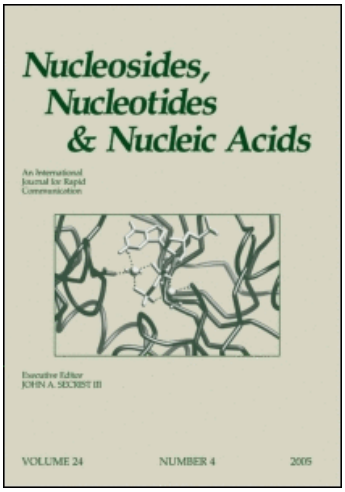

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SOLID-SUPPORTED SYNTHESIS OF 5′-mRNA CAP-4 FROM TRYPANOSOMATIDS

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□ *The unique structure of 5′ mRNA cap from* Trypanosomatids *is the most modified cap found in nature. Here we present the synthesis of cap-4 (m^7 Gpppm$_3{}^{6,6,2'}$ Apm$^{2'}$ Apm$^{2'}$ Cpm$_2{}^{3,2'}$ Up) on a disulfide-tethered solid support. This approach allows obtaining cap-4 more efficiently then previously described. Moreover such modified resin could be a useful tool for affinity purification of* Leishmania *proteins interacting with cap-4. For the final step of synthesis, namely coupling of phosphorylated tetranucleotide with activated 7-methylguanosine 5′-diphosphate two systems were compared. Surprisingly, the coupling in water with Mn^{2+} as a catalyst, gave better results than usually more effective coupling in DMF with $ZnCl_2$.*

Keywords 5′ mRNA Cap-4; disulfide linker; *Leishmania*; oligonucleotide synthesis; solid support

INTRODUCTION

Cap structure, that is, m^{7}GpppN plays important role in various biochemical processes and is used as a tool to study translation, splicing and RNA intercellular transport mechanisms. The recognition of the cap by initiation factor eIF4E is a crucial step in the initiation of protein biosynthesis process. The interactions between cap analogues and eIF4E can be monitored by measuring intrinsic fluorescence quenching of the

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tryptophan residues.[1] Since affinities of cap analogues to the eIF4E differ, they are attractive compounds for studying the molecular basis of cap recognition. Moreover, these differences may have also potential therapeutic significance.

The 5′ cap structure of *Leishmania*, denoted cap-4, is a complex structure that contains unusual modifications on the first four nucleotides—$m^7Gpppm_3{}^{6,6,2'}Apm^{2'}Apm^{2'}Cpm_2{}^{3,2'}U$. The two of four nucleosides: $N^6,N^6,O^{2'}$-trimethyladenosine and $N^3,O^{2'}$-dimethyluridine, were previously unknown in nature.[2] In previous articles, we showed that a synthetically prepared cap-4 analogue was efficiently recognized by an LeishIF4E-1 homologue from *Leishmania*.[3,4] Moreover, this structure is also efficiently recognized by three of four eIF4E isoforms from *Leishmania*—LeishIF4E-1, LeishIF4E-2, and LeishIF4E-4.

Here we describe the chemical synthesis of cap-4 on a disulfide-tethered solid support (Figure 1). This system can be used for affinity purification of *Leishmania* proteins that interact with cap-4. In order to obtain the cap-4 attached to a resin, we synthesized a disulfide-tethered solid support that would be resistant for ammonolysis.[5,6] The 5′-phosphorylated tetramer was prepared by the standard phosphoramidite solid phase method. However, since two of the four monomers for solid phase synthesis (phosphoramidites of $N^6,N^6,O^{2'}$-trimethyladenosine and $N^3,O^{2'}$-dimethyluridine) are not available commercially, we synthesized them according to the standard procedures.[3] As a solid support two commercially available resins were chosen: CPG and TentaGel. The 5′-phosphorylated tetramer was coupled on the resin with an imidazolide derivative of 7-methylguanosine 5′-diphosphate in the presence of 8-fold excess of $ZnCl_2$ (in DMF)[3,7,8] and in the presence of Mn^{2+} (in aqueous solution).[9]

RESULTS AND DISCUSSION

The disulfide linker allowed the RNA chain assembly without cleavage from the support during ammonolysis of base protecting groups; moreover, such linkers also may be reductively cleaved under mild conditions.[5,6] The synthesis of disulfide-tethered linker was performed in similar way like described earlier.[5,6] The amino groups of the standard CPG and TentaGel supports were acylated with dimeric 11-mercaptoundecanoic acid in the presence of N-hydroxysuccinimide, N,N-diisopropylcarbodiimide and 4-(dimethylamino)pyridine. The disulfide bond of dimers was cleaved on solid support by reduction with DTT.

