Tetrahedron Letters, Vol. 34, No. 17, pp. 2723-2726, 1993
Printed in Great Britain

0040-4039/93 $6.00 + .00
Pergamon Press Ltd

SYNTHESIS OF 5′-DEOXY-5′-METHYLPHOSPHONATE LINKED THYMIDINE OLIGONUCLEOTIDES.

Markus P. Böhringer†, Darla Graff and Marvin H. Caruthers*
University of Colorado, Department of Chemistry and Biochemistry, Boulder, CO 80309-0215
†Present address: Pharma Division, F. Hoffmann-La Roche Ltd., CH-4002 Basel, Switzerland

Abstract: A 5′-deoxy-5′-methylphosphonate linked thymidine dinucleotide was synthesized and its 3′-phosphoramidite used to synthesize oligonucleotides.

One of the major requirements for antisense oligonucleotides is stability against enzymatic degradation of the phosphate diester internucleotide linkage.[1] Depending upon the nuclease, the P-O(5′) or P-O(3′) bond is cleaved and the 3′ or 5′ deoxymononucleotide, respectively, is released. Therefore, we expected that a formal replacement of O5′ by a CH_2 group, i.e. substitution of the backbone phosphate by a 5′-deoxy-5′-methylphosphonate (*5′mp*) (Figure 1a, b), would increase stability against nucleases. Further, we assumed that a *5′mp*DNA strand would hybridize with DNA and that the melting temperature (T_m) of such a duplex would be similar to the T_m of the corresponding DNA·DNA duplex. This assumption was based on the observations of Heinemann et al.[2] with *3′mp*DNA where an x-ray analysis and a T_m study revealed only minor influences of a single *3′mp* linkage in a DNA octamer. Recently Stawinski and Szabo have recognized the potential usefulness of this analog as well but have not published any oligonucleotide synthesis strategies.[3]

Within the *5′mp* series, only ribomono- and dinucleotides are described[4,5] (Figure 1 c, d). To obtain these *5′mp* dinucleotides, coupling of two monomers was achieved via the phosphodiester methodology.[6] Since today, the phosphotriester protocol[7,8] for DNA synthesis is regarded as superior to this approach, we expected to improve the coupling yield for the *5′mp* analogs by using the phosphotriester chemistry.

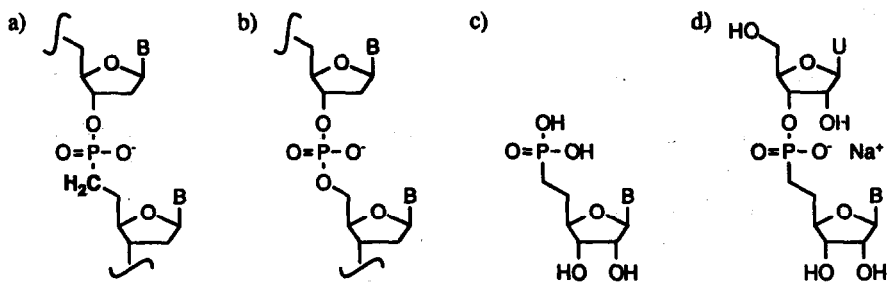

Figure 1. DNA Analogs: a): *5′mp*DNA; b): DNA; c) and d): *5′mp* nucleotides.[4,5] B: A, T, or U.

5′-Deoxy-5′-methylphosphonate linked thymidine deoxyoligonucleotides were synthesized as described in Figure 2. Silylation of 1[6] with TBDMSi-trifluoromethanesulfonate (1.3 eq) in pyridine (Figure 2, reaction (i): 95% 2) followed by treatment with 80% HOAc at 60° afforded 3 in its crystalline form ((ii): 85% 3).[9] The oxidation of 3 to the aldehyde 5 is described by M.-J. Camarasa et al.;[10] but, we never were able to achieve the same results. Much better yields were obtained with a Pfitzner-Moffat oxidation[11] followed *in situ* by protection of the aldehyde 5 as the imidazolidine 4, as described for a similar compound by A. Montgomery et al.[12] ((iii): 90% 4).

