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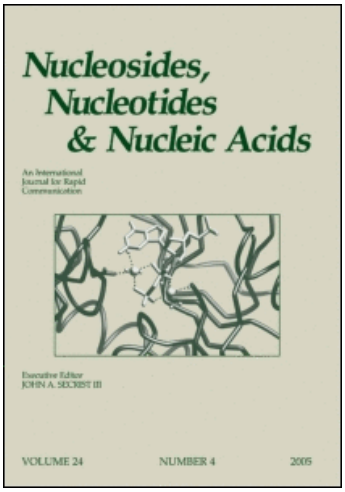

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ONE-POT SYNTHESIS OF TBMPS (*bis* [*tert*-BUTYL)-1 PYRENYLMETHYL-SILYL) CHLORIDE AS A NOVEL FLUORESCENT SILICON-BASED PROTECTING GROUP FOR PROTECTION OF 5′-OH NUCLEOSIDES AND ITS USE AS PURIFICATION HANDLE IN OLIGONUCLEOTIDE SYNTHESIS

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▫ *An efficient and novel synthesis of bis(tert-butyl)-1-pyrenylmethyl-silyl group (TBMPS) has been reported having fluorescent properties conferred by the pyrenyl group. This silyl group being base labile is efficiently used for one-pot protection of the 5′-OH of the nucleosides. While incorporated terminally at the 5′-OH of long sequences viz. AA TGG AGC CAG T and GC TAT GTC AGT TCC CCT TGG TTC TC, this group is also helpful in subsequent purification by HPLC as well as PAGE. Besides these, a labeled dimer (T*T) and a labeled tetramer (T*TTT) were also synthesized to compare the fluorescence properties of short and long labeled sequences. Fluorescence properties of these sequences were studied in detail to find the suitability of the approach.*

INTRODUCTION

Success of antisense methodology[1–3] in recent years for monitoring as well as developing new therapeutic agents against a variety of diseases emphasizes the need for efficient synthetic strategies of oligonucleotides in bulk quantities.[4,5] In polymer-supported synthetic approaches of oligonucleotides, deoxy- as well as ribo-series, the purification of the crude full-length oligonucleotide product is a long-time challenge. For this purpose, a variety of 5′-OH protecting groups have been designed to serve as "purification handles".[6–10] Resolution of the purification

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problem was further facilitated by the incorporation of some fluorescent groups[11] so that the detection of oligonucleotides on thin-layer chromatography or gels down to picomole levels in the visible range became possible.

Silyl protecting groups, being base labile, have proved more advantageous than the acid-labile trityl groups for 5′-OH protection during the synthesis of long-chain oligonucleotides since they avoid repeated acidic treatment, which in turn leads to depurination and eventually to chain cleavage. To overcome this problem, new routes to synthesis of silyl groups having fluorescent as well as nonfluorescent moieties have been effectively employed. Caruthers et al.[12] have reported a number of silyl substituents, which can be regioselectively introduced and deprotected in less than 30 s using TBAF in THF. These groups have been tested for stability in acids and bases. We envisioned that use of silyl groups having fluorescent moieties covalently attached would carry additional advantages for detection of cleavage of the group along with its function as a purification handle.

In efforts towards achieving this dual advantage of a fluorescent purification handle as well as avoidance of acid-labile protecting groups at the 5′ terminus, the present work has been carried out. We chose to synthesize a base-labile hydrophobic silyl group having a fluorescent moiety covalently attached. The design of this group is based on the hypothesis that the group should be able to withstand strong alkaline as well as acidic conditions. The pyrene group, being highly hydrophobic, was selected since using its excitation and emission wavelengths of 346 nm and 390 nm, respectively, for detection purposes would obviate the need to use the DNA-damaging wavelength range of 254–260 nm, the most prevalent range used for DNA detection. Hence, the labeled oligonucleotides may be easily identified and separated as the desired product from the rest of the failed and incomplete sequences, thereby effectively reducing the total cost related to post-synthetic purification.

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MATERIAL AND METHODS

1-Pyrene methanol, di-*tert*-butyl dichlorosilane, *bis*-reagent (2-cyanoethyl-*N*,*N*,*N*′,*N*′-tertaisopropyl phosphoramidite and pyridinium trifluoroacetate were purchased from Aldrich Chemical Co., Milwaukee, WI, 1-Pyrene methanol was recrystallized in DCM prior to use (m.pt. 122°C). 13-mer and 26-mer sequences were synthesized on ABI 292 DNA synthesizer using standard phosphoramidite chemistry and T*(5′-*O*-1,1-*bis*-(*tert*-butyl)-1(1-pyrenylmethyl)-silyl)-thymidine-3′-(*bis*-diisopropylamino,β-cyanoethyl) phosphoramidite (**2**) was coupled in the last cycle of synthesis. Dinucleotide (T*T) and tetramer (T*TTT) were synthesized in a functionalization vessel using standard phosphoramidite chemistry. Solvents were duly purified and dried prior to use. Absorbance studies were conducted on 220S Hitachi spectrophotometer. Fluorescence studies were carried out on SFM 25 KONTRON spectrofluorometer. TLC was done on silica gel G supplied by Merck. FAB-MS was recorded on JEOL SX 102/DA-6000 spectrometer using argon (6 kV, 10 mA) as the FAB gas. ^{1}H NMR and ^{31}P NMR were recorded on 400 MHz using a

