SYNTHESIS OF FUNCTIONALIZED dNTPs FOR ENZYMATIC APPLICATIONS

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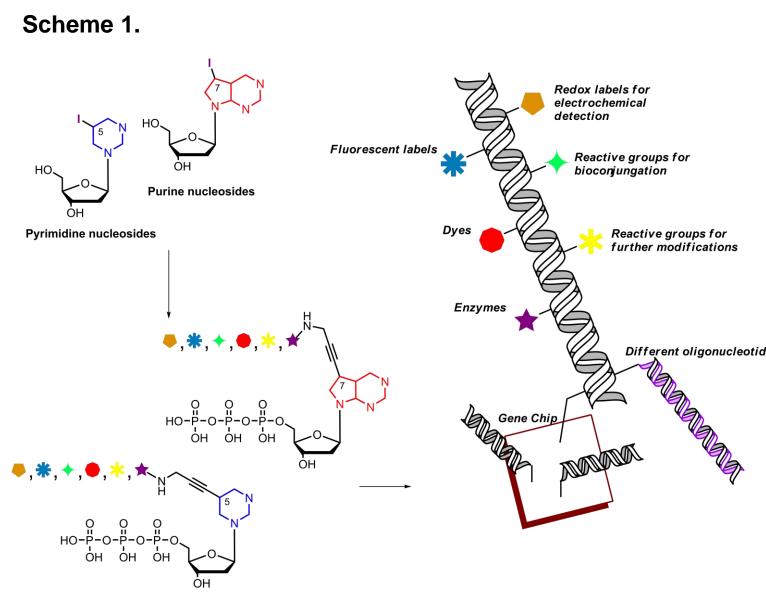
ABSTRACT

Modified nucleotides are of great interest due to their wide application in many biochemical processes. It is known that C5-position of the pyrimidine and C7-position of 7-deazapurine nucleotides are the most favorable for modifications. In this manner modified nucleoside-5'triphosphates are well tolerated by polymerase during enzymatical incorporation into nucleic acid strands. There is a great variety of functionalized molecules that could be used for modification of nucleotides and depending on their nature, labeled nucleic acids are successfully utilized in a number of enzymatic applications (Scheme 1.).1, 2, 3, 4

In the present work we describe an efficient and selective synthesis of amino-, biotin-, azido-functionalized and oligomer-tethered nucleotides and their incorporation during PCR and/or primer extension reactions.

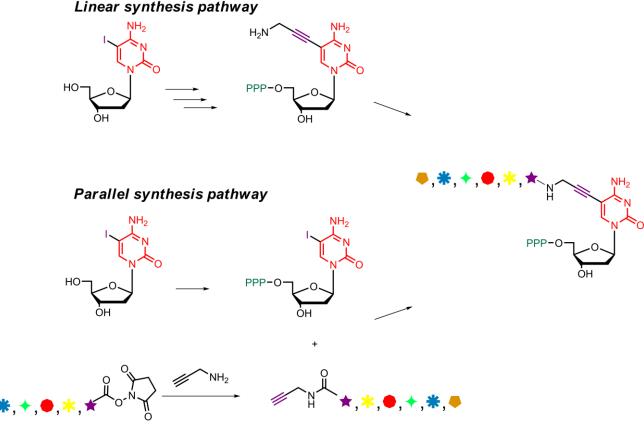
INTRODUCTION

There is a wide array of functionalized molecules that could be used for modification of nucleotides, such as biotin, redox labels for electrochemical detection, fluorescent labels, dyes, many different reactive groups, oligonucleotides, enzymes etc. Depending on the nature of modifications, labeled nucleic acids are successfully utilized in methods such as FISH (Fluorescence In Situ Hybridization), SELEX (Systematic Evolution of Ligands by Exponential Enrichment), GeneChip technology, etc. In addition, this allows to avoid radioactive labelling of DNA in PCR, nick translation, cDNA synthesis or primer extension reactions.3



RESULTS

Scheme 2.



