156. Synthesis, Molecular Recognition, and Enzymology of Oligonucleotides Containing the Non-standard Base Pair between 5-Aza-7-deazaisoguanine and 6-Amino-3-methylpyrazin-2(1*H*)-one, a Donor-Acceptor-Acceptor Purine Analog and an Acceptor-Donor-Donor Pyrimidine Analog

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A 6-aminopyrazin-2(1*H*)-one (pyADD), when incorporated as a pyrimidine-base analog into an oligonucleotide chain, presents a H-bond acceptor-donor-donor pattern to 5-aza-7-deazaisoguanine (puDAA), the complementary donor-acceptor-acceptor purine analog. Reported here are the syntheses of the phosphoramidite of the 2′-deoxyribonucleoside bearing the puDAA base, oligonucleotides containing this nucleoside unit, the enzyme-assisted synthesis of oligoribonucleotides containing the pyADD ribonucleoside, and the molecular-recognition properties of this non-standard base pair in an oligonucleotide context. A series of melting experiments suggests that the pyADD·puDAA base pair contributes to the relative stability of a duplex structure approximately the same as an A·T base pair, and significantly more than mismatches between these non-standard bases and certain standard nucleobases. The pyADD nucleoside bisphosphate is accepted by T4 RNA ligase, but the triphosphate of the pyADD nucleoside was not incorporated by T7 RNA polymerase opposite the puDAA nucleobase in a template. Oligonucleotides containing the pyADD base slowly undergo a reversible first-order reaction, presumably an epimerization process to give the α-D-anomer. These experiments provide the tools for laboratory-based use of the pyADD·puDAA base pair as a component of an oligonucleotide-like molecular-recognition system based on an expanded genetic alphabet.

Introduction. – The standard *Watson-Crick* model for DNA and RNA structure can be extended to include 12 independently replicatable bases joined in 6 base pairs by mutually independent H-bonding patterns [1]. In many cases, chemical behaviors of the non-standard bases, including minor but significant contributions of minor tautomeric forms [2] [3], slow but significant rates of epimerization [4], or decreased stability towards hydrolytic or oxidative degradation [4], can be invoked to explain why individual non-standard bases were not exploited by natural genetic molecules over the course of natural history. In many cases, these properties do not prevent the non-standard base pair having utility in laboratory or clinical environments. *E.g.*, the accessibility of a minor tautomeric form for isoguanosine (implementing a puDDA H-bonding pattern), while causing infidelity in the hands of certain polymerases [3], does not prevent the use of the puDDA·pyAAD pair as an independent recognition element suitable for expanding the genetic lexicon [5], as a tool for incorporating reactive groups at specific positions in an oligonucleotide chain [6], or in diagnostic systems [7] [8].

These results imply that studies of the reactivity of the nucleoside bearing a non-standard nucleobase are not sufficient to define the scope of utility of a nucleobase, but must include studies of the reactivity of oligonucleotides containing the non-standard nucleo-

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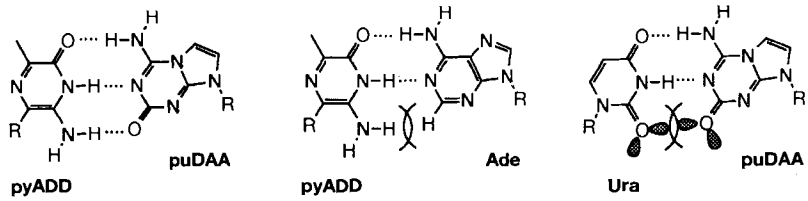
base. To this end, we report the synthesis and investigation of the properties of oligonucleotides and oligonucleotide duplexes incorporating a purine analog (puDAA; 5-aza-7-deazaisoguanine) presenting a donor-acceptor-acceptor H-bonding pattern and a pyrimidine analog (pyADD; 6-aminopyrazin-2(1*H*)-one) presenting an acceptor-donor-donor pattern (*Fig. 1*).

Fig. 1. *Structure of the puDAA·pyADD base pair the puDAA·Ura mismatch and the pyADD·Ade mismatch.* Unfavorable steric or electrostatic interactions expected to occur in a coplanar alignment of the heterocycles of the mismatch structures are indicated.

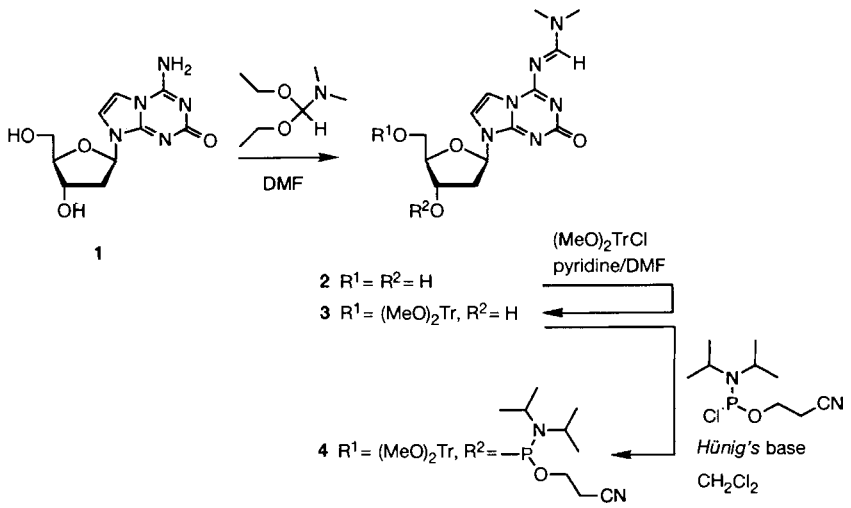


Several properties of the 6-aminopyrazin-2(1*H*)-one heterocycle (pyADD) suggested that it could perform as part of an oligonucleotide-based molecular-recognition system [9]. The heterocycle was found to have a pK_a of *ca.* 8.2 ± 0.1 by UV spectroscopy, permitting it to be uncharged when in an oligonucleotide strand. At equilibrium in H_2O, the desired keto form was found to predominate over the hydroxy form by a factor of *ca.* 2000 under conditions where DNA and RNA polymerases operate [9]. This is substantially better than the tautomer ratio observed with isoguanosine [2], where the minor form has been observed to create tautomer ambiguity with some polymerase systems [3] [10].

The pK_a of the complementary purine analog 5-aza-7-deazaisoguanine (puDAA) was *ca.* 11.2 by potentiometry [11]. In contrast to the pyrimidine analog, minor tautomeric forms were of little concern. Of the three possible tautomers of 5-aza-7-deazaisoguanosine, only the desired form is substituted by the energetically favored exocyclic amino and carbonyl groups. Both of the two undesired tautomers, however, contain a disfavored [12] [13] exocyclic imino group. The puDAA analog, therefore, should show the properties expected of a constituent of an extended genetic alphabet.

Results. – *Synthesis of a Protected Phosphoramidite of the 2′-Deoxyriboside of 5-Aza-7-deazaisoguanine (puDAA).* Preliminary studies showed that the puDAA heterocycle of deoxyribonucleoside **1** decomposed under the conditions normally used to deprotect oligonucleotides prepared by solid-phase synthesis (aq. conc. NH_3/60°/12 h). Therefore, the dimethylformamidine group developed by *Caruthers* and *Matteucci* was chosen [14] [15] to protect the exocyclic NH_2 group of the puDAA nucleobase (*Scheme 1*). This group is selectively introduced in one step to yield **2** without protecting the OH groups. Further, it can be removed under milder conditions than the benzoyl protecting group normally used. In concentrated ammonia at room temperature, conditions recommended for removing phenoxyacetyl groups from A and G (*vide infra*), a half-time for removal of the formamidine group of **2** of *ca.* 5 min was measured. Further, even after prolonged treatment with ammonia at room temperature, only a single nucleoside product was observed.

Scheme 1. *Synthesis of a Protected Phosphoramidite Derivative of the puDAA Deoxyribonucleoside Suitable for Solid-Phase DNA Synthesis*



The nucleobase-protected compound **2** was then dimethoxytritylated in pyridine/DMF [16] to yield **3**, which was converted to the phosphoramidite **4** by the method of *Caruthers* and coworkers [17–19].

Solid-Phase Synthesis of Oligodeoxyribonucleotides Containing the puDAA Deoxyribonucleoside. Oligonucleotides were synthesized on a *Pharmacia Gene Assembler* automated DNA synthesis apparatus following standard conditions, with 1.5 min coupling times for the standard nucleosides, and a 3 min coupling time for the non-standard nucleoside. Deoxyguanosine (dG) and deoxyadenosine (dA) were introduced as their commercially available phenoxyacetyl derivatives, while deoxycytidine (dC) was protected as the isobutyryl amide. Coupling yields, measured by trityl release, were comparable for the standard and non-standard phosphoramidites.

After solid-phase synthesis, the product was released from the resin, and the protecting groups were removed by treatment with concentrated ammonia for 4 h at room temperature [20]. Following initial studies, a new set of deprotection conditions was used, concentrated aqueous ammonia/EtOH 3:1, first at room temperature (3 h) and then at 55–60° (1 h). This appeared to give cleaner products as judged by HPLC. The partially deprotected oligonucleotide was then purified using the 'trityl on'-HPLC procedure. After cleavage of the trityl group, the final product was isolated by 'trityl off'-HPLC.

To verify the incorporation of the puDAA base, the purified oligonucleotides were digested with phosphodiesterase and calf intestinal phosphatase and the products isolated and quantified by HPLC. The experimentally determined compositions of the oligonucleotides corresponded to the theoretical values to within 10%. *Fig. 2* shows a typical result. The identity of the signal assigned to the non-standard nucleoside was confirmed by injection of synthetic puDAA deoxyribonucleoside. Analyses of the synthesized oligonucleotides containing the non-standard puDAA deoxyribonucleoside are shown in *Table 1*.

