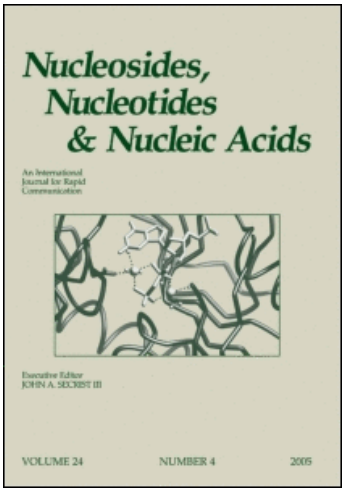

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2'-SUBSTITUTED PHOSPHOROTHIOATE CONTAINING OLIGORIBONUCLEOTIDES: AN APPLICATION TO THE SYNTHESIS AND PURIFICATION OF HAMMERHEAD RIBOZYMES

Anthony DiRenzo[a]; Susan Grimm[a]; Kurt Levy[a]; Peter Haeberli[a]; Lara Maloney[a]; Nassim Usman[a]; Francine Wincott[a]
[a] Department of Chemistry & Biochemistry, Ribozyme Pharmaceuticals Inc,

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2'-SUBSTITUTED PHOSPHOROTHIOATE CONTAINING OLIGORIBONUCLEOTIDES: AN APPLICATION TO THE SYNTHESIS AND PURIFICATION OF HAMMERHEAD RIBOZYMES

Anthony DiRenzo, Susan Grimm, Kurt Levy, Peter Haeberli, Lara Maloney, Nassim Usman and Francine Wincott*

Department of Chemistry & Biochemistry, Ribozyme Pharmaceuticals Inc., 2950 Wilderness Place, Boulder, CO 80301

ABSTRACT A systematic study of the catalytic activity and nuclease stability of selectively modified hammerhead ribozymes has resulted in the identification of a generic motif containing 5 ribose residues and 31 2'-modified sugars (1). This substructure has been further elaborated to include phosphorothioate linkages. Although oligodeoxyribonucleotides containing phosphorothioate linkages have been studied extensively, similarly substituted RNA molecules or ribozymes have not been explored at-length. The synthesis and purification of these ribozymes is discussed (2).

INTRODUCTION

The popularity of nucleic acids as research tools and the emergence of oligonucleotides as therapeutic agents has engendered the need to produce cellularly stable compounds. In recent years, various oligonucleotide modifications of the phosphodiester backbone have been described that endow these molecules with increased nuclease resistance. Much of the work has been in the application of phosphorothioate oligodeoxynucleotides as antisense inhibitors of gene expression (3) with optimal sulfurization accomplished by the use of Beaucage reagent (3H-1,2-benzodithiol-3-one 1,1-dioxide) (4,5).

The focus of our work is on the development of nuclease resistant ribozymes to an appropriate target site. There are few published reports on phosphorothioated oligoribonucleotides. Of the papers that do exist, very few provide experimental details; isolated reports of thiolated 2'-substituted nucleotides (6,7,8) have been published. Additionally, a few studies of oligoribonucleotides (9,10,11) employing either alternative methyl phosphoramidate chemistry (9), H-phosphonate chemistry (10) or manual sulfurization of the oligoribonucleotide (11) have also appeared. Based on early evidence of enhanced stability (and possibly enhanced delivery) of our ribozymes containing strategically placed phosphorothioate linkages (12), a study was undertaken to optimize the automated sulfurization of ribose and other 2'-substituted nucleotides.

Successful synthesis of such ribozymes, in turn necessitated the development of a purification strategy. Typically, anion-exchange HPLC is used to analyze and purify ribozymes (13). Unfortunately, the formation of each phosphorothioate linkage produces 2^n (n = number of linkages) diastereomers. Because there are a limited number of phosphorothioates (P=S) present in the ribozyme the anion-exchange HPLC column is able to partially resolve the diastereoisomers. Consequently, purity analyses of the full length product are complicated. Since the results obtained from HPLC analysis provide an accurate measure of the amount of full length this method can be utilized. However, an orthogonal form of analysis, one that provides the data in a simplified format, should also be employed to ensure purity. We have found capillary gel electrophoresis (14) to be a useful, supplemental analytical tool.

