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RNA-cleaving 10–23 deoxyribozyme with a single amino acid-like functionality operates without metal ion cofactors†

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A series of 10–23 deoxyribozymes (**D2–D9**) containing single amino-acid-bearing nucleosides (thr^{6}dA, hisam^{6}dA, hisam^{5}dU and ncmnm^{5}dU) at positions 4, 5, 8 or 15 of the catalytic core was obtained by chemical synthesis. The deoxyribozymes were screened for their catalytic efficiency, and in the presence of 1 mM Mg^{2+} two of them, containing at position 8 either hisam^{5}dU (**D8**) or ncmnm^{5}dU (**D9**), were found to be RNA nucleases several times more active than their non-modified precursor. Moreover, in the magnesium-free TRIS or PIPES buffers, these enzymes were able to catalyze the cleavage of the phosphodiester bond located between the 5′-GpU-3′ sequence of the complementary RNA substrate. The cleavage reaction proceeded with the highest efficiency at pH > 7.

Introduction

Deoxyribozymes are single-stranded oligodeoxyribonucleotides that can function as specific RNA endonucleases by binding to predetermined sequences of RNA and cleaving the phosphodiester backbone.[1] Highly efficient, sequence-specific cleavage of RNA is a prerequisite for their application, both as general tools for manipulation with RNA[1,2] and as therapeutic agents.[3] The most prominent representative of the RNA-cleaving deoxyribozymes contains the so-called 10–23 catalytic core. This enzyme was originally identified by Santoro and Joyce[4] by *in vitro* selection.[5] It consists of the conserved 15-nucleotide (nt) catalytic core and the substrate recognition arms (6 to 12 nucleotides) of variable sequences that confer target RNA specificity. The cleavage of an RNA substrate occurs between an unpaired purine and paired pyrimidine residues through a divalent-metal-cation-assisted transesterification reaction. The products are two fragments of the substrate RNA: the 5′-terminal fragment with a 2′,3′-cyclic phosphate moiety and the 3′-terminal fragment with a free 5′-hydroxyl function.[6] Despite many successful applications,[1–3] very little is known about the mechanism of the cleavage reaction that is catalyzed by 10–23 deoxyribozyme as the three-dimensional structure of the enzyme-substrate complex in the catalytically active form has not yet been determined.[7] Therefore, our knowledge about the nucleoside units that are essential for the deoxyribozyme activity has been gained mainly from SAR studies that have been performed on systematically mutated enzymes or on modified enzymes that contain analogs of natural nucleosides within the catalytic core.[4,6,8]

It has already been proven that in the catalytic core of 10–23 deoxyribozyme the nucleosides at positions 1–6 and 13–14 are essential for the cleavage activity, while the thymidine unit at position 8 can be deleted or exchanged for any other base without any loss of the catalytic potential.[4,8,9] Trimming of this core by four units (from positions 5–8) led to the generation of a Ca^{2+}-dependent deoxyribozyme.[10–12] Further studies demonstrated that position 8 has a high tolerance for chemical modification. Substitution with azobenzene-modified thymidine in this position provided a tool for the photochemical control of the enzyme activity.[13] In addition, the introduction of the 6-nitropiperonyloxymethyl (NPOM) group at N3 of thymidine located at position 4 or 8 was used to study its influence on the catalytic activity of 10–23 deoxyribozyme by photochemical activation.[14] While the modification at position 4 led to catalytically active deoxyribozyme only after UV irradiation, modifying position 8 resulted in active enzyme independent of UV irradiation. Deoxyribozymes with covalently attached intercalator groups in other positions of the core (*i.e.*, modified unit inserted between dA_{15} and T_{16}) exhibited enhanced RNA cleavage activity.[15]

Mutational analysis showed that the enzyme tolerates 2′-OMe modifications introduced at positions 2, 7, 8, 11, 14 and 15 of the 10–23 catalytic core.[16] Moreover, such deoxyribozymes exhibit remarkably enhanced nucleolytic stability. Besides, modification of the binding arms with 2′-OMe and LNA (Locked Nucleic Acid) units, 3′-inverted nucleosides and phosphorothioate (P_S) internucleotide bonds, led to the chemical stabilization of 10–23 deoxyribozyme in human plasma.[16–18] Phosphorothioate functions were also used to study the importance of the phosphate groups for the catalysis and metal-binding mode. Thus, the P_S-modification was

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introduced into each individual internucleotide bond of the catalytic core of 10–23 deoxyribozyme and such constructs were screened for their cleavage activity in the presence of divalent metal ions (Mg^{2+} and Mn^{2+}).[19] While most of the PS-deoxyribozymes were equally or less active than the parent enzyme, two of these that contained a phosphorothioate bond 5′-adjacent to the units at positions 1 or 8 exhibited enhanced cleavage activity, especially in the presence of manganese ions. The use of stereodefined, diastereoisomerically pure PS-deoxyribozymes, together with a detailed mutational analysis of the nucleoside at position 6, allowed proposal of the structure of the metal binding site at the 10–23 catalytic core.[19] Moreover, analysis of thio and rescue effects for the cleavage reaction catalyzed by 10–23 deoxyribozyme, in which the stereodefined, diastereomerically pure R_P or S_P-phosphorothioate substrates (the phosphorothioate bond was present at the 5′-GU-3′ cleavage site) were used, allowed proposal of a triester-like mechanism for metal-assisted catalysis.[20]

Other studies, which aimed at the generation of catalysts with enhanced cleavage activity, were based on deoxyribozymes that were ''decorated'' with amino acid residues. Such protein-like modifications on the deoxyribozyme unit may provide catalysts with extended functionality. In general, there are two ways to prepare catalytically active nucleic acids that contain modified units. The first method is to use the 5′-triphosphates of modified nucleosides[21–26] for the enzymatic preparation of the pool of oligonucleotides. This is followed by identification of the best enzyme *via in vitro* selection.[5] Modified nucleosides often bear an imidazole ring or an amine function that is able to act as a general base or a metal chelator. Such units can be incorporated into the DNA molecule using mutant DNA polymerases that tolerate functionalized nucleotide triphosphates during the polymerase chain reaction (PCR).[27] Typically, the new functionality is introduced at each occurrence of the particular nucleoside unit within the DNA sequence. However, it is impossible to site-specifically introduce modified nucleosides into oligodeoxyribonucleotide using the *in vitro* selection procedure. The second method uses the synthetic approach, in which phosphoramidite or H-phosphonate derivatives of modified monomers are applied in solid-supported synthesis.[28,29] This approach results in oligonucleotides that have site-specific modifications. Until now, only a few examples of protein-like modified deoxyribozymes have been selected using either the synthetic or the combinatorial approach, which exerted their catalytic activity without metal ion cofactors.[22–23,30–32]

In the present study, we aimed to extend the catalytic activity of 10–23 deoxyribozyme by introducing additional amino acid-like functions (threonine, histamine and glycine) into the catalytic core. Several new nucleosides that were modified with these amino acid residues were site-specifically incorporated into 10–23 deoxyribozyme (Fig. 1). The series of modified enzymes that were obtained by the chemical synthesis were screened for their ribonucleolytic activity under *in vitro* conditions. They were then characterized with respect to their metal ion cleavage dependence and substrate specificity.

Results and discussion

Chemistry

Synthesis of amino acid-modified 2′-deoxyadenosine monomers. Two 2′-deoxyadenosine derivatives were designed that contained either threonine or histamine residues attached to an N-6 exocyclic amino group *via* a carbamate group. The threonyl-modified 2′-dA is an analog of the naturally occurring threonyl-adenosine nucleoside that has been identified in tRNA molecules.[33] The desired threonyl nucleoside **5a** (thr^{6}dA) and the histamyl nucleoside **5b** (hisam^{6}dA) were obtained from a di-*O*-acetyl derivative of 2′-deoxyadenosine (**2**) by activation of the *exo*-amine function with a phenoxycarbonyl residue (Scheme 1). Compound **3** was subjected to NH-substituent exchange with protected threonine or histamine residues, followed by deacetylation of the 2′-deoxyribose hydroxyl functions.

The crucial carbamate **3** was obtained in good yield by treatment of nucleoside **2** with phenoxycarbonyltetrazole,[34] or with the more easily available and less expensive 1-*N*-methyl-3-phenoxycarbonyl-imidazolium chloride.[35] Reaction of **3** with a two-fold molar excess of *O*-*tert*-butyl-dimethylsilyl-L-threonine trimethylsilylethyl ester[36] or with N^{Im}-(4-methoxytrityl)histamine[37] in anhydrous pyridine afforded nucleosides **4a** and **4b** in 69% and 90% yield, respectively. Removal of the acetyl groups from **4a** and **4b** yielded the nucleosides **5a** and **5b**, respectively. The primary 5′-hydroxyl functions of **5a** and **5b** were protected with a 4,4′-dimethoxytrityl group and the resulting derivatives **6a** and **6b** were phosphitylated with 2-chloro-2-cyanoethyl-*N*,*N*-diisopropyl-phosphoramidite. The structure of the monomers **7a** and **7b** was confirmed by ^{31}P NMR and FAB MS analyses.

Synthesis of amino acid-modified thymidine monomers. Two monomeric units, **15a** and **15b**, the derivatives of 2′-deoxyuridine nucleoside modified with histamine or glycine methyl ester residues (the methoxycarbonyl methylamino-methyl group, mcmnm) at position C-5, were obtained in the series of reactions outlined in Scheme 2.

Thus, the 5-methyl group in the acetylated thymidine **9** was oxidized by the use of $K_2S_2O_8$ in the presence of $CuSO_4$ and 2,6-lutidine in acetonitrile–water solution.[38] The resulting 5-formyl-2′,3′-di-*O*-acetyl-2′-deoxyuridine **10** was subjected to reductive amination with N^{Im}-(4-methoxytrityl)histamine or the glycine methyl ester hydrochloride according to our recently described procedure.[39] The desired amine derivative **11a** was obtained in good yield (55%), along with the mixture of isomers **12a**, which are dihydro-2′-deoxyuridine derivatives with enamine-type substituents (yield: 41% of **12a**). In contrary, 5-methoxycarbonyl-aminomethyl derivative **11b** was isolated in 82% yield. The acetyl groups in **11a** and **11b** were removed selectively with Et_3N/MeOH 1 : 9 v/v solution, and the resulting nucleosides **13a** and **13b** were transformed into the fully protected 3′-*O*-phosphoramidities **15a** and **15b** by 5′-*O*-dimethoxytritylation and standard phosphitylation with chloro-2-cyanoethyl-*N*,*N*-diisopropyl-phosphoramidite.

Synthesis of oligonucleotides D1–D9. Four standard phosphoramidite monomers of dA, dC, dG and T, as well as

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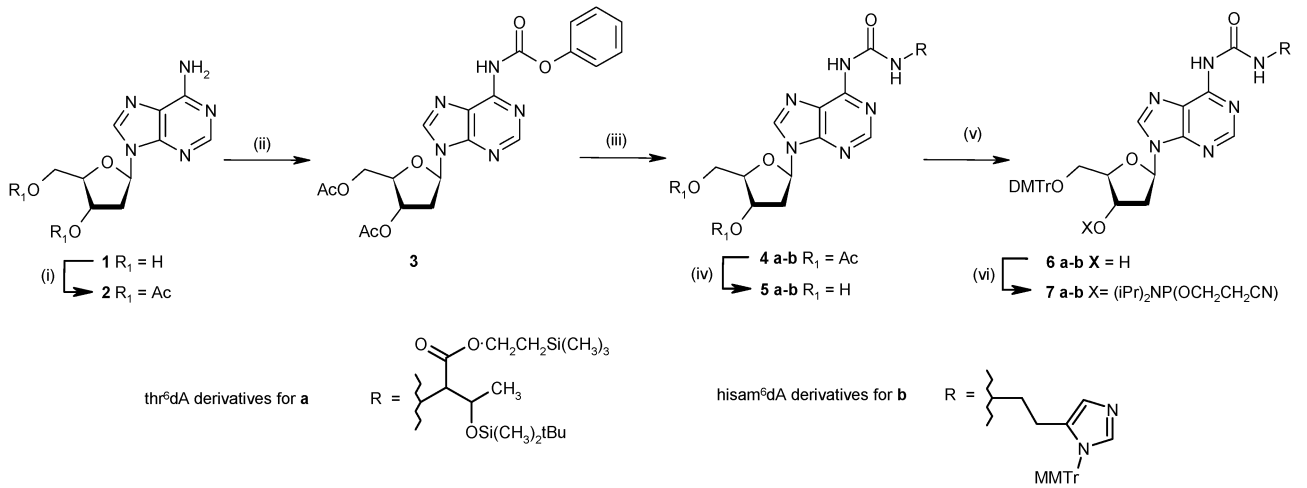
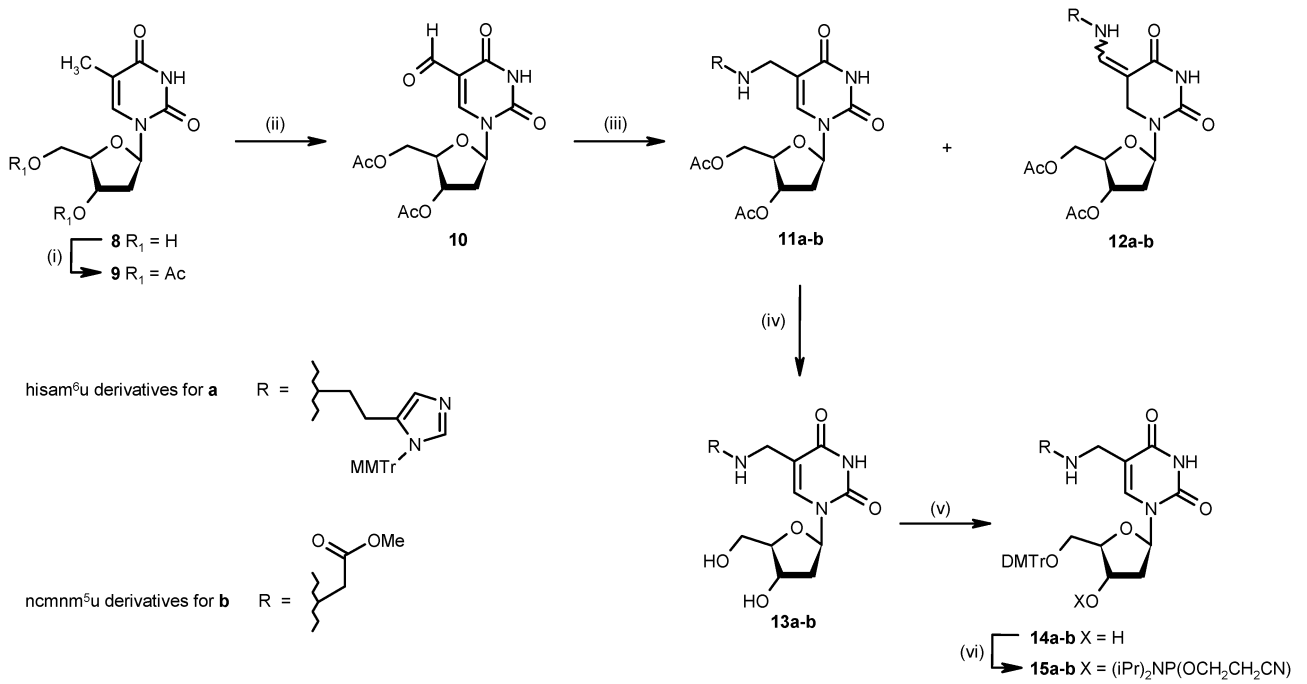


