starting from 5′-allyl uridine achieved the synthesis of a dPTP derivative with a functionalised arm. The triphosphate derivative showed consistent ^{31}P and ^{1}H NMR spectra. The diastereomers were not separated during the course of the synthesis. Subsequent conjugation of the amino side chain with the NHS ester of 5-(6)-carboxy fluorescein provided the fluorescein conjugated nucleotide 6 (denoted as fluorescein-dPTP).

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Incorporation Assays

The ability of fluorescein-dPTP and fluorescein-11-dUTP (11 atom linker between C5 on the base and fluorescein) to compete with the natural dNTP's as substrates for exonuclease free Klenow fragment DNA polymerase I (EFK) was examined using the extension of a ^{33}P-labelled 15 mer primer hybridised to a 24 mer template.

The primer (1 pmol) and template (2 pmol) were hybridised in x2 Klenow buffer (100 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 10 mM β-mercaptoethanol) by heating at 75°C for 3 minutes then allowing to cool to 30°C over at least 30 minutes. To this was added EFK polymerase (0.1 U) and inorganic pyrophosphatase (20 mU) plus a mix of either fluorescein-dPTP and dTTP or fluorescein-11-dUTP and dTTP. The total nucleotide concentration in all the reactions was fixed at 20 μM, but the ratio of the dNTP to the labelled nucleotide was varied. Reactions were incubated at 37°C for 2 minutes before adding formamide/EDTA stop solution. The reactions were then heated at 95°C for 2 minutes before loading samples (4 μl) onto a 20% acrylamide/7M urea gel. After running at 1.8 kV for 2 hours the gel was fixed and dried before exposing to a phosphor screen for analysis and quantification.

The results from the above gel analysis clearly show that the fluorescent nucleotide analogue (fluorescein dPTP) is incorporated approximately 10–15% more efficiently than the corresponding dUTP derivative. However, we have shown that the subsequent extension of primers with fluorescein - dPTP at the 3′ end is approximately two-fold less efficient than the corresponding primer with a dUTP derivative at its 3′ end (unpublished results). Thus as a means of introducing a fluorescein group into an oligo enzymatically with modified nucleotides then the dP derivative is approximately equal to uridine.

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