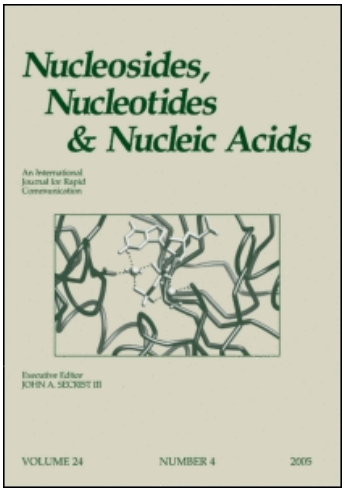

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The Synthesis of 5'-O-Triphosphate-4N-(ω-aminoalkyl)deoxycytidine A Useful Precursor to the Generation of Differently Labeled Triphosphates

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THE SYNTHESIS OF 5'-O-TRIPHOSPHATE-4N-(ω-AMINOALKYL)DEOXYCYTIDINE A USEFUL PRECURSOR TO THE GENERATION OF DIFFERENTLY LABELED TRIPHOSPHATES.

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ABSTRACT

A chemical synthesis is described for 5'-O-triphosphate-4N-[6-(γ-aminopropylamidosuccinylamido)-hexyl]deoxycytidine (13), the substrate for synthesis of 5'-O-triphosphate deoxycytidine derivatives (17-19) labeled with biotin, fluorescein and photoreactive azide.

INTRODUCTION

Non-radioactive labeled nucleotides and oligonucleotides have many potential applications in biochemistry and related fields. For example, they are useful as: (i) hybridization probes for diagnostics[1], (ii) substrates for nucleic acid sequencing[2-5], and (iii) substrates for investigating nucleic acid - nucleic acid or nucleic acid - protein interactions[6-9]. Among the most attractive non-radioactive markers are: biotin[10,11], fluorescent dyes[10], photo-crosslinking reagents[12] and enzymes[13,14]. Labeled oligonucleotides can be synthesized by enzymatic or chemical methods. Chemical methods typically provide oligomers with a functional group easily modified with a marker. Enzymatic methods require a labeled and biological active 5'-O-triphosphate as substrate for biosynthesis of the labeled oligomer[15-17].

This paper reports the complete chemical synthesis of a deoxycytidine triphosphate derivative (13) that can be subsequently modified with a labelling reagent and enzymatically incorporated into a polynucleo-

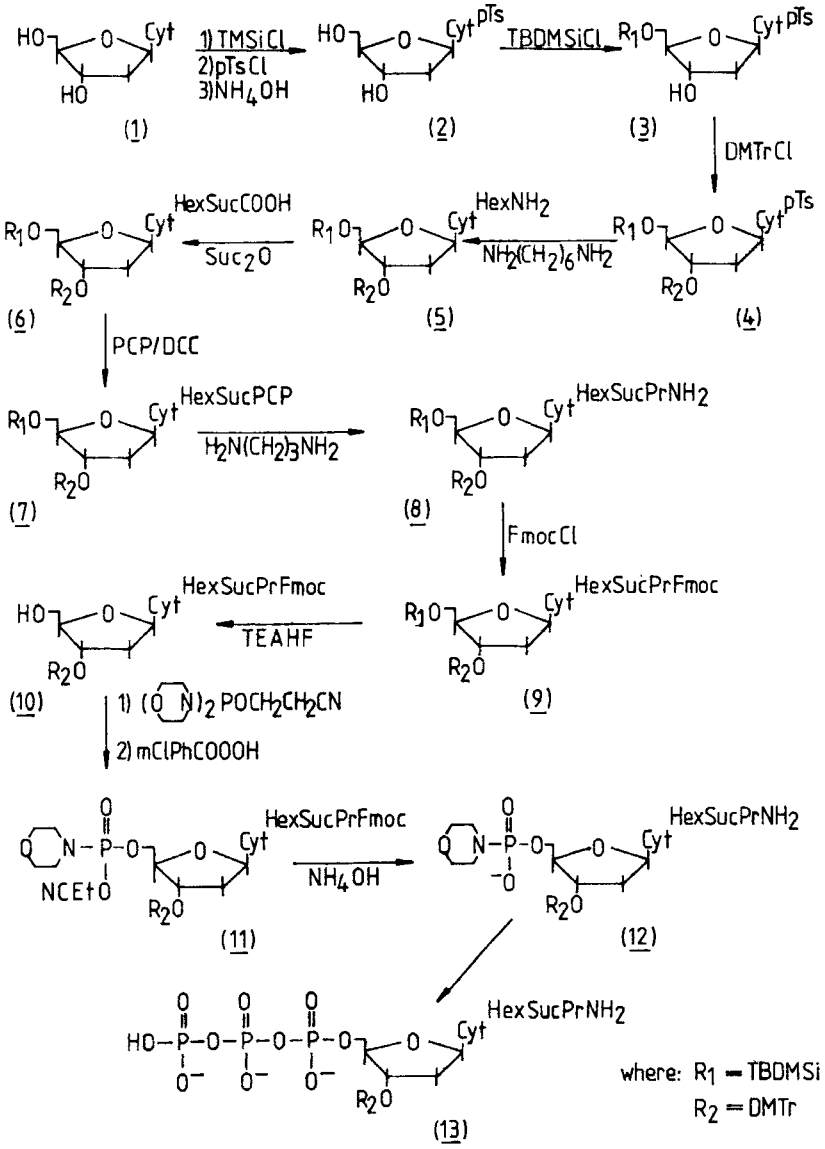


Figure 1: Synthesis of 5'-O-triphosphate-4N-[6-(γ-aminopropylamido-succinylamido)-hexyl]-3'-O-dimethoxytrityldeoxycytidine (13).

tide. It is shown that the derivative (13) can be modified with biotin, fluorescein and azide. The latter provides possibilities for photocrosslinking studies.

