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Achiral Internucleoside Linkages: CH_2-CH_2-NH and NH-CH_2-CH_2 Linkages

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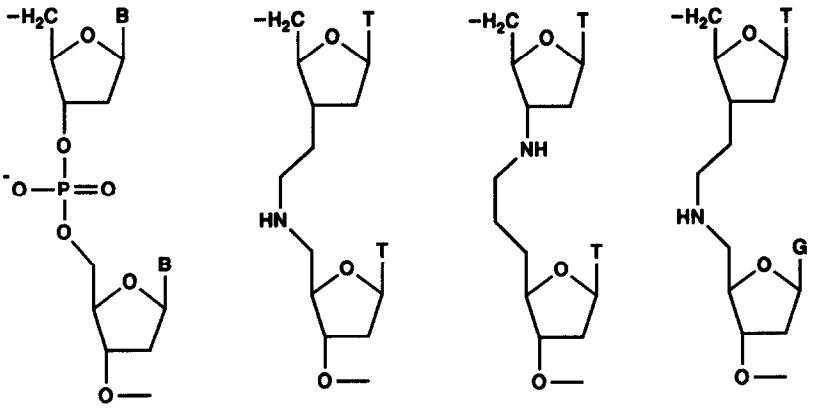
Abstract: The synthesis of CH_2-CH_2-NH and NH-CH_2-CH_2 internucleoside linkages are described. Antisense oligonucleosides containing these dimer modifications hybridized to the sense sequence. Furthermore incorporation of these backbone modifications enhanced the nuclease resistance of the antisense strand.

Recently there has been growing interest in the synthesis of oligodeoxynucleotide analogs with achiral, neutral internucleoside linkages. These analogs of oligo-DNA are expected to modulate transcriptional and translational gene expression.[1,2] Some reports have shown that these analogs possess hypochromicity comparable to the natural oligo-DNA, and bind to complementary DNA or RNA sequences with a high degree of specificity by Watson-Crick base pairing.[3] Further, neutral or positively charged internucleoside linkages such as found in **2-4** might enhance cellular uptake, resistance toward nuclease degradation and duplex formation. In the present communication, two internucleoside modifications wherein the natural O-PO_2^--O linkage (**1**) has been replaced by CH_2-CH_2-NH (**2**) or NH-CH_2-CH_2 (**3**) are described.



1 **2** **3** **4**

Scheme 1 outlines the synthesis of a modified dinucleoside of type **2** which is ready for incorporation into a predetermined DNA sequence. The key reaction for the synthesis of the dimeric unit is the formation of the C-N bond by reductive amination. Further, the synthetic scheme yields the secondary aliphatic nitrogen protected

by a group which is compatible with the standard automated DNA synthetic cycle. Thus, it has proven possible to deprotect the aliphatic amine and other purine and pyrimidine base protecting groups simultaneously at the end of DNA synthesis.

Thymidine aldehyde **5** was prepared from the known 3'-allyl-3'-deoxy-5'-O-*tert*-butyldimethylsilyl thymidine.[4] The allyl compound was regioselectively oxidized with a catalytic amount of OsO_4 as oxidant and 4-methylmorpholine-N-oxide as a co-oxidant to give the diol in 73% yield. The diol was oxidized with $NaIO_4$ to give **5** in almost quantitative yield.[5] Reductive amination of 3'-O-*tert*-butyldimethylsilyl-5'-amino thymidine **6** with thymidine aldehyde **5** was carried out either with $NaCNBH_3$ or with $Na(OAc)_3BH$.[6] Sodium cyanoborohydride mediated amination proceeded in 60-70% yield, but was accompanied by significant reduction of aldehyde **5** to the corresponding primary alcohol. On the other hand, $Na(OAc)_3BH$ delivered the dimer **7** in 85-90% yield with little reduction to the primary alcohol. Then, the aliphatic nitrogen was protected as a trifluoroacetamide by treatment with $(CF_3CO)_2O$-Et_3N. The protected dinucleoside **8** was obtained in more than 90% yield. The protected dimer was desilylated with nBu_4NF and the primary hydroxyl group of the resulting diol **9** was selectively protected with dimethoxytrityl chloride to give **10** in 90% yield. The remaining hydroxyl was transformed to the required phosphoramidite by reacting with 2-cyanoethyl-N,N-diisopropylchlorophosphoramidite.

