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# Synthesis and Preliminary Biochemical Studies with 5'-Deoxy-5'-methylidyne Phosphonate Linked Thymidine Oligonucleotides

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***Abstract:*** Thymidine deoxyoligonucleotides having a 5'-deoxy-5'-methylidyne phosphonate internucleotide linkage were synthesized. Relative to natural DNA, these oligomers were nuclease resistant and formed duplexes with reduced stability. Copyright © 1996 Elsevier Science Ltd

Although a large number of deoxyoligonucleotide analogs have been developed for many applications in biochemistry and molecular biology,[1] there remains a major need to explore new derivatives. For example, in addition to nuclease stability, a significant if not essential criterion for various antisense applications is that the antisense oligomer should induce RNase H activity. Currently only two, phosphorothioate and dithioate DNAs (**2**, **3**, Fig. 1), where one or two non-bridging oxygens of natural DNA (**1**, Fig. 1) are replaced by sulfur, satisfy this criterion. Moreover these two analogs have often been criticized as less than optimal as they bind non-specifically to other macromolecules (perhaps an advantage) or, in the case of phosphorothioates, generate a large number of stereoisomers by standard synthesis protocols.[2] Thus there is a need for additional analogs that are nuclease resistant and stimulate RNase H activity.

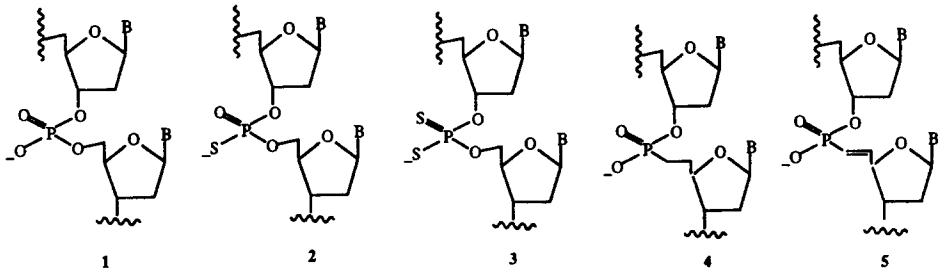


Figure 1

As a direct result of the pathway used for generating 5'-deoxy-5'-methylphosphonate linked DNA[3] (**4**, Fig. 1), the possibility existed that a new analog, 5'-deoxy-5'-methylidyne phosphonate DNA (**5**, Fig. 1) could also be prepared via a similar synthetic route. The rationale for preparing this analog was based upon the generally *trans* or antiperiplanar orientation of the β-torsion angle (rotation around $C_{5'}$-$O_{5'}$) in dinucleotides and DNA.[4] Thus, even though the methylidyne linkage as a $C_{5'}$-$O_{5'}$ would be more rigid than DNA, it should generate a deoxyoligonucleotide whose structure in the *trans* configuration would mimic DNA, potentially be nuclease resistant, and perhaps stimulate RNase H activity as well.

The chemical synthesis of the 5'-deoxy-5'-methylidyne phosphonate linked thymidine dimer and its use to synthesize corresponding deoxyoligonucleotides are outlined in Fig. 2. Commercially available 5'-O-dimethoxytritylthymidine (**6**) was initially silylated with *tert*-butyldimethylsilyltrifluoromethanesulfonate in pyridine to yield **7** (95%), which was followed by treatment with 80% acetic acid to generate **8** (82%). Oxidation of **8** to the corresponding aldehyde was carried out using Pfitzner-Moffat oxidation conditions [5]