RESULTS AND DISCUSSION

The goal of this study was to optimize the automated sulfurization of ribose and other 2'-substituted nucleotides in the context of oligonucleotide synthesis. All experiments were conducted using Beaucage reagent as this was previously demonstrated to produce optimal results and has been widely used in oligodeoxynucleotide synthesis (5).

Dimers were chosen as the initial model, with NMR as the analytical tool to determine extent of sulfurization. Delivery and wait times were reduced separately or in concert to determine minimal conditions resulting in a sulfurization efficiency comparable to the initial standard conditions. The automated synthesis cycles were modified to incorporate the particular sulfurization times followed by an oxidation step such that any remaining phosphite triester linkages would be oxidized into the stable phosphorotriester form. In accordance with current literature convention (5), sulfurization extent was indicated by the average sulfurization efficiency (ASE) defined as follows:

$$\text{Average Sulfurization Efficiency (ASE)} = (\text{PS}/\text{Total})^{1/n-1}$$

PS = integrated ^{31}P NMR values of the P=S diester

Total = integration value of all peaks

n = length of oligo

Once optimal conditions were developed, they were then applied to the synthesis of ribo- and 2'-*O*-Me oligonucleotide 15-mers to assess the efficiency of the protocol on longer oligomers. Finally, the improved conditions were applied to the synthesis of a ribozyme that contained four P=S linkages at the 5'-end. Since the presence of failure sequences would skew the ratio of phosphodiester (P=O) to P=S linkages, the ribozyme required purification prior to analysis by NMR.

Dimers

Our initial sulfurization conditions were based on a protocol which called for delivery of Beaucage reagent (0.05 M), 25.9 equivalents, followed by a 600 s wait time. These conditions are contrasted with those required for DNA synthesis; 6 equivalents and a 30 s reaction time. We were interested in determining if these parameters could be similarly reduced without affecting the quality of the RNA product. A variety of experiments were executed in which the number of equivalents of Beaucage reagent and the reaction times were varied for both ribo- and 2'-*O*-Me nucleotide UC dimers (Table 1). Beaucage reagent consumption could be reduced from 13.8 to 8.6 equivalents, when coupled with reaction times from 300 to 600 s, to achieve complete

TABLE 1: UC Dimer Thiolation Studies

Experiment	2'-Substitution	Delivery	Equiv	Wait Time	ASE(%)
a	OSi	2 x 4 s	13.8	2 x 300 s	100.0
b	OSi	2 x 3 s	10.4	2 x 200 s	100.0
c	OSi	2 x 3 s	10.4	2 x 150 s	100.0
d	OSi	2 x 3 s	10.4	2 x 100 s	95.9
e	OSi	2 x 2 s	6.9	2 x 200 s	89.3
f	**OSi**	**1 x 5 s**	**8.6**	**1 x 300 s**	**100.0**
g	OSi	1 x 5 s	8.6	1 x 150 s	73.7
h	OMe	2 x 4 s	13.8	2 x 300 s	100.0
i	OMe	2 x 3 s	10.4	2 x 200 s	100.0
j	OMe	2 x 3 s	10.4	2 x 150 s	100.0
k	OMe	2 x 2 s	6.9	2 x 200 s	98.6
l	**OMe**	**1 x 5 s**	**8.6**	**1 x 300 s**	**100.0**

Experiments a-g utilized ribose amidites, h-l used 2'-*O*-Me amidites. The equivalents constitute the total amount of Beaucage reagent delivered per sulfurization step. If the reagent was delivered twice, a wait step separated each delivery.

sulfurization efficiency. Incomplete sulfurization, exemplified by the existence of a phosphodiester signal by ^{31}P NMR (δ = 5 ppm), was observed when insufficient reagent (6.9 equivalents) or inadequate reaction times (200 and 300 s) were combined. Clearly the requirements for sulfurization of 2'-substituted linkages are more stringent than for their DNA counterparts. For both types of 2'-substituted nucleotides, a single delivery time of 5 s, 8.6 equivalents of Beaucage reagent, and a single wait step of 300 s was sufficient to provide dimers with 100% ASE. This constitutes a 50% reduction in reaction time and a 67% reduction in reagent usage.