RESULTS AND DISCUSSION

The main aim of our study was the synthesis of 5'-O-triphosphate deoxycytidine, carrying at the C4 atom of the cytosine ring a long chain able to link various markers (see Figure 1). The structure and strategy of synthesis for derivative (13) was chosen for the following reasons: (1) It is a simple and effective method of synthesizing a 4N-alkylated derivative of cytidine[18]. (2) It provides possibilities for attaching linkers of various lengths. Such variable length linkers have proven useful in several previous applications of labeled oligonucleotides[19-22]. In particular, long spacers have been found optimal in several cases[20-22]. (3) It provides a simple and effective method of purification of final products. (4) There is only a small effect of N-alkylation of the base on the thermodynamic stability of duplexes[23,24]. To simplify the discussion of the synthesis of derivatives (17-19), the results have been collected into 5 segments in which the final compounds are derivatives: (4), (8), (10), (13) and (17-19).

1/ Synthesis of 5'-O-tert-butyldimethylsilyl-3'-O-dimethoxytrityl-4N-(p-toluenesulfonyl)deoxycytidine (4).

The first step is the synthesis of 4N-(p-toluenesulfonyl)deoxycytidine (2) by a "transient protection" procedure, analogous to that proposed by Jones[25]. Deoxycytidine is protected by trimethylsilyl groups, and then substituted at the C4 atom of cytosine by p-tolulenesulfonyl moiety[18]. For complete reaction, the reaction mixture has to be warmed to 60°C for 16 h. The trimethylsilyl protecting groups are almost completely stable during aqueous work-up. Complete cleavage requires treatment with an equivolume mixture of pyridine and concentrated aqueous ammonia for 4 h at room temperature. The stability of the trimethylsilyl protecting groups under alkaline conditions supports our earlier suggestion that trimethylsilyl groups are cleaved by pyridinium chloride in aqueous pyridine treatment[26]. Next, the reaction mixture is treated with tert-butyldimethylsilyl chloride[27] following dimethoxytrityl chloride[28] for protection of 5'- and 3'-hydroxyl groups, respectively. At this stage the reaction mixture is purified by silica gel column chromatography.

2/ Synthesis of 5'-O-tert-butyldimethylsilyl-3'-O-dimethoxytrityl-4N-[6-(γ-aminopropylamidosuccinylamido)-hexyl]deoxycytidine (8).

The aim of the reactions that follow is formation of the variable length linker, and therefore the spacer is constructed from shorter segments. This has two advantages: the synthesis is based on simple and commercially available chemicals, and the size and character of the linker is easily varied. In the case described, segments derived from 1,6-diaminohexane, succinic anhydride and 1,3-diaminopropane are applied. The above set of elements form a 17 atom linker corresponding to a length of approximately 25 Å. The first step in the synthesis cycle of the linker is reaction of 4N-(p-toluenesulfonyl)-derivative (4) with 1,6-diaminohexane[29]. A large excess (10 equivalents) of the diamine is used to prevent formation of significant amounts of side-product i.e. 1,6-N,N'-bis[4N-(5'-O-tert-butyldimethylsilyl-3'-O-dimethoxytrityl) deoxycytidyl]-diaminohexane. This large excess of diamine and dilution of the reaction mixture minimizes formation of this by-product to ca. 5%. The second step consists of reaction with succinic anhydride. In the following reaction the unprotected carboxyl group of (6) is converted into pentachlorophenyl ester (7). The last step for construction of the linker is reaction of derivative (7) with 1,3-diaminopropane. Choice of this diamine and N,N-dimethylformamide as the solvent simplifies work-up after completion of the reaction (b.p. 1,3-diaminopropane 140°C). Dilution of the reaction mixture and dropwise addition of the solution of derivative (7) into the solution of 1,3-diaminopropane prevents formation of N,N'-disubstituted derivatives of 1,3-diaminopropane. Attempts to convert derivative (6) into (8) using N,N'-dicyclohexylcarbodiimide give unsatisfactory results. The crude reaction mixture is purified by silica gel column chromatography[30] with the overall yield for this segment 56.2%.

3/ Synthesis of 3'-O-dimethoxytrityl-4N-[6-(9-fluorenylmethoxycarbonyl-amidopropylamidosuccinylamido)-hexyl]deoxycytidine (10).

