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Novel Polyamide Based Nucleic Acid Analogs - Synthesis of Oligomers and RNA-Binding Properties

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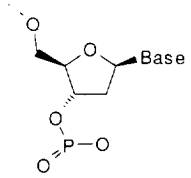
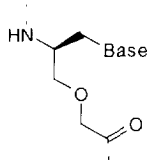
Abstract: *The synthesis of novel polyamide based nucleic acid analogs incorporating monomer units of type* ***I*** *-* ***III*** *has been accomplished using solid-phase strategies based on* N_{δ}*-Fmoc protected building blocks. An oligomer composed of monomer units* ***I*** *exhibited weak, but sequence-specific RNA binding. Improved RNA-binding affinity was observed for analogs incorporating building blocks of type* ***III*** *with 2-(R) stereochemistry, but not in the case of the 2-(S) isomers.* © 1997 Elsevier Science Ltd.

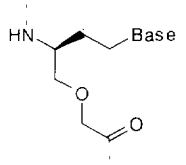
In the preceding paper[1] we have described the synthesis of novel nucleoamino acids, which can serve as building blocks for polyamide based nucleic acid analogs incorporating monomer units of type **I** - **III** (*Fig. 1*).



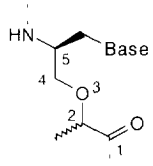
DNA



Base = thymine: **I-T**
Base = cytosine: **I-C**



Base = thymine: **II-T**
Base = cytosine: **II-C**



Base = thymine
2R, 5S: **III-T_R**
2S, 5S: **III-T_S**

Figure 1

These structures form part of a more comprehensive program, which ultimately aims at the identification of polyamide based oligonucleotide analogs that are capable of binding to complementary RNA in a sequence-specific fashion and with similar affinity as natural DNA. Following an iterative design approach, we have initially investigated the RNA-binding properties of oligomers based on relatively simple and flexible monomer units of type **I** and **II**, which according to model building should be able to form heteroduplexes with a complementary RNA strand in an A-type conformation.[2] In a second step we have also explored the effect of additional backbone substituents on the RNA-binding properties of analogs based on **I** by the incorporation of α-methylated monomer units of type **III**. Such methylated oligomers can be expected to exhibit significantly lower conformational flexibility than the corresponding parent compounds containing building blocks **I** exclusively[3] and provided that the presence of substituents α to the amide carbonyl groups does not otherwise interfere with RNA-binding this could lead to improved RNA-binding affinity.

In this communication, we report on the solid-phase synthesis as well as the RNA-binding properties of four different novel polyamide based oligonucleotide analogs **A** - **D** incorporating monomer units of type **I** - **III** (*Fig. 2*). In order to ensure sufficient water solubility for RNA-binding experiments a single lysine residue was attached to the C- as well as the N-terminus of the actual base sequence in all four oligomers.

H-Lys-**ttttctctctctct**-Lys-NH_2

A: **t = I-T, c = I-C**
B: **t = II-T, c = II-C**
C: **t = III-T_R, c = I-C**
D: **t = III-T_S, c = I-C**

