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SYNTHESIS OF DEOXYRIBOTETRANUCLEOTIDES BY THE CYCLIC ENEDIOL PHOSPHORYLATION METHOD

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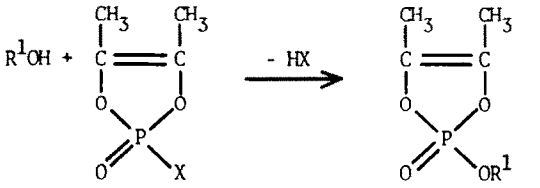
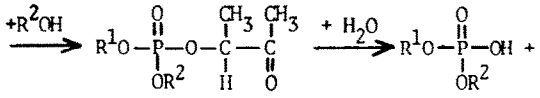
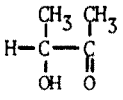
Abstract - Full experimental details are given for the application of the di(1,2-dimethylethenylene)pyrophosphate method of phosphodiester synthesis to the conversion of deoxyribonucleosides into deoxyribodinucleotides in the dG, dA, dC and dT families. N-(1,2-dimethylethenylenedioxyphosphoryl) imidazole is superior to the corresponding pyrophosphate as a reagent for the conversion of deoxyribodinucleotides into deoxyribotetranucleotides.

Unsymmetrical phosphodiesters can be prepared by the following three-step procedure:[1]







To implement this strategy, which eliminates the use of condensing reagents, we have developed several cyclic enediol phosphoryl derivatives (CEP-X).[2] One of these reagents, di(1,2-dimethylethenylene)pyrophosphate (3, Scheme I) has been applied to the preparation of di-, tri- and tetranucleotides based on thymidine.[3] We have now standardized this method and have applied it to the preparation of di- and tetranucleotides containing purine and pyrimidine bases in the deoxyribo-series, 1 and 8.

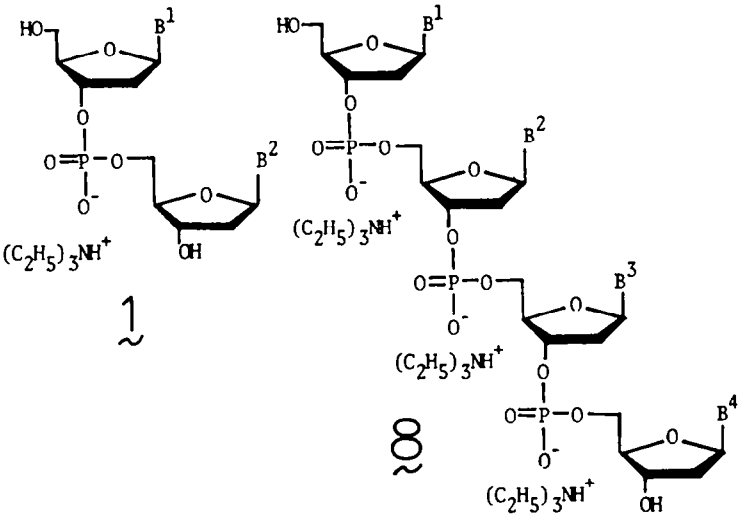
DISCUSSION

The preparation of dinucleotides, 1, is summarized in Scheme I and Table I. The reagent of choice is the pyrophosphate, 3, which is used in conjunction with triethylamine. Under these conditions, the second nucleoside, 5, can have a free 3' position, since the condensation occurs preferentially at the 5' position.[4] As expected, yields are higher when the 3' position of nucleoside 5 is protected. In all cases, the 5' position of the first nucleoside, 2, must be protected. The intermediate cyclic phosphate, 4, is not isolated, while the acyclic triesters, 6 and 7, are purified if they are to be used as blocks in the synthesis of tetranucleotides. The deprotection steps leading to the dinucleotides, 1, have already been described.[3]

