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Synthesis of Oligoribonucleotides Bearing Morpholino-Nucleosides with Carbamate Internucleoside Linkages at the 3'-Terminus

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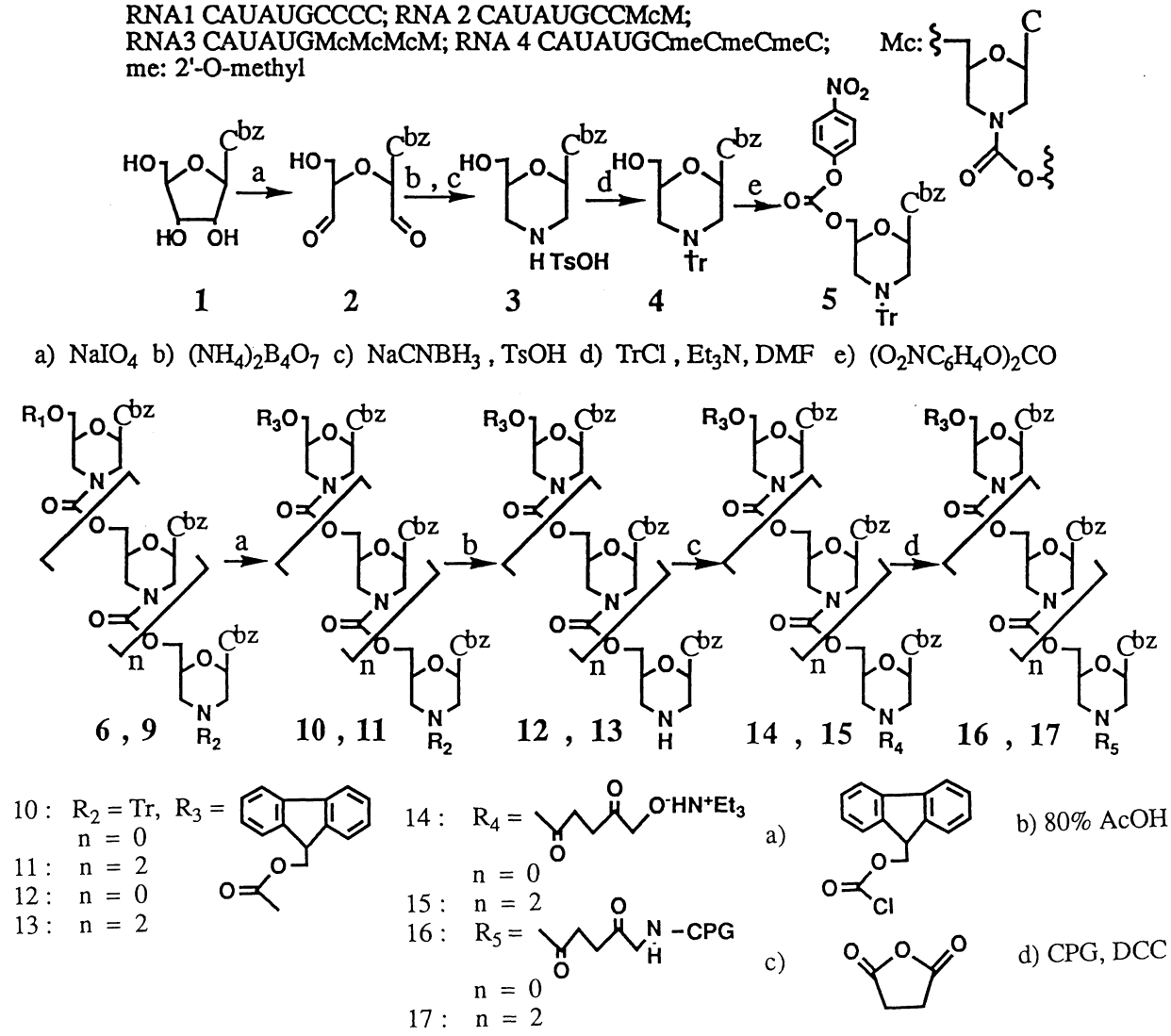
Oligoribonucleotides containing morpholino-nucleosides with carbamate internucleoside linkages at the 3'-terminus were synthesized by the solid phase method. These analogues were examined for stability in a prokaryotic cell-free system.

