Di(*tert*-butyl)[(pyren-1-yl)methoxy]silyl Chloride: Synthesis and Application in Purification of Synthetic Deoxyribonucleotides

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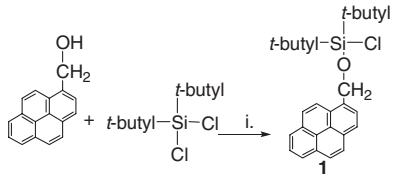
Di(*tert*-butyl)[(pyren-1-yl)methoxy]silyl chloride reacts selectively with the 5′-hydroxy groups in deoxynucleosides and this can be removed under conditions which do not affect other commonly used acid or base labile protecting groups, yet it, is stable to phosphorylation conditions. While incorporated terminally at 5′-OH of the long sequence viz., 26-mer, this group is also helpful in subsequent purification by HPLC as well as PAGE. Fluorescence properties of these sequences were studied to find the suitability of the approach.

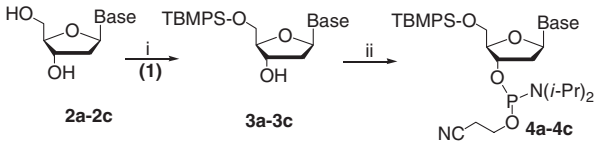
Applications of fluorescent probes in vitro and in vivo are an integral part of modern biochemistry.[1,2] In polymer-supported synthesis of oligonucleotides, the purification of the crude oligonucleotide product is a long time challenge. For this purpose, a variety of 5′-OH protecting groups have been designed, which serve as "purification handles."[3–7] Resolution of the purification problem was further solved by the incorporation of some fluorescent groups[8] so that the detection of oligonucleotides on thin layer chromatography or gels down to picomol level, in the visible range became possible. Caruthers[9] has reported a number of silyl substituents which can be regioselectively introduced and deprotected in less than 30 s with TBAF in THF. These groups have been tested for stability in acids and bases. We predict that use of silyl groups having fluorescent moieties would carry additional advantages for detection of cleavage of the group along with its acting as a purification handle.

Earlier our group has described the use of TBMPS–Cl for the protection of hydroxy group of thymidine.[10] We have applied these same conditions for dA/dC/dG and find that, (a) TBMPS–Cl react preferentially with 5′-hydroxy group; (b) the reaction is rapid even at room temperature; (c) even in the presence of excess TBMPS–Cl reaction occurs preferentially with hydroxy group and not with amino groups; (d) the TBMPS–Cl group is stable to phosphorylation conditions; and (e) the TBMPS–Cl group can be removed with $(n\text{-Bu})_4$NF in THF as describe by Corey and Venkateswarlu[11] without affecting other acid or base labile group on nucleosides. This has facilitated the preparation of number of protected nucleosides.

A general method for the preparation of 5′-protected deoxyribonucleosides has been out in Schemes 1 and 2. Di(*tert*-butyl)[(pyren-1-yl)methoxy]silyl chloride (TBMPS–Cl) (**1**)[11] is prepared by the reaction of 1-pyrenmethanol with di-*tert*-butyldichlorosilane in pyridine and methanolic sodium hydroxide solution (Scheme 1). TBMPS–Cl group was used for protection of 5′-hydroxy group of deoxyribonucleosides viz. N^6-benzoyl deoxyadenosine, N^4-benzoyl deoxycytidine, and N^2-isobutyryl deoxyguanosine in the presence of DMAP to yields (**3a**–**3c**), an example of primary hydroxy group, the 5′-O-protected nucleosides (**3a**–**3c**) were phosphitylated using 2-cyanoethyl bis(*N*,*N*-diisopropyl)phosphorodiamidite



Scheme 1. Synthesis of di(*tert*-butyl)[(pyren-1-yl)methoxy]silyl chloride (1) i) pyridine, 2 M methanolic sodium hydroxide, r.t., 2 h.



