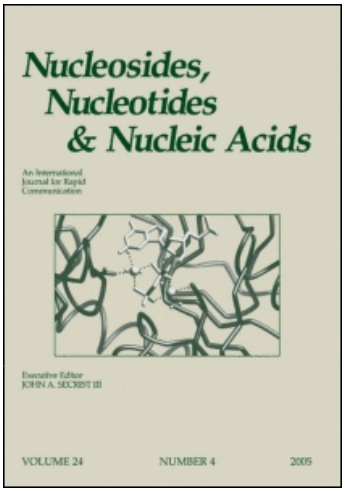


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# Synthesis and Properties of 2'-Deoxycytidine Triphosphate Carrying C-Myc Tag Sequence

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NUCLEOSIDES, NUCLEOTIDES & NUCLEIC ACIDS, 19(10–12), 1543–1552 (2000)

# SYNTHESIS AND PROPERTIES OF 2'-DEOXYCYTIDINE TRIPHOSPHATE CARRYING c-*myc* TAG SEQUENCE.[†]

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**ABSTRACT:** The synthesis of 2'-deoxycytidine triphosphate carrying mercaptoethyl groups at position 4 of cytosine is described. This nucleoside triphosphate was reacted with a maleimido-peptide carrying the c-*myc* tag-sequence to yield a peptide-nucleoside triphosphate chimera. Primer extension studies showed that the nucleoside triphosphate modified with the peptide sequence is incorporated by DNA polymerases opposite guanine.

## Introduction

Non-radioactive DNA labelling systems are gaining acceptance because their sensitivity is comparable to that of radioactive labelling without the problems associated with the handling of radioisotopes.[1] In most of the systems developed recently, the reporter group is incorporated by an enzyme. In these procedures nucleoside triphosphates, modified with non-radioactive labels such as fluorescein, biotin, or digoxigenin, are used instead of, or in combination with, their non-modified counterparts.[1]

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[†] This paper is dedicated to the memory of Professor A. Krayevsky

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Oligonucleotides[2] and peptide nucleic acids[3] labelled with peptide sequences bind to their complementary sequences and are recognized by an antibody raised against the peptide sequence. The great diversity of antibodies raised against peptide sequences and the many possibilities of labelling such antibodies lead us to consider the potential of nucleic acids-peptide conjugates as alternative non-radioactive labelling systems. Here we describe a general method for the preparation of a nucleoside triphosphate carrying peptide sequences. In particular, we describe the attachment of the c-*myc* tag-sequence at position 4 of cytosine. The resulting chimera is accepted by several DNA polymerases as substrate.

## Results and discussion



*Synthesis of nucleoside triphosphates.*

In order to incorporate a peptide sequence into a 2'-deoxynucleoside triphosphate, a cytosine derivative carrying a mercaptoethyl group was selected (Chart 1). As described previously, oligonucleotides carrying a mercaptoethyl group reacted with peptides carrying a maleimido group to give oligonucleotide-peptide conjugates in good yields.[2] Because of the reactivity of thiol groups in the conditions of oligonucleotide synthesis, the mercaptoethyl group was protected with the *tert*-butylthio group. The preparation of the nucleoside triphosphates is illustrated in Chart 1. The starting nucleosides 4-(*p*-nitrophenoxy)-1-(β-D-2-deoxyribofuranosyl)-1H-pyrimidin-2-one and 2´-deoxy-$N^4$-(*tert*-butyldithioethyl)-cytidine were prepared as described.[2,4] In both cases, the triphosphate was introduced by 5' phosphorylation with phosphoryl chloride in trimethyl phosphate[5] followed by the addition of tributylammonium pyrophosphate. The nucleoside triphosphates **1** and **2** were purified by DEAE-Sephadex A-25 chromatography, in 40% yield. Purified nucleoside triphosphates were shown to be homogeneous by reversed phase HPLC and had the expected molecular weight. Conversion of nucleoside triphosphate **1** to triphosphate **2** was attempted but the reaction was slow and accompanied by hydrolysis of triphosphate group (data not shown). For these reasons, phosphorylation of the 2´-deoxy-$N^4$-(*tert*-butyldithioethyl)-cytidine is recommended.

