Synthesis and Enzymic Hydrolysis of Oligoribonucleotides Incorporating 3-Deazaguanosine: The Importance of the Nitrogen-3 Atom of Single Conserved Guanosine Residues on the Catalytic Activity of the Hammerhead Ribozyme

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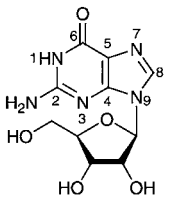
Four base-modified hammerhead ribozyme/substrate complexes were constructed in which single guanosine (**1**) residues were replaced by 3-deazaguanosine (**2**) in the positions G_5, G_8, $G_{L2.1}$, and G_{12}. The base-modified ribozyme complexes were prepared by solid-phase synthesis of oligoribonucleotides employing the novel phosphoramidite **3** derived from **2**. Phosphoramidite **3** carried a phenoxyacetyl group at the amino function and a diphenylcarbamoyl residue at the oxo group of the nucleobase. The 2′-hydroxy group was blocked with a triisopropylsilyl residue. Kinetic analysis of the phosphodiester hydrolysis showed a moderate decrease of the ribozyme catalytic activity when the residues G_5 or G_8 were replaced by 3-deazaguanosine and a 200-fold decrease when G_{12} was substituted. A 6-fold catalytic increase occurred when 3-deazaguanosine was replacing $G_{L2.1}$ in the loop region. The data indicate that the N(3) atom of compound **2**, in particular at position G_{12} is critical for the ribozyme activity.

Introduction. – The hammerhead ribozyme is one of the smallest catalytic RNAs. It has a small catalytic core consisting of ten conserved nucleotides together with interchangeable recognition arms to achieve the sequence-specific cleavage of any target RNA [1–3]. As a result, the transesterification reaction generates two products, a 2′,3′-cyclic phosphate and a 5′-terminal hydroxy group at the cleavage site. The cleavage site was originally defined by a NUH pattern, where N can be any nucleoside and H – the cleavage site of the nucleotide – can be A, U, or C with the most-efficient cleavage occurring at the GUC triplet. This pattern was defined later as the NHH rule, since other triplets such as GAC and GCC can be also cleaved by the hammerhead ribozyme [4]. The hammerhead catalytic activity requires divalent metal ions such as Mg^{2+} or high concentrations of monovalent cations [5]. The precise molecular mechanism of action is not yet defined despite the fact that several X-ray structures of the hammerhead ribozyme with non-cleavable substrates [6–8] and crystallographic studies with trapped intermediates resembling the transition state were performed [9]. The general structure is consistent with the solution studies performed by FRET analysis [10–12]. However, significant Mg^{2+}-dependent conformational mobility of the ribozyme has been detected by time-resolved fluorescence measurements and NMR [13][14]. The structural modification with modified nucleotide analogs identified a number of important functional atoms or groups being involved in the hammerhead catalysis (reviewed in [15][16]). To probe the importance of the N(3) atom of the

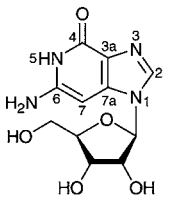
conserved guanosine (**1**) residues of the core region as well as those of the GA platform in the GNRA tetraloop, nucleoside **1** was replaced by 3-deazaguanosine[1]) (**2**) thus eliminating specific proton-acceptor sites that may be involved in critical interactions mediating the folding and the catalysis by the hammerhead ribozyme.

To accomplish this replacement within the hammerhead-ribozyme complex, the 3-deazaguanosine phosphoramidite **3** was synthesized and employed in solid-phase synthesis for the single nucleoside modification of particular guanosine residues. The catalytic activity of the native and the mutated hammerhead-ribozyme complexes was compared and demonstrated the importance of the N(3) position of the G_{12} residue for the hammerhead catalysis. Surprisingly, the replacement of guanosine (**1**) by 3-deazaguanosine (**2**) within the loop sequence ($G_{L2.1}$) resulted in a significant increase in the catalytic activity of the hammerhead ribozyme.



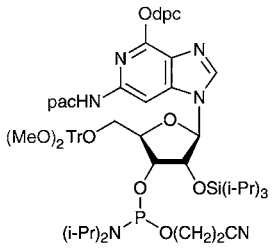
Purine numbering

1



Systematic numbering

2



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Results and Discussion. – 1. *Monomers.* Oligo-2′-deoxyribonucleotides containing 3-deazaguanine have been already synthesized with H-phosphonate of 3-deaza-2′-deoxyguanosine [17]. Alternatively, phosphoramidite chemistry was employed with appropriately functionalized imidazole nucleosides as precursors, which were converted later to the 3-deazaguanine with ammonia containing oligonucleotide on the polymeric level [18]. Oligoribonucleotides incorporating 3-deazaguanosine (**2**) are unknown. For this synthesis, the phosphoramidite building block **3** of the nucleoside **2** was prepared that allows site-specific incorporation of a 3-deazaguanosine moiety into any position of an oligoribonucleotide by standard solid-phase chemistry.

The 3-deazaguanosine (**2**) has already been prepared in 1975 by the *Robins* group [19][20]. This route used ammonia for the six-membered-ring formation. In an alternative route, hydrazine was employed [21]. *Minakawa* and *Matsuda* reported a synthesis that started from AICA-riboside *via* 5-ethynyl-1-(β-D-ribofuranosyl)-1*H*-imidazole-4-carboxamide or -4-carbonitrile [22]. We followed the synthetic route of *Robins* and co-workers. *Vorbrüggen* glycosylation of the trimethylsilyl derivative of methyl 5(4)-(cyanomethyl)-1*H*-imidazole-4(5)-carboxylate (**4**) with 1-*O*-acetyl-2,3,5-tri-*O*-benzoyl-β-D-ribofuranose (**5**) in MeCN in the presence of tin(IV) chloride afforded the intermediate **6** (*Scheme 1*). Treatment of **6** with saturated methanolic ammonia furnished nucleoside **2** [20].

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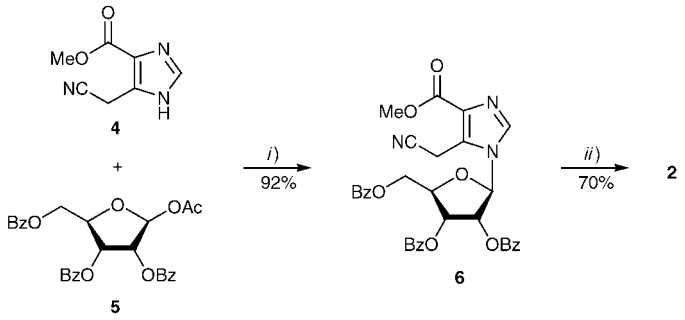
[1]) Purine numbering is used throughout the *Results and Discussion* section; for systematic numbering, see *Exper. Part.*

Scheme 1



i) HMDS (hexamethyldisilazane), $(NH_4)_2SO_4$, 4 h, reflux, $SnCl_4$, MeCN, 3 h, r.t. *ii*) NH_3/MeOH, 48 h, 120°, autoclave. DMTr = $(MeO)_2$ Tr = $(4\text{-}MeOC_6H_4)$PhC.

Compound **2** is more π-electron-rich than the parent guanosine. This is apparent from the pK_a values of protonation and deprotonation of **2**, which are 2.7 (N(7)) and 12.3 (N(1)), respectively [23]. The parent guanosine (**1**) shows pK_a values of 1.9 and 9.2 [23]. The lipophilic character of **2** is decreased over that of **1** as it is found for 7-deazaguanosine and demonstrated by the chromatographic mobility on a reversed-phase (*RP-18*) column. The corresponding 2′-deoxyribonucleosides show similar behavior (*Fig. 1,a*). Differences of **1** and **2** are observed in their CD spectra (*Fig. 1,b*). While the positive lobes observed between 215 and 220 nm are similar for **1** and its 3-deaza analogue **2**, only the latter shows a significant negative trough at 270 nm.