Series	Nucleoside (**2a-2c**)	Yield of **3**	Yield of **4**
a	N^4-benzoyldeoxycytidine	91%	65%
b	N^6-benzoyldeoxyadenosine	89%	68%
c	N^2-isobutyryldeoxyguanosine	94%	81%

Scheme 2. Synthesis of 5′-O-TBMPS deoxyribonucleosides phosphoramidites; i). pyridine, DMAP, TEA, TBMPS–Cl (**1**) ii). Bis reagent/DCM, Py-TFA.

(bis-reagent) and activator pyridinium trifluoroacetate (Py-TFA) in dry DCM to gave the desired phosphoramidite product (**4a**–**4c**) (Scheme 2).[12]

A 2-mer, 4-mer, 13-mer, and 26-mer sequences were synthesized using phosphoramidite chemistry. Modified 5′-*O*-di(*tert*-butyl)[(pyren-1-yl)methoxy]silyl-3′-[(2-cyano-ethyl)-*N*,*N*-diisopropyl]phosphoramidite (of deoxycytidine, deoxyadenosine, and deoxyguanosine) (**4a**–**4c**) were added in last coupling cycle (Figure 1). 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 method in series, absorbance at 260 nm and fluorescence with excitation and emission wavelength at 346 and 390 nm respectively. The fluorescent-labeled oligomers can be easily separated from the non-fluorescent failure sequences. Only the fractions showing both peaks of

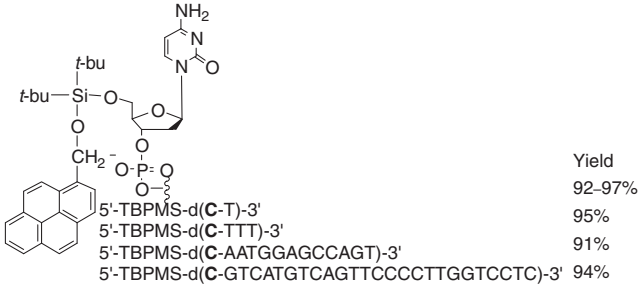


Figure 1. Examples of pyren-labeled oligodeoxyribonucleotides.

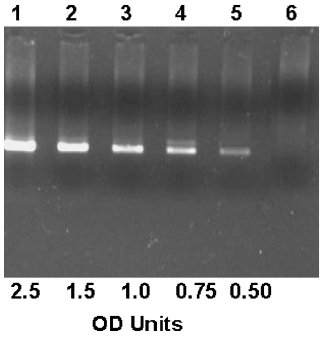


Figure 2. Detection limits: Gel electropherograms; lower panel ON4 (26-mer), volume in each well is 200 μL, lane 1: (2.5 μM); lane 2: (1.5 μM); lane 3: (1.0 μM); lane 4: (0.75 μM); lane 5: (0.50 μM), of 5′-d(**C**^{pl}G TCA TGT CAG TTC CCC TTG GTC CTC)-3′ (26-mer) and lane 6: 26-mer without TBMPS group. Sample were illuminated using a standard laboratory UV-lamp, $\lambda_{max} = 365$ nm. ^{pl} = TBMPS.

absorbance as well as fluorescence were collected and pooled. The fluorescence clearly indicates that the whole peak complex represents products of the correct length. Confirmation of the correct mass has been done by MALDI-TOF mass spectrometry.

The TBMPS group was successfully cleaved by treatment of 2–3 equiv. of tetra-*n*-butylammonium fluoride (TBAF) 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.

The 13-mer and 26-mer both were purified on 8 M urea polyacrylamide gel electrophoresis. As observed with DMTr-protected oligomers, the TBMPS-protected oligomers are well separated from non-fluorescent failure sequences. The fluorescent bands were visible up to at 0.5 OD concentrations by fluorescence on a wet gel at 350 nm and, because of the retarding effect of the TBMPS group; it is well separated from $n-1$ and other failure sequences. The fluorescence studies of all these pyren-labeled oligonucleotides have been carried out (Figure 2).

