122. 7-Deazaadenosine: Oligoribonucleotide Building Block Synthesis and Autocatalytic Hydrolysis of Base-Modified Hammerhead Ribozymes

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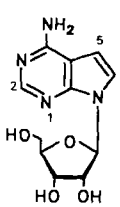
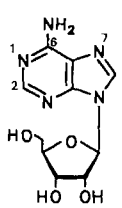
A 7-deazaadenosine (= tubercidin; c^7A; **1**) building block for solid-phase oligoribonucleotide synthesis was prepared. The amino group of **1** was protected with the (dimethylamino)methylidene residue (→ **3**), and the monomethoxytrityl group was introduced at OH–C(5′) (→ **4**). Protection of OH–C(2′) was carried out by silylation, showing that use of the $(i\text{-Pr})_3Si$ group resulted in high 2′-*O*-selectivity (→ **5b**, 80%). Reaction of **5b** with PCl_3 afforded the phosphonate **7** which was used in solid-phase oligoribonucleotide synthesis. The autocatalytic hydrolysis of hammerhead ribozymes using pG-G-G-A-G-U-C-A-G-U-C-C-C-U-U-C-G-G-G-G-A-C-U-C-U-G-A-A-G-A-G-G-C-G-C as substrate strand (S) and modified G-C-G-C-C-G-A-A-A-C-U-C-C-C as enzyme strand (E) was studied. When c^7A replaced A^{13} or A^{14}, a small decrease of catalytic activity was observed, while modification in position A^{15} enhanced the autocatalytic hydrolysis. The results demonstrate, that the atom N(7) of adenosine in any of these positions is not crucial for ribozyme action.

Introduction. – The purine N(7) atoms of nucleoside residues play a crucial role during non-*Watson-Crick* base pairing of ribo- [1] [2] and deoxyribonucleic acids [3] [4]. These N-atoms are also used as proton acceptors by enzymes bound specifically on DNA [5] or RNA [6]. Furthermore, purine N(7) atoms may play an active part in the catalytic activity of RNA.

Replacement of ribonucleoside residues within the so-called hammerhead ribozyme [7] [8] showed that functional groups at the bases [9–12] as well as on the sugar moiety [13–15] are essential for catalytic activity. Since the consensus sequence of the ribozyme (see boxed nucleosides in *Fig. 1*) contains three A residues within the sequence G-C-G-C-C-G-A-A-A-C-U-C-C-C, the replacement of adenosine (A; **2**) by tubercidin (c^7A; **1**) was



1 systematic numbering



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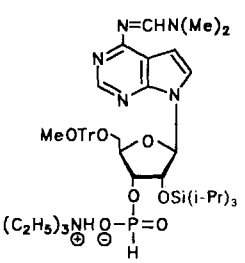

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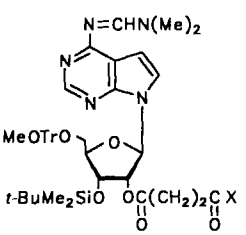

8a X = OH
b X = NH ~ CPG

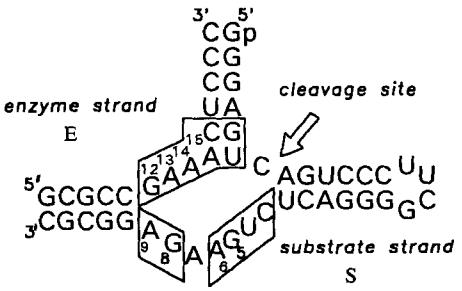

Fig. 1. *Sequence of the hammerhead ribozyme.* Nucleosides in boxes are conserved. The arrow denotes the site of autocatalyzed phosphodiester hydrolysis. Numbering according to a recently developed nomenclature [48].

considered. This may shed more light on the interactions occurring within loop structures of ribozymes and may lead to 'designer ribozymes' with enhanced or altered specificity. For this purpose, tubercidin phosphonate **7**, a building block applicable in solid-phase oligonucleotide synthesis, was prepared which can be used to incorporate c^7A as a structural probe in any position of an RNA fragment. Here, we describe the incorporation of c^7A into the G-A-A-A-C tract of the hammerhead ribozyme (*Fig. 1*) and the influence of this modification on the autocatalytic activity.

Results and Discussion. – *Synthesis of 7-Deazaadenosine Phosphonate* **7**. The synthesis of 2′,5′- and 3′,5′-linked dinucleoside monophosphates containing tubercidin instead of adenosine was described previously [16]. In these studies, which used oligonucleotide chemistry in solution, it appeared that the benzoyl group commonly used for protection of A is too stable for base protection of c^7A, and deblocking requires extended treatment with ammonia [16]. This was later confirmed for oligodeoxyribonucleotides [17].

It is well established that the internucleotide bond of oligoribonucleotides is sensitive to nucleophiles due to the anchimeric assistance of the 2′-hydroxy group [18]. Consequently, harsh ammonia treatment during oligoribonucleotide deprotection should be avoided in contrast to oligodeoxyribonucleotide deprotection. Recently, we employed the (dimethylamino)methylidene group for NH_2 protection in the case of c^7A_d [17]. The same group was now introduced into **1**. The starting material tubercidin (**1**) was synthesized by nucleobase anion glycosylation of 4-chloropyrrolo[2,3-*d*]pyrimidine with 5-*O*-[(1,1-dimethylethyl)dimethylsilyl]-2,3-*O*-(methylidene)-α-D-ribofuranosyl chloride as described earlier [19]. Reaction of **1** with *N*,*N*-dimethylformamide diethyl acetale in DMF gave crystalline **3** in 85% yield. The stability of the protecting group of **3** was tested in 25% NH_3 solution at 40° and led to a $t_{1/2}$ of 31 min. In comparison with the *N*-benzoyl-protected derivative ($t_{1/2}$ (c^7A_d) 65 min at 60° [17]), the (dimethylamino)methylidene (dam) group is *ca.* 10 times more labile than the benzoyl residue and, therefore, suitable for oligoribonucleotide synthesis. Treatment of **3** with (4-methoxytriphenyl)methyl chloride (MeOTrCl) in pyridine afforded **4** in 81% yield (for ^{13}C-NMR spectra, see *Table 1*).

