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Chemical synthesis of an artificially branched hairpin ribozyme variant with RNA cleavage activity

Sergei A. Ivanov,[a] Eugene M. Volkov,[b] Tatiana S. Oretskaya[b] and Sabine Müller*

[a]*Ruhr-Universität Bochum, Fakultät Chemie, Universitätsstrasse 150, 44780 Bochum, Germany*
[b]*Department of Chemistry and Belozersky Institute of Physico-Chemical Biology, Moscow State University, Vorob'evy gory, Moscow 119899, Russian Federation*

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Abstract—Due to the development in the field of RNA synthesis over the past decade of years, preparation of RNA oligonucleotides longer than 50 nucleotides is possible today. In this report, we describe the chemical preparation of a branched RNA molecule with RNA cleavage activity consisting of 81 nucleotides. It is derived from the hairpin ribozyme, a small catalytic RNA occurring in nature. The hairpin ribozyme consists of two separately folded domains (loop A and loop B domain), which can be joined in a number of different ways without loss of activity. In the construct presented here, 2′-deoxy-*N*4-(6-hydroxyhexyl)-5-methylcytidine was introduced to connect the loop B domain with the loop A domain via an artificial branch. The synthesized branched RNA is able to catalyze the cleavage of a number of suitable substrates. Compared with the corresponding non-branched reverse-joined ribozyme it cleaves its substrates only 5-fold slower. Surprisingly, no ligation activity could be detected.

1. Introduction

The past few decades of research have revealed that RNA can catalyze an amazing number of chemical reactions. The structure of a number of ribozymes is known and mechanistic details of RNA catalysis have been elucidated. We can now begin to understand ribozymes well enough to turn them into useful tools. The development of ribozymes for site-directed alteration of an RNA sequence has been a major goal in our laboratory. We have studied the ability of the hairpin ribozyme[1,2] to carry out RNA cleavage and ligation and have combined two hairpin ribozymes into one molecule henceforth dubbed twin ribozyme.[3,4] A twin ribozyme that was engineered by combination of two conventional hairpin ribozymes mediates the removal of a specific fragment from the parent RNA, followed by ligation of a separately added repair oligonucleotide into the resulting gap with 30% yield.[4]

In addition to the conventional hairpin ribozyme, we also used reverse-joined hairpin ribozymes,[5,6] for twin ribozyme engineering (Fig. 1).[3]

Even though these twin ribozymes catalyze the cleavage of an RNA substrate at two specific sites (upper part of Fig. 1), the desired sequence exchange reaction (lower part of Fig. 1) turned out being less efficient (K. Bossmann, S. Müller, unpublished results). The low efficiency may be attributed to the specific structure of the reverse-joined hairpin ribozyme unit in the twin ribozyme. Due to a single stranded linker connecting the loop A domain with the loop B domain (Figs. 1 and 2) the length of the substrate ribozyme duplex on the left of the cleavage site is limited to six base pairs. Upon cleavage the resulting product can easily dissociate making ligation at this site less efficient. We, therefore, re-designed the structure of the so far used reverse-joined hairpin ribozyme HP-RJWTC6 into HP-RJTLB that consists of the loop B domain linked to the loop A domain via a non-natural branch (Fig. 2). In comparison to HP-RJWTC6 that due to its specific structure can form only six base pairs with the 3′-end of its substrate, the branched ribozyme HP-RJTLB is able to bind substrates of any length just by adaptation of the binding arm lengths. Thus, fragments can be more strongly bound and consequently ligated. In this paper, we describe the synthesis and functional characterization of the artificially branched ribozyme.

2. Results and discussion

2.1. Ribozyme design

In the branched ribozyme HP-RJTLB the loop A domain and the loop B domain are joined via a cytidine analogue

Keywords: RNA synthesis; Branched RNA; Reverse-joined hairpin ribozyme.
* Corresponding author. Tel.: +49-234-3227034; fax: +49-234-3214783; e-mail: sabine.w.mueller@rub.de

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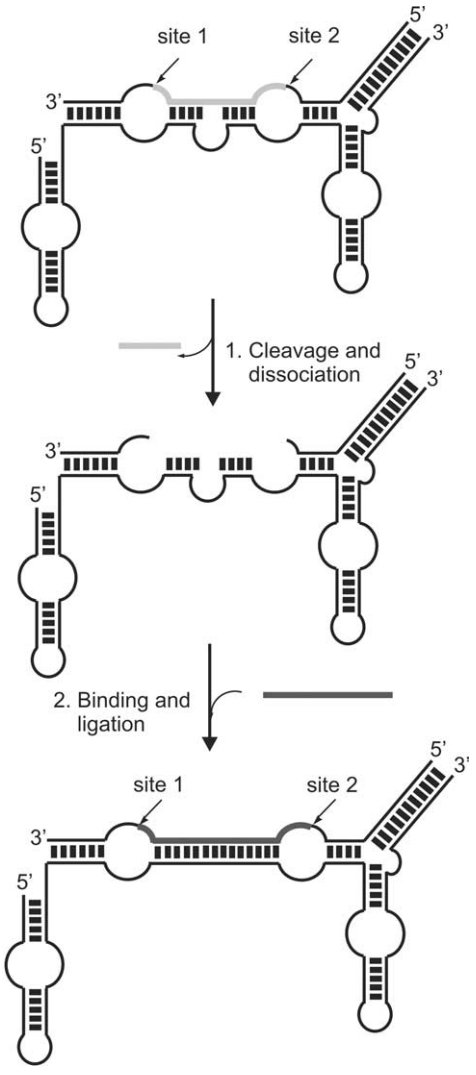

Figure 1. Schematic presentation of site-directed alteration of RNA sequence by an engineered twin ribozyme involving a reverse-joined hairpin ribozyme unit. The twin ribozyme is designed to first cleave the input substrate at two positions to remove the sequence fragment marked in light grey (top). In the second step, a new RNA fragment (dark grey) is bound in the gap left behind and ligated to the remaining fragments of the substrate RNA. For validity of this concept see Ref. 4.

serving as branch point (Fig. 2). The cytidine analogue is modified at C-4 with a linker providing an OH group to be used for assembly of a third RNA chain. The exocyclic amino group at C-4 of cytidine points into the major groove of a double stranded RNA,[7] such that sterical interfering of RNA chains in the branch point is likely to be minimal. Furthermore, the Watson-Crick face of the modified nucleoside is free for base pairing with the opposite guanosine in the substrate strand (Fig. 2). Since the overall structure of an RNA duplex is not affected by a single 2′-deoxy substitution,[8] we decided to use a 2′-deoxy nucleotide instead of the natural ribonucleoside at the branch point to keep preparation and handling of the modified building block as facile as possible. The 2′-deoxy analogue is not located within a conserved sequence and hence will not interfere with ribozyme activity.

