

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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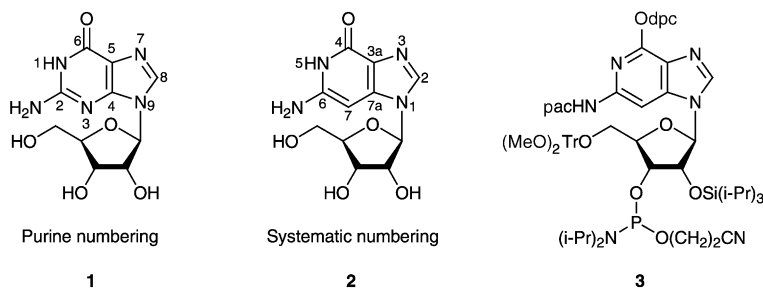
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Four base-modified hammerhead ribozyme/substrate complexes were constructed in which single guanosine (**1**) residues were replaced by 3-deazaguanosine (**2**) in the positions G₅, G₈, G_{12,1}, and G₁₂. The base-modified ribozyme complexes were prepared 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 G₅ or G₈ were replaced by 3-deazaguanosine and a 200-fold decrease when G₁₂ was substituted. A 6-fold catalytic increase occurred when 3-deazaguanosine was replacing G_{12,1} in the loop region. The data indicate that the N(3) atom of compound **2**, in particular at position G₁₂ 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 Mg²⁺ 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 Mg²⁺-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-deazaguanosine¹⁾ (**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 G₁₂ 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.

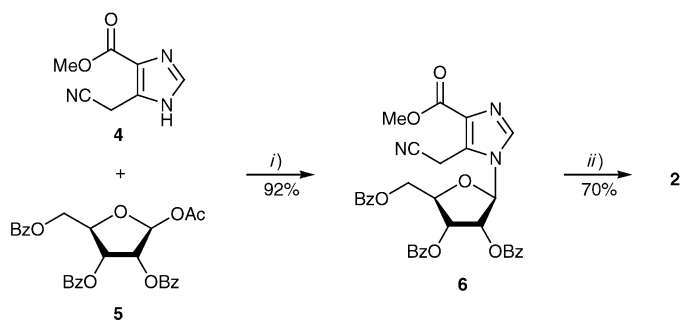


Results and Discussion. – 1. *Monomers.* Oligo-2'-deoxyribonucleotides containing 3-deazaguanine have been already synthesized with H-phosphonate of 3-deaza-2'-deoxyguanosine [17]. Alternatively, phosphoramidite chemistry was employed with appropriately functionalized imidazole nucleosides as precursors, 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].

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

Scheme 1



i) HMDS (hexamethyldisilazane), $(\text{NH}_4)_2\text{SO}_4$, 4 h, reflux, SnCl_4 , MeCN, 3 h, r.t. ii) NH_3/MeOH , 48 h, 120° , autoclave. DMTr = $(\text{MeO})_2\text{Tr} = (4\text{-MeOC}_6\text{H}_4)\text{PhC}$.

Compound **2** is more π -electron-rich than the parent guanosine. This is apparent from the $\text{p}K_a$ 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 $\text{p}K_a$ 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.

