The synthesis and properties of oligoribonucleotide–spermine conjugates†

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Polyamines stabilise nucleic acids against chemical and enzymatic degradation, facilitate the formation of secondary and tertiary structures and enhance cellular uptake. Therefore methods for the syntheses of polyamine–nucleic acid conjugates are of interest. A route for the syntheses of RNA–spermine conjugates has been developed. The polyamine was introduced to the C-5 position of uridine via an ethyl tether and the molecule elaborated into a synthon suitable for oligoribonucleotide assembly. The resultant oligomers were components of the hairpin ribozyme. Characterisation of the spermine-conjugated catalytic RNA revealed that attachment of the polyamine was well tolerated in three of four positions, namely U41, U37 and U34, suggesting that conjugation to C-5 brings about minimal structural perturbation.

Introduction

Polyamines such as putrescine, spermidine and spermine are ubiquitous in biological systems. It is thought that they are involved in the proliferation and differentiation of cells, DNA replication, protein synthesis and the activity of several enzymes.1 The interactions of polyamines with nucleic acids have been studied for many years. These polycationic molecules are known to stabilise DNA against thermal2 and alkaline denaturation,3 enzymatic degradation,4 shear5 and oxidative breakage6 radiation damage,7 and intercalation of aromatic dyes.8 Polyamines have also been shown to have stabilising effects on RNA–DNA hybrids, triple-helical DNA and double-helical secondary structures found in tRNA, rRNA and mRNA.8,9 In addition, polyamines have been demonstrated to effect structural and conformational modifications in nucleic acids,10,11 for example, allowing the hairpin ribozyme to fold into a catalytically competent structure in the absence of metal ions.12 There is also evidence that polyamines can facilitate the cellular uptake of oligodeoxyribonucleotides (ODNs).13–15 Since the therapeutic applications of ODNs as antisense and antigen agents rely on efficient cellular uptake of such molecules, polyamines and polyamine–ODN conjugates may be exploited to improve efficacy.

As a consequence of the properties of polyamines, methods for the synthesis of polyamine–ODN conjugates have received considerable interest.16–35 A variety of nucleic acids with di- and poly-amines attached at different positions have been prepared. In several cases conjugation of a polyamine has been reported to stabilise the resultant ODN duplexes and triplexes compared to their unmodified counterparts. In addition, frequently the tethering of polyamines has also been found to confer nuclease resistance to the oligomer.

Various sites have been used to link polyamines to ODNs. Post-synthetic functionalisation with polyamines has been used to generate a 21-mer homopyrimidine ODN bearing a 5′-polyamine.16 Chattopadhyaya and colleagues have prepared and attached a C-branched spermine derivative to the 5′- and 3′-termini of an ODN or internally to the 2′-position of ara-uridine via a phosphate linkage.17,18

Polyamines have also been introduced into ODNs via conjugation to the heterocyclic bases. In the pyrimidine series Ueda and co-workers introduced the diamine putrescine at the 5-position of thymidine via reduction of the Schiff base formed from a 5-formyl-2′-deoxyuridine derivative and N-phthalaloylputrescine.19 A similar strategy was used by Matsuda and colleagues to attach syn-norspermidine to the end of a butyl linker at the 5-position of 2′-deoxyuridine in a reductiveamination step.20 The same group also reported 2′-deoxyuridine analogues carrying either an N,N-bis(2-aminomethyl)aminomethylcarbamoyl or N,N-bis(3-aminopropyl)aminomethylcarbamoyl linker at the 5-position.21 Kohgo et al. reported the incorporation into ODNs of a novel 2′-deoxyuridine derivative bearing a cyanomethoxy carbonylmethyl group at the 5-position. Post-synthetic functionalisation with monoamines, diamines and the polyamine tris(2-aminoethyl)amine was achieved under mild conditions.22 A 2′-deoxyuridine derivatised at the 5-position with tris(2-aminoethyl)amine was also prepared by the same group from 5-methoxycarbonylmethyl-2′-deoxyuridine.23 The same functionalisation and also the addition of spermine was attained via a post-synthetic method using analogous chemistry.24 Similarly, Haginoya et al. used a post-synthetic modification strategy reacting alkyl diamines with the convertible nucleoside unit 5-methoxycarbonylmethyl-2′-deoxyuridine.25 Prakash et al. tethered triethylenetetramine and spermine to the C-4 position of 2′-deoxy-5-methylcytidine via substitution of a 4-O-(2,4-dimethylphenyl) group.26 Markiewicz et al. prepared 2′-deoxycytidine modified at the C-4 position with spermine following reaction with the 4-p-toluenesulfonylate ester.27 Reductive amination was employed to react 5-formyl-2′-deoxyuridine with putrescine and spermine, yielding conjugation at the 5-position via a methylene group.28

There have also been a number of polyamino-purine conjugates prepared recently. Schmid and Behr attached spermine to the N-2 position of 2′-deoxyguanosine in a post-synthetic fashion via substitution of fluorine.29 Diaz et al. similarly linked spermine plus spermidine and 1-(3-aminopropyl)imidazole to the N-2 position of 2′-deoxyguanosine in a post-synthetic manner.30 The Markiewicz lab described the synthesis of phosphoramidites of 2′-deoxyadenosine and 2′-deoxyguanosine with spermine at the N-6 and N-2 positions, respectively.31,32 Potier et al. also synthesised a 2′-deoxyguanosine phosphor-
amidine with spermine at the N-2 position.\textsuperscript{35,36} Shinozuka et al. used a post-synthetic strategy to obtain ODNs with a branched polyamine conjugated at the C-2 of 2’-deoxyguanosine.\textsuperscript{15}

Ribozymes that are capable of the sequence-specific cleavage of RNA have potential uses in vitro as chemical nucleases and biosensors and in vivo as therapeutic agents for the controlled destruction of mRNA for gene inactivation and the targeted cleavage of viral, oncogene or mutant mRNA.\textsuperscript{37-40} In analogy to ODNs, polyamine-catalytic RNA conjugates may have enhanced nuclease resistance, increased cellular uptake and a more stable tertiary structure. However, although the incorporation of a C5-aminohexylcarbamoyl-2'-OMe uridine monomer and its incorporation into DNA has been described recently,\textsuperscript{41} there has, to our knowledge, been no description of the synthesis of polyamine-nucleic acid conjugates of RNA. Here we describe a method for the syntheses of RNA–spermine conjugates, prepared using a C-5 modified uridine derivative. The resultant oligomers are components of the hairpin ribozyme, and the properties of the resultant spermine-derivatised catalytic RNAs have been characterised.

