Synthesis of 2'-modified nucleotides and their incorporation into hammerhead ribozymes

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Received July 5, 1995; Revised and Accepted September 19, 1995

ABSTRACT

Several 2'-modified ribonucleoside phosphoramidites have been prepared for structure-activity studies of the hammerhead ribozyme. The aim of these studies was to design and synthesize catalytically active and nuclease-resistant ribozymes. Synthetic schemes for stereoselective synthesis of the *R* isomer of 2'-deoxy-2'-*C*-allyl uridine and cytidine phosphoramidites, based on the Keck allylation procedure, were developed. Protection of the 2'-amino group in 2'-deoxy-2'-aminouridine was optimized and a method for the convenient preparation of 5'-*O*-dimethoxytrityl-2'-deoxy-2'-phthalimidouridine 3'-*O*-(2-cyanoethyl-*N,N*-diisopropylphosphoramidite) was developed. During the attempted preparation of the 2'-*O*-*t*-butyldimethylsilyl-3'-*O*-phosphoramidite of arabinouridine a reversed regioselectivity in the silylation reaction, compared with the published procedure, was observed, as well as the unexpected formation of the 2,2'-anhydronucleoside. A possible mechanism for this cyclization is proposed. The synthesis of 2'-deoxy-2'-methylene and 2'-deoxy-2'-difluoromethylene uridine phosphoramidites is described. Based on a '5-ribose' model for essential 2'-hydroxyls in the hammerhead ribozyme these 2'-modified monomers were incorporated at positions U4 and/or U7 of the catalytic core. A number of these ribozymes had almost wild-type catalytic activity and improved stability in human serum, compared with an all-RNA molecule.

INTRODUCTION

Hammerhead ribozymes can catalyze highly specific *trans*-cleavage of any RNA containing the dinucleotide sequence UH (H = C, A or U) (1). Such high specificity suggests the use of these ribozymes as therapeutic agents in gene regulation (2). One of the main obstacles in realizing this potential lies in delivery of the ribozymes to cells. From two existing possibilities, the endogenous expression of a ribozyme delivered via a plasmid or viral vector or exogenous delivery of a chemically synthesized ribozyme itself,

the first method has been explored to a greater extent and has shown promising results (3).

The first problem associated with exogenous delivery of the ribozymes is the instability of oligoribonucleotides in biological fluids. A variety of selective and uniform structural modifications have been applied to oligonucleotides to enhance nuclease resistance (4,5). Recent improvements in the chemical synthesis of RNA (6,7) have led to the ability to similarly modify ribozymes containing the hammerhead core motif (8,9). A complication with ribozymes is that the usual method of oligonucleotide stabilization, using extensive 2'-sugar modifications, can interfere with catalytic activity.

A number of studies have been carried out to determine the number and location of ribose residues required for catalytic activity and to determine sites of nuclease sensitivity (10-13). A consensus '5-ribose model' containing ribose residues at positions G5, A6, G8, G12 and A15.1 (Fig. 1) was found to have wild-type catalytic activity and a half-life >72 h in human serum (14). The optimization of the balance between higher cleavage activity and nuclease resistance in this model required selective modification at positions U4 and U7 (Fig. 1). This prompted us to develop the synthesis of new 2'-modified phosphoramidites and refine the synthesis and protection of known 2'-modified phosphoramidites.

The choice of 2'-modified nucleotides was based on the following. One group of analogs (2'-deoxy-2'-amino and arabino) should closely mimic the conformational and functional (e.g. hydrogen bonding) properties of natural ribonucleotides. The 2'-deoxy-2'-methylene and 2'-deoxy-2'-difluoromethylene nucleotides would conformationally constrain the normal sugar puckering of ribonucleotides. The 2'-deoxy-2'-*C*-allyl modification would be expected to alter the hydrophobic nature of the catalytic core and may also be used to probe the hydrogen bonding network within the core. Comparison of all of these properties in ribozymes containing these modifications would provide information about the relative importance of these factors on cleavage activity and nuclease resistance.

In this report we introduce 2'-deoxy-2'-*C*-allyl nucleotides as a new type of 2'-modification for ribozymes and describe several synthetic routes for the preparation of fully protected 2'-deoxy-2'-*C*-allyl pyrimidine phosphoramidites. In the case of arabinonucleosides we found that the distribution of *t*-butyldimethylsilyl (TBDMS) regioisomers obtained during the silylation

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Figure 1. Sequence of the ribozyme used in this study. Conserved nucleotides within the central core are numbered according to Hertel *et al.* (53). Underlined letters at U4 and U7 indicate positions that were replaced by 2'-substituted nucleotides introduced using phosphoramidites 5, 19, 25, 27 and 29 (Figs 2-5).

of 5'-*O*-dimethoxytrityl (DMT)-arabinouridine differs from that reported by Ogilvie *et al.* (15). Protection of the 2'-amino group in 2'-deoxy-2'-aminouridine was optimized, resulting in the preparation of 5'-*O*-DMT-2'-deoxy-2'-phthalimidouridine 3'-*O*-(2-cyanoethyl-*N,N*-diisopropylphosphoramidite). The synthesis of two conformationally constrained deoxyribose analogs, 2'-deoxy-2'-methylene and 2'-deoxy-2'-difluoromethylene uridine phosphoramidites is also described. Incorporation of these 2'-modified monomers at positions U4 and/or U7 of the catalytic core resulted in ribozymes with high catalytic activity and improved stability in human serum.

RESULTS AND DISCUSSION

Synthesis of 5'-*O*-DMT 2'-deoxy-2'-*C*-allyl uridine & cytidine 3'-*O*-(2-cyanoethyl-*N,N*-diisopropylphosphoramidites)

It has been shown that the incorporation of 2'-*O*-allyl nucleotides at non-essential positions of the hammerhead domain resulted in catalytically active and stable ribozymes (11). At the same time the simultaneous 2'-*O*-allyl modification of positions U4 and U7, which are known to be sensitive to serum nucleases, strongly inhibited cleavage activity. Since 2'-deoxy substitutions at these positions are less detrimental to catalytic activity (16) we decided to explore the properties of 2'-*C*-allyl nucleotides. The synthetic routes for the preparation of phosphoramidites 5 and 8 are shown in Figure 2.

The general synthetic strategy for the preparation of 2'-*C*-allyl derivatives was based on application of the Keck (17) allylation reaction using an appropriate radical donor. Phenoxythiocarbonylation of 3',5'-*O*-protected derivative 1 according to Robins (18) led to thiocarbonate 2 in 85% yield. Fandor *et al.* (19) and Chu *et al.* (20) showed that the Keck allylation proceeded stereospecifically with exclusive formation of the 3'-*R* stereoisomer of the 3'-*C*-allyl derivatives in the case of 5'-*O*-TBDMS-2'-deoxy-3'-*O*-phenoxythiocarbonyl uridine. Based on this, and the considerably less hindered nature of the α face of 3',5'-*O*-tetraisopropylidisiloxane-1,3-diyl nucleosides, the same stereospecificity in the Keck reaction in the case of thiocarbonate 2 was expected. Indeed, allylation of 2 with allyl-tri-*n*-butylstannane in the presence of Bz₂O₂ led to nucleoside 3 in 68% yield. This agrees with the stereochemistry recently reported for the same compound, however, no experimental details were reported (21). A strong NOE was observed between H-1' and the allyl protons and between H-2' and H-3', indicating the *R* configuration at C-2 of 3. Using 2,2'-azobis-(2-methylpropionitrile)

Figure 2. Synthetic scheme for protected 2'-deoxy-2'-*C*-allyl phosphoramidites. Reagents and conditions: (i) PhOC(S)Cl/DMAP/CH₃CN; (ii) allyltributyltin, Bz₂O₂ or AIBN/toluene; (iii) TBAF; (iv) DMT-Cl/pyr; (v) P(OCE)(*N*-i-Pr₂)-Cl; (vi) 1,2,4-triazole, P(O)Cl₃; (vii) a) 29% NH₄OH/dioxane; (vii b) Ac₂O/pyr.

(AIBN) as an initiator the yield was increased to 80% and the reaction could be run on an 80 g scale. Subsequent standard desilylation and tritylation led to 5'-*O*-DMT-derivative 4 in 87% yield, which was then phosphitylated to provide phosphoramidite 5 in 98% yield. Other synthetic routes to 2'-*C*-alkyl-modified nucleotides have been reported recently (22,23).

