Hammerhead Ribozymes Containing Non-Nucleoside Linkers Are Active RNA Catalysts

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Abstract: Twenty-nine hammerhead-like RNA sequences have been synthesized and analyzed for cleavage activity. Each of the 29 sequences contains a portion of the hammerhead ribozyme RNA sequence that includes the sequence-recognizing helices stem I and stem III and most of the conserved catalytic core sequence, but loop II and stem II have been replaced fully or in part by non-nucleoside linker molecules. In some complexes, the nonconserved core nucleoside U7 has also been replaced by appropriate linker molecules. The non-nucleoside linkers include hexaethylene glycol, triethylene glycol, 1,3-propanediol, and an abasic site analogue based upon tetrahydrofuran. All complexes prepared were analyzed under single turnover conditions, and first-order rate constants were obtained to characterize the cleavage reactions. Complexes exhibiting native-like cleavage efficiency were further analyzed under steady-state conditions and were characterized by Michaelis–Menten parameters (K_m and k_{cat}). The results indicate that the four base pairs that constitute loop II can be removed without any significant change in cleavage activity. Loop II and two base pairs from stem II can also be removed, but the activity of these stem-shortened ribozymes is highly dependent upon the sequence of the remaining two base pairs. A two-base pair stem containing the bases $G_{10.1}$–$C_{11.1}$, $C_{10.2}$–$G_{11.2}$ exhibited cleavage activity that was very similar to that of the native complex, while the other three possible stem sequences containing G–C base pairs were all reduced in activity by at least 2 orders of magnitude. Replacement of the nonconserved core nucleoside, U7, with linker molecules did not result in RNA catalysts with remarkable cleavage activity.

Introduction

A variety of RNA-based catalysts have been reported to accelerate the transesterification of specific phosphodiester internucleotide linkages.[1] The transesterification reactions result in cleavage of the target RNA strand, a process that is important for the processing of specific mRNAs;[2] transesterification additionally appears to be a key step in the replication of some plant pathogens.[3] RNA-based cleavage catalysts have the potential to function as an important class of antivirals that rely upon base–base recognition of a target viral RNA sequence with cleavage and subsequent inactivation of the offending viral RNA without harm to the corresponding host sequences. The preparation of synthetic RNA-like molecules with RNA cleavage activity, but simpler to prepare than a complete RNA sequence, could be an important route to the generation of truncated catalysts amenable to detailed structural analyses by NMR[4] or related methods.

The hammerhead complexes represent one class of relatively small RNA catalysts composed of about 40 nucleoside residues that accelerate a simple reaction in which a phosphate ester is transesterified from the 5′-hydroxyl of one nucleoside to the 2′-hydroxyl of the neighboring residue.[5] The reaction appears to follow an S_N2 in-line mechanism,[6] and a magnesium (or manganese) cofactor is required,[5,7] which likely has a structural role and may function as a base to abstract a proton from the active site 2′-OH.[7c,d] The consensus hammerhead ribozyme complexed with its substrate is composed of three helical stems, one of which typically terminates in a hairpin loop.[5,8] There is a core sequence of nine conserved nucleotides that is nominally single-stranded. Sequence mutagenesis of these core residues dramatically reduces catalytic efficiency,[9] presumably by disrupting a particular secondary/tertiary structure that is responsible for binding the metal cofactor(s) that assist(s) in catalyzing the bond-breaking/forming reactions.

It is likely that the catalytically active structure is determined by the specific folding of the core sequence as dictated by the formation of specific hydrogen-bonding interactions between various functional groups located on the bases, carbohydrate or phosphate residues within the core sequence, but structural details of such a structure are lacking. To identify functional groups that may be involved in specific interactions that define the active site of the complex, a number of analogue sequences have been prepared. Nucleoside analogues lacking a specific functional group can be incorporated into an RNA sequence at a preselected site in order to locate a critical interaction. A number of groups have employed nucleoside analogues to locate specific functional groups critical to the efficiency of the cleavage reaction. For example, loss or modification of the 2′-hydroxyls from G5, G8, or U16 dramatically reduces catalytic efficiency, while the hydroxyls at other sites appear to have relatively little effect upon the cleavage rate.[7a,10] The exocyclic amino groups of G5 and G12 are important for catalytic efficiency,[11] as is the N^7-nitrogen

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at A6.[12] Replacement of any of the three guanine residues within the catalytic core by xanthene, isoguanosine, or 2-aminopurine results in complexes of low activity.[11e] While there is general agreement on the importance of specific functional groups, the interpretations of their roles have varied. Many of the identified functional groups have been suggested to be involved in specific hydrogen-bonding interactions or as part of a hydrogen-bonding network; the presence of G/A mismatches has been suggested,[11c,13] without proposing a specific structure; complexation to the metal cofactor has been proposed;[7c,10g,12] and the involvement of at least one functional group as a general base has been suggested.[11c]

Another line of inquiry has involved the simplification of the structure by removing nonconserved nucleosides.[14] In one study, the entire loop and stem II were removed, apparently without significant loss of cleavage activity.[14b] However, the activity for these complexes has been questioned by a more recent report,[14c] in which it was possible to remove two of the base pairs of stem II without significant loss in catalytic activity, but the removal of the entire stem dramatically impacted cleavage rates. These latter experiments have resulted in the suggestion that the $G_{10.1}$–$C_{11.1}$ base pair is required at the base of helix II in order to permit formation of the active complex.

We report on the activity of a series of hammerhead-like catalysts in which non-nucleoside linkers have been used to replace the nonconserved nucleosides in stem and loop II as well as the nonconserved uridine present within the core sequence. A preliminary communication has already appeared describing the initial cleavage activity of some of these materials.[15]

Experimental Section

Materials. The 4,4′-dimethoxytrityl β-cyanoethyl nucleoside phosphoramidites of the four common nucleosides were obtained from Milligen (New Bedford, MA). The four linkers, hexaethylene glycol, triethylene glycol, 1,3-propandiol, and the abasic site analogue prepared as the corresponding 4,4′-dimethoxytrityl β-cyanoethyl phosphoramidite derivatives were synthesized as described elsewhere.[16] The ^{31}P NMR resonances for the four phosphoramidite linker derivatives were 148.6, 148.5, 147.2, and (141.1, 148.0) ppm, respectively. Some of the 1,3-propanediol linker was also obtained from Glen Research (Sterling, VA). Oligonucleotides were synthesized using an Applied Biosystems 381A DNA synthesizer. High-performance liquid chromatography (HPLC) was carried out on an ODS-Hypersil column (0.46 × 25 cm, Shandon Southern, England), using a Beckman HPLC system. ^{1}H NMR spectra were obtained at 300 or 500 MHz on Varian XL-300 or 500 multinuclear spectrometers. ^{31}P NMR spectra were obtained at 121 MHz on the Varian XL-300. Absorption spectra were recorded on a Perkin-Elmer Lambda 3B UV/vis spectrophotometer. HPLC grade acetonitrile and methanol were purchased from Fisher Scientific (Fair Lawn, NJ). [γ-^{32}P]ATP was a product of New England Nuclear/DuPont (Wilmington, DE). T_4 polynucleotide kinase was obtained from New England Biolabs (Beverly, MA). Nuclease S1 is a product of United States Biochemical Corp. (Cleveland, OH). RNase T2 was obtained from Sigma (St. Louis, MO). Calf intestinal alkaline phosphatase and snake venom phosphodiesterase were purchased from Boehringer (Mannheim, Germany). Electroelution was performed with Schleicher & Schuell Elutrap elution chambers and BT1 and BT2 membranes. Sep-Pak cartridges were obtained from Waters/Millipore (Milford, MA). The 2′-*O*-butyl nucleoside phosphoramidites were a generous gift of Dr. Brian Sproat, EMBL Laboratory, Heidleberg, FRG.

