Pergamon

Bioorganic & Medicinal Chemistry Letters 10 (2000) 929–933

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

Synthesis and Hybridization Properties of Polyamide Based Nucleic Acid Analogues Incorporating Pyrrolidine-derived Nucleoamino Acids

Karl-Heinz Altmann,[a,*] Dieter Hüsken,[c] Bernard Cuenoud[b] and Carlos García-Echeverría[a]

[a]*Novartis Pharma AG, TA Oncology Research, WKL-136.4.21, CH-4002 Basel, Switzerland*
[b]*Respiratory Disease TA, Horsham, UK*
[c]*Functional Genomics Area, Basel, Switzerland*

Received 4 January 2000; accepted 17 February 2000

Abstract—N_δ-Fmoc protected nucleoamino acids of type **I** (Base = T, C, A) have been synthesized and employed as building blocks for the construction of novel polyamide based nucleic acid analogues. Homopyrimidine oligomer **A** binds to complementary RNA with significant affinity and in a sequence-specific fashion, while no binding was observed to complementary DNA.

DNA analogues with modified backbone structures have attracted considerable attention as nuclease resistant antisense and antigene agents.[1,2] The majority of modified oligonucleotide analogues that have been investigated in this context are characterized by an *alternating* arrangement of natural phosphodiester and modified internucleoside linkages;[2] however, as illustrated by the discovery of peptide nucleic acids (PNA), which are entirely based on an aminoethyl glycyl backbone, even the complete replacement of the deoxyribose-phosphate backbone in natural oligodeoxyribonucleotides can lead to analogues with superior DNA- and RNA-binding properties.[3] A variety of other polyamide-type structures have subsequently been suggested to represent useful DNA surrogates for possible antisense applications,[4] some of which were reported to possess DNA- and RNA-binding affinities close to those of natural DNA or even PNA.[5]

As part of a comprehensive program directed at the identification of genuinely new (i.e., not PNA-derived) polyamide-based oligonucleotide analogues with RNA-binding affinities at least comparable to those of natural oligodeoxyribonucleotides, we have recently described the synthesis and RNA-binding properties of a series of homopyrimidine 15-mers that were composed of relatively flexible linear δ-amino acids (Fig. 1, **II**–**IV**).[6] As an extension of this previous study we have now investigated

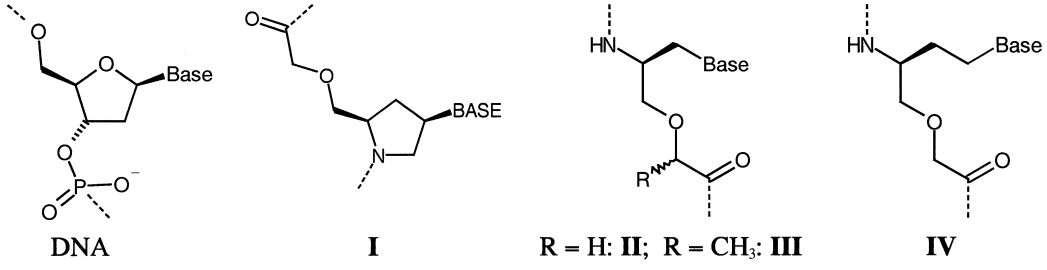


Figure 1.

*Corresponding author. Fax: +41-61-6966246; e-mail: karl-heinz.altmann@pharma.novartis.com