2.2. Synthesis of the branched ribozyme

Our first attempts towards the synthesis of a branched RNA employed the strategy shown in Figure 3, route A. The phosphoramidite building block of thymidine was transformed into the corresponding 4-triazolide **2** following a strategy first introduced by Sung et al.[9] The further strategy involved standard RNA synthesis followed by treatment of the protected RNA on the polymer with hexanolamine to substitute the triazole moiety and to introduce the required functionality for branching.

To evaluate this strategy, we first prepared a short model oligoribonucleotide SI-SLA5 (for sequence details refer to Section 4). After assembly of eight building blocks including the modified monomer **2**, the terminal 5′-OH group was acetylated and the polymer bound protected oligomer was treated with aminohexanol/CH_3CN to substitute the triazole residue at the branching nucleotide. Then, synthesis was continued with stepwise coupling of five more adenosine units at the OH group of the amino hexanol linker to yield the branched oligoribonucleotide. Analysis of SI-SLA5 by enzymatic digestion, however, showed that the oligomer had not the expected nucleoside composition (data not shown). While less cytidines than expected could be identified, the amount of detected adenosine residues was to high. The most plausible explanation for this observation is a side reaction during aminohexanol treatment. Position C-4 of cytidine residues is easy accessible for substitution reactions.[10] Thus, also the natural phenoxyacetyl protected cytidine residue within the synthesized RNA chain may have undergone partial transamination during hexanolamine treatment, such that additional adenosine residues could be attached also at this site. An alternative explanation might be partial removal of the acetyl group at the 5′-terminus of the first synthesised chain during aminohexanol treatment. This however, could be ruled out by further experiments (see below).

In order to circumvent this problem, we applied an alternative strategy focussing on the synthesis of an aminohexanol modified monomer and its incorporation into RNA. For the synthesis of the modified monomer building block **3** (Fig. 3, route B) we basically followed the methodology introduced by Horn and co-workers for synthesis of comb-type oligodeoxyribonucleotides.[11] Briefly, the 5′- and 3′-hydroxyl groups of the sugar moiety were protected with dimethoxytrityl- and *tert*-butyldimethylsilyl functionalities, respectively, following standard procedures.[12–14] The compound was transferred into the 4-triazolo derivative and the triazole group was substituted with hexanolamine. The alcohol group at the linker was converted into a levulinic acid ester followed by removal of the 3′-*O*-TBDMS group. Finally the phosphoramidite was prepared following the standard protocol.[15,16] As demonstrated before, the levulinic acid residue is stable under the conditions of oligonucleotide synthesis.[13,17,18] Vice versa, it can be quantitatively removed with hydrazine hydrate in pyridine/acetic acid, leaving the 2′-*O*-*tert*-butyldimethylsilyl protecting group and the succinyl linker attaching the oligonucleotide to the solid support intact.[19,20] However, the routinely used and easily removable phenoxyacetyl (PAC) groups for N-protection[21] turned out to be too labile under

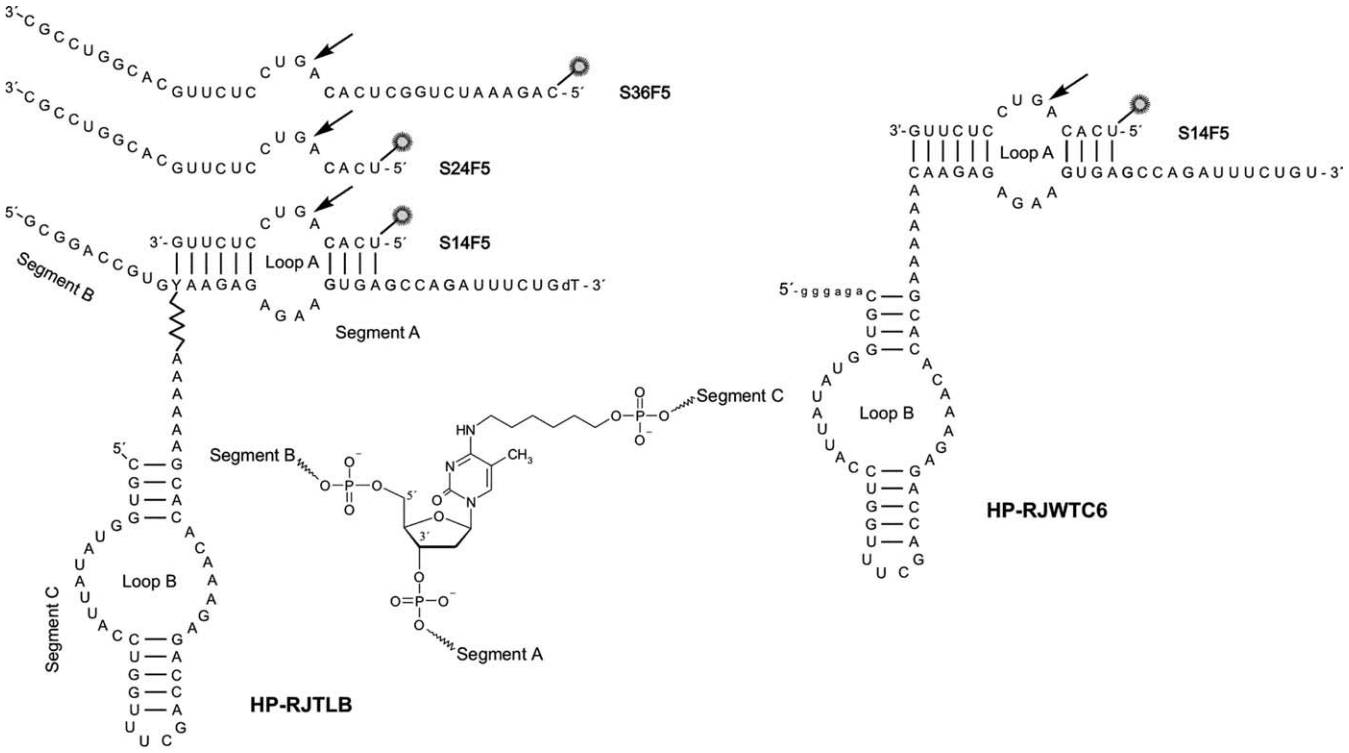

Figure 2. Secondary structure of the branched ribozyme HP-RJTLB with three substrates S14F5, S24F5, S36F5, and of the reverse-joined hairpin ribozyme HP-RJWTC6 with substrate S14F5. All substrates are 5′-end labelled with fluorescein; arrows denote the cleavage site. The structure of the nucleoside at the branch point (marked as Y in HP-RJTLB) is separately shown.