Methods. (i) **RNA Preparation.** The RNA/linker fragments were synthesized using phosphoramidite-based technology[17] modified as described for RNA.[18] Each sequence was treated with 2 mL of 3/1 (v/v) concentrated aqueous NH_4OH/ethanol for 16 h at 55 °C in a screw-cap glass vial. The resulting solution was evaporated to complete dryness and reevaporated from a small amount of ethanol, and the residue was taken up in 0.5–1.0 mL of 1.0 M TBAF in THF and incubated for 17–24 h at ambient temperature protected from light. Next was added 1.5 mL of 1.5 M sodium acetate (pH 6.0), and the resulting solution was extracted twice with 1.5 mL of ethyl acetate. The aqueous phase containing RNA was concentrated to about 0.3 mL, and then 0.9 mL of chilled absolute ethanol was added. The ethanol precipitation was performed after incubation of the solution overnight at −20 °C. The RNA precipitate was collected by centrifugation at 13 000 rpm for 20 min at 4 °C, and the pellet was dissolved in 200–500 μL of distilled water. The crude RNA material was purified by gel electrophoresis using 20% polyacrylamide/1% bisacrylamide/7 M urea gels (14 × 32 cm) in 89 mM Tris–borate buffer and 2 mM Na_2EDTA, pH 8.0 (1× TBE buffer), at 15 mA over night. The product bands were visualized by UV shadowing and excised. The gel pieces were either electroeluted using an Elutrap or extracted by 0.3 M sodium acetate (pH 6.0). The resulting RNA solution was ethanol precipitated or was desalted on a C_{18} Sep-Pak cartridge.

The dodecamer substrate was 5′-end-labeled with [γ-^{32}P]ATP in the following manner. A 100-μL reaction mixture containing 1 A_{260} unit of 12-mer substrate (~0.1 mM), 10 μL of 10× kinase buffer (0.7 M Tris·HCl(pH 7.6), 0.1 M $MgCl_2$, 50 mM DTT), 0.1 mM ATP, 300–600 μCi of [γ-^{32}P]ATP (5 mCi in 33 μL), and 20 units of T_4 polynucleotide kinase was incubated for 60 min at 37 °C. Product isolation was by adsorption on a C_{18} Sep-Pak cartridge. The labeled substrate was repurified by electrophoresis in a 20% polyacrylamide/7 M urea gel (14 × 32 cm). The product band was detected by UV shadowing or autoradiography, cut out, and extracted with 0.3 M sodium acetate (pH 6.0) by the freeze–thaw method (Pieken et al., 1990), followed by desalting through a C_{18} Sep-Pak cartridge. The activity of the labeled substrate was typically 0.01 μCi/pmol.

Oligonucleotide concentrations were determined by assuming a base residue extinction coefficient at 260 nm of 6.6×10^3 $M^{-1}\cdot cm^{-1}$ (ref 19) and no absorbance of linker molecule at 260 nm.

(ii) Purity Analyses. The RNA sequences were analyzed by analytical polyacrylamide gel electrophoresis. Typical migration distances in a 20% acrylamide/1% bisacrylamide/7 M urea gel after 17 h at 250 V were as follows (these samples were all run on the same gel): **1**, 12.3 cm; **2**, 12.3 cm; **3**, 13.0 cm; **4**, 13.6 cm; **5**, 13.4 cm; **6**, 14.6 cm; **7**, 14.7 cm; **17**, 15.6 cm; **18**, 15.6 cm; **19**, 15.9 cm; **20**, 16.4 cm; **21**, 16.4 cm; **22**, 16.8 cm; **23**, 16.8 cm. The RNA fragments eluted as single peaks from a reversed-phase column (ODS-Hypersil) eluted with 50 mM triethylammonium acetate (pH 7.0) and a gradient of acetonitrile (3.5–28% over 15 min).

(iii) Nucleoside Analyses. Nucleotide (or nucleoside) composition and the integrity of the nucleoside 3′–5′ phosphodiester linkage was determined after S1 nuclease (or S1 nuclease and calf intestinal alkaline phosphatase) hydrolysis.

A 10-μL reaction mixture containing 0.5 A_{260} unit of oligomer in 200 mM sodium chloride/5 mM $MgCl_2$/0.1 mM $ZnSO_4$/25 mM sodium

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acetate, pH 5.5, was incubated for 5 min at room temperature with 267 units of S1 nuclease. A 3-μL aliquot was analyzed by HPLC using a 0.46- × 25-cm column of ODS-Hypersil in 20 mM potassium phosphate, pH 5.5, and a gradient of 0–35% methanol (60 min). For nucleoside analyses, 5 μL of 0.1 M Tris·HCl, pH 8.0, and 1 unit of calf intestinal alkaline phosphatase were added to the remaining 7 μL of reaction mixture. Following incubation of the mixture for 60 min at ambient temperature, a 5-μL aliquot was analyzed by HPLC.

The presence of the linkers could be confirmed by treatment of some sequences with RNase T2 followed by calf intestinal alkaline phosphatase. Under these conditions, the linker remained bound to the nucleoside 5′-hydroxyl through a phosphodiester linkage. This assay was not performed for all of the synthesized ribozymes.