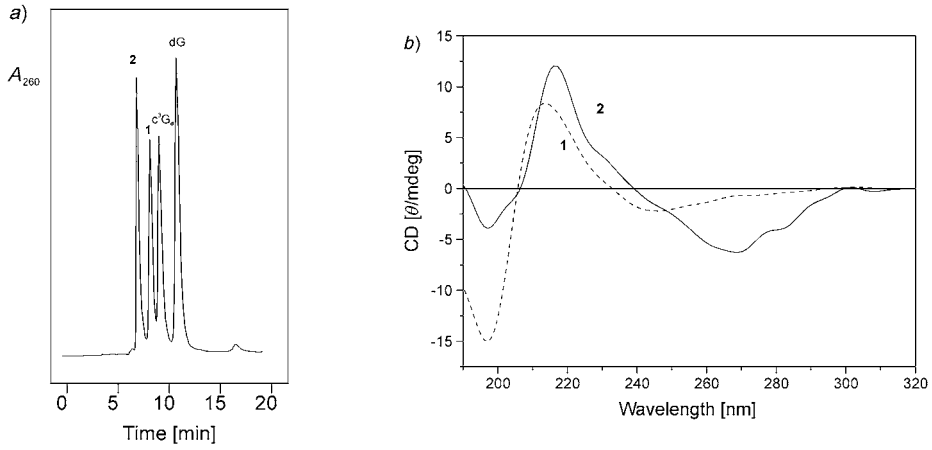
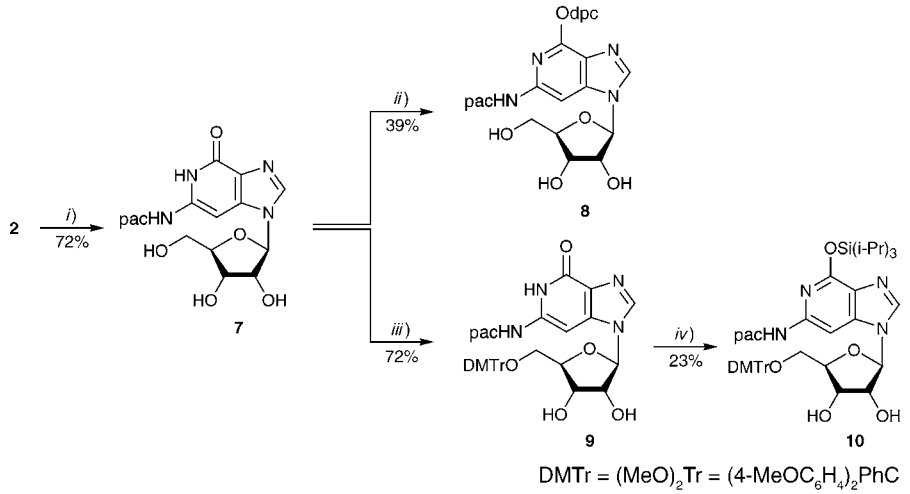


Fig. 1. a) *HPLC Profiles of* c^3G (**2**) *and* c^3G_d *in comparison to their unmodified counterparts G* (**1**) *and dG* (*RP-18* column, 0.5 ml min^{-1}, 0.1M $(Et_3NH)OAc$ buffer with 5% MeCN). b) *CD Spectra of* c^3G (**2**) *and G* (**1**) (in bi-distilled H_2O with 1 mM nucleoside concentration).

Earlier, it was observed for 3-deaza-2′-deoxyguanosine that regular acyl protecting groups used for the 2-amino function are too stable to be used for oligonucleotide synthesis (*Table 1*). Only the phenoxyacetyl(pac)-protected nucleoside or the (dimethylamino)methylidene ($m_2$fa) derivative can be considered for further reactions

[24][25]. However, when this latter group was used [17], problems appeared during the preparation of the phosphoramidite, which resulted from the reactivity of the 6-oxo-group [26]. Thus, the 6-oxo-group was protected. For this purpose, the diphenylcarbamoyl (dpc; $Ph_2NC(=O)$) residue was chosen, which was already used successfully as an oxo-protecting group in the case of guanosine [27][28] and 2′-deoxyisoguanosine [29]. Prior to the 6-oxo group protection, the phenoxyacetyl (pac) group [30] was introduced to block the amino function (**2** → **7**; see *Scheme 2*). Next, it was established whether introduction of the dpc group (**7** → **8**) or that of the 4,4′-dimethoxytrityl ($(MeO)_2Tr$) residue (**7** → **9**) would be the most-effective step to take next. As the latter route gave the better yields, the $(MeO)_2Tr$ derivative **9** was prepared and derivatized further.

Scheme 2



i) 1. HMDS, DMF; 2. pac-Cl, pyridine, r.t., 3 h. *ii*) dpc-Cl, $(i\text{-}Pr)_2EtN$, pyridine, r.t., 6 h. *iii*) $(MeO)_2Tr$-Cl, pyridine, r.t., 4 h. *iv*) $(i\text{-}Pr)_3SiCl$, $AgNO_3$, pyridine/THF, r.t., 20 h. DMTr = $(MeO)_2$ Tr.

Table 1. *Half-Life Values (τ) of Amino-Protected 3-Deazaguanine Derivatives, Measured Spectrophotometrically in 25% Aq. Ammonia Solution*[a])

	τ [min]	λ [nm]
pac^2c^3G (**7**)	190[b]), 4[c])	322
pac^2G_d [30]	15[d])	
$ibu^2c^3G_d$ [17]	500[b])	300
ibu^2G_d [24]	112[b])	300
$m_2fa^2c^3G_d$ [17]	28[b])	320
$m_2fa^2G_d$ [25]	19[b])	300

[a]) pac = phenoxyacetyl, c = deaza, d = 2′-deoxy, ibu = isobutyryl = Me_2CHCO, m_2fa = (dimethylamino)methylene. [b]) Measured at 40°. [c]) Measured at 65° (40% $MeNH_2/H_2O$). [d]) Measured at 20°.

To assure the applicability of the protecting groups during oligoribonucleotide synthesis, the deprotection of compounds **7** and **8** was studied spectrophotometrically in 25% ammonia or 40% aqueous methylamine solution. The overall half-life value for

deprotection of **8** (for both protecting groups) was 4.9 min in 40% $MeNH_2/H_2O$ at 65°. To follow the deprotection stepwise, the reaction was performed at 20° and monitored at 322 nm. According to the pH-dependent UV spectra of 3-deazaguanosine (**2**) (*Fig. 2,a*) and of those of the derivatives **7** and **8** (*Fig. 2,b*), the independent removal of the dpc residue can be followed at this wavelength. Thus, for compound **8**, the half-life of the dpc removal was found to be only 4 min, while that of the pac group was much longer. The rather long half-life time observed for the pac residue of compound **7** was not unexpected as anion formation stabilizes the pac residue. Also the amino group of 3-deazaguanosine is more basic than that of guanosine. A similar observation has been made for 3-deaza-2′-deoxyadenosine [31].