This section discusses the synthesis of derivative (10). First, the ω-amino function of the linker is protected with the base labile 9-fluorenylmethoxycarbonyl group[31]. Second, the tert-butyldimethylsilyl group is removed by triethylammonium fluoride in anhydrous pyridine[32]. Under the conditions of desilylation, i.e. triethylammonium fluoride in tetrahydrofuran or in their mixture with dioxane[33], the reaction is

accompanied by up to 20% cleavage of the 9-fluorenylmethoxycarbonyl group. Derivative (10) is purified by silica gel column chromatography in 69% yield.

4/ Synthesis of 5'-O-triphosphate-3'-O-dimethoxytrityl-4N-[6-(9-fluorenylmethoxycarbonylamidopropylamidosuccinylamido)-hexyl] deoxycytidine (13).

This section presents the reaction which forms the 5'-O-triphosphate function[34-36]. A new method is presented for synthesis of the protected amidoester of the phosphorus. The method is based on application of bis-(morpholino)-2-cyanoethoxyphosphine to the phosphitilation derivative (10) following oxidation, which results in the synthesis of amidodiester (11). This type of phosphine, particularly bis-(diisopropylamino)-2-cyanoethoxyphosphine, is used in the chemical synthesis of DNA and RNA fragments[37-39]. Bis-(morpholino)-2-cyanoethoxyphosphine is synthesized by analogy to bis-(diisopropylamino)-2-cyanoethoxyphosphine. The change concerns the second step of synthesis, in which an equimolar mixture of morpholine and triethylamine is used. The change is essential as morpholine hydrochloride is much more acidic than the diisopropylamine one (pK_a morpholine - 8.7, pK_a diisopropylamine - 10.9). It seems sufficiently acidic to activate bis-(morpholino)-2-cyanoethoxyphosphine leading to decomposition. Due to a very high boiling point (calculated as ca. 400°C under normal pressure), crude phosphine (by ^{31}P NMR ca. 60% pure) is used for phosphitilation. In the presence of 1H-tetrazole, phosphitilation is complete in 60 min. Oxidation of the trisubstituted phosphine with m-chloroperbenzoic acid[40] in chloroform and purification by silica gel column chromatography results in derivative (11) in 87% yield. Next, compound (11) is partially deprotected by concentrated aqueous ammonia in pyridine (1:1 v/v, 16 h at room temperature). Deprotection of the 2-cyanoethyl group occurs in ca. 30 min which is much longer than for cleavage of the same protecting group on phosphotriester center[41]. The 9-fluorenylmethoxycarbonyl, however, requires overnight treatment for complete cleavage.

An alternative method for synthesis of amidoester (12) is based on application of chloromorpholinomethoxyphosphine as the phosphitylating reagent to synthesis of derivative of type (11)[42]. Derivative (11) is then treated with a mixture of pyridine and concentrated aqueous ammonia (1:6 v/v, 48 h at 50°C) to give (12) in satisfactory yield.

TABLE 1. ^{1}H NMR and ^{31}P NMR spectra of derivatives (8-13).

	^{1}H NMR (ppm)						
Derivat.	H-1'	H-5	$-OCH_3$[a]	$-CH_2$[b]	$-CH_2$[c]-	$-CH_3$[d]	aromat[e]
(8)	6.02(m)	5.53(d,8Hz)	3.77(s)	2.55(s)	1.20-1.80; 2.80-3.15	0.77(s)	
(9)	5.82(m)	5.50(d,8Hz)	3.76(s)	2.51(s)	1.15-1.70; 3.00-3.45	0.77(s)	7.25-7.80
(10)	5.92(m)	5.69(d,8Hz)	3.73(s)	2.47(s)	1.10-1.75; 2.80-3.15		7.25-7.80
(11)	5.91(m)	5.67(d,8Hz)	3.77(s)	2.52(s)	1.20-1.75; 2.90-3.35		7.25-7.80

	^{31}P NMR		
	α	β	γ
(11)	7.75; 7.46		
(12)	5.17		
(13)	11.48(d,18.5Hz)	21.51(t,18.5Hz)	8.02(d,19.5Hz)

a- dimethoxytrityl group, b- succinic diamide, c- n-hexyl and n-propyl,
d- tert-butyl of silyl group, e- fluorenylmethoxycarbonyl group

TABLE 2. Characteristics of derivatives (13-19).

Derivative	HPLC[a]		R_f[d]	UV-VIS spectra (nm)[e]	
				minimum	maximum
(13) R_2=DMTr	34.3[b]	51.4[c]	0.11		
(13) R_2=H	13.0	19.5	0.03	259	273
(14)	33.6	50.4	0.29		
(17)	17.1	25.5	0.10	250	272
(15)	33.8	50.7	0.40		
(18)	24.5	36.7	0.16	253	270,482
(16)	38.1	57.2	0.36		
(19)	27.5	41.6	0.14	236	265,478

