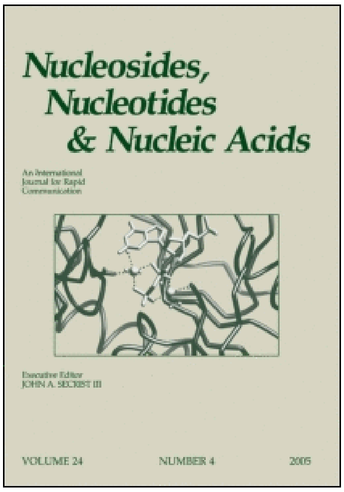

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The Reactions of H-Phosphonates with Bifunctional Reagents. V. Functionalization of Support-Bound Oligonucleotides and Synthesis of Nonradioactive Hybridization Probes

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THE REACTIONS OF H-PHOSPHONATES WITH BIFUNCTIONAL REAGENTS. PART V. FUNCTIONALIZATION OF SUPPORT-BOUND OLIGONUCLEOTIDES AND SYNTHESIS OF NON-RADIOACTIVE HYBRIDIZATION PROBES[a]

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ABSTRACT: Three methods for the functionalization of oligonucleotides with aminoalkyl moieties have been developed and their efficiencies were evaluated in the preparation of non-radioactive hybridization probes.

INTRODUCTION

Base-pairing properties of oligonucleotides provide the underlying principle for an artificial modulation of gene expression and for the detection of specific gene sequences in DNA *via* formation of sequence-specific complexes. In order to serve as a hybridization probe, a synthetic oligonucleotide usually has to be equipped with a radioactive tracer or a reporter group that facilitates its localization. In recent years the growing usage of hybridization probes in molecular medicinal diagnostics, made non-radioactive systems of labeling of oligonucleotides the preferred ones, due to their safety, ease of handling and variety of modes of detection[1,2].

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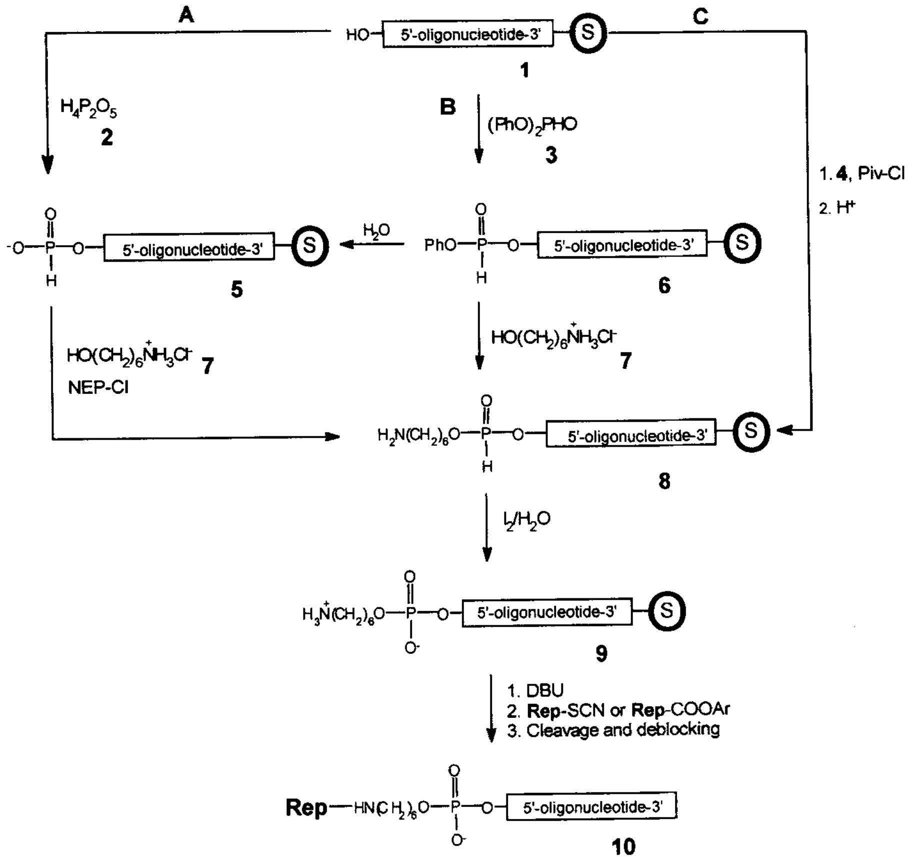
The most common approach to construct a non-radioactive hybridization probe consists of functionalization of an oligonucleotide, *i.e.* the introduction of a new functional group to which a suitable reporter groups can be attached[3,4]. Since a close proximity of a reporter group and nucleobases may impair the hybridization process, various avenues have been pursued to achieve a non-interfering attachment of suitable ligands to oligonucleotides. Irrespective of their position in an oligonucleotidic chain (heterocyclic nucleobases or the phosphate-sugar backbone), the reporter groups are usually joined *via* a suitable linker, to attenuate possible interference during hybridization.