The silylation of **4** with (*t*-Bu)Me_2SiCl under $AgNO_3$ catalysis was already described and afforded the 2′-*O*-silyl derivative in 61% yield, together with the 3′-*O*-isomer (17%) [20]. Recently, an influence of the alkylsilyl function and the base-protecting group on the 2′-*O*-selectivity was observed in the case of 7-deazaguanosine [18]. Therefore, the selectivity in silylation was studied on **4**. At first, standard conditions were used for silylation of

Scheme

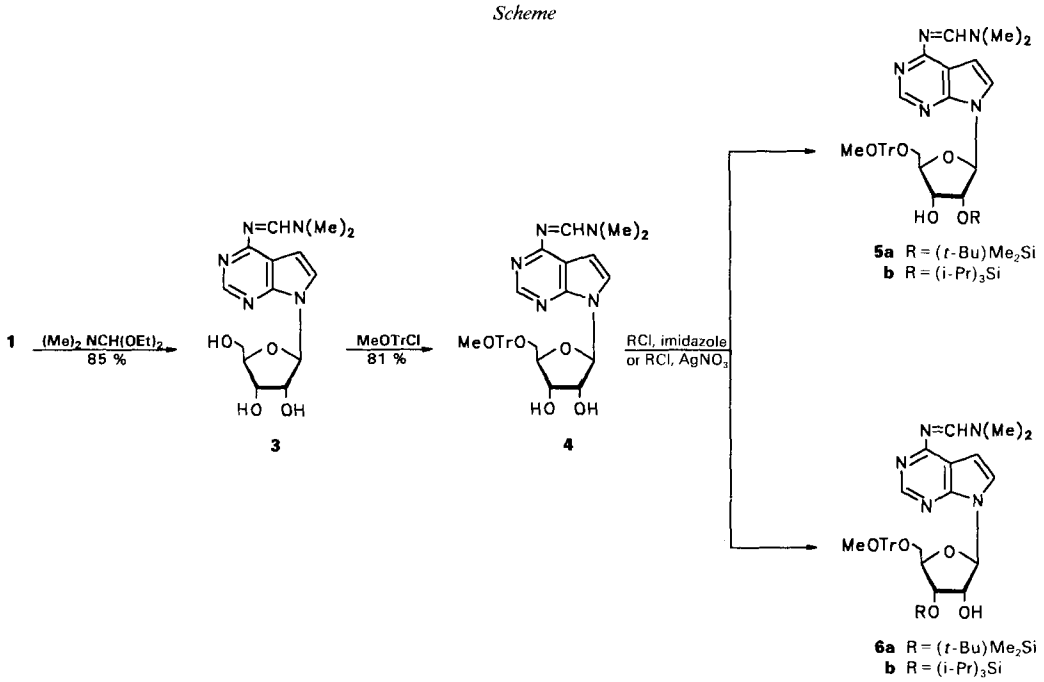

Table 1. *^{13}C-NMR Chemical Shifts* ((D$_6$)DMSO, 23°) *of 7-Deazaadenosine Derivatives.* Systematic numbering.

	C(2)	C(4)	C(4a)	C(5)	C(6)	C(7a)	CH	Me
3[a])	150.8	160.4	111.3	100.1	124.1	151.6	156.8	34.5
4	151.2	160.5	111.3	100.5	123.6	152.0	156.9	34.6
5a	151.2	160.5	111.2	100.7	123.7	152.0	156.9	34.5
6a	151.1	160.5	111.4	100.5	124.0	152.0	156.9	34.6
[b])	151.0	160.9	111.4	100.6	123.7	151.9	156.7	34.4
5b	151.1	160.4	111.3	100.8	123.4	152.2	156.8	34.5
6b	151.1	160.5	111.5	100.5	124.0	151.9	156.9	34.6
8a	151.2	160.6	111.6	100.8	125.1	152.6	156.9	34.6
7	151.1	160.3	111.3	100.8	122.9	152.4	156.8	34.5
	C(1′)	C(2′)	C(3′)	C(4′)	C(5′)	MeO	Me_3CSi or $(Me)_2CHSi$	NCH_2CH_3 or $(CH_2)_2CO_2H$
3[a])	87.2	73.7	70.7	85.0	61.7	–	–	–
4	87.3	73.7	70.5	82.5	64.1	55.1	–	–
5a	87.1	75.8	70.6	83.2	63.9	55.1	17.9	–
6a	87.5	72.9	72.4	82.9	63.5	55.1	18.1	–
[b])	86.8	74.5	72.6	83.5	63.2	55.0	17.7, 17.5	–
5b	87.4	75.4	71.0	83.4	63.9	55.1	11.6	–
6b	87.7	73.1	72.4	83.6	63.3	55.1	11.8	–
8a	86.7	74.4	69.8	82.2	62.6	55.1	17.7	30.0, 29.8
7	86.1	74.5	73.6	83.2	63.8	55.1	11.6	45.3

[a]) According to gated-decoupled spectra. [b]) 2,3-Di-*O*-silylated derivative (MeOTr)dam^{6}A(tbds)$_2^{2',3'}$ (dam = (dimethylamino)methylidene; tbds = (*t*-Bu)Me$_2$Si).

the amidine **4** employing (*t*-Bu)Me_2SiCl and imidazole as catalyst [22]. In this case, an isomeric ratio of **5a**/**6a**/2′,3′-di-*O*-silylated derivative (MeOTr)dam^{6}A(tbds)$^{2',3'}$ of 4.5:3:1 was obtained. Reaction of **4** with (*t*-Bu)Me_2SiCl and $AgNO_3$ [23] in pyridine/THF yielded the 2′-*O*-isomer **5a** in 71% as well as the 3′-*O*-isomer **6a** (17%). Thus, compared to the benzoyl-protected tubercidin (see above), the isomeric ratio was shifted in favor of the 2′-*O*-silylated compound by use of formamidine protection.

Investigations on 7-deazaguanosine [21] also showed that silylation with $AgNO_3$ and (i-Pr)$_3SiCl$ in combination with *N*,*N*-dimethylformamidine protection was best for high 2′-*O*-selectivity. Treatment of **4** with (i-Pr)$_3SiCl$ and $AgNO_3$ afforded compound **5b** in 80% yield, while the 3′-*O*-isomer **6b** was isolated in 8% yield. The 2′-*O*- *vs.* 3′-*O*-selectivity was 10:1 compared to 3.6:1 in the case of N^6-benzoyl-5′-(monomethoxytrityl)tubercidin [20].

The 2′- and 3′-*O*-silyl derivatives could be separated by TLC (*Table 2*). The 2′-*O*-isomer migrated faster as it was found in other cases [24]. The structure of the regioisomers **5a, b** and **6a, b** was confirmed by ^{1}H-NMR, ^{13}C-NMR, and NOE difference spectroscopy (*Tables 1* and *2*).