UNITY_INOVA 400 varian. Oligonucleotide sequences were purified on hplc using whatman reverse-phase C_{18} column using aq. ammonium acetate (0.1 M) as buffer A and 100% CH_3CN as buffer B. The elution was carried out using a linear gradient with a flow rate of 1 mL/min.

Synthesis of 5′-*O*-(1,1-*bis*-(*tert*-Butyl)-1(1-Pyrenylmethyl)Silyl)-Thymidine (1)

To 1-pyrene methanol (0.01 mol; 2.32 g) dissolved in pyridine (10 mL), methanolic sodium hydroxide solution (2 N; 10 mL) was added. While stirring at room temperature, di-*tert*-butyl-dichlorosilane (0.01 mol) was added to it drop by drop in 15 min and the resulting reaction mixture was stirred for 2 h. The reaction was carried out under argon atmosphere. The completion of the reaction was checked on tlc (DCM:MeOH9:1) with appearance of a new fluorescent spot at Rf = 0.95 (1-pyrene methanol has an Rf of 0.4). Predried thymidine (0.005 mol; 1.210 g) dissolved in pyridine (10 mL) was added to it with subsequent addition of DMAP (0.001 mol) and triethylamine (2 mL). The reaction mixture was further stirred for 5 h and then checked on tlc for completion. A fluorescent spot (Rf = 0.8) charring after acid spray indicated the formation of the desired product. The reaction mixture was concentrated to a gum and extracted with DCM (10 mL). The organic phase was added to hot water (20 mL) with immediate formation of a pale yellow amorphous substance. The product was subsequently filtered, dried, and recrystallized in DCM. Yield, 62%; m.p. = 98°C; UV λ_{max}, 260, 346 nm. ^{1}H NMR: 8.45 (d, 1H, pyrene), 8.13 (d, 1H, pyrene), 8.03–7.97 (m, 5H), 7.93 and 7.08 (2H, pyrene), 4.74–4.69 (m, 2H, H-5′), 2.76 (dd, 1H, H-2′), 2.60 (dd, 1H, H-2′), 2.45 (s, 3H, CH_3), 2.16 (s, 3H, CH_3), 0.94 (s, 18 H). FAB-MS(m/z$^+$): 615 (calculated); 614 (found). Elemental analysis (%): Calculated ($C_{34}H_{43}N_2O_6Si$), C, 67.22; H, 7.08; N, 4.61; Found, C, 67.21; H, 7.08; N, 4.6.

Synthesis of 5′-*O*-(1,1-*bis*-(*tert*-Butyl)-1(1-Pyrenylmethyl)Silyl)-Thymidine-3′-(*bis*-Diisopropylamino,β-Cyanoethyl) Phosphoramidite (2)

The above-crystallized product **1** (1 mmol) was dissolved in anhydrous CH_2Cl_2 (10 mL) and *bis*-reagent (2-cyanoethyl-*N*,*N*,*N*′,*N*′-tertaisopropyl phosphoramidite) (0.362 g, 1.2 mmol) was added to it at ambient temperature (Scheme 1). Pyridinium trifluoroacetate (0.232 g, 1.2 mmol) was added to the reaction mixture and the solution was stirred for 4 h. After complete consumption of the starting material (checked on tlc), the solvent was evaporated in vacuo and charged on a short silica column (2.5 × 10 cm; 60–120 mesh). The product was eluted with 60–80% DCM: hexane. Yield: 88%, ^{1}H NMR: 8.45 (d, 1H, pyrene), 8.13 (d, 1H, pyrene), 8.03–7.97 (m, 5H), 7.93 and 7.08 (2H, pyrene), 4.74–4.69 (m, 2H, H-5′), 2.76(dd, 1H, H-2′), 2.60 (dd, 1H, H-2′), 2.45 (s, 3H, CH_3), 2.16 (s, 3H, CH_3), 1.55–1.45 (m, 4H), 1.16–1.07 (m, 9H), 0.94