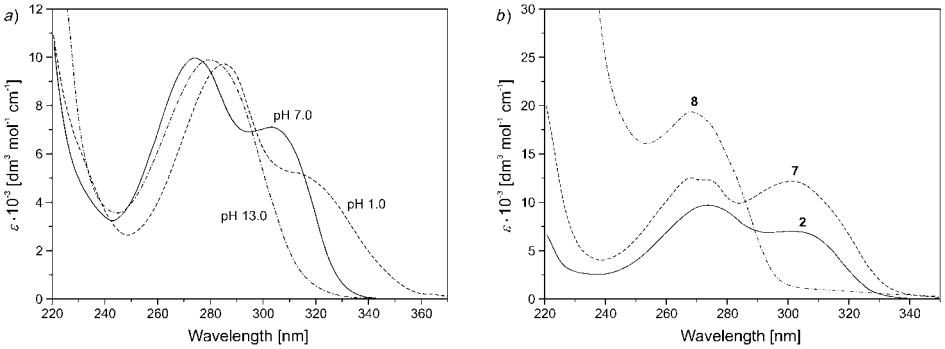
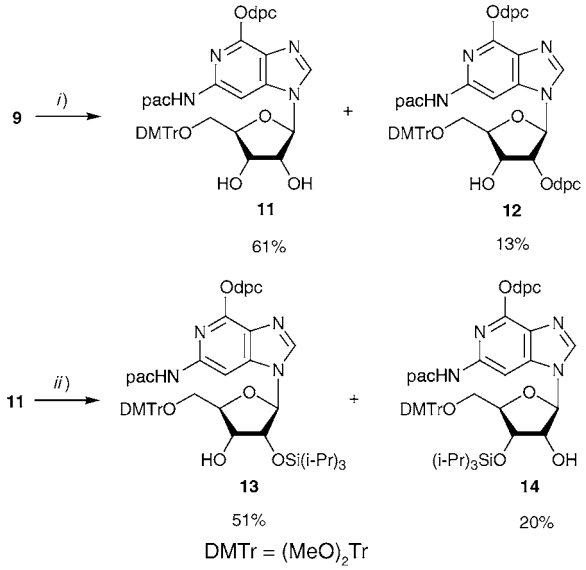


Fig. 2. a) *UV Spectra of 3-deazaguanosine* (**2**) *at pH 1.0, 7.0, and 13.0.* b) *UV Spectra* (MeOH) *of the protected nucleosides* **7** *and* **8** *compared with that of compound* **2**.

Due to the reactivity of the 6-oxo group of **9**, it was not possible to protect the sugar 2′-hydroxy group with the triisopropylsilyl residue without affecting the oxo group of the nucleobase. For instance, silylation of **9** afforded the 6-oxo-protected derivative **10** in moderate yield (*Scheme 2*). Consequently, the 6-oxo function had to be protected before manipulations of the sugar residue took place. Reaction of compound **9** with diphenylcarbamoyl chloride (dpc-Cl) gave the dpc derivative **11** (61% yield) together with the bis-dpc compound **12** (13%) (*Scheme 3*). Afterwards, silylation of compound **11** with $(i\text{-}Pr)_3SiCl$ was performed in the presence of $AgNO_3$ as it was described for other nucleosides [32][33]. The 2′-*O*-silyl derivative **13** was isolated in 51% yield, and the 3′-*O*-isomer **14** in 20%. Phosphitylation of compound **13** under standard conditions [34] resulted in formation of phosphoramidite **3** (75%). All new compounds were characterized by ^1H-, ^{13}C-, and ^{31}P-NMR spectra (see *Table 2* and *Exper. Part*) as well as by elemental analyses. From the NMR spectra of the dpc derivatives, it is apparent that this residue is linked to the 6-oxo group and not to the N(1) atom. Due to aromatization, all ^{13}C-NMR signals of the dpc derivative **8** are shifted significantly in comparison to the mono-protected compound **7**. A similar observation was made for other *O*-dpc-protected guanine nucleosides [27–29].

As the conformational equilibrium of the sugar moiety is controlled by the electronic properties of the base, changes are expected when a 3-deazaguanine replaces guanine within a nucleoside. The sugar conformation (*N vs. S* and around the C(4′)–C(5′) bond) of the nucleosides **1** and **2** as well as of dG, and c^3G_d for comparison

Scheme 3



i) dpc-Cl, $(i\text{-Pr})_2$EtN, pyridine, r.t., 4 h. *ii*) $(i\text{-Pr})_3$SiCl, $AgNO_3$, pyridine/THF, r.t., 20 h.

Table 2. *^{13}C-NMR Chemical Shifts of the 3-Deazaguanosine Derivatives* **2**, **7**–**9**, *and* **11**–**14**[a])

[b]) C(2) [c]) C(6)	C(3) C(7)	C(4)[d]) C(7a)	C(5)[d]) C(3a)	C(6) C(4)	C(8) C(2)	C(1′)	C(2′)	C(3′)	C(4′)	C(5′)
2	147.6	70.5	123.0	142.4	156.5	136.6	88.1	73.8	70.1	85.3	61.2
7	139.6	81.8	128.1	136.7	155.9	139.1	88.2	74.2	70.3	85.7	61.2
8	143.4	94.6	128.7	142.8	146.8	144.1	88.6	73.9	70.3	85.9	61.2
9	139.9	81.6	128.2	136.8	155.9	138.3	88.6	73.7	70.3	81.6	63.5
11	143.6	94.7	128.7	142.9	146.8	144.8	89.0	73.5	70.1	83.5	63.6
12	143.8	94.5	128.8	143.5	146.8	144.7	86.7	76.8	68.6	83.4	63.0
13	143.7	94.3	128.6	143.4	146.9	144.8	90.0	75.5	70.5	84.0	63.4
14	143.7	94.8	128.7	142.9	146.8	144.7	88.9	73.1	72.2	84.4	63.1

[a]) Spectra were measured in (D_6)DMSO. [b]) Purine numbering. [c]) Systematic numbering. [d]) Tentative.

was determined from the vicinal ^3J(H,H) coupling constants of the ^{1}H-NMR spectra measured in D_2O (*Table 3*) by applying the PSEUROT program package (version 6.2) [35][36] or according to *Westhof et al.* [37]. The 3-deazaguanosine shows the same preference of the *anti*-conformation as guanosine (68%) does [38]. *Table 3* indicates that no severe changes are occurring between the modified and the parent nucleosides.

2. *Hammerhead-Ribozymes – Substrate Complexes Containing Single 3-Deazaguanosine Substitutions.* 2.1. *Synthesis.* To probe the importance of the N(3) atom of the conserved guanosine (**1**) residues within the catalytic core of the hammerhead-ribozyme, individual guanosine residues were replaced by 3-deazaguanosine (**2**) in the 53 nucleotide unit of the ribozyme – substrate complex **15** · **16** (see *Table 4*). In addition,

Table 3. *^{1}H-NMR Coupling Constants of the Sugar Protons, the Calculated Pseudorotational Parameters, and the Rotational Equilibrium about the C(4′)–C(5′) Bond of G (1), dG, c^{3}G (2), and c^{3}G$_d$*[a])

	^3J(1′,2′a)	^3J(1′,2′b)	^3J(2′a,3′)	^3J(2′b,3′)	^3J(3′,4′)	^3J(4′,5′a)	^3J(4′,5′b)	%*N*	%*S*	%$\gamma^{(+)g}$	%γ^{t}	%$\gamma^{(-)g}$
G (**1**)	5.80	–	5.00	–	4.00	3.00	4.20	36	64	65	24	11
dG	7.30	6.50	6.30	3.60	3.20	3.60	4.70	29	71	53	30	17
c^{3}G (**2**)	5.65	–	5.00	–	4.40	2.70	4.40	39	61	66	26	8
c^{3}G$_d$	6.80	6.40	6.75	3.90	3.70	3.85	5.15	35	65	45	35	20

