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A Liquid-Phase Process Suitable for Large-Scale Synthesis of Phosphorothioate Oligonucleotides

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Abstract:

A new process for the preparation of large amounts of thioate oligonucleotides in a quasi-classical solution condition is described. This method takes advantage of the use of poly(ethylene glycol) as a soluble, inert support during the synthesis. The quality and amount of the desired oligonucleotides are improved by the use of pre-formed dimeric phosphoramidite as synthons. The easy intermediate purification from moderate excess of reagents allows obtaining very high coupling yields, and, consequently, the efficient production of quite long sequences as those required for their pharmacological applications.

Introduction

The investigation of therapeutic properties of synthetic oligonucleotides, and of their chemically modified analogues, has reached different stages of the clinical trial including the approval of first antisense drug, Vitravene.[1] The current studies, together with the view of a successful commercialization, have triggered the demand of large-scale oligonucleotide synthesis. High coupling efficiency, low reagent consumption, simple and cost-efficient purification are crucial factors for successful production of oligonucleotide-based drugs.[2] Solid-phase automated synthesis has been the method of choice due to ease of operation.[3] However, despite the development of new high-capacity synthesizers, the application of such a technique for the production of large quantities of oligonucleotides still remains problematic. The limitation is mainly due to the heterogeneity of the reaction, which leads to the use of quite large excess of high-cost monomers to achieve high coupling efficiencies. The liquid-phase synthesis was proposed as an answer to the above-mentioned limitation.[4] In this technique, the sequential synthesis is performed, bearing the growing oligonucleotide chain attached to a soluble supporting polymer, and the chain elongation is then carried out in homogeneous media. The polymer-bound growing oligomer is usually recovered from the reaction mixture by precipitation, thus allowing rapid elimination of excess reagent and soluble by-products. In a new liquid-phase method called high efficiency liquid phase (HELP), poly(ethylene glycol) (PEG), with a molecular weight ranging between 5 and 20 kDa, is used as a soluble supporting polymer for oligonucleotide synthesis.[5] The oligonucleotide chain is joined through a succinate linkage. Once completed, the oligonucleotide is released from the support under standard conditions and purified by chromatographic methods. Oligomers of up to 20 nucleotides in length have been synthesized following this approach. This method has also been applied for synthesis of a phosphorothioate analogue[6] in which a nonbridging oxygen is formally replaced by a sulfur atom to overcome the instability of natural DNA towards degradative enzymes.[7] Usually, sulfurization of the phosphite backbone is carried out as a repeating step during each elongation cycle. Thus, conversion of the phosphite moiety to phosphorothioate needs to be performed at the highest efficiency, to achieve high purity of oligonucleotide-based drugs. As a consequence, the improvement of thiolation reaction by the use of new sulfurizing reagents is becoming one of the main aspects of research in this field. Furthermore, the introduction of fully protected phosphorothioate dimers during the coupling step, instead of that of monomers, reduces the number of coupling and thiolation steps (and consequently, the numbers of likely unconverted phosphite linkages) and will certainly result in higher homogeneity of the final phosphorothioate backbone, as recently demonstrated.[8] The feasibility of the HELP method for the large-scale synthesis of phosphorothioate oligonucleotides has been studied, introducing the above-mentioned phosphorothioate dimer blocks and a new sulphurizing reagent. The dimeric synthons are fully protected 3′→5′ phosphorothiate dinucleotides, with a phosphoroamidite group at the 3′-position, as a reacting moiety for coupling with the 5′-hydroxyl of the growing oligonucleotide chain. Sulfurization of the phosphite triester moiety is carried

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out during each synthetic cycle with the recently developed diethyldithiocarbonate disulfide (DDD), which looks more advantageous over traditional sulphurizing reagents.[9] A successful millimolar-range scale of synthesis of a medium-sized oligonucleotide is reported.

Results and Discussion

One of the main features of this research has been the use of a blockmer strategy, as previously indicated. It is expected that the use of blockmers may reduce the levels of shorter deletion sequences derived from the failures during synthesis. The $n - 1$ deletionmer is especially difficult to purify by the use of standard C18 reversed phase HPLC. Since coupling and sulfurization inefficiencies may be the reasons for its formation, a key to reducing this and similar by-products is the use of pre-formed dimeric units in the stepwise synthesis of the oligomer. In this regard, we have focused our attention on the search for the best reaction conditions, as a function of the solubility of these dimers, and subsequent purification procedures, for their utilization in a liquid-phase procedure. A further advantage in the use of dimeric units is the reduction of the number of synthetic steps required. Moreover, it must be remembered that during the liquid-phase procedure all of the intermediate purifications are achieved through a precipitation-and-filtration procedure that leads to a low but unavoidable loss of product. This loss becomes quite important in case of several repeated processes, as those required for the synthesis of longer sequences. For these reasons, the use of preformed dimeric building blocks is obviously quite appealing.