We also prepared the *N*⁴-acetyl cytidine derivative 6 in 90% yield from 2'-*C*-allyl uridine synthon 3 via the 4-(1,2,4-triazol-1-yl)pyrimidin-2(1H)-one intermediate (24), followed by amination and acetylation. Standard desilylation and tritylation led to 5'-*O*-DMT derivative 7, which was converted to phosphoramidite 8 in 75% yield.

When 3',5'-*O*-(tetraisopropylidisiloxane-1,3-diyl)-2'-*O*-phenoxythiocarbonyl-*N*⁴-isobutyryl cytidine was subjected to the analogous allylation conditions formation of the 2'-*C*-allyl derivative was not observed. Examples in the literature revealed that the choice of the protecting group for the exocyclic amine of a nucleoside base is pivotal to the success of radical reactions. Rhei *et al.* (25) were not able to perform the Barton reduction of 2',5'-di-*O*-thexyldimethylsilyl-3'-*O*-phenoxythiocarbonyl cytidine in the case of the *N*⁴-benzoyl and *N*⁴-[2-(nitrophenyl)ethoxycarbonyl] derivatives. On the other hand, *N*⁴-acetyl protection of cytidine seems to be well tolerated (18) under similar conditions. In agreement with these observations, allylation of 3',5'-*O*-(tetraisopropylidisiloxane-1,3-diyl)-2'-*O*-phenoxythiocarbonyl-*N*⁴-acetyl cytidine 9 led to the 2'-*R*-allyl derivative 6 in 15% yield with Bz₂O₂ as radical initiator. The yield of the allylation reaction was improved to 50% using AIBN as the initiator. Cytidine derivative 6 prepared by this route was identical to that obtained from uridine synthon 3.

To simplify the synthesis of phosphoramidites 5 and 8 we also explored the use of 5'-*O*-DMT-3'-*O*-TBDMS uridine 10, a side product in the preparation of standard RNA monomers (6), as a starting material. Phenoxythiocarbonylation of starting synthon 10, in the presence of dimethylaminopyridine (DMAP) according to Robins *et al.* (18), led to thiocarbonate 11 (91%) without noticeable migration (6) of the TBDMS group. The Keck allylation of intermediate 11 with AIBN resulted in the formation of 2'-*C*-allyl derivative 12 (70%) and the related 2'-deoxy by-product (15%). Subsequent desilylation of 12 led to 5'-*O*-

Figure 3. Synthetic scheme for 5'-O-DMT-2'-deoxy-2'-phthalimidouridine phosphoramidite. Reagents and conditions: (i) PhtNCOOC₂H₅, KCN/MeOH; (ii a) PhtNCOOC₂H₅; (ii b) DMT-Cl/Et₃N; (iii) P(OCE)(N-*i*Pr₂)Cl.

DMT derivative **4**, identical to that synthesized from thiocarbonate **2**. Since the starting material for this synthetic route is commercially available, the route represents a less laborious path to key synthon **4**, as well as for other 2'-modified monomers.

Phosphoramidites **5** and **8** were incorporated into hammerhead ribozymes under standard RNA synthesis conditions (6,7) with a coupling efficiency of 98–99%.

Synthesis of 5'-O-DMT-2'-deoxy-2'-phthalimidouridine 3'-O-(2-cyanoethyl-*N,N*-diisopropylphosphoramidite)

During our initial studies on the catalytic activity and nuclease stability of the hammerhead ribozyme we used the Fmoc group for protection (**26**) of the 2'-amino function of 2'-deoxy-2'-aminouridine. Although phosphoramidite **13** (Fig. 3) showed high coupling efficiency and the Fmoc group was completely removed under standard RNA deprotection conditions (6,7), it was not stable in CH₃CN solution at room temperature or in dry form during prolonged storage at –20°C. These drawbacks, which became more problematic for large scale syntheses, prompted us to investigate other protecting groups for the 2'-amino function. We first prepared 2'-deoxy-2'-trifluoroacetamidophosphoramidite **14** according to Benseler *et al.* (27). The coupling efficiency of this phosphoramidite using a 10 min RNA coupling time was only 90%. A better coupling yield could probably be achieved with increased coupling time (28), however, longer reaction times often lead to side reactions that lower the overall yield of the oligonucleotide (7).

Since the strong electronegativity of the trifluoroacetyl group could be responsible for the lower coupling efficiency of phosphoramidite **14** we prepared 2'-deoxy-2'-acetamidophosphoramidite **15** by selective deacylation of the known 2'-acetamido-3',5'-di-*O*-acetyl-2'-deoxyuridine (29) (NH₃/EtOH, 24 h, room temperature), followed by dimethoxytritylation and phosphitylation in 70% overall yield.

When phosphoramidite **15** was incorporated at positions U4 and U7 of a stabilized hammerhead motif (Fig. 1) we routinely observed coupling efficiencies in the 98.5–99% range with a standard 10 min coupling time. Unfortunately, nucleoside compositional analysis of these ribozymes, deprotected under standard (NH₄OH/EtOH, 3:1, 65°C, 4 h) or forced (CH₃NH₂/NH₄OH, 1:1, 65°C, 15 min) conditions, revealed the presence of 2'-deoxy-2'-acetamidouridine instead of 2'-deoxy-2'-aminouridine, which correlates with the high stability of 2'-deoxy-2'-amidonucleosides towards aminolysis (30). Incorporation of 2'-acetamido nucleotides as potential conjugate linkers has recently been published (31).

It appeared that the *N*-phthaloyl group, used for *N*⁶-protection of 2'-deoxyadenosine (**32**) and later to protect aliphatic primary amines in oligonucleotide conjugates (**33,34**) was an alternative. We first prepared phosphoramidite **19**, starting from 2'-deoxy-2'-aminouridine **16** (29) using 3',5'-intermediate protection (**35**), which led to the 3',5'-*O*-(tetraisopropylidisiloxane-1,3-diyl) derivative in 50% yield. Subsequent introduction of the *N*-phthaloyl group by Neffkins' method (36), desilylation, dimethoxytritylation and phosphitylation, provided phosphoramidite **19**. Since the overall yield of this multistep procedure was low (20%) we investigated alternate approaches, concentrating on the selective introduction of the *N*-phthaloyl group (37) without acylation of the 5'- and 3'-hydroxyls. When 2'-deoxy-2'-aminouridine was reacted with 1.05 eq. *N*-(ethoxycarbonyl)phthalimide in *N,N*-dimethylformamide (DMF) overnight with subsequent treatment with 1.0 eq. Et₃N (1 h), TLC indicated that 10–15% 2'-*N*-5'-*O*- or 2'-*N*-3'-*O*-bisphthaloyl derivatives were formed, with the major component being *N*-phthaloyl derivative **17** (80–85%). The *N,O*-bis by-products could be selectively and quantitatively converted to **17** by treatment of the crude reaction mixture with cat. KCN/MeOH (38). However, chromatographic isolation of pure intermediate **17** proved to be difficult, due to the low lipophilicity of this nucleoside. Finally, we developed a convenient 'one pot' procedure for the synthesis of key intermediate **18** that involved selective *N*-phthaloylation followed by dimethoxytritylation with DMT-Cl/Et₃N. This procedure resulted in the preparation of 5'-O-DMT derivative **18** in 85% overall yield. Standard phosphitylation of **18** produced phosphoramidite **19** in 85% yield.

Phosphoramidite **19** was then tested in incorporation and deprotection of RNA oligonucleotides containing 2'-NH₂ residues. The oligonucleotide CUN-PhtGAU_{2'-O}-MeGAU_{2'-O}-Me-AU_{2'-O}-MeA, which models the C3–A9 region of the stabilized hammerhead catalytic core (Fig. 1), was first prepared with phosphoramidites **13** (Fmoc) and **19** (Pht). Using a 10 min coupling time phosphoramidite **19** had a 3–5% better coupling efficiency than did **13**. After standard deprotection [NH₄OH/EtOH, 3:1, 65°C, 4 h, tetrabutylammonium fluoride (TBAF), 24 h] the test oligonucleotides were subjected to nucleoside compositional analysis. The profiles of both oligonucleotide digests were identical, demonstrating full deprotection of the Pht group. Several hammerhead ribozymes containing 2'-deoxy-2'-aminouridine at positions U4 and U7 were also prepared using phosphoramidites **13** and **19**. Following deprotection these ribozymes had the same nucleoside composition and cleavage activity, again indicating full deprotection.