[a]) Measured in D_2O at 30°; r.m.s. ≤ 0.4 for all calculations; $\Delta J_{max} \leq 0.5$ Hz.

a non-conserved residue in the GAAA loop was substituted as well (*Fig. 3*). Each of these oligonucleotides contains a single substitution at positions G_5, G_8, G_{12}, or $G_{L2.1}$. The oligonucleotide synthesis was performed on a solid support employing the phosphoramidite **3**. The coupling conditions were according to a protocol applied for the phosphoramidite of 1-deazaadenosine (0.1M phosphoramidite, 0.1M 5-(ethylthio)-1*H*-tetrazole, coupling time 450 s). The coupling efficiency of **3** was 90–93%, which is slightly below that of the unmodified building blocks but still in an acceptable range for an efficient assembly of the target oligonucleotides **17**–**20** (see *Table 4*; *cf.* also *Exper. Part* and *Fig. 3*). The choice of the protecting groups for compound **3** proved to be adequate, since complete deblocking was achieved with 40% $MeNH_2/H_2O$ at 65° (15 min). The MALDI-TOF-MS analysis was performed with the deprotected oligonucleotides. The M^+ data were in accordance with the calculated values.

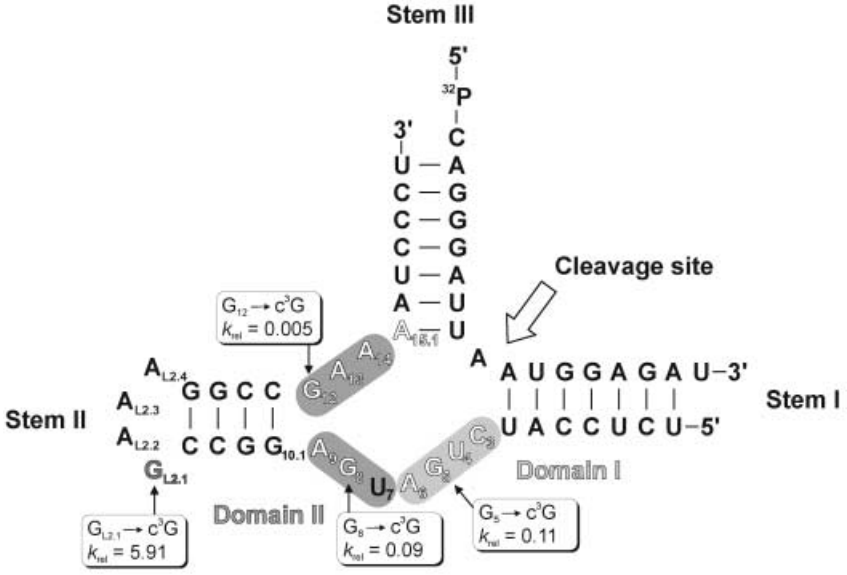


Fig. 3. *Structure of the hammerhead RNA complex* **15**/**16** *showing the sites of the 3-deazaguanosine incorporation and the relative cleavage rates*

Table 4. *Oligoribonucleotide Sequences of the Enzymes* **16**–**20** *and the Substrate* **15**

5′-[^{32}P]p(C-A-G-G-G-A-U-U-A-A-U-G-G-A-G-A-U)-3′ (**15**)
5′-(U-C-U-C-C-A-U-C-U-G-A-U-G-A-G-G-C-C-G-A-A-A-G-G-C-C-G-A-A-A-A-U-C-C-C-U)-3′ (**16**)
5′-(U-C-U-C-C-A-U-C-U-G-A-U-**2**-A-G-G-C-C-G-A-A-A-G-G-C-C-G-A-A-A-A-U-C-C-C-U)-3′ (**17**)
5′-(U-C-U-C-C-A-U-C-U-G-A-U-G-A-G-G-C-C-G-A-A-A-G-G-C-C-**2**-A-A-A-A-U-C-C-C-U)-3′ (**18**)
5′-(U-C-U-C-C-A-U-C-U-**2**-A-U-G-A-G-G-C-C-G-A-A-A-G-G-C-C-G-A-A-A-A-U-C-C-C-U)-3′ (**19**)
5′-(U-C-U-C-C-A-U-C-U-G-A-U-G-A-G-G-C-C-**2**-A-A-A-G-G-C-C-G-A-A-A-A-U-C-C-C-U)-3′ (**20**)

2.2. *Ribozyme Activity.* To probe the importance of the N(3) atom of the conserved guanosine residues on the catalytic activity of the hammerhead-ribozyme complex, the cleavage reaction of the native 53 oligonucleotide was compared with that of the modified complexes containing single guanosine replacements by compound **2** (*Table 4*). The 3-deazaguanosine residues can participate in *Watson-Crick* or *Hoogsteen* base pairing, with the exception that the H-bonding involving N(3) is eliminated (*Fig. 4*). In cases where N(3) is participating in specific interaction(s) being necessary for an efficient cleavage, a significant reduction in the catalytic efficiency can be expected. *Fig. 4* shows the potential donor and acceptor positions for the modified nucleoside as well as the electron densities of 3-deazaguanine (**2**) as well as of guanine (**1**). The electron densities were calculated on the HF/6-31G* level using HyperChem 5 (*Hypercube Inc.*, FL, USA). The data indicate that **1** and **2** differ in their electron densities at the atoms located at the positions 2, 3, and 4 [39]. According to this, the H-bonding *via* N(3) is eliminated, and the 2-amino group becomes more basic (*i.e.*, a less-efficient proton donor).

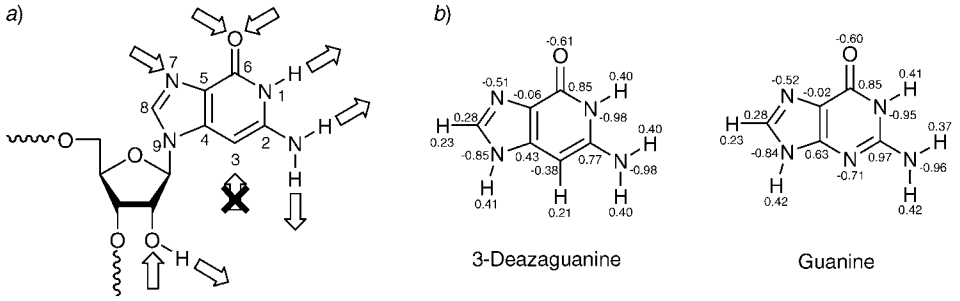


Fig. 4. a) *Proton acceptor/donor pattern of 3-deazaguanosine* (**2**) *and* b) *electron densities* (in electron units) *of 3-deazaguanine and guanine determined by* ab initio *calculations at the 6-31G* level*

2.3. *Guanine → 3-Deazaguanine Substitutions in Domain II.* The results of several X-ray analyses demonstrated that the hammerhead ribozyme is composed of two structural domains: a 'uridine turn' (domain I; C_3 to A_6) and a tandem $G \cdot A$ mismatch (domain II; U_7 to A_9 and G_{12} to A_{14}) located at the junction of three helices [6] (*cf. Fig. 3*). The domain II is strictly conserved, and its Mg^{2+} complex pulls stems II and III into the alignment required for catalysis. Two symmetric metal-binding sites associated with domain II were identified by biochemical methods as well as by NMR and X-ray analysis [11][40–46]. These include the less-defined A_{13} site [42] and the site A_9 to

$G_{10.1}$, where functional groups involved in coordination of metal were characterized in detail [44–46]. According to the X-ray data, N(3) of the guanosine residue G_{12} is involved in H-bonding with the 6-amino group of the adenosine residue A_9 (see *Fig. 5*) The same type of interaction is observed for the residues G_8 and A_{13}, although in this case, O–C(4′) of the furanose moiety of G_8 is participating in an additional H-bond with the 6-amino group of A_{13}, while in the $G_{12} \cdot A_9$ pair, the 2′-hydroxy group of G_{12} serves as another H-bond donor.