**Results and discussion**

**Synthesis of a spermine–uridine conjugate suitable for RNA synthesis**

Several sites of attachment of polyamines to RNA are possible including the 5’- and 3’-termini, the 2’-hydroxyl group or other sites within the ribose unit, the phosphate or the nucleobase. Placement of a polyamine within a folded RNA structure is more likely to be achieved via internal, rather than 5’- or 3’-terminal, attachment. Since the 2’-hydroxyl group is commonly involved in RNA tertiary interactions, conjugation to this site may inhibit the formation of RNA three-dimensional structure. Other sites of sugar attachment are more synthetically demanding, whereas linking to the phosphate groups involves the formation of highly labile phosphate triesters. Thus we elected to create a polyamine conjugate linked to the nucleobase. The C-5 position of uridine was chosen for derivatisation since this does not interfere with the formation of Watson–Crick hydrogen bonds. When designing the uridine–spermine conjugate we considered an alkyl linker between the polyamine and the nucleobase to be vital to allow conformational flexibility of the entire polyamine. Thus, an ethyl linker was selected, since a high yielding and efficient synthesis of hydroxethyluracil has been reported previously.\textsuperscript{42}

Our first attempted route to a spermine–polyamine conjugate suitable for RNA synthesis is illustrated in Scheme 1. Hydroxethyluracil (I)\textsuperscript{43} was employed in a Vorbrüggen-type\textsuperscript{44} coupling reaction to afford the perbenzoylated nucleoside 2 but in variable yield (30–80%), possibly due to the poor solubility of the starting chromophore. As expected this procedure yielded only the β-isomer. Protection of the hydroxyl group of hydroxyethyluracil (1) was not required as this could be achieved by increasing the amount of trimethylsilyl chloride used to generate the silylated nucleobase. Subsequent oxidation of the hydroxyl group of 2 afforded the aldehyde 3 suitable for conjugation of the polyamine via reductive amination. Oxidation of the primary alcohol proceeded in highly variable but typically rather poor yield (35%) using Swern oxidation conditions, but failed entirely using pyridinium chlorochromate. Using a similar strategy to that described by Ueda and colleagues for the synthesis of a putrescinylglycidylamine derivative,\textsuperscript{45} reaction of the aldehyde 3 with spermine to form a Schiff base, followed by reduction with NaBH$_4$ afforded a polyamine linked nucleoside. Without purification of the polyamine conjugate, the sugar benzoyl groups were removed with aqueous ammonia and then the amines were masked with trifluoroacetyl groups to produce the protected spermine conjugate 4. The overall yield for these four steps was 17%. In addition to the target material bearing four trifluoroacetyl groups (compound 4), significant amounts of a nucleoside with three trifluoroacetyl groups and one benzoyl group was also isolated. This latter side product most likely results from the transfer of a benzoyl group from the sugar to the polyamine and illustrates that it would be preferable to not have acyl hydroxyl protection in place during reductive amination. Attempts to introduce a dimethoxytrityl group to the spermine conjugate 4 to generate 5 proved unsuccessful, despite the addition of silver nitrate, DMAP, excess dimethoxytrityl chloride (DmtCl) and gentle warming. The tritylation failed for unknown reasons.

As an alternative route to the polyamine linker reagent, addition of the dimethoxytrityl group prior to introduction of the spermine moiet was investigated. In order to achieve selective 5’-O-dimethoxytritylation, the 5’-hydroxyl side chain of compound 1 was protected with tert-butyldimethylsilyl chloride (tBDMSCl) to afford 6, which was utilised in Vorbrüggen coupling to generate 8 (Scheme 2). The use of tBDMS-protected hydroxethyluracil 6, rather than hydroxethyluracil itself, increased the yields and the reliability of the glycosyl bond-forming, probably due to the drastically improved solubility of the silylated nucleobase. The products of this reaction included both tBDMS-protected nucleoside 8 and some material that had lost the silyl protecting group. This latter material was therefore reprotected prior to isolation. This procedure gave reproducible yields of 68%.

The Lewis acid used in the coupling reaction presumably results in the partial removal of the tBDMS-protecting group from 8. To increase the acid stability of the protecting group and avoid desilylation the use of the more acid-stable tert-
venting loss of the 5'-hydroxyl group to generate the common 5'-terminal uridine dimer, r(U*pG), where U* is the polyamine conjugate (19). RNase I catalyses the transesterification of the resultant nucleotides to the corresponding free nucleosides suitable for RP HPLC analysis. It is noteworthy that whilst this enzyme-catalysed reaction proceeds, it does so at a severely reduced rate suggesting that the polyamine modification confers some degree of nuclease resistance. Further studies of the substrate properties of the polyamine conjugates with a range of nucleases are in progress.

RNase I catalyses the transesterification of RNA molecules to yield products containing one or more 3'-O-tBDMS-3'-O-phosphoramidite and 3'-O-IDMS-2'-O-phosphoramidite have been isolated. Such a mixture would result if silyl migration had occurred during phosphenylation. The eventual consequences of the use of this mixture in oligoribonucleotide synthesis would be the generation of 2'-5' linked RNA in addition to the normal 3'-5' linked species. Alternatively, the four phosphorus resonances could be the result of the presence of more than one conformation of each phosphoramidite diastereoisomer. This might explain the differences in peak ratios observed in differing solvents, as the characteristics of a particular solvent might be expected to adjust conformational equilibria. Attempts to confirm the presence of a conformational equilibrium using variable-temperature NMR were unsuccessful due to decomposition of the phosphoramidite due to oxidation.