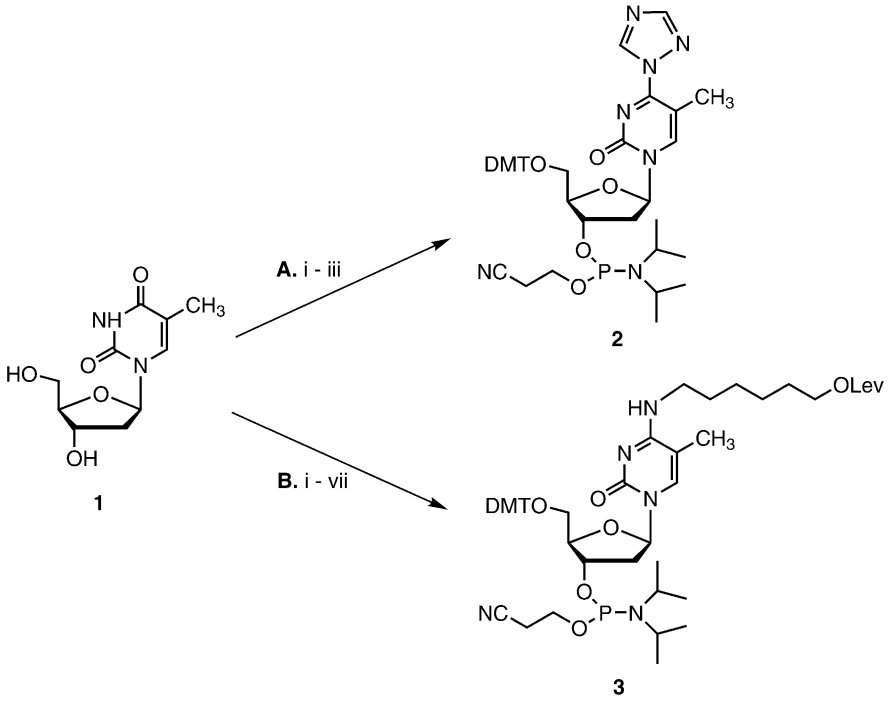

Figure 3. Synthesis of alternative phosphoramidite building blocks **2** and **3** for synthesis of the branched RNA. Route A: (i) DMTCl, DMAP, pyridine, 1.5 h, rt; (ii) chloro-(2-cyanoethoxy)-diisopropylaminophosphine, EtN(*iso*-Pr)$_2$, THF, 2 h, rt; (iii) 1,2,4-triazole, $POCl_3$, Et_3N, CH_3CN, 1 h, 0 °C→rt. Route B: (i) DMTCl, DMAP, pyridine, 1.5 h, rt; (ii) TBDMSCl, imidazole, DMF, 12 h, rt; (iii) 1,2,4-triazole, $POCl_3$, Et_3N, CH_3CN, 1 h, 0 °C→rt; (iv) H_2N–$(CH_2)_6$–OH, CH_3CN, 1 h, rt; (v) (a) levulinic acid, DCC, pyridine, 5 h, rt, levulinic anhydride, DMAP, pyridine, 1.5 h, rt; (vi) TBAF–$3H_2O$, THF, 15 min, rt; (vii) chloro-(2-cyanoethoxy)-diisopropylaminophosphine, EtN(*iso*-Pr)$_2$, THF, 2 h, rt.

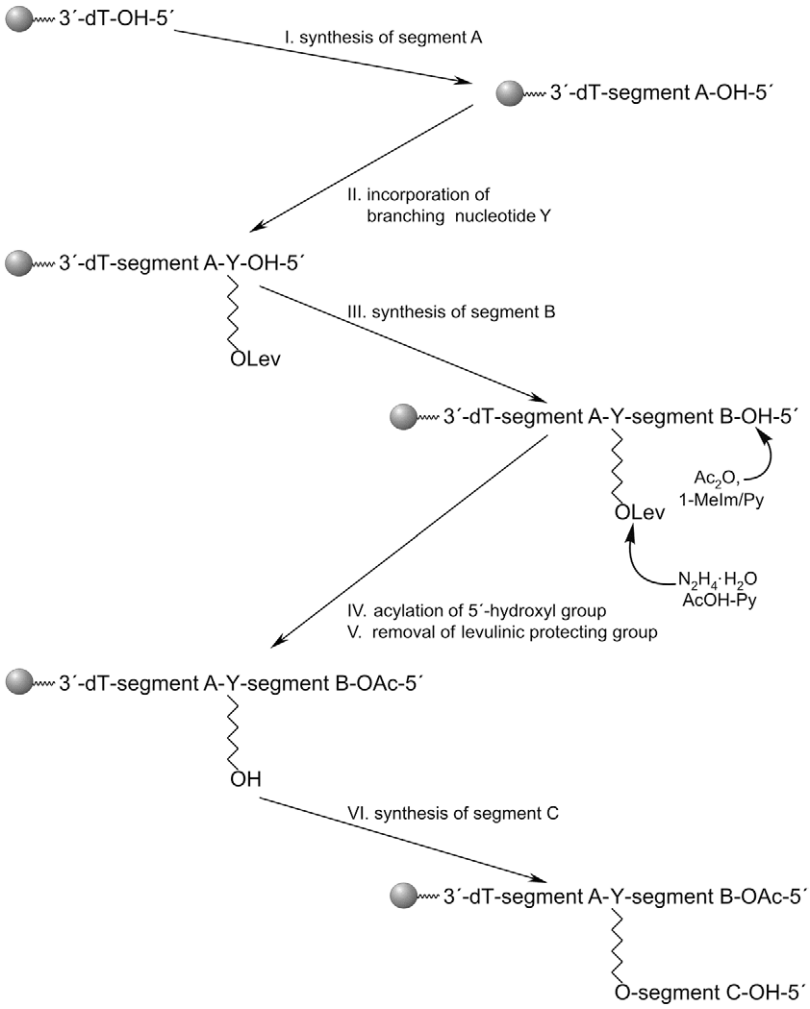

Figure 4. Synthesis scheme of the branched RNA HP-RJTLB. Sequences of segments A, B and C as well as the structure of Y are shown in Figure 2. Details of synthesis are given in the text.

the conditions of hydrazinolysis. Therefore, more stable N-protecting groups were used: benzoyl for A and C,[17] and dimethylformamidine for G.[21,22] The half life of *N*-benzoyl protected adenosine and cytidine under the conditions of hydrazinolysis was determined to be about 3 and 5 h, respectively, while the dimethylformamidine group was stable for the time of observation. Removal of the levulinyl group by hydrazinolysis takes about 5 min, hence leaving the major part of the N-protected nucleobases intact.