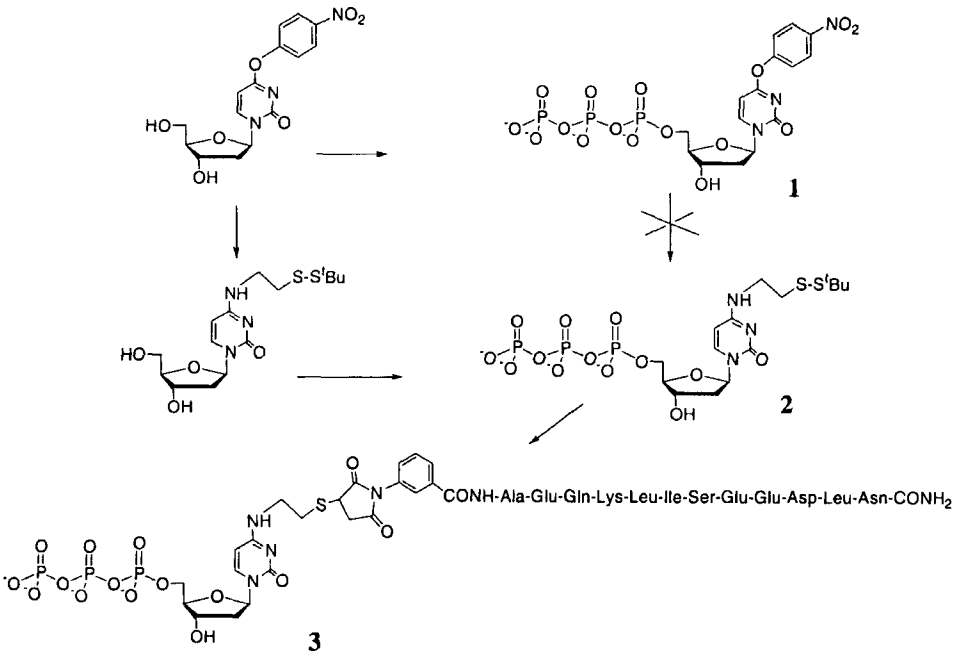


CHART 1. Preparation of nucleoside triphosphates **1**-**3**.



Nucleoside triphosphate **2** was treated with DTT to remove the *tert*-butylthio group. The reaction at neutral pH and room temperature was slower than in oligonucleotide deprotection conditions (conc. $NH_3$, 55 °C), but the formation of a single product with the expected molecular weight was observed. The unprotected nucleoside triphosphate was easily separated from the unreacted compound **2** and the excess of DTT by HPLC. The removal of the *tert*-butylthio group was judged to be near 60% after 3 hours. No attempts were made to change the pH or increase the temperature to increase the yield due to the lability of the triphosphate group. The desired nucleoside triphosphate carrying the free mercapto group was immediately mixed with a solution of maleimido peptide prepared as described.[2] To prevent side reactions between amino acids side chains and the maleimido group[6], the maleimido peptide was cleaved from the support just before use. Conjugation of maleimido-peptide with the thiol containing nucleoside triphosphate was performed at pH 6.5, for 3 hours at room temperature. One major product was obtained that was isolated by reversed-phase HPLC. The nucleoside triphosphate **3** carrying the peptide was characterized by mass spectrometry.

*Primer extension experiments.*

The incorporation of modified nucleoside triphosphates **1-3** by DNA polymerases, was analysed with one fluorescence-labelled primer and two different templates (Table 1).

Figure 1 shows the insertion of nucleoside triphosphates **1-3** by several DNA polymerises, including non-thermostable polymerases (Klenow fragment and T7 Sequenase version 2.0$^{TM}$) and thermostable polymerases (Vent$_R$$^{TM}$ (exo -), Tth, Taq).

Primer extension reactions with dTTP and nucleoside triphosphate **1** were performed with template M22 to study the incorporation opposite A, whereas reactions with dCTP and nucleoside triphosphates **2** and **3** were performed with template M22C to analyze incorporation opposite G.

Nucleoside triphosphate **1** with the *p*-nitrophenyl group at position 4 was not incorporated by Taq and Klenow, and the primer was degraded (lane 2). Sequenase and Tth incorporate one unit (lane 3) and Vent incorporated more than one of these units (lane 2).

Nucleoside triphosphate carrying the *t*-butyldithioethyl group (**2**) was incorporated by Sequenase (lane 5), Tth (lane 5), Taq (lane 4) and Vent (lane 4). Again Vent was able to incorporate more than one unit, and the primer was degraded by Klenow (lane 5).

Nucleoside triphosphate carrying the c-*myc* peptide (**3**) was incorporated by Sequenase (lane 6) and Vent (lane 5) as shown by the strong band with very low mobility. With Vent, a second faint band with a mobility equal to a 42-base oligonucleotide was observed (see arrow) indicating the incorporation of a second unit of the peptide-nucleotide chimera. Taq gave a weaker band (lane 5) whereas Tth and Klenow showed no sign of incorporation (lanes 6).

dCTP was more efficiently incorporated than nucleoside triphosphate **3** and nucleoside triphosphate **2** opposite G. The lower efficiency of incorporation of modified dCTP derivatives may be due to steric hindrance, since the modification is in a position involved in base pairing, but also the use of a short linker may play a role. Further adjustments, such as longer linkers and an alternative position of the modification, may improve the efficiency of incorporation of these compounds.

