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Large Scale, Liquid Phase Oligonucleotide Synthesis by Alkyl H-phosphonate Approach

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Abstract—A new approach to the liquid phase synthesis of oligonucleotide is described, it is based on oxidative coupling using alkyl H-phosphonate synthon and polyethylene glycol (PEG_{5000}) as a soluble support. Nucleoside alkyl H-phosphonate undergoes oxidative coupling in presence of NBS. The use of polyethylene glycol as a soluble polymeric support preserves some convenient features of the solid phase synthesis with new interesting advantages. This liquid phase method appears effective in terms of speed and coupling yield and can be evaluated for the production of large amount of oligonucleotide (100 μM).

Introduction

Synthetic oligonucleotides are moving out of the research laboratories and into practical biochemical application.[1–3] The possible use of oligonucleotide and their analogues in chemotherapy[4] has recently made their large scale synthesis a matter of considerable importance. Although the demand for relatively large quantities of material has so far been met mainly by the scaling-up of solid phase synthesis, we believe that if specific oligonucleotide sequence becomes licensed as a drug and multikilogram quantities of it are required, liquid phase synthesis is likely to become the method of choice. Over past years the liquid phase method has been proposed as an alternative to the well known solid phase synthesis of oligonucleotide. It utilizes, as a handle for the growing chains, a polymeric support (PEG) subsequently soluble in the reaction media, the polymer is freed from the unreacted reagent and soluble by product by crystallization.[5–7] Since the technique avoids any heterogeneity due to insolubility of support, scaling up of the process can be reasonably foreseen.

On other hand the great potential of the new therapeutic methodologies based on oligonucleotide derivatives justifies any effort aimed at the development of an innovative system of production. At present nucleoside 3′-phosphoramidite[8–12] and nucleoside 3′H-phosphonate[13,14] are commonly used as the starting nucleotidic unit for the synthesis of oligonucleotides. A major emphasis of our research has been to develop an alternative synthetic strategy with a minimum number of steps and aimed at high yield and better purity.[15] In view of this, we reinvestigate the use of nucleoside alkyl H-phosphonate as a synthon for oligonucleotide synthesis.[20,21] Nucleoside alkyl H-phosphonate undergoes oxidative coupling with unprotected 5′ hydroxyl group in presence of N-bromosuccinimide (NBS) as an activating agent.

Results and Discussion

The oxidative coupling of nucleosides is a relatively new methodology,[16,17] and in continuation of our work in DNA chemistry,[15,18] we report here the nucleoside 3′-alkyl H-phosphonate (**1**) as a versatile starting material for oligonucleotide synthesis via oxidative coupling. 5′-*O*-Acetyl thymidine-3′-benzyl-H-phosphonate has been described by Todd[19] for the synthesis of dithymidine, however, it has not been generally used for the synthesis of oligonucleotides in solution or in solid phase.

Nucleoside 3′-alkyl H-phosphonates are stable compounds which form as a by-product during the preparation of nucleoside alkyl phosphoramidites. For the present studies nucleoside-alkyl H-phosphonates were prepared conveniently from the corresponding phosphoramidites (Scheme 1), and yields of the products are shown in Table 1. These compounds have not generally been found useful in oligonucleotide synthesis.

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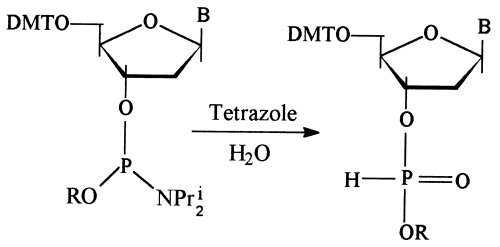


R = -CH_3 or -CH_2CH_2CN

B = Nucleoside bases

Scheme 1. R=-CH_3 or -CH_2CH_2CN; B = nucleoside bases.