followed by *in situ* protection of the aldehyde as the imidazolidine (**9**, 93%). In the Wittig reaction, the aldehyde (**10**), freshly generated by the treatment of the imidazolidine with *p*-toluenesulfonic acid, was coupled with ylide reagent **13** (prepared by quaternization of triphenylphosphine with **12**)[3] to yield **14** (64%).[6] The $C_{5'}$-$C_{6'}$ double bond in **14** was assigned as *trans* based upon the NMR spectra ($J_{5',6'}$ = 17.5 Hz). NMR analysis of compounds (**15**,**18**) generated from this synthon revealed no isomerization of the *trans* linkage. However, attempts to crystallize **14** failed and thus further structural analysis by X-ray crystallography was not possible. Selective deprotection of one *o*-chlorophenyl group of **14** was achieved by treatment with a mixture of 2-nitrobenzaldoxime and 1,1,3,3-tetramethylguanidine in aqueous dioxane to yield **15** (75%).[7,8] The dinucleotide (**16**, 61%)[9] as a 1:1 mixture ($^{31}$P NMR) of the two diasteromers was next generated by condensation in anhydrous $CH_2Cl_2$ of **6** (1 eq.) with **15** (1 eq.) using 2,4,6-triisopropylbenzenesulfonylchloride (5 eq.) and 1-methylimidazole (11 eq.) as coupling agent. Since suitable, selective conditions for removal of the 3'-silyl group could not be found without affecting the *o*-chlorophenyl protection on phosphate, both the 5'- and 3'-protecting groups of **16** were removed simultaneously with 0.5 M HCl in methanol to yield **17** (50%)[10] and the resulting product converted to **18** (66%)[11] using dimethoxytritylchloride and tetrabutylammonium perchlorate in dry pyridine. Finally **19** was prepared from **18** with 2-cyanoethyl-N,N-diisopropylchlorophosphoramidite using published procedures.[12] After chromatography and precipitation, the amidite **19** was isolated in 56% yield as a 1:1:1:1: mixture of its four diastereomers ($^{31}$P NMR).[13]

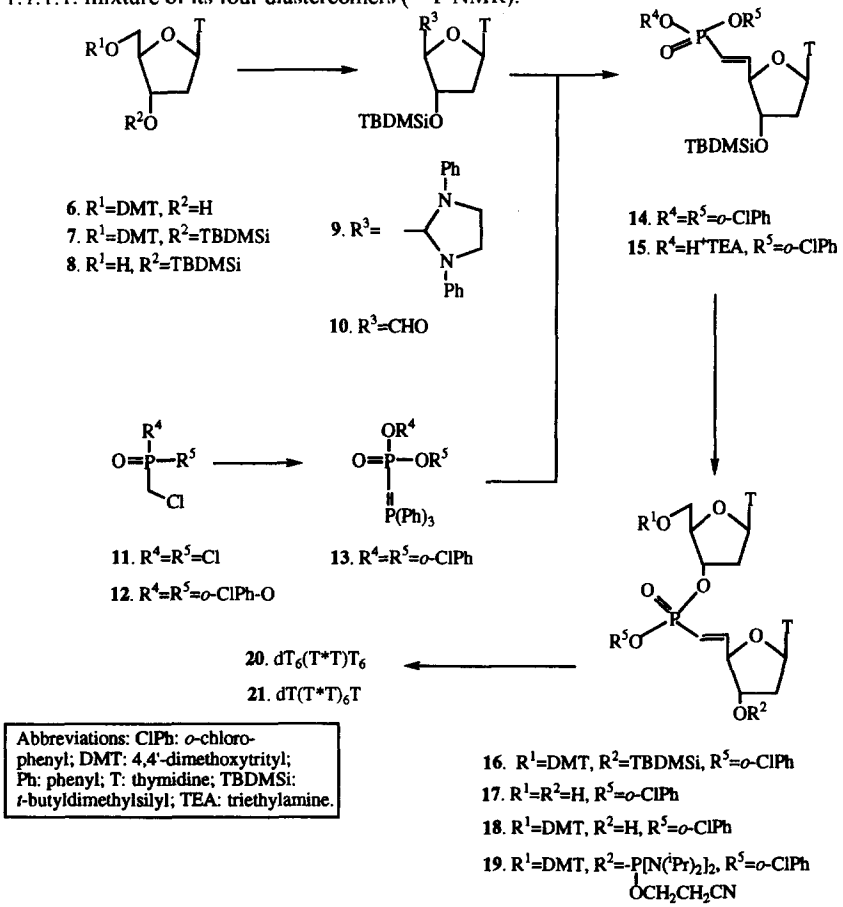


Figure 2. Synthesis of 5'-Deoxy-5'-methylidyne Phosphonate Di- and Deoxyoligonucleotides. T*T indicates an internucleotide linkage corresponding to **5** in Figure 1.