Table 2. *Chromatographic Mobilities and Selected NMR Data of the 2′- and 3′-*O*-Silyl Derivatives* **5a, b** *and* **6a, b**

	R_f[a])	δ (2′-OH) [ppm]	δ (3′-OH) [ppm]	δ (H–C(1′)) [ppm]	NOE (H–C(4′)) [%]	NOE (H–C(1′)) [%]
5a	0.40	–	5.04	6.19	3.5 (3′-OH)	–
6a	0.25	5.31	–	6.10	1.5 (MeSi)	3.5 (2′-OH)
5b	0.55	–	5.07	6.23	3.3 (3′-OH)	–
6b	0.40	5.31	–	6.10	–	2.3 (2′-OH)

[a]) Silica-gel TLC glass plates (*Sil G-25 UV_{254}, Merck*), elution with AcOEt.

In the ^{1}H-NMR spectra, the signal for OH–C(2′) of the 3′-*O*-regioisomers **6a, b** is always shifted more downfield compared to OH–C(3′) of the 2′-*O*-silylated nucleosides **5a, b**. Moreover, the H–C(1′) signal appears at lower ppm values for **6a, b**. In the ^{13}C-NMR spectra, the characteristic downfield shift for C(2′) and C(3′) is observed in the case of **5a, b** and **6a, b**, respectively (*Table 1*) [25] [26]. Using NOE difference spectroscopy for the determination of the silylation position (*Table 2*), a NOE at OH–C(3′) of 3% is obtained after irradiation of H–C(4′) of the 2′-*O*-protected **5a, b**, while irradiation of H–C(1′) led to no NOE, which is the opposite for the 3′-*O*-isomers **6a, b**.

Since ribonucleoside phosphonates show higher reactivity during the coupling reaction than phosphoramidites and are stable to hydrolysis and oxidation [27], compound **5b** was treated with PCl_3 and 1*H*-1,2,4-triazole in CH_2Cl_2 [28] affording the phosphonate **7** in 93% yield. It was characterized by ^{1}H-, ^{13}C-, and ^{31}P-NMR spectroscopy as well as by elemental analysis. Starting from tubercidin (**1**), the overall yield for the synthesis of phosphonate **7** was 51%.

Solid-Phase Synthesis of the Oligonucleotides **10–12** *and Influence of A* vs. c^7A *Replacement on the Hydrolysis of the Hammerhead Ribozyme* **9**. Solid-phase oligoribonucleotide synthesis was carried out on a *ABI* synthesizer using phosphonate **7** together with the phosphonates of the regular nucleosides [29] [30]. The cycle times for detritylation, coupling, activation, and oxidation followed the protocol recently published [21]. The trityl groups at the 5′-end of the oligomers were removed, and the oligomers **10–12** cleaved from the solid support. The base was deprotected with 25% NH_3/EtOH 3:1.

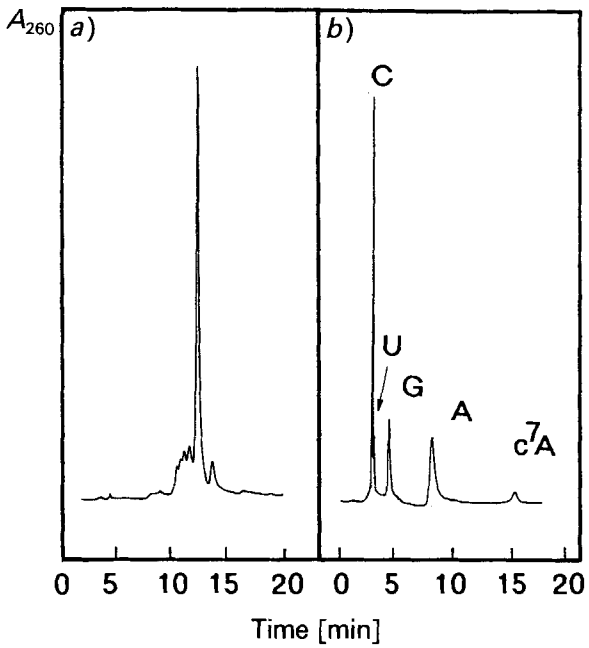

Fig. 2. *HPLC Profiles of G-C-G-C-C-G-A-c⁷A-A-C-U-C-C-C* ([(c⁷A)¹⁴]E; **11**) *obtained on a* RP-18 *column:* a) *crude oligomer after desilylation and desalting;* b) *after enzymatic hydrolysis with snake-venom phosphodiesterase followed by alkaline phosphatase.* Conditions, see *Exper. Part.*

Desilylation was accomplished with Bu_4NF/THF within 16 h at room temperature. Desalting with an anion-exchange cartridge resulted in almost pure oligomers (*Fig. 2a*). Further purification was carried out by reversed-phase HPLC. The purified oligomers were again desalted and lyophilized to a white powder. The compositions of the synthesized oligomers were confirmed by HPLC after enzymatic digestion (*Fig. 2b*).

G-C-G-C-C-G-A-A-A-C-U-C-C-C (E, **9**)

G-C-G-C-C-G-c⁷A-A-A-C-U-C-C-C ([(c⁷A)¹³]E, **10**)

G-C-G-C-C-G-A-c⁷A-A-C-U-C-C-C ([(c⁷A)¹⁴]E, **11**)

G-C-G-C-C-G-A-A-c⁷A-C-U-C-C-C ([(c⁷A)¹⁵]E, **12**)

The synthesis of oligoribonucleotides with tubercidin (**1**) at the 3′-end which are not described within this manuscript required the immobilization on the solid support. For this purpose the 3′-*O*-silylated compound **6a** was reacted with succinic anhydride to give **8a**. This was coupled with amino-linked controlled pore glass (CPG ~ NH_2) by a slightly modified procedure as described for 2′-deoxynucleotides yielding **8b** [31]. The loading was determined UV-spectrophotometrically after detritylation and amounted to 60 μmol/g.

RNA hairpins containing G-A-A-A loop have unusual stability partly due to a G·A base pair, which is formed by H-bonding of the exocyclic NH_2 group and N(3) in G to the NH_2 and N(7) of the last adenosine [1] (see **D** in *Fig. 3*). Since a G-A-A-A sequence is also present in the hammerhead ribozyme in a region not involved in a *Watson-Crick* duplex, G·A base pairing might be involved in formation of the secondary structure of this RNA.