15-mers

Starting with the optimal dimer parameters, a series of 15-mers were synthesized with an all phosphorothioate backbone to ensure these conditions were valid for a longer oligonucleotide. We determined that for

oligoribonucleotide 15-mers, 8.6 equivalents of Beaucage reagent, with a dramatically reduced reaction time of 250 s provided the same ASE achieved with the standard conditions (13.8 eq, two 300 s wait steps), 99.6% (Table 2). For the 2'-*O*-Me 15-mer counterparts, the optimal conditions were achieved with 8.6 equivalents of the sulfurizing reagent and a 300 s reaction time. In this case the ASE was 99.7%, which was also identical to the ASE obtained using the original conditions.

Ribozyme Synthesis

The optimal reaction parameters were applied to the synthesis of ribozyme **1**, directed at a site in the c-*myb* mRNA (12). Over-expression of c-*myb* has been implicated in the hyperproliferation of smooth muscle cells following stimulation. Restenosis, or post-operative occlusion of arteries following coronary angioplasty, is characterized by excessive proliferation of medial smooth muscle cells. Ribozymes that are capable of cleaving c-*myb* mRNA have been shown to inhibit cell proliferation.

5'- $g_s u_s u_s$ $u_s$uc cc<u>U</u> GAu Gag gcc gaa agg ccG aaA uuc ucc iT -3'

Ribozyme **1**

Lower case letters represent 2'-*O*-Me, upper case letters represent ribonucleotides, underlined letters are 2'-*C*-allyl substituted nucleotides and iT represents an inverted thymidine residue that is attached to the following nucleotide through a 3'-3'-phosphodiester linkage. The subscripts represent the four internucleotidic linkages at the 5'-end that are thiolated, while the other 32 residues contain phosphodiester linkages. The sulfurization synthesis cycle incorporates the sulfurization/cap/ox cycle used in the dimer syntheses. Analysis by ^{31}P NMR should result in a P=O/P=S ratio of 8:1.

Ribozyme **1**, synthesized on a 2.5 μmol scale, utilized 8.6 equivalents of Beaucage reagent with a single 300 s reaction time for each sulfurization cycle. Subsequent deprotection (13) provided 378.6 AU of crude total RNA.

Ribozyme Analysis

Anion-exchange HPLC reveals the problem when analyzing oligomers containing a limited number of phosphorothioate linkages (Figure 1a). The

TABLE 2: 15-mer Thiolation Studies

Experiment	2'-Substitution	Delivery	Equiv	Wait Time	ASE(%)
m	OSi	2 x 4 s	13.8	2 x 300 s	99.6
n	**OSi**	**1 x 5 s**	**8.6**	**1 x 250 s**	**99.6**
o	OMe	2 x 4 s	13.8	2 x 300 s	99.7
p	OMe	1 x 5 s	8.6	1 x 250 s	99.6
q	**OMe**	**1 x 5 s**	**8.6**	**1 x 300 s**	**99.7**

Experiments m-n utilized ribose amidites, o-q used 2'-*O*-Me amidites. The equivalents constitute the total amount of Beaucage reagent delivered per sulfurization step. If the reagent was delivered twice, a wait step separated each delivery.