Chain assembly was carried out as shown in Figure 4. We used a 1000 Å CPG support (instead of the routinely used 500 Å), to avoid sterical hindrance of reaction sites by growing branched oligonucleotide chains.

Synthesis of segment A (Fig. 4, step I) was performed using the standard procedure of automated RNA synthesis. The branching nucleotide was incorporated with small changes to the standard protocol. A higher excess of **3** and an extended coupling time ensured 99.5% coupling efficiency (Fig. 4, step II, for details refer to Section 4). Synthesis was continued to assemble segment B, as before following the standard protocol (Fig. 4, step III). Next, the 5′-OH group at the end of segment B was capped with an acetyl group to prevent further reaction (Fig. 4, step IV). To initiate the synthesis of segment C, the levulinic acid group at the modified cytidine base was removed (Fig. 4, step V) followed by assembly of segment C (Fig. 4, step VI). Synthesis of all three segments of the branched oligonucleotide was achieved with yields as usual for completely unmodified non-branched oligoribonucleotides.

2.3. Deprotection, purification and characterization

Removal of base protecting groups and β-cyanoethyl groups at the phosphates as well as cleavage from the polymer support was accomplished using saturated methanolic ammonia, followed by treatment with TEA-3HF/DMF (3:1) to remove silyl groups at the 2′-OH.[23] In order to avoid exposure of the branched RNA to long heating in ammonia, the first of these two steps was carried out at ambient temperature, however, for prolonged time. The deprotected oligonucleotide was precipitated from *n*-butanol and purified by electrophoresis through a 20% denaturing polyacrylamide gel at 60 °C. We evaluated these conditions as being essential to achieve sufficient resolution of the desired product band (Fig. 5).

Due to the presence of branched and non-branched species it was not possible to predict the migration properties of the

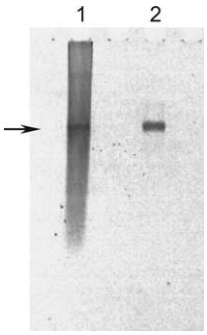

Figure 5. Gel electrophoretic analysis (20% polyacrylamide) of HP-RJTLB. Lane 1: crude product of HP-RJTLB synthesis. The arrow denotes the product band. Lane 2: HP-RJTLB after purification and reloading onto the gel.

product RNA. Therefore, the strongest band was cut out and the RNA was eluted from the gel. In order to prove the identity of the product, the obtained RNA was subjected to nucleoside composition analysis. For this purpose samples were digested with both nuclease P1 and alkaline phosphatase and the resulting nucleosides were separated and identified by HPLC (Fig. 6). In the presence of nuclease P1 the phosphodiester bond between the aminolinker hydroxyl group and the neighbouring nucleoside was totally hydrolyzed. We found snake venom phosphodiesterase being less efficient in catalyzing this reaction (data not shown). 2′-Deoxy-*N*4-(6-hydroxyhexyl)-5-methylcytidine could be identified in the digestion mixture (marked as Y in Fig. 6), which confirms successful incorporation of this modified nucleoside into the analyzed oligonucleotide. Integration of peak areas normalized to specific extinction coefficients revealed a nucleoside composition which agrees very well with theoretically calculated values, demonstrating the superior quality of the synthesized branched RNA.

2.4. Cleavage experiments and kinetic characterization

Since HP-RJTLB is derived from a catalytic RNA structure we next asked if activity has been preserved in the branched ribozyme. To this end, we studied the ability of HP-RJTLB to cleave three substrates of different length: S14F5, S24F5 and S36F5 (Fig. 2), and compared the results with the non-branched reverse-joined hairpin ribozyme HP-RJWTC6. All three substrates carry a fluorescein label at the 5′-end to allow for reaction analysis with an automated DNA sequencer as demonstrated previously.[3] Reactions were carried out in the presence of 2 mM spermine, since we have found previously that activity of reverse-joined hairpin ribozymes is considerably improved in the presence of the polyamine.[24] All substrates are cleaved by HP-RJTLB with the same specificity as observed for HP-RJWTC6. The results are summarized in Table 1.

The short substrate S14F5 as well as S24F5 that extends over the branch point are cleaved by HP-RJTLB with only about 5-fold lower rates than observed with the non-branched ribozyme HP-RJWTC6. Interestingly, cleavage of the substrate S36F5 that is fully paired with the ribozyme proceeds with rather slow rate, k_{react} for HP-RJTLB is another 5-fold down compared with the other two substrates S14F5 and S24F5. A similar result has been obtained with HP-RJWTC6: S36F5 is cleaved 3-fold slower than the two shorter substrates. Also, the end-point of the reaction is rather low. Only 7% of the total S36F5 substrate is cleaved by HP-RJTLB, 45% by HP-RJWTC6. This result might be attributed to an increased ligation activity of both ribozymes with the long substrate. The 5′-terminus of S36F5 forms a contiguous 16 base pair helix with the 3′-terminus of the ribozyme. As mentioned above the natural hairpin ribozyme is also an efficient ligase and it has been shown earlier that structural stabilisation shifts the cleavage/ligation equilibrium towards ligation. Fedor and co-workers have