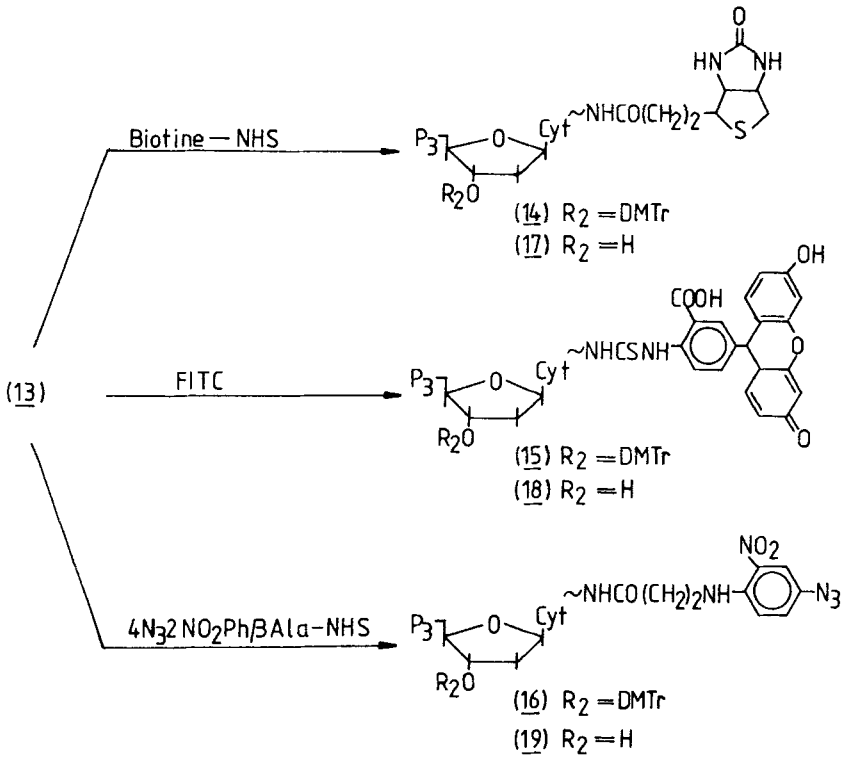
a - buffer A -0.1 M triethylammonium acetate pH=7; buffer B - buffer A/acetonitrile (1:3v/v; gradient -0-100% B in 50 min.; flow rate - 1.0 mL/min. column - C_8 (Aquapore) 250x4.6 mm.
b - retention time in minutes
c - % acetonitrile in the mixture buffers A and B
d - isopropanol/ammonia/water (65:10:25v/v/v)
e - in anhydrous methanol

The last reaction of this set is synthesis of the 5'-O-triphosphate function. Derivative (12) is treated with N,N-dimethylformamide solution of di(tri-n-butylammonium)-pyrophosphate[43]. The reaction is complete in 4 h at 50°C. Longer treatment results in formation of significant amounts of 5'-O-diphosphate. This could be a result of hydrolysis of the final product (13) or attack of the pyrophosphate salt on the γ-phosphorus of derivative (13)[44].

The reaction mixture is worked-up and purified by silica gel column chromatography with yield of 45.6%. The work-up and purification methods described significantly simplify synthesis of triphosphate (13). Some characteristic data, including ^1H and ^{31}P NMR spectra are presented in Tables 1 and 2.

5/ Labeling derivative (13) - Synthesis of derivatives (17-19) and their incorporation into DNA.

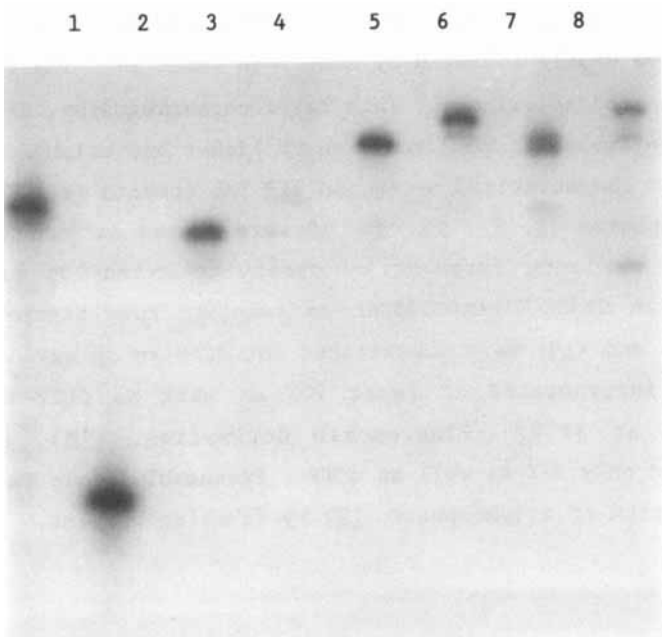
The derivative of 5'-O-triphosphate deoxycytidine (13) above was used as substrate for three different types of labeling: (i) with biotin



where: FITC - fluorescein 5-isothiocyanate, Biotin-NHS - biotin-N-hydroxysuccinimide ester, $4N_32NO_2Ph\beta Ala$-NHS - 4-azide-2-nitrophenyl-β-alanine-N-hydroxysuccimide ester, P_3 - 5'-O-triphosphate function, ~-hexylsuccinylpropyl linker.