Table 1. Yield of nucleoside-alkyl H-phosphonate

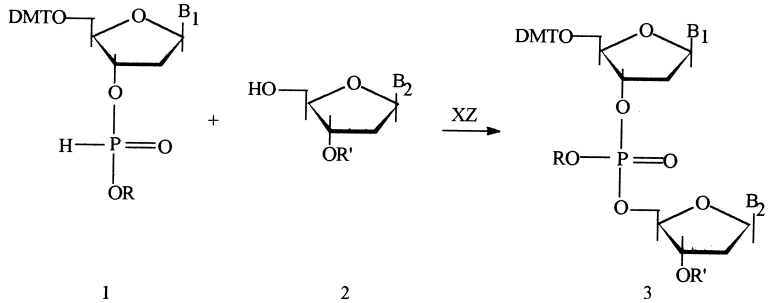
Sl no.	B	R	% Yield
1a	T	CH_2CH_2CN	92
1b	C	CH_2CH_2CN	90
1b	A	CH_2CH_2CN	87
1d	G	CH_2CH_2CN	85
1e	C	CH_3	95
1f	G	CH_3	89
1g	A	CH_3	87

They have been reported to be completely resistant to activation by powerful condensing agent such as diphenyl phosphorochloridites, in the presence or absence of *N*-methylimidazole, 2,4,6-triisopropylbenzenesulfonyl chloride or 2,4,6-triisopropylbenzenesulfonyl tetrazolides. Attempted reaction of these compounds with an unprotected 5′-hydroxyl group did not produce detectable amounts of phosphitetriester.[20,21] Nucleoside 3′-alkyl H-phosphonates have been used for the synthesis of oligonucleotides containing an interphosphoramidate group.[22] Although these compounds are stable, we observed that they become highly reactive in the presence of an activating agent like I_2 or NBS, and undergo oxidative coupling (Scheme 2).

The condensation of **1** (0.11 mmol) with a 3′-*O*-acetyl nucleoside (0.1 mmol), in the presence of a coupling agent (NBS (0.12 mmol)/I_2 (0.12 mmol)/CCl_4 (excess)) at room temperature in CH_3CN:triethylamine (4:1 v/v) gave dinucleoside phosphate (**3**) as the only detectable product (Scheme 2). The yields of **3** depends on the coupling agent used, with NBS, 85% yield of **3** was obtained within 5 min. When I_2 (0.12 mmol) was used as a coupling agent reaction was completed in about 10 min with a 74% yield of **3**. The UV, ^{1}H NMR, and HPLC analyses indicated that the heterocyclic moiety and all protecting groups (DMTr, -COR, -OCH_3, -OCH_2CH_2CN) remained unaffected. ^{31}P NMR shows a singlet at $\delta = 2.21$ indicating that the product is a phosphotriester and not a phosphitetriester. Additionally, the reverse phase HPLC elution time of the product matches with the authentic sample prepared by the solid phase phosphoramidite method.

A plausible mechanism is shown in Scheme 3, where by **4** is expected to be formed as a highly reactive intermediate that reacts immediately with the free hydroxyl group of a nucleoside to give the desired product. The mechanism has been studied by ^{31}P NMR (XZ = NBS) and **4** was found to be the only intermediate.

To set up the synthetic protocol for the PEG supported synthesis of oligonucleotides via β-CE-ethyl H-phosphonates. The effects of different coupling agents, solvents and bases on coupling efficiency were studied by preparing a dimer d(AT). The results are shown in Table 2. When I_2 was employed as a coupling agent the complete removal of the reagent becomes difficult due to its high affinity for PEG. The four dimer d(TT), d(AT), d(GT) and d(CT) were then prepared using NBS as a coupling agent, in the CH_3CN:TEA system. The influence of the excess of these compounds and NBS, and time of the reaction were carefully studied; particular attention was paid to the minimum amount of reagent required for the maximum yield. From these studies 98–99% yield in the phosphate bond formation