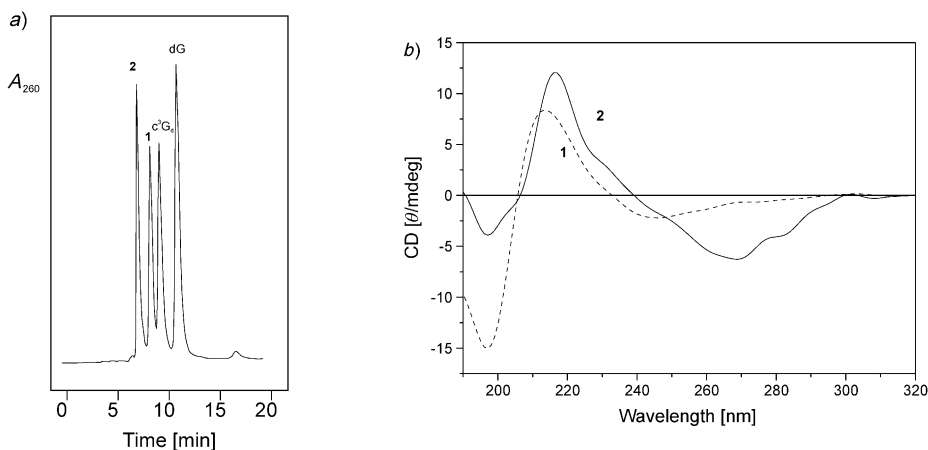
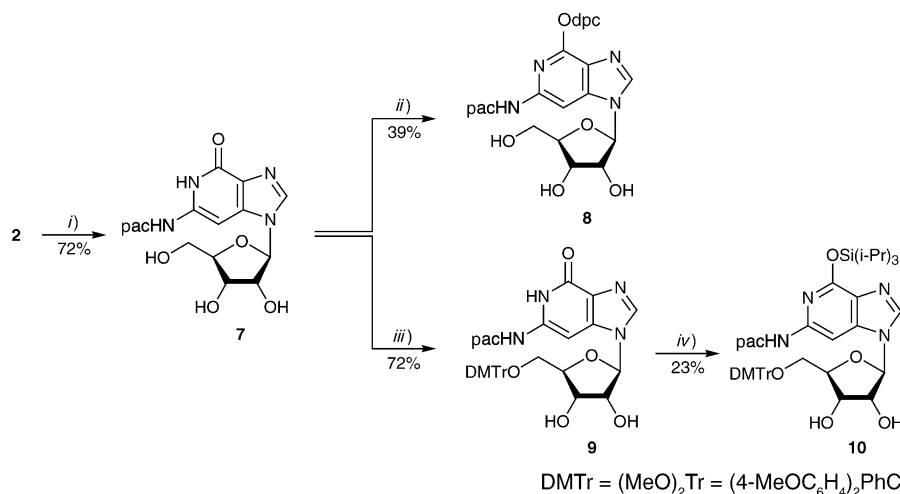


Fig. 1. a) HPLC Profiles of c^3G (**2**) and c^3G_a in comparison to their unmodified counterparts **G** (**1**) and **dG** (RP-18 column, 0.5 ml min^{-1} , 0.1 M $(\text{Et}_3\text{NH})\text{OAc}$ buffer with 5% MeCN). b) CD Spectra of c^3G (**2**) and **G** (**1**) (in bi-distilled H_2O with 1 mM nucleoside 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 I). Only the phenoxyacetyl(pac)-protected nucleoside or the (dimethylamino)methylidene (m_2fa) 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; $\text{Ph}_2\text{NC}(=\text{O})$) 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** $\rightarrow$ **7**; see *Scheme 2*). Next, it was established whether introduction of the dpc group (**7** $\rightarrow$ **8**) or that of the 4,4'-dimethoxytrityl ((MeO) $_2$ Tr) residue (**7** $\rightarrow$ **9**) would be the most-effective step to take next. As the latter route gave the better yields, the (MeO) $_2$ Tr 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-Pr) $_2$ EtN, pyridine, r.t., 6 h. iii) (MeO) $_2$ Tr-Cl, pyridine, r.t., 4 h. iv) (i-Pr) $_3$ SiCl, 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 ² c ³ G (7)	190 ^b), 4 ^c)	322
pac ² G _d [30]	15 ^d)	
ibu ² c ³ G _d [17]	500 ^b)	300
ibu ² G _d [24]	112 ^b)	300
m ₂ fa ² c ³ G _d [17]	28 ^b)	320
m ₂ fa ² G _d [25]	19 ^b)	300

^a) pac = phenoxyacetyl, c = deaza, d = 2'-deoxy, ibu = isobutyryl = Me $_2$ CHCO, m₂fa = (dimethylamino)methylene. ^b) Measured at 40°. ^c) Measured at 65° (40% MeNH $_2$ /H $_2$ O). ^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% MeNH₂/H₂O 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].

