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# IMPROVED SYNTHESIS OF OLIGONUCLEOSIDE METHYLPHOSPHONATE ANALOGS

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**Abstract:** Solid-phase synthesis using pent-4-enoyl nucleoside *(PNT)* phosphonamidites allow for the facile preparation of oligonucleoside methylphosphonates, methylphosphonothioates and their chimeric analogs. With *PNT* phosphonamidites, nucleobase and phosphate deprotection, as well as, cleavage from the support can all be accomplished by treatment of the support-bound oligonucleotide with $NH_4OH$ (28%, rt, 1 h).

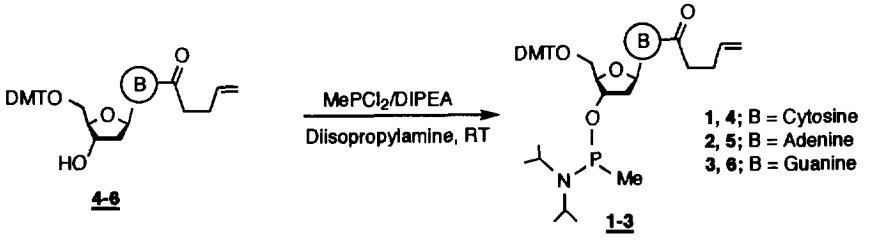

Oligonucleoside methylphosphonates (PMe-oligos) have long been studied as modulators of gene expression.[1,2] Being non-ionic, they are readily taken up by cells and have been shown to hybridize in a sequence-specific manner with either single- or double-stranded nucleic acids.[1,3] Although the duplex of PMe-oligos with complementary RNA is not a substrate for RNase H, gene regulation has been clearly demonstrated with these analogs. Interestingly however, the duplexes of RNA with chimeric oligonucleotides, which contain segments of methylphosphonates and phosphorothioate linkages, are reported to be substrates for RNase H.[2,4] Recent work from our laboratory has revealed that the chimeric analogs of the 25-mer oligodeoxyribonucleoside phosphorothioate GEM 91® incorporating methylphosphonate and phosphorothioate (PS) segments are potent anti-HIV agents and show improved pharmacokinetic profile.[5]

The P-Me oligos are routinely synthesized on a polymeric[6a]/solid-support[6b] using phosphoramidite chemistry.[6c] Because the methylphosphonate linkages are inherently susceptible to hydrolysis by base, prolonged treatment with 28% ammonium hydroxide, the reagent generally used to deprotect the nucleobases and to effect the cleavage of the oligonucleotide from the support, cannot be employed. This limitation has necessitated several modifications in the preparation of P-Me oligonucleotides[6a] and their chimeric analogs. Two major changes in the synthesis and deprotection protocol include (a) the use of *N*-isobutyryl-protected cytidine nucleoside phosphonamidite monomers, in conjunction with $dA^{bz}$ and $dG^{iBu}$ monomers; the deprotection is effected by initial exposure of the oligomer to 10% ammonium hydroxide in acetonitrile/ethanol (1/1) at room temperature, followed by extended reaction with ethylenediamine; and (b) pretreatment of the protected oligonucleotide with hydrazine hydrate in pyridine/acetic acid followed by prolonged exposure (depending on the synthesis scale) to ethylenediamine/ethanol (1/1). Notwithstanding the inconvenience of these two-step deprotection protocols, in our hands, the large-scale synthesis of the chimeric oligonucleotides (which contain segments of different internucleotidic linkages) presented additional practical difficulties. For example, the $dG^{iBu}$-methylphosphonamidite monomer is not soluble in acetonitrile, a solvent used with most other amidite monomers, but only in anhydrous peroxide-free THF or $CH_3CN/CH_2Cl_2$ (1/1). Consequently, during the automated solid-phase synthesis of chimeric analogs, we have found that the amidite delivery lines and columns of the DNA synthesizer need to be thoroughly washed with the appropriate solvents to displace any residual acetonitrile, before switching the β-cyanoethyl phosphoramidite monomers for the