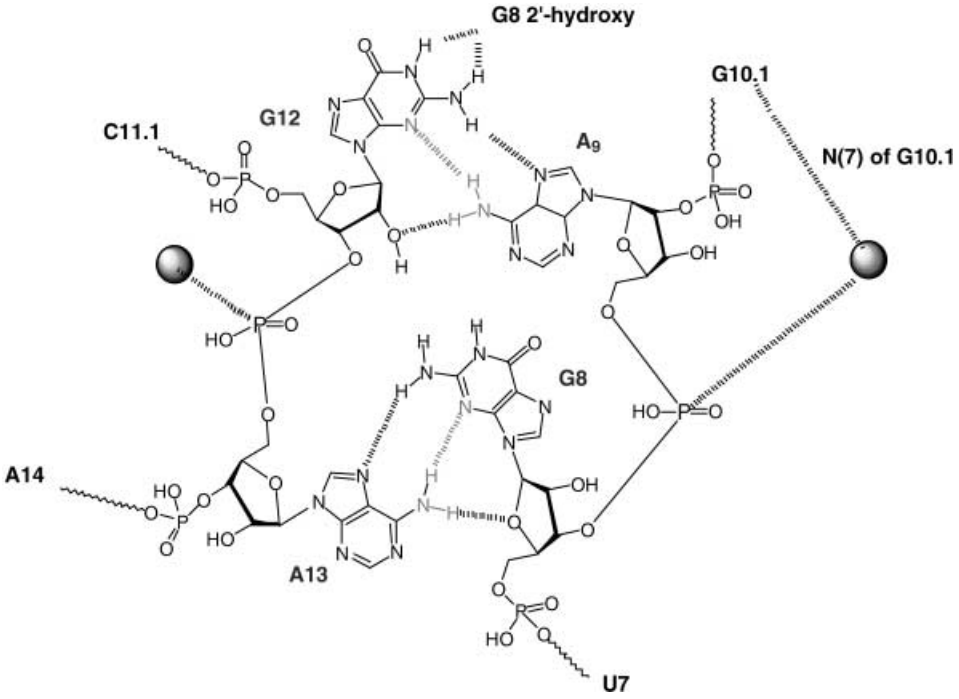


Fig. 5. *Hydrogen-bonding pattern of domain II of the hammerhead-ribozyme complex, with donor and acceptor sites of guanine and adenine and the sugar moieties.* The pattern follows X-ray data described recently [44] [45].

Interestingly, the $G \rightarrow c^3G$ substitution of G_8 and G_{12} resulted in different outcomes. In the case of the G_8 substitution, the decrease of the cleavage of the ribozyme–substrate complex is only 10-fold compared to 200-fold for the G_{12} substitution (*Fig. 3*). This most likely indicates that N(3) of the guanosine residue G_{12} is involved in H-bonding being critical for the hammerhead catalysis, while N(3) of G_8 is less critical. The odd base pair $G_8 \cdot A_{13}$ observed in the ground state of the ribozyme–substrate complex identified by X-ray analysis is probably rearranged during the transition towards the productive complex in which $N(3)(G_8) \cdots H_2N(A_{13})$ H-bonds are not critical for the activity or are compensated by new interactions. It is also notable that the G_8 adjacent to U_7 is the beginning of the flexible uridine turn. This may account for

the tolerance in geometry of the conserved $G_8 \cdot A_{13}$ pair. A significantly larger effect of 3-deazaguanine substitution is found for the $G_{12} \cdot A_9$ pair. That might be related to a precise positioning of adjacent metal-binding sites at A_9 to $G_{10.1}$. This is implicated by a direct participation in the cleavage reaction [46].

Our results on differential sensitivity of $G_{12} \cdot A_9$ and $G_8 \cdot A_{13}$ base pairs toward 3-deazaguanine substitutions are in agreement with those of *McLaughlin* and co-workers [47] as well as with those of *Seela* and co-workers [48] regarding the pair $G_8 \cdot A_{13}$. In contrast to the results of *McLaughlin* and co-workers, which obtained no change when A_9 was replaced by 7-deazaadenosine, we observed 200-fold-reduced activity when guanine was replaced by 3-deazaaguanine in the $A_9 \cdot G_{12}$ interaction. The relative insignificance of the potential H-bonds in the $G_8 \cdot A_{13}$ pair correlates well with the finding for adenosine → 7-deazaadenosine substitution [47][48] as well as with those for the replacement of guanosine by 3-deazaguanosine described in the present work. The results obtained for the substitution of G_{12} by 3-deazaguanosine and of A_9 by 7-deazaadenosine in the $A_9 \cdot G_{12}$ pair probably indicate that the H-bond of N(3) with the 6-amino group which is found for the ground state by X-ray analysis (*Fig. 5*) is absent during transition to the active complex. The N(3) of the G_{12} residue might find another binding partner during this transition.

2.4. *Guanine → 3-Deazaguanine Substitution of G_5.* The replacement of the G_5 residue in the domain I and its role in hammerhead catalysis is a subject of continuing controversy. Any substitution of the base moiety of this nucleobase residue by hypoxanthine, 2-aminopurine, purine, isoguanine, xanthine [49], and 7-deazaguanine [47][48] resulted in significant (>1000 fold) loss of the cleavage activity in the ribozyme–substrate complex. According to the first two X-ray structures [44][45], this residue interacts only with solvent molecules. Our data, observed for the substitution of G_5 with 3-deazaguanosine, results in only a modest (ninefold) decrease of the cleavage rate (*cf. Fig. 3*), placing 3-deazaguanosine into the rare category of non-invasive modification as it was observed for 7-deazaguanosine. It also confirms that the *Watson-Crick* pairing site of G_5 rather than the *Hoogsteen* site of this guanosine residue is involved in interactions crucial for the activity of the hammerhead catalysis. The recently identified metal-binding site near G_5 [50], with potential outer-sphere coordination to the *Watson-Crick* site of G_5, supports this finding.

2.5. *Guanine → 3-Deazaguanine Substitution of $G_{L2.1}$.* An unexpected result was observed when 3-deazaguanosine was replacing $G_{L2.1}$ in the GAAA tetra loop (*cf. Fig. 3*). A sixfold increase in cleavage rate was observed. To the best of our knowledge, this is only the second example of enhancement of the hammerhead ribozyme activity produced by a modification [51]. We have demonstrated recently that the GNRA tetra loop is not optimal for the hammerhead catalysis. The GUUA loop confers increased cleavage rates for this type of ribozyme [52]. At the same time, 1-deazaadenosine at position L2.3 of the GAAA part has no effect on catalysis [53]. It is possible that $G_{L2.1}$ is indirectly involved in a conformational change from the ground state to the productive ribozyme–substrate complex. Additional modifications at this position are needed to confirm this idea.

