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PHOSPHOCYTIDINES AS VERSATILE 3' PROTECTING GROUPS IN TRIESTER SYNTHESIS OF OLIGODEOXYRIBONUCLEOTIDES

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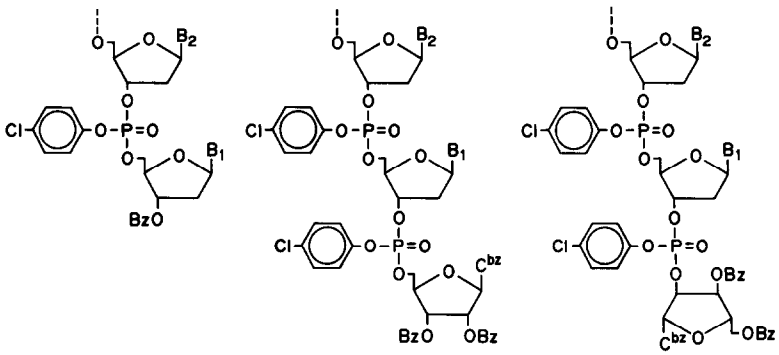
Summary: Any oligodeoxyribonucleotide triester block with a 3' aryl phosphate end can be protected by condensing its phosphodiester terminal with the 2'-hydroxyl group of $\underline{N}^4,\underline{O}^{3'},\underline{O}^{5'}$-tribenzoylcytidine. As the final step in deprotection of the modified block, mild treatment with Pb^{++} ion cleaves the new 3'-2' linkage in such a way that the original 3' terminal phosphate group of the oligomer is transferred to the ribonucleoside. This procedure eliminates the need for specialized terminator blocks in triester synthesis.

Biological applications of chemically synthesized oligodeoxyribonucleotides generally require that the molecules possess a free 3'-hydroxyl terminus. However, most synthetic schemes are based on the use of suitably protected deoxyribonucleoside 3' phosphates as the construction units, so that generation of a non-phosphorylated 3' end poses a special problem. Usually, this has been dealt with by introducing one of the four 3'-$\underline{O}$-acyldeoxyribonucleosides as a 3' terminator. In schemes that employ dimers as synthetic units the situation is still more complicated since an additional sixteen terminators are then necessary. Both strategies lead to oligomers having the structure I (Figure 1); these molecules can be deprotected to yield the desired products dN-dN_n-dN directly.

In order to minimize the number of synthetic units required for constructing defined sequence oligonucleotides, we originally proposed[1] the use of the single ribonucleoside $\underline{N}^4,\underline{O}^{2'},\underline{O}^{3'}$-tribenzoylcytidine as a universal 3' terminator that can be added to the 3' phosphodiester end of a triester block, giving molecules of the type II (Figure 1). After deprotection, the resulting dN-dN_n-dN-rC can be converted into dN-dN_n-dNp by periodate oxidation of the 2',3'-cis-hydroxyl system followed by β-elimination[2,3]. Enzymatic dephosphorylation then gives dN-dN_n-dN. This two-step conversion procedure provides good yields of 3'-hydroxyl terminated oligomers but has proved inconvenient in practice, particularly when applied to large scale preparations, and now we only use $\underline{N}^4,\underline{O}^{2'},\underline{O}^{3'}$-tribenzoylcytidine for generating molecules with a 3' terminal phosphate.

As a simpler alternative, we have investigated the properties of an "inverted" cytidine residue. This is joined to the end of the oligodeoxyribonucleotide block through a 3'-2' phosphotriester linkage (III, Figure 1), formed by condensing the block's terminal phosphodiester with $\underline{N}^4,\underline{O}^{3'},\underline{O}^{5'}$-tribenzoylcytidine[4]. In this case, after deprotection, the cytidine residue in the resulting dN-dN_n-dN(3'-2')rC possesses a free neighboring 3'-hydroxyl function which, by intramolecular attack on the internucleotide phosphodiester linkage at elevated pH, promotes breakdown into cytidine 2',3'-cyclic phosphate and dN-dN_n-dN.