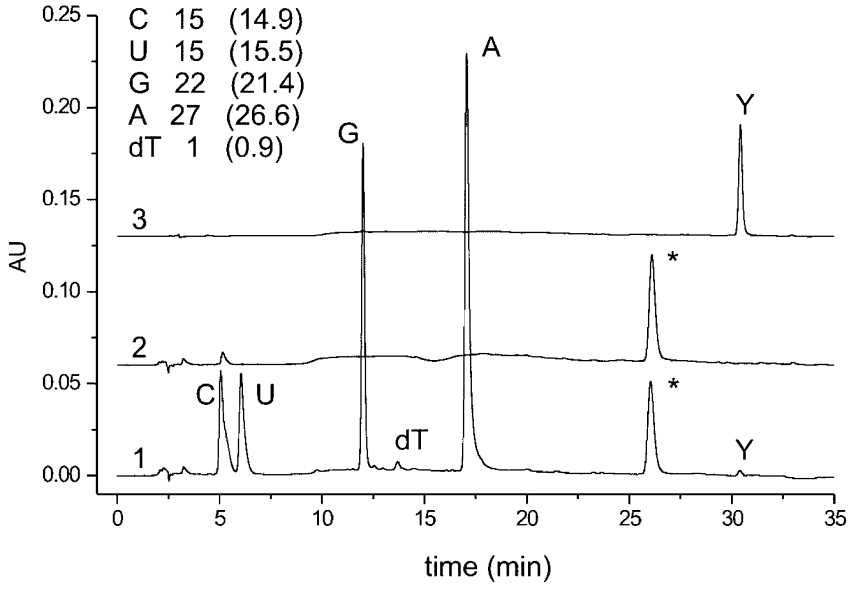

Figure 6. Nucleoside composition analysis of HP-RJTLB by reverse phase HPLC. Lane 1: digestion mixture of synthesized HP-RJTLB. The peak marked with an asterisk results from a component of the digestion buffer or the enzyme (see also lane 2). Lane 2: enzymatic digestion mixture without RNA as control. Lane 3: marker nucleoside Y: 2′-deoxy-N4-(6-hydroxyhexyl)-5-methylcytidine. The calculated and experimentally found (in parenthesis, data results from three independent digestion experiments) base composition for HP-RJTLB is shown.

Table 1. Kinetic constants for cleavage reactions of HP-RJTLB and HP-RJWTC6

Substrate	HP-RJTLB			HP-RJWTC6		
	K_1, nM	$k_{react}\times10^2$ min^{-1}	End-point of reaction (%)	K_1, nM	$k_{react}\times10^2$ min^{-1}	End-point of reaction (%)
S14F5	71.0±10.3	2.4±0.1	80	12.8±2.4	13.0±0.4	95
S24F5	84.3±11.5	2.3±0.1	70	93.0±15.6	12.4±0.7	90
S36F5	152.7±31.2	0.044±0.004	7	46.5±11.9	3.8±0.2	45

demonstrated that cleavage takes place rapidly only when cleavage products dissociate rapidly and that cleavage rates fall when cleavage products remain bound in stable base paired helices. This was taken as evidence that bound products are re-ligated.[25–28] What holds for the conventional hairpin ribozyme also applies to reverse-joined hairpin ribozymes, ligation activity increases with the length of duplexes (S. Ivanov, S. Müller, unpublished observations). Therefore, the observed slow cleavage of S36F5 by HP-RJTLB and HP-RJWTC6 might be interpreted as enhanced ligation due to stable bound cleavage products and in consequence re-ligation. This interpretation gains further support by the observation, that both ribozymes cleave only a fraction of the long substrate, while cleavage of short substrates went on further (Table 1). To additionally support this result we carried out ligation experiments with both ribozymes providing the two cleavage fragments as substrates (Fig. 7).

Whereas as expected, the reverse-joined ribozyme HP-RJWTC6 readily ligated the two fragments (yield: 11%), very surprisingly no ligation activity could be detected for HP-RJTLB. The ligation reaction has been carried out several times with varied concentrations of the two fragments and of the ribozyme as well as under differing reaction conditions. However, in no case ligation activity could be detected. Obviously, the low activity of the branched ribozyme HP-RJTLB for cleavage of the long substrate S36F5 is not the result of re-ligation. Comparison of HP-RJTLB with HP-RJWTC6 shows that the branched ribozyme HP-RJTLB cleaves all three substrates to a smaller extent than HP-RJWTC6 and the cleaved fraction decreases with the length of substrates (Table 1). This might imply that a fraction of HP-RJTLB is trapped in inactive conformations. With increasing length of the substrate the ability to fold into the required active structure may be decreased, such that only a small fraction of the substrate can be cleaved. Accordingly, also ligation would be hampered with long substrates. Strikingly, cleavage might occur only from temporarily folded species that are insufficiently stable to allow for ligation. Extending the linker that connects the two domains in the branch point might help to improve ribozyme folding in a way that the two domains have more conformational freedom to be

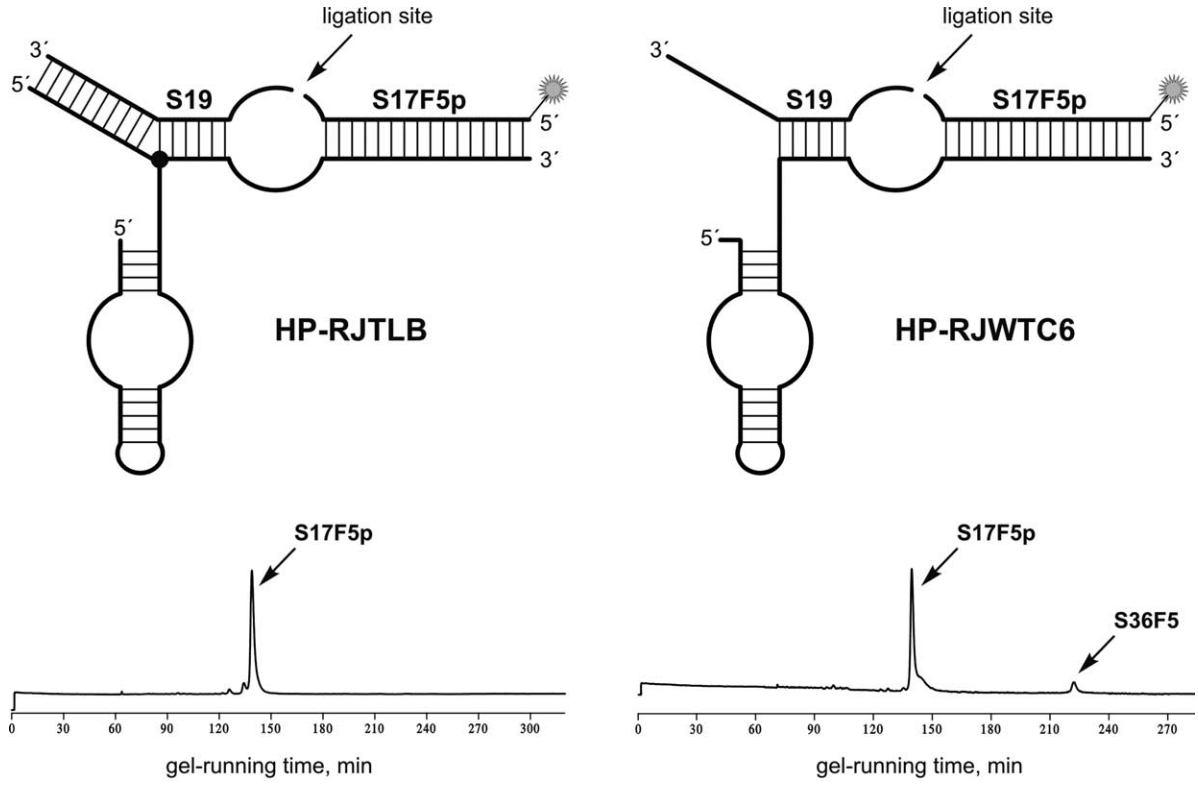

Figure 7. Schematic presentation of the ligation assay and data analysis. The substrate S17F5p carries a 2′,3′-cyclic phosphate and is ligated with S19 to yield the product S36F5.