The nucleotide derivatives modified on the sugar moiety or the phosphate group have been used to inhibit viral gene expression or degradation by nucleases. The modified nucleoside derivatives were explored further in more recent studies by several groups. For example, Morvan,[1] Sproat,[2] and Stirchak[3] have reported the synthesis of oligonucleotides containing α-anomeric nucleoside, 2'-O-methylribonucleoside, and molpholino -nucleoside derivatives. Among these nucleoside derivatives, the oligoribonucleotides containing morpholino-nucleosides linked by a carbamoyl group were first synthesized and then examined for their ability to inhibit exonuclease activity of nuclease contaminating a prokaryotic cell-free system.[4-8]

We examined the synthesis of the leader sequences of mRNA coding phage Qβ A-protein bearing morpholino-cytidine with carbamate internucleoside linkages at the 3'-terminus. Preparation of the morpholino building blocks were synthesized according to the method by Stirchak et al.[4] (Scheme 1). Next, we have examined the preparation of the controlled pore glass (CPG) loading morpholino blocks (**6,9**). Here, the key point to success is to find appropriate protecting groups for the 6'-hydroxyl group, because of use of acid-labile protecting group for the imino group. In order to overcome this problem, we examined the use of an base-labile protecting group, 9-fluorenylmethoxycarbonyl (Fmoc) group.[9,10] The reaction of 9-fluorenylmethoxycarbonylation of morpholino blocks (**6,9**) with Fmoc-Cl gave the corresponding **10** and **11** in good yields. The compounds **10** and **11** were treated with 80% aqueous acetic acid at room temperature for 1.5 h to give the corresponding **12** and **13** which were used in the next succinylation after



Scheme 1.

purification. Compounds **12** and **13** were succinylated with succinic anhydride (1.5 molar equiv.) at 35-40 °C for 40 h in the absence of a base catalyst, such as 4-dimethylaminopyridine (DMAP), to avoid the removal of the 5'-O-Fmoc groups during the reaction. The succinylated compounds **14** and **15** were obtained in 70% and 74% yields after separation by silica gel column chromatography. Next, the succinylated derivatives **14** and **15** on CPG were loaded at room temperature for 16 h in the presence of 1,3-dicyclohexylcarbodiimide (DCC) in the absence of base catalysts such as triethylamine and DMAP for the same reason as described above. The amount of **16** and **17** loaded was 11 and 12 μmol/g, respectively, as estimated from the absorbance at 305 nm after treatment by 0.1 M piperidine/CH_3CN.

The reaction was carried out on a small column of nucleoside-function-

alised glass (**16** and **17**) (0.2 μM) with a Millipore Cyclone Plus DNA synthesizer. We demonstrated the elongation cycle as described follows: (1) treatment with 0.1 M piperidine/CH_3CN for 2 min to generate the 6'-free hydroxyl group,[9] (2) washing [CH_3CN, 30 s], (3) coupling [0.1 M 5'-O-dimethoxytrityl-2'-O-t-butyldimethyisilyl-N-protected nucleoside β-cyanoethyl phosphoramidite units[11] and 0.3 M 1H-tetrazole in CH_3CN for 10-15 min], (4) washing [CH_3CN, 70 s], (5) oxidation [0.1 M I_2 in THF/luitidine/H_2O, 30 s], (6) washing [CH_3CN, 30 s], (7) capping [solution A: acetic anhydride/luitidine/THF, solution B: N-methylimidazole/THF, 60 s],(8) washing [CH_3CN, 70 s], (9) 6'-deblocking [3% Cl_3COOH/CH_2Cl_2, 24 s], (10) washing [CH_3CN, 70 s], (11) coupling. The utility of morpholino-nucleoside oligomers with the carbamate (Mc) internucleoside linkages are now demonstrated by the synthesis of CAUAUGCCMcM (RNA 2) and CAUAUGMcMcMcM (RNA 3) containing a leader sequence of mRNA coding phage Qβ A-protein.