To investigate this further we therefore synthesised a ribo-nucleoside dimer, r(U*+G), where U* is the polyamine conjugate. 31P NMR of this dimer indicated the presence of a single phosphorus species. Moreover when the dimer was subjected to enzymatic analysis using RNase I and alkaline phosphatase it was completely degraded to guanosine and the uridine–polyamine conjugate 19 as monitored by RP HPLC. It is noteworthy that whilst this enzyme-catalysed reaction proceeds, it does so at a severely reduced rate suggesting that the polyamine modification confers some degree of nuclease resistance. Further studies of the substrate properties of the polyamine conjugates with a range of nucleases are in progress.

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The synthesis of hairpin ribozymes containing the uridine–polyamine conjugate

The syntheses of oligoribonucleotides containing the polyamine conjugate were carried out using standard procedures employing 2′-O-TBDMS monomers rather than the simple doublet seen for compound 15. Furthermore, accurate mass data were consistent with those expected for compounds 16–18 and the mass spectra of these compounds indicated the absence of other mass ions. This allows the possibility of a mixture of two compounds that might be the result of formation of the Schiff base with either secondary or primary amino groups of the polyamine to be ruled out and is again consistent with a conformational equilibrium of a single species.

Characterisation of the spermine-modified hairpin ribozymes

A three-stranded hairpin ribozyme was used in this study (Fig. 1). This consists of a substrate and two ribozyme strands, strand A and strand B. The sequence is identical to a ribozyme employed by Burke and co-workers and has been optimised to minimise alternative conformers. A 5′-fluorescein moiety was added to the substrate to allow the amount of product to be quantified using a fluorescence detector following denaturing HPLC separation of the substrate and products. The addition of a 5′-fluorophore to the hairpin ribozyme substrate has been shown previously not to change the catalytic parameters of the ribozyme. The catalytic parameters of the ribozyme used in this study (K_M = 7.1 ± 1.6 nM, k_cat/Mg^{2+} = 0.3 ± 0.01 min^{-1}) with Mg^{2+} cofactor; K_M = 5.4 ± 1.4 nM, k_cat/Mg^{2+} = 0.3 ± 0.01 min^{-1} with spermine as the sole cofactor) are similar to other three-stranded ribozymes.

Evaluation of the polyamine hairpin ribozyme conjugates

Table 1 Rate constants of unmodified and modified ribozymes with or without the presence of 10 mM Mg^{2+} at 100 nM [S] and 20 nM [E]

<table>
<thead>
<tr>
<th>Modification</th>
<th>With Mg^{2+}</th>
<th>Without Mg^{2+}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unmodified</td>
<td>0.30</td>
<td>0.009</td>
</tr>
<tr>
<td>U*34</td>
<td>0.41</td>
<td>0.008</td>
</tr>
<tr>
<td>U*37</td>
<td>0.20</td>
<td>0.004</td>
</tr>
<tr>
<td>U*41</td>
<td>0.30</td>
<td>0.002</td>
</tr>
<tr>
<td>U*42</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

The addition of 10 mM MgCl_2, 40 mM Tris HCl pH 7.5) with substrate and ribozyme concentrations of 100 and 20 nM, respectively. The results of this analysis are shown in Table 1. Substitution of either of U34, U37 or U41 with the polyamine conjugate [Fig. 1(c)] is tolerated well, producing rates of reaction comparable to the wild-type hairpin ribozyme. However, the replacement of U42 with the spermine–uridine derivative [Fig. 1(c)] was severely inhibitory to reaction. As U42 plays a critical role in the loop A–loop B interface, making five hydrogen bonds with other nucleosides within loops A and B, it is likely that attachment of the polyamine prevents the correct folding of the ribozyme essential for catalysis. As exogenous spermine is capable of supporting hairpin ribozyme catalysis in the absence of divalent metal ions, the rates of reaction of the spermine-modified ribozyme substrate have been investigated in the absence of magnesium ions (Table 1). None of the conjugates produced a significant rate of reaction in the absence of divalent metal ions, suggesting that the polyamine is not correctly positioned to produce a catalytically competent structure in the
absence of divalent metal ions. The finding that in three of the four positions tested, attachment of spermine to the C-5 of uridine is tolerated, suggests that this is a suitable position for conjugation.

Conclusions

We have reported the synthesis of the phosphoramidite of a C5-modified uridine analogue that allows the preparation of RNA–spermine conjugates. The characterisation and catalytic properties of four different hairpin ribozymes containing the analogue in place of conserved uridines have been described. Three of the modified ribozymes show similar kinetic parameters to the wild-type sequence, whilst the substitution of U42 which is at the interface between loops A and B results in a large decrease in activity. However, the requirement for magnesium ion cofactors for all four spermine-modified ribozymes was not decreased compared to the wild-type ribozyme. Alternative sites of conjugation, longer tethers for linking to the polyanime and the use of C-branched polyanimes may allow reduction or elimination of hairpin ribozyme divalent metal ion cofactor requirements. The finding that in three of the four positions tested, attachment of spermine to the C-5 of uridine is tolerated, suggests that this is a suitable position for conjugation. The ability to attach spermine to ribozymes without loss of activity may find application for the use of ribozymes in vivo due to the known propensity for the cellular uptake of polyamines.