Table 1. *Oligonucleotide Sequences Containing puDAA and Their Relative Nucleoside Composition as Analyzed by HPLC After Digestion to Deoxyribonucleosides and Relative Nucleoside Composition of the pyADD-Containing Penta- and Hexaribonucleotide as Determined by HPLC after Digestion to Ribonucleosides*[a])

Name	Sequence (5′–3′ orientation)[b])	Relative nucleoside composition				
		d[puDAA][b])	dC	dG	dT	dA
OL-J1	d(GCT[puDAA]TCTCCCTATAGTGAGTCGTATTA)	1.0(1)	6.0(6)	5.0(5)	9.6(10)	4.4(5)
OL-J2	d(GCC[puDAA]TCTCCCTATAGTGAGTCGTATTA)	1.0(1)	7.2(7)	5.0(5)	8.8(9)	4.3(5)
OL-J3	d(GCT[puDAA]CCTCCCTATAGTGAGTCGTATTA)	1.1(1)	7.2(7)	5.0(5)	9.0(9)	4.9(5)
OL-J4	d(GCC[puDAA]CCTCCCTATAGTGAGTCGTATTA)	1.0(1)	7.8(8)	5.0(5)	7.8(8)	4.7(5)
OL-J5	d(TCGG[puDAA]TCCG)	1.0(1)	3.0(3)	3.0(3)	2.1(2)	0.0(0)
OL-J6	d(TTT[puDAA]TCCG)	n.d.	n.d	n.d.	n.d.	n.d.
		[pyADD][b]) ($5\beta + 6\beta$)	C	G	A	
	CGGA[pyADD]	1.0(1)	0.9(1)	2.0(2)	0.9(1)	
	CGGA[pyADD]A	1.0(1)	0.9(1)	2.0(2)	2.0(2)	

[a]) Theoretical values in parentheses; n.d. = not determined. [b]) For convenience, the hyphen between the nucleoside symbols representing the phosphodiester link is omitted. d[puDAA] represents the 2′-deoxyribonucleoside containing the nucleobase 5-aza-7-deazaisoguanine (puDAA) and [pyADD] the ribonucleoside containing the nucleobase 6-amino-3-methylpyrazin-2(1*H*)-one (pyADD).

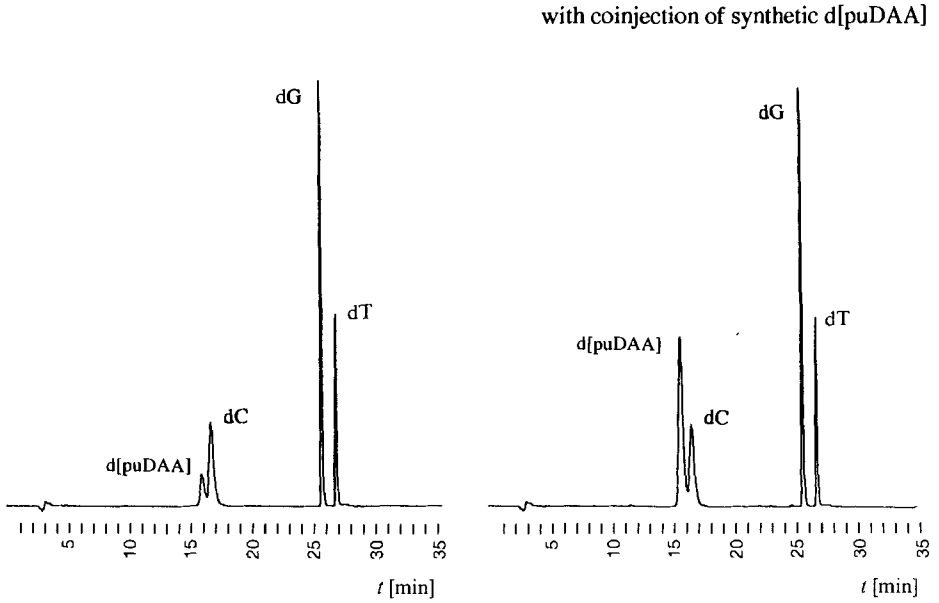


Fig. 2. *HPLC Analysis of the nonadeoxyribonucleotide d[T-C-G-G-[puDAA]-T-C-C-G] after digestion to mononucleosides.* HPLC, see *Exper. Part.*

Synthesis of Oligoribonucleotides Containing the pyADD Ribonucleoside. Because of the sensitivity of the pyADD nucleobase to base-catalyzed decomposition (see accompanying report [4]), a new set of conditions for preparing oligoribonucleotides was developed. These followed earlier work by *Uhlenbeck*, *Gumport*, and others using T4 RNA

ligase as a synthetic tool [21–24]. T4 RNA Ligase couples an acceptor oligonucleotide, which is preferably at least a trinucleoside diphosphate carrying a free 3′-OH group attached to a ribose (*Scheme 2*), with a donor, which can be as short as a single nucleoside 3′,5′-bisphosphate, although longer oligonucleotides are readily accepted if they are 5′-phosphorylated. ATP is a cosubstrate and provides the energetic driving force of the reaction. Removal of the 3′-phosphate of the product by treatment with alkaline phosphatase creates a new 3′-end, which can then serve as an acceptor in the subsequent coupling reaction. The substrate specificity of T4 RNA ligase is rather broad [23] [24]. However, uridine is not preferred in the three 3′-terminal positions of the acceptor.

The starting point for the synthesis of ribonucleotides containing the pyADD base (6-amino-3-methylpyrazin-2(1*H*)-one) was a synthetic tetranucleotide with the sequence C-G-G-A, known to be a satisfactory acceptor for T4 RNA ligase [25]. Exploratory experiments suggested that the best products would be obtained by elongating this tetranucleotide stepwise according to *Scheme 2*. Following each coupling, the product was isolated by HPLC. The yield of isolated product following each step was *ca.* 30–50% (ligation plus dephosphorylation). A total of 1.5 nmol of octaribonucleotide was isolated at the end of the sequence.

Scheme 2

	Ligase		Phosphatase	
C-G-G-A + p[pyADD]p	⟶	C-G-G-A-[pyADD]p	⟶	C-G-G-A-[pyADD]
C-G-G-A-[pyADD] + pAp	⟶	C-G-G-A-[pyADD]-Ap	⟶	C-G-G-A-[pyADD]-A
C-G-G-A-[pyADD]-A + pAp	⟶	C-G-G-A-[pyADD]-A-Ap	⟶	C-G-G-A-[pyADD]-A-A
C-G-G-A-[pyADD]-A-A + pAp	⟶	C-G-G-A-[pyADD]-A-A-Ap	⟶	C-G-G-A-[pyADD]-A-A-A

Each product was characterized by HPLC (*Fig. 3*). The pentanucleotide and hexanucleotide were digested as before and the products quantitated (*Fig. 4* and *Table 1*). The identity of the pyADD ribonucleoside as a β-D-furanosyl sugar was established by comparison to authentic material; a small amount of rearrangement arose during the digestion and was also observed when pure pyADD ribonucleoside was incubated under the digestion conditions. Further, the pyADD heterocycle in its keto tautomeric form has a characteristic absorption at 360 nm. A convenient routine measure of the ratio of non-standard and standard nucleobases can, therefore, be obtained by UV spectroscopy, measuring the ratio of absorbance at 260 and 360 nm (see *Exper. Part*). Comparison of the UV spectrum of the octanucleotide in aqueous buffer with model compounds containing *N*-methyl and *O*-methyl heterocycles (*Fig. 5*) suggested that the pyADD base lies preferentially in the desired keto tautomeric form when incorporated into an oligonucleotide single strand [9].

Molecular Recognition Properties and Stability of Oligonucleotides Containing the pyADD and puDAA Bases. The ability of the pyADD and puDAA bases to selectively form pairs within an oligonucleotide context was examined under conditions of 'high salt' (1M NaCl), to compensate for the short length of the synthesized oligonucleotides. Duplex stability was measured by a change in UV absorption (260 nm) as a function of temperature (a 'melting study' [26]). The melting temperatures, collected for several heating cooling cycles, are reported in *Table 2*. For comparison purposes and to analyze mismatched pairs, oligonucleotides incorporating only natural bases (prepared by standard methods; see *Exper. Part*) were synthesized and their melting temperatures measured.

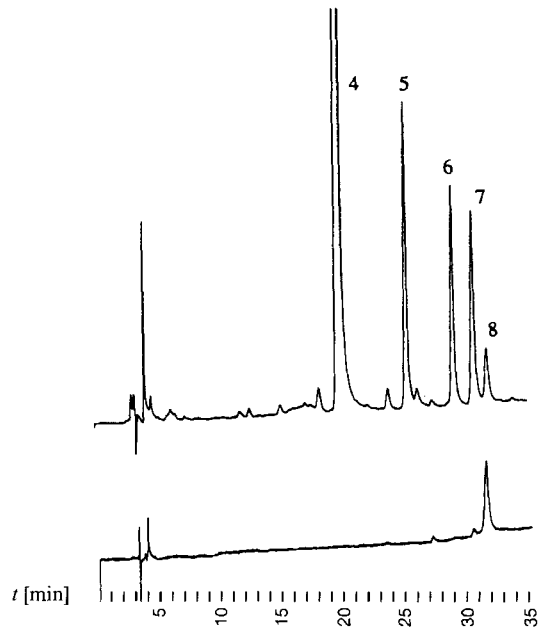


Fig. 3. *HPLC of a mixture of all intermediate synthetic oligoribonucleotides and the isolated pure octaribonucleotide end product.* 4 = C-G-G-A, 5 = C-G-G-A-[pyADD], 6 = C-G-G-A-[pyADD]-A, 7 = C-G-G-A-[pyADD]-A-A, 8 = C-G-G-A-[pyADD]-A-A-A. HPLC, see *Exper. Part.*

with coinjection of synthetic pyADD

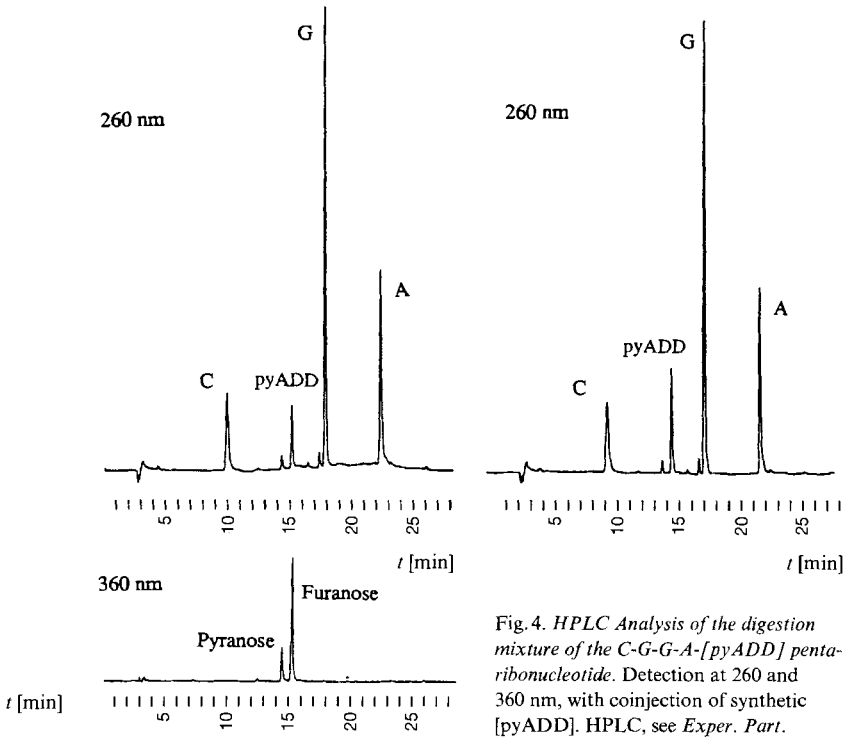


Fig. 4. *HPLC Analysis of the digestion mixture of the C-G-G-A-[pyADD] pentaribonucleotide.* Detection at 260 and 360 nm, with coinjection of synthetic [pyADD]. HPLC, see *Exper. Part.*