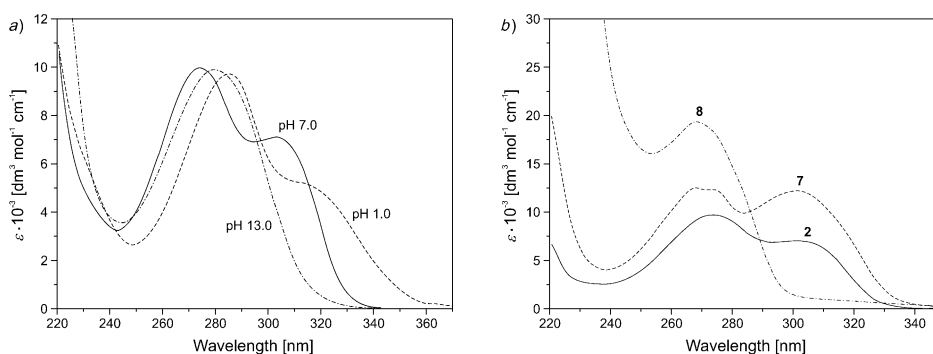
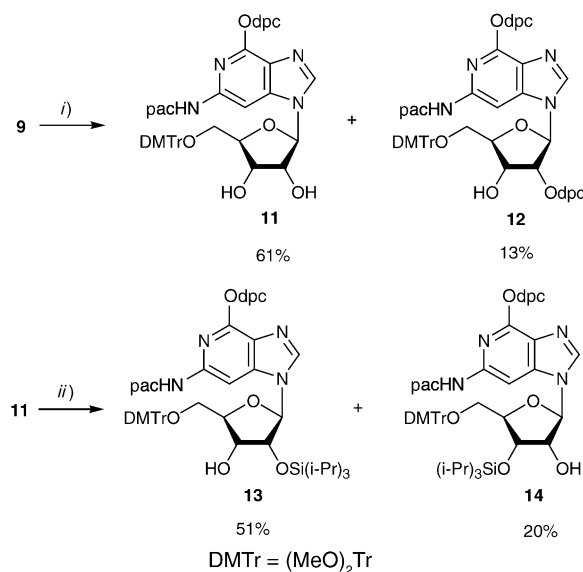


Fig. 2. a) UV Spectra of 3-deazaguanosine (**2**) at pH 1.0, 7.0, and 13.0. b) UV Spectra (MeOH) of the protected nucleosides **7** and **8** compared with that of compound **2**.

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-Pr)₃SiCl was performed in the presence of AgNO₃ 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-, ¹³C-, and ³¹P-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 ¹³C-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 c³G_d for comparison

Scheme 3



i) dpc-Cl, (i-Pr)₂EtN, pyridine, r.t., 4 h. ii) (i-Pr)₃SiCl, AgNO₃, pyridine/THF, r.t., 20 h.

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

^{b)}	C(2)	C(3)	C(4) ^{d)}	C(5) ^{d)}	C(6)	C(8)	C(1')	C(2')	C(3')	C(4')	C(5')
^{c)}	C(6)	C(7)	C(7a)	C(3a)	C(4)	C(2)					
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 (D₆)DMSO. ^{b)} Purine numbering. ^{c)} Systematic numbering. ^{d)} Tentative.

was determined from the vicinal ³J(H,H) coupling constants of the ¹H-NMR spectra measured in D₂O (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. ^1H -NMR Coupling Constants of the Sugar Protons, the Calculated Pseudorotational Parameters, and the Rotational Equilibrium about the $\text{C}(4')\text{--}\text{C}(5')$ Bond of G (1), dG , c^3G (2), and c^3G_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	% $\gamma^{(+)\text{g}}$	% γ'	% $\gamma^{(-)\text{g}}$
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^3G (2)	5.65	–	5.00	–	4.40	2.70	4.40	39	61	66	26	8
c^3G_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; $\Delta J_{\text{max}} \leq 0.5$ 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 $\text{G}_{\text{L}2.1}$. 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)-1H-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% $\text{MeNH}_2/\text{H}_2\text{O}$ 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.