Experimental

Dichloromethane (DCM), pyridine and acetonitrile were dried under reflux from calcium hydride and then distilled and stored over 3 Å molecular sieves under argon. DMF and DMSO were obtained as anhydrous solvents from Aldrich. Triethylamine (TEA) and diisopropylethylamine (DIPEA) were dried by distillation from KOH pellets. Dry MeOH was obtained as anhydrous solvents from Aldrich. Triethylamine (TEA) and diisopropylethylamine (DIPEA) were dried by distillation from magnesium turnings. Diethyl ether was obtained by distillation from magnesium turnings. All spectra were run on Bruker AC-250 and AMX-400 spectrometers. All coupling constants are quoted in Hertz. All reactions were carried out under argon and in the absence of dioxygen. Dichloromethane (DCM), pyridine and acetonitrile were dried under reflux from calcium hydride and then distilled and stored over 3 Å molecular sieves under argon. DMF and DMSO were obtained as anhydrous solvents from Aldrich. Triethylamine (TEA) and diisopropylethylamine (DIPEA) were dried by distillation from KOH pellets. Dry MeOH was obtained as anhydrous solvents from Aldrich. Triethylamine (TEA) and diisopropylethylamine (DIPEA) were dried by distillation from magnesium turnings. Diethyl ether was obtained by distillation from magnesium turnings. All spectra were run on Bruker AC-250 and AMX-400 spectrometers. All coupling constants are quoted in Hertz.

Anhydrous DMMP (0.13 mL, 1.8 mmol) in dry DCM (0.4 mL) was added to a stirred solution of oxalyl chloride (0.08 mL, 0.9 mmol) in dry DCM (2 mL) under argon at −78 °C. After 2 min, hydroxyl nucleoside 2 (dried over P2O5 under high vacuum) (0.5 g, 0.8 mmol) in dry DCM/DMMP (0.8 mL/0.5 mL) was added over 5 min, the solution was then stirred for 15 min and anhydrous TEA (0.58 mL, 4.2 mmol) was added. The reaction was then stirred for 5 min and allowed to warm to r.t. over 1 h before being partitioned between water (10 mL) and DCM (10 mL). The organic layer was then washed successively with 10 mL each of brine, 1% aq. HCl solution, water, dilute aq. Na2CO3 solution (5%) and finally water. The organic layer was then dried (MgSO4) and evaporated and the crude product purified by silica gel chromatography (eluent gradient 10−25%, EtOAc in DCM) to yield 3 as a pale yellow solid (0.18 g, 36% yield). 1H NMR (250 MHz, CDCl3): δ 2.94 (2H, s, C5-CH2CHO), 4.57 (1H, dd, J = 3.5, 12.1, 51-H), 4.65 (1H, dd, J = 3.5, 6.2, 41-H), 4.82 (1H, dd, J = 2.6, 12.1, 51-H), 5.69 (1H, t, J = 6.1, 21-H), 5.85 (1H, dd, J = 6.0, 3.5, 31-H), 6.36 (1H, d, J = 6.4, 11-H), 7.28–7.60 (10H, m, ArH) and 7.90 (4H, m, ArH), 8.05 (2H, m, ArH), 8.53 (1H, s, NH) and 9.42 (1H, t, J = 11.1, C5-CH2CHO); 13C NMR (250 MHz, CDCl3): δ 37.0, 66.1, 71.4, 73.4, 80.6, 87.3, 113.3, 128.4, 128.6, 128.9, 129.7, 129.9, 130.3, 133.8, 137.0, 150.4, 164.0, 165.4; Electrospray-MS: 599 (M+H)+, Acc. Mass: 599.1676, C25H33N2O4 requires 599.1666 deviation 1.1 ppm.

5-[15-Trifluorouacemido-3,7,12-tris-(N-trifluoroacetyl)-3,7,12-triazapentadecyl]-uridine 4

Aldehyde nucleoside 3 (1 g, 0.33 mmol) was evaporated from dry pyridine (3 × 5 mL), then spermine (1.69 g, 8.4 mmol), anhydrous MeOH (5 mL) and dry pyridine (5 mL) were added and the solution stirred at r.t. under argon for 16 h until no starting material remained. Sodium borohydride (0.13 g, 3.3 mmol) was then added and after 1.5 h the solvent was evaporated. The resulting solid was then suspended in aq. NH4 solution (S.G. = 0.88) (10 mL), stirred for 2.5 h and then evaporated to dryness. After co-evaporation with dry pyridine (3 × 5 mL) the material was dissolved in dry pyridine (10 mL) and trifluoroacetic anhydride (5.66 mL, 40.1 mmol) added to the stirred solution at 0 °C under argon over 15 min. The reaction was then allowed to warm to r.t. and after 19 h was cooled in ice and MeOH (5 mL) was added. The solvent was then evaporated and the crude product partitioned between water (25 mL) and EtOAc (25 mL). The organic layer was dried (MgSO4) and evaporated and the crude product purified by silica gel chromatography (eluent 5−25% MeOH in DCM) to yield a moist, orange solid. This material was purified twice by silica gel chromatography (eluent 2.5−25% MeOH in DCM) to yield 4 as a pale yellow foam (0.25 g, 17% yield). 1H NMR (250 MHz, CD3OD): δ 1.70 (4H, m, CH2CH2N), 1.87–2.15 (4H, m, CH2CH2N), 2.62–2.75 (2H, m, C5-CH2CH2N), 3.31–3.39 (2H, m, C5-CH2CH2N), 3.43–3.71 (12H, m, CH2N), 3.75–3.86 (1H, m, 51-H), 3.87–3.94 (2H, m, 51-H), 4.02–4.14 (1H, m, 41-H), 4.14–4.30 (3H, m, 21-H and 31-H), 5.94 (1H, d, J = 4.0, 11-H), 8.05 (1H, d, J = 13.1, 16-H); 13C NMR (250 MHz, CD3OD): δ 24.9, 25.5, 26.8, 27.2, 29.2, 37.9, 38.3, 45.4, 45.8, 46.4, 47.1, 47.6, 62.1, 71.2, 75.9, 86.3, 90.6, 112.1, 113.7, 116.5, 119.4, 139.8, 152.4, 158.5, 163.4, 165.8; Electrospray-MS: 879 (M+Na)+, Acc. Mass: 879.2182, C25H33N2O4F3Na requires 879.2199 deviation 1.9 ppm. 

