Methyl Phosphotriester Oligonucleotides: Facile Synthesis Using *N*-Pent-4-enoyl Nucleoside Phosphoramidites

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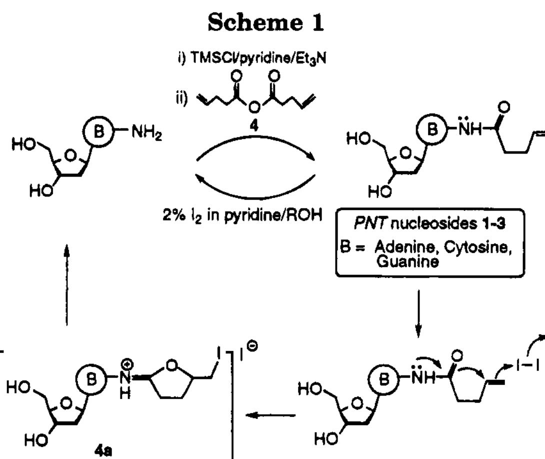
The exciting prospects presented by nucleic acid-based therapeutics[1ab] has sparked the search for analogs superior to phosphoric diester (PO) oligonucleotides. To be a viable therapeutic entity, it is desirable that the oligonucleotide analogs possess these attributes: (a) they should undergo sequence-specific hybridization with complementary target RNA or DNA, (b) they should show resistance toward degradation mediated by nucleases, (c) they must display favorable pharmacokinetic and pharmacodynamic profiles as well as good bioavailability, and (d) they should have pharmaceutical properties appropriate for formulation into suitable dosage forms.

Amongst the various analogs hitherto synthesized and evaluated, the phosphorothioate (PS) oligonucleotides targeted against a host of viral genomes as well as against cancer have advanced to various stages of clinical development.[1b] There is also considerable interest in "chimeric" PS oligonucleotides which contain 2′-OMe ribonucleotide and nucleoside methyl phosphonate segments.[1b] Compared to the PS- and methylphosphonate oligonucleotides, only limited studies of other interesting and novel *uncharged isosteres of* phosphoric diesters have been reported. Specifically, *phosphotriester* analogs, as exemplified by *O*-methyl phosphate (POOMe) and *O*-methyl phosphorothioate (PSOMe) oligonucleotides, either alone or as chimerics, have not been rigorously evaluated for biological activity and biophysical properties because their labile nature has eluded a practical strategy especially for routine synthesis.[2a–c]

Herein, we report a general approach toward the synthesis of POOMe and PSOMe oligonucleotides which uses *N*-pent-4-enoyl (PNT)[3ab] protected nucleosides **1–3**, in conjunction with phosphoramidite chemistry.[4] The flexibility of our synthetic methodology is demonstrated by the preparation of chimeric molecules incorporating these novel phosphotriester segments at predetermined sites in the backbone of a target oligonucleotide. An added bonus is that the herein described strategy also provides access to natural and modified support-bound oligonucleotides, which have potential applications in combinatorial chemistry and in oligonucleotide-based "affinity" columns.[5]

Scheme 1



Incorporation of the PNT group on dA, dG, and dC was carried out using pent-4-enoic anhydride **4** following the transient protection of the 3′- and 5′-hydroxyl groups as their trimethylsilyl ethers (Scheme 1).[6] The PNT group is readily removed by treatment with iodine reagent (2% in pyridine/H_2O or MeOH, 98/2, 30 min). Presumably, the deprotection is mediated by the initial electrophilic addition of iodine to the terminal olefin and capture of the resulting cation by the carboxamide group to give the imido ether intermediate **4a** (Scheme 1).[3a] Addition of water or methanol to **4a** results in its fragmentation to give the deprotected nucleoside. Thus, both incorporation and removal of the *PNT* group in nucleosides are readily accomplished.

The PNT deoxyribonucleosides **1–3** were then employed in the synthesis of β-cyanoethyl- (CEPNT) and methoxy- (MEPNT) 3′-*O*-(phosphoramido)-5′-*O*-(4,4′-dimethoxytriphenyl)methyl) (DMT) monomers by standard methodologies[7] and fully characterized. Initially, the CEPNT as well as MEPNT monomers were used to synthesize 2′-deoxyribodinucleoside PO and PS diesters on a controlled pore glass (CPG) support by using standard phosphoramidite chemistry,[7] except that t-BuOOH (1 M in toluene)[2b] was substituted for iodine in the oxidation cycle and removal of the base-protecting groups was done with iodine reagent (2% in pyridine/H_2O, 98/2, 30 min).[8a] Treatment of the CPG-bound dinucleotides with 28% NH_4OH (2 h, rt) or anhydrous K_2CO_3/MeOH (0.05 M, rt, 8 h)[2a,8b] gave the corresponding dinucleotides (yields 96–98%, as evaluated by reversed-phase HPLC[9a]), identical with authentic samples prepared under standard conditions using dA^{Bz}, $dG^{i\text{-}Bu}$, dC^{Bz}, and T β-cyanoethyl phosphoramidite monomers.