Here we represent two different synthesis pathways for biotin-11-dCTP synthesis. Conventional method to obtain such products is a linear five-step synthesis.^{1, 2}. We have established more effective and less time-consuming, three-step synthesis of biotin-11-dCTP (Scheme 2).

Biotinylated dCTP synthesis

Linear synthesis pathway

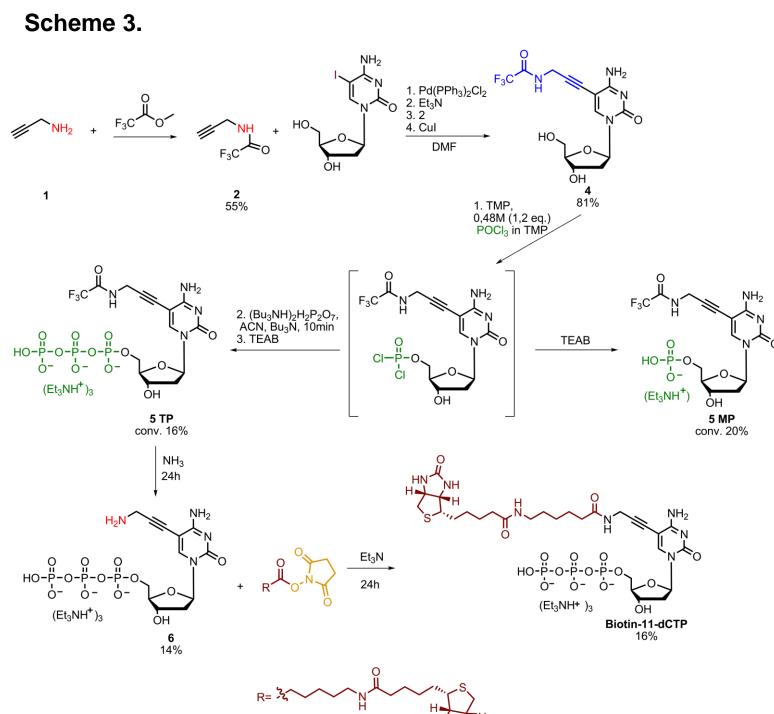


Table 1. Phosphorylation reaction conditions

No.	Additive reagents	Solvent	Reaction time 1 st step	Reaction conditions	Conversion to 5 MP	Conversion to 5 TP
1.	-		24 h	-5 – -10°C → r. t.	Trace	-
2.	10 eq. POCl ₃	TMP	24 h	-5 – -10°C → r. t.	Trace	-
3.	2 eq. Py		24 h	-5 – -10°C → r. t.	15%	-
4.	2eq. Py	ACN	24 h	-5 – -10°C → r. t.	Trace	-

In order to synthesize 5-(3-(2,2,2-trifluoroacetamido)prop-1-ynyl)-dCTP (5 TP) different phosphorylation reaction conditions were examined (Scheme 3, Table 1). During the five steps linear synthesis biotin-11-dCTP was obtained in 16% yield.

Parallel synthesis pathway

The linear synthesis pathway requires long time to implement and provides low yields. Accordingly, we established a more effective and less time-consuming synthesis route to obtain functionalized nucleotides (Sheme 2). Elaborating which, the limiting monophosphorylation step of iodinated nucleosides was optimized. (Table 2, Scheme 4).

Scheme 4.