Scheme 1 Synthesis of the phosphoramidite monomers of the modified 2′-deoxyadenosine units. *Reagents and conditions*: (i) Ac_2O, pyridine, 85%; (ii) 1-methyl-3-phenoxycarbonyl-imidazolium chloride, CH_2Cl_2, room temp., 75%; (iii) for **4a**, trifluoroacetate of *O*-*tert*-butyldimethylsilyl-L-threonine trimethylsilylethyl ester, pyridine, 35 °C, 69%; for **4b**, N^{Im}-(MMTr)-histamine, pyridine, 35 °C, 78%; (iv) 10 M NH_3/MeOH, room temp., for **5a**, 75%, for **5b**, 78%; (v) DMTrCl, pyridine, room temp. for **6a**, 71%, for **6b**, 63%; (vi) chloro-2-cyanoethoxy-*N*,*N*-diisopropylamine-phosphoramidite, DIPEA, CH_2Cl_2, room temp., for **7a**, 57%, for **7b**, 59%.



Scheme 2 Synthesis of the phosphoramidite monomers of the modified 2′-deoxyuridine derivatives: (i) Ac_2O, pyridine, 85%; (ii) $K_2S_2O_8$, $CuSO_4 \cdot 2H_2O$, 2,6-lutidine, CH_3CN–H_2O 1 : 1 v/v, 65 °C, 57%; (iii) direct reductive amination: for **11a**: 1.2 eq. N^{Im}-(MMTr)-histamine trifluoroacetate, 1.2 eq. Et_3N, 1.2 eq. $NaBH(OAc)_3$, CH_2Cl_2, room temp., and an additional 0.5 equiv. $NaBH(OAc)_3$ after 2 h, overall reaction time 4 h, 55%; for **11b**: 1.2 eq. glycine methyl ester hydrochloride, 1.2 eq. Et_3N, 1.2 eq. $NaBH(OAc)_3$, CH_2Cl_2. room temp., and additional 0.5 eq. $NaBH(OAc)_3$ after 2 h, overall reaction time 4 h, 82%; (iv) Et_3N/MeOH 1 : 9 v/v, room temp. for **13a**, 72%, for **13b**, 66%; (v) DMTrCl, pyridine, room temp., for **14a**, 61%, for **14b**, 68%; (vi) chloro-2-cyanoethoxy-*N*,*N*-diisopropylamine-phosphoramidite, DIPEA, CH_2Cl_2, room temp., for **15a**, 56%, for **15b**, 51%.

phosphoramidite derivatives of the modified 2′-deoxyadenosine (**7a**,**b**) and 2′-deoxyuridine (**15a**,**b**) were used for the synthesis of the site-specifically modified oligonucleotides of the 10–23 deoxyribozyme sequence (Fig. 1).

The monomers **7a**,**b** were used to introduce the amino acid-modified dA units at the positions 5 or 15, while **15a**,**b** were used to introduce the corresponding 2′-deoxyuridine units at the positions 4 or 8 of the deoxyribozyme catalytic core. According to earlier reports, the nucleoside units at positions 4, 5 and 15 are conserved and crucial for high catalytic activity.[8] In contrast, position 8 was reported to be less important for the function of the deoxyribozyme and could

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Table 1 The sequences of 10–23 deoxyribozymes bearing modifications at the selected position of the catalytic core (**D2–D9**) and the sequence of the substrate (**S1**). The MALDI TOF data of the oligonucleotides and the yield of the synthesis at the 1 μmol scale are given. The oligonucleotide substrate is composed of two short stretches of DNA in the recognition arms (small letters) and a 5′-GU-3′ ribonucleotide dimer at the cleavage site

No.	Position of modification	Sequences of oligonucleotides	M–H$^-$ calc. [*m*/*z*]	M–H$^-$ obtain. [*m*/*z*]	Yield [OD]
D1	unmod.10–23	5′-agg gtt gag gct agc tac aac gat cat ctg t-3′	9571	9568	61.8
D2	5/thr^{6}dA	5′-agg gtt gag gct (thr^{6}dA)gc tac aac gat cat ctg t-3′	9718	9713	65.5
D3	5/hisam^{6}dA	5′-agg gtt gag gct (hisam^{6}dA)gc tac aac gat cat ctg t-3′	9711	9708	58.7
D4	15/thr^{6}dA	5′-agg gtt gag gct agc tac aac g(thr^{6}dA)t cat ctg t-3′	9718	9714	61.2
D5	15/hisam^{6}dA	5′-agg gtt gag gct agc tac aac g(hisam^{6}dA)t cat ctg t-3′	9711	9707	55.6
D6	4/ncmnm^{5}dU[a]	5′-agg gtt gag gc(ncmcm^{5}dU) agc tac aac gat cat ctg t-3′	9643	9640[a]	67.3
D7	4/hisam^{5}dU[b]	5′-agg gtt gag gc(hisam^{5}dU) agc tac aac gat cat ctg t-3′	9680	9718[b]	56.5
D8	8/ncmnm^{5}dU[a]	5′-agg gtt gag gct agc (ncmcm ^{5}dU)ac aac gat cat ctg t-3′	9643	9640[a]	60.4
D9	8/hisam^{5}dU[b]	5′-agg gtt gag gct agc (hisam^{5}dU)ac aac gat cat ctg t-3′	9680	9717[a]	56.5
S1	Substrate	5′–aca gat gaG Uca acc ct-3′	5179	5176	72.2
S1′	Non-hydrolyzable substrate	5′–aca gat gag uca acc ct-3′	5147	5144	80.0

[a] Conversion of the methyl ester of the glycine residue to the amide function under the oligonucleotide deprotection conditions. [b] Acetylation of the histamine amine function under the oligonucleotide synthesis conditions.

be modified without loss of function.[4,6,8] The syntheses were performed on a 1 μmol scale using an Applied Biosystems 394 synthesizer under conditions recommended by the manufacturer. The resulting oligonucleotides were cleaved from the solid support, deprotected and purified by HPLC using the routine two-step procedure[40] (Table 1).

The molecular masses of deoxyribozymes **D1–D9** were confirmed by MALDI-TOF mass spectrometry analysis, while 95 + % purity was assessed using polyacrylamide gel electrophoresis (PAGE), followed by PhosphorImager quantification. The sequence of the parent 10–23 deoxyribozyme (**D1**) was designed to cleave the RNA substrate homologous to BACE1 (aspartyl protease Asp2) mRNA at positions 1801–1817. The sequences of deoxyribozymes **D2–D9**, bearing amino acid-like residues at the selected positions, and the respective sequence of a short substrate **S1** that is homologous to the target site of BACE mRNA are given in Table 1. The designed oligonucleotide substrate is composed of two short stretches of DNA in the recognition arms and a 5′-GU-3′ ribonucleotide dimer at the cleavage site. Such chimeric substrates are cleaved equally efficiently in the deoxyribozyme catalyzed reactions, as are the corresponding fully RNA substrates.[41] It should be noted that, during the synthesis, the secondary amine function of the histamine-modified 2′-deoxyuridine (as in oligomers **D7** and **D9**) was acetylated at the capping steps and the acetyl group was not removed under standard deprotection conditions. In the case of dA derivative (as in oligomers **D3** and **D5**), the less nucleophilic amine group of the histamine residue, being a part of carbamate function, even if acetylated during the synthesis, was successfully deprotected at the final ammonolysis step. Similarly, the NH group of the side chain of ncmnmd^{5}U unit was deprotected in the final step of the **D6** and **D8** preparation.

For further studies, these acetylated units were used. Moreover, the treatment of oligonucleotides **D6** and **D8** with ammonia resulted in the transformation of the methyl ester of the glycine residues into the corresponding amides. Therefore, these two deoxyribozymes carried amidocarboxymethylaminomethyl (ncmnm) substituents at the modified units located at positions 4 and 8 of the catalytic core, respectively.

The hybridization properties of the modified deoxyribozymes D2–D9 complexed with the non-hydrolyzable substrate S1′. Melting temperature experiments were performed to analyze the influence of the protein-like substituents on the thermodynamic parameters of the enzyme/substrate complexes. Non-hydrolyzable oligodeoxyribonucleotide **S1′** of the sequence that is homosequential to the target site was used in these experiments to prevent cleavage of the substrate. The melting temperatures (T_m) and the ΔH, ΔS and ΔG values of the dissociation of enzyme/substrate complexes are given in Table 2. The majority of complexes demonstrated hybridization parameters and binding affinities that were similar to the parent, non-modified complex **D1**/**S1′**. The complexes of the deoxyribozymes **D2** and **D4**, which were modified with threonine at position 5 or 15, respectively, were slightly less stable than **D1**/**S1′** (ΔT_m = 1.7–1.8 °C). This was possibly due to repulsion between the negatively charged carboxyl function of the threonyl residue and the phosphate backbone of the substrate. In contrast, the complexes of deoxyribozymes **D8** and **D9** were found to be slightly more stable (if any) than the remaining screened complexes, based on the values of T_m and ΔG.

Enzymatic studies

Cleavage activity of the modified deoxyribozymes in the presence of the metal ion cofactor. The deoxyribozymes **D2–D9** were screened for their catalytic activity in the cleavage reaction performed under single turnover conditions and in the presence of 10 mM magnesium chloride. The cleavage efficiency was determined by calculating the percentage of the radioactivity of the 5′-radiolabeled product and the non-cleaved substrate (after separation by polyacrylamide gel electrophoresis). The resulting data are shown at Fig. 2a. In 20 min reactions, the enzymes **D4**, **D6**, **D8** and **D9** were equally as active as **D1**. In contrast, the **D3** and **D5** enzymes, which contain hisam^{6}dA at positions 5 and 15, respectively, were significantly less active (only ~20% cleavage). The enzymes **D2** and **D7**, which were modified at position 5 with threonine and at position 4 with histamine residues, partially

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Table 2 The thermodynamic parameters for the dissociation of the complexes of deoxyribozymes **D1–D9** with the oligodeoxyribonucleotide **S1′** substrate

Complex **S1′/D**	T_m/°C	ΔH/kcal mol^{-1}	ΔS/cal K^{-1} mol^{-1}	ΔG/kcal mol^{-1}
D1	44.0	−65.55	−180.25	−9.65
D2	42.3	−72.60	−203.21	−9.57
D3	44.3	−73.30	−204.14	−9.99
D4	42.2	−72.62	−203.29	−9.57
D5	43.3	−70.47	−196.03	−9.67
D6	43.4	−71.85	−200.00	−9.82
D7	43.8	−73.59	−205.45	−9.87
D8	44.4	−79.81	−224.49	−10.18
D9	44.2	−78.60	−220.90	−10.08

Conditions: 3 μM deoxyribozyme; buffer: 10 mM TRIS; pH 7.5; 100 mM NaCl; 10 mM $MgCl_2$, T_m standard error ±0.2 °C.

lost their catalytic activity. The same reaction, when performed at a lower magnesium ion concentration (3 mM) and for a shorter time (5 min), gave more informative results (Fig. 2b). Two modified deoxyribozymes **D8** and **D9**, which contain at position 8 nucleosides ncmnm^{5}dU or hisam^{5}dU, respectively, exhibited enhanced catalytic activity in comparison to **D1**. It means that the protein-like residues at position 8 of the catalytic core allow to manipulate the nucleolytic acitivity of the parent 10–23 enzyme. Moreover, our results confirm that position 8 of the 10–23 catalytic core is highly tolerant for chemical modifications. In contrast, 10–23 deoxyribozyme hardly tolerates protein-like decorations at positions 4, 5 and 15. We might expect some loss of the activity of the enzyme modified at positions 4 and 5, since, according to earlier reports, these positions are crucial for the 10–23 catalytic core.[8] Surprisingly, as the nature of a nucleoside at position 15 is not important for the activity of 10–23 deoxyribozyme, we rather expected that decoration of this unit with amino acid residues would not reduce the enzyme activity and even might enhance its catalytic potential. The observed loss of activity might originate from the close proximity of the bulky amino acid residues to the scissile phosphate bond.

