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Synthesis of 1-Deoxy-1-*C*-(*p*-Aniline)-β-D-Ribofuranose and Its Incorporation into Hammerhead Ribozymes

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Abstract: *p*-Aniline*C*-ribofuranoside **11** was prepared in 5 steps from 1-bromo-4-lithiobenzene (**1**) and D-ribono-1,4-lactone **2**. A three step derivatization of **11** yielded 3'-*O*-phosphoramidite **14** which was incorporated into ribozymes using the solid phase phosphoramidite procedure.

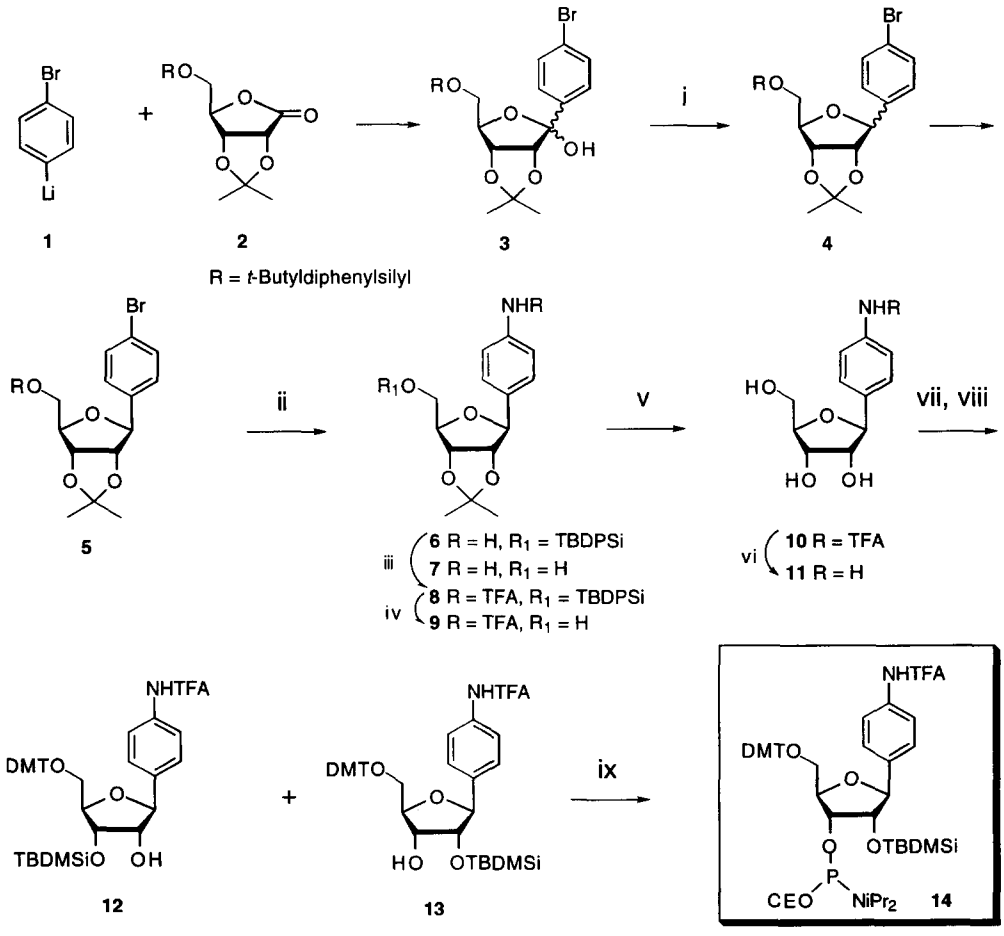
As part of our studies[1-4] on the molecular mechanism of action of hammerhead ribozymes, we were interested in the effect of incorporation of base modified nucleosides into the hammerhead domain as well as into the RNA substrate. In their mutagenesis study Uhlenbeck and colleagues[5] argued that the poor cleavage of NUG target sequences by hammerhead ribozymes could be the result of the formation of a base pair between G in the cleavage site (G17) and the C3 residue of the catalytic core. To test this hypothesis, incorporation of nucleoside analogs that lack H-bonding capabilities into the C3 position of the catalytic core was desirable. If the disruption of this hypothetical G-C base pair could be achieved without significant changes in geometry of the transition state, one could obtain hammerhead ribozymes that efficiently cleave UG sites. Recently, the crystal structure of a hammerhead ribozyme was reported,[6] suggesting that the C3 amino group plays a role in coordinating a possibly catalytic $Mg(H_2O)_5^{2+}$ ion. Additional evidence that the amino group of C3 is essential was obtained recently through chemical modification studies.[7] Therefore, we synthesized a cytidine analog that retains the amino group implicated in $Mg(H_2O)_5^{2+}$ binding and yet is devoid of any additional hydrogen bonding functionalities. Here we describe the synthesis of *p*-aniline *C*-ribofuranoside (Fig 1), a 1,3-deaza analog of cytidine, and its incorporation into ribozymes.

