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# Synthesis and use of labelled nucleoside phosphoramidite building blocks bearing a reporter group: biotinyl, dinitrophenyl, pyrenyl and dansyl

A.Roget[1], H.Bazin[1] and R.Teoule[1,2]

[1]CIS Bioindustries, BP 6, F-91192 Gif-sur-Yvette and [2]Laboratoires de Chimie, Departement de Recherche Fondamentale, Centre d'Etudes Nucléaires de Grenoble, 85 X, F-38041 Grenoble Cédex, France

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## ABSTRACT

The synthesis of protected nucleoside phosphoramidites bearing various markers such as biotinyl, dinitrophenyl, dansyl and pyrenyl groups are reported. These labelled deoxynucleosides phosphoramidites were used for solid phase oligonucleotide synthesis in the same way than the usual protected phosphoramidites without any change in the synthetic cycle and the deprotection step. The new labelled building blocks described herein have been used in conjunction with the labile base protected phosphoramidites ('PAC phosphoramidites') which allowed mild ammonia deprotection, especially recommended for the dinitrophenyl-labelled oligonucleotides. Multiple labelling (i.e. 10 to 20 biotins) can be efficiently and easily performed, on the same oligonucleotide which results in an increase of sensitivity. The polylabelled oligonucleotides are chemically well defined and gave increased signal and low background coloration for *in situ* hybridisation.The modified oligonucleotides can still be kinased in the normal way as the reporter groups are on the heterocycles.

## INTRODUCTION

Non radioactive labelling of oligonucleotides and DNA offers important potential advances to molecular biology methods, medical diagnostics and DNA sequencing.

In recent years several systems have emerged for preparing these probes. Biotin, one of the most prominent markers has been incorporated by nick-translation using DNA polymerase and biotinylated nucleoside triphosphate (1). DNA was also tagged with dinitrophenyl dATP derivatives (2). The labelling of oligodeoxynucleotides with biotin was achieved by primer extension reaction using E. coli DNA polymerase (3). Biotinylated (4) or fluorescent (4, 5) RNA were prepared by $T_4$ RNA ligase. Another technique uses the terminal deoxynucleotidyl transferase and tailing at the 3'-end of a nucleic acid with either a biotinylated nucleotide (6) or thiouridine and post-labelling with $\alpha$-haloacetamido derivatives of chemical labels (7) or a nucleotide bearing an amino group which is tagged in a second step with different reporter groups (8). DNA or RNA were also labelled by a photoactivable analog of biotin (9) or by using the bisulfite catalyzed transamination of cytidine to prepare biotinyl (10) or fluorescent (11) probes.

None of these methods can be used to prepare well defined oligonucleotide probes and especially the 5'-labelled one. The chemical labelling of the 5'-end of an oligonucleotide was performed by creating a carbamate linkage (12) or by synthesis of a 5'-phosphorimidazolyl oligonucleotide (13, 14, 15) and in a subsequent step introduction of the reporter group. The use of phosphonate (16) or phosphoramidite (17, 18) derivatives of a linear aliphatic amine or a cyclic phosphoramide is also described. A 5'-N-protected 5'-amino-5'-deoxythymidine phosphoramidite was used to introduce different fluorescent labels (20).

By these methods only one reporter group is incorporated at the 5'-end of an oligonucleotide.

The use of phosphoramidites bearing a N-protected aminoalkyl group either at C-5 position (21, 22, 23) or C-4 position (24) of a pyrimidine or at C-8 position of deoxyadenosine (25) was also investigated. All these derivatives require an additional step to introduce the reporter group. These derivatives were also used to prepare enzyme linked oligonucleotides (26, 27) which gives a simplified hybridization procedure but increases the revelation time.

The guideline of our research was to develop new products which can be successfully and routinely used to prepare mono or polylabelled oligonucleotides directly on an automatic synthesizer.

The general strategy involves the preparation of labelled deoxynucleoside phosphoramidites. These building blocks are covalently attached to the growing synthetic oligonucleotide by the same procedure as the classical synthesis.