Kinetic analysis of the cleavage reaction catalyzed by **D8** and **D9**, carried out at 1 mM magnesium concentration (similar to that found in the cell) in PBS buffer (140 mM phosphate buffered saline) containing 100 mM NaCl, at 37 °C, under single turnover conditions (*i.e.*, a 100-fold molar excess of the enzyme over the substrate), has shown that the k_{obs} values of 0.137 ± 0.003 and 0.166 ± 0.010 min^{-1}, respectively, were 3.3 and 4.0-fold higher than for **D1** (k_{obs} 0.042 ± 0.003 min^{-1}) (Fig. 3). These data allow for important conclusion that introduction of a single protein-like function at this position results in enhancement of the catalytic activity. Among the two screened protein-like substituents (histamine and glycine), the histamine residue had a more positive effect. One can assume that because this residue bears an imidazole substituent, it may operate as a general base (the pK_a is 6.0 at 25 °C[42]). Therefore, it may catalyze the *trans*-esterification reaction *via* activation of the 2′-OH group involved in the cleavage of the phosphodiester linkage,[43] supporting the triester-like mechanism.[20]

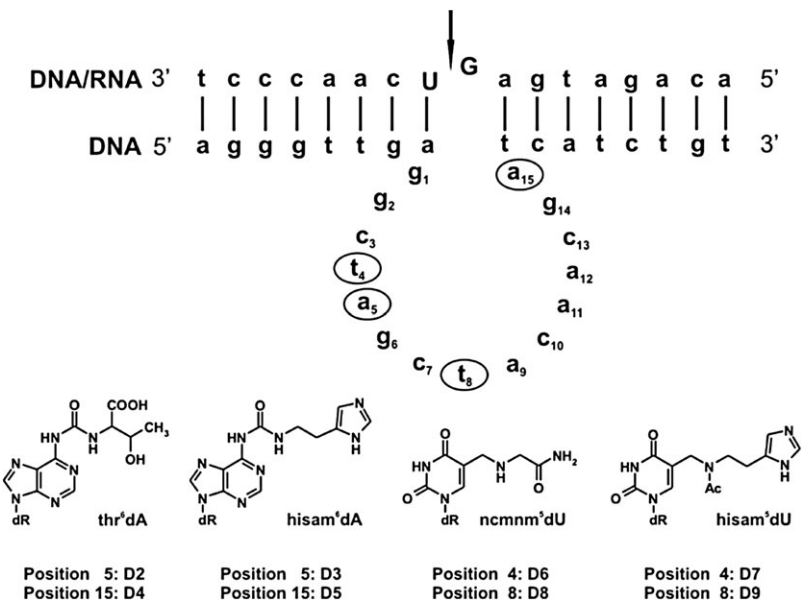

Fig. 1 The sequence of the 10–23 deoxyribozyme/substrate and the structures of modified nucleosides incorporated into the catalytic core. The target substrate is a chimeric DNA–RNA oligonucleotide that is homosequential to the mRNA of BACE1 (nucleotides 1801–1817). Lower case letters abbreviate the 2′-deoxyribonucleosides, and upper case letters abbreviate the ribonucleosides.

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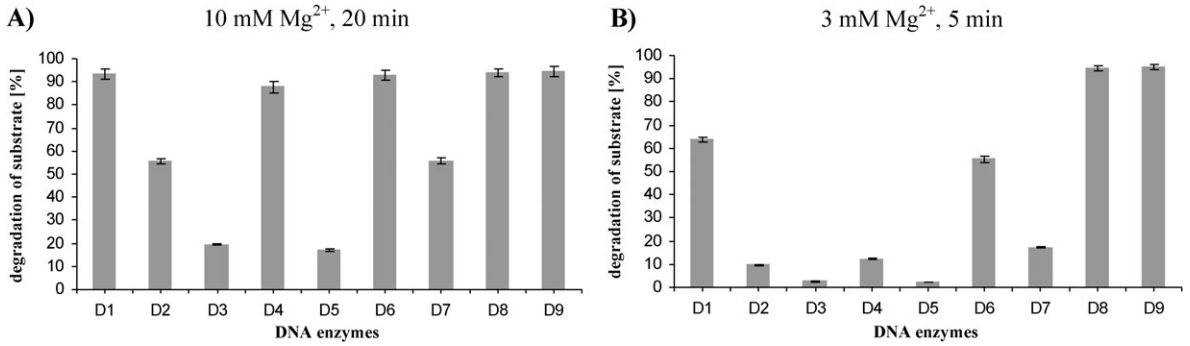

Fig. 2 Comparison of the cleavage activity of the modified deoxyribozymes D2–D9 and unmodified 10–23 (**D1**) towards substrate S in PBS buffer (pH 7.5) at 37 °C: (a) at 10 mM Mg^{2+}, after 20 min; (B) at 3 mM Mg^{2+}, after 5 min. The ^{32}P-labeled substrate S was incubated in the presence of a 100-fold excess of each enzyme. The cleavage reactions were analyzed by electrophoresis on a 20% denaturing polyacrylamide gel. All values given are the average of two independent experiments; the error bars indicate ±SD.

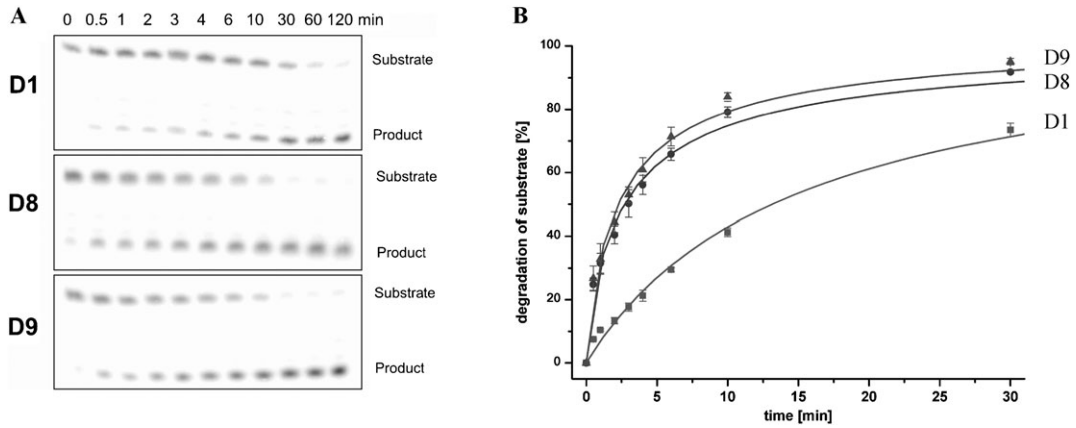

Fig. 3 Kinetic analysis of the cleavage reaction catalyzed by modified deoxyribozymes **D8** and **D9** and by the parent 10–23 deoxyribozyme (**D1**) under single turnover conditions. The cleavage reactions were performed in PBS buffer (pH 7.5) containing 100 mM NaCl and 1 mM Mg^{2+} with 0.1 μM 5′-end ^{32}P-labeled substrate and 10 μM deoxyribozyme at 37 °C. (A) The electrophoretic analysis of the cleavage reactions, (B) the time course of the substrate cleavage ν (k_{obs} = 0.042 ± 0.003 min^{-1} for **D1**, 0.137 ± 0.003 for **D8** and 0.166 ± 0.010 for **D9**).

The cleavage potential of the modified deoxyribozymes without the metal ion cofactor. Many studies have shown that certain deoxyribozymes can operate *in vitro* without divalent metal ions. However, they require either the presence of an amino acid cofactor (*e.g.*, the histidine–dependent DNAzyme[44]) or low pH which assists the intra-strand general acid/base catalysis,[45] or high concentrations of monovalent ions.[46] Moreover, obtained by *in vitro* selection modified deoxyribozymes that have been decorated with protein-like residues are shown to be catalytically active in the absence of metal ion cofactors. However, the cleavage efficiency of these enzymes is rather low. Recent studies by Perrin's group,[22,31] have demonstrated that enzymes that contain three different side chains, mimicking histidine, lysine and arginine, achieve remarkable catalysis parameters.

The authors selected deoxyribozyme Dz9-86, which cleaves the phosphate linkage at ribocytidine embedded in a DNA strand with a rate constant of 0.13 min^{-1}.[22] Dz9-86 is not sensitive to the ionic strength of the buffer or to the nature of the monovalent ion (Na^+, K^+ or Li^+). Reselection from an extended (N40) library delivered Dz10-66, which operates at the highest rate for the M^{2+}-independent self-cleavage reaction that has been reported to date for any nucleic acid catalyst (k_{obs} = 0.63 ± 0.04 min^{-1} at 37 °C).[31] This enzyme was also effective under multiple turnover conditions and cleaved in *trans* an RNA substrate with the catalytic efficiency k_{cat} = 0.20 ± 0.01 min^{-1}, K_M = 382 ± 1 nM. These encouraging results prompted us to investigate whether the 10–23 deoxyribozymes **D3**, **D5–D9**, which carry a glycine or histamine function, maintain any activity in the absence of divalent metal ion cofactors.

(a) The catalytic activity of modified deoxyribozymes in the absence of magnesium ions. The cleavage reactions were performed under single turnover conditions in two different buffers: 20 mM TRIS containing 100 mM NaCl or PBS, both at pH 7.5, at 37 °C for 24 h. The reactions were carried out in the presence of $MgCl_2$ (3 mM) or absence of divalent metal ions. We used 4 mM EDTA to prevent any divalent ion contamination. The electrophoretic analysis of the reaction products is shown in Fig. 4.

As expected, the parent deoxyribozyme **D1** was inactive under the conditions listed above for the reactions carried out in the absence of Mg^{2+} ions, regardless of the buffer used. Similarly, the modified enzymes **D3**, **D5**, **D6** and **D7** were inactive in the reactions carried out in the absence of Mg^{2+}

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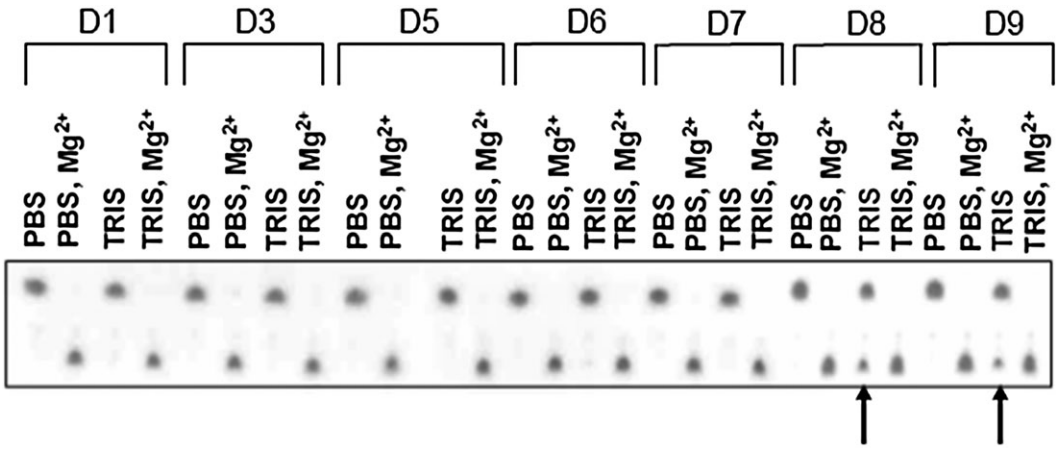

Fig. 4 PAGE analysis of the products generated from the ^{32}P-labeled substrate by the modified 10–23 deoxyribozymes in PBS containing $MgCl_2$ (3 mM) or in the absence of metal ions, and in 20 mM TRIS, pH 7.5, containing NaCl (100 mM) and $MgCl_2$ (3 mM) or in the absence of magnesium ions. The 4 mM EDTA was added to the buffers to assure the absence of Mg^{2+} ions. The reactions were performed using 10 μM 10–23 deoxyribozyme and 0.1 μM substrate at 37 °C for 24 h. The radiolabeled product and substrate were separated using electrophoresis in a 20% denaturing polyacrylamide gel.

ions. The **D3**, **D5** and **D7** enzymes were less active than **D1** in previous experiments carried out at 3 mM magnesium ions for 5 min (see Fig. 2B). Interestingly, the last two deoxyribozymes of the screened series, **D8** and **D9**, produced the cleavage product in TRIS buffer in the absence of magnesium ions, whereas the substrate remained intact in the buffer solution without enzyme (data not shown). The cleavage activity of **D8** and **D9** was lower (only ~30% substrate was cleaved) than their activity in the presence of 3 mM Mg^{2+}, where complete substrate cleavage was observed. In contrast, neither **D8** nor **D9** were catalytically active in PBS buffer that was deprived of Mg^{2+} ions (Fig. 4). This observation indicates that even a single amino acid function located at a specific site of the catalytic core may be sufficient to facilitate the cleavage of the phosphate bond within the RNA chain.

