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# SYNTHESIS OF OLIGONUCLEOTIDES VIA PHOSPHORAMIDITE APPROACH UTILIZING 2-DIPHENYLMETHYLSILYLETHYL (DPSE) AS A PHOSPHORUS PROTECTING GROUP

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***Abstract:*** *2-Diphenylmethylsilylethyl (DPSE) is a new protecting group for internucleotidic bonds in the synthesis of oligodeoxyribo nucleotides by the phosphoramidite approach. This group is stable to acidic conditions and can be removed under mild β-fragmentation conditions using aqueous ammonium hydroxide.*

## Introduction

The advent of the "antisense" therapeutic principle has inspired several groups to extend their work beyond the synthesis of modified oligodeoxyribonucleotides to investigation of the potential of small nucleic acid sequences as therapeutic agents. This inspiration is self-evident from the volume of publications reported.[1-34] A major advantage of the antisense strategy lies in potential specificity of action. In principle, an antisense oligodeoxynucleotide can be designed to target any single gene within the human genome, creating specific therapeutics for any disease for which a causative gene is known. The strategy is proving to be extremely promising and a key question has been the feasibility of manufacturing sizable amounts of oligonucleotides. In order to develop cost-effective synthetic process, issues related to fast and efficient synthesis, automatability, scalability and product purification are being investigated with renewed attention. The introduction of the phosphite triester method by Letsinger has greatly aided[35,36] this effort and several groups have studied in detail the application of different phosphitylating reagents. Important improvements were made by Beaucage and Caruthers[37] who introduced *N,N*-diisopropylaminophosphoramidites. In the Letsinger-

Beaucage-Caruthers synthesis, the amidite moiety acts as a stabilizing factor and reacts with another hydroxyl containing unit only upon activation with a weak acid like tetrazole or amine hydrochlorides. Although the activation process is widely used in the synthesis of DNA and RNA, its mechanism still remains to be proved. Since the introduction of phosphoramidite synthons, there have been several successful reports[2,4] on the synthesis of oligonucleotides via the phosphoramidite approach using novel phosphate triester intermediate protecting groups. However there still remain some crucial problems of the removal of protecting groups from internucleotidic phosphates. The deprotection should preferably proceed through a mechanism which does not involve the phosphorus center to eliminate the possibility of internucleotidic bond degradation. The problems may be solved in principle by finding more useful protecting groups which can be removed under mild conditions. Few current phosphate protecting groups satisfy these requirements. For example, 2-cyanoethyl,[38-40] 2,2,2-trichloro-1,1-dimethylethyl,[41,42] *p*-nitrophenylethyl,[43-46] and allyl[47,48] groups were reported by several laboratories.

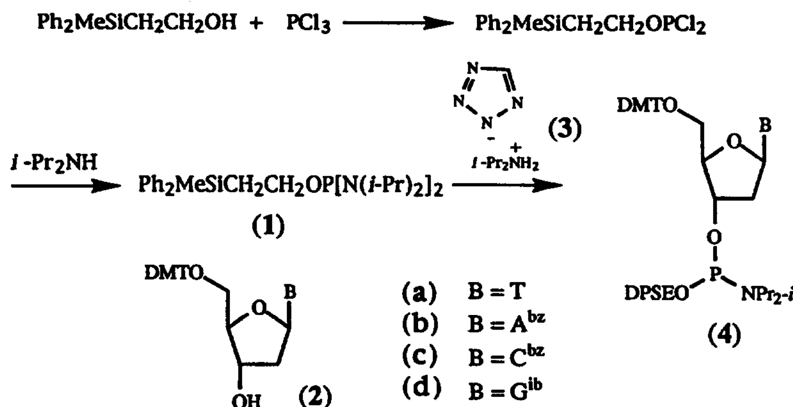
Recently we communicated a novel method[49] for the protection of internucleotidic phosphates using 2-diphenylmethylsilylethyl as a protecting group. In this paper, we wish to report the full details of our results wherein a) the easily accessible reagent bis[*N,N*-diisopropylamino]-2-diphenylmethylsilylethoxyphosphine (**1**) has excellent phosphitylating properties and b) the resulting diphenylmethylsilylethyl (DPSE) protecting group can be removed under mild conditions using aqueous ammonium hydroxide.

