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SYNTHESIS OF 2'-C-α-DIFLUOROMETHYLARAURIDINE AND ITS 3'-O-PHOSPHORAMIDITE INCORPORATION INTO A HAMMERHEAD RIBOZYME

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Abstract: The 2'-C-difluoromethylated nucleoside **4** was synthesized starting from uridine. **4** was then converted to the 3'-O-phosphoramidite derivative **5** and was incorporated into a hammerhead ribozyme (**7**). The cleavage characteristics of the modified oligonucleotide have been analysed.

Development of ribozyme analogues which are resistant against ribonuclease degradation is an important step toward practically useful applications of this class of oligonucleotides. Systematical replacement of the 2'-OH groups of the ribonucleosides in the catalytic core of the hammerhead ribozyme with deoxynucleosides or 2'-O-alkylribonucleosides allowed the identification of G5,A6, G8, G12 and A15.1 or I15.1[1] as those positions where the presence of 2'-OH groups is essential for cleavage[2-5]. These essential 2'-hydroxyls are involved in important hydrogen bond interactions stabilising the tertiary structure of the hammerhead[6-9]. Additionally, it has been demonstrated that obtaining a 2'-OH group at position U4 results in a cleavage activity similar to the wild-type hammerhead ribozyme[10-12]. A pyrimidine ribonucleoside, in contrast to purine ribonucleosides though, cannot be tolerated in such a chemically modified ribozyme analogue, since it will be rapidly cleaved by endonucleases[13,14] A series of investigations have been undertaken so far to identify nucleoside analogues for the substitution of ribonucleosides in the catalytic core of the hammerhead ribozyme, which will provide endonucleolytic stability in addition to good cleavage activity[15-21]. The most prominent development was the use of 2'-amino-2'-deoxyuridine as a U4 replacement[13,22]. We were looking for alternative ways to stabilize a hammerhead ribozyme without compromising its catalytic activity. The difluoromethyl group is a good mimic for a hydroxy group

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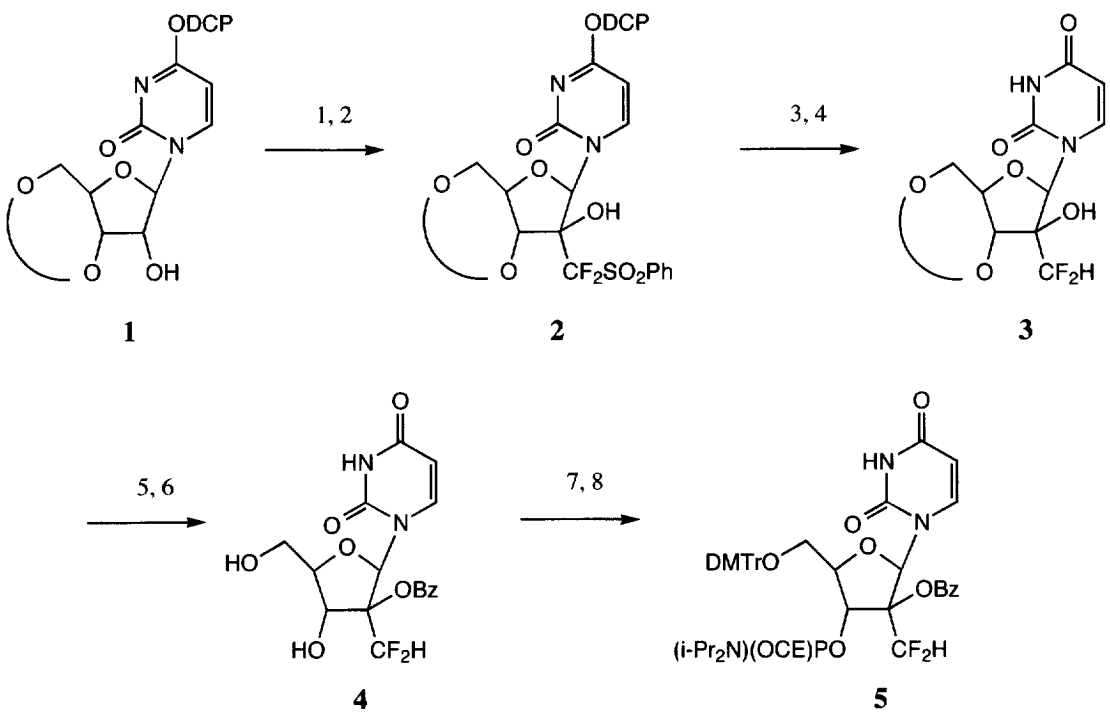
due to its H-bond donor and acceptor abilities[23]. Therefore, a hammerhead ribozyme containing a 2'-C-difluoromethyl substituted uridine at position U4 ought to show good cleavage activity. On the other hand, the modified ribozyme should be stable to endonucleolytic attack at this position. To test our hypothesis, we have synthesized the uridine derivative **4**, converted it into a phosphoramidite building block **5** and inserted it into a modified hammerhead ribozyme. The synthesis of the 2'-C-difluoromethylarauridine and its conversion to the phosphoramidite was performed according to figure 1[19].