(b) Activity of D8 and D9 in HEPES, PIPES and EPPS buffers. As the enzymes **D8** and **D9** exhibited their cleavage ability only in TRIS buffer, and not in PBS buffer, we examined whether the functional groups of the buffer affect the catalysis. As PBS and TRIS have no functional groups in common, for further experiments we selected three other buffers that are typically used in biochemical experiments, *i.e.*, HEPES, PIPES and EPPS (Fig. 5). These contain hydroxyalkyl and/or alkylsulfonyl residues. The cleavage reactions catalyzed by **D8** and **D9** that were performed in the absence of Mg^{2+} exhibited a small amount of the cleavage product in PIPES buffer only, and not in HEPES nor EPPS buffers (Fig. 5). It is rather hard to explain these results, as HEPES contains the hydroxyalkyl functional group that is also present in TRIS and the ethylsulfonyl moiety that is present in PIPES. Thus, if the functional groups of the buffers play any catalytic role, one would expect that HEPES should assist the cleavage reaction. The control reactions that were performed either in HEPES, PIPES or EPPS, but without deoxyribozymes, did not afford cleavage products (Fig. 5). Nonetheless, one cannot exclude the possibility that the primary amino function of TRIS molecule facilitates the catalysis, although in this case the cleavage product should be observed both in the control reaction performed without deoxyribozymes and in all screened cleavage reactions performed in the presence of the remaining deoxyribozymes.

(c) Kinetics of the cleavage reactions catalyzed by D8 and D9 in Mg^{2+}-free buffers (TRIS and PIPES). The results of the kinetic analysis of the reactions catalyzed by **D8** and **D9** in TRIS and PIPES buffers at pH 7.5 or 8.0 in the absence of magnesium ions are shown in Table 3.

The highest k_{obs} values of 0.85 ± 0.49 and 1.25 ± 0.54 min^{-1} were observed for the reactions catalyzed by **D8** and **D9**, respectively, carried out in 20 mM TRIS buffer at pH 8.0. These values are 20 and 30 times greater than the k_{obs} value for

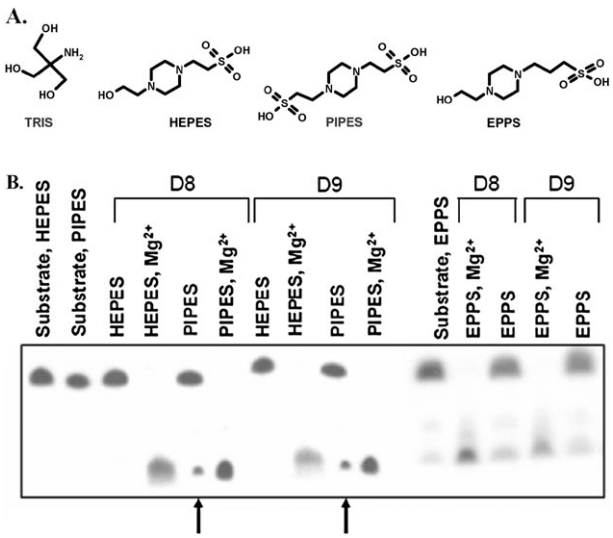

Fig. 5 (A) The structures of buffer salts used in the study: TRIS, HEPES, PIPES and EPPS. (B) A comparison of the cleavage activity of the modified deoxyribozymes **D8** and **D9** in HEPES, PIPES and EPPS buffers, pH 7.5, in the presence and absence of Mg^{2+} ions, at 37 °C for 24 h. The cleavage products in the Mg^{2+}-free system are indicated by arrows.

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Table 3 Single-turnover rate constants (min^{-1}) of the cleavage reactions catalyzed by deoxyribozymes **D8** and **D9** that were carried out in the absence of Mg^{2+}. The measurements were done in triplicate. ±SD the standard deviations

	TRIS		PIPES	
Enzyme	pH 7.5	pH 8.0	pH 7.5	pH 8.0
D8	0.42 ± 0.06	0.85 ± 0.49	0.20 ± 0.09	0.35 ± 0.17
D9	0.65 ± 0.19	1.25 ± 0.54	0.35 ± 0.01	0.79 ± 0.65

the reaction catalyzed by **D1** (0.0415 ± 0.0025 min^{-1}) performed in the presence of 1 mM Mg^{2+} at pH 7.5. Moreover, in each case **D9** has 1.5–2.0 times higher k_{obs} values than **D8**, and both enzymes are approximately twice as active in TRIS buffer as in PIPES buffer. These data indicate that the hisam^{5}dU unit at position 8 better facilitates the catalysis than the ncmcm ^{5}dU unit, and that for any further applications TRIS buffer should be selected. Interestingly, the cleavage reactions catalysed by **D8** and **D9** were less efficient in TRIS buffer at concentrations above 50 mM (data not shown). The oligomers **D8** and **D9**, which bear only single protein-like functionalities, are good candidates for further structure optimization to become potential tools for applications in cellular and *in vivo* experiments. Their kinetic parameters are comparable to those for the recently described Dz10-66[31] that was modified with as much as three different functionalities.

(d) The pH and temperature dependence of the cleavage activity of D8 and D9 in TRIS buffer. The cleavage reactions were performed in TRIS buffer, in which both enzymes operate without magnesium ions at the highest efficiency. The results depicted in Fig. 6 indicate that the activity of both enzymes is pH-dependent. Both enzymes are inactive at pH < 6, and are most active at pH > 7.0. The temperature did not appear critical, and this differs our 10–23 deoxyribozymes that were modified with amino acid residues from previously selected deoxyribozymes, which were catalytically optimal at lower temperatures (*e.g.*, 13 °C for $Dz9_{25}$-11).[30]

(e) Substrate specificity. The activities of **D8** and **D9** towards the sixteen substrates that had the general sequence 5′-acagatgaN1N2caaccct-3′ and that contained all possible two-nucleotide mutations in the cleavage site (N1 and N2 = A, G, U or C) were determined in the presence or absence of Mg^{2+} ions in TRIS buffer, pH 7.5, at 37 °C for 24 h. The cleavage yields (listed in Fig. 7) allow the following conclusions: (i) only the substrate containing the 5′-GU-3′-dinucleotide at the cleavage site is processed by the **D8** and **D9** enzymes in the Mg^{2+}-free buffer. (ii) In the presence of magnesium ions, **D8** and **D9** are as active as **D1** in processing the substrates containing the GU, GA, AU, GC, UU, AA and GG dinucleotides at the cleavage site. (iii) Elevated activity of **D8** and **D9** is observed towards the substrates containing the UG, AG, AC UA, CU and AA dinucleotides which are poor substrates for **D1**. We assume that these amino acid-like modified enzymes **D8** and **D9** will be also active towards all-RNA substrates, similarly to the parent **D1**.[1–3,19]

Conclusions

In this paper we describe the synthesis of dA modified with threonine (thr^{6}dA) or histamine (hisam^{6}dA) residues as well as 2′-dU units containing glycine (ncmnm^{5}dU) or histamine (hisam^{5}dU) residues. The modified nucleosides were transformed into 3′-phosphoramidites and, together with standard monomers, were used for the synthesis of oligonucleotides of 10–23 deoxyribozyme sequence. This enzyme is known as an efficient RNA cleaving enzyme operating in the presence of divalent metal ions. In the studies we introduced single amino acid-bearing nucleoside units at positions 4, 5, 8 or 15 of the catalytic core. The obtained deoxyribozymes **D2–D9** were screened for their catalytic efficiency, and in the presence of Mg^{2+} two of them, **D8** and **D9**, containing at position 8 either hisam^{5}dU or ncmnm^{5}dU, respectively, were found to be RNA nucleases slightly more active than their non-modified **D1** precursor. Recent studies[21–22,31] have shown that certain deoxyribozymes when decorated with multiple protein-like residues can operate *in vitro* in the absence of divalent metal ions. In our case, single amino acid residue containing deoxyribozymes **D8** and **D9** were able to catalyze the Mg^{2+}-free cleavage of the phosphodiester bond located between the 5′-GpU-3′ sequence of the complementary RNA substrate. The cleavage reaction was pH-dependent and proceeded with the highest efficiency at pH > 7 in TRIS or PIPES buffer only. We do not have any rational explanation, why these two buffers facilitate the catalysis. It might be that only in these buffers the amino acid base residues of the enzyme **D8** or **D9** are close enough to the reacting 2′-OH group to activate it and promote the transesterification reaction.

Thus, in summary, by the synthetic approach we identified single protein-like modified 10–23 deoxyribozymes, which in the presence of Mg^{2+} ions are more active than their parent

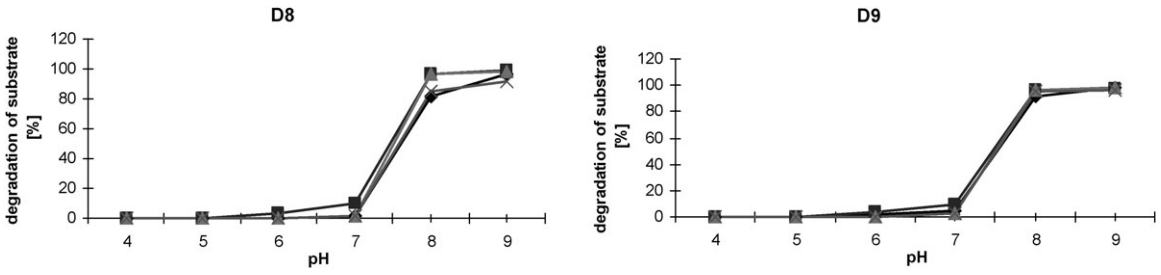

Fig. 6 The effects of temperature and pH on the Mg^{2+}-free cleavage reactions catalyzed by modified deoxyribozymes **D8** and **D9** (the percentage of the cleaved substrate is given on the *y* axis). The reactions were carried out under single turnover conditions in TRIS buffer at pHs ranging from 4 to 9 that contained 100 mM NaCl and 4 mM EDTA at 25 (×), 30 (▲), 37 (◆) and 43 °C (■) for 24 h.

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D9	D8	D1 non-modified	
97.0	97.8	99.0	GG/Mg^{2+}
0	0	0	GG
95.7	93.8	53.0	AA/Mg^{2+}
0	0	0	AA
3.4	2	0	CC/Mg^{2+}
0	0	0	CC
98.0	98.0	100.0	UU/Mg^{2+}
0	0	0	UU
95.0	97.0	55.0	CU/Mg^{2+}
0	0	0	CU
9.3	6.0	0	UC/Mg^{2+}
0	0	0	UC
0	0	0	CG/Mg^{2+}
0	0	0	CG
99.0	98.0	99.0	GC/Mg^{2+}
0	0	0	GC
50.0	48.0	19.0	UA/Mg^{2+}
0	0	0	UA
99.0	98.0	99.0	AU/Mg^{2+}
0	0	0	AU
5.6	4.0	2.0	CA/Mg^{2+}
0	0	0	CA
29.0	27.0	4.0	AC/Mg^{2+}
0	0	0	AC
36.4	29.0	3.0	AG/Mg^{2+}
0	0	0	AG
22.3	17.7	3.5	UG/Mg^{2+}
0	0	0	UG
100.0	99.0	100.0	GA/Mg^{2+}
0	0	0	GA
100.0	100.0	100.0	GU/Mg^{2+}
33.0	28.5	2.9	GU

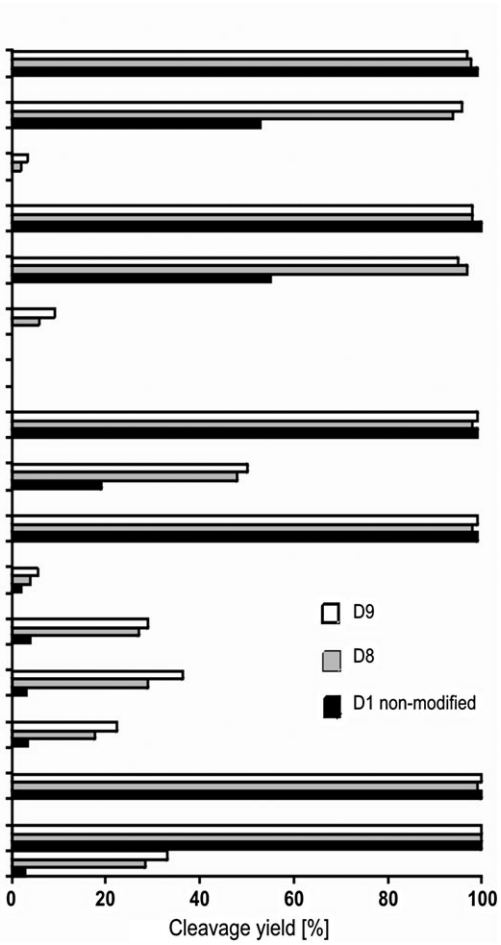

Fig. 7 The yields of the cleavage reaction of the acagatgaN1N2caaccct oligonucleotides, where N1 and N2 = A, G, U or C units, catalyzed by deoxyribozymes **D1**, **D8** and **D9**. The cleavage reactions were carried out in TRIS buffer at pH 7.5 and either using 3 mM Mg^{2+} ions or in the Mg^{2+}-free system (with 4 mM EDTA) for 24 h, at 37 °C.

precursor and can operate in a magnesium-free systems cleaving the phosphodiester bond in chimeric DNA–RNA substrates.