## RESULTS AND DISCUSSION

*Synthesis of the 2'-deoxycytidine building block :*

The literature survey showed three main methods for the preparation of 4-N-aminoalkyl-2'-deoxy-cytidine itself or its 5-C methyl analogue:

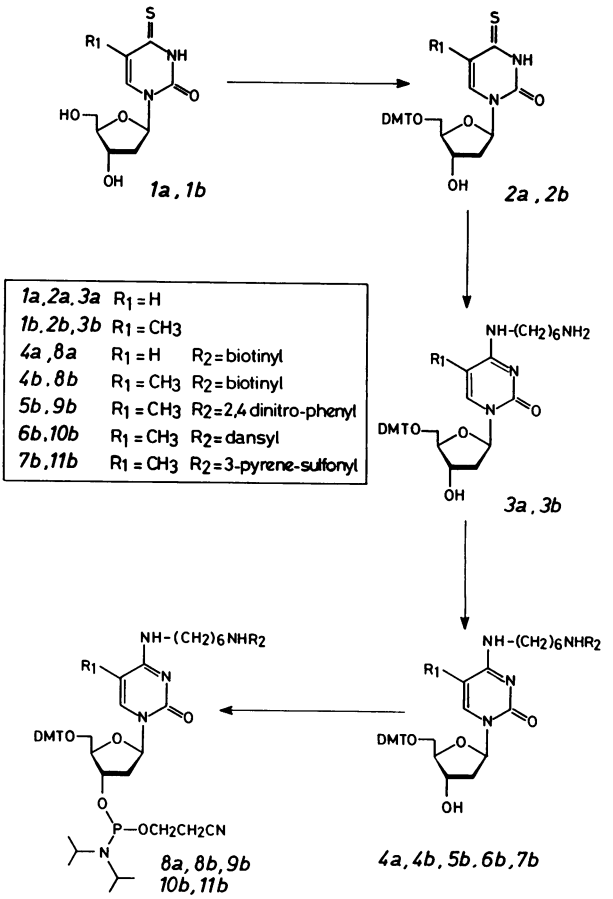
a) Introduction of a leaving group, triazolyl (28), tetrazolyl (29), nitrotriazolyl (30) through the reaction of a phosphorochloridate (28, 29) an arenesulfonyl chloride (31) or phosphoryl chloride (32) on deoxyuridine or thymidine. The intermediate can be either treated with the diamine or converted to a 4-O-nitrophenyl derivative (33, 34) which is subsequently treated with the diamine.

b) The bisulfite catalysed transamination (35, 36) or displacement on 4-N-tosyl-deoxycytidine derivative (34).

c) The displacement of sulphur from 4-thio derivatives with a diamine (38, 39).

The first method gives a reported overall yield (from nucleoside to N-protected form of *3a* or *3b*) ranging from 20 to 40%, the second method is rather attractive (starting from deoxycytidine) and often used in conjunction with silyl protecting groups gives c.a 35% overall yield.

In this work we selected a method (outlined in the enclosed scheme) which has the advantage to use readily accessible reagents. No stringent dry conditions are needed and the method is easy to scale-up. Crystalline 3',5'-di-O-acetyl-2'-deoxyuridine (or thymidine) was converted to its corresponding 4-thio derivative in 81% yield using phosphorus pentasulfide in dioxane (38) followed by deprotection with ammonia in methanol at room temperature to give *1a* or *1b* in almost quantitative yield. Then *1a* and *1b* were treated with dimethoxytrityl chloride to give *2a* and *2b* in 86 and 77% yield respectively. The thio derivatives *2a* and *2b* were treated with 4 to 5 molar excess of diamine in ethanolic solution at 60°C overnight to give respectively *3a* and *3b* in 85% yield. The 4-N-aminoalkyl-deoxycytidine derivatives *3a* and *3b* were reacted with biotin-N-hydroxysuccinimide ester in dimethylformamide to give *4a* and *4b* in 75 and 79% yield respectively. The phosphitylation (40, 41) of *4a* and *4b* gave rise to the biotinylated phosphoramidites *8a* and *8b* in 85 and 87% yield respectively after precipitation from cold hexan. The reaction of 4-N-aminoalkyl-deoxycytidine derivative *3b* with 1-fluoro-2,4-dinitrobenzene, dansyl chloride and pyrenesulfonyl chloride gave the labelled compounds *5b*, *6b* and *7b* in 79, 82 and 73% yield respectively. The phosphitylation (40, 41) followed by flash




chromatography and precipitation from hexan offered the labelled phosphoramidites *9b*, *10b* and *11b* in 91, 91 and 85% yield respectively. The structure and purity of all compounds were assigned on the basis of proton and phosphorus NMR , mass spectrum analysis (FAB or EI) and UV spectra.

