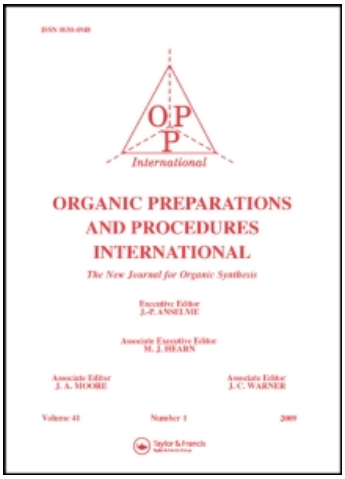

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FLUOROUS SILYL PROTECTING GROUP FOR 5'-HYDROXYL PROTECTION OF OLIGONUCLEOSIDES

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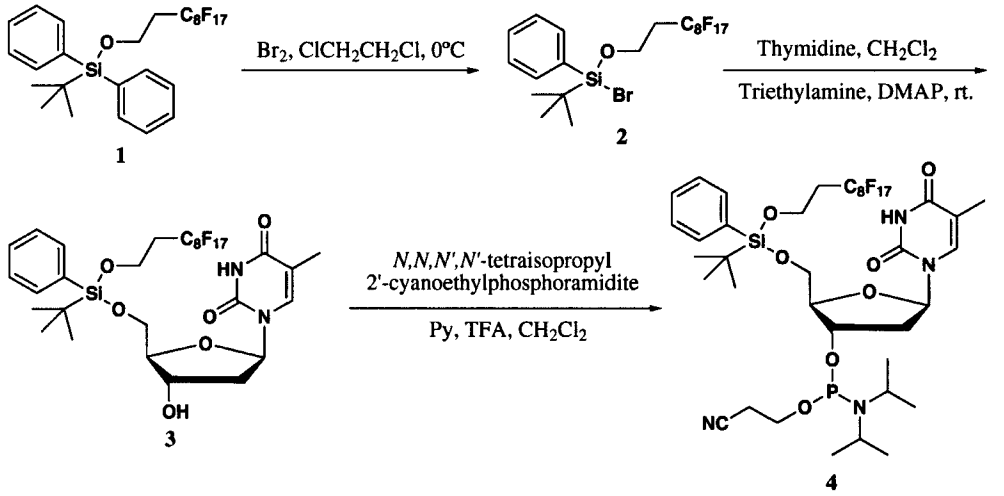
The success of antisense oligonucleotides[1] as drugs has led to increased demand for the production of oligonucleotides on a large scale.[2] With the approval of first oligonucleotide analog as an antiviral drug (*Vitravene*™ of ISIS Pharmaceuticals-Ciba vision) and with several of oligonucleotide-based therapeutics being at various stages of clinical evaluation, the search for and development of a cost-effective method for oligonucleotide synthesis on a multikilogram scale become imperative.[3-5]

The purification and yield improvement are the two very challenging tasks in the field of oligonucleotide synthesis. Therapeutic uses of antisense oligonucleotides require efficient synthesis and purification. However, on solid-support synthesis the final yield of the desired sequence is greatly affected by purification cycles. The idea contemplated in the present communication was to carry out the solid-support synthesis in such a way that the desired sequences, after cleavage from the support, may be easily separated from the failed (n-1) and truncated sequences by liquid-liquid extraction or by simple precipitation, thus avoiding tedious and costly chromatographic separations. To implement the concept, the "Fluorous Tags" carrying high content of fluorine may be used as "purification handles", which impart excessive hydrophobicity to the tagged molecule. Fluorous chemistry has recently been recognized due to its uniqueness for liquid-liquid triphasic separations, *viz.* organic, fluorous solvent and water, or passing through the silica gel, while the fluorous content will bind to the gel and can be eluted selectively from fluorous reverse phase silica gel using fluorous solvent.[6] The same chemistry, if applied in the oligonucleotide synthetic post purification, might be helpful in cutting the cost involved in purification manifold.