Figure 2

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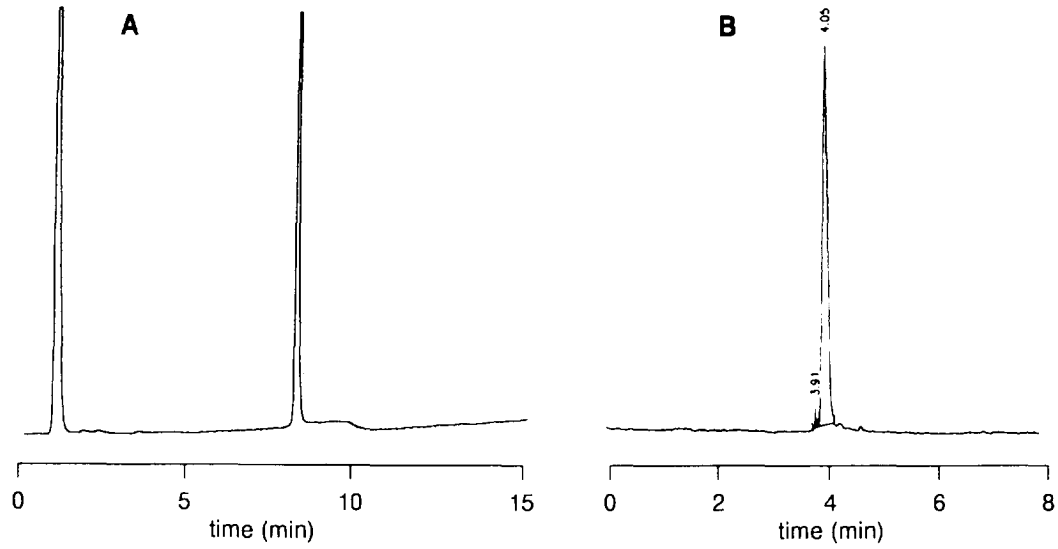

Figure 3. A) Analytical reversed-phase HPLC chromatogram of the purified oligomer **B** on a C_{18} Nucleosil column (250 x 4 mm; 5 μm; 300 Å): linear gradient over 15 min of MeCN-0.09% TFA and H_2O-0.1% TFA from 1:19 to 1:1, flow rate 1.0 ml/min, detection at 215 nm. **B**) Electropherogram of the purified oligomer **B**: running buffer 0.1 M phosphate buffer pH 2.5; detection at 191 nm.

Oligonucleotide analogs **A-D** were synthesized on a 4-(2',4'-dimethoxyphenyl-aminomethyl)-phenoxy resin (copoly(styrene-1% DVB); f≈ 0.24 mmol/g)[4] employing Nδ-9-fluorenylmethoxycarbonyl (Fmoc-) protected nucleoamino acid building blocks.[1] Allyloxycarbonyl (Alloc-) or benzyloxycarbonyl (Cbz-) groups were used to protect the N^4-amino function of cytosine derivatives of type **I** and **II**, respectively,[1] while lysine residues were incorporated into the growing polyamide chain as the Nα-Fmoc-Nε-*tert*-butoxycarbonyl derivative (Fmoc-Lys(BOC)-OH). Chain elongation was achieved by 2-(2-oxo-1(2H)-pyridyl))-1,1,3,3-tetramethyluronium tetrafluoroborate (TPTU)[5] mediated single couplings (2- or 3-fold excess of nucleoamino acid, 3.0 equiv. of TPTU) in the presence of diisopropylethylamine (3.3 equiv.) in N-methylpyrrolidin-2-one, followed by capping of unreacted amino groups with acetic anhydride (Ac_2O/DMA/pyridine 1:8:1).[6] The coupling time was set to 90 min, including 30 min at 40 °C. In general, coupling efficiencies were > 95%, as determined by recording the UV absorption of the fulvene-piperidine adduct formed upon removal of the $N_δ$-Fmoc protecting group (λ = 299.8 nm, ε = 7800 M^{-1} x cm^{-1})[7] with 20% piperidine/DMF. In order to ensure complete deprotection of the terminal amino group, on-line monitoring of the Fmoc-cleavage step proved to be of crucial importance, as cleavage rates varied as a function of sequence position. The 20% piperidine/DMF treatment interval was thus adjusted in each deprotection step according to the observed lability of the protecting group. Depending on the base protection strategy, two different protocols were used to accomplish cleavage of the completed oligonucleotide analog from the solid-support and side chain deprotection. When the allyloxycarbonyl group was used as cytosine protecting group (oligomers **A**, **C**, and **D**), the following protocol was employed: i) DCM (4 x 1 min); ii) $Pd(Ph_3P)_4$, trimethylsilylmorpholine, and trimethylsilylacetate in DCM (3 h; Ar atmosphere);[8] iii) DCM (4 x 1 min); iv) 0.05 M sodium diethyldithiocarbamate in DMF containing 0.5% of DIEA (4 x 1 min); v) DCM (4 x 0.5 min); vi) MeOH (4 x 0.5 min); vii) DCM (4 x 0.5 min); viii) cleavage from the solid-support with TFA/H_2O (95:5, v/v), 2 h at RT. Cbz-protecting groups (oligomer **B**) were removed in solution with TFMSA/TFA/DMS/m-cresol (1:10:6:2, v/v/v/v; 3 h at RT.)[9] after cleavage of the protected oligomer from the solid-support with TFA/H_2O (95:5, v/v; 3 x 30 min). The crude compounds were subjected to analysis and purification on standard diphenyl or C_{18} reversed-phase columns eluting with an acetonitrile-water gradient. The purity of the oligomers was verified by diphenyl or C_{18} reversed-phase analytical HPLC (see *Fig. 3* for

