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RAPID PREPARATION OF HEXANUCLEOTIDE TRIESTER BLOCKS FOR USE IN POLYDEOXYRIBONUCLEOTIDE SYNTHESIS

G. R. Gough*, M. J. Brunden, J. G. Nadeau, and P. T. Gilham

Department of Biological Sciences, Purdue University, West Lafayette, IN 47907, U.S.A.

Summary: Hexanucleotide phosphotriester blocks suitable for use in the construction of polydeoxyribonucleotides can be synthesized in high yield by a rapid procedure involving simple extractive purification of intermediates.

The dual objectives of speed and ease of assembly of oligodeoxyribonucleotides have been accomplished in a number of recently introduced solid support systems[1-4]. However, the solid phase approach despite its convenience is economically unattractive for large-scale production of oligomers since a considerable excess of incoming mono- or oligonucleotide is required in each chain extension step. As a way to overcome this disadvantage, we described measures by which the unused nucleotide units in a solid phase triester synthesis could be recovered from spent reaction mixtures as their barium salts and recycled[5]. Nevertheless, we felt that to produce the larger quantities of high-purity oligo- and polynucleotides necessary for physicochemical studies, an improved methodology for rapid phosphotriester block synthesis in solution would be more desirable.

Reaction mixtures for formation of the phosphotriester internucleotide linkage in solution generally contain a slight excess of a 3' phosphodiester over the 5'-hydroxyl component, together with a condensing agent. If, upon completion of the coupling, the unreacted excess of phosphodiester and the condensing agent could be quantitatively removed, the only species remaining would be the target phosphotriester ready for further chain elongation. We have noted[5] that the powerful coupling agent p-toluenesulfonyl nitrotriazole (TSNT)[6] is quickly hydrolyzed in aqueous pyridine to water-soluble products. Moreover, when N-protected 5'-O-dimethoxytrityl nucleoside-3' p-chlorophenyl phosphates serve as phosphodiester components, they can be extracted from organic solvents into water after removal of the lipophilic trityl group[7]. These observations form the basis of a scheme (Figure 1) for rapid (< 1 day) large-scale synthesis of fully protected hexanucleotide blocks, using the readily available mononucleotide barium salts $[(MeO)_2Tr]dN'\text{-}(ClPh).\frac{1}{2}Ba^{++}$ and dinucleotides $[(MeO)_2Tr]dN'^{o}dN'^{o}(CNEt)$[8,9].

Hexanucleotides I-IV (Table 1) were prepared using the procedure outlined in Figure 1 and described in detail in Footnote 10. The construction of hexamer V was further simplified by using the dinucleotide $[(MeO)_2Tr]dT^{o}dT\text{-}(ClPh)$ in place of the two internal monomer additions[12]. Finally, a fully protected hexanucleoside pentaphosphate (VI) terminating with a 3'-benzoyl group was assembled starting with the dinucleoside phosphate $[(MeO)_2Tr]dbzA^{o}dT(Bz)$. VI is an example of a block designed to be used at the 3' end of a synthetic sequence.

For estimation of their purity, 3 mg samples of the hexanucleotides were deprotected to the form $dN\text{-}dN_4\text{-}dN\text{-}(ClPh)$ by successive treatments with (a) pyridine:Et_3N (2:1 v/v, 3 ml, 6 hr, 25°) followed by evaporation in vacuo; (b) tetramethylguanidinium pyridinealdoximate[13] (1 M in