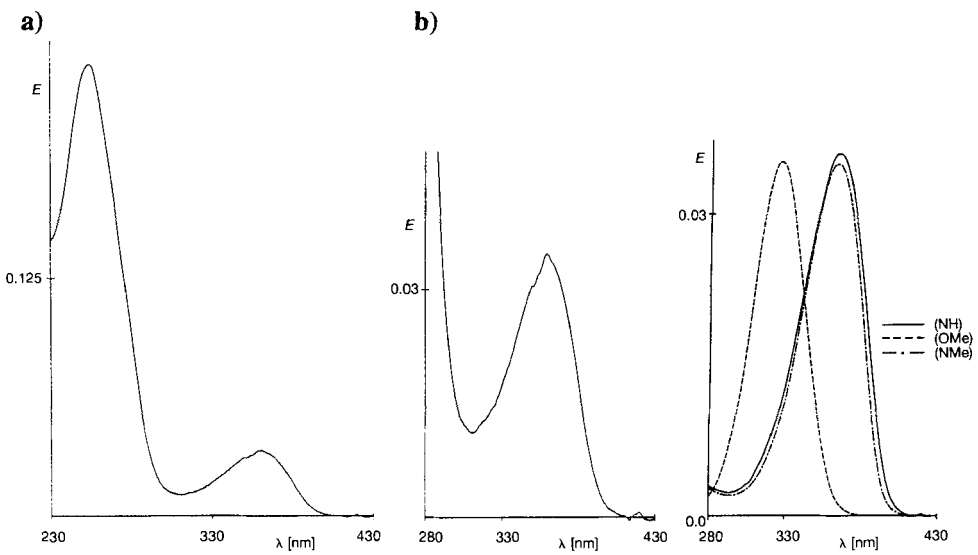


Fig. 5. a) *UV Spectrum of the C-G-G-A-[pyADD]-A-A-A octaribonucleotide in aqueous buffer and* b) *comparison of a segment of the UV spectrum of the octaribonucleotide C-G-G-A-[pyADD]-A-A-A with those of the free NH, the O-methylated, and the* N-*methylated pyrazine model compounds described previously* [9]. The noise of the signal in the octaribonucleotide spectrum is due to instrumental limitations.

Table 2. *UV-Spectroscopically Determined Melting Temperatures [°C] of the Oligonucleotide Duplexes Formed from C-G-G-A-X-A-A-A and d[T-T-T-Y-T-C-C-G]*[a])

		X		
		C	U	[pyADD]
Y:	dG	*36*	17	< 5
	dA	5	*28*	17[b])
	d[puDAA]	< 5	13	*27*

[a]) **X** and **Y** denote the nucleosides shown in the table. Conditions: UV measurements at 254 nm; buffer: 10 mM NaH_2PO_4 (adjusted with aq. NaOH soln. to pH 7.0), 1.0M NaCl, 0.1 mM EDTA; heating rate 1°/min; conc. of oligonucleotides *ca.* 1.3 μM. [b]) Only one measurement performed.

The absorption at 360 nm, characteristic of the keto tautomeric form of the pyADD base, was also observed in the duplex, suggesting that the pyADD base forms a *Watson-Crick* pair with the puDAA base, as expected. Over time, however, the total hyperchromicity, but not the apparent melting temperature, changed in melting experiments involving the oligonucleotide containing the pyADD ribonucleoside (for a typical result, see *Fig. 6,a*). To analyze this unusual behavior, samples of the oligonucleotide mixture were analyzed by HPLC before and after the heating cycle in the melting experiment (*cf. Fig. 6,b* with *Fig. 6,c*). Whereas the puDAA-containing oligodeoxyribonucleotide remained unchanged during the melting-temperature experiments, the pyADD-containing oligoribonucleotide partially reacted to a new oligoribonucleotide product, which retained the UV absorption at 360 nm typical for the pyADD heterocycle (not shown). This

new product was attributed to the slow epimerization of the pyADD riboside, an epimerization that was observed in the nucleoside derivative of the base (see accompanying report [4] and below, *Fig. 7*). The lower hyperchromicity (but unaltered melting temperature) suggested that the oligoribonucleotide containing the pyADD riboside in the α-D-configuration did not form a stable duplex under these conditions.

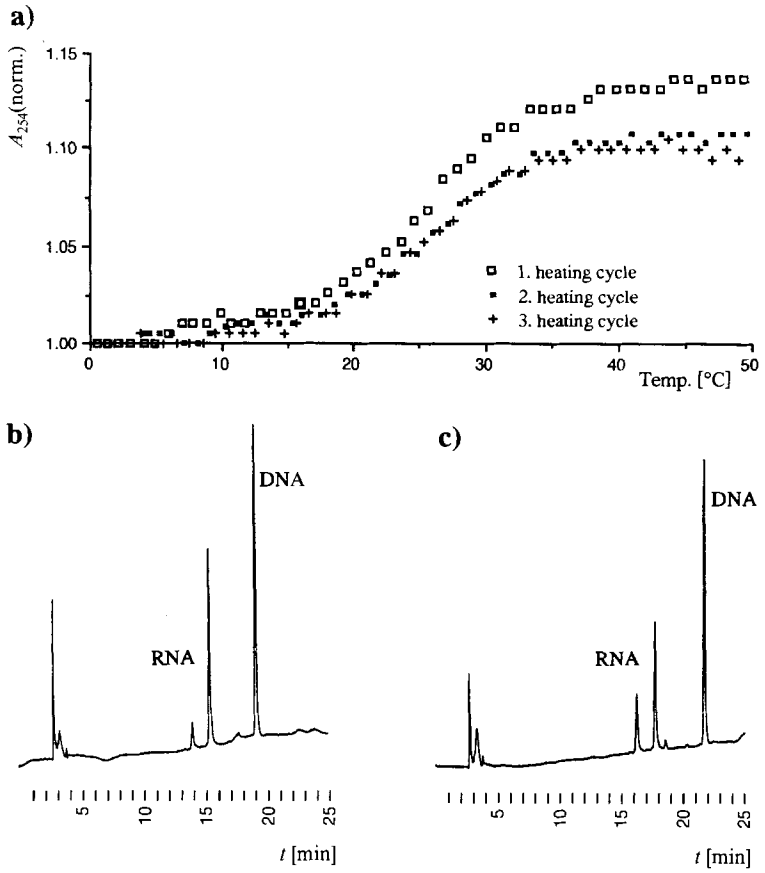


Fig. 6. *UV-Spectroscopically determined melting curves and HPLC analysis of the oligonucleotide duplex formed from oligoribonucleotide C-G-G-A-[pyADD]-A-A-A and oligodeoxyribonucleotide d[T-T-T-[puDAA]-G-T-T-C]:* a) *UV melting curves of three consecutive heating cycles measured with a single probe* (for exper. conditions, see *Footnote a* in *Table 2*); b) *HPLC analysis of the oligonucleotide solution used in* Fig. 6, a, *before the first heating cycle;* c) *HPLC analysis of the oligonucleotide solution used in* Fig. 6, a, *after two heating cycles.* The signals in the HPLC with a retention time of *ca.* 3 min are due to the Tm buffer. HPLC conditions: *Supelco* column, *LC18-S* anal. particle size 5 μm, 25 cm × 4.6 mm; elution: 0.1M (Et_3NH)OAc, pH 7.0; gradient: 5–20% MeCN in 30 min; flow rate: 1.0 ml/min.; UV detection at 260 nm.

As the epimerization reaction is expected to be reversible, the newly formed oligoribonucleotide product was isolated by HPLC and again incubated under HPLC analysis at pH 7 and 50° (*Fig. 7, a* and *b*). The result was the same mixture of oligonucleoside products as observed when the equilibrium was approached in the opposite direction.

Analysis of the HPLC data provided an equilibrium distribution of 66 ± 5% to 34 ± 5% (β-D/α-D) for the epimers of the pyADD riboside embedded into this oligoribonucleotide, and a pseudo-first-order velocity constant of 0.070 min^{-1} ($t_{½(pseudo)}$ = 10 min for the reaction converting the α-D-epimer to the β-D epimer) at 50.5 ± 0.5° in 0.1M $(Et_3NH)OAc$ buffer (pH 7.1) containing 10% MeCN (*Fig. 7c*).