An appropriately protected dinucleoside possessing a $NH\text{-}CH_2\text{-}CH_2$ linkage was synthesized as depicted in **Scheme 2.** Subunits **12** and **13** were coupled using $Na(OAc)_3BH$ to produce dimer **14** in 82% yield.[7] Compound **14** was further functionalized for DNA synthesis as described above to give **17.** Compound **14** was chosen as a model for the deprotection of trifluoroacetamide. The deprotection was accomplished in almost quantitative yield by treating with ammonia at 60°C for 6 h.

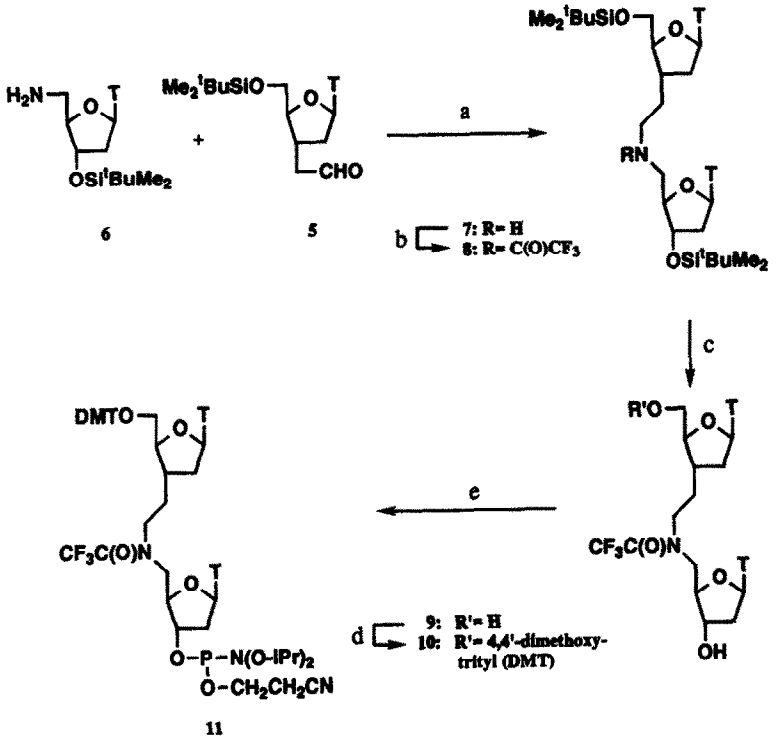
With slight modification, the above synthetic sequence could be extended to the preparation of mixed base heterodimers. For example the $CH_2\text{-}CH_2\text{-}NH$ modification incorporating thymine and guanine bases **4** was prepared simply by utilizing the N^2-isobutyryl guanine analog of **6** in step a, **Scheme 1** and substituting 1-trifluoroacetyl imidazole for $(CF_3CO)_2O$-Et_3N in step b. With this heterodimer, it was found that $(CF_3CO)_2O$-Et_3N produced a considerable amount of depurination whereas 1-trifluoroacetyl imidazole gave the desired product in consistently high yield without any detectable depurination.

Phosphoramidites **11** and **17** were incorporated into an 11-mer polythymidine sequence. The coupling yields with **11** and **17** were consistently in the range of 96-99% in automated DNA synthesis as determined by DMT assay. The polythymidine strands were treated with ammonia at 60°C for 6 h and deprotection of trifluoroacetamide was found to be quantitative. The oligo sequences were analyzed by negative FAB-MS spectra and the expected deprotonated molecular ions $(MW\text{-}1)^-$ were observed.