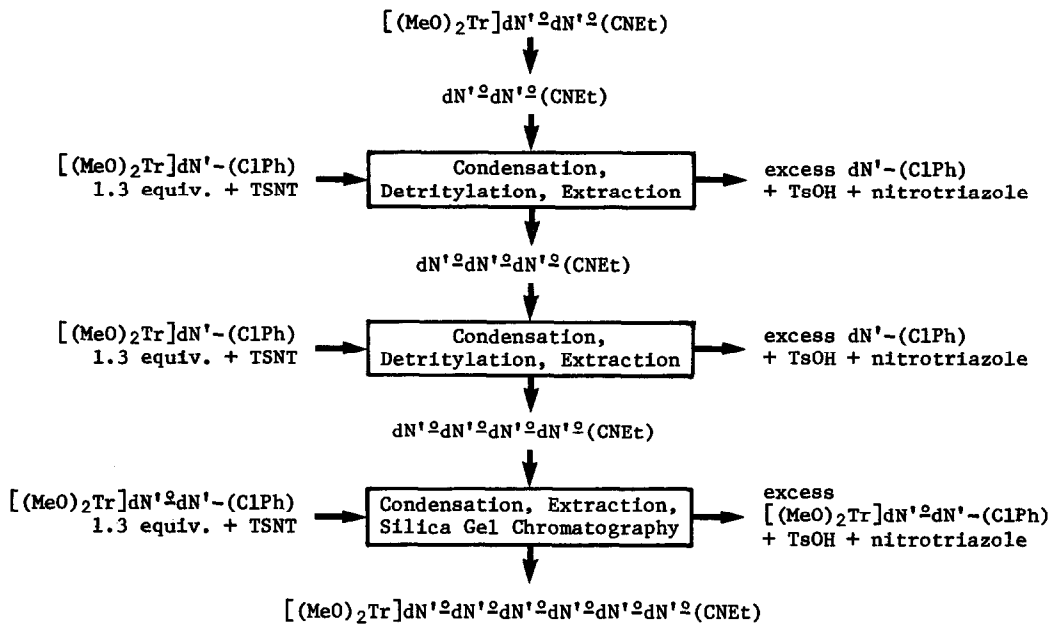

FIGURE 1: Scheme for solution phase triester synthesis of hexanucleotides

50% aqueous dioxane, 60 µl, 2 days at 25°); (c) concentrated NH_4OH (3 ml, 3 days, 25°) followed by evaporation to dryness; and (d) 80% acetic acid (3 ml, 25 min, 25°) followed by removal of solvent. The residue in each case was dissolved in 20% EtOH (1 ml) and the pH was adjusted to ca. 7 with NH_4OH. Hexamer VI was deprotected similarly with the omission of step (a) to give d(A-T-C-G-A-T). Aliquots of each solution were desalted (Sephadex G-10) and analyzed by HPLC on a column of cross-linked polyethyleneimine (PEI) on microparticulate silica[14]. The hexanucleotides were exceptionally pure; a typical elution profile is shown in Figure 2A for hexamer II.

The usefulness of these blocks for preparing longer oligonucleotides is demonstrated by the synthesis of d(T-G-T-A-T-C-A-T-C-G-A-T) from I and VI. Hexamer VI (0.0073 mmol) was detritylated with 3 ml of 2% benzenesulfonic acid in $CHCl_3$:MeOH (7:3 v/v) for 25 min at 0°, then partitioned between $CHCl_3$ (25 ml) and 5% $NaHCO_3$ (25 ml) containing t-BuOH (5 ml). The organic

TABLE 1		Sequence	Yield[a]
[(MeO)2Tr]dN'ºdN'ºdN'ºdN'ºdN'ºdN'º(CNEt):	I	TGTATC	61%
	II	GTTGAC	64%
	III	CCAACC	75%
	IV	TTGACG	53%
	V	TTTTTT	72%
[(MeO)2Tr]dN'ºdN'ºdN'ºdN'ºdN'ºdN'(Bz):	VI	ATCGAT	74%

