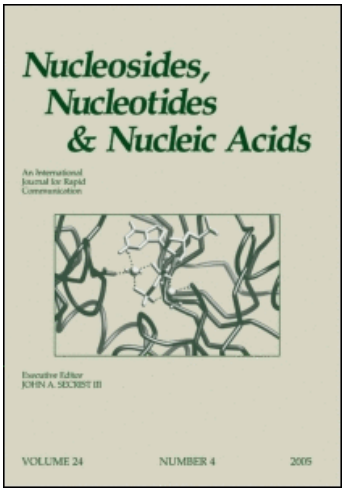

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Replacement of the Phosphodiester Bond Between U4 and G5 in the U-Turn of a Chemically Modified Hammerhead Ribozyme by an Amide Bond

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REPLACEMENT OF THE PHOSPHODIESTER BOND BETWEEN U4 AND G5 IN THE U-TURN OF A CHEMICALLY MODIFIED HAMMERHEAD RIBOZYME BY AN AMIDE BOND

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ABSTRACT: The phosphodiester bond between U4 and G5 in the U-turn of a chemically modified hammerhead ribozyme was substituted by an amide backbone without compromising the ribozyme's cleavage activity. Furthermore, the modified ribozyme proved to be completely stable against endonucleolytic digestion at this position.

Recently, we reported the successful use of a 2'-C-difluoromethylarauridine derivative in the catalytic core of a chemically modified hammerhead ribozyme[1]. During the course of our search for novel nucleoside analogues for the U4 activity-vs.-stability problem[2-11], we developed the notion that an optimal U4 substitute must contain the 2'-hydroxyl function of the ribose moiety, as well as the uracil aglycon. On the other hand, to gain the desired stability against endonucleolytic degradation of the phosphodiester backbone at 3', the optimal U4 substitute must be modified to not allow RNase A (an endonuclease) degradation. Other than modifying the 2'-substituent of uridine, it should be possible to achieve this goal by a suitable modification to the phosphate backbone between U4 and G5.

Crystal structure of the hammerhead ribozyme[12-15] suggested that there were no essential hydrogen bonds between the phosphate group at 3' of U4 and other parts of the catalytic core of the hammerhead / substrate complex. Therefore, we decided to synthesize a chemically modified hammerhead ribozyme that contained an amide-3-type backbone[16-18] between U4 and G5 to test our hypothesis. We chose this alternative backbone for its

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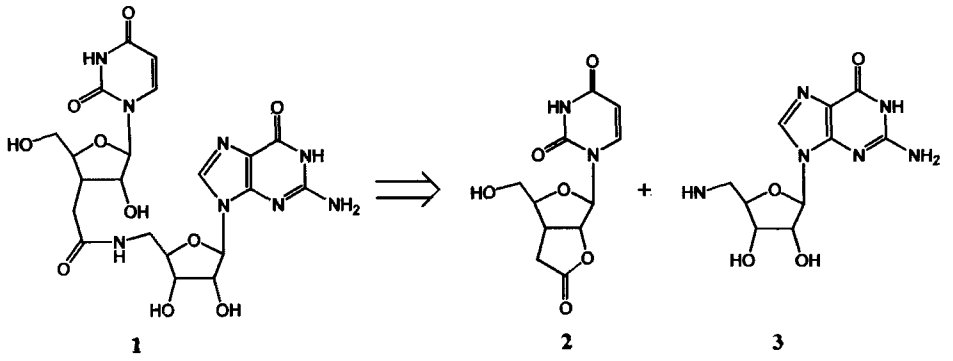
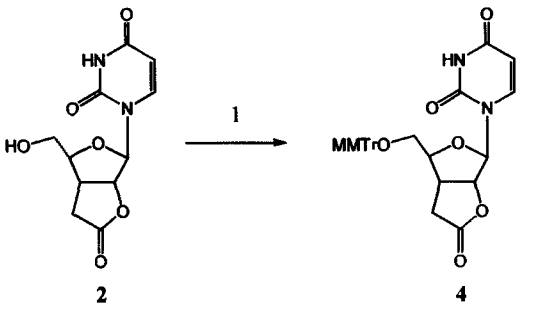
##) Research laboratory closed.