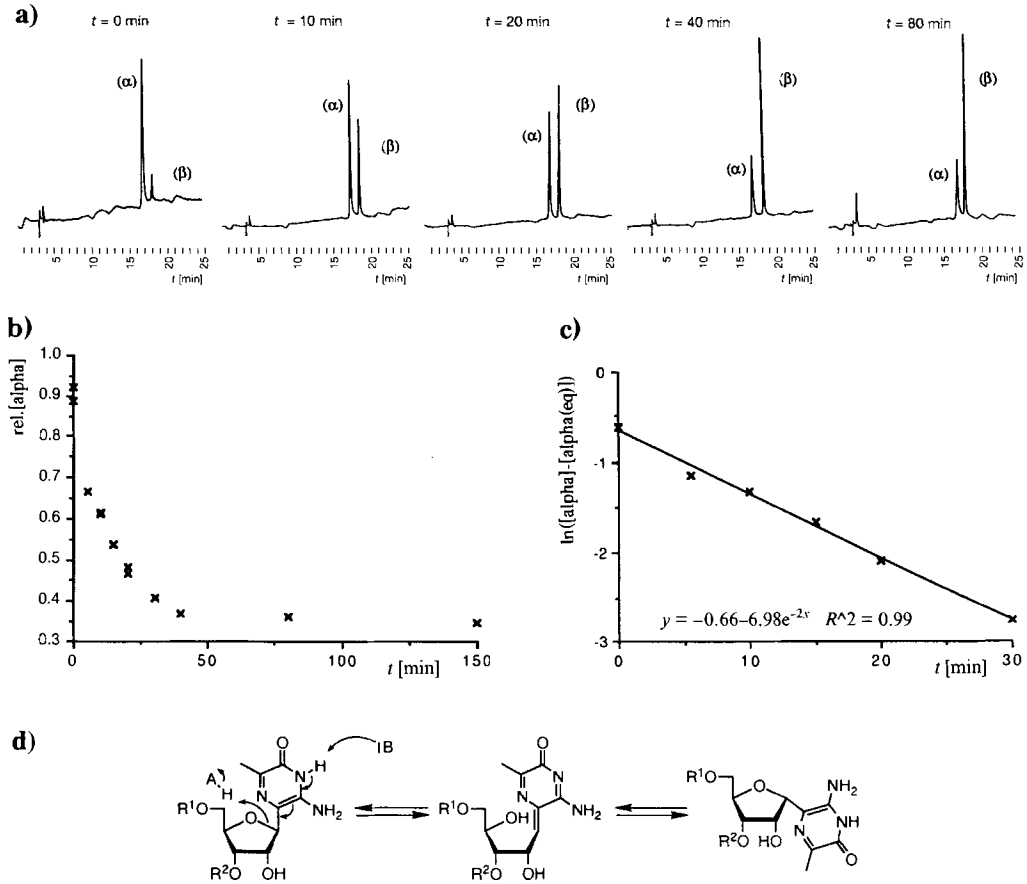


Fig. 7. *Backward reaction of the isolated oligoribonucleotide C-G-G-A-[pyADD]-A-A-A* (symbolized as α or alpha), *presumably containing the α-D-epimer of the pyADD ribonucleoside* (conditions: 50.5 ± 0.5°; 0.1M $(Et_3NH)OAc$ buffer (pH 7.1)/10% MeCN): a) *HPLC analysis* (HPLC conditions, see *Fig. 6*); b) *time course calculated with the data from* Fig. 7, a (rel. conc. of the presumed α-D-epimer containing oligoribonucleotide as a function of time; data of two independent measurements are overlayed); c) *analysis of the data from* Fig. 7, a, *as a reversible first-order process: ln([alpha] – [alpha(eq)]) as a function of time;* d) *suggested mechanism of the presumed epimerization reaction of the pyADD ribonucleoside embedded into an oligoribonucleotide*

The observed reversible first-order process and the observation of an analogous process for the free pyADD ribonucleoside is not an absolute proof that the dynamic process observed with oligonucleotides containing the pyADD nucleobase involves the proposed epimerization. The proposal is, however, consistent with similar results ob-

tained with a 5′-phosphorylated *C*-nucleoside [27]. Only the two epimeric furanosyl compounds are formed, while pyranosyl compounds, observed in the free pyADD nucleoside [4], are excluded due to the unavailability of a free 5′-OH group in the oligonucleotide. Further, oligonucleotides lacking the pyADD nucleobase did not display similar behavior.

In vitro *Transcription Experiments with T7 RNA Polymerase. In vitro* transcription reactions using T7 RNA polymerase were performed to analyze whether the pyADD·puDAA base pair could be incorporated enzymatically into oligonucleotide duplexes. In addition, the possibilities of enzymatically induced formation of the mismatches between adenine (puDA) and pyADD, and uracil (pyADA) and puDAA were examined (see *Fig. 1*, *Table 2*, and discussion below).

For experiments with T7 RNA polymerase, the natural promoter sequence extracted from the bacteriophage T7 genome (positions 18521 to 18560) was used ([28] and ref. cit. therein on p. 500). Either puDAA or adenine, as a control, was incorporated at position +7, followed by three further nucleotides to analyze for elongation after incorporation of the corresponding pyrimidine or pyrimidine analog. Several template sequences placing puDAA or adenine in different sequence contexts were prepared (*Fig. 8*).

OL-0:	5′-d(TAATACGACTCACTATA**G**)-3′[a])			
OL-1:	3′-d(ATTATGCTGAGTGATATCCCTC***T***	***A***	***T*****CG**)-5′[a])	
OL-2:	3′-d(ATTATGCTGAGTGATATCCCTC***T***	***A***	***C*****CG**)-5′[a])	
OL-3:	3′-d(ATTATGCTGAGTGATATCCCTC***C***	***A***	***T*****CG**)-5′[a])	
OL-4:	3′-d(ATTATGCTGAGTGATATCCCTC***C***	***A***	***C*****CG**)-5′[a])	
OL-J1:	3′-d(ATTATGCTGAGTGATATCCCTC***T***	***[puDAA]***	***T*****CG**)-5′[a])	
OL-J2:	3′-d(ATTATGCTGAGTGATATCCCTC***T***	***[puDAA]***	***C*****CG**)-5′[a])	
OL-J3:	3′-d(ATTATGCTGAGTGATATCCCTC***C***	***[puDAA]***	***T*****CG**)-5′[a])	
OL-J4:	3′-d(ATTATGCTGAGTGATATCCCTC***C***	***[puDAA]***	***C*****CG**)-5′[a])	
position:	−17	+1	+7	+10

[a]) For convenience, the hyphen between the nucleoside symbols representing the phosphodiester link is omitted.

Fig. 8. *Oligonucleotides used to prepare templates for* in vitro *transcription with T7 RNA polymerase.* OL-0 forms together with one of the eight other oligodeoxyribonucleotides the double-stranded promoter region from −17 to +1. A double strand is absolutely required in this region, whereas the transcribed part of the sequence can be read from a single strand. The coding region of OL-1 to OL-4 and OL-J1 to OL-J4 is depicted in bold face. Sequences OL-1 to OL-4 and OL-J1 to OL-J4 are identical except for the nearest neighbors surrounding the single coding adenosine and [puDAA], respectively (region in italics). Note, that the last base of OL-0 in position +1 is replaced by the first transcribed nucleotide (GTP).

OL-0 was annealed to a template-promoter oligonucleotide by mixing in equimolar amounts, quickly heating the resulting solution to 60°, and cooling the mixture slowly over a period of *ca.* 15 min to room temperature. *In vitro* run off transcription was performed following the method of *Uhlenbeck* and coworkers using γ-^{32}P-labelled GTP [29], as adopted in earlier work from our laboratory [3] [10] [30]. The products were resolved by denaturing polyacrylamide gel electrophoresis, and the reaction products were visualized by autoradiography. The relative concentrations of oligonucleotide products formed were calculated from the fact that each oligomer begins with G, and that the radiolabelled γ-phosphate group is retained only on the first nucleotide.

The possible formation of pyADD·adenine mismatches was investigated using promoters containing oligonucleotides OL-1 to 4 which place a single adenine at the critical position +7 in the template. A series of reactions were run (see *Fig. 9,a*, and results not shown). In the first [pyADD][2]) triphosphate was present, and UTP was lacking. Full-length product would arise only through mispairing of A with [pyADD], conceivable through a two-bond *Watson-Crick* pair (as is formed between A and U). Second, a reference reaction was run where all four standard triphosphates were present. The full length product is expected, together with the customary addition of one or two nucleotides to the product that are not encoded by the template [29] [31]. The third incubation lacked both [pyADD] triphosphate and UTP. The expected product is the hexamer resulting from aborted transcription at base 7. The fourth incubation contained both UTP and [pyADD] triphosphate, to test for a possible inhibition of incorporation of UTP by [pyADD] triphosphate.

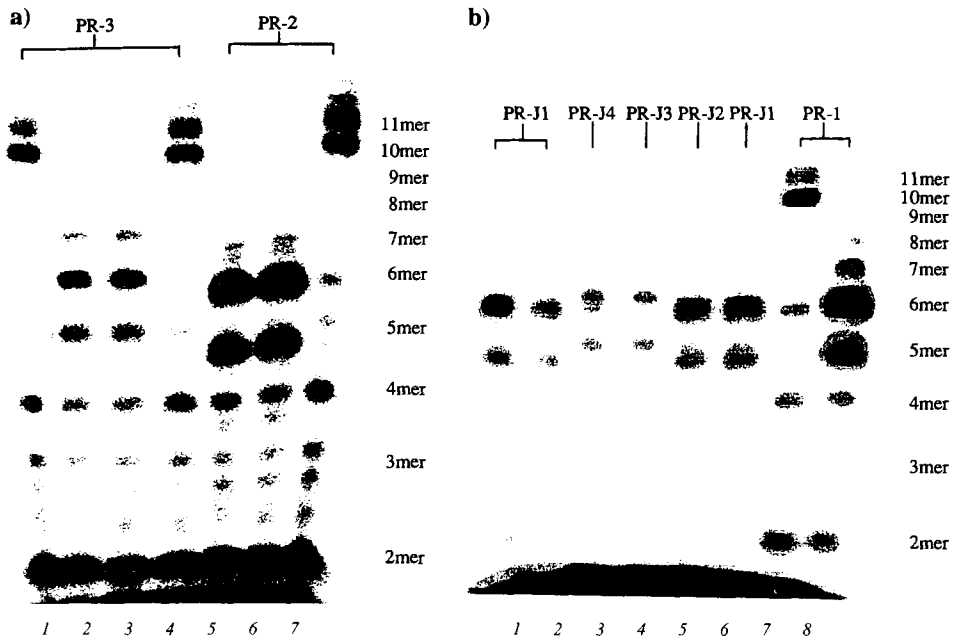


Fig. 9. In vitro *Transcription reactions with T7 RNA polymerase:* a) *Reactions at pH 8.1 using promotors containing only standard nucleosides,* i.e., *PR-2* (OL-0 + OL-2) *and PR-3* (OL-0 + OL-3) *with the following NTPs added:* Lane 1: *G, A, C, U, and [pyADD]*[2]), Lane 2 *and* 5: *G, A, C, and [pyADD]*, Lane 3 *and* 6: *G, A, and C, and* Lane 4 *and* 7: *G, A, C, and U* (the experiments performed with promotors PR-1 (OL-0 + OL-1) and PR-4 (OL-0 + OL-4) which yielded identical results are not shown). b) *Reactions at pH 7.0 with templates containing [puDAA]* (in Lane 1 and *2*, Mn^{2+} and Cd^{2+}, respectively, were added in a final conc. of 1 mM to the reaction buffer; no DTT was added in these cases) i.e., *PR-J1* (OL-0 + OL-J1), *PR-J2* (OL-0 + OL-J2), *PR-J3* (OL-0 + OL-J3), *PR-J4* (OL-0 + OL-J4), *and PR-1* (OL-0 + OL-1) *with the following NTP's added:* Lanes 1–6: *G, A, C, U, and [pyADD]*, Lane 7: *G, A; C, and U and* Lane 8: *G, A, and C*

[2]) [pyADD] represents the ribonucleoside containing the nucleobase 6-amino-3-methylpyrazin-2(1*H*)-one (pyADD) and d[puDAA] the 2′-deoxyribonucleoside 2′-deoxyribonucleoside **1**.