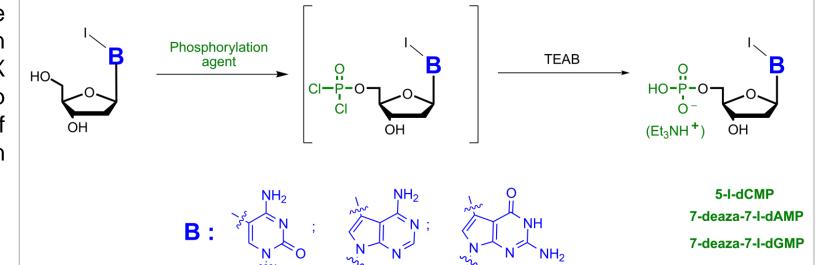
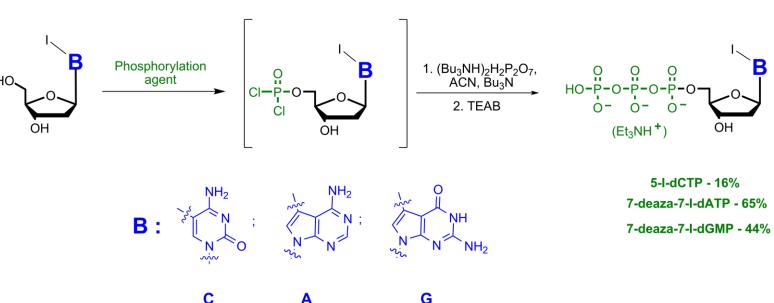


Table 2. Monophosphorylation reaction conditions

Nucleoside	Phosphorylation agent	Quantity of phosphorylation agent (eq.)	Solvent	Additive reagents	Temperature	Reaction time	Conversion to	В
	О			-	-5 – -10 °C	4h	35 %	
	CI—P-CI CI	1.2	TMP	WDI	r. t.	70 min	19 %	
				NBu ₃	r. t.	30 min	34 %*	
		1.2	TMP	2 eq. NBu ₃	r. t.	3 h	0 %*	
				2 eq. NBu ₃	r. t.	3 h	0 %*	
5-I-dC	O O CI—P-O-P-CI	3	Py:Dioxane (1:2)	-	r. t.	5 min	37 %*	
				2 eq. NBu ₃	r. t.	2 h	48 %*	
	ĊI ĊI			2 eq. NBu ₃	0 °C	4.5 h	70 %*	
		3 +1	ACN	2 eq. Py	0 °C	4.5 h	60 %*	
		(after 3h)		1 eq. Proton sponge	0 °C	4.5 h	69 %*	
	O CI-P-CI CI	2.2 TMP	-	-5 – -10	5 h	18 %		
			TMP	2 eq. NBu ₃	-5 – -10 °C	5 h	35 %	
		3.2		2 eq. NBu ₃	-20 °C	3 h	26 %	
			TMP	2 eq. NBu ₃	-1020 °C	3 h	0 %*	
				2 eq. Py	-1020 °C	3 h	0 %*	
		1.2		1 eq. Proton sponge	-1020 °C	3 h	0 %*	
				2 eq. NBu ₃	r. t.	3 h	0 %*	
				2 eq. NBu ₃	0 °C	4.5 h	14 %*	
	0 0	3+3		2 eqv Py	0 °C	4.5 h	62 %*	
7-deaza-7-l- dA	O O CI—P-O-P-CI CI CI	(after 3 h)	ACN	1 eq. Proton sponge	0 °C	4.5 h	53 %*	
.		3+3		4 eq. Py + 4 eq. (after 30 min)	0 °C	1.5 h	20 %*	
		(after 30 min)		1 ek. Proton sponge + 1 eq. (after 30 min)	0 °C	1.5 h	39 %	
		3 + 1 (after 1 h 20 min)		-	0 °C	3 h	88%*	
	O CI—P—CI CI	2.2		-	-5 – -10 °C	5 h	11 %*	
				2 eq. NBu ₃	-5 – -10 °C	5 h	25 %	
		2 + 1.2	TMP	2 eq. NBu ₃	-20 °C	3 h	56 %	
		(after 2 h)						
		1.2	TMP	2 eq. NBu ₃	r. t.	3 h	0 %*	
7-deaza-7-	0 0	3+1		2 eq. NBu ₃	0 °C	4.5 h	29 %*	
I-dG	CI-P-O-P-CI			2 eq. Py	0 °C	4.5 h	17 %*	
	O O CI—P-O-P-CI CI CI	(after 3 h)	ACN	1 eq. Proton sponge	0 °C	4.5 h	29 %*	
		3+1		_	0 °C	3 h	8 %*	
		(after 1 h 20 min)						