*Oligonucleotide synthesis*

A series of tagged oligonucleotides were prepared using the new phosphoramidites described above. High coupling yield (>97%) of these labelled building blocks were achieved using 0.5 M tetrazole in acetonitrile on an automatic synthesizer with a coupling time of 1 minute, the concentration of the modified amidites being in the range of 0.1 to 0.2 M in acetonitrile solution. The crude oligonucleotides from ammonia deprotection were either purified on reversed phase HPLC or PAGE after removal of the DMT group.

Phosphoramidites with labile base protection :'PAC amidites' (42) were used for the synthesis of probes bearing the DNP group especially in the preparation of penta-dinitrophenylated probes.

*Stability of the labelling*

The stability of the linkage between the aminoalkyl chain and the reporter moiety was

investigated under the condition required for the final deprotection step of a synthetic oligonucleotide (50−60°C, 28% ammonia, 16 H). The labelled free nucleosides obtained by removal of the dimethoxytrityl group from *4b*, *5b* and *6b* were used as simple model compounds.

The dinitrophenyl derivative displayed the following physical data λ max = 364, 270 nm, $R_t$ = 66′. The nucleoside was treated with 28% ammonia at 55°C for 16 H.

After removal of the ammonia, the HPLC analysis was carried out using the same condition as above. The analysis showed two new compounds which co-migrated respectively with 2,4-dinitrophenol ($R_t$ = 45′, λ max = 359,256 nm) and 4-N-(6-aminohexyl)-2′-deoxycytidine ($R_t$ = 22′). Integration of the peak showed c.a. 30% removal of the dinitrophenyl group. These compounds were isolated and displayed UV consistent with the assigned structure.

The same type of experiment was run on the free nucleoside obtained by removing the dimethoxytrityl group from *4b*, *6b*. The biotinylated nucleoside ($R_t$ = 44.5′) and the dansylated one ($R_t$ = 80.3′) were submitted to ammonia treatment. The HPLC analysis was run using the same condition as above and no degradation was noted.

*Use of the biotinylated probes provided by the phosphoramidite approach*

The phosphoramidites described above were used to produce a great variety of polylabelled oligonucleotides complementary to peptides mRNAs including corticotropin releasing factor, prodynorphin, vasopressin, calcitonin, calcitonin gene related peptide, vasopressin intron I, RD2 receptor, propiocortin, oxytocin, ... These probes were used for mRNAs detection on sections of plastic embedded tissues (44). For example the oligonucleotide probe 45 nucleotides long with a tail of 10 biotins at its 5′ end complementary to the sequence of the vasopressin mRNA corresponding to the first fifteen aminoacids of neurophysin II was prepared. Its efficiency was tested in parallel with the same 45-mers biotinylated at its 3′-end by addition of a tail with biotin-dUTP as described in (44). Both probes were revealed with streptavidine-alkalin phosphatase, then BCIP and NBT. Incubation with the 5′ ten biotins probe obtained by phosphoramidite mediated synthesis provided a much higher signal than with the 3′ probe tailed with biotin-dUTP in staining magnocellular neurones (43). The use of biotinylated probe on semi-thin sections presents many advantages. The tissues can be stored for months embedded in Araldite before treatment without any loss of the signal (43, 44)

## EXPERIMENTAL

All solvents were of analytical grade. Alcohol-free dichloromethane stabilized with 2-methyl-2-butene was used for synthesis. $^1$H-NMR spectra (200 MHz) were recorded on a Brucker AC 200 spectrometer, $^{31}$P-NMR spectra (101 MHz) on a Brucker WM 250. Chemical shift (δ) values are given as positive downfield shift from tetramethysilane (internal standard), or phosphoric acid (external standard). Analytical UV spectra were recorded on a Beckman UV 530 spectrometer, ε quoted to nearest 100 units. Mass spectra were recorded on a KRATOS M 50. Oligonucleotide synthesis was performed on an Applied Biosystems 381 A DNA synthesizer, using the preconized procedure for modified building blocks. Short column chromatography (45) was run on silica gel G 60 (Merck), pyridine or triethylamine (c.a 0.5%) was added to the elution solvent to prevent loss of dimethoxytrityl group. Thin layer chromatography was performed on plastic sheets, silica 60 F (254), 0.2mm layer (Merck) using the following solvent systems:

(A) methanol-chloroform (10:90, V/V)

(B) ammonia-methanol-chloroform (5:15:80, V/V)
(C) triethylamine-ethylacetate-dichloromethane (5:45:50, V/V)
(D) methanol-chloroform (5:95, V/V)

The HPLC analyses were performed on a Varian 5000 using the following condition: (Nucleosil 10 μm, C-18 column, $CH_3CN$ in TEAA 0.05 M, 10% (10'), 10−40% (40'), 1.5 ml/min).

4-Dimethylaminocinnamaldehyde-ethanolic sulfuric acid spray was used to visualize biotin derivatives (46) on TLC. The M 13 SS mp 18 was from Boehringer, nitrocellulose membranes from Schleicher and Schull, streptavidine-alkaline phosphatase conjugate, BCIP (5-Bromo-4-chloro-3-indolyl phosphate) and NBT (p-Nitro-Tetrazolium blue) from B.R.L.

*5'-O-(4,4-Dimethoxytrityl)-4-thio-2'-deoxyuridine* (2a) : The 4-thiodeoxynucleoside *1a* (4.08 g, 16.75 mmoles) was dried by coevaporation with 2×50 ml of dry pyridine. It was dissolved in 150 ml of anhydrous pyridine and chilled. Then 4,4'-dimethoxytrityl chloride (6.23 g, 18.4 mmol) was added and the reaction was kept overnight at 4°C. After completion of the reaction (checked by T.L.C. on silica plates) 1 ml of methanol was added and after 40 min the reaction mixture was concentrated to one fourth of the volume. The residue was taken up in 500 ml of dichloromethane, washed with 3×500 ml of saturated $NaHCO_3$ and 500 ml of water. The organic layer was dried over $Na_2SO_4$, evaporated to dryness and coevaporated with toluene. The product was purified on silica gel column with a gradient of dichloromethane-methanol (0 to 4%). The product was obtained as a yellow foam with a yield of : 7.01 g (77%). Rf = 0.69 (solvent A). UV (Ethanol) : max : 331 nm (ε = 17300) ; λ max : 234 nm (16200). MS ($FAB^+$) : m/e = 569 $(M+Na)^+$. $^1$H-NMR ($CD_3OD$) : 7.65 (*d*, J = 7.7 Hz, 1 H) 6-H ; 7.43−7.21 (*m*, 9 H) DMT ; 6.86 (*m*, 4 H) DMT ; 6.15 (*dd*, J = 6.2 Hz, 1 H) 1'-H ; 5.97 (*d*, J = 7,7 Hz, 1 H) 5-H ; 4.48 (*m*, 1 H) 3'-H ; 4.0 (*m*, 1 H) 4'-H ; 3.75 (*s*, 6 H) $OCH_3$-DMT ; 3.39 (*m*, 2 H) 5'-HaHb; 2.33 (*m*, 2 H) 2'HaHb.

*5'-O-(4,4'-Dimethoxytrityl)-4-thio-2'-deoxythymidine (2b) :* Crude 4-thio deoxythymidine *1b* (0.64 g, 2.5 mmoles) was treated as *1a*. Yield of *2b* : 1.20 g (86%). Rf = 0.69 (solvent A). UV (Ethanol) : λ max 335 nm (ε = 19200), 235 nm (ε = 25200). $^1$H-NMR ($C_3COCD_3$) : 7.74 (*s*, 1 H) 6-H ; 7.52−7.24 (*m*, 9 H) DMT ; 6.90 (*m*, 4 H) DMT ; 6.31 (*dd*, J = 6.7 Hz, 1 H) 1'-H ; 4.61 (*m*, 1 H) 3'-H ; 4.09 (*m*, 1 H) 4'-H ; 3.78 (*s*, 6 H) $OCH_3$-DMT ; 3.40 (*m*, 2 H) 5'-Ha, Hb ; 2.42 (*m*, 2 H) 2'Ha,Hb ; 1.66 (*s*, 3 H) 5-$CH_3$.

