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The use of barium salts of protected deoxyribonucleoside-3' p-chlorophenyl phosphates for construction of oligonucleotides by the phosphotriester method: high-yield synthesis of dinucleotide blocks

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ABSTRACT

A new experimental approach to the synthesis of polydeoxyribonucleotides via the phosphotriester method involves construction of oligonucleotide blocks by direct use of the easily prepared barium salts of $O^{5'}$,N-protected deoxyribonucleoside-3' p-chlorophenyl phosphates as the key monomers in condensation reactions. The procedure has been demonstrated by the rapid synthesis in high yield and purity of all sixteen fully protected dinucleotides $[(MeO)_2Tr]dN'^{o}dN'^{o}(CNEt)$, (where dN' = dT, dbzC, dbzA, or dibG; ^{o} = diesterified p-chlorophenyl phosphate). This set of molecules constitutes a "syllabary" for the preparation of defined sequence oligonucleotides.

INTRODUCTION

We recently reported[1] simplified methods for preparation by the modified phosphotriester approach[2,3] of oligonucleotide blocks that can be used in the synthesis of defined sequence polynucleotides. The key intermediates in the new strategy are base-protected 5'-O-dimethoxytrityldeoxyribonucleoside-3' p-chlorophenyl phosphates which are isolated by a barium salt precipitation procedure. These nucleoside phosphodiesters, after conversion to their tetraethylammonium salts, can be employed for the phosphorylation of suitable nucleotide derivatives possessing 5'-hydroxyl groups, under the action of a new class of condensing agents, the arylsulfonyl nitroimidazoles. With these techniques we prepared moderately large quantities of two dinucleotide blocks which, in conjunction with appropriate monomers, provided the basic materials for synthesis of an icosanucleotide analogous to the sequence at the ends of Rous sarcoma virus 35S RNA. We have now determined that the barium salts of the protected nucleoside phosphodiesters can be used directly in syntheses of this type, and have demonstrated the efficacy of the new procedure by rapidly preparing in high yield all 16 fully protected dinucleotide blocks of the form $[(MeO)_2Tr]dN'^{o}dN'^{o}(CNEt)$.[4]

SYNTHESIS OF FULLY PROTECTED DINUCLEOTIDES FROM MONONUCLEOTIDE BARIUM SALTS

The pure barium salts of 5'-O-dimethoxytrityl protected deoxyribonucleoside-3' p-chlorophenyl phosphates (I, Fig.1) were prepared in high yield by our previously reported method,[1] slightly modified to decrease the time required for the phosphorylation reaction. These stable, easily handled solids were the basis for synthesis of the 16 possible fully protected dinucleotides, which were prepared in groups of four from a common 3'-terminal nucleotide. Mononucleotide barium salt (I) was converted in 16 hr to the fully protected triester II by reaction with 2-cyanoethanol in the presence of the condensing agent 1-(p-toluenesulfonyl)-4-nitroimidazole.[1] II, without isolation, was detritylated using excess benzenesulfonic acid and the resulting 5'-hydroxyl component III was divided into four equal parts. These were separately phosphorylated with different nucleotide barium salts (I), the four reactions running simultaneously. The resulting dimers IV were then isolated by silica gel column chromatography. Three more runs, using the other mononucleotide barium salts as 3' termini, generated the remaining 12 dinucleotides. Minor complications arose in syntheses of dinucleotides containing deoxyguanosine residues. One problem concerned the deoxyguanosine nucleotide III which

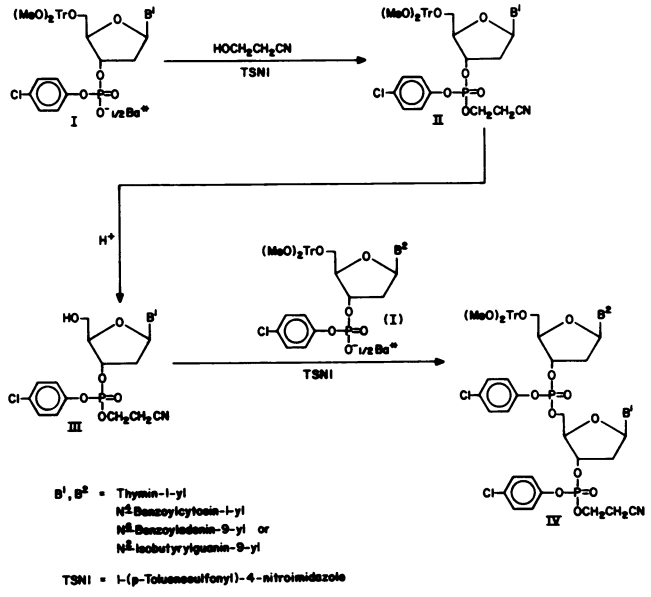

Figure 1. Synthesis of fully protected dinucleotides from protected mononucleotide barium salts.

