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Replacement of the Phosphodiester Linkage in Oligonucleotides by an Amide: Effect of Backbone Length on Duplex Stability with RNA Complement

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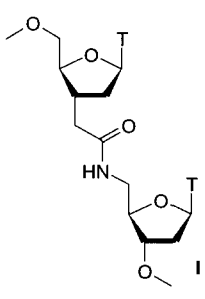
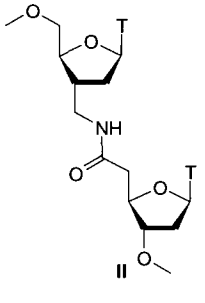
Abstract: Five dimers containing amide linkages instead of the natural phosphodiester linkage were synthesized and incorporated into oligonucleotides. The length of the amide backbone was varied. The hybridization properties of the modified oligonucleotides with RNA complements and their conformational analysis are described and compared to previously reported amide containing oligonucleotides. In addition, the synthesis of a thioamide phosphoramidite dimer is reported.

Recently we reported replacement of the phosphodiester linkage in oligonucleotides for their use in antisense strategy with a number of amide isomers.[1] We disclose the synthesis and hybridization properties of oligonucleotides containing new amide dimers with shorter and longer backbones compared to **I** and **II**.[2]



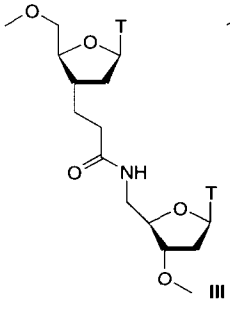
I



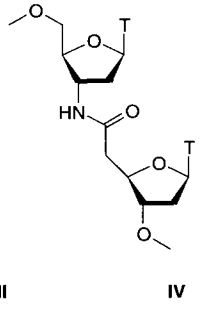
II

In addition we report the synthesis of thioamide modification **VII**. Amide replacement by thioamides have been extensively studied in peptides.[3] While the overall geometry of the thioamide is similar to the one of the amide (the Z-conformation is preferred over the E-conformation),[4] several differences are noticeable.

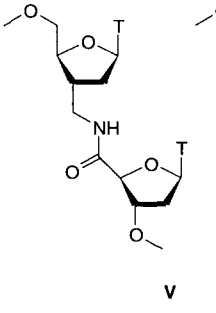
The longer C=S bond as well as the larger Van der Waals radius of the sulfur atom do restrict the rotational freedom of the neighboring torsional angles. The NHC(S)-proton (pK = 11-13) is more acidic than the amide-proton,[5] influencing hydrogen bonding interactions and solvation. For those reasons, thioamide containing modification **VII** appeared to be an attractive analog, however, predictions whether **VII** would show improved binding properties towards RNA could not be made.



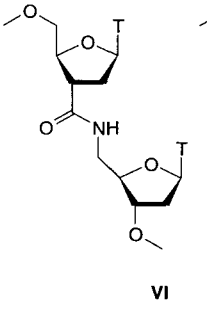
III



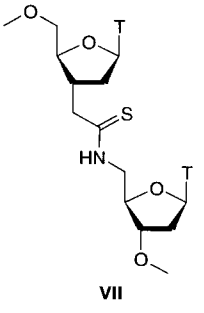
IV



V



VI



VII

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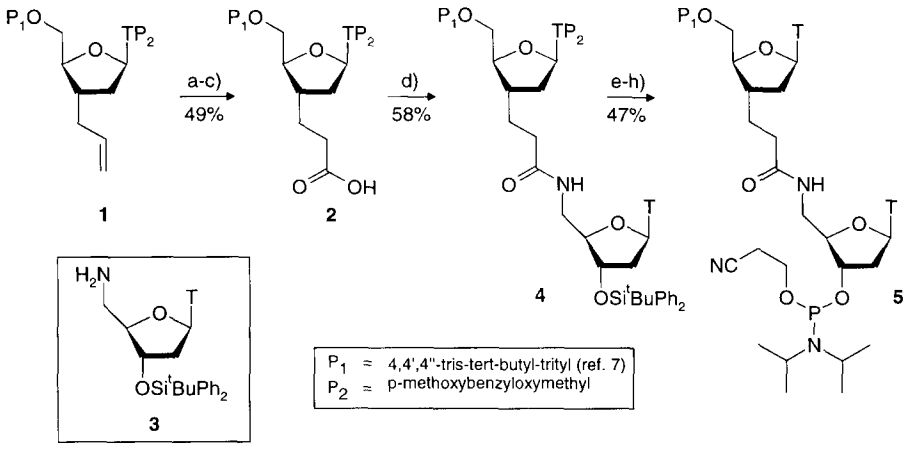
The synthesis of dimers **III-VI** is outlined in Schemes 1 and 2 and follows the general strategy that we developed for the synthesis of dimers **I** and **II**.[1] The amines and carboxylic acids have been