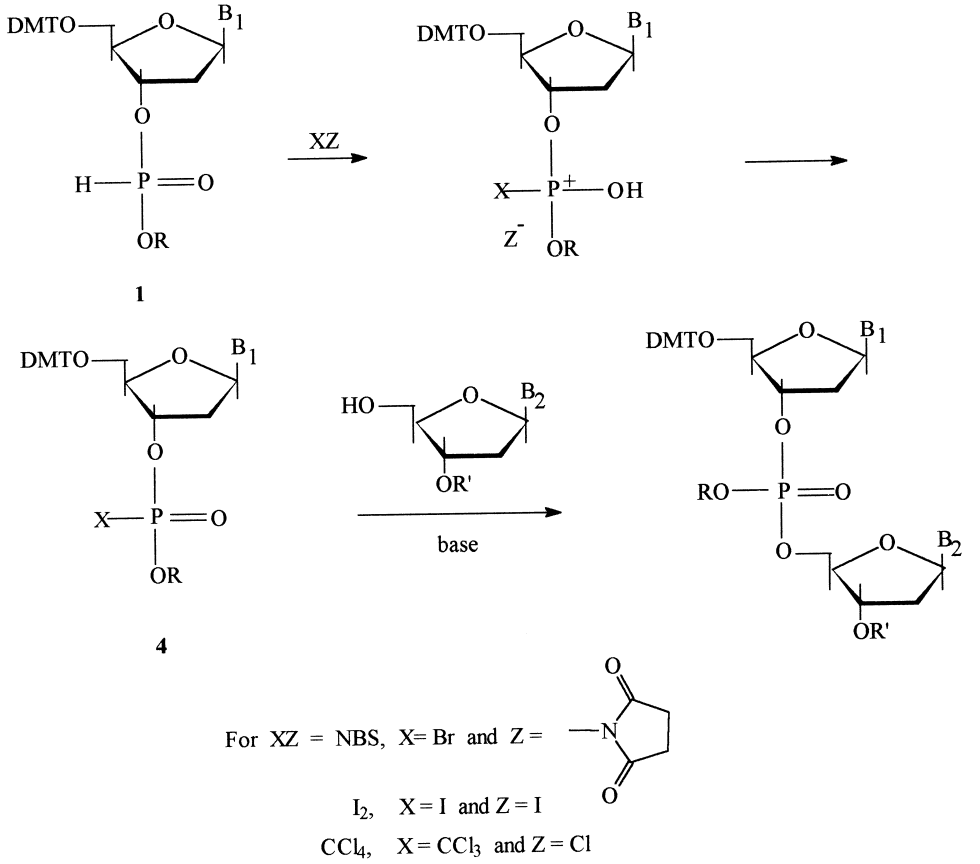
XZ = Coupling agent, NBS or I_2 or CCl_4

R = -CH_3 or -CH_2CH_2CN

R' = -OAc or -PEG

B_1 and B_2 are nucleoside bases.

Scheme 2. XZ = coupling agent, NBS or I_2 or CCl_4; R = -CH_3 or -CH_2CH_2CN; R′ = -OAc or -PEG; B_1 and B_2 are nucleoside bases.



Scheme 3.

was observed by 2.5 times excess of alkyl H-phosphonate and 5 times excess of NBS as judged by UV analysis (Table 3). ^{31}P NMR of d(AT) shows a singlet at δ 2.21 which indicates the product to be phosphotriester and not phosphitetriester; also the reverse phase HPLC elution time of the product matches with the authentic sample prepared by the solid phase phosphoramidite method (Fig. 1).

From all these studies a standard synthetic protocol for synthetic cycle as shown in Table 4 was set up.

Synthesis of d(ACGGGCCCGT) (100 μM)

The feasibility of the method was tested by the synthesis of d(ACGGGCCCGT) using the method described. The DMT absorption values have shown coupling efficiency of 98%. Finally deprotection, separation from PEG, and purification by NAP column afforded decamer 92% homogenous to HPLC (Fig. 2a). The overall yield of decamer was 75%. The product has been characterized by PAGE and HPLC elution time using the authentic sample of oligomer having the same sequence and prepared independently by solid phase method using phosphoramidite approach (Fig. 2b). Hydrolysis (snake venom phosphodiesterase and alkaline phosphatase) shows no base modification or wrong linkages as the oligomer afforded dA, dC, dG and dT in the predicted amount.

The results outline a method whereby alkyl H-phosphonate can be used as synthon for large scale, liquid phase synthesis of oligonucleotide. The synthons are easily prepared via readily scalable procedure and

Table 2. Coupling efficiency using different coupling agent, for the preparation of d(AT)

Coupling agent[a]	Coupling efficiency (%)		
	CH_2Cl_2/Py (4:1 v/v)	CH_3CN/Py (4:1 v/v)	CH_3CN/TEA (4:1 v/v)
CCl_4	60[b]	62	69
I_2	72	75	79
NBS	90	96	99

[a]10 times excess of both the coupling agent and nucleoside, β-CE-H-phosphonate were used.
[b]CCl_4 was used instead of CH_2Cl_2 as a solvent.