Next, the CPG-bound **9–12** and "free" POOMe dinucleosides **5–8** were synthesized using phosphoramidite

(1) (a) Uhlmann, E.; Peyman, A. *Chem. Rev.* **1990**, *90*, 544–84. (b) Agrawal, S.; Iyer, R. P. *Curr. Opin. Biotech.* **1995**, *6*, 12–19 and references therein.

(2) For recent efforts in the synthesis of POOMe oligonucleotides, see: (a) Kuijpers, W. H. A.; Huskens, J.; Koole, L. H.; van Boeckel, C. A. A. *Nucl. Acids Res.* **1990**, *18*, 5197–5205. (b) Alul, R. H.; Signman, C. H. N.; Zhang, G.; Letsinger, R. L. *Nucl. Acids Res.* **1991**, *19*, 1527–32. (c) Hayakawa, Y.; Hirose, M.; Hayakawa, M.; Noyori, R. *J. Org. Chem.* **1995**, *60*, 925–30.

(3) (a) For the use of PNT protecting group in the synthesis of aminosugars and other elegant studies see: Debenham, J. S.; Madsen, R.; Roberts, C.; Fraser-Reid, B. *J. Am. Chem Soc.* **1995**, *117*, 3302–03 and references therein. (b) *O*-Pentenyl glycosides have been employed as glycosyl donors; e.g., see: Ellervik, U.; Magnusson, G. *Acta Chem. Scand.* **1993**, *47*, 826–28.

(4) For a review see: Beaucage, S. L.; Iyer, R. P. *Tetrahedron* **1992**, *48*, 2223–2311.

(5) Atkinson, T.; Gillam, S.; Smith, M. *Nucl. Acids Res.* **1988**, *16*, 6232.

(6) Ti, G. S.; Gaffney, B. L.; Jones, R. A. *J. Am. Chem. Soc.* **1982**, *104*, 1316–19.

(7) Beaucage, S. L. In *Protocols for Oligonucleotides and Analogs*; Agrawal, S., Ed.; Humana Press: Totowa, NJ, 1993; Vol. 20, pp 33–61.

(8) (a) In the synthesis of PO-dimers, using MEPNT monomers, demethylation of the support-bound dinucleoside methyltriesters was carried out with 2-mercaptobenzothiazole.[7] (b) Under these conditions, removal of the β-cyanoethyl phosphate protecting group is also achieved.[2a]

(9) (a) For details of analysis and purification by HPLC see: Iyer, R. P.; Yu, D.; Agrawal, S. *Bioorg. Chem.* **1995**, *23*, 1–21. (b) It is important to avoid the use of I_2/pyr/*water* during deprotection to prevent demethylation of the phophotriester; thus, under our experimental protocol, less than 0.5% of the products correspond to PO dinucleosides. (c) Under our experimental protocol, less than 0.5% of the products correspond to PO or PS dinucleosides.

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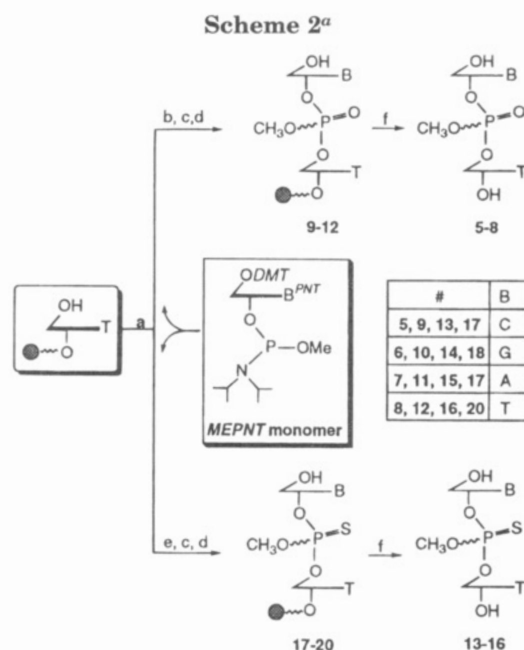
Scheme 2[a]



[a] Key: (a) 1*H*-tetrazole; (b) *t*-BuOOH (1 M in toluene); (c) DCA/DCM; (d) I_2 (2% in pyr/MeOH 98/2); (e) 3*H*-benzodithiol-3-one 1,1-dioxide; (f) anhyd K_2CO_3/MeOH (0.05 M).

chemistry by employing the appropriate MEPNT monomers (Scheme 2). Following the 1*H*-tetrazole-mediated formation of the deoxyribodinucleoside phosphites, oxidation of the internucleotidic phosphite linkage was carried out using *tert*-butyl hydroperoxide (1 M in toluene). Subsequent exposure of the CPG-bound dinucleotides to the iodine reagent (2% in pyridine/MeOH, 98/2, 30 min) completely removed the base-protecting groups to give the CPG-bound dinucleoside methyl phosphates **9–12**. Cleavage from the support using anhydrous K_2CO_3 (0.05 M in methanol, rt, 8 h) gave the triesters **5–8** as R_p and S_p diastereomeric mixtures (yields of 95–97%) which were characterized (see the supporting information).[9b] Unlike their PO counterparts, the POOMe dinucleosides **5–8** were resistant to hydrolysis by *snake venom phosphodiesterase* and *nuclease P1*. The assignment of absolute configuration to the diastereomers was done by correlation with R_p and S_p PSOMe dinucleosides **13–16** (*vide infra*) (see the supporting information).