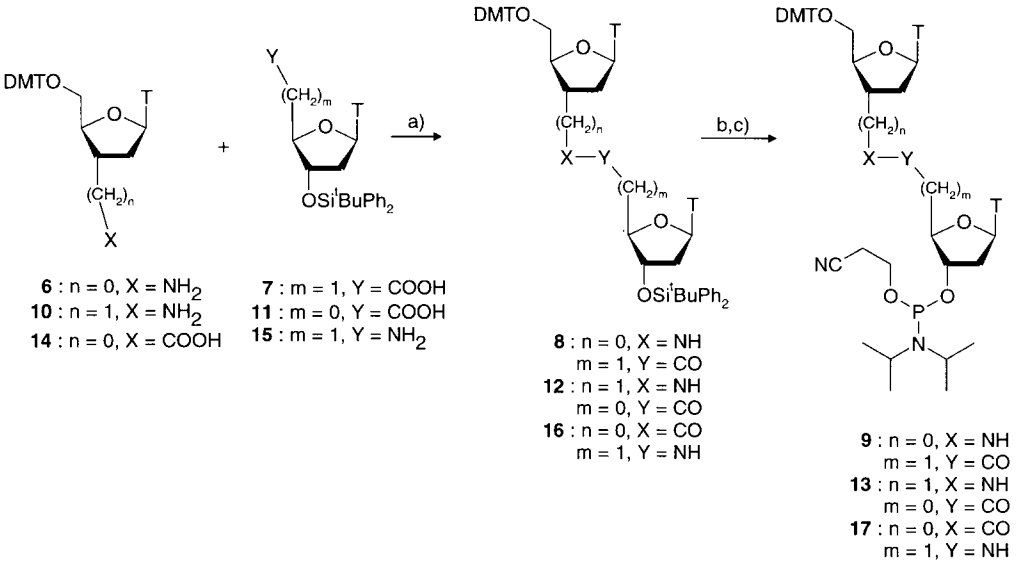


Scheme 1. a) 1.5 eq. 9-BBN, THF, -50°C, 2.5h; 1.2 eq. NaOH, 3.5 eq. H_2O_2 (30%), EtOH, 50°C, 1h. b) 2 eq. $(COCl)_2$, 4 eq. DMSO, CH_2Cl_2, -55°C, 0.5h, then 5 eq. Et_3N, -55°C → r.t.. c) 6 eq. $NaClO_2$, 4 eq. 2-methyl-2-butene, 4 eq. NaH_2PO_4, $tBuOH:H_2O$ (10:6), r.t., 0.3h. d) 1 eq. **2**, 1.1 eq. Et_3N, 1.1 eq. O(1H-benzotriazol-1yl) -N,N,N',N', tetramethyluronium-tetrafluoroborate, 0.5 eq. N-hydroxybenztriazole, CH_3CN, r.t., 1.5h, then add 1.5 eq. **3**, 1.5 eq. Et_3N, r.t., 3h. e) 10 eq. DDQ, $CH_2Cl_2:H_2O$ (20:1), r.t., 12h. f) 1.2 eq. TTTrCl, 1.2 eq. Et_3N, pyridine, r.t., 2 d. g) 1.5 eq. nBu_4NF, 1.5 eq. AcOH, r.t., 1.25h. h) 3.0 eq. $((iPr_2)N)_2POCH_2CH_2CN$, 5 eq. $(iPr)_2NH_2^+$tetrazole$^-$, CH_2Cl_2, r.t., 1.5h.