## Results and Discussion

### Synthesis of Nucleoside 3'-*N,N*-Diisopropylphosphoramidites.

Due to the low cost and commercial availability of trimethylsilylethanol, we first examined the synthesis of bis[*N,N*-diisopropylamino]-2-trimethylsilylethoxyphosphine as a phosphitylating agent. Treatment of trimethylsilylethanol[50] with phosphorous trichloride in anyhdrous ether gave the trimethylsilylethylphosphorodichloridite as a colorless viscous liquid, which on further treatment with diisopropylamine gave the diamidite in 86% yield as a colorless viscous liquid ($^{31}$P NMR $\delta$ 124). However treatment of the diamidite with 5'-dimethoxytritylthymidine did not give the expected amidite product ($^{31}$P NMR $\delta$ 13.63 and 13.96). The trimethylsilylethyl group was found to be too labile under a variety of conditions during subsequent condensation reactions. Similar observations have been reported by Honda and Hata.[51] Next we turned our attention to the more stable diphenylmethylsilylethyl group. The favourable properties of diphenylmethylsilylethyl as a protecting group have been amply demonstrated by Honda and Hata in the synthesis[51] of oligonucleotides using the phosphotriester approach. In order to find out if these properties could be extended to the phosphoroamidite approach, we set out to synthesize the phosphitylating reagent (**1**). To a 1.35 molar excess of

phosphorus trichloride in anhydrous ether was added dropwise, during 30 min at 0°C, 2-diphenylmethylsilylethanol[50] in ether. After 3 h at room temperature , the solution was concentrated to remove excess phosphorus trichloride and redissolved in anhydrous ether.




To this solution at 0°C was added a solution of diisopropylamine in ether. After stirring at room temperature overnight, the reaction mixture was filtered and concentrated to afford the phosphitylating agent in almost quantitative yield. The crude phosphine was treated with 5'-dimethoxytrityl thymidine (2a) in the presence of diisopropylammonium tetrazolide (3) in acetonitrile. After 3 h at room temperature, the usual aqueous work-up followed by silica gel column chromatography gave phosphoramidite (4a) in 77% yield.

We investigated the formation of thymidine phosphoramidite using different catalysts. Use of 1H-tetrazole and 5-(4-nitrophenyl)-1H-tetrazole as activators gave 71% and 80% yield of phosphoramidite (4a) respectively. $^{31}P$ NMR($CDCl_3$) of phosphoramidite (4a) showed two characteristic signals corresponding to a diastereomeric mixture and no 3'-3' dinucleoside phosphite could be detected. Similarly the phosphoramidites of deoxycytidine, deoxyadenine, and deoxyguanine (4b-d) were prepared in 68-74 % yields.

Table 1. Yields and $^{31}P$ NMR data of phosphoramidites (4a-d)

| Compound | Yield (%) | $^{31}P$ NMR Chemical Shift (ppm) |
|---|---|---|
| 4a | 77 | 146.09, 146.32 |
| 4b | 68 | 146.09, 146.48 |
| 4c | 74 | 146.28, 146.68 |
| 4d | 65 | 145.48, 146.18 |

Chemical shifts are given in ppm relative to an external standard of 85% $H_3PO_4$.

The synthetic utility of phosphoramidites (**4**) were initially demonstrated by the preparation of four phosphodiester homo-dimers, d(TpT), d(CpC), d(ApA) and d(GpG) (yields > 99%), on solid support. Oxidations were effected using 0.1 M solution of iodine in tetrahydrofuran/pyridine/water (8:4:2, v/v/v). The compounds were characterized using $^{31}P$ NMR ($\delta$ -0.3 ppm) and compared by tlc and HPLC with the same homo-dimers independently synthesized[52] using cyanoethoxy protection on the phosphate backbone and found to be identical.

