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Synthesis and Incorporation of Methyleneoxy(methylimino) Linked Thymidine Dimer into Antisense Oligonucleosides+

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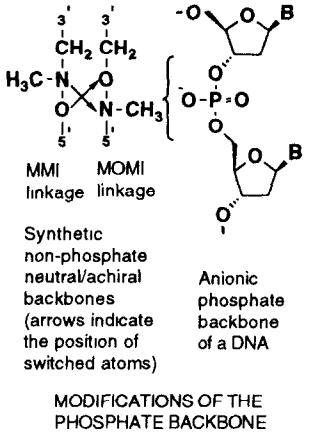
Abstract: A convenient synthesis of a thymidine (T) nucleoside dimer (T-3'-CH_2-O-NCH_3-5'-T) **12** has been accomplished via a nucleoside coupling reaction. An alternative synthesis of 3'-deoxy-3'-*C*-hydroxymethylthymidine is described. The new dimer and methodology is useful for the development of backbone-modified antisense oligonucleosides.

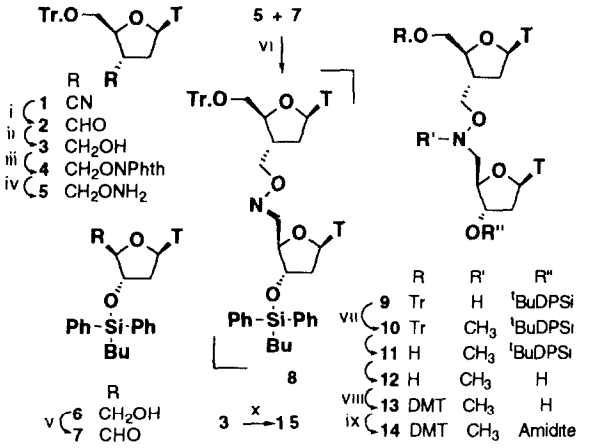
The selective inhibition of gene expression by antisense oligonucleotides (AO) and their derivatives provides an opportunity for the rational design of therapeutic agents.[1] The most important factor in the effectiveness of AO lies in their ability to hybridize, via Watson-Crick base pairing, with a complementary region in a target mRNA. Although AO are routinely made using an automated DNA synthesizer, their therapeutic applications have been hampered by difficulties, such as degradation by nucleases,[2] insufficient cellular penetration and distribution,[3] and the high cost of solid-support synthesis.[4] These difficulties could be resolved by modifications of the phosphate backbone.[5] For example, phosphorothioates[6] (PT) provided enhanced nuclease stability and methylphosphonates[7] (MP) are stable to nucleases and may enhance cellular uptake due to their neutral characters. However, both the PT and MP backbone modifications introduce phosphorus chirality that leads to a mixture of $2^{(n-1)}$ diastereomers. In order to circumvent the chirality and cost problems, we[8] and others[9] have replaced the natural anionic phosphate linkage with a neutral and achiral non-phosphate linkage (**Figure 1**). Generally, cellular nucleases recognize and cleave the natural sugar-phosphate linkage, whereas it is believed that the unnatural non-phosphate backbone modifications will not serve as substrates.[5] Thus in search for superior backbone linkages, we recently reported methylene(methylimino) backbone (MMI) as a novel linker with potential AO applications.[8] The MMI linkage was not cleaved by nucleases, MMI-modified AO hybridize to their complementary RNA effectively, while maintaining a high level of base pair specificity. Also, a neutral backbone linkage such as MMI may enhance the uptake of AO in a manner similar to that of neutral MP analogs.

In order to explore the structure-activity relationship (SAR) between AO backbone modifications and their biochemical and biophysical properties, we synthesized methyleneoxy(methylimino) backbone (MOMI) as a positional isomer of MMI linkage. We believe that a simple atom switch in the MMI linker as shown in **figure 1** would retain an acceptable internucleosidic distance. In the present work we wanted to assess the importance of the structural changes in the backbone and their effects on antisense properties by comparing the MMI and MOMI linkers.

Figure 1



Scheme 1. SYNTHESIS OF THE DIMER[a]