Experimental

General experimental data

Dichloromethane and pyridine were dried under reflux from calcium hydride. Thin layer chromatography was done on Merck coated plates $60F_{254}$. Silica gel used for column chromatography was from Merck (particle size 30–60 μm). The ^{1}H NMR spectra were recorded on a Bruker Avance DPX-250 spectrometer and chemical shifts are reported in δ values relative to tetramethylsilane (^{1}H) or 85% phosphoric acid (^{31}P) as an external standard. All coupling constants are quoted in Hertz. The FAB MS spectra were recorded on a Finnigan Mat 95 and MALDI-TOF spectra on a Voyager Elite Instrument.

N6-Modified-2′-deoxyadenosines

3′,5′-Di-*O*-acetyl-N^6-phenoxycarbonyl- 2′-deoxyadenosine (3). (a) With 1-phenoxycarbonyltetrazole.[34] 3′,5′-Di-*O*-acetyl-2′-deoxyadenosine (1.16 g, 3.5 mmol) was dissolved in anhydrous dioxane (35 mL) and treated with 1-phenoxycarbonyltetrazole (2.0 g, 10.5 mmol) at 35 °C. The reaction was monitored with TLC ($CHCl_3$–MeOH 90 : 10 v/v) and after 24 h, when no nucleoside substrate was detected, the reaction mixture was evaporated to dryness and the residue was chromatographed on silica gel column using 0–2% methanol in chloroform. Nucleoside **3** was obtained as white foam (1.20 g, 75%).

(b) With 1-methyl-3-phenoxycarbonylimidazolium chloride.[35] 3′,5′-Di-*O*-acetyl-2′-deoxyadenosine (1.68 g, 5 mmol) was dissolved in dry CH_2Cl_2 (50 mL) and then 1-methyl-3-phenoxycarbonylimidazolium chloride (3.6 g, 15 mmol) was added. After the reaction mixture was stirred for 2 h at r.t., the solution was evaporated in vacuum and the residue dissolved in small amount of EtOAc. Purification on a silica gel column using the same solvent as the eluent gave the nucleoside **3** (1.71 g, 75%).

TLC R_f: 0.63 ($CHCl_3$–MeOH 90 : 10), 0.42 (AcOEt).

δ_H (250 MHz, $CDCl_3$; Me_4Si) 2.07 (3H, s, CH_3COO), 2.14 (3H, s, CH_3COO), 2.67 (1H, ddd, $J_{H2',H3'}$ = 2.5 Hz, $J_{H2',H1'}$ = 6.0 Hz, J_{gem} = 14.2 Hz, H2′), 2.98 (1H, ddd, $J_{H2'',H3'}$ = 6.5 Hz, $J_{H2'',H1'}$ = 7.7 Hz, J_{gem} = 14.2 Hz, H2″), 4.27–4.49 (3H, m, H4′, H5′, H5″), 5.44 (1H, m, H3′), 6.49 (1H, dd, $J_{H1',H2'}$ = 6.1 Hz, $J_{H1',H2''}$ = 7.7 Hz, H1′), 6.80–7.47 (5H, m, PhO), 8.26 (1H, s, H2), 8.81 (1H, s, H8), 9.34 (1H, bs, CON*H*).

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3′,5′-Di-*O*-acetyl-N6-[(1*S*,2*R*)-1-{2-(trimethylsilyl)ethoxy]-carbonyl}-2-{[(*tert*-butyl)dimethylsilyl]oxy}propylaminocarbonyl]-2′-deoxyadenosine (4a). 3′,5′-di-*O*-acetyl-N^6-phenoxycarbonyl-2′-deoxyadenosine (650 mg, 1.4 mmol) was treated with 2 molar excess of trifluoroacetate of *O*-*tert*-butyl-dimethylsilyl-L-threonine trimethylsilylethyl ester (1.75 g, 2.8 mmol) in anhydrous pyridine (10 mL) at 35 °C. After 72 h, TLC analysis ($CHCl_3$–MeOH, 98 : 2) revealed no starting nucleoside in the reaction mixture. The solvent was evaporated and the residue, after coevaporation with toluene (2 × 15 mL), was chromatographed over silica gel column using 0–2% methanol in chloroform. 3′,5′-*O*-Acetyl-N^6-threonylcarbonyl-2′-deoxyadenosine derivative was obtained as white foam (690 mg, 69%).

TLC R_f: 0.09 ($CHCl_3$–MeOH 98 : 2); 0.40 (AcOEt–MeOH 98 : 2).

m/*z* (MS, FAB) 695.4 $[M + H]^+$, 693.4 $[M - H]^-$ ($C_{30}H_{50}Si_2N_6O_9$ requires 694.3); *m*/*z* (HRMS, FAB) 695.3233 ($[M + H]^+$ $C_{30}H_{51}Si_2N_6O_9$ requires 695.3256).

δ_H (250 MHz, $CDCl_3$; Me_4Si) 0.03 (9H, s, $(CH_3)_3Si$), 0.04 (3H, s, CH_3Si), 0.09 (3H, s, CH_3Si), 0.94 (9H, s, $(CH_3)_3CSi$), 0.96–1.06 (2H, m, $SiCH_2CH_2$), 1.27 (3H, d, $J_{CH\gamma,CH\beta}$ = 6.3 Hz, CH_3-γ), 2.09 (3H, s, CH_3COO), 2.15 (3H, s, CH_3COO), 2.65 (1H, ddd, $J_{H2',H3'}$ = 2.6 Hz, $J_{H2',H1'}$ = 6.0 Hz, J_{gem} = 14.0 Hz, H2′), 3.02 (1H, ddd, $J_{H2'',H3'}$ = 6.3 Hz, $J_{H2'',H1'}$ = 7.9 Hz, J_{gem} = 14.0 Hz, H2″), 4.10–4.64 (7H, m, CH_2CH_2O, H4′, H5′, H5″, CH-β CH-α), 5.45 (1H, m, H3′), 6.45 (1H, dd, $J_{H1',H2'}$ = 6.0 Hz, $J_{H1',H2''}$ = 7.9 Hz, H1′), 8.17 (1H, s, H2), 8.22 (1H, bs, NHCO), 8.54 (1H, s, H8), 10.04 (1H, d, *J* = 9.3 Hz, CONHCH).

δ_C NMR (63 MHz, $CDCl_3$) −5.19 (CH_3Si), −4.10 (CH_3Si), −1.44 ($(CH_3)_3Si$), 17.45 ($(CH_3)_3CSi$), 17.99 ($SiCH_2CH_2$), 20.86 (CH_3COO), 21.04 (CH_3COO), 21.28 (CH_3-γ), 25.72 ($(CH_3)_3CSi$), 37.40 (C2′), 59.70 (CH-α), 63.77 (CH_2CH_2O), 63.86 (C5′), 68.98 (CH-β), 74.69 (C3′), 82.83 (C4′), 84.92 (C1′), 121.17 (C5), 141.84 (C8), 150.77 (C4), 151.39 (C2), 154.76 (C6), 170.37 (CH_3COO), 170.57 (CH_3COO), 171.20 ($COOCH_2$).

N^6-[(1*S*,2*R*)-1-{[2-(trimethylsilyl)ethoxy]carbonyl}-2-{[(*tert*-butyl)dimethylsilyl]oxy}propylaminocarbonyl]-2′-deoxyadenosine (5a). Protected nucleoside **4a** (556 mg, 0.8 mmol) was treated with saturated ammonia in MeOH (~10 M, 17 mL) and stirred at r.t. for 24 h. The solvent was then evaporated to give an oil residue which was purified by silica gel chromatography (eluent 0–10% MeOH in $CHCl_3$). Deacetylated nucleoside **5a** was obtained as white foam (370 mg, 75%).

TLC R_f: 0.34 ($CHCl_3$–MeOH 90 : 10); 0.36 (AcOEt–MeOH 90 : 10).

m/*z* (MS, CI) 611.3 $[M + H]^+$($C_{26}H_{46}N_6O_7Si_2$ requires 610.3).

δ_H (250 MHz, $CDCl_3$; Me_4Si), 0.00 (9H, s, $(CH_3)_3Si$), 0.01 (3H, s, CH_3Si), 0.07 (3H, s, CH_3Si), 0.90 (9H, s, $(CH_3)_3CSi$), 0.92–1.02 (2H, m, $SiCH_2CH_2$), 1.25 (3H, d, $J_{CH\gamma,CH\beta}$ = 6.2 Hz, CH_3-γ), 2.37 (1H, m, H2′), 3.02 (1H, m, H2″), 3.80–4.56 (7H, m, CH_2CH_2O, H4′, H5′, H5″, CH-β, CH-α), 4.79 (1H, m, H3′), 6.44 (1H, dd, $J_{H1',H2'}$ = 5.6 Hz, $J_{H1',H2''}$ = 9.0 Hz, H1′), 8.17 (1H, s, H2), 8.47 (1H, s, H8), 8.64 (1H, bs, NHCO), 10.00 (1H, d, $J_{NH\alpha,CH\alpha}$ = 9.1 Hz, CONHCH).

3′,5′-Di-*O*-acetyl-N^6-{2-[N^1-(4-methoxytrityl)-imidazol-4-yl]-ethylaminocarbonyl}-2′-deoxyadenosine (4b). 3′,5′-Di-*O*-acetyl-N^6-phenoxycarbonyl-2′-deoxyadenosine (**3**) (271 mg, 0.6 mmol) was dried by repeated co-evaporation from anhydrous pyridine (2 × 5 mL) before being dissolved in the further portion of the solvent (4 mL). N^{Im}-(4-methoxytrityl)histamine[37] (460 mg, 1.2 mmol, 2.0 equiv.), also pre-dried by co-evaporation from anhydrous pyridine, was added to the stirring solution of **3** and the reaction mixture was left overnight at 35 °C. As no substrate nucleoside was detected on TLC plate ($CHCl_3$–MeOH, 90 : 10) after this period, the reaction mixture was concentrated in vacuum and co-evaporated with toluene (3 × 5 mL) to remove the traces of pyridine. The crude product was purified by silica gel chromatography (eluent 0–5% MeOH in $CHCl_3$) yielding protected nucleoside **4b** as cream-colored foam (350 mg, 78%).

TLC R_f: 0.44 ($CHCl_3$–MeOH 90 : 10); 0.28 (AcOEt–MeOH 90 : 10).

m/*z* (MS, FAB) 745.3 $[M + H]^+$, 743.1 $[M - H]^-$, ($C_{40}H_{40}N_8O_7$ requires 744.3); *m*/*z* (HRMS, FAB) 745.3078 ($[M + H]^+$ $C_{40}H_{41}N_8O_7$ requires 745.3082).

δ_H (250 MHz, $CDCl_3$; Me_4Si) 2.08 (3H, s, CH_3COO), 2.14 (3H, s, CH_3COO), 2.63 (1H, ddd, $J_{H2',H3'}$ = 2.5 Hz, $J_{H2',H1'}$ = 6.0 Hz, J_{gem} = 14.2 Hz, H2′), 2.90 (2H, t, *J* = 6.7 Hz, $ImCH_2$), 3.02 (1H, ddd, $J_{H2'',H3'}$ = 6.3 Hz, $J_{H2'',H1'}$ = 8.0 Hz, J_{gem} = 14.2 Hz, H2″), 3.66–3.76 (2H, m, CH_2NH), 3.77 (3H, s, OCH_3), 4.31–4.47 (3H, m, H4′, H5′, H5″), 5.46 (1H, dt, $J_{H3',H2'}$ = $J_{H3',H4'}$ = 2.3 Hz, $J_{H3',H2''}$ = 6.1 Hz, H3′), 6.46 (1H, dd, $J_{H1',H2'}$ = 5.9 Hz, $J_{H1',H2''}$ = 8.0 Hz, H1′), 6.66 (1H, d, $^4J_{H5,H2}$ = 1.4 Hz, Im-CH-5), 6.73–7.35 (14H, m, MMTr), 7.39 (1H, d, $^4J_{H2,H5}$ = 1.4 Hz, Im-CH-2), 8.27 (1H, s, H2), 8.31 (1H, bs, NHCO), 8.34 (1H, s, H8), 9.56 (1H, t, $J_{NH\alpha,CH\alpha}$ = 5.6 Hz, $CONHCH_2$).

δ_C (63 MHz, $CDCl_3$) 20.97 (CH_3COO), 21.13 (CH_3COO), 28.89 ($ImCH_2$), 37.57 (C2′), 40.17 (CH_2NH), 55.44 (OCH_3), 63.91 (C5′), 74.71 (C3′), 75.00 (MMTr–CPh_3), 82.90 (C4′), 84.92 (C1′), 113.38 (MMTr-o′), 118.92 (Im-CH-5), 121.02 (C5), 128.13 (MMTr-o,p), 129.82 (MMTr-m), 131.29 (MMTr-m′), 134.69 (Im-CH-4), 138.64 (Im-CH-2), 138.75(MMTr-i′), 141.22 (C8), 142.93 (MMTr-i), 150.13 (C4), 150.64 (NHCONH), 151.43 (C2), 154.01 (C6), 159.23 (MMTr-p′), 170.47 (CH_3COO), 170.62 (CH_3COO).

N^6-{2-[N^1-(4-methoxytrityl)-imidazol-4-yl]ethylaminocarbonyl}-2′-deoxyadenosine (5b). Acetylated nucleoside **4b** (335 mg, 0.45 mmol) was treated with saturated ammonia in MeOH (~10 M, 9.5 mL) and stirred at r.t. for 24 h. After this period, TLC analysis revealed no starting protected nucleoside. Evaporation of the solvent and purification of the residue by column chromatography (eluent 0–15% MeOH in $CHCl_3$) gave nucleoside **5b** as creamy foam (230 mg, 78%).