0960-894X/00/$ - see front matter
PII: S0960-894X(00)00121-9

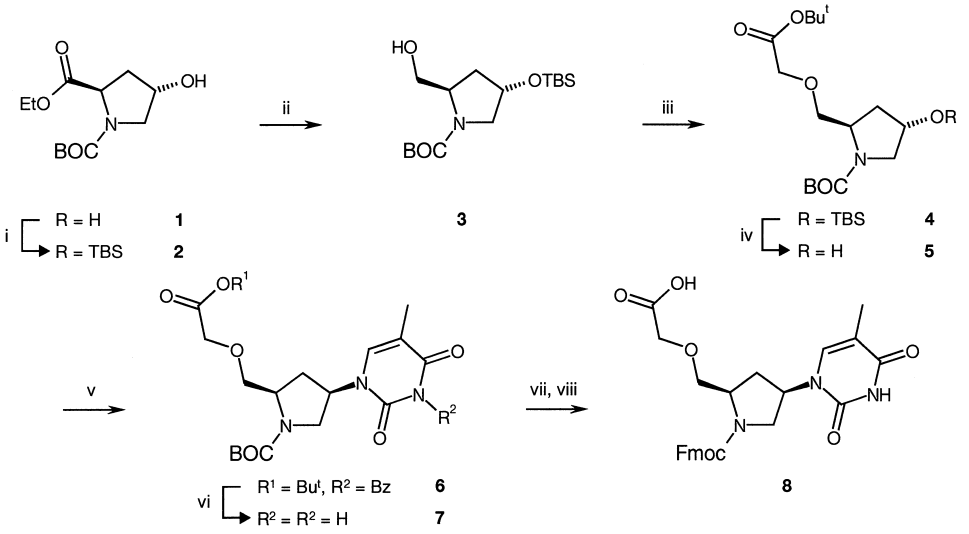
a conformationally more rigid type of polyamide-based DNA analogue incorporating pyrrolidine-derived monomer units **I** (Fig. 1).[7]

In this communication we want to report on the synthesis of protected nucleoamino acids[8] of type **I** as well as a corresponding homo-pyrimidine oligomer and provide some preliminary data on the RNA- and DNA-binding properties of this novel type of oligonucleotide analogue.

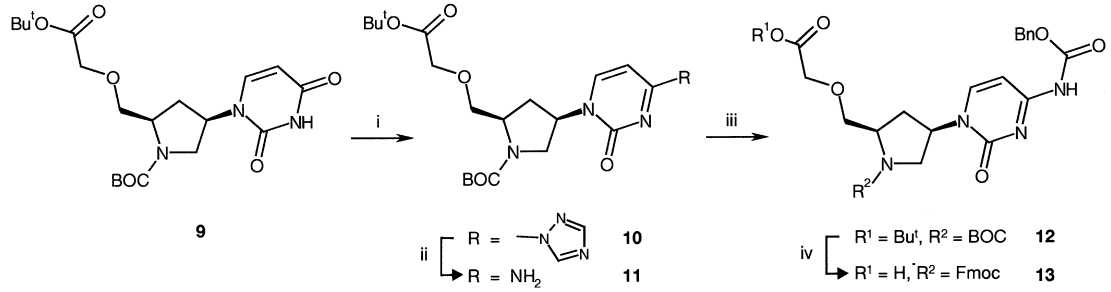
The synthesis of nucleoamino acids of type **I** was based on alcohol **5** as the central common intermediate, which can be obtained in a four step sequence from known D-hydroxyproline derivative **1**[9] in 69% overall yield (Scheme 1). The conversion of **5** into fully protected nucleoamino acids was achieved through alkylation of appropriately protected nucleic acid base derivatives (N^3-Bz-thymine or -uracil) or nucleic acid base precursors (6-chloropurine) under *Mitsunobu* conditions.[6a,10] These alkylation products were subsequently elaborated into N_δ-9-fluorenylmethoxycarbonyl-(Fmoc-)protected nucleoamino acids through base conversion (where required) followed by appropriate protecting group manipulations. In the case of fully protected thymine derivative **6** (Scheme 1) this involved simultaneous removal of the base protecting group and hydrolysis of the *tert*-butyl ester moiety by treatment with aqueous base, acid catalyzed cleavage of the N_δ-BOC protecting group, and finally reprotection of the free amino acid by reaction with Fmoc-OSu[11] to provide **8** in 35% overall yield (based on **5**).

