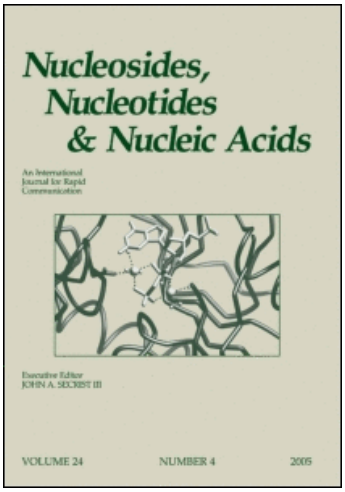

Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

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Online publication date: 09 August 2003

To cite this Article Klöpffer, A. E. and Engels, J. W.(2003) 'The Effect of Universal Fluorinated Nucleobases on the Catalytic Activity of Ribozymes', Nucleosides, Nucleotides and Nucleic Acids, 22: 5, 1347 — 1350
To link to this Article: DOI: 10.1081/NCN-120022962
URL: http://dx.doi.org/10.1081/NCN-120022962

NUCLEOSIDES, NUCLEOTIDES & NUCLEIC ACIDS
Vol. 22, Nos. 5–8, pp. 1347–1350, 2003

The Effect of Universal Fluorinated Nucleobases on the Catalytic Activity of Ribozymes

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ABSTRACT

Four fluoro modified universal nucleobases have been synthesized. The universal nucleobases **1** and **2**, containing a 2,4-difluorobenzene as nucleobase and a 4,6-difluorobenzimidazole, respectively, were chemically incorporated into a selected hammerhead ribozyme sequence which has already been retrovirally expressed as an anti-HIV ribozyme to investigate their effect on the catalytic activity of the ribozymes. The substitution of the natural nucleosides with either **1** or **2** results only in a small decrease of the catalytic activity. The K_m value for the monosubstituted ribozyme with a 2,4-difluorobenzene is $309\,nM^{-1}$, the corresponding k_{cat} is $2.91 \cdot 10^{-3}\ min^{-1}$. A disubstituted hammerhead ribozyme carrying one of each modification has also been synthesized. For a further stabilization of the ribozyme/substrate complex 2′-(β-aminoethoxy) modified fluorinated nucleosides **15** and **16** have been developed.

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DOI: 10.1081/NCN-120022962
1525-7770 (Print); 1532-2335 (Online)

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INTRODUCTION

It has been shown that fluorinated nucleobase analogues **1** and **2** can act as universal bases and therefore do not discriminate between the four natural nucleobases.[1] Furthermore the incorporation of the fluorine leads to a stabilization of the investigated 12mer RNA duplexes compared to the oligoribonucleotides carrying the benzene or the benzimidazole modification, respectively.[2] To determine whether the nucleosides **1** and **2** effect the catalytic activity of ribozymes we incorporated them into hammerhead ribozymes and determined the kinetic parameters.[3] To further improve the catalytic cleavage reaction of the modified ribozymes through electrostatic interactions we developed the nucleosides **15** and **16** and present an optimized synthetic pathway for these compounds. Thus, it should be possible to direct the ribozymes against the HIV polymerase gene containing point mutations (''hot spots'') without the loss of activity due to mismatch base pairs.

THE EFFECT ON RIBOZYME CATALYSIS

The ribonucleosides with a 2,4-difluorobenzene **1** or a 4,6-difluorobenzimidazole **2** as base analogues for a pyrimidine or a purine scaffold, respectively, were synthesized according to literature.[2] The corresponding phosphoramidites have been prepared using the recently developed TOM chemistry.[4] The hydrolytic RNA cleavage reactions were catalyzed by 10 mM $MgCl_2$ and carried out at 37°C under single turnover conditions. Cy5 labelling of the substrate allowed the fast and efficient kinetic analysis on an ALF Express® DNA Sequencer.

The substitution of either $^{2.4}U$ or $^{15.4}A$[5] with the nucleobases **1** or **2**, respectively, (Fig. 1) resulted only in a small reduction of the catalytic efficiency of the hammerhead ribozyme compared to the unmodified one. The K_m value obtained for the ribozyme containing the 2,4-difluorobenzene modification is $309\,nM^{-1}$ with an initial rate constant k_{cat} of $2.9 \cdot 10^{-3}$ min^{-1}. Comparable results were obtained by the reaction with the ribozyme containing the 4,6-difluorobenzimidazole modification on position 15.4. The doubly modified ribozyme showed decreased catalytic activity.

IMPROVED SYNTHESIS OF 2′-MODIFIED RIBONUCLEOSIDES

To enhance the rate of association between the ribozyme and the substrate we further developed 2′-modified fluorinated nucleobases. The 2′-position was modified with an β-aminoethoxy group[6] to yield the nucleosides **15** and **16** that are protonated under physiological conditions. The electrostatic interaction between the protonated amino group and the negatively charged RNA phosphodiester backbone should lead to an additional stabilization of helix I or III in the catalytically active three dimensional conformation of the hammerhead ribozyme.