proved to be moderately soluble in water, a property that resulted in some loss of material when the detritylation mixture was subjected to aqueous extraction as part of the regular procedure to remove benzenesulfonic acid. Consequently, for dinucleotides with deoxyguanosine at the 3' terminus, the general method was modified to include intermediate isolation of the monomer $[(MeO)_2Tr]dibG^{ø}(CNEt)$. Less benzenesulfonic acid was then required for detritylation, and aqueous extraction was unnecessary since small quantities of the acid, as its pyridinium salt, can be carried through into the subsequent condensation reactions without apparent adverse effects.

A further difficulty was encountered in early trials of reactions involving deoxyguanosine derivatives, regardless of the method of synthesis. During the second condensation (III + I → IV), significant amounts of a side product were formed. This material was first observed in syntheses leading to the d(NG) series of dinucleotides where it appeared on thin layer chromatography as a band with a slightly higher mobility than the fully protected dimer. It was later also detected in lesser amounts in the d(GN) series. However, it proved to be labile and disappeared when the post-condensation reaction mixtures were treated for 40 min with aqueous pyridine. In order to determine its nature, we isolated by silica gel column chromatography a small quantity of the side product generated in a reaction for the synthesis of fully protected d(AG). On treatment with aqueous pyridine it broke down into $[(MeO)_2Tr]dbzA^{ø}dibG^{ø}(CNEt)$ together with a product that contained a dimethoxytrityl group and remained at the origin on TLC plates. Moreover, when the material was partially deprotected with ammonia and analyzed as discussed in the following section, it was converted to $[(MeO)_2Tr]dA\text{-}dG\text{-}(ClPh)$ and a substance that had the same spectral properties and chromatographic retention volume as $[(MeO)_2Tr]dA\text{-}(ClPh)$. On the basis of these results we suggest that the side product arises from attack of activated monomer $[(MeO)_2Tr]dbzA\text{-}(ClPh)$ on the guanine moiety of $[(MeO)_2Tr]dbzA^{ø}dibG^{ø}(CNEt)$, leading to the formation of a "branched" trinucleotide.[5] Since related contaminants can be found in all dinucleotide reactions involving deoxyguanosine residues, we have incorporated a post-condensation treatment with aqueous pyridine into their work-up as a routine procedure.

Using the above methods the sixteen dinucleotides were prepared in high yield as shown in Table 1.

DEPROTECTION AND ANALYSIS OF THE DINUCLEOTIDES

The purity of the fully blocked dinucleotides was examined in two ways. First, thin-layer chromatography on silica gel plates showed each dimer

Table 1. Yields and Chromatographic Retention Volumes of Dinucleotide Blocks

Nucleotide Sequence	Fully Protected Dinucleotide	Yield[a] %	$\bar{V}$ (ml) for [(MeO)$_2$Tr]dN-dN-(ClPh)[b]
CC	[(MeO)$_2$Tr]dbzC^{o}dbzC^{o}(CNEt)	93	83
AC	[(MeO)$_2$Tr]dbzA^{o}dbzC^{o}(CNEt)	91	80
TC	[(MeO)$_2$Tr]dT^{o}dbzC^{o}(CNEt)	92	82
GC	[(MeO)$_2$Tr]dibG^{o}dbzC^{o}(CNEt)	88	111
CA	[(MeO)$_2$Tr]dbzC^{o}dbzA^{o}(CNEt)	91	86
AA	[(MeO)$_2$Tr]dbzA^{o}dbzA^{o}(CNEt)	92	84
TA	[(MeO)$_2$Tr]dT^{o}dbzA^{o}(CNEt)	89	86
GA	[(MeO)$_2$Tr]dibG^{o}dbzA^{o}(CNEt)	85	109
CT	[(MeO)$_2$Tr]dbzC^{o}dT^{o}(CNEt)	88	88
AT	[(MeO)$_2$Tr]dbzA^{o}dT^{o}(CNEt)	91	87
TT	[(MeO)$_2$Tr]dT^{o}dT^{o}(CNEt)	90	92
GT	[(MeO)$_2$Tr]dibG^{o}dT^{o}(CNEt)	83	111
CG	[(MeO)$_2$Tr]dbzC^{o}dibG^{o}(CNEt)	81	123
AG	[(MeO)$_2$Tr]dbzA^{o}dibG^{o}(CNEt)	83	108
TG	[(MeO)$_2$Tr]dT^{o}dibG^{o}(CNEt)	82	116
GG	[(MeO)$_2$Tr]dibG^{o}dibG^{o}(CNEt)	75	155