Most commercially available reporter groups are active aryl carboxyesters or isothiocyanates, which can be conveniently attached to oligonucleotides *via* an appropriate linker carrying a primary amino group. The required functionalization can thus be achieved by the introduction of a modified nucleotide moiety (bearing a suitable functional group in nucleobase moieties[5,6] or in the sugar-phosphate backbone[7-10]) or other non-nucleotidic synthons into an oligonucleotidic sequence, as terminal units. To facilitate the process of preparation of functionalized oligonucleotides, we wished to develop a synthetic protocol, which would make use of cheap, commercially available chemicals, and would require minimal number of manual operations. Also, to make the method as versatile as possible, we considered it highly desirable that (i) the functionalization and the attachment of a reporter group would be independent of the approach used for the synthesis of an oligonucleotide and (ii) the modification and the attachment of the reporter group to the oligonucleotide should be carried out on a solid support, to simplify the isolation of an oligonucleotidic hybridization probe.

RESULTS AND DISCUSSION

1. FUNCTIONALIZATION OF SUPPORT-BOUND OLIGONUCLEOTIDES

Recently, we have conducted a detailed investigation of the reactions of H-phosphonate mono- and diester with amino alcohols, to get some insight into the chemoselectivity during condensation[11], oxidative coupling[12], and transesterification[13] oligonucleotides (Scheme 1). Method A, commences with the introduction of an H-phosphonate monoester function to the 5'-end of an oligonucleotidic chain (intermediate processes involving these compounds. These studies enabled us to design three synthetic approaches, based on H-phosphonate chemistry, for the 5'-terminal



1a = T_{10} obtained with phosphoramidite method
1b = T_{10} obtained with H-phosphonate method
4 = N-(4,4'-dimethoxytrityl)-6-aminohexyl-H-phosphonate
S = CPG-LCAA
NEP-Cl = 2-chloro-5,5-dimethyl-2-oxo-2λ^5-1,3,2-dioxaphosphinane
10a, Rep = fluorescein
10b, Rep = biotin

SCHEME 1

functionalization of **5**), followed by its esterification with an amino alcohol to produce the 5'-aminoalkyl-modified oligonucleotide **8**. Method B, consists of the introduction of an aryl H-phosphonate moiety at the 5'-end of an oligomer by means of diphenyl H-phosphonate. Transesterification of the produced intermediate **6** with an amino alcohol should lead to the functionalized oligonucleotide **8**, while its hydrolysis (if so desired), to the 5'-phosphonylated oligonucleotide **5**. The third variant (method C), makes use of a suitable non-nucleotidic synthon **4** containing an aminoalkyl moiety for the attachment to the 5'-OH function of an oligonucleotide *via* a standard condensation reaction.

