Pergamon

Bioorganic & Medicinal Chemistry Letters, Vol. 6, No. 22, pp. 2663-2668, 1996

0960-894X/96 $15.00 + 0.00

PII: S0960-894X(96)00498-2

MIXED BACKBONE OLIGONUCLEOTIDES CONTAINING INTERNUCLEOTIDIC PRIMARY PHOSPHORAMIDATE LINKAGES

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Abstract. Mixed backbone oligonucleotides (MBOs) which contain segments of primary phosphoramidate linkages (PO-NH_2) in conjunction with either phosphoric diesters (PO), or phosphorothioates (PS) were prepared. Thermal denaturation of the duplexes with RNA and DNA reveal that they form stable duplexes which display cooperative melting profiles. Preliminary stability studies reveal that the PO-NH_2 linkage is resistant to serum nucleases. Thus, these MBOs represent novel antisense molecules. Copyright © 1996 Elsevier Science Ltd

Over the past few years there has been considerable progress in the use of oligonucleotides as potential therapeutic and diagnostic agents,[1a-c] and as unique tools in understanding cellular processes at the molecular level. The attractive features of the oligonucleotide-based drug design are (a) its universal appeal, which enables targeting of a wide variety of diseases, and (b) its simplicity in that it employs fairly well understood principles governing molecular recognition (Watson-Crick and Hoogsteen modes of base-pairing). The recent progress in the development of phosphorothioate oligonucleotides as antisense agents is a testament to the therapeutic potential of these molecules. In the quest for second generation therapeutics, novel oligonucleotide analogs that embody these properties are being sought: (a) enhanced resistance to cellular nucleases; (b) higher selectivity towards the intended molecular target; (c) improved cellular uptake; (d) ability to destroy the intended target by harnessing the host RNase H or alternatively by encoding in itself a hybridization-triggered cleaving agent; (e) unique pharmaceutical properties, which enables site- and tissue-specific delivery (f) lesser polyanion-related side effects; and (g) oral bioavailability with favorable pharmacokinetic profile. Our studies reveal that MBOs (Figure 1) incorporating judicious combination of oligonucleotides with ionic and non-ionic segments in a

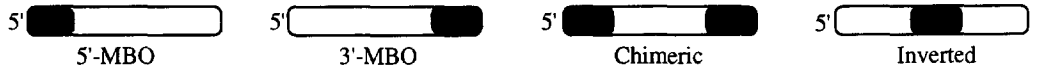


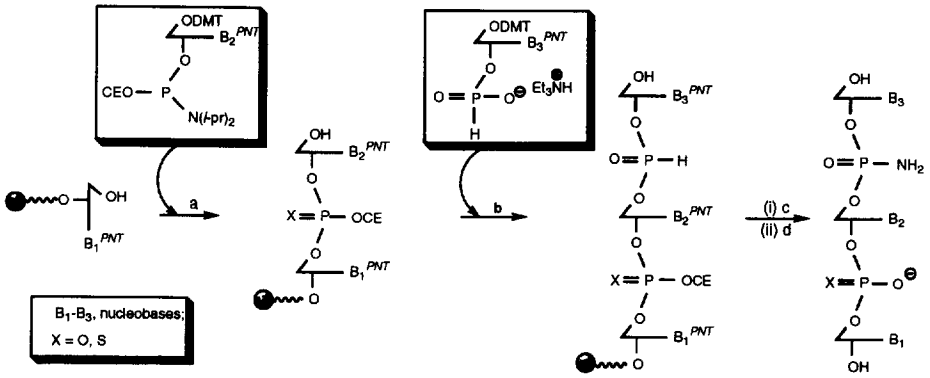
Figure 1. Mixed backbone oligonucleotide designs: The shaded area represents non-ionic segments while the open area represents ionic segments.

modular oligonucleotide design, have the potential for improved antisense properties.[2] In this regard, our recent focus has been on the synthesis of non-ionic analogs that contain internucleotidic primary phosphoramidate linkages (P[O]NH_2). Though non-ionic, these phosphodiester isosteres are expected to be water soluble, and have the potential for cooperative hydrogen-bonding interaction with the backbone of a complementary strand[3,4] and the consequent enhancement of the affinity for the target.