FIG. 1:



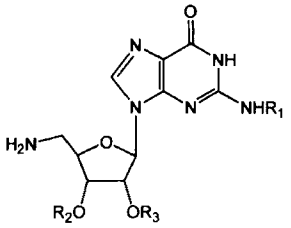
excellent chemical stability and good hybridization properties in addition to its compatibility with the phosphoramidite approach of DNA / RNA synthesis. Additionally, the chemistry for introducing this backbone modification into nucleoside phosphoramidite building blocks had been described[16-18]. For the synthesis of the desired phosphoramidite derivative of dinucleoside **1**, retrosynthetic analysis yielded the uridine synthon **2** and the guanosine synthon **3** (FIG. 1). The synthesis of **2** was known[19] and its 5'-protection should pose no problem. We chose the monomethoxytrityl group for 5'-protection which could be kept throughout the following synthetic steps to the dimer phosphoramidite.

FIG. 2:



1; MMTrCl, pyridine (87%)

FIG. 3:



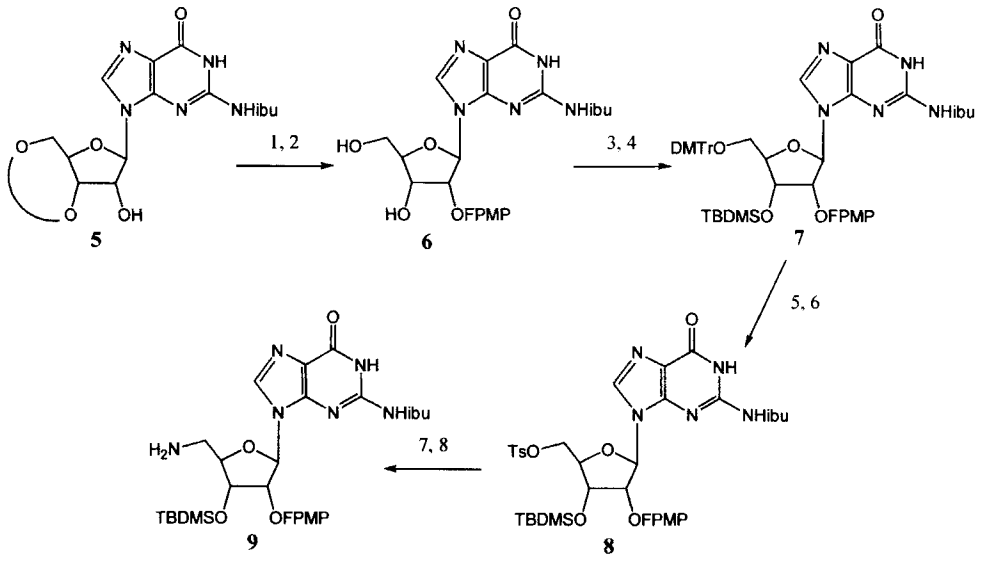
3: $R_1 = R_2 = R_3 = H$

9: R_1 = ibu
R_2 = TBDMS
R_3 = FPMP

For protection of the guanosine synthon **3**, R_2 must be selectively cleavable in the presence of R_1 and R_3. Furthermore, R_1, R_2 and R_3 must be stable under forcing S_N2 conditions introducing the azide function and during its reduction to the amino group and R_1 and R_3 must be cleavable under mild conditions post RNA synthesis. After some experimental work, the following design proved viable (FIG. 3).

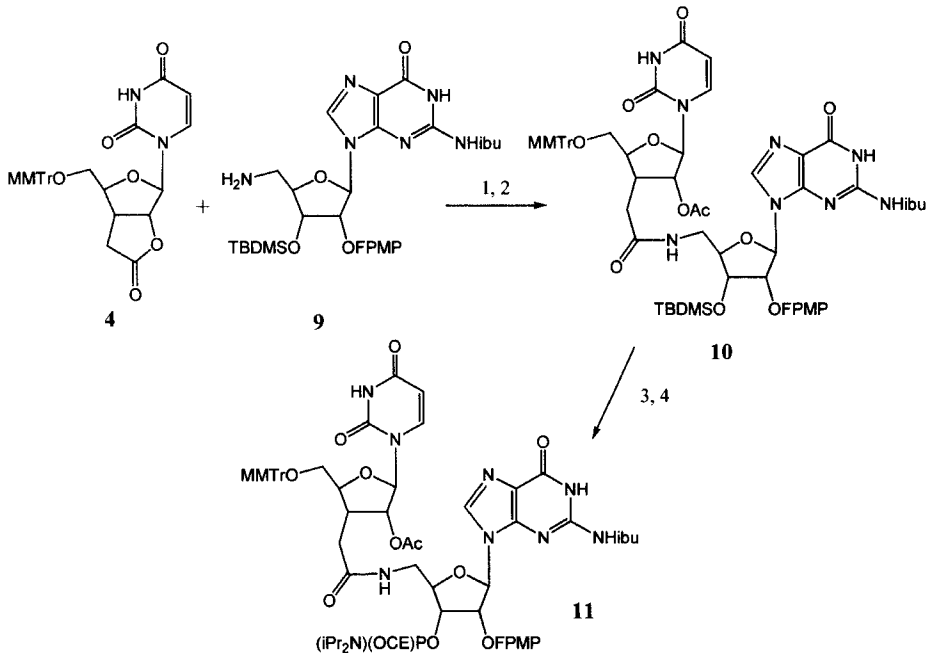
3',5'-O-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)-N2-isobutyroylguanosine[20] (**5**, FIG. 4) was converted to the 2'-O-FPMP derivative followed by fluoride ion assisted removal of