Figure 2: The labeling of 5'-O-triphosphate (13)

(by biotin-N-hydroxysuccinimide ester)[45] (ii) with fluorescein (by fluorescein 5-isothiocyanate) and (iii) with a derivative of photoreactive azide (by 4-azide-2-nitrophenyl-β-alanine-N-hydroxysuccinimide ester)[46] (see Figure 2). Both anhydrous and aqueous conditions were tested for labeling. For anhydrous conditions, 0.01 M solution of derivative (13) in a mixture of N,N-dimethylformamide and triethylamine is used to complete the labeling reaction by 2 equivalents of the labeling reagents (30 min at 40°C). However, the solubility of substrate (13) is a problem in this anhydrous approach. This inconvenience is solved with the aqueous condition for labeling. In this case 0.025 M



Lane 1 - 5'phosphorylated template, Lane 2 - 5'phosphorylated primer; in the next lanes primer extension in the presence, Lane 3 - dCTP, Lane 4 - without dCTP, Lane 5 - (13), Lane 6 - (17), Lane 7 - (19), Lane 8 - (18).

Figure 3: PAGE analysis of primer extension with triphosphates (13), (17), (18), and (19).

solution of derivative (13) in a mixture of N,N-dimethylformamide, water and triethylamine (8:1:1 v/v/v), and 2 equivalents of fluorescein 5-isothiocyanate are used to complete the labeling in 4 h at 40°C. For the active esters of biotin and photoreactive azide, 5 equivalents of these reagents are necessary to bring the labeling to completion in 1 h at 40°C. After completion of labeling, the reaction mixture is dried and detritylated with 80% aqueous acetic acie (1 h at room temperature). The reaction mixtures are purified by chromatography on silica gel plates (isopropanol/ammonia/water=7:1:2 v/v/v). The yields for anhydrous and aqueous conditions of labeling are similar. For example, with the aqueous method of labeling, the overall yields determined spectrophotometrically after labeling, detritylation and purification, are 43.6%, 53.9% and 76.1% for derivatives of biotin, azide and

fluorescein, respectively. Detritylation and labeling of derivative (13) could be easily followed by HPLC (see Table 2). The final products (17-19) were characterized by thin layer chromatography, HPLC and UV-VIS spectra (see Table 2). The structure of linker and triphosphate function were earlier characterized by ^{1}H and 31P NMR spectra (see Table 1).

Triphosphates 13, 17, 18, and 19 were tested as substrates for DNA polymerase I Klenow fragment by observing extension of the primer TCGCGCCG with CTAGCCTTGAACGGCGCGA as template (see Figure 3)[47]. When (13), (17), and (19) were substituted for dCTP in an extension mixture, they were incorporated at least 90% as well as dCTP during a 2 h incubation at 37°C. Fluorescein derivative, (18), however, was incorporated only 35% as well as dCTP. Presumably, this is due to grade of modification of triphosphate (18) by labeling reagent.

CONCLUSIONS

The above method of synthesizing a derivative of 5'-O-triphosphate deoxycytidine (13) with a linear linker is attractive for several reasons: (1) simple and commercially available chemicals are used, (2) reactions proceed smoothly and can be easily scaled up, (3) simple and effective methods of purification and analysis are used, (4) a simpler method for the synthesis of phosphormorpholidate (12) is introduced, (5) a different character and length of the linker can be composed, (6) derivative (13) is a universal substrate for different labeling reagents, and (7) triphosphates (13 R_2=H), (17), (18), and (19) are effeciently incorporated into DNA.

EXPERIMENTAL

General part

All solvents used in reactions were purified and dried according to earlier published procedures[33,38]. Thin layer chromatography was performed on Merck F_{254} silica gel (System A and B) and silanized silica RP-2 (Merck) (System C) plates in the following solvents: A-chloroform/methanol (9:1 v/v), B-benzene/acetone (6:4 v/v) and C-acetone/water (7:3 v/v). Short column chromatography was performed on silica gel Merck H60 in chloroform containing methanol or in a mixture of isopropanol/ammonia/water. Spectra were measured on: Carl Zeiss Jena UV-VIS spectrophotometer and Jeol 90 FX Fourier transform NMR instrument for ^{1}H and ^{31}P nuclei (90 and 34.6 MHz respectively) using TMS and 85%

H_3PO_4 as internal and external standards. Di(tri-n-butylammonium)-pyrophosphate was prepared according to the Khorana procedure[43].

The primer (dTCGCGCCG) and the template (dCTAGCCTTGAACGGCGCGA) used in extension experiment were synthesized by phosphoramidite method[42].

Synthesis of bis-(morpholino)-2-cyanoethoxyphosphine.

To a stirred and cooled at 0°C solution of trichlorophosphorous (240 mM, 21.0 mL) in 15 mL of anhydrous acetonitrile, a solution of 2-cyano-ethanol (35 mM, 2.40 mL) in 12 mL of anhydrous acetonitrile was added dropwise over 10 min and stirred for the next 45 min at room temperature. The reaction mixture was evaporated to oil and diluted in 200 mL of dry diethyl ether. A mixture of triethylamine (80 mM, 11.24 mL) and morpholine (80 mM, 6.97 mL) in 100 mL of dry diethyl ether was added dropwise to the solution over 30 min, and left overnight at room temperature. The solid was filtered off and the filtrate was quenched with water. The organic layer was dried over sodium sulfate and evaporated to oil. The phosphine was used as ca. 1 M acetonitrile solution. The yield was ca. 80% and the purity of the phosphine determined by ^{31}P NMR spectroscopy was ca. 60% (^{31}P NMR phosphine -130.85 ppm).