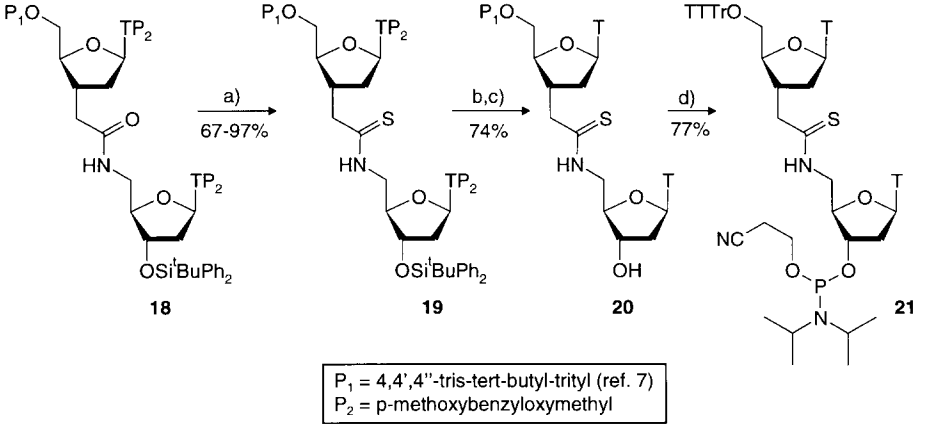
synthesized in our laboratories previously,[1,6] with the exception of compound **2** (Scheme 1). It was derived from hydroboration with oxidative workup of olefin **1**, without interference of the 5'- and base-protecting groups, followed by stepwise oxidation to the carboxylic acid. Amide coupling proceeded smoothly to give dimer **4**. During DDQ mediated oxidative deprotection reaction of the p-methoxybenzyloxymethyl group on the thymidine base, de-tris-tert-butyl-tritylation was observed. It was found to be advantageous to allow for complete removal of the tris-tert-butyl-trityl group, and reprotect the 5'-alcohol with TTTrCl.[7] Desilylation of **4** followed by formation of the phosphoramidite resulted in compound **5**. In a similar manner dimers **9**, **13** and **17** were synthesized (Scheme 2).

The synthesis of the thioamide **20** started with amide dimer **18**, having both thymidine protected with p-methoxybenzyloxymethoxy groups and which was synthesized as previously described.[1b] Conversion of the amide **18** to the thioamide **19** was accomplished through treatment with PCl_5 and Et_3N, resulting in the intermediate imino chloride, which was reacted with excess of Li_2S. Other conditions [$(CF_3CO)_2O$, $NaSH{\cdot}H_2O$, pyridine;[8] PCl_5, Et_3N, $NaSH{\cdot}H_2O$, CH_2Cl_2;[9] Lawesson reagent;[10] PCl_5/thiourea/$POCl_3$;[11]] were unsuccessful. Selective deprotection of both p-methoxybenzyloxymethoxy masked thymidines on **19** could be achieved without interference with the thioamide by successive treatment with DDQ and nBu$_4$NF, resulting in **20**. ROESY ^{1}H-NMR of **20** displayed no cross peak between H-6' and H-5" confirming a *trans* thioamide conformation.

The phosphoramidites **5**, **13**, and **17** were incorporated into oligonucleotides and purified on reverse phase HPLC (RPC-18, DMT on. Conditions: Buffer "A": Et_3NHOAc, pH 7; buffer "B": 50 mM Et_3NHOAc, pH 7, 70% CH_3CN. Gradient: 15-45% B in 55 min.).[12] Molecular weight of oligonucleotides was confirmed by MALDI-TOF MS[13] and the melting temperatures of the duplexes with their RNA complements were determined (4 μM each strand, 10 mM phosphate pH 7.0 (Na salts) 100 mM total Na^+, 0.1 mM EDTA) as summarized in Table 1.[1b] In contrast to the efficient incorporation of our amide dimers into oligonucleotides that we have always observed in previous cases, incorporation of phosphoramidites **9** and **21** were unsuccessful after several attempts.