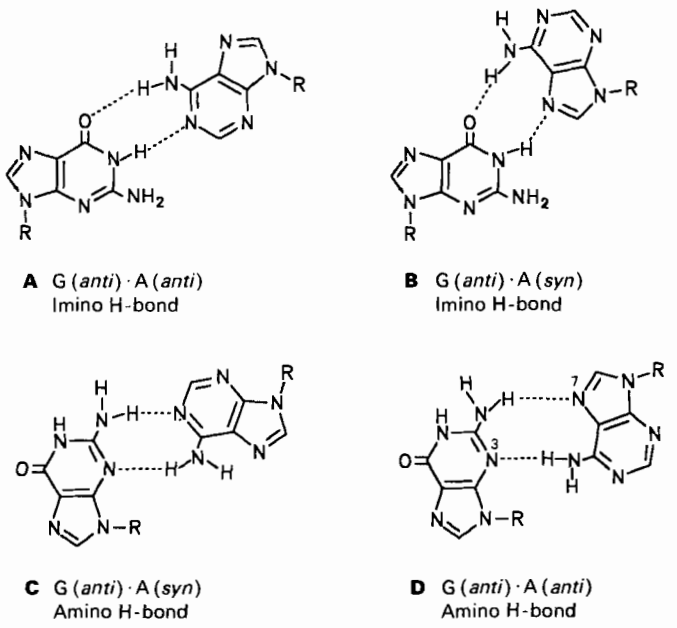

Fig. 3. *Possible structures for a G · A mismatch*

Recent studies on the replacement of selected G residues by inosine or A residues by nebularine in the hammerhead ribozyme showed that the structure may be stabilized by two intrastrand G · A base pairs [9] [12]. *Fig. 3* shows the four possible H-bonding schemes for a G · A mismatch.

To investigate the role of the adenine N(7) atoms in the structure and catalytic activity of the hammerhead ribozyme, we determined the relative phosphodiester hydrolysis rates using the oligonucleotides **10**–**12** or the parent oligomer E (**9**) together with the 35-mer substrate strand pG-G-G-A-G-U-C-A-G-U-C-C-C-U-U-C-G-G-G-G-A-C-U-C-U-G-A-A-G-A-G-G-C-G-C (S). The substrate strand S was synthesized as previously described [9] (*Fig. 1*). Stock solutions of the oligoribonucleotides were prepared and preheated to 90° for 1 min to disrupt aggregation states potentially formed during RNA storage [32]. The cleavage reactions were performed using 2 nM enzyme strand, *i.e.* **9**–**12**, in *Tris*-HCl buffer (50 mM, pH 7.5) containing $MgCl_2$ (10 mM). The mixtures were pre-incubated [33] and the reactions initiated by addition of [5′-^{32}P]-labeled substrate strand (20 nM) [34] and incubated at 25°. Within 120 min, several aliquots were removed, quenched by addition of stop mix (50 mM EDTA, 7M urea, 0.04% xylene xyanol, 0.04% bromophenol blue), and analyzed by polyacrylamide gel electrophoresis. Bands were visualized by autoradiography and analyzed by scanning densitometry. The data from these experiments are plotted in *Fig. 4*. From the initial velocities, the relative cleavage rates were calculated on the basis of first-order rate (see *Table 3*). It can be seen that the replacement of A at position 13 or 14 (A^{13} or A^{14}) by c^7A decreases the autocatalytic product formation 4-fold and 8-fold, respectively, whereas the incorporation of c^7A in position 15 slightly enhances the hydrolysis rate.

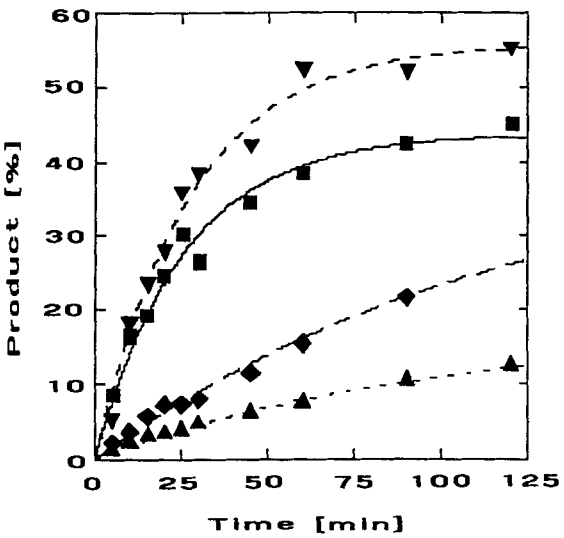

Fig. 4. *Time course of product formation upon autocatalytic phosphodiester hydrolysis of the substrate strand of the hammerhead ribozyme by the parent enzyme strand E* (**9**; ■) *and the modified enzyme strands [(c⁷A)¹³]E* (**10**; ◆), *[(c⁷A)¹⁴]E* (**11**; ▲) *and [(c⁷A)¹⁵]E* (**12**; ▼). Conditions, see *Exper. Part.*

Table 3. *Kinetic Parameters of Hammerhead Ribozyme Hydrolysis Containing the Parent* **9** *or the c⁷A-Modified Enzyme Strands* **10–12**[a])

Enzyme	k [min^{-1}]	k_{rel}
G-C-G-C-C-G-A-A-A-C-U-C-C-C (E; **9**)	0.206	1.00
G-C-G-C-C-G-c⁷A-A-A-C-U-C-C-C ([(c⁷A)¹³E]; **10**)	0.050	0.24
G-C-G-C-C-G-A-c⁷A-A-C-U-C-C-C ([(c⁷A)¹⁴E]; **11**)	0.026	0.13
G-C-G-C-C-G-A-A-c⁷A-C-U-C-C-C ([(c⁷A)¹⁵E]; **12**)	0.256	1.24

[a]) Measured in 50 mM *Tris*-HCl buffer containing 10 mM $MgCl_2$ at 25°; enzyme-strand concentration, 2 nM; substrate-strand concentration, 20 nM.