Table 3. Effect of excess equivalent of NBS, and β-CE-H-phosphonate on coupling efficiency

Sl no.	Equivalent of NBS	Equivalent of β CEH-phosphonate	Coupling efficiency (%)
1	10.0	10.0	98
2	5.0	5.0	98
3	5.0	2.5	98
4	2.5	5.0	96
5	2.5	2.5	95

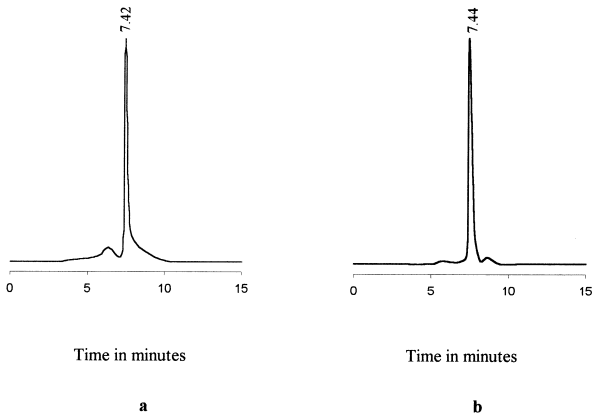


Figure 1. Reverse phase HPLC of d(AT). (a) Synthesized on PEG support by nucleoside β-CE-H-phosphonate approach. (b) Synthesized on solid phase by phosphoramidite approach.

Table 4. Oligonucleotide synthetic cycle

Step	Reagent	Quantity	Time (min)
Detritylation	3% DCA in DCM	20 ml	15
Coupling	β-CE-H-phosphonate (0.1M) in CH_3CN	2.5 equiv	5
	NBS (0.5 M) in CH_3CN	5.0 equiv	
Capping	Ac_2O	1.0 mL	3
	2,6-Lutidine	1.0 mL	
	NMI	1.0 mL	
	in CH_3CN	10.0 mL	

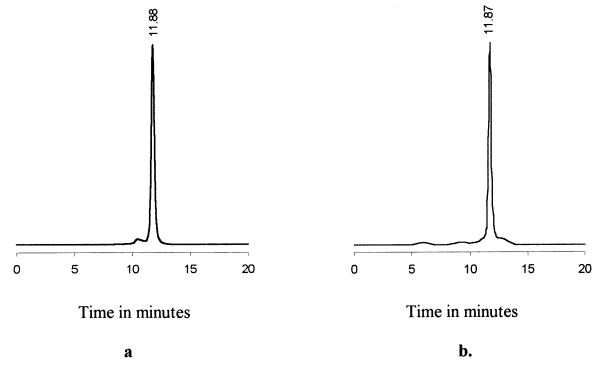


Figure 2. Reverse phase HPLC of d(ACGGGCCCGT). (a) Synthesized on PEG support by nucleoside β-CE-H-phosphonate approach. (b) Synthesized on solid phase by phosphoramidite approach.

appears to be stable towards normal laboratory condition. A particularly useful feature as well is the observation that activation with NBS or I_2 directly yield the dinucleoside phosphate as the only detectable product.

Conclusion

The described method offers the following advantages.

1. The reaction in the presence of an activating agent is fast with good yield of coupling.
2. The method is less time-consuming as it does not require an additional oxidation step, thereby reduces one step per synthetic cycle as compare to classical strategy. This is most important because the weight reduction of about 1% of the total amount has been observed during the repeated precipitation and filtration step.
3. Use of a cheap activating agent as compared to 1*H*-tetrazole used in the phosphoramidite method is an additional advantage.
4. The synthesis is performed in a homogenous solution where lower excess of reagent is required and appreciable cost saving are possible.
5. Large amounts of oligomer can be obtained from one synthetic run.
6. The synthesis is easily monitored by non-destructive spectrophotometric method.

