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SYNTHESIS AND INCORPORATION OF 2'-AMINO ACID CONJUGATED NUCLEOTIDES INTO RIBOZYMES

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Abstract: 2'-Amino-2'-deoxyuridine (**1**) was conjugated with *N*-Fmoc protected L-amino acids. 5'-*O*-Dimethoxytritylation followed by 3'-*O*-phosphitylation afforded 3'-*O*-phosphoramidites **4a-d**. These monomers were incorporated into ribozymes using the solid phase phosphoramidite method. The catalytic activity of these ribozymes is reported.

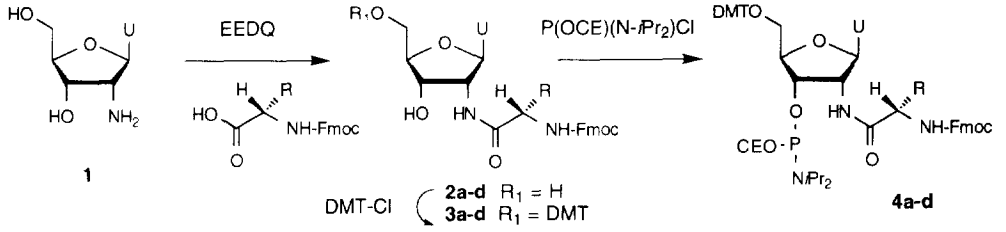
The highly sequence-specific endoribonuclase activity of hammerhead ribozymes[1,2] suggests their use as therapeutic agents for the inhibition of gene expression. There are two approaches to realize this potential, exogenous delivery of chemically synthesized ribozymes or endogenous expression of ribozyme constructs in afflicted cells. Only the first approach offers flexibility in attaching different ligands and inserting modified monomers at any desired position. Exogenous delivery provides a basis to address the three major limitations to the use of oligonucleotides as therapeutic agents: nuclease stability, targeting to specific cells and transport through cellular membranes.

As part of our efforts to increase the efficacy of chemically synthesized ribozymes *in vivo*, we were interested in the effects of a covalent attachment of various ligands to ribozymes. We hoped to preserve nuclease stability and cleavage activity[3] while increasing cellular uptake and ribozyme localization into desired subcellular compartments.

In particular, we were interested in aminoacyl-conjugated ribozymes. This choice, though arbitrary, was based on several criteria: (a) aminoacyl-oligonucleotide conjugates occur naturally in the genome of certain RNA and DNA viruses where an amino acid-nucleic acid linkage occurs through a phosphodiester bond to serine, tyrosine, or threonine;[4] (b) aminoacyl nucleosides are an important structural element of a family of aminoacyl-tRNA molecules;[5] (c) DNA-enzyme conjugates have been used as sequence-specific, oligonucleotide-directed nucleases;[6-8] (d) conjugation of poly L-lysine to synthetic oligonucleotides improves oligonucleotide transport into cells;[9,10] (e) several short peptides serve as localization signals for some cell compartments;[11] (f) a 2'-amido bond is expected to be chemically stable and not to interfere with oligonucleotide synthesis;[12] (g) both on the monomer level or post-synthetically, aminoacyl moieties can serve as biodegradable linkers[13] for the attachment of a wide variety of chemical species.

For delivery purposes, different ligands have been attached to the 3'- or 5'-ends of antisense oligonucleotides. This strategy cannot be applied directly to ribozymes since it interferes with their cleavage activity.[14] We and others previously demonstrated that the site-specific introduction of certain 2'-modified nucleotides into ribozymes provides increased nuclease resistance without reducing catalytic activity.[3,15-17] It has also been shown that the U4 and U7 positions in the catalytic core can tolerate a number of modifications including 1-deoxy-D-ribofuranose (the "abasic" nucleoside).[18] Based on these observations, the influence on cleavage activity of 2'-aminoacyl conjugated ribozymes was investigated. Readily available 2'-amino-2'-deoxyuridine (**1**)[19] was used to attach different amino acids to the nucleoside through an amide bond.