N_δ-Fmoc-N^4-benzyloxycarbonyl-protected cytosine derivative **13** (Scheme 2) was prepared via uracil **9** (obtained from **5** and N^3-Bz-uracil followed by cleavage of the N^3-Bz protecting group with aq ammonia/dioxane in 51% overall yield).[12] Conversion of **9** into triazolide **10** followed by treatment of **10** with aq ammonia/dioxane[6a,13] provided cytosine derivative **11**, whose exocyclic amino function was protected with a benzyloxycarbonyl (Cbz) group by reaction with Cbz-OBt.[14] Concomitant cleavage of the BOC- and *tert*-butyl ester moieties and reprotection of the δ-amino group gave **13** in 18% overall yield for the five step sequence from **9**.

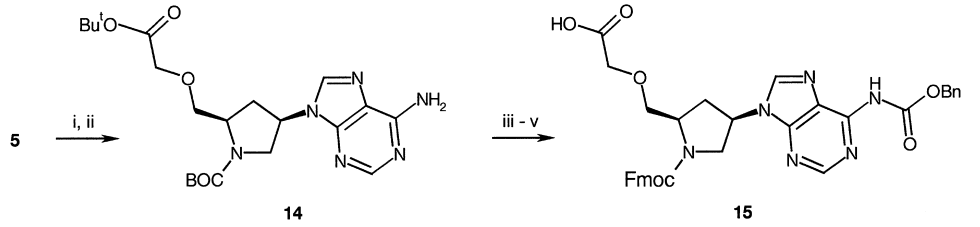
Preparation of N_δ-Fmoc-N^6-Cbz-protected adenine derivative **15** initially involved reaction of **5** with 6-chloropurine as an adenine surrogate (Scheme 3).[10] Although the resulting N^9-alkylated 6-chloropurine could not be obtained in entirely pure form (contamination by residual *N*,*N*′-diethoxycarbonyl hydrazine) treatment of this material with aq ammonia at 60 °C provided adenine

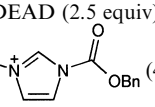


Scheme 1. (i) TBS-Cl (1.5 equiv), imidazole (2.2 equiv), Et_3N, DMF, rt, 2 h, quant. (ii) $LiBH_4$ (3.5 equiv), THF, 0°, 2.5 h, 86%. (iii) $BrCH_2COOBu^t$, (2.5 equiv), Bu_4NHSO_4 (0.25 equiv), benzene/50% NaOH 1/1, 2 h, 88%. (iv) TBAF (2.5 equiv), THF, 0°, 1 h, 91%. (v) N^3-Bz-thymine (2 equiv), DEAD (2.5 equiv), Ph_3P (2.5 equiv), THF, 0°, 7 h, 63%. (vi) 2N NaOH/MeOH/DMF 1.2/3/4 (4 equiv OH^-), rt, 3.5 h, 77%. (vii) CF_3COOH, rt, 45 min. (viii) Fmoc-OSu (1.15 equiv), Na_2CO_3 (1.6 equiv), dioxane/H_2O 1/1, 73% (2 steps).



Scheme 2. (i) $POCl_3$ (3.5 equiv), triazole (22.5 equiv), Et_3N (23 equiv), CH_3CN, rt, 43%. (ii) Concd NH_3/dioxane 1/3, rt, 93%. (iii) Cbz-OBt (1.3 equiv), Et_3N (1.3 equiv), CH_2Cl_2, rt, 82%. (iv) a) CF_3COOH, rt; b) Fmoc-OSu (1.2 equiv), Na_2CO_3 (2.5 equiv), dioxane/H_2O 6/4, rt, 55% (2 steps).



Scheme 3. (i) DEAD (2.5 equiv), Ph_3P (2.5 equiv), 6-Cl-purine (2 equiv), THF, 0 °C, 1 h, rt, 48 h. (ii) Concd NH_3/dioxane 1/1, 60°, 24 h, 75% (2 steps). (iii)  (4 equiv), rt, 16h, 70%. (iv) CF_3COOH, rt, 3 h, 60%. (v) Fmoc-OSu (1.15 equiv), Na_2CO_3 (2.3 equiv), dioxane/H_2O 1/1, rt, 48 h, 62%.

derivative **14** in a satisfactory 75% overall yield based on **5**. **14** was then elaborated into **15** by the same sequence of reactions as described above for the preparation of cytosine derivative **13**. It should be noted, however, that in contrast to **11** (Scheme 2) protection of the exocyclic amino group of **14** with a Cbz-group could only be achieved by means of the Rapaport reagent,[15] whereas neither Cbz-OBt nor Cbz-Cl, even in large excess, resulted in any of the desired acylation product.