Experimental

Polyethylene glycol monomethyl ether-5000 (Fluka) was purified by crystallization from dichloromethane (DCM): diethyl ether and dried under vacuum over KOH pellets. HPLC grade acetonitrile (E. Merck, Germany), was predried over activated 4 Å molecular sieves and distilled over CaH_2. 4-Dimethylaminopyridine (DMAP), dicyclohexylcarbodiimide (DCC), *N*-methylimidazole (NMI) and 1-*H*-tetrazole were purchased from Aldrich. All the nucleoside phosphoramidites were commercially available from Glen research, USA. Anhydrous diethyl ether was purchased from Spectrochem, India. All glass equipments were oven dried and stored in a dessicator over KOH pellets.

NMR spectra were recorded on a Bruker AMX 500 spectrometer, HPLC were carried out on a Perkin–Elmer, using RP C-18 column, with Et_3N:HOAc (pH = 7) and CH_3CN gradient of 1%/min starting from 0%, flow rate 1 mL/min. Polyacrylamide gel electrophoresis (PAGE) was performed on a Hoefer instrument. TLC were performed on a precoated silica gel sheet 60 F_{254} (Merck, Germany).

Synthesis of 5′-*O*-(*p*,*p*′-dimethoxytrityl)2′-deoxynucleoside 3′-(2 cyanoethyl)/methyl H-phosphonate. 5′-*O*-(*p*,*p*′-Dimethoxytrityl)2′-deoxynucleoside 3′-(2 cyanoethyl): methyl *N*,*N*-diisopropylphosphoramidite (1 mmol), was dissolved in CH_3CN (10 mL). 0.30 mL of H_2O and 0.10 g of tetrazole were added, the mixture was stirred at rt and the reaction was followed by TLC (solvent system: ethyl acetate:petroleum ether (8:2)). After completion of the reaction, solution was concentrated and the residue was partitioned between DCM and water. The organic phase was dried over anhydrous Na_2SO_4 and evaporated to yield **1** (87–93%).

1a. 5′-*O*-(*p*,*p*′-Dimethoxytrityl) thymidine 3′-(2 cyanoethyl) H-phosphonate (yield = 92%). ^{1}H NMR (CD_3CN): δ 1.48 (s, 3H, CH_3), 2.15 (t, 2H, CH_2CN), 2.60 (m, 2H, $H_{2'}$), 2.90–2.95 (m, 2H, $H_{5'}$), 3.45–3.52 (m, 2H, CH_2O), 3.80 (s, 6H, CH_3,OAr), 4.31–4.36 (m, 2H, $H_{3'}$, $H_{4'}$), 6.41 (t, 1H, $H_{1'}$), 6.92 (d, 4H, $J = 8.5$ Hz, protons *ortho* to OCH_3 of DMTr), 7.26–7.52 (m, 9H, Ar), 7,60 (s, 1H, H-6),

6.27 and 7.73 (d, 1H, H-P, $J=730$ Hz). ^{31}P NMR (CD_3CN) δ 7.19 (d, $J=729$ Hz).

1b. 5′-*O*-(*p*,*p*′-Dimethoxytrityl) 2′-deoxycytidine 3′-(2 cyanoethyl) H-phosphonate (yield = 90%). ^{1}H NMR (CD_3CN): δ 2.61 (m, 1H, $H_{2'}$), 3.01 (m, 5H, $H_{2''}$, CH_2CN, $H_{5'}$), 3.57 (m, 2H, CH_2O), 3.85 (s, 6H, CH_3O-Ar), 4.36–4.43 (m, 1H, $H_{4'}$), 4.47 (m, 1H, $H_{3'}$), 6.30 (m, 1H, $H_{1'}$), 6.98 (d, 4H, $J=8.5$ Hz, protons *ortho* to OCH_3 of DMTr), 7.29–7.70 (m, 14H, Ar-H), 8.21 (d, 1H, H-6), 6.35 and 7.77 (d, 1H, $J=710$ Hz, H-P). ^{31}P NMR (CD_3CN): δ 7.16 (d, $J=708$ Hz).