**Stability of the DPSE group.**

To check the stability of the DPSE group in presence of aqueous ammonium hydroxide, DPSE protected homo-dimers d(TpT), d(CpC), d(ApA) and d(GpG) were analyzed after cleavage from solid support by $^{31}P$ NMR at 55°C. Complete deprotection takes place within 10 min. These results demonstrate that removal of the DPSE group is a fast and selective process, as cleavage of the internucleotide bond was not observed. However, removal of the DPSE group from oligonucleotides appears to be slower and complete deprotection was observed after treatment of the product with a mixture of concentrated ammonia and ethanol (9/1 v/v) at 55°C for 20 h.

**Synthesis of homo-thymidine dodecamer:** The utility of DPSE protection was extended to synthesis of a homo-thymidine dodecamer which is the typical length of an antisense oligonucleotide on a solid support. The following elongation cycle shown in Table 2 was utilized.

Table 2. Elongation Cycle - Solid phase synthesis of homothymidine dodecamer

| Steps | Solvents and reagents | Time (sec) | Vol. (mL) |
|---|---|---|---|
| 1 | $CH_3CN$ | 10 | 0.55 |
| 2 | $C_2H_4Cl_2$ | 35 | 1.2 |
| 3 | 3% dichloroacetic acid in dichloroethane | 95 | 3.2 |
| 4 | $CH_3CN$ | 35 | 1.9 |
| 5 | (4a) (0.2 M solution) in $CH_3CN$ + Tetrazole (0.4 M solution) in $CH_3CN$ | 60 | 0.17 + 0.17 |
| 6 | $CH_3CN$ | 10 | 0.55 |
| 7 | Cap A + Cap B | 10 | 0.25 + 0.25 |
| 8 | $CH_3CN$ | 5 | 0.2 |
| 9 | Iodine/water/pyridine/tetrahydrofuran | 10 | 0.5 |
| 10 | $CH_3CN$ | 10 | 0.55 |

During the synthesis of oligonucleotides on solid support we observed formation of considerable amount of (n-1) products. We attributed this to the inefficient removal of

the dimethoxytrityl cation after detritylation by deblocking solution. This in combination with the reversability of the detritylation reaction leads to the accumulation of (n-1) products. Use of 3% solution of dichloroacetic acid in dichloroethane (DCE) in place of dichloromethane (DCM) eliminated this problem. The overall coupling efficiency was found to be 98.5% (for 0.2 M solution of the phosphoramidite used) as determined by the usual spectrophotometric quantitation of released *p,p'*-dimethoxytriphenylmethyl cation. After the synthesis of dodecamer, the controlled-pore glass was treated with a mixture of concentrated ammonium hydroxide and ethanol (9:1, v/v) at 55°C for 24 h to afford the deprotected oligonucleotide. Ethanol was included to increase the solubility of the partially deprotected product. The longer deprotection time reflects the steric crowding around the silicon atom within the oligonucleotide. Also, the partially deprotected oligomers still bearing the DPSE group are sufficiently liphophilic to prevent oligomers from going into the solution.

