Amide-Modified Oligonucleotides with Preorganized Backbone and Furanose Rings: Highly Increased Thermodynamic Stability of the Duplexes Formed with their RNA and DNA Complements

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Abstract. The amide backbone modification C3'-CH_2-CONH-C5' has been further modified by introducing a methyl at C5', either in *R* or in *S* configuration. Only the *S* stereoisomer can adopt the required geometry to fit into a duplex with complementary RNA. Additional O-methyl groups at C2' of the furanose generate antisense oligonucleotides with considerably improved binding affinity to complementary RNA (ΔT_m up to 4.4 °C per modification).

Replacement of phosphodiester linkages by amide bonds leads to modified oligonucleotides displaying higher affinity for an RNA target and very good resistance towards nucleases.[1] We have shown that the parent amide modification **I** (Figure 1) could be further improved by the introduction of 2'-OMe substituents on both furanose rings.[2]

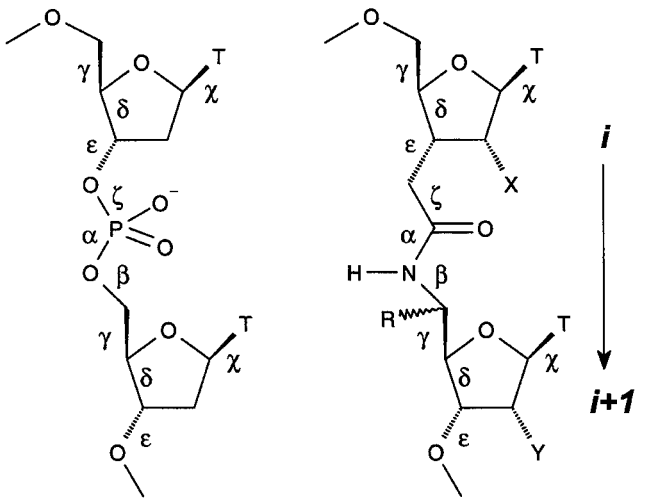
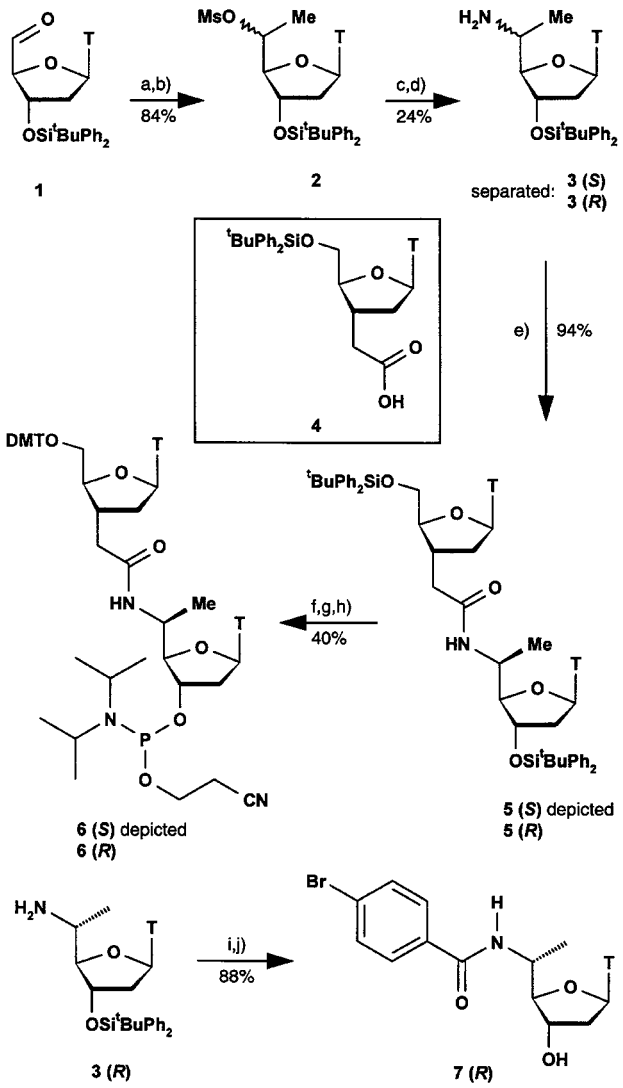