Although many *N*-substituted oligonucleoside phosphoramidate analogs are well known,[1c] the corresponding unsubstituted analog (i.e., that having the P[O]NH_2 linkage) is reportedly difficult to synthesize due to the hydrolysis of this linkage[5a-b] during the deprotection of N-acyl protected nucleobases with aqueous ammonium hydroxide. Nevertheless, a few dinucleoside analogs have been synthesized.[4,5a-h]

We have recently reported a high yield synthesis of these analogs in conjunction with the recently developed *N*-pent-4-enoyl (PNT) protected nucleoside *H*-phosphonates.[6a] In continuation of our report, we present here the synthesis, biophysical properties, and stability studies of MBOs that contain phosphoric diester (PO) or phosphorothioate (PS) segments alongside $P(O)NH_2$ segments.[7]

Synthesis of trinucleoside MBOs. Scheme 1 illustrates the automated solid-phase synthesis of a trinucleoside MBO containing PO and $P(O)NH_2$ linkages wherein phosphoramidite chemistry[8a] was used to



Scheme 1. Synthesis of MBOs; Key: (a) phosphoramidite cycle using tert-butyl hydroperoxide as the oxidizing agent and 3*H*-1,2-benzodithiole-3-one-1,1-dioxide as the sulfurizing reagent[8b]; (b) H-phosphonate synthesis cycle; (c) CC_4/satd. NH_3 in dioxane (1/1); (d) satd. NH_3 in DMF, 65 °C, 20 h.

build the PO linkage and H-phosphonate chemistry[9] was employed for the $P(O)NH_2$ linkage. During the synthesis of PO segment, the oxidation of the internucleotidic phosphite linkage was carried out with *tert*-butyl hydroperoxide instead of iodine reagent to prevent premature removal of the PNT protecting group.[6b] Following the synthesis, the removal of the PNT groups was done by treatment with iodine/pyridine/MeOH followed by exposure of the support-bound trinucleotide to saturated ammonia in DMF to effect the removal of the phosphate protecting group, and the cleavage of the trinucleotide from the support. The product trimers, isolated as a mixture of two diastereomers by reversed-phase HPLC, were characterized by ^{31}P NMR in which a pair of signals corresponding to the $P(O)NH_2$ segment appeared at ca. δ 12.5 ppm, and that corresponding to the PO segment appeared at -0.5 ppm, respectively. However, extension of this strategy for the trimer MBO (that contains the PS segment) resulted in the formation of the desulfurized product. Thus, in the ^{31}P NMR of the product, the characteristic peak at δ 52 ppm corresponding to the PS segment was absent, but instead the presence of a peak at δ -1 ppm was noted presumably corresponding to a PO linkage. Evidently, during the conversion of the H-phosphonate to phosphoramidate with CCl_4/saturated NH_3 in dioxane, (Scheme 1) elimination of the β–cyanoethyl protecting group had occurred to give the phosphorothioate diester, which suffered desulfurization during the removal of the PNT group with I_2/pyr/MeOH.[10] This problem was circumvented by avoiding the use of iodine for the removal of PNT group, but instead subjecting the support-bound trinucleotide to prolonged exposure to saturated NH_3 (anhydrous) in DMF (65 °C for 20 h) to effect both the removal of the PNT group, and the β-cyanoethyl phosphate protecting group, as well as, to cleave the trimer from the support, all in a single operation. Figure 2 shows the ^{31}P NMR of representative trimers prepared using this procedure. Several trimers incorporating different combinations of dC, dA, and dG were prepared

and characterized. In all cases, complete deprotection of the nucleobases had been achieved while preserving the integrity of the backbones (also *vide infra* for longer oligonucleotides). It is pertinent to mention that the "naked" phosphoramidate linkage was apparently stable to reagents employed in phosphoramidite or H-phosphonate chemistry.[11] This allowed the site specific incorporation of this linkage within the oligonucleotide chain, and subsequent chain elongation by using either phosphoramidite or H-phosphonate chemistry.

