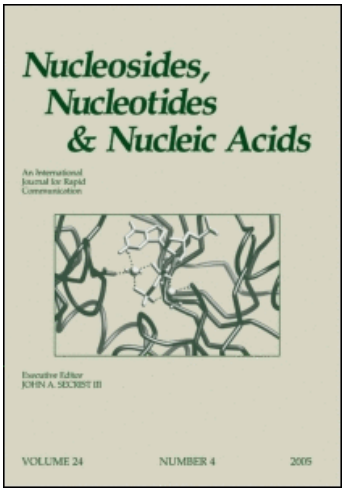

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Synthesis and Properties of Non-Hammerhead RNA Using 1-(2-Chloroethoxy)-ethyl Group for the Protection of 2'-Hydroxyl Function

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SYNTHESIS AND PROPERTIES OF NON-HAMMERHEAD RNA USING 1-(2-CHLOROETHOXY)-ETHYL GROUP FOR THE PROTECTION OF 2'-HYDROXYL FUNCTION#

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Abstract. The new type protecting group, 1-(2-chloroethoxy)ethyl (Cee) group has been employed for the protection of the 2'-hydroxyl functions of ribonucleoside residues in the synthesis of oligoribonucleotides by the phosphoramidite approach on a solid support, using an acid-labile 5'-dimethoxytrityl group. This group is completely stable under the acidic conditions required to remove the 5'-terminal protecting groups in oligonucleotide synthesis on a solid support, and yet is easily removable under the similar conditions to that of the tetrahydropyranyl (Thp) in the region of pH 2-3 for the final unblocking step. The synthesis of the Cee-protected ribonucleoside 3'-phosphoramidite units proceed smoothly. The Cee-protected ribonucleoside 3'-phosphoramidite units were evaluated in the synthesis of oligoribonucleotides of up to 20 mer residues. The autocleavage of a precursor RNA from bacteriophage T4 (p2Sp1 RNA; precursor of species 1) does not contain the "hammerhead" sequence required for the authocleavage of other known self-cleaving RNA.

INTRODUCTION

Recently, ribonucleic acids (RNAs) chemistry has grown with the important discovery of self-splicing RNA[1], antisense RNA in the translation control of the expression of genes[2], recombinant RNA technology[3], the formation of "lariats" during the processing of pre-RNA[4], involvement of a chloroplast glutamate tRNA in chlorophyll biosynthesis[5], and self-cleavage of viroid RNAs[6].

The chemical synthesis of oligoribo- and poly-ribonucleotides on a solid support is more elaborate and time-consuming than the oligo- and poly-deoxyribonucleotides primarily because of the need to protect the 2'-hydroxyl function of ribonucleosides. The choice of the protecting group for the 2'-hydroxyl function is a crucial point in the polyribonucleotide synthesis and it should be completely stable under the conditions required

for the removal of the 5'-protecting groups in the solid-phase synthesis of oligoribonucleotides. Finally, it must be removed at the end of synthesis by a procedure that does not cause internucleotidic cleavage or phosphoryl migration under the deprotection conditions.

The tert-butyldimethylsilyl and o-nitrobenzyl groups as the 2'-hydroxyl function have recently been described by Ogilvie et al. and Tanaka et al. for use with the phosphoramidite approach of long chain synthesis. The tert-butyldimethylsilyl group has been combined with an acid labile 5'-protecting group and used for the synthesis of the 43 and 77mers using the phosphoramidite approach.[7] The o-nitrobenzyl group has also been combined an acid-labile 5'-protecting group and used for the synthesis of oligoribonucleotides of up to 34mer using the phosphotriester[8], phosphoramidite[9], and H-phosphonate[10] approaches. The use of 2'-O-tetrahydropyranylated ribonucleosides for the synthesis of several 8mers on a solid support has been recently demonstrated by Caruthers.[11] We have also investigated the use of substituted aryl ethers such as 4-methoxy-[12] and 3,4-dimethoxybenzyl[13] groups as the 2'-hydroxyl functions with some success. Quite recently, Stawinski et al.[14], as well as Ogilvie et al.[15] demonstrated the lability of the 2'-O-silylated oligoribonucleotides towards concentrated ammonia due to the undesired removal of the TBDMS moiety, and subsequent loss of the silyl groups and cleavage of the phosphodiester bonds, during the alkaline removal of the base and phosphate protecting groups. However, these side reactions were suppressed in the ethanol containing ammonia solution or methanolic anhydrous ammonia.

