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SYNTHESIS OF PYRIDINONE RIBONUCLEOSIDE 3'-*O*-PHOSPHORAMIDITES AND THEIR INCORPORATION INTO OLIGORIBONUCLEOTIDES

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Abstract: Protected pyridin-2- and pyridin-4-one ribonucleosides **3** and **9** were synthesized using a one-pot reaction of silylated bases with 1-*O*-acetyl-tri-*O*-benzoyl-β-D-ribofuranose (**2**) in the presence of $CF_3SO_3SiMe_3$. The nucleosides were converted in 4 steps into 3'-*O*-phosphoramidites **7** and **11** which were incorporated into hammerhead ribozyme substrates using solid-phase phosphoramidite chemistry.

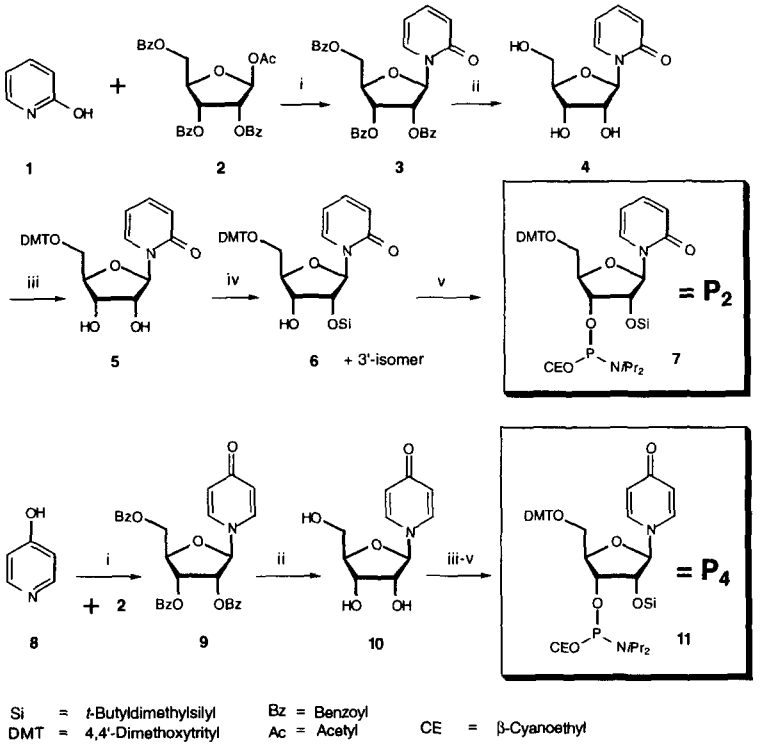
As part of our studies[1-3] on the mechanism of action of hammerhead ribozymes[4] we were interested in the effect of incorporation of base modified nucleosides into the hammerhead domain, as well as into the RNA substrate. In particular, we were interested in universal base analogs[5] that behave indiscriminately towards opposite bases, as well as in hydrophobic base analogs and/or base analogs lacking certain H-bonding capability. We[1,2] and others[6] reported the incorporation of abasic nucleoside analogs into ribozymes. Surprisingly, several ribozymes containing 1-deoxy-D-ribofuranose instead of uridine at the U4 and/or U7 position of the catalytic core demonstrated high cleavage activity.[2] By designing additional analogs that retain close structural and steric relationships to the natural bases, but display novel hydrogen-bonding patterns and different sugar pucker, new data on mechanism of action of hammerhead ribozyme could be generated. Here we report the synthesis of two isomeric "3-deaza" pyrimidine analogs and their incorporation into hammerhead ribozyme substrates.

Pyridin-2- and pyridin-4-one ribonucleosides were first prepared by Pischel and Wagner[7] by condensation of silver salts of 2- and 4-hydroxypyridine with 1-chloro-2,3,5-tri-*O*-benzoyl-D-ribofuranose to afford *O*-glycosides, followed by O,N-rearangement in boiling toluene in the presence of $HgBr_2$. The same compounds were also synthesized by the Hilbert-Johnson reaction of 2- and 4-ethoxypyridines with a 1-chloro sugar.[7] Later Vorbrüggen *et al.*[8] applied the silyl Hilbert-Johnson reaction catalyzed by $SnCl_4$ to the synthesis of pyridinone nucleosides. While silylated 2-hydroxypyridine (**1**) (Figure 1) reacted smoothly with 1-*O*-acetyl-2,3,5-tri-*O*-benzoyl-β-D-ribofuranose (**2**) to give a high yield of *N*-1-riboside, the analogous reaction of 4-hydroxypyridine (**8**) took place only under forced conditions and in a moderate yield. An improvement in the synthesis of ribonucleosides was reported[9] by switching the Friedel-Crafts catalyst from $SnCl_4$ to trimethylsilyl triflate (TMSTfl) which has lower Lewis acidity compared to $SnCl_4$. Consequently, higher yields of desired *N*-1-nucleosides are obtained in the case of more basic silylated heterocycles like cytosine and 4-hydroxypyridine.