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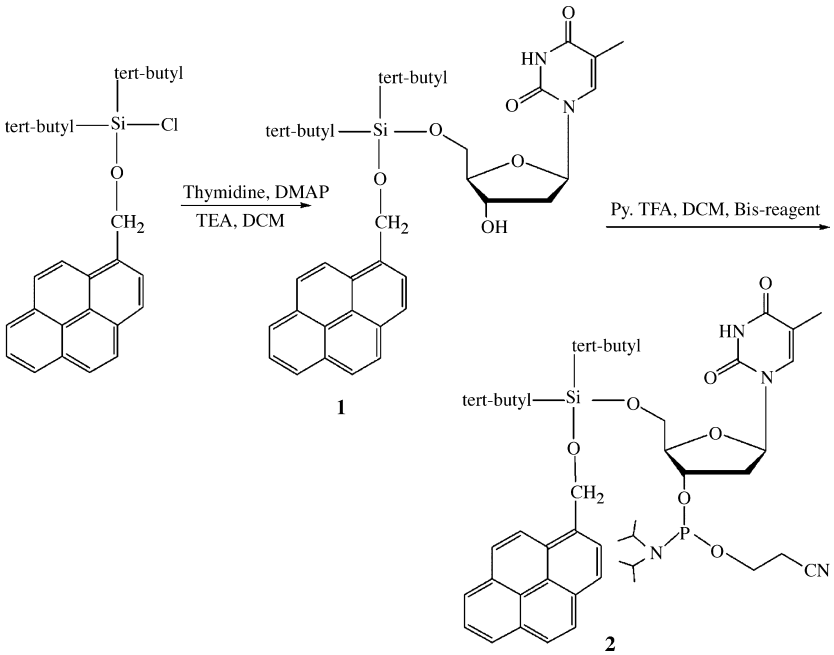

SCHEME 1 Synthesis of 5′-*O*-(1,1-*bis*-(*tert*-butyl)-1(1-pyrenylmethyl)silyl)-thymidine-3′-(*bis*-diisopropylamino, β-cyanoethyl) phosphoramidite (**2**).

(s, 18 H), ^{31}P NMR (CD_3CN): 151.0 ppm. Elemental analysis (%): Calculated ($C_{42}H_{59}N_4O_7SiP$), C, 63.4; H, 7.43; N, 7.05; Found, C, 63.22; H, 7.42; N, 7.1.

Synthesis of Pyrene-Labeled Oligonucleotides

A dinucleotide T*T, a tetramer T*TTT and T*AA TGG AGC CAG T and T*GC TAT GTC AGT TCC CCT TGG TTC TC (T* represents the labeled unit) were synthesized using standard phosphoramidite chemistry. All these sequences were further deprotected from CPG support using 30% ammonia for 12 h at 55°C. The sequences were further purified by reverse-phase HPLC using dual detection methods in series, absorbance at 260 nm and fluorescence with excitation and emission wavelength at 346 nm and 390 nm, respectively. The fluorescent-labeled oligonucleotides can be easily separated from the nonfluorescent truncated and failed sequences. Only the fractions showing both peaks of absorbance at 260 nm as well as fluorescence emission at 390 nm were collected and pooled. Confirmation of the correct mass was done by MALDI-TOF mass spectrometry. The TBMPS group was successfully cleaved by using 0.1 M tetrabutylammonium fluoride in THF for 3 min at r.t. The purified oligonucleotides after deprotection did not show any absorbance peak at 346 nm and any fluorescence emission at 390 nm.

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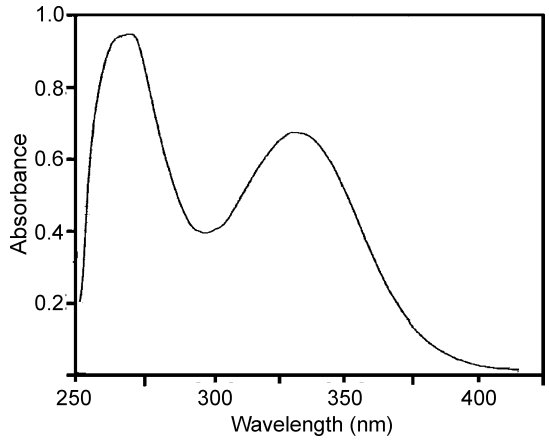

FIGURE 1 Absorbance spectrum of 5′-*O*-(1,1-*bis*-(*tert*-butyl)-1(1-pyrenylmethyl)silyl)-thymidine (**1**) in DCM (λ_{max} at 260 nm and 346 nm).

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The 13-mer and 26-mer sequences were purified on 8 M denaturing urea polyacrylamide gel electrophoresis. The fluorescent bands were visible up to at 0.5 OD units by fluorescence on a wet gel at 350 nm.