Synthesis of 4-azide-2-nitrophenyl-β-alanine.

To β-alanine (9.29 mM, 0.830 g) dried under vacuum for a few hours, were added 29 mL of anhydrous dimethylsulfoxide and triethylamine (27.3 mM, 3.93 mL) following 4-fluoro-3-nitrophenylazide (9.29 mM, 1.691 g). The mixture was stirred for 42 h at 60-62°C. The reaction mixture was evaporated and dissolved in 180 mL warm ethanol. Then 25 mL of water were added and the mixture was concentrated to the point of crystallization. The mixture was warmed and left in the refrigerator overnight. Crystals were filtered off and dried. The yield for two crystallizations was 77.5% (1.844 g): mp. 141-143°C; IR - 2130 cm^{-1} (νN_3), 1720 cm^{-1} (νCO); ^1H NMR (DMSO-d_6) 8.22 (1H,t,J=5.9Hz,NH), 7.67-7.09 (3H,m, substituted phenyl system) 3.59 (2H,k,J=6.3Hz,βCH_2), 2.62 (2H,t,J=6.6Hz, αCH_2).

Synthesis of 4-azide-2-nitrophenyl-β-alanine-N-hydroxysuccinimide ester.

4-Azide-2-nitrophenyl-β-alanine (0.50 mM, 0.126 g) was co-evaporated with anhydrous dioxane and dissolved in 2.50 mL of anhydrous dioxane.

Then N-hydroxysuccinimide (0.55 mM, 0.062 g) and N,N'-dicyclohexylcarbodiimide (0.06 mM, 0.123 g) were added and stirred at room temperature for 30 min. The precipitate was filtered off and the solution quenched with water and extracted with chloroform (3x5 mL). The combined organic layers were dried and evaporated. The residue was dissolved in 15 mL warm isopropanol, concentrated to the point of crystallization, and left in the refrigerator. Crystals were filtered off, washed with isopropanol and dried. The yield of synthesis was 80.6% (0.141 g): mp. 147-148°C; IR - 2120 cm^{-1} (νN_3), 1740 cm^{-1}, 1790 cm^{-1} and 1830 cm^{-1} (ν CO); ^1H NMR (DMSO-d_6) 8.21 (3H,t,J=5.1Hz,NH), 7.73-7.17 (3H,m,substituted phenyl system), 3.76 (2H,k,J=6.2Hz,βCH_2), 3.09 (2H,t;J=6.8Hz,αCH_2), 2.81 (4H,s,CH_2).

Synthesis of 5'-O-(tert-butyldimethylsilyl)-4N-[6-(aminopropylamido-succinylamido)-hexyl]-3'-O-dimethoxytrityldeoxycytidine (9).

a/ Synthesis of 4N-(p-toluenesulfonyl)deoxycytidine (2).

Cytidine hydrochloride (10 mM, 2.62 g) was evaporated with anhydrous pyridine. Next, trimethylchlorosilane (40 mM, 1.90 mL) was added to a solution of deoxycytidine in 60 mL anhydrous pyridine. After 30 min., when reaction was completed. The mixture was concentrated to about 40 mL and p-toluenesulfonyl chloride (20 mM, 3.80 g) was added and left for 16 h at 60°C. The reaction mixture was filtered off and the solution was quenched with saturated aqueous sodium bicarbonate and extracted with chloroform (3x100 mL), dried with sodium sulfate and evaporated. To the residue, 20 mL of pyridine and 20 mL of concentrated aqueous ammonia were added. Desilylation was complete in 4 h at room temperature. The reaction mixture was evaporated and co-evaporated a few times with anhydrous pyridine. R_f values: 0.35 (A), 0.12 (B) and 0.89 (C).

b/ Synthesis of 5'-O-(tert-butyldimethylsilyl)-4N-(p-toluenesulfonyl) deoxycytidine (3).

The residue was dissolved in 40 mL of anhydrous pyridine and tert-butyldimethylsilyl chloride (12 mM, 1.80 g) was added. After 3-4 h the reaction was complete and saturated aqueous sodium bicarbonate was added and the reaction mixture extracted with chloroform (3x100 mL), dried and evaporated. The residue was co-evaporated with anhydrous pyridine. R_f values: 0.63 (A), 0.56 (B) and 0.64 (C).

c/ Synthesis of 5'-O-(tert-butyldimethylsilyl)-4N-(p-toluenesulfonyl)-3'-O-dimethoxytrityldeoxycytidine (4).

Dimethoxytrityl chloride (12 mM, 4.05 g) was added to a solution of residue in 20 mL of pyridine and left overnight. The reaction mixture was quenched with aqueous sodium bicarbonate, extracted with chloroform (3x100 mL) and evaporated. Traces of pyridine were removed by co-evaporation with toluene. The reaction mixture was partially purified by silica gel column chromatography. The eluant solvent was chloroform/diethyl ether (7:3 v/v). R_f values: 0.97 (A), 0.93 (B) and 0.34 (C).

d/ Synthesis of 5'-O-(tert-butyldimethylsilyl)-4N-(6-aminohexyl)-3'-O-dimethoxytrityldeoxycytidine (5).