The SH function of resin (**1**) was treated with 2-[(4,4′-dimethoxytrityl) oxy]ethyl 2-pyridyl disulfide and gave modified resin (**2**) ready to use in automated synthesis (Figure 2). According to the dimethoxytrityl (DMTr) cation assay, the loading was 60 μmol/g (for CPG) and 160 μmol/g (for TentaGel).

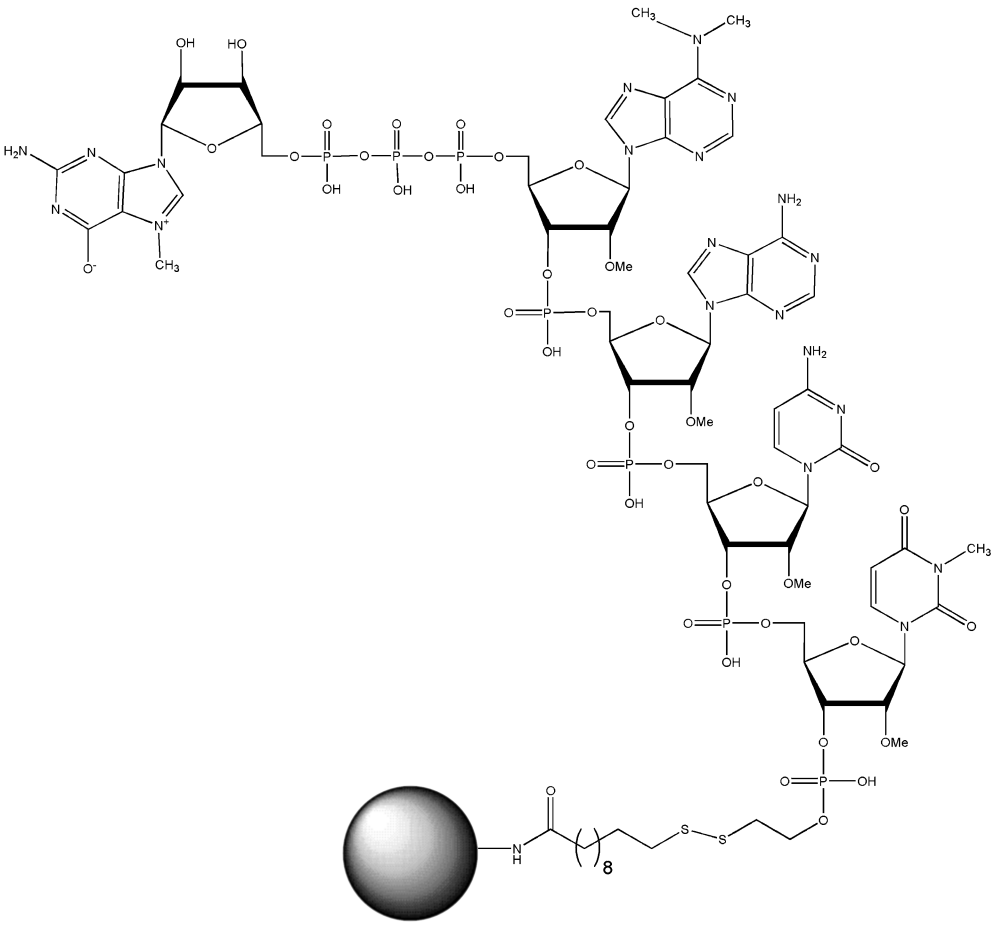

FIGURE 1 The structure of cap-4 on a disulfide-tethered solid support.