*5'-O-(4,4'- Dimethoxytrityl)-4-N-[6-aminohexyl]-2'-deoxycytidine (3a) :* To a solution of *2a* (3.71 g, 6.8 mmoles) in 40 ml of absolute ethanol, was added 1,6-diaminohexane (3.95 g, 34 mmoles). The reaction was left 16 H at 60°C in a tightly stopped flask. After cooling and evaporation of the solvent, the residue was taken up in 200 ml of chloroform, washed with 3×200 ml of 0.1 N sodium hydroxide solution and thoroughly washed with water up to neutrality. The organic layer, dried over $Na_2SO_4$, was evaporated to dryness. The product was purified over short silica column with a gradient of methanol in dichloromethane. Elution with 15% of methanol offered compound *3a* as a white foam with a yield of : 3.65 g (85%). UV (Ethanol) : max : 273 nm (ε = 13700) ; λ max : 235 nm (ε = 26600). MS ($FAB^+$) : m/e = 629 $(M+H)^+$. $^1$H-NMR ($CD_3OD$) : 7.81 (*d*, J = 8.0 Hz, 1 H) 6-H ; 7.43−7.21 (*m*, 9 H) DMT ; 6.85 (*m*, 4H) DMT ; 6.24 (*dd*, J = 6.3 Hz, 1 H) 1'-H ; 5.54 (*d*, 1 H) 5-H ; 4.49 (*m*, 1 H) 3'-H ; 4.00 (*m*, 1 H) 4'-H; 3.78 (*s*, 6 H) $OCH_3$−DMT ; 3.41 (*m*, 4 H) 5'-HaHb, 1-$CH_2$ (Hex) ; 2.70 (*t*, 2 H) 6-$CH_2$ (Hex ; 2.42 (*m*, 1 H) 2-Ha ; 2.18 (*m*, 1 H) 2'-Hb ; 1.70−1.30 (*m*, 8 H) 2 to 5-$CH_2$ (Hex).

*5'-O-(4,4'-Dimethoxytrityl)-4-N-[6-aminohexyl]-5-methyl-2'-deoxycytidine (3b)* : The compound *2b* (3.81 g, 6.8 mmoles) was treated as described for *2a*. The yield after purification was 3.72 g (85%). Rf = 0.5 (solvent B). MS ($FAB^+$) : m/e = 643 $(M+H)^+$. $^1$H-NMR ($CD_3OD$) : 7.68 (*s*, 1 H) 6-H) ; 7.44 −7.18 (*m*, 9 H) DMT ; 6.84 (*m*, 4 H) DMT ; 6.34 (*dd*, J = 6.5 Hz, 1 H) 1'-H ; 4.50 (*m*, 1 H) 3'-H ; 4.01 (*m*, 1 H) 4'-H; 3.71 (*s*, 6 H) $OCH_3$-DMT ; 3.46 −3.29 (*m*, 4 H) 5'-HaHb, 1-$CH_2$ (Hex) ; 2.6 (*t*, J = 6.8 Hz, 2 H) 6-$CH_2$ (Hex) ; 2.38−2.25 (*m*, 2 H) 2' - HaHb) ; 1.60−1.45 (*m*, 11 H) 5-$CH_3$ ; 2 to 5-$CH_2$ (Hex).