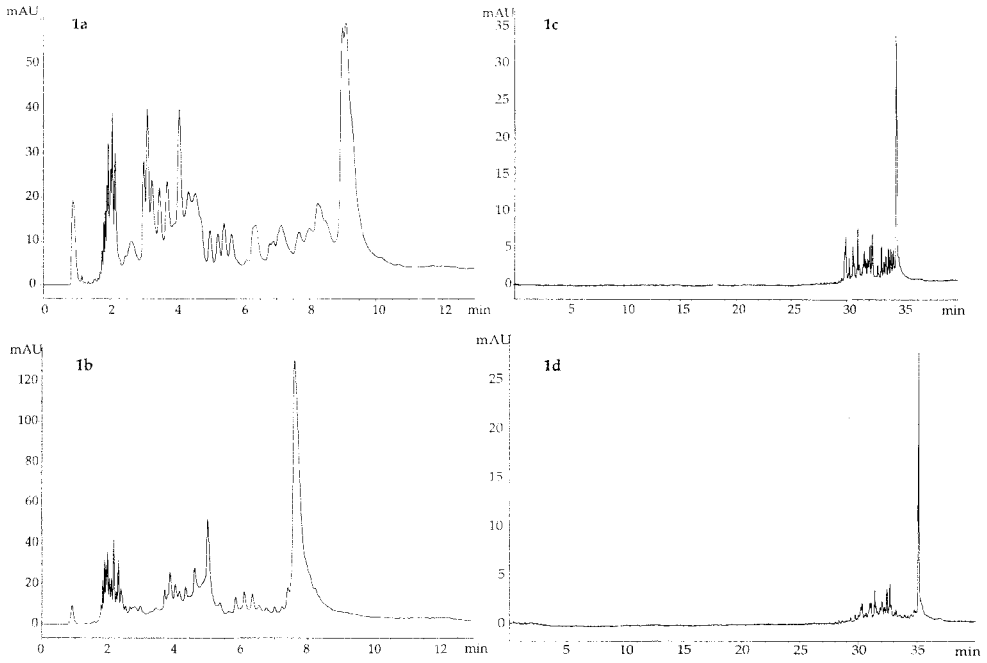

FIGURE 1: a + b) Chromatograms of crude ribozymes, with and without phosphorothioates, analyzed by anion-exchange HPLC a) Chromatogram of phosphorothioate containing ribozyme **1**. b) Chromatogram of ribozyme **2**. c + d) Electropherograms of crude ribozymes, with and without phosphorothioates, analyzed on a Hewlett Packard ^{3D}CE. c) Electropherogram of phosphorothioate containing ribozyme **1**. d) Electropherogram of ribozyme **2**.

full length product of ribozyme **1** appears as a number of broad peaks which, when combined, integrate to 30.3%. This result is contrasted by the chromatogram of the all phosphodiester analogue, ribozyme **2** (Figure 1b). In this case, a single sharp peak which integrates to 37.3%, is observed for the full length product. Fortunately, analysis by capillary gel electrophoresis (CGE) provides a fully resolved, unambiguous peak for the full length product regardless of the presence of phosphorothioates. Although the profiles appear different, the actual amount of full length product detected by both methods is similar. By CGE the synthesis of ribozyme **1** yielded 32.2% (121.9 AU) full length product (Figure 1c). Similarly, analysis of the non-thiolated ribozyme **2** by CGE (Figure 1d) also provided the same value for full length product (39.2%) as obtained from anion exchange HPLC.

Ribozyme Purification

Historically, oligomers for pharmaceutical applications are DNA derived molecules, typically 15- to 25-mers that are fully thiolated. These compounds are amenable to one step trityl-on reversed phase purification (15). Because the compounds we are synthesizing are longer and of an RNA nature (35- to 40-mers), a single reversed phase purification does not provide product of sufficient purity. Trityl-on reversed phase chromatography is routinely used as the first step in our large scale purification process. This procedure generates no more than 75% full length product (13). Fortunately, on a small scale, we have found that a one step, trityl-off anion exchange method provides ribozymes of sufficient purity and yield, typically greater than 85%.

The trityl-off polymer, ribozyme **1**, was purified by anion exchange chromatography to yield 69.2 AU of the desired product (Figure 2a). Analysis of the ribozyme indicated a purity of 90% by CGE (Figure 2b), with an overall recovery of 56.8% of the full length product.

^{31}P NMR Analysis

Two purified samples of ribozyme **1**, synthesized using both the old (Rz **1b**) and optimized (Rz **1a**) thiolation protocols, were analyzed by ^{31}P NMR.

