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Synthesis and evaluation of an alkyne-modified ATP analog for enzymatic incorporation into RNA

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ABSTRACT

Alkyne-modified nucleoside analogs are useful for nucleic acid localization as well as functional and structural studies because of their ability to participate in copper-catalyzed azide/alkyne cycloaddition (CuAAC) reactions. Here we describe the synthesis of the triphosphate of 7-ethynyl-8-aza-7-deaza-adenosine (7-EAATP) and the enzymatic incorporation of 7-EAA into RNA. The free nucleoside of 7-EAA is taken up by HeLa cells and incorporated into cellular RNA by endogenous RNA polymerases. In addition, 7-EAATP is a substrate for both T7 RNA polymerase and poly (A) polymerase from *Escherichia coli* in vitro, albeit at lower efficiencies than with ATP. This work adds to the toolbox of nucleoside analogs useful for RNA labeling.

Nucleoside analogs are important for studying nucleic acid structure and function.[1] Derivatives bearing terminal alkynes are particularly useful since they allow for further modification by copper-catalyzed azide/alkyne cycloaddition (CuAAC) reactions.[2–7] Nucleoside analogs can be incorporated into oligonucleotides enzymatically or by chemical synthesis.[2–11] Previously, our lab introduced 7-ethynyl-8-aza-7-deazaadenosine (7-EAA) into RNA using chemical synthesis with a 7-EAA phosphoramidite.[2] X-ray crystallography of RNA duplexes bearing 7-EAA showed this analog forms a stable base pair with uridine and does not perturb the helical geometry of the duplex while projecting the 7-ethynyl group into the RNA duplex major groove (Fig. 1).[3] Furthermore, the high CuAAC reactivity of 7-EAA in RNA suggested it could be a useful probe of RNA structure and localization.[3] Other alkyne-modified adenosine derivatives have been reported, but less is known about their effects on RNA structure.[4,11] Enzymatic incorporation of 7-EAA using RNA polymerases would allow for the synthesis of longer RNAs with more complex structures. In addition, since 7-EAA is an adenosine analog, its triphosphate is a mimic of ATP and could function as a substrate (or co-substrate) for other ATP-dependent enzymes.[13–15] For these reasons, we prepared 7-EAATP and evaluated it as a substrate for two different ATP-utilizing enzymes: T7 RNA polymerase and *Escherichia coli* poly (A) polymerase. In addition, we showed that HeLa cells treated with the 7-EAA nucleoside produce RNAs reactive in CuAAC reactions indicating 7-EAA uptake and intracellular conversion to the triphosphate in human cells.

Enzymatic reactions of 7-EAA-containing RNAs showed that certain enzymes (e.g., AMV reverse transcriptase and an active site mutant of human editing enzyme ADAR2) recognized 7-EAA in RNA as adenosine.[3] Given the similarity between 7-EAA and toyocamycin, a natural product known to be incorporated into cellular RNA,[16] we believed 7-EAA could be taken up by human cells, converted to the triphosphate and incorporated into cellular RNAs via RNA polymerases.[4–6] In order to test this idea, the 7-EAA nucleoside was required. This compound was originally synthesized by Seela and coworkers.[17] Here we prepared the molecule with an optimized two step synthesis from 2′,3′,5′-tri-*O*-benzoyl-7-iodo-8-aza-7-deazaadenosine (**1**) (Scheme 1).[18] The unprotected nucleoside product was then added to tissue culture media supporting the growth of HeLa cells and these cells were incubated for an additional 12 h followed by RNA isolation. We then performed a CuAAC reaction on the isolated RNA using a biotin azide. The resulting RNAs were resolved on a denaturing agarose gel, transferred to a nylon membrane and probed for the presence of biotin (Fig. 2). Biotin signal was observed from RNAs isolated from the cell sample grown in the presence of 7-EAA. Various lengths of RNAs were observed on the biotin detection image, suggesting different lengths of RNA were labeled with 7-EAA within the cell. In contrast, RNAs isolated from the cell sample grown in the absence of 7-EAA showed no biotin signal. To establish that absence of signal was not caused by absence of material, the same membrane was imaged using ethidium bromide detection. RNAs isolated from