(iv) Kinetic Studies. (a) Stoichiometric Cleavage Analysis. These analyses were performed using procedures similar to those described earlier for the native complex.[9c] Two 25-μL solutions containing either 1.2 μM ribozyme or 0.2 μM substrate in 50 mM Tris·HCl, pH 7.5, were heated to 95 °C for 1 min and cooled at 25 °C for 15 min. Each solution was adjusted to 10 mM $MgCl_2$ and incubated at 25 °C for 15 min. The reaction was initiated by mixing the two solutions (final ribozyme concentration, 0.6 μM; final substrate concentration, 0.1 μM). Aliquots of 5–7 μL were withdrawn at appropriate time intervals ranging from 0–60% total cleavage, and the reaction was quenched by the addition of an equal volume of 50 mM Na_2EDTA/7 M urea/10% glycerol/0.05% xylene cyanol/0.05% Bromophenol Blue. For the faster cleaving ribozymes (half-lives near 0.7 min), aliquots were taken every 10–15 s for a 60–90 s time period. The extents of cleavage were analyzed by electrophoresis in 20% polyacrylamide/1% bisacrylamide/7 M urea gels (14 × 16 cm) in 89 mM Tris–borate buffer and 2 mM Na_2EDTA, pH 8.0. After autoradiography, the substrate and product bands were excised and lyophilized to dryness, and the amount of radioactivity was determined by scintillation counting. For each complex, the fraction of cleaved substrate was normalized to the percentage of cleavage after extended incubation (usually longer than 10 half-lives) before plotting. The logarithm of the unreacted fraction was plotted against time, and the data points were fitted using a linear least-squares analysis. The cleavage half-lives ($t_{1/2}$) were used to obtain first-order rate constants ($k_f = 0.693/t_{1/2}$). For any given ribozyme, kinetic parameters from experiment to experiment were found to vary by no more than a factor of 2.

(b) Catalytic Cleavage Analysis. The procedures described below are similar to those described by others (refs 7d and 17). Solutions with different substrate concentrations were prepared separately in a 25-μL volume containing 50 mM Tris·HCl (pH 7.5). Each solution was heated separately to 95 °C for 1 min and cooled to 25 °C for 15 min. A solution of 4 nM ribozyme in 50 mM Tris·Hcl (pH 7.5) was also preheated and cooled. Each solution was then adjusted to 10 mM $MgCl_2$ and incubated at 25 °C for 15 min. The reaction was initiated by addition of a 25-μL ribozyme solution to the 25-μL substrate solutions, which resulted in a final ribozyme concentration of 2 nM. Six substrate concentrations were used, varying from a final concentration of 10–300 nM depending on the individual sequences. Aliquots of 4 μL were taken from the reaction mixture at various times, quenched, and analyzed as described above. Values up to 15% cleavage were used in the calculation of the kinetic parameters. K_m and V_{max} values were obtained from linear Lineweaver–Burk plots, from Eadie–Hofstee plots, and by fitting the velocity and substrate concentration data to as hyperbolic function. The variations in kinetic parameters, K_m, k_{cat}, and k_{cat}/K_m, for different batches of isolated ribozyme are about a factor of 2.

Results

We have examined the cleavage activity of shortened ribozymes, in which loop II and stem II (see Figure 1) have been truncated and replaced in whole or in part with non-nucleoside linkers. Three simple linkers of different lengths have been employed: 1,3-propanediol, triethylene glycol, and hexaethylene glycol. Further variation in the distance between the nucleoside residues bridged by the linker molecule(s) was achieved with the incorporation of more than one linker, bridged by a simple phosphodiester linkage.

(i) Oligonucleotide Synthesis and Purification. The three linker molecules were protected as the 4,4′-dimethoxytrityl derivatives and then converted to the corresponding phosphoramidites using procedures similar to those described elsewhere.[16] RNA synthesis, deprotection, and purification proceeded by standard methodology.[17,18] The linker phosphoramidites could be incorporated into the RNA sequence at the desired site with efficiencies comparable to those of the common nucleoside phosphoramidites based upon the yield of the dimethoxytrityl cation. After deprotection, preparative polyacrylamide gel electrophoresis (PAGE) on denaturing gels was employed for purification of the sequences. Each ribozyme was electroeluted from the gel, precipitated, and stored at −20 °C. Analysis of the sequences by analytical PAGE or HPLC indicated that each RNA fragment was present as a single species. The migration distances for the RNA/linker sequences were directly related to the number of nucleoside residues present, but the presence of additional phosphodiester residues in the linkers often resulted in small variations in the distance of migration.

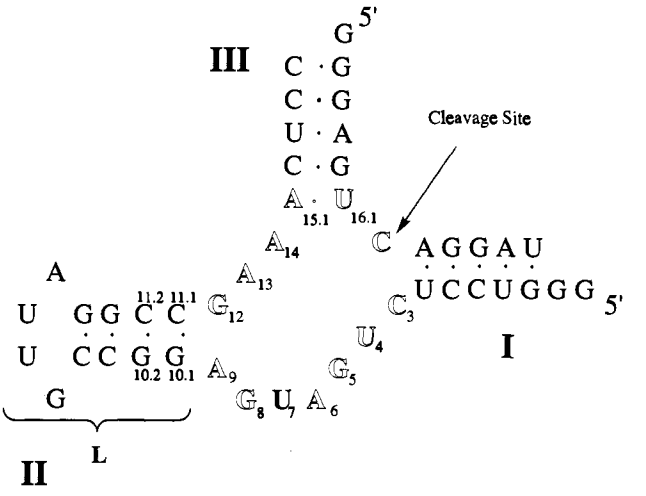


Figure 1. Diagram of the 34-mer ribozyme complexed to the 12-mer substrate. Outlined residues represent the conserved core sequence. The non-nucleoside linkers (L) are employed to replace loop/stem II.

The presence of the linker within the RNA sequence could be confirmed after hydrolysis with RNase T2. This enzyme has a substrate requirement for a 2′-hydroxyl at the cleavage site. Hydrolysis of the 3′-hydroxyl/linker junction occurred normally by RNase T2, but the phosphodiester at the linker/5′-hydroxyl site was not a substrate for the enzyme (Figure 2). After further treatment of the RNase T2-treated mixture with calf intestinal alkaline phosphatase, the linker remained tethered to the 5′-nucleoside monophosphate residue, as illustrated in Figure 2 for the 1,3-propanediol linker in complex **5**. This residue could be resolved by HPLC and its identity confirmed by co-injection of the appropriate standard.

