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SYNTHESIS OF 1-DEOXY-D-RIBOFURANOSE PHOSPHORAMIDITE & THE INCORPORATION OF ABASIC NUCLEOTIDES IN STEM-LOOP II OF A HAMMERHEAD RIBOZYME

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Abstract The synthesis of 1-deoxy-D-ribofuranose-3-(2-cyanoethyl *N,N*-diisopropylphosphoramidite) **9** from D-ribose and its incorporation in a hammerhead ribozyme is described. Ribozymes containing 4, 6 or 8 abasic nucleotides, **H**, in Loop and Stem II have wild-type catalytic activity.

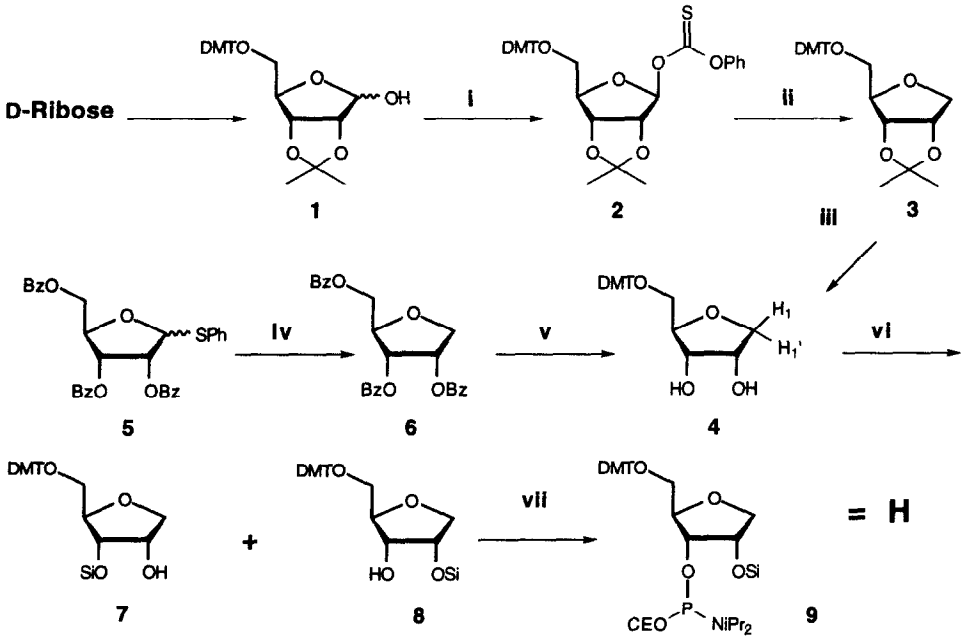
Hammerhead ribozymes[1] are one of the smallest catalytic RNAs with sequence-specific endoribonuclease activity. Their highly specific cleavage activity suggests their use as therapeutic agents for the inhibition of gene expression. Structure-activity studies, based on the incorporation of base,[2] sugar[3] and backbone[4] modified nucleotides into the hammerhead domain, has allowed the determination of some functionally important residues in this ribozyme motif. As part of our studies on the molecular mechanism of action of hammerhead ribozymes, we were interested in the effect of incorporation of abasic sites **H** (Figure 2) in the Stem-Loop II region of a hammerhead ribozyme model sequence.

The synthesis of 1-deoxy-D-ribofuranose phosphoramidite **9** is shown in Figure 1. The synthesis of the related phosphoramidite of 1,2-dideoxy-D-ribofuranose has been described.[5,6] Our initial efforts concentrated on the deoxygenation of synthon **1**, prepared by a "one pot" procedure from D-ribose.[7] Phenoxythiocarbonylation of acetonide **1** under Robins' conditions[8] led to the β-anomer **2** ($J_{1,2}$ = 1.2 Hz) in modest yield (45-55%). Radical deoxygenation using Bu_3SnH/AIBN resulted in the formation of the ribitol derivative **3** in 50% yield. Subsequent deprotection with 90% CF_3COOH (10 min) and introduction of a dimethoxytrityl group led to the key intermediate **4** in 40% yield.[9]

The low overall yield of this route prompted us to investigate a different approach to the synthesis of **4**. Phenylthioglycosides, successfully employed in the Keck reaction,[10] appeared to be an alternative. However, it is known that free-radical reduction of the corresponding glycosyl bromides with participating acyl groups at the C2-position can result in the migration of the 2-acyl group to the C1-position (depending on Bu_3SnH concentration[11]). Therefore we subjected phenylthioglycoside **5** (prepared from commercially available 1-*O*-acetyl-2,3,5-tri-*O*-benzoyl-D-ribofuranose according to Ferrier[12]) to radical reduction with Bu_3SnH (6.1 eq.) in the presence of Bz_2O_2 (2 eq.) resulting in the isolation of tribenzoate **6** in 63% yield. Subsequent debenzoylation and dimethoxytritylation led to synthon **4** in 70% yield. Introduction of the TBDMS group, using standard conditions, resulted in the formation of a 4:1 ratio of 2- and 3-isomers **8** and **7**. The two regioisomers were separated by silica gel chromatography. The 2'-*O*-*t*-butyldimethylsilyl derivative **8** was phosphitylated to provide phosphoramidite **9** in 82% yield.