In this paper, the synthesis of oligonucleotide sequences covalently attached to a silyl fluorescent moiety as purification handle has been carried out. The synthesis is a simple one-step procedure and the in situ product formed i.e. di(*tert*-butyl)[(pyren-1-yl)methoxy]silyl chloride (**1**) is subsequently used for 5′-OH protection of nucleosides. Thus, a one-pot synthesis is possible for protection of all of the nucleosides (**2a**–**2c**). The nucleosides protected with 5′-TBPMS group tend to crystallize easily therefore product can be purified without expensive column chromatography. The monomer is obtained in fairly good yield. The acid stability and the base stability of the monomer show its usefulness that it can withstand the basic condition while cleaving the sequence from the support.

All the fluorescence studies were carried out at 0.04 OD concentrations. Since the oligomers were readily soluble in DMF buffer, therefore, all the spectra were recorded in it (10 mM phosphate buffer (pH 7.0), 20% v/v dimethylformamide (DMF) and 0.2 M NaCl). DMF is reported to be an effective solvent for controlling hybridization stringency as well as having a similar effect as that of formamide and for enhancing the fluorescence of pyren labels.[13,14] The fluorescence studies showed a peculiar phenomenon. Four different lengths of sequences i) dimer; ii) tetramer; iii) 13-mer, and iv) 25-mer

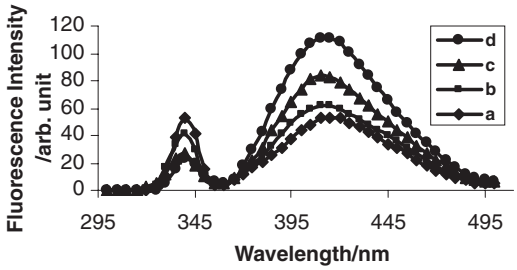


Figure 3. The relative fluorescence spectra of 1) 5′-d(**C**^{pl}T)-3′ shown by (a); 2) 5′-d(**C**^{pl}TTT)-3′ shown by (b); 3) 5′-d(**C**^{pl}AAT GGA GCC AGT)-3′ shown by (c) and 5′-d(**C**^{pl}G TCA TGT CAG TTC CCC TTG GTC CTC)-3′ shown by (d) in 10 mM phosphate buffer (pH 7.0), 20% v/v DMF and 0.2 M NaCl by exciting at 346 nm. ^{pl} = TBMPS.

was used for studies to visualize the effect of length on the fluorescent properties when covalently attached to sequences (Figure 1). At the dimer and tetramer level the excitation intensity and the emission intensity were more or less same. But at 13-mer and 26-mer levels the excitation peaks were found to be too diminished whereas the emission peaks were found to be having significant intensity (Figure 3). This shows that pyrene at 5′-terminus position is sterically unhindered which gives a better emission intensity, which makes it detectable through fluorescence even at as much low concentration. This would enable to identify easily the desired product in a complex mixture, where some of the sequences may get truncated in the synthesis cycle.

In conclusion we found that TBPMS–Cl react selectively with the hydroxy groups of nucleosides and preferentially with the 5′-hydroxy group. Because of the compatibility of TBPMS group with other acid and base labile groups, almost complete manipulation of protecting group is now possible. The TBMPS group is stable to phosphorylation. The nucleosides protected with 5′-TBPMS group tend to crystallize easily therefore product can be purified without expensive column chromatography. Purification of 5′-TBMPS-labeled oligonucleotides of different length viz. 2-mer, 4-mer, 13-mer, and 26-mer is easily achieved using this approach. Thus, the desired sequences may be purified from the rest of $(n-1)$ failure sequences at 0.5 OD unit on denaturing polyacrylamide gel aids in quantitative recovery of the full length product (Figure 2).

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