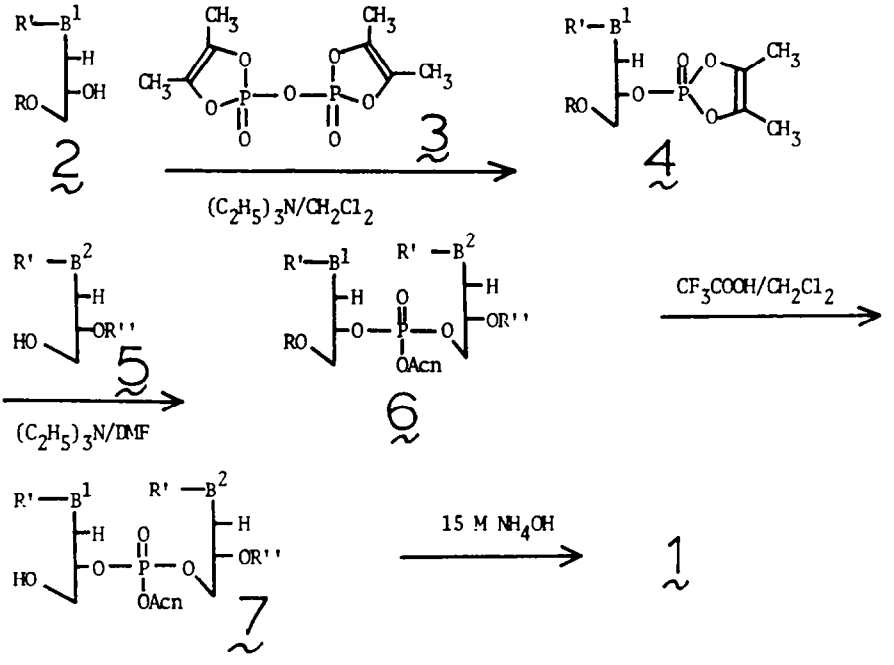
The preparation of tetranucleotides, 8, is summarized in Scheme II and Table II. The preferred reagent is the phosphoimidazole, 10, which results in higher yields of tetranucleotide than the pyrophosphate, 3. In these syntheses, protection at the 3' position of the second dinucleotide, 12, is highly desirable. The acyclic triesters, 13 and 14, are not purified and the respective deprotection steps

are the same as in the dinucleotide synthesis. As before, the cyclic triester, 11, is not isolated.

15, with acetic acid, which causes only detritylation at position 5'. The resulting diester is treated with ammonium hydroxide to



Scheme I. Acn = $CH_3 \cdot CO \cdot CH(CH_3)-$; R = $(p\text{-}CH_3O.C_6H_4)(C_6H_5)_2C-$;



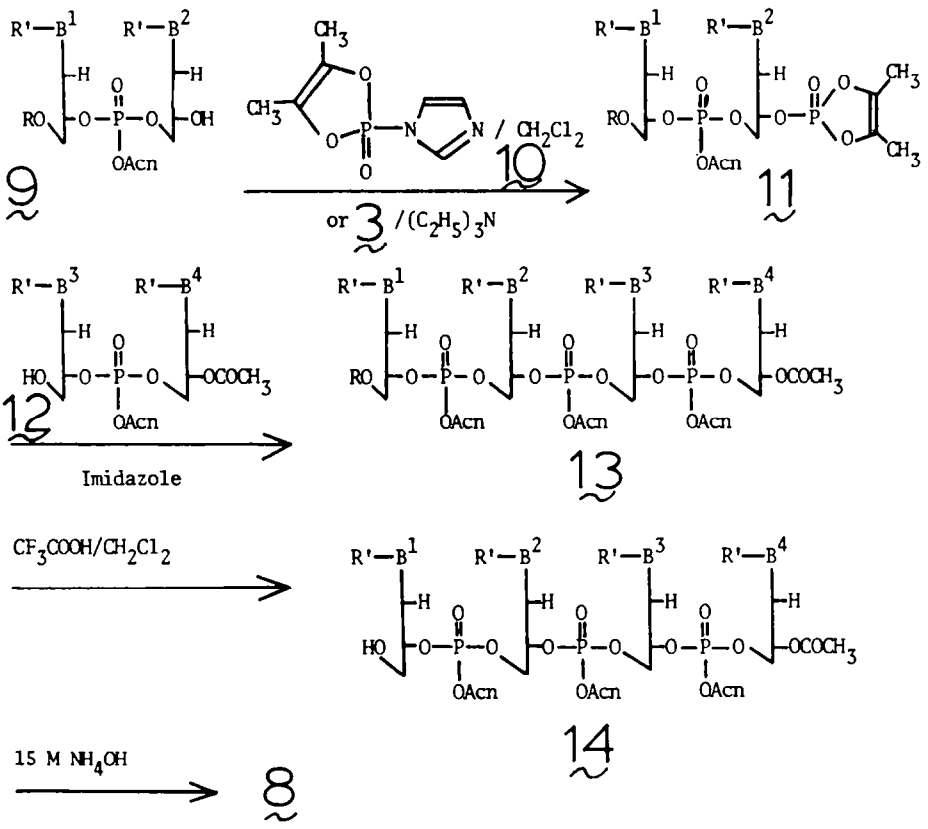
An alternative deprotection sequence involves treatment of triester 13 with triethylamine in aqueous pyridine, which removes only the acetoinyl or 3-oxo-2-butyl group (Acn). The resulting partially protected diester, 15, (formula not shown) is treated with ammonium hydroxide, which removes all protection except that at the 5' position. A final detritylation of the resulting diester using acetic acid leads to the tetranucleotide, 8. Another sequence treats the partially protected diester, yield the tetranucleotide, 8. These alternatives do not affect significantly the yields of 8, but provide more flexibility in the utilization of intermediates for other possible sequences.