On the other hand, the recent studies shown[16,17] that an acid-labile 2'-acetal protecting group was insufficiently stable under acidic conditions required for the removal of either 5'-dimethoxytrityl[18] or 9-phenylxanthen-9-yl[19] groups to be useful for chain elongation on solid supports. However, Hata et al.[20] have synthesized the oligoribonucleotides (10 and 13mers) using two different 5'-O-protecting groups [9-phenylxanthen-9-yl or (4-methoxy)phenylxanthen-9-yl] in combination with the 2'-O-tetrahydropyranyl group.

In order to overcome this problem, a few workers have explored a new acetal protecting group for 2'-hydroxyl functions or have searched a procedure without acid treatment during chain elongation. Reese et al.[21] have investigated the use of a new type of acetal group such as 1-[(2-chloro-4-methyl)-phenyl]-4-methoxypiperidin-4-yl group designed to be stable under the conditions required for the removal of a pixyl group but cleavable under mild conditions at pH 2.0. Recently, Ohtsuka et al.[22] have reported the synthesis of a 21mer by the phosphoramidite approach using the base-labile levulinyl[23] and tetrahydrofuranyl groups, respectively, for the

protection of 2'-O- and 5'-O-hydroxyl functions. More recently, Gait et al.[24] have reported that a potentially useful protecting combination of the 9-fluorenylmethoxycarbonyl group (Fmoc)[25] for 5'-protection and 4-methoxytetrahydropyran-4yl (Mthp)[26] for 2'-protection was very effective for the synthesis of oligoribonucleotides than the procedure described by Ohtsuka et al.. However, these approaches introduces some problems, such as the low selectivity of introduction to the 5'-position of the levulinyl and Fmoc groups and the unsatisfactory for the preparation of phosphoramidite units. Further, it is possible that during the removal of 5'-Fmoc and levulinyl groups, some of the 2-cyanoethyl groups on the phosphotriester bonds of the support-bound oligonucleotide chain are removed.

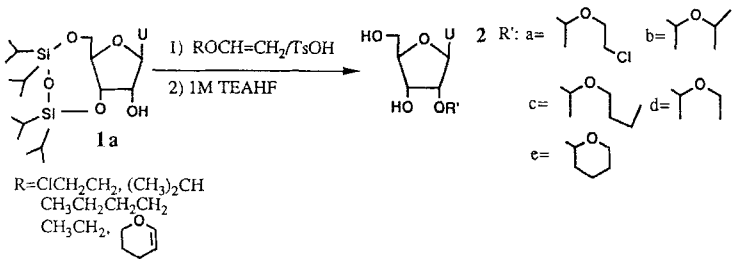
In our continuing studies[27] to develop the acetal groups as the 2'-protecting groups for the solid phase synthesis of oligoribonucleotides using the phosphoramidite approach,[28] we have found that 1-(2-chloroethoxy)ethyl (Cee) group is stable under the acidic conditions required for the complete removal of a DMTr group at every cycle of oligoribonucleotide addition; however, under mild hydrolytic conditions (pH 2.0), the Cee group is removed.[29] The results of this study have been used successfully to synthesize a series of oligoribonucleotides of up to 20 residues.

Results and Discussion

Synthesis and properties of 2'-O-acetal uridine derivatives

First we examined the synthesis of uridine derivatives (**2a-c**) bearing three different 2'-acetal protecting groups. Further, we carried out investigation without separation of the diastereoisomers. The reaction between 3',5'-O-(tetraisopropyldisiloxane-1,3-diyl)-uridine (**1a**)[30] and alkyl vinyl ethers in the presence of p-toluenesulfonic acid and the conversion of the products obtained into **2a-c**[31] (80-83%) are indicated in outline in Scheme I. 2'-O-Acetal uridine derivatives (**2d,e**) were prepared according to the published procedure[32,33].