(ii) Stoichiometric Cleavage Activity. To simplify the hammerhead structure, we began by eliminating the four nucleosides in the loop region. Replacement of these four residues by hexaethylene glycol or bis(triethylene glycol) phosphate produced complexes that functioned under stoichiometric cleavage conditions essentially as efficiently as the native complex [relative cleavage rates (k_{rel}) of 0.74 and 0.65 for **1** and **2**, respectively].[15] Some reduction in cleavage activity was observed with the incorporation of tris(propanediol) bisphosphate (**3**, $k_{rel} = 0.11$), and more dramatic losses in activity were observed for the corresponding bis(propanediol) phosphate (**4**, $k_{rel} = 0.0068$) and propanediol (**5**, $k_{rel} = 0.0034$) linkers.[13]

An additional truncation of stem/loop II was performed by the construction of sequences which lacked all four residues of the loop as well as an additional four residues (two base pairs) of the stem structure. Initially we prepared a sequence containing the hexaethylene glycol linker and the two G_{10}–C_{11} base pairs at the base of the native stem II (see Figure 1 and compound **6**, Table 1). This complex exhibited cleavage activity 45-fold lower than that of the native complex ($k_{rel} = 0.022$). However, if the base pair tethered by the hexaethylene glycol linker was reversed

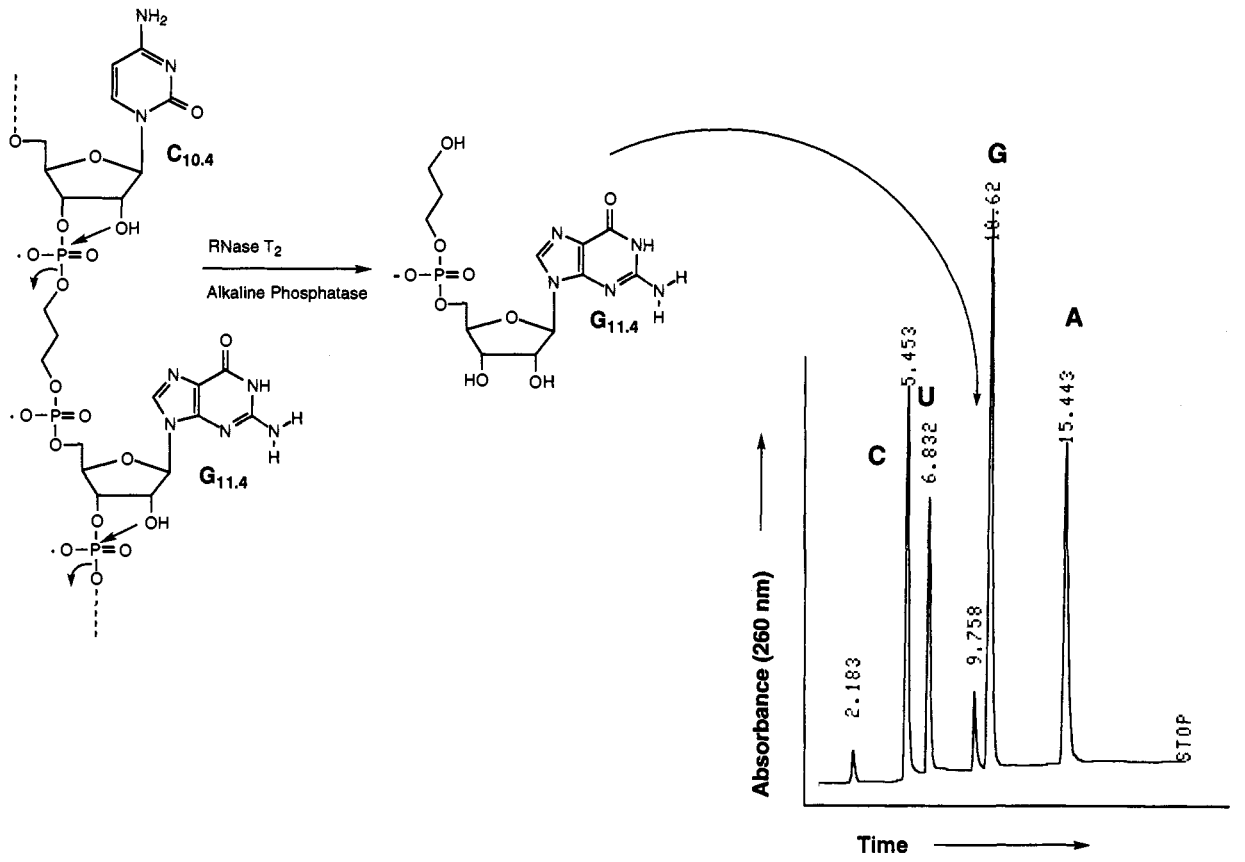


Figure 2. Nucleoside analysis of complex **5** in which the propanediol linker bridges $C_{10.4}$ and $G_{11.4}$. The identification of the propanediol residue tethered to GMP was confirmed by co-injection of the independently prepared standard.

($G_{10.2}$–$C_{11.2}$ to $C_{10.2}$–$G_{11.2}$), the cleavage activity was at least 2 orders of magnitude faster, essentially indistinguishable from that of the native sequence (k_{rel} = 1.1) (see **7**, Table 1). This relationship between stem sequence and activity (for two-base-pair stems) was observed for three different sets of linker complexes (see Table 1). In each case, the complex containing the stem sequence composed of the two base pairs $G_{10.1}$–$C_{11.1}$, $C_{10.2}$–$G_{11.2}$ was more active than the one in which the base pair $C_{10.2}$–$G_{11.2}$ was reversed to $G_{10.2}$–$C_{11.2}$ (compare sequences **6**, **8**, and **10** in Table 1 with **7**, **9**, and **11**, respectively).

Additional linker/ribozymes were prepared containing the hexaethylene glycol linker and variations in the sequence of the two-base-pair stem (see Table 2). Of the four complexes composed of each of the possible arrangements of G–C (C–G) base pairs (see **6**, **7**, **12**, and **13**, Table 2), only the complex containing the $G_{10.1}$–$C_{11.1}$, $C_{10.2}$–$G_{11.2}$ base pairs was as active as the native ribozyme. The remaining three complexes were all much less efficient than the native complex in cleavage activity. However, if the base pair $C_{10.2}$–$G_{11.2}$ in complex **7** was replaced by $U_{10.2}$–$A_{11.2}$, maintaining the same purine/pyrimidine orientation (**14**, Table 2), nearly normal activity was retained (k_{rel} = 0.37).

Three complexes were prepared in which the stem was further shortened such that a single base pair remained ($G_{10.1}$–$C_{11.1}$). Whether these two residues actually exist as a Watson–Crick base pair in the active complexes could not be determined. The sequence containing the hexaethylene glycol linker was the most active of these complexes (**17**, k_{rel} = 0.10) but was 1 order of magnitude less active than the native ribozyme. The corresponding complexes containing the bis(triethylene glycol) phosphate linker (**18**, k_{rel} = 0.045) or the tris(propanediol) bisphosphate linker (**19**, k_{rel} = 0.029) were significantly less active.[15]

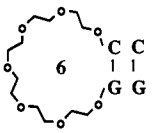
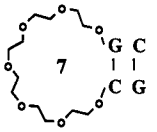
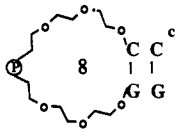
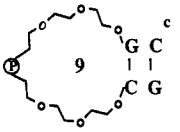
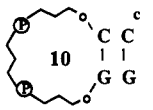
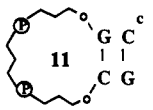
The final group of sequences prepared in this series includes those where the entire stem II and loop II have been removed, and these are illustrated in Table 3. In spite of the observation that the three complexes of nominally a one-base-pair stem (see above) were relatively poor catalysts, a linker of appropriate size tethering A_9 and G_{12} might still permit the core sequence to fold into the active site complex. Six complexes were prepared in which the linkers varied in size from 17–19 atoms (see **20** and **21**, Table 3) to only three atoms (**25**, Table 3). None of these complexes exhibited significant cleavage activity. The most effective catalysts of this group (**20**, **21**, and **23**) were still 200-fold less efficient than the native complex.