TLC R_f: 0.45 ($CHCl_3$–MeOH 85 : 15); 0.25 (AcOEt–MeOH 80 : 20).

m/*z* (MS, FAB) 661.5$[M + H]^+$, 659.3 $[M - H]^-$, ($C_{36}H_{36}N_8O_5$ requires 660.3).

δ_H (250 MHz, $CDCl_3$; Me_4Si) 2.42 (1H, ddd, $J_{H2',H3'}$ = 1.9 Hz, $J_{H2',H1'}$ = 5.7 Hz, J_{gem} = 13.5 Hz, H2′), 2.91 (1H, ddd, $J_{H2'',H3'}$ = 5.2 Hz, $J_{H2'',H1'}$ = 7.7 Hz, J_{gem} = 13.5 Hz, H2″), 3.05 (2H, t, *J* = 6.5 Hz, $ImCH_2$), 3.77

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(3H, s, OCH_3), 3.78–4.03 (4H, m, CH_2NH, H5′, H5′′), 4.19 (1H, m, H4′), 4.84 (1H, m, H3′), 6.42 (1H, dd, $J_{H1',H2'}$ = 5.9 Hz, $J_{H1',H2''}$ = 7.7 Hz, H1′), 6.75 (1H, d, $^4J_{H5,H2}$ = 0.7 Hz, Im-C*H*-5), 6.76–7.38 (15H, m, MMTr, Im-C*H*-2), 8.32 (1H, s, H2), 8.40 (1H, s, H8), 8.55 (1H, bs, N*H*CO), 9.58 (1H, t, $J_{NH\alpha,CH\alpha}$ = 5.5 Hz, CON*H*CH_2).

5-Modified 2′-deoxyuridines

3′,5′-Di-*O*-acetyl-5-{2-[N1-(4-methoxytrityl)-imidazol-4-yl]-ethylaminomethyl}-2′-deoxyuridine (11a). 5-Formyl-3′,5′-di-*O*-acetyl-2′-deoxyuridine (**1**) (340 mg, 1 mmol) was dried by repeated co-evaporation with anhydrous CH_2Cl_2 (2 × 10 mL) and finally dissolved in the same solvent (7 mL). To this stirred solution, N^{Im}-(4-methoxytrityl)histamine (460 mg, 1.2 mmol, 1.2 equiv.) and Et_3N (167 μL, 1.2 mmol) was added followed by immediate addition of $NaBH(OAc)_3$ (254 mg, 1.2 mmol). After 2 h at room temp., when TLC analysis ($CHCl_3$–MeOH-90 : 10, v/v) revealed some remaining aldehyde **10**, the second portion of $NaBH(OAc)_3$ (106 mg, 0.5 mmol) was added. After stirring for an additional 2 h, the reaction was quenched with $NaHCO_3$ (10 mL, 5% aq. solution) and then extracted with CH_2Cl_2 (3 × 20 mL). The combined organic phases were dried ($MgSO_4$), filtered and concentrated in vacuum. The oily residue was chromatographed on a silica gel column with increasing amounts (from 0 to 25%) of CH_3OH in $CHCl_3$. The corresponding fractions (checked on TLC with ninhydrine test) were collected and evaporated to give **11a** (390 mg, 55%) and **12a** as a mixture of *Z* and *E* isomers (290 mg, 41%).

11a: TLC R_f: 0.32 ($CHCl_3$–MeOH 90 : 10); 0.11 (AcOEt–MeOH 90 : 10).

m/*z* (MS, FAB) 708.4 $[M + H]^+$, 706.5 $[M - H]^-$, ($C_{39}H_{41}N_5O_8$ requires 707.3); *m*/*z* (HRMS, FAB) 708.3052 ($[M + H]^+$ $C_{39}H_{42}N_5O_8$ requires 708.3033).

δ_H (250 MHz, $CDCl_3$; Me_4Si) 2.10 (3H, s, CH_3COO), 2.11 (3H, s, CH_3COO), 2.30 (1H, ddd, $J_{H2',H3'}$ = 6.4 Hz, $J_{H2',H1'}$ = 8.6 Hz, J_{gem} = 14.5 Hz, H2′), 2.43 (1H, ddd, $J_{H2'',H3'}$ = 2.0 Hz, $J_{H2'',H1'}$ = 5.7 Hz, J_{gem} = 14.5 Hz, H2′′), 2.80 (2H, t, *J* = 7.0 Hz, ImCH_2), 2.99 (2H, t, *J* = 7.0 Hz, CH_2NH), 3.57 (1H, d, J_{gem} = 13.7 Hz, one of CH_2-7), 3.66 (1H, d, J_{gem} = 13.8 Hz, one of CH_2-7), 3.81 (3H, s, OCH_3), 4.21 (1H, m, H4′), 4.28 (1H, dd, $J_{H5'',H4'}$ = 3.5 Hz, J_{gem} = 12.0 Hz, H5′′), 4.39 (1H, dd, $J_{H5',H4'}$ = 4.7 Hz, J_{gem} = 12.0 Hz, H5′), 5.22 (1H, dt, $J_{H3',H2''}$ = $J_{H3',H4'}$ = 2.0 Hz, $J_{H3',H2'}$ = 6.4 Hz, H3′), 6.30 (1H, dd, $J_{H1',H2''}$ = 5.7 Hz, $J_{H1',H2'}$ = 8.6 Hz, H1′), 6.59 (1H, d, $^4J_{H5,H2}$ = 1.2 Hz, Im-C*H*-5), 6.77–7.37 (14H, m, MMTr), 7.38 (1H, d, $^4J_{H2,H5}$ = 1.4 Hz, Im-C*H*-2), 7.66 (1H, s, H6).

δ_C (63 MHz, $CDCl_3$) 21.08 (2x*C*H_3COO), 27.56 (Im*C*H_2), 37.34 (C2′), 45.52 (C7), 48.85 (*C*H_2NH), 55.47 (O*C*H_3), 64.03 (C5′), 74.58 (C3′), 75.05 (MMTr-CPh_3), 82.52 (C4′), 85.30 (C1′), 111.73 (C5), 113.45 (MMTr-o′), 118.74(Im-*C*H-5), 128.19 (MMTr-o,p), 129.82 (MMTr-m), 131.30 (MMTr-m′), 134.64 (Im-*C*H-4), 138.13 (MMTr-i′), 138.64 (C6),138.71 (Im-*C*H-2), 142.88 (MMTr-i), 150.43 (C2), 159.25 (MMTr-p′), 163.47 (C4), 170.51 (CH_3*C*OO), 170.65 (CH_3*C*OO).

12a: TLC R_f: 0.48, 0.63 ($CHCl_3$–MeOH 90 : 10); 0.23, 0.41 (AcOEt–MeOH 90 : 10).

m/*z* (MS, FAB) 708.3 $[M + H]^+$, 706.4 $[M - H]^-$ ($C_{39}H_{41}N_5O_8$ requires 707.3); *m*/*z* (HRMS, FAB) 708.3048 ($[M + H]^+$ $C_{39}H_{42}N_5O_8$ requires 708.3033).

δ_H (250 MHz, $CDCl_3$; Me_4Si) 2.08 (3H, s, CH_3COO od *Z*), 2.09 (3H, s, CH_3COO of *Z*), 2.03–2.19 (10H, m, 2xCH_3COO of *E*, H2′, H2′′ of *Z* and *E*), 2.77 (2H, t, *J* = 6.7 Hz, ImCH_2 of *Z*), 2.79–2.88 (2H, m, ImCH_2 of *E*), 3.48–3.60 (4H, m, CH_2NH of *Z* and *E*), 3.81 (3H, s, OCH_3 of *Z*), 3.82 (3H, s, OCH_3 of *E*), 3.68–3.98 (4H, m, CH_2-6 of *Z* and *E*), 3.99–4.39 (6H, m, H4′, H5′, H5′′ of *Z* and *E*), 5.06–5.19 (3H, m, H3′ of *Z* and *E*, CH_2N*H*CH of *E*), 6.30 (1H, dd, $J_{H1',H2''}$ = 5.9 Hz, $J_{H1',H2'}$ = 8.9 Hz, H1′ of *Z*), 6.41 (1H, dd, $J_{H1',H2''}$ = 5.9 Hz, $J_{H1',H2'}$ = 8.8 Hz, H1′ of *E*), 6.60 (1H, d, $^4J_{H5,H2}$ = 1.0 Hz, Im-C*H*-5 of *Z*), 6.61 (1H, d, $^4J_{H5,H2}$ = 1.0 Hz, Im-C*H*-5 of *E*), 6.70 (1H, d, $J_{H7,NH}$ = 13.1 Hz, H7 of *Z*), 6.79–7.41 (31H, m, MMTr, N*H*-3 of *Z* and *E*, Im-C*H*-2 of *E*), 7.43 (1H, d, $^4J_{H2,H5}$ = 1.3 Hz, Im-C*H*-2 of *Z*), 7.46 (1H, m, H7 of *E*), 8.43 (1H, m, CH_2N*H*CH of *Z*).

δ_C (63 MHz, $CDCl_3$) 21.00 (*C*H_3COO of *E* and *Z*), 21.06 (*C*H_3COO of *E* and *Z*), 28.90 (Im*C*H_2 of *E*), 30.09 (Im*C*H_2 of *Z*), 32.69 (C2′of *E*), 33.30 (C2′ of *Z*), 36.84 (C6 of *E*), 40.32 (C6 of *Z*), 48.75 (*C*H_2NH of *Z*), 49.34 (*C*H_2NH of *E*), 55.42 (OCH_3 of *Z* and *E*), 63.94 (C5′ of *E*), 64.16 (C5′ of *Z*), 74.34 (C3′ of *E*), 74.46 (C3′ of *Z*), 75.10 (MMTr-CPh_3 of *Z*), 75.22 (MMTr-CPh_3 of *E*), 80.44 (C4′of *Z*), 80.66 (C4′ of *E*), 84.20 (C1′ of *Z*), 84.59 (C1′ of *E*), 85.00 (C5 of *Z*), 87.43 (C5 of *Z*), 113.44 (MMtr-o′ of *Z* and *E*), 118.91 (Im-*C*H-5 of *E*), 119.60 (Im-*C*H-5 of *Z*), 128.17 (MMTr-o of *Z* and *E*, MMTr-p of *Z*), 128.44 (MMTr-p of *E*), 129.74 (MMTr-m of *Z* and *E*), 131.23 131.30 (MMTr-m′of *Z* and *E*), 134.31 (Im-*C*H-4 of *E*), 134.43 (Im-*C*H-4 of *Z*), 137.18 (MMTr-i′ of *Z*), 137.75 (MMTr-i′ of *E*), 138.57 (Im-*C*H-2 of *E*), 138.83 (Im-*C*H-2 of *Z*), 142.61 (MMTr-i of *E*), 142.70 (MMTr-i of *Z*), 146.72 (C7 of *E*), 150.15 (C7 of *Z*), 153.09 (C2 of *E*), 153.81 (C2 of *Z*), 159.25 (MMTr-p′ of *Z* and *E*), 164.91 (C4 of *E*), 166.71 (C4 of *Z*), 170.63 (CH_3*C*OO of *Z* and *E*), 171.09 (CH_3*C*OO of *Z* and *E*).

5-{2-[N^1-(4-methoxytrityl)-imidazol-4-yl]ethylaminomethyl}-2′-deoxyuridine (13a). Acetylated nucleoside **11a** (354 mg, 0.5 mmol) was treated with Et_3N/MeOH (1 : 9 v/v, 4 mL) at r.t. for 24 h. After this period, TLC analysis revealed no starting protected nucleoside. The reaction mixture was concentrated to an oil residue, re-dissolved in CH_2Cl_2 and applied onto a silica gel column. Elution was performed with a gradient of 0–15% MeOH in $CHCl_3$ to give nucleoside **13a** as foam (225 mg, 72%).

TLC R_f: 0.24 ($CHCl_3$–MeOH 85 : 15); 0.05 (AcOEt–MeOH 80 : 20).

m/*z* (MS, FAB) 624.3 $[M + H]^+$, 622.2 $[M - H]^-$ ($C_{35}H_{37}N_5O_6$ requires 623.3).

δ_H (250 MHz, $CDCl_3$; Me_4Si) 2.24 (1H, m, H2′), 2.43 (1H, ddd, $J_{H2'',H3'}$ = 1.4 Hz, $J_{H2'',H1'}$ = 5.5 Hz, J_{gem} = 14.0 Hz, H2′′), 2.75 (2H, t, *J* = 6.7 Hz, ImCH_2), 2.93 (2H, t, *J* = 6.8 Hz, CH_2NH), 3.52 (1H, d, J_{gem} = 14.1 Hz, one of CH_2-7), 3.60 (1H, d, J_{gem} = 14.1 Hz, one of CH_2-7), 3.81 (3H, s, OCH_3), 4.19–4.32 (2H, m, H4′, H5′′), 4.37 (1H, dd, $J_{H5',H4'}$ = 4.4 Hz, J_{gem} = 12.0 Hz, H5′), 5.21 (1H, dt, $J_{H3',H2''}$ = $J_{H3',H4'}$ = 2.0 Hz, $J_{H3',H2'}$ = 6.5 Hz, H3′), 6.30 (1H, dd, $J_{H1',H2''}$ = 5.6 Hz, $J_{H1',H2'}$ = 8.7 Hz, H1′), 6.58 (1H, d,

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$^4J_{H5,H2}$ = 1.2 Hz, Im-C*H*-5), 6.77–7.34 (14H, m, MMTr), 7.35 (1H, d, $^4J_{H5,H2}$ = 1.2 Hz, Im-C*H*-2), 7.56 (1H, s, H6).