Figure 1: Synthesis of the 2'-C-Difluoromethylarauridine 3'-O-Phosphoramidite, 5

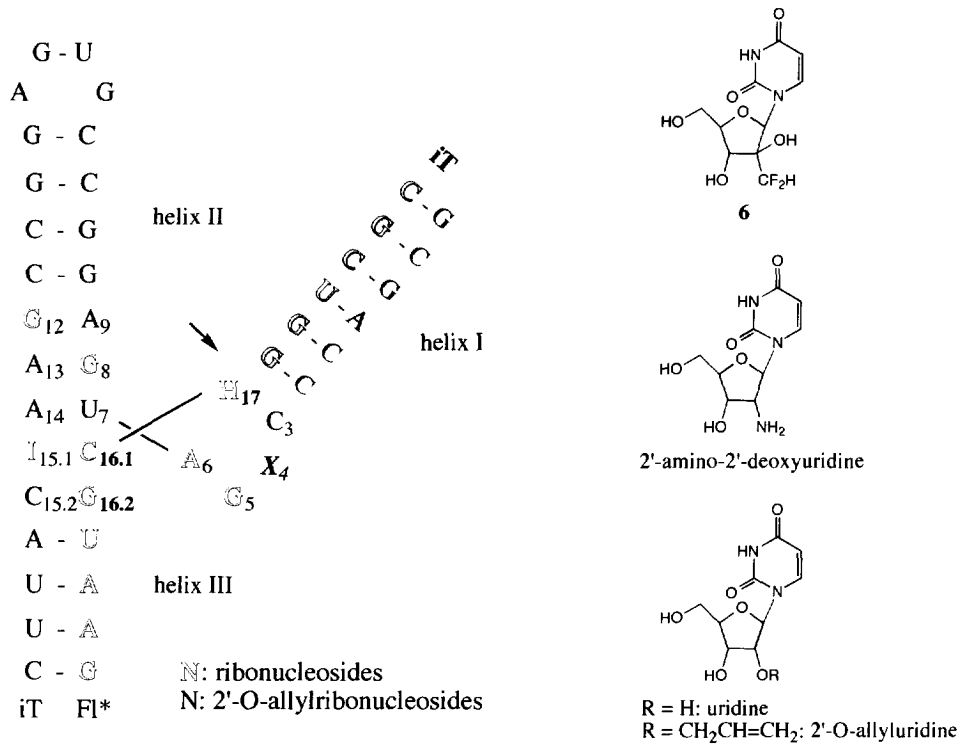


Reagents: 1, Dess-Martin periodinane, 1,2-dichloroethane (95%); 2, $PhSO_2CF_2H$, $LiN(TMS)_2$, HMPA, THF (49%); 3, 2-nitrobenzaldoxime, tetramethylguanidine, CH_3CN (64%); 4, Na-Hg, Na_2HPO_4, MeOH (45%); 5, BzCl, pyridine; 6, TBAF-AcOH, THF (75% from **3**); 7, DMTrCl, pyridine (76%), 8, (i-Pr_2N)(OCE)PCl, DIPEA, 1,2-dichloroethane (65%)