(iii) **Stability of Shortened Stem II.** Helix stability with two-base-pair or one-base-pair stems was a concern for the overall stability and activity of these complexes. The largely inactive complexes containing nominally one base pair (**17**–**19**) or no base pairs (**20**–**25**) in stem II argue for the presence of a stable base-paired structure at the base of stem II to assist in organization of the active site. Poor stability of some of the two-base-pair stems could also result in disruption of the active site and reduction in catalytic efficiency. We attempted to estimate differences in helix stability for complexes varying in the number of base pairs in helix II from thermal melting studies, but the absorbance vs temperature curves were not well-defined and could not be effectively interpreted with regard to the stability of stem II. We attempted to simply enhance the stability of the short helices to determine whether enhanced stability impacted cleavage activity. To provide further stabilization of these shortened stem structures, two types of experiments were performed: (i) salt effects and (ii) helix-stabilizing modifications.

(a) **Salt Effects.** Increasing the salt concentration could stabilize the complexes by two mechanisms. Shielding the negatively charged phosphate diesters with an appropriate counterion has long been known to stabilize nucleic acid structures. In addition to their utility in charge shielding, the polyglycol linkers used in this study are similar in structure to crown ethers.[20] It is not clear that the polyglycol has the ability to bind cations

(20) Weber, E.; Vogtle, F. In *Host Guest Complex Chemistry Macrocycles*; Weber, E., Vogtle, F., Eds.; Springer-Verlag: New York, 1985; pp 1–42.

Table 1. Cleavage Activity for Linker/Ribozymes Containing a Shortened Stem II[a]

	k_f (min^{-1})	k_{rel}	P_∞[b]
	0.024	0.022	0.73
	1.16	1.1	0.87
	0.0061	0.0056	0.82
	0.66	0.63	0.82
	0.0026	0.0024	0.69[d]
	0.83	0.75	0.80

[a] Single turnover cleavage with [ribozyme] = 0.6 μM and [substrate] = 0.1 μM. [b] P_∞ = percent product at t_∞ ($t_\infty = 10t_{1/2}$). [c] P represents the phosphodiester linkage. [d] $P_\infty = 8.5t_{1/2}$.

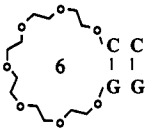
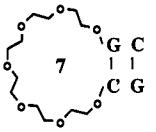
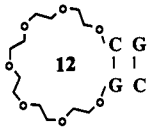
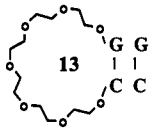
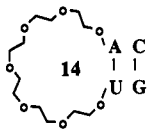
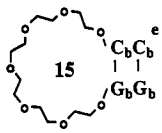
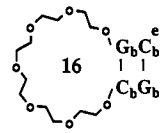
in the fashion expected for a crown ether. The present structures appear very "crownlike" in the illustrations of Tables 1–5, but in fact the distance between the terminal phosphates of a helical structure would result in the polyglycol adopting a rather extended conformation since a minimum of nine atoms is required to bridge the base of an RNA helix.[16c] Open-chain crown ethers (podands) are not particularly effective in binding cations in aqueous solution,[21] but at high salt concentrations, low-affinity binding to the polyglycol might still assist in stabilizing the structure of stem II and result in formation of the active complex.

We examined the activity of the complexes containing a single $G_{10.1}$–$C_{11.1}$ base pair in stem II (**17**–**19**) at constant magnesium ion concentration (10 mM) and varying concentrations of potassium chloride (0, 0.05, and 1.0 M). All three complexes exhibited reduced cleavage activity as the potassium chloride concentration increased. For example, the native complex exhibited half-lives of 0.35, 0.41, and 1.0 min at 0, 0.05, and 1.0 M KCl, respectively. By comparison, complex **17** exhibited half-lives of 4.6, 8.2, and 44 min at 0, 0.05, and 1.0 M KCl, respectively, and complexes **18** and **19** exhibited properties that were very similar to those of **17**.

(b) Helix-Stabilizing Modifications. Helices containing 2′-*O*-alkyl substitutions (particularly butyl) exhibit enhanced helix stabilization.[21] Ribozymes containing a number of 2′-*O*-alkyl nucleosides have been reported to function as active cleavage catalysts.[22] In the present case, we attempted to stabilize two of

(21) Personal communication from Brian Sproat, but see also ref 22.
(22) Paolella, G.; Sproat, B. S.; Lamond, A. I. *EMBO J.* **1992**, *5*, 1913–1919.

Table 2. Effect of Sequence on the Cleavage Activity of Linker/Ribozymes Containing a Shortened Stem II[a]

	k_f (min^{-1})	k_{rel}	P_∞[b]
	0.024	0.022	0.69
	1.16	1.1	0.87
	0.0036	0.0033	0.68[c]
	0.00044	0.0004	0.44[d]
	0.41	0.37	0.76
	0.012	0.011	0.92
	0.16	0.15	0.93

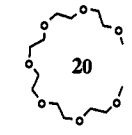
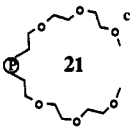
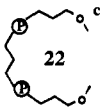
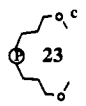
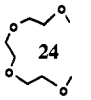
[a] Single turnover cleavage with [ribozyme] = 0.6 μM and [substrate] = 0.1 μM. [b] P_∞ = percent product at t_∞ ($t_\infty = 10t_{1/2}$). [c] $P_\infty = 9t_{1/2}$. [d] $P_\infty = t_{1/2}$. [e] C_b = 2′-*O*-butylcytidine, G_b = 2′-*O*-butylguanosine.

the two-base-pair stem complexes containing the $G_{10.1}$–$C_{11.1}$ base pair. The two complexes differed significantly in activity (compare **6** with **7**, Table 2). We prepared two analogue complexes in which the four bases composing the stem structure were modified as the 2′-*O*-butyl derivatives, generating **15** and **16** (Table 2). Modification of the 2′-hydroxyls of sequence **7** (k_f = 1.16 min^{-1}) with butyl groups resulted in **16** (k_f = 0.16 min^{-1}), which was some 7-fold less active. By comparison, modifications of **6** (k_f = 0.024 min^{-1}) with butyl groups resulted in **15** (k_f = 0.012 min^{-1}), about 2 orders of magnitude less active than **7** (or the native ribozyme) (see Table 2).