I II III

FIGURE 1: B = thymin-1-yl, $\underline{N}^4$-benzoylcytosin-1-yl, $\underline{N}^6$-benzoyladenin-9-yl or $\underline{N}^2$-isobutyrylguanin-9-yl.

Such neighboring group participation in the hydrolysis of phosphodiesters is well-known from the example of alkaline degradation of RNA. However, the strong alkali required also causes some deamination of cytidine residues[5], so we sought milder conditions. Divalent metal ions have been reported to depolymerize RNA at room temperature and at pH values near 7[6,7]. With this in mind we synthesized oligodeoxyribonucleotides with "inverted" cytidine termini[8] and examined the removal of the phosphocytidine residue using Pb^{++} ion, which has been described as a particularly effective depolymerizing agent[7]. As a simple test case we first prepared dT-dT_{10}-dT. The projected synthesis of this 3'-hydroxyl terminated dodecamer from hexamers would normally require two different blocks, one with a 3'-phosphate terminus and a second with a protected 3'-hydroxyl group. However, using the new strategy, only the precursor [$(MeO)_2$Tr]dTºdTºdTºdTºdTºdTº(CNEt)[10,11] was necessary.

The hexamer was decyanoethylated[12] and divided into two equal parts. One half (0.038 mmol) was preactivated in pyridine (400 µl) with toluenesulfonyl nitrotriazole (TSNT, 0.15 mmol) for 10 min, then treated with $\underline{N}^4$,$\underline{O}^{3'}$,$\underline{O}^{5'}$-tribenzoylcytidine (0.115 mmol) for 2 hr. The product, [$(MeO)_2$Tr]dTºdTºdTºdTºdTºdT3'º2'[(5'Bz)rbzC(3'Bz)], was isolated in 78% yield by silica gel column chromatography, detritylated, and condensed with the remaining portion of the decyanoethylated hexamer, again using TSNT. After workup and chromatography, fully protected dT-dT_{10}-dT3'-2'rC was obtained in 85% yield. The material was deprotected with 1.0 M tetramethylguanidinium pyridinealdoximate (2 days, 25°), concentrated ammonia (3 days, 25°), and 80% acetic acid (20 min, 25°), then salts were removed by a Sephadex G-10 column. A sample of the cytidine terminated oligomer (40-400 A_{260} units in 1 ml of water) was adjusted to pH 7-8 with ammonia and treated with lead acetate (38 mg, 0.1 mmol) at 37° for 18 hr. Dowex ion-exchange resin (Bio-Rad AG50W-X2(Na^+), 100-200 mesh, 1 ml) was then added and the suspension was stirred until most of the precipitated lead salts had dissolved (0.5 hr). The entire mixture was transferred to the top of a column (1 X 5 cm) of the same resin, which was washed with water (50 ml). The eluate was concentrated $\underline{in\ vacuo}$ and the dT-dT_{10}-dT was isolated by chromatography on a column of cross-linked polyethyleneimine (PEI) on microparticulate silica[11]. The overall yield, based on 0.038 mmol of the original protected hexamer, was 47%.

The product was chromatographically indistinguishable from a commercial sample of dT-dT_{10}-dT (P.-L. Laboratories), and was completely degradable by snake venom phosphodiesterase and by spleen phosphodiesterase, indicating the presence of free hydroxyl groups at both 3' and 5' ends. Analysis of the dodecamer using the spleen enzyme method described earlier[11] gave a hyperchromicity of 9.3% at 260 nm (pH 6.5, 25°) and the ratio dTp:dT=11.1:0.96.

