# A trinucleotide cap analog bearing a locked nucleic acid moiety: synthesis, mRNA modification, and translation for therapeutic applications

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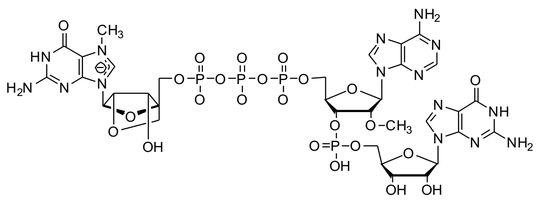
## Abstract



A new trinucleotide cap analog with the general formula m$^{7(LNA)}$G(5′)ppp(5′)A$_m$pG, which contains a locked nucleic acid (LNA) moiety, is reported. This new cap analog outperforms the standard trinucleotide cap, m$^7$G(5′)ppp(5′)A$_m$pG, and anti-reverse cap analog (ARCA), m$_2^{7,3'\text{-}O}$G(5′)ppp(5′)G, by 5-fold in terms of its translational properties.

## Introduction

Cellular and eukaryotic viral mRNA synthesized by RNA polymerase contains the distinct cap structure m$^7$G(5′)ppp(5′)N, where N is any nucleotide. A 7-methylguanosine residue is connected to the 5′ end of the transcribed RNA via a 5′-5′ bridge. This unique cap structure plays an important role in mRNA metabolism. Using the synthetic dinucleotide analog m$^7$G(5′)ppp(5′)G for *in vitro* synthesis of 5′-capped mRNAs generates products with forward [m$^7$G(5′)ppp(5′)G(pN)$^n$] and reverse [G(5′)ppp(5′)m$^7$G(pN)$^n$] orientations due to the presence of 3′-OH groups on both guanosine moieties. However, an anti-reverse cap analog (ARCA) paired with an m$^7$G moiety incorporates exclusively in the forward orientation if the 3′-OH or 2′-OH group on the moiety is chemically modified. This results in a more than two-fold increase in translation efficiency over the standard cap analog.

The synthesis and molecular application of various trinucleotide cap analogs with translational properties that are superior to those of dinucleotide cap analogs has been reported. It should be noted that the presence of a 2′-*O*-methyl group on the first transcribed nucleotide significantly affects differentiation between self and non-self RNA during viral infection. It also contributes to the sensing of non-self RNA and suppresses viral replication and pathogenesis.

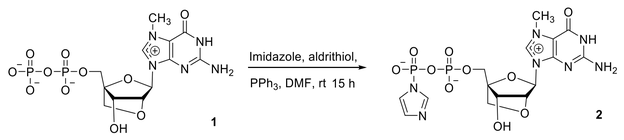
mRNA for the Moderna and Pfizer-BioNTech vaccines for the severe acute respiratory syndrome coronavirus SARS-CoV-2 is transcribed *in vitro* and contains a cap 1 structure. Since emergency use authorization was issued for the vaccines by the Food and Drug Administration (FDA), demand for new trinucleotide cap analogs to develop mRNA vaccines for other infectious diseases has grown. We hypothesized that a new trinucleotide cap analog bearing an LNA moiety could be a useful molecular biology tool for the development of new mRNA vaccines and mRNA transfection applications, such as anticancer immunization, protein production, and gene therapy.

Herein, we report the first synthesis and biological evaluation of a trinucleotide cap analog that has the general structure m$^{7(LNA)}$G(5')ppp(5')A$_m$pG and bears an LNA moiety.

