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Modified Oligonucleotides. Effect of 4 vs 5-atom Chimeric Internucleoside Linkages on Duplex Stability.

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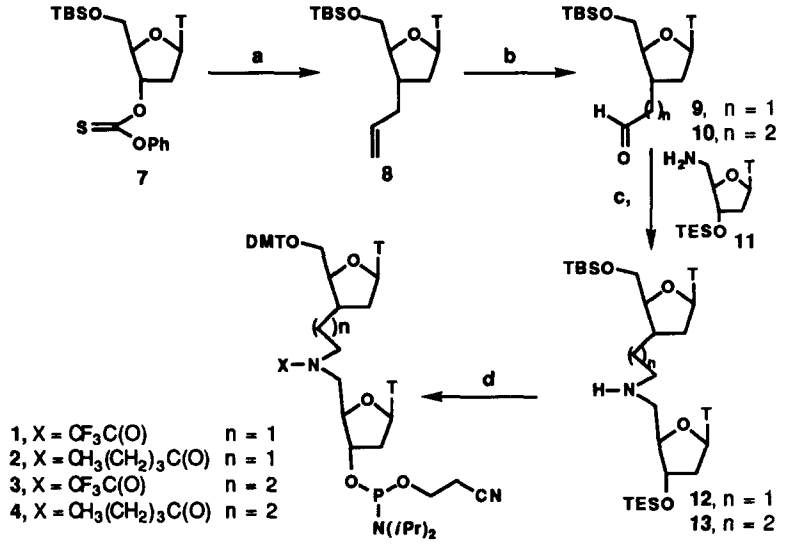
Abstract: The question whether a four or a five atom non phosphorus based internucleoside link leads to stronger binding of the resulting chimeric oligonucleotides to complementary sequences has been examined by comparing melting temperatures of relevant duplexes.

Inhibition of gene expression by antisense oligonucleotides has gained much attention as a promising drug design concept since it was first introduced by Zamecnik *et al.* in 1978.[1,2] Natural phosphodiester oligonucleotides suffer from serious problems such as poor nuclease resistance and low cellular uptake. Since 1978, some 2,000 publications have focused on problems related to various aspects of the antisense approach such as improvement of nuclease resistance and cellular uptake. Much research has been focused on modification at the phosphorus atom, to make phosphodiester analogs such as phosphorothioates, phosphoramidates, and methylphosphonates.[2a] Many of these phosphorus modifications,[3] however, generate mixtures of 2^n diastereomers, where *n* is the number of modified linkages in a given oligonucleotide sequence. Another approach, which avoids this problem, involves replacement of the normal phosphodiester linkage by neutral or cationic, achiral, *non-phosphorus* connectors. A variety of linkages have been examined in that context: amide,[4] amine,[5] formacetal and formthioacetal,[6] methylhydroxylamine,[7] and peptide nucleic acid (PNA).[8]

We were struck by the fact that all of the binding data on non phosphorus internucleotide tethers involves oligonucleotides in which 4-atom connectors have been used to replace the natural 4-atom phosphate ones. In fact, because P-O bonds are longer than C-O, C-N, or C-C bonds, it is not a priori obvious *whether there is any particular merit to the universally used 4- rather than a five atom replacement.* To answer this question, we chose to examine dimers **1-6,** which include the previously reported **1** and **5,** which were then used to construct oligodeoxythymidine 10-mers in which one of the natural phosphodiester linkage is replaced by either a 4- or a 5-atom tether embodying a (previously unreported) secondary amine, as well as the corresponding N-valeryl derivatives. The melting temperatures (T_m) of the duplexes formed between the variously modified 10-mers with Poly(dA) and Poly(A) were then compared.

The preparation of dimers **1-6** all started from the known 3'-allyl-5'-*tert*-butyldimethylsilyl-2'-deoxy thymidine **8**,[9] made from **7** by trapping the radical intermediate in the Barton deoxygenation with allyltributyltin, a reaction we were able to improve so that it could be done on an eight gram scale[10].

Dihydroxylation of **8** with the standard catalytic OsO_4/N-methylmorpholine oxide procedure, followed by treatment with sodium periodate, then gave the two carbon aldehyde **9** in good yield. Reductive amination of the aldehyde **9** with amine **11**, by reaction with sodium cyanoborohydride at pH 6 (using bromcresol green as indicator)[11] in methanol gave dimer **12** in 64% yield. From this dimer, acylation of the secondary amine, removal of the silyl protecting groups, dimethoxytrityl protection of the primary hydroxyl, followed by standard phosphoramidation[12] then gave dimers **1** or **2**, depending on the acylating reagent.