The effect of the novel internucleoside linkages on duplex stability with single strand DNA (ssDNA) has been investigated through thermal melting (T_m) studies. Initially polythymidine strands containing a single incorporation of the modified dimers **2** and **3** were chosen to probe the effect of these dimers on duplex stability since changes observed in T_m with poly T/deoxyribo-poly A sequences are expected to be more pronounced than in duplexes containing mixed base sequences.[8]

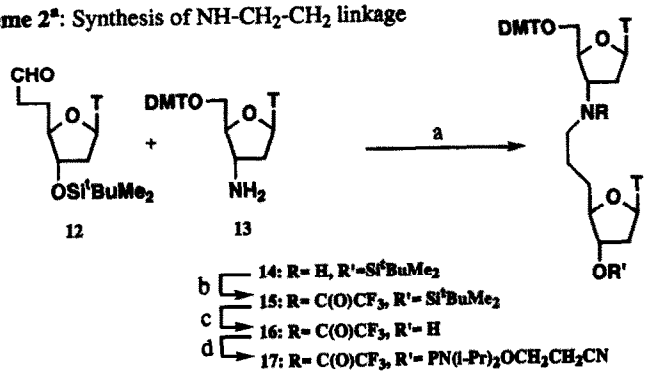
Thermal melting curves of strands **19-22** with a deoxyribo-A_{11} sequence showed a characteristic single sigmoid transition. The shape of the curves coupled with the observed net hypochromicity suggest only the formation of duplex without significant perturbation.

Scheme 1[a]: Synthesis of CH_2-CH_2-NH linkage



[a]Reagents and conditions: (a) $Na(OAc)_3BH$, $ClCH_2CH_2Cl$, 25°C, 85%; (b) $(CF_3CO)_2O$-Et_3N, CH_2Cl_2, 92%; (c) nBu_4NF, THF, 25°C, 90%; (d) DMTCl, Et_3N, pyr., DMAP, 86%; (e) $EtN(i\text{-}Pr)_2$, 2-cyanoethyl N,N-diisopropyl-chlorophosphoramidite, CH_2Cl_2, 25°C, 75%.

Scheme 2[a]: Synthesis of NH-CH_2-CH_2 linkage



[a]Reagents and conditions: (a) $Na(OAc)_3BH$, $ClCH_2CH_2Cl$, 25°C, 82%; (b) $(CF_3CO)_2O$-Et_3N, CH_2Cl_2, 90%; (c) nBu_4NF, THF, (d) $EtN(i\text{-}Pr)_2$, 2-cyanoethyl N,N-diisopropylchlorophosphoramidite, CH_2Cl_2, 25°C, 75%.

Table 1: Melting Temperatures (T_m)[a]

Oligomer	T_m (°C)	ΔT_m (°C)
18: d-TpTpTpTpTpTpTpTpTpTpT	32.5	
19: d-**T-CH2-CH2-NH-T**pTpTpTpTpTpTpTpTpT	29.0	-3.5
20: d-TpTpTpTpTpTpTpTpTpT**-CH2-CH2-NH-T**pT	27.1	-5.4
21: d-**T-NH-CH2-CH2-T**pTpTpTpTpTpTpTpTpT	30.6	-1.9
22: d-TpTpTpTpTpTpTpTpTpT**-NH-CH2-CH2-T**pT	28.0	-4.5
23: d-GpGpGpTpGpTpGpTpGpTpTpApGpCpGpGpG	68.0	
24: d-GpGpGpTpGpTpGpTpGpT**-CH2-CH2-NH-T**pApGpCpGpGpG	64.3	-3.7
25: d-GpGpGpTpGpTpGpTpGpT**-NH-CH2-CH2-T**pApGpCpGpGpG	64.8	-3.2

(a) Melting Temperatures (T_m) measured at 5.0 μM oligomer concentration containing 100 mM NaCl, 10 mM Na_2HPO_4 (pH= 7.0) and 0.1 mM EDTA. Melting curves were recorded in steps of 0.5 °C/min.