In order to assess the hybridization affinity of oligomers of type **I** to complementary RNA and DNA, oligonucleotide analogue **A** was prepared as a simple model system, employing Fmoc-protected nucleoamino acids **8** and **13** as building blocks:[16]

H-Lys-**ttt ttc tct ctc tct**-Lys-NH_2 (**A**) **t,c = I-T, I-C**

Lysine residues were attached to the N- as well as the C-terminus of the actual base sequence in order to ensure adequate solubility in UV-melting experiments. Oligomer synthesis was performed on a 4-(2′,4′-dimethoxyphenyl-aminomethyl)-phenoxy resin (copoly(styrene)-1% DVB; $f \approx 0.24$ mmol/g),[17] with lysine residues being incorporated into the growing polyamide chain as the N_α-Fmoc-N_ϵ-*tert*-butoxycarbonyl derivative (Fmoc-Lys(BOC)-OH). Chain elongation was achieved by 2-(2-oxo-1(2*H*)-pyridyl))-1,1,3,3-tetramethyluronium tetrafluoroborate (TPTU)[18]-mediated single couplings (2- or 3-fold excess of nucleoamino acid, similar excess of TPTU) in the presence of ethyl-diisopropyl-amine (3.3 equiv) in *N*-methyl-pyrrolidinone, followed by capping of unreacted amino groups with acetic anhydride (Ac_2O/DMA/pyridine 1/8/1). The coupling time was set to 90 min, including 30 min at 40 °C.[19] 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 with 20% piperidine/DMF ($\lambda = 299.8$ nm, $\epsilon = 7800$ $M^{-1} \times cm^{-1}$).[20] 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. After cleavage of the protected oligomer from the solid support with TFA/H_2O (95/5, v/v; 3.5 h), the Cbz-protecting groups on cytosine were removed in solution at rt (TFMSA/TFA/DMS/m-cresol (1/10/6/2, v/v/v/v),[22] 3.5 h). The crude compounds were subjected to analysis and purification on a standard C_{18} reversed-phase column eluting with an acetonitrile–water gradient. The purity of the oligomer was verified by C_{18} reversed-phase analytical HPLC and was found to be >95%. In addition, the identity of the final product was assessed by mass spectral (matrix-assisted laser desorption ionization time-of-flight mass spectrometry, MALDI-TOF) analysis, which gave the expected molecular weight (4179.4 versus 4178.4 (calc)).

The interactions of oligomer **A** with complementary RNA and DNA were investigated in UV-melting experiments and the results of these studies are summarized in Table 1. Slow heating of a 1/1 mixture of **A** and its antiparallel[23] RNA complement immediately after mixing produced a biphasic melting curve with two cooperative transitions around 33 and 68 °C, respectively. Upon subsequent cooling of the mixture only the low temperature transition was still observed (33.2 °C); likewise, re-heating resulted in a cooperative melting curve with only a single transition at 33.4 °C. Although the origin of the high-temperature transition in the initial heating cycle is currently unresolved, the cooperative nature of the melting curve as well as its full reversibility with regard to the low-temperature transition clearly indicate that **A** is able to bind to its antiparallel RNA complement, r[$(AG)_5A_5$], in a cooperative fashion. The presence of a single mismatched base in the RNA target sequence (r[$(AG)_4ACA_5$]) resulted in a decrease in melting temperature of ≈10 °C (Table 1) which confirms that binding is (Watson–Crick) sequence-specific. In addition, the results of titration experiments indicate that complex formation between **A** and r[$(AG)_5A_5$] occurs with 1/1 stoichiometry.[24] Based on the difference in T_m-values between the **A**/r[$(AG)_5A_5$] complex and the corresponding natural DNA/RNA duplex ($\Delta T_m \approx -19$ °C, Table 1), it is clear that **A** binds to complementary antiparallel RNA with lower affinity than the corresponding oligodeoxyribonucleotide, d[$(T)_5(CT)_5$]; however, the resulting ΔT_m-value of between −1.2 and −1.3°/residue should be compared to similar or significantly more *negative* values that have been reported for a variety of differently modified DNA analogues, which are more closely related to the structure of natural DNA than oligomer **A**.[1,2] It should also be noted that the complex between **A** and r[$(AG)_5A_5$] is