## Results and discussion

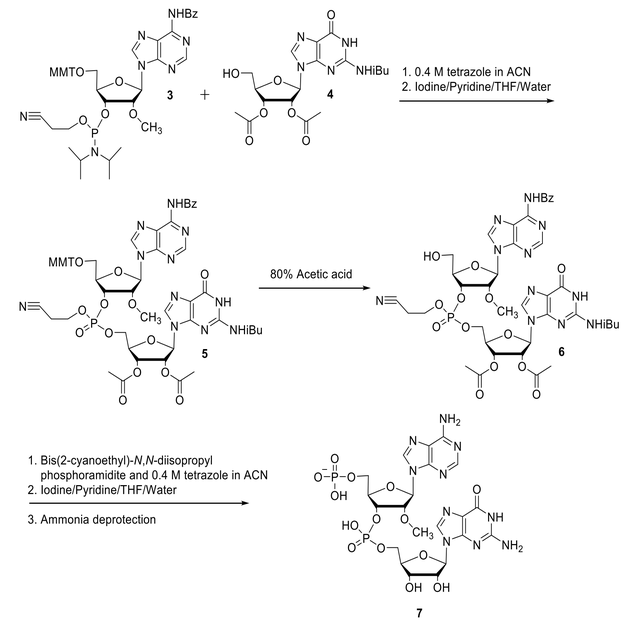
The intermediate m$^{7(LNA)}$ImGDP **2** required for synthesis of the LNA-substituted trinucleotide cap structure m$^{7(LNA)}$G(5')ppp(5')A$_m$pG **8** was obtained from m$^{7(LNA)}$GDP **1** as depicted in Scheme 1.

**Scheme 1. Synthesis of m$^{7(LNA)}$ImGDP 2.**



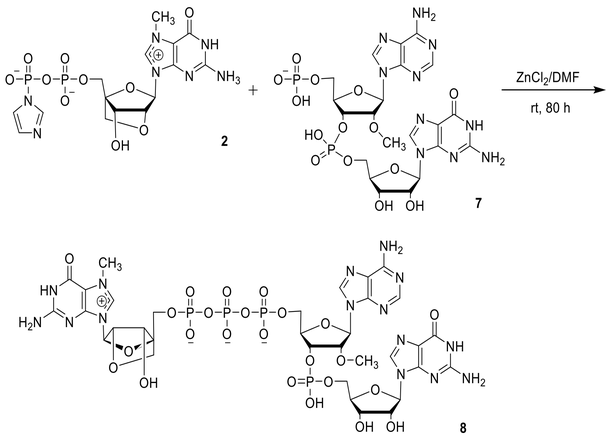

The intermediate pA$_m$pG **7** required to synthesize the LNA trinucleotide cap m$^{7(LNA)}$G(5')ppp(5')A$_m$pG **8** was obtained via liquid-phase synthesis in three steps as shown in Scheme 2.

**Scheme 2. Synthesis of dinucleotide pA$_m$pG 7.**




The coupling reaction of pA$_m$pG **7** with m$^{7(LNA)}$ImGDP **2** in the presence of a zinc chloride catalyst in DMF produced m$^{7(LNA)}$G(5′)ppp(5′)A$_m$pG **8** at 55% yield and >99% purity, as evidenced by HPLC. The structure of **8** was thoroughly characterized by $^1$H NMR, $^{31}$P NMR, and mass spectrometry.

**Scheme 3. Synthesis of LNA-substituted trinucleotide cap analog 8.**




### mRNA transcription

- The capping efficiency of LNA-substituted trinucleotide cap **8** was compared to that of the standard trinucleotide cap analog (GAG), m$^7$G(5')ppp(5')A$_m$pG **9**, and the anti-reverse cap analog (ARCA), m$_2^{7,3'\text{-}O}$G(5')ppp(5')G **10** (Figure 1), using an *in vitro* transcription system.
- The mRNA yields from *in vitro* transcription are shown in Figure 2.