Table1: Sequence of primer and templates used.

| | | |
|---|---|---|
| Primer | M18 | 5'Fluorescein-CAG CGG GAG CAC GAA TAA |
| Templates | M22 | 3'GTC GCC CTC GTG CTT ATT AAAA |
| | M22C | 3'GTC GCC CTC GTG CTT ATT GGGG |

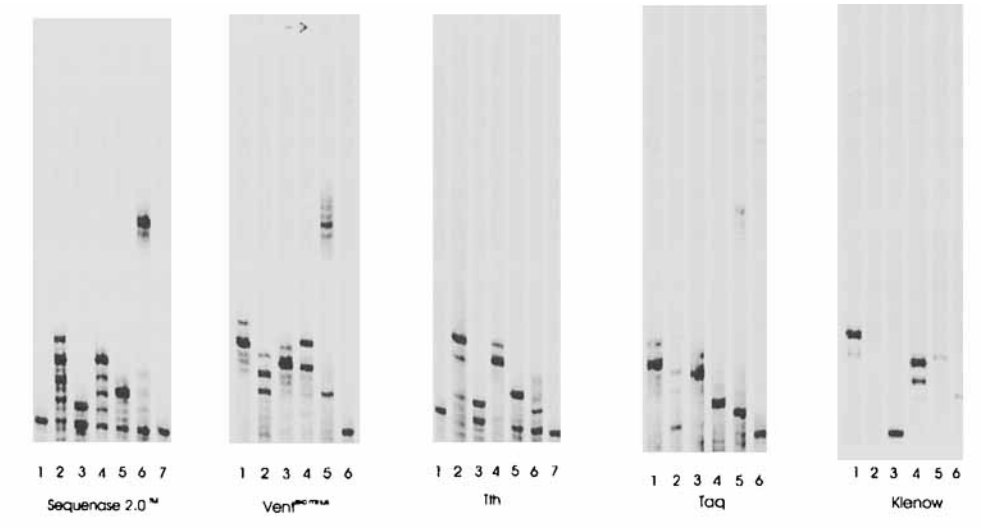


FIGURE 1. Gel data for the insertion of modified nucleoside triphosphates (at 5 $\mu$M concentration) by several DNA polymerases. The sequences of templates and primer are shown in Table 1. The reaction conditions are reported in detail in the Experimental section. Primer extension reactions with dTTP and nucleoside triphosphate **1** were performed with template M22 and primer M18, whereas reactions with dCTP and nucleoside triphosphates **2** and **3** were performed with template M22C and primer M18. The arrow shows a faint band with a mobility equal to a 42-bases oligonucleotide observed in the primer extension reaction performed with Vent polymerase. The products present in each lane are detailed in the following table.

| Enzyme | Lane 1 | Lane 2 | Lane 3 | Lane 4 | Lane 5 | Lane 6 | Lane7 |
|---|---|---|---|---|---|---|---|
| T7 Seq. | M18 | dTTP | **1** | dCTP | **2** | **3** | M18 |
| Vent | dTTP | **1** | dCTP | **2** | **3** | M18+ladd | |
| Tth | M18 | dTTP | **1** | dCTP | **2** | **3** | M18 |
| Taq | dTTP | **1** | dCTP | **2** | **3** | M18+ladd | |
| Klenow | dTTP | **1** | M18 | dCTP | **2** | **3** | |

M18+ladd: M18 primer mixed with a single base ladder starting at 35 bases

In summary, we describe the preparation of a dCTP derivative carrying a peptide sequence linked through position 4. In spite of the use of a short linker, this compound is accepted as dNTP substrate by Sequenase and Vent DNA polymerases and is incorporated opposite to G. These results open the possibility to use peptide-nucleoside triphosphate chimeras for the enzymatic incorporation of peptide sequences into DNA. The resulting DNA-peptide chimeras may have important uses as non-radioactive probes and other applications such as gene delivery.[7]

## Experimental section

Abbreviations used: DMF, N-N-dimethylformamide; DTT, dithiothreitol; OD, optical density units at 260 nm; TEAA, triethylammonium acetate, TEAB, triethylammonium bicarbonate, TFA, trifluoroacetic acid. HPLC was performed on a Waters chromatograph. The products were purified by using a PRP-1 (Hamilton, 305 x 7 cm) column. Buffer A was 5% acetonitrile in 0.1 M aqueous TEAA pH 6.5. Buffer B was 70% acetonitrile in aqueous TEAA (pH 6.5). A 30 min gradient from 0 to 60% B was used.