In parallel to our experiments, *Fu* and *McLaughlin* recently reported on the tubercidin modification in the A tract of another ribozyme structure and obtained similar results regarding the A[13] and A[14] residues located in the loop region of the A tract [35]. However, in contrast to our results, they also noted a decrease in autocatalytic activity when the A residue located in the stem region (position 15) was replaced by c⁷A. Here, a slightly enhanced cleavage rate was observed as it was found upon replacement of G[12] by (c⁷G)[12] [36]. An enhancement of enzymatic activity for c⁷A replacement of A was observed in another case, namely in the polymerization of a c⁷ADP/ADP mixture with polynucleotide phosphorylase; in this case c⁷A was more efficiently incorporated than A [37]. Differences observed in the values of k_{rel} for the autocatalytic activity of hammerhead ribozymes are not unusual. Ribozymes having only slightly different structures often have different kinetic parameters, and the effects of modifications are not always the same. *E.g.*, quite different effects were observed for deoxynucleotide substitution

[38–40]. Nevertheless, the discrepancy in the effect of $(c^7A)^{15}$ substitution of A^{15} between our results and those of *Fu* and *McLaughlin* [35] is surprising. One possible explanation is that in their case the N^6-benzoyl group was not completely removed during ammonia deprotection.

Considering the different physical properties of 7-deazaadenosine (**1**) compared to adenosine (**2**), the relatively small effects on ribozyme-catalytic activity on replacement of A by c^7A ($[(c^7A)^{13}]E$, $[(c^7A)^{14}]E$) are also surprising since the nucleosides **1** and **2** show many differences in their physicochemical behavior. The higher π-electron density of **1** leads to a higher pK value (5.3) compared to **2** (3.5) [41]. Moreover, the exocyclic NH_2 group of **1** is more basic and, therefore, a less efficient proton donor, a phenomenon which destabilizes the $c^7A \cdot U$ base pairs in homopolymers [42]. These properties are compensated, in part, by the higher basicity of N(1) of **1** and, therefore, the better proton-acceptor properties of this N-atom. ^{15}N-NMR Experiments showed that N(1) is the exclusive proton acceptor site of **1** [19]. In addition, the altered N-pattern of **1** results in differences in the strength and direction of the dipole moment. Finally, **1** is more hydrophobic than **2** and has a shorter and extremely stable N-glycosylic bond [43]. All these properties may stabilize or destabilize the A tract in the hammerhead ribozyme, depending on the position of incorporation, and decrease or enhance the autocatalytic activity.

In conclusion, the replacement of A residues by c^7A within the A tract of the enzyme strand of the hammerhead ribozyme is not a severe event with respect to autocatalytic activity. Depending on the position of substitution, the activity is not drastically reduced, and in one case, it is even enhanced. This suggests that N(7) of the adenosine residues A^{13}, A^{14}, and A^{15} do not play a vital role within the ribozyme structure. As the base-pairing patterns **A** and **B** for the G·A mismatch (see *Fig. 3*) can be excluded from NMR experiments [44] [45], only interactions as shown in **C** can be discussed. With regard to the synthesis of 'designer ribozymes' with altered catalytic activity which were recently developed by *in-vitro* selection [46], 7-deazapurines may be good candidates for incorporation.

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Experimental Part

General. See [21]. Flash chromatography (FC) and TLC: solvent systems CH_2Cl_2/MeOH 9:1 (*A*), AcOEt (*B*), light petroleum ether/AcOEt/Et_3N 10:88:2 (*C*), MeCN/H_2O 9:1 (*D*), CH_2Cl_2/Et_3N 98:2 (*E*), and CH_2Cl_2/MeOH/Et_3N 88:10:2 (*F*). Prep. HPLC: *reversed-phase RP-18 Lichrosorp* column (250 × 4 cm); *Merck-Hitachi* HPLC apparatus with one pump (model *655-A-12*) connected with a proportioning valve, a variable-wavelength monitor (model *655 A*), a controller (model *L-5000*), and an integrator (model *D-2000*); solvent systems consisting of 0.1M $(Et_3NH)OAc$ (sterile, pH 7.5)/MeCN 95:5 (I) and MeCN (II); gradient, 0–20% II in I within 20 min. Microanalyses were performed by '*Mikroanalytisches Labor Beller*', Göttingen, Germany.

*4-{[(Dimethylamino)methylidene]amino}-7-(β-*D*-ribofuranosyl)-7*H*-pyrrolo[2,3-*d*]pyrimidine* (**3**). Under Ar, **1** (266 mg, 1 mmol) in DMF (5 ml) was stirred with *N,N*-dimethylformamide diethyl acetale (2 ml) for 1 h at 50°. After evaporation, the residue was co-evaporated with MeOH and applied to FC (silica gel, column 20 × 3 cm, *A*): **3** (273 mg, 85%). Pale yellow crystals (acetone). M.p. 164°. TLC (*A*): R_f 0.60. UV (MeOH): 223 (18500), 260 (11900), 312 (23800). ^{1}H-NMR ((D_6)DMSO): 3.12, 3.17 (2*s*, Me_2N); 3.6 (*m*, H–C(5′)); 3.89 (*m*, H–C(4′)); 4.10 (*m*, H–C(3′)); 4.44 (*m*, H–C(2′)); 5.13 (*d*, OH–C(3′)); 5.23 (*t*, OH–C(5′)); 5.33 (*d*, OH–C(2′)); 6.08 (*d*, H–C(1′)); 6.55

(*d*, H–C(5)); 7.51 (*d*, H–C(6)); 8.33 (*s*, H–C(2)); 8.83 (*s*, N=CH). Anal. calc. for $C_{14}H_{19}N_5O_4$ (321.3): C 52.34, H 5.96, N 21.79; found: C 52.18, H 6.16, N 21.78.

*4-{[(Dimethylamino)methylidene]amino}-7-{5-O-[(4-methoxytriphenyl)methyl]-β-*D*-ribofuranosyl}-7H-pyrrolo[2,3-*d*]pyrimidine* (**4**). To a soln. of **3** (320 mg, 1 mmol) in pyridine (10 ml) were added $(i\text{-}Pr)_2EtN$ (650 µl, 4 mmol) and (4-methoxytriphenyl)methyl chloride (463 mg, 1.5 mmol). After stirring at 50° for 3 h, MeOH (10 ml) and 5% aq. $NaHCO_3$ soln. (10 ml) were added. The aq. layer was extracted 3 times with CH_2Cl_2, the org. layer dried (Na_2SO_4) and evaporated, and the residue applied to FC (column 30 × 3 cm, *A*): colorless amorphous **4** (480 mg, 81%). TLC (*A*): R_f 0.45. UV (MeOH): 232 (37200), 256 (19200), 312 (24400). ^{1}H-NMR ((D$_6$)DMSO): 3.1 (*m*, H–C(5′)); 3.13, 3.19 (2*s*, Me_2N); 3.73 (*s*, MeO); 4.05 (*m*, H–C(4′)); 4.21 (*m*, H–C(3′)); 4.48 (*m*, H–C(2′)); 5.20 (*d*, OH–C(3′)); 5.45 (*d*, OH–C(2′)); 6.18 (*d*, H–C(1′)); 6.55 (*d*, H–C(5)); 6.87–7.42 (*m*, MeO*Tr*, H–C(6)); 8.34 (*s*, H–C(2)); 8.85 (*s*, N=CH). Anal. calc. for $C_{34}H_{35}N_5O_5$ (593.7): C 68.79, H 5.94, N 11.80; found: C 68.62, H 6.07, N 11.74.