1,6-diaminohexane (100 mM, 11.60 g) was added to the solution of (4) in 50 mL of pyridine, and left for 24 h at 70°C. Saturated aqueous sodium bicarbonate was added and extracted with chloroform (3x100 mL). The combined organic layers were washed with water (2x100 mL), dried and evaporated. Traces of pyridine were removed by co-evaporation with toluene. R_f values: 0.00 (A), 0.00 (B) and 0.02 (C).

e/ Synthesis of 5'-O-(tert-butyldimethylsilyl)-4N-[6-pentachlorophenyl-succinylamido)-hexyl]-3'-O-dimethoxytrityldeoxycytidine (7).

The residue was dissolved in 40 mL of chloroform (methanol free) and succinic anhydride (12 mM, 1.20 g) and 4-dimethylaminopyridine (1 mM, 0.126 g) were added. Reaction was complete in 15 min and pentachlorophenol (12 mM, 3.17 g) and N,N'-dicyclohexylcarbodiimide (12 mM, 2.47 g) were added. The reaction was complete in 3-4 h. Solid dicyclohexylurea was filtered off, and the solution worked-up with saturated aqueous sodium bicarbonate and extracted with chloroform (3x100 mL). The combined chloroform layers were washed with 0.5 M aqueous sodium dihydrogen phosphate, dried and evaporated. R_f values of derivatives: (6) 0.19 (A), 0.00 (B), 0.70 (C) and (7) 0.87 (A), 0.24 (B) and 0.21 (C).

f/ Synthesis of 5'-O-(tert-butyldimethylsilyl)-4N-[6-(aminopropylamido-succinylamido)-hexyl]-3'-O-dimethoxytrityldeoxycytidine (8).

Substrate (7) was co-evaporated with anhydrous N,N-dimethylformamide and dissolved in 100 mL of anhydrous N,N-dimethylformamide. To this

stirred solution, a solution of 1,3-diaminopropane (50 mM, 4.15 mL) in 100 mL of anhydrous N,N-dimethylformamide was added dropwise over 15 min. After 15 min, reaction was complete and the mixture was evaporated and then co-evaporated with N,N-dimethylformamide (2x10 mL). The reaction mixture was purified by silica gel column chromatography. The column was eluted with chloroform/methanol (97:3 v/v) and followed by chloroform/methanol (7:3 v/v). Overall yield (for steps a-f) was 5.08 g (56.2%). R_f values: 0.00 (A), 0.00 (B) and 0.02 (C).

Synthesis of 4N-[6-(9-fluorenylmethoxycarbonylamidopropylamidosuccinylamido)-hexyl]-3'-O-dimethoxytrityldeoxycytidine (10).

Derivative (8) (0.50 mM, 0.452 g) was co-evaporated with anhydrous pyridine (2x2 mL) and dissolved in 2.5 mL of anhydrous pyridine and 9-fluorenylmethoxychloroformate (0.55 mM, 0.142 g) was added and stirred for 2 h at room temperature. The mixture was worked-up with saturated aqueous sodium bicarbonate, extracted with chloroform (3x100 mL), dried and evaporated. The residue was co-evaporated with anhydrous pyridine and 5 mL of 0.5 M triethylammonium fluoride in pyridine was added and left for 48 h at room temperature. The mixture was quenched with saturated aqueous sodium bicarbonate and extracted with chloroform (3x50 mL). The organic layers were dried, evaporated and co-evaporated with toluene. The residue was purified by silica gel column chromatography. The column was eluted with chloroform/methanol (96:4 v/v). The total yield for these two reactions was 0.350 g (69.3%). R_f values of derivatives: (9) 0.31 (A), 0.40 (acetone), 0.24 (C) and (10) 0.23 (A), 0.15 (acetone), 0.43 (C).

Synthesis of 4N-[6-(9-fluorenylmethoxycarbonylamidopropylamidosuccinylamido)-hexyl]-5'-O-(2-cyanoethyl,morpholenyl)phosphate-3'-O-dimethoxytrityldeoxycytidine (11).

Substrate (10) (0.306 mM, 0.309 g) and 1H-tetrazole (0.306 mM, 0.022 g) were dried under vacuum for a few hours. Next, 1 mL of anhydrous, freshly distilled dichloromethane was injected through a rubber septum into the reaction mixture followed by 1.84 mL of 1 M bis-(morpholylo)-2-cyanoethoxyphosphine in acetonitrile. The mixture was stirred for 1 h at room temperature. Next, 25 mL of 0.1 M m-chloroperbenzoic acid in chloroform was added. The reaction was complete in 1-2 min and worked-

up with saturated aqueous sodium bicarbonate and extracted with chloroform (3x10 mL). The combined organic layers were dried and evaporated. The residue was purified by silica gel column chromatography using chloroform/methanol (96:4 v/v). The yield was 0.32 g (87.4%). R_f values of derivatives: (derivative P (III)) 0.34 (A), 0.56 (acetone), 0.43 (C) and (11) 0.30 (A), 0.29 (acetone) and 0.38(C).