We used the one-pot procedure[10] for the synthesis of pyridinone nucleosides **3** and **9** from silylated bases **1** and **8** and 1-*O*-acetyl sugar **2** in the presence of TMSTfl (Figure 1).[11] This procedure proved to be particularly suitable for the synthesis of pyridin-2-one nucleoside **3** since silylated 2-hydroxypyridine is a volatile compound (b.p. 63 °C/12 mm Hg)[12] that is not easily dried by evaporation and coevaporation with toluene, a requirement when hexamethyldisilazane and/or $(CH_3)_3SiCl$ are used for the preparation of the silylated base. Protected nucleoside **3**, obtained in 98% yield after flash chromatography (2-10% MeOH in CH_2Cl_2) was saponified using $NaOCH_3/CH_3OH$ to give **4**[13] in 91% yield. Dimethoxytrityl protection of the 5'-OH under standard conditions (DMT-Cl, DMAP, Et_3N, Pyr) afforded, after chromatography (0.5-10% MeOH in CH_2Cl_2) the 5'-*O*-DMT derivative **5** in 76% yield. Selective protection of the 2'-OH using *t*-butyldimethylsilyl chloride proceeded in the

presence of $AgNO_3$ and pyridine in THF[14] to afford a mixture of 2'-*O*-TBDMSi, 3'-*O*-TBDMSi isomers and some 2',3'-bis-*O*-TBDMSi compound. Separation of these products using flash chromatography (20-50% EtOAc in hexanes) afforded a faster running 2'-*O*-TBDMSi isomer **6**[15] in 69% yield and slower running 3'-*O*-TBDMSi isomer in 17% yield. The structures of these isomers were unequivocally determined using a series of homodecoupling ^1H NMR experiments. Phosphitylation of **6** using 2-cyanoethyl *N,N*-diisopropyl-chlorophosphoramidite in the presence of *N,N*-diisopropylethylamine and 1-methylimidazole yielded the desired 3'-*O*-phosphoramidite **7**, ^{31}P NMR in $CDCl_3$ δ 148.0 and 147.7 ppm for two *P*-diastereoisomers, respectively.

Figure 1. Synthesis of Pyridin-2(4)-one Nucleoside 3'-*O*-Phosphoramidites



Reagents and Conditions: *i*: *N,O*-bis(trimethylsilyl)acetamide (BSA)/TMSTfl/CH_3CN, 70 °C, *ii*: NaOMe/MeOH, *iii*: DMT-Cl/DMAP/Et_3N/Pyr, *iv*: TBDMSi-Cl/$AgNO_3$/Pyr/THF, *v*: P(OCE)(N-*i*Pr_2)Cl/DIPEA/1-MeIm.

Protected pyridin-4-one nucleoside **9**[8] was prepared in 93% yield in an analogous way to that for the synthesis of **3**. It is worth noting that procedure of Vorbrüggen *et al.*,[9] in our hands, resulted in a poor yield of the desired *N*-1-nucleoside caused by the competing formation of the *O*-4-riboside and decomposition products.[9] Debenzoylation of **9** using NaOMe yielded **10**[8] in 84% yield. 5'-*O*-Dimethoxytritylation under the conditions used for the synthesis of **5** yielded 5'-*O*-dimethoxytrityl derivative in 67% yield. Selective protection of the 2'-OH with TBDMSi group proceeded as for pyridin-2-one nucleoside **5**, yielding the mixture of 2'-, 3'- and 2',3'-bis substituted nucleosides. Careful separation of this mixture by column chromatography using 0.5-5% MeOH in

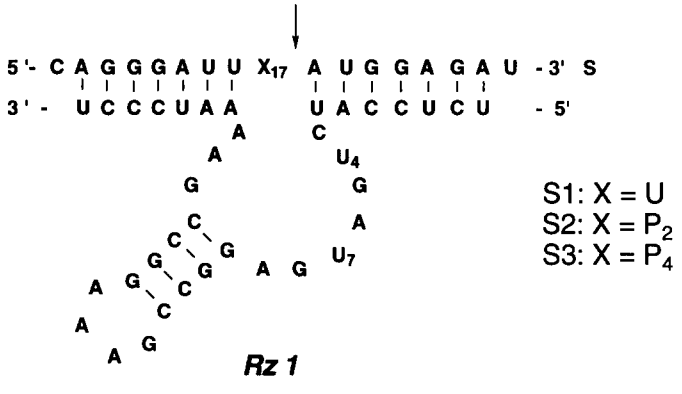
EtOAc for elution yielded the desired faster moving 2'-*O*-TBDMSi isomer[16] in 41% yield and slower moving 3'-*O*-isomer in 36% yield. Phosphitylation proceeded smoothly to give 3'-*O*-phosphoramidite **11** in 94% yield, ^{31}P NMR in $CDCl_3$ δ 150.5 and 147.3 ppm for two P-diastereoisomers, respectively.