Absorbance and Fluorescence Studies

Absorbance studies of all the purified oligonucleotide showed desired λ_{max} at 260 nm and 346 nm (see Figure 1 for compound 1). The fluorescence studies could not be done in methanol and water as the small oligos were found to be only sparingly soluble in these solvents. Since the oligos were found to be readily soluble

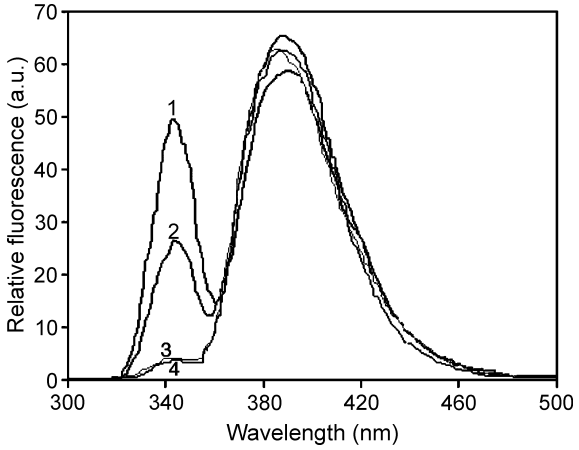

FIGURE 2 The relative fluorescence spectra of 1) T*T, 2) T*TTT, 3) T*AA TGG AGC CAG T, and 4) T* GC TAT GTC AGT TCC CCT TGG TTC TC.

in DMF buffer, therefore, all the spectra were recorded using it [10 mM phosphate buffer (pH 7.0) in 20% v/v dimethylformamide (DMF) and 0.2 M NaCl]. DMF is reported to have an additional advantage of an effective solvent for controlling hybridization stringency as well as having a similar effect as that of formamide and for enhancing the fluorescence of pyrene-labeled products.[13,14] Furthermore, a water-miscible organic solvent may influence the fluorescence quantum efficiency of pyrene through pyrene-solvent dipole-dipole interaction. Emission spectra were recorded by exciting at 346 nm wavelength (Figure 2). All the studies were carried out at 0.1 calibrations and at high voltage of 300.

Acid and Base Stability

Acid stability of the pyrene-labeled nucleoside was checked by treating the compound with 0.01 M HCl. 5′-*O*-(1,1-*bis*-(*tert*-butyl)-1(1-pyrenylmethyl)silyl)-thymidine (**1**) was dissolved in DCM (0.5 mL) containing HCl (0.01 M) and then stirred for 24 h at r.t. Intactness of the compound was confirmed by TLC as well as absorbance studies. The base stability of the group was checked at 55°C in the presence of 30% ammonia for 15 h (regular time course for the cleavage of oligos from the solid support). The compound was found to be 100% stable during the above-mentioned period.

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RESULTS AND DISCUSSION

In the present communication, we have synthesized oligonucleotide sequences covalently attached to a silyl fluorescent moiety as a purification handle. The synthesis is a simple one step procedure and the in situ product formed, i.e., 1,1-*bis*-(*tert*-butyl)-1-pyrenylmethyl-silyl chloride is subsequently used for 5′-OH protection of nucleosides. Thus, a one-pot synthesis is possible for protection of all of the nucleosides (Scheme 1). The 5′-*O*-protected thymidine, being highly hydrophobic in nature, was obtained in crystallized form and is, therefore, suitable for long-term storage. The acid stability and the base stability of the monomer enhances its utility since it can withstand the basic condition while cleaving the sequence from the support. All the fluorescence studies were carried out at 0.04 OD/ml concentration. Four different lengths of sequence were used for the studies: 1) dimer, 2) tetramer, 3) 13-mer, and 4) 25-mer to visualize the effect of length on the fluorescent properties when covalently attached to sequences. The fluorescence studies showed a peculiar phenomenon. At the dimer and tetramer level, the excitation intensity and the emission intensity were similar. However, at the 13-mer and 26-mer levels, the excitation peaks were observed to be very diminished whereas the emission peaks were observed to be have high intensity (Figure 2). This indicates that pyrene at the 5′-terminus position is sterically unhindered, which results in a better emission intensity and allows for fluorescent detection even in the nanomolar range. This strategy would enable the easy identification of the desired product in a complex

mixture where the desired full-length sequence can be easily purified from the shorter, incomplete synthesis products.

CONCLUSIONS

- We have demonstrated successful one-pot synthesis of 5′-*O*-(1,1-*bis*-(*tert*-butyl)-1(1-pyrenylmethyl)silyl) nucleoside in a < 60% yield by simple and efficient purification facilitated by the presence of a bulky hydrophobic group.
- Purification of 5′-*O*-1, 1-*bis*-(*tert*-butyl)-1′-pyrenylmethyl-silyl-nucleoside labeled oligonucleotides of different lengths, viz. 2-mer, 4-mer, 13-mer and 25-mer is easily achieved using this approach.
- Visualization of labeled oligonucleotides at 0.5 OD units on a denaturing polyacrylamide gel aids in quantitative recovery of the full-length product from the truncated and failed sequences, reducing the cost involved in post-synthetic purification.

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