Figure 1. Synthesis of 2'-(Aminoacyl)amino-2'-Deoxy Uridine 3'-*O*-Phosphoramidites



a R = CH_3 **b** R = Ph **c** R = $(CH_2)_4$NH-Fmoc **d** R = $CH_2COOBzl$

Direct condensation of **1** with *N*-protected amino acids (purchased from BACHEM Bioscience Inc.) in the presence of *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ)[20] or 1-isobutyloxycarbonyl-2-isobutyloxy-1,2-dihydroquinoline (IIDQ)[21] as a condensing agent, followed by purification, afforded 2'-aminoacyl-conjugated compounds **2a-d** in good yields[22] (Figure 1, Table 1). Because the 9-fluorenylmethoxycarbonyl (Fmoc) group is compatible with solid phase phosphite triester RNA synthesis and deprotection conditions,[23,17] it was used to protect the amine of the amino acid moiety. There was no evidence in the ^{1}H NMR spectra (not shown) of **2a-d** that racemization of the aminoacyl-α-carbon occurred under the above condensation conditions. Protection of the 5'-hydroxyl with the 4,4'-dimethoxytrityl (DMT) group,[24] followed by standard 3'-*O*-phosphitylation,[25] provided 3'-*O*-phosphoramidites **4a-d** in high yields (Table 1). All phosphoramidites were purified by flash silica gel chromatography and isolated as colorless foams. The presence of only two expected singlets in the ^{31}P NMR spectra, corresponding to two *P*-diasteroisomers, confirmed that no racemization took place during this three-step synthesis.

We subjected 2'-aspartyl derivative **2d** to conc. NH_4OH-EtOH 3:1 at 55 °C for 4 h to determine whether the isomeric integrity of this compound would be preserved during the standard base deprotection of oligonucleotides. ^{1}H NMR spectrum (not shown) of the product revealed cleavage of the *N*-Fmoc group and conversion of the ester to an amide. In addition, racemization yielded a 1:1 mixture of diastereoisomers. Racemization did not occur in the case of the other amino acid derivatives **2a-c**. Therefore, milder basic conditions for base deprotection are needed to preserve the chiral integrity of the aspartic acid residue. No cleavage of any of the aminoacyl residues in **2a-d** was observed under the basic deprotection conditions.

Table 1. Yields and Physicochemical Characteristics of 2-4.

Compound	2a	2b	2c	2d	3a	3b	3c	3d	4a	4b	4c	4d
Yield (%)	86	91	92	66	72	83	77	64	73	73	84	70
Elution Solvent[a]	A	A	B	B	C	C	C	C	D	E	E	E
δ ^{31}P NMR[b]	---	---	---	---	---	---	---	---	149.3	149.3	149.5	149.8
									147.6	146.7	147.5	147.9

[a]linear gradient for purification on silica gel; A=2-10% MeOH in CH_2Cl_2; B=1-5% MeOH in CH_2Cl_2; C=1-2% MeOH in CH_2Cl_2; D=50-75% EtOAc in hexanes; E=1-2% EtOH in CH_2Cl_2; [b]($CDCl_3$, ppm) two singlets for two P-diastereoisomers, respectively.

Phosphoramidites **4a-d** were incorporated into our stabilized hammerhead ribozyme motif[3] (***Rz 1*** in Figure 2) that contains five ribose residues, twenty-nine 2'-*O*-Me residues, two 2'-amino-2'-deoxyuridine residues and a 3'-3'-linked nucleotide "cap" (inverted thymidine, iT). Standard RNA synthesis and deprotection procedures[26] were followed with average stepwise coupling yields of ~98%. Matrix-assisted laser desorption (MALDI-TOF) mass spectrometry of the fully deprotected ribozymes was used to verify their structures. The $[M-H]^-$ peaks were in the 0.2-0.3% accuracy range.

