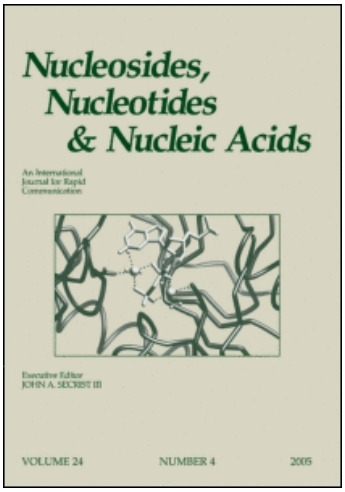

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New Affinity Resin for Purification of Cap-Binding Proteins

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NEW AFFINITY RESIN FOR PURIFICATION OF CAP-BINDING PROTEINS

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▫ *Cap binding proteins, which recognize the cap structure present at 5′ termini of RNA polymerase II transcripts, have been routinely isolated and purified using affinity resins with mononucleotide cap analogs attached. Here we present a new methodology in which dinucleotide cap analog, m^7GpppG, has been linked to the EAH-Sepharose. The method is based on derivatization of 2′, 3′-cis diol of the second nucleotide within the cap structure, with levulinic acid, and subsequent coupling of resulted acetal through its carboxylic group with aminohexyl-agarose.*

INTRODUCTION

The cap structure (m^7GpppN, N-any nucleoside) present at the 5' termini of all eukaryotic mRNAs is recognized by cytoplasmatic cap-binding protein eIF4E during the rate-limiting step of protein initiation.[1] In the nucleus, the cap structure of nascent eukaryotic RNA polymerase II transcripts (e.g., pre-mRNA, U snRNA) is bound by heterodimeric cap binding complex CBC.[2] Rapid and effective purification of various cap-binding proteins has been accomplished by using affinity chromatography based on mononucleotide cap analogues covalently attached to a solid matrix. Commercially available m^7GTP-Sepharose, containing the cap analogue coupled through γ-*p*-aminophenyl ester, has been widely used for isolation and purification of different cap-binding proteins, both recombinant and from natural sources.[3] Attachment of the mononucleotide analogue, 7-methyl-guanosine di- or triphospate, provides the resin with high affinity towards the eIF4E protein. It has been shown that di- or triphosphates of 7-methylguanosine (m^7GDP,

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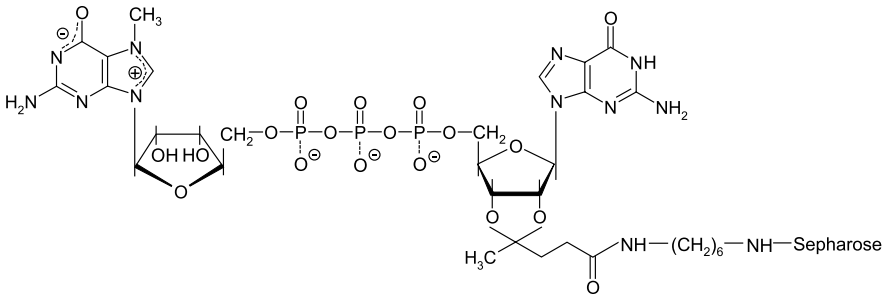


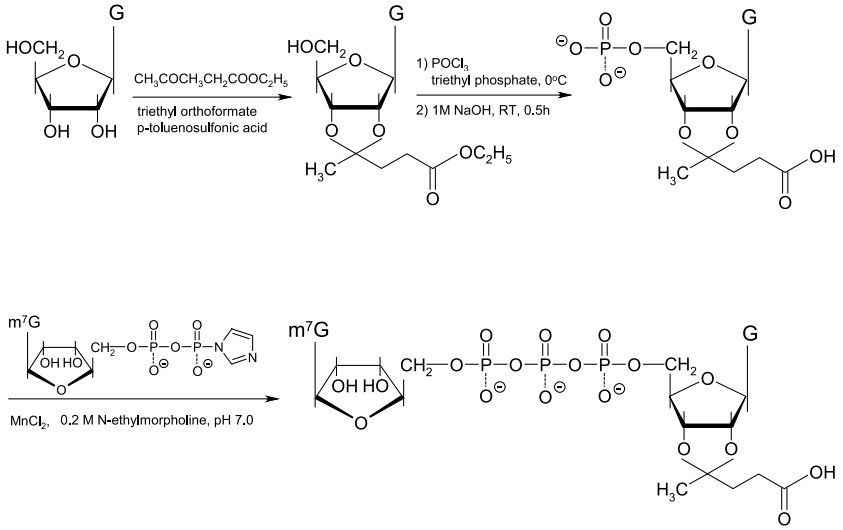
FIGURE 1 Affinity resin, m^7GpppG-Sepharose.