positioned in an optimal orientation to each other and to interact via a specific net of hydrogen bonds that is known to stabilize the three-dimensional complex.[29]

3. Conclusion

A ribozyme 81 nucleotides in length was prepared by chemical synthesis and the modified nucleoside 2′-deoxy-*N*4-(6-hydroxyhexyl)-5-methylcytidine was introduced in order to create an artificial branch at a pre-defined position of the RNA chain. The branched ribozyme is functional: cleavage rates were only 5-fold lower compared with the corresponding non-branched ribozyme. To the best of our knowledge, HP-RJTLB is the first example of a chemically synthesised artificially branched catalytic RNA. Even though, HP-RJTLB in its present form does not catalyze ligation of RNA fragments and, therefore, is not suitable to be used as building block of a twin ribozyme for RNA repair,[4] the herein presented results demonstrate, that the existing strategies for chemical preparation of RNA allow for the development of new catalytic structures that cannot be prepared enzymatically. This considerably increases the range of structural modification for analysis and functional design of RNA molecules. We expect our results to greatly enhance the prospects of synthetic RNA molecules in molecular biology and biochemistry studies. Further investigations into optimization of the structure of reverse-joined hairpin ribozymes for the twin ribozyme approach[4] are in progress.

4. Experimental

4.1. General methods

NMR studies were carried out on a Bruker AM 300 NMR spectrometer at 300 K using approx. 30 mM sample solutions in $CDCl_3$. ^1H, ^{13}C and ^{31}P NMR spectra were recorded at 300, 75 and 121 MHz, respectively. Chemical shifts were measured in relation to tetramethylsilane ($\delta=0$ ppm) for ^1H NMR and ^{13}C NMR spectra and 85% phosphoric acid ($\delta=0$ ppm) for ^{31}P NMR spectra. Mass spectra (FAB or ESI) of compounds were taken with a Quadrupole Mass Spectrometer HP 5995 A. Analytical HPLC was performed using an Eurospher 100 C18 column (4.6×250 mm, 5 μm) (Knauer GmbH, Germany). Gravity chromatography was performed on a 2.5×15 cm column packed with silica gel 60 (0.063–0.200 mm, Merck, Germany) with an appropriate solvent as eluent. Thin-layer chromatography (TLC) of reaction mixtures and purified compounds was carried out using pre-coated silica gel 60 F_{254} plates (Merck).

4.2. Reagents and solvents

Reagents and solvents for preparation of modified phosphoramidites were purchased from Sigma-Aldrich Chemie GmbH (Germany) and Merck (Germany), fully protected ribonucleoside phosphoramidites and long-chain alkylamine CPG-columns for RNA-synthesis were purchased from Glen Research (USA). Substrate RNAs were 5′-end labelled using 'fluoreprime' fluorescein amidite (Amersham Pharmacia Biotech). Buffers for ribozyme digestion and substrate cleavage were prepared using autoclaved deionized water, filtered through a sterile 0.2 μm pore filter, and stored at −20 °C prior to use.

4.2.1. Preparation of modified phosphoramidite building blocks 5′-*O*-(4,4′-dimethoxytrityl)-4-(1,2,4-triazolyl)-thymidine-3′-*O*-(2-cyanoethyl-*N*,*N*-diisopropylphosphoramidite) 2. 1,2,4-Triazole (780 mg, 11.29 mmol) was suspended in dry CH_3CN (15 ml), with intensive stirring, in an ice bath. Dry triethylamine (1.8 ml, 12.95 mmol) was added followed by dropwise addition of $POCl_3$ (0.24 ml, 2.70 mmol). The mixture was left to react for 45 min and then filtered through cellulose into a solution of 5′-*O*-(4,4′-dimethoxytrityl)-thymidine-3′-*O*-(2-cyanoethyl-*N*,*N*-diisopropylphosphoramidite) (447 mg, 0.60 mmol) in dry CH_3CN (1 ml). After 1 h at room temperature quantitative conversion of the educt into product (TLC analysis) was observed. Ethylacetate (100 ml) was added to the reaction mixture and the organic layer was washed with an equal volume of 10% aqueous $NaHCO_3$ followed by brine. The aqueous solution was back-extracted with ethylacetate. The organic layers were combined, dried over Na_2SO_4, filtered and concentrated to dryness in vacuo resulting in yellow oil. The viscous material was purified on a silica gel column under isocratic conditions using $Et_3N/CHCl_3$ (0.5:99.5, v/v) as eluent. The desired fractions were pooled and evaporated to obtain **2** as white foam (392 mg, 82.0%). TLC analysis: R_f (**2**)=0.73 (both isomers) ($AcOEt/Et_3N/CH_2Cl_2$, 34:8:58, v/v). ^1H NMR ($CDCl_3$): $\delta=9.21$ (s, 1H, H-triazolyl), 8.29 (d, 1H, H–C6), 8.01 (d, 1H, H-triazolyl), 7.35–7.20 (m, 9H, H-phenyl), 6.76 (d, 4H, H-phenyl), 6.27 (q, 1H, H–C1′), 4.61 (m, 1H, H–C3′), 4.05 (m, 1H, H–C4′), 3.72 (s, 6H, CH_3O), 3.62–3.45 (m, 1H, H_a-C5′; 2H CH_2OP; 2H H–C *i*-Pr), 3.29 (m, 1H, H_b-C5′), 2.77 (m, 1H, H_a-C2′), 2.56 (t, 1H, H–CHCN), 2.35 (m, 1H, H_b-C2′; 1H, H–CHCN), 1.87, 1.86 (ss, 3H, CH_3–C5), 1.18–0.97 (m, 12H, CH_3 *i*-Pr). ^{13}C NMR ($CDCl_3$): $\delta=158.7$ (C phenyl), 158.1 (C4), 153.9 (C2), 153.3, 146.7 (C triazolyl), 145.0 (C phenyl), 144.2 (C6), 135.2 (C phenyl), 130.2–127.2 (CH phenyl), 117.4 (CH_2CN), 113.4–113.3 (CH phenyl), 105.8 (C5), 87.5, 87.4, 86.9 (Cq, C1′,C4′), 72.1 (C3′), 62.3 (C5′), 58.0 (CH_2OP), 55.2 (OCH_3), 41.2 (C2′), 43.2 (CH_2CN), 24.6 (CH-*i*Pr), 20.2 ($(CH_3)_2$*i*Pr), 16.4 (CH_3–C5). ^{31}P NMR ($CDCl_3$): $\delta=$ 150.6, 150.0. FAB-MS, *m*/*z*: Calcd ($M+Na^+$) 818.87, found 818.40 ($M=C_{42}H_{50}O_7N_7P$).