Synthesis of 1-(2-*O*-acetyl-5-*O*-DMT- β -D-arabinofuranosyl) uracil 3'-*O*-(2-cyanoethyl-*N,N*-diisopropylphosphoramidite)

It has been shown that the internucleotide linkage between arabinonucleosides is resistant to hydrolysis by some nucleases (39). At the same time, altering the configuration at C2' in arabino- versus ribonucleosides has only a slight effect on the furanose bond distances and angles (40). The only noticeable difference is a forbidden high-*anti* orientation of the base in arabinonucleosides, despite the fact that *syn-anti* interconversion can still take place (41). Recently it was reported (42) that oligoarabinonucleotides can hybridize to complementary RNA and DNA exhibiting T_m values comparable with, or in some cases higher than, the corresponding unmodified oligomers.

High yield synthesis of the 5'-*O*-DMT-2'-*O*-Ac-3'-*O*-phosphoramidite **25**, by direct phosphorylation of **20** followed by acetylation, has been reported (43). The yield for this method of preparing arabinonucleosides is, however, lower in the case of the purines *N*⁶-benzoyl adenosine (43) and *N*²-isobutyryl guanosine (data not shown) and requires separation of the desired 3'-*O*-phosphoramidite from 2'-*O*- and 2',3'-*O*-bis-phosphoramidite by-products. A more general regiospecific route, using the same 2'-*O*-TBDMS protection used for ribonucleosides (6,44), would allow preparation of the desired phosphoramidites from a 5'-*O*-DMT-*N*-protected precursor in two steps (Fig. 4, Path A). Ogilvie *et al.* (15) reported a general procedure for the preparation of TBDMS-protected arabinonucleosides suitable for conversion into 3'-*O*-phosphoramidites for incorporation into oligonucleotide chains. During the silylation of arabinonucleosides with free 2'- and 3'-hydroxyl groups the authors discovered that a change of solvent [dimethoxyethane (DME) versus tetrahydrofuran (THF)] and base (triethylamine versus pyridine) dramatically changed the ratio of 3'- versus 2'-*O*-TBDMS isomers. We decided to apply the same strategy (Fig. 4, Path A, **22**–**24**) to the preparation of suitably protected arabinouridine 2'-*O*-TBDMS-3'-*O*-phosphoramidite **24** for incorporation into ribozymes.

The reaction of 1- β -D-arabinofuranosyl uracil with DMT-chloride in pyridine proceeded in a selective manner to give 5'-*O*-DMT derivative **20**, as reported (15). An attempt to selectively silylate (15) the 2'-OH group of **20** using *t*-butyldimethylsilylchloride/silver nitrate in DME as solvent, in the presence of Et₃N as base, yielded 3'-*O*-TBDMS derivative **22** as the major product (71%, R_f 0.47 in CH₂Cl₂/MeOH, 10:1) and, surprisingly, the 5'-*O*-DMT-2,2'-anhydro-1- β -D-arabinofuranosyl uracil derivative **23** (9% yield, R_f 0.42 in CH₂Cl₂/MeOH, 10:1). The expected 2'-*O*-TBDMS isomer **21** was not detected as a product in this reaction. The structure of **22** was unequivocally proven by a ¹H double resonance experiment. D₂O exchange of the 2'-hydroxyl group of **22** followed by irradiation of the anomeric proton caused conversion of the 2'-proton signal from a doublet ($J_{2',1'} = 4.4$ Hz, $J_{2',3'} = 4.4$ Hz) to a doublet ($J_{2',3'} = 4.4$ Hz), confirming the presence of the free hydroxyl at the 2'-position. The structure of the anhydro derivative **23** was confirmed by complete deprotection using 80% acetic acid followed by TBAF/THF and comparing the resulting compound with authentic 2,2'-anhydrouridine (46). It is noteworthy that 1-(5-*O*-DMT- β -D-arabinofuranosyl)-*N*⁴-isobutyryl cytosine was silylated with TBDMS-Cl/AgNO₃/Et₃N in DME to yield the 3'-*O*-TBDMS isomer as a major product and the 2'-*O*-TBDMS isomer as a minor product (data not shown).

Figure 4. Synthetic scheme for 5'-*O*-DMT-2'-*O*-acetyl-1- β -D-arabinofuranosyl uracil 3'-*O*-phosphoramidite and proposed reaction mechanism for the formation of 5'-*O*-DMT-2,2'-anhydro-1- β -D-arabinofuranosyl uracil. Reagents and conditions: (i) TBDMS-Cl/AgNO₃/DME/Et₃N; (ii a) Ac₂O/pyr; (ii b) TBAF; (iii) P(OCE)(N-*i*Pr₂)Cl.

To elucidate the mechanism of 2,2'-anhydronucleoside formation, **22** was subjected to the above silylation conditions. A 2,2'-anhydronucleoside **23** was isolated in 6% yield, along with starting **22**. The most likely mechanism for the observed cyclization is depicted in Figure 4. Silver ion coordination of the 2'-hydroxyl and 2-carbonyl group of uracil is followed by selective silylation of the uracil C2-enolate. A favorably positioned 2'-OH group can now displace the C2-*O*-TBDMS group forming the 2,2'-anhydro structure.

Intrigued by the difference between our results and those previously published (15) regarding the 2'-*O*- and 3'-*O*-TBDMS isomer distribution under 'selective' 2'-*O*-silylation conditions, we tried the standard silylation conditions used in the ribose series (THF, pyridine, AgNO₃) (45) to silylate 5'-*O*-DMT nucleoside **20**. Once again the 3'-*O*-TBDMS isomer **22** was obtained exclusively and no **21** (2'-*O*-TBDMS) could be detected by TLC or ¹H NMR. Formation of the 2,2'-anhydro derivative **23** was not observed.

Because of the failure to synthesize the desired 2'-*O*-silylated derivative **23**, we decided to convert intermediate **22**, in two steps, to phosphoramidite **25** (Fig. 4, Path B). Acetylation of **25** followed by removal of the 3'-*O*-TBDMS group with TBAF provided **24** in 81% yield. Reaction of **24** with cyanoethyl *N,N*-diisopropylchlorophosphoramidite using the standard procedure yielded phosphoramidite **25** in 84% yield (43). Phosphoramidite **25** was incorporated into hammerhead ribozymes using standard RNA synthesis conditions (6,7) with a coupling efficiency of 98–99%.

Synthesis of 5'-*O*-DMT 2'-deoxy-2'-methylene and 2'-deoxy-2'-difluoromethylene uridine 3'-*O*-(2-cyanoethyl-*N,N*-diisopropylphosphoramidite)

Assuming that imposing conformational restrictions on the sugar pucker of riboses might provide increased nuclease resistance without reducing catalytic activity of ribozymes, we turned our

Figure 5. Synthetic scheme for the synthesis of 2'-deoxy-2'-methylene and 2'-deoxy-2'-difluoromethylene uridine 3'-O-phosphoramidites. Reagents and conditions: (i) P(OCE)(N-*i*Pr₂)Cl.

attention to the synthesis of 2'-deoxy-2'-methylene and difluoromethylene derivatives of ribonucleosides. It has been shown by X-ray crystal structure analyses that the C1', C2' and C3' in 2'-deoxy-2'-methylene pyrimidine nucleosides are nearly co-planar (47).

We prepared the starting nucleosides **25** and **26** according to published procedures (48,49) and converted them to phosphoramidites **27** and **29** as shown in Figure 5 in 50 and 42% overall yields respectively.

When nucleotides **27** and **29** were incorporated at positions U4 and U7 of the ribozyme shown in Figure 1 we observed a coupling efficiency comparable with that of phosphoramidites **5**, **19** and **25**, but the overall yield of full-length product was lower by 15–20%. We assume that the known acid lability (48,49) of 2'-deoxy-2'-(difluoro)methylene nucleosides could be responsible for partial chain scission during repeated detritylation steps.

Strategy for ribozyme modification

Analysis of the literature suggests that the major pattern of degradation of hammerhead ribozymes in serum is consistent with RNase A-like endoribonuclease cleavage at pyrimidine-containing sites (50). Therefore these sites should be the primary targets for modification aimed at increasing stability.

We used the 5-ribose model shown in Figure 1 (14), in which the five purine ribose residues A15.1, G12, G8, A6 and G5 are maintained and all other residues are 2'-*O*-Me nucleotides except for those at U4 and/or U7, as our model system for evaluating catalytic activity and nuclease resistance. Phosphoramidites **5**, **19**, **25**, **27** and **29** were incorporated at positions U4 and/or U7.