1-Bromo-4-lithiobenzene (**1**) was prepared from 1-bromo-4-iodobenzene or 1,4-dibromobenzene and BuLi and reacted with 5-*O*-*t*-butyldiphenylsilyl-2,3-*O*-isopropylidene-D-ribono-1,4-lactone (**2**), as described for the preparation of analogous 1-C-phenyl ribofuranoside.[4,8] Reduction of the crude α/β mixture of hemiacetals **3** using Et_3SiH and $BF_3 \bullet Et_2O$ in acetonitrile proceeded stereoselectively to afford a 1:4 mixture of α/β anomers **4**. Column chromatographic separation yielded pure β anomer **5** in 34% overall yield from **1**.[9] Aminolysis of **5** in liquid ammonia catalyzed by CuI gave aniline C-ribofuranoside **6** in 63% yield.[10] Variable amounts of 5'-*O*-desilylated derivative **7** were generated during amination, caused by traces of moisture in the reaction mixture.

We investigated several acyl protecting groups for the protection of the aniline amino group. Acetyl, phenoxyacetyl and *N,N*-dimethyformamidine groups were too sta-

ble under standard ammonia oligoribonucleotide deprotection conditions.[11] The *N*-phthaloyl group was too labile to be practical during preparation and isolation of the aniline phosphoramidite **14**. The TFA group had the desired lability (*N*-TFA of **10** was removed completely in 4 h with conc. ammonia/ethanol 3:1 at 65 °C). Trifluoroacetylation of **6** afforded **8** in high yield. The 5′-*O*-TBDPSi group of **8** was removed with tetrabutylammonium fluoride and subsequent acid catalyzed removal of the isopropylidene group afforded **10** in good yield (colorless crystals from ethyl acetate, mp 171-172 °C).

Figure 1. Synthesis of 1-Deoxy-1-C-(*p*-Aniline)-β-D-Ribofuranose 3′-*O*-Phosphoramidite



Reagents and Conditions: i) Et_3SiH/ $BF_3 \cdot Et_2O$/ CH_3CN, -40 °C, ii) liq. NH_3/CuI/ 115 °C, iii) $(CF_3CO)_2O$/Pyr, iv) 1 M TBAF/THF, v) 70% aq. AcOH/ reflux, vi) conc. NH_4OH/EtOH 3:1/ 65 °C, vii) DMT-Cl/DMAP/Et_3N/Pyr, viii) TBDMSi-Cl/Pyr/$AgNO_3$/THF, ix) P(OCE)(N*i*Pr_2)Cl/DIPEA/1-Me-Imidazole.

It is worth noting that complete cleavage of the isopropylidene group of *N*-TFA derivative **9** was effected in 15 min in refluxing 70% aqueous acetic acid while 60 min was required for the deprotection of **7** that has a free *p*-amino group. In addition, extensive decomposition of the starting material took place. Protonation of the amino function is the probable cause of the observed change in the reactivity of **7** compared to *N*-protected derivative **9**. In model studies on *N*-deprotection conditions, **10** was treated with ammonia in ethanol at elevated temperatures and free *C*-ribofuranoside **11**[12] was obtained as a chromatographically pure syrup that resisted crystallization. Aqueous methylamine (40%), used to remove *N*-acyl groups from oligoribonucleotides[13] failed to deprotect **10**.

To prepare phosphoramidite **14**, *C*-aniline ribofuranoside **10** was selectively 5'-*O*-dimethoxytritylated (70% yield) and then silylated with *t*-butyldimethylsilyl chloride in the presence of $AgNO_3$. A 1:1 mixture of a slower migrating 3'- and a faster migrating 2'-isomer **12** and **13**, respectively, was obtained. These two isomers could only be separated when a large amount of base (10% Et_3N, v/v) was added to the chromatographic solvent mixture.[14] The structures of these two isomers were determined using homonuclear decoupling experiments. The 2'-*O*-silyl isomer **13**[15] was phosphitylated under standard conditions to give 3'-*O*-phosphoramidite **14** (^{31}P NMR ($CDCl_3$): δ 148.8 (s), 151.5 (s)) in 74% yield. The new *C*-nucleoside analog **11** showed good stability towards bases, acids and TBAF, the conditions encountered during oligoribonucleotide synthesis and deprotection.