2-[(4,4′-Dimethoxytrityl)oxy]ethyl 2-Pyridyl Disulfide was obtained in a two-step reactions as described earlier involving synthesis of 2-hydroxyethyl 2-pyridyl disulfide from 2,2′-dithiodipyridine and mercaptoethanol and protection of OH-group with 4,4′-dimethoxytrityl chloride.[5] 5′-O-dimethoxytrityl-N^6,N^6,$O^{2'}$-trimethyladenosine-3′-O-(2-cyanoethyl)-N,N-diisopropylphosphoramidite was prepared as described earlier.[3] 5′-O-dimethoxytrityl-N^3,$O^{2'}$-dimethyluridine-3′-O-(2-cyanoethyl)-N,N-diisopropylphosphoramidite was obtained from 5′-O-dimethoxytrityl-N^3,$O^{2'}$-dimethyluridine in the presence of *N,N*-diisopropylethylamine and 2-cyanoethoxy *N,N*-diisopropylaminochlorophosphine in anhydrous acetonitrile.

After removal of the DMTr group, the tetranucleotide sequence was assembled by normal phosphoramidite chemistry and the 5′-hydroxy function in tetranucleotide was phosphitylated with 3-DMTrO-2,2-

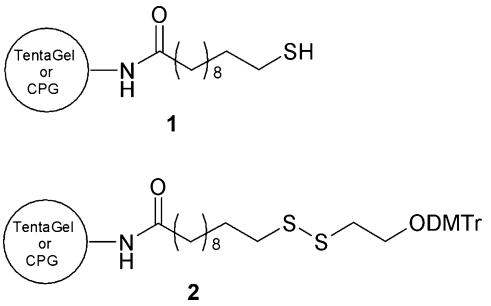

FIGURE 2 Structure of the supports.

bis(ethoxycarbonyl)propyl 2-cyanoethyl *N,N*-diisopropylphosphoramidite and oxidized to a phosphate triester.[10] The base and phosphate protections were removed by standard DMTr-off ammonolysis. The HPLC control of the ammonolysis of the protective groups have shown partial lost of tetranucleotide from CPG resin. We finished the ammonolysis when the concentration of deprotected oligonucleotide was about 90% (according to the HPLC analysis). In the case of tetranucleotide on TentaGel resin, we haven't observed the cleavage of the product from the resin during ammonolysis. The support-anchored oligonucleotide 5′-phosphate was then treated with 7-methylguanosine 5′-diphosphate imidazolide in DMF in the presence of 8-fold excess of $ZnCl_2$ (Figure 3) or in aqueous solution in the presence of Mn^{2+}. Surprisingly, the latter method gave better results in coupling reaction.

We monitored the capping reaction using HPLC (the disulfide bond was finally cleaved with DTT and the 2-mercaptoethyl group was allowed to

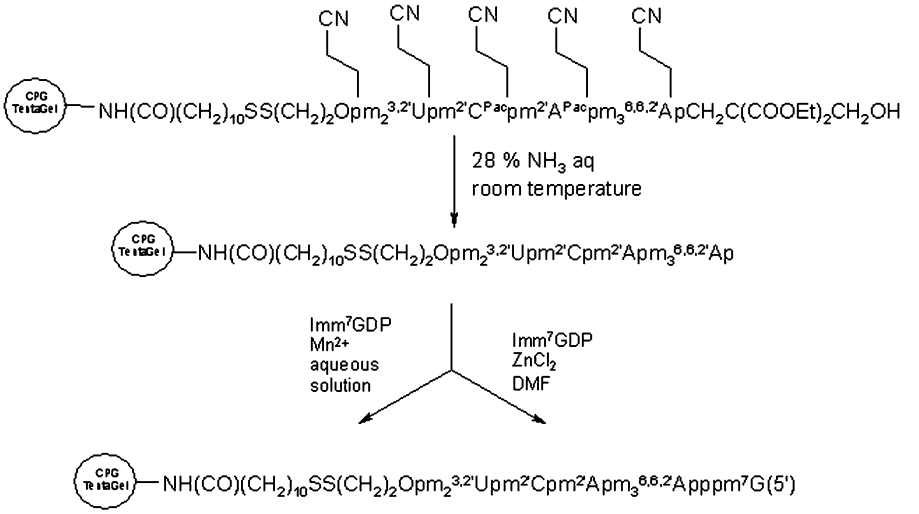

FIGURE 3 The synthesis of cap-4 on disulfide-tethered solid support.

eliminate in aqueous solution of methanol containing 0.01% triethylamine). On the basis of HPLC the yield of the capping step was 50%, (in the case of TentaGel resin).

The cap-4 on solid support (the structure shown on Figure 1.) will be used in affinity chromatography of eIF4E homologues from *Leishmania.*

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