*4-{[(Dimethylamino)methylidene]amino}-7-{2-O-[(1,1-dimethylethyl)dimethylsilyl]-5-O-[(4-methoxytriphenyl)methyl]-β-*D*-ribofuranosyl}-7H-pyrrolo[2,3-*d*]pyrimidine* (**5a**). To a soln. of **4** (146 mg, 0.25 mmol) in dry pyridine (5 ml) was added $AgNO_3$ (64 mg, 0.38 mmol) and, after 5 min, (*t*-Bu)Me_2SiCl (38 mg, 0.25 mmol) in dry THF (10 ml) under exclusion of light and moisture. After 10 h stirring, again (*t*-Bu)Me_2SiCl (20 mg, 0.13 mmol) was added and stirring continued for another 14 h. AgCl was filtered off and the filtrate treated with 5% aq. $NaHCO_3$ soln. (20 ml). The aq. layer was extracted twice with CH_2Cl_2, the combined org. layer dried (Na_2SO_4) and evaporated, and the residue applied to FC (column 30 × 2 cm, *B*). From the slower migrating zone, **6a** (30 mg, 17%) was isolated, and from the faster one **5a** (125 mg, 71%). Colorless amorphous solid. TLC (*B*): R_f 0.4. UV (MeOH): 232 (36600), 258 (18600), 311 (23800). ^{1}H-NMR ((D$_6$)DMSO): −0.07, 0.2 (2*s*, Me_2Si); 0.71 (*s*, *t*-BuSi); 3.12, 3.17 (2*s*, Me_2N); 3.2 (*m*, H–C(5′)); 3.74 (*s*, MeO); 4.07 (*m*, H–C(4′)); 4.14 (*m*, H–C(3′)); 4.60 (*m*, H–C(2′)); 5.04 (*d*, OH–C(3′)); 6.19 (*d*, H–C(1′)); 6.54 (*d*, H–C(5)); 7.45–6.85 (*m*, 14 arom. H, H–C(6)); 8.32 (*s*, H–C(2)); 8.84 (*s*, N=CH). Anal. calc. for $C_{40}H_{49}N_5O_5Si$ (707.9): C 67.86, H 6.98, N 9.89; found: C 67.86, H 6.95, N 9.79.

*4-{[(Dimethylamino)methylidene]amino}-7-{3-O-[(1,1-dimethylethyl)dimethylsilyl]-5-O-[(4-methoxytriphenyl)methyl]-β-*D*-ribofuranosyl}-7H-pyrrolo[2,3-*d*]pyrimidine* (**6a**). A soln. of **4** (146 mg, 0.25 mmol) in pyridine (5 ml) was treated with 1*H*-imidazole (45 mg, 0.66 mmol) and (*t*-Bu)Me_2SiCl (50 mg, 0.33 mmol). The mixture was stirred for 24 h and the reaction stopped by addition of sat. aq. $NaHCO_3$ soln. (20 ml). Workup as described for **5a** and FC (column 30 × 3 cm, *B*) gave 3 zones. From the 2nd zone, **5a** (72 mg, 41%) was obtained. Evaporation of the slowest migrating zone yielded **6a** (50 mg, 28%). Colorless solid. TLC (*B*): R_f 0.25. UV (MeOH): 232 (36600), 258 (18700), 311 (24300). ^{1}H-NMR ((D$_6$)DMSO): 0.01, 0.05 (2*s*, Me_2Si); 0.82 (*s*, *t*-BuSi); 3.08, 3.16 (2*s*, Me_2N); 3.4 (*m*, H–C(4′)); 3.72 (*s*, MeO); 3.97 (*m*, H–C(4′)); 4.36 (*m*, H–C(3′)); 4.58 (*m*, H–C(2′)); 5.29 (*d*, OH–C(2′)); 6.08 (*d*, H–C(1′)); 6.53 (*d*, H–C(5)); 6.83–7.43 (*m*, 14 arom. H, H–C(6)); 8.31 (*s*, H–C(2)); 8.81 (*s*, N=CH). Anal. calc. for $C_{40}H_{49}N_5O_5Si$ (707.9): C 67.86, H 6.98, N 9.89; found: C 67.86, H 7.02, N 9.98.

*4-{[(Dimethylamino)methylidene]amino}-7-{2,3-O-bis[(1,1-dimethylethyl)dimethylsilyl]-5-O-[(4-methoxytriphenyl)methyl]-β-*D*-ribofuranosyl}-7H-pyrrolo[2,3-*d*]pyrimidine* ((MeOTr)dam^{6}A(tbds)$_2^{2',3'}$). Evaporation of the fast migrating zone (see **6a**) yielded **7a** (18 mg, 8.8%). Colorless foam. TLC (*B*): R_f 0.60. UV (MeOH): 232 (37200), 258 (18900), 311 (24200). ^{1}H-NMR ((D$_6$)DMSO): −0.45, −0.18, −0.05, 0.00 (4*s*, 2 Me_2Si); 0.64, 0.80 (2*s*, 2 *t*-BuSi); 3.06, 3.12 (2*s*, Me_2N); 3.69 (*s*, MeO); 3.99 (*m*, H–C(4′)); 4.18 (*m*, H–C(3′)); 4.68 (*m*, H–C(2′)); 6.06 (*d*, H–C(1′)); 6.51 (*d*, H–C(5)); 6.80–7.39 (*m*, 14 arom. H, H–C(6)); 8.23 (*s*, H–C(2)); 8.78 (*s*, N=CH). Anal. calc. for $C_{46}H_{63}N_5O_5Si_2$ (822.2): C 67.20, H 7.72, N 8.52; found: C 67.32, H 7.91, N 8.41.