(iv) Steady-State Kinetic Parameters. Complexes that exhibited activity similar to that of the native complex under stoichiometric conditions were further studied under multiple turnover conditions. After heat denaturation and preincubation of both ribozyme and substrate with the magnesium cofactor, the complexes examined in this study exhibited cleavage rates that were first order in ribozyme concentration. The linker/ribozymes could be saturated with substrate and were amenable to analysis by Michaelis–Menten kinetics.

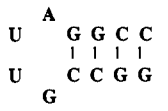
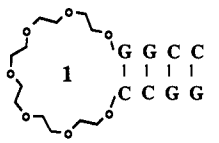
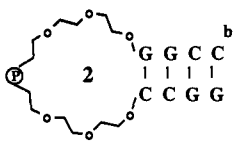
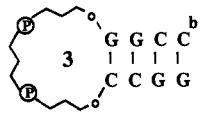
Three complexes were examined in which the entire loop sequence was replaced by a linker, but these sequences retained a full four-base-pair stem II (**1**, **2**, and **3**, Table 4). The two complexes containing either a hexaethylene glycol linker (**1**) or

Table 3. Linker/Ribozymes Lacking Loop II and Stem II[a]

	k_f (min^{-1})	k_{rel}	P_∞[b]
 20	0.0052	0.0047	0.71
 21	0.0055	0.0050	0.71
 22	n.c.[d]	0	0
 23	0.0060	0.0055	0.74
 24	<0.0004		
 25	<0.0001		

[a] Single turnover cleavage with [ribozyme] = 0.6 μM and [substrate] = 0.1 μM. [b] P_∞ = percent product at t_∞ ($t_\infty = 10t_{1/2}$). [c] P represents the phosphodiester linkage. [d] n.c. = no cleavage observed after an 18-h period of incubation.

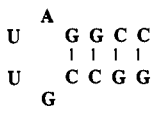
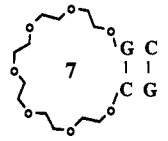
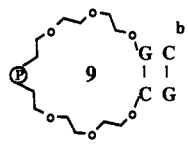
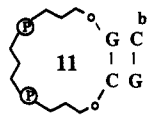
Table 4. Steady-State Kinetic Parameters for Linker/Ribozymes Containing a Full Four-Base-Pair Stem[a]

	K_M (nM)	k_{cat} (min^{-1})	k_{cat}/K_M (nM^{-1} min^{-1})	k_{cat}/K_M (rel)
	51	1.5	0.030	1.0
 1	63	1.9	0.031	1.0
 2	40	2.0	0.049	1.6
 3	57	0.15	0.0026	0.087

[a] For conditions of multiple turnover experiments, see Experimental Section. [b] P represents the phosphodiester linkage.

a bis(triethylene glycol) phosphate linker (**2**) exhibit kinetic parameters that are indistinguishable from those of the native complex (Table 4). By comparison, complex **3** containing the tris(propanediol) bisphosphate linker exhibits a K_m of 57 nM, identical to that of the native complex, but the k_{cat} value is reduced by 1 order of magnitude to 0.15 min^{-1}. As a result of the reduced k_{cat} value, the overall catalytic efficiency as expressed by the apparent bimolecular rate constant (k_{cat}/K_m) is reduced by about 1 order of magnitude (k_{cat}/K_m = 0.087).

Table 5. Steady-State Kinetic Parameters for Linker/Ribozymes Containing a Shortened Stem II[a]

	K_M (nM)	k_{cat} (min^{-1})	k_{cat}/K_M (nM^{-1} min^{-1})	k_{cat}/K_M (rel)
	51	1.5	0.030	1.0
 7	230	2.5	0.011	0.37
 9	165	0.98	0.0060	0.20
 11	196	1.3	0.0067	0.22

[a] For conditions of multiple turnover experiments, see Experimental Section. [b] P represents the phosphodiester linkage.

Three complexes containing a shortened two-base-pair stem were studied under multiple turnover conditions. In all three cases, the stem sequence contained the $G_{10.1}$–$C_{11.1}$, $C_{10.2}$–$G_{11.2}$ base pairs and one of the same three linkers used in complexes **1**, **2**, and **3** (above). The three complexes containing a truncated stem II (**7**, **9**, and **11**, Table 5) all exhibit an increased K_m term relative to the native complex. The increased K_m value varies from 3- to 5-fold for the three complexes. Conversely, the k_{cat} terms are essentially unchanged from that of the native complex. The slight increase in K_m has relatively little effect on overall catalytic efficiency, with the k_{cat}/K_m values for the three complexes reduced only 3- to 5-fold from that of the native complex.

(v) Replacement of the Nonconserved Pyrimidine, U7. U7 is the only nonconserved nucleoside residue present in the catalytic core sequence; it can be replaced with any of the other three common nucleosides with only moderate effects upon cleavage activity.[9e] These observations suggest that the base residue at this site does not participate in any interactions critical to substrate binding or transition-state stabilization. It is possible to cross-link the base residue of U7 to a 6-thioinosine residue placed in the substrate sequence.[23] Similar cross-links have not been reported for the corresponding A7, G7, or C7 complexes. The presence of a cross-link to this site reflects an orientation in which U7 is spatially near the substrate sequence but does not by itself indicate the presence of any critical interactions involving this residue. Deletion of the 2′-hydroxyl from the carbohydrate at this site by substitution with 2′-deoxyuridine (U7dU) results in a moderate increase in catalytic efficiency, indicating that the hydroxyl is not critical for RNA cleavage.[24]

We attempted to simplify the core sequence by replacement of U7 with two linker molecules (see Figure 3). In one case, we incorporated a tetrahydrofuran residue (see **28**) that has been

(23) Woisard, A.; Favre, A.; Clivio, P.; Fourrey, J.-L. *J. Am. Chem. Soc.* **1992**, *114*, 10072–10074.
(24) Tanaka, H.; Hosaka, H.; Takahashi, R.; Imamura, Y.; Takai, K.; Yokoyama, S.; Takaku, H. *Nucleic Acids Res. Symp. Ser.* **1993**, *29*, 175–176.