[a] Reagents and conditions: (i) 1 equiv of **1**, 2.5 equiv of DIBAL-H (1 M in toluene), THF, 0° C, 24h, 55% of **2**, (ii) 1 equiv of **2**, 3.5 equiv of $NaBH_4$ in EtOH/H_2O (75 25, v/v), room temp., 2h, 83% of **3**; (iii) 1 equiv of **3**, 1.3 equiv of HONPhth, 1.3 equiv of Ph_3P, 1.5 equiv of DIPAD, THF, 0° C -> room temp, 20h, 79% of **4**; (iv) 1 equiv of **4**, 1.5 equiv of H_3CNHNH_2, CH_2Cl_2, room temp., 2h, 70 % of **5**, (v) CrO_3/Ac_2O/Pyridine/DMF/CH_2Cl_2, 70% of **7**, see ref.15 for experimental details; (vi) 1 equiv of **5**, 1.8 equiv of **7**, 1.2% AcOH/CH_2Cl_2, room temp., 2h, dimer **8** (not isolated) -> 6 equiv of $NaBH_3CN$, AcOH, room temp., 2h, 60% of **9**; (vii) 1 equiv of **9**,16 5 equiv of HCHO, 6 equiv of $NaBH_3CN$, AcOH, room temp , 30 min, dimer **10** (not isolated) ->aq HF (48%)/CH_3CN (5.95, v/v), dimer **11** was not isolated -> nBu4NF (1ml,1 M in pyridine)/pyridine (10 ml), room temp , 1h, 65% (over 3-steps) of **12**; (viii) 1 equiv of **12**, 3 equiv of DMTCl, pyridine, room temp., 24h, 71% of **13**; (ix) 1 equiv of **13**,1.3 equiv of 2-cyanoethyl N,N,N',N'-tetraisopropylphosphorodiamidite, 0.7 equiv of N,N-diisopropylammonium tetrazolide/CH_2Cl_2, room temp , 6h, 75% of **14**; (x) 0 09 N HCl/MeOH, 16h, 82%.

A straightforward synthesis of MOMI linkage was visualized by an extension of the coupling chemistry developed for the synthesis of the MMI linkage.[8] Retrosynthetic analysis of the desired dimer **12** indicated that a 3′-branched *C*-hydroxymethyl nucleoside **3** would serve as a key intermediate. A recent synthesis of **3** as the unprotected nucleoside used a multistep convergent approach in which thymine was coupled to a functionalized carbohydrate moiety.[10] We report a convenient synthesis of 1-[2′,3′-dideoxy-3′-*C*-(hydroxymethyl)-β-*D*-erythro-pentofuranosyl]thymine (**15**), from 3′-deoxy 3′-α-cyanothymidine[11] (**1**) in good yield. A DIBAL-H reduction of **1** led to the blocked aldehyde[12] **2** in 55% isolated yield (**scheme 1**). Further reduction of **2** with $NaBH_4$ in EtOH furnished **3** (83%). Detritylation of **3** gave **15**, which was identical in all respects to the literature reports.[10]

A Mitsunobu reaction[13] of **3** with *N*-hydroxyphthalimide gave **4** (79%), which on hydrazinolysis provided a 70% yield of*O*-amino nucleoside **5**, the top fragment of the dimer **12**. The bottom fragment was derived from 3′-*O*-(*t*-butyldiphenylsilyl)thymidine[14] (**6**) which on oxidation[15] with CrO_3/pyridine /Ac_2O gave the expected 5′-aldehyde derivative **7**. An acid catalyzed coupling[8] between **5** and **7** (freshly prepared) gave an oxime intermediate **8**, which was then reduced ($NaBH_3CN$/AcOH) in situ to the dimer **9**. Reductive alkylation with HCHO/$NaBH_3CN$/AcOH of the amino group in the linker of dimer **9** furnished MOMI-linked protected dimer **10**. Conventional deprotection of **10** gave 3′-de(oxyphosphinico)-3′-[methyleneoxy(methylimino)]-thymidylyl-(3′→5′)-5′-deoxythymidine (**12**), which was fully characterized[16] by ^1H NMR, FAB MS and elemental analysis.

The 3′-α- and 4′-β-configuration of the MOMI linker between the two sugar moieties was established by COSY and NOESY studies.

Table 1. Comparative hybridization data on backbone modified oligodeoxynucleosides

Oligo-mers[a]	Sequence d(5′→3′)[b]	T_m[c] (Type of linkage) [d]	
		MMI[h]	MOMI
I[e]	GpCpGpTpTpTpTpT*TpTpTpTpTpGpCpG	49.4	48.9
II[e]	GpCpGpTpTpTpT*TpT*TpTpTpTpGpCpG	49.7	47.5
III[e]	GpCpGpTpTpT*TpT*TpT*TpTpTpGpCpG	48.2	44.2
IV[e]	GpCpGpTpT*TpT*TpT*TpT*TpTpGpCpG	--	42.3
V[e]	GpCpGpT*TpT*TpT*TpT*TpT*TpGpCpG	50.8	40.2
VI[f]	CpTpCpGpTpApCpT*TpT*TpCpCpGpGpTpCpC	57.4	54.3
VII[g]	CpGpApCpTpApTpGpCpApApTpT*TpC	43.6	43.8

[a]All oligomers were hybridized with complementary RNA; [b]p = natural phosphate linkage, * = modified linkage; [c]Absorbance vs. temperature profiles were measured at 4 mM of each strand in 100 mM Na^+, 10 mM phosphate, 0.1 mM EDTA, pH 7.0 (see ref.20 for experimental details), T_m data are averages of at least 3 experiments; [d]see figure 1 for the structure of MMI and MOMI linkages; [e]T_m of unmodified sequence was 50.2°C; [f]T_m of unmodified sequence was 56.7°C; [g]T_m of unmodified sequence was 44.1°C; [h]partial data was reported in reference 8.