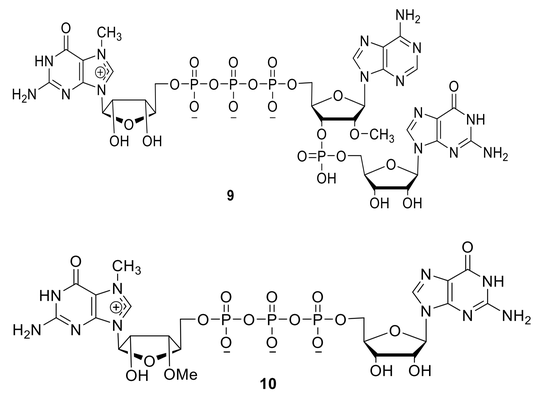


**Figure 1.** Chemical structures of standard trinucleotide cap GAG **9** and ARCA **10**.

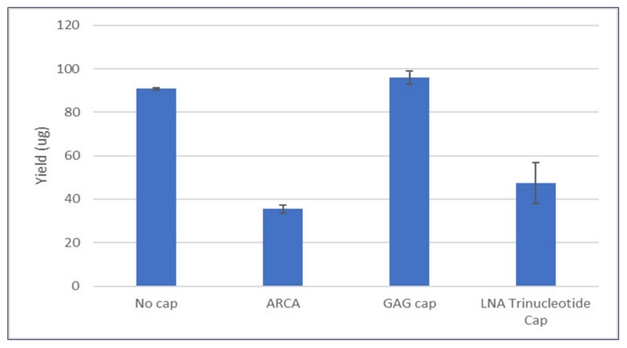



**Figure 2.** mRNA transcription yields obtained with LNA-substituted trinucleotide cap **8**, GAG **9**, and ARCA **10**.

- The data showed that LNA-substituted trinucleotide cap 8 had a capping efficiency of 53%, while standard trinucleotide cap 9 had a capping efficiency of 95% (Figure 3). ARCA 10 had a capping efficiency of 83%.
- The lower capping efficiency of LNA-substituted trinucleotide cap **8** was probably due to modification of the LNA.

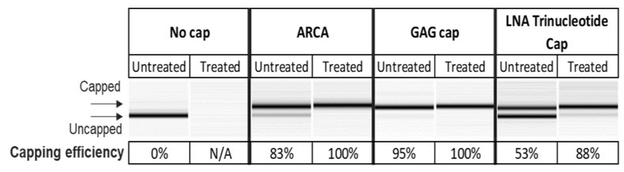



**Figure 3.** Capping efficiencies of LNA-substituted trinucleotide cap **8**, GAG **9**, and ARCA **10**.

### Translation efficiency

- Translation efficiency was evaluated with each cap analog in JAWSII cells from an immortalized mouse immature dendritic cell line.
- The cell culture was assayed for GFP fluorescence by flow cytometry analysis.
- The data showed that expression of the purified LNA-substituted trinucleotide-capped mRNA transcripts was 5 times higher than expression of the GAG- and ARCA-capped mRNA transcripts in the dendritic cell line (Figure 4).
- The remarkable translational properties of LNA-substituted trinucleotide cap **8** were probably due to the exclusive formation of forward-capped mRNA transcripts that produced a homogeneous population of mRNA molecules.
- It is likely that a conformational preference for a C3′-endo (N-type)[28] LNA structure and greater intracellular stability due to LNA modification promoted higher translation efficiency compared to that of mRNA capped with standard trinucleotide cap **9** and ARCA **10**.

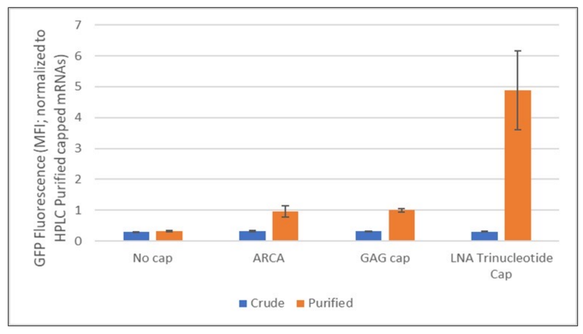



**Figure 4**. Translation efficiency with LNA-substituted trinucleotide cap **8**, GAG **9,** and ARCA **10**.

## Conclusion

- This is the first report of synthesis of a new trinucleotide cap analog bearing an LNA moiety.
- *In vitro* transcription of the new LNA cap analog generates mRNA cap 1 structures.
- A capping assay indicates this new trinucleotide LNA cap analog is a substrate for T7 RNA polymerase.
- The new trinucleotide LNA cap analog outperforms control cap analogs 5-fold in terms of its translational properties.
- Based on the higher translation efficiency, this new LNA-substituted trinucleotide cap analog is a potentially useful molecular biology tool for mRNA vaccine production and mRNA transfection applications like anticancer immunization, protein production, and gene therapy.

## References

1. Senthilvelan A, Vonderfecht T, Shanmugasundaram M, Pal I, Potter J, Kore AR (2021) *Org Lett* 23:4133-4136.

2. Shanmugasundaram M, Senthilvelan A, Kore AR (2017) *Curr Org Chem* 21:2530-2560.