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NUCLEASE RESISTANT HAMMERHEAD MOTIF: FROM '5-RIBO' TO '3-RIBO' MODEL

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Abstract: Previously developed '5-ribo' nuclease stabilized hammerhead motif was further refined by systematic incorporation of 1-(β-D-xylofuranosyl) adenine (xA) and 1-(β-D-xylofuranosyl) guanine (xG) in the place of conserved ribopurine residues of the catalytic core. Modified ribozymes substituted with xA at positions A15.1 and A6 demonstrated catalytic activity close to the parent stabilized ribozyme. Analogous guanosine substitutions at positions G5, G8 and G12 substantially lowered catalytic rates.

Hammerhead ribozymes are small oligoribonucleotides that can catalyze highly specific trans-cleavage of any RNA containing dinucleotide sequence UH (H = C, A or U).[1] Such high specificity suggests the use of these ribozymes as therapeutic agents in gene regulation.[2] One of the main obstacles in realizing this potential lies in instability of oligoribonucleotides in biological fluids. An additional challenge with ribozymes is that the common approach of oligonucleotide stabilization using uniform 2'-sugar or backbone modifications usually interferes with catalytic activity. Extensive studies have been carried out to determine the number and location of ribose residues required for catalytic activity.[3-6] A consensus '5-ribose' model, containing ribose residues at positions G5, A6, G8, G12 and A15.1 in 2'-*O*-Me-background (Rz A, Figure 1) was found to have close to wild-type catalytic activity and a half-life >72 h in human serum.[7] Further investigation of similar motif in animal models indicated that 5-ribo residues appeared to be initial sites of nuclease degradation. We reasoned that additional stabilization at these positions might be beneficial for pharmacodynamic properties of the potential drug candidates.

Our initial strategy focused on systematic replacement of adenosine and guanosine residues at '5-ribose' positions with related 2'-F and 2'-NH_2 analogs. We expected that such modifications could preserve[6] some of the hydrogen-bonding pattern of the parent 2'-OH groups that seem to be critical for the maintenance of catalytic activity. Unfortunately, in all cases, these 2'-F and 2'-NH_2-A and -G modified ribozymes demonstrated significantly reduced (>50-fold) cleavage activity. It became apparent that alternative strategy will be needed: the conserved 2'-OH at positions G5, A6, G8, G12, and A15.1 should be left unmodified; at the same time other positions of ribofuranose moiety (or phosphodiester backbone) of these conserved nucleotides should be modified to provide nuclease resistance while maintaining cleavage activity of modified ribozymes.

One modification that may satisfy the above criteria is β-D-xylofuranosyl sugar modification.[8,9] It differs from its natural β-D-ribofuranosyl counterpart in that the 3'-OH group now occupies β or "up" position of the furanose ring. The trans configuration of 2'- and 3'-hydroxyls in these analogs should efficiently prevent phosphodiester cleavage through the postulated 2',3'-cyclic phosphate intermediate and thus increase the ribozyme resistance to ribonucleolytic cleavage.

Seela *et al.*[10] described the synthesis of 2'-deoxy-β-D-xylofuranosyl nucleoside 3'-phosphoramidites and 3'-phosphonates and their incorporation into deoxyoligonucleotides. Here we report an efficient method for the preparation of 1-(β-D-xylofuranosyl) purine 3'-phosphoramidites in three steps from the commercially available 5'-*O*-dimethoxytrityl-2'-*O*-*t*-butyldimethylsilyl nucleosides (Scheme 1).

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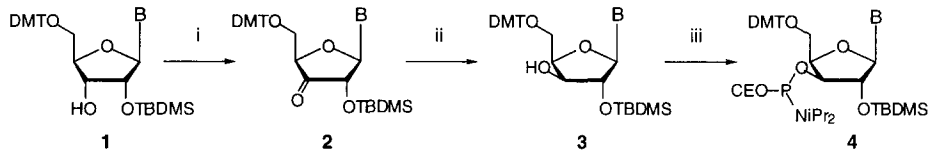
Scheme 1. Synthesis of β-D-xylofuranosyl purine 3'-phosphoramidites.



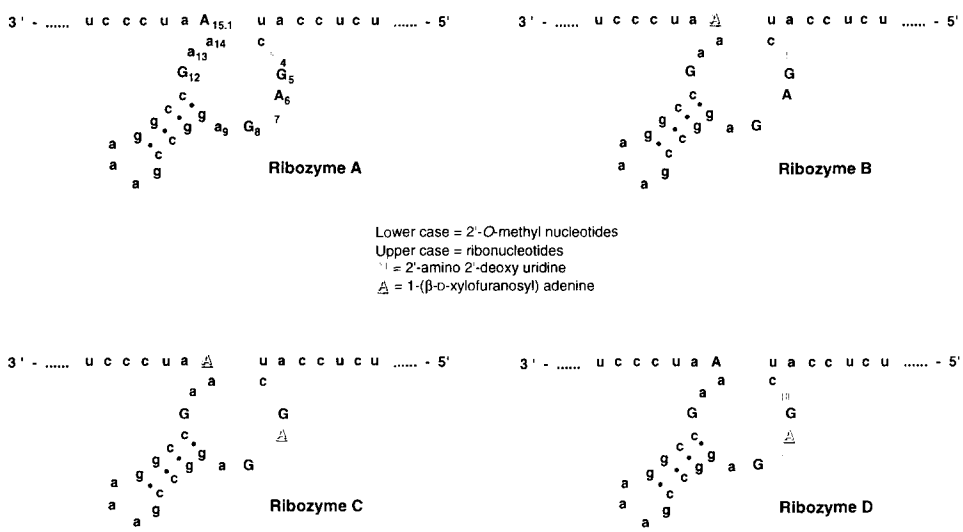
DMT = 4,4'-dimethoxytriphenylmethyl
TBDMS = *t*butyldimethylsilyl
Bz = benzoyl
i-Bu = isobutyryl