It was then interesting to explore the relative stabilities of these acetal groups in 2'-O-acetal derivatives (**2a-e**) under the acidic conditions to evaluate their possible use in the chemical synthesis of oligoribonucleotides in conjunction with other acid-labile protecting groups on the pentose sugar. The relative rates of removal of the acetal groups from the corresponding 2'-O-acetal derivatives (**2a-e**) are shown in Table 1. It can be seen from the Table 1 that **2a** may be used as a 2'-protecting group in view of its relatively high stability even under the acidic condition (1.0% dichloroacetic acid in CH_2Cl_2) required for the



Scheme I

TABLE 1. THE RELATIVES RATES OF HYDROLYSIS OF ACETAL GROUPS FROM URIDINE DERIVATIVES (**2a-e**)[a].

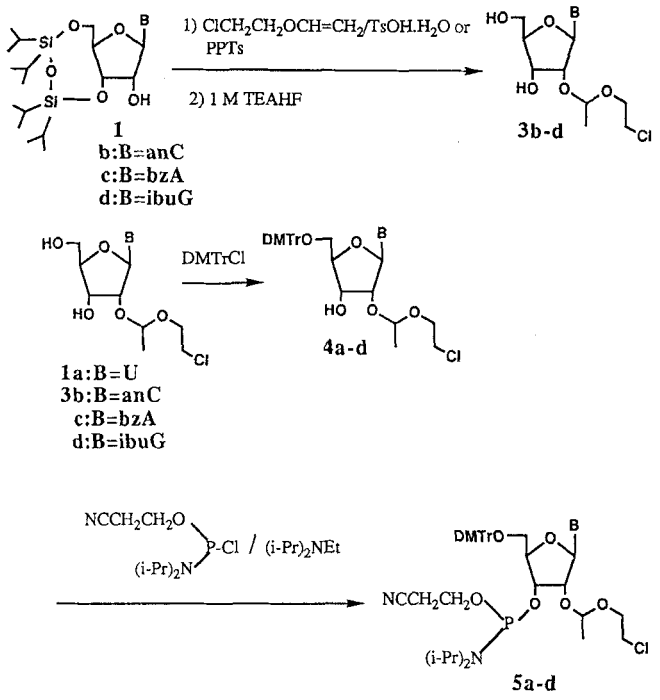
Comp.	1.0% DCA in CH_2Cl_2		0.01 N HCl (pH 2.0)	
	$t_{1/2}$(min)	t_∞(min)	$t_{1/2}$(min)	t_∞(min)
2a	420	960	96	360
2b	-	30 sec	1	4
2c	2	5	12	34
2d	20 sec	3	5	18
2e	90	273	32	150

a) These reactions were carried out with diastereomeric mixture of 2'-O-acetal uridine derivatives (**2**) at room temperature. The reactions were monitored by TLC and the reverse phase TLC.

complete removal of a 5'-O-DMTr group. Especially, in case of **2a**, no hydrolysis can be detected after 10 min.[34] However, **2a** has hydrolysis properties similar to that of the Thp or Mthp protecting groups under the relatively mild conditions, for example, by treatment with 0.01 N hydrochloric acid (pH 2.0) at room temperature.

Preparation of ribonucleoside 3'-O-phosphoramidite units

The N-acyl-2'-O-1-(2-chloroethoxy)ethyl-ribonucleosides **3b-d** were key intermediates for the preparation of ribonucleoside 3'-O-phosphoramidite units. The cytidine derivative **3b** was obtained from 3',5'-O-(tetra-isopropyldisiloxane-1,3-diyl)-N-anisoylcytidine (**1b**) in 87% yield by reaction with 2-chloroethyl vinyl ether in CH_2Cl_2 in the presence of p-toluenesulfonic acid followed by treatment with 1M triethylammonium hydrogen fluoride (TEAHF)[35] as previously described for the similar tetrahydropyranyl derivative. However, the 2'-O-1-(2-chloroethoxy)-ethylation of **1c and d** did not proceed smoothly under the conditions described above. In the presence of pyridinium p-toluenesulfonate (PPTS)[36] in CH_2Cl_2, **1c and d** reacted almost quantitatively with 2-chloroethyl vinyl



Scheme 2

ether and the conversion of products obtained into **3c and d** (83 and 81%) are indicated in Scheme II. The location of the 1-(2-chloroethoxy)ethyl group were determined by ^{1}H-NMR. Further, evidence of the site of the Cee group was obtained by determining the structure of the oligoribonucleotide with hydrolysis of ribonuclease T_2 as described later in this paper.