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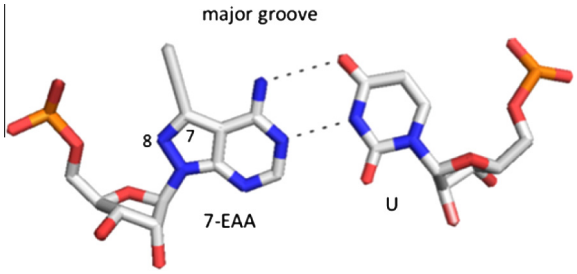
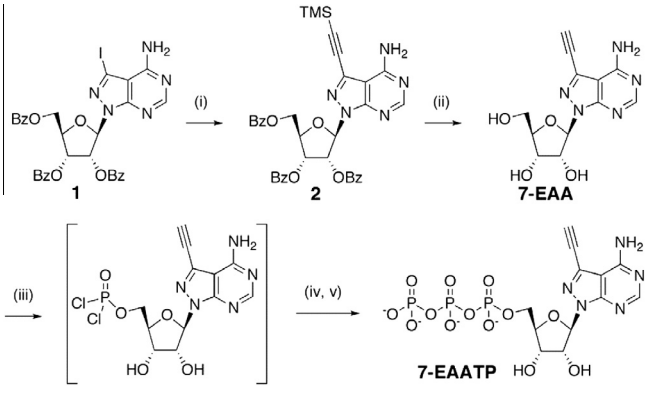


Figure 1. 7-Ethynyl-8-aza-7-deazaadenosine (7-EAA) base pairs with uridine (U) in duplex RNA and projects a terminal alkyne into the major groove.[3]

the cell sample grown without 7-EAA showed an intense signal with ethidium bromide detection. In addition, to confirm the signal observed was indeed from modified RNA, we treated the isolated RNA samples with ribonuclease V1 prior to the Northern blot detection. The results indicated the signal was generated by RNA. Together these data show that 7-EAA was taken up by HeLa cells and incorporated into cellular RNA. We also carried out a pulse/chase experiment to assess the turnover of 7-EAA-modified RNA in HeLa cells and compared this to 5-ethynyl uridine (EU)-modified RNA turnover. These experiments indicate 7-EAA-modified RNA is longer lived in these cells with a $t_{1/2}$ approximately 2-fold greater that EU-modified RNA (Supporting information).

To test directly the efficiency of transcription with the triphosphate of 7-EAA, we synthesized this compound (Scheme 1, 7-EAATP). Different approaches have been reported for the synthesis of nucleoside triphosphates chemically and enzymatically.[9,19–21] Compared with other methods, the one-pot synthesis from unprotected nucleoside to nucleoside triphosphate has the advantages of minimizing functional group protection, simplifying the synthetic procedure and decreasing the number of purification steps.[19,20] Therefore we synthesized 7-EAATP from 7-EAA using the one-pot synthesis procedure shown in Scheme 1. The purified 7-EAATP was later used in transcription reactions in vitro. T7 RNA polymerase was chosen for these experiments since it is readily available and widely used in biochemistry laboratories. A 34 nucleotide (nt) DNA template was designed for in vitro transcription reactions bearing a T7 promoter sequence and a template for the synthesis of a 17 nt RNA transcript containing one A (Fig. 3A). The transcription reaction was performed in the presence of α-[^{32}P]GTP enabling the detection of the final transcription product using storage phosphor autoradiography (Fig. 3B). To evaluate the extent of 7-EAA incorporation into the T7 transcript, we isolated the labeled 17 nt RNA



Scheme 1. Synthesis of 7-EAATP. Reagents and conditions: (i) TMS–ethyne, Pd$(PPh_3)_4$, CuI, Et_3N, THF, 87%; (ii) DBU, MeOH, 60%; (iii) $(MeO)_3PO$, proton sponge, $POCl_3$, −15 °C; (iv) $(HNBu_3)_2H_2P_2O_7$, NBu_3, DMF, −15 °C; (v) TEAB, rt, 11% (three steps).