During this investigation, two different kinds of supporting monomethoxy polyethylenglycol (MPEG) were selected—a lower molecular weight MPEG (5000 Da) for the synthesis of a shorter oligonucleotide (an 8mer), and a higher molecular weight MPEG (10 000 Da) for the synthesis of a 15mer. In both cases the starting MPEG functionality, a primary OH group, has been derivatized by reaction with a 3′-succinate derivative, through a standard DCC activation, of the dimer of choice. The yield of functionalization was similar to that obtained with a monomer (97%, instead of 97.5%); a slightly lower efficiency (93%) was observed with the reaction on MPEG 10 000.

As a first study, the minimum number of equivalents required for maximum coupling efficiency was investigated. To estimate the minimum amount of dimer phosphoramidite required for nearly quantitative reaction, synthesis of a 4-mer was performed with different amounts of reagents; 2.5 equiv of amidite resulted in a coupling efficiency of 96−97%, whereas the use of 3.0 equiv led to an almost quantitative yield (99−100%). Consequently, this higher amount of reagent, even if economically less advantageous, was selected for all synthetic steps to get higher yield of the final product.

The sulfurization step represented one of the key reactions in the entire process. In this reported method the new sulfurizing reagent, that is, diethyldithiocarbonate disulfide (DDD), was employed. This reagent worked efficiently, using 10-fold excess and a contact time of 15 min, in acetonitrile solvent. Excess reagent was removed by standard procedure, namely precipitation from ether and subsequent filtration. A further modification over the previously reported procedure [6] is the capping step. To reduce any possible deprotection of the cyanoethyl group from the phosphorothioate triester linkage, the overall step was performed at 0 °C.

The first synthetic study was performed on lower molecular weight MPEG. In this case, only a shorter oligonucleotide was produced, since the liquid-phase approach demands that there be a correct ratio between the dimension of the soluble supporting polymer and that of the oligomer to be synthesized. This is due to the logical need that the supporting unit must always "support" the synthesized product, and not vice versa. A lower molecular weight offers an additional advantage of a higher number of sites to be derivatized per gram. In fact, the MPEG 5,000 corresponds to 0.2 mmol of free OH groups for each gram of polymeric chains. Doubling the molecular weight will allow only for one-half of the reacting groups for the same amount of MPEG.

To verify the feasibility of the proposed process on a large scale, 10 g of starting MPEG 5000 was employed in the *first millimolar-scale synthesis of an oligonucleotide on a bench.* It is noteworthy that the HELP synthesis of oligonucleotides will allow for a convenient scaling up of the entire procedure, at least in principle, since it operates in fully homogeneous conditions as do most other organic reactions. This must be kept in mind if a convenient industrial procedure has to be devised for the preparation of commercial amounts of oligonucleotides. The following oligonucleotide sequence was synthesized as an example of a potential commercially interesting product. The sequence, namely, d[PS(TTGGGGTT)], was obtained by three consecutive cycles of the steps demanded for the oligonucleotide growing. A description of a single cycle is reported in Scheme 1. As previously indicated, the initial dimer has been introduced as the 3′-succinate dimer. The main synthetic data, as well as the recovered amount of product, after the single cycle, and at the end of the entire process, are reported in Table 1. It is clearly highlighted that, even with the use of the dimeric units, the overall survey of the results is in agreement with other findings already obtained with this approach.[10]

As a further investigation, the synthesis of a fully thioate 15mer, expected to be of interest for its application as new drug, was performed on a MPEG 10,000 support. 2.0 g of polymer, for a total of 0.2 mmol of maximum obtainable oligonucleotide, was utilized. During the entire process the pre-constructed dinucleotides have been employed, save the introduction of a single guanosine residue in position 13 (the final oligonucleotide presents an odd number of residues).