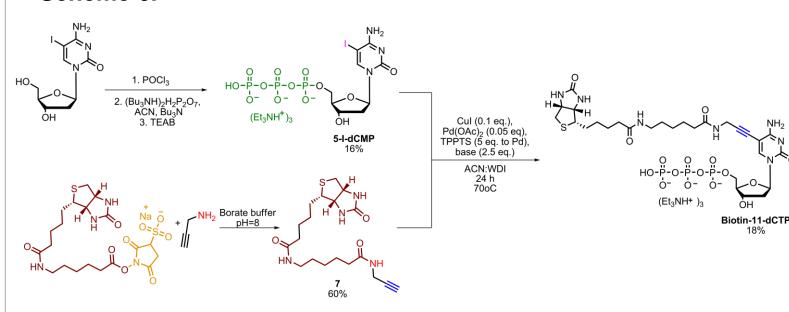
Scheme 5.

(after 1 h 20 min)



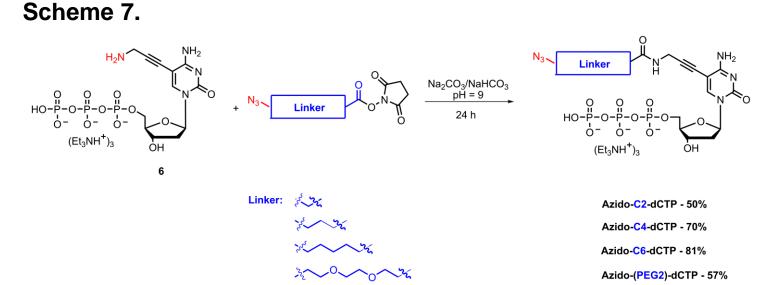
To obtain 5-I-dCMP and 7-deaza-7-I-dAMP the best results were achieved when pyrophosphoryl chloride was employed. On the contrary, the best conditions for synthesis of 7-deaza-7-I-dGMP were utilizing phosphorus oxychloride in TMP. It is worth mentioning, that in all cases pyrophosphoryl chloride in TMP gave no conversion to corresponding monophosphate (Sheme 4, Table 2). According to optimized monophosphorylation conditions corresponding 5'-triphosphates were synthesized.

Scheme 6.



Utilizing aqueous-phase Sonogashira coupling reaction the biotin-11-dCTP was successfully synthesized giving 18% yield. Biotin-11-dCTP was successfully incorporated into 3'-end of DNA strands, moreover, it perfectly worked during PCR.

Synthesis of azido-dCTPs bearing different linkers and their tethering to DNA oligomers



½~~0~0~0~1×

Efficient incorporation of modified nucleotides, during DNA synthesis is highly dependable on the size of attached label. More importantly, the lenght of linker, between nucleotide heterocyclic base and label, has the significant impact on incorporation. The linker should be long enough to reduce label steric hinderance and changes of nucleotide steric structure. At the same time, it has to be short enought to avoid back-folding onto the DNA strand. Moreover, the terminal functional groups of the linker must be tolerated by DNA polimerase enzymes. Properly designed linker will allow to incerporate nucleotides bearing large labels. Accordint to this we chose five different linkers and synthesysed corresponding azido group bearing cytidine triphosphates (Scheme 7).

Scheme 8.