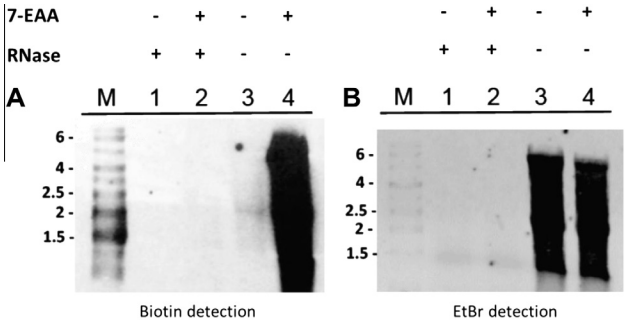


Figure 2. 7-EAA is taken up by HeLa cells and incorporated into cellular RNA. (A) Detection of biotin-labeled RNA after click reaction with biotin–azide (M) biotinylated molecular weight markers in kb, (1) sample isolated from cells grown in absence of 7-EAA, treated with RNase V1; (2) sample isolated from cells grown in the presence of 100 μM 7-EAA, treated with RNase V1; (3) sample isolated from cells grown in the absence of 7-EAA, no RNase V1 treatment; (4) sample isolated from cells grown in presence of 100 μM 7-EAA, no RNase V1 treatment. (B) Same as in (A) with ethidium bromide (EtBr) detection instead of biotin detection.

product and performed a CuAAC reaction followed by analysis using polyacrylamide gel electrophoresis (Fig. 3C). RNA generated from a transcription reaction containing 7-EAATP was clearly shifted to a new position in the gel after the CuAAC reaction. Indeed, 87% of the RNA was shifted (Fig. 3C, lane 3), suggesting that at least this amount of the RNA sample contained 7-EAA. Importantly, no shift was observed for RNA generated from a transcrip-

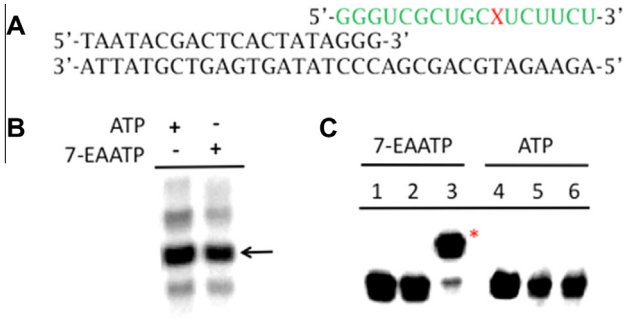


Figure 3. Synthesis of 17 nt RNA by in vitro transcription with 7-EAATP and T7 RNA polymerase. (A) T7 RNA polymerase transcription template for synthesis of 17 nt RNA containing one 7-EAA or A residue (X). (B) Products of T7 polymerase transcriptions with template shown in (A) containing 5 mM ATP or 7-EAATP, α-^{32}P GTP and allowed to proceed for 2 h at 37 °C. (Arrow indicates major transcription product.) (C) CuAAC reaction with 17 nt RNAs. Lanes 1–3: product from transcription with 7-EAATP, Lanes 4–6: product from transcription with ATP. Lanes 1 and 4: no CuAAC reaction reagents added, Lanes 2 and 5: CuAAC reagents lacking azide, Lanes 3 and 6: CuAAC reagents plus azide. (*) indicates position of CuAAC product.

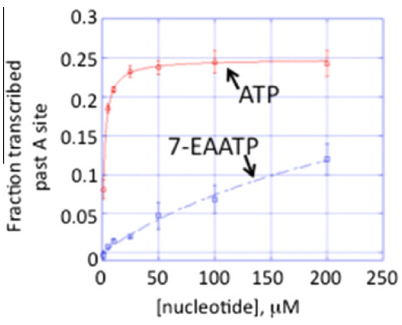


Figure 4. Comparison of T7 RNA polymerase efficiency with template shown in Figure 3A using varying concentrations of 7-EAATP or ATP.