In Table 2 the main synthetic data are reported, together with the overall amount of recovered product. The results are in line with the expected values. The lower average yield observed with the MPEG 10 000 can be attributed to a reduced reactivity of the growing chain toward the end of

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Scheme 1. **Liquid-phase synthesis of phosphorothioate oligonucleotides on MPEG support (HELP method) using dimeric synthons**

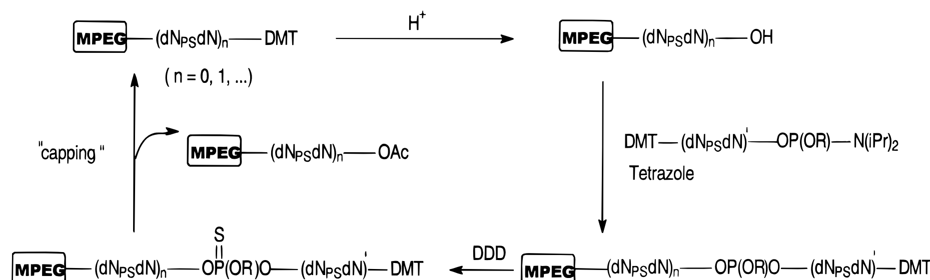

Table 1. **Synthetic data of thioate d(TTGGGGTT) on MPEG 5000**

overall yield	90.0%
average yield	96.5%
overall recovery	74.5%

Table 2. **Synthetic data of thioate 15mer on MPEG 10 000**

overall yield	58.0%
average yield	92.5%
overall recovery	66.0%

Table 3. **Estimated reagent and solvent consumption for a millimolar-scale synthesis of the thioate 15mer on 10.0 g of MPEG 10 000 (maximum theoretical amount produced: 5.175 g)**

MTBE	45.0 L
AcCN	2.5 L
DCE	3.5 L
EtOH	14.0 L
dimeric amidites	15.0 g
tetrazole	4.0 g
DDD	12.5 g

the synthesis. This behavior cannot be foreseen; hence, as a general rule, the yield of each coupling step must be always carefully evaluated to verify if a further recycling of this step is demanded for a quantitative reaction.

In Table 3, a rough evaluation of the expected amount of reagents and solvent required for the synthesis of the 15mer on a millimolar scale is reported. These values are derived from the extrapolation of the measured amount of the materials used in the above-described synthesis. As expected, a larger amount of solvent is required for the repeated precipitation-and-filtration steps needed to complete the synthesis. However, an easy recovering process of the solvents used can be, and must be, easily devised as well as for the recovering of the unused excess of dimers.

In Table 4 the time required to complete a single step, and the overall synthesis, is reported. It can be safely assumed that the reaction time will not increase as result of a further scaling up of the process. On the other hand, it is also obvious that the intermediate purification will demand an increased time as a function of the increased amount of material to be precipitated and filtered. However, we can reasonably assume that the total time needed for the production of an even larger amount of product will be acceptable and will not greatly overcome that expected for a large-scale production of

Table 4. **Estimated average production time for the preparation of 1.0 mmol of thioate 15mer on MPEG 10,000**

Reaction Times (Single Step)	
detritylation[a]	15 min (+ 15 min)
coupling[b]	5 to 15 min
sulphurisation	15 min
capping	3 min
overall time	53 to 68 min
Intermediate Purification Times (Single Step)	
precipitation with MTBE	4 (+1)[c] × 20 min
recrystallization from EtOH	60 min
overall time	up to 3.0 h
Overall Time	
time for single synthetic cycle	2.5 h
time for 15mer synthesis	35 (+14)[d] h

[a] Second acidic treatment is often demanded for a complete DMT removal. [b] Larger reaction time can be required as the oligonucleotide chain length is growing. [c] Further step is required if a second acidic treatment occurs [d] One average hour per cycle can be reasonably added on the whole, to account for some reactions asked to be repeated.

oligonucleotides on a solid-phase support, when possible.

The final oligonucleotide is detached from the MPEG support and freed from all of the protecting groups, following the basic and acidic conditions of standard oligonucleotide synthesis.