No incorporation of [pyADD] was observed opposite to adenosine in the template, regardless of the bases flanking the adenosine nucleotide at position 7. Note, that in agreement with reported observations [29], even when the highly conserved consensus promoter (reaching from position −17 to +6 relative to the start site of RNA synthesis) is used, the polymerase still produces abortive products of up to hexamer length. Addition of the [pyADD] triphosphate led to a weak nonspecific inhibition of the transcription reaction (*Fig. 9,a*, *cf. Lane 1* with *Lane 4*), but not a change in the relative product concentrations. This effect might be due to residual buffer salts or traces of impurities in the preparation of the non-standard triphosphate.

Oligonucleotides OL-J1 to J4 were used to investigate the possibility of the formation of the puDAA·pyADD base pair and of a puDAA·uracil mismatch catalyzed by T7 RNA polymerase (*Fig. 9,b*). No incorporation of uridine or the pyADD nucleoside opposite to d[puDAA][2]) in the template could be observed at pH 7.0 (*Fig. 9,b*, Lanes 3–6). No incorporation was also observed at pH 8.1 (data not shown), in the presence of 10% DMSO (data not shown), or in the presence of heavy metal salts (*Fig. 9,b*, *Lanes 1* and *2*), which decrease the substrate specificity of T7 RNA polymerases [32–34] and *E. coli* RNA polymerase [35] [36].

Discussion. – Qualitatively, the UV melting studies reported here show that the puDAA·pyADD base pair contributes selectively towards the specific binding of complementary oligonucleotide strands. These results confirm the essential details of the *Watson-Crick* base-pairing model with this pair, which meets both size complementarity and H-bonding complementarity rules, but on a previously uninvestigated heterocyclic ring system.

Quantitative investigations of the duplex pairs showed the expected results with the standard nucleobases. A central G·C base pair, joined by three H-bonds, contributed more to the duplex stability than a central A·U base pair, joined by only two H-bonds [37]. Further, the G·U 'wobble' mismatch [38] contributed more to duplex stability than the A·C mismatch (*Table 2*).

These results provided a framework for interpreting the stability of duplexes containing the puDAA·pyADD base pair. An oligonucleotide duplex containing a central non-standard puDAA·pyADD pair was slightly less stable than the same duplex with a central A·U pair, even though the first is joined by three H-bonds and the second by only two (*Fig. 1*).

Several structural features of the puDAA·pyADD base pair might account for this unexpected result. First, the pyADD heterocycle is connected to the ribose *via* a C–C bond ('*C*-glycoside') instead of the standard C–N bond. *C*-Glycosides pair with lower overall stability than analogous *N*-glycosides; *e.g.*, oligonucleotide duplexes containing the *C*-glycoside *N*-methylpseudouridine in a base pair with adenosine show lower melting temperatures than the analogous duplexes where thymidine is paired with adenosine [39]. The structural origin of this effect is unclear as the C–C bond is only slightly longer than the C–N bond of the standard nucleosides.

Second, although the 6-aminopyrazin-2(1*H*)-one heterocycle resides predominantly in its keto form in aqueous solution, the nucleobase in nonpolar media (*e.g.*, dioxane) prefers the enol tautomer [9]. As the inside of a double helix has a microscopic dielectric that is almost certainly lower than that of pure H_2O, the propensity of the pyADD

heterocycle to adopt an enol tautomer (more than the standard nucleobase uracil) in nonpolar environments might contribute to the lower apparent stability of the pyADD·puDAA base pair.

Mismatches involving the non-standard nucleobases showed the expected behavior. Both the d[puDAA]·U and the dA·[pyADD] mismatches contributed less to duplex stability than the corresponding d[puDAA]·[pyADD] match (*Table 2*). Both of these mismatches are presumably joined by two H-bonds (*Fig. 1*). If the nucleobases are in a coplanar alignment, they are, however, at the same time destabilized due to unfavorable nonbonding interactions. Opposing carbonyl O-atoms on the d[puDAA]·U base pair present free electron pairs that must be repulsive. For the dA·[pyADD] base pair, a H-atom of the exocyclic NH_2 group of the pyADD base is expected to interact unfavorably with H–C(2) of adenine (*Fig. 1*). These destabilizing effects might be minimized by twisting of the base-pair mismatches, which would not completely abolish H-bonding between the two complementary functional groups, but might lead to overall lower stability.

Whereas the base-pairing properties of the puDAA·pyADD pair, therefore, allow the use of this non-standard base pair for laboratory applications, the observed instability of the pyADD nucleoside towards epimerization is a major draw back. Note, however, that carbocyclic ribose analogs in which the ring O-atom is replaced by CH_2 or CF_2 would not suffer from this instability.

Although the puDAA·pyADD base pair seems to contribute to the thermodynamic stability of a duplex, no evidence was found that the base pair could be incorporated into a duplex by template-directed polymerization catalyzed by T7 RNA polymerase. The *C*-nucleoside character of the pyADD ribonucleoside cannot exclusively be responsible for this failure, as a template containing methylpseudouridine directs the T7 RNA polymerase-mediated incorporation of the *C*-nucleoside formycin [40]. In addition, non-standard base pairs presenting new H-bonding patterns have already been successfully transcribed with T7 RNA polymerase. *E.g.*, xanthosine ([puADA]) is incorporated opposite a pyDAD *C*-nucleoside in the template [30] and isoguanine ([puDDA]) is incorporated opposite of isocytidine ([pyAAD]) in the template [3] [10] by T7 RNA polymerase.

T7 RNA Polymerase has never been found to incorporate a non-standard pyrimidine analog presented as a triphosphate into an oligonucleotide duplex product. All attempts to incorporate the pyDAD ribonucleoside triphosphate [41] and the pyAAD ribonucleoside triphosphate (isoCTP) [3] opposite the corresponding purine base failed. Both natural pyrimidine bases, uracil and cytosine (pyADA and pyDAA, resp.) are substituted with a carbonyl group in position 2 of the heterocycle, presenting a lone pair of electrons in the minor groove. Similarly, both purines present a lone pair of electrons on N(3), which is located at approximately the same position. In contrast to this, all three pyrimidine analogs not incorporated, pyADD (this study), pyDAD, and pyAAD are substituted with an exocyclic amino group at this position. If T7 RNA polymerase is able to recognize the lone pair of electrons presented by the natural bases in the minor groove (*e.g. via* H-bonding; concept of 'minor groove scanning' proposed by *Steitz* for DNA polymerases [42]), the non-standard pyrimidine analogs presenting a H-bond donor function at this position may, therefore, not be accepted as substrates. In the recently reported crystal structure of DNA polymerase beta, the enzyme appears to make pre-

cisely this sort of interaction [43]. Further work with *in vitro* transcription reactions using T7 RNA polymerase and the pyDAA·puADD base pair [44] must be undertaken to explore this hypothesis.

Experimental Part

1. *General.* See accompanying report [4]. All chemicals were purchased from *Fluka*, except for cyanoethyl *N,N*-diisopropylchlorophosphoramidite, which was from *Aldrich*, and adenosine 3′,5′-diphosphate, adenosine 2′,5′-diphosphate, and ATP (disodium salt), which were from *Sigma*. The nucleoside triphosphate stock solns. (0.1M) for *in vitro* transcription reactions were from *Pharmacia*. [γ-^{32}P]GTP (aq. soln. with > 5000 μCi/mmol) was from *Amersham International*. The following enzymes were from *Boehringer Mannheim:* alkaline phosphatase from calf intestine, 86 μl/432 μg; phosphodiesterase from *Crotalus durissus, ca.* 1.5 U/mg; RNAse inhibitor, 40 u/μl (special quality for molecular biology). T4 RNA Ligase, 20000 U/ml, was from *New England Biolabs* and T7 RNA polymerase, 60000 u/ml ('FPLC pure'), from *Pharmacia*.