*4-{[(Dimethylamino)methylidene]amino}-7-{5-O-[(4-methoxytriphenyl)methyl]-2-O-[tris(1-methylethyl)silyl]-β-*D*-ribofuranosyl}-7H-pyrrolo[2,3-*d*]pyrimidine* (**5b**). As described for **5a**, with **4** (146 mg, 0.25 mmol), pyridine (5 ml), $AgNO_3$ (65 mg, 0.38 mmol), $(i\text{-}Pr)_2SiCl$ (50 µl, 0.25 mmol), THF (5 ml), and $(i\text{-}Pr)_3SiCl$ (26 µl, 0.13 mmol; stirring for 20 h). After FC (column 30 × 2 cm, *C*), evaporation of the faster migrating zone yielded **5b** (150 mg, 80%). Colorless solid. TLC (*B*): R_f 0.55. UV (MeOH): 231 (37500), 257 (19100), 311 (23900). ^{1}H-NMR ((D$_6$)DMSO): 0.81–0.91 (*m*, i-PrSi); 3.11, 3.17 (2*s*, Me_2N); 3.3 (*m*, H–C(5′)); 3.74 (*s*, MeO); 4.09 (*m*, H–C(4′)); 4.20 (*m*, H–C(3′)); 4.80 (*m*, H–C(2′)); 5.07 (*d*, OH–C(3′)); 6.23 (*d*, H–C(1′)); 6.54 (*d*, H–C(5)); 6.88–7.44 (*m*, 14 arom. H, H–C(6)); 8.30 (*s*, H–C(2)); 8.84 (*s*, N=CH). Anal. calc. for $C_{43}H_{55}N_5O_5Si$ (750.0): C 68.86, H 7.39, N 9.34; found: C 68.85, H 7.48, N 9.33.

*4-{[(Dimethylamino)methylidene]amino}-7-{5-O-[(4-methoxytriphenyl)methyl]-3-O-[tris(1-methylethyl)silyl]-β-*D*-ribofuranosyl}-7H-pyrrolo[2,3-*d*]pyrimidine* (**6b**). From the slower migrating zone (see **5b**), **6b** (15 mg, 8%) was obtained. Colorless foam. TLC (*B*): R_f 0.4. UV (MeOH): 231 (37200), 257 (18600), 312 (24300). ^{1}H-NMR ((D$_6$)DMSO): 1.0–1.1 (*m*, i-PrSi); 3.10, 3.13 (2*s*, Me_2N); 3.4 (*m*, H–C(5′)); 3.71 (*s*, MeO); 4.02 (*m*, H–C(4′)); 4.50 (*m*, H–C(3′)); 4.63 (*m*, H–C(2′)); 5.31 (*d*, OH–C(2′)); 6.10 (*d*, H–C(1′)); 6.54 (*d*, H–C(5));

6.82–7.41 (*m*, 14 arom. H, H–C(6)); 8.30 (*s*, H–C(2)); 8.81 (*s*, N=CH). Anal. calc. for $C_{43}H_{55}N_5O_5Si$ (750.0): C 68.86, H 7.39, N 9.34; found: C 68.78, H 7.36, N 9.31.

4-{[(Dimethylamino)methylidene]amino}-7-{3-O-[(1,1-dimethylethyl)dimethylsilyl]-5-O-[(4-methoxytriphenyl)methyl]-β-D-ribofuranosyl}-7H-pyrrolo[2,3-d]pyrimidine 2-Succinate (**8a**). A soln. of **6a** (71 mg, 0.1 mmol) in pyridine (2 ml) was treated with 4-(dimethylamino)pyridine (6 mg, 50 μmol), Et_3N (83 μl, 0.6 mmol), and succinic anhydride (30 mg, 0.3 mmol). The mixture was stirred for 3 days at r.t., evaporated, and co-evaporated with toluene. The residue was dissolved in CH_2Cl_2, the org. layer washed with 5% aq. $NaHCO_3$ soln., dried (Na_2SO_4), and evaporated, and the residue applied to FC (column 20 × 2 cm, *D*). Evaporation of the main zone yielded **8a** (60 mg, 74%). Colorless solid. TLC (*D*): R_f 0.4. UV (MeOH): 232 (36500), 258 (18300), 311 (23800). ^{1}H-NMR ((D$_6$)DMSO): 0.00, 0.07 (2*s*, Me_2Si); 0.82 (*s*, *t*-BuSi); 2.4 (*m*, 2 CH_2); 3.1 (*m*, H–C(5′)); 3.15, 3.21 (2*s*, Me_2N); 3.72 (*s*, MeO); 4.01 (*m*, H–C(4′)); 5.04 (*m*, H–C(3′)); 5.94 (*m*, H–C(2′)); 6.27 (*d*, *J* = 5.3, H–C(1′)); 6.62 (*d*, *J* = 3.5, H–C(5)); 6.86–7.52 (*m*, 14 arom. H, H–C(6)); 8.41 (*s*, H–C(2)); 8.87 (*s*, N=CH).

4-{[(Dimethylamino)methylidene]amino}-7-{5-O-[(4-methoxytriphenyl)methyl]-2-O-[tris(1-methylethyl)-silyl]-β-D-ribofuranosyl}-7H-pyrrolo[2,3-d]pyrimidine 3-(Triethylammonium Phosphonate) (**7**). To a soln. of PCl_3 (43 μl, 0.5 mmol) and *N*-methylmorpholine (543 μl, 5.0 mmol) in dry CH_2Cl_2 (5 ml) was added 1*H*-1,2,4-triazole (264 mg, 3.82 mmol). After 30 min stirring at r.t., the mixture was cooled to 0° and a soln. of **5b** (75 mg, 0.1 mmol) in dry CH_2Cl_2 (2 ml) added dropwise over 10 min. The mixture was stirred for another 20 min at 0° and then hydrolyzed with 1M aq. $(Et_3NH)HCO_3$ (*TBK*, pH 7.5; 10 ml). The aq. layer was extracted twice with 5 ml of CH_2Cl_2, and the org. layer dried (Na_2SO_4) and evaporated. Chromatography (column 30 × 2 cm, *E* (150 ml), then *F* (250 ml)) yielded, after washing with 0.1M *TBK* (6 × 5 ml), drying (Na_2SO_4), and co-evaporation with acetone, **7** (85 mg, 93%). Colorless foam. TLC (silica gel, *F*): R_f 0.70. UV (MeOH): 232 (36800), 257 (18800), 312 (24200). ^{1}H-NMR ((D$_6$)DMSO): 0.74–0.86 (*m*, i-PrSi); 1.2 (*m*, CH_3CH_2); 3.0 (*m*, CH_3CH_2); 3.10, 3.16 (2*s*, Me_2N); 3.3 (*m*, H–C(5′)); 3.73 (*s*, MeO); 4.32 (*m*, H–C(4′)); 4.62 (*m*, H–C(3′)); 4.92 (*m*, H–C(2′)); 6.25 (*d*, H–C(1′)); 6.53 (*d*, H–C(5)); 6.74 (*d*, H–P); 6.85–7.43 (*m*, arom. H, H–C(6)); 8.26 (*s*, H–C(2)); 8.82 (*s*, N=CH). ^{31}P-NMR ((D$_6$)DMSO): 2.37. Anal. calc. for $C_{49}H_{71}N_6OPSi$ (915.2): C 64.31, H 7.82, N 9.18; found: C 63.74, H 7.82, N 8.92.