Particular attention has been made to the removal, and the possible recovery, of the supporting polymer, that could also interfere during the chromatographic purification. The precipitation of the oligonucleotide from the cool acetone suspension of the crude deblocked mixture allows for the recovery of 90−95% of MPEG, and near the overall oligonucleotide, separately. The high solubility of polymer in acetone, and the high insolubility of the oligonucleotide product, sustains these favorable results. The chromatographic purification has been attempted both by reverse phase (RP) as well as strong anion-exchange (IE) HPLC. In our hands the separation of shorter sequences looks to be quite inefficient by RP-HPLC, while the IE chromatography gives a clearer pattern and an easier process. In Figure 1 the IE exchange of the 8mer synthesized on MPEG 5000 and of the 15mer obtained with a MPEG 10 000, both crude and purified, are reported. The desalting was performed by a low-pressure gel-filtration process on a Sephadex G-25 resin, or by a RP-HPLC on a C18 column. An alternative ultrafiltration process through a 1000 MW cutoff membrane has been exploited, but the recovering of product was time-consuming, with a final recovery lower than 80%. In our

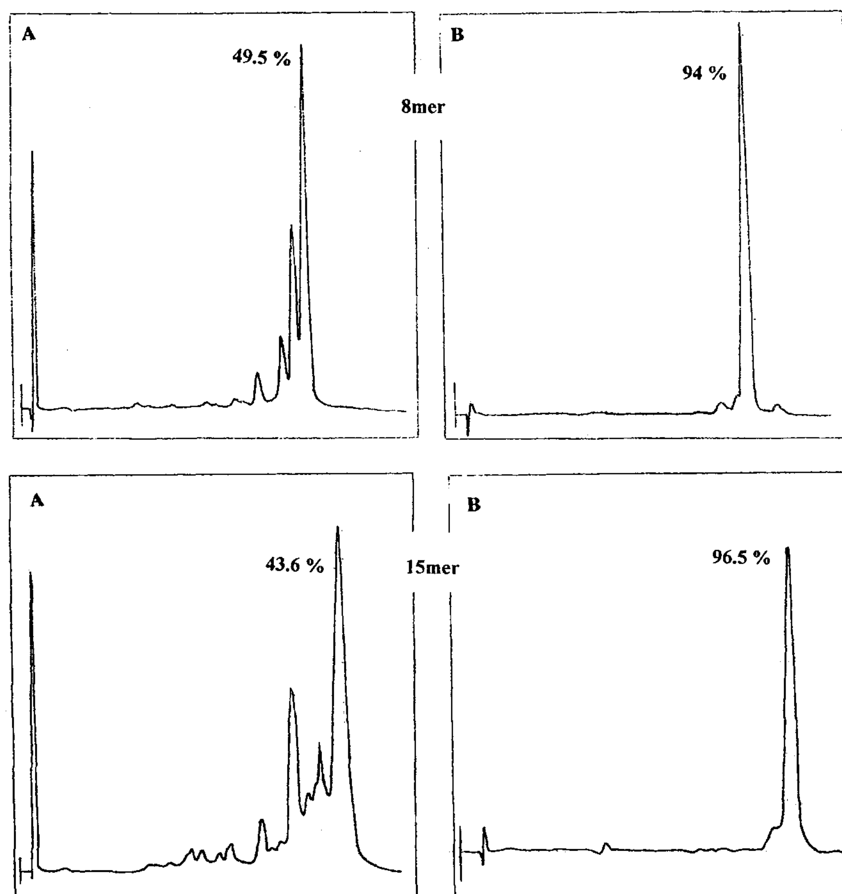

Figure 1. **IE-HPLC of crude (A) and purified (B) oligonucleotides synthesized on a large scale, liquid-phase process by using dimeric synthons.**

hands, a precipitation with cold 2-propanol of the oligonucleotide from its aqueous solution[11] failed, since the product remained mostly in solution, possibly due to its short sequence.

Conclusions

The large-scale synthesis of phosphorotioate oligonucleotides, as well as of natural sequences, can be efficiently performed by the adoption of this new time-saving use of dimeric synthons on a liquid-phase process. The quality of the final product is satisfying, as expected from a process requiring a reduced number of synthetic steps. On the basis of the overall feature of the method, and on the basis of the acquired experience, it can be safely foreseen that a larger-scale process as demanded for a commercial production will be really effective.

Among the pros of the described procedure it must be recalled:

a. An almost complete derivatization of the starting MPEG support with a dimeric 3′-succinate block was achieved.

b. An average and overall yield fully comparable with those observed in solid-phase based approaches was observed.

c. A reasonable amount of high quality dimeric reagents is demanded for high-yielding synthesis.

d. An efficient deblocking procedure and contemporary removal of the polymeric support, whose recovering can be devised, was obtained.

e. An equitable overall working time, especially if projected toward a very high scale process, was achieved.