Ribozyme catalytic activity is reported in Table 1 as a substrate cleavage half-time (t_A) at 40 nM ribozyme concentration, reflecting the time required for cleavage of 50% of the matched substrate. The stability of ribozymes to nuclease degradation was assessed in human serum, using 5'-³²P-labeled ribozymes. Ribozyme stability is reported in Table 1 as the stability half-life time in human serum (t_S). For comparison of one ribozyme's stability and activity to another we have defined a parameter, β , which is the ratio of the stability and activity half-times compared to a reference, Rz **1** (Table 1). Thus in Table 1

$$\beta(\text{Rz } n) = [t_S/t_A(\text{Rz } n)]/[t_S/t_A(\text{Rz } 1)]$$

A larger β represents an improvement either in ribozyme stability, activity or both relative to Rz **1**.

Analysis of the cleavage activity and nuclease resistance of ribozymes **1–17** (Table 1) demonstrated that extensive 2'-*O*-Me substitution (30 out of 36 residues) resulted in a highly active Rz **1** (t_A 1 min, the same as for an all-RNA ribozyme). At the same

time Rz **1** was not stable in human serum, providing a major degradation product that corresponds to cleavage at position U4, the only pyrimidine nucleotide lacking 2'-modification (14). When U4 in Rz **1** was modified by introduction of an additional 2'-*O*-Me nucleotide (Rz **2**) a dramatic increase in serum stability was achieved (t_S 260 min), but catalytic activity was decreased 4-fold (t_A 4 min versus 1 min for Rz **1**).

Optimization of modifications at positions U4 and U7 by introducing 2'-arabino, 2'-NH₂, 2'-*C*-allyl, 2'-=CH₂ and 2'-=CF₂ nucleotides in combination with 2'-*O*-Me substitutions (Rzs **3–17**) gave significant increases in nuclease resistance for these ribozymes, while having variable effects on catalytic activity.

Table 1. Cleavage activity and stability of ribozymes **1–17**

Rz	2'-Modification ^a (U4/U7)	Activity t_A (min) ^b	Stability t_S (min) ^c	$\beta(\text{Rz } n)$
1	OH/ <i>O</i> -Me	1	0.1	1
2	<i>O</i> -Me/ <i>O</i> -Me	4	260	650
3	araOH/ <i>O</i> -Me	8.5	500	580
4	<i>O</i> -Me/araOH	6.5	350	540
5	araOH/araOH	6.5	320	500
6	NH ₂ / <i>O</i> -Me	10	500	500
7	<i>O</i> -Me/NH ₂	5.5	500	900
8	NH ₂ /NH ₂	2	300	1500
9	<i>C</i> -Allyl/ <i>O</i> -Me	3	>500	>1700
10	<i>O</i> -Me/ <i>C</i> -Allyl	3	300	1000
11	<i>C</i> -Allyl/ <i>C</i> -Allyl	3	300	1000
12	=CH ₂ / <i>O</i> -Me	6.5	250	380
13	<i>O</i> -Me/=CH ₂	8	320	400
14	=CH ₂ /=CH ₂	8.5	250	300
15	=CF ₂ / <i>O</i> -Me	4.5	400	900
16	<i>O</i> -Me/=CF ₂	5.5	250	220
17	=CF ₂ /=CF ₂	>15	380	250

^aModifications follow the numbering scheme shown in Figure 1.

^bTime required to cleave 50% of a short substrate. Reactions were carried out at 1 nM substrate and 40 nM ribozyme concentrations.

^cStability half-life of a ribozyme in human serum. Times <1 min are estimated and may be shorter.

Certain correlations between the type of modification and ribozyme activity were noted. Introduction of the 2'-deoxy-2'-methylene and 2'-deoxy-2'-difluoromethylene modifications, which significantly distort the normal ribose ring pucker, resulted in ribozymes with reduced activity (Rz **12–14** and Rz **15–17**). When both positions U4 and U7 were modified with these nucleotides a more pronounced negative effect was observed (Rz **14** and **17**). Forced planarization of the ribofuranose ring in 2'-deoxy-2'-methylene and 2'-deoxy-2'-difluoromethylene nucleotides could cause a distortion in the optimal phosphate backbone geometry of the critical C3–U7 'U turn' (51), thus resulting in a decrease in cleavage activity.

The high activity of Rz **8** (U4 and U7 = 2'-NH₂-U) is in agreement with the recently published observation that incorporation of 2'-NH₂-U into both the U4 and U7 positions rescues the activity of uniformly 2'-fluoro-substituted ribozymes at pyrimidine sites (26). Interestingly, only the double modification (U4/U7 = NH₂/NH₂) provided a highly active ribozyme, indicating that both 2'-NH₂ groups could participate in a hydrogen bonding network. At the same time the relatively high catalytic activity of the 2'-*C*-allyl-modified Rz **9–11** complicates the hydrogen bond-

ing network proposed by Heidenreich *et al.* (50). This may indicate the importance of hydrophobic interactions in the 'U turn' (51).

Incorporation of arabinonucleotides at positions U4 and U7 resulted in ribozymes (Rz 3–5) with decreased catalytic activity. Inverting the stereochemistry at C2' of nucleotides U4/U7 probably disrupts, or unproductively alters the formation of, a hydrogen bonding network. The importance of ribo versus arabino stereochemistry at position G5, but not at position G8, was recently described (52).

In summary, we have identified two candidate ribozymes with the best combination of cleavage activity and nuclease stability, Rz 8 (U4/U7 = 2'-NH₂-U) and Rz 9 (U4 = 2'-C-allyl, U7 = 2'-O-Me-U). These ribozymes had a >1500-fold improvement in combined activity and stability over the wild-type all-RNA ribozyme. A complete analysis of the catalytic activity–stability correlation in human serum of these and other modified ribozymes has recently appeared (14).

MATERIALS AND METHODS

The solid-phase synthesis of oligoribonucleotides and ribozymes and their deprotection were performed according to published methods (6,7). Reagents were purchased from Aldrich and 5'-O-DMT-3'-O-TBDMS uridine from ChemGenes Inc. (Waltham, MA). Commercially available anhydrous solvents (Aldrich) were employed without purification.

¹H and ³¹P nuclear magnetic resonance (NMR) spectra were obtained on a Varian Gemini-400 spectrometer equipped with a Varian 5 mm multinuclear probe. ¹H NMR spectra were referenced to the internal CHCl₃ signal in the samples, δ = 7.24 p.p.m. ³¹P NMR chemical shifts were referenced to an external 85% H₃PO₄ standard, δ = 0 p.p.m. CDCl₃ (Cambridge Isotopes) was used as lock. Full NMR data on non-key intermediates may be obtained from the authors.

Nucleoside compositional analysis, ribozyme cleavage and stability assays were performed as described (14).

General method A: desilylation of nucleosides

3',5'-O-(Tetraisopropylidisiloxane-1,3-diyl) nucleoside (1.0 mmol) was dissolved in dry THF (10 ml). A 1 M solution of TBAF in THF (1.7 ml) was added and the reaction mixture was stirred at room temperature until complete disappearance of the starting material was observed by TLC (~20 min). The reaction mixture was concentrated *in vacuo* and the residue triturated with petroleum ether. The product was purified by column chromatography.

General method B: 4,4'-dimethoxytritylation of nucleosides

The nucleoside (1.0 mmol) was co-evaporated with dry pyridine (2 × 10 ml) and dissolved in dry pyridine (10 ml). DMT-Cl (1.1 mmol) was added and the reaction mixture was stirred at room temperature until all starting material had been consumed (6–10 h). MeOH (1 ml) was added and the reaction mixture was stirred for an additional 10 min, evaporated to dryness, co-evaporated with toluene (2 × 50 ml) and dissolved in CH₂Cl₂ (100 ml). Subsequent standard work-up (sat. NaHCO₃ and brine extraction, Na₂SO₄ drying) and column chromatography gave the desired compound.

General method C: preparation of nucleoside phosphoramidites

To a cooled (0°C) solution of DMT-protected nucleoside (1 mmol) and *N,N*-diisopropylethylamine (2 mmol) in dry CH₂Cl₂ (20 ml), 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite (1.25 mmol) was added drop-wise under Ar. The mixture was stirred at room temperature until all starting material was consumed (~3–5 h), cooled to 0°C and quenched with dry MeOH (2.0 ml). The reaction mixture was stirred for 5 min and evaporated to a syrup under reduced pressure (40°C bath temperature). Column chromatography of the residue gave the desired compound.