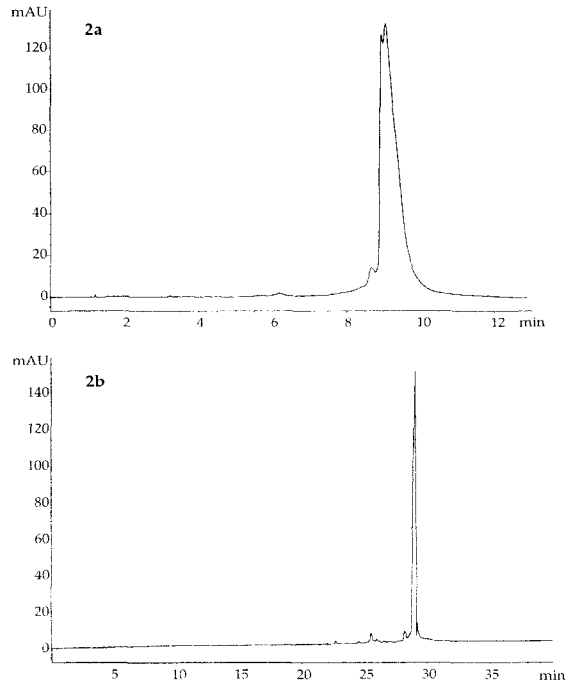

FIGURE 2: a) Chromatogram of purified, phosphorothioate containing ribozyme **1**. b) Electropherogram of purified, phosphorothioate containing ribozyme **1**.

Inspection of the spectra indicated that the ratios of the integrals of the signals at δ 56.5 ppm (P=S) and δ 0 ppm (P=O) were equivalent (Figure 3). As expected, the ratio of the P=S linkage resonances, integrated relative to the P=O resonances, was 8:1. This result validated our thiolation protocol for the synthesis of phosphorothioate containing ribozymes.

Catalytic Activity

Following development of this new protocol for the synthesis of phosphorothioated ribozymes, it was necessary to ascertain its effect on the catalytic activity of our ribozymes. Additionally, we were interested in determining the effect of phosphorothioates on catalytic activity. Ribozyme **1** was synthesized using the old thiolation protocol (Rz **1b**) and the new

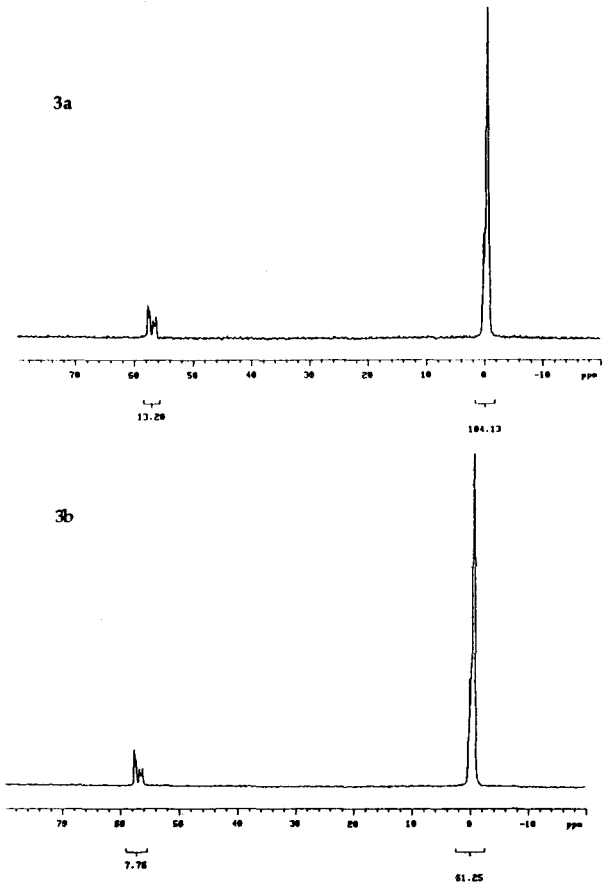

FIGURE 3: a) ^{31}P NMR spectra of purified ribozyme **1a**, synthesized using the optimized sulfurization protocol. b) ^{31}P NMR spectra of purified ribozyme **1b**, synthesized using the old sulfurization protocol.

truncated procedure (Rz **1a**). Furthermore, ribozyme **2** was also assayed to provide a comparison of the cleavage activity of thiolated and non-thiolated ribozymes. All the ribozymes were deprotected (13), HPLC purified and assayed for their cleavage rate. The results are shown in Figure 4. The graph depicts cleavage of the substrate, over time, by the ribozymes. All ribozymes, regardless of the method of thiolation, or the presence of phosphorothioate linkages, exhibited equal catalytic activity.

