Synthesis and incorporation of carbocyclic nucleosides into a hammerhead ribozyme domain–RNase resistance and catalytic activity

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A hammerhead ribozyme domain incorporating (−)-carbodine 8a and (−)-aristeromycin 8b at selected positions, manifests increased RNase resistance and exhibits significant catalytic activity.

RNA enzymes or ribozymes are endonucleases which cleave, in a sequence dependent manner, their RNA substrates.[1] Among natural ribozymes, the hammerhead motif, as found in the self-cleaving domain of plus-strand satellite RNA from tobacco ringspot virus,[2] has served to engineer *trans* acting ribozymes.[3] Because of its simplicity the hammerhead system has received a great deal of atttention focusing at the elucidation of its three dimensional structure,[4] the determination of the kinetic parameters of the cleavage reaction[5] as well as the crucial role of the conserved residues located in the central core[6] (Fig. 1). Many efforts in hammerhead ribozymology are directed at developing therapeutically active ribozymes. This might be achieved by the construction of synthetic sequences, amenable to exogeneous cell delivery, manifesting a maximum catalytic activity and an increased resistance to RNases. A variety of modifications have been proposed to avoid 3′-exonuclease degradation.[7] However, for the elaboration of endonuclease resistance ribozymes considerable hurdles need to be overcome. The conserved central core is particularly exposed to these nucleases. As an alternative to the previously proposed modifications we report here the exchange of a number of nucleosides of the ribozyme central core by their carbocyclic analogues. As a prerequisite for this study we devised a rapid and efficient access to the carbocyclic nucleosides (−)-carbodine **8a** and (−)-aristeromycin **8b**[9] starting from lactone (−)-**1**[10] as outlined in Scheme 1. Both nucleoside analogues were further elaborated to their appropriate 3′-phosphoramidite derivatives **7a,b** in view of their incorporation into RNA sequences by chemical synthesis. Thus, **8a** and **8b** could be readily introduced in place of cytidine at the cleavage site (position 17) of the normal substrate **S** (C_{17}) and **S(A)** (A_{17}) of ribozyme **R** to give **S(8a)** and **S(8b)**, respectively. Similarly, one single (−)-aristeromycin unit was incorporated in place of adenosine residues A_6, A_{14} and $A_{15.1}$ of the central core of **R** leading to the three analogues **R(6)**, **R(14)** and **R(15.1)** (Fig. 1).

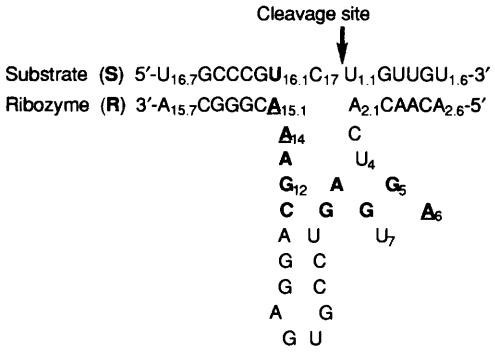
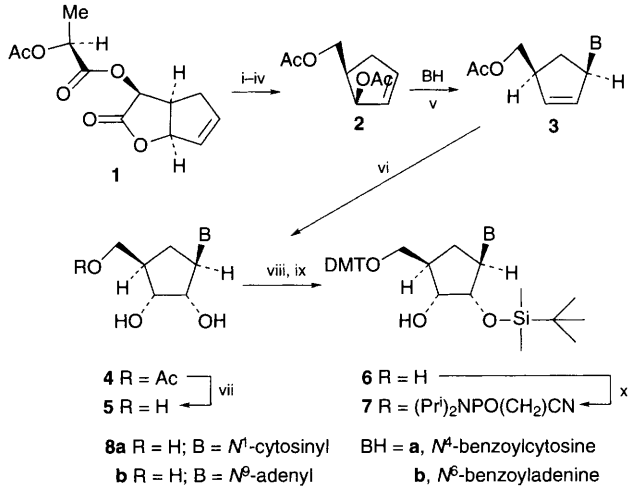


Fig. 1 Hammerhead ribozyme domain. Bold letters indicate consensus residues. Underlined letters indicate the positions of **R** which have been substituted by (−)-aristeromycin **8b**.

In a first series of experiments we compared the initial digestion rates of normal [**S** and **S(A)**] and modified [**S(8a)** and **S(8b)**] substrates by various endonucleases and alkali (Table 1, Fig. 2). In all cases an increased cleavage resistance at the substituted position was observed. This could be partly due to the higher p*K*a value of the 2′-OH upon O-4′ substitution and/or ring conformation alteration, cyclopentane being more flexible than furan.[11] The resistance was found to be particularly strong in the case of RNases Φm and CL3. Interestingly, ribonuclease digestion of the phosphodiester linkage on the 5′-side of the modified base is also reduced, with the exception of RNase T2



Scheme 1 *Reagents and conditions*: i, $LiAlH_4$, THF; ii, $NaIO_4$, MeOH–H_2O; iii, $NaBH_4$, MeOH; iv, Ac_2O, DMAP, pyridine, 40%, 4 steps; v, BH, Cs_2CO_3, $Pd(PPh_3)_4$, DMF, 55 °C, 40–45%; vi, OsO_4, TMNO, acetone–H_2O, 30%; vii, NaOH, THF–H_2O, 95%; viii, dimethoxytrityl chloride, pyridine; ix, *tert*-butyldimethylsilyl chloride, imidazole, DMF; x, 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite, *N,N*-diisopropylethylamine, *N*-methylimidazole, CH_2Cl_2, 30%, 3 steps.

