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Synthesis of the first double-functionalized dinucleotide mRNA cap analogue for its specific labeling

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ABSTRACT

The modification of various important nucleotide-based molecules (such as nucleotides, RNA, DNA, oligonucleotides) with fluorophores, affinity tags and reactive moieties is of enormous utility for studying their localization, structure and dynamics, as well as diverse biological functions involving their interacting partners. Herein, we report chemical methodology in which the dinucleotide mRNA cap analogue is doubly modified within its second nucleotide. The prepared dinucleotide contains an alkyne at the *N*2-position of guanine, and levulinic acid within the ribose moiety. Such modifications may be further used for specific labeling of the cap, for instance with a fluorophore that will allow the molecule to be tracked inside the cell and an attachment cell-penetrating peptide that will help to deliver it to the area of interest. Exemplar molecules were attached in order to demonstrate the utility of the newly synthesized cap analogue.

Interest in the study of RNA, DNA and their fragments have grown steadily over the previous decades.[1] Chemical modifications of nucleotides are often indispensable, not only for localization, structure and dynamics studies, but also for research on the biomolecular interactions of parent compounds with protein partners.[2] General approaches to obtain nucleotides bearing diverse "reporters" at specific locations are based on the selective insertion of functional groups that are responsible for the characteristic chemical reactions of those molecules. For this reason, it is mandatory that the functional groups have different chemical properties. Additionally, labels should not influence the biological properties of the (oligo)nucleotide or nucleic acid, in other words it is important that both are placed at the correct distance from each other and are attached to the appropriate parts of the molecule.

Among widely studied nucleotide-like compounds are analogues of the 5′ end of mRNA, the cap, that in its basic form consists of a 7-methylguanosine connected *via* a 5′,5′-triphosphate bridge with the second nucleotide (usually G).[3] The 5′-end of RNA plays an essential role in several processes during gene expression and serves as a signal for engaging proteins required for those processes.[4] To date numerous modified cap analogues have been tested and used in various biological and biophysical studies to elucidate processes in which it is involved.[5] Apart from analogues with modifications that altered their biological properties,[6] these studies have employed derivatives containing additional single functionalization with an attached probe or other specific molecule.[7]

Besides the undeniable role of cap analogues in explaining the molecular processes associated with gene expression, these compounds have been intensively examined as effective inhibitors of translation that could be used as anti-cancer drugs.[8] The idea of applying such nucleotide derivatives emerged with reports that numerous cancer cells exhibit elevated level of eukaryotic initiation factor 4E (eIF4E).[9] The interaction of eIF4E with the mRNA cap structure is crucial and a rate-limiting step of protein synthesis, and for that reason it can be exploited as a target for discovery.[10] In this context, the need for covalent attachment to therapeutic molecules, e.g. biophysical probes that will allow to follow them inside the cell as well as other molecules that will increase, for example, their cell membrane permeability is desirable. To obtain such molecules and to open up new research opportunities, herein, we describe the first methodology to synthesize double functionalized dinucleotide mRNA cap analogues. In addition, the work demonstrates the use of such a functionalized cap analogue for the attachment of two compounds - a fluorescent dye and a model peptide.

If a particular biologically active compound is used for biophysical and biological studies, labels should be introduced in a manner that does not influence the functionality of the compound. For this reason, in our case, it was highly important to consider which parts of the 5′ end of mRNA are involved in protein interaction. The cap

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structure binds to the eIF4E protein mainly by charge-related interactions inside the cap-binding center, and are accomplished by: sandwich cation-π stacking of methylated guanine with protein aromatic side-chains, hydrogen bonds between nitrogen atoms N1 and/or N2 of the m^7G base and acidic residues, and salt bridges between the negatively charged phosphate chain of the cap and basic residues of eIF4E.[5d–g] Knowing this, the ribose moiety of the second nucleoside and the second base of the cap structure are the most favorable sites to conjugate with the two different labels. To make this possible, the cap analogue was modified with functional groups complementary to the functionality of the probes. The proposed strategy included insertion of functional groups that were compatible with "click chemistry" and the carbodiimide reaction of an activated carboxyl group with primary amines or alcohols. Click chemistry was chosen as it is a widely used reaction for various applications, due to the fact that it usually proceeds rapidly and selectively in a neutral environment (pH 6–8) at ambient temperature (20–30 °C).[11] For the first alkyne/azide functionalization, the exocyclic NH_2 group present within the second base of the cap was selected. This type of derivative (N2-modified guanosine) has already been synthesized for modification of the first methylated nucleotide in the cap structure and evaluated as potential translation inhibitors, whereas now this methodology was used to modify the second non-methylated base in the dinucleotide cap mRNA.[12]