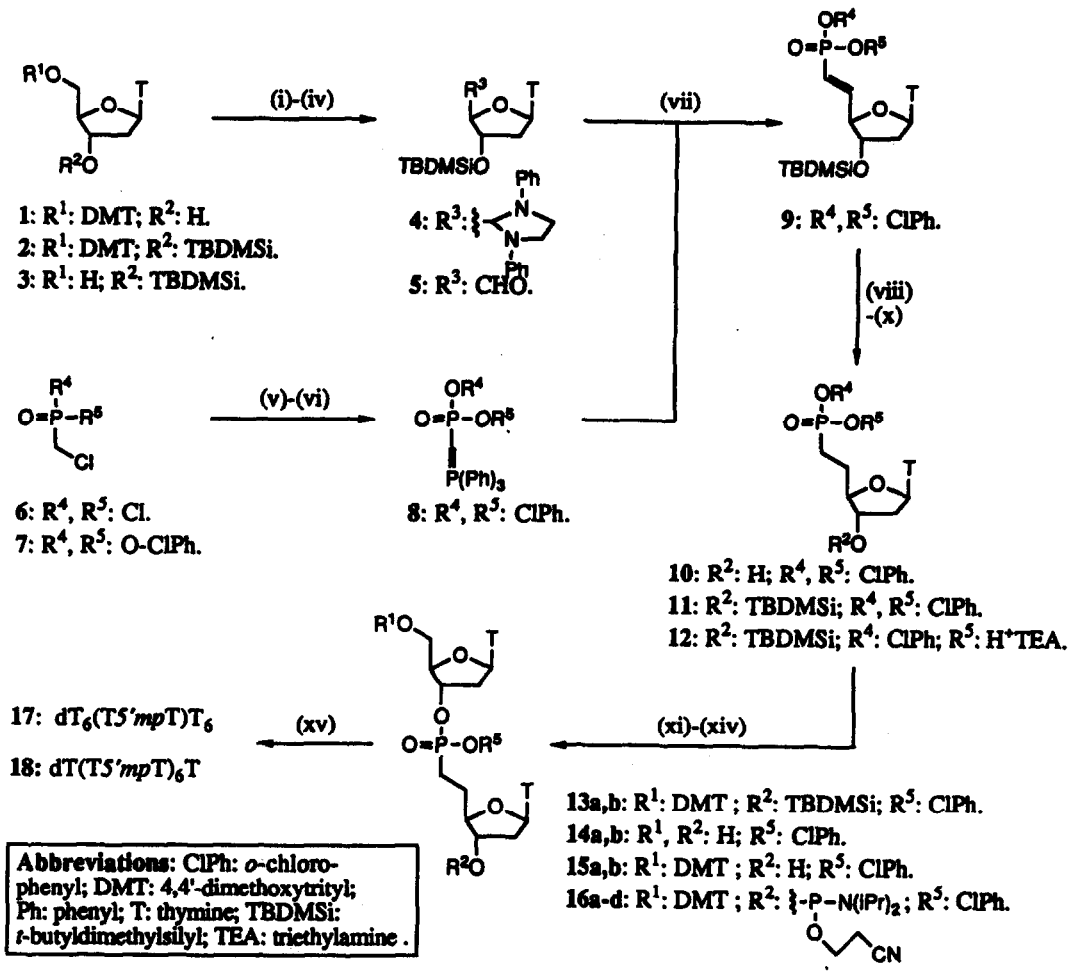

Figure 2. Synthesis of *5'mp* Linked Di- and Oligonucleotides. (Analytical data in references and notes[13])