In the present communication, fluorous alkoxysilyl (base labile) ethers were investigated. Base labile silyl protecting groups have proved to be more advantageous than the acid labile trityl groups for 5'-OH protection during the synthesis of long chain oligonucleotides since they do not require repeated acidic treatment, which leads to depurination, eventually resulting in chain cleavage. Although, the $(C_6F_{13}CH_2CH_2)_3Si$ group[7] has a high content of fluorine, it is reported to be acid labile. Since the *bis*-alkoxysilyl ethers[8,9] are reported to be more acid stable, Curran *et al.* used the group for protection of primary and secondary alcohols and obtained good yields. They reported *tert*-butylphenyl-1H,1H,2H,2H-heptafluorodecyloxysilyl (BPFOS)[10] to be more acid stable than the $(C_6F_{13}CH_2CH_2)_3Si$ group. It was envisioned that the BPFOS group might be efficiently used for the protection of 5'-hydroxyl of nucleosides, an example of primary hydroxyl group. The 5'-*O*-protected nucleoside, after subsequent phosphorylation could be incorporated at the final synthesis cycle of the oligonucleotide, thereby tagging the desired sequence with fluorous group. This fluorous group should act as a protecting group as well as a phase tag, which would presumably help in further purifications. The fluorous synthesis schemes allow for easy purification or removal of highly fluorinated intermediates or reagents.



Synthesis of 5'-*O*-*tert*-Butylphenyl-1H,1H,2H,2H-perfluorodecyloxysilyl thymidine 3'-*O*-phosphoramidite (**4**)

Scheme

One further step towards a cost effective synthesis of oligonucleotides was explored by changing the conventional phosphitylating reagents. The usual procedure for preparing amidite building blocks involves phosphitylation of free hydroxyl group with *N,N*-diisopropyl 2-cyanoethylchlorophosphoramidite in presence of activators such as 1*H*-tetrazole. Since *N,N*-diisopropyl 2-cyanoethylchlorophosphoramidite is expensive and highly reactive, the use of *N,N,N',N'*-tetraisopropyl 2-cyanoethylphosphoramidite, a relatively stable and less expensive alternative was suggested.[11] Similarly, 1*H*-tetrazole being toxic, costly and reportedly explosive was replaced with inexpensive and less toxic pyridine trifluoroacetate as alternative activator in the process of phosphitylation.[11]

In all, five oligonucleotide sequences of different lengths (*see Table*) were synthesized by standard phosphoramidite chemistry. The fluorous tagged monomer was incorporated in them during the last synthesis cycle (T* represents the fluorous tagged thymidine). To check the stability of fluorous-tagged oligonucleotide under standard basic conditions, oligo-nucleotides were treated with 30% aqueous ammonia for 6 h at 50°C. After precipitation with isopropanol/ethanol, the pellets of oligonucleotides were dried and lyophilized. The molecular weight was confirmed using *MALDI-TOF* for the sequences in order to confirm the integrity of the fluorous silyl group after the standard basic treatment. Their structure was confirmed by mass spectrometry MS (MALDI) and the results are tabulated in the Table.

Table. Retention Time and MW of Five Oligonucleotide Sequences Synthesized

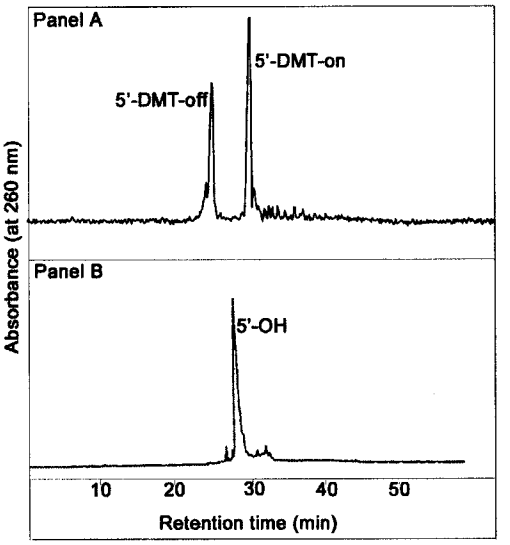
Sequences	Retention time (Rt)[a] (In min)	MW (MALDI) Calcd.	Observed
5'-T*ACGT-3'	7.8	3005.51	3005.11
5'-T*ACGTACGT T-3'	12.4	5443.44	5443.22
5'-T*AATGGAGCCAG T-3'	18.5	6954.54	6954.22
5'-T*TTCAGTCCCACCTC CC-3'	24.2	8711.88	8711.09
5'-T*GCTATGTCAGTTCCCCT T-3'	28.8	9770.85	9770.51

a) The HPLC was performed after deprotection of the fluorous handle.