5-[2-(tert-Butyldimethylsilyloxy)ethyl]-uracil 6

Hydroxethyluracil 1 \textsuperscript{4} (10 g, 64.1 mmol) was dissolved in anhydrous DMF (200 mL) under argon. Imidazole (7.85 g, 0.12 mol) and tert-butyldimethylsilyl chloride (tBDMSCl) (8.99 mL, 34.6 mmol) were added and the reaction stirred at r.t. for 17.5 h. After work-up, the reaction was partitioned between DCM (800 mL) and dilute aqueous NaHCO\textsubscript{3} solution, washed further with aq. NaHCO\textsubscript{3} (34.3 mmol), and finally SnCl\textsubscript{4} (4.05 mL, 34.3 mmol) in dry acetonitrile (40 mL) were added. The cloudy solution was then stirred overnight at r.t. before heating to an oil bath temperature of 70 °C over 1 h. Some material dissolved but a precipitate remained so the reaction was worked up by addition of DCM (350 mL) and saturated aq. NaHCO\textsubscript{3} solution (275 mL). The aqueous layer was further extracted with DCM (150 mL) and the combined organic extracts were washed with brine (150 mL), dried (MgSO\textsubscript{4}) and evaporated. Following gel chromatography (eluent 10% MeOH in DCM) which gave debenzoylated nucleoside 7 (14.4 g, 60% yield). 1H NMR (250 MHz, CDCl\textsubscript{3}): \( \delta \) 9.02 (9H, s, tBu), 2.86–2.18 (1H, m, C5-\textsuperscript{3}Si) and 1.98–1.83 (2H, m, C5-\textsuperscript{4}Si). 13C NMR (250 MHz, CDCl\textsubscript{3}): \( \delta \) 19.3, 27.1, 29.9, 62.5, 109.0, 128.3, 130.2, 133.6, 135.4, 139.7, 151.9, 164.9; Electrospray-MS: 395 (M+H\textsuperscript{+}); Acc. Mass: 395.1973. C\textsubscript{12}H\textsubscript{13}N\textsubscript{5}O\textsubscript{3}Si requires 395.2321 deviation 0.3 ppm.

5-[2-(tert-Butyldimethylsilyloxy)ethyl]-2',3',5'-tri-O-benzyl-uridine 10

Protected nucleoside 8 (29.7 g, 41.6 mmol) was treated with saturated ammonia in MeOH (400 mL) and stirred at r.t. for 30 h. The solvent was then evaporated to give an orange-coloured oil which was purified by silica gel chromatography (eluent 4–10% MeOH in DCM) which gave debenzoylated nucleoside 9 as a white foam (13.8 g, 82% yield). 1H NMR (250 MHz, CD\textsubscript{2}OD): \( \delta \) 0.00 (6H, s, SiMe), 0.84 (9H, s, tBu), 2.47 (2H, t, \( J = 6.4 \)) (H, m, C5-CH\textsubscript{2}CH\textsubscript{2}OSi), 3.67–3.74 (3H, m, C5-CH\textsubscript{2}CH\textsubscript{2}OSi) and 5.37–5.40 (1H, s, ArH); 13C NMR (250 MHz, CD\textsubscript{2}OD): \( \delta \) 13.4, 19.2, 26.5, 31.6, 62.3, 62.5, 71.4, 75.8, 86.4, 90.6, 112.4, 140.1, 152.6, 165.9; Electrospray-MS: 403 (M+H\textsuperscript{+}); Acc. Mass: 403.1909. C\textsubscript{12}H\textsubscript{13}N\textsubscript{5}O\textsubscript{3}Si requires 403.2001 deviation 2.2 ppm.
Electrospray-MS: 527 (M + H)+, Acc. Mass: 527.2209, C_{27}H_{36}N_{2}O_{4}Si requires 527.2214 deviation 0.8 ppm.

5-[(2-tert-Butyldimethylsilyloxy)ethyl]-5′-O-(4,4′-dimethoxytrityl)-uridine 12

Nucleoside 10 (5.76 g, 14.3 mmol) was treated with DmtCl (5.81 g, 17.2 mmol) in dry pyridine (55 mL) under argon at rt. After 18 h a further amount of DmtCl (0.97 g, 2.86 mmol) was added. After 1 h, water (150 mL) and DCM (150 mL) were added and the aqueous layer further extracted with DCM (2 × 150 mL). The combined organic layers were dried (MgSO₄) and evaporated and the residual solvent removed by co-evaporation with toluene (2 × 50 mL). Purification by silica gel chromatography (eluent 0%–3% MeOH in DCM containing 0.2% TEA) gave 12 as a yellow foam (7.51 g, 74% yield). ¹H NMR (250 MHz, CDCl₃): δ 0.96 (6H, s, SiMe), 0.74 (9H, s, t-Bu), 1.96–2.07 (1H, m, C₅-CH₃CH₂OSi), 2.25–2.35 (1H, m, C₅-CH₃CH₂OSi), 2.32–3.32 (1H, dd, J = 3.1, 10.7, 5′-H), 3.38–3.43 (1H, dd, J = 2.3, 10.5, 5′-H), 3.45–3.57 (2H, m, C₅-CH₃CH₂OSi), 3.71 (6H, s, ArOCH₃), 4.16–4.20 (2H, m, 3′-H and 4′-H), 4.27 (1H, t, J = 4.3, 2′-H), 5.76 (1H, d, J = 4.0, 1′-H), 6.76 (4H, d, J = 8.9, ArH), 7.11–7.34 (10H, m, ArH and H6), 7.53 (1H, s, NH); ¹C NMR (250 MHz, CDCl₃): δ = 54.8, 18.2, 25.9, 30.4, 55.2, 61.0, 62.8, 70.4, 75.3, 83.9, 86.7, 90.5, 112.0, 113.3, 127.0, 128.0, 130.1, 133.5, 134.5, 137.3, 144.5, 151.3, 158.6, 164.1; Electrospray-MS: 705 (M + H)+, Acc. Mass: 705.3228, C_{27}H_{36}N_{2}O_{4}Si requires 705.3207 deviation 2.7 ppm.