Scheme 2. a) 1 eq. **7**, **11** or **14**, 1.1 eq. Et_3N, 1.1 eq. O(1H-benzotriazol-1yl) -N,N,N',N', tetramethyluronium-tetrafluoroborate, 0.5 eq. N-hydroxybenztriazole, CH_3CN, r.t., 1.5h, then add 1.5 eq. **6**, **10** or **15**, 1.5 eq. Et_3N, r.t., 6h. b) 1.1 eq. TBAF, r.t., 1.5h. c) 3.0 eq. $((iPr_2)N)_2POCH_2CH_2CN$, 5 eq. $(iPr)_2NH_2^+$tetrazole$^-$, CH_2Cl_2, r.t., 1.5h. **6**→**9**: a) 93%, b) 98%, c) 65%. **10**→**13**: a) 90%, b) 96%, c) 76%. **14**→**17**: a) 67%, b) 79%, c) 54%



Scheme 3. a) 1.5 eq. PCl_5, 5 eq. Et_3N, CH_2Cl_2, r.t., 3h, then Li_2S, r.t, 1h. b) 10 eq. DDQ, CH_2Cl_2:H_2O (20:1), r.t., 0.5h. c) 1.5 eq. nBu$_4$NF, THF, r.t., 1h. d) 3.0 eq. $((iPr_2)N)_2POCH_2CH_2CN$, 5 eq. $(iPr)_2NH_2^+$tetrazole$^-$, CH_2Cl_2, r.t., 5h.

Interestingly, the modifications **III**, **V** and **VI**, incorporated into oligonucleotides, exhibited lower melting points compared to the parent amide modifications **I** and **II** (modifications **I** and **II** exhibited average ΔT_m/mod (°C) of +0.4 and 0°C, respectively). It appears that the longer modification (**III**), having 5 atoms between the two sugar residues instead of 4 atoms, is more easily accommodated in the duplex structure compared to the modifications containing the shorter backbones (**V**, **VI**). Our results obtained using mixed sequence oligonucleotides are in qualitative agreement with the ones reported by Stork *et al.* for modification **III**.[2] However, these authors observed lower ΔT_m/mod. than us due to their use of a T_{10}-oligonucleotide having a single modification in the middle of the sequence. In our experience, the use of poly-dT oligonucleotides does not allow a quantitative comparison between various backbone modifications.

Table 1. ΔT_m/mod (°C) for the backbone modifications **III-IV**.

Sequence		III	V	VI
1) TTTt-t$(CT)_5$	RNA	-1.1	-1.8	-5.0
2) GCG$(t\text{-}t)_5$GCG	RNA	-2.6		

Molecular mechanics (MM) and dynamics (MD) studies have been carried out for compounds **III** and **VI**. Conformational analysis was performed on RNA·DNA octamer duplexes with one modified linkage between the middle residues of the DNA strand. MD trajectories were recorded on 14mer RNA·DNA duplexes with five alternating modified linkages in the DNA strand, using the respective lowest-energy conformers as starting structures. All computational procedures were identical to those used in all our work on modified backbones in oligonucleotides.[1,14]

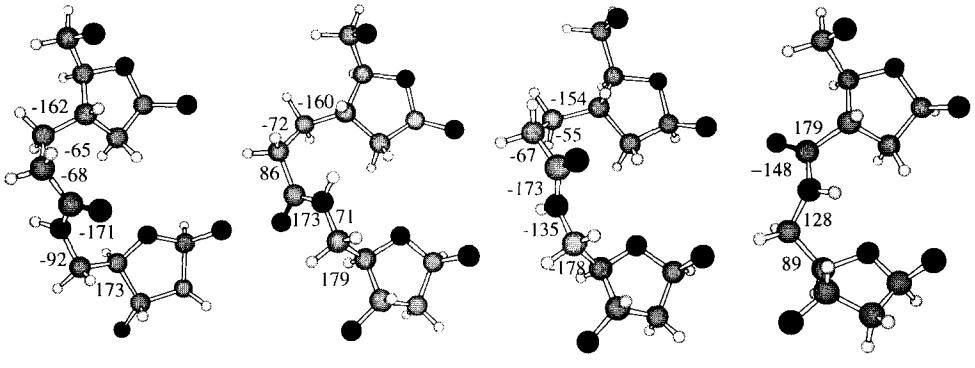


Figure 1. Left: three lowest-energy conformations for modification **III**; right: lowest energy conformation for modification **VI**. For clarity, only the N1 atoms of the bases are shown.

