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A NEW METHOD FOR THE SYNTHESIS OF CAPPED OLIGORIBONUCLEOTIDES BY USE OF AN APPROPRIATELY PROTECTED 7-METHYLGUANOSINE DIPHOSPHATE DERIVATIVE AS A DONOR FOR THE TRIPHOSPHATE BOND FORMATION

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Abstract. A 7-methylguanosine 5'-diphosphate derivative (1) protected with the acid-labile 4,4',4"-trimethoxytrityl and methoxymethylene groups was synthesized and used as a donor component for the triphosphate bond formation to give a capped hexaribonucleotide, $m^7G^{5'}$pppGUAUUA, in an improved yield.

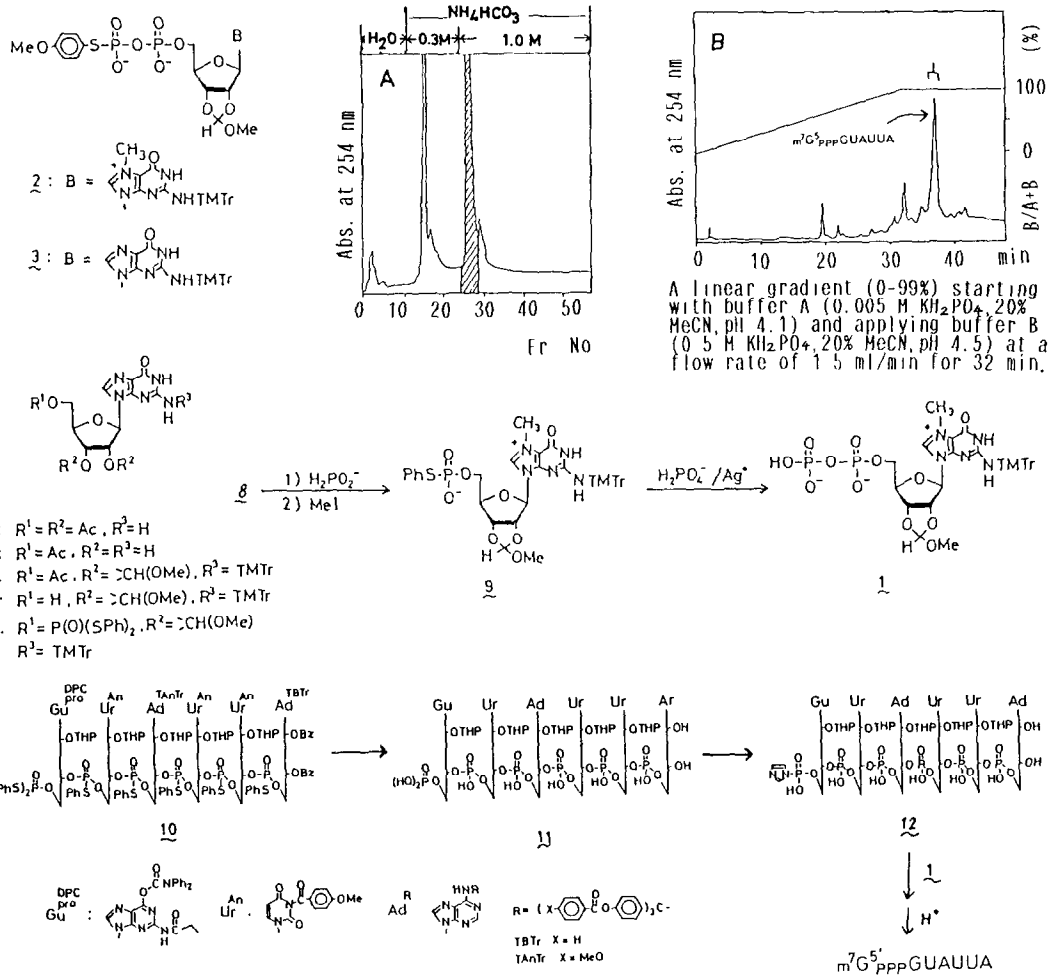
The cap structure discovered from eukaryotic mRNAs in 1975 by Furuichi and Miura[1] is known to make an important role in not only protection from digestion by exonucleases[2,3] but also regulation of peptide synthesis at the stage of formation of initiation complexes with ribosomes.[4,5] Because of the extreme base-labile property of the cap structure its chemical synthesis must be designed in a manner where all protecting groups should be removed under neutral or acidic conditions after construction of the cap structure from a capping reagent and an oligoribonucleotide component. This limited situation makes it more difficult to apply the usual strategy used for oligoribonucleotide synthesis to capped oligoribonucleotides. We[6-11] and other research groups[12,13] have reported several methods for preparation of the minimum-sized cap structure, $m^7G^{5'}$pppN (N=nucleoside). In these cases, satisfactory yields were obtained. However, application of these methods to the synthesis of capped oligoribonucleotides unexceptionally resulted in complex mixtures so that the desired products were isolated only in extremely poor yields.[9,11] For example, the isolated yield of $m^7G^{5'}$pppAUGACC was only 5%.[11] Therefore, we have searched for alternative more effective procedures for the capping reaction of oligoribonucleotides. In this paper, we wish to report a new promising approach to such capped oligoribonucleotides by the use of an appropriately protected 7-methylguanosine 5'-diphosphate derivative (1).