In one approach, four residues in Loop II were replaced with 2'-aminoacylated nucleotides (***Rz 2a-d***); in the other approach, U4 and U7 nucleotides in the catalytic core were replaced with 2'-aminoacylated nucleotides (***Rz 3a-d***). The cleavage activities of these ribozymes are given in Table 2. The results show that the incorporation of 2'-aminoacyl uridines in Loop II (***Rz 2a - 2d***) had no effect on catalytic activity compared to control ribozyme ***Rz 1***. Incorporation of 2'-phenylalanyl uridine into the catalytic core (***Rz 3b***) resulted in only a 2-fold decrease in catalytic activity compared to control ribozyme ***Rz 1***, while the other aminoacyl substitutions reduced activity ~10-fold. Studies on the nuclease stability and cellular uptake of these modified ribozymes are in progress and will be reported elsewhere.

Figure 2. Hammerhead Ribozymes Containing 2'-Aminoacyl Uridine Sites

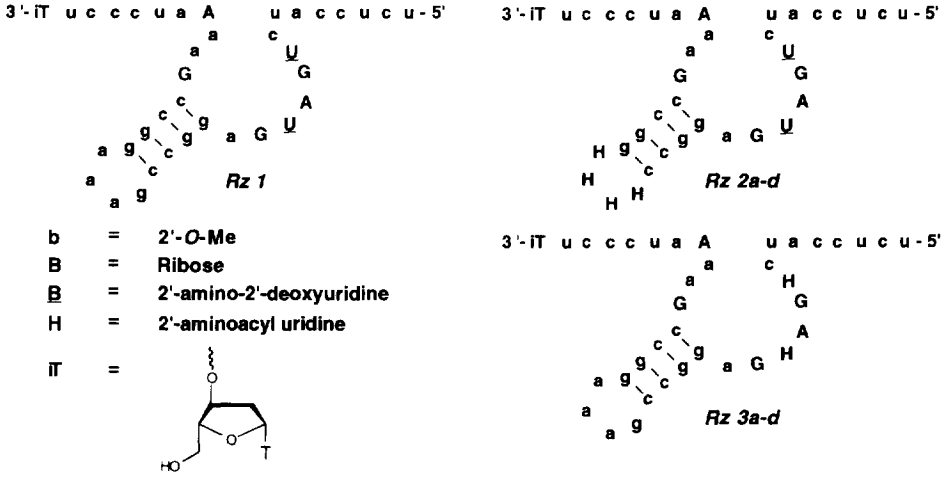


Table 2. Cleavage Activity of Amino Acid Conjugated Ribozymes[a]

Rz	$t_{1/2}$ (min)	*Rz*	$t_{1/2}$ (min)
1	1.5	***1***	1.5
2a (4 Ala Loop)	1.5	***3a*** (U4,7 = Ala)	13.0
2b (4 Phe Loop)	2	***3b*** (U4,7 = Phe)	3.0
2c (4 Lys Loop)	1	***3c*** (U4,7 = Lys)	11.5
2d (4 Asn Loop)	1	***3d*** (U4,7 = Asn)	8.0

[a]Ribozymes were tested at 8 nM concentration (substrate concentration was ≤ 1 nM) at 37 °C in 50 mM Tris-Cl, pH 7.5 and 10 mM $MgCl_2$, $t_{1/2}$ = time required to cleave 50% of a short substrate.