It is worth nothing that depyridination of 1-(2-deoxy-β-D-ribofuranosyl)-pyridin-2-one in a buffer solution at 60 °C was speculated by Strazewski and Tamm[17]. This prompted us to determine stability of the pyridine nucleoside analogs **4** and **10** under acidic and basic conditions, i.e. how suitable they are for the oligonucleotide synthesis and deprotection conditions. We subjected nucleoside analogs **4** and **10** to 80% aqueous acetic acid at rt for 24 hours as well as to conc. ammonia-methanol 3:1 at 65 °C for 18 hours. In each case the starting material was recovered intact (judged by TLC and ^1H NMR).

Phosphoramidites **7** and **11** were incorporated into oligoribonucleotides using standard protocol for synthesis and deprotection[18,19] with ~98% average stepwise coupling yields at 300 sec. coupling time which corresponded to 65% yield of the full length material for 15 mers S2 and S3.

To verify incorporation of unaltered pyridine nucleoside analogs **4** and **10** in modified oligoribonucleotides used in this study we also subjected aliquot's of gel purified[3] substrates S2 and S3 to the nucleoside compositional analysis[20] which demonstrated absence of any additional modified nucleosides except **4** (S2) and **10** (S3).

Figure 2. Hammerhead Ribozyme and Substrates Containing Pyridinone Residues



Considerable attention has been devoted recently to the functional group modification studies of the nucleotides in the catalytic core of hammerhead domain.[21] At the same time, elucidation of the structural requirements for the nucleotide in the cleavage site (N17) has received less attention.[22] We incorporated nucleotides **7** and **11** into position 17 (S2 and S3 respectively, Figure 2) and compared the cleavage rates for these substrates relative to the cleavage of a substrate containing U at the cleavage site (S1), using wild type Rz 1 under single turnover conditions.

Interestingly, the substrate with pyridin-2-one at the cleavage site (S2) was cleaved almost 2 times faster than the "U" substrate S1 and 20 times faster than substrate S3 containing pyridin-4-one. Several factors can contribute to this difference: (a) an altered syn-anti equilibrium in nucleotides **7** and **11** compared to uridine, (b) different ribose puckering, (c) altered ability for stacking interactions due to the changed polarity of the heterocycle, (d) change in the pKa of the 2'-OH in the modified nucleotides.

Analysis of ^{1}H-NMR spectra (D_2O) of pyridin-2-one -riboside **4** showed an up field shift of H-1' (δ 6.17 ppm) compared to uridine (5.90 ppm), at the same time the chemical shift of H-2'-remained unchanged (δ 4.37 ppm for **4** vs. 4.34 for uridine) indicating subtle changes in the syn-anti equilibrium.[23] Analysis of syn-anti equilibrium in nucleoside **10** as well as comparative analysis of nucleosides **10** and **4** is not possible due to the symmetry of pyridin-4-one heterocycle.

Table 1. Cleavage Rates for Substrates S1-S3 and Sugar Puckering of Modified Nucleosides

Nucleoside	$J_{1',2'}$(Hz)[a]	N_x (%)[b]	K_{obs} (min^{-1})[c]	Substrate
Uridine	4.4	50.7	1.17	S1
Pyridin-2-one 4	3.4	65.2	2.01	S2
Pyridin-4-one 10	5.6	33.2	0.095	S3

[a]^{1}H-NMR spectra were recorded at 25 °C in D_2O. [b]Nx is the % population of N conformer (C3'-endo-C2'-exo), determined from the equation[24] %N = 100(7.9- $J_{1',2'}$)/6.9. [c]Substrates S 1-3 were tested with Rz 1 (Fig. 2) at [Rz] = 100nM, [S] = 1nM, 50 mM Tris-Cl pH 7.7, 25 °C, 10 mM Mg^{2+}.

Table 1 gives estimates of sugar puckering for pyridine-2-one-riboside **4**, pyridine-4-one riboside **10** and uridine based on the concept of pseudorotation[25] along with the cleavage rates (K_{obs}) for modified short substrates. Analysis of the data in Table 1 indicates that an increase in mole fraction of N conformer (C3'-endo-C2'-exo) in the order **10>Urd>4** is associated with an increase in the cleavage rate of the substrates with these modifications in the cleavage site. This observation is tempered by the fact that the S-N equilibrium is characterized by a low (1-2 kcal/mol) energy barrier,[26] and that the observed sugar pucker on the nucleoside level may differ from the one in the complete substrate. Nevertheless, this data could indicate favorable sugar puckering for stabilization of the transition state in hammerhead cleavage.