From 5-[(2-tert-Butyldimethylsilyloxy)ethyl]-5′-O-(4,4′-dimethoxytrityl)-uridine 13, TBAF (1 M solution in THF; 10.7 mL, 10.7 mmol) was added to nucleoside 13 (5.9 g, 7.12 mmol) under argon at rt. The solution was stirred for over 42 h and a further 4.27 mL (4.27 mmol) TBAF were added in 1.42 mL (1.42 mmol) aliquots. The reaction was then worked up by addition of DCM (150 mL) and DCM (150 mL). The aqueous layer was further extracted with DCM (2 × 50 mL) and the organic layers were combined, dried (Na₂SO₄) and evaporated. Purification by silica gel chromatography (eluent 4%–6% MeOH in DCM containing 0.2% TEA) gave 13 as a cream-coloured foam (3.1 g, 75% yield) with identical spectral data to those reported above.

5-′O-(4,4′-Dimethoxytrityl)-uridine-5′-acetaldehyde 15

Via modified Swern oxidation. A two-necked 100 mL round bottom flask fitted with a 50 mL dropping funnel was charged with argon and cooled to −60°C in a dry ice/acetonitrile bath. Dry DCM (6.5 mL) followed by oxalyl chloride (0.18 mL, 2.04 mmol) were added with stirring, followed by dry DMSO (0.29 mL, 4.04 mmol) in dry DCM (2.5 mL). After 2 min, nucleoside 14 (1.09 g, 1.86 mmol) in dry DCM (11 mL) containing dry TEA (0.32 mL, 2.27 mmol) was added via the dropping funnel over 5 min. After a further 15 min dry TEA (1.01 mL, 7.25 mmol) was added and 5 min later the reaction was partitioned between cold saturated aq. NaHCO₃ solution (50 mL) and cold DCM (20 mL). The aqueous layer was further extracted with cold DCM (2 × 20 mL) and the combined organic layers were co-evaporated with toluene (10 mL). Purification by silica gel chromatography (eluent 3% MeOH in DCM containing 0.1% TEA) gave a pale yellow foam that was partitioned between saturated aq. NaHCO₃ solution (50 mL) and DCM (20 mL). The aqueous layer was further extracted with DCM (2 × 20 mL) and the combined organic layers were dried (Na₂SO₄) and evaporated to give 15 as a pale yellow foam (0.67 g, 61% yield). ¹H NMR (250 MHz, CDCl₃): δ 2.48 (2H, bs, C₅-CH₃CHO), 3.43 (1H, d, J = 8.9, 5′-H), 3.48 (1H, d, J = 8.9, 5′-H), 3.70 (6H, s, ArOCH₃), 4.09–4.11 (1H, m, 4′-H), 4.30–4.41 (2H, m, 2′-H and 3′-H), 5.90 (1H, d, J = 3.4, 1′-H), 6.74 (4H, d, J = 8.5, ArH), 7.14–7.29 (10H, m, ArH and H6), 7.82 (1H, s, NH), 9.21 (1H, s, C₅-CH₃CHO); ¹C NMR (250 MHz, CDCl₃): δ 19.2, 26.9, 50.3, 55.2, 61.9, 62.9, 70.3, 75.1, 84.0, 86.7, 90.8, 111.7, 133.3, 127.0, 127.6, 128.0, 128.1, 129.6, 130.1, 133.6, 133.8, 135.5, 135.6, 137.3, 144.6, 151.2, 158.6, 163.8; Electrospray-MS: 829 (M + H)+, Acc. Mass: 829.3500, C_{27}H_{36}N_{2}O_{4}Si requires 829.3520 deviation 2.5 ppm.

5-(2-Hydroxyethyl)-5′-O-(4,4′-dimethoxytrityl)-uridine 14

From 5-[(2-tert-Butyldimethylsilyloxy)ethyl]-5′-O-(4,4′-dimethoxytrityl)-uridine 12. Tetrabutylammonium fluoride (TBAF) (1 M solution in THF, 14 mL, 14 mmol) was added to nucleoside 12 (8.2 g, 11.6 mmol) under argon at rt. Over a 42 h period, the reaction was stirred and further TBAF solution (11.7 mL, 11.7 mmol) was added in five portions. The reaction was worked up by addition of water (250 mL) and DCM (250 mL) and the aqueous layer was further extracted with DCM (2 × 100 mL). The combined organic layers were dried (MgSO₄) and evaporated and the residue purified by silica gel chromatography (eluent 5%–7% MeOH in DCM containing 0.2% TEA) to give 14 as a cream-coloured foam (6.7 g, 98% yield). ¹H NMR (250 MHz, CDCl₃): δ 1.90 (2H, bs, C₅-CH₃CH₂OH), 3.25–3.43 (4H, m, C₅-CH₃CH₂OH and 5′-H), 3.68 (6H, s, ArOCH₃), 4.07 (1H, bs, 4′-H), 4.29 (2H, bs, 2′-H and 3′-H), 5.86 (1H, d, J = 3.4, 1′-H), 6.74 (4H, d, J = 8.6, ArH), 7.09–7.31 (10H, m, ArH and H6), 7.53 (1H, s, NH); ¹C NMR (250 MHz, CDCl₃): δ 30.2, 55.2, 61.0, 63.0, 70.6, 74.7, 83.8, 86.8, 89.0, 112.5, 113.3, 127.1, 128.0, 128.2, 130.2, 135.4, 135.5, 137.8, 144.4, 151.3, 158.6, 165.0; Electrospray-MS: 613 (M + Na)+, Acc. Mass: 613.2164, C_{27}H_{34}N_{2}O_{4}Na requires 613.2162 deviation 0.3 ppm.