m^7GTP) bind to eIF4E more than 10-fold stronger than the dinucleotide 5′–5′ triphosphates of type m^7GpppN.[4] A similar relationship was obtained from earlier quantitative assessments of various cap analogues as inhibitors of in vitro translation.[5] Human nuclear CBC exhibits different cap analogue recognition specificity as it binds dinucleotide cap analogue m^7GpppG about 30-fold more efficiently than m^7GTP or m^7GDP.[6] Because of that, we anticipate that m^7GpppN dinucleotide coupled to the sepharose matrix could provide more suitable affinity resin for isolation and/or purification of CBC.

We have developed a new methodology to connect dinucleotide cap analogs, for instance m^7GpppG, to the sepharose (Figure 1) in a way allowing to maintain the basic structural features of cap necessary for the recognition by cap-binding proteins. Our approach is based on derivatization of 2′,3′-*cis* diol of the second nucleotide in cap structure with levulinic acid and subsequent coupling of resulted acetal through its carboxylic group with aminohexyl-agarose.

RESULTS

Synthesis of functionalized m^7GpppG ligand with levulinic acid is shown in Scheme 1. The first step was to convert guanosine into 2′,3′-*O*-[1-(3-etoxy-3-oxopropyl)ethylidene]guanosine (Lew(OEt)Guo). As we were unable to obtain the above compound following the method described by Seela and Waldek,[7] we have developed a modified procedure. It relies on reaction of guanosine with ethyl levulinate in the presence of *p*-toluenosulfonic acid and triethyl orthoformate. Next, Lew(OEt)Guo was phosphorylated with phosphorus oxide trichloride in triethyl phosphate according to Yoshikawa's procedure.[8] After subsequent saponification requiring use of 0.5 M NaOH, final LewGMP was obtained with total 55% yield compared to 30% according to Seela and Waldek.[7] The pyrophosphate bond formation was achieved by coupling reaction between imidazole-activated m^7GDP and LewGMP in water solution in the presence of manganese(II) ions.[9] The product was separated from reaction mixture by ion-exchange chromatography on DEAE-Sephadex in HCO_3^- form. Overall reaction yield was about 35%. The purity of final product was determined by analytical HPLC on both ion exchange



SCHEME 1 Synthesis of functionalized m^7GpppG ligand.

and reverse-phase column. Identification of m^7GpppG derivative was based on ^{1}H, ^{31}P NMR, and mass spectra. It is noteworthy that the coupling occurred exclusively through the phosphate group. The coupling of the above dinucleotide ligand with agarose containing 1,6-diaminohexyl spacer groups (EAH-Sepharose) was achieved by carbodiimide method using water-soluble condensation reagent, N-ethyl-N′-(3′-dimethyl-aminopropyl)-carbodiimide hydrochloride (EDC).

Determination of the amount of ligand covalently linked to EAH-Sepharose was performed by slightly modified procedure described earlier.[7] It relies on acidic hydrolysis of bound nucleotide at 100°C and measurement of the extinction at 260 nm. Comparing this value with those obtained for starting commercial polymer and ligand alone, it was possible to estimate the quantity of ligand bound to the matrix. In our case it was 28 μmol ligand per 1 g of dry resin. The newly synthesized affinity resin is being currently tested.

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