Figure 1. Amide modifications **I** → **V**

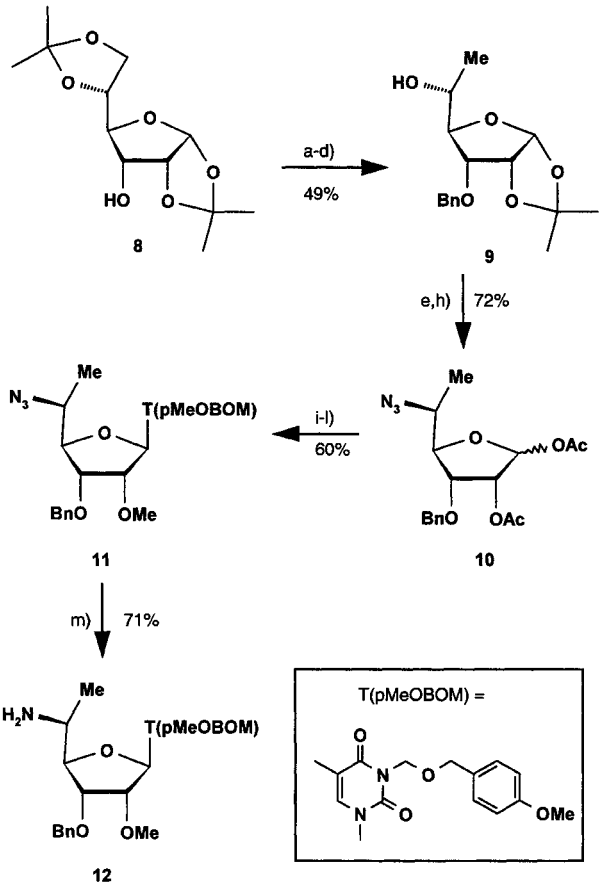
Our molecular modeling studies revealed that the amide backbone can adopt various low-energy conformations, the two lowest being separated by ca. 0.5 kcal·mol^{-1} and differing by the orientation of the plane of the amide bond.[3] We suggested that the conformational freedom of the backbone could be restricted by the introduction of a 5'-methyl (5'-Me) substituent. This increase in the preorganization of the backbone should translate into an increase in T_m of the corresponding duplex with RNA. However, we postulated that the 5'-Me group with the *S* configuration would be much more favorable compared to the 5'-(*R*)-Me substituent.[4] We report here that these hypotheses were fully validated experimentally.

The synthesis of the 5'-(*S*)-Me and 5'-(*R*)-Me amide dimers **6** are depicted in Scheme 1. Treatment of the aldehyde **1** with excess of methyl Grignard reagent led to a mixture of 5'-(*S*)-Me and 5'-(*R*)-Me derivatives.[4] The diastereomers could not be separated on a preparative scale by column chromatography prior to the stage of amine **3**. The absolute configuration at C5' was determined by X-ray diffraction of the crystalline derivative **7*R***.



Scheme 1. a) 2.05 eq. MeMgBr (3M, ether), THF, -78°C→RT, 3h. b) 1.4 eq. MsCl, pyridine, 0°C, 1.5h. c) 5 eq. NaN_3, DMF, 65°C, 5h. d) 2 eq. $SnCl_2.H_2O$, MeOH, RT, 16h; separation of **3(*S*)** and **3(*R*)**. e) 1 eq. **4**, 1.1 eq. O-(1H benzotriazol-1-yl)-N,N,N',N'-tetramethyl uronium tetrafluoroborate, 0.5 eq. hydroxybenzotriazole, 1.1 eq. Et_3N, MeCN, 0°C, 1h, then 1 eq. **3(*S*)** or **3(*R*)**, 1.1 eq. Et_3N, 0°C→ RT, 14h. f) 2.2 eq. nBu_4NF, THF, RT, 4h. g) 2.8 eq. DMTCl, pyridine, RT, 14h. h) 3 eq. $((iPr_2)N)_2POCH_2CH_2CN$, 5 eq. $(iPr)_2NH_2^+$tetrazole$^-$, CH_2Cl_2, RT, 12h. i) 1 eq. 4-bromobenzoic acid, 1.1 eq. O-(1H benzotriazol-1-yl)-N,N,N',N'-tetramethyl uronium tetrafluoroborate, 0.5 eq. hydroxy-benzotriazole, 1.1 eq. Et_3N, MeCN:THF (1:2), RT, 1h, then 1 eq. **3(*R*)**, 1.5 eq. Et_3N, 0 °C→ RT, 15h. j) 1.1 eq. nBu_4NF, THF, RT, 3h

Melting temperature measurements (T_m) confirmed our hypothesis that only the 5'-(*S*)-Me substituent had a positive effect on the thermodynamic stability of the duplexes with an RNA complement.