On a DNA synthesizer and using the phosphoramidite methodology, $dT_6(T^*T)T_6$ (**20**), $dT(T^*T)_6T$ (**21**), $dT_{14}$ and $dA_{14}$ were prepared with **19** and commercially available 2-cyanoethylphosphoramidites of thymidine and deoxyadenosine. The coupling time was extended with **19** from 60 sec to 155 sec. Prior to ammonia treatment, the modified sequences still linked to the support were treated with 0.5 M solution of 2-nitrobenzaldoxime and tetramethylguanidine in dioxane/$H_2O$, 3:1 to remove the *o*-chlorophenyl protecting groups.[7] Following removal of the remaining protecting groups and cleavage from the support,[12b] deoxyoligonucleotides were purified by ion exchange HPLC (Nucleogen DEAE 60-7) and desalted by Sep-Pak® cartridges. Analysis of crude reaction mixtures by HPLC (Fig. 3a) or denaturing polyacrylamide gel electrophoresis revealed that the coupling efficiency with **19** was essentially the same as for unmodified deoxynucleoside phosphoramidites as only one major product corresponding in length to the expected oligomer was obtained. Based upon these results, and also $^{31}$P-NMR, this internucleotide linkage is similar to the natural phosphate diester found in DNA as it is completely stable toward synthesis and deprotection conditions, including 0.5 M HCl and concentrated ammonium hydroxide. Moreover the isolated oligomers are very soluble and stable toward storage for months in aqueous solutions at pH 7.0. Synthesis of oligomers having all four bases and the 5'-deoxy-5'-methylidyne phosphonate linkage should be possible using this approach. However earlier work with natural DNA indicates that activation of purine deoxynucleotides with 2,4,6-triisopropylbenzenesulfonyl chloride leads to lower yields than with the pyrimidines.[14] Similar yield reductions would be expected as well via this approach.

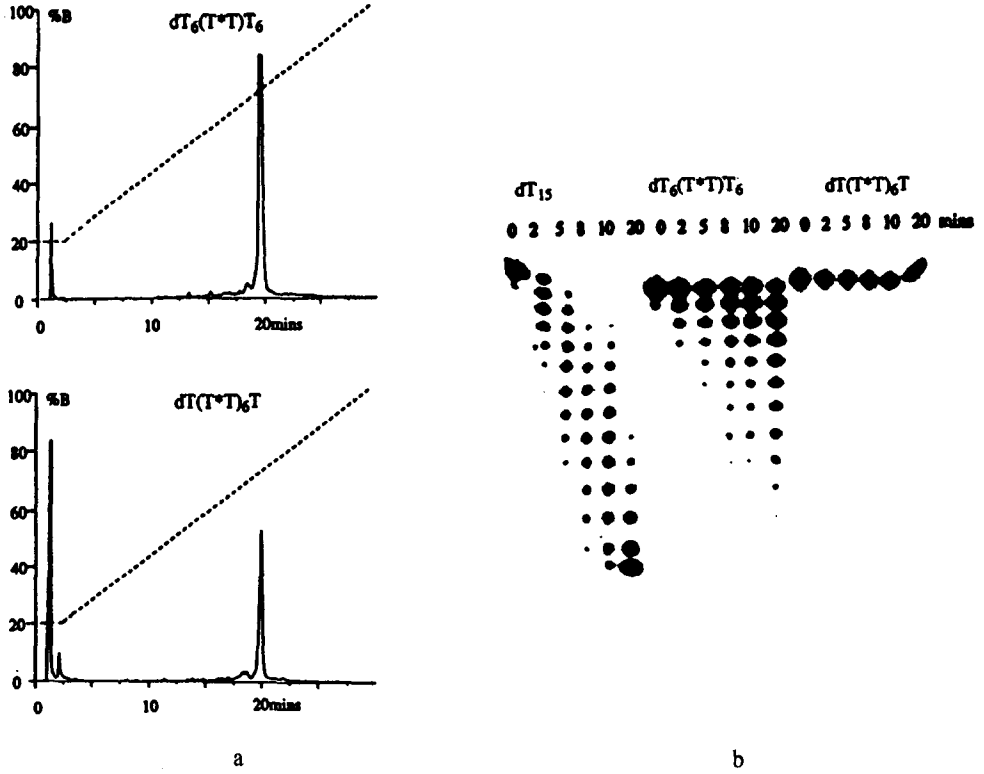


Figure 3. a) HPLC Chromatograms of Crude Deoxyoligonucleotides. Eluants: A: 20 mM NaOAc, pH 6.0, 40% $CH_3CN$, 60% $H_2O$; B: 0.7 M LiCl, 20 mM NaOAc, pH 6.0, 40% $CH_3CN$, 60% $H_2O$. Flow: 1.5 ml/min.

b) Denaturing Polyacrylamide Gel Electrophoresis Analysis of Degraded Deoxyoligonucleotides. 5'-$^{32}$P end-labeled deoxyoligonuclotides were treated with snake venom phosphodiesterase in 25 mM Tris, pH 9.2, 0.006% Triton X-100 and aliquots analyzed over 20 min.