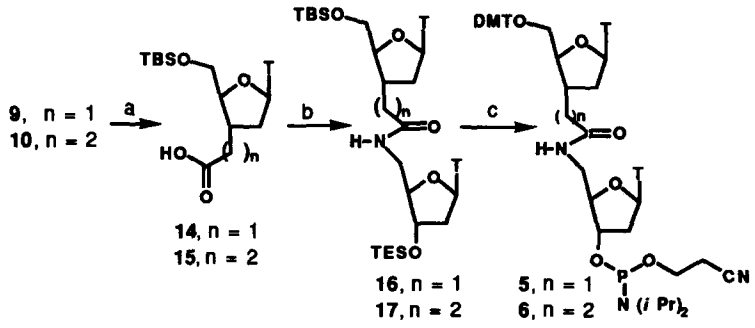


Reagents and conditions:
a. 6.0 eq Allyltributyltin, 0.4 eq. AIBN, toluene, 80 °C, 4-6 , 84%. **b.** For **12**: (i) 0.02 eq OsO_4, 1.5 eq NMO, acetone-H_2O (4:1), RT, 94 %; (ii) 1.2 eq $NaIO_4$, THF-H_2O (3:1), RT, 88 %. For **13**: (i) BH_3, THF, 0 °C, 64 %. (ii) 1.5 eq Dess-Martin periodinane, CH_2Cl_2, 88 %. **c.** 1.2 eq amine **11**, 2 eq $NaCNBH_3$, MeOH, 3A M.S., pH 6, 65 %. **d.** For **1** and **3**: (i) $(CF_3CO)_2O$, Et_3N, CH_2Cl_2, 0 °C, 84-88%. (ii) TBAF, THF, RT. (iii) DMTCl, DMAP, Et_3N, Pyr., 85-94% for 2 steps. (iv) 1.2 eq (i-Pr)$_2$NP(Cl)OCH_2CH_2CN, i-Pt_2NEt, CH_2Cl_2, 85%. For **2** and **4**: (i) valeric anhydride, Et_3N, CH_2Cl_2, RT. (ii), (iii) as above, 68-77% for 3 steps. (iv) as above, 85%.

The same 3'-deoxy-3'-allyl intermediate **8** was used in the construction of the related 5-atom linked dimers, starting with hydroboration (64%), followed by Dess-Martin periodinane oxidation[13] to the three carbon aldehyde **10**. Dimers **3** and **4** were then prepared as for **1** and **2**.

The linkers in dimers **1** and **3** become secondary amines when the trifluoroacetyl group is removed after incorporation in a modified oligonucleotide. Dimers **2** and **4** have a neutral amide linkage in which the amide carbonyl is *external* to the linkage. For comparison with amide linkers in

which the amide carbonyl is part of the link, dimers **5** and **6**[4] were also prepared. Aldehydes **9** (n =1) and **10** (n = 2) were oxidized, in quantitative yield, to the corresponding carboxylic acids **14** (n = 1) and **15** (n = 2) with sodium chlorite, using 2-methyl-2-butene as HOCl scavenger[14]. Carboxylic acids **14** (n = 1) and **15** (n = 2) were coupled[15] with amine **11** to give dimers **16** (n = 1, 65%) and **17** (n = 2, 77%). Desilylation and selective dimethoxytritylation, followed by standard phosphoramidation gave the desired dimers **5** (n = 1) and **6** (n = 2).



Reagents and conditions:
a. 6.0 eq $NaClO_2$, 4.0 eq KH_2PO_4, 10 eq $(CH_3)_2C{=}CHCH_3$, t-BuOH-H_2O (2:1), quantitative;
b. 0.5 eq N-hydroxybenzotriazole, 1.1 eq TBTU, 1.1 eq N-methylmorpholine, CH_3CN, rt, 30 min, then 1.2 eq amine **11**, 1.5 eq N-methylmorpholine, CH_3CN, rt, 5 min (**16**, 65%, **17**, 77%);
c. (1) TBAF, THF, rt, (2) DMTCl, DMAP, Et_3N, Pyr (over the two steps, 63% from **16** and 80% from **17**); (3) 1.2 eq $(i\text{-}Pr)_2NP(Cl)OCH_2CH_2CN$, $(i\text{-}Pr)_2NEt$, CH_2Cl_2, 87%.

Dimers **1** to **6** were each inserted in the center of a 10-mer $\overline{TTTTT}_L\overline{TTTTT}$ by the usual automated oligonucleotide synthesis procedure and the denaturation temperatures of the duplexes formed by each modified 10-mer with poly(dA), as well as with poly(A), were measured (Table).

Table : Melting temperature(T_m) of duplexes*

EXP	Linker structure in $\overline{TTTTT}_L\overline{TTTTT}$	Poly(dA)		Poly(A)	
	3' 5'	T_m, °C	ΔT_m, °C	T_m, °C	ΔT_m, °C
1	$-OPO_2^-O-$	29.3	-	26.4	-
2	$-CH_2CH_2NH-$	19.5	-9.8	13.3	-13.1
3	$-CH_2CH_2CH_2NH-$	20.2	-9.1	17.0	-9.4
4	$-CH_2CH_2N(ac)$#	23.2	-6.1	18.8	-7.6
5	$-CH_2CH_2CH_2N(ac)-$	19.3	-10.0	17.8	-8.6
6	$-CH_2C(O)NH-$	26.5	-2.8	24.4	-2.0
7	$-CH_2CH_2C(O)NH-$	25.3	-4.0	22.3	-4.1

*The thermal dissociation experiments were done in 10mM sodium phosphate buffer, 0.15 M NaCl, pH = 7.0. #ac = valeryl.

Although *all* non-phosphorus links tested here result in decreased binding to a complementary native sequence, our results show that, with 2 of the 3 connectors we examined, a 4 atom link resulted in better binding than a 5. It is, however, intriguing that the 5-atom *secondary amine* connector (entries 2 and 3) appears less unfavorable than the corresponding 4 atom one.

Acknowledgments

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