4.2.2. 2′-Deoxy-5′-*O*-(4,4′-dimethoxytrityl)-*N*4-(6-hydroxyhexyl-(*O*-levulinyl))-5-methyl-cytidine 3′-*O*-(2-cyanoethyl-*N*,*N*-diisopropylphosphoramidite) 3. The modified nucleoside was prepared mainly as described by Horn et al.[11] and converted into the phosphoramidite building block. Briefly, the procedure involved protection of 5′- and 3′-hydroxyl groups of 2′-deoxyuridine followed by conversion of the base into the 4-triazolo derivative. Next, the triazolo group was substituted with 6-amino-hexanol and the alcohol group at the linker was converted into a levulinic acid ester. Finally the 3′-*O*-TBDMS group was removed for preparation of the 3′-*O*-phosphoramidite. 2′-deoxy-5′-*O*-(4,4′-dimethoxytrityl)-*N*4-(6-hydroxyhexyl-(*O*-levulinyl))-5-methyl-cytidine 3′-*O*-(2-cyanoethyl-*N*,*N*-diisopropylphosphoramidite) **3** was obtained as light yellow foam (392 mg, 83.2%). TLC analysis: R_f (**3**)=0.66, 0.75

(both isomers) (n-C_6H_{14}/AcOEt/Et_3N, 5:25:2.4, v/v). ^{1}H NMR ($CDCl_3$): δ=7.58 (s, 1H, H–C6), 7.33–7.15 (m, 9H, H-phenyl), 6.75 (d, 4H, H-phenyl), 6.39 (m, 1H, H–C1′), 4.52 (m, 1H, H–C3′), 4.02 (m, 1H, H–C4′), 3.99 (t, 2H, CH_2-Lev), 3.73, 3.72 (ss, 6H, CH_3O), 3.51–3.42 (m, 4H, CH_2; 2H, CH_2OP, 2H H–C i-Pr), 3.22 (m, 2H, H–C5′), 2.70–2.66 (m, 2H, CH_2-Lev), 2.56–2.47 (m, 1H, H_a-C2′; 1H, H–CHCN), 2.31 (t, 1H, H–CHCN), 2.18 (m, 1H, H_b-C2′), 2.12 (s, 3H, CH_3-Lev), 1.55 (m, 4H, CH_2), 1.37 (s, 3H, CH_3-C5), 1.30 (m, 4H, CH_2), 1.22–0.93 (m, 12H, CH_3 i-Pr). ^{13}C NMR ($CDCl_3$): δ=206.8 (Me–CO), 172.8 (O–CO), 163.1 (C4), 158.6 (C phenyl), 156.2 (C2), 144.4 (C phenyl), 137.1 (C6), 135.5 (C phenyl), 130.2–127.0 (CH phenyl), 117.6 (CH_2CN), 113.3–113.2 (CH phenyl), 101.7 (C5), 86.6, 85.5 (Cq, C1′,C4′), 72.1 (C3′), 64.5 (C5′), 58.1 (CH_2OP), 55.3 (OCH_3), 46.9 (C2′), 45.7 (CH_2-linker), 43.2 (CH_2CN), 40.9 (CH_2-linker), 37.9 (CH_2-Lev), 29.9, 29.2 (CH_2-linker), 28.5 (CH_3-Lev), 28.0 (CH_2-Lev), 26.4, 25.6 ($2CH_2$-linker), 24.7–24.5 (CH-iPr), 20.1–19.4 ((CH_3)$_2$iPr), 12.4 (CH_3–C5). ^{31}P NMR ($CDCl_3$): δ=150.1, 149.5. FAB-MS, m/z: Calcd ($M+Na^+$) 965.10, found 965.03 ($M=C_{51}H_{68}O_{10}N_5P$).

4.3. RNA synthesis

Oligoribonucleotides were synthesized by the phosphoramidite method essentially as described previously.[3,30] An automated DNA/RNA synthesizer (Gene Assembler Special, Pharmacia) was used for chain assembly at a 1 μmol scale.

The model oligoribonucleotide SI-SLA5 ($^{AAAAA-}_{CGUG-}$XAA(dT) with X=4-triazolo-dT), was synthesized on a 500 Å CPG support. The modified phosphoramidite **2** was dissolved in dry acetonitrile to give a 0.12 M concentration and filtered through 0.45 μm Teflon filters prior to use. Synthesis was performed 'trityl off'. Phenoxyacetyl groups were used for protection of the amino functions of adenine, cytosine and guanine. Unmodified phosphoramidites were coupled using the standard protocol.[30] The coupling time for **2** was extended from 5 min (standard protocol) to 10 min. The following nucleotides CGUG were coupled without changes to the standard protocol. Prior to synthesis of the side-chain (A)5, the terminal 5′-OH group of the main-chain was acetylated by manually treating the polymer support five times with 100 μl Capping A solution (Ac_2O in pyridine) and 100 μl Capping B solution (1-methylimidazole in pyridine), each turn taking 10 s. The column was washed thoroughly with acetonitrile (5 ml), then removed from the synthesizer and treated with 10 ml of a freshly prepared solution of 0.33 M aminohexanol in acetonitrile for 45 min (solution was slowly passed through the column using a syringe). The column again was washed thoroughly with anhydrous acetonitrile (10 ml), transferred back to the synthesizer and washed again with anhydrous acetonitrile (5 ml). Assembly of the side-chain was carried out following the standard protocol.