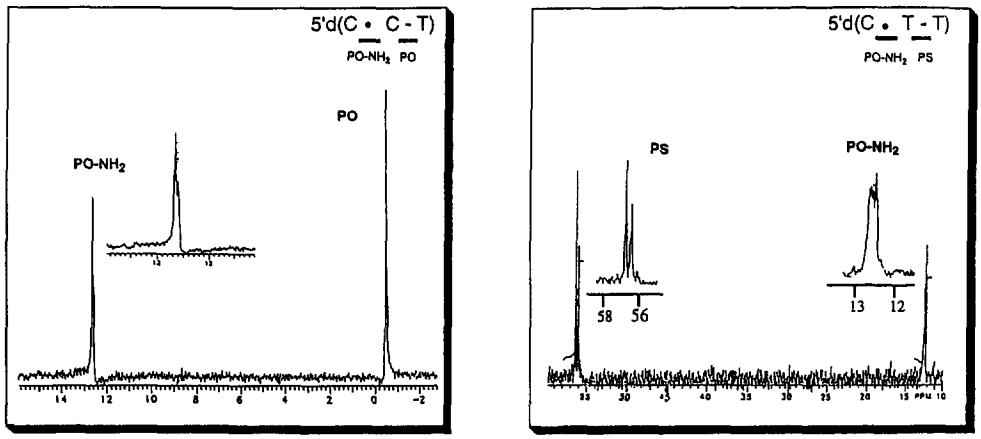


Figure 2. ^{31}P NMR (D_2O, 85% H_3PO_4, external standard) of trinucleoside MBOs.

Synthesis of MBOs

To make the synthetic methodology applicable for longer oligonucleotides, and in order to ascertain that complete base and phosphate deprotection had occurred without any attendant base-modifications, we prepared the MBO **1**, which contained a single $P(O)NH_2$ linkage at the 5'-end. The oligonucleotide was purified by preparative PAGE, desalted, and subjected to base-composition analysis. The HPLC analysis (Figure 3) of the enzyme digest showed that the bases were present in the correct integral ratio along with the dinucleoside T-T $P(O)NH_2$ which was resistant to nuclease-mediated degradation under these conditions.

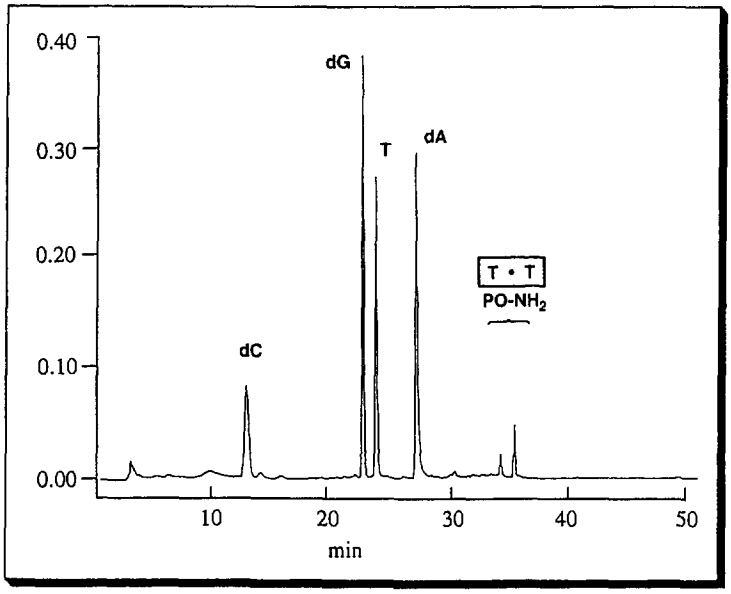


Figure 3. HPLC profile of the products resulting from enzymatic digestion of the MBO **1** with *snake venom phosphodiesterase* and *alkaline phosphatase*.

5'd T • T -GAT GCA CT
PONH2 PO

1

We next undertook the synthesis of MBOs **2-5** wherein the PO-NH_2 segments were placed at defined positions in an oligonucleotide. The sequence of the MBO chosen for this study was GEM 91®, a 25-mer oligonucleotide targeted against the *gag* mRNA of HIV-1. Following their synthesis on a 10 μmol scale and deprotection, as before, the oligonucleotides were purified either by preparative PAGE (DMT-off synthesis) or by reversed-phase HPLC (DMT-on synthesis)[12] followed by detritylation using Dowex (H^+).[13] Figure 4 shows the ^{31}P NMR of a representative MBO (panel A) and the analytical PAGE (panel B) of the purified MBOs. As expected, these MBOs were relatively more soluble in water compared to the corresponding MBOs with methyl phosphonate linkages.