Table 1: Melting temperatures (T_m's) of complexes formed between oligonucleotide analogs **A** - **D** and complementary RNA.[a]

	Oligonucleotide or oligonucleotide analog[b]					
RNA complement	**A**	**B**	**C**	**D**	d[$(TC)_5T_5$]	d[$T_5(CT)_5$]
r[$(AG)_5A_5$]	15.2	~ 7.5[c]	23.2	~ 9.0[c]	52.4	-
r[$A_5(GA)_5$]	16.9	~ 6.4[c]	24.4	n. d.[d]	-	53.3
r[$(AG)_4ACA_5$][e]	~ 5[c]	n. d.[f]	10.0	n. d.[d]	-	40.9

[a]T_m's were determined in 10 mM phosphate buffer, pH 7, 100 mM Na^+, at 4 μM strand concentrations.[11] [b]*Cf. Fig. 1*. [c]Approximate values. [d]Not determined. [e]Antiparallel RNA complement containing a single mismatch.

oligomer **B**). In addition, the identity of the final products was assessed by mass spectral (matrix-assisted laser desorption ionization time-of-flight mass spectrometry, MALDI-TOF) analyses, which gave the expected molecular weights for all four oligonucleotide analogs.[10]

As illustrated by the UV-melting data summarized in *Table 1* oligomer **A** is capable of binding to complementary antiparallel[12] RNA (r[$(AG)_5A_5$]) in a cooperative and (Watson-Crick) sequence-specific fashion, with the resulting complex exhibiting a T_m-value of 15.2°. Binding of **A** to its parallel[12] RNA complement (r[$A_5(GA)_5$]) is also observed and occurs with comparable affinity, but it is not clear whether this process in fact involves formation of a parallel **A**/RNA duplex. The specific nature of the sequences investigated in this study even for complementary RNA with an overall *parallel*[11] orientation to **A** allows for the formation of a 11-base pair *antiparallel* duplex based on Watson-Crick base pairs between residues 5 to 11 of **A** and 5 to 11 of r[$A_5(GA)_5$].[13,14] Greatly reduced binding of **A** as well as **C** is observed to a (antiparallel) 15-mer oligoribonucleotide containing a single mismatched base (*Table 1*), thus confirming the sequence-specific nature of their interaction with r[$(AG)_5A_5$]. This latter finding clearly rules out the possibility that complex formation between **A**/**C** and their fully matched RNA complement is mainly the result of non-specific electrostatic interactions involving the positively charged terminal lysine residues and the negatively charged RNA backbone, respectively.[15] As indicated by a difference in melting temperature (ΔT_m) of 37.2° between the (antiparallel) **A**/RNA complex and the wild-type DNA/RNA duplex (T_m = 52.4°), the affinity of **A** for its complementary RNA is rather weak; however, the resulting ΔT_m-value of - 2.5°/modification should be compared to similar or even more negative values that have been reported for modified DNA/RNA heteroduplexes containing a variety of differently modified DNA strands, which are much more closely related to the structure of natural DNA than oligomer **A**.[16] As indicated by the reduced T_m-values of the **B**/RNA complexes and also by a lower cooperativity of the dissociation process, oligomer **B** binds to complementary RNA with significantly lower affinity than **A**. This difference may be ascribed to the increased flexibility of oligonucleotide analogs that are based on side chain homologated monomer units of type **II**, but additional parameters may also contribute to the reduction in *RNA*-binding affinity observed for oligomer **B**.[17]