The crystalline imidazolidine **4** was converted to the aldehyde **5** by treatment with *p*-toluenesulfonic acid ((iv): 76% **5**).[14] In a Wittig reaction, the freshly prepared aldehyde **5** was converted with **8** to **9** ((vii): 83% **9**). The C5'-C6' double bond in **9** appeared to be trans because of the coupling constants in the ^{1}H-NMR spectra ($J_{5'6'}$=17.5 Hz). The Wittig reagent **8** was derived from **7**[15] by quarternization of triphenylphosphine and obtained in its crystalline form ((vi): 45% **8**).[4b] Under treatment of **9** with H_2-Pd in MeOH and 1% HOAc, the C5'-C6' double bond was reduced ((viii): 97% **10**) and, unfortunately, the 3'-O-protecting group, TBDMSi, was cleaved. Therefore, **10** was silylated to **11** using the same conditions as conversion of **1** to **2** ((ix): 87% **11**). Selective deprotection of one ClPh group of **11** was achieved by treatment with a mixture of 2-nitrobenzaldoxime and 1,1,3,3-tetramethylguanidine (1.2 eq each) in dioxane/H_2O, 3:1[16] ((x): 98% **12**). In CH_2Cl_2, **12** was converted with 1 eq **1**, 5.7 eq 2,4,6-triisopropylbenzenesulfonyl chloride and 11.4 eq 1-methylimidazole to **13a,b** ((xi): 78% **13a,b**) which was obtained as a 1:1 mixture (^{31}P-NMR) of the two diastereomers (characterized by ^{31}P, ^{13}C and ^{1}H-NMR and tlc). Attempted cleavage of the TBDMSi group of **13a,b** with $Bu_4NF{\cdot}3H_2O$ resulted

in a rapid cleavage of the ClPh. Thus, **13a,b** was deprotected with 0.5 M HCl in MeOH at 40° to obtain **14a,b** ((xii): 93% **14a,b**). With an excess of DMTCl and Bu_4NClO_4 (2.8 eq each), **15a,b** was prepared ((xiii): 76% **15a,b**).[17] Finally, **16a-d** was derived from **15a,b** with 2-cyanoethyl-N,N-diisoprophylchlorophosphoramidite ((xiv): 76% **16a-d**).[18] After chromatography and precipitation, the amidite **16a-d** was isolated as 1:1:1:1 mixture of its 4 diastereomers (^{31}P-NMR).

On a DNA synthesizer and using the phosphoramidite methodology,[19] dT_6(T5′*mp*T)T_6 (**17**), dT(T5′*mp*T)$_6$T (**18**), and dT_{14} were prepared with **16a-d** and commercially available 2-cyanoethylphosphoramidite of thymidine as building blocks. The coupling time was extended from 53 sec to 138 sec. The trityl assays of all three syntheses did not differ. The 5′-O-DMT groups were then cleaved from the fully assembled oligonucleotides still linked to the solid support. For dT_6(T5′*mp*T)T_6 and dT(T5′*mp*T)$_6$T, deprotection at phosphorus and cleavage from the support were achieved by treatment with a 0.5 M solution of 2-nitrobenzaldoxime and tetramethylguanidine in dioxane/H_2O, 3:1,[16,20] followed by the usual NH_3 treatment. All oligonucleotides were purified by ion exchange HPLC and desalted by Sep-Pak® cartridges. Chromatograms of the crude material revealed equal coupling efficiency for dT(T5′*mp*T)$_6$T, dT_6(T5′*mp*T)T_6 and dT_{14} (Figure 3.a). Analytical HPLC of the purified oligonucleotides indicated 98% purity for dT(T5′*mp*T)$_6$T, dT_6(T5′*mp*T)T_6 and dT_{14}. Polyacrylamide gel electrophoresis under native and denaturing conditions yielded similar results (Figure 3.b)

T_m studies, nuclease digestion and RNase H stimulation experiments for the potential use of 5′*mp*DNA as an antisense oligonucleotide are currently underway.