[a]Determined by spectrophotometric assay of the dimethoxytrityl cation[11]

layer was extracted once more with the $NaHCO_3$:t-BuOH mixture and then with 10% NaCl (25 ml):t-BuOH (5 ml). The chloroform solution was dried (Na_2SO_4), filtered and evaporated. The residue was rendered anhydrous by co-evaporation with pyridine and combined with decyanoethylated hexamer I (0.0094 mmol kept in 3 ml of pyridine:Et_3N (2:1 v/v) for 6 hr at 25° followed by evaporation in vacuo). After further co-evaporation with pyridine, the mixture was treated in 250 mg of anhydrous pyridine with TSNT (35 mg). At the end of an hour, hydrolysis, extraction, and silica gel chromatography were performed as described in Footnote 10; the product was eluted from the column with $CHCl_3$:MeOH (96:4 v/v). The dodecamer was deprotected with pyridinealdoxime, ammonia, and acetic acid, then desalted and analyzed on the PEI-silica column (Figure 2B). Preparative chromatography on the same column gave 207 A_{260} units of pure material (29% based on VI). The oligonucleotide was completely degraded by snake venom and spleen phosphodiesterases, and its base composition was verified[15]. After 5' end labeling with ^{32}P, it traveled as a single spot on two-dimensional electrophoresis-homochromatography and its sequence was confirmed by mobility shift analysis (Figure 2C)[16,17]. The overall yield of the isolated dodecamer compares favorably with those obtained in much more elaborate syntheses of similar molecules.

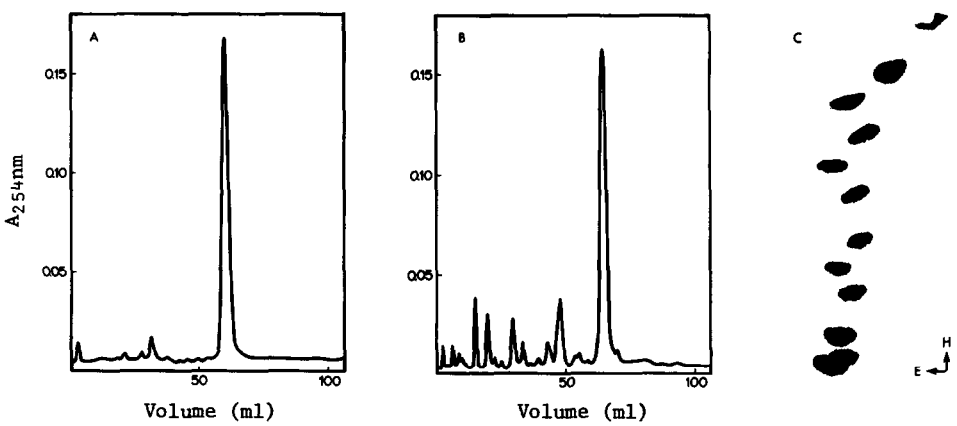

FIGURE 2. A: Chromatographic elution profile for d(G-T-T-G-A-C)-(ClPh)
B: Chromatographic elution profile for d(T-G-T-A-T-C-A-T-C-G-A-T)
C: Mobility shift pattern for d(T-G-T-A-T-C-A-T-C-G-A-T)

The methods reported here produce good yields of high quality hexanucleotides which can be used for the synthesis of extended polynucleotides. The reactions can be scaled up without difficulty, and the strategy of using preformed dinucleotides at the termini with internal monomer additions may be easily adapted to the construction of either longer or shorter blocks.