The fluorous-tagged oligonucleotides were readily precipitated when suspended in deionized water due to enhanced hydrophobicity. They are sparingly soluble in CH_3CN and surprisingly insoluble in FC-72 (perfluoro-*n*-hexane; fluorous solvent). It appears that, as the molecular weight of the tagged molecules increases, their solubility decreases in the fluorous solvent. Thus the desired sequences may be purified from the rest of the *(n-1)* failure oligonucleotides by suspending and stirring the oligonucleotides in water after cleavage from the solid support and deprotection. The precipitated out mass may then be separated by simple vacuum filtration.

Deprotection of the fluorous tag of the sequences was achieved by treatment of the oligonucleotides with solution of tetrabutylammonium fluoride (0.6 M) in THF (0.5 mL) for 3 h. The solvent was evaporated and the residue was partitioned between FC-72 and water. The sequences are retained in the aqueous layer while the fluorous group passes in the FC-72 layer. Upon concentration, the aqueous phase gave the desired sequences in better than 90% yield. The sequences were further purified by HPLC and matched with the routinely made phosphoramidite sequences. HPLC was performed using Waters MS C_{18} (4.8 x 50 mm) column and a gradient of 5-25% of CH_3CN (in 100 mM triethylammonium acetate; pH 7.0), temp. 30°C, flow rate: 1 mL/min, λ_{max} 260 nm. (For r.t., see *Table*)

In conclusion, a process has been developed which does not require tedious chromatographic techniques for purification at any step. Different length of sequences were chosen to



*(**Panel A**) HPLC Chromatogram of 19-mer Sequence (5'-TGCTATGTCAGTTCCCCT T-3') showing the mixture of 5'-DMT-on and 5'-DMT-off through conventional method using phosphoramidite chemistry.*
*(**Panel B**) Chromatogram of the same sequence synthesized by tagging with fluorous handle (5'-T*GCTATGTCAGTTCCCCT T-3') and after subsequent purification.*

Fig. 1

prove the viability of the fluorous protecting groups and to optimize the length of sequences that may be purified easily using liquid-liquid extraction. The maximum length used is of 19-mer, an ideal length for antisense oligos. Easy precipitation of oligonucleotides and 100% recovery in water after deprotection of fluorous tag, proves the approach to be viable. The strategy is found to be appropriate for the small sequences up to 20-mer, the ideal length of oligonucleotide for antisense technology. In addition, the use of pyridinium trifluoroacetate as activator instead of using the standard 1*H*-tetrazole and *N,N,N',N'*-tetraisopropyl 2-cyanoethylphosphoramidite as phosphitylating reagent instead of *N,N*-diisopropyl 2-cyanoethylchlorophosphoramidite are one further step towards "green" and safer chemistry.[12]

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EXPERIMENTAL SECTION

1H,1H,2H,2H-Perfluorodecanol-1 was purchased from Strem Chemicals, Newburyport, MA, USA. *tert*-Butyldiphenylsilyl chloride was purchased from Aldrich Chem. Co, USA. FC-72 was purchased from Acros Organics, NJ, USA. Imidazole and 4-(dimethylamino)pyridine were

recrystallized prior to use. Sequences were synthesized on ABI 392 automated DNA synthesizer. ^{1}H NMR and ^{31}P NMR were recorded on 400 MHz using a UNITY/INOVA 400 'VARIAN'.

***tert*-Butyldiphenyl-1H,1H,2H,2H-perfluorodecylsilyl Ether (TBDPS) (1).**- A solution of *tert*-butyldiphenylsilyl chloride (3.64 g, 13.25 mmol) 1H,1H,2H,2H-perfluorodecanol-1(12.05 mmol, 5.59 g), DMAP (0.6 mmol, 73 mg) and imidazole (1.151 g, 16.9 mmol) in CH_2Cl_2 (25 mL) was stirred at r. t. overnight. Then 25 mL CH_2Cl_2 was added and the solution was washed successively with water, 1M HCl and with brine. Drying the organic layer over Na_2SO_4 and evaporation of the solvent yielded the TBDPS ether as colorless oil (7.2 g, 90%) ^{1}H NMR ($CDCl_3$): δ 7.69-7.66 (m, 4H), 7.45-7.38 (m, 4H), 3.96 (t, 2H), 2.45-2.25 (m, 2H), 1.07 (s, 9H).