*5'-O-(4',4'-Dimethoxytrityl)-4-N-[6-N-biotinylaminohexyl]-2'-deoxycytidine (4a)* : To a solution of *3a* (3.15 g, 5 mmoles) in 50 ml of DMF, biotin-N-hydroxysuccinimide ester (1.7 g, 5 mmoles) was added. The reaction was kept 3 hours at 25°C under stirring. Most of the solvent was removed in vacuo. The residue was taken up in 500 ml of chloroform and extracted with 10% aqueous $NaHCO_3$ and then water. The organic layer was evaporated to dryness. The crude material was purified by silicagel chromatography using a gradient of methanol in dichloromethane. Elution with 4% of methanol gave the pure product as a hard glass. Yield of *4a* : 3.2 g (75%).Rf = 0.30 (Solvent A). MS ($FAB^+$): m/e = 855 $(M+H)^+$ ; 877 $(M+Na)^+$. $^1$H-NMR ($CD_3OD$) : 7.82 (*d*, J = 8.0 Hz, 1 H) 6-H ; 7.43−7.22 (*m*, 9 H) DMT ; 6.86 (*m*, 4 H) DMT ; 6.23 (*dd*, J = 6.0 Hz) 1'-H; 5.56 (*d*, 1 H) 5-H, 4.46 (*m*, 2 H) 3'-H, 4-H (Biot) ; 4.26 (*dd*, 1 H) 3-H (Biot) ; 3.98 (*m*, 1 H) 4'-H ; 3.77 (*s*, 6 H) $OCH_3$-DMT ; 3.38 (*m*, 5 H) 5'-HaHb, 1-$CH_2$ (Hex), 2-H (Biot) ; 3.15 (*t*, 2 H) 6-$CH_2$ (Hex) ; 2.9 (*dd*, 1 H) 5-Ha (Biot) ; 2.68 (*d*, 1 H) 5-Hb (Biot); 2.39 (*m*, 1 H) 2'-Ha ; 2.24 (*m*, 1 H) 2'-Hb ; 2.18 (*t*, 2 H) 9-$CH_2$ (Biot) ; 1.62−1.26 (*m*, 12 H) 7, 8-$CH_2$ (Biot), 2 to 5-$CH_2$ (Hex).

*5'-O-(4,4'-Dimethoxytrityl)-N-4-(6-Biotinyl-aminohexyl)-5-methyl-2'-deoxycytidine (4b)* : The biotinylation of *3b* (0.41 g, 0.67 mmoles) was performed as described for *4a*. The yield of purified compound was 0.44 g (79%).MS ($FAB^+$) : m/e = 869 $(M+H)^+$ ; 892 $(M+Na^+)$. $^1$H-NMR ($CD_3OD$) : 7.68 (*s*, 1 H) 6-H ; 7.44−7.21 (*m*, 9 H) ; 6.67−6.62 (*m*, 4 H) DMT ; 6.33 (*dd*, J = 6.4 Hz, 1 H) 1'-H ; 4.43 (*m*, 1 H) 4-H (Biot) ; 4.25 (*dd*, 1 H) 3-H (Biot) ; 4.00 (*m*, 1 H) 4'-H ; 3.75 (*s*, 6 H) $OCH_3$-DMT ; 3.46−3.35 (*m*, 5 H) 2-H, 5'-HaHb, 1-$CH_2$ ; 3.16 (*m*, 2 H) 6-$CH_2$ ; 2.88 (*dd*, 1 H) 5-Ha ; 2.67 (*d*, 1 H) 5-Hb ; 2.35 (*m*, 2 H) 2'- HaHb ; 2.19 (*t*, 2 H) 9-$CH_2$ ; 1.70−1.28 (*m*, 15 H) 5-$CH_3$, 7,8-$CH_2$, 2 to 5-$CH_2$.

*5'-O-(4,4'-Dimethoxytrityl)-4-N-[6-N-biotinylaminohexyl]-2'-deoxycytidine-3'-O-(2-cyanoethyl)-N,N-diisopropylphosphoramidite (8a)* : After dissolution in the minimal volume of dichloromethane compound *4a* (854 mg, 1 mmole) was dried by coevaporation with anhydrous acetonitrile. To the dried foam were added 10 ml of dichloromethane, diisopropylammonium tetrazolide (85 mg, 0.5 mmole), bis( diisopropyl-amino)-2-cyanoethoxyphosphine (340 µl, 1.2 mmole). The reaction was kept 2 hours at room temperature under stirring, diluted to 50 ml with dichloromethane and extracted with 3×50 ml of saturated $NaHCO_3$ and 50 ml of brine. After drying over $Na_2SO_4$ the organic layer was evaporated to dryness. The foamy residue was dissolved in 5 ml of dichloromethane and precipitated from 100 ml of cold hexane (−78°C) to give a white powder. Yield of *5a* : 897 g (85%).MS ($FAB^+$) : m/e = 1055 $(M+H)^+$, 1077 $(m+Na)^+$. $^{31}$P-NMR ($CD_3CN$) : 148.29, 148.07.