In addition, a fully protected decamer with the sequence d(CCAGTGTTC)3'-2'rC was constructed from pentamers corresponding to the sequences d(CCAGT) and d(GTTC)3'-2'rC, using analogous procedures. After deprotection and Pb^{++} treatment, the overall yield of d(C-C-A-G-T-G-T-T-C) was 54% (based on the block d(GTTC)). The molecule was completely degradable by each of the phosphodiesterases and analysis as before gave a hyperchromicity of 19.8% at 260nm (pH 6.5, 25°) and base ratios of dCp:dAp:dTp:dGp:dC=2.1:1.1:3.0:1.9:0.9. Figure 2 shows the chromatographic elution profiles obtained from aliquots taken during the deprotection of d(CCAGTGTTC)3'-2'rC just before the addition of lead acetate and immediately after the removal of the lead ions. It is clear that the pyridinealdoximate and ammonia treatments already cause a partial loss of the phosphocytidine residue and that the lead treatment results in the complete removal of the terminator. A number of other oligomers, representing molecules ending with all four deoxyribonucleosides, have now been prepared using this strategy. In each case, despite some differences in rates, the phosphocytidine group was completely removed by overnight reaction with Pb^{++}.

Accordingly, cytidine residues connected by 2'-3' phosphate linkages[13] to the ends of oligodeoxyribonucleotides constitute a useful method of protection, replacing four nucleoside and sixteen dinucleoside phosphate terminators and, in effect, removing the necessity for specialized terminal blocks in phosphotriester syntheses. A further advantage of this methodology over other systems that also employ acidic removal of trityl blocking groups lies

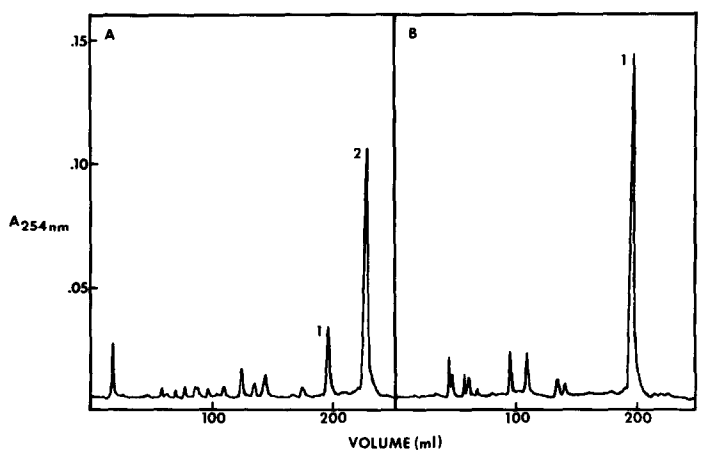


FIGURE 2: Chromatographic elution profiles of the products present before Pb^{++} treatment (A), and after Pb^{++} treatment (B), in the preparation of d(CCAGTGTTC). Peak identification: 1, d(CCAGTGTTC); 2, d(CCAGTGTTC)3'-2'rC. Column: cross-linked PEI-silica (1 X 25 cm). Solvent: 240 ml of aqueous 0.05 M KH_2PO_4 containing 15% (v/v) MeCN and a linear gradient of 0-0.2 M $(NH_4)_2SO_4$ at pH 6.0; flow rate: 4 ml/min.

in the synthesis of molecules of the form dN-dN_n-dA, where a cytidine 2'-phosphate terminator helps to protect the adjacent N-benzoyldeoxyadenosine against depurination by maintaining it in an internal position, with two flanking phosphotriester groups, until the end of the synthesis. Such internal units are reported to be considerably more resistant to acidic cleavage of the glycosyl bond than N^6-benzoyl-$O^{3'}$-acyldeoxyadenosine terminators[14], a factor that becomes more significant during the multiple cycles of detritylation conditions to which the 3'-terminal regions of longer synthetic oligonucleotides are inevitably exposed.

Acknowledgements

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8. We chose cytidine for a terminator because of the high mobility of its protected derivatives during silica gel chromatography, a property which facilitates the separation of excess nucleoside from oligonucleotides after condensation. Moreover, phosphocytidine residues are second only to phosphouridine with respect to their rate of alkaline hydrolysis in RNA[9].
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10. The symbol ² denotes a p-chlorophenyl phosphotriester linkage.
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13. We have synthesized molecules with phosphocytidine attached via a 3'-3' linkage in a similar fashion, using N^4,$O^{5'}$-dipivaloyl-$O^{2'}$-benzoylcytidine[4]. The ribonucleotide in this configuration is also readily released by Pb^{++} ion.
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