Conclusions. – An efficient synthesis for ribozyme–substrate constructs is described based on the novel phosphoramidite **3** of 3-deazaguanosine (**2**), protected

at the 2-amino group of the base with a phenoxyacetyl residue and at the 6-oxo function with a diphenylcarbamoyl group. Single replacements of guanosine residues G_8, G_{12}, G_5, and $G_{L2.1}$ by 3-deazaguanosine (**2**) and analysis of the kinetic data established the following: *i*) a differential sensitivity of the $G_{12} \cdot A_9$ and $G_8 \cdot A_{13}$ base pairs towards 3-deazaguanosine substitution and the importance of the base pairing mediated by the N(3) atom of G_{12} for hammerhead catalysis, *ii*) the confirmation that the *Watson-Crick* pairing site of G_5 is involved in interactions important for catalysis, whereas N(3) is not, and *iii*) a surprising increase of the ribozyme–substrate-complex cleavage rate when $G_{L2.1}$ was substituted by 3-deazaguanosine. The significant decrease of the catalytic activity of the ribozyme–substrate complex, in particular the almost complete loss when the G_{12} residue was replaced, is in accordance with the X-ray structure.

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Experimental Part

General. Chemicals were purchased from *Aldrich*, *Sigma*, or *Fluka* (*Sigma-Aldrich Chemie GmbH*, Deisenhofen, Germany). The solvents were of laboratory grade. TLC: silica gel *60* F_{254} aluminium sheets (0.2-mm layer; *Merck*, Darmstadt, Germany). Column flash chromatography (FC): silica gel *60* (*Merck*, Darmstadt, Germany); at 0.4 bar ($4 \cdot 10^4$ Pa). M.p.: *Büchi-SMP-20* apparatus (*Büchi*, Flawil, Switzerland); uncorrected. UV Spectra: *U-3200* spectrometer (*Hitachi*, Tokyo, Japan); λ_{max} (ε) in nm. NMR Spectra: *Avance-DPX-250* or *AMX-500* spectrometer (*Bruker*, Karlsruhe, Germany); 250.13 (^{1}H) and 500.14 MHz (^{1}H; 125.13 MHz for ^{13}C), resp.; chemical shifts δ in ppm rel. to $SiMe_4$ as internal standard; *J* values in Hz. The elemental analyses were performed by *Mikroanalytisches Laboratorium Beller*, Göttingen, Germany.

Protecting-Group Stability. The stability of the protecting groups was determined spectrophotometrically with a *U-3200* spectrometer (*Hitachi*, Japan), heated with an *RC-6-CP* thermostat (*Lauda*, Germany) at a defined temperature. UV-Overlay spectra of compounds **7** and **8** were measured, followed by time scans at that wavelength where the highest absorbance change was observed.

*1,5-Dihydro-6-[(phenoxyacetyl)amino]-1-(β-*D*-ribofuranosyl)-4*H*-imidazo[4,5-*c*]pyridin-4-one* (**7**). To a suspension of 3-deazaguanosine (**2**; 1.5 g, 5.3 mmol) in dry DMF (50 ml), 1,1,1,3,3,3-hexamethyldisilazane (9 ml, 42.7 mmol) was added, and the mixture was stirred for 30 min at r.t. The resulting clear soln. was evaporated to give an oil, which was dissolved in anh. pyridine (50 ml). Phenoxyacetyl chloride (0.88 ml, 6.4 mmol) was added under stirring. After 3 h, the soln. was cooled (ice bath), diluted with H_2O (6 ml), and kept for 15 min. Then, the mixture was treated with 25% aq. NH_3 soln. (6 ml), stirred for another 15 min, and concentrated to 10 ml. H_2O (150 ml) was added, the aq. soln. washed with CH_2Cl_2 (50 ml, twice), the org. phase evaporated, and the residue co-evaporated with toluene (50 ml) and MeOH (50 ml) and then applied to FC (silica gel, column 10×3 cm, CH_2Cl_2/MeOH 4:1). The content of the main zone was crystallized from EtOH: **7** (1.6 g, 72%). Colorless crystals. M.p. 190–192°. R_f (CH_2Cl_2/MeOH 8:2) 0.2. UV (MeOH): 268 (13200), 274 (13100), 302 (13200). ^{1}H-NMR ((D$_6$)DMSO): 3.62 (*m*, 2 H–C(5′)); 3.97 (*m*, H–C(4′)); 4.09 (*m*, H–C(3′)); 4.32 (*m*, H–C(2′)); 4.75 (*s*, CH_2); 5.10 (br. *s*, OH–C(5′)); 5.33 (br. *s*, OH–C(3′)); 5.58 (br. *s*, OH–C(2′)); 5.67 (*d*, $^3J = 6.3$, H–C(1′)); 6.98–7.07 (*m*, H–C(7), 3 arom. H); 7.35 (*t*, $^3J = 7.7$, 2 arom. H); 8.23 (*s*, H–C(2)); 10.27 (br. *s*, NH), 11.33 (br. *s*, NH). Anal. calc. for $C_{19}H_{20}N_4O_7$ (416.38): C 54.81, H 4.84, N 13.46; found: C 54.89, H 4.91, N 13.39.

*4-[(Diphenylcarbamoyl)oxy]-*N^6*-(phenoxyacetyl)-1-(β-*D*-ribofuranosyl)-1*H*-imidazo[4,5-*c*]pyridin-6-amine* (**8**). Compound **7** (200 mg, 0.48 mmol) was dried by co-evaporation with anh. pyridine (5 ml) and suspended in dry pyridine (5 ml). To this suspension, diphenylcarbamic chloride (250 mg, 1.1 mmol) and (i-Pr)$_2$EtN (180 µl, 1.3 mmol) were added, and the mixture was stirred for 6 h at r.t. This soln. was poured into 5% aq. $NaHCO_3$ soln. (20 ml), the org. phase extracted with CH_2Cl_2 (20 ml, twice), the combined org. phase dried (Na_2SO_4) and evaporated, and the residue adsorbed on silica gel and applied to FC (silica gel, column 10×2 cm,

CH_2Cl_2/MeOH 9:1): **8** (115 mg, 39%). Pale yellow powder. R_f (CH_2Cl_2/MeOH 9:1) 0.5. UV (MeOH): 228 (43100), 269 (17700), 275 (sh, 16700). ^{1}H-NMR ((D$_6$)DMSO): 3.63 (*m*, 2 H–C(5′)); 3.99 (*m*, H–C(4′)); 4.10 (*m*, H–C(3′)); 4.39 (*m*, H–C(2′)); 4.81 (*s*, CH_2); 5.09 (*t*, ^3J = 5.2, OH–C(5′)); 5.32 (*d*, ^3J = 4.7, OH–C(3′)); 5.61 (*d*, ^3J = 6.6, OH–C(2′)); 5.84 (*d*, ^3J = 6.3, H–C(1′)); 6.93–7.47 (*m*, 15 arom. H); 8.28 (*s*, H–C(7)); 8.61 (*s*, H–C(2)); 10.73 (br. *s*, NH). Anal. calc. for $C_{32}H_{29}N_5O_8$ (611.60): C 62.84, H 4.78, N 11.45; found: C 62.93, H 4.78, N 11.24.