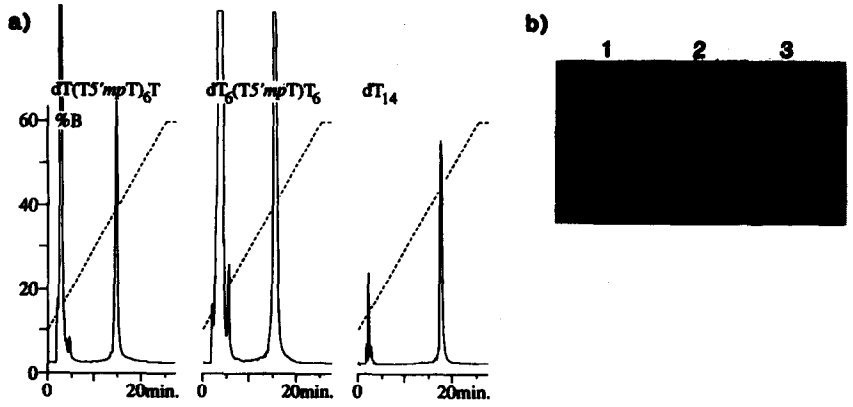

Figure 3. a) HPLC Chromatograms of Crude Oligonucleotides. Column: Nucleogen 60-7 DEAE. Eluents: A: 20 mM NaOAc, pH 6, 40% CH_3CN, 60% H_2O; B: 0.7 M LiCl, 20 mM NaOAc, pH 6, 40% CH_3CN, 60% H_2O. Flow: 1 ml/min. b) Denaturing polyacrylamide gel electrophoresis. 20% acrylamide, 19:1 crosslinking, 8 M urea. 5′-end labelings were with T4-polynucleotide kinase using γ-^{32} ATP. Lane 1: dT_{14}, Lane 2: dT(T5′*mp*T)$_6$T, Lane 3: dT_6(T5′*mp*T)T_6.

Acknowledgements We wish to thank R. Barkley for the FAB mass spectral analyses. This work was supported by NIH (GM25680 and GM21120). This is paper 38 on Nucleotide Chemistry. Paper 37 is Wiesler, W. T.; Marshall, W. S.; Caruthers, M. H. in Oligonucleotide Synthesis Protocols, Methods in Molecular Biology (Ed. S. Agrawal), The Humana Press, Inc. in press.