3′,5′-Di-*O*-acetyl-5-methoxycarbonylaminomethyl-2′-deoxyuridine (11b). 5-Formyl-3′,5′-di-*O*-acetyl-2′-deoxyuridine (**10**) (340 mg, 1 mmol) was dried by repeated co-evaporation with anhydrous CH_2Cl_2 (2 × 10 mL) and finally dissolved in the same solvent (7 mL). To this stirred solution, hydrochloride of glycine methyl ester (180 mg, 1.5 mmol, 1.5 equiv.) and Et_3N (210 μL, 1.5 mmol) was added followed by immediate addition of $NaBH(OAc)_3$ (255 mg, 1.2 mmol). After 2 h at room temp., when TLC analysis ($CHCl_3$–MeOH-90 : 10, v/v) revealed some remaining aldehyde **10**, the second portion of $NaBH(OAc)_3$ (106 mg, 0.5 mmol) was added. After stirring for an additional 2 h, the reaction was quenched with $NaHCO_3$ (10 mL, 5% aq. solution) and then extracted with CH_2Cl_2 (3 × 20 mL). The combined organic phases were dried ($MgSO_4$), filtered and concentrated in vacuum. The oily residue was chromatographed on a silica gel column with increasing amounts (from 0 to 5%) of CH_3OH in $CHCl_3$. The corresponding fractions (checked on TLC with ninhydrine test) were collected and evaporated to give **11b** (340 mg, 82%) and **12b** as a mixture of Z and E isomers (33 mg, 8%).

11b: TLC R_f: 0.45 ($CHCl_3$–MeOH 90 : 10); 0.22 (AcOEt–MeOH 95 : 5).

m/*z* (MS, FAB) 414.2 $[M + H]^+$, 412.2 $[M - H]^-$, ($C_{17}H_{23}N_3O_9$ requires 413.1); *m*/*z* (HRMS, FAB) 414.1513 ($[M + H]^+$ $C_{17}H_{24}N_3O_7$ requires 414.1507).

δ_H (250 MHz, $CDCl_3$; Me_4Si) 2.12 (3H, s, C*H*$_3$COO), 2.14 (3H, s, C*H*$_3$COO), 2.20 (1H, m, H2′), 2.47 (1H, ddd, $J_{H2'',H3'}$ = 1.6 Hz, $J_{H2'',H1'}$ = 5.5 Hz, J_{gem} = 14.2 Hz, H2′′), 3.42 (2H, s, CH_2NH), 3.56 (2H, s, CH_2-7), 3.72 (3H, s, OCH_3), 4.22–4.35 (2H, m, H4′, H5′′), 4.40 (1H, dd, $J_{H5',H4'}$ = 4.4 Hz, J_{gem} = 12.1 Hz, H5′), 5.23 (1H, dt, $J_{H3',H2''}$ = $J_{H3',H4'}$ = 1.6 Hz, $J_{H3',H2'}$ = 6.6 Hz, H3′), 6.34 (1H, dd, $J_{H1',H2''}$ = 5.5 Hz, $J_{H1',H2'}$ = 8.8 Hz, H1′), 7.53 (1H, s, H6).

δ_C (63 MHz, $CDCl_3$) 20.60 (*C*H$_3$COO), 20.72 (*C*H$_3$COO), 37.12 (C2′), 45.27(C7), 49.67 (*C*H$_2$NH), 51.66 (O*C*H$_3$), 63.80 (C5′), 74.29 (C3′), 82.09 (C4′), 84.88 (C1′), 112.77 (C5), 136.52 (C6), 150.41 (C2), 163.43 (C4), 170.25 (CH$_3$*C*OO), 170.35 (CH$_3$*C*OO), 172.27 (*C*OOCH$_3$).

12b: TLC R_f: 0.50, 0.60 ($CHCl_3$–MeOH 90 : 10); 0.32, 0.50 (AcOEt–MeOH 95 : 5).

m/*z* (MS, FAB) 414.3 $[M + H]^+$, 412.2 $[M - H]^-$ ($C_{17}H_{23}N_3O_9$ requires 413.14).

δ_H (250 MHz, $CDCl_3$; Me_4Si) 2.07–2.22 (16H, m, 2 × C*H*$_3$COO, H2′, H2′′ of *Z* i *E*), 3.77 (3H, s, OCH$_3$ of *E*), 3.63–3.88 (2H, m, one of CH_2-6 of *Z* i *E*), 3.77 (3H, s, OCH$_3$ of *Z*), 3.90–4.40 (11H, m, one of CH_2-6, CH_2NH, H4′ of *Z* and *E*, H5′, H5′′ of *Z*, H5′′ of *E*), 4.47 (1H, dd, $J_{H5',H4'}$ = 7.0 Hz, J_{gem} = 11.1 Hz, H5′ of *E*), 5.06–5.19 (2H, m, H3′ of *Z* and *E*), 5.61 (1H, m, CH$_2$N*H* of *E*), 6.31 (1H, dd, $J_{H1',H2''}$ = 6.1 Hz, $J_{H1',H2'}$ = 8.6 Hz, H1′ of *Z*), 6.40 (1H, m, H1′ of *E*), 6.65 (1H, d, $J_{H7,NH}$ = 12.8 Hz, H7 of *Z*), 7.38 (1H, dt, $^4J_{H7,H6}$ = 1.4 Hz, $J_{H7,NH}$ = 14.4 Hz, H7 of *E*), 7.45 (1H, s, N*H*-3 of *Z*), 7.47 (1H, s, N*H*-3 of *E*), 8.48 (1H, m, CH$_2$N*H* of *Z*).

δ_C (63 MHz, $CDCl_3$) 21.04 (*C*H$_3$COO of *Z* and *E*), 21.10 (*C*H$_3$COO of *Z* and *E*), 33.40 (C2′ of *Z* and *E*), 36.35 (C6 of *E*), 40.25 (C6 of *Z*), 49.40 (*C*H$_2$NH of *Z*), 49.50 (*C*H$_2$NH of *E*), 52.52 (OCH$_3$ of *E*), 52.68 (O*C*H$_3$ of *Z*), 64.12 (C5′ of *E*), 64.17 (C5′ of *Z*), 74.37 (C3′ of *Z*), 74.42 (C3′ of *E*), 80.50 (C4′ of *Z*), 81.18 (C4′ of *E*), 84.26 (C1′ of *Z*), 84.54 (C1′ of *E*), 88.19 (C5 of *Z*), 90.52 (C5 of *E*), 145.68 (C7 of *E*), 149.18 (C7 of *Z*), 152.60 (C2 of *E*), 153.37 (C2 of *Z*), 164.88 (C4 of *E*), 166.93 (C4 of *Z*), 170.70 (CH$_3$*C*OO of *Z* and *E*), 170.77 (CH$_3$*C*OO of *Z* and *E*), 171.81 (*C*OOCH$_3$ of *Z* and *E*).

5-Methoxycarbonylaminomethyl-2′-deoxyuridine (13b). Starting nucleoside **11b** (320 mg, 0.77 mmol) was treated with Et_3N/MeOH (1 : 9 v/v, 6 mL) at r.t. After 24 h, when TLC analysis showed no protected nucleoside the reaction mixture was concentrated to an oil residue, re-dissolved in CH_2Cl_2 and applied onto a silica gel column. Elution was performed with a gradient of 0–20% MeOH in $CHCl_3$ to give nucleoside **13b** as white foam (165 mg, 66%).

TLC R_f: 0.45 ($CHCl_3$–MeOH 90 : 10); 0.22 (AcOEt–MeOH 95 : 5).

m/*z* (MS, FAB) 330.2 $[M + H]^+$, 328.2 $[M - H]^-$, ($C_{13}H_{19}N_3O_7$ requires 329.1).

δ_H (250 MHz, D_2O) 2.28–2.48 (2H, m, H2′, H2′′), 3.50 (2H, s, CH_2-α), 3.57 (2H, s, CH_2-7), 3.73 (3H, s, OCH_3), 3.76 (1H, m, H5′′), 3.85 (1H, dd, $J_{H5',H4'}$ = 3.2 Hz, J_{gem} = 12.5 Hz, H5′), 4.04 (1H, m, H4′), 4.47 (1H, m, H3′), 6.27 (1H, t, $J_{H1',H2''}$ = $J_{H1',H2'}$ = 6.6 Hz, H1′), 7.86 (1H, s, H6).

General procedure for 5′-*O*-dimethoxytritylation:

The starting nucleoside (0.35 mmol) was dried by repeated coevaporation from anhydrous pyridine (2 × 4 mL) before being finally dissolved in further portion of the solvent (4 mL). 4,4′-Dimethoxytrityl chloride (124 mg, 0.39 mmol) was added and the reaction mixture was stirred at r.t. overnight. After this period, a further portion of dimethoxytrityl chloride (0.1 equiv.) was added and after 4 h, water (7 mL) and $CHCl_3$ (15 mL) were added and the aqueous layer was extracted with $CHCl_3$ (3 × 15 mL). The combined organic layer was dried over $MgSO_4$ and evaporated to dryness. Residual pyridine was removed by evaporation with toluene (3 × 10 mL) to yield an oily crude product which was purified by silica gel chromatography (eluent 0–10% MeOH in $CHCl_3$ containing 0.1% Et_3N) to provide corresponding 5′-*O*-dimethoxytrityl derivative as a slightly yellow foam.

5′-*O*-(4,4′-Dimethoxytrityl)-N^6-[(1*S*,2*R*)-1-{[2-(trimethylsilyl)-ethoxy]-carbonyl}-2-{[(*tert*-butyl)dimethylsilyl]oxy}propylamino-carbonyl]-2′-deoxyadenosine (6a). 225 mg, yield 71%; TLC R_f: 0.64 ($CHCl_3$–MeOH 90 : 10); 0.54 (AcOEt–MeOH 95 : 5).

m/*z* (MS, CI) 913.2 $[M + H]^+$ ($C_{47}H_{64}N_6O_9Si_2$ requires 912.4).

δ_H (250 MHz, $CDCl_3$; Me_4Si), 0.03 (9H, s, (CH_3)$_3$Si), 0.05 (3H, s, CH_3Si), 0.10 (3H, s, CH_3Si), 0.95 (9H, s, (CH_3)$_3$CSi), 1.01 (2H, t, *J* = 8.5 Hz, SiCH_2CH$_2$), 1.28 (3H, d, $J_{CH\gamma,CH\beta}$ = 6.2, CH_3-γ), 2.57 (1H, m, H2′), 2.85 (1H, m, H2′′), 3.35 (1H, dd, $J_{H5'',H4'}$ = 3.3 Hz, J_{gem} = 10.3 Hz, H5′′), 3.42 (1H, dd, $J_{H5',H4'}$ = 5.3 Hz, J_{gem} = 10.3 Hz, H5′), 3.79 (6H, s, 2xOCH_3), 4.12–4.62 (5H, m, CH$_2$CH_2O, C*H*-β, C*H*-α, H4′), 4.69 (1H, m, H3′), 6.46 (1H, t, $J_{H1',H2'}$ = $J_{H1',H2''}$ = 6.5 Hz, H1′), 6.79–7.41 (13H, m, DMTr), 7.96

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(1H, bs, N*H*CO), 8.07 (1H, s, H2), 8.48 (1H, s, H8), 10.03 (1H, d, $J_{NH\alpha,CH\alpha}$ = 9.4 Hz, CON*H*CH).

5′-*O*-(4,4′-Dimethoxytrityl)-N^6-{2-[N^1-(4-methoxytrityl)-imidazol-4-yl]ethylaminocarbonyl} -2′-deoxyadenosine (6b). 212 mg, yield 63%; TLC R_f: 0.45 ($CHCl_3$–MeOH 90 : 10); 0.41 (AcOEt–MeOH 85 : 15).

m/*z* (MS, FAB) 962.9 $[M + H]^+$, 961.3 $[M - H]^-$, ($C_{57}H_{54}N_8O_7$ requires 962.4).

δ_H (250 MHz, $CDCl_3$; Me_4Si) 2.56 (1H, m, H2′), 2.91 (1H, m, H2′′), 3.05 (2H, t, *J* = 6.5 Hz, ImC*H*$_2$), 3.40–3,43 (2H, m, C*H*$_2$NH), 3.76 (9H, s, 3 × OC*H*$_3$), 3.64–3.84 (2H, m, H5′, H5′′), 4.13 (1H, m, H4′), 4.72 (1H, m, H3′), 6.47 (1H, m, H1′), 6.75 (1H, s, Im-C*H*-5), 6.74–7.40 (28H, m, DMTr, MMTr, Im-C*H*-2), 7.91 (1H, bs, N*H*CO), 8.10 (1H, s, H2), 8.28 (1H, s, H8), 9.50 (1H, t, $J_{NH\alpha,CH\alpha}$ = 5.7 Hz, CON*H*CH$_2$).

5′-*O*-(4,4′-Dimethoxytrityl)-5-{2-[N^1-(4-methoxytrityl)-imidazol-4-yl]ethylaminomethyl}-2′-deoxyuridine (14a). 197 mg, yield 61%; TLC R_f: 0.53 ($CHCl_3$–MeOH 85 : 10); 0.32 (AcOEt–MeOH 80 : 20).

m/*z* (MS, FAB) 926.3 $[M + H]^+$, 924.2 $[M - H]^-$, ($C_{56}H_{55}N_5O_8$ requires 925.4).