FIG. 4:



1; FPMP reagent[24], CF_3COOH, THF (72%); 2; TBAF, THF (68%); 3; DMTrCl, pyridine (88%); 4; TBDMSCl, imidazole, pyridine (92%); 5; TsOH, CH_2Cl_2-MeOH (85%); 6; TsCl; pyridine (75%); 7; LiN_3, DMF (62%); 8; H_2, Pd/C, ethylacetate (95%)

FIG. 5:



1; imidazole, pyridine, 80°C (68%); 2; Ac_2O, 2,6-lutidine, DMAP, THF (96%); 3; TBAF, AcOH, THF (64%); 4; $(iPr_2N)(OCE)PCl$, DIPEA, 1,2-dichloroethane (72%)

the TIPDSi group to give **6**. 5'-O-dimethoxytritylation and subsequent 3'-O-silylation with TBDMSCl led to intermediate **7**. Cleavage of the 5'-O-DMTr group followed by tosylation yielded the guanosine derivative **8**. Replacement of the tosyl group using lithium azide in DMF and eventual hydrogenation gave the desired synthon **9**.

As expected[21,22], coupling of the synthons occured without major difficulties although the yield was not optimized. Subsequent acetylation of the uridine 2'-hydroxyl yielded **10**. Removal of the guanosine 3'-O-TBDMS group and subsequent phosphitylation under standard conditions gave the U4-G5 dinucleoside synthon 11 (FIG. 5).

The U4G5 dimer phosphoramidite was incorporated into two uniformly 2'-O-allyl modified hammerhead ribozymes (**12**: $I_{15.1}$ and **16**: $A_{15.1}$) instead of nucleotides U_4 and G_5 under standard conditions (coupling yield >95%). The crude oligonucleotides were deprotected and purified according to a standard in-house procedure[23]. Additionally, the 2'-O-FPMP group was deprotected by a final treatment with acid to give **12** and **16**[24,25].

To verify whether the U_4G_5-dinucleoside was accepted at position U_4G_5 in the modified hammerhead ribozymes **12** and **16**, we compared their cleavage activities on the different fluorescein-labelled substrates (**20** – **23**)[26] with the corresponding ribozymes **13** – **15** (NCH-type) and **17** – **19** (NUH-type) containing uridine, 2'-amino-2'-deoxyuridine and 2'-O-allyluridine at position *X4*. Sequences of ribozymes and substrates (normalface: 2'-O-allylnucleotides; **boldface: ribonucleotides**; T: thymidine):

Ribozymes:

iT-3'-CUUAC**N**$_{15.1}$A$_{14}$A$_{13}$**G**$_{12}$CCGGAGUGCCGGA$_9$**G**$_8$U$_7$**A**$_6$**G**$_5$*X*$_4$C$_3$CCAGCG-5'

12: $N_{15.1}$ = I, G_5X_4 = dimer (**1**)
13: $N_{15.1}$ = I, X_4 = uridine
14: $N_{15.1}$ = I, X_4 = 2'-aminouridine
15: $N_{15.1}$ = I, X_4 = 2'-O-allyluridine
16: $N_{15.1}$ = A, G_5X_4 = dimer (**1**)
17: $N_{15.1}$ = A, X_4 = uridine
18: $N_{15.1}$ = A, X_4 = 2'-aminouridine
19: $N_{15.1}$ = A, X_4 = 2'-O-allyluridine

Substrates:

F*-5'-**GAAUG**$_{16.2}$**Y**$_{16.1}$**H**$_{17}$**GGUCGC**-3'-iT

20: $Y_{16.1}$ = C, H_{17} = A
21: $Y_{16.1}$ = C, H_{17} = C
22: $Y_{16.1}$ = U, H_{17} = A
23: $Y_{16.1}$ = U, H_{17} = C

The cleavage experiments were performed under single turnover conditions as described earlier[1,26]. We employed these near physiological conditions, since it became clear from the earlier experiments that the cleavage characteristics with different U_4 analogues were dependent on the magnesium ion concentration and on the cleavage type ($N_{15.1}$ / $N_{16.2}Y_{16.1}H_{17}$)[1,26,27]. In TABLE 1, the catalytic activity of the selected ribozymes (**12** – **19**) are compared using $t_{1/2}$ (time in minutes required for 50% cleavage of the respectively 5'-fluorescein labeled short substrate **20** – **23**).