The general procedure of the preparation of N-acyl-5'-O-DMTr-2'-O-Cee-nucleoside 3'-O-phosphoramidite units (**5a-d**) is shown in Scheme II. The protected ribonucleosides **4a-d** were dissolved in dry CH_2Cl_2 to which was added diisopropylethylamine followed by 2-cyanoethyl N,N-diisopropylchloro-phosphoramidite[37] at 0°C. After 2h at room temperature, the workup was a extraction between ethyl acetate and saturated sodium chloride. The crude products were purified by short column chromatography on silica gel. However, this reaction was unsatisfactory. When THF was used in place of CH_2Cl_2, the reaction proceed smoothly and gave the phosphoramidite units (**5a-d**) in 82-87% yields. The ^{31}P-NMR data clearly shown that there are, as expected, two pair of diastereomeric signals for all of the phosphoramidite units, thereby establishing the isomeric purity of these compounds. These diastereomers should have no influence on the coupling reaction.

Synthesis of ribonucleoside resin

We examined the synthesis of 5'-O-DMTr-2'-O-Cee-ribonucleoside 3'-O-succinates (**6a-c**) required to attach the commercially available long chain alkylamine CPG. Compounds **4a-c** were treated with scuccinic anhydride and a catalytic amount of DMAP in pyridine for 2 h.[38] TLC analysis indicated that the reaction was complete. Following workup, the ribonucleoside monosuccinates **6a-c** were obtained in good yields after the separation of silica gel column chromatography. The LCAA-CPG was treated with **6**, dicyclohexylcarbodiimide (DCC), and triethylamine in the presence of a catalytic amount of DMAP in DMF for 24 h. The extent of nucleosides loading was 44.0 μmol per gram for **7a** functionalisation, 39.5 μmol per gram for **7b** functionalisation and 37.5 μmol per gram for **7c** functionalisation, as estimated from the DMTr cation release after treatment with 1.0% dichloroacetic acid in CH_2Cl_2.

Solid phase synthesis of oligoribonucleotides.

The procedure for synthesis are a modification of a procedure previously described.[38] The reaction was carried out on a small column of nucleoside-functionalised glass (0.2 μmoles) with a Applied Biosystems Model 381A DNA synthesizer. We showed the following elongation cycle to be effective: treatment with (1) 5'-unblocking [1.0% $Cl_2CHCOOH$ in CH_2Cl_2, 90 sec], (2) washing [CH_3CN, 30 sec], (3) coupling [0.07 M ribonucleoside phosphoramidite (**5a-d**) and 0.210 M tetrazole in CH_3CN for 15-20 min], (4) washing [CH_3CN, 30 sec], (5) oxidation [0.1 M iodine in THF/ lutidine/water (40:10:1), 60 sec], (6) wasing [CH_3CN, 70 sec], (7) capping [solution A: THF/lutidine/acetic anhydride (8:1:1, v/v), solution B: 0.27 M DMAP in THF, 60 sec], (8) washing [CH_3CN, 70 sec].

The utility of ribonucleoside 3'-phosphoramidites (**5**) in which the 2'-hydroxyl functions are protected with the Cee group is now demonstrated by the synthesis of 2-20 mers consisting of the homopolymers of cytidylic and uridylic acids, the 20mer (AGUAUAAGAGGACAUAUGCA), and a precursor RNA from bacteriophase T4 (p2Sp1 RNA, CGUUUCGUACAAACAC)[39]. In all cases, the average coupling yields were ranging from 95 to 98%.

The 2'-O-tetrahydropyranylated cytidine 3'-O-phosphoramidite[11] was used in the synthesis of rC_{18}. The avarage coupling yield from the 2'-O-Thp amidite was similar, 95%, when compared with 95% yield obtained with the 2'-Cee amidite **5b**. As will be described bellow, the yield of the fully deprotected oligomer was slightly higher in the case of the 2'-O-Cee protected oligomer.