Special attention must be also put to the following points:

a. A large amount of precipitating solvent is required, but a properly devised side-collecting and recycling can be reasonably arranged.

b. A careful evaluation of the intermediate condensation yields is strictly required after each step to ensure a minimum amount of failure sequences, since some low-yield coupling can be unexpectedly found as a function of length and type of the growing sequence.

c. The amount of obtainable product will be a function of the size of the supporting MPEG that must be carefully selected to ensure a full solubilisation of the product during the whole process.

d. Absolute care in drying all solvents and reagents, mainly during the coupling step, is needed.

Experimental Section

General. Acetonitrile (AcCN), *tert*-butyl methyl ether (TBME), 1,2-dichloroethane (DCE) and pyridine were stored under argon, over 4 Å molecular sieves activated at 350 °C for 3−4 h, and cooled under vacuum. Poly(ethylene glycol) monomethyl ether (MPEG) was purchased from Shearwater and stored at 4−7 °C. All other chemicals were used as

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purchased. Diethyldithiocarbonate disulfide (DDD), as well as the phosphoramidite dimers, was provided by ISIS Pharmaceuticals.

Reagent solutions. 1*H*-tetrazole in AcCN. Typically, 1H-tetrazole (0.5 M, 2 g), in a bottle sealed with a rubber cap, was dissolved in dry AcCN (57.1 mL) and injected with a syringe. The solution was stored under argon, at room temperature.

DDD in AcCN. Typically, DDD (0.5 M, 1.21 g) was dissolved in dry AcCN (10 mL), in a dark bottle. The solution was stored under argon, at 4−7 °C.

TCA (6%, w/v) in DCE. Detritylation solution was prepared before use by dissolving TCA (6 g) in dry DCE (100 mL). The unused solution was stored in a dark bottle at 4−7 °C.

5′-DMT-d[phosphorothioate dimer]-3′-phosphoramidite (0.2 M) in AcCN. Typically, the required 5′-DMT-phosphorothioate dimer 3′-phosphoramidite, in a dark bottle sealed with rubber cap, was dissolved with the proper amount of dry AcCN and injected with a syringe. The solutions were stored under argon, at −20 °C.

TLC was performed on precoated silica gel sheets 60 F_{254}, eluted with chloroform/ethanol, 9:1 (v/v; +0.5% pyridine), visualized using a UV light source (254 nm), and developed by spraying 60% perchloric acid/ethanol, 3:2 (v/v). DMT-bearing compounds show orange-coloured spots. MPEG-bearing compounds were also detected by exposure to iodine vapour.

UV spectra were recorded on a Perkin-Elmer Lambda 5 spectrophotometer. Chromatographic analysis and purification were performed with a HPLC Gilson 306, a HPLC Jasco 880, and a Pharmacia FPLC system.

Water content of solvents was measured with a coulometer Metrohm Karl-Fisher Automat E547. Glassware used for oligonucleotide synthesis was dried in an oven (110 °C) and stored in a desiccator over KOH pellets, prior to use. Polypropylene sterile syringes were single-used.

Functionalisation of MPEG with Succinate Dimer Block

Synthesis of 5′-DMT-(thymidine-3′-*β*CE-phosphorothioate-5′-thymidine)-3′-succinate. 5′-DMT- (thymidine-3′-*β*CE-phosphorothioate-5′-thymidine)-3′-OH [5′-DMT-d(T_{PS}T)-3′$_{Succ}$-OH] (3 g, 3.3 mmol) was co-evaporated with dry pyridine (2 × 20 mL) and dissolved in dry pyridine (14 mL) in a 250 mL round-bottom flask. DMAP (0.202 g, 1.65 mmol) was then added, and succinic anhydride (0.33 g, 3.3 mmol) was slowly added during 30 min, under stirring. The solution was protected against light and stirred, under an argon atmosphere, at room temperature for 24 h. The reaction was monitored by TLC. The solution was evaporated to dryness and then co-evaporated with toluene (3 × 20 mL) to remove traces of pyridine. The thick oil obtained was dissolved in dichloromethane (30 mL) and washed in a 250 mL separatory funnel with 10% citric acid (w/v, 60 mL), previously cooled in an ice bath. The aqueous solution was extracted with dichloromethane (2 × 20 mL), and the organic layers were pooled and washed with water (2 × 50 mL). The organic layer was dried over anhydrous Na_2SO_4 and evaporated to dryness. The thick oil obtained was dissolved in dichloromethane/0.5% pyridine (v/v, 14 mL) and dropped into a stirred mixture of *n*-hexane/ether, 1:1 (v/v, 140 mL), cooled in ice bath. A white powder was recovered after filtration through a sintered funnel and dried under vacuum over KOH pellets. The product (1.88 g) was stored at 4−7 °C.