Table 1. Melting temperatures (T_m's) of complexes between oligomer **A** and complementary RNA and DNA[a]

	RNA/DNA complement				
	r[$(AG)_5A_5$]	r[$A_5(GA)_5$]	d[$(AG)_5A_5$]	d[$A_5(GA)_5$]	r[$(AG)_4ACA_5$]
T_m (°C):	34.0[c] (33.2[d])	34.5[c] (34.9[d])	n.c.[e]	n.c.[e]	23.3
T_mWT (°C):[b]	52.4	53.3	42.8	43.2	42.9

[a]T_m's were determined in 10 mM phosphate buffer, pH7, 100 mM Na^+, 0.1 mM EDTA at 4 μM strand concentrations (for details cf. ref 21).
[b]T_m's for the corresponding natural (antiparallel) DNA/RNA and DNA/DNA duplexes.
[c]Transition-temperature obtained upon slow heating (0.5 °C/min) of a 1/1 mixture of oligomer **A** and the corresponding RNA complement from −5 to 95° after initial slow heating and subsequent slow cooling over the same temperature range. In the case of **A**/r[$(AG)_5A_5$] two cooperative transitions were observed at 33.5 and 68.4 °C, respectively, upon immediate slow heating of a freshly prepared 1/1 mixture. The latter transition was not observed upon subsequent cooling or re-heating of the same sample (cf text).
[d]Transition-temperature obtained upon slow cooling of a 1/1 mixture of oligomer **A** and the corresponding RNA complement from 95 to −5 °C after initial slow heating over the same temperature range.
[e]No cooperative transition observed.

significantly more stable than the corresponding complexes incorporating oligonucleotide analogues of types **II**–**IV** (cf. Fig. 1).[6b] On the other hand, Lowe et al.[5a] have recently reported that oligonucleotide analogues related to those of type **I** by the replacement of the -CH_2O- unit with an amide group -C(O)NH- supposedly bind to natural RNA as well as DNA with similar affinities as PNA (and thus more tightly than natural DNA). The difference between Lowe's system and our structure **I** could conceivably be caused by an increase in conformational flexibility in the latter, which would entropically disfavor hybrid formation (cf., however, ref 25).

Cooperative binding of **A** to its parallel[23] RNA complement (r[$A_5(GA)_5$]) was also observed and occurs with comparable affinity as for complementary RNA with an antiparallel alignment (Table 1). Most notably, only a single transition was observed under all experimental conditions and the binding curve was fully reversible upon heating and cooling without any indication of hysterisis. It is not clear, however, whether the interaction between **A** and r[$A_5(GA)_5$] in fact involves formation of a parallel **A**/RNA duplex, as binding of **A** to r[$A_5(GA)_5$] could also occur through the formation of an 11-base pair antiparallel duplex based on Watson–Crick base pairs between residues 5 to 15 of **A** and 15 to 5 of r[$A_5(GA)_5$].[26]

In contrast to complementary RNA, cooperative binding of **A** to complementary DNA, either in an antiparallel or parallel fashion, was *not* observed. This is contrary to the behaviour of the corresponding oligomers of type **II**–**IV** (Fig. 1), for which complexes with complementary DNA appear to be of comparable stability as those with RNA.[6b]

In summary, we have achieved the synthesis of a new polyamide based oligonucleotide analogue incorporating thymine- and cytosine-derived building blocks of type **I**. Homo-pyrimidine oligomer **A** exhibits sequence-specific binding to complementary RNA, but not DNA. As nucleoamino acids of type **I** easily lend themselves to further chemical modification (e.g., through introduction of additional substituents on the pyrrolidine ring or at the position α to the carboxy group; cf. also ref 6b), these building blocks could represent an appropriate template for the design of DNA analogues exhibiting further improved RNA-binding properties.