1.1. Method A: phosphonylation with H-pyrophosphonate followed by condensation with an amino alcohol.

For the phosphonylation of a support-bound oligonucleotide we have chosen a mild, moderately reactive pyridinium salt of H-pyrophosphonic acid (**2**). The reagent, which has been successfully used in the synthesis of nucleoside H-phosphonate monoesters[14], can readily be prepared by reaction of phosphonic acid (1.0 molar equiv.) and pivaloyl chloride (Piv-Cl) (0.5 molar equiv.) in pyridine and used as such for the phosphonylation. In a typical experiment (Protocol A), the model oligonucleotide **1** [5'-HO-$(T)_{10}$, 0.1 μM], attached to the glass support (CPG-LCAA) was treated with 0.2 M pyridinium H-pyrophosphonate in pyridine (0.2 mL). The reaction went to completion within 2 h, as judged from the polyacrylamide gel electrophoresis (PAGE) (EXPERIMENTAL, Figure 1, lane 2). After the deprotection with 33% aq. ammonia, the desired 5'-H-phosphonylated decathymidine nonaphosphate **5** was produced in >90% yield.

Recently, a similar reagent system, phosphonic acid/Piv-Cl, has been used for the phosphonylation of oligonucleotides on a solid support, but only with a moderate success[15]. The problems experienced by those authors [the extensive formation of di(oligonucleotide-5'-yl) H-phosphonates] can most likely be traced to the use of 3-fold molar excess of pivaloyl chloride over phosphonic acid in the reagent system. Under such reaction conditions, the putative intermediate formed is tripivaloyl phosphite[16], which may produce an excessive cross-linking of oligonucleotidic chains or promote other side reactions[15].

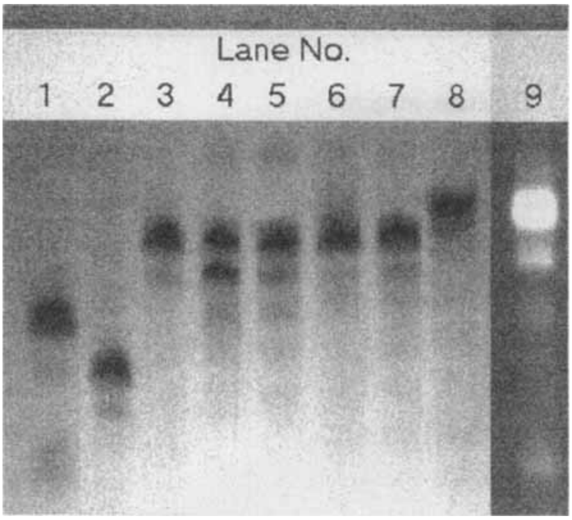


FIG. 1.

Lane 1 - decathymidine nonaphosphate **1b**.
Lane 2 - 5'-H-phosphonate oligonucleotide **5**.
Lane 3 - **9** obtained from **1b** by method A, followed by the ammonia treatment.
Lane 4 - **9** obtained from **1a** (method A), followed by the ammonia treatment.
Lane 5 - **9** obtained from **1a** (method A), deprotected with methylamine.
Lane 6 - **9** obtained from **1b** (method B), deprotected with ammonia.
Lane 7 - **9** obtained from **1b** (method C).
Lane 8 - **10b**.
Lane 9 - **10a**, UV_{366} shadowed.

For the transformation of the produced 5'-H-phosphonylated oligonucleotide **5** to the aminoalkyl-modified oligonucleotide **8**, we adapted a method, previously developed by us for the synthesis of nucleoside aminoalkyl-H-phosphonate in solution[11]. To this end, the oligonucleotide 5'-H-phosphonate **5** (attached to a solid support) was treated with an excess (50 molar equiv.) of 6-aminohexan-1-ol hydrochloride[17] in pyridine in the presence of 2-chloro-5,5-dimethyl-2-oxo-2λ^5-1,3,2-dioxaphosphinane (NEP-Cl) as a condensing agent, and then subjected to oxidation.