Oxidation of 3',5'-O-(tetraisopropyldisiloxane-1,3-diyl)-4-O-(2,6-dichlorophenyl)uridine[24], compound **1** with the Dess-Martin periodinane and subsequent alkylation with difluoromethylphenylsulfone[25] diastereoselectively gave the difluoromethylene derivative **2**. Removal of the aglycon protective group followed by the reductive cleavage of the phenylsulfonyl group resulted in the difluoromethyl derivative **3**. Subsequent benzoylation of the 2'-hydroxyl function and finally fluoride-ion mediated removal of the TIPDSi group gave the nucleoside **4**. 5'-

hydroxyl protection with the DMTr group and subsequent 3'-O-phosphitylation yielded the desired phosphoramidite synthon **5**. **5** was inserted at position X_4 into the modified hammerhead ribozyme analogue (**7**) under routine conditions (coupling yield > 95%). The crude oligonucleotide was deprotected and purified according to Innovir's routine in-house procedure[26]. To verify whether the 2'-C-difluoromethyl substituted uridine derivative **6** is accepted at position U4 in a modified hammerhead ribozyme (**7**), we have compared its cleavage activity on different fluorescein-labeled substrates (**11-13**) with ribozymes **8-10** containing uridine, 2'-amino-2'-deoxyuridine and 2'-O-allyluridine at position U4 respectively (Figure 2).

Figure 2: Sequence of the ribozymes (7, 8, 9, 10) and substrates (11, 12, 13)



7: X = **6**; **8:** X =uridine; **9:** X = 2'-amino-2'-deoxyuridine; **10:** X = 2'-O-allyluridine
11: H = A; **12:** H = C; **13:** H = U

The experiments were performed under single turnover conditions with a 10-fold excess of ribozyme over substrate (2.5 μM ribozyme, 250 nM substrate) at pH 7.4, 37°C in the presence of 1 mM Mg^{2+}. We were employing near physiological conditions[27,28], since it became clear from earlier experiments that the cleavage characteristics with different U4 analogues are dependent on the magnesium ion concentration[12,29,30]. The

cleavage kinetics of the ribozymes **7-10** (Figure 3) show more or less the same order of activity (X_4: 2'-OH > 2'-NH_2 ≥ 2'-CF_2H >> 2'-O-Allyl), irrespective of the targeted cleavage triplets GCA, GCC and GCU. The cleavage data suggest that in fact the difluoromethyl group is responsible for the activity of **7**, since Beigelman et al.[17] have demonstrated that arauridine at position X_4 of a hammerhead ribozyme - even in the presence of 10 mM Mg^{2+} - accounts for a dramatic decrease in activity. However the acceptance of the 2'-C-difluoromethyl substituted uridine derivative differs with respect to the target-triplets, i.e. on the substrate containing GCA (**11**) the activity of **7** is only moderately decreased compared to **8** (k_2 = 0.87 min^{-1}). In fact the GCA site does not seem to be very critical to modifications at the 2'-position of U4 at all, since 2'-amino-2'-deoxyuridine is accepted very well and even changing to 2'-O-allyluridine still results in an acceptable cleavage activity[5]. On the other hand the cleavage activity of **7** on substrates containing triplets GCC (**12**) and GCU (**13**) is considerably