a B = A^{Bz}
b B = $G^{i\text{-}Bu}$

Reagents and Conditions: (i) CrO_3/Pyr/Ac_2O/CH_2Cl_2, rt, 1 h; (ii) $NaB(OAc)_3H$/EtOH, rt, overnight; (iii) 2-Cyanoethyl-*N,N*-diisopropylchlorophosphoramidite/1-MeIm/DIPEA/CH_2Cl_2, rt, 2 h.

Oxidation of **1a,b** with CrO_3/pyridine/acetic anhydride followed by highly selective reduction of the resulting 3'-ketones **2a,b** with sodium triacetoxyborohydride afforded the protected xylofuranosyl nucleosides **3a,b** in moderate yields.[11] The xylo configuration in intermediates **3a,b** was evident from characteristic[9] decrease in coupling constants $J_{1',2'}$ (1.2 Hz in **3a** *vs* 5.6 Hz in **1a**) and $J_{2',3'}$ (2.8 Hz in **3a** vs 5.6 Hz in **1a**), respectively. Phosphitylation under standard conditions yielded 3'-phosphoramidites **4a,b** in 70-80% yield.[12] These monomers were incorporated into ribozymes using solid phase phosphoramidite chemistry. The coupling time for xylofuranosyl monomers was extended to 20 min, using S-ethyltetrazole as an activator. Average stepwise coupling yield was 96-98%. At the end of the synthesis oligonucleotides were cleaved from the solid support and base-deprotected using 40% aq methylamine for 10 min at 65 °C followed by TEA/3HF reagent for the removal of the 2'-*O*-silyl protection.[13] Finally, ribozymes were purified by anion exchange HPLC.

Figure 1. Hammerhead ribozymes containing β-D-xylofuranosyl adenine substitution



According to our strategy adenosines and guanosines of the stabilized Rz A were systematically replaced by xylofuranosyl analogs (xA and xG) and cleavage activity of modified Rz's was determined at a single turnover conditions[7] for a matched substrate 5'-CAG GGA UUA AUG GAG AU. Adenosine replacements at positions A15.1 and A6, singly (Rz B and Rz D) or in combination (Rz C) (Figure1), were well tolerated; modified ribozymes retained the cleavage activity similar to that of the parent Rz A (Table 1). On the contrary, guanosine replacement at position G12 (Rz E) reduced catalytic activity 5-fold while replacements at G5 (Rz F) and G8 (Rz G) abolished the activity.

Table 1. Relative Rates of the Catalytic Activity of xA and xG Modified Ribozymes

β-D-xylofuranosyl A modified ribozymes			β-D-xylofuranosyl G modified ribozymes		
Ribozyme	**K_{obs} (min^{-1})**	**K_{rel}**	**Ribozyme**	**K_{obs} (min^{-1})**	**K_{rel}**
Rz A	3.4	1	Rz A	3.4	1
Rz B	2.9	0.85	Rz E	0.7	0.2
Rz C	2.3	0.68	Rz F	0.002	0.0006
Rz D	2.7	0.79	Rz G	0.004	0.001
Reaction conditions: 1μM ribozyme, <1 nM substrate, 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 37 °C.					

The HPLC of the enzymatic digest of Rz B indicated the presence of xA2'OMeA dimer (t_R 22.11 min) while the enzymatic digest of a double substituted Rz C indicated the presence of both xA2'OMeA (t_R 22.11 min) and xA2'NH_2U (t_R 17.51 min) dimers, respectively (Figure 2). Enzymatic digest of Rz A under the same conditions demonstrated complete hydrolysis to its constituent nucleosides.