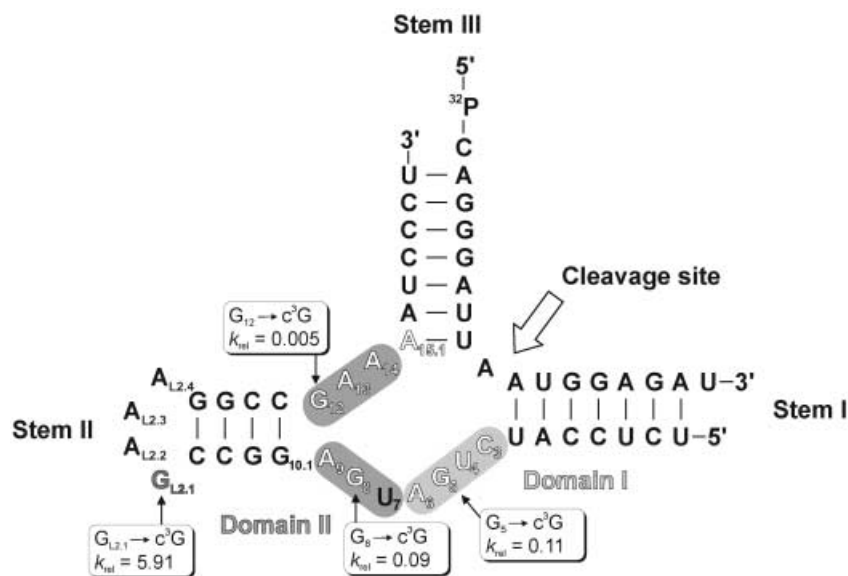
Fig. 3. Structure of the hammerhead RNA complex **15/16** showing the sites of the 3-deazaguanosine incorporation and the relative cleavage rates

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-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-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-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-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-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).

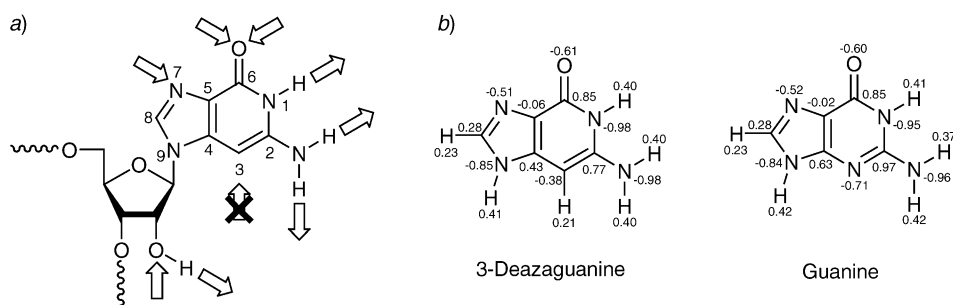


Fig. 4. a) Proton acceptor/donor pattern of 3-deazaguanosine (**2**) and b) electron densities (in electron units) of 3-deazaguanine and guanine determined by ab initio calculations at the 6-31G* level

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; C₃ to A₆) and a tandem G · A mismatch (domain II; U₇ to A₉ and G₁₂ to A₁₄) located at the junction of three helices [6] (*cf.* Fig. 3). The domain II is strictly conserved, and its Mg²⁺ 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 A₁₃ site [42] and the site A₉ 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.