[a] Yield of the fully protected dinucleotide is based on the nucleoside phosphodiester barium salt used for the 3' end, except in the NG series, where yield is based on [(MeO)$_2$Tr]dibG^{o}(CNEt).
[b] Anion-exchange retention volume of partially deprotected dinucleotide.

running as a single band or, in some cases, as two or three closely adjacent bands representing resolution of some of the four possible diastereomers. A more stringent test of purity was carried out by partially deprotecting each dinucleotide with ammonia. This treatment removes the 3'-terminal cyanoethyl group, the p-chlorophenyl group from the internucleotide phosphotriester linkage, and the blocking groups from the bases. The resulting compounds [(MeO)$_2$Tr]dN-dN-(ClPh) were analyzed by high-resolution anion-exchange column chromatography. Each dinucleotide ran as a single peak accompanied by only trace amounts of material in the mononucleotide elution range. This range was established by chromatographing authentic samples of the various mononucleotides dN-(ClPh) and [(MeO)$_2$Tr]dN-(ClPh), since these would be the deprotected equivalents of contaminants most likely to be found in the dinucleotide products. The purities of the dimers, based on optical density measurements at 254 nm, were estimated to lie between 96 and 99%.

PREPARATION OF THE BARIUM SALT OF A PROTECTED DINUCLEOTIDE

The fully protected dinucleotides must be decyanoethylated in every case before addition to the 5' end of a growing oligonucleotide. An obvious extension of the present work, therefore, resides in using the dinucleotides as their preformed 3'-p-chlorophenyl phosphate barium salts for such chain elongation. As a preliminary test of the feasibility of preparing these compounds, we decyanoethylated $[(MeO)_2Tr]dT^{o}dT^{o}(CNEt)$ using triethylamine in pyridine and precipitated the resulting $[(MeO)_2Tr]dT^{o}dT$-(ClPh) as its barium salt. The integrity and purity (> 99%) of this material were demonstrated by partial deprotection followed by ion-exchange chromatographic analysis. The same compound was also conveniently synthesized by phosphorylating the dimer $[(MeO)_2Tr]dT^{o}dT$ and precipitating the product in a manner analogous to that used for preparation of the mononucleotide barium salts. Agarwal and Riftina[6] have recently reported an extremely rapid procedure for synthesizing various dinucleoside monophosphates of the type $[(MeO)_2Tr]dN'^{o}dN'$, which in light of the above can be readily adapted to our own strategy.

CONCLUSION

The readily prepared barium salts of $O^{5'}$,N-protected deoxyribonucleoside-3' p-chlorophenyl phosphates and their capacity for direct use in condensation reactions provide a facile route to the sixteen fully protected dimers $[(MeO)_2Tr]dN'^{o}dN'^{o}(CNEt)$. These dinucleotides, together with the four mononucleotide barium salts and one 3'-terminal blocking group (such as the removable ribonucleoside $bzC(OBz)_2$[1]), constitute a complete and easily acquired set of synthetic units for rapid construction of oligodeoxyribonucleotides of any desired sequence.