Starting from the universal nucleobases **3** and **4** we protected the 5′- and the 3′-hydroxy group simultaneously with the Markiewicz reagent *t*-isopropyldisiloxane dichloride (Fig. 2). After the reaction with methyl bromoacetate the nucleosides **7**

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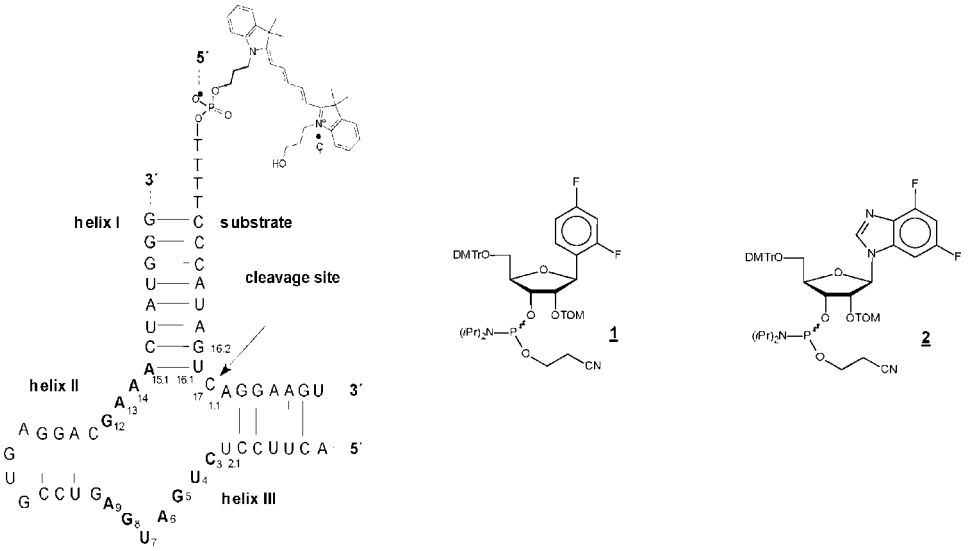

Figure 1. Secondary structure of the Cy5 labeled hammerhead ribozyme and modified nucleosides.

and **8**, respectively, were reduced with $LiBH_4$ in THF/MeOH 8 : 2. The corresponding alcohols **9** and **10** were tosylated with *p*-toluenesulfonyl chloride and afterwards converted into the azides **13** and **14**. In a final step the 2′-(β-aminoethoxy) - substituted nucleosides **15** and **16** were obtained by reduction of the azides with $SnCl_2$ in methanol.

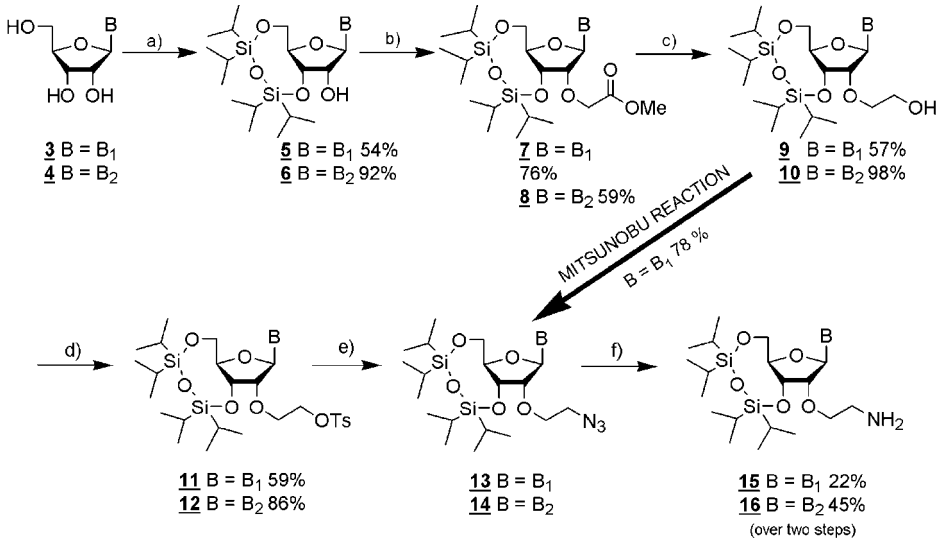

Figure 2. $B_1 = 2,4$-difluorobenzene, $B_2 = 4,6$-difluorobenzimidazole a) T*i*PDSiCl_2, pyridine b) $BrCH_2COOMe$, NaH, THF c) $LiBH_4$, THF/MeOH 8 : 2 d) *ρ*-TsCl, DMAP, NEt_3, CH_2Cl_2 e) NaN_3, DMF f) $SnCl_2$, MeOH.

We optimized the synthesis of the 2′- modified nucleosides by converting the alcohol **9** directly into the azide via the MITSUNOBU reaction[7] using sodium azide, DEAD (diethylazodicarboxylate) and triphenylphosphane.

ACKNOWLEDGMENTS

We want to thank the ''Fonds der Chemischen Industrie'' and the BMBF for a Ph.D. fellowship and the SFB 579 for financial support. A.K. would also like to thank Dr. U. Scheffer for the work on the ALF® Express System.

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