Oligonucleosides **19** and **21** showed a drop of 3.5° and 1.9°C respectively in T_m when the modifications **2** and **3** were incorporated at the 5'-end. Oligonucleosides **20** and **22** showed a more pronounced drop of 5.4° and 4.5° when the modifications were incorporated at the 3'- end. Next, the effect of modifications **2** and **3** on duplex stability when incorporated at the middle of a mixed base sequence was investigated. Oligonucleosides **24** and **25** showed a drop of 3.7° and 3.2°C in T_m respectively with a typical sigmoid transition. These results are consistent with the formation of duplex structure since a single mismatch in a duplex of comparable length is expected to show a drop of 5-8°C in T_m.[9]

Table 2 offers a comparison of the effects of incorporation of dimers **2** ,**3** and other backbone modified dimers reported in the literature on duplex stability with ssDNA. Although no direct comparison is possible because of variation in the component bases of the dimers and variation in the sequences in which the dimers are incorporated, a general trend can be established. Oligonucleosides containing linkage **3** showed less perturbation on duplex stability than linkage **2**. ΔT_m resulting from incorporation of linkage **3**, the formacetal and the sulfamate linkages is approximately -1.5 to -3.0°C with ssDNA.

Table 2: Effect of selected achiral modifications on duplex stability with ssDNA

Linkage	ΔT_m in ^oC (ssDNA) per incorporation
T-NH-CH_2-CH_2-T	-1.9 to -4.5
T-CH_2-CH_2-NH-T	-3.8 to -5.4
T-O-CH_2-O-T	-3.0[10]
G-O-SO_2-NH-A	-1.5[2g]
T-O-C(O)-NH-CH_2-T	No duplex formed[11]

Nuclease stability of oligonucleosides **18-22** was evaluated in 1% fetal bovine serum-containing media (RPMI 1640) at 37^oC with time. Oligonucleosides were purified from the serum by extraction and analyzed by HPLC using an anion exchange column. Results were analyzed for peak retention times and areas.

Table 3: Nuclease Stability with 3'-exo nuclease

Oligonucleoside	$T_{1/2}$ in min
18: d-$T_pT_pT_pT_pT_pT_pT_pT_pT_pT$	3
19: d-**T-CH_2-CH_2-NH-**$T_pT_pT_pT_pT_pT_pT_pT_pT$	3
20: d-$T_pT_pT_pT_pT_pT_pT_pT_pT$**-$CH_2$-$CH_2$-NH-**$T_pT$	4
21: d-**T-NH-CH_2-CH_2-**$T_pT_pT_pT_pT_pT_pT_pT_pT_pT$	3
22: d-$T_pT_pT_pT_pT_pT_pT_pT_pT$**-NH-$CH_2$-$CH_2$-**$T_pT$	111

Oligonucleoside **18,** having a half-life of 3 minutes, was the standard by which other oligonucleosides were compared in terms of exonuclease stability. HPLC analysis of oligonucleoside strand **22** showed a rapid cleavage of the 3'-terminal phosphodiester bond. The remaining oligonucleoside had a half-life of 111 min. thus, indicating enhanced resistance of the modified linkage to the 3'-exonuclease known to be present in fetal bovine serum. Oligonucleoside **20** also showed rapid hydrolysis of the 3'-terminal phosphodiester bond, without the remainder of the molecule retaining resistance to the enzyme. Oligonucleosides **19** and **21** were also investigated for 3'- exonuclease stability. Not unexpectedly, these oligonucleosides did not show any enhanced nuclease resistance.

In conclusion, an efficient and practical syntheses of internucleoside linkages **2** and **3** were demonstrated. These modified dimers were efficiently incorporated into oligonucleoside sequences by automated synthesis. Trifluoroacetamide protecting group was introduced to automated DNA synthesis. Oligonucleosides containing these dimers hybridized to the ssDNA with ΔTm's corresponding to other linkages reported in the literature. Incorporation of these dimers showed enhancement of nuclease resistance, however, as observed with methyl phosphonates a single dimer incorporation may not be sufficient for greatly enhanced nuclease resistance.

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