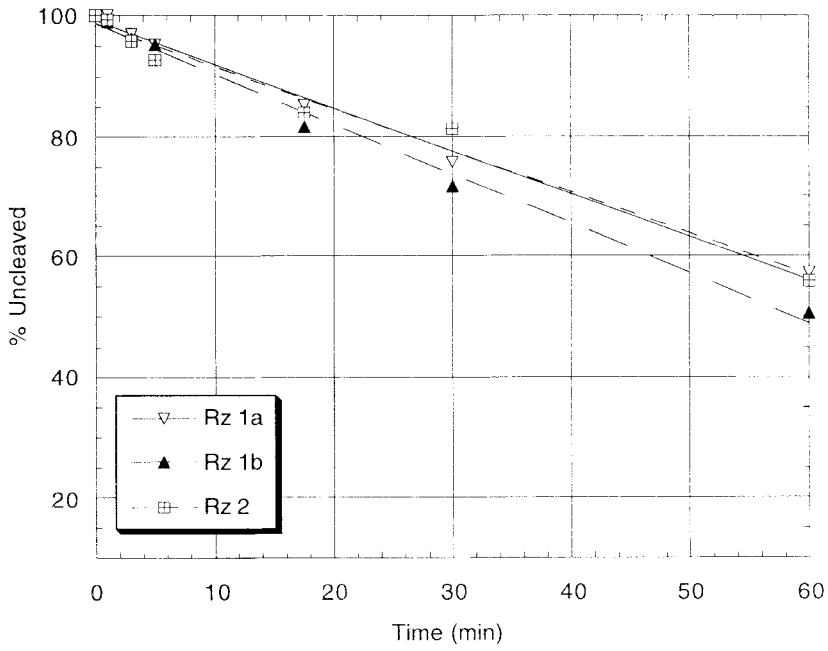

FIGURE 4: Comparative cleavage activity of ribozyme **1a**, synthesized using the optimized sulfurization protocol, ribozyme **1b**, synthesized using the old sulfurization protocol and ribozyme **2**. Assays were carried out in ribozyme excess.

CONCLUSIONS

Many new modifications have been incorporated into ribozymes in pursuit of compounds that are both active and stable in biological fluids. The development of these modified ribozymes has resulted in an expansion of the technologies typically used to provide oligoribonucleotides. In this paper we have optimized the synthesis, analysis and purification of phosphorothioate containing ribozymes. The actual sulfurization protocol has been improved by reducing both the amount of reagent utilized and the reaction times. A one step purification procedure has been developed that provides ribozyme with the appropriate ratio of phosphorothioate to phosphodiester linkages, as demonstrated by ^{31}P NMR. Concurrently, we have established the utility of CGE as an orthogonal method of analysis for phosphorothioate containing

oligonucleotides. Finally, it has clearly been demonstrated that no loss of quality or activity results from these changes.

EXPERIMENTAL

General

All analytical HPLC analyses were performed on a Hewlett Packard 1090 HPLC with a Dionex NucleoPac® PA-100 column, 4 x 250 mm, at 50 °C. Flow rate was 1.5 mL/min, Buffer A = 20 mM $NaClO_4$, 1 mM Tris pH 9.3, Buffer B = 300 mM $NaClO_4$, 1 mM Tris pH 9.3 and the gradient was as follows:

Time (min)	%B
0.50	45.0
12.50	75.0
12.60	100.0
14.10	100.0
14.60	0.0
15.00	0.0

All CGE analyses were performed on a Hewlett Packard ^{3D}CE with a J & W μPAGE™-5 (5% T, 5% C) polyacrylamide gel-filled column, 75 μm I.D. x 75 cm, 50 cm effective length, 100 mM Tris-Borate, 7 M Urea, pH = 8.3, and J & W μPAGE™ Buffer (100 mM Tris-Borate, 7 M Urea, pH = 8.3). Samples were electrokinetically injected using -13 KV for 3-10 s, run at -13 KV and detected at 260 nm.