Recent publications[5-7] describe existing procedures for oligonucleotide synthesis. Some of these methods have yielded DNA segments significantly larger than those available by the CEP-X method in its present form. However, the techniques described here represent a

simple and reproducible way of preparing natural and unnatural small oligonucleotides by a short sequence of standardized steps.

Enzymatic degradation of tetranucleotides by snake venom phosphodiesterase was carried out by standard procedures; the expected ratio nucleoside/nucleoside-5'-phosphate was observed

Scheme II. Acn = $CH_3.CO.CH(CH_3)-$; R = $(p\text{-}CH_3O.C_6H_4)(C_6H_5)_2C-$



EXPERIMENTAL SECTION

Pyridine was dried by refluxing over, and distilling from calcium hydride. Dichloromethane was distilled from P_2O_5. Dimethylformamide (DMF) was purified by fractional distillation. All were stored over 4A molecular sieves. Triethylamine was refluxed over, and distilled from sodium, and was stored over sodium hydroxide. Purifications of triesters were carried out by preparative TLC on 20 x 20 cm precoated silica gel plates (2 mm-thick, PLC 60 F-254, Merck Cat. No. 5766). Four plates were utilized to purify the products from reactions at a 1 mmol scale; one plate at an ~ 0.25 mmol scale. Each plate was developed two or three times with the same solvent system. Analytical TLC was performed on HPTLC silica gel plates, Merck Cat. No. 5628. High performance liquid chromatography (HPLC) was carried out using a Varian 8500 liquid chromatograph on a Whatman Partisil PXS 10/25 SAX column (25 cm x 4.6 mm id.). Detection was by UV at 260 nm. Authentic nucleotide samples obtained from Sigma Chemical Co. or P & L Biochemicals were used as reference. The protected nucleosides were made by known procedures.[8-11] The conversion of 5'-protected into 3'-protected dinucleotide triesters was carried out by successive treatments with acetic anhydride/pyridine, water and trifluoroacetic acid/dichloromethane, as described.[5] by HPLC.

Synthesis of 5'-Protected Dinucleotide Triesters. The 5'-O-p-methoxytritylnucleoside was dried by three successive evaporations from pyridine (5 mL), and was dissolved in dichloromethane (2 mL) containing one molequiv of triethylamine. A solution of di(1,2-dimethylethenylene)pyrophosphate[2] (one molequiv) in dichloromethane (2 mL) was added at 20° C. The solution was kept at 20° C for 2 h, and the solvent was evaporated (1 mm at 20° C). The residue was dissolved in DMF (2 mL), and the solution was treated with a solution containing the 5'-unprotected nucleoside (one molequiv; dried by evaporation from pyridine), and triethylamine (two molequiv), in DMF (2 mL). The solution was kept at 0° C for 15 h and at 20° C for 2 h. The solvent was evaporated (1 mm at 20° C), and the residue was dissolved in dichloromethane for purification by PLC. The compound was extracted from silica by means of $CHCl_3/CH_3OH$, 3/1, or CH_3COCH_3/H_2O, 20/1. The extract was evaporated, the residue was dissolved in dichloromethane, and the solution was filtered to remove traces of silica. The filtrate was concentrated to 1-2 mL and ether or petroleum ether was added to obtain a powder suitable for filtration.

Syntheses utilizing a "second nucleoside" component protected at 3'-OH were carried out in dichloromethane solution instead of DMF.

Attempts to purify the 5'-protected

dinucleotide triesters by silica gel column chromatography utilizing $CHCl_3/CH_3OH$ as developing solvent were unsatisfactory due to partial decomposition of the triesters.