A reported procedure[17] was utilized for the a sequential dimethoxytritylation and phosphitylation of **12** to provide **14** in high overall yield. Using an automated DNA synthesizer,[18] amidite **14** was incorporated into a standard 16-mer oligodeoxynucleoside sequence from one to five times with an average coupling efficiency of 98% for each dimer incorporation. The oligomers bearing 3′-CH_2-O-N(CH_3)-5′ linkages were purified by HPLC and exhibited a single band on polyacrylamide gel after purification. The structural identity of oligomers I-VII was confirmed by two methods: (a) HPLC analysis of the enzymatic degradation[19] of I exhibited the expected ratios of nucleosides and the T*T dimer **12**, and (b) ^1H and ^{31}P NMR analysis of a tetramer TpT*TpT to confirm the integrity of the MOMI linkage after automated synthesis. A comparative hybridization study[20] with oligomers containing MMI and MOMI linkages is summarized in **table 1**. The study indicates that the oligomer containing MOMI linkages have a lower affinity for duplex formation with their complementary RNA (average ΔT_m/modification = -1.44° C; whereas an average ΔT_m/modification for MMI linkage = -0.3° C). Preliminary studies to evaluate the nuclease resistance[2] of 3′-capped oligomer VII exhibited a half-life of ~2 h with 10% heat inactivated fetal calf serum (FCS), whereas as the half-life of unmodified oligomer is ~0.5 h. The MMI modified linkage exhibited a half-life of 14 h in FCS.[8] Studies are in progress to determine the reason for the less efficient[21] hybridization (T_m) and lower nuclease resistance of MOMI modified oligomers compared with MMI modified oligomers.

In summary, initial results indicate that a subtle change in the structure of MMI linker may alter the biochemical and biophysical properties of AO dramatically. A convenient synthesis of MOMI-linked dimer **12** was accomplished, and it demonstrates the versatility of the coupling methodology developed for backbone modification. Also, a new achiral, neutral linkage was successfully incorporated into oligodeoxynucleosides.

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References and Notes

[+]We refer to modified oligonucleotides that have the phosphorus atom removed as *oligonucleosides*.

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16. **12**: ^{1}H NMR (T1 represents 3'-substituted thymidine moiety and T2 represents 5'-substituted thymidine moiety) (DMSO-d_6) δ.11.27 (br s, 2, N*H*), 7.85 (s, 1, T1C$_6$*H*), 7.51(s, 1, T2C$_6$*H*), 6.15 (pseudo t, 1, T2C$_1$'*H*, $J_{1',2'}$ = 7.8Hz, $J_{1',2''}$ = 4.5 Hz), 6.0 (pseudo t, 1, T1C$_1$'*H*, $J_{1',2'}$ = 6.9 Hz, $J_{1',2''}$ = 4.5 Hz), 5.32 (br s, 1, C$_3$'O*H*), 5.09 (br s, 1, C$_5$'O*H*), 4.17 (m, 1, T2C$_3$'*H*), 3.90 (m, 1, T2C$_4$'*H*), 3.76-3.66 (m, 4, T1C$_4$'*H*, T1C$_5$'*H*, C$_3$"C*H*$_2$), 3.60-3.52 (m, 1, T1C$_5$"*H*), 2.82 (m, 2, T2C$_5$'*H*, $_5$"*H*), 2.57 (s, 3, NC*H*$_3$), 2.47 (m, 1, T1C$_3$'*H*), 2.23-2.02 (m, 4, C$_{2',2''}$*H*), 1.81 (s, 3, C$_5$C*H*$_3$), 1.78 (s, 3, C$_5$C*H*$_3$). Anal. Calcd for $C_{22}H_{31}N_5O_9 \cdot 0.5\ H_2O$: C, 50.96; H, 6.22; N, 13.50. Found: C, 51.01; H, 6.22; N, 13.19. FAB MS: m/z 510 $(M+H)^+$.
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21. A plausible reason for this effect may be the close contact between the sterically bulky 5'-N(CH$_3$) moiety of the MOMI linkage and the C$_6$H of the thymine base. As a result of this proximity the thymine base may be pushed into a conformation not suitable for optimum base-pairing. This close contact does not exist in the MMI linker, which contains a relatively small oxygen atom in the same position.