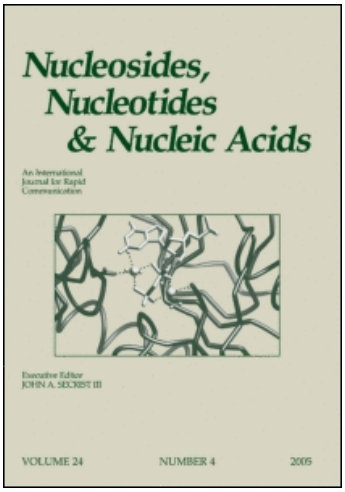

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Synthesis, Biophysical and Biological Evaluations of Novel Antisense Oligonucleosides Containing Dephosphono-Internucleosidic Linkages

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SYNTHESIS, BIOPHYSICAL AND BIOLOGICAL EVALUATIONS OF NOVEL ANTISENSE OLIGONUCLEOSIDES CONTAINING DEPHOSPHONO-INTERNUCLEOSIDIC LINKAGES

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ABSTRACT: Efficient large-scale syntheses of **m**ethylene(**m**ethyl**i**mino) (MMI) linked mixed base nucleosidic dimers have been accomplished. These dimers were successfully incorporated into deoxyoligonucleotides by automated solid-support synthesis. The hybridization properties, nuclease stability, RNase H mediated cleavage and *in vitro* biological activity of novel chimeric oligomers have been studied. The biophysical and biological evaluation of these chimeric oligomers containing MMI linkages suggests that MMI is a promising chemical modification of the backbone linkage for the construction of antisense molecules useful as therapeutics.

In search of novel antisense oligonucleotides (AO) that contain a neutral and achiral linkage replacing the natural phosphodiester linkage, we have discovered the **m**ethylene(**m**ethyl**i**mino) backbone **3** [MMI; 3′-$CH_2N(CH_3)OCH_2$-4′] as an alternative linkage.[1] The AO containing MMI linkages when compared to their natural counterparts phosphodiester **1** (P=O) are expected to have the following advantages. Backbone modified AO: (i) are amenable to the large-scale synthesis; (ii) are stable to cellular nuclease degradation; (iii) are able to enter the target cells; (iv) have the ability to form more stable duplexes with complementary RNA; and (v) demonstrate biological activity. We consider phosphorothioate (P=S) **2** as the first generation AO, which has entered human clinical trials recently. Uncharged oligomers, such as MMI linked oligomers may become an important class of the second generation of AO. Our preliminary results with nuclease studies indicate that an alternating P=O and MMI linked oligomer provide significant protection from cleavage by endo- and exo- nucleases. In addition, these AO, compared to the unmodified AO or P=S AO, exhibit an increased affinity towards their respective complementary RNA targets.[2] In order to construct AO with alternating P=O and MMI linkages one must prepare a set of sixteen dimers (**3**) from eight nucleoside building-blocks (**5**, **6**; Figure 1).