Deprotection of 5'-Protected Dinucleotide Triesters. The purified 5'-protected dinucleotide triester (6, 0.5 mmol) was dissolved in 500 mL of a 0.2% solution of trifluoroacetic acid in dichloromethane, at 0° C. After 20 min at 0°, the acid in the solution was neutralized by addition of pyridine. The solution was evaporated (30 mm and 1 mm at 20° C) and the residue was thoroughly triturated with ether to extract monomethoxytrityl-containing by-products and pyridinium trifluoroacetate. The remaining 5'-unprotected dinucleotide triester, 7, was subjected to further deprotection steps, with or without an intervening purification step.

A suspension of the 5'-unprotected dinucleotide triester (7, 0.5 mmol) in 50 mL of 15 M ammonium hydroxide was kept at 50-55° C for 6 h in a sealed tube. The solution was evaporated (at 20°, 1 mm), the residue was dissolved in water, and the solution was applied to a 30 x 2.2 cm column of DEAE-cellulose, (HCO_3^- form). Isocratic elution with 0.005 M triethylammonium bicarbonate, followed by application of a linear gradient (0.005-0.30 M) of the same buffer gave a solution of the dinucleotide salt. The buffer was evaporated (1 mm at 20° C), the remaining buffer was removed by several evaporations of the residue with methanol, and the residue was dissolved in a small volume of methanol. A relatively large volume of THF was added to the methanol solution to precipitate the dinucleotide, which was filtered and dried (1 mm at 20° C). The dinucleotide triethylammonium salt, 1, (Table II) was obtained as a white powder. Only one peak was observed upon analysis of the sample by HPLC. The synthetic dinucleotide was compared with an authentic sample of the compound.

Synthesis of 5'-Protected Tetranucleotide Triesters by Means of Di(1,2-dimethylethenylene) pyrophosphate (3). The procedure was analogous to that described above for the synthesis of the 5'-protected dinucleotide triesters. The following quantities of reagents and solvents were employed. First step: 5'-O-p-methoxytrityldinucleotide triester, (9, 0.1 mmol), dichloromethane (1 mL), triethylamine (1.5 molequiv), di(1,2-dimethylethenylene) pyrophosphate (3, 1 molequiv). Second step: DMF (1 mL), 5'-unprotected 3'-O-acetyldinucleotide triester (12, 1 molequiv), triethylamine (2.5 molequiv), DMF (1 mL). The coupling reaction was allowed to proceed at 0° C for 36 h, and at 20° C for 2 h. The crude 5'-O-p-methoxytrityl-3'-O-acetyltetranucleotide triester (13) was submitted to deprotection steps without purification.

Synthesis of 5'-Protected Tetranucleotide Triesters by Means of N-(1,2-dimethylethenylenedioxyphosphoryl)imidazole (10). The reagent 10, was freshly generated by addition of 2-chloro-4,5-dimethyl-2-oxo-1,3,2-dioxaphospholene[2] (0.1 mmol) to a solution of imidazole (2 molequiv) in dichloromethane (1 mL) at 20° C. The mixture was filtered through a sintered glass funnel to remove imidazolium chloride, which was washed with a few drops of dichloromethane. The filtrate containing 10 was added to a stirred solution of the 5'-O-p-methoxytrityldinucleotide triester (9) in dichloromethane (1 mL) at 20° C. After 2 h at 20° C, the solution was treated with a solution of the 5'-unprotected 3'-O-acetyldinucleotide triester (12, 1 molequiv) in dichloromethane (1 mL). The solution was kept at 0° C for 24 h, and at 20° C for 2 h. The solvent was evaporated (1 mm at 20° C) to yield the crude 5'-O-p-methoxytrityl-3'-O-acetyltetranucleotide (13), which was submitted to deprotection steps without purification.

Procedure I for Deprotection of 5'-Protected Tetranucleotide Triesters. The crude 5'-protected tetranucleotide triester (13, 0.1 mmol) was dissolved in 100 mL of a 0.2% solution of trifluoroacetic acid in dichloromethane, and the solution was kept for 20 min at 0°. The work up was analogous to that described above for deprotection of the 5'-protected dinucleotide triesters. The resulting crude 3'-O-acetyl-tetranucleotide triester (14) was treated with 15 M ammonium hydroxide, and the mixture was worked up as in the dinucleotide case. The resulting tetranucleotide, 8, was purified by DEAE-cellulose chromatography as in the dinucleotide case. The identity and purity of the tetranucleotides was established by HPLC and by enzymatic degradation.

