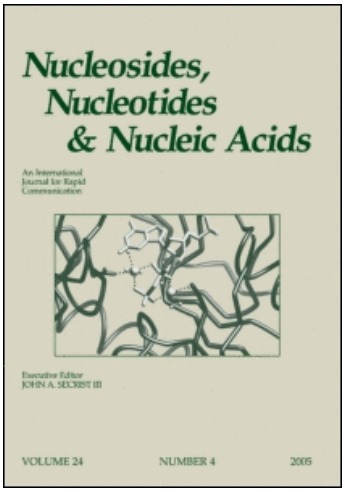

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SYNTHESIS OF 6-AZA- & 6-METHYL-PYRIMIDINE RIBONUCLEOSIDE PHOSPHORAMIDITES AND THEIR INCORPORATION IN HAMMERHEAD RIBOZYMES

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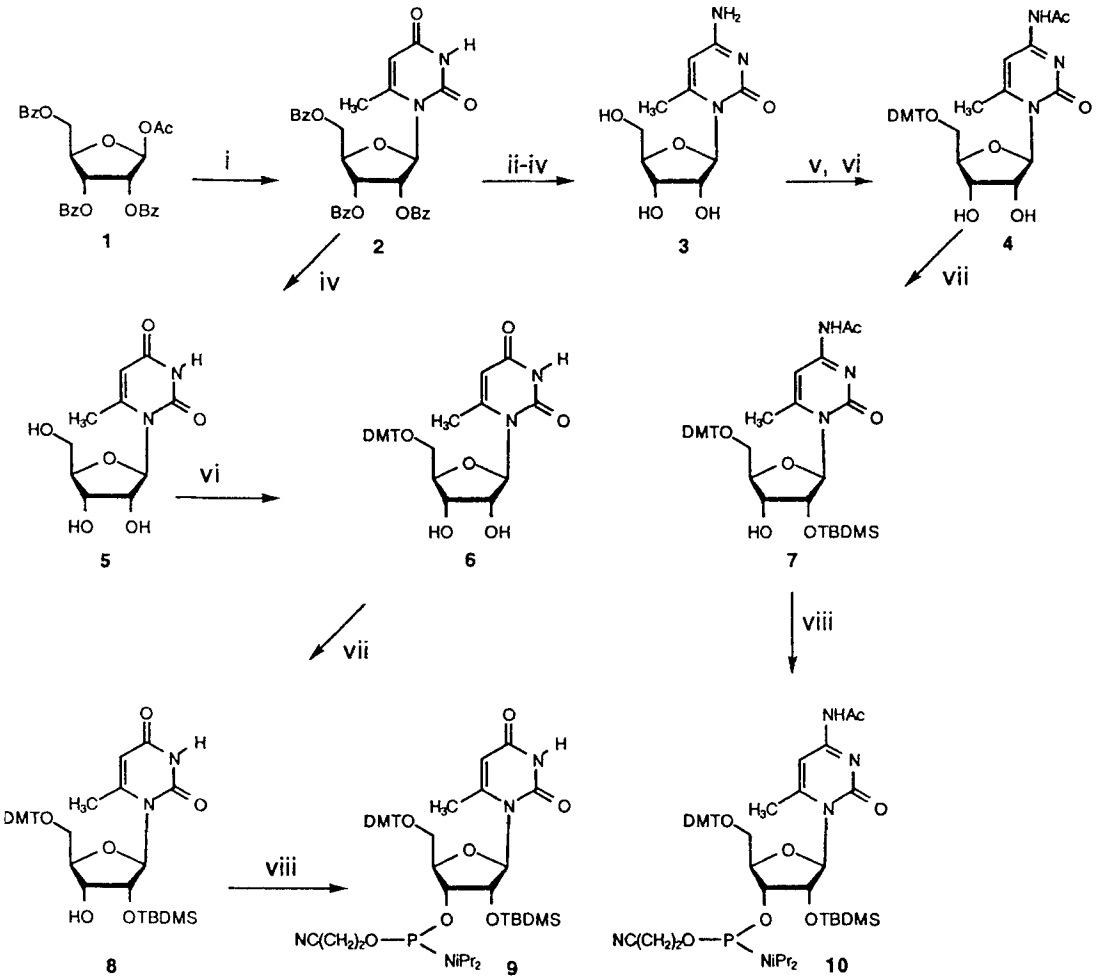
Abstract: The synthesis of phosphoramidites of 6-modified pyrimidine ribonucleosides and their incorporation into hammerhead ribozymes and influence on nuclease stability and catalytic activity is described.

As a part of our studies on the structure-activity relationships and molecular mechanism of action of hammerhead ribozymes, we were interested in the effect of the incorporation of pyrimidine nucleotides modified at the 6-position in a hammerhead ribozyme. These heterocyclic modifications alter the *syn-anti* conformation around the glycosidic bond[1-3] and may affect Watson-Crick base pairing at specific positions. They may also provide nuclease resistance since the 6 position is often required for base-specific nuclease binding. We describe the synthesis of 6-methyl- and 6-aza-pyrimidine ribonucleoside phosphoramidites **9, 10** and **16, 17** 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.