phosphonamidite monomers. Otherwise, the inadvertent presence of acetonitrile causes the precipitation of the dG-methylphosphonamidite monomer in the delivery lines and column. Special precautions are also required for storing any unused THF solutions of the monomer. These practical considerations prompted us to evaluate alternate nucleobase protecting groups that could be readily incorporated and expeditiously removed. In this context, the advantages of the pent-4-enoyl *(PNT)*[7a] nucleosides in the synthesis of different classes of oligonucleotides, using both phosphoramidite[7b] and *H*-phosphonate methodologies,[7c] have been recently disclosed by us. Originally designed with an intent to provide both support-bound and free oligonucleotide analogs, the *PNT* group[7b] is being recognized as a versatile nucleobase protecting group for a number of reasons: (a) as a universal nucleobase protecting group, *PNT* group is readily incorporated into nucleobases; and (b) it is removed under neutral conditions ($I_2$/pyr/ROH), as well as, under basic but mild conditions (0.05 M $K_2CO_3$, 4 to 8 h or 28% $NH_4OH$, rt, 15 min to 1 h).[8] The aforementioned problems during the synthesis of chimeric oligonucleotides containing methylphosphonate linkages, prompted us to evaluate the suitability of the *PNT* nucleoside methylphosphonamidites in the synthesis of the chimeric oligonucleotides. Reported herein are the multiple advantages of the *PNT* nucleobase protecting group in the synthesis of methylphosphonate and chimeric oligonucleotides which contain segments of phosphoric diester (PO), phosphorothioate (PS), methylphosphonate {P(O)Me} and methylphosphonothioate {P(S)Me}[9] internucleotidic linkages.

The requisite nucleoside methylphosphonamidite monomers **1-3**, bearing the *PNT* nucleobase protecting group, were prepared (Scheme 1) by the initial addition of *N,N*-diisopropylamine to dichloromethylphosphine followed sequentially by addition of the appropriate 5'-*O*-DMT-*PNT*-nucleosides[7b] **4-6** in a one pot reaction.

**Scheme 1**




Each of the *PNT* nucleoside methylphosphonamidite monomers **1-3** thus obtained was purified by column chromatography and fully characterized. These monomers were obtained as white powders and were stored at -20 °C until ready for use. We were also gratified to find that the monomers **1-3** were readily soluble in anhydrous acetonitrile. The phosphonamidites **1-3** were then employed in the synthesis of methylphosphonate dimers 5'd[AT], d[CT] and d[GC] using phosphoramidite chemistry. Thus, following the formation of the internucleotidic methylphosphonite P (III) linkages, the oxidation to the corresponding methylphosphonates was effected using *tert*-butyl hydroperoxide (1 M in toluene). The support-bound dimers were then treated with aqueous ammonium hydroxide (28%, 1 h, rt) to remove the *PNT* group and to also effect the cleavage of the dimers from the support. When analyzed by HPLC,[10a] the dimers (obtained in yields of 94-96% as a mixture of $R_p$, $S_p$ diastereomers) were found to be identical to authentic samples prepared from commercial phosphonamidite monomers[10b] using both *tert*-butylhydroperoxide as well as iodine, as the

oxidants, and employing the reported deprotection conditions. Additional structure proof for the dimers was obtained from $^{31}P$ NMR and MALDI-TOF mass spectra (Fig. 1). Thus, the multiple advantages of employing