δ_H (250 MHz, $CDCl_3$; Me_4Si) 2.19–2.42 (2H, m, H2′, H2′′), 2.48–2.67 (4H, m, ImC*H*$_2$, C*H*$_2$NH), 3.05 (1H, d, J_{gem} = 14.0 Hz, one of CH_2-7), 3.11 (1H, d, J_{gem} = 14.0 Hz, one of CH_2-7), 3.35 (1H, dd, $J_{H5'',H4'}$ = 3.8 Hz, J_{gem} = 10.6 Hz), 3.42 (1H, dd, $J_{H5',H4'}$ = 3.8 Hz, J_{gem} = 10.6 Hz), 3.74 (6H, s, 2xOCH_3), 3.80 (3H, s, OCH_3), 3.99 (1H, q, $J_{H4',H5'}$ = $J_{H4',H5''}$ = 3.8 Hz, H4′), 4.50 (1H, m, H3′), 6.31 (1H, t, $J_{H1',H2''}$ = $J_{H1',H2'}$ = 6.5 Hz, H1′), 6.52 (1H, d, $^4J_{H5,H2}$ = 1.0 Hz, Im-C*H*-5), 6.75–7.43 (28H, m, DMTr, MMTr, Im-C*H*-2), 7.57 (1H, s, H6).

5′-*O*-(4,4′-Dimethoxytrityl)-5-methoxycarbonylaminomethyl-2′-deoxyuridine (14b). 150 mg, yield 68%; TLC R_f: 0.32 ($CHCl_3$–MeOH 90 : 10); 0.22 (AcOEt–MeOH 95 : 5).

m/*z* (MS, FAB) 632.2 $[M + H]^+$, 630.2 $[M - H]^-$, ($C_{34}H_{37}N_3O_9$ requires 631.3).

δ_H (250 MHz, $CDCl_3$; Me_4Si) 2.29 (1H, m, H2′), 2.40 (1H, ddd, $J_{H2'',H3'}$ = 4.1 Hz, $J_{H2'',H1'}$ = 6.7 Hz, J_{gem} = 14.4 Hz, H2′′), 2.93–3.15 (4H, m, C*H*$_2$NH, C*H*$_2$-7), 3.37 (1H, dd, $J_{H5',H4''}$ = 3.4 Hz, J_{gem} = 10.5 Hz, H5′′), 3.47 (1H, dd, $J_{H5',H4'}$ = 3.6 Hz, J_{gem} = 10.5 Hz, H5′), 3.65 (3H, s, OCH_3), 3.79 (6H, s, 2xOCH_3), 4.02 (1H, m, H4′), 4.55 (1H, m, H3′), 6.36 (1H, t, $J_{H1',H2''}$ = $J_{H1',H2'}$ = 6.7 Hz, H1′), 6.79–7.44 (13H, m, DMTr), 7.66 (1H, s, H6).

General procedure for 3′-*O*-phosphitylation

The starting dimethoxytritylated nucleoside (0.2 mmol) was dried in vacuum overnight before being dissolved in anhydrous CH_2Cl_2 (2 mL). The solution was then placed under argon and anhydrous diispropylethylamine (140 μL, 0.8 mmol, 4 equiv.) was added followed by the dropwise addition of 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite (67 μL, 0.3 mmol, 1.5 equiv.). After 1 h at room temp, distilled MeOH (150 μL) was added and the mixture was stirred for 2 min. After then, more CH_2Cl_2 (15 mL) was added and the organic layer was washed with 5% aqueous $NaHCO_3$ solution (5 mL), dried over $MgSO_4$, and evaporated to dryness. The residue was purified by silica gel column chromatography eluting with the mixture of solvents specified for each compound, as described below. The collected fractions containing the pure product were concentrated to dryness to obtain white foam.

3′-*O*-(2-Cyanoethyl-*N*,*N*-diisopropylaminophosphinoxy)-5′-*O*-(4,4′-dimethoxytrityl)-N^6-[(1*S*,2*R*)-1-{[2-(trimethylsilyl)ethoxy]-carbonyl}-2-{[(*tert*-butyl)dimethylsilyl]oxy} propylaminocarbonyl]-2′-deoxyadenosine (7a). Eluting mixture: CH_2Cl_2 : ethyl acetate : triethylamine (10 : 10 : 1).

127 mg, yield 57%; TLC R_f: 0.32 (CH_2Cl_2–AcOEt–Et_3N 10 : 10 : 1 v/v/v).

m/*z* (MS, FAB) 1113.7 $[M + H]^+$, 1111.9 $[M - H]^-$; ($C_{56}H_{81}N_8O_{10}PSi_2$ requires 1112.5); *m*/*z* (HRMS, FAB) 1113.5402 ($[M + H]^+$ $C_{56}H_{82}N_8O_{10}PSi_2$ requires 1113.5430).

^{31}P NMR (101 MHz, C_6D_6; ext. H_3PO_4) 148.9, 149.2.

3′-*O*-(2-Cyanoethyl-*N*,*N*-diisopropylaminophosphinoxy)-5′-*O*-(4,4′-dimethoxytrityl)-N^6-{2-[N^1-(4-methoxytrityl)-imidazol-4-yl]-ethylaminocarbonyl}-2′-deoxyadenosine (7b). Eluting mixture: Petroleum ether : acetone 3 : 1, 2 : 1, 1 : 1, 1 : 2 v/v (100 mL of each).

137 mg, yield 59%; TLC R_f: 0.15 (CH_2Cl_2–AcOEt–Et_3N 10 : 10 : 1 v/v/v).

m/*z* (MS, FAB) 1163.7 $[M + H]^+$, 1162.0 $[M - H]^-$ ($C_{66}H_{71}N_{10}O_8P$ requires 1162.5); *m*/*z* (HRMS, FAB) 1163.5235 ($[M + H]^+$ $C_{66}H_{72}N_{10}O_8P$ requires 1163.5272).

^{31}P NMR (101 MHz, C_6D_6; ext. H_3PO_4) 149.2, 149.3.

3′-*O*-(2-Cyanoethyl-*N*,*N*-diisopropylaminophosphinoxy)-5′-*O*-(4,4′-dimethoxytrityl)-5-{2-[N^1-(4-methoxytrityl)-imidazol-4-yl]-ethylaminomethyl}-2′-deoxyuridine (15a). Before purification on silica gel column compound **15a** was precipitated from petroleum ether at −78 °C. Eluting mixture: methanol : CH_2Cl_2 (5 : 95 v/v) with 5% of triethylamine.

126 mg, yield 56%; TLC R_f: 0.09 (benzene–CH_2Cl_2/Et_3N 7 : 2 : 1 v/v/v).

m/*z* (MS, FAB) 1126.0 $[M + H]^+$, 1124.7 $[M - H]^-$, ($C_{65}H_{72}N_7O_9P$ requires. 1125.51); *m*/*z* (HRMS, FAB) 1126.5172 ($[M + H]^+$ $C_{65}H_{73}N_7O_9P$ requires 1126.5207).

^{31}P NMR (101 MHz, C_6D_6; ext. H_3PO_4) 148.0, 148.8.

3′-*O*-(2-Cyanoethyl-*N*,*N*-diisopropylaminophosphinoxy)-5′-*O*-(4,4′-dimethoxytrityl)-5-methoxycarbonylaminomethyl-2′-deoxyuridine (15b). Eluting mixture: petroleum ether : acetone 3 : 1, 2 : 1, 1 : 1, 1 : 2 v/v (100 mL of each).

85 mg, yield 51%; TLC R_f: 0.29 (benzene–CH_2Cl_2–Et_3N 7 : 2 : 1 v/v/v); *m*/*z* (MS, FAB) 832.2 $[M + H]^+$, 830.8 $[M - H]^-$ ($C_{43}H_{54}N_5O_{10}P$ requires 831.4); *m*/*z* (HRMS, FAB) 832.3655 ($[M + H]^+$ $C_{43}H_{55}N_5O_{10}P$ requires 832.3687).

^{31}P NMR (101 MHz, C_6D_6; ext. H_3PO_4) 149.1, 149.2.

Oligonucleotide synthesis (DNA synthesis)

Oligonucleotides were synthesized on the 1 μmol scale using standard protocols on an Applied Biosystems 394 automated synthesizer with reagents, columns and phosphoramidities from Glen Research, Sterling, VA. The syntheses were performed using 0.10 M acetonitrile solution of canonical phosphoramidities and 0.15 M solutions of modified units.

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Non-modified oligonucleotide was deprotected by conc. aqueous ammonia/ethanol 3 : 1, v/v treatment for 4 h at 55 °C while those containing modified units were deprotected at room temp. overnight.

All oligonucleotides with DMTr group at the 5′-end were purified using reverse-phase HPLC (Hamilton PRP-1, 250 × 10 mm) using a flow rate of 3 ml min^{-1} with a gradient of 0–40% CH_3CN in 0.1 M triethylammonium bicarbonate, pH 7.0 for 30 min. Purified oligonucleotides were then detritylated (50% AcOH at room temp. for 0.5 h, followed by evaporation) and re-purified by HPLC as above using gradient 0–40% CH_3CN in 0.1 M triethylammonium bicarbonate, pH 7.0 for 15 min. The molecular mass of the oligonucleotides was confirmed by MALDI-TOF mass spectrometry and the purity was checked by RP-HPLC or polyacrylamide (20%/7 M urea) gel electrophoresis.

Oligonucleotide labeling

The substrate oligonucleotide of RNA/DNA chimeric sequence was 5′-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. A mixture containing 10 mM Tris/HCl (pH 8.5), 10 mM $MgCl_2$, 7 mM 2-mercaptoethanol, 30 μM (0.1 A_{260} unit) oligonucleotide, 1 μL (10 μCi) of [γ-^{32}P]ATP and T4 polynucleotide kinase (6 units) was incubated for 30 min at 37 °C, and then heat denatured and stored at −20 °C.

Enzymatic assay for cleavage activity in the presence of magnesium ions

The **S1** substrate cleavage reactions were performed under single-turnover conditions with the enzyme in 100-fold molar excess over the substrate. The 5′-labeled substrate (at concentrations 0.1 μM) was incubated with series of deoxyribozymes **D2–D9** (10 μM) in PBS buffer (pH 7.5) containing 10 mM or 3 mM $MgCl_2$, at 37 °C. After various time intervals 10 μL aliquots were withdrawn and cleavage reaction was stopped by addition of 33 or 4 mM EDTA, respectively, and by cooling in ice. Before electrophoresis 8 μL of formamide containing 0.03% bromophenol blue and 0.03% xylene cyanol was added to each sample, and the cleavage products were separated from non-cleaved substrate by electrophoresis in 20% polyacrylamide gel under denaturing conditions (with 7 M urea). The amount of the product was determined by a PhosphorImager (Molecular Dynamics).

General procedure for the cleavage of substrate in Mg^{2+}-free systems

The **S1** substrate cleavage reactions catalyzed by **D1**, **D3**, **D5**, **D6–D9** were performed under single turnover conditions in PBS or 20 mM TRIS buffer (pH 7.5) containing 100 mM NaCl and 4 mM EDTA, at 37 °C for 24 h. The **S1** substrate cleavage reactions catalyzed by **D8** and **D9** were performed analogously in 20 mM PIPES, HEPES or EPPS buffer (pH 7.5) containing 100 mM NaCl and 4 mM EDTA, at 37 °C, 24 h, or alternatively in 20 mM TRIS buffer (pH 7.5) at 25, 30 at 37 or 43 °C for 24 h. **D8** and **D9**-assisted cleavage reactions were also performed with a set of sixteen DNA/RNA chimeric substrates 5′-aca gat gaN1 N2ca acc ct-3′ where N1 and N2 are A,G,C or U and lower case letters correspond to 2′-deoxyribonucleotide units (these substrates are not listed in Table 1). As a control analogous reactions were carried out in the presence of 3 mM $MgCl_2$. All the reactions were carried out according to the procedure similar to that described above. The cleavage extent was determined by PAGE analysis.

Kinetic measurements

The 5′-labeled substrate (at concentrations 0.1 μM) was incubated with deoxyribozymes **D1**, **D8** or **D9** (10 μM) in PBS buffer (pH 7.5) containing 1 mM $MgCl_2$, or with deoxyribozymes **D8** or **D9** (10 μM) in 20 mM TRIS or PIPES buffers (pH 7.5 or 8.0) with 100 mM NaCl and without magnesium ions, in the presence of 4 mM EDTA, at 37 °C. After various time intervals 10 μL aliquots were withdrawn and cleavage reaction was stopped by addition of 4 mM EDTA (for the reaction carried out in the presence of Mg^{2+}), and by cooling in ice. Before electrophoresis 8 μL of formamide containing 0.03% bromophenol blue and 0.03% xylene cyanol was added to each sample, and the cleavage products were separated from non-cleaved substrate by electrophoresis in 20% polyacrylamide gel under denaturing conditions (with 7 M urea). The amount of the product was determined by a PhosphorImager and the observed rate constants (k_{obs}) were calculated from a pseudo-first-order reaction equation: $Y = [EP]\,[1 - \exp(-k_{obs} \times t)]$, where Y is the percentage of the cleaved product at time t; EP is the endpoint showing the percentage of cleaved product at the plateau of reaction. Reactions were carried out near to completion. Endpoints between 80 and 90% were used in kinetic analysis. In all cases, good fits to the appropriate kinetic model were obtained, with $R^2 > 0.96$. The k_{obs} values for cleavage of the substrate by modified deoxyribozymes represent mean values of three independent experiments and errors indicate standard deviations (± SD) between individual experiments.

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