For synthesis of the branched ribozyme HP-RJTLB, CPG with a pore size of 1000 Å was used. The branching nucleotide building block **3** was dissolved in dry acetonitrile at a concentration of 0.2 M and filtered through 0.45 μm Teflon filters prior to use. Synthesis was performed 'trityl off'. Benzoyl was used for N-protection with adenine and cytosine, dimethylformamidine with guanine. Unmodified phosphoramidites were coupled using the standard protocol.[30] For coupling of the branching nucleotide the following changes to the protocol were made: a 40-fold excess of phosphoramidite over the solid phase was used and the coupling time was extended from 5 to 24 min. The coupling reaction was carried out in two steps: first, 100 μl of the 0.2 M phosphoramidite solution were cycled over the column and coupling was allowed to proceed for 12 min. Immediately after this, the same amount of fresh phosphoramidite solution was transferred to the column and the coupling reaction was extended for another 12 min. The following nucleotides (segment B) were coupled without changes to the standard protocol. Prior to synthesis of segment C (see Figs. 2 and 4), the terminal 5′-OH group of segment B was acetylated by treating the polymer support manually five times with 100 μl Capping A solution (Ac_2O in pyridine) and 100 μl Capping B solution (1-methylimidazole in pyridine), each turn taking 3 s. The column was washed thoroughly with acetonitrile (5 ml), then removed from the synthesizer and treated with 6 ml of a freshly prepared solution of 0.5 M hydrazine hydrate in pyridine/acetic acid (4:1, v/v) for 5 min (solution was slowly passed through the column using a syringe). The column again was washed thoroughly with anhydrous acetonitrile (7 ml) and transferred back to the synthesizer. Assembly of segment C was carried out again following the standard protocol.

Fluorescein labelled RNA substrates (S14F5, S24F5 and S36F5) were synthesized on CPG 500 Å, as described.[30]

Deblocking was performed using saturated methanolic ammonia for 22 h at 25 °C with the branched RNAs SI-SLA5 and HP-RJTLB or over 12 h at ambient temperature with substrate RNAs S14F5, S24F5 and S36F5. Removal of the 2′-O-silyl-protecting groups was achieved by treating the samples with triethylamine trihydrofluoride in DMF (3:1, v/v, 0.8 ml) for 1.5 h at 55 °C. The reaction was quenched by addition of water (0.2 ml) and the RNA chains were precipitated from n-butanol.

The branched RNAs were purified by 20% denaturing (7 M urea) PAGE (acrylamide/bis-acrylamide 19:1) on 8×10 cm glass plates at 60 °C in TBE buffer at 100 V. The gel was soaked in ethidium bromide solution; visualization of RNA was achieved by irradiation with UV-light (254 nm). The band corresponding to the desired product was excised from the gel and eluted with 2 M $LiClO_4$ overnight at room temperature. The oligonucleotide then was precipitated from acetone. Fluorescein labelled RNA substrates were purified using 20% denaturing polyacrylamide gels. Bands corresponding to the desired products were visualized both at 254 and 366 nm, excised from the gel and treated as described above.

4.4. Nucleoside composition analysis

Oligoribonucleotides (0.08 O.D.$_{260}$) were dissolved in 10 μl of nuclease P1 buffer (40 mM AcONa, 2 mM $(AcO)_2Zn$ pH 5.3) and a freshly prepared solution of nuclease P1 from Penicillium citrinum in the same buffer (10 μl, 10 μg/ml) was added. After incubation at 37 °C for 12 h, 10× dephosphorylation buffer (4 μl), alkaline phosphatase from

calf intestine (3 μl, 3 U) and water (13 μl) were added to obtain a final volume of 40 μl. Reaction was allowed to proceed for 3 h at 37 °C. Individual reaction mixtures were analysed by HPLC using a RP-18 column with buffer A (0.1 M ammonium acetate, pH 6.5), and a gradient of buffer B (0.1 M ammonium acetate, pH 6.5, 60% CH_3CN): 0–15% B over 20 min, 15–40% B over 30 min and 40–100% over 2 min at 37 °C and a flow rate of 1 ml/min.

Peak areas in conjunction with extinction coefficients at 260 nm (C: 7.4, U: 9.9, G: 11.5, A: 15.4, d*T*: 8.7, in l/mmol cm) were used to calculate relative concentrations of monomers.

4.5. Cleavage reactions

Cleavage reactions were performed under single-turnover conditions and each experiment was repeated at least once. A mixture of ribozyme (20–220 nM) and substrate (10 nM) in Tris–HCl buffer (15 mM, pH 7.5) was heated at 90 °C for 1 min followed by incubation at 32 °C for 15 min. Magnesium chloride was added to a concentration of 10 mM and the mixture was left at 32 °C for another 10 min. The cleavage reaction was started by addition of spermine (pH 7.5, final concentration 2 mM). The final volume of the reaction mixture was 20 μl. Aliquotes (3 μl) were removed at six suitable time intervals and the reaction was quenched by addition to 3 μl of stop-mix (10 mM EDTA in 90% formamide). Samples were heated to 90 °C for 1 min, then immediately cooled on ice and subsequently subjected onto a 15% denaturing gel (7 M urea) using an ALF DNA sequencer as described previously.[3] Initial rates of reactions were obtained from a plot of product formation against time within the linear phase of reaction. Kinetic parameters were determined from linear curve fitting using Eaddie–Hofstee plots.

4.6. Ligation reactions

For ligation the two following substrates were used: GUC CUC UUG CAC GGU CCG C (S19) and 5′-fluorescein labelled Φ-CAG AAA UCU GGC UCA CAcp (S17F5p), which carries a 2′, 3′-cyclic-phosphate group. Ligation was performed with equimolar concentrations of substrates and ribozyme (200 nM). A mixture of ribozyme and 19-mer in Tris–HCl buffer (15 mM, pH 7.5) was heated at 90 °C for 1 min followed by incubation at 32 °C for 15 min. Then the 17-mer and $MgCl_2$ (final concentration of $MgCl_2$ 10 mM) were added and incubated at 32 °C for another 10 min. The reaction was started by addition of spermine (pH 7.5) to a concentration of 2 mM. The final volume of the reaction mixture was 20 μl. Aliquotes (3 μl) were removed after 30, 60 and 120 min. The reaction was quenched by pipetting the aliquots into 3 μl of stop-mix (10 mM EDTA in 90% formamide). Samples were heated to 90 °C for 1 min, then immediately cooled on ice and subsequently subjected onto a 15% denaturing gel (7 M urea) using an ALF DNA sequencer as described.[3]

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