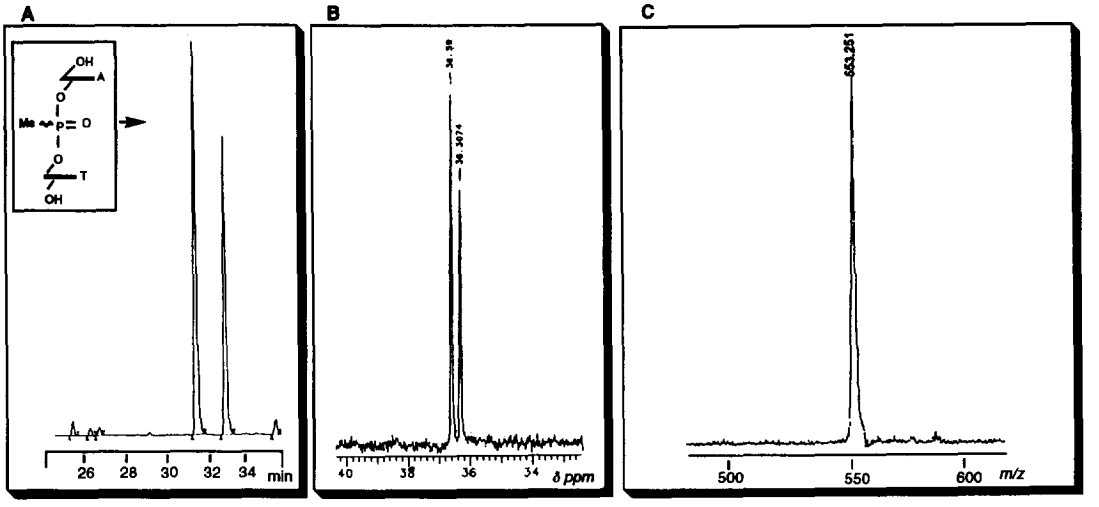



**Figure 1.** Characterization of d[AT] methylphosphonate. **Panel A.** Reversed-phase HPLC[10b] profile of the crude dimer. **Panel B.** $^{31}P$-NMR spectrum ($D_2O$, 85% $H_3PO_4$ as the external standard). **Panel C.** MALDI-TOF mass spectrum. The molecular ion peak is at 553.25 (calcd for $C_{21}H_{28}N_7O_9P$ 553.12).

the *PNT* methylphosphonamidite monomers in the synthesis of methylphosphonates were clearly revealed by these preliminary studies.

We also prepared methylphosphonothioate dimers[9] 5'd[AT], 5'd[CT] and 5'd[GT]. Thus, following the formation of the internucleotidic methylphosphonite P (III) linkages, the oxidation to the corresponding methylphosphonothioates was effected using 3*H*-1,2-benzodithiole-3-one-1,1-dioxide.[11] The support-bound dimers were then treated with aqueous ammonium hydroxide (28%, 1 h, rt) to remove the *PNT* group and to also effect the cleavage of the dimers from the support. When analyzed by HPLC (Fig. 2), the dimers (obtained in yields of 92-96% as a mixture of $R_p$, $S_p$ diastereomers) were found to be identical to authentic methylphosphonothioates prepared from commercial phosphonamidite monomers,[10b] employing the reported deprotection conditions.[6a]

To further evaluate the use of the *PNT* methylphosphonamidite monomers, the synthesis of the chimeric oligonucleotides, **7-10**, incorporating segments of PO and P(O)Me linkages was undertaken on a 1-10 $\mu$mol scale (Fig. 3). The sequences **7-10** contain both purine and pyrimidine bases in different segments along the oligonucleotide chain. Following the synthesis, the support-bound oligonucleotides were treated with aq. $NH_4OH$ (28%, 1 h, ambient temperature). This treatment was sufficient to remove the nucleobase and phosphate protecting groups as well as to cleave the oligonucleotides from the support. This was in conformity with our earlier experiments with *PNT* nucleosides, which had revealed that the *PNT* group is removed by treatment with 28% $NH_4OH$ in (15 min - 1 h) at ambient temperature. HPLC analysis of the oligonucleotides obtained using the *PNT* monomers and commercial monomers indicated a higher ratio of product/failure sequences with the former compared to the latter (data not shown). Additionally, the evaluation of the HPLC-

purified chimeric oligonucleotides **7-10** by polyacrylamide gel electrophoresis (PAGE) and capillary gel electrophoresis revealed, in each case, that the *PNT*-derived **7-10** had the same electrophoretic mobility as the

