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Practical Chemical Synthesis of RNA Fragments. Improvement in the Preparation of Ribonucleosidephosphoramidite Units

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Highly pure ribonucleoside phosphoramidite units were prepared and three 20 long RNA fragments were synthesized on the solid phase by use of these starting compounds.

Recently, Ogilvie et al.[1] obtained good results for the long RNA sequence assembly on the solid supports by use of the t-butyldimethylsilyl group for the protection of the 2'-hydroxyl on the ribonucleotide molecules. This 2'-protecting group was frequently employed by several research groups.[2] On the other hand, various acetal type blocking groups, such as Thp group,[3a] 4-methoxytetrahydropyranyl (Mthp) group,[3b] tetrahydrofuranyl (Thf) group,[3c] and 1-[(2-chloro-4-methyl)phenyl]-4-methoxypiperidinyl (Ctmp) group,[3d] were introduced into the 2'-hydroxyl function. Using the 2'-Thp group, we have previously reported the practical and rapid synthesis of the medium sized RNA fragments via the phosphoramidite approach.[4] For the 5'- hydroxyl protection, the use of the Pix[5a] and Mox[5b] groups, which are rather labile 5'-protecting group in the trityl type blocking groups, gave satisfactory results in the solid phase synthesis.

In this paper, we wish to report that three 20-nucleotide long RNA fragments ($C_{19}A$, $U_{19}A$, CUCGUCCUCGCCGGCUAGUA) were synthesized by use of an automatic synthesizer (ABI DNA synthesizer Model 381 A) via the cyanoethylphosphoramidite method.

Our previous paper[4] showed that the hydrolyzed products (^{31}P-NMR, 13 ppm) could not be completely removed by chromatography in the case of the adenosine and cytidine phosphoramidite units, and these derivatives were employed sufficiently for the successive coupling reaction by the manual procedure. However, the preparation of highly pure starting compounds

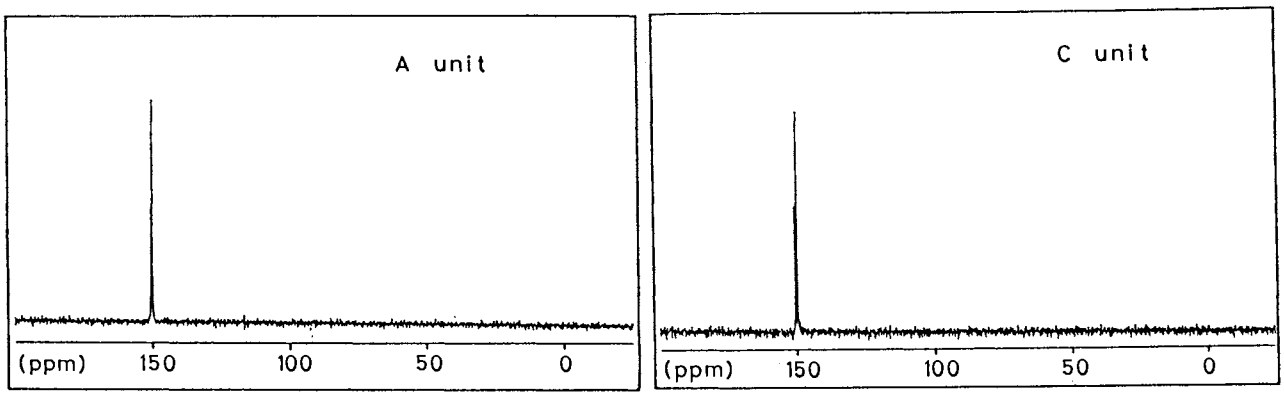

Fig. 1. ^{31}P-NMR spectra of the a) adenosine phosphoramidite unit and b) cytidine phosphoramidite unit.

are required for the synthesis of relatively long RNA fragments by using the automatic synthesizer. In order to remove the side products completely, we searched silica gel media and elution systems for chromatography which have the appropriate separation efficiency. As a result we decided to employ chromatographical purification on silica gel (Merck Kiesel gel 60, 70-230 mesh) using the mixed solution (CH_2Cl_2 - C_6H_{14}, 2% NEt_3), in which each organic solvent was employed after purification by distillation. These amidite units were obtained by the above purification system with excellent purity of 99% determined by ^{31}P-NMR (500 MHz). Especially, for adenosine and cytidine derivatives, as shown in Fig. 1 the hydrolyzed products could not be detected on the ^{31}P-NMR spectra. The automatic synthesis of RNA fragments was performed under the following conditions: 1) detritylation step; 2% dichloroacetic acid (DCA) in CH_2Cl_2 for 60 s. (This condition is used for the 0.2 μmol synthetic scale) 2) coupling step; 0.2 M phosphoramidite unit and 0.4 M tetrazole in CH_3CN for 5 min. 3) oxidation step; 0.1 M I_2 in THF / pyridine /H_2O, 7:2:1 for 30 s. 4) capping step; 0.25 M Ac_2O / 1-methylimidazole / lutidine in THF for 20 s. The time consumed for elongation cycle was ca. 13 min. The coupling overall yields are listed in Table 1.