Acknowledgements

We thank A. Garnier, R. Wille, V. von Arx, and M.-L. Piccolotto for technical assistance.

References and Notes

1. For recent reviews on antisense technology and the importance of structural modifications of oligonucleotides for antisense applications cf., e.g.: a Crooke, S. T. *Med. Res. Rev*. **1996,** *16*, 319. (b) Matteucci, M. D. *Persp. Drug Disc. Des*. **1996**, *4*, 1. (c) Altmann K.-H.; Cuenoud, B.; von Matt, P. In *Applied Antisense Oligonucleotide Technology*; Stein, C. A.; Krieg, A. M., Eds.; Wiley-Lyss: New York, 1997; pp 73–107.
2. For recent reviews on backbone modified oligonucleotides cf., e.g.: (a) De Mesmaeker, A.; Häner, R.; Martin, P.; Moser, H. E. *Acc. Chem. Res*. **1995**, *28*, 366. (b) De Mesmaeker, A.; Altmann, K.-H.; Waldner, A.; Wendeborn, S. *Curr. Opinion Struct. Biol*. **1995**, *5*, 343.
3. For recent reviews on PNA cf., e.g.: (a) Nielsen, P. *Curr. Opin. Struct. Biol*. **1999**, *9*, 353. (b) Hyrup; B.; Nielsen, P. E. *Bioorg. Med. Chem*. **1996**, *4*, 5.
4. Cf., e.g.: (a) Schah, V. J.; Cerpa, R.; Kuntz, I. D.; Kenyon, G. L. *Bioorg. Chem*. **1996**, *24*, 201. (b) Petersen, K. H.; Buchardt, O.; Nielsen, P. E. *Bioorg. Med. Chem. Lett*. **1996**, *6*, 793. (c) Lenzi, A.; Reginato, G.; Taddei, M. *Tetrahedron Lett*. **1995**, *36*, 1713; Lenzi, A.; Reginato, G.; Taddei, M.; Trifilieff, E. *Tetrahedron Lett*. **1995**, *36*, 1717. (d) Garner, P.; Yoo, J. U. *Tetrahedron Lett*. **1993**, *34*, 1275. (e) Lewis, I. *Tetrahedron Lett*. **1993**, *36*, 5697. (f) Hudziak, R. M.; Barofsky, E.; Douglas, F.; Weller, D. L.; Huang, S.-B.; Weller, D. D. *Antisense Nucleic Acid Drug Dev*. **1996**, *6*, 267. For a review on earlier attempts to construct polyamide-based nucleic acid analogues see: Jones, S. A. *Int. J. Biol. Macromolecules* **1979**, *1*, 194.
5. (a) Lowe, G.; Vilaivan, T.; Westwell, M. S. *Bioorg. Chem*. **1997**, *25*, 321. (b) Jordan, S.; Schwemler, C.; Kosch, W.; Kretschmer, A.; Stropp, U.; Schwenner, E.; Mielke, B. *Bioorg. Med. Chem. Lett*. **1997**, *7*, 687.
6. (a) Altmann, K.-H.; Schmit Chiesi, C.; García-Echeverría, C. *Bioorg. Med. Chem. Lett*. **1997**, *7*, 1119. (b) García-Echeverría, C.; Schmit Chiesi, C.; Hüsken, D.; Altmann, K.-H. *Bioorg. Med. Chem. Lett*. **1997**, *7*, 1123.
7. Related structures have recently been described (cf. ref. 5).
8. The term nucleoamino acid is meant to signify amino acids with side chains bearing nucleic acid bases.
9. Baker, G. L.; Fritschel, S. J.; Stille, J. R.; Stille, J. K. *J. Org. Chem*. **1981**, *46*, 2954.