Procedure II for Deprotection of 5'-Protected Tetranucleotide Triesters. The crude 5'-protected tetranucleotide triester (13; 0.1 mmol) was dissolved in a mixture of pyridine and water (1:1 v/v; 2 mL) containing triethylamine (6 molequiv). The solution was stirred at 20° C for 3 h, and the solvent was evaporated (1 mm at 20° C) to yield the crude 5'-O-p-methoxytrityl-3'-O-acetyltetranucleotide diester (15). This compound, 15, was dissolved in 15 M ammonium hydroxide (10 mL), and the solution was kept at 50° C for 6 h in a sealed tube. The solvent was evaporated (1 mm at 20° C) to give the crude 5'-O-p-methoxytrityl-tetranucleotide diester lacking any protection at the 3'-position of the ribose and at the base. This compound was dissolved in 80% acetic acid (3 mL), and the solution was stirred at 20° C for 5 h. The solvent was evaporated (1 mm at 20° C), and the residue was triturated with ether to remove the tritylated by-product. The tetranucleotide, 8, was purified by DEAE-cellulose chromatography.

Procedure III for Deprotection of 5'-Protected Tetranucleotide Triesters. This procedure was designed to provide intermediate tetranucleotide diester salts, having protection at the 3'-position of ribose and at the base but with an unprotected 5'-position. The first step was identical to that described under procedure II. The second step involved treatment of the crude 5'-O-p-methoxytrityl-3'-O-acetyltetranucleotide diester (15) with 80% acetic acid at 20° C for 5 h to give the partially protected diester. This intermediate can be purified or converted into the tetranucleotide 8 with 15 M ammonium hydroxide at 50° C for 6 h.

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Table I. Synthesis of Deoxyribodinucleotides, 1, by the Di(1,2-dimethylethenylene)pyrophosphate Method (Scheme I, 1 mmol scale), 2 = First Nucleoside; 5 = Second Nucleoside; 6 = 5'-Protected Triester; 7 = 5'-Unprotected Triester

2[a]	5[b]	6[c] % Yield; Solv.	7 % Yield Solv.	1[d] % Yield
dC	dC	66 ; A	86 ; A	82
dC	dC_{Ac}	86 ; A	91 ; A	...
dC	dT	67 ; A	90 ; A	85
dC	dT_{Ac}	83 ; A	83 ; A	...
dT	dC	65 ; A	88 ; A	83
dT	dC_{Ac}	80 ; A	90 ; A	...
dC	dA	66 ; A	...[e]	80
dC	dG	65 ; A	...	82
dG	dG	54 ; B	...	77
dG	dG_{ib}	72 ; C	...	...
dG	dT	55 ; B	...	60
dT	dG	62 ; B	...	61

[a]R' = Amino-group protection: benzoyl for dC and dA, isobutyryol for dG in all cases. [b]R'' = 3'-OH protection as noted: ac, acetyl; ib, isobutyryol. [c]Yields after purification by preparative TLC with solvents A = $CH_3CO_2C_2H_5$/CH_3COCH_3/H_2O, 7/3/1; B = CH_2Cl_2/i-C_3H_7OH, 3/1; C = $CHCl_3$/CH_3OH, 8/1. Samples dried for 24 h at 25° C (0.1 mm) prior to elemental analysis; C, H, N found within 0.4% of calculated value. [d]Yields after purification by chromatography on DEAE cellulose, utilizing a linear gradient, 0.005 - 0.30 M of $(C_2H_5)_3NH^+CO_3H^-$. Purity of dinucleotide established by HPLC (isocratic elution with 0.05 M KH_2PO_4 at pH 4.5), and comparison with an authentic dinucleotide sample. [e]Triester 7 was not purified; yield of dinucleotide 1 was based on triester 6 (two steps).

Table II. Synthesis of Deoxyribotetranucleotides, 8, by the Di(1,2-dimethylethenylene) pyrophosphate and N-(1,2-dimethylethenylenedioxyphosphoryl)imidazole Methods (Scheme II, 0.2-0.4 mmol scale). 9 = First Dinucleotide Triester; 12 = Second Dinucleotide Triester

9[a]	12	8[b] % Yield
	Pyrophosphate Method	
dC, dC	dC, dC	25
dC, dT	dC, dT	24
dT, dT	dC, dC	26
dT, dG	dT, dG	21
	Phosphoimidazole Method	
dT, dG	dT, dG	37
dT, dG	dG, dT	38
dT, dG	dT, dT	44

[a]Amino-group protection (R') as in Table I. [b]Yields after purification (as in Table I) based on first dinucleotide 9. The 5'-protected and 5'-unprotected triesters, 13 and 14, were not purified.

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