Most interestingly, the replacement of thymine derived building blocks **I-T** (*Fig. 1*) in **A** by α-methylated derivatives **III-T_R** in **C** leads to a significant enhancement in RNA-binding affinity, as indicated by a ΔT_m-value of + 7.9° (or + 0.79°/modification) between **C** and **A** (*Table 1*).[18] Assuming this effect to be approximately additive and also to be independent of the nature of the bases involved, the complex between a *fully* α-methylated analog of **C** and its complementary RNA would be expected to exhibit a T_m of ≈ 28°, corresponding to a ΔT_m/modification value of only - 1.6° with respect to the wild-type DNA/RNA duplex. In contrast, substituting diastereoisomeric thymine derivative **III-T_S** for **I-T** in oligomer **A** (oligomer **D**) results in a substantial decrease in melting temperature for the **D**/RNA complex. This indicates that the presence of additional substituents α to the amide carbonyl in nucleic acid analogs of type **I** in the case of a *S*-configuration may sterically interfere with RNA binding.

In summary, we have achieved the synthesis of novel polyamide based oligonucleotide analogs incorporating four different types of nucleoamino acid building blocks. Based on the RNA-binding data obtained for oligomers **A** and **C** it would appear that monomer units of type **I** could serve as an appropriate template for the construction of DNA analogs exhibiting even more favorable RNA-binding properties than **C**. Studies along these lines are in progress.