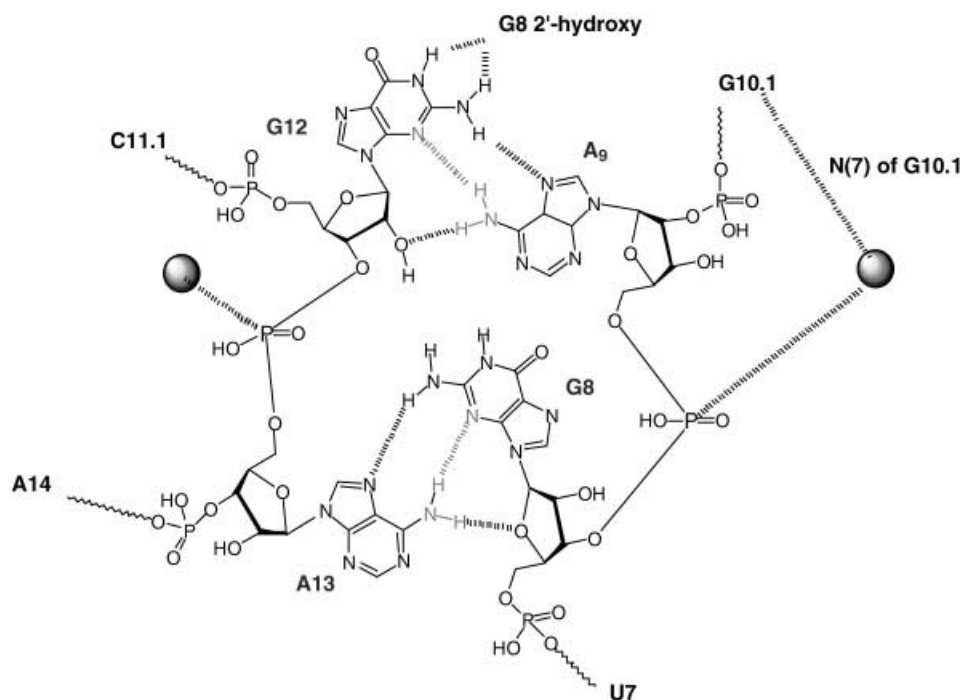


Fig. 5. Hydrogen-bonding pattern of domain II of the hammerhead-ribozyme complex, with donor and acceptor sites of guanine and adenine and the sugar moieties. The pattern follows X-ray data described recently [44][45].

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) $\cdots$ H₂N(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 $\rightarrow$ 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 $\rightarrow$ 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 $\rightarrow$ 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 G₈, G₁₂, G₅, and G_{L2.1} by 3-deazaguanosine (**2**) and analysis of the kinetic data established the following: *i*) a differential sensitivity of the G₁₂·A₉ and G₈·A₁₃ base pairs towards 3-deazaguanosine substitution and the importance of the base pairing mediated by the N(3) atom of G₁₂ for hammerhead catalysis, *ii*) the confirmation that the Watson-Crick pairing site of G₅ 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 G₁₂ 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 F₂₅₄ aluminium sheets (0.2-mm layer; Merck, Darmstadt, Germany). Column flash chromatography (FC): silica gel 60 (Merck, Darmstadt, Germany); at 0.4 bar (4 · 10⁴ 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 (¹H) and 500.14 MHz (¹H; 125.13 MHz for ¹³C), resp.; chemical shifts δ in ppm rel. to SiMe₄ 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)-4H-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 anhyd. 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 H₂O (6 ml), and kept for 15 min. Then, the mixture was treated with 25% aq. NH₃ soln. (6 ml), stirred for another 15 min, and concentrated to 10 ml. H₂O (150 ml) was added, the aq. soln. washed with CH₂Cl₂ (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, CH₂Cl₂/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 (CH₂Cl₂/MeOH 8 : 2) 0.2. UV (MeOH): 268 (13200), 274 (13100), 302 (13200). ¹H-NMR ((D₆)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*, CH₂); 5.10 (*br. s.*, OH–C(5′)); 5.33 (*br. s.*, OH–C(3′)); 5.58 (*br. s.*, OH–C(2′)); 5.67 (*d*, ³*J* = 6.3, H–C(1′)); 6.98–7.07 (*m*, H–C(7), 3 arom. H); 7.35 (*t*, ³*J* = 7.7, 2 arom. H); 8.23 (*s*, H–C(2)); 10.27 (*br. s.*, NH), 11.33 (*br. s.*, NH). Anal. calc. for C₁₉H₂₀N₄O₇ (416.38): C 54.81, H 4.84, N 13.46; found: C 54.89, H 4.91, N 13.39.