2. *Buffers and Solutions for Enzymatic Reactions.* All buffers and solns. for enzymatic work were prepared according to [45]. Double deionized water from a *Skan-Nanopur* water purification system was autoclaved for 20 min at 120° before use. *Digestion buffer:* 100 mM *Tris*·HCl, 20 mM $MgCl_2$ adjusted to pH 8.2 with aq. KOH soln. *Ligation buffer* (2×): 0.2M HEPPS (*N*-[2-hydroxyethyl]piperazine-*N*′-[3-propanesulfonic acid]), 40 mM $MgCl_2$, 3.0 mM ATP, 6.0 mM DTT (dithiothreitol), 20 μg/ml BSA (bovine serum albumin), adjusted to pH 8.25 with aq. soln. NaOH, 1:1 dilution with pH 8.18. *pAp Standard solution:* 20 mM of p5′A3′p in H_2O (an estimate of p5′A3′p/p5′A2′p 3:2 obtained by ^{31}P-NMR was used for the calculation of the p5′A3′p conc.; total pAp conc. by UV spectroscopy). *p[pyADD]p Standard solution:* 30 mM of p5′[pyADD]3′p in H_2O (ε = 10000 at 360 nm; based on the ε of a model compound which contains the same heterocycle). T_m *Buffer:* 1.0M NaCl, 10 mM NaH_2PO_4, 0.1 mM EDTA (ethylenediaminetetraacetic acid), with aq. NaOH soln. adjusted to pH 7.0. *NTP Standard solution* (40 mM): 0.1M NTP soln. (80 μl) was diluted with DEPC (diethyl dicarbonate) treated H_2O (120 μl) and stored at −20°. *Stop buffer* (7M urea): urea (4.20 g), bromophenol blue sodium salt (2 mg), and xylene cyanole FF (2 mg) were dissolved in sterile TBE 1× (10 ml). *TBE (5×) Buffer: Tris* base (54 g), boric acid (27.5 g), and 0.5M EDTA of pH 8.0 (20 ml) were adjusted to 1 l with H_2O. *TMS Buffer pH 8.1: Tris*·HCl (15.76 g), $MgCl_2$ (10.17 g), and spermidine (2.5 ml of a 1M soln. in H_2O) were dissolved in H_2O, the pH was adjusted to 8.1 with aq. NaOH soln., and the total volume brought to 500 ml with H_2O. Autoclaved aliquots were stored at −20°.

3. *Protected Phosphoramidite of the puDAA Deoxyribonucleoside. 8-(2′-Deoxy-β-*D*-ribofuranosyl)-4-{[(dimethylamino)methylidene]amino}imidazo[1,2-*a*]-1,3,5-triazin-2(8*H*)-one* (**2**). Deoxynucleoside **1** (100 mg, 0.37 mmol; synthesized as described in [46]) was suspended in abs. DMF (2.0 ml) and dimethylformamide diethyl acetal (0.7 ml) added at r.t. The resulting slightly cloudy soln. was stirred for 6.5 h at r.t. The solvents were removed in a 'Kugelrohr' oven at 50°. The residue was co-evaporated with toluene and then dried under high vacuum overnight. Chromatography (silica gel (10 g), CH_2Cl_2/MeOH 9:1 then 8:2) yielded **2** (88 mg, 73%). Light yellow solid. ^{1}H-NMR ((D_6)DMSO): 2.17 (*ddd*, *J* = 3.1, 6.1, 13.2, 1 H–C(2′)); 2.40 (*ddd*, *J* = 5.8, 7.7, 13.2, 1 H–C(2′)); 3.15 (*d*, *J* = 0.8, 3 H, Me_2N); 3.24 (*d*, *J* = 0.5, 3 H, Me_2N); 3.49–3.59 (*m*, 2 H–C(5′)); 3.81–3.83 (*m*, H–C(4′)); 4.30–4.33 (*m*, H–C(3′)); 5.07 (*dd*, *J* = 5.5, 5.6, OH–C(5′)); 5.30 (*d*, *J* = 4.2, OH–C(3′)); 6.16 (*dd*, *J* = 6.2, 7.6, H–C(1′)); 7.42 (*s*, H–C(6), H–C(7)); 8.819–8.821 (*m*, Me_2N–C*H*=N). ^{1}H-NMR ((D_6)DMSO, D_2O exchange): 2.19 (*ddd*, *J* = 3.1, 6.2, 13.3, 1 H–C(2′)); 2.41 (*ddd*, *J* = 5.9, 7.6, 13.3, 1 H–C(2′)); 3.16 (*d*, *J* = 0.8, 3 H, Me_2N); 3.24 (*d*, *J* = 0.5, 3 H, Me_2N); 3.51 (*dd*, *J* = 4.2, 11.8, 1 H–C(5′)); 3.57 (*dd*, *J* = 4.3, 11.8, 1 H–C(5′)); 3.82–3.84 (*m*, H–C(4′)); 4.31–4.34 (*m*, H–C(3′)); 6.17 (*dd*, *J* = 6.3, 7.4, H–C(1′)); 7.42 (*d*, *J* = 2.9, H–C(7)); 7.44 (*dd*, *J* = 0.3, 2.9, H–C(6)); 8.80–8.81 (*m*, Me_2N–C*H*=N). ^{13}C-NMR ((D_6)DMSO): 34.95 (*q*, MeN); 38.85 (*t*, C(2′)); 41.04 (*q*, MeN); 61.54 (*t*, C(5′)); 70.53 (*d*, C(3′)); 82.78 (*d*, C(4′)); 87.52 (*d*, C(1′)); 107.39 (*d*, C(6)); 114.96 (*d*, C(7)); 150.29 (*s*, C(2)); 153.70 (*s*, C(8a)); 158.92 (*d*, Me_2N–*C*H=N); 162.11 (*s*, C(4)). FAB-MS (3-NOBA): 667 (38, $[M_2 + Na]^+$), 645 (18, $[M_2 + 1]^+$), 345 (62, $[M + Na]^+$), 323 (100, $[M + 1]^+$).

*8-[2′-Deoxy-5′-*O*-(4,4′-dimethoxytrityl)-β-*D*-ribofuranosyl]-4-{[(dimethylamino)methylidene]amino}-imidazo[1,2-*a*]-1,3,5-triazin-2(8*H*)-one* (**3**). Protected deoxynucleoside **2** (75 mg, 0.23 mmol) was dried over P_2O_5 under high vacuum for 2 days and then dissolved with warming in abs. DMF (3.5 ml). The soln. was cooled to r.t. and a soln. of dimethoxytrityl chloride (103 mg, 0.32 mmol) in abs. pyridine (0.9 ml) added. After stirring for 9.5 h at r.t., the reaction was quenched with a mixture of MeOH/pyridine 1:1 (0.2 ml), and the solvents were removed under high vacuum. Chromatography (silica gel (10 g), CH_2Cl_2/MeOH 100:1, 100:5, and 100:10, each containing 1% Et_3N) yielded **3** (127 mg, 66%) as a microcrystalline solid with a trace of Et_3N as impurity. This was used

directly without further purification. ^{1}H-NMR ($CDCl_3$): 2.43–2.48 (*m*, 1 H–C(2′)); 2.76–2.81 (*m*, 1 H–C(2′)); 3.18 (*d*, J = 0.6, 3 H, Me_2N); 3.22 (*s*, 3 H, Me_2N); 3.28 (*dd*, J = 3.7, 10.5, 1 H–C(5′)); 3.42 (*dd*, J = 3.0, 10.5, 1 H–C(5′)); 3.78 (*s*, 2 MeO); 4.23–4.24 (*m*, H–C(4′)); 4.70–4.73 (*m*, H–C(3′)); 5.45 (br. *s*, OH); 6.48 (*dd*, J = 6.2, 6.3, H–C(1′)); 6.80–6.98 (*m*, 4 H_o); 7.04 (*d*, J = 2.8, 1 H, H–C(6) or H–C(7)); 7.10 (*d*, J = 2.8, 1 H, H–C(6) or H–C(7)); 7.18–7.44 (*m*, 9 arom. H); 8.98 (*s*, Me_2N–C*H*=N). ^{13}C-NMR ($CDCl_3$): 34.45 (*q*, MeN); 41.46 (*t*, C(2′)); 41.81 (*q*, MeN); 55.24 (*q*, MeO); 63.67 (*t*, C(5′)); 71.29 (*d*, C(3′)); 83.84 (*d*, C(1′) or C(4′)); 86.01 (*d*, C(1′) or C(4′)); 86.54 (*s*, $(MeOC_6H_4)_2$PhC); 107.09 (*d*, C(6)); 113.17 (*d*, C_o(Ph)); 114.86 (*d*, C(7)); 126.86 (*s*, $C_p(MeOC_6H_4)$); 127.85, 128.33 (2*d*, C_o,$C_m(MeOC_6H_4)$); 130.20, 130.21 (2*d*, C_m(Ph)); 135.69, 135.80 (2*d*, C_p(Ph)); 144.61 (*s*, $C_{ipso}(MeOC_6H_4)$); 150.20 (*s*, C(2)); 154.65 (*s*, C(8a)); 158.51 (*s*, C_{ipso}(Ph)); 159.78 (*d*, Me_2N–C*H*=N); 165.01 (*s*, C(4)). FAB-MS (3-NOBA): 625 ($[M + 1]^+$).