Figure 1

Synthesis of 2-*O*-t-Butyldimethylsilyl-5-*O*-Dimethoxytrityl-3-*O*-(2-Cyanoethyl-N,N-diisopropylphosphoramidite)-1-Deoxy-D-Ribofuranose 9

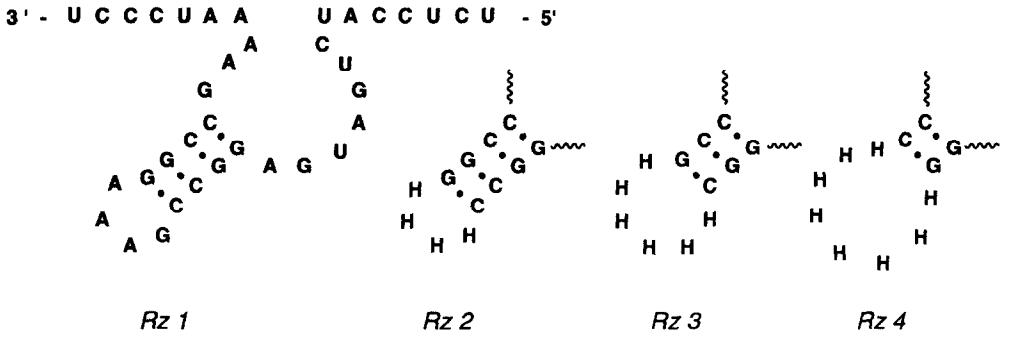


Si = *t*-Butyldimethylsilyl
DMT = 4,4'-Dimethoxytrityl
CE = β-Cyanoethyl

Reagents and Conditions: *i)* PhOC(S)-Cl/DMAP, *ii)* Bu_3SnH/AIBN, *iii)* CF_3COOH, DMT-Cl/Pyr, *iv)* Bu_3SnH/Bz_2O_2, *v)* 2M NaOH/Pyr/MeOH, DMT-Cl/Pyr, *vi)* TBDMS-Cl/$AgNO_3$, *vii)* P(OCE)(N-iPr_2)Cl

Figure 2

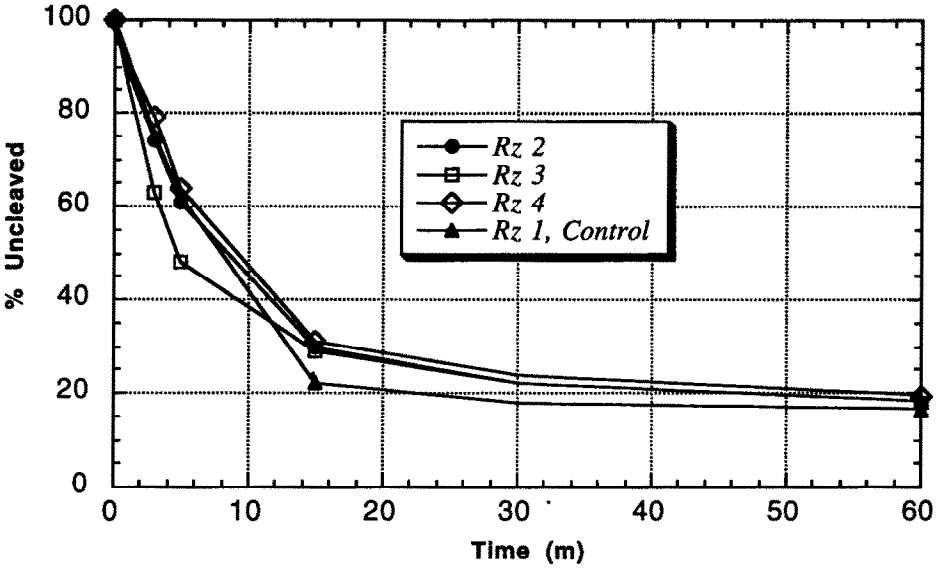
Hammerhead Ribozymes Containing Abasic Nucleotides H



Four, six and eight abasic residues were incorporated into the hammerhead ribozyme shown in Figure 2 utilizing standard RNA synthesis protocols with coupling efficiencies of 98.5%.[13]