Protocol A

Function, reagents	Volume (mL)	Time (min)
1. wash - pyridine	8 x 0.1	1
2. phosphonylation - 0.2 M H-pyrophosphonic acid in pyridine	4 x 0.1	120
3. wash - pyridine	8 x 0.1	1
4. coupling - 6-aminohexan-1-ol x HCl (0.2 M) in pyridine (0.04 mL) followed by NEP-Cl (0.3 M) in pyridine (0.08 mL)	0.12 total	4
5. wash - pyridine	8 x 0.1	1
6. oxidation - 3% I_2 in pyridine/H_2O 9:1 (v/v)	2 x 0.1	10
7. wash - pyridine	8 x 0.1	1

When the oligonucleotide **1b**, obtained *via* the H-phosphonate approach, was functionalized according to Protocol A and split from the support using conc. ammonia, the desired aminoalkyl-oligonucleotide **9** was produced nearly quantitatively, as judged from PAGE analysis of the crude reaction mixture (Figure 1, lane 3). Somewhat surprisingly, when the starting oligonucleotide **1a** synthesized *via* the phosphoramidite method (2-cyanoethyl as a phosphate protecting group) was functionalized according to the above protocol, we observed the formation of considerable amounts (~40-80%) of a side product, with electrophoretical mobility close to that of **9** (Figure 1, lane 4). This phenomenon, which was independent of the protocol used for the functionalization, will be discussed in detail later on in this paper.

1.2. Method B: phosphonylation with diphenyl H-phosphonate followed by transesterification with an amino alcohol.

In this approach for the functionalization of a support-bound oligonucleotide **1**, we have exploited the ease of formation of nucleoside aryl H-phosphonates and their susceptibility to transesterification[13,19]. The key reagent, which enabled us to carry a complete functionalization procedure without using a coupling agent, was diphenyl H-phosphonate **3**. Treatment of the oligonucleotide **1** with **3** in pyridine (0.5 M) readily produced (~5 min) the putative oligonucleotide-5'-yl phenyl H-phosphonate **6**. The latter was too unstable to be characterized, but from its clean hydrolysis [pyridine/H_2O 9:1

Protocol B

Function, reagents	Volume (mL)	Time (min)
1. wash - pyridine	8 x 0.1	1
2. phosphonylation - 0.5 M diphenyl H-phosphonate in pyridine	0.1	5
3. wash - pyridine	8 x 0.1	1
4. functionalization - transesterification with 6-aminohexan-1-ol x HCl (0.2 M) in pyridine	0.1	60
5. wash - pyridine	8 x 0.1	1
6. oxidation - 3 % I_2 in pyridine/H_20 9:1 (v/v)	2 x 0.1	10
7. wash - pyridine	8 x 0.1	1

(v/v), 5 min] to a stable oligonucleotide 5'-H-phosphonate **5** (PAGE, not shown), we infer that it was formed nearly quantitatively. To prevent the cross-linking of oligonucleotidic chains [the formation of symmetrical di(oligonucleotide-5'-yl) H-phosphonate diesters], it was essential to use a considerable excess (250 molar equiv.) of diphenyl H-phosphonate **3** for the phosphonylation[20]. The transesterification of the produced oligonucleotide-5'-yl phenyl H-phosphonate **6** with 6-aminohexan-1-ol hydrochloride (0.2 M solution in pyridine, ~60 min) to form the 5'-aminoalkyl H-phosphonate **8**, also proceeded efficiently, as judged from the quantitative formation (after oxidation with I_2/pyridine/H_2O) of the functionalized oligonucleotide **9** (Figure 1, lane 6).

The protocol, which we arrived to after the optimization of the individual synthetic steps (dodecathymidine nonaphosphate **1b** was used as a model compound), is as follows:

1.3. Method C: attachment of a non-nucleotidic synthon.

The third approach investigated made use of a non-nucleotidic synthon, N-protected aminoalkyl H-phosphonate **4**, which was attached to the 5'- terminus of an oligonucleotide **1** *via* standard condensation. Since the condensation step seldom poses any problem, the utility of such types of approaches usually is limited by the availability of a suitable synthon[21]. For this purpose, we have developed simple and efficient method[22] for the synthesis of N-(4,4'-dimethoxytrityl)-6-aminohexyl H-phosphonate **4**. This is based on commercial substrates and can be easily adapted for the preparation of other derivatives of this type. The synthesis commences with transesterification of diphenyl H-phosphonate with 6-aminohexan-1-ol to produce di(6-aminohexyl) H-phosphonate diester, that upon addition of water smoothly hydrolyses and affords a crystalline 6-aminohexyl H-phosphonate monoester. From this, the final synthon **4** was obtained *via* 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) promoted 4,4'-dimethoxytritylation, followed by simple chromatographic purification[22].