The cleavage of the ribozymes **12** – **19** (TABLE 1) showed more or less the same order of activity (X_4 = uridine ≥ X_4G_5 = dinucleoside > X_4 = 2'-aminouridine >> X_4 = 2'-O-allyluridine), irrespective of the targeted cleavage triplets GCA, GCC, GUA and GUC. The data clearly showed that in fact the dimer containing ribozyme was almost as active as the U_4 = uridine containing ribozyme and at least equally as active as the corresponding U_4 = 2'-aminouridine containing ribozyme. However, the acceptance of the U_4G_5 dinucleoside containing ribozymes differed with respect to the target-triplets, i.e. on the substrates containing GCA (**20**) and GUA (**22**) the activity of **12** respectively **16** was moderately decreased compared to **13** (**17**) but larger or equal to **14** (**18**). Even the U_4 = 2'-O-allyluridine

TABLE 1:

ribozyme	substrate	$t_{1/2}$ (min.)	U_4 substituent	cleavage type
12	**20**	2	U_4G_5 dinucleoside	I / GCA
13	**20**	1	uridine	I / GCA
14	**20**	2	2'-aminouridine	I / GCA
15	**20**	25	2'-O-allyluridine	I / GCA
12	**21**	9	U_4G_5 dinucleoside	I / GCC
13	**21**	7	uridine	I / GCC
14	**21**	16	2'-aminouridine	I / GCC
15	**21**	n.d.	2'-O-allyluridine	I / GCC
16	**22**	3	U_4G_5 dinucleoside	A / GUA
17	**22**	1	uridine	A / GUA
18	**22**	4	2'-aminouridine	A / GUA
19	**22**	25	2'-O-allyluridine	A / GUA
16	**23**	12	U_4G_5 dinucleoside	A / GUC
17	**23**	7	uridine	A / GUC
18	**23**	18	2'-aminouridine	A / GUC
19	**23**	n.d.	2'-O-allyluridine	A / GUC

containing ribozymes (**23** and **27**) exhibited moderate cleavage activities with substrates containing GYA triplets. On the other hand, the cleavage activity of **12** – **19** on substrates containing triplets GCC (**21**) and GUC (**23**) was considerably lower. We demonstrated before[1] that these type of triplets (i.e. GCC, GCU, GUC, GUU) were much more selective for substitution at U_4. Although the activities of the U_4G_5 dinucleoside containing ribozymes **12** and **16** were somewhat lower than the cleavage activities of the corresponding U_4 = uridine containing ribozymes, they still showed a significantly higher activity than the corresponding ribozymes containing U_4 = 2'-aminouridine. So far though, these effects have only been reported in uniformly 2'-O-allylated ribozymes and it remains to be seen whether these discriminating effects on 2'-modified uridines also occur in uniformly 2'-O-methylated and / or wild-type hammerhead ribozymes.

Additionally, the ribozymes **12** and **16** exhibited real turnover in the respective experiment with a short substrate (data not shown). Finally, we checked the stability of **12** and **16** against degradation by RNase A, compared to **13** – **15** and **17** – **19**. This experiment indicated complete stability of **12** and **16** (as well as **14**, **15**, **18** and **19**) to endonucleolytic

digestion in contrast to **13** and **17** which were completely degraded within seconds (data not shown).

In summary, we have developed a new solution for the U_4 activity-vs.-stability problem in hammerhead ribozymes. The substitution of U_4 and G_5 in a chemically modified hammerhead ribozyme by a G_5U_4 dinucleoside analogue containing an amide linkage seemed to be especially useful where the cleavage triplet in the targeted mRNA was one of the strongly discriminating types NYC and especially NYU. To date, it is the only modification in these cases that approaches the cleavage activities of the endonucleolytically unstable U_4 = uridine containing ribozymes. Full experimental details of the synthetic chemistry and ribozyme kinetics will be published in due course.

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