4-[(Diphenylcarbamoyl)oxy]-N⁶-(phenoxyacetyl)-1-(β-D-ribofuranosyl)-1H-imidazo[4,5-c]pyridin-6-amine (8**).** Compound **7** (200 mg, 0.48 mmol) was dried by co-evaporation with anhyd. pyridine (5 ml) and suspended in dry pyridine (5 ml). To this suspension, diphenylcarbamoyl chloride (250 mg, 1.1 mmol) and (i-Pr)₂EtN (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. NaHCO₃ soln. (20 ml), the org. phase extracted with CH₂Cl₂ (20 ml, twice), the combined org. phase dried (Na₂SO₄) and evaporated, and the residue adsorbed on silica gel and applied to FC (silica gel, column 10 × 2 cm,

$\text{CH}_2\text{Cl}_2/\text{MeOH}$ 9:1): **8** (115 mg, 39%). Pale yellow powder. R_f ($\text{CH}_2\text{Cl}_2/\text{MeOH}$ 9:1) 0.5. UV (MeOH): 228 (43100), 269 (17700), 275 (sh, 16700). $^1\text{H-NMR}$ ((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 $\text{C}_{32}\text{H}_{29}\text{N}_5\text{O}_8$ (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]-4H-imidazo[4,5-c]pyridin-4-one (**9**). Compound **7** (840 mg, 2.0 mmol) was dried by co-evaporation with anhyd. pyridine (20 ml) and dissolved in dry pyridine (20 ml) under gentle heating. To this soln., $(\text{MeO})_2\text{Tr-Cl}$ (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, $\text{CH}_2\text{Cl}_2/\text{MeOH}$ 9:1): **9** (1.04 g, 72%). Colorless solid. R_f ($\text{CH}_2\text{Cl}_2/\text{MeOH}$ 9:1) 0.4. UV (MeOH): 270 (sh, 12100), 275 (12800). $^1\text{H-NMR}$ ((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 $\text{C}_{40}\text{H}_{38}\text{N}_4\text{O}_9$ (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]-N⁶-(phenoxyacetyl)-4-[[tris(1-methylethyl)silyl]oxy]-1H-imidazo[4,5-c]pyridin-6-amine (**10**). To a soln. of **9** (210 mg, 0.29 mmol) in anhyd. pyridine (3 ml), AgNO_3 (74 mg, 0.44 mmol) was added under stirring at r.t. After 10 min, a soln. of $(i\text{-Pr})_3\text{SiCl}$ (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 $(i\text{-Pr})_3\text{SiCl}$ (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, $\text{CH}_2\text{Cl}_2/\text{acetone}$ 7:3): **10** (61 mg, 23%). Colorless foam. R_f ($\text{CH}_2\text{Cl}_2/\text{MeOH}$ 9:1) 0.4. UV (MeOH): 233 (sh, 37000), 269 (25300), 275 (25900), 302 (21600). $^1\text{H-NMR}$ ((D_6) DMSO): 1.09 (*d*, $^3J = 7.5$, 3 Me_2CH), 1.49 (*d*, $^3J = 7.5$, 3 Me_2CH), 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 $\text{C}_{49}\text{H}_{58}\text{N}_4\text{O}_9\text{Si}$ (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]-N⁶-(phenoxyacetyl)-1H-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]-N⁶-(phenoxyacetyl)-1H-imidazo[4,5-c]pyridin-6-amine (**12**). To a soln. of **9** (850 mg, 1.18 mmol) in anhyd. pyridine (15 ml), diphenylcarbamoyl chloride (410 mg, 1.8 mmol) and $(i\text{-Pr})_2\text{EtN}$ (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, $\text{CH}_2\text{Cl}_2/\text{acetone}$ 4:1). The slow migrating main zone furnished **11** (650 mg, 61%). Colorless solid. R_f ($\text{CH}_2\text{Cl}_2/\text{acetone}$ 4:1) 0.4. UV (MeOH): 228 (62100), 269 (20700), 274 (sh, 20200). $^1\text{H-NMR}$ ((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 $\text{C}_{53}\text{H}_{47}\text{N}_5\text{O}_{10}$ (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 ($\text{CH}_2\text{Cl}_2/\text{MeOH}$ 9:1) 0.4. UV (MeOH): 229 (59400), 269 (18100), 274 (17500). $^1\text{H-NMR}$ ((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 $\text{C}_{66}\text{H}_{56}\text{N}_6\text{O}_{11}$ (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]-N⁶-(phenoxyacetyl)-1H-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]-N⁶-(phenoxyacetyl)-1H-imidazo[4,5-c]pyridin-6-amine (**14**). To a soln. of **11** (500 mg, 0.55 mmol) in anhyd. pyridine (5 ml),