Vorbrüggen glycosylation of 6-methyluracil[4] at 0 °C in the presence of trimethylsilyl trifluoromethane sulfonate gave the nucleoside derivative **2** (Figure 1) in 75% yield. The latter was debenzoylated to give 6-methyl uridine (**5**). Subsequent standard dimethoxytritylation, *t*-butyldimethylsilylation and phosphitylation yielded uridine amidite **9**. Protected 6-methyluridine **2** was converted into the corresponding cytidine derivative **3** using a triazolide intermediate.[5] 6-Methylcytidine (**3**) was N^4-acetylated using the "transient protection" procedure[6] and, without separation, dimethoxytritylated to give compound **4** in 74% yield. Standard *t*-butyldimethylsilylation and phosphitylation led to the cytidine phosphoramidite **10.**

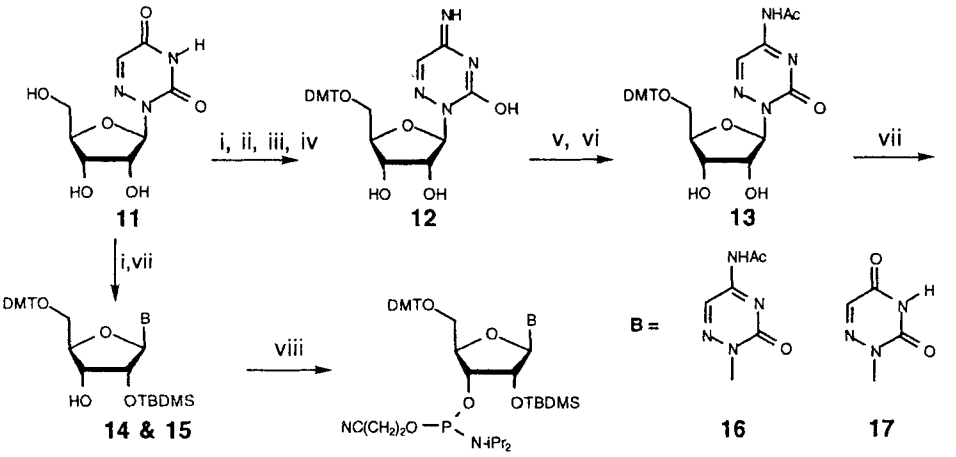
6-Aza-uridine phosphoramidite **17** was synthesized from 6-aza-uridine (**11**) using the standard steps of dimethoxytritylation, *t*-butyldimethylsilylation and phosphitylation



Reagents and Conditions: *i)* 6-Me-Ura^{TMS}, $CF_3SO_3SiMe_3$, 0 °C; *ii)* 1,2,4-triazole, $POCl_3$; *iii)* NH_4OH/dioxane; *iv)* 2M NaOH/Pyr/MeOH; *v)* Me_3Si-Cl/Pyr, then Ac_2O; *vi)* DMT-Cl/Pyr; *vii)* TBDMS-Cl/$AgNO_3$/Pyr/THF; *viii)* 2-Cyanoethyl-*N,N*-diisopropylchlorophosphoramidite, DIPEA/CH_2Cl_2.

FIGURE 1

Synthesis of 6-Methyl-Uridine & Cytidine Phosphoramidites



Reagents and Conditions: *i)* DMT-Cl/Pyr; *ii)* Ac_2O/Pyr; *iii)* 1,2,4-triazole, $POCl_3$, Et_3N; *iv)* NH_4OH/dioxane; *v)* Me_3Si-Cl/Pyr; *vi)* Ac_2O/Pyr; *vii)* TBDMS-Cl, $AgNO_3$, Pyr/THF; *viii)* 2-Cyanoethyl-*N,N*-diisopropyl-chlorophosphoramidite, DIPEA/CH_2Cl_2.