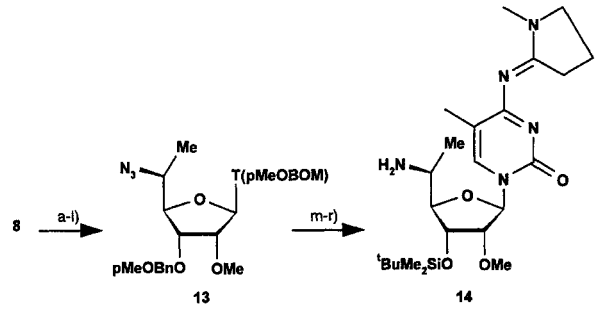


Scheme 2. a) 1.1 eq. NaH, 1.5 eq. BnBr, 0.05 eq. nBu_4NI, THF, 0→25°C. b) 90% AcOH, H_2O, 40 °C, 2.5h. c) 1.4 eq. TsCl, 2 eq. Et_3N, 2.4 eq. pyridine, 0.1 eq. DMAP, CH_2Cl_2, 0→25°C, 2.5h. d) 3 eq. NaI, 1.5 eq. nBu_3SnH, 0.1 eq. AIBN, DME, 80°C, 1.5h. e) 2.5 eq. TsCl, 3 eq. Et_3N, 0.1 eq. DMAP, pyridine, 70 °C, 3.5h. f) 2.0 eq. NaN_3, DMF, 80 °C, 1.5h. g) CF_3COOH, CH_2Cl_2, H_2O, RT, 16h. h) 5.0 eq. Ac_2O, pyridine. i) 1.3 eq. thymine, 2.2 eq. N,O-bis(trimethylsilyl)acetamide, 2.0 eq. TMSOTf, CH_3CN, 50°C, 3.5h. j) 1.8 eq. pMeOBOMCl, 2.0 eq. DBU, 0°C→ RT, 2h. k) 4 eq. NaOMe, MeOH, 0°C→ RT. l) 3 eq. NaH, 10 eq. MeI, THF, 0 °C, 1h. m) 1.2 eq. $SnCl_2 \cdot H_2O$, MeOH, 0°C→ RT, 16h

Consequently, we developed a stereoselective synthesis of the 5'-(*S*)-Me derivatives carrying an additional 2'-OMe substituent (Scheme 2). Alcohol **9** was obtained in high overall yield after 3'-O-benzylation, hydrolysis of the 5',6'-ketal, selective tosylation of the resulting 6'-primary alcohol and reduction. The tosylation of the secondary alcohol **9** was achieved at 70°C, followed by its substitution with NaN_3. The remaining 1',2'-ketal was hydrolyzed. Thymine was stereoselectively introduced.[5] The methylation of the 2'-OH function required the initial protection of the thymine with the p-methoxy-BOM group.

For the evaluation of our new backbone modification in mixed sequences, we needed not only the thymidine building block **12**, but also the 5-MeC analog **14**. The conversion of the azide **11** into the corresponding 5-MeC derivative **14** was successfully realized. Unfortunately, the cleavage of the 3'-O-benzyl group in the amide dimer by hydrogenolysis was always accompanied by substantial reduction of the C=C bond of the 5-MeC ring. Therefore, we had to repeat the synthesis of the 5-MeC building block starting from **8** using the p-MeO-benzyl group for the protection of the 3'-OH function (Scheme 3).

The modifications **IV** and **V** were introduced into oligonucleotides as T*T and T*MeC dimers using the phosphoroamidites **16**, **17** and **19**, **20**, (Scheme 4).[6]