Initial biochemical analysis demonstrated that the 5'-deoxy-5'-methylidyne phosphonate linkage is surprisingly resistant to degradation with snake venom phosphodiesterase (Fig. 3b). Thus under conditions where unmodified $dT_{15}$ is essentially completely degraded, $dT(T^*T)_6T$ remains undigested by this enzyme. Even an oligomer having one 5'-deoxy-5'-methylidyne linkage, $dT_6(T^*T)T_6$, is significantly stabilized against degradation. Under conditions (150 mM NaCl, 10 mM $Na_2HPO_4$, 1 mM $NaN_3$, pH 7.0) where $dT_{14}$:$dA_{14}$ had a $T_m$ of 36°C, this analog formed less stable duplexes. For example, the $T_m$ was 33°C for $dA_{14}$:$dT_6(T^*T)T_6$ having one 5'-deoxy-5'-methylidyne phosphonate linkage. With $dT(T^*T)_6T$, which contains six of these linkages, the corresponding $T_m$ was less than 20°C. Thus despite the enhanced resistance towards snake venom phosphodiesterase, the reduced duplex stability due to this analog may limit its utility for antisense research. However, the biochemical potential of this analog remains undetermined until RNase H experiments have been completed.

**Acknowlegements.** We wish to thank R. Barkley for the FAB mass spectral analyses. This work was supported by NIH (GM25680). This is paper 44 on nucleotide chemistry. Paper 43 is Greef, C. H.; Seeberger, P. H.; Caruthers, M. H.; Beaton, G.; Bankaitis-Davis, D. *Tetrahedron Lett.*, in press.

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6. Analytical data on **14**: $^{31}P$ NMR ($CDCl_3$): δ 11.1. $^1H$ NMR ($CHCl_3$): δ 9.62 (s, br. 1H, NH), 7.15-7.50 (m, 8H, Cl-Ph), 7.14 (m, 1H, H-5'), 6.45 (m, 1H, H-6'), 6.20 (m, 1H, H-1'), 4.20-4.50 (m, 2H, H-3", 4'), 2.00-2.40 (m, 2H, H-2', 2"), 1.96 (s, 3H, Me), 0.91 (s, 9H, tBu), 0.08 (s, 6H, $SiMe_2$). FAB MS 653 ($M^+$).
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8. Analytical data on **15**: $^{31}P$ NMR ($CD_3OD$): δ 8.13. $^1H$ NMR ($CH_3OD$): δ 7.00-7.70 (m, 5H, Ar, H-6), 7.0 (m, 1H, H-5'), 6.35 (m, 1H, H-6"), 6.30 (m, 1H, H-1'), 5.0 (s, 1H, OH), 4.00-4.30 (d, 2H, H-3", 4"), 3.10 (m, 2H, $C\underline{H}_2CH_3$), 2.00-2.20 (m, 2H, H-2', 2"), 1.80 (s, 3H, Me), 1.20 (m, 3H, $CH_2C\underline{H}_3$), 0.90 (s, 9H, tBu), 0.05 (d, 6H, $SiMe_2$). FAB MS 541 ($M^+$-1).
9. Analytical data on **16**: $^{31}P$ NMR ($CDCl_3$): δ 15.2, 15.1. FAB MS 1068 ($M^+$-1).
10. Analytical data on **17**: $^{31}P$ NMR ($CD_3OD$): δ 17.4, 17.3. $^1H$ NMR ($CD_3OD$): δ 8.0 (s, 2H, 2 x H-6), 7.00-7.60 (m, 4H, Cl-Ph), 6.4 (m, 2H, 2 x H-1'), 5.45 (s, 2H, 2 x H-3'), 4.20-4.60 (m, 4H, 2 x H-4',5'), 2.40-2.80 (m, 4H, 2 x H-2', H-2"), 2.0 (s, 6H, Me). FAB MS 653 ($M^+$).
11. Analytical data on **18**: $^{31}P$ NMR ($CDCl_3$): δ 15.2, 14.8. FAB MS 954 ($M^+$-1).
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13. Analytical data on **19**: $^{31}P$ NMR ($CDCl_3$): δ 149.6, 149.4, 15.0, 14.9. FAB MS 1155 ($M^+$).
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