3',5'-O-(Tetraisopropylidisiloxane-1,3-diyl)-2'-O-phenoxythio-carbonyl uridine (2). To a stirred solution of 1 (15.1 g, 31 mmol) and DMAP (7.57 g, 62 mmol) in CH₃CN (50 ml) a solution of phenylchlorothionoformate (5.15 ml, 37.2 mmol) in CH₃CN (50 ml) was added drop-wise and the reaction mixture was stirred at room temperature for 8 h. TLC (EtOAc/hexane, 1:1) showed disappearance of starting material. The reaction mixture was evaporated, the residue dissolved in CHCl₃ (300 ml), washed with water (100 ml) and brine (100 ml), the organic layer dried over sodium sulfate, filtered and evaporated to dryness. The residue was purified by flash chromatography on silica gel with EtOAc/hexane, 2:1 as eluent to give 16.44 g (85%) of 2. ¹H NMR (CDCl₃) δ 0.92–1.11 (28H, *i*-Pr, m), 4.02–4.12 (2H, 4'-H, 5'-H, m), 4.26 (1H, 5''-H, d, J_{5',5''} = 13.2), 4.56 (1H, 3'-H, dd, J_{3',2'} = 4.7), 5.73 (1H, 5-H, d, J_{5,6} = 8.0), 5.94 (1H, 1'-H, s), 6.02 (1H, 2'-H, d, J_{2',3'} = 4.7), 7.12–7.45 (5H, Ph, m), 7.73 (1H, 6-H, d, J_{6,5} = 8.0), 8.45 (1H, N3-H, bs).

3',5'-O-(Tetraisopropylidisiloxane-1,3-diyl)-2'-deoxy-2'-C-allyl uridine (3). To a refluxing solution, under Ar, of 2 (5 g, 8.03 mmol) and allyltributyltin (12.3 ml, 40.15 mmol) in dry toluene (200 ml) was added portion-wise Bz₂O₂ (0.5 g) over 1 h. The resulting mixture was allowed to reflux under Ar for an additional 7–8 h. The mixture was evaporated and purified by flash chromatography on silica gel with EtOAc/hexane, 1:3 as eluant to provide 2.82 g (68.7%) of 3. ¹H NMR (CDCl₃) δ 0.93–1.13 (28H, *i*-Pr, m), 2.23 and 2.27 (2H, 6'-H and 6''-H, m), 2.32 (1H, 2'-H, m), 3.95 (1H, 4'-H, ddd, J_{4',3'} = 8.2, J_{4',5'} = 3.0, J_{4',5''} = 2.9), 4.00 (1H, 5'-H, dd, J_{5',5''} = 13.0), 4.13 (1H, 5''-H, dd), 4.52 (1H, 3'-H, dd, J_{3',2'} = 7.0), 5.12 (1H, 8'-H, d, J_{8',7'} = 10.0), 5.16 (1H, 9'-H, d, J_{9',7'} = 17.3), 5.70 (1H, H5, d, J_{5,6} = 8.0), 5.81 (1H, 1'-H, d, J_{1',2'} = 2.0), 5.95 (1H, 7-H', dddd, J_{7',6'} = 6.0, J_{7',6''} = 8.0), 7.73 (1H, 6-H, d, J_{6,5} = 8.0), 8.45 (1H, N3-H, bs).

An analogous large scale reaction was run with 2 (80.0 g, 0.13 mol) and allyl tributyltin (203 ml, 0.65 mol) in dry toluene (2000 ml). A solution of AIBN (5.38 g, 32.5 mmol) in toluene (500 ml) was added portion-wise over 2 h followed by refluxing under Ar (8 h). Column chromatography yielded 53.2 g (80%) of 3.

5'-O-DMT-2'-deoxy-2'-C-allyl uridine (4). Intermediate 3 (1.25 g, 2.45 mmol) was desilylated using general procedure A and after purification on a silica gel column in a gradient of CHCl₃-CHCl₃/MeOH, 9:1 was dimethoxytritylated using general procedure B. The title compound was purified by flash chromatography on silica gel with EtOAc/hexane, 1:1 as eluant to give 1.18 g (79%) of 4 as a white foam. ¹H NMR (CDCl₃) δ 2.29 (1H, 6'-H, m), 2.41–2.56 (2H, 6''-H and 2'-H, m), 3.45 (2H, 5'-H and 5''-H, d, J_{4',5'} = 3.1, J_{4',5''} = 3.1), 3.83 (6H, OCH₃, s), 4.13 (1H, 4'-H, dd, J_{4',3'} = 8.0, J_{4',5'} = 3.1), 4.46 (1H, 3'-H, m), 5.15 (1H, 8'-H, d, J_{8',7'} = 10.0), 5.20 (1H, 9'-H, d, J_{9',7'} = 17.3),

5.42 (1H, 5-H, d, $J_{5,6}$ = 8.0), 5.84 (1H, 7'-H, dddd, $J_{7',6'} = 6.0$, $J_{7',6''} = 8.0$), 6.14 (1H, 1'-H, d, $J_{1',2'}$ = 8.0), 6.85–7.40 (13H, DMT, m), 7.69 (1H, 6-H, d, $J_{6,5}$ = 8.0), 8.45 (1H, N3-H, bs).

5'-O-DMT-2'-deoxy-2'-C-allyl uridine 3'-O-(2-cyanoethyl-N,N-diisopropylphosphoramidite) (5). Compound **4** (2.93 g, 5.14 mmol) was phosphitylated according to general method C. Flash chromatography on silica gel using a 10–60% gradient of EtOAc in hexane containing 1% Et₃N as eluant gave 3.90 g (98.5%) of **5** as a white foam. ¹H NMR (CDCl₃) δ 1.40–1.00 (6H, CH(CH₃)₂, m), 2.20 (2H, CH₂CN, t, J = 6.7), 2.65 (2H, CH₂CN, t, J = 6.7), 2.40–2.60 (2H, 6''-H and 2'-H, m), 3.90–3.40 (5H, H-5', H-5'', CH₂O, CH(CH₃)₂, m), 3.80 (3H, OCH₃, s), 3.83 (3H, OCH₃, s), 4.30 (1H, H-4', m), 4.55 (1H, H-3', m), 5.05–5.20 (2H, 8'-H, 9'-H, m), 5.40 (1/2 H, 5-H, d, $J_{5,6}$ = 8.0), 5.44 (1/2H, 5-H, d, $J_{5,6}$ = 8.0), 5.80 (1H, 7'-H, m), 6.12 (1/2 H, H-1', d, $J_{1',2'}$ = 7.8), 6.08 (1/2 H, H-1', d, $J_{1',2'}$ = 7.8), 7.40–6.80 (13H, DMT, m), 7.72 (1/2 H, H-6, d, $J_{6,5}$ = 8.0), 7.68 (1/2 H, H-6, d, $J_{6,5}$ = 8.0), 8.20 (1H, N3-H, bs). ³¹P NMR (CDCl₃) δ 151.05, 152.96.