The effect of incorporation of nucleotides **7** and **11** in the catalytic core on hammerhead ribozymes will be reported in due course.

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11. In a typical procedure 2- or 4-hydroxypyridine (**1**) or (**8**) (2.09 g, 22 mmol), 1-*O*-acetyl-2,3,5-tri-*O*-benzoyl-β-D-ribofuranose (**2**) (10.08 g, 20 mmol) and N,O-bis-trimethylsilylacetamide (5.5 ml, 22 mmol) were dissolved in dry CH_3CN (100 ml) under argon at 70 °C (oil bath) and the mixture stirred for 10 min. $CF_3SO_3SiMe_3$ (5.5 ml, 28.5 mmol) was added and the mixture stirred for additional hour for **1** or four hours for **8**. Standard work up and column chromatography afforded target compounds.

^1H NMR ($CDCl_3$) for **3**: δ 8.21-7.35 (m, 17H, Bz, 2-Py), 6.69 (d, $J_{1',2'}$=4.4, 1H, H-1'), 6.60 (d, J = 8.8, 1H, 2-Py), 6.14 (m, 1H, 2-Py), 5.98 (dd, $J_{3',2'}$=5.7, $J_{3',4'}$ =5.8, 1H, H-3'), 5.89 (dd, 1H, H-2'), 4.95 (dd, $J_{4',5'}$=2,9, $J_{5',5''}$=12.2, 1-H, 5'-H), 4.85 (m, 1H, H-4'), 4.76 (dd, $J_{4',5'}$=4,0, $J_{5',5''}$=12.2,1-H, 5''-H)

^1H NMR ($CDCl_3$) for **9**: δ 8.32-7.42 (m, 17H, Bz, 4-Py), 6.36 (m, 2H, 4Py), 5.86 (dd, 1H, H-2'), 5.80 (d, $J_{1',2'}$=4.9, 1H, H-1'), 5.71 (dd, $J_{3',2'}$=5.3, $J_{3',4'}$ =5.2, 1H, H-3'), 4,98 (dd, $J_{4',5'}$=2.7, $J_{5',5''}$=12.5, 1-H, 5'-H) 4.89 (m, 1H, H-4'), 4.78 (dd, $J_{4',5''}$=3.0, $J_{5',5''}$=12.5, 1-H, 5''-H)

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15. Selected ^1H NMR ($CDCl_3$ + D_2O) for **6**: δ 6.19 (d, $J_{1',2'}$=1.7, 1H, H-1'), 4.42 (dd, $J_{3',2'}$=4.7, $J_{3',4'}$ =7.4, 1H, H-3'), 4.34 (dd, 1H, H-2'), 4.14 (m, 1H, H-4'), 1.00 (s, 9H, *t*-Bu), 0.38 (s, 3H, Me), 0.25 (s, 3H, Me),

16. Selected ^1H NMR (acetone-d_6 + D_2O) data for 2'-*O*-TBDMSi isomer: δ 5.51 (d, $J_{1',2'}$=6.2, 1H, H-1'), 4.54 (dd, $J_{2',3'}$=5.2, 1H, H-2'), 4.39 (dd, $J_{3',4'}$=5.4, 1H, H-3'), 4.29 (m, $J_{4',5'}$=2.9, 1H, H-4'), 0.97 (s, 9H, *t*-Bu), 0.18 (s, 3H, Me), 0.09 (s, 3H, Me).

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20. The substrates were converted to nucleosides by incubation of 0.3 A_{260} units of oligonucleotide with 10 units of P1 nuclease (EC 3.1.30.1; Boehringer Mannheim) and 2 units of calf intestinal alkaline phosphotase

(EC 3.1.3.1; Boehringer Mannheim) in 30 mM NaOAc, 1 mM $ZnSO_4$, at pH 5.2 (total volume = 100 µl) overnight at 50 °C. The digested material was injected directly onto a C18 coumn (Waters, Symmetry, ODS 4.6 x 250 mm), and nucleosides were separated by gradient of buffer A (50 mM potassium phosphate, pH 7.0) and B (95% MeOH-5% water); 0-90% B in A over 35 min. The retention times were compared with monomer standards. Since 2-pyridinone nucleoside has λ_{max} at 300nm[7] the absorbance was monitored at 300 and 265 nm. At these conditions the following elution times (min) were observed: S2 2-Pyridinone (11.07) λ_{max} 299.8nm; S3 4-Pyridinone (6.78) λ max 265.3nm.

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