There are several possible combinations for construction of capped oligoribonucleotides. Our previous approach involved the triphosphate bond formation based on activation of the β-phosphoryl group of the pyrophosphorylating reagent, p-MeOPhSppG_{mM}^{TMTr} (2), having the 4,4',4"-trimethoxytrityl (TMTr) and methoxymethylene (mM) groups at the guanine and ribose moieties,

respectively. However, this reaction mode gave a lot of degradation products derived from the activated 7-methylguanosine 5'-diphosphate derivative. The activation of the β-phosphate residue of 2 led to its self decomposition since it had the reactive dissociated α-phosphate residue in the same molecule. To avoid such a complex reaction we previously added imidazole to the reaction mixture to trap the extremely reactive β-methaphosphate intermediate for its conversion into a more stable but still reactive β-phosphorylimidazole intermediate.[11] Although this modification gave considerably improved results, capped oligoribonucleotides were obtained only in poor yields. In consideration of these facts, we decided to examine the the capping reaction in which the 5'-terminal phosphate of oligoribonucleotide components was activated and an appropriately protected 7-methylguanosine 5'-diphosphate was used as a donor molecule.

A most accessible donor molecule for the capping reaction is a hydrolized product 1 of 2. Since it is known that the TMTr group tends to be lost considerably even at the stage of methylation of the precursor p-MeOPhSppG$^{TMTr}_{mM}$ (3), the diphosphate 1 can not be obtained in high yield by this method. Therefore, we synthesized 1 by the following new strategy: 2',3',5'-Tri-O-acetylguanosine (4)[14] was selectively deacylated by a modification of Ishido's procedure[15] to give 5'-O-acetylguanosine (5: mp 188.5-189 °C (MeOH)) in 61% yield. Trimethylsilyl chloride-catalyzed methoxymethylenation[16] of 5 with methyl orthoformate in DMF for 4.5 h followed by tritylation with TMTrCl in pyridine for 30 min gave compound 6 in 60% yield. Deacetylation of 6 with butylamine-MeOH (1:1, v/v) for 6 h gave the 5'-free guanosine derivative (7) in 71% yield. Phosphorylation of 7 with cyclohexylammonium S,S-diphenyl phosphorodithioate (PSS)[17] in the presence of 1-H-tetrazole and isodurenedisulfonyl dichloride (DDS)[18] in pyridine for 30 min gave the 5'-phosphorylated product (8) in 77% yield. Treatment of 8 (0.053 mmol) with 2.5M phosphinic acid[19] in pyridine (1.35 ml) and triethylamine (0.18 ml) at 40 °C for 1 h[20] followed by methylation gave the 7-methylguanylic acid derivative (9) in 85% yield. When needed, compound 9 was converted to the diphosphate derivative 1 by treatment with phosphoric acid in the presence of silver nitrate in pyridine for 1 h followed by treatment with hydrogen sulfide and subsequent extractive workup. The donor 1 thus obtained was found to be pure without contamination of the corresponding detritylated product. It was noteworthy that during a series of reactions described above the TMTr group was completely stable.

To demonstrate the utility of this new donor, a partially protected hexaribonucleotide 5'-phosphate (10) was synthesized as an accepter molecule according to the published procedure.[21] The synthesis of the parent fully protected hexaribonucleotide derivative (11) involved the use of the base-labile 4,4',4"-tris(benzoyloxy)trityl (TBTr)[22] and 4,4',4"-tris(p-anisoyloxy)trityl (TAnTr)[21] as the protecting groups of the amino groups of internal and 3'-terminal adenosine units, respectively. Reaction of 10 (octylammonium salt, 5 μmol) with carbonyldiimidazole[23] (0.1 mmol) in DMF (0.5 ml) for 1 h



gave the phosphorimidazolidate derivative (11). This compound was allowed to react *in situ* with 1 (25 μmol) in dry DMF (0.6 ml) for 21 h. After the reaction was completed, the mixture was evaporated and treated with 0.01 M HCl (pH 2.0) in dioxane-water (2:3, v/v) for 25 h. Chromatography of the mixture on a Sephadex A-25 column (see Fig. A) gave $m^7G^{5'}$pppGUAUUA (211 A_{259}, 61%) as crude material with a purity of *ca* 70%. Further purification by anion-exchange HPLC (Whatman partisil 10-SAX) (see Fig. B) gave the pure capped hexaribonucleotide in an overall yield of 16% from (10). Although considerable loss of material was observed during HPLC purification, the present capping reaction gave a very simple separation profile in both Sephadex A-25 and Whatman 10 SAX column chromatography. The structure of the synthetic capped hexaribonucleotide was confirmed by enzymatic assay with snake venom phosphodiesterase and nuclease P_1. The former gave a mixture of pm^7G, pG, pU, and pA in the expected ratio. The latter gave a mixture of $m^7G^{5'}$pppG, pU, and pA in the correct ratio.

In conclusion, the method described here would provide a new promising access to longer capped oligoribonucleotide fragments which could be utilized for biological studies in connection with the mechanism of peptide synthesis in eukaryotic cell.

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