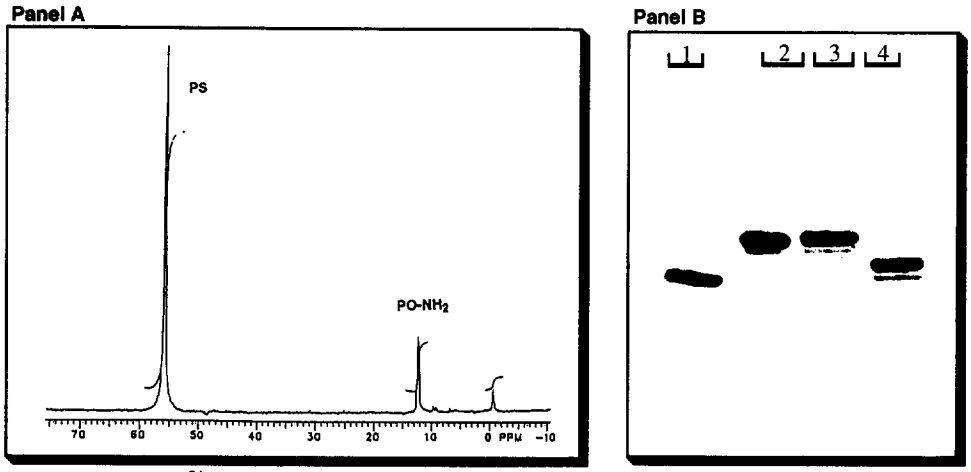


Figure 4. **Panel A,** ^{31}P NMR (D_2O, 85% H_3PO_4, external standard) of crude MBO **4**; **Panel B,** Analytical PAGE profile of the purified MBOs **3 - 5** (lanes 2-4) and **6** (Lane 1).

Biophysical properties of MBOs.

For the biophysical evaluation, T_ms of the duplexes formed between the MBOs and the corresponding complementary DNA and RNA were determined by measurements of the absorbance at λ_{260} nm vs temperature. For comparison, the T_ms of the duplexes formed between GEM 91 ("all PS" analog **6**) and the corresponding "all PO" analog (**7**) with DNA and RNA were also determined. All complexes showed a monophasic melting transition, which is characteristic of oligonucleotides. The T_ms, which represent the average of at least two determinations (forward and backward melting curves), are revealed in Table 1. The following trends in the T_ms are noteworthy: (a) in the case of the duplex formed between the MBO **4** and the complementary DNA, the T_m value was the same as that of **6**; (b) in the case of MBOs **2**, **3**, and **5**, the incorporation of the P(O)NH_2 linkage produced a marginal drop in the T_m (0.5 to 0.7 °C per P[O]NH_2 substitution) compared to **6** and **7**; and (c) in the case of the DNA/RNA duplex formed between MBOs with complementary RNA, a slight drop in T_m was seen (~ 0.5 to 1 °C per substitution), compared to that with **6**. Furthemore, the plots of absorbance vs temperature profiles showed cooperative melting profiles associated with almost identical hypochromicity changes in the cases of the duplexes of MBOs with DNA, as well as, those of **6** and **7** with DNA (data not shown). Overall, however, this preliminary data does not suggest that there is any increase in the stability of the duplexes formed between MBOs containing the P(O)NH_2 linkages and complementary DNA/RNA due to the

anticipated reduction in phosphate to phosphate interstrand repulsion, or potential interstrand hydrogen-bonding interactions.

