# Morpholino-Linked Ribozymes: A Convergent Synthetic Approach

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Hammerhead ribozymes are naturally occurring self-cleaving RNA enzymes[1] that can be engineered as *trans*-cleaving molecules.[2] Advances in the solid-phase chemical synthesis of RNA[3] have allowed for the site-specific introduction of various chemical modifications in ribozymes to provide nuclease resistance without hampering catalytic activity.[4] Although the average stepwise coupling efficiency of ribonucleotides has been increased to ≥97.5%,[3b] iterative RNA synthesis leads to relatively moderate overall yields (∼40% on a 37-mer), which is further diminished by the inherent difficulty in purification of the full-length ribozyme.[3b] An alternative approach to overcoming these limitations is to synthesize two half-ribozymes using known solid-phase methodologies and to chemically ligate these moieties through a covalent linkage. This approach has several advantages over the iterative procedure with respect to yields (>70% on a 19-mer) and ease of purification of the full-length ribozyme product from the halves. The primary requirement is that the site of conjugation must not interfere with the ribozyme core to ensure that full catalytic activity is retained. It is now well-established that the stem II/loop II of the hammerhead ribozyme is not essential for catalytic activity.[5] Therefore, appropriate chemical functionalities which allow post-synthetic ligation of the two halves could replace the standard GAAA tetra loop II.

We synthesized analogs of RPI.2972 (Table 1), a chemically stabilized ribozyme targeted against site 575 of *c-myb* mRNA that inhibits smooth muscle cell proliferation with an $IC_{50}$ of approximately 75 nM.[6] The half-ribozymes, **1** and **2**, used in this study contained a modified 5 base-pair stem II and the appropriate reactive groups at the termini (Figure 1). We selected the morpholino moiety[7] as the linkage between the 5′- and 3′-half-ribozymes since this motif can easily be obtained in aqueous solution by reductive amination[8] of the periodate-oxidized[9] 3′-uridilyl-5′-half-ribozyme **4** with the 5′-amino-hexyl-3′-half-ribozyme **2**.

**Table 1.** Cleavage Rate of Substrate **3** by Ribozymes **7**, **8**, and RPI.2972

| ribozyme | $K_{obs}$ ($min^{-1}$)[a,b] |
|---|---|
| **7** | 0.013 |
| **8** | 0.1445 |
| RPI.2972[c] | 0.023 |

[a] Over 1 h, 80%, 85%, and 60% of substrate **3** was cleaved with ribozymes RPI.2972, **8**, and **7**, respectively. [b] [Rz] = 500 nM, [**3**] ∼ 1 nM, 50 nM Tris·HCl pH 8.0, 25 °C, 40 nM $Mg^{2+}$. [c] $g_S u_S u_S u_S$uc cc*U* Gau Gag gcc gaa agg ccG aaA uuc ucc iB, S = phosphorothioate linkage.