Table 1 Ratio of the initial cleavage rates of substrates **S** or **S(A)** *vs.* those of the corresponding modified substrates **S(8a)** or **S(8b)** at positions 17 and 16.1 by alkali and RNases

	OH^-	U2 (A)	T2 (ns)	A (C, U)	Φm (A, U)	CL3 (C)
Position 17						
S/S(8a)	5	—	10	6	—	400
S(A)/S(8b)	5	5	3	—	25	—
U 16.1						
S/S(8a)	3	—	1	14	—	nd
S(A)/S(8b)	3	nd	1	—	2	—

Letters in brackets indicate enzyme specificity. Ns = non specific. The amount of cleavage at a given position was corrected by taking account of cleavage on the 5′-side. Nd = not determined.

(Table 1). This might result from the removal of hydrogen bonding between O-4′ and 2′-OH of the residue in 5′.[11] Similar effects were observed qualitatively upon digestion of modified ribozymes **R(6)**, **R(14)** and **R(15.1)**.

The k_{cat} values obtained upon cleavage of **S(8a)** and **S(8b)** by ribozyme **R** were similarly 800-fold reduced when compared to those obtained with unmodified substrates **S** and **S(A)** (Table 2). It is unlikely that the modification of the p*K*a of the 2′-OH and/or a ring conformational change could have such an important effect.[11] Therefore, this effect should be largely ascribed to the removal of the nπ perpendicular interaction between O-4′ of C_{17} (or A_{17}) and the plane of A_6, as seen in the crystal structure.[4h] If correct, this interpretation would stress the importance of this interaction for the catalytic activity.

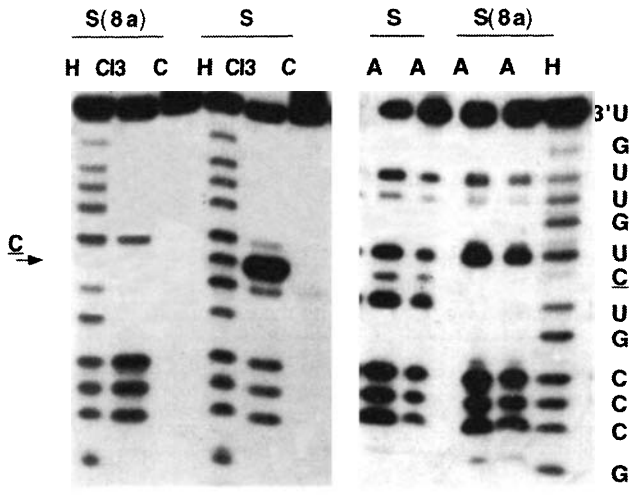


Fig. 2 RNase digestion profiles of the 5′-^{32}P labelled substrates **S** and **S(8a)**. Lanes Cl3: Limited RNase CL3 digestion. Lanes A: Limited RNase A digestion (two enzyme concentrations). Lanes H: Partial alkaline hydrolysis. Lanes C: Control. The underlined letter indicates the position which has been substituted by (−)-carbodine.

Table 2 Kinetic parameters for modified ribozyme-substrate systems

Ribozyme	Substrate	k_{cat}/min^{-1}	K_m/ nmol dm^{-2}	k_{cat}/K_m (dm^3 μmol^{-1} min^{-1})
R[a]	**S**	15	750	20
R[b]	**S(8a)**	0.018	1*	18
R[a]	**S(A)**	1.6	125	13
R[b]	**S(8b)**	0.002	0.25*	8
R(6)[b]	**S**	0.56	26	21
R(14)[a]	**S**	1.1	240	4.5
R(15.1)[a]	**S**	7	320	22

The cleavage reactions were performed at 37 °C in 20 mmol dm^{-3} $MgCl_2$, 50 mmol dm^{-3} Tris.HCl (pH 8). The k_{cat} and K_m values were determined by: [a] multiple turnover experiments; [b] under single turnover conditions. The same k_{cat} values were obtained under multiple turnover conditions with variations well below 30%. The dissociation constant k_{-1} of the ribozyme–substrate complex **R-S(8b)** was measured to be 3×10^{-3} min^{-1} by pulse-chase experiments. (ref. 5) .The K_m values marked by an asterisk were calculated according to $K_m = (k_{-1} + k_{cat})/k_1$ assuming $k_1 = 2.10^7$ mol dm^{-3} min^{-1}.

When the capacity of **R(6)**, **R(14)** and **R(15.1)** to cleave substrate **S** was examined, it was observed (Table 2) that the k_{cat} value was moderately decreased for **R(15.1)** and more significantly diminished for **R(14)** and **R(6)**. (−)-Aristeromycin substitution at position 6 of **R(6)** should alter the hydrogen bonding network[4h] at this position, thus perturbing the catalytic pocket. In the crystal structure, A_{14} and $A_{15.1}$ are placed at the junction of helices II and III and are connected to the catalytic pocket by non canonical base pairing.[4h] We surmise that the increased basicity of the heterocycle[12] at the substituted position modulates its donor-acceptor capacity. Substitution at position 14 should reinforce the hydrogen bonds between N-1 of A_{14} and the 2′-OH group of U_7, limiting the flexibility of the catalytic pocket as indeed observed in solution[4b,4g] and thus impairing the catalytic activity.

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