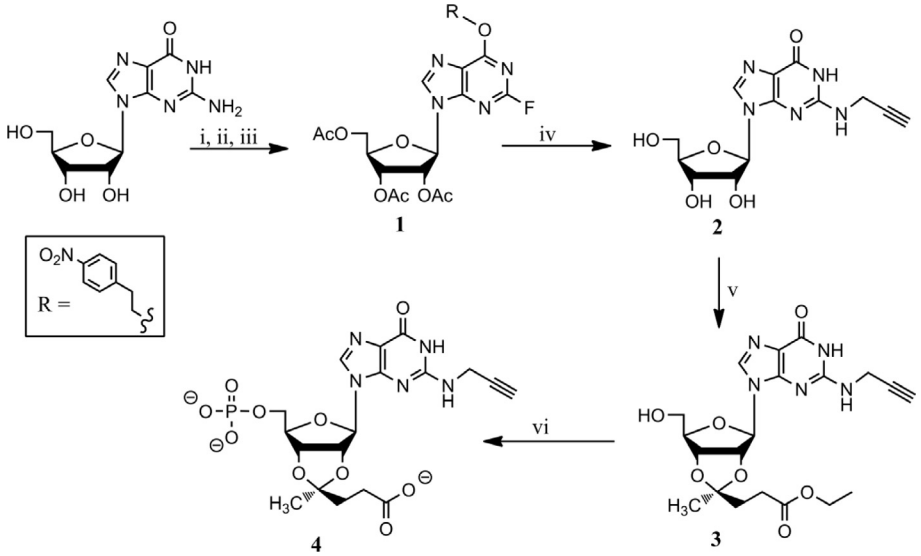
The second modification to the carboxylic group was also introduced within the non-methylated nucleotide, but in a site that does not cause congestion with the N2 position. Among different possibilities we proposed to functionalize the cap analogue by derivatization of the 2′,3′–*cis* diol of the second ribose with levulinic acid. We chose this functionalization since: a) the introduced reactive group is not present within the natural compound, which provides high selectivity, b) the coupling reaction can be successfully carried out under aqueous conditions, and c) such modifications had been previously used for the preparation of ligands for affinity resins and employed to isolate the cap specific eIF4E protein.[13]

The main difficulty in obtaining a double modified molecule is that it requires attachment of two different fragments that can strongly influence the properties (e.g. solubility) of the molecule which may lead to the need for alteration or adaptation of procedures that were previously developed. Therefore, it should be considered which probe is introduced first, and how it might affect the following step(s) of the synthesis.

The guanosine bearing alkyne modification was chosen to be performed first according to a procedure that was previously reported by our group.[12b] This method is based on the transformation of guanosine into its fully protected form, then subsequent fluorination and substitution of the obtained derivative to give the N2 modified guanosine analogue (Scheme 1).

Briefly, dried guanosine was subjected to triacetylation with acetic anhydride (Ac_2O) in the presence of triethylamine (TEA) and *N*,*N*-(dimethylamino)pyridine (DMAP), followed by protection of the O^6 group of guanosine with a *p*-nitrophenylethyl (NPE) group using the Mitsunobu reaction as previously described.[12] Obtained in this manner, 2′,3′,5′-tri-*O*-acetyl-O6-[2-(4-nitrophenyl)ethyl]guanosine was subjected to diazotization and fluorination reactions under anhydrous conditions with *t*-butyl nitrite and HF in pyridine. *N*2-fluoro-2′,3′,5′-O-triacetyl-O6-[2-(4-nitrophenyl)ethyl]inosine (**1**) was then converted to alkyne derivative (**2**) by nucleophilic substitution with commercially available 2-propyn-1-amine. In the next step the *N*2 modified guanosine was treated with ethyl levulinate in the presence of *p*-toluenesulfonic acid and triethyl orthoformate to afford 2′,3′-*O*-[1-[2-(ethoxycarbonyl)ethyl]ethylidene]guanosine with a propargyl substituent at the N2 position (**3**) in high yield. Compound **3** was not isolated from the reaction mixture but directly converted to derivative **4** by 5′ phosphorylation using phosphorus trichloride oxide ($POCl_3$) in trimethyl phosphate at 4 °C and alkaline hydrolysis. The resulting mononucleotide **4** was purified by ion-exchange chromatography (DEAE-Sephadex). Notably, prior to ion-exchange chromatography all steps did not require sophisticated purification methods, only simple precipitation of the product, washing and drying.

The next step included coupling the functionalized mononucleotide with 7-methyguanosine-5′-diphosphate (m^7GDP). The most popular synthetic pathway leading to formation of the 5′,5′-triphosphate bridge in the cap structure is based on a strategy in



Scheme 1. Synthesis of double functionalize mononucleotide **4**; Reagents and conditions: i) Ac_2O (3.1 equiv.), DMAP (0.1 equiv.), TEA (7.8 equiv.), MeCN (3.9 mL), 0 °C, 98%; ii) 2-(4-nitrophenyl)ethanol (1.5 equiv.), PPh_3 (1.5 equiv.), DIAD (1.2 equiv.), anhydrous toluene (8.1 mL), 48 h, RT, 70%; iii) 70% HF in pyridine (235 equiv.), anhydrous pyridine (3.8 mL), *t*-butyl nitrite (2.5 equiv.), −35 °C, 85%; iv) a) 2-propyn-1-amine (2 equiv.), DMSO; b) 33% dimethylamine solution in absolute EtOH (0.05 equiv.), 70%; v) ethyl levulinate (16 equiv.), triethyl orthoformate (2.4 equiv.), *p*-toluenesulfonic acid (0.6 equiv.), 5 h, RT, 97%; vi) a) $POCl_3$ (2.5 equiv.), trimethyl phosphate (42 equiv.), 5 h, 4 °C; b) 1 M NaOH (2 mL), 35%.