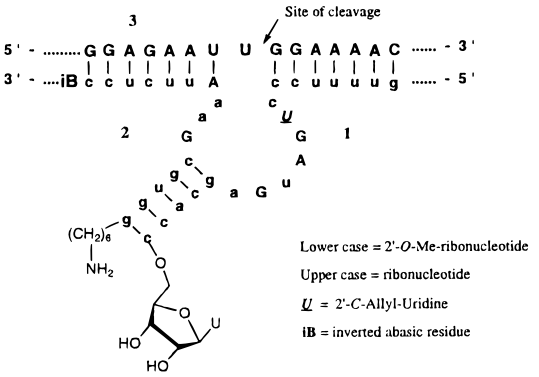


**Figure 1.** Structure of the half-ribozymes **1** and **2** and of the substrate **3**.

Half-ribozymes **1** and **2** were synthesized and purified according standard methods.[3b] The 3′-uridilyl-5′-half-ribozyme, **1**, (150 $\mu$M) was dissolved in sodium $N_2$-acetamido-2-iminodiacetate (ADA) buffer (100 mM, pH 6.0) and subjected to oxidative cleavage with 2 molar equivalents of a 100 mM aqueous solution of sodium periodate[10a,b] (Figure 2A). After 2 h, the acyclic 2′,3′-dialdehyde derivative **4** was formed quantitatively, as confirmed by HPLC monitoring[11a] (Figure 2B). ES-MS analysis, performed on purified **1** (calcd 6490.2, found 6489.1) and **4** (calcd 6488.2, found 6489.2), did not allow direct identification since **1** and **4** differed only by 2 atomic mass units (amu). However, the presence of the 3′-phosphoryl-5′-half-ribozyme, **5**, (calcd 6263.1, found 6262.6) in the ES-mass spectrum of **4** confirmed the dialdehydic structure of **4**. Product **5** resulted from the $E_2$-elimination[10b] of **6** occurring during the ammonium acetate[12] precipitation of **4** at pH 8 (Figure 2A). As expected, **5** (retention time = 5.3 min) was only observed in desalted samples of **4** (retention time = 5.5 min) (Figure 2C)[11b] thus corroborating that the desalting procedure[12] was responsible for the base-catalyzed $\beta$-elimination.

The crude, oxidized mixture containing **4** and an excess of $NaIO_4$ was then directly mixed with the 5′-aminohexyl-3′-half-ribozyme **2** under reductive amination conditions[8] (Figure 3). The unreacted sodium periodate was not quenched with a *cis*-diol source[10b,13] as the resulting aldehydes might have competed

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(11) Column Dionex NucleoPac PA-100, 4.0 × 250 mm: (a) 125−185 mM $NaClO_4$ in 1 mM Tris·HCl pH 9.3, 50 °C over 9 min; (b) 125−215 mM $NaClO_4$ in 1mM Tris·HCl pH 9.3, 50 °C over 12 min.
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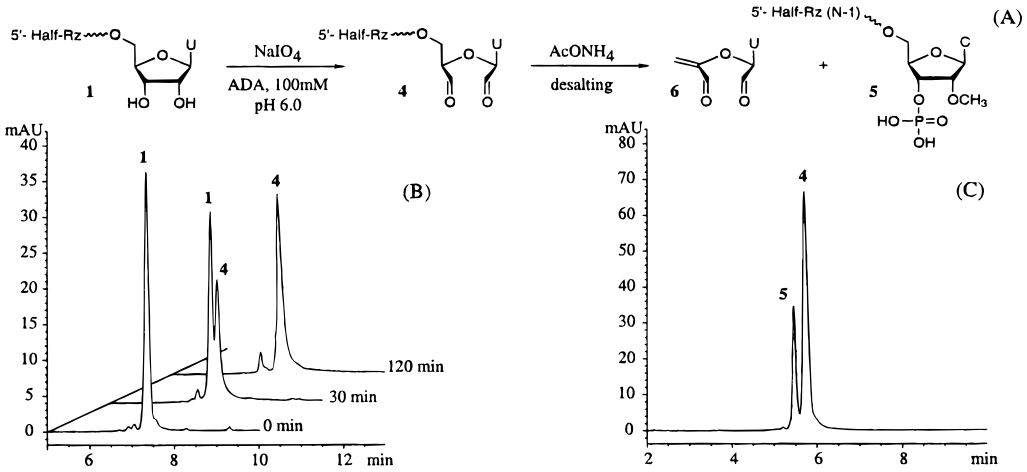


**Figure 2.** (A) Oxidative cleavage and $E_2$ elimination reaction of **1**. (B) Anion-exchange HPLC analysis of reaction of **1** with $NaIO_4$ after 0, 30, and 120 min. (C) Anion-exchange HPLC analysis of ammonium acetate desalted **4**.