The crude product was purified by flash chromatography, using a silica gel (Merck, Kieselgel 60, 70−230 mesh) column (100 × 35 mm), eluted with a stepwise gradient of chloroform/methanol (+0.5% pyridine), 0−10%. Each fraction was checked with TLC. Product-containing fractions were pooled and evaporated to dryness, dissolved in dichloromethane (10 mL) and added dropwise to a stirred solution of *n*-hexane/ether, 1:1 (100 mL), cooled in ice bath. A white powder was recovered after filtration through a sintered funnel and dried under vacuum over KOH pellets. The product (1.16 g) was stored at 4−7 °C.

Synthesis of 5′-DMT-(thymidine-3′-*β*CE-phosphorothioate-5′-thymidine)-3′$_{Succ}$−MPEG. 5′-DMT-d(T_{PS}T)-3′$_{Succ}$-OH (0.916 g, 0.9 mmol) was co-evaporated with dry AcCN (2 × 10 mL) and dissolved in dry DCE/0.5% pyridine (v/v, 3 mL) in a 100 mL round-bottom flask. The solution was then cooled in an ice bath, and DCC (0.103 g, 0.5 mmol) was added under stirring. After 15 min the solution was filtered through a sintered funnel, poured into a 250 mL round-bottom flask containing MPEG 5000 (1.5 g, 0.3 mmol) previously co-evaporated with dry AcCN (2 × 10 mL), and dissolved in dry DCE/0.5% pyridine (v/v, 7.5 mL). NMI (0.05 mL, 0.6 mmol) was added under stirring, and the solution was protected against light and stirred, under an argon atmosphere, at room temperature for 24 h. The reaction was monitored by TLC, since MPEG-bound derivatives always show an $R_f = 0$. The solution was filtered through a sintered funnel, in a 250 mL round-bottom flask, and the reaction volume was reduced to one-third by evaporation and cooled in an ice bath. MTBE (70 mL) was then added dropwise to the cooled solution, under vigorous stirring, during 20 min. A white powder was obtained, filtered through a sintered funnel, extensively washed with dry ether, and dried under vacuum over KOH pellets. The complete removal of the unreacted excess of reagents was ascertained by TLC; under these conditions 5′-DMT-d(TPST)-3′$_{Succ}$−MPEG has an $R_f = 0$. The crude product was suspended in EtOH (100 mL) at 38 °C. A white residue (mainly unreacted dimeric succinate) remained undissolved. The mixture was rapidly filtered, and the filtrate was allowed to stand at 5−7 °C for 1 h. A white powder was recovered by filtration through a sintered funnel. The product (1.58 g) was stored at 4−7 °C.

The degree of functionalisation was estimated spectrophotometrically as follows: an aliquot of product (5 mg) was dissolved in 60% perchloric acid/ethanol, 3:2 (v/v, 10 mL), the resulting solution was diluted 10-fold, and the absorbance at 498 nm was measured. The loading of MPEG was calculated (in μmol/g) from the equation: [A_{498} (10 mm cell) × 10 × 14.3]/mg of weighed support.

Functionalisation: 161 μmol/g (theoretical 166.6 μmol/g).

When MPEG 10 000 was employed, for each gram of polymer, dissolved in 2.5 mL of DCE/0.5% pyridine 0.303 g of d(T_{PS}T)-3′$_{succ}$ were used.

Fuctionalisation: 84 μmol/g (theoretical 90.9 μmol/g).

Acetylation of 5′-DMT- (thymidine-3′-βCE-phosphorothioate-5′-thymidine)-3′$_{Succ}$−MPEG (Capping). 5′-DMT-d(TPST)-3′$_{Succ}$−MPEG (1.58 g) was dissolved in dry AcCN (8 mL) in a 250 mL round-bottom flask. The solution was cooled in an ice bath and 2,6-lutidine (0.8 mL), NMI (0.8 mL) and acetic anhydride (0.8 mL) were added under stirring. The solution was left to react at 0 °C for 3 min. MTBE (70 mL) was added dropwise, under vigorous stirring. A white powder was recovered after filtration through a sintered funnel, extensively washed with ether, and dried under vacuum over KOH pellets.

Elongation of the Oligonucleotide Chain.

As an example, the detailed procedure for the synthesis of a 4mer, starting from the 2mer linked to the supporting polymer, is described. The growing of the chain follows exactly the same order of reactions, up to the desired sequence length.