3',5'-O-(Tetraisopropylidisiloxane-1,3-diyl)-2'-deoxy-2'-C-allyl-N⁴-acetyl cytidine (6) from 2'-C-allyl uridine, 3. Triethylamine (6.35 ml, 45.55 mmol) was added drop-wise to a stirred ice-cooled mixture of 1,2,4-triazole (5.66 g, 82 mmol) and phosphorous oxychloride (0.86 ml, 9.11 mmol) in anhydrous CH₃CN (50 ml). To the suspension was added **3** (2.32 g, 4.55 mmol) in CH₃CN (30 ml) drop-wise and the reaction mixture was stirred for 4 h at room temperature and concentrated *in vacuo* to a minimal volume (not to dryness). The residue was dissolved in CHCl₃ (150 ml) and washed with water (50 ml), saturated aq. NaHCO₃ (50 ml) and brine (50 ml). The organic layer was dried over Na₂SO₄ and the solvent removed *in vacuo*. The resulting foam was dissolved in 1,4-dioxane (50 ml) and treated with 29% aq. NH₄OH (50 ml) overnight at room temperature. TLC (CHCl₃/MeOH, 9:1) showed complete conversion of the starting material to the desired cytidine derivative. The clear solution was evaporated, dried by co-evaporation with anhydrous pyridine (2 × 50 ml) and acetylated with acetic anhydride (0.52 ml, 5.46 mmol) in pyridine (50 ml) overnight. The reaction mixture was quenched with MeOH (5 ml), evaporated and the residue dissolved in CHCl₃ (100 ml) and washed with aq. NaHCO₃ (50 ml) and brine (50 ml). The organic layer was dried over Na₂SO₄, evaporated to dryness and purified by flash chromatography on silica gel (3% MeOH in CHCl₃) to provide 2.3 g (90%) of compound **6** as a white foam. ¹H NMR (CDCl₃) δ 0.93–1.13 (28H, *i*-Pr, m), 2.25 (3H, Ac, s), 2.27 and 2.62 (2H, 6'-H and 6''-H, m), 2.35 (1H, 2'-H, m), 4.00–4.03 (2H, 5'-H, 4'-H, m), 4.21 (1H, 5''-H, dd, $J_{5'',5'} = 13.0$, $J_{5'',4'} = 2.2$), 4.46 (1H, 3'-H, m, $J_{3',2'} = 7.96$), 5.12 (1H, 8-H', d, $J_{8',7'} = 10.0$), 5.16 (1H, 9'-H, d, $J_{9',7'} = 17.3$), 5.81 (1H, 1'-H, s), 6.08 (1H, 7'-H, dddd, $J_{7',6'} = 6.0$, $J_{7',6''} = 8.0$), 7.40 (1H, 5-H, d, $J_{5,6}$ = 8.0), 8.27 (1H, 6-H, d, $J_{6,5}$ = 8.0), 9.40 (1H, N3-H, bs).

3',5'-O-(Tetraisopropylidisiloxane-1,3-diyl)-2'-O-phenoxythiocarbonyl-N⁴-acetyl cytidine (9). 3',5'-O-(Tetraisopropylidisiloxane-1,3-diyl)-N⁴-acetyl cytidine (1.0 g, 1.89 mmol) was phenoxythiocarbonylated analogously to the preparation of **3**. The title compound was purified by flash chromatography on silica gel with EtOAc/hexane, 2:1 as eluant to provide 0.96 g (76%) of product **9**. ¹H NMR (CDCl₃) δ 0.91–1.23 (28H, *i*-Pr, m), 2.24 (3H, Ac, s), 4.02 (1H, 5''-H, dd, $J_{5'',5'} = 13.0$, $J_{5'',4'} = 2.4$), 4.14–4.29 (2H, 5'-H, 4'-H, m), 4.46 (1H, 3'-H, dd, $J_{3',4'} = 9.2$),

6.02 (1H, 1'-H, s), 6.01 (1H, 2'-H, d, $J_{2',3'}$ = 4.4), 7.09–7.39 (5 H, Phe, m), 7.40 (1H, 5-H, d, $J_{5,6}$ = 7.3), 8.18 (1H, 6-H, d, $J_{6,5}$ = 7.2), 9.45 (1H, N3-H, bs).

3',5'-O-(Tetraisopropylidisiloxane-1,3-diyl)-2'-deoxy-2'-C-allyl-N⁴-acetyl cytidine (6). Compound **9** (5.1 g, 7.68 mmol) was allylated analogously to the preparation of **3** using AIBN. The title compound was purified by flash chromatography on silica gel with a 15–50% gradient of EtOAc/hexane as eluant to provide 2.14 g (49.4%) of **6**. ¹H NMR spectrum identical to the spectrum of the product obtained from 2'-C-allyl uridine (**3**), *vide supra*.

5'-O-DMT-2'-deoxy-2'-C-allyl-N⁴-acetyl cytidine (7). Compound **7** was prepared from **6** analogously to the preparation of uridine derivative **4** from **3**. Yield 55%. ¹H NMR (CDCl₃) δ 2.25 (3H, Ac, s), 2.27 and 2.55 (2H, 6'-H and 6''-H, m), 2.35 (1H, 2'-H, m), 3.72 (2H, 5'-H, 5''-H, m), 3.81 (6H, OCH₃, s), 4.18 (1H, 4'-H, m), 4.43 (1H, 3'-H, m), 5.06 (1H, 8'-H, d, $J_{8',7'} = 10.0$), 5.10 (1H, 9'-H, d, $J_{9',7'} = 17.3$), 5.84 (1H, 7'-H, dddd, $J_{7',6'} = 6.0$, $J_{7',6''} = 8.0$), 6.27 (1H, 1'-H, d, $J_{1',2'}$ = 6.8), 7.39 (1H, 5-H, d, $J_{5,6}$ = 8.0), 7.24–7.32 (13H, DMT, m), 8.06 (1H, 6-H, d, $J_{6,5}$ = 8.0), 9.40 (1H, N3-H, bs).

5'-O-DMT-2'-O-phenoxythiocarbonyl-3'-O-t-butyltrimethylsilyl uridine (11). Compound **6** (5.0 g 7.57 mmol) was phenoxythiocarbonylated analogously to the preparation of **2**. The title compound was purified by flash chromatography on silica gel in CH₂Cl₂ as eluant to provide 5.51 g (91.3%) of **11**. ¹H NMR (CDCl₃) δ 0.95 (9H, *t*-Bu, s), 0.11 (3H, CH₃, s), 0.04 (3H, CH₃, s), 3.57 (2H, 5'-H, 5''-H, m, $J_{5',4'} = 2.4$, $J_{5'',4'} = 2.8$, $J_{5',5''} = 11.0$), 3.86 (6H, OCH₃, s), 4.07 (1H, 4'-H, m), 4.83 (1H, 3'-H, dd, $J_{3',4'} = 2.8$, $J_{3',2'} = 5.2$), 5.44 (1H, 5-H, d, $J_{5,6}$ = 8.0), 5.99 (1H, 2'-H, dd, $J_{2',1'} = 6.4$, $J_{2',3'} = 5.2$), 6.46 (1H, 1'-H, d, $J_{1',2'}$ = 6.4), 6.89–7.79 (18H, DMT, Phe, m), 7.88 (1H, 6-H, d, $J_{6,5}$ = 8.0), 7.95 (1H, N3-H, bs).

5'-O-DMT-2'-deoxy-2'-C-allyl-3'-O-t-butyltrimethylsilyl uridine (12). Compound **11** (5.5 g, 6.9 mmol) was allylated analogously to the preparation of **3** using AIBN. The title compound was purified by flash chromatography on silica gel with a 0–30% gradient of EtOAc/hexane as eluant to provide 3.38 g (70.0%) of product **12**. ¹H NMR (CDCl₃) δ 0.95 (9H, *t*-Bu, s), 0.11 (3H, CH₃, s), 0.04 (3H, CH₃, s), 2.23 (1H, 6'-H, m), 2.38–2.52 (2H, 6''-H and 2'-H, m), 3.46 (2H, 5'-H and 5''-H, m, $J_{5',4'} = 2.5$, $J_{5'',4'} = 3.2$, $J_{5',5''} = 10.8$), 3.86 (6H, OCH₃, s), 4.13 (1H, 4'-H, dd, $J_{4',3'} = 8.0$, $J_{4',5'} = 3.2$, $J_{4',5''} = 2.5$), 4.46 (1H, 3'-H, m), 5.15 (1H, 8'-H, d, $J_{8',7'} = 10.0$), 5.20 (1H, 9'-H, d, $J_{9',7'} = 17.3$), 5.44 (1H, 5-H, d, $J_{5,6}$ = 8.0), 5.81 (1H, 7'-H, dddd, $J_{7',6'} = 6.0$, $J_{7',6''} = 8.0$), 6.14 (1H, 1'-H, d, $J_{1',2'}$ = 8.0), 6.88–7.52 (13H, DMT, m), 7.76 (1H, 6-H, d, $J_{6,5}$ = 8.0), 8.17 (1H, N3-H, bs).