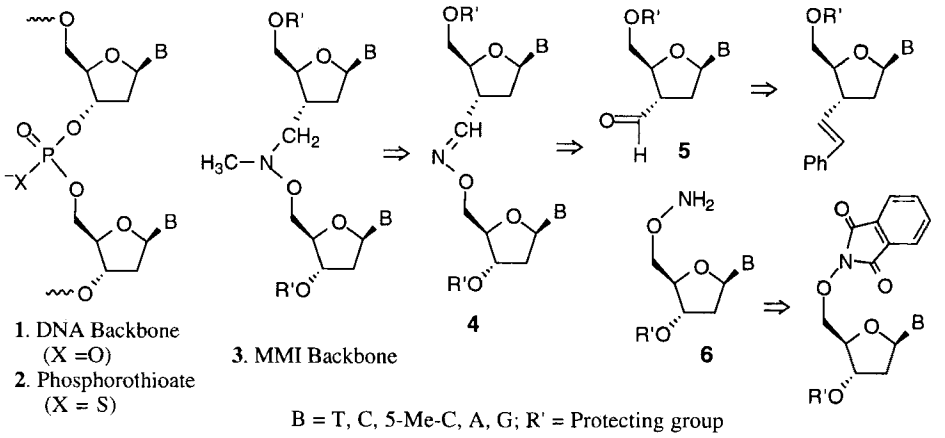

Figure 1

Chemistry: The dimers containing MMI linkages were synthesized by a convergent approach in which commercial 2′-deoxynucleosides (T, C, A, G) were appropriately functionalized with a 5′-*O*-amino and or a 3′-*C*-formyl groups *via* a series of reactions. An efficient large-scale synthesis of all eight building-blocks (**5**, **6**: B = T, C, A, G) utilizing novel chemistries has been accomplished for the first time.[3] Coupling of **5** and **6** may potentially provide all 16 possible oxime dimers **4**, which provides 16 corresponding MMI dimers **3**. We have synthesized[4] several (T*C, T*C^{Me}, C*T, C^{Me}*T C*C, C^{Me}*C^{Me}, A*C, T*G, G*C; C^{Me} = 5-Me-C) mixed base MMI dimers **3**, containing both purines and pyrimidines and incorporated certain mixed base dimers into AO *via* automated synthesis. An average coupling yield of 90-99% was obtained for all AO made. The AO were purified by preparative HPLC and the final purity and composition were checked by CE and electrospray MS analysis.

Hybridization: Chimeric AO containing MMI linked dimers were found to hybridize to their complementary RNAs (4 μM of each strand, pH 7, 100 mM NaCl, 0.1 mM EDTA) as effectively as the unmodified DNAs with a high level of base pair specificity.[2] AO composed of an alternating P=O and MMI linkage exhibited a ΔT_m (modified DNA•RNA) of ~ +1.0° C/modification compared to the parent P=S AO. The Watson–Crick base-pair specificity of AO containing MMI linkages was found to be better than the wild-type DNA. The change in entropy (Δ S) measured for the duplexes (modified DNA•RNA) was higher than the unmodified DNA•RNA duplex, suggesting that MMI has conformational rigidity similar to the P=O linkage.

Nuclease studies: The nuclease stability of oligomers containing MMI linkages (placed at various positions) have been studied using purified exo- (SVPD) and endo- (S1)

nucleases.[5] Placement of the MMI linkages at the 3′-end of an oligomer provided enhanced stability to the oligomer from degradation by SVPD, compared to the unmodified DNA. In a similar manner, an alternating P=O and MMI linked oligomer (19-mer) displayed high stability ($t_{1/2}$ >24 h) in the presence of endonuclease. We believe that the MMI linkage manifested an increase in stability of the internal P=O linkage due to the conformational change of the backbone. This property of the MMI linkage may have a pharmacological advantage towards designing superior antisense oligomers.

RNase H studies: One of the mechanisms by which AO cause reduction in mRNA levels is through the action of RNase H (an endonuclease that cleaves mRNA strand of DNA•RNA duplexes). Our studies with fully alternating P=X (X = O, S) and MMI linked oligomers indicated that such an arrangement was not a substrate for RNase H when hybridized to a complementary RNA. However, RNase H activity was maintained when MMI linkages were placed away from the cleavage site. Thus, a chimeric (Gap: MMI•P=S•MMI) oligomer displayed similar or better antisense potencies in inhibiting Ha-ras or PKC-α expression, compared with the corresponding unmodified P=S oligomer.[6]

Structural studies: NMR and modeling studies confirmed that the 3′-CH_2 substituent of the MMI linked nucleosidic dimer was responsible for shifting the pseudorotation of that sugar towards high *N*-conformation compared to the natural deoxynucleoside.[7] We believe that increased binding affinity of MMI linked oligomers towards complement RNA is due to the higher population of the RNA-like (*N*-pucker) conformation of the sugar moiety. The CD studies of an oligomer containing five MMI linkages alternating with P=O suggest an RNA-type conformation.

Conclusions: Our results demonstrate that MMI linked AO are significantly resistant to nuclease degradation, provide better affinity and specificity toward RNA targets, exhibit biological activity without interfering with RNase H activity and adopt a preorganized structure with an RNA-like conformation. Clearly, the initial data on MMI modified AO suggest that second generation AO containing achiral and neutral linkages may exhibit substantially improved pharmacokinetic and pharmacodynamic properties. Additionally, backbone modifications in combination with 2′-sugar and base modifications may further enhance the activity of AO.[8]

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