*8-[2′-Deoxy-5′-O-(4,4′-dimethoxytrityl)-β-*D*-ribofuranosyl]-4-{[(dimethylamino)methylidene]amino}-imidazo[1,2-a]-1,3,5-triazin-2(8*H*)-one 3′-[(2-Cyanoethyl) Diisopropylphosphoramidite]* (**4**). The tritylated deoxynucleoside **3** (125 mg, 0.20 mmol) was dried over P_2O_5 under high vacuum for 3 days, and then dissolved in abs. CH_2Cl_2 (1.0 ml). After addition of diisopropylethylamine (137 µl, 0.80 mmol), 2-cyanoethyl diisopropylchlorophosphoramidite (54 µl, 0.24 mmol) was added dropwise at r.t. After 1.5 h, Et_3N (0.2 ml) was added, the resulting precipitate dissolved in the chromatography-elution mixture (1.0 ml) and chromatographed (silica gel (10 g), CH_2Cl_2/MeOH 95:5, containing 1% Et_3N) to yield **4** (mixture of 2 diastereoisomers; **a**/**b** 109 mg, 66%). Amorphous white solid. ^{1}H-NMR (arbitrary numbering of the two diastereoisomers; $CDCl_3$): 1.16–1.18 (*m*, 24 H, 2 Me_2CH (**4a**/**b**)); 2.42–2.65 (*m*, 8 H, H–C(2′), CH_2CN (**4a**/**b**)); 3.166 (*s*, 3 H, Me_2N); 3.167 (*s*, 3 H, MeN); 3.21 (*s*, 6 H, MeN); 3.30–3.34 (*m*, 2 H, 2 H–C(5′) (**4a**/**b**)); 3.42 (*dd*, J = 3.2, 10.5, 1 H, 1 H–C(5′) (**4a**)); 3.48 (*dd*, J = 3.0, 10.5, 1 H, 1 H–C(5′) (**4b**)); 3.53–3.69, 3.71–3.88 (2*m*, 8 H, 2 Me_2C*H*, CH_2OP (**4a**/**b**)); 3.787, 3.792, 3.793 (3*s*, 12 H, MeO (**4a**/**b**)); 4.14–4.19 (*m*, 2 H, H–C(4′) (**4a**/**b**)); 4.65–4.71 (*m*, 2 H, H–C(3′) (**4a**/**b**)); 6.45–6.49 (*m*, 2 H, H–C(1′) (**4a**/**b**)); 6.81–6.85 (*m*, 8 H, H_o (**4a**/**b**)); 6.98 (*d*, J = 2.8, 1 H, H–C(6) or H–C(7) (**4a**)); 7.05 (*d*, J = 2.8, 1 H, H–C(6) or H–C(7) (**4a**)); 7.08–7.09 (*m*, 2 H, H–C(6), H–C(7) (**4b**)); 7.20–7.34, 7.41–7.44 (2*m*, 18 arom. H (**4a**/**b**)); 8.99, 9.00 (2*s*, 2 H, Me_2N–C*H*=N (**4a**/**b**)). ^{13}C-NMR ($CDCl_3$): 20.20 (*td*, J = 7.1, *C*H_2CN (**4a**)); 20.43 (*td*, J = 7.2, *C*H_2CN (**4b**)); 24.47, 24.53, 24.55, 24.57, 24.59, 24.61, 24.63, 24.64 (8*q*, *Me*$_2$CH); 35.36 (*q*, MeN); 40.05 (*t*, C(2′)); 41.74 (*q*, MeN); 43.16, 43.24, 43.26, 43.34 (4*d*, Me_2*C*H); 55.24, 55.27 (*q*, MeO); 58.18 (*td*, J = 18.7, CH_2OP (**4a**)); 58.25 (*td*, J = 18.5, CH_2OP (**4b**)); 63.95, 63.23 (2*t*, C(5′)); 72.80 (*dd*, J = 16.0, C(3′) (**4a**)); 73.60 (*dd*, J = 17.1, C(3′) (**4b**)); 83.34, 83.40 (2*d*, C(1′)); 85.30 (*dd*, J = 6.1, C(4′) (**4a**)); 85.40 (*dd*, J = 3.6, C(4′) (**4b**)); 86.61, 86.62 (2*s*, $(MeOC_6H_4)_2$PhC); 107.43, 107.45 (2*d*, C(6)); 113.20 (*d*, C_o(Ph)); 113.98, 133.99 (2*d*, C(7)); 117.49, 117.75 (2*s*, CN); 126.99, 127.03 (2*s*, $C_p(MeOC_6H_4)$); 127.90, 128.24, 128.31 (3*d*, C_o,$C_m(MeOC_6H_4)$); 130.15, 130.18, 130.21, (3*d*, C_m(Ph)); 135.49, 135.52, 135.54, 135.57 (4*d*, C_p(Ph)); 144.41, 144.42 (2*s*, $C_{ipso}(MeOC_6H_4)$); 150.59, 150.67 (2*s*, C(2)); 154.40, 154.41 (2*s*, C(8a)); 158.62, 158.63 (2*s*, C_{ipso}(Ph)); 159.73 (*d*, Me_2N–C*H*=N); 164.26, 164.31 (2*s*, C(4)). ^{31}P-NMR ($CDCl_3$): 148.8, 149.0. FAB-MS (3-NOBA): 847 ($[M + Na]^+$), 825 ($[M + 1]^+$).

4. *Oligodeoxyribonucleotide Synthesis.* The DNA oligonucleotides containing only standard nucleobases were synthesized according to standard procedures (2-cyanoethyl diisopropylphosphoramidite method; 1.3 µmol scale; *Gene Assembler Plus-Pharmacia-LKB* DNA synthesizer), deprotected in conc. ammonia overnight at 60°, and purified as exemplified below for the puDAA-containing oligonucleotides. The DNA oligonucleotides containing the non-standard puDAA nucleobase were prepared analogously, except that the puDAA phosphoramidite was coupled with a reaction time of 3.0 min, double the time used for coupling of the standard nucleosides. These oligonucleotides were deprotected and released from the support by incubation with saturated ammonia in H_2O/EtOH 3:1 for 3 h at r.t. followed by an additional 1 h at 50°. The support was removed by centrifugation and the ammonia soln. removed by rotary evaporation (30–40°) using a dry ice/i-PrOH cold trap.

The oligonucleotides were purified by HPLC using the 'trityl on' procedure (*Supelco, LC18-S*, particle size 5 µm, 25 cm × 10 mm; elution solvents: 0.1M (Et_3NH)OAc pH 7.0/MeCN 99:1(*A*), 0.1M (Et_3NH)OAc pH 7.0/MeCN 60:40(*B*), MeCN(*C*), 0.1M (Et_3NH)OAc pH 7.0/MeCN 80:20(*D*); flow rate, 6.0 ml/min, gradient see below; UV detection at 260 nm). The product-containing fractions were combined, the solvent removed under high vacuum, and the residue twice taken up in H_2O (2 ml) and lyophilized to remove all salts.

The product was detritylated by adding 80% AcOH (300 µl). EtOH (300 µl) was then added, and the solvents removed under high vacuum. The residue was taken up with H_2O (1 ml) and the trityl alcohol removed by extracting with Et_2O (3 × 1 ml). The oligonucleotides were then purified by 'trityl off' HPLC. Product-containing fractions were combined and the solvents evaporated. Finally the product was dissolved in H_2O (2 ml) and then lyophilized. This step was repeated to remove all salts. The resulting product was dissolved in sterile water (0.5–1.0 ml) and quantified by UV spectroscopy. All synthesized oligonucleotides were checked for purity by anal. HPLC.

Oligonucleotide sequences, solvent gradients (percentage of buffer *B*, *C*, or *D* in *A*) during HPLC purification and corresponding retention time are given below:

OL-J1: (5′–3′)d(G-C-T-[puDAA]-T-C-T-C-C-C-T-A-T-A-G-T-G-A-G-T-C-G-T-A-T-T-A); gradient 'trityl-on' 15–20% in 5 min to 26% in 25 min to 40% in 5 min (buffer *C*), t_R 23 min; gradient 'trityl-off' 45–55% in 30 min (buffer *D*), t_R 12 min.

OL-J2: (5′–3′)d(G-C-C-[puDAA]-T-C-T-C-C-C-T-A-T-A-G-T-G-A-G-T-C-G-T-A-T-T-A); gradient 'trityl-on' 40–50% in 5 min to 57% in 25 min to 100% in 5 min (buffer *B*), t_R 21 min; gradient 'trityl-off' 45–58% in 30 min (buffer *D*), t_R 12 min.

OL-J3: (5′–3′)d(G-C-T-[puDAA]-C-C-T-C-C-C-T-A-T-A-G-T-G-A-G-T-C-G-T-A-T-T-A); gradient 'trityl-on' 40–50% in 5 min to 55% in 25 min to 100% in 5 min (buffer *B*), t_R 18 min; gradient 'trityl-off' 45–50% in 25 min to 70% in 5 min (buffer *D*), t_R 14 min.

OL-J4: (5′–3′)d(G-C-C-[puDAA]-C-C-T-C-C-C-T-A-T-A-G-T-G-A-G-T-C-G-T-A-T-T-A); gradient 'trityl-on' like for OL-J1, t_R 21 min; gradient 'trityl-off' 45–50% in 25 min (buffer *D*), t_R 12 min.

OL-J5: (5′–3′)d(T-C-G-G-[puDAA]-T-C-C-G); gradient 'trityl-on' 40–50% in 10 min to 60% in 25 min to 100% in 5 min (buffer *B*), t_R 30 min; gradient 'trityl-off' isocratic 39%(buffer *D*), t_R 10 min.

OL-J6: (5′–3′)d(T-T-T-[puDAA]-T-C-C-G); gradient 'trityl-on' 40–50% in 5 min to 90% in 25 min (buffer *B*), t_R 23 min; gradient 'trityl-off' 5–20% in 30 min (buffer *C*) (purified on an anal. otherwise identical column; flow 1 ml/min), t_R 21 min.

5. *Characterization of puDAA-Containing Oligodeoxyribonucleotides.* A small aliquot (*ca.* 0.2 *OD* at 254 nm) of the oligonucleotide was dissolved in digestion buffer (0.7 ml) and incubated with phosphodiesterase (5 μg) at 37° for 30 min. Alkaline phosphatase (5 μg) was added and the reaction continued for 30 min at 37°. The relative nucleotide composition was determined by HPLC analysis of aliquots of the digestion mixture *Supelco, LC18-S* anal., particle size 5 μm, 25 cm × 4.6 mm, elution solvents: 0.05M K_2HPO_4 pH 7.0(*A*), 0.05M K_2HPO_4 pH 7.0/MeOH 60:40(*B*); flow rate: 1.0 ml/min, gradient: 0% *B* for 10 min, to 60% *B* in 18 min, then at 60% *B* for 7 min. UV detection at 260 nm; t_R [min]: d[puDAA] 15.4, dC 16.2, dT 26.1, dG 27.3, dA 30.9). The following absorption coefficients (260 nm) were used: d[puDAA] 7500, dC 7300, dA 15400, dT 8800, dG 11700 [47]. The identity of the d[puDAA] signal was demonstrated by coinjection of synthetic d[puDAA] (**1**).

6. *Enzymatic Oligoribunucleotide Synthesis Using T4 RNA Ligase.* General protocol: H_2O (2.0 μl), ligation buffer (5.0 μl), RNA acceptor (1.0 μl, 1.0 mM), and ribonucleoside bisphosphate (1.0 μl, 2.0 mM) were mixed and an aliquot of the mixture removed for HPLC analysis. T4 RNA Ligase (1.0 μl, 20 U) was added and the reaction incubated for 4–6 h at 37° under HPLC control of aliquots of the mixture. Alkaline phosphatase was added directly to the mixture and the resulting soln. again incubated for 45 min at 37°. The mixture was diluted to 500 μl and microfiltered and the product isolated by HPLC (*Supelco, LC18-S* anal., particle size 5 μm, 25 cm × 4.6 mm; elution solvents: 0.1M $(Et_3NH)OAc$ pH 7.0(*A*), pure MeCN(*B*); flow rate 1.0 ml/min.; gradient, 5–11% *B* in *A* in 34 min, UV detection at 260 nm, for pyADD at 360 nm). Product-containing fractions were pooled and lyophilized. For removal of the volatile buffer salts, the product was twice dissolved in H_2O and again lyophilized. The RNA product was quantified by UV spectroscopy, dissolved in the appropriate amount of sterilized H_2O and then used for the next reaction cycle. In early reactions, the reaction volumes were up to 10 fold higher; the same relative concentration of all components was used, however.