The attachment of synthon **4** to oligonucleotide **1b** was brought about in acetonitrile/pyridine (1:1, v/v), using Piv-Cl as a condensing agent. After (i) coupling (1 min), followed by (ii) oxidation with iodine under aqueous conditions, (iii) removal of the DMT group, and (iv) the final deprotection with conc. ammonia, the 5'-aminoalkyl-functionalized oligonucleotide **9** was produced as a major product (Figure 1, lane 7). The corresponding synthetic protocol is shown below.

Protocol C

Function, reagents	Volume (mL)	Time (min)
1. wash - pyridine	8 x 0.1	1
2. phosphonylation - 0.2 M solution of **4** in acetonitrile/pyridine 1 : 1 (v/v) - 0.04 mL, followed by 0.3 M Piv-Cl in the same solvent - 0.08 mL	0.12	1.5
3. wash - pyridine	2 x 0.1	0.25
4. oxidation - I_2 in pyridine/H_20 9:1 (v/v)	2 x 0.1	10
5. wash - CH_2Cl_2	8 x 0.1	1
6. removal of DMT - 3% DCA in $C_2H_4Cl_2$	16 x 0.1	2
7. wash - CH_2Cl_2	8 x 0.1	1

2. ATTACHMENT OF THE NON-RADIOACTIVE TRACERS TO THE 5'-END OF AMINOALKYL-MODIFIED OLIGONUCLEOTIDES

As a final step in the synthesis of non-radioactive probes, a suitable reporter group is attached to functionalized oligonucleotides.

In preliminary experiments, when the aminoalkylated oligonucleotide **9** was treated with fluorescein isothiocyanate or biotin 4-nitrophenyl ester in pyridine, the tagged oligonucleotides **10** were usually obtained in rather poor yields (~ 20%, PAGE), irrespective of the excess of the reagents used or the reaction time. We assumed, that the most likely reason for inefficiencies of these reactions could be the zwitter-ionic structure of the 5'-aminoalkyl phosphate moiety, in which the amino function is protonated. To generate the free amino function, the support-bound aminoalkyl-modified oligonucleotide **9** was pre-washed with 0.2 M solution of DBU in pyridine and then subjected to the reaction with fluorescein isothiocyanate (0.03 M in pyridine). As expected, the attachment of fluorescein became much more efficient (30 min) and the tagged oligonucleotide **10a** was found to be the predominant product in the crude reaction mixture (Figure 1, lane 9). Also, by using an analogous protocol with biotin 4-nitrophenyl ester, a considerable improvement in the yield of the biotin-tagged oligonucleotide **10b** (Figure 1, lane 8) was achieved.

The above protocol for tagging functionalized oligonucleotides seems to be rather general and applicable to other commercially available derivatives of common reporter groups, *e.g.* rhodamine isothiocyanate, digoxigenin-3-O-methylcarbonyl-ε-aminocapronic acid-N-hydroxysuccinimide ester, *etc.*