Detritylation of 5′-DMT-d(TPST)-3′$_{Succ}$−MPEG. 5′-DMT-TPST-3′$_{Succ}$−MPEG (5.56 g, 0.9 mmol) was dissolved in dry DCE (50 mL) in a 1 L round-bottom flask. TCA (6%, w/v, 50 mL) in DCE was added dropwise under vigorous stirring. After 15 min the solution was cooled in an ice bath, and the MPEG−oligonucleotide precipitated by dropwise addition of MTBE (350 mL). The mixture was filtered through a sintered funnel and the precipitate washed extensively with ether. The white powder was then dried under vacuum over KOH pellets. If detritylation was incomplete, the TCA treatment was repeated, following the above procedure. TCA traces in the fully detritylated product were eliminated by recrystallisation from DCE/MTBE (50 mL/350 mL). The product was dried under vacuum over KOH pellets. The product (5.22 g) was stored at 4−7 °C.

Synthesis of 5′-DMT-d(GPSG-3′-CE-phosphite-TPST)-3′$_{Succ}$−MPEG. 5′-HO-d(TPST)-3′$_{Succ}$−MPEG (5.22 g) was co-evaporated with dry AcCN (3 × 20 mL) and dried under vacuum, in a 1 L three-necked round-bottom flask sealed with rubber caps and provided with a three-way stopcock. Argon was flushed for 4−5 min. The dried residue was dissolved in AcCN (13.5 mL) and injected with a syringe through the septum. To the stirring solution, 0.2 M 5′-DMT-2′−d(G^{i}BuPSG^{i}Bu)-3′-phosphoramidite (13.5 mL, 2.7 mmol) and 0.5 M 1*H*-tetrazole (18 mL, 9 mmol) were simultaneously added by syringes. The solution was stirred, under argon atmosphere, at room temperature for 5 min. The solution was cooled in an ice bath, and MTBE (350 mL) was added dropwise, under vigorous stirring. A white powder was recovered after filtration through a sintered funnel, rapidly washed with ether, and dried under vacuum, over KOH pellets. The crude product was recrystallised from EtOH (300 mL) until the TLC did not reveal any orange spot at R_f > 0, testing that any excess reagent has been removed. The white powder obtained was dried under vacuum, over KOH pellets. The product (6.3 g) was stored at 4−7 °C.

Synthesis of 5′-DMT-d(GPSG-3′-βCE-phosphothioate-TPST)-3′$_{Succ}$−MPEG. 5′-DMT-d(GPSG-phosphite-TPST)-3′$_{Succ}$−MPEG (6.3 g, 0.9 mmol), placed in a 1 L round-bottom flask, was dissolved in dry AcCN (31 mL). DDD (0.5 M, 18 mL, 9 mmol) was added under stirring. The solution was stirred at room temperature for 15 min and then ice-cooled. MTBE (400 mL) was added dropwise, under vigorous stirring. The white precipitate was recovered by filtration through a sintered funnel, washed with ether, and dried under vacuum, over KOH pellets. The product (6.26 g) was stored at 4−7 °C.

Acetylation of 5′-DMT-d(GPSGPSTPST)-3′$_{Succ}$−MPEG (Capping). 5′-DMT-d(GPSGPSTPST)-3′$_{Succ}$−MPEG (6.26 g) was dissolved in dry AcCN (30 mL) in a 1 L round-bottom flask. 2,6-Lutidine (3 mL), NMI (3 mL), and acetic anhydride (3 mL) were added under stirring. The solution was left to react at 0 °C for 3 min. Capped 5′-DMT-d(GPSGPSTPST)-3′$_{Succ}$−MPEG was precipitated from the ice-cooled solution with MTBE (400 mL) added dropwise. The mixture was filtered through a sintered funnel and the precipitate extensively washed with ether and dried under vacuum over KOH pellets. The product (6.19 g) was stored at 4−7 °C.

The degree of functionalisation was estimated spectrophotometrically as follows: an aliquot of product (5 mg) was dissolved in 60% perchloric acid/ethanol, 3:2 (v/v, 10 mL), the resulting solution was diluted 10-fold, and the absorbance at 498 nm was measured.