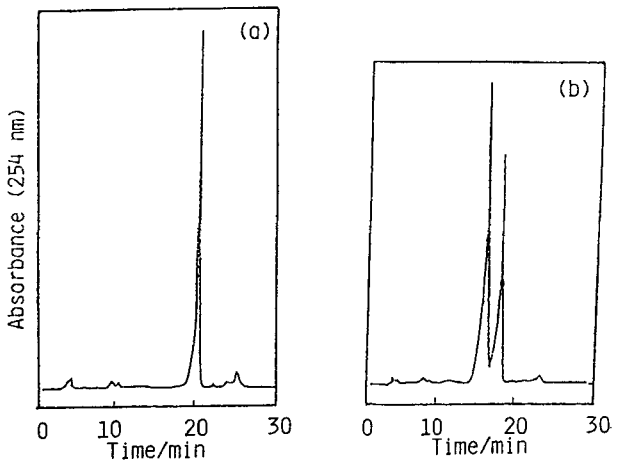


FIGURE 1. HPLC analysis of the crude mixture containing rU3'p5'U after deprotection (a), using TSKgel oligo-DNA RP column with a linear gradient of 50% aqeous methanol (0-50%, during 30 min) in 0.05 M ammonium phosphate (pH 7.0), and after addition of rU2'p5'U (b), using the same gradient.

Deprotection of Chemical Synthesized Oligoribonucleotides.

In order to study both internucleotidic cleavage and phosphoryl migration under the deprotection conditions, the dimer, rUpU was treated first with ammonia and then with 0.01 N HCl (pH 2.0). The 2',5'-protected dimer was dissolved in 0.01 N HCl and the pH adjusted to 2.0 by addition of 0.1 N HCl. The mixture was stirred at room temperature for 6 h and neutralized with a dilute ammonia solution, followed by the reverse phase HPLC analysis (Figure 1). Integration of the main peak in Figure 1a reveals that the dimer, rUpU account for 98% of the total absorbance at 254 nm. Further, no isomerization to uridylyl-(2'-5')-uridine can be detected under the deprotection conditions (Figure 1b). The ratio of rUp and rU were estimated by the reversed phase HPLC after digestion of rUpU with ribonuclease T_2 and was found to be rUp:rU=1.00:1.04 (theoretical, 1.00:1.00) (Figure 2). Under the condition of digestion, ribonuclease T_2 did not cleave a uridylyl-(2'-5')-uridine. No peak corresponding to rUpU was observed in the chromatogram of the digests, indicating the absence of any 2'-5' internucleotidic bonds.

All of the sequences synthesized above were treated in a similar manner with ammonia at 55°C for 5-8 h to cleavage of the reversible bond

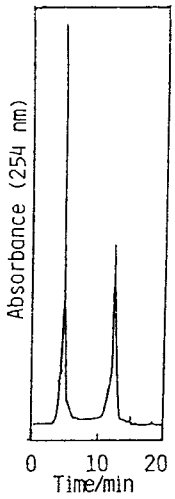


FIGURE 2. Analysis of the products after hydrolysis of rUpU with ribonucleoase T_2 on a TSKgel oligo-DNA RP column.

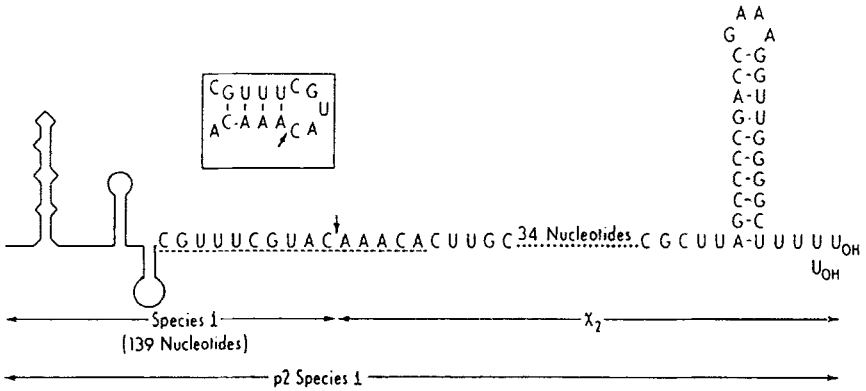


FIGURE 3. Primary and secondary structure of p2Sp1 RNA.[39]

TABLE 2. ISOLATED YIELDS IN THE SYNTHESIS OF OLIGORIBONUCLEOTIDES.