EXPERIMENTAL

Materials and Methods

5'-O-Dimethoxytritylthymidine and 5'-O-dimethoxytrityl-N^2-isobutyryldeoxyguanosine were synthesized by published procedures.[7,8] The dimethoxytrityl derivatives of N^4-benzoyldeoxycytidine and N^6-benzoyldeoxyadenosine were purchased from Collaborative Research, Inc. 2-Cyanoethanol (Aldrich Chemical Co.) was distilled under reduced pressure and stored over molecular sieves (Type 4A). The condensing agent 1-(p-toluenesulfonyl)-4-nitroimidazole (TSNI) was prepared as previously described.[1]

Eastman Chromagram sheets (silica gel with fluorescent indicator) were

used for thin-layer chromatography. The completeness of condensation reactions was monitored by TLC using 2% or 5% MeOH in $CHCl_3$ (v/v) as developing solvents. Dimethoxytrityl-containing compounds were detected by exposing the plates to the vapor from concentrated HCl. Silica gel chromatography of fully protected dinucleotides was carried out at 3° on columns (2.4 X 30 cm) of SilicAR CC-7 Special (Mallinckrodt) packed in chloroform, and partially deprotected dinucleotides were chromatographed on the anion-exchange material AS Pellionex SAX, obtained from Whatman, Inc.

Barium Salts of Protected Deoxyribonucleoside-3' p-Chlorophenyl Phosphates (I)

For 5 mmol: 1,2,4-Triazole (2.075 g, 30 mmol) was dissolved with stirring in warm anhydrous dioxane (50 ml). The solution was cooled to 25° and treated with triethylamine (4.2 ml, 30 mmol) followed by p-chlorophenyl phosphorodichloridate[9] (2.46 ml, 15 mmol). The thick slurry was stirred for 45 min then filtered to remove $Et_3NH^+Cl^-$. The filtrate was mixed with a solution of 5 mmol of protected nucleoside (5'-O-dimethoxytrityl derivative of dT, dbzC, dbzA, or dibG) in anhydrous 2,6-lutidine (10.5 ml) and the mixture was allowed to stand at 25° until TLC showed the phosphorylation to be complete (approximately 2 hr). It was then added dropwise with stirring to water (250 ml). After standing for 1 hr, the clear solution was poured into vigorously stirred aqueous barium chloride (10 g of $BaCl_2.2H_2O$ in 1 liter of water) at 3°. The resulting suspension was gently warmed to 30° without allowing the barium salt to settle. Upon formation of a filterable solid, the mixture was again cooled to 3°, stirred for 1 hr, then filtered through a coarse sinter under gravity flow. The collected precipitate was washed thoroughly with water and dried in vacuo over P_2O_5. The barium salts obtained by this procedure were white, non-hygroscopic powders, chromatographically homogeneous on TLC in $CHCl_3$-MeOH (80:20), and stable on prolonged storage at 0°. Barium 5'-O-dimethoxytritylthymidine-3' p-chlorophenyl phosphate: Anal. Calcd. for $C_{37}H_{35}ClN_2O_{10}PBa_{0.5}.H_2O$: C, 54.14; H, 4.54; N, 3.41; P, 3.77. Found: C, 53.84; H, 4.67; N, 3.40; P, 3.77. Barium 5'-O-dimethoxytrityl-N^6-benzoyldeoxyadenosine-3' p-chlorophenyl phosphate: Anal. Calcd. for $C_{44}H_{38}ClN_5O_9PBa_{0.5}.4H_2O$: C, 53.49; H, 4.69; N, 7.09; P, 3.14. Found: C, 53.50; H, 4.63; N, 6.77; P, 2.93. Barium 5'-O-dimethoxytrityl-N^4-benzoyldeoxycytidine-3' p-chlorophenyl phosphate: Anal. Calcd. for $C_{43}H_{38}ClN_3O_{10}PBa_{0.5}.2.5H_2O$: C, 55.12; H, 4.63; N, 4.49; P, 3.31. Found: C, 55.34; H, 4.92; N, 4.52; P, 3.54. Barium 5'-O-dimethoxytrityl-N^2-isobutyryldeoxyguanosine-3' p-chlorophenyl phosphate: Anal. Calcd. for $C_{41}H_{40}ClN_5O_{10}PBa_{0.5}.4H_2O$: C, 50.77; H, 4.98; N, 7.22; P, 3.19. Found: C, 50.54; H, 5.05; N, 7.23; P, 3.55.