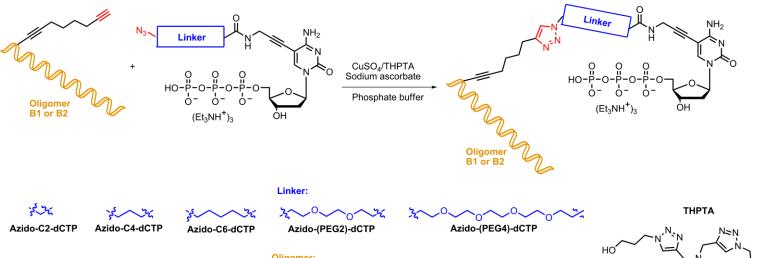


Table 3. Results of Click reaction

Oligomer	Azide	Reaction time	Product	Conversion to product
	Azido-C2-dCTP	50 min	B1-C2-dCTP	81 %
	Azido-C4-dCTP	1 h 30 min	B1-C4-dCTP	76 %
B1	Azido-C6-dCTP	1 h	B1-C6-dCTP	70 %
	Azido-PEG2-dCTP	1 h	B1-PEG2-dCTP	72 %
	Azido-PEG4-dCTP	1h 30 min	B1-PEG4-dCTP	80 %
	Azido-C2-dCTP	50 min	B2-C2-dCTP	82 %
	Azido-C4-dCTP	3 h	B2-C4-dCTP	51 %
B2	Azido-C6-dCTP	1 h	B2-C6-dCTP	74 %
	Azido-PEG2-dCTP	2 h	B2-PEG2-dCTP	66 %
	Azido-PEGA-dCTP	1 h 30 min	B2-DEGA-dCTD	68 %

Applying *Click* reaction ten different oligomer-tethered nucleotides were synthesized. Reaction mixtures were analyzed with liquid chromatography (Sheme 8, Table 3). Liquid chromatography with mass spectrometry was used for all products

Primary incorporation experiments of precursors and *Click* reaction products have been made. For primer extension reaction two different substrates (Figure. 1) and polymerases (Phu exo- and Tag) has been chosen. Experiments were carried out by incubating reaction mixtures for 1 h at 60°C temperature. Electrophoresis were performed utilizing denaturing urea polyacrylamide gel (Urea PAGE 15%).

Figure 1. Schemes of a primer extension reaction

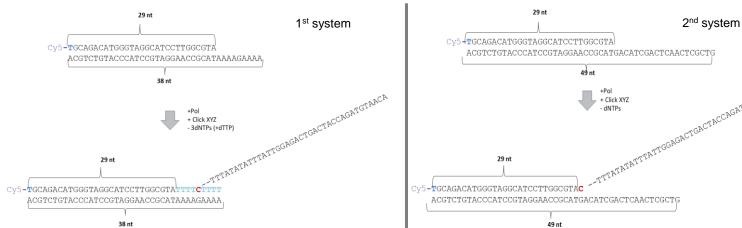
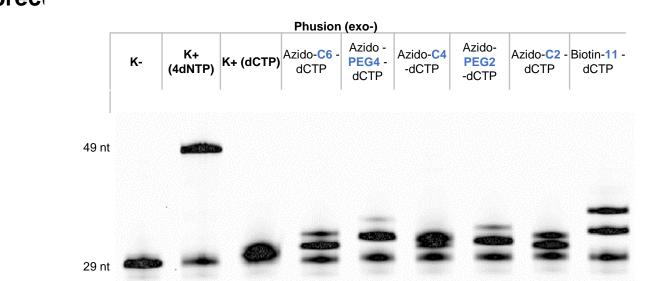


Figure 2. Results of a primer extension reaction using



Primer extension reaction was carried out according to 2nd system.

