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# Feature of Internucleotidic 2',3'-Cyclicphosphate Intermediates During Liquid and Solid Phase Synthesis of RNA Fragments and Practical Synthesis of Octaadenylate in the Phosphoramidite Approach

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Internucleotidic cyclic phosphate intermediates were found to be hydrolyzed completely to give no 2'-5' linkages under the usual conditions for deprotection in RNA synthesis.

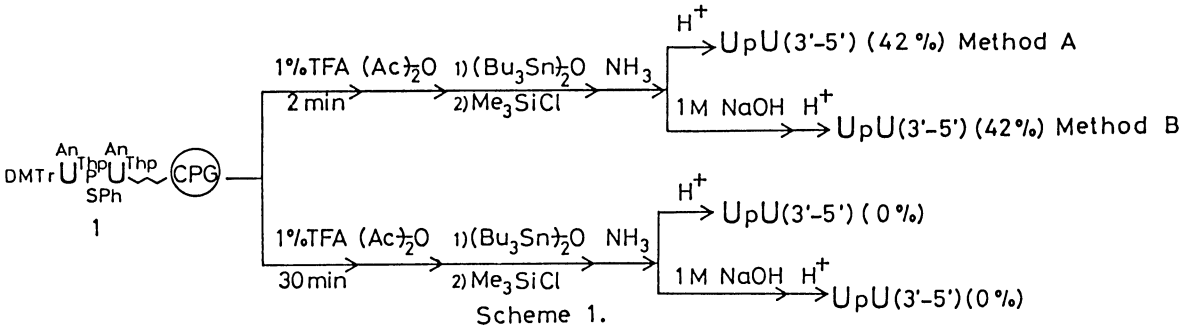
In RNA synthesis, the tetrahydropyranyl group(Thp) has been used as a 2'-hydroxyl protecting group both in the liquid and solid phase approaches.[1,2] When the 5'-hydroxyl group is protected with dimethoxytrityl (DMTr) or pixyl in combination with the Thp group, selective removal of the former is required for chain elongation in the 5'-direction. Recent two papers[3,4] suggested that the acid lability of the Thp group caused 3'→2' phosphoryl isomerization via a cyclic phosphotriester intermediate so that synthetic oligoribonucleotides would be contaminated with undesired 2'-5'-linked regioisomers. However, oligoribonucleotides synthesized on polymer supports by the use of the DMTr and Thp groups have been proved to be free of 2'-5' linkages[5,6] as evidenced by enzymatic assay with nuclease $P_1$ which interacts exclusively with 3'-5' natural linkage. In this paper, we wish to report that such 2'-5' linkages are completely hydrolyzed during treatment with ammonia used for removal of N-acyl protecting groups which is performed before removal of the remaining 2'-Thp group.

A fully protected dimer (UpU) on CPG (1), which was obtained in 95% yield (trityl cation assay) via the phosphotriester approach,[5,6] was treated with 1% TFA in $CH_2Cl_2$ at r.t for 2 min as shown in Scheme 1. These conditions were required for half cleavage of the 2'-Thp group. The capping reaction was successively carried out. Part of this CPG gel was used for the usually deprotection procedure (Method A).[5,6] (see Scheme 1) Independently, the same gel was treated with (1 M NaOH for 3 h) before the acid treatment in order to remove 2'-5' linkages (Method B) since such strong alkaline conditions are known to lead to complete hydrolysis of RNA fragments.[7] Although we expected that the UpU (3'-5') was contaminated with UpU (2'-5') more or less in the case of Method A, the dimer isolated by paper chromatography was the only native 3'-5' linked UpU. The yield of UpU (3'-5') derived from Method A was the same as obtained in the case of Method B.