General Method for Synthesis of Quartets of Fully Protected Dinucleotides (IV)

The barium salt of that protected deoxyribonucleoside-3' p-chlorophenyl phosphate (I, 2 mmol) destined to constitute the 3' end of a set of four dinucleotides was freed from water by several additions and evaporations of dry pyridine, then dissolved in a total of 5 g of anhydrous pyridine and treated with 2-cyanoethanol (10 mmol) and TSNI (6 mmol). After 16 hr at 25°, the mixture, which often set to a gel, was dissolved in pyridine (25 ml) and added to ethyl acetate (200 ml). The resulting suspension in ethyl acetate was extracted with 0.1 N sodium bicarbonate (3 X 120 ml) followed by 10% aqueous NaCl (120 ml). The EtOAc layer was concentrated to an oil from which pyridine was removed by evaporation with toluene (60 ml). The residue was treated at 0° with 60 ml of a 2% (w/v) solution of benzenesulfonic acid in chloroform-methanol (7:3, v/v). After 25 min, the mixture was neutralized with 5% $NaHCO_3$ and added to ethyl acetate (200 ml). Following extraction with 5% $NaHCO_3$ (60 ml) and water (60 ml), the EtOAc layer was treated with pyridine (30 ml), evaporated to ca 20 ml, and divided into four equal portions, each representing 0.5 mmol of III. To each of the four separate solutions was added a different protected deoxyribonucleoside-3' p-chlorophenyl phosphate barium salt (I, 0.65 mmol). The solutions were rendered anhydrous by additions and evaporations of pyridine, then each residue was dissolved in a total of 2 g of dry pyridine and treated with TSNI (2 mmol). After 16-18 hr at 25°, the mixtures were diluted with pyridine (6 ml), and, in the case of dinucleotides containing deoxyguanosine, the suspensions were further treated with 3 ml of H_2O for 40 min. Each condensation reaction mixture was separately transferred into ethyl acetate (75 ml), and washed with 5% $NaHCO_3$ (30 ml) followed by 10% NaCl (30 ml). The EtOAc layers were concentrated to thick oils, then dissolved in chloroform (3 ml each) and chromatographed on silica gel columns. Elution with 300 ml of $CHCl_3$ followed by 600 ml of $CHCl_3$-MeOH (98:2) yielded the fully protected dinucleotides, which were obtained as foams after evaporation of appropriate fractions from the columns. Yields (Table 1) were based on the constant weights of these materials after drying in vacuo (0.1 mm).

The dinucleotides were stored at <-20°. In the case of the d(GN) series, slight losses of dimethoxytrityl groups were observed following chromatographic isolation. This breakdown can be prevented by maintaining the presence of small amounts of pyridine in the samples after their emergence from the columns and during subsequent storage.

Modified Procedure for Synthesis of Fully Protected Dinucleotides Containing a 3'-Terminal dG Residue

5'-O-Dimethoxytrityl-N^2-isobutyryldeoxyguanosine-3' p-chlorophenyl phosphate barium salt (2 mmol) was converted to its fully protected triester [$(MeO)_2$Tr]dibG^{ib}(CNEt) by treatment with 2-cyanoethanol and TSNI in the usual manner. Upon completion of the cyanoethylation, the reaction mixture was dissolved in pyridine (10 ml) and added to ethyl acetate (200 ml). The suspension in ethyl acetate was extracted with 0.1 N $NaHCO_3$ (3 X 120 ml), then with 10% aqueous NaCl (3 X 120 ml). The EtOAc layer was evaporated to a thick oil which was dissolved in chloroform (5 ml) and chromatographed on silica gel. Elution of the column with 600 ml of $CHCl_3$ followed by 600 ml of $CHCl_3$-MeOH (98:2) gave pure [$(MeO)_2$Tr]dibG^{ib}(CNEt) in 96% yield.

For conversion to dinucleotides, this material was detritylated by treatment with 1.66 equivalents of benzenesulfonic acid, as a 2% solution in $CHCl_3$-MeOH (7:3), for 25 min at 0°. The deprotection was terminated by the addition of pyridine (30 ml). The mixture was then evaporated to ca 20 ml and divided into four equal portions. These four solutions were each treated with a different protected nucleoside phosphodiester barium salt (I, 1.3 equiv.) and, following removal of water by co-evaporation with pyridine, TSNI (2.6 equiv.) was added in the usual way. After 16-18 hr at 25°, the reaction mixtures were treated with pyridine (3 ml) and water (1.5 ml) and allowed to stand for 40 min. Ethyl acetate extraction and silica gel chromatography were then carried out as usual to isolate the four dinucleotides.