Calculated ε at 260 nm and results of HPLC (t_R in min) and UV analysis: C-G-G-A: ε 46100, t_R 19.8; C-G-G-A-[pyADD]: ε 49400, t_R 25.3, yield 48%, *A*(260 nm)/*A*(360 nm) 3.8; C-G-G-A-[pyADD]-A: ε 64800, t_R 29.0, yield 40%, *A*(260 nm)/*A*(360 nm) 4.6; C-G-G-A-[pyADD]-A-A: ε 80200, t_R 30.7, yield 46%, *A*(260 nm)/*A*(360 nm) 5.6; C-G-G-A-[pyADD]-A-A-A: ε 95600, t_R 31.8, yield 31%, *A*(260 nm)/*A*(360 nm) 6.5. For the calculation of ε, the extinction coefficients of the deoxyribunucleosides were used (see above). The ε of pyADD (3300 at 260 nm; 10000 at 360 nm) was from a model compound containing the pyrazine heterocycle. The final yield of RNA octamer was 1.5 nmol.

7. *Characterization of pyADD-Containing Oligoribonucleotides.* The synthesized penta- and hexaribonucleotides were digested to the ribonucleosides and analyzed by HPLC as described above for the oligodeoxyribonucleotides (*Supelco, LC18-S* anal. particle size 5 μm, 25 cm × 4.6 mm; elution solvents: 0.05M K_2HPO_4 pH 7.0(*A*), 0.05M K_2HPO_4 pH 7.0/MeOH 80:20(*B*); flow rate, 1.0 ml/min; gradient: 0% *B* for 3 min, to 100% *B* in 14 min then at 100% *B* for 8 min; UV detection at 260 nm, for pyADD at 360 nm). For ε values, see above. Detection at 360 nm was used for quantification of pyADD, and the identity of the pyADD peak was confirmed by coinjection with synthetic pyADD ribonucleoside.

8. *Deprotection and Purification of Oligoribonucleotides Containing Standard Nucleotides Only.* Two RNA octamers were purchased from *MWG-Biotech* in 5′-*O*-$(MeO)_2$Tr- and 2′-*O*-Fpmp-protected form. Following the instructions of the manufacturer, the oligonucleotides were deprotected by incubation in the provided depro-

tection soln. (500 µl, pH 2–3) for 24 h at r.t. The soln. was cooled to 0°, neutralized with Et_3N (60 µl), and microfiltered prior to HPLC purification, which was performed as described above for the pyADD-containing octaribonucleotides.

9. In vitro *Transcription Using T7 RNA Polymerase*. General protocol following [29] [31]: H_2O (3 µl), TMS buffer (4.0 µl; 200 mM *Tris* · HCl, 100 mM $MgCl_2$, 5 mM spermidine), DTT soln. (1.0 µl; 100 mM), RNAse inhibitor (1.0 µl), NTP's (5.0 µl; 1:1:1:1, 10 mM/NTP), *Triton X-100* soln. (1.0 µl; 0.2%), DNA template (1.0 µl; 27 pmol), BSA soln. (1.0 µl; 1 mg/ml), [γ-^{32}P]GTP (1.0 µl; 1 µCi), and T7 RNA polymerase (2 µl, 140 U) were incubated at 37° for 20 min. To stop the reaction, stop buffer (12 µl) was added and the resulting mixture heated to 67° for 3 min. The mixture was separated on a denaturing 20% polyacrylamide gel, and the products were analyzed by autoradiography using the Phosphorimager.

REFERENCES

[1] S. A. Benner, R. K. Allemann, A. D. Ellington, L. Ge, A. Glasfeld, G. F. Leanz, T. Krauch, L. J. MacPherson, S. E. Moroney, J. A. Piccirilli, E. Weinhold, *Cold Spring Harbor Sym. Quant. Biol.* **1987,** *52,* 53.
[2] J. Sepiol, Z. Karzimierczuk, D. Shugar, *Z. Naturforsch., C* **1976,** *31,* 361.
[3] C. Y. Switzer, S. E. Moroney, S. A. Benner, *Biochemistry* **1993,** *32,* 10489.
[4] J. J. Voegel, S. A. Benner, *Helv. Chim. Acta* **1996,** *79,* 1863.
[5] J. D. Bain, C. Switzer, A. R. Chamberlin, S. A. Benner, *Nature (London)* **1992,** *356,* 537.
[6] Y. Tor, P. B. Dervan, *J. Am. Chem. Soc.* **1993,** *115,* 4461.
[7] T. Horn, C. A. Chang, M. L. Collins, *Tetrahedron Lett.* **1995,** *36,* 2033.
[8] T. Horn, C. A. Chang, M. L. Collins, *Nucleosides Nucleotides* **1995,** *14,* 1023.
[9] J. J. Voegel, U. v. Krosigk, S. A. Benner, *J. Org. Chem.* **1993,** *58,* 7542.
[10] C. R. Switzer, S. E. Moroney, S. A. Benner, *J. Am. Chem. Soc.* **1989,** *111,* 8322.
[11] J. J. Voegel, Dissertation, ETH No. 10691, Zürich, 1994.
[12] J. Elgureo, C. Marzin, A. R. Katritzky, P. Linda, *Adv. Het. Chem. Supplement 1* **1976,** 502.
[13] A. R. Katrizky, M. Karelson, P. A. Harris, *Heterocycles* **1991,** *32,* 329.
[14] L. J. McBride, R. Kierzek, S. L. Beaucage, M. H. Caruthers, *J, Am. Chem. Soc.* **1986,** *108,* 2040.
[15] B. C. Froehler, M. D. Matteucci, *Nucleic Acids Res.* **1983,** *11,* 8031.
[16] H. Schaller, G. Weimann, B. Lerch, H. G. Khorana, *J. Am. Chem. Soc.* **1963,** *85,* 3821.
[17] S. L. Beaucage, M. H. Caruthers, *Tetrahedron Lett.* **1981,** *22,* 1859.
[18] L. J. McBride, M. H. Caruthers, *Tetrahedron Lett.* **1983,** *24,* 245.
[19] M. J. Gait 'Oligonucleotide Synthesis: A Practical Approach', IRL Press, Oxford, 1984, p. 25.
[20] J. C. Schulhof, D. Molko, R. Teoule, *Nucleic Acids Res.* **1987,** *15,* 397.
[21] J. R. Barrio, M. G. Barrio, N. J. Leonard, T. E. England, O. C. Uhlenbeck, *Biochemistry* **1978,** *17,* 2077.
[22] T. E. England, O. C. Uhlenbeck, *Biochemistry* **1978,** *17,* 2069.
[23] O. C. Uhlenbeck, R. I. Gumport, *Enzymes* **1982,** *15,* 31.
[24] R. I. Gumport, O. C. Uhlenbeck, 'Gene Amplification and Analysis', Vol. 2, 'Structural Analysis of Nucleic Acids', Eds. J. G. Chirikjian and T. S. Tapas, Elsevier/North-Holland, New York–Amsterdam–Oxford, 1981, p. 313.
[25] C. G. o. N. A. Synthesis, *Sci. Sin.* **1978,** *21,* 687.
[26] W. Saenger, 'Principles of Nucleic Acid Structure', Springer Verlag, New York, 1984.
[27] R. W. Chambers, V. Kurkov, R. Shapiro, *Biochemistry* **1963,** *2,* 1192.
[28] J. J. Dunn, F. W. Studier, *J. Biol. Chem.* **1983,** *166,* 477.
[29] J. F. Milligan, D. R. Groebe, G. W. Witherell, O. C. Uhlenbeck, *Nucleic Acids Res.* **1987,** *15,* 8783.
[30] J. A. Piccirilli, T. Krauch, S. E. Moroney, S. A. Benner, *Nature (London)* **1990,** *343,* 33.
[31] J. F. Milligan, O. C. Uhlenbeck, *Methods Enzymol.* **1989,** *180,* 51.
[32] M. Chamberlain, J. Ring, *J. Biol. Chem.* **1973,** *248,* 2235.
[33] M. Chamberlain, J. McGrath, L. Waskell, *Nature (London)* **1970,** *228,* 227.
[34] F. Conrad, A. Hanne, R. K. Gaur, G. Krupp, *Nucleic Acis Res.* **1995,** *23,* 1845.
[35] D. B. Ludlum, *Biochim. Biophys. Acta* **1971,** *247,* 412.
[36] D. B. Ludlum, R. C. Wilhelm, *J. Biol. Chem.* **1968,** *243,* 2750.
[37] W. Saenger, 'Principles of Nucleic Acid Structure', Springer Verlag, New York, 1984, pp. 145–149.
[38] W. Saenger, 'Principles of Nucleic Acid Structure', Springer Verlag, New York, 1984, p. 155.

[39] I. Rosenberg, J.F. Soler, Z. Tocik, W.-Y. Ren, L.A. Ciszewski, P. Kois, K.W. Pankiewicz, M. Spassova, K.A. Watanabe, *Nucleosides Nucleotides* **1993,** *12,* 381.
[40] J.A. Piccirilli, S.E. Moroney, S.A. Benner, *Biochemistry* **1991,** *30,* 10350.
[41] T. Krauch, Dissertation, ETH No. 8940, Zürich, 1989.
[42] T. Steitz, in 'Biological Organization: Macromolecular Interactions at High Resolution', Eds. R.M. Burnet and H.J. Vogel, Adademic Press, New York, 1987, p.45.
[43] H. Pelletier, M.R. Sawaya, A. Kumar, S.H. Wilson, J. Kraut, *Science* **1994,** *264,* 189.
[44] U. von Krosigk, S.A. Benner, *J. Am. Chem. Soc.* **1995,** *117,* 5361.
[45] J. Sambrook, E.F. Fritsch, T. Maniatis, 'Molecular Cloning', Cold Spring Harbor Laboratory Press, New York, 1989.
[46] J.J. Voegel, M.M. Altorfer, S.A. Benner, *Helv. Chim. Acta* **1993,** *76,* 2061.
[47] F. Seela, S. Lampe, *Helv. Chim. Acta* **1991,** *74,* 1790.