1c. 5′-*O*-(*p*,*p*′-Dimethoxytrityl) 2′-deoxyadenosine 3′-(2 cyanoethyl) H-phosphonate (yield = 87%). ^{1}H NMR (CD_3CN): δ 3.02 (t, 2H, CH_2CN), 3.09 (m, 2H, $H_{2'}$), 3.54–3.57 (m, 2H, $H_{5'}$), 3.59 (t, 2H, CH_2O), 3.91 (s, 6H, CH_3O), 4.51 (m, 1H, $H_{4'}$), 4.59 (m, 1H, $H_{3'}$), 6.76 (m, 1H, $H_{1'}$), 6.98 (d, 4H, $J=8.6$ Hz, protons *ortho* to OCH_3 of DMTr), 7.36–7.62 (m, 14H, Ar-H), 8.56 (s, 1H, H-8), 8.69 (s, 1H, H-2), 6.46 and 7.9 (d, 1H, $J=720$ Hz). ^{31}P NMR (CD_3CN): δ 7.06 (d, $J=721$ Hz).

1d. 5′-*O*-(*p*,*p*′-Dimethoxytrityl)2′-deoxyguanosine 3′-(2 cyanoethyl) H-phosphonate (yield = 85%). ^{1}H NMR (CD_3CN): δ 1.12 (d, 6H, $2CH_3$), 2.35 (m, 1H, CHCO), 2.58–2.75 (m, 4H, CH_2CN, $H_{2'}$), 3.26–3.47 (m, 4H, CH_2O, $H_{5'}$), 3.75 (s, 6H, CH_3O-Ar), 4.17 (m, 1H, $H_{4'}$), 4.24–4.30 (m, 1H, $H_{3'}$), 6.15 (m, 1H, $H_{1'}$), 6.75 (d, 4H, $J=8.6$ Hz, protons *ortho* to OCH_3 of DMTr), 6.74–7.42 (m, 9H, Ar-H), 7.73 (s, 1H, H-8), 6.22 and 7.67 (d, 1H, $J=725$ Hz, P-H). ^{31}P NMR (CD_3CN): δ 6.62 (d, $J=726$ Hz).

1e. 5′-*O*-(*p*,*p*′-Dimethoxytrityl)2′-deoxycytidine 3-methyl H-phosphonate (yield = 95%). ^{1}H NMR ($CDCl_3$): δ 2.38 (m, 1H, $H_{2'}$), 2.90 (m, 1H, $H_{2''}$), 3.47–3.2 (m, 3H, $H_{3'}$, $2H_{5'}$), 3.71 (m, 3H, CH_3OP), 4.34 (m, 1H, $H_{4'}$), 6.30 (m, 1H, $H_{1'}$), 6.86 (d, 5H, $J=8.6$ Hz protons *ortho* to OCH_3, of DMTr, H-5), 7.23–7.51 (m, 14H, Ar-H) 6.12 and 7.54 (d, 1H, $J=710$ Hz, P-H), 8.18 (dd, $J=4.5$ Hz and 7.1 Hz, H-6), 8.8 (br, 1H, NHCO). ^{31}P NMR ($CDCl_3$): δ 7.74 (d, $J=710$ Hz).

1f. 5′-*O*-(*p*,*p*′-Dimetboxytrityl)2′-deoxyadenosine 3′-methyl H-phosphonate (yield = 89%). ^{1}H NMR ($CDCl_3$): δ 2.80 (m, 1H, $H_{2'}$), 3.11 (m, 1H, $H_{2''}$), 3.45 (m, 2H, $H_{5'}$), 3.78 (s, 6H, CH_3O-Ar), 3.80 (m, 3H, CH_3O-P), 4.4 (m, 1H, $H_{4'}$), 6.52 (m, 1H, $H_{1'}$), 6.80 (m, 4H, $J=8.5$ Hz protons *ortho* to OCH_3 of DMTr), 7.00–7.50 (m, 9H, ArH), 6.17 and 7.55 (d, 1H, $J=690$ Hz, P-H). ^{31}P NMR ($CDCl_3$): δ 7.87 (d, $J=692$ Hz).

