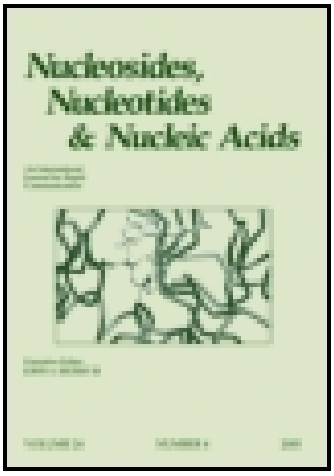

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2′-O-Methyl Thiomethyl Modifications in Hammerhead Ribozymes

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2'-*O*-METHYLTHIOMETHYL MODIFICATIONS IN HAMMERHEAD RIBOZYMES

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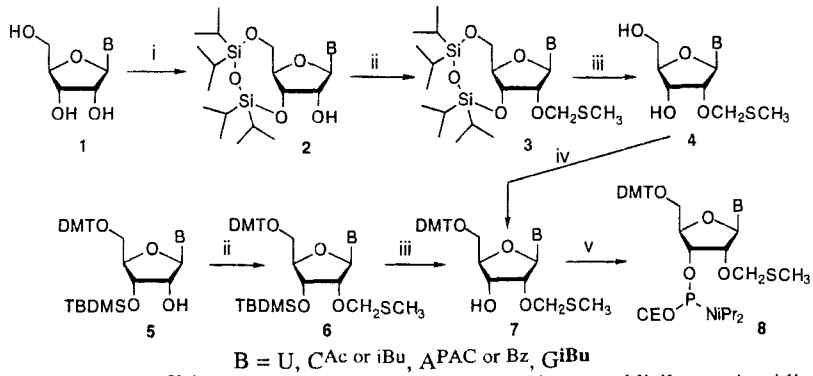
Abstract: The synthesis of all four phosphoramidites of 2'-*O*-methylthiomethyl ribonucleosides and their incorporation into hammerhead ribozymes and influence on nuclease stability and catalytic activity is described.

As part of our studies on the structure-activity relationships and molecular mechanism of action of hammerhead ribozymes[1-3] we were interested in the effect of the incorporation of nucleotides having a 2'-*O*-methylthiomethyl group (MTM) in a hammerhead ribozyme model sequence. MTM modifications could provide enhanced nuclease resistance which is very important in creating oligonucleotide therapeutics. Also, the hydrophobic nature of MTM group could have a positive effect on cell delivery of an oligonucleotide therapeutic to its target.

We describe here the synthesis of all four 2'-*O*-MTM nucleoside phosphoramidites **8** (B = U, $C^{Ac\ or\ iBu}$, $A^{PAC\ or\ Bz}$, G^{iBu}) and their incorporation into a 36-mer hammerhead ribozyme by solid phase RNA synthesis. The resulting modified ribozymes were tested for their catalytic activity and nuclease stability in human serum.

Methylthiomethyl ethers are well-established as protecting groups for alcohol functionalities.[4] The standard procedure involving direct preparation of MTM ethers from alcohols using acetic acid-acetic anhydride in dimethylsulfoxide[5] and its recent modification in nucleosides[6], requires long reaction times and strong acidic conditions. Alternatively, mild conversion of various alcohols to MTM ethers using methyl sulfide-benzoyl peroxide in the presence of 2,6-lutidine[7] and its successive application to deoxynucleosides[8] has been reported. Application of this method to the 3',5'-protected ribonucleosides **2** or commercially available derivatives **5** resulted in formation MTM ethers **3** or **6** respectively with 55-70% yields (Fig 1). The major by-product in this reaction was identified as a 2'-keto derivative (20% in case of U). Compounds **3** were deprotected using TBAF/THF resulting in 2'-*O*-MTM nucleosides **4.** Subsequent standard dimethoxytritylation led to



B = U, $C^{Ac \text{ or } iBu}$, $A^{PAC \text{ or } Bz}$, G^{iBu}

Reagents and conditions: *i)* 1,3-di-chloro-1,1,3,3-tetraisopropyldisiloxane/pyridine; *ii)* $(CH_3)_2S$, Bz_2O_2, 2,6-lutidine/MeCN-CH_2Cl_2; *iii)* TBAF/THF; *iv)* DMT-Cl/ pyridine; *v)* 2-cyanoethyl N,N-diisopropyl chlorophosphoramidite

FIGURE 1
Synthesis of 2'-*O*-Methylthiomethyl Ribonucleoside Phosphoramidites

5'-*O*-dimethoxytrityl-2'-*O*-MTM nucleosides **7**, which were converted to the corresponding phosphoramidites **8**. Alternatively, compounds **6** were deprotected with TBAF in THF to give the phosphitylation precursors identical to those prepared from derivatives **4.**

Phosphoramidites **8** were incorporated into ribozymes using standard protocols[9,10] for solid phase RNA synthesis. The presence of intact 2'-*O*-MTM nucleosides in ribozyme sequences and therefore resistance of thioether function in **8** to iodine oxidation during RNA synthesis was proved by base-compositional analysis.[2]