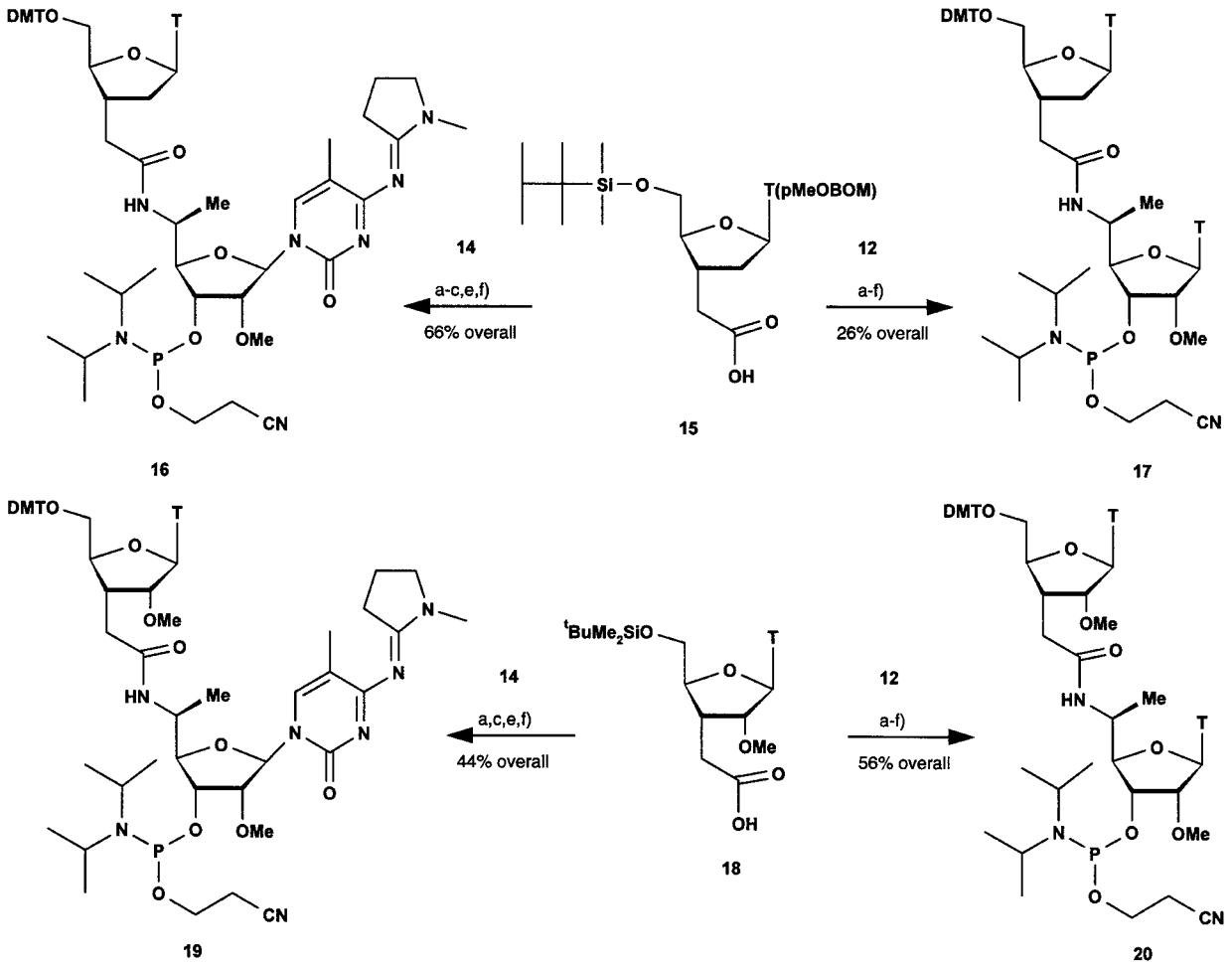
Scheme 3. a) 1.5 eq. NaH, 1.5 eq. pMeOBnCl, 0.05 eq. nBu_4NI, THF, RT 12h, 95%. For reaction conditions b-l) see Scheme 2: b) 89%. c) 82%. d) 80%. e) 80%. f) 88% g) 47%. h) 97%. i) 86%. j) 80% k) 94%. l) 99%. m) 2.4 eq. DDQ, CH_2Cl_2:H_2O (20:1), RT, 3h, 77%. n) 1.1 eq. $^tBuPh_2SiCl$, 1.5 eq. imidazole, CH_2Cl_2, RT, 12h, 86%. o) 22.5 eq. triazole, 23.5 eq. Et_3N, 2.5 eq. $POCl_3$, MeCN, 0→25°C, 3h. p) NH_3 (25% in H_2O), dioxane, 60°C, 3h, 81% over two steps. q) 5.0 eq. N-methylpyrrolidinone dimethylacetal, THF:pyridine (1:1), RT, 20h, 80%. r) H_2, Pd/C, MeOH, RT, 6h, 63%