All of the sequences synthesized above were treated with conc.NH_4OH/EtOH (3:1v/v) at 30 °C for 24 h to cleave the reversible bond and remove the exocyclic amino acyl protecting groups. In the final deprotection, the TBDMS was removed from a partially protected oligomer by treatment with

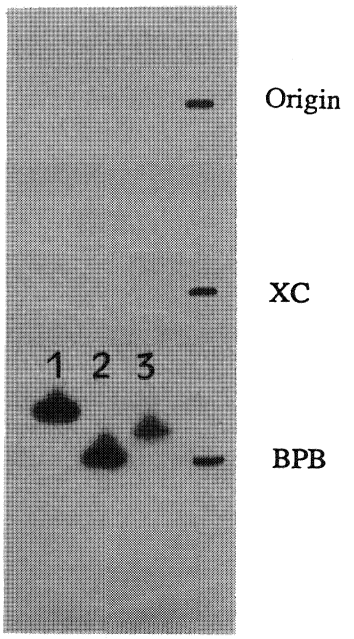


Fig. 1. 20% Polyacrylamide gel electrophoresis containing 7M urea of ^{32}P labelled oligoribonucleotide analogues.
Lane 1:RNA1, lane 2:RNA 3, lane 3: RNA2.

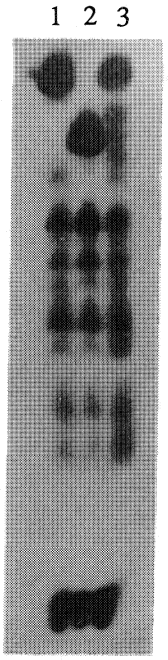


Fig. 2. 20% polyacrylamide gel electrophoresis containing 7M urea of oligoribonucleotide analogues.
lane 1: RNA 4, lane 2: RNA 3, lane 3:RNA1.

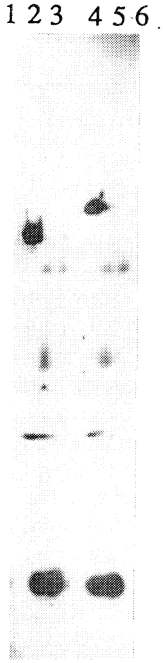


Fig. 3. Stability of analogues in a procaryotic cell free system.
lane 1-3: RNA3, lane 4-6: RNA 1, lane 1,4: (without S30 extract), lane 2,5: 30 min, lane 3,6: 60 min.

tetrabutylammonium fluoride/THF at room temperature for 24 h. The crude oligomer was purified by an OPC cartridge column and 20% polyacrylamide gel electrophoresis. Interestingly, the bands corresponding to RNA 2 and 3 containing morpholino-nucleoside oligomer moved faster than the corresponding to RNA 1. This phenomenon might be due to ion charge number of phosphate groups on RNAs (Fig. 1). Furthermore, the RNA 3 structure was analyzed by alkaline hydolysis (Fig. 2). The 3'-cleaved fragments formed by alkaline hydrolysis have different bands in the corresponding position to the 6 mer from 5'-terminus that is identical to the RNA structure containing a morpholino -nucleoside oligomer with carbamate linkages at the 3'-terminus (Fig. 2, Lane 2).

Finally, RNA 1, RNA 3, and RNA 4 (CAUAUGCmeCmeCmeC)[12] were examined for stability in a prokaryotic cell-free system[5] (Fig. 3). ^{32}P-Labeled RNAs were incubated with a S30 extract from *E. coli*, MRE 600 in 1 mM Tris-HCl buffer (pH 8.0), and 0.1 mM Na_2-EDTA (pH 8.0) at 37 °C. We found that the RNA containing modified nucleoside derivatives at the 3'-terminus were degraded completely by nucleases containing a prokaryotic cell-free protein synthesizing system. These findings suggested that the modified nucleoside derivatives can not be used to study the mechanism of translation in a prokaryotic cell.

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