5'-O-DMT-2'-deoxy-2'-C-allyl-N⁴-acetyl cytidine 3'-O-(2-cyanoethyl-N,N-diisopropylphosphoramidite) (8). Compound **7** (0.8 g, 1.31 mmol) was phosphitylated using general method C. Flash chromatography on silica gel using CHCl₃/EtOH, 98:2 containing 2% Et₃N as eluant provided 0.91 g (85%) of product **8** as a white foam. ¹H NMR (CDCl₃) δ 1.00–1.40 (6H, CH(CH₃)₂, m), 2.30 (2H, CH₂CN, t, J = 6.7), 2.75 (2H, CH₂CN, t, J = 6.7), 2.20–2.60 (2H, 6''-H and 2'-H, m), 3.40–3.90 (5H, H-5', H-5'', CH₂O, CH(CH₃)₂, m), 3.81 (3H, OCH₃, s), 4.28 (3/4 H, H-4', m), 4.34 (1/4 H, H-4', m), 4.60 (3/4 H, H-3', m), 4.50 (1/4 H, H-3', m), 5.05–5.20 (2H, 8-H', 9'-H, m), 5.42 (1H, 5-H, d, $J_{5,6}$ = 8.0), 5.85 (1H, 7'-H, m), 6.25 (1H, d, H-1', $J_{1',2'}$ = 6.8), 7.39 (1H, 5-H, d, $J_{5,6}$ = 8.0), 7.40–6.80 (13H, DMT, m), 8.08 (3/4 H, H-6, d, $J_{6,5}$

= 7.8), 8.02 (1/4 H, H-6, d, $J_{6,5}$ = 7.8), 9.10 (1H, NHAc, bs). ^{31}P NMR (CDCl_3) δ 148.74, 149.77.

5'-O-DMT-2'-deoxy-2'-acetamidouridine 3'-O-(2-cyanoethyl-N,N-diisopropylphosphoramidite) (15). 5'-O-DMT-2'-deoxy-2'-acetamidouridine (2.0 g, 3.4 mmol) prepared by dimethoxytritylation of 2'-deoxy-2'-acetamidouridine (29,31) according to general method B was further phosphitylated according to general method C. Flash column chromatography on silica gel using a 20–30% gradient of THF in CH_2Cl_2 containing 1% Et_3N as eluant provided 2.1 g (78%) of compound **15** as a white foam. ^1H NMR (CDCl_3) δ 1.10–1.21 (6H, $\text{CH}(\text{CH}_3)_2$, m), 2.03 (3H, Ac, s), 2.47 (2H, CH_2CN , t, J = 6.3), 2.69 (2H, CH_2CN , t, J = 6.3), 3.36–3.88 (5H, H-5', H-5'', CH_2O , $\text{CH}(\text{CH}_3)_2$, m), 3.78 (3H, OMe, s), 3.82 (3H, OMe, s), 4.23 (1H, H-4', m), 4.49 (1H, H-3', m), 4.83 (1H, H-2', dd), 5.44 (1/2 H, H-5, d, $J_{5,6}$ = 8.0), 5.48 (1/2 H, H-5, d, $J_{5,6}$ = 8.0), 6.12 (1/2 H, H-1', d, $J_{1',2'}$ = 8.3), 6.16 (1/2 H, H-1', d, $J_{1',2'}$ = 8.3), 6.80–7.53 (13H, DMT, NHAc, m), 7.65 (1/2 H, H-6, d, $J_{6,5}$ = 8.0), 7.75 (1/2 H, H-6, d, $J_{6,5}$ = 8.0), 8.05 (1H, N3-H, bs). ^{31}P NMR (CDCl_3) δ 152.72, 150.75.

5'-O-DMT-2'-deoxy-2'-phthalimidouridine (18), one pot method. 2'-Deoxy-2'-aminouridine (1.0 g, 4.1 mmol) (29) was co-evaporated twice from dry DMF (50 ml) and dried *in vacuo*. The residue was dissolved in dry DMF (50 ml) and *N*-carbethoxyphthalimide (1.0 g, 4.3 mmol) was added and the solution stirred overnight. TLC (10% EtOH in CHCl_3) showed 90% conversion to faster moving products and Et_3N (57 ml, 0.4 mmol) was added. After 1 h additional Et_3N (855 μl , 6.16 mmol) was added, followed by DMT-Cl (1.53 g, 4.53 mmol). The reaction mixture was left for 16 h at room temperature. TLC (10% EtOH in CHCl_3) showed >90% conversion to product. The reaction was quenched by addition of EtOH (20 ml). Solvent was removed *in vacuo* and the residue was washed with 5% aq. NaHCO_3 solution (500 ml) and extracted with EtOAc (2 $\times$ 200 ml), dried, evaporated *in vacuo* and the residue purified by flash chromatography using an EtOAc/hexane gradient. The product eluted at 80–85% EtOAc to provide 2.17 g (85%) of **18** as a white foam. ^1H NMR ($\text{DMSO}-d_6$) δ 3.33 (2H, H-5', H-5'', m, $J_{5',4'} = 6.4$, $J_{5'',4'} = 1.2$, $J_{5',5''} = 10.5$), 4.36 (1H, H-4', m, $J_{4',3'} = 7.2$), 4.44 (1H, H-3', t, $J_{3',2'} = 7.6$), 4.96 (1H, H-2', dd, $J_{2',1'} = 4.0$), 5.58 (1H, H-5, d, $J_{5,6} = 8.0$), 5.72 (1H, OH-3', d, $J_{\text{OH},3'} = 6.3$, exchangeable with D_2O), 6.53 (1H, H-1', d, $J_{1',2'} = 4.0$), 7.72–7.87 (4H, Pht, m), 11.4 (1H, N3-H, bs).

5'-O-DMT-2'-deoxy-2'-phthalimidouridine 3'-O-(2-cyanoethyl-N,N-diisopropylphosphoramidite) (19). Phosphitylation of 5'-O-DMT derivative **18** was performed as for the *N*-2'-acetyl compound **15** to provide phosphoramidite **19** in 87% yield. ^1H NMR (CDCl_3) δ 0.08–1.10 (6H, $\text{CH}(\text{CH}_3)_2$, m), 2.30 (2H, CH_2CN , t, J = 6.4), 2.58 (2H, CH_2CN , t, J = 6.3), 3.86 (3H, OMe, s), 3.88 (3H, OMe, s), 3.90–3.16 (5H, H-5', H-5'', CH_2O , $\text{CH}(\text{CH}_3)_2$, m), 4.70 (1H, H-4', m), 4.85 (1H, H-3', m), 5.06 (1H, H-2', m), 5.40 (1H, H-5, d, $J_{5,6} = 8.0$), 5.55 (1H, H-5, d, $J_{5,6} = 8.0$), 6.55 (1H, H-1', d, $J_{1',2'} = 4.0$), 6.92–7.87 (18H, Pht, DMT, m), 8.10 (1H, N3-H, bs). ^{31}P NMR (CDCl_3) δ 152.92, 152.49.

1-(3-O-*t*-Butyldimethylsilyl-5-O-DMT- β -D-arabinofuranosyl) uracil (22) and 2,2'-anhydro-1-(3-O-*t*-butyldimethylsilyl-5-O-DMT- β -D-arabinofuranosyl) uracil (23). To a solution of **20** (15) (17.0 g, 31.1 mmol) in DME (400 ml) were added Et_3N (34.7 ml, 248.8 mmol) and AgNO_3 (15.5 g, 91.3 mmol). The mixture was stirred for 1 h at room temperature and then TBDMS-Cl (14.1 g, 93.4 mmol) was added. The reaction mixture

was stirred for 4 h at room temperature and filtered into 5% aq. NaHCO_3 (1 l). The resulting solution was extracted with CHCl_3 (3 $\times$ 400 ml), the combined organic layers were dried and the solvent removed *in vacuo*. The residual syrup was purified by flash chromatography on silica gel using a 2–10% MeOH gradient in CH_2Cl_2 . Compound **22** eluted first and was obtained after removal of solvents as a colorless foam (16.5 g, 71%). ^1H NMR (CDCl_3) δ 0.02 (3H, CH_3 , s), 0.08 (3H, CH_3 , s), 0.83 (9H, *t*-Bu, s), 3.35 (1H, H-5'', dd, $J_{5'',5'} = 10.9$, $J_{5'',4'} = 3.1$), 3.63 (1H, 2'-OH, d, $J_{\text{OH},2'} = 6.4$, exchangeable with D_2O), 3.70 (1H, H-5', dd, $J_{5',5''} = 10.9$, $J_{5',4'} = 2.8$), 3.95 (1H, H-4', m), 3.81 (6H, OCH_3 , s), 4.17 (1H, H-3', t, $J_{3',2'} = 4.6$), 4.26 (1H, H-2', m), 5.48 (1H, H-5, d, $J_{5,6} = 8.1$), 6.13 (1H, H-1', d, $J_{1',2'} = 4.4$), 7.42–6.85 (13H, DMT, m), 7.94 (1H, H-6, d, $J_{6,5} = 8.1$), 8.81 (1H, N3-H, d, exchangeable with D_2O).