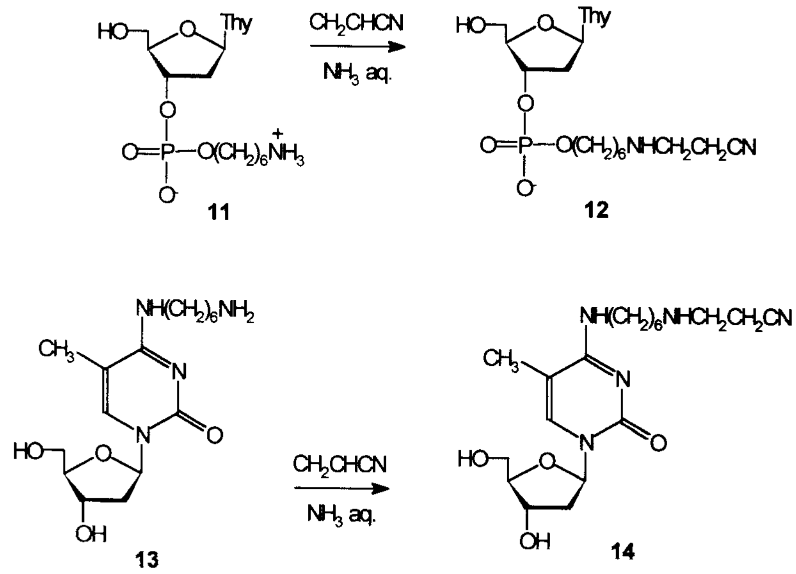
3. POSSIBLE SIDE REACTIONS DURING THE DEPROTECTION OF THE AMINOALKYL-FUNCTIONALIZED OLIGONUCLEOTIDES OF TYPE 9

On several occasions we have observed that oligonucleotides functionalized according to Protocol A - C, afforded (after treatment with conc. ammonia) the desired oligonucleotide **9** as a major (or exclusive) product only when the starting oligonucleotide **1** was produced by the H-phosphonate method (oligomer **1b**). In the instance of oligonucleotides **1a**, prepared *via* the phosphoramidite approach and used in the form of 2-cyanoethyl phosphotriesters for the functionalization, a considerable amount of a side product, with an electrophoretical mobility close to that of **9**, was formed. We assumed as likely that acrylonitrile generated during the removal of 2-cyanoethyl group (the β-elimination process) from the oligonucleotide, may react with the amino function of the 5'-aminoalkyl moiety in **9** to form stable N-alkylated derivatives.

The validity of this assumption was first checked on nucleoside 3'-aminoalkyl phosphate[11] **11** and aminoalkyl nucleoside[23] **13** as models, having aminoalkyl moieties in different chemical environments (Scheme 2). Both compounds were found to react quantitatively with acrylonitrile (10 molar equiv.) in concentrated aqueous ammonia (15 min, TLC) affording, N-alkylated species **12** and **14**, respectively, which structures were confirmed by ^{1}H NMR spectroscopy. In analogous experiments, but in the presence of methylamine (40% aq.)[18] used instead of ammonia, neither compound **11** nor **13** underwent any detectable N-alkylation, probably due to rapid scavenging of acrylonitrile by methylamine. It is worth to note that in concentrated aq. ammonia the unprotected nucleosides (dA, dC, dG and T) remained unaffected when treated with acrylonitrile (10 equiv., 12 h).

Experiments on oligonucleotides confirmed these findings. When the purified (PAGE) 5'-aminoalkyl-modified oligonucleotide **9** (5'-aminoalkyl phosphorylated T_{10}, obtained by the H-phosphonate method) was treated in 33% aq. ammonia with acrylonitrile (10 molar equiv.) for 2 h, it was quantitatively converted to an oligomer with the electrophoretical mobility identical to that observed for the side product. This result strongly suggested that oligonucleotides bearing aminoalkyl moiety may undergo N-alkylation with acrylonitrile to produce N-(2-cyanoethyl)-6-aminohexyl oligonucleotide analogues. In agreement with this, the oligonucleotides bearing 5'-[N-(4,4'-dimethoxytrityl)]-6-aminohexyl phosphate moiety (irrespective of the method of their synthesis) were completely resistant to alkylation by acrylonitrile (10 molar equiv., 33% aq. ammonia, several hours). As expected, the formation of this side product was significantly suppressed, when methylamine (40 %, aq.)[18] was used instead of ammonia for the deprotection (Figure 1, lane 5).