Sequence	Length	Yields[a]
rCCCCCCCCCCCC	12	3.50 A_{260} unit (25%)
rCCCCCCCCCCCCCCCCCC	18	1.96 A_{260} unit (14%)
rCCCCCCCCCCCCCCCCCC	18	1.40 A_{260} unit (10%)[b]
rUGUCGGUC	8	6.34 A_{260} unit (36%)
rGACCGUCA	8	5.34 A_{260} unit (27%)
rGACCGACA	8	4.89 A_{260} unit (30%)
rAGUAUAAGAGGACAUAUGCA	20	2.01 A_{260} unit (9%)
rCGUUUCGUACAAACAC	16	10.56 A_{260} unit (18%)[c]

a) The overall yield from the 3'-terminal ribonucleoside on CPG. b) The coupling reaction was carried out by use of DMTranCThp-3'-O-phosphoramidite unit. c) This sequence is the self-cleavage site of p2Sp1 RNA (CGUUUCGUAC-AAACAC) (Figure 3).[39]

and to complete the removal of the exocyclic amino acyl protecting groups, and then 0.01 N-HCl (pH 2.0) at room temperature for 8-30 h. The reaction was then neutralized with a dilute ammonia solution and analyzed by the reverse phase HPLC. Further, the crude oligomer was checked by analytical polyacrylamide gel electrophoresis. It is cear from the gel results that the crude oligomers were already pure, indicating the effectiveness of the Cee group. The purity of the oligoribonucleotides of up to 20 residues was assessed by electrophoresis on a 20% polyacrylamide gel containing 7M urea, and when a single band could not be detected, the purification was conducted again by the polyarylamide gel electrophoresis. Isolated yields of deprotected oligoribonucleotides after the reverse phase HPLC or PAGE are as shown in Table 2. ***It is noteworthy that comparison of the isolated yields showed syntheses using 2'-O-Cee protection to be 4% higher than those employing 2'-O-Thp protection.***

It is important to ensure the validity of sample purified that no base modification or phosphodiester bond isomerization had occurred. For example, the deprotected octamer, rGACCGUCA was complete digested with RNase T_2 and then treated with alkaline phosphatase. The reverse-phase HPLC analysis showed complete conversion into rA, rC, rU , and rG in the expected proportions. The base sequences of the 20mer was confirmed by partial enzymatic degradation of the 5'-end-labeled oligomer on polyacrylamide gel electrophoresis. The autoradiogram of the gel is shown in substaniates the expected sequence (Figure 4).

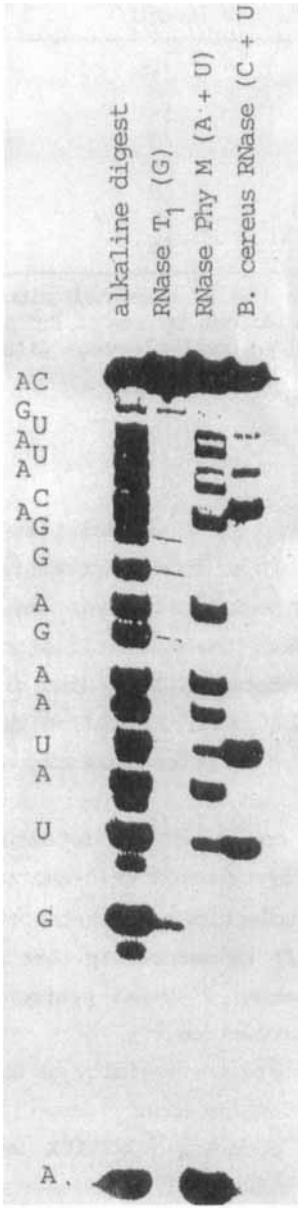


FIGURE 4. Autoradiogram of 20%PEG for RNA sequence. The 5'-end-^{32}p-labeled RNA 20mer was partially digested with ribonucleases according to the method of Donis-Keller.