Figure 3 shows a time course of the cleavage of a 17-mer RNA substrate 5'- CAG GGA UUC AUG GAG AU -3' (cleavage site in bold). The ribozyme and substrate concentrations were 8 and 1 nM respectively. Substitution of Loop II by four abasic residues (*Rz 2*) had no effect on catalytic activity. Interestingly the shortening of Stem II and enlargement of Loop II that occurred in the case of six (*Rz 3*) and eight (*Rz 4*) residue substitutions also had no effect on activity. We,[14] and others,[15] have shown that the replacement of Loop II by hexaethylene glycol spacers (20 atoms with 2 phosphates) also has little effect on catalysis, although the number, and types, of atoms replaced were not the 24 atoms and 4 phosphates of a normal tetraloop. Moreover, prolonged preincubation in the presence of $MgCl_2$ was required for hexaethylene glycol linker-modified ribozymes to obtain maximum activity,[15] reflecting the nonisosteric nature of the linker. In Ribozyme 2 we have an exact abasic replacement of Loop II maintaining all the back-bone atoms. In the cases of Ribozymes 3 and 4 there is still an exact replacement of the backbone although two base-pairs have been lost in Stem II. This data is in agreement with the observation that the shortening of Stem II from 4 to 2 base pairs has no effect on catalytic activity.[16]

Figure 3.



Substrate Cleavage of Abasic Nucleotide Containing Ribozymes at 8 nM: Cleavage of 5'- CAG GGA UUC AUG GAG AU -3' by the hammerhead ribozymes shown in Figure 2 at 37 °C in 50 mM Tris-Cl, pH 7.5 and 10 mM $MgCl_2$. The substrate concentration was ~1 nM.

These results support the hypothesis that the majority of the Stem II/Loop II region of the hammerhead ribozyme serves a general structural role in maintaining or allowing a certain conformation in the single-stranded catalytic core. There are no specific required base-base or base-metal interactions in this stem-loop.

Experimental

General

NMR-spectra were acquired on a Varian Gemini 400 spectrometer in either chloroform-d or dimethylsulfoxide-d_6 at 24 °C. Oligoribonucleotide syntheses were performed on an ABI-394 synthesizer.

2,3,5-Tri-O-Benzoyl-1-Deoxy-D-Ribofuranose 6

Thiophenyl-2,3,5-tri-*O*-benzoyl-D-ribofuranose[12] (5.95 g, 10.73 mmol) was dissolved, under argon, in dry toluene (70 mL) and Bu_3SnH (17.16 mL 63.79 mmol) was added. A solution of Bz_2O_2 (5.15 g, 21.46 mmol) in dry toluene (50 mL) was added to the refluxing reaction solution over 1 h. The reaction mixture was then allowed to reflux under argon for an additional 6 h. The solvent was removed *in vacuo*, the residue dissolved in CH_2Cl_2 and washed with $Na_2S_2O_3$, $NaHCO_3$ and brine. The organic layer was dried over Na_2SO_4 and evaporated to dryness. The crude mixture was purified by flash chromatography on silica gel using CH_2Cl_2 followed by CH_2Cl_2/2% MeOH as eluent. The appropriate fractions were collected, evaporated to dryness and dissolved in a minimal volume of CH_2Cl_2. The CH_2Cl_2 solution was then added dropwise to cooled light petroleum ether (1 L). The resulting precipitate was filtered to give 3.0 g (62.5%) of **6** as a white powder. ^1H NMR ($CDCl_3$): δ 4.18 (1H, H1, dd, $J_{1,1'}$ = 10.3, $J_{1,2}$ = 4.0), 4.52 (1H, H1', d, $J_{1',1}$ = 10.3), 4.52-4.62 (2H, H4 and H5, m), 4.74 (1H, H5, dd, $J_{5,5'}$ = 11.2, $J_{4,5}$ = 2.4), 5.67 (1H, H3, m), 5.82 (1H, H2, m).

5-O-Dimethoxytrityl-1-Deoxy-D-Ribofuranose 4

To a cooled (-15 °C) solution of **6** (2.93 g, 6.5 mmol) in a mixture of pyridine (60 mL) and methanol (10 mL) was added an ice-cooled 2M aq. solution of sodium hydroxide (9.75 mL) with stirring. The reaction mixture was stirred at -10 to -15 °C for an additional 30 min and then neutralized to pH 7 with Dowex 50 (Pyr^+). The resin was filtered and washed with H_2O:pyridine/4:1 (200 mL). The combined mother liquor and washings were evaporated to dryness and dried by multiple coevaporation with dry pyridine. The residue was redissolved in dry pyridine (75 mL) and dimethoxytrityl chloride (2.42 g, 7.15 mmol) was added and the reaction mixture left overnight at RT. The reaction was quenched with methanol (25 mL) and evaporated to dryness. The residue was dissolved in CH_2Cl_2, washed with saturated aq. $NaHCO_3$ and brine. The organic layer was dried over Na_2SO_4 and the solvent removed *in vacuo*. The residue was purified by flash chromatography on silica gel using 2% MeOH in CH_2Cl_2 as eluent to give 2 g (70%) of **4**. ^1H NMR (DMSO-d_6): δ 2.91-3.10 (2H, H5 and H5', m, $J_{5,5'}$ = 9.9, $J_{5,4}$ = 5.0, $J_{5',4}$ = 2.84), 3.58 (1H, H1 or H1', m), 3.72-3.75 (8H, 2 x OCH_3, H1 or H1', H4, m), 3.92-3.99 (2H, H2 and H3, m), 4.78 (2H, 2-OH and 3-OH, m, exchangeable with D_2O).