Synthesis of 4N-[6-(aminopropylamidosuccinylamido)-hexyl]-5'-O-triphosphate-3'-O-dimethoxytrityldeoxycytidine (13).

Derivative (12) (0.267 mM, 0.325 g) was treated with 4 mL of a mixture of pyridine and concentrated aqueous ammonia (1:4 v/v) for 16 h at room temperature. Next, the reaction mixture was evaporated and co-evaporated (2x3 mL) with anhydrous N,N-dimethylformamide. To the residue, 1.5 mL of 1 M di(tri-n-butylammonium)-pyrophosphate in N,N-dimethylformamide was added and co-evaporated (3x3 mL) with toluene. After 4 h at 50°C, 10 mL saturated aqueous sodium bicarbonate and 10 mL pyridine were added to the reaction mixture and evaporated. The residue was co-evaporated with pyridine (2x10 mL) and suspended in 10 mL of anhydrous pyridine. The solid was filtered off and washed with pyridine. The solution was evaporated and co-evaporated with toluene (2x5 mL). The residue was macerated with chloroform (3x20 mL) and dried off. The derivative (13) was purified by silica gel column chromatography. The starting eluent was a mixture of isopropanol/concentrated aqueous ammonia/water (90:5:5 v/v/v). The content of ammonia was the same during the whole separation process. The ratio of water to isopropanol was increased stepwise by 5% per each 50 mL of the solvent mixture. The derivative (13) was eluted by the mixture containing 35% water. The yield 0.163 g, (45.6%). R_f values derivative: (12) 0.00 (A), 0.00 (B), 0.11 (C) and (13) - see Table 2.

The reaction of the labeling derivative (13) - the synthesis derivatives (17-19).

a/ anhydrous condition

Biotin-N-hydroxysuccinimide ester ($5.6x10^{-3}$ mM, 2.10 mg) or fluorescein-5-isothiocyanate ($5.6x10^{-3}$ mM, 2.17 mg) or 4-azide-2-nitrophenyl-β-alanine-N-hydroxysuccinimide ester ($5.6x10^{-3}$ mM, 1.97 mg)

were added to the solution of 5'-O-triphosphate (13) (2.8×10^{-3} mM, 3.31 g) in 0.25 mL of a mixture of N,N-dimethylformamide and triethylamine (9:1 v/v) and kept for 30 min at 40°C.

b/ aqueous condition

To a solution of derivative (13) (3.0×10^{-3} mM, 3.45 mg) in 0.132 mL of a mixture of N,N-dimethylformamide, triethylamine, and water (8:1:1 v/v/v), biotin-N-hydroxysuccinimide ester (15×10^{-3} mM, 5.60 mg) or fluorescein-5-isothiocyanate (6×10^{-3} mM, 2.33 mg) or 4-azide-2-nitrophenyl-β-alanine-N-hydroxysuccinimide ester (15×10^{-3} mM, 5.20 mg) were added and kept at 40°C for 4 h for fluorescein derivative and 1 h in the case of biotin and azide derivatives.

The next part of the procedure was identical for both conditions. The reaction mixture was dried and treated with 80% aqueous acetic acid for 1 h at room temperature. The reaction was neutralized to pH=6-7 with dilute aqueous ammonia, dried down again, and separated on silica gel plates in isopropanol/ammonia/water (65:10:25 v/v/v). The proper spots were scrapped off and the silica gel was washed with methanol/water (1:1 v/v). Overall yields determined spectrophotometrically for the anhydrous condition were 43.6%, 53.9% and 76.1%, respectively, for the derivatives of biotin, azide and fluorescein. Similar yields were found for the aqueous conditions. The characteristics of final products (17-19) are collected in Table 2.

Extension of DNA primer.

The primer dTCGCGCCG (750 pM) and the template dCTAGCCTTGAACGGCGCGA (750 pM) in 75 μL of buffer containing 50 mM Tris•HCl pH=7.5, 50 mM NaCl and 10 mM $MgCl_2$ were heated to 90°C for 2 min, transferred to a 70°C heating block and cooled to room temperature over 1 h. The primer-template solution was aliquated into 2 μL fractions to which were added 2 μL each of dGTP and dTTP at 0.5 mM, 2 μL of 0.05 mM dATP, 2 μL of 1.7 μM [α-^{32}P] dATP, and 2 μL of 0.5 mM dCTP or analoges (13), (17), (18), or (19). The total volume was increased to 19 μL by adding Klenow buffer, and reaction was started by adding 1 μL (5U) of Klenow fragment (New England Biolabs). Reactions were incubated for 2 h at 37°C, stopped by adding 20 μL of formamide loading buffer and run on a 40 cm, 20% polyacrylamide gel.

Abbreviations:

TMSiCl - trimethylsilyl chloride, p-TsCl - p-toluenesulfonyl chloride, TBDMSiCl - tert-butyldimethylsilyl chloride, DMTrCl - 4,4'-dimethoxytrityl chloride, Suc_2O-succinic anhydride, PCP - pentachlorophenol, DCC - N,N'-dicyclohexylcarbodiimide, FmocCl - 9 fluorenylmethoxychloroformate, TEAHF - triethylammonium fluoride.

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