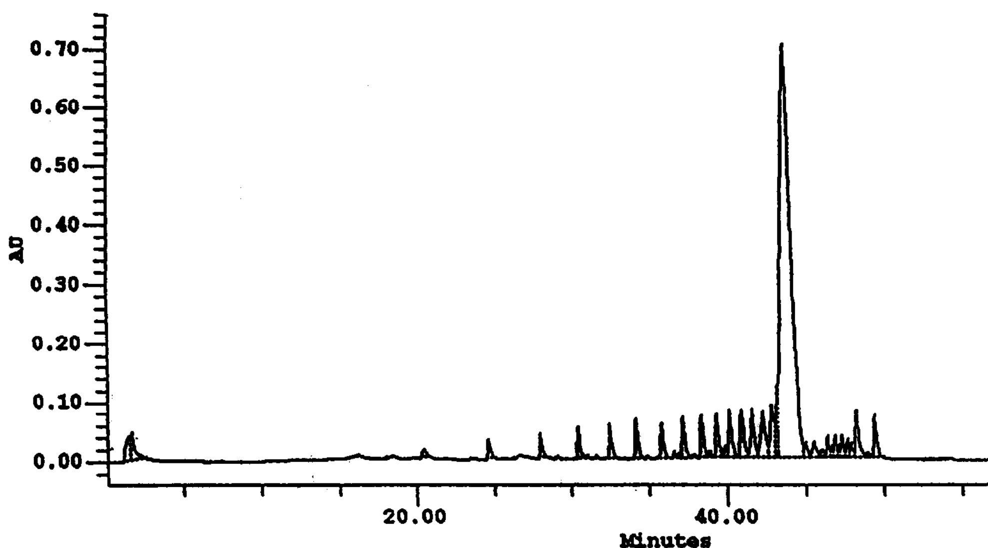



Fig. 1 HPLC analysis of the synthesized homo-thymidine dodecamer was performed on GEM-PAK FAX column, using a linear gradient of buffer A and Buffer B (0% B for 5'; 0-100% B in 45'). Buffer A = 2M NaCl in 50 mM Tris/methanol, 90/10, pH10, and buffer B = 50 mM Tris/methanol, 90/10, pH10.

The product was analysed by a strong ion-exchange HPLC and polyacrylamide gel electrophoresis (PAGE) which are shown in Fig. 1 and 2 respectively. The chromatographic profile shows that all reactions in the synthetic cycle proceed efficiently. The synthesized product has retention time identical on HPLC and polyacrylamide gel electrophoresis to the authentic oligomer synthesized using β-cyanoethylphosphoramidite chemistry.

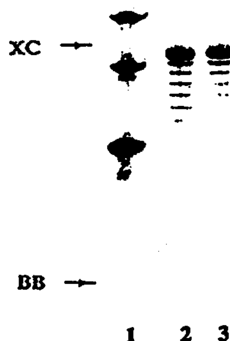



Fig. 2 PAGE analysis of homo-thymidine dodecamer. Bromophenol Blue (BB) and Xylene Cyanol (XC) were used as length markers. Lane 1 - length markers: 12, 18 and 27-mer; Lane 2 - $T_{20}$ synthesized using cyanoethoxy (CE) group and Lane 3 - $T_{20}$ synthesized using DPSE group.

We have investigated the synthesis of homothymidine dodecamer using different concentrations. It was observed that coupling yields were better at 0.2 M phosphoramidite concentration in acetonitrile (Fig. 3). Due to the bulky nature of the DPSE group, longer coupling time was necessary for efficient coupling. An investigation of coupling time showed that better results were obtained at 100 sec./coupling (Fig. 4).

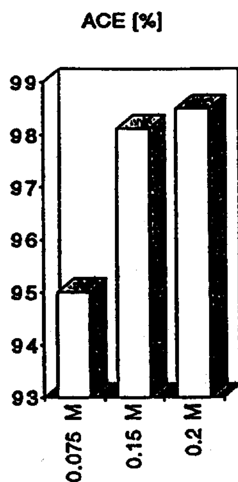



Fig. 3

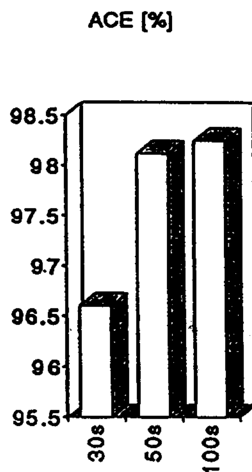



Fig. 4

In summary, the DPSE is a suitable protecting group for internucleotidic phosphate protecting group. Compared with other common protecting groups, as for example the cyanoethoxy group which is removed via a β-elimination mechanism, it is to be noted that the DPSE group can be easily removed using ammonium hydroxide via a β-fragmentation mechanism.