*1-{5′-*O*-[Bis(4-methoxyphenyl)phenylmethyl]-β-*D*-ribofuranosyl}-1,5-dihydro-6-[(phenoxyacetyl)amino]-4*H*-imidazo[4,5-*c*]pyridin-4-one* (**9**). Compound **7** (840 mg, 2.0 mmol) was dried by co-evaporation with anh. pyridine (20 ml) and dissolved in dry pyridine (20 ml) under gentle heating. To this soln., $(MeO)_2$Tr-Cl (815 mg, 2.4 mmol) was added under stirring, and the reaction was continued for 4 h at r.t. Then, the mixture was quenched by the addition of MeOH (5 ml), stirred for further 30 min, and then concentrated to half of its volume. The soln. was diluted with CH_2Cl_2 (100 ml), washed with 5% aq. $NaHCO_3$ soln. (50 ml, twice) and brine (50 ml), dried (Na_2SO_4) and evaporated and the residue co-evaporated with toluene (2 × 30 ml) and purified by FC (silica gel, column 4 × 10 cm, CH_2Cl_2/MeOH 9:1): **9** (1.04 g, 72%). Colorless solid. R_f (CH_2Cl_2/MeOH 9:1) 0.4. UV (MeOH): 270 (sh, 12100), 275 (12800). ^{1}H-NMR ((D$_6$)DMSO): 3.19 (*m*, 2 H–C(5′)); 3.71 (*s*, 2 MeO); 4.12 (*m*, H–C(3′), H–C(4′)); 4.78 (*m*, H–C(2′)); 4.73 (*s*, CH_2); 5.33 (*d*, ^3J = 5.0, OH–C(3′)); 5.73 (*d*, ^3J = 4.1, OH–C(2′)); 5.75 (*d*, ^3J = 3.8, H–C(1′)); 6.81–7.38 (*m*, 19 arom. H, H–C(7)); 8.13 (*s*, H–C(2)); 10.17 (br. *s*, NH); 11.32 (br. *s*, NH). Anal. calc. for $C_{40}H_{38}N_4O_9$ (718.75): C 66.84, H 5.33, N 7.80; found: C 66.89, H 5.61, N 7.76.

*1-{5′-*O*-[Bis(4-methoxyphenyl)phenylmethyl]-β-*D*-ribofuranosyl}-*N^6*-(phenoxyacetyl)-4-{[tris(1-methylethyl)silyl]oxy}-1*H*-imidazo[4,5-*c*]pyridin-6-amine* (**10**). To a soln. of **9** (210 mg, 0.29 mmol) in anh. pyridine (3 ml), $AgNO_3$ (74 mg, 0.44 mmol) was added under stirring at r.t. After 10 min, a soln. of (i-Pr)$_3$SiCl (66 µl, 0.31 mmol) in dry THF (4 ml) was added with stirring under exclusion of light and moisture. The reaction was continued for 6 h, and another portion of (i-Pr)$_3$SiCl (33 µl, 0.15 mmol) was added. Stirring was continued for another 16 h. The AgCl was filtered off, the filtrate diluted with 5% aq. $NaHCO_3$ soln. (5 ml), the aq. phase extracted with CH_2Cl_2 (10 ml, twice), the combined org. phase washed with H_2O (10 ml) and brine (20 ml), dried (Na_2SO_4), and evaporated, and the residue applied to FC (silica gel, column 4 × 10 cm, CH_2Cl_2/acetone 7:3): **10** (61 mg, 23%). Colorless foam. R_f (CH_2Cl_2/MeOH 9:1) 0.4. UV (MeOH): 233 (sh, 37000), 269 (25300), 275 (25900), 302 (21600). ^{1}H-NMR ((D$_6$)DMSO): 1.09 (*d*, ^3J = 7.5, 3 *Me*$_2$CH), 1.49 (*d*, ^3J = 7.5, 3 Me$_2$C*H*), 3.20 (*m*, 2 H–C(5′)); 3.71 (*s*, 2 MeO); 4.10 (*m*, H–C(3′), H–C(4′)); 4.51 (*s*, H–C(2′)); 4.81 (*s*, CH_2), 5.32 (*d*, ^3J = 3.8, OH–C(3′)); 5.69 (*d*, ^3J = 5.0, OH–C(2′)); 5.84 (*d*, ^3J = 4.7, H–C(1′)); 6.77–7.36 (*m*, 18 arom. H); 7.99 (*s*, H–C(7)); 8.28 (*s*, H–C(2)); 9.59 (br. *s*, NH). Anal. calc. for $C_{49}H_{58}N_4O_9Si$ (875.09): C 67.25, H 6.68, N 6.40; found: C 67.27, H 6.62, N 6.44.

*1-{5′-*O*-[Bis(4-methoxyphenyl)phenylmethyl]-β-*D*-ribofuranosyl}-4-[(diphenylcarbamoyl)oxy]-*N^6*-(phenoxyacetyl)-1*H*-imidazo[4,5-*c*]pyridin-6-amine* (**11**) *and 1-{5′-*O*-[Bis(4-methoxyphenyl)phenylmethyl]-2′-*O*-(diphenylcarbamoyl)-β-*D*-ribofuranosyl}-4-[(diphenylcarbamoyl)oxy]-*N^6*-(phenoxyacetyl)-1*H*-imidazo[4,5-*c*]pyridin-6-amine* (**12**). To a soln. of **9** (850 mg, 1.18 mmol) in anh. pyridine (15 ml), diphenylcarbamic chloride (410 mg, 1.8 mmol) and (i-Pr)$_2$EtN (0.3 ml, 2.2 mmol) were added at r.t. The mixture was stirred for 4 h and then poured into 5% aq. $NaHCO_3$ soln. (15 ml). This soln. was extracted with CH_2Cl_2 (30 ml, twice), the org. phase washed with H_2O (20 ml) and brine (20 ml), dried (Na_2SO_4), and evaporated, and the residue purified by FC (silica gel, column 4 × 10 cm, CH_2Cl_2/acetone 4:1). The slow migrating main zone furnished **11** (650 mg, 61%). Colorless solid. R_f (CH_2Cl_2/acetone 4:1) 0.4. UV (MeOH): 228 (62100), 269 (20700), 274 (sh, 20200). ^{1}H-NMR ((D$_6$)DMSO): 3.23 (*m*, 2 H–C(5′)); 3.67 (*s*, 1 MeO); 3.68 (*s*, 1 MeO); 4.12 (*m*, H–C(4′), H–C(3′)); 4.51 (*m*, H–C(2′)); 4.79 (*s*, CH_2); 5.34 (*d*, ^3J = 5.0, OH–C(3′)); 5.76 (*d*, ^3J = 6.0, OH–C(2′)); 5.93 (*d*, ^3J = 4.4, H–C(1′)); 6.76–7.48 (*m*, 28 arom. H); 8.31 (*s*, H–C(7)); 8.52 (*s*, H–C(2)); 10.77 (br. *s*, NH). Anal. calc. for $C_{53}H_{47}N_5O_{10}$ (913.97): C 69.65, H 5.18, N 7.66; found: C 69.56, H 5.16, N 7.70.

The faster-migrating zone gave **12** (165 mg, 13%). Colorless solid. R_f (CH_2Cl_2/MeOH 9:1) 0.4. UV (MeOH): 229 (59400), 269 (18100), 274 (17500). ^{1}H-NMR ((D$_6$)DMSO): 3.26 (*m*, 2 H–C(5′)); 3.67 (*s*, 1 MeO); 3.68 (*s*, 1 MeO); 3.93 (*m*, H–C(4′)); 4.50 (*m*, H–C(3′)); 4.79 (*s*, CH_2); 5.53 (*t*, ^3J = 4.4, H–C(2′)); 5.87 (*d*, ^3J = 5.7, OH–C(3′)); 6.12 (*d*, ^3J = 3.5, H–C(1′)); 6.75–7.48 (*m*, 38 arom. H); 8.21 (*s*, H–C(7)); 8.53 (*s*, H–C(2)); 10.79 (br. *s*, NH). Anal. calc. for $C_{66}H_{56}N_6O_{11}$ (1109.18): C 71.47, H 5.09, N 7.58; found: C 71.67, H 5.19, N 7.35.