AgNO₃ (140 mg, 0.83 mmol) was added under stirring at r.t. A soln. of (i-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-Pr)₃SiCl (62 µl, 0.29 mmol) was added. After 12 h, the AgCl was filtered off, the filtrate diluted with 5% aq. NaHCO₃ soln. (10 ml), the aq. phase extracted with CH₂Cl₂ (20 ml, twice), the combined org. phase washed with H₂O (20 ml) and brine (30 ml), dried (Na₂SO₄), and evaporated, and the residue applied to FC (silica gel, column 4 × 10 cm, CH₂Cl₂/AcOEt/petroleum ether 3:1:1). The faster migrating zone yielded **13** (300 mg, 51%). Colorless foam. *R*_f (CH₂Cl₂/AcOEt/petroleum ether 3:1:1) 0.4. UV (MeOH): 229 (59900), 269 (20100), 274 (sh, 19600). ¹H-NMR ((D₆)DMSO): 0.81–0.91 (*m*, 3 Me₂CH₂); 3.28 (*m*, 2 H–C(5')); 3.69 (*s*, 2 MeO); 4.14 (*m*, H–C(3'), H–C(4')); 4.69 (*t*, ³*J* = 4.2, H–C(2')); 4.77 (*s*, CH₂); 5.33 (*d*, ³*J* = 6.0, OH–C(3')); 5.98 (*d*, ³*J* = 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 C₆₂H₆₇N₅O₁₀Si (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 (CH₂Cl₂/AcOEt/petroleum ether 3:1:1) 0.45. UV (MeOH): 229 (61900), 269 (20600), 274 (sh, 20200). ¹H-NMR ((D₆)DMSO): 0.95–0.98 (*m*, 3 Me₂CH₂); 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*, CH₂); 5.66 (*d*, ³*J* = 6.3, OH–C(2')); 5.91 (*d*, ³*J* = 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 C₆₂H₆₇N₅O₁₀Si (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]-N⁶-(phenoxyacetyl)-1H-imidazo[4,5-c]pyridin-6-amine 3'-(2-cyanoethyl diisopropylphosphoramidite) (3). A stirred soln. of **13** (300 mg, 0.28 mmol) in anhyd. CH₂Cl₂ (9 ml) was pre-flushed with Ar and treated with (i-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 (CH₂Cl₂/acetone 98:2). After 4 h, the mixture was diluted with CH₂Cl₂ (40 ml), washed with 5% aq. NaHCO₃ soln. (20 ml, twice) and brine (20 ml), dried (Na₂SO₄), and evaporated and the residue co-evaporated with CH₂Cl₂ (20 ml) and purified by FC (silica gel, column 3 × 10 cm, CH₂Cl₂/acetone 98:2): **3** (267 mg, 75%). Colorless foam. *R*_f (CH₂Cl₂/acetone 98:2): *R*_f 0.45, 0.5. ³¹P-NMR (CDCl₃): 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 ³²P-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 MgCl₂. 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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