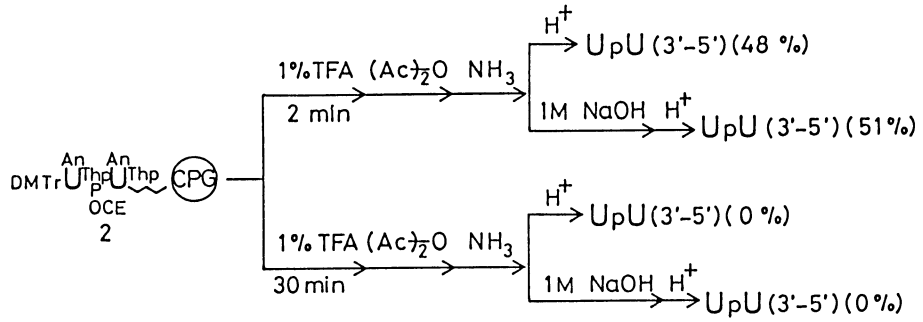
Next, the first TFA treatment was carried out for 30 min, whereupon the 2'-Thp group would be completely removed. The same manipulations were carried out

as shown in Scheme 1. To our surprize, neither 2'-5' nor 3'-5' UpU could be obtained under these conditions. The same phenomenon was observed in the case of the fully protected dimer on CPG (2), which was synthesized in 97% yield (trityl cation assay) via the phosphoramidite approach[8] (Scheme 2).

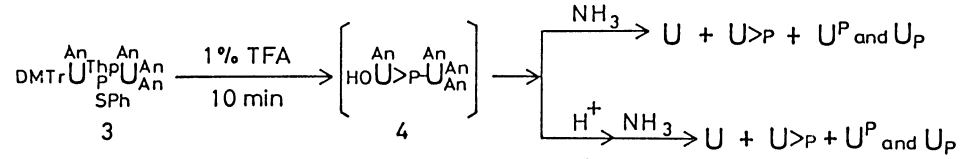
To clarify hydrolytic process of 2',3'-cyclic phosphate intermediates postulated in the 3'→2' phosphoryl migration, a cyclic phosphate intermediate (4) was prepared by the reaction of a fully protected UpU derivative (3) with 1% TFA in $CH_2Cl_2$ followed by evaporation of all the volatile reagent and solvent.




Scheme 1.




Scheme 2.




Scheme 3.

Table 1. The conditions and results of hydrolysis of the cyclic phosphate(4)

| Entry | Acid treatment | Ammonia treatment | Yield of products / % UpU | Uridine | 2',3'- Cyclic phosphate | 2' and 3'- Monophosphate |
|---|---|---|---|---|---|---|
| 1 | — | c) | 0 | 92 | 37 | 27 |
| 2 | a) | c) | 0 | 95 | 32 | 40 |
| 3 | b) | c) | 0 | 96 | 23 | 72 |
| 4 | — | d) | 0 | 96 | 61 | 13 |
| 5 | b) | d) | 0 | 89 | 31 | 60 |

a) 0.01 M HCl : dioxane (5:1, v/v) 30 min.
b) 80%acetic acid : dioxane (5:1, v/v) 30 min.
c) 60 °C, 6 h and r.t, 12 h.
d) r.t, 24 h.
e) The yields were estimated by UV of the products isolated by paper chromatography.

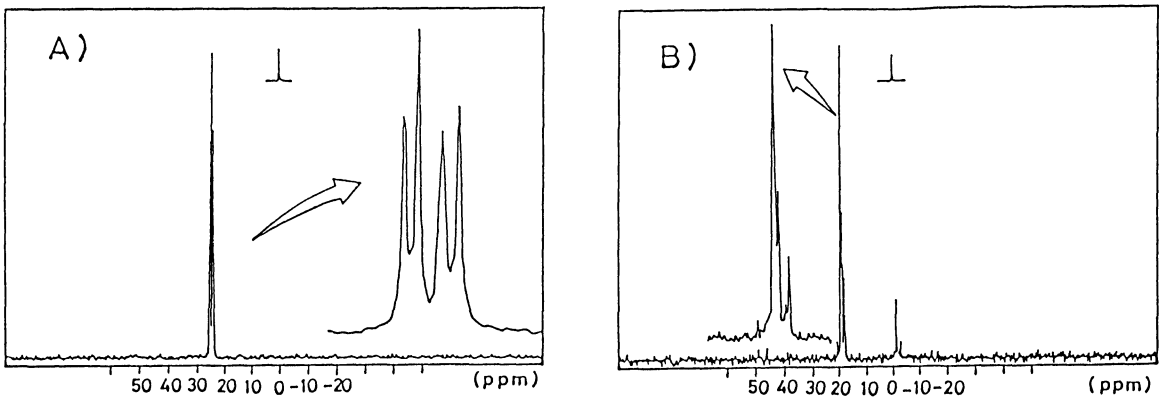



Fig. 1. $^{31}P$ NMR spectra ($CDCl_3$) of A) a fully protected dimer (UpU) and B) the reaction mixture (4) in Scheme 3.