The Table summarizes the melting temperatures of the duplexes formed between the modified oligonucleotides **II-V** and their RNA (sequences A-C) and DNA (sequence D) complements.[7] The 5'-(*S*)-Me substituent in **II** had a very positive effect on the thermal stability of the duplex with RNA: an increase in T_m of +1.0 to +1.4°C per modification **II** was observed. In the parent amide modification **I**, the average ΔT_m/mod. = +0.4°C was favorable, compared to the wild type (ΔT_m/mod. = 0). The increase in the thermodynamic stability obtained with the 5'-(*S*)-Me substituent is in agreement with our assumption that it would preorganize the backbone into the lowest energy conformation suitable for hybridization.

In contrast, the 5'-(*R*)-Me substituent strongly destabilized the duplex formed with an RNA complement. A decrease in T_m as large as -3.6 to -4.9°C per modification was observed for **III**. These results suggest that the 5'-(*R*)-Me substituent induces a less favorable conformation of the backbone which does not fit easily into the duplex structure.

Even more gratifying were the results obtained for the modifications **IV** and **V**, which combine the effect of 5'-(*S*)-Me substituent (backbone preorganization) with the conformationally restricted 2'-OMe-furanose rings (sugar preorganization). A very large increase in T_m was reached with modification **IV** (ΔT_m/mod. = +3.4 to +3.7°C) where the ***i+1*** sugar unit carried a 2'-OMe substituent. In this modification **IV**, both five membered rings adopted preferentially the C-3' endo puckering mode. Indeed, the ***i*** residue (even without a 2'-OMe substituent) has been found to prefer a puckering mode closer to the C3'-endo range.[3] For the corresponding modification without the 5'-(*S*)-Me substituent (equivalent to **IV** with R = H) an average ΔT_m/mod. of +2.2°C was obtained.[2] The present results confirmed the additivity of the substituent effects on the backbone and sugar conformations and, consequently, on the melting temperature of the duplexes. A similar trend was deduced for the modification **V** where the 2'-OMe substituents on both sugar residues definitely induce a C3'-endo puckering mode. The additivity of the substituent effects (5'-(*S*)-Me and 2'-OMe substituents) translated into a spectacular increase in T_m of +3.0 to +4.4°C per modification **V**. These values compare very favorably with the ones obtained for the parent modification without 5'-(*S*)-Me substituent (equivalent to **V** with R=H; ΔT_m/mod. = +2.6 to +3.0°C).[2]

The compatibility of the lowest energy conformation ***a*** (see modeling part) adopted by the amide backbone carrying a 5'-(*S*)-Me substituent with the C3'-endo puckering mode of both sugar residues results in this very high increase in thermodynamic stability of the duplex formed between these modified oligonucleotides and their RNA complement.



Scheme 4. a) 1 eq **15** or **18**, 1.1 eq. O-(1H benzotriazol-1-yl)-N,N,N',N'-tetramethyl uronium tetrafluoroborate, 0.5 eq. hydroxybenzotriazole, 1.1 eq. Et_3N, MeCN, RT, 1h, then 1 eq. **12** or **14**, 1.1 eq. Et_3N, RT, 16h. b) 3.1 eq. DDQ, CH_2Cl_2, H_2O, RT, 3h. c) 3 eq. nBu_4NF, THF, RT, 3h. d) H_2, Pd/C, MeOH, RT, 12h. e) 1.5 eq. DMTCl, 3.0 eq. Et_3N, pyridine, RT, 5h. f) 3eq. $((iPr_2)N)_2POCH_2CH_2CN$, 5 eq. (iPr) $_2NH_2^+$tetrazole$^-$, CH_2Cl_2, RT, 12h