The additional $-CH_2-$ in **III** compared to **I** leads to a set of low-energy conformers in a very narrow range (11 distinct conformers within 3.5 kcal·mol^{-1}). The geometry of the three lowest minima (all within 0.2 kcal·mol^{-1}) is depicted in Figure 1. The left conformation was used as starting geometry for a 100 picoseconds MD trajectory, the results of which are depicted graphically in Figure 2. The average MD structure is strongly perturbed, with a number of base pairs broken. In contrast, for the amide modification **I**, the duplex was stable over the entire MD trajectory under identical conditions.[1b] However, no considerable strain is apparent in the structures of modification **III** shown in Figure 1. We conclude tentatively that the broad

range of low-energy conformations leads to an increase of conformational transitions in the duplex and that the motion (entropy) overcomes the base-pairing energy (enthalpy).

The conformational freedom of the short linkage in **VI** is restricted to 3 low-energy geometries within 4 kcal·mol^{-1}. In all three, the amide bond is strongly distorted, resulting in torsion angles deviating ca. 30° from planarity. A conformer with *cis* amide bond is found to have a planar peptide bond geometry (2°), but the resulting structure has a number of other unfavorable distortions and nonbonded interactions eventually leading to an energy of ca. 9 kcal·mol^{-1} higher than the lowest-energy conformer shown in Figure 1. The low melting point observed for RNA·DNA duplexes modified by this linkage can easily by tracked down to the highly unfavorable conformation this backbone has to adopt in order to allow for *Watson-Crick* base pairing. The average MD structure might qualitatively look more favorable than in the case of **III**, but the restricted conformational freedom does not allow the structure to escape from the initial lowest-energy, although strongly distorted, conformational domain. Still, the start of base-pair breaking can be observed in the MD average structure in Figure 2.

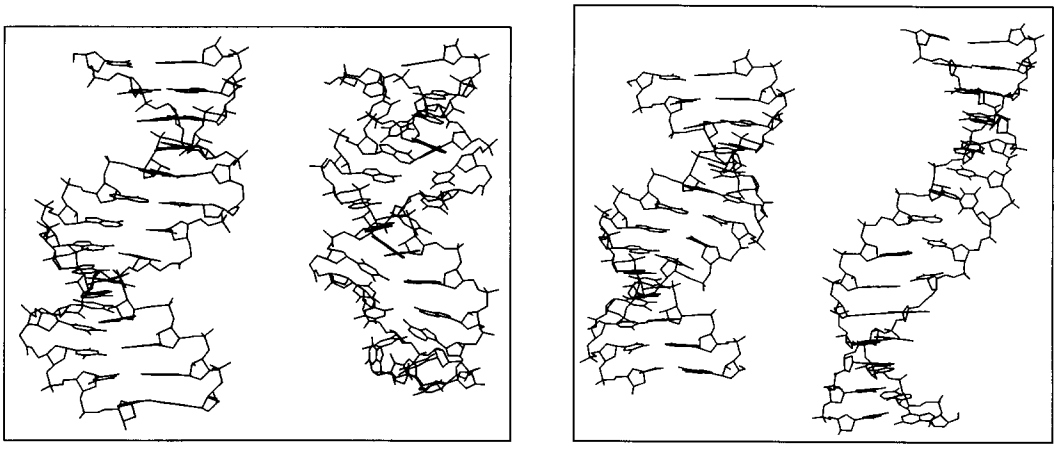


Figure 2. Molecular dynamics of **III** (A) and **VI** (B). The respective starting structures are shown at left in each frame, the MD average at right.

In conclusion, our molecular mechanics and molecular dynamics studies are in good agreement with the experimental data (T_m's) of the duplexes formed between an RNA and a modified 2'-deoxy oligonucleotide containing backbone modifications **III**, **V** and **VI**. These modifications having either an additional methylene group (**III**), or one methylene group less (**V**,**VI**) compared to the parent amide modifications **I** and **II** showed substantial duplex destabilization. The modification **VI** is the most destabilizing amide backbone we observed, so far. The increase for the thermodynamic stability of the duplexes observed for the amide modification **I** and **II** resulted from the restricted number of low energy conformations adopted by the backbone which were compatible with the overall helical structure. This favorable preorganization of the backbone cannot be realized by the corresponding longer or shorted backbones.

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