Anal. Calcd for $C_{26}H_{23}F_{17}OSi$: C, 46.20; H, 3.41. Found: C, 46.28; H, 3.40

***tert*-Butylphenyl-1H,1H,2H,2H-perfluorodecyloxysilyl Bromide (2).**- Bromine (13.25 mmol; 0.75 mL) was added dropwise to a solution of TBDPS (**1**) (11.05 mmol) in 1,2-dichloroethane (75 mL) at 0°C. The reaction mixture was stirred at rt overnight. Distillation at reduced pressure yielded the product as colorless oil. ^{1}H NMR ($CDCl_3$): δ 7.69-7.65 (m, 2H), 7.48-7.39 (m, 3H); 4.11-4.06 (m, 2H), 2.47-2.35 (m, 2H), 1.01 (s, 9H).

5'-*O*-*tert*-Butylphenyl-1H,1H,2H,2H-perfluorodecyloxysilyl Thymidine (3).- To a solution of compound **2** (2.42 g, 3.3 mmol) in CH_2Cl_2 (15 mL), thymidine (3 mmol), triethylamine (14.2 mmol) and 4-(dimethylamino)pyridine (0.15 mmol) were added and the reaction mixture was stirred at rt overnight. CH_2Cl_2 (15 mL) was added and the mixture was washed with $NaHCO_3$ solution. The organic phase was dried over Na_2SO_4, and the solvent was evaporated in vacuo. The resulting oil was chromatographed through a short silica column (CH_2Cl_2: hexane 60:40). The desired batches showed UV (λ_{max} 260 nm) positive product and charring after exposure to sulfuric acid fumes on TLC. The desired batches were pooled and evaporated to afford **3** (2.04 g, 86%) as a brown oil but was obtained as a light brown solid. ^{1}H NMR ($CDCl_3$): δ 7.65-7.6 (m, 2H), 7.48-7.39 (m, 3H), 4.11 (t, 2H), 2.47-2.35 (m, 2H), 2.01 (s, 9H); 4.74-4.69 (m, 2H, H5'), 2.76 (dd, 1H, H2'); 2.60 (dd, 1H, H2'), 2.45 (s, 3H, CH_3); 2.45 (s, 3H, CH_3); 2.16, (s, 3H, CH_3). HRMS (EI) *m/z*: Calcd for $C_{30}H_{31}O_6N_2F_{17}Si$: 866.65. Found: 866.61.

Anal. Calcd for $C_{30}H_{31}F_{17}N_2O_6Si$: C, 42.95; H, 3.69; N, 3.34. Found: C, 42.82; H, 3.67; N, 3.30

5'-*O*-*tert*-Butylphenyl-1H,1H,2H,2H-perfluorodecyloxysilyl Thymidine-3'-*O*-phosphoramidite (4).- Product **3** (2.04 g, 2.3 mmol) was suspended in dry CH_2Cl_2 (8 mL) and *N,N,N',N'*-tetraisopropyl 2'-cyanoethylphosphoramidite (0.78 g, 2.56 mol) was added at ambient temperature. Pyridinium trifluoroacetate (0.57 g, 2.56 mmol) was added to reaction mixture and the solution was stirred for 4 h. On complete consumption of the starting material (checked through TLC using a gradient CH_2Cl_2: hexane:50:50), the entire reaction mixture was transferred directly to the top of a short silica column (2.5 x 10 cm, 60-120 mesh). The product was eluted with CH_2Cl_2: hexane (60:40). The desired batches showed UV (λ_{max} 260 nm) positive product and

charring after exposure to sulfuric acid fumes on TLC. The appropriate fractions were collected and pooled and the solvent was evaporated to furnish the product as a light brown solid. ^{31}P NMR (CD_3CN): δ 151.0, HRMS (EI) *m/z:* Calcd for $C_{39}H_{48}O_8N_4F_{17}PSi$: 1082.8444. Found 1082.6661.

Anal. Calcd for $C_{39}H_{48}F_{17}O_7PSi$: C, 45.13; H, 4.63; N. 5.40. Found: C, 45.10; H, 4.55; N, 5.34

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