CPG-Linked Tubercidin Derivative **8b** *from* **8a**. A soln. of **8a** (84 mg, 0.1 mmol) in 1,4-dioxane (5% pyridine; 2 ml) was treated with 4-nitrophenol (21 mg, 0.15 mmol) and dicyclohexylcarbodiimide (41 mg, 0.2 mmol). The mixture was stirred overnight and dicyclohexylurea filtered off. The filtrate was added to a suspension of amino-linked silica gel (*Fractosil 200*/450 μmol NH_2/g; *Merck*) in dry DMF (2 ml). After shaking for 4 h, Ac_2O (60 μl) was added and shaking continued for another 30 min. Silica gel **8b** was filtered off, washed with DMF, EtOH, and Et_2O, and dried *in vacuo*. The amount of covalently linked nucleoside **8a** was determined after the release of monomethoxytrityl cation from the support (5 mg). Upon treatment with 0.1M TsOH in MeCN (1 ml), the loading was 60 μmol/g modified CPG.

Solid-Phase Synthesis of the Oligoribonucleotides **10–12**. See [21]. Compounds **10–12** were synthesized using the phosphonates of [(MeO)$_2$Tr]bz^{6}Atbds$^{2'}$, [(MeO)$_2$Tr]ib^{2}Gtbds$^{2'}$, [(MeO)$_2$Tr]bz^{4}Ctbds$^{2'}$, [(MeO)$_2$Tr]Utbds$^{2'}$ which were commercial products of *Chem Genes* (USA) and **7**. CPG supports of the unmodified ribonucleosides were purchased from *Milligen* (Eschborn, Germany). Oligoribonucleotide synthesis was carried out on an automated DNA synthesizer, model *380 B* (*Applied Biosystems*, Weiterstadt, Germany) on a 1-μmol scale using a synthesis cycle described earlier [21]. The oligomers were synthesized with deblocking of the OH group at the 5′-end and were cleaved from the solid support with 25% NH_3/EtOH 3:1.

Deprotection and Purification of Synthetic Oligoribonucleotides. See [21]. Deprotection of the base was carried out by treatment with 25% NH_3/EtOH 3:1 at 50° for 16 h. The soln. was evaporated and co-evaporated with abs. EtOH. Removing of the silyl groups was accomplished by treatment with 1.1M Bu_4NF/THF (1 ml; *Aldrich*, USA) for 16 h at r.t. All H_2O used for further purification was sterilized by autoclaving (120°, 2 h) or filtration through a *PV 050/3 Vacuflo* filtration apparatus (*Schleicher & Schüll*, Germany). All glass- and plasticware used for the deprotected oligoribonucleotides was autoclaved. Desilylation was stopped by addition of 0.1M $(Et_3NH)HCO_3$ (*TBK*; 10 ml, pH 7.0) followed by desalting using a *Diagen*-tip-*500* anion-exchange cartridge (*Diagen*, Düsseldorf, Germany). The oligonucleotides were eluted with 2M *TBK* buffer (pH 8.0), evaporated, and dissolved in 500 μl of sterile H_2O. Further purification was performed by HPLC. To avoid the formation of secondary structures, the oligoribonucleotides were heated to 95°, quickly cooled down to 0°, and injected. The main peak was collected, evaporated to 5 ml. An *Oligo-Pak* cartridge (*Millipore*, Germany; autoclaved) was prewashed with MeCN, 0.05M $(Et_3NH)OAc$ (pH 7.0)/MeCN 1:1 and 0.05M $(Et_3NH)OAc$ (5 ml each).

The soln. from HPLC purification was applied to the cartridge washed with 0.05M $(Et_3NH)OAc$ (5 ml). Then the oligoribonucleotides were eluted with MeOH/MeCN/H_2O 1:1:1 (5 ml) and dried on a *Speed-Vac* concentrator to a white powder.

Enzymatic Hydrolysis of the Oligoribonucleotides. The oligomers (0.3 A_{260} units) were dissolved in 0.1M *Tris*-HCl buffer (pH 8.3, 200 µl) and treated with snake-venom phosphodiesterase (EC 3.1.4.1, *Crotalus durissus*; *Boehringer Mannheim*, Germany; 6 µg) at 37° for 45 min and alkaline phosphatase (EC 3.1.3.1, calf intestine; *Boehringer Mannheim*, Germany; 2 µg) for 30 min at 37°. The mixture was analyzed by reversed-phase HPLC (*RP-18*, solvent I): t_R 3.7 (C), 4.2 (U), 6.4 (G), 8.8 (A), 14.2 (c^7A) min. Quantification was made on the basis of the peak areas which were divided by the extinction coefficient of the nucleosides (ε_{260}: 15300 (A), 7600 (C), 12200 (G), 10200 (U), 9100 (c^7A)).

Autocatalytic Ribozyme Phosphodiester Hydrolysis. Stock solns. of enzyme (20 nM) in *Tris*-HCl buffer (50 mM, pH 7.5) and [^{32}P]-labelled substrate [34] (200 nM) were preheated separately to 90° for 1 min and then cooled to 25°. After 15 min, the reaction was started with $MgCl_2$ (10 mM final concentration). After preincubation for 15 min at 25°, the cleavage reactions were performed using the following concentration: 20 nM of the substrate strand S, 2 nM of the ribozyme strands **9–12**, 50 mM *Tris*-HCl (pH 7.5), and 10 mM $MgCl_2$. Aliquots (10 µl) were removed after 5, 10, 15, 20, 25, 30, 45, 90 and 120 min and quenched by addition of stop mix (20 µl; 7M urea, 50 mM EDTA, 0.04% bromophenol blue, 0.04% xylencyanol in H_2O). Products were separated by 20% denaturating polyacrylamide gel electrophoresis [47] and bands visualized by autoradiography. Autoradiographs were scanned using a molecular-dynamics laser scanning densitometer.

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