*5'-O-(4,4'-Dimethoxytrityl)-4-N-[6-N-biotinylaminohexyl]-2'-deoxycytidine-5-methyl-3'-O-(2-cyanoethyl)-N,N-diisopropylphosphoramidite (8b)* : This compound was prepared from *4b* (0.26 g, 0.3 mmole) according to the procedure used for *8b*. Yield 0.28 g (87%). MS

(FAB$^+$) e/m = 1069 (M+H)$^+$, 1091 (M+Na)$^+$. $^{31}$P-NMR ($CD_3CN$) : 148.97.

*5'-O-(4,4'-Dimethoxytrityl)-4-N-[6-N-(2,4-dinitrophenyl)-aminohexyl]-5-methyl-2'-deoxycytidine (5b) :* The compound *3b* (0.94 g, 1.46 mmole) was dissolved in pyridine and 2,4-dinitrofluorobenzene (0.3 g, 1.60 mmole) was added. After few minutes the reaction was complete. The reaction mixture was poured in aqueous hydrogen carbonate solution and then extracted with chloroform. The organic layer was further washed with water and evaporated to dryness. The residue was taken-up in dichloromethane and loaded on to a short silicagel column. The column was eluted with a gradient of methanol in dichloromethane (up to 5%, V/V). Yield : 0.93 g (79%). MS (FAB$^+$) : m/e = 839 (M+Na)$^+$. UV (Ethanol) : λ max 270 nm (ε = 17200), 364 (ε = 17000). $^1$H-NMR ($CDCl_3$) : 9.1 (*d*n H = 2.5 Hz, 1 H) DNP ; 8.26 (*dd*, J = 9.5 Hz, 1 H) DNP ; 7.68 (*s*, 1 H) 6-H ; 7.42−7.21 (*m*, 9 H) DMT ; 6.90 (*d*, 1 H) DNP ; 6.80 (*m*, 4 H) DMT; 6.44 (*dd*, J = 6.0 Hz, 1 H) 1'-H ; 4.57 (*m*, 1 H) 3'-H ; 4.10 (*m*, 1 H) 4'-H ; 3.78 (*s*, 6 H) $OCH_3$-DMT ; 3.55−3.30 (*m*, 6 H) 5'-HaHb, 1-$CH_2$, 6-$CH_2$ ; 2.56 (*m*, 1 H) 2'-Ha ; 2.22 (*m*, 1 H) 2'-Hb ; 1.90−1.40 (m, 11 H) 2 to 5-$CH_2$ (Hex), 5-$CH_3$.

*5'-O-(4,4'-Dimethoxytrityl)-4-N-[6-N-(2,4-dinitrophenyl)-aminohexyl]-5-methyl-2'-deoxycytidine-3'-O-(2-cyanoethyl)-N,N-diisopropylphosphoramidite (9b) :* The compound *5b* (0.41 g, 0.51 mmole) was phosphitylated as described for *4a* (reaction time 90 mn). The crude reaction product was taken up in a dichloromethane-hexane mixture 8:2, V/V, containing 2% of triethylamine and purified on a short silicagel column. The column was then eluted with a gradient of solvent C in dichloromethane. The relevant fractions were combined, evaporated and taken-up in toluene. A precipitation from cold hexane offered *9b* as a yellow powder. Yield 0.47 g (91%). Rf = 0.66 (Solvent C). $^{31}$P($CD_3CN$) : 149.20.