Table 1. Comparative T_m Data of GEM 91® Analogs 2 - 7

Seq. #	Sequence	T_m¶	T_m§
2	5' C•T•C•T•C•GCA CCC ATC TCT CTC CTT CT PO-NH_2 PO	61.3	-
3	5' C•T•C•T•CGC ACC CAT CTC TCT CCT TCT PO-NH_2 PS	53.1	63.3
4	5' C•T•C TCG CAC CCA TCT CTC TCC TT•C•T PO-NH_2 PS PO-NH_2	55.3	63.8
5	5' CTC TCG CAC CC•A•T•C•T CTC TCC TTC T PS PO-NH_2 PS	52.0	59.8
6	5' CTC TCG CAC CCA TCT CTC TCC TTC T PS	55.0	65.1
7	5' CTC TCG CAC CCA TCT CTC TCC TTC T PO	64.8	-

¶ Against complementary DNA (30-mer) (PO); §Against complementary RNA (25-mer) (PO). All T_ms represent the average of at least two determinations forward and backward.

Nuclease stability of phosphoramidates.

The nuclease-mediated degradation of a phosphodiester group in an oligonucleotide is contingent upon the presence of the negative charge on the phosphate, which apparently provides an anchor for metal-ion binding and subsequent nucleophilic attack to cleave the internucleotidic bond. Presumably, by depriving the essential ion-anchoring domain for nuclease action, or perhaps due to reduced affinity for the enzyme, many non-ionic oligonucleotides become resistant to hydrolyis by nucleases. However, the primary phosphoramidate linkage differs from other non-ionics in that the PO-NH_2 group (a) can bind with metal ions and become susceptible to nucleolytic attack, and (b) can also potentially hydrogen bond with an acceptor site of the enzyme making it susceptible to those nucleases which employ nucleophilic or acid-base catalysis (e.g., RNases).[14] Our studies reveal (Figure 5) that under conditions in which a phosphodiester linkage is degraded, the primary phosphoramidate linkage in a dinucleotide was resistant to the action of serum nucleases. Detailed stability studies using MBOs **2-5** against a variety of nucleases are in progress.

In conclusion, this is the first report of the synthesis and biophysical properties of MBOs that contain PS and PO-NH_2 segments. Our preliminary studies reveal that these MBOs linkages are novel antisense molecules. Large-scale synthesis of these MBOs for biological and pharmacological evaluations is under way and will be reported in due course.

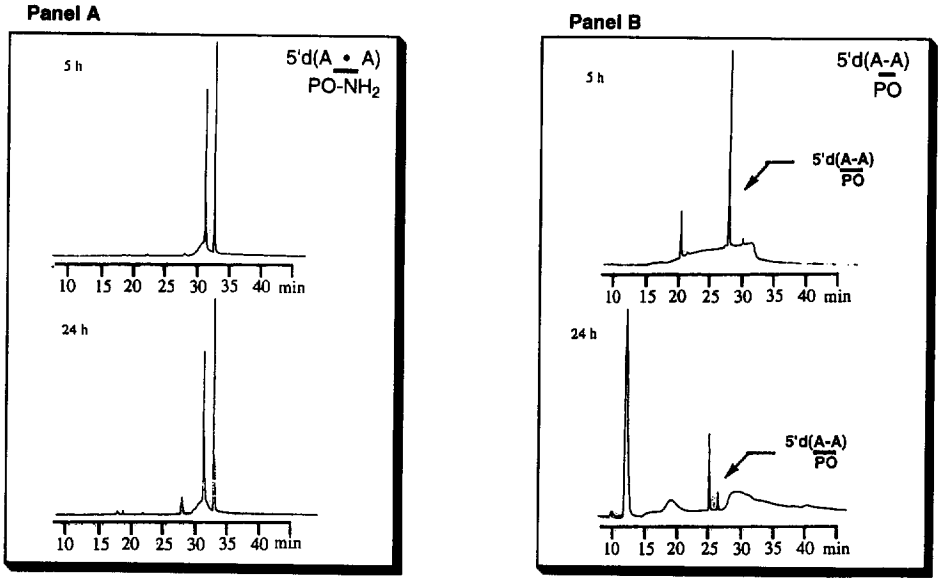


Figure 5. Reversed-phase HPLC profiles of phosphoramidates and phosphoric diesters following incubation in serum; **Panel A**, d(A[PONH$_2$]A); **Panel B**, d(A[PO]A).

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(*Received in USA* 21 *August* 1996; *accepted* 10 *October* 1996)