In conclusion, we have developed three approaches, based on H-phosphonate chemistry, for the functionalization of support-bound oligonucleotides. They are of comparable efficiency and make use of substrates that are commercially avaiable or easy to be prepared. The chemical principles underlying these methods are, however, different and this can be exploited in the instances, when particular requirements concerning the reaction conditions would be necessary. For a standard synthesis of non-radioactively labeled oligonucleotidic hybridization probes the approach involving diphenyl H-



Thy = thymine

SCHEME 2

phosphonate for the generation of highly reactive aryl alkyl H-phosphonate diesters as intermediates, seems to be the method of choice due to its simplicity, efficiency, and versatility. Using this method several fluorescein and biotin tagged oligonucleotidic hybridization probes [*e.g.* biotin-d(CAGGGACAATGTGGT) - a probe for the detection of genes encoding cysteine proteases of *Fasciola hepatica*, biotin-d(GATCTTCAGT) - a probe for studies of interactions within the anticodon loop of tRNA^{Phe} and fluorescein-d(CATACACCTCCAGCACCTAA) - a probe for the detection of human papillomavirus type 16 (HPV 16)] have been synthesised and successfully used in biological studies.

EXPERIMENTAL

^{1}H and ^{31}P NMR spectra were recorded on Varian Unity 300 BB VT spectrometer with references TMS (internal reference) and 5% of H_3PO_4 in D_2O (external reference), respectively. Pyridine and acetonitrile (LabScan) were dried over 4A molecular sieves until the content of water was less than 20 ppm (coulometric measurement with

Metrohm 684 KF Coulometer). Methylene dichloride (POCH) for column chromatography was passed through Al_2O_3 (neutral). Dichloroacetic acid (Fluka) and pivaloyl chloride (Merck) were distilled before use. Diphenyl H-phosphonate (Aldrich), 6-aminohexan-1-ol (Aldrich), acrylonitrile (Aldrich), fluorescein isothiocyanate isomer I and biotin *p*-nitrophenyl ester were commercial grade and used without additional purification. 2-Chloro-5,5-dimethyl-2-oxo-2λ^5-1,3,2-dioxaphosphinane[24] (NEP-Cl), N-(4,4'-dimethoxytrityl)-6-aminohexyl H-phosphonate[22] **4**, thymidin-3'-yl 6-aminohexyl phosphate[11] **11** and N^4-(6-aminohexyl)-5-methylcytidine[23] **13** were prepared according to published procedures.

Oligodeoxynucleotides were synthesised using standard H-phosphonate[25,26] or phosphoramidite[27] methodologies. Functionalizations of the support-bound oligonucleotides were performed using a home-made, computer controlled machine, according to the protocols specified in the text. High performance liquid chromatography (HPLC) was conducted on Hypersil 5µ ODS (4.6 mm x 250 mm) using a linear gradient of solvent B (acetonitrile/0.1 M TEAA 1:1 v/v) in A (0.1 M TEAA) 15 - 70% in 30 min. Polyacrylamide (20 %) gel electrophoresis (PAGE) of modified oligonucleotides was run under denaturating conditions (7 M urea) and UV shadowing was used for the visualization.

Preparation of samples of modified oligonucleotides for analysis. After phosphonylation, functionalization or the attachment of a reporter group, the oligonucleotide was cleaved off from the support and deprotected with ammonia (33%, aq.) or methylamine (40%, aq.) at room temperature. After two hours samples were concentrated and the remaining residues subjected to PAGE (1.0 OD). The results are shown below on Figure 1.

General procedure for the attachment of non-radioactive tracer - synthesis of 10a and 10b. 10a. The column containing functionalized oligonucleotide **9** was washed with 0.2 mL of pyridine/DBU (9:1 v/v) and treated with a solution (0.03 M in pyridine) of fluorescein isothiocyanate isomer I (0.2 mL, 60 molar equiv., 30 min). After washing the support with acetonitrile, the fluorescein-tagged oligonucleotide **10a** was cleaved and deprotected (33% aq. ammonia treatment, 2 h). The solvent and excess ammonia were evaporated (Speed-vac), dissolved in water and 1 OD of the crude product was subjected for PAGE analysis which showed nearly quantitative conversion of **9** to **10a** (Figure 1, lane 9). With above procedure fluorescein labeled HPV 16 probe purified by means of PAGE was obtained with 57% yield.