Deprotection and Analysis of the Dinucleotide Blocks

Each fully protected dinucleotide (ca 3 mg) was dissolved in pyridine (2 ml) and treated with 7 M NH_4OH (4 ml). After 3 days at 25°, pyridine was removed by several additions and evaporations of dilute ammonia. Finally, the deprotected material was stored at -20° in 40% aqueous ethanol (0.6 ml) containing one drop of concentrated NH_4OH. For analysis, a sample (30-50 µl) of the solution of dinucleotide was evaporated to dryness in the presence of triethylamine, redissolved in 40% ethanol, and chromatographed on a column (0.4 X 50 cm) of AS Pellionex SAX using a 200 ml gradient of 0-0.2 M NH_4Cl (pH 8) in 40% ethanol at 20 ml/hr. The partially deprotected dinucleotides [$(MeO)_2$Tr]dN-dN-(ClPh) appeared as single peaks accompanied by only trace amounts (< 4%) of mononucleotide material, and their retention volumes ($\bar{V}$) are given in Table 1. All possible mononucleotide contaminants, such as dN-(ClPh) and [$(MeO)_2$Tr]dN-(ClPh), have retention volumes in the range 28 to 58 ml; representative examples dC-(ClPh), [$(MeO)_2$Tr]dA-(ClPh), [$(MeO)_2$Tr]dC-(ClPh),

and [(MeO)$_2$Tr]dG-(ClPh) have $\bar{V}$ values of 28, 42, 44, and 58 ml, respectively.

Preparation of the Barium Salt of the Dinucleotide [(MeO)$_2$Tr]dT$^{\underline{o}}$dT-(ClPh)

(a) Fully protected dinucleotide [(MeO)$_2$Tr]dT$^{\underline{o}}$dT$^{\underline{o}}$(CNEt) (60 mg, 0.05 mmol) was dissolved in dry pyridine (2 ml) and treated with anhydrous triethylamine (1 ml). After 6 hr at 25°, the mixture was evaporated several times with pyridine to ca 1/3 of its original volume. The residual solution was added dropwise to vigorously stirred aqueous barium chloride (200 mg of $BaCl_2.2H_2O$ in 20 ml water). The suspension of barium salt was centrifuged and the precipitate was washed with water (2 X 20 ml). It was dried in vacuo over P_2O_5 to give an off-white solid (57 mg, > 90% yield). A sample (3 mg) of this material was deprotected as described above. Ion-exchange chromatography showed the product to be identical with the dinucleotide generated by direct ammonia deprotection of [(MeO)$_2$Tr]dT$^{\underline{o}}$dT$^{\underline{o}}$(CNEt), and indicated a purity of >99%.

(b) 5'-O-Dimethoxytritylthymidine-3' p-chlorophenyl phosphate barium salt (1 mmol), rendered anhydrous by several evaporations with pyridine, was treated in 3-4 g of dry pyridine with thymidine (1.3 mmol) and TSNI (2 mmol). After 20 hr at 25°, the mixture was transferred into chloroform (100 ml) which was extracted with 0.1 M triethylammonium bicarbonate (3 X 40 ml). The organic layer was dried (Na_2SO_4), evaporated, and applied to a 60 ml sintered glass funnel containing 50 ml of silica gel. The short column was washed with $CHCl_3$ (125 ml), then with increasing concentrations of MeOH in $CHCl_3$; the product (86% yield) was eluted with $CHCl_3$ containing ca 2% MeOH. This material was converted to its 3'-p-chlorophenyl phosphate barium salt by the same technique used to prepare the mononucleotide barium salts. About 0.7 mmol of [(MeO)$_2$Tr]dT$^{\underline{o}}$dT-(ClPh) barium salt was obtained and was shown, by partial deprotection and ion-exchange chromatography, to be identical with the material prepared as described in (a), and to have a purity of > 97%. The absence of 3'-3' phosphodiester linkages in this product was demonstrated by its complete degradation to thymidine upon detritylation followed by treatment with spleen phosphodiesterase and calf intestinal phosphatase.

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4. dN' represents dT, dbzC, dbzA, or dibG, and p-chlorophenyl phosphotriester linkages are denoted by the symbol º (see ref. 1).
5. We have also deliberately synthesized a representative example of this unusual side product by condensing $[(MeO)_2Tr]dT$-(ClPh) barium salt with the *fully protected* dinucleotide $[(MeO)_2Tr]dT^{º}dibG^{º}$(CNEt), and are attempting to discover the nature and position of the proposed linkage to guanine. This apparent lack of full protection of the base in dibG may well be a factor contributing to the low yields commonly encountered in chemical syntheses of oligonucleotides containing guanine residues.
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