## EXPERIMENTAL

**Materials.** Trimethylsilylethanol (Aldrich), diphenylmethylsilylethanol (Fluka), phosphorus trichloride (Aldrich), 1H-tetrazole(Aldrich), 5-(4-nitrophenyl)-1H-tetrazole (Chem-Impex) were used as received. Diisopropylamine, and triethylamine were dried by heating at reflux over calcium hydride (5 g/L), distilled and used. Acetonitrile was dried by heating at reflux over $K_2CO_3$ (5g/L), distilled, and stored over 3A molecular sieves before use. All other solvents used were of the highest commercial grade obtainable. T.L.C. were performed using Whatman Silica Gel 60A Diamond KGF plates. Compounds were visualized by illuminating tlc plates under UV light (254 nm) and/or by spraying with 10% methanolic sulfuric acid followed by heating. Evaporations were carried out at 30-40°C using a rotary evaporator and a vacuum pump coupled to a vacuum controller. 5'-dimethoxytrityl-$N^2$-isobutyryl-2'-deoxyguanosine, 5'-dimethoxy-trityl-$N^4$-benzoyl-2'-deoxycytidine, 5'-dimethoxytrityl-$N^6$-benzoyl-2'-deoxyadenosine, 5'-Dimethoxytritylthymidine were purchased from Chem-Impex and used as received. All gel electrophoresis reagents were purchased from Boheringer Mannheim Biochemicals. Stains-All dye was from Eastman Kodak.

**General Methods.** $^{31}P$ NMR spectra were recorded, at ambient temperature, on a Varian Gemini-200 (79.990 MHz) or Varian Unity-400 (159.981 MHz) spectrometer with an external capillary containing 85% $H_3PO_4$ used as a reference. For anion-exchange chromatography, a Waters Millennium 2010 Chromatography Manager as solvent programmer, a Waters 996M Photodiode Array Detector, a NEC 486/33i microprocessor-controlled data system were employed. Samples were analyzed at 25°C on a GEN-PAK FAX column, using a linear gradient of buffer A and buffer B (0% B for 5'; 0-100% B for 45'). Buffer A = 2M NaCl in 50 mM Tris/methanol, 90/10, pH10, and buffer B = 50 mM Tris/methanol, 90/10, pH10. Flow rate 0.7ml/min. Oligonucleotides were synthesized on an Applied Biosystems Model 394B automated DNA synthesizer. DMT protecting group was removed on the solid support by performing additional detrytilation step.

**Gel Electrophoresis.** Analytical gel electrophoresis was accomplished using 1 mm thick 20% acrylamide gel (containing 7.5 M urea) at 50-55°C. Samples were run at 40 watts (ca. 1000-1100 volts). Position of the oligonucleotide was visualized by staining with Stains-All.

**Diphenylmethylsilylethyl *N,N*-diisopropylphosphoramidite.**

A 500 mL three-necked flask equipped with a magnetic stirrer, glass stopper and an inlet for argon was assembled under argon atmosphere. All glassware were dried in an oven at 120°C for 1 h. The reaction flask was charged with anhydrous ether (150 mL) and

phosphorous trichloride (9.27 g; 67.5 mmol). Diphenylmethylsilylethanol (12.12 g; 50 mmol) in ether (50 mL) was added to the reaction flask slowly with stirring at 0°C (ice cooling) using pressure-equalized addition funnel. After addition was complete, ice bath was removed and the reaction stirred for 3 h. The reaction mixture was transferred to a 500 mL flask and concentrated under reduced pressure. To this colorless product in anhydrous ether (200 mL) was added diisopropylamine (57.7 mL) at 0°C under argon. After the addition was complete, stirring was continued at room temperature for 16 h (overnight). The reaction mixture was filtered and concentrated to afford a colorless viscous liquid. $^{31}P$ NMR ($CDCl_3$) of this product showed a major peak at δ 123.4 ppm.