Figure 3. Results of a primer extension reaction (B1-C6-dCTP)

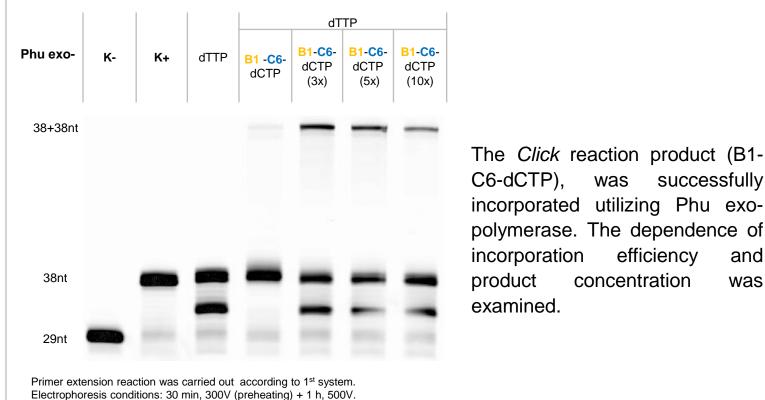
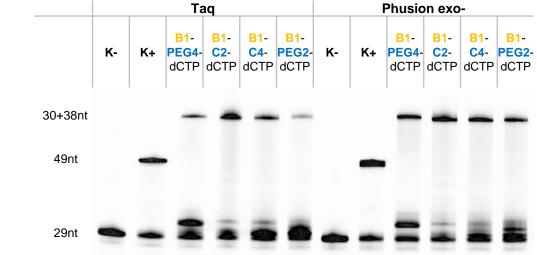


Figure 4. Results of a primer extension reaction (B1-linker-

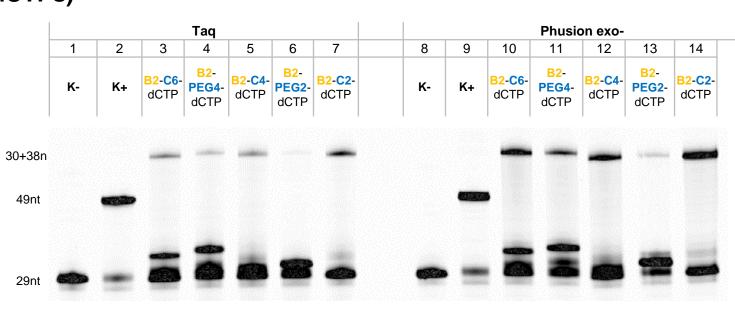


Primer extension reaction was carried out according to 2nd system Electrophoresis conditions: 30 min. 300V (preheating) + 1 h. 500V.

dCTPs)

Azido-(PEG4)-dCTP - 90%

Figure 5. Results of a primer extension reaction (B2-linkerdCTPs)



It has been shown that all azido group bearing dCTPs were successfully incorporated. Moreover, polymerases were able to incorporate dNTPs tethered to such bulky molecules as oligomers and neither linker nor oligomer structures did not influenced incorporation. However Phusion exo- polymerase gave better primer extension results then Tag polymerase.

CONCLUSIONS

In order to examine our constructed, more effective and less time consuming synthesis route for modified nucleotides synthesis, screening of phosphorylation reactions has been carried out. Consequently most suitable reaction conditions were identified for synthesis of 5-I-dCTP, 7-deaza-7-I-dATP and 7-deaza-7-I-dGTP. Hereafter, during three step synthesis pathway labeled nucleotide (biotin-11-dCTP) was obtained. In comparison the conventional five

was successfully synthesized. We found propargylamino- fragment bearing nucleoside 5'triphosphates to be efficient precursors for further modifications. In order to examine the dependance of incorporation efficiency on linker lenght during DNA synthesis, we used different linkers bearing azido-NHS-esters and synthesize terminal azido-group bearing dCTPs. Furthermore, azido-dCTPs and biotin-11-dCTP were

step synthesis pathway was applied, subsequently biotin-11-dCTP

Additionally, utilizing two different terminal alkine group bearing oligonucleotides, we successfully synthesised ten oligomer-tethered dCTPs. Applying Phu exo- and Taq polymerases we performed successful Click products incorporation during the synthesis of DNA strand.

successfully incorporated into DNA strand, moreover, biotin-11-

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dCTP perfectly worked during PCR.

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TRADEMARKS/LICENSING

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