The PSOMe dinucleosides **13–16** were synthesized by employing MEPNT monomers (Scheme 2), wherein oxidative sulfurization of the internucleotidic phosphite linkage was carried out with 3*H*-benzodithiol-3-one 1,1-dioxide.[10] Subsequent exposure of the CPG-bound dinucleotides to the iodine reagent (2% in pyridine/MeOH, 98/2, 30 min) gave the base-deprotected support-bound PS-OMe dinucleosides **17–20**. Cleavage from the support, using anhydrous K_2CO_3/MeOH (0.05 M, 8 h) gave **13–16** as R_p, S_p diastereomeric mixtures (yields of 94–98%) which were characterized (see the supporting information).[9c] The analogs **13–16** were resistant to hydrolysis by *snake venom phosphodiesterase* and *nuclease P1*. The assignment of absolute configurations to the R_p and S_p diastereomers of **13–16** and **5–8** was done as described[11] (see the supporting information).

The CEPNT and MEPNT monomers were also used to prepare chimeric trinucleotides having a single PO or PS deoxyribonucleoside linked to POOMe or PSOMe

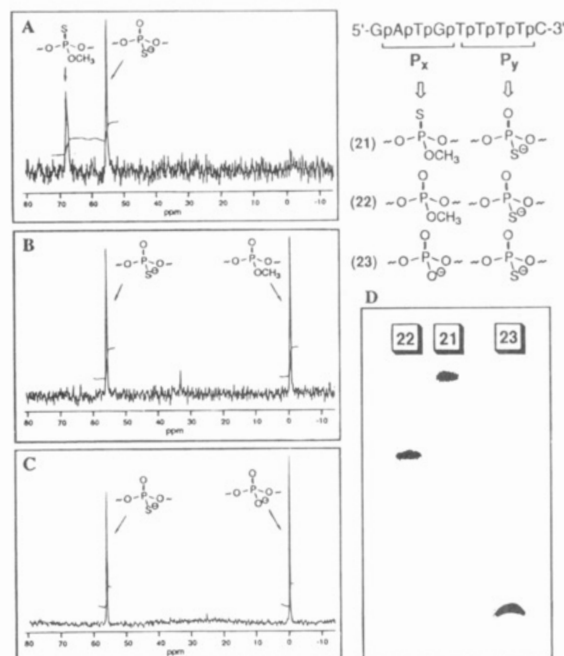


Figure 1. Panel A: ^{31}P-NMR (D_2O) spectrum of chimeric nonanucleotide (**21**). Panel B: ^{31}P-NMR (D_2O) spectrum of chimeric nonanucleotide (**22**). Panel C: ^{31}P-NMR (D_2O) spectrum of nonanucleotide (**23**). Panel D: PAGE profile of **21–23**.

deoxyribodinucleosides. For this purpose, the appropriate POOMe and the PSOMe segment was assembled using the MEPNT monomer while the PO or the PS segment was constructed with CEPNT monomers. The trimers, a mixture of four diastereomers, thus obtained were characterized by ^{31}P-NMR and ^{1}H-NMR (see the supporting information). This strategy was then extended to the synthesis of support-bound and free nonanucleotide chimeras, having the sequence 5′-GATGTTTTC **21** and **22**. In each case, analysis by ^{31}P-NMR (Figure 1) showed that the methyl phosphotriester and PS segments were present in the correct relative proportion, confirming that our mild deprotection conditions preserved the structural integrity of these base-sensitive molecules. Additionally, analysis by polyacrylamide gel electrophoresis (PAGE) showed that the chimeras **21** and **22** had slower mobility compared to the POPS nonanucleotide **23** having the identical sequence (Figure 1).

With a practical synthetic methodology to these novel molecules in hand, we are currently undertaking the large-scale synthesis and evaluation of biophysical properties as well as biological activities of oligonucleotides bearing the triester segments at predetermined sites. Applications involving the use of CPG-bound natural and modified oligonucleotides in diagnostics, in affinity chromatography, and combinatorial chemistry as well as studies with PNT nucleoside H-phosphonates[12] and synthesis of RNA will be reported in due course.

Supporting Information Available: NMR spectral data and analytical data for *PNT* nucleosides **1–3**; NMR spectral data and FAB-MS for *MEPNT* and *CEPNT* nucleosides; HPLC profile, spectral data, and MALDI-TOF mass spectra of d(GT) [POOMe], d(AT) [PSOMe]; assignment of absolute configurations to R_p, S_p TT (POOMe, PSOMe); NMR spectra of chimoric trinucleotides; synthesis cycle for preparation of **21–23** (11 pages).

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