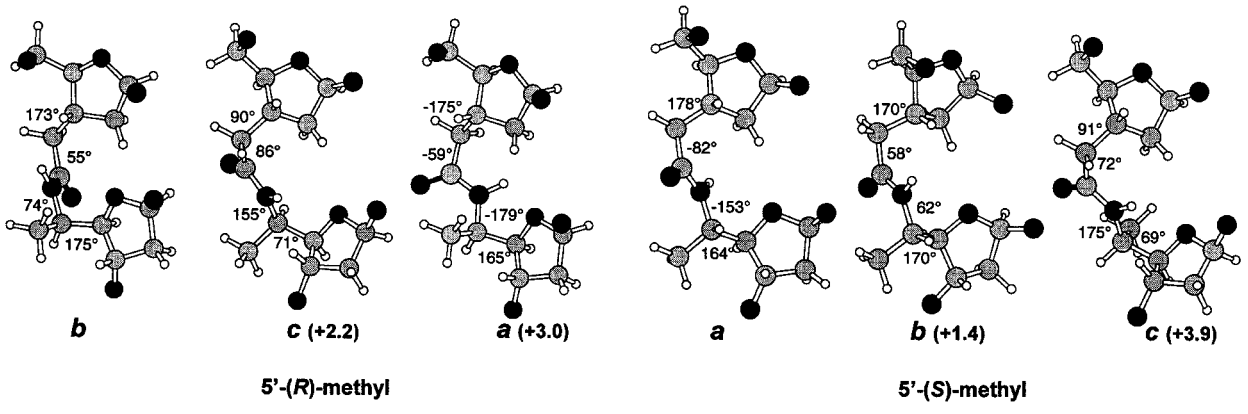


Figure 2. Three lowest-energy structures (from conformational analysis) of **III** (left) and **II** (right). The values in parentheses are energies in kcal·mol^{-1}, relative to the respective lowest-energy conformation

Consequently, this substitution pattern allowed us to drastically improve the affinity of our initial amide backbone modification **I** for its RNA target. Similar conclusions can be drawn for a DNA complement (sequence D).

Molecular mechanics (MM) and molecular dynamics (MD) investigations were carried out on all four modifications reported here using the procedures described before.[3,8,9]

For the backbone structures **II**, **IV**, and **V**, the computed low-energy conformations follow the order as in the original amide structure **I**.[3] For

Table. Melting temperatures (T_m and ΔT_m) for wild type and modified oligonucleotides [a)]

	Modified oligonucleotide [5'→3']	T_m [°C] Wild type	ΔT_m [°C] [b)] II	III	IV	V
A	TTTT*TCTCTCTCTCT	51.6	+1.0	-3.6	+3.4	+3.0
B	T*TT*TT*MeCT*MeCT*MeCT*MeCT*MeCT	56.9			+3.7	
C	GCGT*TT*TT*TT*TT*TGCG	50.2	+1.4	-4.9	+3.3	+4.4
D	GCGT*TT*TT*TT*TT*TGCG	54.1	+0.4	-5.6	+2.2	+2.4

[a)] A - C with RNA complement, D with DNA complement. [b)] ΔT_m = [T_m (modified oligonucleotide) - T_m (wild type)]/(number of modified dinucleosides)

the 5'-(*R*)-Me isomer **III**, the usual order of conformations for this type of amide backbone is perturbed (Figure 2, left three conformations). Conformer ***b*** is the lowest-energy structure, favored over ***c*** by >2 kcal·mol^{-1} while conformation ***a*** is higher by 3 kcal·mol^{-1}. The effects leading to this different order of low-energy conformations can be multiple. The computations reveal that the 5'-(*R*)-Me stereoisomer does not fit the scheme of the other modifications based on the amide structure **I**. Its clearly disastrous influence on the duplex stability is a further hint that the conformation ***a***, or at least the distribution of conformers, proposed for the amide **I** backbone modification is the preferred one.

The molecular dynamics simulations were carried out on 14*mer* hybrid duplexes d(CT12C). r(GA12G) in which the DNA strand had five standard phosphodiester linkages replaced by five amide backbone modifications: CTT*TT*TT*TT* TT*TTC. This corresponds closely to sequence C in the Table.

Globally, the observations during the MD simulations are in agreement with previously reported data for the amide **I** modification alone[3] and also for the effects of 2'-OMe substituents on ***i*** and ***i+1*** residues.[2] While none of the structures undergoes considerable fluctuations during the MD investigations, the overall rigidified geometry for **V** leads to remarkably stable MD trajectory with moderate oscillations around the starting geometry and a continuous perfect maintenance of all Watson-Crick base pairs (Figure 3).

Figure 3. Molecular dynamics structures of **V**. From left to right: refined MD starting geometry, geometry with time-averaged coordinates, final geometry after 100 ps of MD

In conclusion, we have disclosed here new backbone modifications displaying exceptionally high affinity for their RNA targets and strong resistance towards nucleases. They found, in our laboratories, very promising applications for the antisense strategy.

References and Notes

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