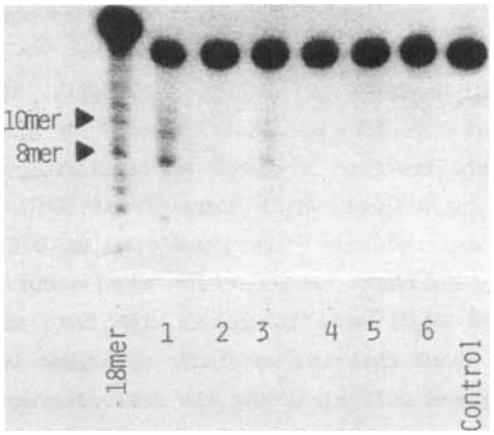


FIGURE 5. The requirements for the cleavage reaction of p2Sp1 RNA (CGUUUCGUACAAAACAC). The lane 1,2,3,4, and 5 were incubated for 2 h at 37°C. The lane 6 was incubated 2h at 0°C as a temperature. The minimal reaction, lane 1 contained only 0.5% Briji, 170mM-NH_4Cl and RNA. The lanes 2 and 4 were the same as the total assay (lane 3) expect that either Briji 58 or NH_4Cl were not included in the reaction mixture. The total reaction, lane 1, contained 170mM-NH_4Cl, 0.2mM-Na_2EDTA (pH 7.0), 1mM-$MgCl_2$, 25mM-Tris-maleate buffer (pH 5.25) and 0.5% Briji 58.

From these analyses it is clear that the 3'-5' phosphodiester bonds of oligoribonucleotides is preserved during the synthesis and deprotection procedures.

Self Cleavage of a Precursor RNA from Bacteriophage T4

One viroid and several and satellite RNAs have been shown to possess a site-specific self-cleavage reaction.[6] These RNAs undergo the cleavage reaction in the presence of Mg^{++} at neutral pH. The self-cleavage domain has been postulated to form a three-stemmed secondary structure. Some "hammer-head" short RNAs have been prepared using T7 RNA polymerase and the consensus sequences were proved to be important for self-cleavage.[40] Watson et al. have recently reported that a precursor of an RNA molecule from T4-infected E. coli cells (p2Sp1 RNA; Figure 3) has the capacity of cleave itself in a specific position.[39] This self-cleavage reaction requires at least a monovalent cation and is aided by non-ionic detergents. To prove that the self-cleavage reaction occurred autolytically, we studied the selective hydrolysis of RNA using a chemical synthesized 16mer (CGUUUCGUACAAACAC) having a sequence similar to C130-C145 of the p2Sp1 RNA.

Figure 5 shows the requirements for the self-cleavage reaction of p2Sp1 RNA. The complete reaction mixture included the ^{32}P-labeled 16mer, 170 mmol-NH_4Cl, 0.2 mmol-Na_2EDTA (pH 7.0), 1 mmol-$MgCl_2$, 25 mmol-Tris-maleate buffer (pH 5.25) and 0.5% Brij 58. Lane 3 shows 16mer cleavage after 2 h at 37°C in the complete reaction mixture. No reaction occurred without the Briji 58 (Lane 2) or without NH_4Cl (Lane 4) at 37°C. When the complete reaction mixture was incubated for same time at 0°C, no reaction was observed (Lane 6). Further, the reaction also occurred when only 0.5% Brij58 and 170 mmol-NH_4Cl were incubated with the 16mer (Lane 1). ***This experiment clearly shows that the non-ionic detergent Brij58 and 170 mmol-NH_4Cl are necessary and sufficient for the self-cleavage of p2Sp1 RNA, and that the reaction is temperature-dependent. We have further shown that this sequence does not contain the "hammer-head" structure and consensus sequence proposed for the viroid RNA self-cleavage.***

Acknowledgments

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#This paper is dedicated to Professor Colin B. Reese on the occasion of his 60th birthday.

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