All ^{31}P NMR spectra were recorded on a Varian Gemini 400 spectrometer operating at 161.947 MHz referenced externally with H_3PO_4. T_1 values for phosphodiester and phosphorothioate peaks were determined using arrayed longitudinal relaxation times to achieve optimal sensitivity for our studies. Determined T_1 values for phosphodiester and phosphorothioate peaks were 0.65 and 0.71 seconds, respectively. A D1 value of 4 seconds was used during acquisition based on the determined T_1 values. As such, typical experiments achieved a signal to noise value of 180 after 15,000 transients.

Synthesis of RNA and Ribozymes Containing Phosphorothioate Linkages

The general procedures for RNA synthesis and deprotection have been described previously (13,16). Syntheses were conducted on a 394 (ABI)

synthesizer using a modified 2.5 μmol scale protocol with a 5 min coupling step for 2′-*O*-TBDMS protected nucleotides and 2.5 min coupling step for 2′-*O*-methylated nucleotides. Table 3 outlines the amounts and the contact times of the reagents used in the synthesis cycle. A 6.5-fold excess of phosphoramidite and a 24-fold excess of *S*-ethyl tetrazole relative to polymer-bound 5′-hydroxyl was used in each coupling cycle. For those linkages which are phosphorothioate, the iodine solution is replaced with a 0.05 M solution of 3H-1,2-benzodithiol-3-one 1,1-dioxide in acetonitrile. Beaucage reagent (0.05 M in acetonitrile) was made up from the solid reagent obtained from American International Chemical, Inc.

RNA and Ribozyme Purification

The crude, fully deprotected material was diluted to 20 mL with Milli Q water. The sample was injected onto a Pharmacia Mono Q® 16/10 m m column with 60% buffer A (20 mM NaCl, 1 mM Tris, pH 9.3 in 10% CH_3CN). A gradient from 65-80% buffer B (800 mM NaCl, 1 mM Tris, pH 9.3 in 10% CH_3CN) in 28 min at a flow rate of 8 mL/min was used to elute the RNA. Fractions were analyzed by HPLC and those containing full length product ≥80% by peak area were pooled for desalting. The pooled fractions were applied to a SepPak cartridge (C_{18}) that was prewashed successively with CH_3CN (10 mL), $CH_3CN/MeOH/H_2O$:1/1/1 (10 mL) and Milli Q H_2O (20 mL). Following sample application, the cartridge was washed with Milli Q H_2O (20 mL) to remove the salt. Product was then eluted from the column with $CH_3CN/MeOH/H_2O$:1/1/1 (10 mL) and dried.

The purified ribozymes were then precipitated according to the following protocol. To the ribozyme in Milli Q water (0.5 mL) was added 3 M sodium acetate (50 μL, pH 5.2) followed by addition of absolute EtOH (2.5 mL). The mixture was cooled to -20 °C for 1 h and then centrifuged at 4 °C, at 10,000 RPM for 20 min. The solution was decanted, 80% EtOH (2.0 mL) was added to the pellet and the sample was centrifuged at 4 °C, at 10,000 RPM for 20 min. The solution was decanted and the resulting pellet was then dried.

TABLE 3: 2.5 μmol SYNTHESIS CYCLE

Reagent	Equivalents	Amount	Wait Time*
Phosphoramidite	6.5	163 μL	5/2.5 min
S-Ethyl Tetrazole	23.8	238 μL	5/2.5 min
Acetic Anhydride	100	233 μL	5 s
N-Methyl Imidazole	186	233 μL	5 s
TCA	83.2	1.73 mL	21 s
Iodine	8.0	1.18 mL	45 s
Beaucage Reagent	8.6	430 μL	300 s
Acetonitrile	NA	6.67 mL	NA

* Wait time does not include contact time during delivery. Where two coupling times are indicated the first refers to RNA coupling and the second to 2'-*O*-methyl coupling.