FIGURE 2

Synthesis of 6-Aza-Uridine & Cytidine Phosphoramidites

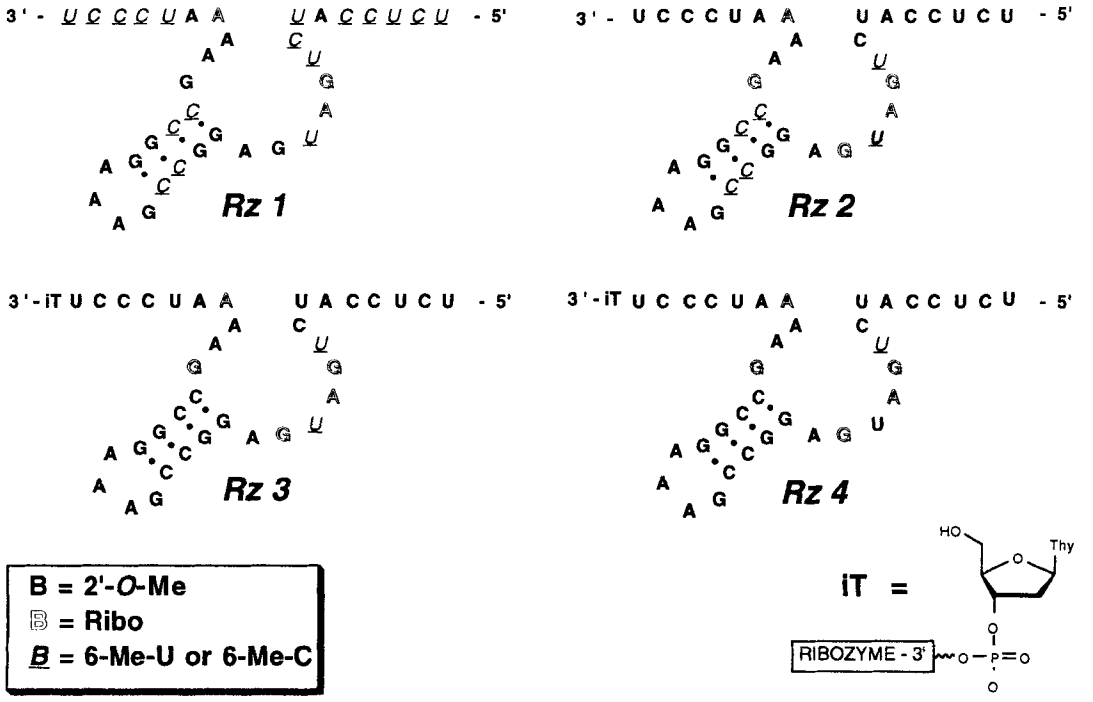


FIGURE 3

Hammerhead Ribozymes Containing 6-Methyl-Uridines & Cytidines

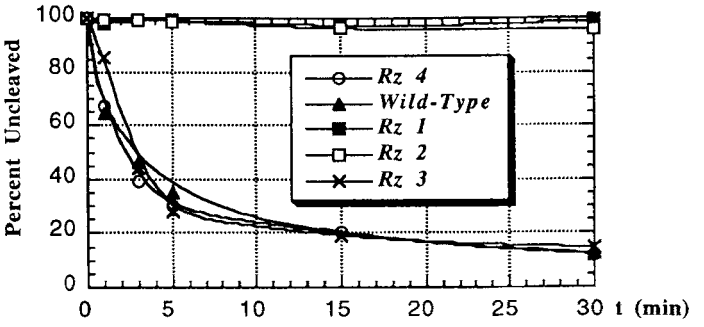


FIGURE 4

Cleavage Activity of Ribozymes Containing 6-Me-Uridines & Cytidines

(Figure 2). To obtain the 6-aza-cytidine amidite, 6-aza-uridine (**11**) was dimethoxytritylated and acetylated (without intermediate isolation) to give 5'-*O*-dimethoxytrityl-2',3'-di-*O*-acetyl-6-aza-uridine in 75% yield. Amination of this compound through the corresponding triazolide intermediate[5] led to 6-aza-cytidine **12** in 50% yield. The latter was N^4-acetylated *via* the "transient protection" procedure[6] to give 5'-*O*-DMT-N^4-acetyl-6-aza-cytidine **13**. The diol **13** was then silylated and phosphitylated to give 6-aza-cytidine phosphoramidite **17.** The structures of all compounds synthesized were confirmed by NMR spectroscopy.

6-Methyluridine and 6-methylcytidine were incorporated into the hammerhead ribozymes shown in Figure 3. Figure 4 shows a time course of ribozyme cleavage of a 17-mer RNA substrate sequence 5'- AGG GAU UCA UGG AGA -3'.

Total substitution of all C's and U's (***Rz 1***) resulted in complete loss of catalytic activity. The ribozyme with 6-Me-C substituted Stem II and 6-Me-U modifications at the U4 and U7 positions of the catalytic core (***Rz 2***) also had no cleavage activity. This data indicates that 6-Me-pyrimidine nucleosides, that exist preferably in the *syn*- conformation, most probably affect duplex formation and thus inactivate the ribozyme. However, ribozymes modified only at U4 or at both U4 and U7 in the catalytic core (***Rz 3*** and ***Rz 4***) still have almost wild-type cleavage activity.

We compared the stability of ***Rz 4*** (U4 = 6-Me-U; U7 = 2'-*O*-Me-U) to a control Rz (U4 = ribo U; U7 = 2'-*O*-Me-U, not shown) in human serum. The control Rz was instantaneously cleaved providing degradation products corresponding to cleavage at position U4. In contrast ***Rz 4*** remained intact after a 24 h incubation (approximate half-life ~40 h), providing an improvement in stability of more than 3 orders of magnitude.

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