10b. The column containing functionalized oligonucleotide **9** was pre-washed with DBU (as for **10a**) and then treated with a solution (0.03 M in pyridine) of biotin *p*-nitrophenyl ester (50 molar equiv.) containing 3% (v/v) of DBU (30 min). The further work-up and analysis as described for **10a**. PAGE analysis, see Figure 1, lane 8. Biotin labeled d(CAGGGACAATGTGGT) synthesized with above procedure was purified by means of HPLC and obtained with 62% yield.

Thymidin-3'-yl N-(2-cyanoethyl)-6-aminohexyl phosphate (**12**). To a solution of thymidin-3'-yl 6-aminohexyl phosphate **11** (0.20 g, 0.5 mmol) in 10 mL of conc. aqueous ammonia, was added 0.33 mL acrylonitrile (5.0 mmol, 10 molar equiv.). When starting material **11** disappeared (15 min., TLC), the solvent and excess of acrylonitrile were evaporated under reduced pressure yielding pure product **12** which was precipitated from excess of diethyl ether. Yield, 0.20 g (85%). R_f = 0.74 [iPrOH/H_2O/conc. NH_3 85:10:5 (v/v/v)]; ^{1}H NMR δ_H (CD_3OD) 1.45 (4H, m, $CH_2CH_2CH_2CH_2CH_2CH_2$), 1.65 (4H, m, $CH_2CH_2CH_2CH_2CH_2CH_2$), 1.88 (3H, s, 5-CH_3), 2.27 and 2.46 (2H, 2 m, 2'-H_2), 2.81 (2H, t, J = 6.9 Hz, CH_2CN), 2.85 (2H, t, J = 7.2 Hz, $CH_2CH_2CH_2NHCH_2$), 3.12 (2H, t, J = 6.0 Hz, CH_2CH_2CN), 3.30 (2H, m, 5'-H_2), 3.89 (2H, dt, J_{HH} = 6.0 Hz, J_{HP} = 6.5 Hz, $POCH_2CH_2$), 4.15 (1H, m, 4'-H), 4.82 (1H, m, 3'-H), 6.30 (1H, m, 1'-H), 7.86 (1H, br s, 6-H). ^{31}P NMR δ_P (CH_3OH) 0.15 ppm (q, $^3J_{HP}$ = 6.8 Hz).

N^4-[N-(2-cyanoethyl)-6-aminohexyl]-5-methylcytidine (**14**). To a solution of N^4-6-aminohexyl-2'-deoxy-5-methylcytidine **13** (0.16 g - 0.5 mmol) in 10 mL of conc. aqueous ammonia, 0.33 mL of acrylonitrile (5.0 mmol, 10 molar equiv.) was added. When starting material **13** disappeared (15 min., TLC), the solvent and excess of acrylonitrile were evaporated under reduced pressure. Product **14** was isolated by silica gel column flash chromatography using a linear gradient (0–20 %) of methanol in methylene dichloride containing triethylamine (1% of volume). The appropriate fractions were collected and after evaporation of the solvent, pure product **14** was obtained as white powder. Yield, 0.16 g (86%) Rf = 0.22 [$CHCl_3$/CH_3OH/Et_3N 85:10:5 (v/v/v)]; ^{1}H NMR δ_H (CD_3OD) 1.51 (4H, m, $CH_2CH_2CH_2CH_2CH_2CH_2$), 1.60 (4H, m,

$CH_2CH_2CH_2CH_2CH_2CH_2$), 1.96 (3H, s, 5-CH_3), 2.15 and 2.26 (2H, 2 m, 2'-H_2), 2.59 (2H, t, J = 6.9 Hz, CH_2CN), 2.63 (2H, t, J = 6.9 Hz, $CH_2CH_2CH_2NHCH_2$), 2.86 (2H, t, J = 7.5 Hz, CH_2CH_2CN), 3.36 (2H, t, J = 7.5 Hz, $NHCH_2CH_2CH_2$), 3.76 (2H, m, 5'-H_2), 3.92 (1H, m, 4'-H), 4.39 (1H, m, 1'-H), 7.75 (1H, br s, 6-H).

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