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Synthesis of 5'-Phosphonate Linked Thymidine Deoxyoligonucleotides

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Abstract: 5'-Deoxy-5'-phosphonothymidine was synthesized and coupled with 3'-deoxy-3'-hydroxymethylthymidine to yield a 3'-C-O-P-5' linked thymidine dinucleotide. This dimer as its 3'-phosphoramidite was used to synthesize deoxyoligonucleotides. Copyright © 1996 Elsevier Science Ltd

Deoxyoligonucleotides with modified internucleotide linkages have many uses in biochemistry.[1] The most studied analogs to date are those where a methyl group or sulfur atom (Fig. 1: type 1 DNA; X = Me, S; Y = O) replaces a nonbridging oxygen atom in the phosphate ester linkage. These deoxyoligonucleotides are resistant to nucleases and retain their ability to form stable duplexes with natural RNA or DNA. There are limitations. One arises because the phosphorus center is rendered chiral. In order to overcome this problem, achiral analogs have been synthesized either by replacement of the nonbridging oxygen atoms with sulfur (Fig. 1: type 1 DNA; X, Y = S),[2] or by substituting a bridging oxygen atom (Fig. 1: type 2 DNA; W or Z = S, CH_2, NH; W or Z = O).[3,4] Here we report the synthesis of an achiral 5'-phosphono-linked thymidine dimer (3'-C-O-P-5' linkage) and its incorporation into deoxyoligonucleotides (Fig. 1, part D). Within this analog, the phosphonate moiety is displaced by one chemical bond relative to the position of phosphorus as found in natural DNA. In a manner similar to earlier work on positional isomers of amide linked oligomers,[5] our objective is to study the biochemical consequences of this displacement.

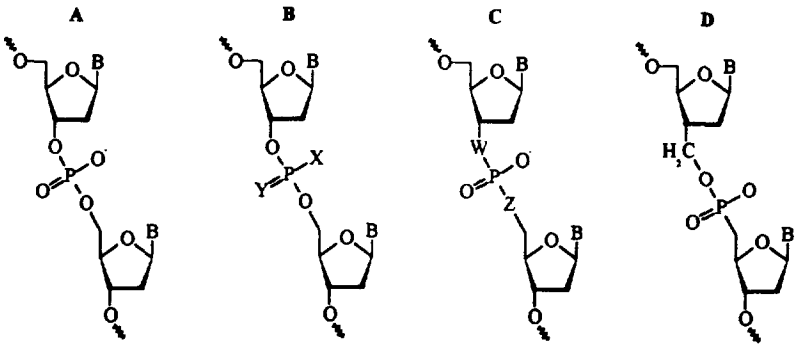

Fig. 1. DNA analogs: A) DNA; B) type 1 DNA; C) type 2 DNA; D) 5'-phosphonate DNA.

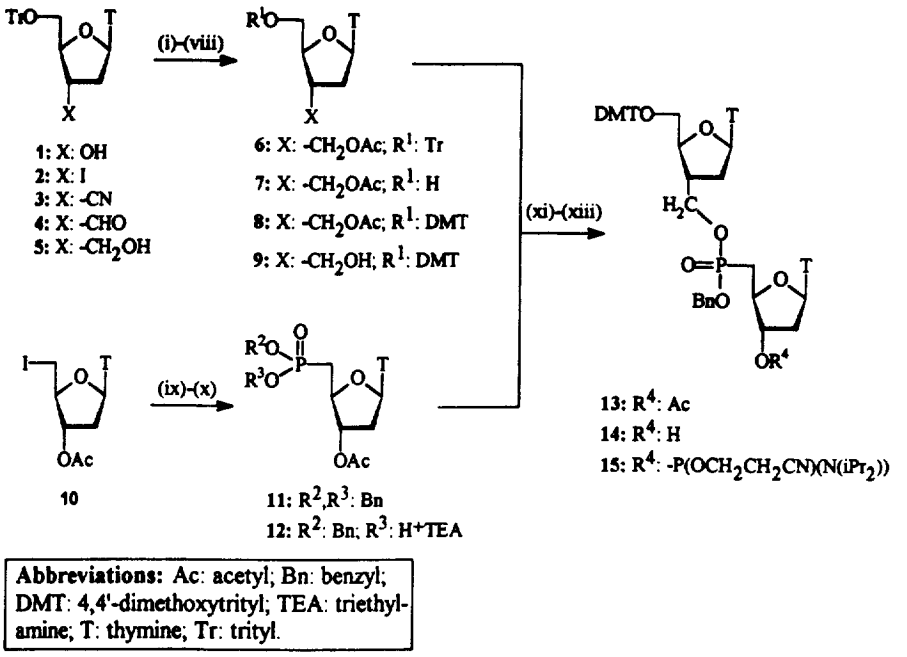

Fig. 2. Synthesis of 5'-phosphono linked dinucleotide.