Deblocking of Oligonucleotide from the Support

Cleavage of Succinyl Linkage and Removal of Protecting Groups. This treatment afforded the cleavage of the succinyl linkage with MPEG, the removal of cyanoethyl-protecting groups from the phosphorothioate backbone, the removal of the *N*-isobutyryl-protecting group from guanosine, and the removal of the *N*-benzoyl-protecting group from adenosine and cytidine. 5′-DMT-oligonucleotide-3′$_{Succ}$−MPEG was dissolved in 30% NH_3 (5 mL × 20 mg) in a tightly closed glass container and left, without stirring, at 60 °C for 10 h. The solution was evaporated to dryness, the reside was rinsed with water and evaporated, and the procedure was repeated until there was no ammonia odor. The residue was dissolved in water (5 mL) and extracted with ether (4 × 5 mL). The aqueous layer was freeze-dried, and the residue was dissolved in acetic acid/water, 4:1 (v/v, 5 mL x 20 mg), in a 100 mL round-bottom flask, and stirred at room temperature for 30 min. The resulting yellow solution was extracted with ether (5 × 5 mL) and the aqueous layer evaporated to dryness. The residue was rinsed with water and evaporated, and the procedure was repeated until acetic acid odor was removed. The residue was dissolved in water, freeze-dried, and stored at −20 °C.

Recovery of MPEG after Oligonucleotide Removal from Support and Deprotection. Recovery of MPEG from the crude mixture was achieved by precipitation of the oligomers from aqueous solution with acetone. In a typical procedure, a solid mixture of the final oligonucleotide, failed sequences, and deblocked MPEG was dissolved in a minimum amount of deionized water and cooled in an ice-bath.

About 20 vols of acetone were added dropwise, under stirring; after 1 h of stirring, the resulting precipitate was separated from the solution by centrifugation at 5 °C (at 4500 rpm for 20 min), redissolved in water, and freeze-dried. The upper, settled solution was concentrated under vacuum and freeze-dried. Usually, about 95% of the oligomer is recovered from the solution phase, while more than 90% of the oligonucleotide is present in the precipitate.

If a 5′-DMT-protected oligonucleotide has to be collected, a 0.5% ammonia solution in water is used as dissolving phase to avoid detritylation.

Purification and Analysis of the Oligonucleotides.

Analytical IE-FPLC. Analytical ion-exchange FPLC was performed using a Pharmacia Mono Q HR 5/5 column operating on a Pharmacia FPLC System. The sample (2 mg) was dissolved in water (1.5 mL), filtered through a 0.22 μm sterile filter, diluted 10-fold with start buffer, and degassed by centrifugation. The resulting solution (100 μL) was injected.

Mobile phase A: 0.1 M NaCl (pH 11.5). Mobile phase B: 3.0 M NaCl (pH 11.5). Elution gradient: 0% B 5 min hold, 0−100% B in 35 min. Flow rate: 1.0 mL/min. Temperature: rt. UV detector: 260 nm.

Analytical RP-HPLC. Reversed-phase HPLC was performed using a Supelco Progel-TSK Oligo-DNA-RP column (15 cm × 4.6 mm i.d.) operating on a Gilson HPLC System. The sample (2 mg) was dissolved in water (1.5 mL), filtered through a 0.22 μm sterile filter, diluted 10-fold with start buffer, and degassed by centrifugation. The resulting solution (100 μL) was injected.

Mobile phase A: 0.05 M TEAAc (pH 7.0). Mobile phase B: 0.05 M TEAAc (pH 7.0)/AcCN, 20:80 (v/v). Elution gradient: 10−40% B in 40 min. Flow rate: 1.0 mL/min. Temperature: rt. UV detector: 260 nm.

Preparative IE-FPLC. Ion-exchange purification was performed using a Pharmacia Mono Q HR 5/5 column operating on a Pharmacia FPLC System. Completely deblocked 15mer was dissolved in start buffer, filtered through a 0.22 μm sterile filter, and degassed by centrifugation (5 min).

Mobile phase A: 0.1 M NaCl (pH 11.5). Mobile phase B: 3.0 M NaCl (pH 11.5). Elution gradient: 20% B 3 min hold, 20−80% B in 22 min. Flow rate: 1.0 mL/min. Temperature: rt. UV detector: 280 nm.

The desalting of the collected peak has been performed by a reverse phase chromatographic process, using a Pharmacia PepRPC HR 5/5 column operating on a Pharmacia FPLC System. Mobile phase A: water. Mobile phase B: methanol. Elution gradient: 0% A 15 min hold, 0−100% B in 15 min. Flow rate: 0.7 mL/min. UV detector: 260 nm.

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