5-[Trifluoroacetoamido-3,7,12-tris-((N-trifluoroacetyl)-3,7,12-triazapentadecyl]-5'-O-(4,4'-dimethoxytrityl)-uridine 16

Compound 15 (0.67 g, 1.14 mmol) was treated with spermine (1.15 g, 5.7 mmol) in dry pyridine and MeOH (3.5 mL each) under argon at rt. After stirring for 15 h, NaBH₄ (91 mg, 2.4 mmol) was added and the mixture was heated at 90°C for 30 min. The reaction was quenched with NaHCO₃ (0.54 mmol) and filter. The filtrate was concentrated under reduced pressure to give a red-brown oil which was purified by silica gel chromatography (eluent 2-50% MeOH in DCM containing 0.1% TEA) to give the polyamine conjugate 16 as a pale brown foam (0.75 g, 57% yield). 1H NMR (250 MHz, CDCl₃); δ 1.60–1.96 (9H, m, CH₂(CH₂)₃N), 2.15–2.28 (1H, m, CH₂(CH₂)₃N), 3.06–3.62 (16H, m, CH₂CH₂N), 2.15–2.5' (H, s, ArOH), 4.20 (1H, bs, 4'-H), 4.35–4.58 (2H, m, 2'-H and 3'-H), 5.89–5.92 (1H, m, 1'-H), 6.80–6.84 (4H, m, ArH), 7.20–7.40 (10H, m, ArH and H6), 7.55–7.84 (2H, m, NH); 13C NMR (250 MHz, CDCl₃); δ 23.4, 25.3, 26.0, 26.3, 29.3, 36.2, 36.6, 43.4, 44.1, 45.4, 45.6, 46.8, 54.8, 61.0, 70.0, 74.9, 83.7, 86.4, 89.9, 109.9, 110.5, 112.9, 114.2, 117.1, 120.2, 126.8, 127.6, 129.7, 134.8, 137.3, 143.9, 150.6, 156.4, 157.1, 158.3, 162.4; 19F NMR (250 MHz, CDCl₃); δ -76.5, -76.4, -76.4, -69.9, -69.9, -69.9, -69.3, -69.3; 1H NMR (CDCl₃ × 2, deuterated) δ -76.5, -76.4, -76.4, -69.9, -69.9, -69.9, -69.3, -69.3, -69.3, -69.3; Electrospray-MS: 1181 (M + Na)+, Acc. Mass: 1181.3536, C₂₅H₂₄N₂O₁₁F₁₁SiP requires 1181.3506 deviation 2.6 ppm.

5-(15-Amino-3,7,12-triazapentadecyl)-uridine 19

Nucleoside 4 (0.1 g, 0.12 mmol) was treated with methanolic ammonia (100 mL) at 55°C for 20 h. The solution was then removed in vacuo and the residue was partitioned between water (10 mL) and EtOAc (10 mL). The aqueous layer was further extracted with EtOAc (2 × 10 mL) before it was evaporated to dryness in vacuo to yield the deprotected polyamine conjugate 19 as a brown oil (40 mg, 72% yield). This was used as an HPLC standard. 1H NMR (250 MHz, CD3OD); δ 1.80 (4H, m, CH₂CH₂N), 2.05–2.18 (4H, m, CH₂CH₂N), 2.69–2.75 (2H, t, J = 6.7, C₅H₅CH₃CN), 3.02–3.24 (14H, m, CH₅N), 3.73–3.79 (1H, dd, J = 2.9, 12.4, 5'-H), 3.85–3.91 (1H, dd, J = 2.6, 12.4, 5'-H), 4.00–4.02 (1H, m, 4'-H), 4.15–4.22 (2H, m, 2'-H and 3'-H), 5.89 (1H, d, J = 4.0, 1'-H), 8.04 (1H, s, H6); Electrospray-MS: 473 (M + H)+, Acc. Mass: 473.3073, C₁₉H₁₄N₄O₂ requires 473.3088 deviation 3.1 ppm.

Oligonucleotide synthesis

RNA synthesis was carried out on an ABI-294 synthesiser on a 1μmol scale using 2'-O-tert-butyldimethylsilyl-3'-O-(2-cyanoethyl diisopropylphosphoramide) monomers and solid supports with phenoxycetyl protection for adenine and guanosine and acetyl protection for cytidine (Glen Research). 5'-FAM substrates were prepared using 5'-fluorescein reagent (Glen Research) (Fig. 1b). The syntheses were carried out using standard RNA synthesis procedures except for the use of 5'-benzylmercaptotetrazole (EMP Biotech) instead of tetrazelate. Deprotection of the oligonucleotides was carried out by treatment with NH₄OH/EtOH (3:1) at 55°C for 6 h and then TBAF (1 mL) for 20 h followed by desalting using Sephadex NAP10 columns. The following oligonucleotides were prepared, where U* represents a polyamine-conjugated uridine synthesised using phosphoramidite 18: 5'-FAM substrate, 14-mer, M = 4828.08, F₂₅₀ nm = 146.0 μM cm⁻²; 5'-FAM-UCG CAG UCA UAU UU-3'; tricyclic riboamide A, 30-mer, M = 10369.48, F₂₅₀ nm = 401.2 μM cm⁻²; 5'-AAA UGA AGA AGC GAA CCA GAG CAG AAG CAC ACG CC-3'; riboamide strand B, 21-mer, M = 6729.09, F₂₅₀ nm = 238.2 μM cm⁻²; modified riboamide strand B synthesised using phosphoramidite 18 in place of a single uridine residue, M = 6957.44, F₂₅₀ nm = 238.2 μM cm⁻², U* where U* is replaced by the conjugate U*, U** where U** is replaced by the conjuga-
gate, U₄¹ is replaced by the conjugate and U₄² where U₄₂ is replaced by the conjugate.