Ribozyme sequences and sites of 2'-*O*-MTM nucleosides incorporation are shown in Fig 2. Figure 3 shows a time course of ribozyme cleavage of a 17-mer RNA substrate containing the recognition sequence 5'- AGG GAU UAA UGG AGA -3'. All tested ribozymes (***Rz 1-4***) demonstrated enhanced cleavage rate under single-turnover conditions comparing to control (U4=U7=2'-aminouridine). For the most active ***Rz 3*** (2'-*O*-MTM-C's in Stem II) and ***Rz 4*** (U4=U7=2'-*O*-MTM-U) the values of k_2 (rate of the chemical step) and K_M were determined (Fig 4). It is noticeable, that even extensive substitution with 2'-*O*-MTM residues (***Rz 1*** and ***Rz 2***) provide highly active motifs. The dependence of rate constants on concentration for these ribozymes is shown on Fig 4.

To determine relative nuclease stability of 2'-*O*-MTM *vs* 2'-*O*-Me modifications we tested the stability of predominantly 2'-*O*-Me ***Rz 2*** containing 2'-*O*-MTM residues in "nuclease sensitive" positions U4 and U7[2] in human serum. Ribozyme remained intact af-

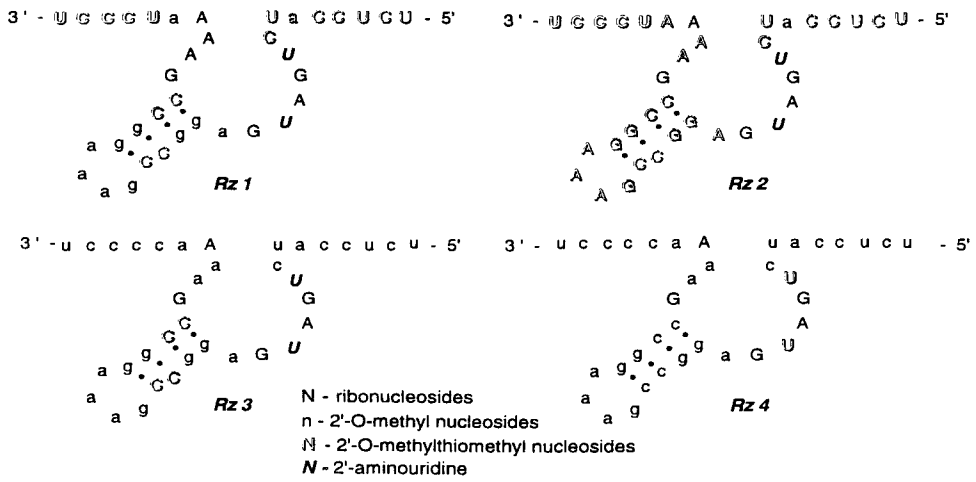


FIGURE 2
Hammerhead Ribozymes Containing 2'-*O*-MTM-Ribonucleosides

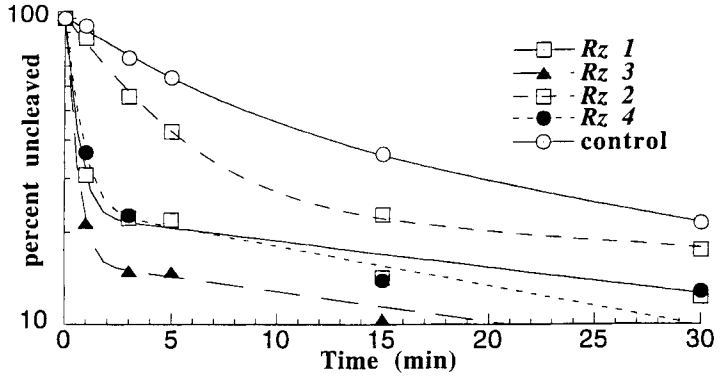


FIGURE 3
Cleavage Activity of Ribozymes Containing 2'-*O*-MTM Nucleosides

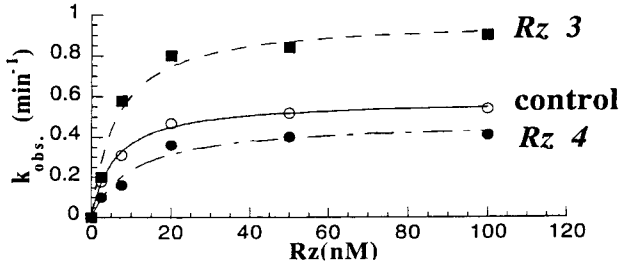


FIGURE 4
Cleavage Rates for Ribozymes Containing 2'-O-MTM Nucleosides

ter a 24 h incubation providing no degradation products corresponding to cleavage at position U4 or U7 or any other site demonstrating that ribozymes containing 2'-*O*-MTM-residues have equal or greater nuclease stability compared to those with 2'-*O*-Me modifications.

	CONTROL	*Rz 3*	*Rz 4*
$k_2(min^{-1})$	0.62 ± 0.04	1.2 ± 0.2	0.73 ± 0.06
K_M (nM)	6.9 ± 0.6	9.4 ± 1.1	22 ± 3

In summary 2'-*O*-MTM modification represents a promising alternative to 2'-*O*-Me in providing nuclease resistant and highly active hammerhead ribozymes.

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