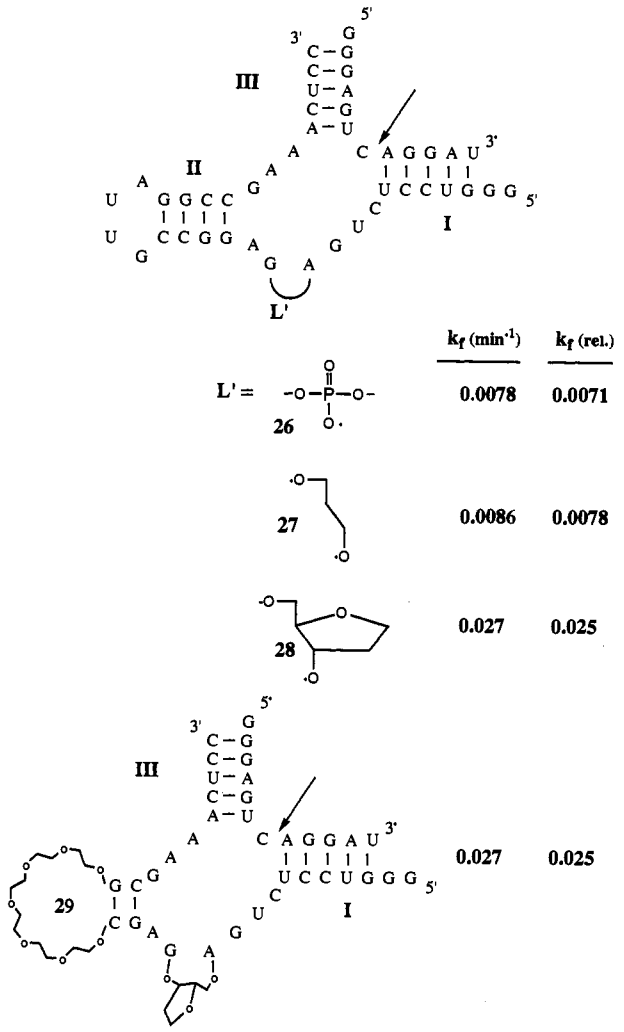


Figure 3. Complexes and cleavage rates resulting from the replacement of the nonconserved core nucleoside U7 by linker molecules. Complex **29** is formed by replacing 8 nucleosides of loop/stem II and U7 by linker molecules.

used in previous studies as an abasic site analogue.[16a] The second linker was 1,3-propanediol (see **27**), identical to that described above. A third complex was prepared in which U7 was simply deleted and A6 and G8 were connected only with a phosphodiester linkage (**26**). A final complex was prepared in which U7, loop II, and a portion of stem II were all replaced by non-nucleoside linkers (**29**). None of these four complexes exhibited remarkable cleavage activity.

Discussion

The 34-mer ribozyme bound to the 12-mer substrate (Figure 1) was the complex chosen for modification by non-nucleoside linkers. Previous work by Fedor and Uhlenbeck[25] indicated that this ribozyme exhibits high catalytic efficiency, with a K_m value of 47 nM and a k_{cat} of 1.5 min^{-1}. Kinetic analysis of this complex indicates that the k_{cat} parameter is representative of the chemical cleavage step. The incorporation of simple non-nucleoside organic linkers based upon oligomeric ethylene glycol or similar materials into the 34-mer ribozyme has permitted us to prepare simplified hammerhead-like molecules of reduced size. In the extreme cases, the 34-nucleotide ribozyme was reduced in length by 12 nucleosides (complete elimination of the sequences that constitute loop II and stem II) to generate a 22-nucleotide sequence. Although the resulting 22-nucleotide complexes are not themselves efficient RNA cleavage catalysts, some of the linker ribozymes in which loop II and only a portion of stem II had been excised are as efficient at RNA cleavage as is the native 34-mer ribozyme.

(i) Cleavage Activity of Linker/Ribozymes Composed of a Four-Base-Pair Stem II. The replacement of the four nucleotides of loop II by hexaethylene glycol (**1**), bis(triethylene glycol) phosphate (**2**), or tris(propanediol) bisphosphate (**3**) appeared to have relatively little effect upon cleavage activity under stoichiometric conditions in comparison with the activity of the native 34-mer. Fragments with cleavage activity similar to that of the native complex exhibited half-lives of 30–40 s under the described conditions. By taking data points every 10 s, only four to five data points were obtianed during the periods of 0–60% total cleavage used to characterize these complexes under stoichiometric conditions. In these cases, more detailed characterization of cleavage activity was obtained from the steady-state analyses.

Shortening of the linker by incorporating the bis(propanediol) phosphate (**4**) or a single molecule of propanediol (**5**) drastically reduced RNA cleavage efficiencies. Previous reports have indicated that nine carbon atoms is the minimum linker length necessary to bridge the terminal carbons atoms of a simple RNA duplex.[16b-d] The linker in complex **4** contains a total of nine atoms, but the introduction of a charged phosphodiester may impact the ability of the linker to effectively span the terminal phosphate of helix II without some helix distortion. The very short linker employed in **5** likely disrupts the hydrogen-bonding interactions in one or more of the base pairs composing stem II. The lack of structural integrity of helix II in the presence of the short linkers present in **4** and **5** likely accounts for the observed loss in cleavage activity.

Steady-state kinetic analyses of **1**–**3** (Table 4) permitted additional characterization of the cleavage characteristics of the "active" complexes. The native complex exhibits a K_m of 51 nM and a k_{cat} of 1.5 min^{-1}, and these parameters are virtually identical with those obtained for the enzymatically prepared complex[26] as well as those reported for other chemically synthesized samples of this ribozyme.[10,11e] One attractive structural characteristic for the ribozyme–substrate complex would involve the folding of loop II/stem II back across the catalytic core sequence such that the loop nucleosides could provide hydrogen-bonding interactions in much the same way as is observed for the loop structures in tRNA^{Phe}.[26] However, the hexaethylene glycol-containing (**1**) and bis(triethylene glycol) phosphate-containing (**2**) ribozymes, lacking these loop residues, exhibit kinetic parameters that are virtually unchanged from those of the native complex. This observation is consistent with previous reports[14c] indicating that the loop II sequence can be altered without dramatic changes in cleavage activity and additionally suggests that no critical interactions take place with the nucleosides in this loop. The study of similar ribozymes by Thomson et al.[28] also indicates that four-base-pair ribozymes containing similar linkers result in nearly normal cleavage activity. The ability to completely replace the four loop II nucleoside residues with a simple organic linker argues for a three-dimensional structure of the ribozyme–substrate complex in which loop II plays no role in substrate recognition or transition-state stabilization. With linker/ribozyme **3**, containing the tris(propanediol) bisphosphate linker, a 10-fold reduction in catalytic activity relative to the native sequence is observed, primarily as a result of a reduction in the k_{cat} parameter. This third linker is only two atoms shorter than the hexaethylene glycol linker of (**1**) or four shorter than the bis(triethylene glycol) phosphate linker (**2**), but the presence of two charged phosphodiester residues

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may increase ionic repulsive forces that destabilize the stem sequence and ultimately reduce the ability of the complex to stabilize the transition state. This latter result differs from the results of a recent report,[28] in which a similar complex exhibits nearly normal kinetic parameters.