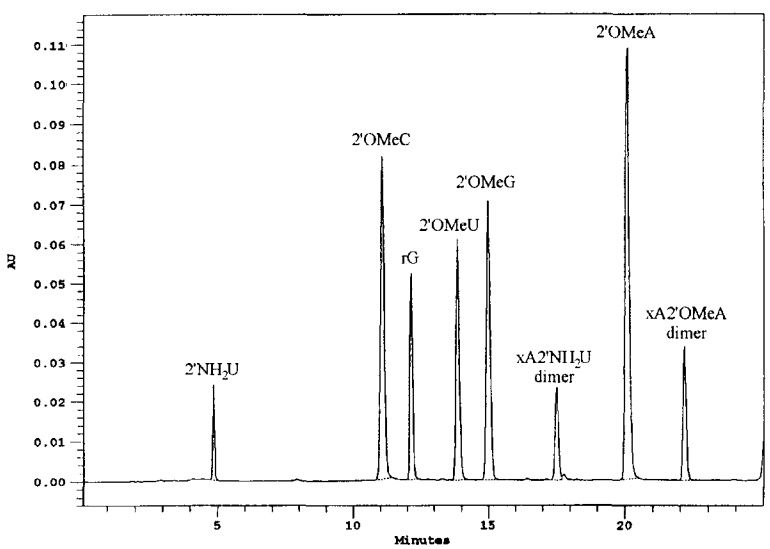


Figure 2. HPLC profile after enzymatic tandem hydrolysis of Rz C with nuclease P_1 followed by alkaline phosphatase. Waters Symmetry C18, 4.0 x 250 mm. Mobile phase: A = 25 mM KH_2PO_4, B = CH_3CN. Flow rate: 1 ml/min. Gradient: 4% B at 10 min.; 10% B at 20 min.; 100% B at 23 min.

In conclusion, this study demonstrated that the xylofuranosyl modification of adenosine residues at positions A6 and A15.1 of the stabilized ribozyme catalytic core does not impart ribozyme activity while at the same time it improves its resistance towards nucleolytic cleavage. This will allow for the further reduction of the number of conserved ribo-residues that are necessary for maintaining high catalytic activity in nuclease resistant hammerhead motif.

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References and Notes

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11. In a typical procedure 2'-*O*-TBDMS-5'-*O*-DMT ribonucleoside (2 mmol) was added to a pre-mixed solution of CrO_3 (600 mg), pyridine (1 mL) and acetic anhydride (0.6 mL) in CH_2Cl_2 (15 mL) and the reaction mixture was stirred at rt for 1 h. Ethyl acetate (100 mL) was then added and the mixture filtered through a Celite pad. The filtrate was concentrated *in vacuo* (40 °C), ethyl acetate (100 mL) was added and the mixture filtered slowly through the mixture of silica gel and Florisil (1:1, 40 g). The filtrate was concentrated *in vacuo* (40 °C) and used directly in the next step.
The above material was dissolved in ethanol (30 mL) and $NaB(OAc)_3H$ (848 mg, 2 equiv.) was added. The reaction mixture was stirred at rt overnight and the solvent removed *in vacuo*. The residue was partitioned between ethyl acetate and brine, organic layer was washed with 5% aq $NaHCO_3$ solution, dried (Na_2SO_4) and evaporated to a colorless foam. Purification by flash silica gel column chromatography using CH_2Cl_2/MeOH or CH_2Cl_2/THF mixtures yielded pure products in 50-65% yields (based on the starting ribo nucleosides).
^1H NMR ($CDCl_3$) for **4a**: 8.94 (brs, 1 H, NH), 8.75 (s, 1 H, H-8), 8.24 (s, 1 H, H-2), 8.09-6.77 (m, 18 H, aromatic), 5.91 (d, $J_{1',2'}$ = 1.6, 1 H, H-1'), 5.47 (d, $J_{OH,3'}$ = 8.4, 1 H, 3'-OH), 4.51 (s, 1 H, H-2'), 4.36 (dd, $J_{4',3'}$ = 8.0, $J_{4',5'}$ = 5.2, 1 H, H-4'), 4.06 (dd, $J_{3',2'}$ = 2.8, $J_{3',4'}$ = 8.0, 1 H, H-3'), 3.80 (s, 3 H, OMe), 3.77 (s, 3 H, OMe), 3.61 (d, $J_{5',4'}$ = 5.2, 2 H, H-5'), 0.89 (s, 9 H, *t*-Bu), 0.08 (s, 3 H, Me), 0.07 (s, 3 H, Me); **4b**: 7.83 (s, 1 H, H-8), 7.52-6.80 (m, 13 H, aromatic), 5.62 (d, $J_{1',2'}$ = 3.2, 1 H, H-1'), 4.56 (m, 1 H, H-2'), 4.32 (m, 1 H, H-4'), 4.10 (m, 1 H, H-3'), 3.77 (s, 6 H, 2xOMe), 3.56 (dd, $J_{5',5''}$ = 10.5, $J_{5',4'}$ = 4.4, 1 H, H-5'), 3.44 (dd, $J_{5'',5'}$ = 10.5, $J_{5'',4'}$ = 5.0, 1 H, H-5"), 2.25 (m, 1 H, *i*-Bu), 1.12 (d, J = 6.8, 3 H, *i*-Bu), 1.05 (d, J = 6.8, 3 H, *i*-Bu), 0.87 (s, 9 H, *t*-Bu), 0.08 (s, 3 H, Me), 0.03 (s, 3 H, Me).
12. ^{31}P NMR ($CDCl_3$): **4a** δ 151.0 (s) and 149.3 (s); **4b** δ 151.5 (s) and 148.3 (s).
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