r(U*G) Dimer
The dimer r(U*G) was prepared on an ABI-294 synthesiser as described above, employing polyamine monomer 18. Following detritylation the functionalised solid support was heated in conc. aq. NH₄H₂OAc (3:1) at 55°C for 6 h. After removal of the support the solution was evaporated and the residue treated with TEA-3HF (1 mL) for 20 h. After quenching with water (0.2 mL) the sample was precipitated by addition of 1-butanol (15 mL) at −20°C. The sample was then recovered by centrifugation for 10 min at 3500 rpm and the solvent decanted. The residue was dissolved in sterile water and the resulting material was purified by reverse-phase (RP) HPLC on a Jones C18 column (25 cm × 4.6 mm). Buffer A = 100 mM triethylammonium acetate solution, pH 7, buffer B (100 mM triethylammonium acetate solution, pH 7, 50% acetonitrile) with a gradient of 0–15% B in 20 min. The retention time of the dimer was 13.5 min. The sample was concentrated to 500 µL to give pure dimer (4.9 OD₂₆₀ units). MALDI-TOF MS: 819 (M + H)⁺. 3¹P NMR (250 MHz, D₂O; δ −0.49).

Enzymatic digestion of r(U*G) dimer
0.25 OD₂₆₀ units of r(U*G) dimer were incubated with 10 µL of RNase I (1000 units, New England Biolabs) in a solution of 10 mM Tris HCl (pH 8.0) and 100 mM NaCl at 37°C. After 3 h the reaction solution was adjusted to 10 mM MgCl₂ and 1 µL alkaline phosphatase (0.1 units, Sigma) was added. Monitoring by HPLC, further aliquots of RNase I (total volume used was 18 µL) were added over 34 h until the dimer was completely digested.

The reaction was monitored by RP HPLC using the same gradient as employed for purification of the dimer. Retention times: 5-(15-amino-3,7,12-triazapentadecyl)-uridine (19) 3.8 min, r(U*G) dimer 13.5 min, rG 15.5 min.

Purification of oligoribonucleotides
Modified and unmodified ribozyme strands A and B were purified by denaturing preparative 20% PAGE, excised from the gel and extracted by soaking overnight in ammonium acetate buffer [1–2 mL, 0.5 M NH₄H₂OAc, 1 mM EDTA (pH 6.5)] and then desalted using Sep-Pak columns.
The 5'-FAM substrate was purified by reverse-phase HPLC (RP HPLC): on a Jones C18 column (25 cm × 4.6 mm) using the following conditions: buffer A: 100 mM triethylammonium acetate solution, pH 6.5, 5% acetonitrile; buffer B: 100 mM triethylammonium acetate solution, pH 6.5, 5% acetonitrile. Gradient of 0–75% B in 25 min: retention time approx. 18 min. Following RP HPLC purification, the oligonucleotide was desalted by extensive dialysis against water.

All oligoribonucleotides were analysed by MALDITOF MS and molecular weights were found to be within 4 Da of the calculated value.

Determination of Michaelis–Menten parameters
With MgCl₂, A stock solution of the wild-type ribozyme strands A and B (50 nM each) in 40 mM Tris HCl (pH 7.5) and a separate stock solution of the 5'-FAM substrate (1 µM) were prepared. The ribozyme solution was denatured at 90°C for 1 min and then placed in a water bath set at 37°C for 10 min. The concentration of MgCl₂ in the ribozyme stock solution was adjusted to 10 mM and it was incubated for a further 15 min at 37°C. During this 15 min the substrate stock solution was denatured at 90°C for 1 min and then placed in the water bath set at 37°C for 10 min. The appropriate quantity of ribozyme was added to the reaction mixtures containing MgCl₂ (final concentration 10 mM) and Tris HCl (pH 7.5) (final concentration 40 mM) before initiating the reaction with the suitable amount of substrate and brief vortexing. The final concentrations of substrate were 1–400 nM and for the ribozyme 0.1–20 nM. The reactions were monitored by the removal of an appropriately sized aliquot at suitable time intervals. The samples were immediately quenched by addition to 50 mM EDTA (pH 7.5) (5 × aliquot volume). The samples were analysed by denaturing HPLC (dHPLC) (DNA Sep⁺ Column, Transgenomic Inc.) using the following conditions: buffer A: 2.5 mM tetrabutylammonium bromide, 0.1% acetonitrile, 2 mM EDTA, pH 7.5; buffer B: 2.5 mM tetrabutylammonium bromide, 70% acetonitrile, 2 mM EDTA, pH 7.5. The retention time of the fluorescent product and substrate were approximately 5 and 7.5 min, respectively. The initial rates of reaction at the different substrate concentrations were determined and the kinetic parameters were calculated by non-linear regression fitting to the Michaelis–Menten equation [eqn. (1)] using KaleidaGraph (Synergy Software)

\[
y = \frac{k_{\text{cat}} [S]}{[E] + K_M + [S]} \tag{1}
\]

where y = initial rate of reaction, [E] = total concentration of ribozyme, [S] = concentration of substrate.

With spermine. The experiments were done in the same way as above with spermine (10 mM) replacing MgCl₂.

Rate of reaction of the polyamine-modified hairpin ribozymes
The four modified ribozymes were tested for cleavage activity with and without the presence of 10 nM MgCl₂ using 100 nM substrate and 20 nM ribozyme in 40 mM Tris HCl pH 7.5. The reactions were carried out in the same way as the Michaelis–Menten experiments above except for the experiments with no MgCl₂ where water was added to the ribozyme instead.

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