Acknowledgements

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References and Notes

1. Altmann, K.-H.; Schmit-Chiesi, C.; Garcia-Echeverría, C.; preceding paper in this issue.
2. For a comprehensive discussion of nucleic acid structures see: W. Saenger, "*Principles of Nucleic Acid Structure*", Springer, New York 1984.
3. The presence of α-methyl groups in carboxylic acid derivatives can impose significant conformational constraints. It is, e. g. a well known fact that the conformational space accessible to an alanyl residue in polypeptides is substantially smaller than for a glycyl residue, which in large part is a consequence of unfavorable interactions between the α-methyl group and the adjacent carbonyl oxygen. Likewise, the presence of α-methyl groups in poly(lactic acid) leads to greatly reduced flexibility of this polymer: Flory, P. J. "Statistical Mechanics of Chain Molecules", Interscience, New York 1969.
4. Rink, H. *Tetrahedron Lett.* **1987**, *28*, 3787 - 3790. Syntheses were carried out on a semi-automated peptide synthesizer using 140 - 190 mg of resin.
5. Knorr, R.; Trzeciak, A.; Bannwarth, W.; Gillesen, D. *Tetrahedron Lett.* **1989**, *30*, 1927 - 1930.
6. Efficient incorporation of the *N*-terminal lysine residue in general required double-coupling.
7. Atherton, E.; Sheppard, R. C. In *Solid Phase Peptide Synthesis: A Practical Approach*; IRL Press, Oxford 1989.
8. Merzouk, A.; Guibé, F.; Loffet, A. *Tetrahedron Lett.* **1992**, *33*, 477 - 480. Formation of allylamine by-products was observed when $Pd(PPh_3)_4$ was used in a solution of $CHCl_3$ containing 5% acetic acid and 2.5% *N*-methyl-morpholine: Kates, S. A.; Daniels, S. B.; Albericio, F. *Anal. Biochem.* **1993**, *212*, 303 - 310.
9. Tam, J. P; Heath, W. F.; Merrifield, R. B. *J. Am. Chem. Soc.* **1986**, *108*, 5242 - 5251.
10. Experimentally determined molecular masses were within 0.1% of the expected values (positive or negative-ion mode): **A**, 3787.1 (calc. 3787.8); **B**, 3996.7 (calc. 3996.2); **C**, 3925.9 (calc. 3926.1); and **D**, 3926.9 (calc. 3926.1).
11. The terms "antiparallel" and "parallel" RNA complements refer to the Watson-Crick alignment of oligomers **A** - **D** in the N→C direction with the RNA in the 3'→5' ("antiparallel") and 5'→3' ("parallel") direction.
12. Concentrations of oligomers **A** - **D** were determined using the corresponding nucleotide extinction coefficients (ε_{260}) for thymine and cytosine derivatives, respectively (*cf.* Puglisi, J. D. *Methods Enzymol.* **1989**, *180*, 304 - 325). For further details regarding T_m measurements *cf.*: Lesnik, E. A.; Guinosso, C. J.; Kawasaki, A. M.; Sasmor, H.; Zounes, M.; Cummins, L. L.; Ecker, D. J.; Cook, P. D.; Freier, S. M. *Biochemistry* **1993**, *32*, 7832 - 7838.
13. One might argue that the similar stabilities of the complexes of **A** with $r[(AG)_5A_5]$ (15 Watson-Crick base pairs) and $r[A_5(GA)_5]$, respectively, render the existence of an 11-base pair duplex between **A** and its parallel RNA complement a rather unlikely possibility. However, it should be kept in mind that no significant stability difference exists between the duplexes of $d[T_5(CT)_5]$ with $r[(AG)_5A_5]$ and $r[A_5(GA)_5]$ (Tm-values of 52° and 48°, respectively).
14. Binding of **A** to antiparallel and parallel DNA was also observed, albeit with lower affinity and with less cooperative melting than for the **A**/RNA complexes (T_m-values = 9.4° for **A**/$d[(AG)_5A_5]$ and 11.4° for **A**/$d[A_5(AG)_5]$ *vs.* 42.8° and 43.2° for $d[T_5(CT)_5]$/$d[(AG)_5A_5]$ and $d[(CT)_5T_5]$/$d[A_5(AG)_5]$, respectively).
15. Likewise, no cooperative melting was observed upon heating a solution of single-stranded **A**.
16. For recent reviews *cf.*: a. De Mesmaeker, A.; Häner, R.; Martin, P.; Moser, H. E. *Acc. Chem. Res.* **1995**, *28*, 366 - 374. b. De Mesmaeker, A.; Altmann, K.-H.; Waldner, A.; Wendeborn, S. *Curr. Opinion Struct. Biol.* **1995**, *5*, 343 - 355.
17. The notion that parameters other than side chain flexibility may contribute to differences in RNA- and DNA-binding affinity between **A** and **B** is borne out by the fact that the complex of **B** with parallel DNA ($d[A_5(AG)_5]$) exhibits a T_m of 21.7° *vs.* only 11.9° for the corresponding **A**/DNA duplex. The affinity of **B** is significantly higher for its parallel than its antiparallel DNA complement (T_m = 21.7° vs. ~ 6.8°).
18. We have not determined the stoichiometry of the complexes of **A** and **C** with complementary RNA. As all melting curves were indicative of a single cooperative transition and no hysterisis was observed upon cooling, we assume that **A** as well as **C** bind to RNA with 1/1 stoichiometry. In contrast, the binding of an isosequential *PNA* to RNA is characterized by strong hysterisis in UV melting experiments (D. Hüsken, K.-H. Altmann, unpublished data). Nevertheless, we cannot exclude the possibility that **A** and/or **C** form 2/1 complexes with complementary RNA. However, in either case the data presented in this work demonstrate that binding occurs in a *defined* and *sequence specific* fashion.

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