### 4-(*p*-Nitrophenoxy)-1-(β-D-2-deoxyribofuranosyl)-1H-pyrimidin-2-one 5'-triphosphate (1).

4-(*p*-Nitrophenoxy)-1-(β-D-2-deoxyribofuranosyl)pyrimidin-2(1H)-one[2,4] (70 mg, 0.2 mmol) was dissolved in 0.5 ml of trimethyl phosphate, and the mixture was cooled on an ice-bath. $POCl_3$ (25 ml, 0.26 mmol) was added, and the mixture was stirred for 90 min at 4 °C . A solution of tributylammonium pyrophosphate (452 mg, 1 mmol) in 2 ml of DMF was added to the mixture together with 0.2 ml of tributylamine. After 5 min of stirring, 5 ml of a 1M TEAB aqueous solution was added and the mixture was concentrated to dryness. The resulting triphosphate was purified on a DEAE-Sephadex A-25 column (6 x 1.4 cm) with a 0.05 -0.8 M TEAB gradient. The desired triphosphate eluted at approx. 0.4 M TEAB. Yield: 660 OD units (0.076 mmol, 38%). HPLC : retention time 18 min (<95%). UV (water) λmax 280 nm. Mass spectra (electrospray) : 588.9 (expected for $C_{15}H_{18}N_3O_{16}P_3$ 589.2)

**2´-Deoxy-$N^4$-(*tert*-butyldithioethyl)-cytidine 5'-triphosphate (2).**

The triphosphate of 2´-deoxy-$N^4$-(*tert*-butyldithioethyl)-cytidine[2] (72 mg, 0.2 mmol) was prepared as described above. The resulting triphosphate was purified on a DEAE-Sephadex A-25 column (6 x 1.4 cm) with a 0.05 -0.8 M TEAB gradient. Compound **2** eluted at approx. 0.4 M TEAB. Yield: 616 OD units (0.080 mmol, 40%). HPLC : retention time 20 min (<95%). UV (water) λmax 266 nm. Mass spectra (electrospray) : 615.0 (expected for $C_{15}H_{28}N_3O_{13}S_2P_3$ 615.4)

**2´-Deoxy-$N^4$-($NH_2$CO-Asn-Leu-Asp-Glu-Glu-Ser-Ile-Leu-Lys-Gln-Glu-Ala-benzoyl-3-maleimidyl-thioethyl)-cytidine 5'-triphosphate (3).**



2´-Deoxy-$N^4$-(*tert*-butyldithioethyl)-cytidine 5'-triphosphate (30 OD units) was dissolved in 0.3 ml of 50 mM TEAA (pH 6.5) and 0.1 ml of a 1M DTT solution were added. The reaction was followed by reversed phase HPLC. Retention time of the starting material **2** was 20 min and the desired unprotected nucleoside triphosphate was 13.5 min (electrospray MS: 548 (M+Na) expected for $C_{11}H_{19}N_3O_{13}SP_3$ 526.1). After 3 hours at room temperature (60% conversion by HPLC), the reaction mixture was injected in the HPLC column and the products were separated obtaining 12 OD units of the nucleoside triphosphate carrying the unprotected thiol and 10 OD units of the starting material.

The HPLC fraction containing the nucleoside triphosphate wtih a free thiol function was mixed with a solution of the c-*myc* peptide carrying a maleimido group prepared as follows: 4 mg of peptide-support prepared as described[2] was treated with 1 ml of 95% TFA in water for 2 hours at room temperature. The resulting mixture was filtered and concentrated to dryness. The residue was dissolved in 1 ml of 0.1M TEAA (pH 6.5) and the pH was adjusted to pH 6.5.

The mixture of nucleoside triphosphate with the free thiol and the maleimido-peptide was incubated at room temperature for 3 hours and the analysis by reversed phase HPLC show the presence of a single new product that was purified by HPLC. Yield: 6 OD units (20%). HPLC : retention time 16.2 min (<95%). UV (water) λmax 266 nm. Mass spectra (electrospray): 2112.7 (expected for $C_{80}H_{123}N_{21}O_{38}SP_3$ 2111.6)

**Primer extension experiments.**

Two DNA template oligomers, 22 nucleotides long, were prepared using standard solid-phase phosphite-triester protocols : M22 3'GTC GCC CTC GTG CTT ATT AAA A 5' and M22C 3'GTC GCC CTC GTG CTT ATT GGG G 5'. Both templates contain the 18-nucleotide sequence complementary to the following fluorescence-labelled primer : M18 5' Fluorescein-CAG CGG GAG CAC GAA TAA 3'.