Further elution afforded compound **23** as a colorless foam (1.9 g, 10%). ^1H NMR (CDCl_3) δ 0.09 (3H, CH_3 , s), 0.15 (3H, CH_3 , s), 0.89 (9H, *t*-Bu, s), 2.98 (1H, H-5'', dd, $J_{5'',5'} = 10.2$, $J_{5'',4'} = 6.7$), 3.07 (1H, H-5', dd, $J_{5',5''} = 10.2$, $J_{5',4'} = 5.8$), 3.83 (6H, OCH_3 , s), 4.25 (1H, H-4', m), 4.51 (1H, H-3', m), 5.06 (1H, H-2', d, $J_{2',1'} = 5.8$), 6.01 (1H, H-5, dd, $J_{5,6} = 7.4$, $J_{5,\text{NH}} = 1.6$), 6.12 (1H, H-1', d, $J_{1',2'} = 5.8$), 7.37–6.82 (14H, DMT, H-6, m).

1-(2-O-Acetyl-5-O-DMT- β -D-arabinofuranosyl) uracil (24). Compound **22** (5.0 g, 7.57 mmol) was dissolved in a mixture of pyridine (25 ml) and Ac_2O (5 ml). The solution was set aside at room temperature for 3 h. MeOH (20 ml) was added and the mixture evaporated under reduced pressure to a syrup which was partitioned between 5% aq. NaHCO_3 (100 ml) and CH_2Cl_2 (100 ml). The organic layer was separated and the aqueous layer extracted with CH_2Cl_2 (100 ml). The organic layers were combined, dried (Na_2SO_4) and concentrated to a syrup. The syrup was dissolved in THF (30 ml) and 1 M TBAF/THF (20 ml) was added. The solution was stirred for 2 h at room temperature and the solvents removed *in vacuo*. The residue was partitioned between 5% aq. NaHCO_3 (100 ml) and CH_2Cl_2 (100 ml). The organic layer was separated and the aqueous layer extracted with CH_2Cl_2 (100 ml). The combined organic layers were dried and evaporated *in vacuo*. The residue was purified by flash chromatography on silica gel using a 50–80% hexane/EtOAc gradient to provide compound **24** (3.6 g, 81%) as a colorless foam. ^1H NMR (CDCl_3) δ 2.00 (3H, Ac, s), 3.45 (1H, H-5'', dd, $J_{5'',5'} = 10.5$, $J_{5'',4'} = 3.2$), 3.53 (1H, H-5', dd, $J_{5',5''} = 10.5$, $J_{5',4'} = 2.7$), 4.07 (1H, H-4', m), 4.37 (1H, H-3', t, $J_{3',2'} = 4.5$), 5.25 (1H, H-2', dd, $J_{2',1'} = 5.5$, $J_{2',3'} = 4.5$), 5.54 (1H, H-5, d, $J_{5,6} = 8.1$), 6.35 (1H, H-1', d, $J_{1',2'} = 5.5$), 7.45–6.86 (13H, DMT, m), 7.71 (1H, H-6, d, $J_{6,5} = 8.1$), 9.15 (1H, N3-H, bs, exchangeable with D_2O).

1-(2-O-Acetyl-5-O-DMT- β -D-arabinofuranosyl) uracil 3'-O-(2-cyanoethyl-N,N-diisopropylphosphoramidite) (25). Compound **24** (750 mg, 1.27 mmol) was phosphitylated according to general procedure C. Flash chromatography using a 50–80% hexane/EtOAc gradient containing 1% Et_3N provided compound **25** (840 mg, 84%) as a white foam. ^1H NMR (CDCl_3) δ 1.21–1.10 (6H, $\text{CH}(\text{CH}_3)_2$, m), 1.92 (3/2 H, Ac, s), 1.93 (3/2 H, Ac, s), 2.44 (2H, CH_2CN , t, J = 6.4), 2.64 (2H, CH_2CN , t, J = 6.3), 3.88–3.36 (5H, H-5', H-5'', CH_2O , $\text{CH}(\text{CH}_3)_2$, m), 3.82 (3H, OCH_3 , s), 3.83 (3H, OCH_3 , s), 4.20 (1H, H-4', m), 4.46 (1H, H-3', m), 4.58 (1H, H-3', m), 5.53–5.42 (2H, H-5, H-2', m), 6.31 (1/2 H, H-1', d, $J_{1',2'} = 4.2$), 6.32 (1/2 H, H-1', d, $J_{1',2'} = 4.7$), 7.47–6.85 (13H, DMT, m), 7.50 (1/2 H, H-6, d, $J_{6,5} = 8.2$), 7.62 (1/2 H, H-6, d, $J_{6,5} = 8.2$), 8.36 (1H, N3-H, bs). ^{31}P NMR (CDCl_3) δ 152.92, 152.49.

5'-O-DMT-2'-deoxy-2'-methylene uridine 3'-O-(2-cyanoethyl-N,N-diisopropylphosphoramidite) (**27**). Compound **26** (0.43 g, 0.8 mmol) obtained by standard dimethoxytritylation of the known 2'-deoxy-2'-methylene uridine (**47**) was further phosphitylated according to general procedure C. Flash chromatography using a 25–70% EtOAc/hexane gradient containing 1% Et₃N as eluant provided compound **32** (0.45 g, 75%) as a white foam. ¹H NMR (CDCl₃) δ 1.21–1.03 (6H, CH(CH₃)₂, m), 2.34 (2H, CH₂CN, t, J = 6.4), 2.64 (2H, CH₂CN, t, J = 6.4), 3.88–3.36 (5H, H-5', H-5'', CH₂O, CH(CH₃)₂, m), 3.81 (3H, OCH₃, s), 3.83 (3H, OCH₃, s), 4.02 (1/2H, 4'-H, m), 4.08 (1/2H, 4'-H, m), 5.10 (1H, 3'-H, m), 5.45 & 5.70 (2H, =CH₂, s s), 5.30 (1H, H-5, d, J_{5,6} = 8.0), 6.70 (1/2 H, 1'-H, s), 6.68 (1/2 H, 1'-H, s), 6.80–7.45 (13H, DMT, m), 7.68 (1/2 H, H-6, d, J_{6,5} = 8.0), 7.82 (1/2 H, H-6, d, J_{6,5} = 8.0), 8.15 (1H, N3-H, bs). ³¹P NMR (CDCl₃) δ 151.73 and 152.64.

Synthesis of 5'-O-DMT-2'-deoxy-2'-difluoromethylene uridine 3'-O-(2-cyanoethyl-N,N-diisopropylphosphoramidite) (**29**). Compound **28** (0.58 g, 1 mmol) obtained by standard dimethoxytritylation of the known 2'-deoxy-2'-difluoromethylene uridine (**49**) was further phosphitylated according to general procedure C. Flash chromatography using a 20–70% EtOAc/hexane gradient containing 1% Et₃N as eluant provided compound **29** (0.45 g, 58%) as a white foam. ¹H NMR (CDCl₃) δ 1.20–1.03 (6H, CH(CH₃)₂, m), 2.44 (2H, CH₂CN, t, J = 6.4), 2.68 (2H, CH₂CN, t, J = 6.4), 3.88–3.36 (5H, H-5', H-5'', CH₂O, CH(CH₃)₂, m), 3.79 (3H, OCH₃, s), 3.81 (3H, OCH₃, s), 4.05 (1H, 4'-H, m), 4.55 (1/2 H, 3'-H, m), 4.65 (1/2 H, 3'-H, m), 5.21 (1/2 H, H-5, d, J_{5,6} = 8.0), 5.24 (1/2 H, H-5, d, J_{5,6} = 8.0), 5.98 (1/2 H, 1'-H, s), 6.04 (1/2 H, 1'-H, s), 7.55–6.80 (13H, DMT, m), 8.02 (1/2 H, H-6, d, J_{6,5} = 8.0), 8.07 (1/2H, H-6, d, J_{6,5} = 8.0), 8.43 (1H, N3-H, bs). ³¹P NMR (CDCl₃) δ 151.32 and 151.73.

ACKNOWLEDGEMENTS

We thank Dr A.Modak for preparation of phosphoramidites **27** and **29**, Anthony DiRenzo, Chris Shaffer and Dr Fran Wincott for the design of model oligoribonucleotides and synthesis of ribozymes and stimulating discussions on deprotection studies, Carolyn Gonzalez for ribozyme cleavage assays and Kristy Jensen for serum stability assays.

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