Table 1. Oligoribonucleotide synthesis on the solid phase support

RNA fragments	Overall yield/%	Isolated amounts(OD)	Isolated yield/%[a)]
(1)CCCCCCCCCCCCCCCCCCCA	90	9.8	33
(2)UUUUUUUUUUUUUUUUUUUA	91	15.5	39
(3)CUCGUCCUCGCCGGCUAGUA	88	11.1	30

a)These yields were estimated by assuming the hypochromicity[6)] of 3.4% for fragment 1, 2.1% for fragment 2, and 6.8% for fragment 3.

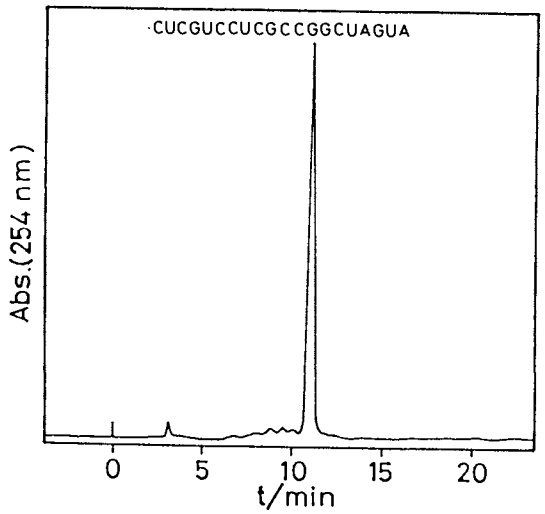

Fig. 2. Reversed phase HPLC profile of a 20 mer, fragment (3) after prepurification with SEP-PAK C_{18}.

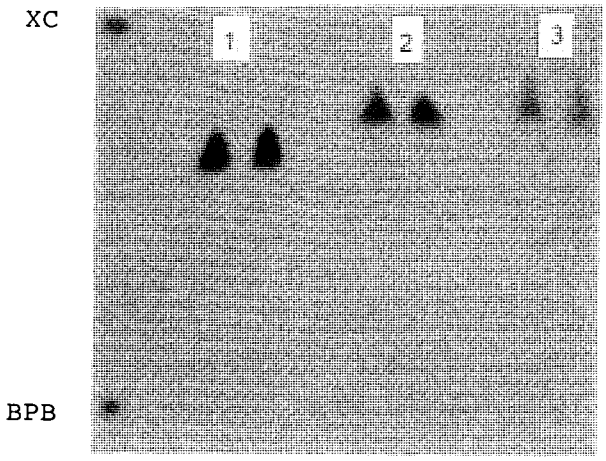

Fig. 3. 20% polyacrylamide gel electrophoresis of the deprotected RNA fragments.lane 1; C_{19}A, lane 2; U_{19}A,lane 3;CUCGUCCUCGCCGGCUAGUA.

For each fully protected RNA fragment on CPG the average coupling efficiency was ca. 99%. Deprotection of these fully protected oligoribonucleotides was performed by the method reported previously.[4] Figure 2 shows the reversed phase HPLC (μBondasphere C_{18}) profile of the fragment (3) after prepurification with SEP-PAK C_{18}. This fragment was obtained as the relatively pure form. Similarly, fragment (1) and (2) were observed as the simple HPLC profiles.(Data not shown.) Purification of these RNA fragments were performed by the reversed phase HPLC. The isolated amounts and yields of the RNA fragments are listed in Table 1. Figure 3 shows gel electrophoresis in the presence of 7 M urea after ^{32}P-labelling of the 5'-terminus of the RNA fragments. Our 5'-detritylation condition for removal of Pix and Mox groups on the automatic synthesizer was sufficiently employed for the synthesis of RNA fragments consisting of high ratio of pyrimidine base nucleotide. The sequence analysis of these 20 mers was performed by the method of Donis-Keller[7] and the isolated products were completely digested with nuclease P_1 to give expected monomers in the correct ratios.

We concluded that the relatively long RNA fragments were synthesized by using the automatic synthesizer with our strategy, which is the combined use of the 2'-Thp group and 5'-Pix and Mox groups by the cyanoethylphosphoramidite approach, although preparation of pure ribonucleoside phosphoramidite units was required.

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