Figure 3: Comparison of the cleavage activities of 7 - 10 on substrates 11 – 13

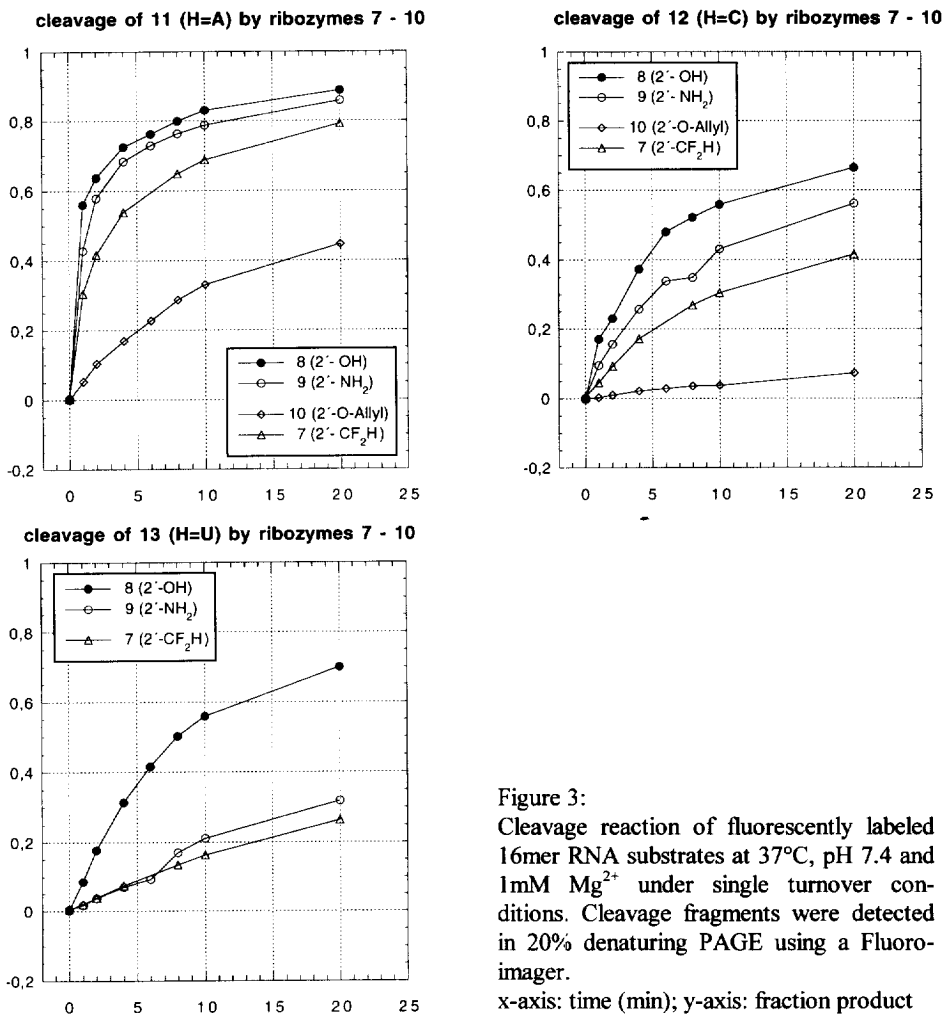


Figure 3:
Cleavage reaction of fluorescently labeled 16mer RNA substrates at 37°C, pH 7.4 and 1mM Mg^{2+} under single turnover conditions. Cleavage fragments were detected in 20% denaturing PAGE using a Fluoro-imager.
x-axis: time (min); y-axis: fraction product

lower. Especially the ribozyme-substrate complex containing a GCC site seems to react very critically to changes at the 2'-position of U4. Here the cleavage activity of ribozymes **7** and **9** is significantly decreased compared to **8** and the 2'-O-allyluridine containing **10** gives practically no cleavage at all. This sensitivity for 2'-substitution at U4 was also found for the corresponding GUC triplet containing substrate[5,27].

Finally, we have checked the stability of **7** against degradation by RNase A compared to **8**, **9** and **10**. This experiment resulted in the total stability of **7** (as well as **9** and **10**) to endonucleolytic digestion in contrast to **8**, which was completely degraded within seconds (data not shown)[13].

In summary, we have found that the 2'-C-difluoromethyl substituted uridine derivative **6** can be substituted for uridine at position U4 and results in cleavage activities close to 2'-amino-2'-deoxyuridine or similar to 2'-C-allyl-2'-deoxyuridine[16,17] (data not shown), known 2'-modified uridines employed in the endonucleolytic stabilization of modified hammerhead ribozymes.

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