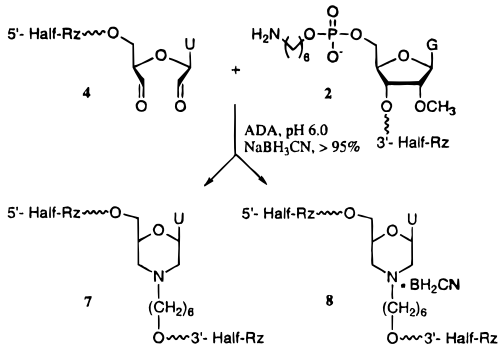


**Figure 3.** Synthesis of the morpholino-linked ribozymes **7** and **8**.

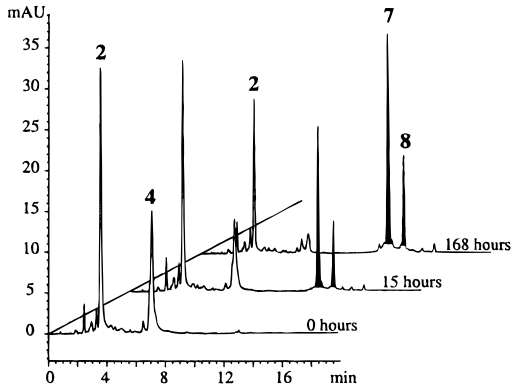


**Figure 4.** Anion-exchange HPLC analysis of the reductive alkylation of **2** and **4** after 0, 15, and 168 h.

with **4** in the reductive alkylation reaction of **2**. Moreover, since the 3′-end of **2** contained an inverted abasic residue,[14] no *cis*-diol functionalities susceptible to undesired oxidative cleavage were present. Typically, **2** (600 $\mu$M) was added to the crude **4** in 100 mM ADA buffer pH 6.0. The transient Schiff base adduct could not be formed unless a 5 molar excess of aqueous $NaBH_3CN$ (500 mM) was introduced (Figure 4), leading to the concomitant formation of the cross-linked products **7** and **8** in a 3 to 1 ratio. Seven days were necessary to allow the coupling reaction to reach completion (>95%), determined by the disappearance of the limiting reagent **4**. Reducing the molar excess of **2** from 4 equiv to nearly a stoichiometric amount (1.5 equiv) did not change the course of the reaction. After purification, **7** was identified as the morpholino-linked ribozyme on the basis of ES-MS analysis (calcd 11723.7, found 11724.8). Interestingly, the ES-MS of compound **8** exhibited a mass signal higher than **7** by 38.8 amu. This suggested that the higher mass product **8** was a cyanoborane adduct of the tertiary nitrogen atom of the morpholino moiety (calcd 11762.7, found 11763.6). Similar amine−cyanoborane complexes have been observed previously under $NaBH_3CN$-mediated reductive amination[15] or by direct addition of cyanoborane.[16] To confirm the identity of compound **8**, we prepared $^{13}$C-labeled $NaBH_3{}^{13}CN$ from $Na^{13}CN$, according to the procedure of Hui,[17] and repeated the reductive amination of **4** on a 2 $\mu$mol scale. As expected, **7** and **8** were produced. $^{13}$C-NMR performed on the two products clearly showed a singlet at 126.8 ppm for **8**, confirming the presence of a cyanoborane adduct whereas this signal could not be observed in the $^{13}$C spectrum of **7**.

Once the morpholino-linked ribozymes were synthesized and characterized it was critical to ascertain the effect of this chemical cross-link on the rate of catalytic cleavage. Ribozymes **7** and **8**, as well as the control RPI.2972, were assayed[4c] for their cleavage rate on short substrate **3** (Figure 1). The cleavage activity of the morpholino-linked ribozyme **7** was very similar to that of the control (Table 1), confirming that one can extensively modify the loop II/stem II region without hampering catalytic efficiency. Interestingly, **8** cleaved substrate **3** six times faster than the control, RPI.2972, which differs slightly from **7** and **8** in the stem II region. Such enhancement of catalytic rate has since been confirmed in two other ribozymes targeted against sites within stromelysin mRNA. Work is currently in progress to determine the effect of the cyanoborane adduct on magnesium chelation. Furthermore, alternative chemistries applicable to segmented assembly are under extensive investigation. This approach to ribozyme synthesis can be used to produce hammerhead and larger ribozyme motifs such as hairpin, Hepatitis Delta Virus, or VS ribozymes.

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