**5'-O-(4,4'-dimethoxytrityl)thymidine-3'-O-(2-diphenylmethylsilylethyl *N,N*-diisopropyl phosphoramidite).**

A 250 mL two-necked flask equipped with a magnetic stirrer, a gas inlet for argon, and a septum was assembled under an argon atmosphere. All glassware were dried at 120°C for 1 h. The flask was charged with 5'-O-(4,4'-dimethoxytrityl)thymidine (3.81 g; 7 mmol) and 5-(4-nitrophenyl)-1H-tetrazole (1.07 g; 5.6 mmol). Anhydrous acetonitrile (50 mL) was added. To this stirred mixture under argon at room temperature was added a solution of diphenylmethylsilylethyl *N,N*-diispropylphosphoramidite (4.96 g; 10.5 mmol) in acetonitrile (50 mL). After stirring for 2 h, tlc showed disappearance of starting nucleoside. The reaction mixture was filtered and the filtrate diluted with ethyl acetate (100 mL), washed once with cold saturated sodium bicarbonate solution, brine and dried ($MgSO_4$). The dried solution was concentrated under reduced pressure to afford a viscous foamy liquid. The crude product was purified by flash chromatography using silica gel. A gradient solvent system consisting of ethyl acetate and hexane was used. Triethylamine (1%) was used throughout the purification. The fractions corresponding to the product were combined and concentrated to afford a pale yellow viscous foamy liquid (10.25 g; 80%). $^{31}P$ NMR ($CDCl_3$) δ 145.48, 146.17; Anal. Calcd for $C_{52}H_{62}N_3O_8PSi$: C, 68.18; H, 6.82; N, 4.59. Found: C, 68.56; 6.97; N, 4.71.

***N*2-Isobutyryl-5'-O-(4,4'-dimethoxytrityl)-2'-deoxyguanosine-3'-O-(2-diphenylmethyl-silylethyl *N,N*-diisopropylphosphoramidite).**

A 250 mL two-necked flask equipped with a magnetic stirrer, a gas inlet for argon, and a septum was assembled under an argon atmosphere. All glassware were dried at 120°C for 1 h. The flask was charged with *N*2-isobutyryl-5'-O-(4,4'-dimethoxytrityl)-2'-deoxyguanosine (3.195 g; 5 mmol) and diisopropyl ammonium tetrazolide (0.684 g; 4 mmol). Anhydrous acetonitrile (50 mL) was added. To this stirred mixture under argon at room temperature was added a solution of diphenylmethylsilylethyl *N,N*-diispropylphosphoramidite (3.543 g; 7.5 mmol) in acetonitrile (50 mL). After stirring for 2 h, tlc (100% ethyl acetate) showed disappearance of starting nucleoside. The reaction mixture was filtered and the filtrate diluted with ethyl acetate (100 mL), washed once with cold saturated sodium bicarbonate solution, brine and dried ($MgSO_4$). The dried solution was concentrated under reduced pressure to afford a viscous foamy liquid. The crude product was purified by flash chromatography using silica gel. A gradient solvent system consisting of ethyl acetate and hexane was used. Triethylamine (1%) was used throughout the purification. The fractions corresponding to the product were combined and concentrated to afford a pale yellow viscous foamy liquid (3.6 g; 65%). $^{31}P$ NMR

($CDCl_3$) δ 145.48, 146.18; Anal. Calcd for $C_{56}H_{67}N_6O_8PSi$: C, 66.51; H, 6.68; N, 8.31. Found: C, 66.13; 6.82; N, 8.54.

**$N^6$-Benzoyl-5'-O-(4,4'-dimethoxytrityl)-2'-deoxyadenosine-3'-O-(2-diphenylmethyl silylethyl *N,N*-diisopropylphosphoramidite).**

A 250 mL two-necked flask equipped with a magnetic stirrer, a gas inlet for argon, and a septum was assembled under an argon atmosphere. All glassware were dried at 120°C for 1 h. The flask was charged with $N^6$-benzoyl-5'-O-(4,4'-dimethoxytrityl)-2'-deoxyadenosine (3.285 g; 5 mmol) and diisopropylammonium tetrazolide (0.684 g; 4 mmol). Anhydrous acetonitrile (50 mL) was added. To this stirred mixture under argon at room temperature was added a solution of diphenylmethylsilylethyl *N,N*-diispropylphosphoramidite (2.835 g; 6 mmol) in acetonitrile (50 mL). After stirring for 2 h, the reaction mixture was filtered and concentrated to afford a viscous foamy material. The crude product was purified by flash chromatography using silica gel. A gradient solvent system consisting of ethyl acetate and hexane was used. Triethylamine (1%) was used throughout the purification. The fractions corresponding to the product were combined and concentrated to afford a viscous foamy liquid (3.81 g; 68%). $^{31}P$ NMR ($CDCl_3$) δ 146.09, 146.48; Anal. Calcd for $C_{59}H_{65}N_6O_7PSi$: C, 68.85; H, 6.37; N, 8.17. Found: C, 68.19; 6.12; N, 8.24.