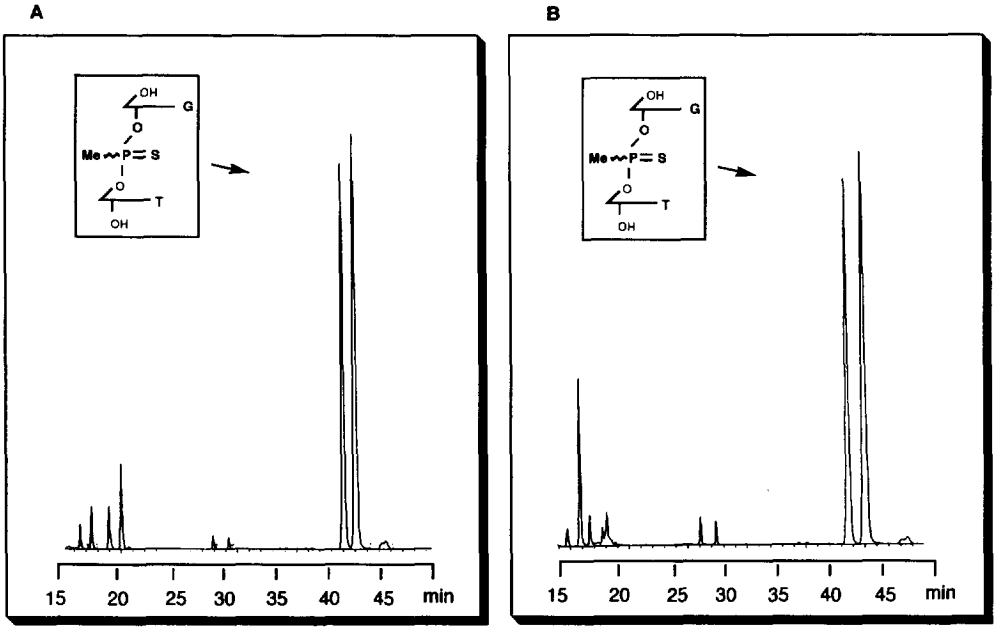



**Figure 2.** Characterization of d[GT] methylphosphonothioate dimer. **Panel A.** Reversed-phase HPLC[10a] profile of the crude dimer derived from *PNT* amidites. **Panel B.** Reversed-phase HPLC profile of the crude dimer derived from commercial amidites.[10b]

**7** 5'-A*C*G*A*C*TTTTTTTTT

**8** 5'-T*A*T*C*C*A*T*A*T*G*TTTTTTTTT

**9** 5'-TTTTTTTTTC*T*A*G*TTT TT

**10** 5'-TTTTTC*T*A*G*TTTTTTTTT

**Figure 3.** Chimeric oligonucleotide sequences **7-10**. The internucleotidic linkages shown with * are methyl phosphonates. All other linkages are PO linkages. The sequences were synthesized on a Biosearch 8700 synthesizer using standard synthesis cycles except that when using the appropriate *PNT*-deoxynucleoside (dA, dC, dG) methylphosphonamidites, *tert*-butylhydroperoxide (1 M in toluene) was used as the oxidant.

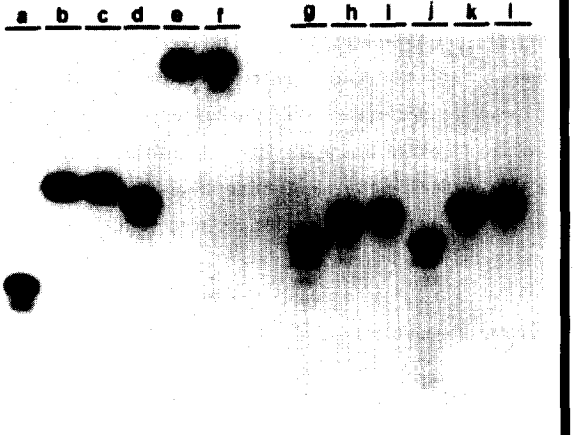



**Figure 4.** Autoradiography following PAGE (20%, 8 M urea) of $^{32}$P-labeled oligonucleotides **7-10.** Lanes **c, f, i, l** correspond to **7-10** respectively, prepared using *PNT* methyl phosphonamidites. The lanes **b, e, h, k** correspond to **7-10** prepared using commercial methylphosphonamidites. The oligonucleotides **7-10** having all PO linkages are included as standards (lanes **a, d, g, j** respectively).

sequences prepared using commercial monomers with reported synthesis/deprotection protocols.[6] A sample of each oligonucleotide **7-10** was also either 5' (analogs **9, 10**)- or 3' (analogs **7, 8**)-end labeled using [$\gamma$-$^{32}$P]ATP/*polynucleotide* kinase or with [$\gamma$-$^{32}$P]ddATP/*terminal deoxynucleotidyl transferase* respectively, and subjected to PAGE followed by autoradiography (Fig. 4). For comparison of the electrophoretic mobilities, identical sequences which were synthesized using commercial monomers are included.

We next prepared chimeric oligonucleotides **11-13** (Fig. 5) containing segments of PO and P(S)Me, as well as PS and P(S)Me internucleotidic linkages. Figure 6 shows the PAGE profiles of these oligonucleotides. Again, for comparison, identical sequences that were synthesized using commercial monomers are included.

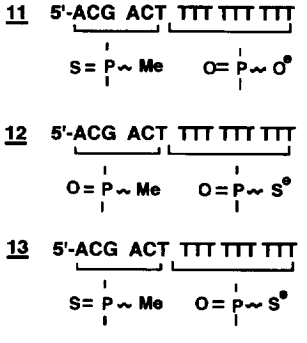



**Figure 5.** Sequences of chimeric oligonucleotides - having P(O)Me, P(S)Me, PO and PS internucleotidic linkages - prepared using *PNT* nucleoside methylphosphonamidites. The sequences were synthesized on a BIOSEARCH 8700 Synthesizer using standard synthesis cycles except that oxidations were performed by using either tert-butylhydroperoxide or 3*H*-benzodithiole-3-one- 1,1-dioxide[11] as appropriate.