Acknowledgements

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9. dN' represents dT, dbzC, dbzA, or dibG; ^{o} denotes p-chlorophenyl phosphotriester linkages.
10. Syntheses of oligonucleotides I-IV are exemplified by the preparation of I: The fully protected dinucleotide $[(MeO)_2Tr]dT^{o}dbzC^{o}(CNEt)$, (0.03 mmol), was detritylated by treatment for 25 min at 0° with 3 ml of 2% benzenesulfonic acid in $CHCl_3$:MeOH (7:3 v/v). The acid was neutralized with pyridine (100 µl), then the solution was added to $CHCl_3$ (25 ml) and 5% $NaHCO_3$ (25 ml) containing 5 ml of t-BuOH. The alcohol served to retain the detritylated oligonucleotide in the organic phase. After shaking the mixture, the aqueous layer was removed with a pipette. The organic layer was extracted twice more with 5% $NaHCO_3$ (25 ml):t-BuOH (3 ml), then twice with 10% NaCl (25 ml):t-BuOH (3 ml), before drying it over Na_2SO_4. The solution was filtered, then $CHCl_3$ was removed in vacuo. The residue was rendered anhydrous by co-evaporation with dry pyridine (3 X 5 ml) and combined with 0.039 mmol of the barium salt of $[(MeO)_2Tr]dbzA-(ClPh)$. Following further evaporation of pyridine (3 X 3 ml), the mixture was treated in 250 mg pyridine with TSNT (35 mg). After 1 hr at 25°, pyridine (1 ml) and water (100 µl) were added at 0°. The cold solution was allowed to warm up to room temperature; after 1 hr it was evaporated until ca. 100 mg pyridine remained. The oily residue was detritylated with benzenesulfonic acid solution (20 ml, 30 min at 0°), then the mixture was quenched with pyridine (300 µl) and added to 5% $NaHCO_3$(25 ml) containing t-BuOH (5 ml). The volume of the organic phase was adjusted to 25 ml by addition of $CHCl_3$. Extraction was performed as described above. After drying, filtration, and removal of $CHCl_3$, the detritylated trinucleotide $dbzA^{o}dT^{o}dbzC^{o}(CNEt)$ was combined in pyridine with 0.039 mmol of $[(MeO)_2Tr]dT-(ClPh).\frac{1}{2}Ba^{++}$, then dried by co-evaporation and treated in pyridine (250 mg) with TSNT (35 mg). After 1 hr, the mixture was hydrolyzed, evaporated, detritylated and extracted as before. The resulting $dT^{o}dbzA^{o}dT^{o}dbzC^{o}(CNEt)$ was mixed with 0.039 mmol of dinucleotide $[(MeO)_2Tr]dT^{o}dibG-(ClPh)$. The latter had been prepared in advance from the fully protected dimer $[(MeO)_2Tr]dT^{o}dibG^{o}(CNEt)$ by treatment with pyridine:Et_3N (2:1 v/v, 3 ml) for 6 hr at 25°, followed by co-evaporation with pyridine (3 X 3 ml). The mixture of dimer and tetramer was treated with TSNT (35 mg) in pyridine (250 mg) for 1 hr at 25°. After hydrolysis of the condensing agent, the solution was diluted with $CHCl_3$ (50 ml) and extracted twice with 5% $NaHCO_3$ (25 ml) and twice with 20% NaCl (25 ml), adding t-BuOH (5 ml) at each step. The organic phase was dried and evaporated, then co-evaporated with pyridine (2 X 5 ml) until less than 100 mg of pyridine remained. The residue was dissolved in $CHCl_3$ (10 ml) and loaded onto a column (0.9 X 30 cm) of SilicAR CC-7 (Mallinckrodt) packed in $CHCl_3$ and maintained at 0°. After a 100 ml $CHCl_3$ wash, the hexanucleotide was eluted with $CHCl_3$:MeOH (97:3 v/v). It emerged as a well-defined band preceded by small amounts of faster-running impurities which were discarded. Hexamer products were kept at -70°; those with a dibG residue at the 5' end were stored in anhydrous pyridine to prevent detritylation (see Ref. 8).
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15. The oligonucleotide (1.5 A_{260} units) dissolved in 1 ml of 0.01 M sodium 2-(N-morpholino)-ethanesulfonate(pH 6.5) was treated with 0.25 units of spleen phosphodiesterase. After the A_{260} stopped increasing, the mixture was chromatographed on a column (0.4 X 20 cm) of Dowex 1 ion-exchange resin (AG1-X2, -400 mesh, Bio-Rad Laboratories) using 200 ml of 10% EtOH (v/v) containing a linear gradient of 0.05 M NaCl-0.005 M NH_4OH to 0.5 M NaCl-0.05 M NH_4OH at 20 ml/hr. The products were analyzed spectrophotometrically; dCp:dAp:dTp:dGp:dT = 2.0:2.9:4.2:1.9:0.9. For the dodecamer, hyperchromicity at 260nm = 28% and $\varepsilon_{260} \approx 99{,}200$.
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