*5'-O-(4,4'-Dimethoxytrityl)-4-N-[6-dansylaminohexyl]-5-methyl-2'-deoxycytidine (6b) :* A solution of dansyl chloride (0.3 g, 1.1 mmoles) in 2 ml of dimethylformamide was added slowly to *3b* (0.64 g, 1 mmole) in 5 ml of dimethylformamide containing 1.5 ml of saturated aqueous hydrogen carbonate solution. After 30 min, 1 ml of 20% ammonia solution was added, and after 15 min the reaction mixture was evaporated nearly to dryness and the residue was precipitated from water. The precipitate was collected by centrifugation, taken-up in chloroform, and washed with water. The organic layer was concentrated and loaded onto a short silicagel column, which was eluted with a gradient of methanol in dichloromethane. Elution with 4% methanol-dichloromethane V/V offered 0.72 g (82%) of *6b* as a bright-yellow foam. Rf = 0.34 in Solvent D. UV (Ethanol): 270nm (ε = 12000), λ max=335nm (ε = 3300) ; MS (FAB$^+$) : m/e = 898 (M+Na$^+$). $^{31}$P-NMR($CD_3CN$) 149.34. $^1$H-NMR ($CD_3COCD_3$) : 8.62 (*m*, 2 H) ; 8.34 (*d*, 1 H) ; 7.68 (*m*, 3 H) dansyl; 7.60−7.30 (*m*, 10 H) DMT, 6-H ; 7.0 (*m*, 4 H) DMT ; 6.59 (*dd*, J = 6.0 Hz) 1'-H; 4.69 (*m*, 1 H) 3'-H ; 4.20 (*m*, 1 H) 4'-H ; 3.88 (*s*, 6 H) $OCH_3$-DMT ; 3.50 (*m*, 2 H) 5'-HaHb ; 3.49 (*m*, 2 H) 1-H (Hex) ; 3.42 (*m*, 2 H) 6-H (Hex) ;2.96 (*s*, 6 H) $CH_3$-dansyl; 2.52 (*m*, 1 H) 2'-Ha ; 2.38 (*m*, 1 H) 2(-Hb ; 1.69 (*s*, 3 H) 5-$CH_3$ ; 1.55−1.27 (*m*, 8 H) 2 to 5-$CH_2$ (Hex).

*5'-O-(4,4',-Dimethoxytrityl)-4-N-[6-N-dansyl-aminohexyl]-2'-deoxycytidine-3'-O-(2-cyanoethyl)-N,N-diisopropylphosphoramidite (10b) :* The compound *6b* (0.41 g, 0.47 mmole) was treated as described for *5b*. The precipitation gave *10b* as a powder.Yield: 0.36 g (91%). Rf = 0.49 (Solvent C). UV (Ethanol) : λ max 335 nm (ε = 3300) ; 270 nm (Sh) (ε = 12000). MS (FAB$^+$) : m/e = 1106 (M+Na$^+$). $^{31}$P-NMR ($CD_3CN$): 149.34.

*5'-O-(4,4'-Dimethoxytrityl)-4-N-[6-N-(3-pyrenesulfonyl)-aminohexyl]-5-methyl-2'-deoxy*

*cytidine (7b) :* The compound *3b* (0.32 g, 0.5 mmole) was treated with 3-pyrenesulfonyl chloride (prepared from sodium 3-pyrene sulfonate and phosphorus oxychloride and used without further purification). The procedure was the same as described for *6b*. The yield of pure *7b* was 0.33 g (73%). Rf = 0.28 (Solvent A). UV (Ethanol): 242 nm (32000), 278 nm (25300), 350 nm (23600). MS ($FAB^-$) : M-H = 905. $^1$H-NMR ($CD_3COCD_3$) : 9.20 (*d*, 1 H) Pyr ; 8.70 (d, 1 H) Pyr ; 8.40−8.03 (*m*, 7 H) Pyr; 7.70−7.30 (*m*, 10 H) DMT, 6-H ; 6.90 (*m*, 4 H) DMT ; 6.54 (*dd*, J = 6.0 Hz, 1 H) 1′-H ; 4.65 (*m*, 1 H) 3′-H ; 4.10 (*m*, 1 H) 4′-H ; 3.76 (*s*, 6 H) $OCH_3$; 3.39 (*m*, 2 H) 5′-HaHb ; 3.09 (*m*, 2 H) 1-$CH_2$ (Hex) ; 2.90 (*m*, 2 H) 6-$CH_2$ (Hex); 2.42 (*m*, 1 H) 2′-Ha; 2.26 (*m*, 1 H) 2′-Hb ; 1.51 (*s*, 3 H) 5-$CH_3$ ; 1.45−1.20 (*m*, 8 H) 2 to 5-$CH_2$ (Hex).

*5′-O-(4,4′-Dimethoxytrityl)-4-N-[6-N-(3-pyrenesulfonyl)-aminohexyl]-5-methyl-3′-O-(2-cyanoethyl)-N,N-diisopropylphosphoramidite (11b) :* The compound *7b* (0.30 g, 0.33 mmole) was phosphitylated and purified as for *9b*. Yield 0.31 g (85%). MS ($FAB^+$) : m/e = 1106 $(M+Na)^+$. $^{31}$P-NMR($CD_3CN$) : 149.06.

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