*1-{5′-*O*-[Bis(4-methoxyphenyl)phenylmethyl]-2′-*O*-[tris(1-methylethyl)silyl]-β-*D*-ribofuranosyl}-4-[(diphenylcarbamoyl)oxy]-*N^6*-(phenoxyacetyl)-1*H*-imidazo[4,5-*c*]pyridin-6-amine* (**13**) *and 1-{5′-*O*-[Bis(4-methoxyphenyl)phenylmethyl]-3′-*O*-[tris(1-methylethyl)silyl]-β-*D*-ribofuranosyl}-4-[(diphenylcarbamoyl)oxy]-*N^6*-(phenoxyacetyl)-1*H*-imidazo[4,5-*c*]pyridin-6-amine* (**14**). To a soln. of **11** (500 mg, 0.55 mmol) in anh. pyridine (5 ml),

$AgNO_3$ (140 mg, 0.83 mmol) was added under stirring at r.t. A soln. of $(i\text{-}Pr)_3SiCl$ (125 µl, 0.58 mmol) in dry THF (8 ml) was introduced after 5 min under exclusion of light and moisture. The reaction was continued for 8 h, before a second portion of $(i\text{-}Pr)_3SiCl$ (62 µl, 0.29 mmol) was added. After 12 h, the AgCl was filtered off, the filtrate diluted with 5% aq. $NaHCO_3$ soln. (10 ml), the aq. phase extracted with CH_2Cl_2 (20 ml, twice), the combined org. phase washed with H_2O (20 ml) and brine (30 ml), dried (Na_2SO_4), and evaporated, and the residue applied to FC (silica gel, column 4×10 cm, CH_2Cl_2/AcOEt/petroleum ether 3:1:1). The faster migrating zone yielded **13** (300 mg, 51%). Colorless foam. R_f (CH_2Cl_2/AcOEt/petroleumether 3:1:1) 0.4. UV (MeOH): 229 (59900), 269 (20100), 274 (sh, 19600). ^{1}H-NMR ((D$_6$)DMSO): 0.81–0.91 (*m*, 3 Me_2CH_2); 3.28 (*m*, 2 H–C(5′)); 3.69 (*s*, 2 MeO); 4.14 (*m*, H–C(3′), H–C(4′)); 4.69 (*t*, $^3J = 4.2$, H–C(2′)); 4.77 (*s*, CH_2); 5.33 (*d*, $^3J = 6.0$, OH–C(3′)); 5.98 (*d*, $^3J = 4.4$, H–C(1′)); 6.79–7.47 (*m*, 28 arom. H); 8.28 (*s*, H–C(7)); 8.54 (*s*, H–C(2)); 10.74 (br. *s*, NH). Anal. calc. for $C_{62}H_{67}N_5O_{10}Si$ (1070.31): C 69.57, H 6.31, N 6.54; found: C 69.35, H 6.15, N, 6.55.

The slower migrating zone gave isomer **14** (118 mg, 20%). Colorless foam. R_f (CH_2Cl_2/AcOEt/petroleum-ether 3:1:1) 0.45. UV (MeOH): 229 (61900), 269 (20600), 274 (sh, 20200). ^{1}H-NMR ((D$_6$)DMSO): 0.95–0.98 (*m*, 3 Me_2CH_2); 3.25 (*m*, 2 H–C(5′)); 3.68 (*s*, 2 MeO); 4.12 (*m*, H–C(4′)); 4.28 (*m*, H–C(3′)); 4.55 (*m*, H–C(2′)); 4.78 (*s*, CH_2); 5.66 (*d*, $^3J = 6.3$, OH–C(2′)); 5.91 (*d*, $^3J = 4.7$, H–C(1′)); 6.78–7.48 (*m*, 28 arom. H); 8.31 (*s*, H–C(7)); 8.53 (*s*, H–C(2)); 10.77 (br. *s*, NH). Anal. calc. for $C_{62}H_{67}N_5O_{10}Si$ (1070.31): C 69.57, H 6.31, N 6.54; found: C 69.65, H 6.38, N 6.56.

*1-{5′-*O*-[Bis(4-methoxyphenyl)phenylmethyl]-2′-*O*-[tris(1-methylethyl)silyl]-β-*D*-ribofuranosyl}-4-[(diphenylcarbamoyl)oxy]-*N^6*-(phenoxyacetyl)-1*H*-imidazo[4,5-*c*]pyridin-6-amine 3′-(2-Cyanoethyl Diisopropylphosphoramidite)* (**3**). A stirred soln. of **13** (300 mg, 0.28 mmol) in anh. CH_2Cl_2 (9 ml) was pre-flushed with Ar and treated with $(i\text{-}Pr)_2EtN$ (0.14 ml, 1.0 mmol) followed by 2-cyanoethyl diisopropylphosphoramidochloridite (0.19 ml, 0.85 mmol) at r.t. The reaction was monitored by TLC (CH_2Cl_2/acetone 98:2). After 4 h, the mixture was diluted with CH_2Cl_2 (40 ml), washed with 5% aq. $NaHCO_3$ soln. (20 ml, twice) and brine (20 ml), dried (Na_2SO_4), and evaporated and the residue co-evaporated with CH_2Cl_2 (20 ml) and purified by FC (silica gel, column 3×10 cm, CH_2Cl_2/acetone 98:2): **3** (267 mg, 75%). Colorless foam. R_f (CH_2Cl_2/acetone 98:2): R_f 0.45, 0.5. ^{31}P-NMR ($CDCl_3$): 149.9, 153.3.

Oligonucleotides: Synthesis and Properties. The oligoribonucleotides were synthesized on polystyrene supports (ABI) on an *Applied-Biosystems-394*-DNA/RNA synthesizer (Foster City, CA, USA). The synthesis and the deprotection of the oligonucleotides **15**–**20** (*Table 4*) were performed as described previously [53][54]. The final oligonucleotides were purified by anion-exchange HPLC and characterized by MALDI-TOF-MS .The measured masses for the enzyme **16** and for the modified enzymes **17**–**20** is in accordance with the calculated values.

Substrate-Cleavage Assay. Ribozyme oligonucleotides and 5′-end ^{32}P-labeled substrates were heated separately in the reaction buffer at 95° for 1 min, quenched on ice and then equilibrated to the final reaction temp. (37°) prior to starting the reactions. Reactions were carried out with an excess of the enzyme and were initiated by mixing equal volumes (20 µl) of the substrate (final concentration < 1 nM) and ribozyme (1 µM) in 50 mM Mes buffer, pH 6.5, 10 mM $MgCl_2$. Aliquots (4 µl) were removed at various times (5 s to 2 h), quenched in 8 µl of formamide loading buffer (95% formamide, 20 mM EDTA), and loaded onto a 15% polyacrylamide TBE (89 mM tris-borate, 2 mM EDTA) gel containing 7M urea. The fraction of substrate and product present at each time point was determined by quantification of scanned images with a *Molecular Dynamics PhosphorImager*. The ribozyme-cleavage rates were obtained from the plots of the fraction of the substrate remaining *vs.* time using a nonlinear, least-squares fit to a double-exponential curve (*KaleidaGraph*, *Synergy Software*, Reading, PA, USA). The initial-rate section of the curve represented 80–90% of the total reaction; thus, the observed cleavage rates (k_{obs}) were taken from the rate constant for the first exponential. Relative rates of cleavage (k_{rel}) were calculated by dividing the observed cleavage rate by the cleavage rate of unmodified ribozyme (0.52 min^{-1}). The total extent of cleavage was always $> 70\%$.

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