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References and Notes

1. Uhlenbeck, O. C. *Nature* **1987**, *328*, 596.
2. Haseloff, J.; Gerlach, W. L. *Nature*, **1988**, *334*, 585.
3. Beigelman, L.; McSwiggen, J.; Draper, K.; Gonzales, C.; Jensen, K.; Karpeisky, A.; Modak, A.; Matulic-Adamic, J.; DiRenzo, A.; Haeberli, P.; Sweedler, D.; Tracz, D.; Grimm, S.; Wincott, F.; Usman, N. *J. Biol. Chem.* **1995**, *270*, 25702.
4. Vartapetian, A. B.; Bogdanov, A. A. *Prog. Nucleic Acids Res. Mol. Biol.* **1987**, *34*, 209.
5. *Biochemistry*, Stryer, L., 3rd Ed.; Freeman W.H.: New York, 1981; p 733.
6. Zuckermann, R. N.; Corey, D. R.; Schultz, P. G. *J. Amer. Chem. Soc.* **1988**, *110*, 1614.
7. Zuckermann, R. N.; Schultz, P. G. *J. Amer. Chem. Soc.* **1988**, *110*, 6592.
8. Zuckermann, R. N.; Schultz, P. G. *Proc. Natl. Acad. Sci. USA* **1989**, *86*, 1766.
9. Lemaitre, M.; Bayard, B.; Lebleu, B. *Proc. Natl. Acad. Sci. USA* **1987**, *84*, 648.
10. Leonetti, J. P.; Degols, G.; Lebleu, B. *Bioconjugate Chem.* **1990**, *1*, 149.
11. Goldfarb, D. S.; Gariepy, J.; Schoolnik, G.; Kornberg, R. D. *Nature* **1986**, *322*, 641.
12. Hendrix, C.; Devreese, B.; Rozenski, J.; Van Aerschot, A.; De Bruyn, A.; Van Beeumen, J.; Herdewijn, P. *Nucleic Acids Res.* **1995**, *23*, 51.
13. Kawaguchi, T.; Sakairi, H.; Kimura, S.; Yamaguchi, T.; Saneyoshi, M. *Chem. Pharm. Bull.* **1995**, *43*, 501.
14. Simultaneous attachment of 'inverted T' residue to 3'- and 5'-ends of the stabilized motif[3] resulted in a considerable reduction of ribozyme cleavage activity (L. Beigelman *et al.* unpublished results).
15. Paolella, G.; Sproat, B. S.; Lamond, A. I. *EMBO J.* **1992**, *11*, 1913.
16. Pieken, W. A.; Olsen, D. B.; Benseler, F.; Aurup, H.; Eckstein, F. *Science* **1991**, *253*, 314.
17. Heidenreich, O.; Benseler, F.; Fahrenholz, A.; Eckstein, F. *J. Biol. Chem.* **1994**, *269*, 2131.
18. Beigelman, L.; Karpeisky, A.; Matulic-Adamic, J.; Gonzales, C.; Usman, N. *Nucleosides Nucleotides* **1995**, *14*, 907.
19. Verheyden, J. P. H.; Wagner, D.; Moffatt, J. G. *J. Org. Chem.* **1971**, *36*, 250.
20. Belleau, B.; Malek, G. *J. Amer. Chem. Soc.* **1968**, *90*, 1651.
21. Kiso, Y.; Kai, Y.; Yajima, H. *Chem. Pharm. Bull.* **1973**, *21*, 2507.
22. In a typical procedure 2'-amino-2'-deoxyuridine (**1**)[19] (1.0 g, 4.1 mmol), EEDQ (2.0 g, 8.2 mmol) and *N*-Fmoc amino acid (6.2 mmol, 1.5 eq) were dissolved in DMF (50 mL) and the reaction mixture was stirred at 50 °C for 24 hours. The clear solution was then concentrated to a syrup under reduced pressure and chromatographed on a silica gel column.
23. Nielsen, J.; Brenner, S.; Janda, K. D. *J. Amer. Chem. Soc.* **1993**, *115*, 9812.
24. In a typical procedure 2'-(aminoacyl)amino-2'-deoxy uridine (**2**) (3 mmol) was dissolved in dry pyridine (40 mL) and DMT-Cl (1.32 g, 3.9 mmol) was added. The reaction mixture was stirred at rt for 24 h. MeOH was then added and the solution concentrated under reduced pressure. The residual syrup was partitioned between CH_2Cl_2 and 5% aq. $NaHCO_3$, the organic layer washed with brine, dried (Na_2SO_4) and evaporated to dryness *in vacuo*. Column silica gel chromatography of the residue afforded pure products **3** as yellowish foams.
25. Atkinson, T.; Smith, M. In *Oligonucleotide Synthesis: A Practical Approach*; Gait, M.J., Ed.; IRL: Oxford, 1984, p 35-81.
26. Wincott, F.; DiRenzo, A.; Shaffer, C.; Grimm, S.; Tracz, D.; Workman, C.; Sweedler, D.; Gonzalez, C.; Scaringe, S.; Usman, N. *Nucleic Acids Research* **1995**, *14*, 2677.

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