10. Jenny, T. F.; Horlacher, J.; Previsiani, N.; Benner, S. A. *Helv. Chim. Acta*. **1993**, *76*, 248.
11. Sigler, G. F.; Fuller, W. D.; Chaturverdi, N. C.; Goodman, M.; Verlander, M. *Biopolymers* **1983**, *22*, 2157.
12. In related alcohol substrates, direct *Mitsunobu* reaction with cytosine or N^4-isobutyryl cytosine predominantly resulted in *O*-2 alkylation (Altmann, K.-H., unpublished results).
13. Divakar, K. J.; Reese, C. B. *J. Chem. Soc. Perkin Trans. 1* **1982**, 1171
14. Wünsch, E.; Graf, W.; Keller, O.; Keller, W.; Wersin, G. *Synthesis* **1986**, 958.
15. Watkins, B. E.; Kiely, J. S.; Rapaport, H. *J. Org. Chem.* **1982**, *104*, 5702. For use of this reagent in the synthesis of PNA building blocks cf.: Egholm, M.; Behrens, C.; Christensen, L.; Berg, R. H.; Nielsen, P. E.; Buchardt, O. *J. Chem. Soc., Chem. Commun*. **1993**, 800.
16. Using the oligomerization/purification protocol described in this work, we have also prepared an oligomer H-Lys-**cau aau cau caa ucu**-Lys-NH_2 (**u** = **I-U**), which incorporates pyrimidine- as well as purine-derived building blocks.
17. Rink, H. *Tetrahedron Lett*. **1987**, *28*, 3787. Syntheses were carried out on a semi-automated peptide synthesizer using 140–190 mg of resin.
18. Knorr, R.; Trzeciak, A.; Bannwarth, W.; Gillesen, D. *Tetrahedron Lett*. **1989**, *30*, 1927.
19. Efficient incorporation of the N-terminal lysine residue in general required double-coupling.
20. Atherton, E.; Sheppard, R. C. In *Solid Phase Peptide Synthesis: A Practical Approach*; IRL Press, Oxford, 1989.
21. Concentrations of oligomer **A** 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). 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.
22. Tam, J. P.; Heath, W. F.; Merrifield, R. B. *J. Am. Chem. Soc*. **1986**, *108*, 5242.
23. The terms 'antiparallel' and 'parallel' RNA/DNA complement refer to the Watson–Crick alignment of oligomer **A** in the N→C direction with the RNA (DNA) in the 3′→5′ ('antiparallel') and 5′→3′ ('parallel') direction, respectively.
24. Titration experiments were performed at 260 nm and 280 nm by mixing 4 μM solutions of r[$(AG)_5A_5$] and **A** at molar fractions of **A** ranging from 1 to 0 in steps of 0.1 (i.e., molar fractions of **A** were 1, 0.9, 0.8, etc.). Absorbance was measured at rt either directly 15 min after mixing or after prior heating to 85 °C for 5 min. At 280 nm, no significant differences in absorbance were found between heated and unheated samples, with a trough in absorbance being observed for a 1/1 ratio of r[$(AG)_5A_5$] and **A** in both cases. At 260 nm absorbance versus molar fraction **A** curves were biphasic; the slope of the curves increased significantly for ratios of r[$(AG)_5A_5$]/**A** < 0.5 (0.29° →0.56° for the unheated and 0.11→0.68 °C for the heated samples). These data strongly suggest that the complex between **A** and r[$(AG)_5A_5$] is of 1/1 stoichiometry.
25. Independent of Lowe's work (ref 5a) we have also prepared an analogue of **A** with amide linkages -C(O)NH- substituting for all ether linkages -CH_2O- in **A**. In our hands, under the experimental conditions referred to in Table 1, a 1/1 mixture of this oligomer with r[$(AG)_5A_5$] upon initial heating gave a reasonably cooperative melting curve with an apparent T_m of 30 °C. However, no cooperative transition was observed upon cooling the sample. Furthermore, subsequent re-heating did not reproduce the original transition curve, but merely resulted in a virtually monotonous increase in absorption.
26. One could 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[$A_5(GA)_5$] (T_m-values of 52 and 48°, respectively).