Non-thermostable polymerases.

*Klenow fragment.* Prior to the polymerase reaction, primer and template were annealed as follows: 2 µL (2 pmol) of template (M22 or M22C) solution, 1 µl (1 pmol) of primer (M18) solution and 2 µl of 10x Filling-In buffer (Amersham Life Sciences, USA) were mixed, two drops of mineral oil were added and the mixture was rapidly heated to 95°C and allowed to cool slowly to room temperature (2 hours). To the solution, 13 µl of water, 1µl of 0.1 mM solution of the corresponding nucleoside triphosphate and 1 µl (5 U) of DNA polymerase (Klenow fragment, Amersham) solution were added. Reactions were incubated at 36°C for 15 min and terminated by the addition of 3 µl of 0.5 M EDTA followed by heating to 95°C for 2 min. Reaction samples were diluted with the addition of 1 ml of water. Aliquots of 20 µl were taken, concentrated to dryness and analysed on 14% denaturing polyacrylamide gel on an ABI 373A DNA sequencer.

*T7 Sequenase version 2.0*$^{TM}$: Enzyme, reaction and dilution-buffer were from Amersham Life Science, USA. Prior to reaction, the enzyme was brought to a concentration of 1.25 U/µl with dilution buffer. 2 µl (2pmol) template, 1 µl (1pmol) primer and 4 µl reaction buffer (5x) were mixed, 2 drops of mineral oil were added, the mixture was rapidly heated to 95°C and allowed to cool to room temperature within 2 h. Water (9µl), the appropriate dNTP (0.1 mM, 1 µl) and enzyme (4µl, 5U) were added, and the mixture was incubated at 36°C for 30 min. The reaction was stopped by addition of 3 µl of 0.5 M EDTA and heating to 95°C for 2 min.

*Unmodified T7 polymerase* under comparable conditions resulted in degradation of the primer (data not shown).

Thermostable polymerases

*Vent*$_R^{TM}$*(exo-):* Enzyme and buffer were from New England Biolabs Inc. 2 $\mu$l (2pmol) template, 1 $\mu$l (1pmol) primer and 2 $\mu$l reaction buffer (10x ThermoPol reaction buffer) were mixed, 2 drops of mineral oil were added, the mixture was rapidly heated to 95°C and allowed to cool to room temperature within 2 h. Water (10$\mu$l), the appropriate dNTP (0.1 mM, 1 $\mu$l), 25mM $MgCl_2$ (1.5 $\mu$l ) and enzyme (2.5 $\mu$l, 5 U ) were added and the mixture was incubated at 70°C for 45 min. The reaction was stopped by addition of 3 $\mu$l of 0.5 M EDTA and kept on ice.

*Tth.* Enzyme and buffer were from Promega, USA. 2 $\mu$l (2pmol) template, 1 $\mu$l (1pmol) primer and 2 $\mu$l reaction buffer (10x Chelate Buffer) were mixed, 2 drops of mineral oil were added, the mixture was rapidly heated to 95°C and allowed to cool to room temperature within 2 h. Water (11$\mu$l), the appropriate dNTP (0.1 mM, 1 $\mu$l), 25mM $MgCl_2$ (2 $\mu$l ) and enzyme (1 $\mu$l, 5 U ) were added and the mixture was incubated at 70°C for 45 min. The reaction was stopped by addition of 3 $\mu$l of 0.5 M EDTA and kept on ice.

*Taq.* Enzyme and buffer were from Promega, USA. 2 $\mu$l (2pmol) template, 1 $\mu$l (1pmol) primer and 2 $\mu$l reaction buffer ( 10x Taq DNA polymerase buffer containing 15 mM $MgCl_2$) were mixed, 2 drops of mineral oil were added, the mixture was rapidly heated to 95°C and allowed to cool to room temperature within 2 h. Water (13$\mu$l), the appropriate dNTP (0.1 mM, 1$\mu$l) and enzyme (1$\mu$l, 5U) were added and the mixture was incubated at 70°C for 45 min. The reaction was stopped by addition of 3 $\mu$l of 0.5 M EDTA and kept on ice.

All samples were diluted to 1ml with water, 20 $\mu$l were evaporated to dryness and analysed on a 14% denaturing PAGE gel on the 373A ABI DNA-Sequencer.

Screen shots of the gel images produced by the DNA-Sequencer were taken, red, green and blue canals were separated, the blue canal image was converted to grey-scale and inverted.

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