Phosphoramidite **14** was incorporated into a 36-mer all RNA hammerhead at positions U4, U7 and C3 with a coupling efficiency comparable to that of unmodified ribonucleoside monomers (>97%). IE HPLC analysis of the ribozymes after deprotection[11] revealed two peaks around the t_R of the full length product. Both compounds were analyzed by ESI MS and the results clearly showed[16] that the faster eluting peak has an additional acetyl group attached to it, while the slower peak represents the fully deprotected oligomer. This finding can be explained by partial exchange of the TFA group with an acetyl group during repetitive capping steps with acetic anhydride, simular to observation made by Chaix *et al.*[17] when the labile phenoxyacetyl group was used for the protection of guanine residues. To verify structural integrity of the ribozymes the slower peak corresponding to the anticipated fully deprotected oligomer was subjected to the nucleoside compositional analysis[2] which demonstrated presence of intact **11** (t_R= 6.7 min) and absence of its N^{ac} derivative (t_R = 18.27 min), as well as any other modified nucleosides.

Incorporation of analog **11** at positions U4 and C3 of all RNA hammerhead ribozymes resulted in more than 20 fold reduction in cleavage activity; at the same time, ribozymes where U7 was replaced with **11** reproducibly showed 3 fold increase in catalytic rates. A study is in progress to optimize the incorporation of phosphoramidite **14** into oligonucleotides, as well as structure/activity studies of ribozymes containing this modified nucleoside.

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8. In a typical procedure 1,4-dibromobenzene (10 mmol) was dissolved in anhydrous ether (15 mL) under argon and to the stirred solution was added BuLi (3.64 mL of 2.5 M solution in hexane). The solution was stirred at rt for 40 min, than cooled to -78 °C. A solution of **2** (8.3 mmol) in anhydrous THF (10 mL) was then added dropwise with stirring and the reaction continued for 1 h at -78 °C. The mixture was warmed to room temp. and stirred an additional 1 h, then quenched with water and extracted with ether. The organic layer was washed with brine, dried (Na_2SO_4) and concentrated to a syrup. This material was used without further purification in the next step.

9. Selected ^1H NMR ($CDCl_3$) data for **5**: δ 4.83 (d, $J_{1',2'}$=5.4, 1H, H1'), 4.79 (dd, $J_{3',4'}$=3.7, $J_{3',2'}$=6.6, 1H, H3'), 4.45 (app t, $J_{2',3'}$=6.6, 1H, H -2'), 1.60 (s, 3H, Me), 1.35 (s, 3H, Me).

10. Selected ^1H NMR ($CDCl_3$) data for **6**: δ 4.79 (m, 2H, H1', H3'), 4.50 (app t, $J_{2',3'}$=6.3, 1H, H2'), 3.68 (br s, 2H, NH_2) 1.60 (s, 3H, Me), 1.35 (s, 3H, Me).

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12. ^1H NMR (D_2O) data for **11**: δ 4.75 (d, $J_{1',2'}$=7.7, 1H, H1'), 4.20 (app t, $J_{3',2'}$=5.3, 1H, H3'), 4.09 (app t, $J_{2',3'}$= 5.3, 1H, H2'), 3.86 (dd, $J_{5',4'}$=3.7, $J_{5',5''}$=12.3, 1H, H5'), 3.79 (dd, $J_{5'',4'}$=5.4, $J_{5'',5'}$=12.3, 1H, H5'').

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14. The most successful solvent system for separation of isomers **12** and **13** was a 1-10% gradient of dichloromethane in hexane containing 10% Et_3N.

15. Selected ^1H NMR ($CDCl_3$) data for 2'-O-TBDMSi isomer **13**: δ 4.73 (d, $J_{1',2'}$=7.2, 1H, H1'), 4.20 (m, 1H, H4'), 4.14 (dd, $J_{2',1'}$=7.2, $J_{2',3'}$=5.2, 1H, H2'), 4.10 (m, 1H, H3'), 0.86 (s, 9H, *t*-Bu), 0.10 (s, 3H, Me), -0.19 (s, 3H, Me).

16. MS/ESI$^-$ for the faster peak calc. MW 11514.9 found 11515.9, slower peak calc. MW 11472.8 found 11472.5.

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