REFERENCES AND NOTES

1. a) Cohen, J. S. *Oligodeoxynucleotides: Antisense Inhibitors of Gene Expression*; MacMillan Press Limited: London, 1989; pp.1-6. b) Stein, C. A.; Cohen, J. S. *Cancer Res.* **1988**, *48*, 2659-2668. c) Toulmé, J.-J.; Hélene, C. *Gene* **1988**, *72*, 51-58.
2. Heinemann, U.; Rudolph, L.-N.; Alings, C.; Morr, M.; Heikens, W.; Frank, R.; Blöcker, H. *Nucleic Acids Res.* **1991**, *19*, 427-433.
3. Stawinski, J.; Szabo, T. "International Symposium on Synthetic Oligonucleotides: Problems and Frontiers of Practical Application," Moscow, Russia, June 23-30, 1991, p. 72.
4. a) Jones, G. H.; Moffat, J. G. *J. Am. Chem. Soc.* **1968**, *90*, 5537-5538. b) Jones, G. H.; Hamamura, E. K.; Moffat, J. G. *Tetrahedron Lett.*, **1968**, 5731-5734.
5. Jones, G. H.; Albrecht, H. P.; Damodaran, N. P.; Moffat, J. G. *J. Am. Chem. Soc.* **1970**, *92*, 5510-5511.
6. Khorana, H. G. *Pure Appl. Chem.* **1968**, *17*, 349-381.
7. Reese, C. B. *Tetrahedron* **1978**, *34*, 3143-3179 and references cited therein.
8. Miller, P. S.; Reddy, M. P.; Murakami, A.; Blake, K. R.; Lin, S.-B.; Agris, C. H. *Biochemistry* **1986**, *25*, 5092-5097.
9. The nucleoside **3** is described using another approach: a) Ogilvie, K. K.; Iwacha, D. J. *Tetrahedron Lett.* **1973**, 317-319. b) Ogilvie, K. K. *Can. J. Chem.* **1973**, *51*, 3799-3807.
10. Camarasa, M.-J.; De las Heras, F. G.; Pérez-Pérez, M. J. *Nucleosides and Nucleotides* **1990**, *9*, 533-546.
11. Pfitzner, K. E.; Moffat, J. G. *J. Am . Chem. Soc.* **1965**, *87*, 5661-5670.
12. Montgomery, J. A. ; Thomas, H. J. *J. Org. Chem.* **1981**, *46*, 594-598.
13. Selected analytical data: ^{31}P-NMR (^{31}P) were recorded on a Varian VRX 300s. Chemical shifts in ppm refer to external H_3PO_4. Solvent is $CDCl_3$ unless otherwise mentioned. FAB positive and negative mass spectra (MS) were taken on a VG 7070 EQ-HF. Only the molecule mass peaks and their relative intensities are presented. **2**: MS: 659 (M^+, 2). **3**: MS: 355 ($(M-H)^-$, 61). **4**: MS: 549 (M^+, 5). **7**: ^{31}P: 13.3 (s). MS: 351 (M^+, 100). **8**: ^{31}P: 22.2 (d, J = 47.6, 1P, P=O); 29.1 (d, J = 47.6, 1P, $P(Ph)_3$). MS: 577 (M^+, 20). **9**: ^{31}P: 11.8 (s). MS: 651 ($(M-H)^-$, 20). **10**: ^{31}P: 27.2 (s). MS: 545 (M^+, 1); 543 (M^+, 4); 541 (M^+, 5). **11**: ^{31}P: 26.8 (s. MS: 655 (M^-, 7). **12**: ^{31}P: 22.6 (s). MS: 545 (M^-, 55); 543 (M^-, 100). **13a**: ^{31}P: 30.3 (s, 0.5P). **13b**: ^{31}P: 30.4 (s, 0.5P). **13a,b**: MS: 1072 (M^-, 10); 1070 (M^-, 19). **14a,b**: ^{31}P (CD_3OD): 32.0 (s, 0.5P); 32.1 (s, 0.5P). MS: 658 (MH^+, 3); 656 (MH^+, 8). **15a,b**: ^{31}P: 30.0 (s, 0.5P); 30.1 (s, 0.5P). MS: 957 (M^+, 50). **16a-d**: ^{31}P: 29.84, 29.86, 30.07 and 30.11 (4s, 4 x 0.25P, P(V)); 149.10 (s, 0.5P, P(III)); 149.13 and 149.16 (2s, 2 x 0.25P, P(III)). MS: 1156 $(M-H)^-$, 14).
14. Jones, G. H.; Taniguchi, M.; Tegg, D.; Moffat, J. G. *J. Org. Chem.* **1979**, *44*, 1309-1317.
15. McCall, M. A.; McConnell, R. L. *US patent* **1959**, 2,900,405.
16. Reese, C. B.; Zard, L. *Nucleic Acids Res.* **1981**, *9*, 4611-4626.
17. Reddy, M. P.; Rampal, J. B.; Beaucage, S. L. *Tetrahedron Lett.* **1987**, *28*, 23-26.
18. Driller, H.; Seela, F. *Helv. Chim. Acta* **1988**, *71*, 1191-1198.
19. a) Letsinger, R. L.; Finnan, J. L.; Heavner, G. A.; Lunsford, W. B. *J. Am. Chem. Soc.* **1975**, *97*, 3278-3279. b) Letsinger, R. L.; Lunsford, W. B. *J. Am. Chem. Soc.* **1976**, *98*, 3655-3661. c) Beaucage, S. L.; Caruthers, M. H. *Tetrahedron Lett.* **1981**, *22*, 1859-1862. d) McBride, L. J.; Caruthers, M. H. *Tetrahedron Lett.* **1983**, *24*, 245-248.
20. Sproat, B. J.; Gait, M. J. Solid-phase Synthesis of Oligodeoxyribonucleotides by the Phosphotriester Method. In *Oligonucleotide Synthesis*; Gait, M. J. IRL Press Limited: Oxford, Washington DC, 1985; pp. 83-115.

(Received in USA 30 September 1992; accepted 16 February 1993)