(ii) **Cleavage Activity of Linker/Ribozymes Containing a Shortened Stem II.** A recent report by Tuschl and Eckstein[14c] indicated that the sequence of stem II can be reduced with only moderate effects upon cleavage efficiency. Additionally, these authors note that reversing the $G_{10.1}$–$C_{11.1}$ base pair to $C_{10.1}$–$G_{11.1}$ in the native complex reduces catalytic efficiency by at least 2 orders of magnitude (relative k_{cat}/K_m = 0.004). In the present work, a reduction in the size of stem II in combination with the use of a non-nucleoside linker has mixed effects upon cleavage activity (see Table 1). The first of the stem II two-base-pair ribozymes prepared contained the two G_{10}–C_{11} base pairs at the base of stem II and a simple linker to close the helix. This sequence exhibited only poor cleavage activity (Table 1). However, if the two base pairs from the center of the stem, $G_{10.2}$–$C_{11.2}$ and $C_{10.3}$–$G_{11.3}$, were employed with a simple linker, essentially native-like cleavage activity was exhibited (Table 1). This relationship between the two stem-shortened ribozymes was observed with two additional examples in which different linkers were used to close stem II with either of the two-base-pair sequences (Table 1).

Tuschl and Eckstein[14c] have reported that the $G_{10.1}$–$C_{11.1}$ base pair at the base of stem II must be kept constant in order to maintain significant catalytic activity. However, upon further examination of the stem sequence in the present series of linker/ribozymes (see Table 2), this requirement does not always result in the desired activity. With a series of two-base-pair stem sequences bridged with the hexaethylene glycol linker, the only stem that maintains significant activity is the one noted above with the $G_{10.1}$–$C_{11.1}$, $C_{10.2}$–$G_{11.2}$ sequence (**7**). Reversing the $G_{10.1}$–$C_{11.1}$ base pair results in a dramatic loss of cleavage activity (**13**), but so does reversing $C_{10.2}$–$G_{11.2}$ to $G_{10.2}$–$C_{11.2}$ and forming a stem sequence that conserves the $G_{10.1}$–$C_{11.1}$ base pair (**6**, Table 2). Finally, maintaining the $G_{10.1}$–$C_{11.1}$ base pair as suggested by Tuschl and Eckstein[14c] and incorporating a $U_{10.2}$–$A_{11.2}$ base pair into a two-base-pair stem regenerates activity similar to that of the native sequence (**14**). These results suggest a definite preference for a pyrimidine–purine/purine–pyrimidine stem for ribozymes in which the stem has been shortened to two base pairs. But the reasons for this preference remain unclear. The most active ribozyme (**7**) contains the G–C stem sequence that is thermodynamically most stable.[29] But stem stability cannot explain the differences in activity, since the A–U-containing stem is less stable than all four of the G–C-containing two-base-pair stems.

The preceding discussion assumes that the ribozymes containing two complementary base pairs in stem II with the helix closed by an appropriate linker will actually exist with a two-base-pair "minihelix" as stem II in the classical structure (see Figure 1). This assumption seems reasonable since the complexes in which stem II is completely absent (see Table 3) exhibit no significant cleavage activity, but small helices containing two base pairs, by themselves, do not exhibit exceptional stability. We were unable to confirm helix stability using T_m measurements. Attempts to further stabilize the nominally two-base-pair helices by incorporation of 2′-*O*-butyl nucleosides into both strands of the minihelix did not dramatically enhance activity. The presence of the butyl groups should enhance helix stability, based upon experiments with longer sequences,[22] but no examples have been reported for minihelices containing 2′-*O*-alkyl substitution. Attempts to enhance helix stability through salt effects were also largely ineffective in enhancing reactivity. But increasing the KCl concentration is rather indiscriminate, and futher stabilization of helices I and III could alter the rate-limiting step by reducing the rate of product release.

The three stem II-shortened linker ribozymes examined under steady-state cleavage conditions (**7**, **9**, and **11**, Table 5) were all effective RNA cleavage catalysts with overall catalytic efficiencies as expressed by k_{cat}/K_m within 5-fold of that exhibited by the native ribozyme. This slight reduction in efficiency in all three cases was a function of the K_m parameter and suggests that the presence of stem II may be important to properly orient the ribozyme for substrate binding. An increased K_m term has also been observed by Thomson et al. for a similar series of stem II-shortened ribozymes in which the stem is closed by simple linker molecules.[28]

(iii) The Nonconserved Pyrimidine, U7. Previous studies have shown that the uridine residue at position 7 can be replaced by C, G, or A with only moderate effects upon cleavage activity,[9e] and elimination of the 2′-hydroxyl at this site results in a moderate increase in activity. These observations suggested that the nucleoside at this position might function indiscriminately, for example simply as a spacer, to permit organization of the catalytic core into an active site complex. Removal of this residue from the sequence resulted in a complex exhibiting very poor activity, an observation consistent with the residue functioning as a spacer (Figure 3). However, the two linker molecules incorporated at this site to provide the same spacing between A6 and G8 as does the ribose of U7 did not regenerate native-like activity.

The final complex prepared for this study incorporated both types of linkers in order to reduce the size of loop/stem II and eliminate U7 (**29**, Figure 3). Although the loss of the U7 residue was expected to reduce cleavage activity (as observed with **28**), it was uncertain whether further modification of loop/stem II with hexaethylene glycol would result in an additional reduction in cleavage activity. In fact, ribozyme **29** appeared to function with cleavage efficiency that was virtually indistinguishable from that of **28** (Figure 3). Although the activity of **29** is not remarkable (50-fold lower than that of the native complex), this experiment does suggest the possibility of incorporating multiple non-nucleoside residues of appropriate design into the ribozyme sequence without generating any detrimental nonspecific interactions that further reduce the catalytic activity of these materials.

Conclusions

Loop II and a portion of stem II (two base pairs) of the hammerhead ribozyme complex can be replaced with non-nucleoside linkers without significant changes in catalytic activity. With the two-base-pair stems, a decided sequence preference is observed. Ribozymes containing the stem sequence $G_{10.1}$–$C_{11.1}$, $C_{10.2}$–$G_{11.2}$ function as the most effective catalysts, with the related stem sequence $G_{10.1}$–$C_{11.1}$, $U_{10.2}$–$A_{11.2}$ also an effective RNA cleavage agent. Other G–C-containing stem sequences of two base pairs were poor catalysts. Replacement of the nonconserved U7 within the catalytic core with simple linkers did not result in ribozymes with effective cleavage activity.

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(29) Thermodynamic parameters for adjacent base pairs have been recently determined.[27] $\Delta G°_{37}$ values for the three-base-pair sequences,

5′- GC 5′-CG 5′-CC
CG-5′ GC-5′ GG-5′

are −3.4, −2.0, and −2.9 kcal mol^{-1}, respectively.