Synthesis of the 5'-phosphono linked dinucleotide (Fig. 2) begins with the preparation of an appropriately protected 3'-hydroxymethylthymidine (**9**). Following the initial conversion of **1** to **2** using standard procedures, 3'-cyano-3'-deoxythymidine (**3**) was prepared with good stereospecificity using the method of Parkes and Taylor[6] (33% yield from thymidine of the purified erythro isomer). The nitrile was reduced to the aldehyde **4** by adding diisobutylaluminum hydride (2 eq.) to a solution of **3** in dry THF at 70°C and the resulting imine hydrolyzed by adding 0.5 M aqueous acetic acid (57%). Sodium borohydride (10 eq.) was used to reduce **4** to the corresponding alcohol **5** (89%). In order to use the dimer for automated solid phase synthesis, the trityl group was exchanged via a four step procedure with the more labile dimethoxytrityl (DMT). Accordingly **5** was acetylated with acetic anhydride in pyridine to yield **6** and the resulting nucleoside detritylated using 80% aqueous acetic acid at reflux to yield **7**. Further conversion to **9** involved protecting the 5'-hydroxyl of **7** with dimethoxytrityl chloride and removal of the acetyl group from **8** with ammonia in methanol. The overall yield from these four steps was 71%.[7]

Phosphonate deoxynucleosides have been prepared via either a Wittig[4] or Arbuzov[8] reaction. For example, when trialkylphosphite is used in an Arbuzov reaction with 5'-iodouridine, the product is the corresponding dialkylphosphono nucleoside.[8] Unfortunately, alkyl esters are not easily removed from this

phosphonate.[9] As a consequence we chose to investigate benzyl protection. 3'-O-Acetyl-5'-deoxy-5'-iodothymidine (0.25 mmol) was added to 4 ml tribenzylphosphite[10,11] and the Arbuzov reaction allowed to proceed for 20 h at 120-125°C. After cooling, the reaction mixture was loaded on to a silica gel column in chloroform and dibenzylbenzylphosphonate eluted using chloroform. Further elution with chloroform/methanol mixtures led to the isolation of **10** (25%) and 3'-O-acetyl-5'-dibenzylphosphonothymidine (**11**, 45%).[12] The selective removal of one benzyl group from **11** was completed by treatment with thiophenol in triethylamine/dioxane.[2a] Purification by silica gel chromatography gave **12** as the triethylammonium salt (79%).[13]

Further conversion of **9** and **12** to the appropriate dinucleotide synthon was completed in three steps. Coupling of **9** (1.5 eq.) and **12** (1 eq.) using 2,4,6-triisopropylbenzenesulfonyl chloride (3 eq.) and 1-methyl-imidazole (6 eq) in pyridine gave **13** (62%) as a 1:1 mixture of diastereomers.[14] Cleavage of the acetyl group from **13** with ammonia in methanol (5 M) generated **14** (87%)[15] which was converted by standard procedures[16] to **15** (89%).[17]

Table 1. Synthesized sequences and melting temperature

	Sequence	T_m (°C)	ΔT_m (°C)
(A)	5'-TTTTTTTTTTTTTTTT-3'	40.0	—
(B)	5'-TTTTTTTT*TTTTTTTT-3'	36.4	3.6
(C)	5'-TTTTTTT*TT*TTTTTTT-3'	36.0	2.0
(D)	5'-T*TTTTTTTTTTTTTT-3'	40.0	0.0

T = thymidine; * 3'-C-O-P-5' linker; T_m = melting temperature; ΔT_m = change in T_m per modification.

Deoxyoligonucleotides **A-D** were prepared on solid supports by standard phosphoramidite methodology[16] using **15** and commercially available thymidine phosphoramidite. Benzyl deprotection at phosphorous was achieved by treatment with thiophenol in triethylamine/dioxane and deoxyoligonucleotide was cleaved from the support with ammonium hydroxide. All deoxyoligonucleotides were purified by reverse phase on cartridges (COP, Cruachem).® Further purification after removal of the dimethoxytrityl group by HPLC on an anion exchange column yields one major peak corresponding to A-D for each synthesis. The ability of these modified deoxyoligonucleotides to form a duplex with complementary deoxyoligoadenosine of the same length was examined by UV melting point measurements (Table 1). The melting point of modified duplexes

were reduced by 0-3.6°C per modification. Studies on the stability of these oligomers toward nucleases and on their ability to stimulate RNase H activity are currently underway.

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11. ^{31}P NMR ($CDCl_3$) δ 140.54.
12. ^{31}P NMR ($CDCl_3$) δ 28.47, 28.96. FAB^+ MS 529 (M+1).
13. ^{31}P NMR ($CDCl_3$) δ 18.25, 18.05. FAB^+ MS 540 (M+1).
14. ^{31}P NMR ($CDCl_3$) δ 28.57, 28.29. FAB^+ MS 979 (M+1).
15. ^{31}P NMR ($CDCl_3$) δ 34.39, 34.29. FAB^+ MS 937 (M+1).
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17. ^{31}P NMR ($CDCl_3$) δ 150.35, 150.25, 149.99, 149.77, 29.43, 29.15, 28.87, 28.30.

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