1g. 5′-*O*-(*p*,*p*′-Dimethoxytrityl) 2′-deoxyguanosine 3′ methyl H-phosphonate (yield = 87%). ^{1}H NMR ($CDCl_3$): δ 1.12 (d, 6H, $2CH_3$), 2.40 (m, 1H, CHCO), 2.8 (m, 2H, $H_{2'}$), 3.12 (m, 2H, $H_{5'}$), 3.63 (m, 3H, CH_3OP), 3.78 (s, 6H, CH_3OAr), 3.81 (m, 1H, $H_{4'}$), 4.24 (m, 1H, $H_{3'}$), 6.16 (m, 1H, $H_{1'}$), 6.81 (d, 4H, aromatic protons *ortho* to CH_3O of DMTr, $J=8.8$ Hz), 7.15–7.36 (m, 9H, ArH), 7.82 (s, 1H, H-8), 6.05 and 7.47 (d, 1H, $J=710$ Hz, P-H). ^{31}P NMR ($CDCl_3$): δ 8.54 (d, $J=712$ Hz).

Functionalisation of PEG

One gram of PEG was dehydrated by coevaporation with anhydrous CH_3CN and dissolved in 10 mL of DCM. 0.6 mmol of 5′-*O*-DMT-2′-deoxynucleoside-3′-*O*-succinate was dissolved in 5 mL of DCM and 0.33 mmol of dicyclohexylcarbodiimide (DCC) was added under stirring at 0 °C. After 15 min the solution was filtered, poured into the PEG solution and 0.33 mmol of DMAP was added. The final solution was concentrated and left to react for 24 h, at rt. The solution was filtered and the modified polymer precipitated under stirring at 0 °C with an excess of diethyl ether (about 10 vol). It was further purified by crystallization from DCM/diethyl ether. The -OH group have been esterified in 70–75% yield, which corresponds to 140–150 μM of nucleoside/g of PEG, as determined by measuring the DMT absorption at 489 nm.

The unreacted OH groups of PEG were acetylated by reacting with 10 mL of acetonitrile solution containing 10% of acetic anhydride, 10% 2,6-lutidine and 10% *N*-methylimidazole (v/v). The reaction mixture was kept at room temperature under stirring for 3 min. The polymer was precipitated from the ice-cooled solution with diethyl ether, filtered and dried under vacuum over KOH pellets.

General procedure for oligonucleotide assembly

Detritylation. One gram of 5′-DMT-nucleoside 3′-*O*-PEG was dissolved in 10 mL of DCM. 10 ml of 6% dichloroacetic acid (DCA) in DCM was added dropwise under stirring. After 15 min the polymer was precipitated at 0 °C, with diethyl ether (200 mL), washed with diethyl ether and then filtered. The extent of deblocking was controlled qualitatively by TLC (no orange colour present with acidic spraying) and quantitatively by UV analysis. If some DMT is still present, the DCA treatment was repeated. The final polymer was recrystallized from DCM/diethyl ether and dried under vacuum over KOH pellets.

Condensation. One gram of 5′-OH-nucleoside-3′-*O*-PEG was coevaporated 3 times with anhydrous acetonitrile, dried under high vacuum and dissolved in CH_3CN:TEA (1.5:1.0 v/v), to the solution were added 2.5 equiv of alkyl H-phosphonate from a 0.1 M solution in CH_3CN and 5.0 equiv of NBS from a 0.5 M solution in CH_3CN. The solution was stirred under nitrogen atmosphere for 5 min. The polymer was precipitated from the ice cooled solution with 10 vol of ether and recrystallized from CH_3CN:ether. The yield of the reaction was evaluated spectroscopically from the DMT absorption, after a TLC analysis to ascertain the complete removal of reagents and by-products.

Capping. The unreacted 5′-OH groups were acetylated by reacting with 10 mL of CH_3CN solution containing 10% of acetic anhydride, 10% of 2,6-lutidine and 10% of *N*-methylimidazole (v/v). The reaction mixture was kept at room temperature under stirring for 3 min. The PEG was precipitated from the ice-cooled solution with

diethyl ether, filtered and dried under vacuum over KOH pellets.

Deprotection and purification. The final PEG-oligonucleotide (DMT-off) was dissolved in 30% aqueous ammonia and reaction mixture was allowed to stand at 60 °C for 24 h after cooling the aqueous solution was extracted three times with 25 mL of ether. The oligonucleotide was separated from PEG by precipitation with an excess of methanol as described[3] (where PEG is soluble), and following centrifugation.

Acknowledgements

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