The $^{31}P$ NMR spectrum of 3 in $CDCl_3$ showed four peaks at 25.04 25.24, 26.06, and 26.55 ppm which were assigned to diastereoisomers based on the chiral centers of the internucleotidic phosphate and Thp groups. In contrast to this spectrum, the $^{31}P$ NMR spectrum of the residue obtained in the above experiment showed a set of two peaks at 18.75 and 19.13 ppm which was due apparently to the phosphorus chirality. These values are similar to that of ethyl ethylene phosphate (17 ppm).[9] When 4 thus obtained was treated with concd. ammonia at room temperature or at 60 °C plus at room temperature (entries 1 and 4 in Table 1), UpU was not obtained either. Reese[3] reported that treatment of a cyclic phosphate similar to 4 with 80% acetic acid gave a mixture of UpU (2'-5')(24%), UpU (3'-5')(19%), Up(2'), Up(3'), Up(2',3'- cyclic)(21%), and U derivatives(26%) respectively. Therefore, the cyclic phosphate 4 was first treated with 80% acetic acid at room temperature for 30 min (entries 3 and 5) or hydrochrolic acid at room temperature for 30 min (entry 2) and then treated with concd. ammonia as shown in Scheme 3. However, even in these cases no UpU dimers were formed. Only degradation products were obtained.

Next, to see to what extent hydrolysis of UpU(3'-5') occured under the same conditions as used in condition c of entry 2, UpU(3'-5') was treated with concd. ammonia at 60 °C for 6 h plus at room temperature for 12 h. Consequently, 70% of UpU(3'-5') was hydrolyzed to Up and U. A similar treatment of UpU(3'-5') with concd. ammonia at room temperature for 24 h resulted in 50-60% degradation of UpU(3'-5').

These results showed that, if a $N^3$-anisoylated UpU(3'-5') derivative was formed in ca. 19% yield[10] in the reaction of 4 with 80% acetic acid,[3] it should remain unchanged to an extent of 6% upon the successive ammonia treatment. However, our results obtained by the present experiments showed that UpU(3'-5') could not be detected even in a 100 OD scale. Although it is still unclear why 4 was converted all to degradation products in our hand, it can be said that, even if the Thp group was eliminated during removal of the 5'-DMTr group, oligoribonucleotides finally isolated could be purified as 2'-5' linkage-free material by simple treatment with ammonia.

Based on these findings, we synthesized octaadenylate ($A_8$) via the phosphoramidite approach. A phosphitylating reagent, bis(diisopropylamino)-2-cyanoethoxyphosphine (5) was synthesized by a modification of the procedure of Caruthers[11] and van Boom et al.[12] The adenosine amidite unit DMTr$A^{bz}$(2'-THP)(3'-p(OCEt)($NiPr_2$)) ($^{31}$P-NMR ($CDCl_3$): 150.74, 150.50, 149.82 ppm) was obtained in 90% yield by the usual phosphitylation of 5'-O-(4,4'-dimethoxytrityl)-2'-O-(tetrahydropyran-2-yl)-$N^6$-benzoyladenosine with 5.[12] The synthesis of $A_8$ was carried out according to a method similar to that described in deoxyribonucleotide synthesis by Caruthers,[11] the use of 1% TFA in $CH_2CH_2$ (5 s X 3) for removal of the DMTr group, and additional manipulation of drying in vacuo for 10 min after removal of the DMTr group.[13] The average yield was estimated to be 98.3% by the trityl cation assay. This fully protected octamer on CPG was first treated with ammonia (60 °C, 6 h and r.t, 12 h) followed by acid treatment (pH 2.0, 24 h). The crude $A_8$ was purified with reversed phase HPLC, to give homogeneous $A_8$ in an isolated yield of 24%. It is also confirmed that this octamer was completely hydrolyzed with nuclease $P_1$ to A and pA in the correct ratio as expected.

These results suggest that the combination use of the 2'-Thp with the 5'-DMTr group is practical for the synthesis of medium-size (≈20 mer) RNA fragments.

References

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10) This estimation was based on the assumption that the same products contribution as reported by Reese was expected.
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13) The reaction time for condensation was 5 min and the time required for one cycle was ca. 20 min.

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