2-O-t-Butyldimethylsilyl-5-O-Dimethoxytrityl-1-Deoxy-D-Ribofuranose 8

To a stirred solution of **4** (1.85 g, 4.19 mmol) in dry THF (50 mL), was added pyridine (1.35 mL, 16.76 mmol) and $AgNO_3$ (1.71 g, 10.06 mmol). After 10 min, *t*-butyldimethylsilyl chloride (0.76 g, 5.03 mmol) was added and the reaction mixture was stirred at RT for 6 h. The resulting suspension was filtered into 5% aq. $NaHCO_3$ (100 mL). The solution was extracted with CH_2Cl_2 (2 x 100 mL). The combined organic layer was washed with brine, dried over Na_2SO_4 and evaporated to dryness. The residue was purified by flash chromatography on silica gel with hexanes-ethyl acetate (2:1) as eluent to yield 1.4 g of the desired compound **8**, 0.37 g of 3-silyl isomer **7** and 0.26 g of the bis-silyl derivative. ^1H NMR (DMSO-d_6): δ 2.95 (1H, H5, dd, $J_{5,5'}$ = 9.7, $J_{5,4}$ = 4.0), 3.11 (1H, H5', bd, $J_{5,5'}$ = 9.7), 3.57 (1H, H1, dd, $J_{1,1'}$ = 9.04, $J_{1,2}$ = 3.4), 3.72 (6H, OCH_3, s), 3.76 (2H, H3 and H4, m), 3.96 (1H, H1', dd, $J_{1,1'}$ = 9.04, $J_{1',2}$ = 0.72), 4.18 (1H, H2, dd, $J_{2,3}$ = 7.2), 4.49 (1H, 2-OH, d, $J_{2,2\text{-OH}}$ = 5.4, exchangeable with D_2O).

2-O-t-Butyldimethylsilyl-5-O-Dimethoxytrityl-3-O-(2-Cyanoethyl-N,N-diisopropylphosphoramidite)-1-Deoxy-D-Ribofuranose 9

To an ice-cooled stirred solution of **8** (1.16 g, 2.11 mmol) in dry CH_2Cl_2 (20 mL) under argon was added, dropwise *via* syringe, a premixed solution of *N,N*-diisopropylethylamine (0.76 mL, 2.24 mmol) and 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite (0.58 mL, 2.61 mmol) in CH_2Cl_2 (3 mL). Simultaneously, *via* another syringe *N*-methylimidazole (0.14 mL, 1.74 mmol) was added and stirring was continued for 2 h at RT. The reaction mixture was again ice-cooled and quenched with dry methanol (15 mL). After 5 min stirring, the mixture was concentrated *in vacuo* (40 °C) and purified by flash chromatography on silica gel using hexanes-ethyl acetate (6:1) containing 1% NEt_3 as an eluent, yielding 1.3 g (82%) of **9** as a white foam. ^{31}P NMR ($CDCl_3$): δ ppm: 150.312, 150.753.

Synthesis of Hammerhead Ribozymes Containing 1-deoxy-D-ribofuranose

The method of synthesis and deprotection procedures used followed that for normal RNA synthesis as described in reference 13. The average stepwise coupling yields were ~98%.

Ribozyme Activity Assay

Purified ^{32}P-5'-end labeled 17-mer RNA substrate and purified ^{32}P-5'-end labeled 36-mer ribozymes, in 50 mM Tris-Cl, pH 7.5 and 10 mM $MgCl_2$, were both heated to 95 °C, quenched on ice and equilibrated at 37 °C, separately. The final ribozyme concentrations were 8 nM and the final substrate RNA concentrations were ~1 nM. Total reaction volumes were 50 µL. Reactions were initiated by 1/1 mixing of substrate and ribozyme stock solutions, 16 nM and 2 nM respectively. Aliquots of 5 µL were removed at time points of 1, 5, 15, 30 and 60 min. Each aliquot was quenched in formamide loading buffer and loaded onto a 15% denaturing polyacrylamide gel for analysis. Quantitative analyses were performed using a phosphorimager (Molecular Dynamics®).

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