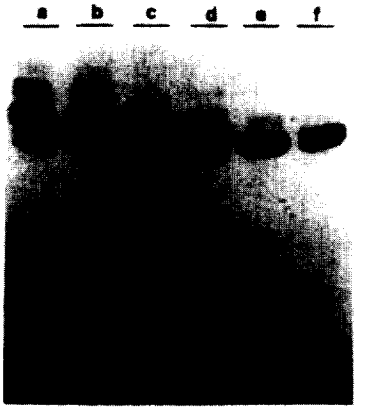



**Figure 6.** PAGE (20%, 8 M urea) profile of oligonucleotides **11-13**. Lanes **b, d, f** correspond to **11-13** respectively prepared using *PNT* methyl phosphonamidites. The lanes **a, c, e** correspond to **11-13** respectively, prepared using commercial methyl-phosphonamidites.

In conclusion, our studies reveal that *PNT* deoxynucleoside methylphosphonamidites are versatile synthons in the preparation of P-Me oligonucleotides and their chimeric analogs. Synthesis of different oligonucleotides using the *PNT* synthons and evaluation of other deprotection conditions/reagents are in progress in order to make the methodology more generally applicable in oligonucleotide synthesis.

***General Experimental Procedures: Synthesis of the phosphonamidites* 1-3.**

Methyldichlorophosphine (3 mmol) was dissolved in anhydrous methylene chloride (5 mL) under argon. Anhydrous diisopropylamine (6 mmol) was added to the solution *via* syringe at room temperature. The reaction mixture was rapidly stirred and a solution of 5'-*O*-DMT *PNT* nucleoside[7b] (1 mmol) in anhydrous methylene

chloride (5 ml) containing *N,N*-diisopropylethylamine (1.5 mmol) was added. After the reaction was allowed to continue for 20 min, anhydrous methanol (0.5 mL) was added to destroy any residual chlorophosphonite. The reaction mixture was poured into 5% aqueous sodium bicarbonate, and the product extracted with methylene chloride (3 X 20 mL). The combined extracts were dried over anhydrous $Na_2SO_4$, evaporated to dryness and the residue purified by silica gel column chromatography. Elution gave the desired product amidites as white foams after drying in vacuo (yields: **1**, 84%; **2**, 92%; **3**, 54%). $^{31}P$ NMR ($CDCl_3$) (trimethylphosphate, $\delta = 0$, as the external standard) $\delta$ **1**, 119.01, 120.55; **2**, 117.70, 119.13; **3**, 116.85, 117.98 ppm ($R_p/S_p$, ca. 60/40, diastereomeric mixture); FAB-MS ($M^+$ ion): **1**, calcd for 756.88, found 757.0; **2**, calcd for 780.94, found 781.0; **3**, calcd for 796.91, found 797.0.

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7. (a) The *PNT* protecting group was first used in the synthesis of amino sugars, see: Debenham, J. S.; Madsen, R.; Roberts C.; Fraser-Reid, B. *J. Am. Chem. Soc.* **1995**, *117*, 3302; (b) Iyer, R. P.; Yu, D.; Ho, N.-H.; Devlin, T.; Agrawal, S. *J. Org. Chem.* **1995**, *60*, 8132; (c) Iyer, R. P.; Devlin, T.; Habus, I.; Ho, N.-H.; Yu, D.; Agrawal S. *Tetrahedron Lett.* **1996**, *37*, 1539.
8. It is of interest to note that the *PNT* group in nucleosides (dC, dA, dG) is readily removed using 28% $NH_4OH$ or 0.05 M $K_2CO_3$/MeOH (15 min to 1 h, rt). Certain acyl protecting groups, particularly in the case of the purine nucleosides, are reportedly difficult to remove[6c] and require prolonged treatment with 28% $NH_4OH$ to effect complete deprotection, especially when synthesizing oligonucleotides. Experiments are under way to gain insight into the mechanism of deprotection using *PNT* nucleosides. For further enhancement of the rate of deprotection using *PNT* nucleosides other reagents are also being evaluated.
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