**$N^4$-Benzoyl-5'-O-(4,4'-dimethoxytrityl)-2'-deoxycytidine-3'-O-(2-diphenylmethyl silylethyl *N,N*-diisopropylphosphoramidite).**

A 250 mL two-necked flask equipped with a magnetic stirrer, a gas inlet for argon, and a septum was assembled under an argon atmosphere. All glassware were dried at 120°C for 1 h. The flask was charged with $N^4$-benzoyl-5'-O-(4,4'-dimethoxytrityl)-2'-deoxycytidine (3.169 g; 5 mmol) and diisopropylammonium tetrazolide (0.684 g; 4 mmol). Anhydrous acetonitrile (50 mL) was added. To this stirred mixture under argon at room temperature was added a solution of diphenylmethylsilylethyl *N,N*-diispropylphosphoramidite (3.543 g; 7.5 mmol) in acetonitrile (50 mL). After stirring for 2 h, tlc (100% ethyl acetate) showed disappearance of starting nucleoside. The reaction mixture was filtered and the filtrate diluted with ethyl acetate (100 mL), washed once with cold saturated sodium bicarbonate solution, brine and dried ($MgSO_4$). The dried solution was concentrated under reduced pressure to afford a viscous foamy liquid. The crude product was purified by flash chromatography using silica gel. A gradient solvent system consisting of ethyl acetate and hexane was used. Triethylamine (1%) was used throughout the purification. The fractions corresponding to the product were combined and concentrated to afford a viscous foamy liquid (4.09 g; 74%). $^{31}P$ NMR (($CDCl_3$) δ 146.28, 146.68; Anal. Calcd for $C_{58}H_{65}N_4O_8PSi$: C, 69.30; H, 6.52; N, 5.57. Found: C, 69.92; 6.83; N, 5.71.

**Synthesis of dimers**

The synthesis of homo-dimer of thymidine was performed as follows: 5'-O-Dimethoxytritylthymidine bonded to CPG was taken in a glass reactor, and a dichloromethane solution of 2% dichloroacetic acid (15 mL) was added to deprotect the 5'-hydroxyl protecting group. The product was washed with dichloromethane and then

with dry acetonitrile. Then, a 0.2 M solution of 5'-O-dimethoxytrityl)thymidine-3'-O-(2-diphenylmethylsilylethyl *N,N*-diisopropylphosphoramidite) in acetonitrile (5 mL) and a 0.4 M solution of 1H-tetrazole in acetonitrile (5 mL) were added and reacted at room temperature for 5 minutes. The excess solution was filtered and the product washed with acetonitrile. Then a 0.1 M solution of iodine in tetrahydrofuran/pyridine/water (8:4:2, v/v/v) was added and reacted at room temperature for 5 min. After filtering the excess reagents, this oxidation step was repeated one more time. The support was then washed with acetonitrile (10 mL) and dried. The support containing the dimer was treated with 30% aqueous ammonium hydroxide solution for 90 min. and then at 55°C for 1 h. Concentration of the aqueous solution afforded the desired product. $^{31}P$ NMR (($CDCl_3$) $\delta$ -0.3 ppm.

The synthesis of homodimers of dC, dA and dG were performed in a similar manner. In these cases, deprotection was carried out by incubation with $NH_4OH$ at 55°C for 10 h to deprotect the exocyclic amino protecting groups.

**Synthesis of homo-thymidine dodecamer**

The synthesis of homo-thymidine dodecamer was performed on a 1 µmol scale using the cycle and conditions mentioned earlier in the discussion. Also, the oligonucleotide thus synthesized was characterized as discussed earlier.

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