Ribozyme Activity Assay

Ribozymes and 5'-^{32}P-labeled substrate were heated separately in reaction buffer (50 mM Tris-Cl, pH 7.5; 10 mM $MgCl_2$) to 95 °C for 2 min, quenched on ice, and equilibrated to 37 °C prior to starting the reactions. Reactions were carried out in enzyme excess, and were started by mixing ~1 nM substrate and 40 nM ribozyme to a final volume of 50 μL. Aliquots of 5 μL were removed at 1, 5, 15, 30, 60 and 120 min, quenched in formamide loading buffer, and loaded onto 15% polyacrylamide/8 M urea gels. The fraction of substrate and cleavage product present at each time point was determined by quantitation of scanned images from a Molecular Dynamics PhosphorImager®. Ribozyme cleavage rates were calculated from plots of the fraction of substrate remaining *vs* time using a double exponential curve fit (Kaleidagraph, Synergy Software).

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REFERENCES

1. Beigelman, L.; McSwiggen, J.; Draper, K.; Gonzalez, C.; Jensen, K.; Karpeisky, A.; Modak, A.; Matulic-Adamic, J.; DiRenzo, A.; Haeberli, P.; Tracz, D.; Sweedler, D.; Grimm, S.; Wincott, F.; Thackray, V.; Usman, N. *J. Biol. Chem.* **1995**, *270*, 25702-25708.
2. A preliminary report of this work was presented; Wincott, F.; DiRenzo, A.; Levy, K.; Haeberli, P.; Grimm, S.; Usman, N. *J. Cell. Biochem.* **1995**, *19A*, 218.
3. Uhlmann, E.; Peyman, A. *Chem. Rev.* **1990**, *90*, 563-584.
4. Iyer, R.P.; Phillips, L.R.; Egan, W.; Regan, J.B.; Beaucage, S. *J. Org. Chem.* **1990**, *55*, 4693-4699.
5. Wyrzykiewicz, T.K.; Ravikumar, V.T. *Bioorg. Med. Chem. Lett.* **1994**, *4*, 1519-1522.
6. Chiang, M.-Y.; Chan, H.; Zounes, M.; Freier, S.M.; Lima, W.F.; Bennett, C.F. *J. Biol. Chem.* **1991**, *266*, 18162-18171.
7. Kawasaki, A.M.; Casper, M.D.; Freier, S.M.; Lesnik, E.A.; Zounes, M.C.; Cummins, L.L.; Gonzalez, C.; Cook, P.D. *J. Med. Chem.* **1993**, *36*, 831-841.
8. Shibahara, S.; Mukai, S.; Morisawa, H.; Nakashima, H.; Kobayashi, S.; Yamamoto, N. *Nucleic Acids Res.* **1989**, *17*, 239-252.
9. Morvan, F.; Rayner, B.; Imbach, J.-L. *Tetrahedron Lett.* **1990**, *31*, 7149-7152.
10. Agrawal, S.; Tang, J.Y.; Sun, D.; Sarin, P.S.; Zamecnik, P.C. *Antisense Strategies* in *Annals New York Academy of Sciences*, R. Baserga and D. Denhardt, Editors, **1992**, *660*, 3-10.
11. Slim, G.; Gait, M.J. *Nucleic Acids Res.* **1991**, *19*, 1183-1188.
12. Jarvis, T.C.; Alby, L.J.; Beaudry, A.A.; Wincott, F.E.; Beigelman, L.; McSwiggen, J.A.; Usman, N.; Stinchcomb, D.T. *RNA*, **1996**, 2, 419-428.
13. Wincott, F.; DiRenzo, A.; Shaffer, C.; Grimm, S.; Tracz, D.; Workman, C.; Sweedler, D.; Gonzalez, C.; Scaringe, S.; Usman, N. *Nucleic Acids Res.* **1995**, *23*, 2677-2684.
14. DeDionisio, L. *J. Chromatography*, **1993**, *652*, 101-108.
15. Zon, G. *Oligonucleoside Phosphorothioates*, in *Protocols for Oligonucleotides and Analogs*, S. Agrawal, Editor. **1993**, Humana Press: Totawa, NJ. p. 165-189.
16. Usman, N.; Ogilvie, K.K.; Jiang, M.-Y.; Cedergren, R.J. *J. Am. Chem. Soc.*, **1987**, *109*, 7845-7854.

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