Methods xxx (2011) xxx–xxx

Contents lists available at ScienceDirect

Methods

journal homepage: www.elsevier.com/locate/ymeth

Optimized method for the synthesis and purification of adenosine – Folic acid conjugates for use as transcription initiators in the preparation of modified RNA

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ARTICLE INFO

Article history:
Available online xxxx

Keywords:
Folic acid
Adenosine monophosphate (AMP)
Affi-gel boronate
In vitro transcription
T7 RNA polymerase
RNA labeling

ABSTRACT

We present an optimized synthetic strategy for the attachment of molecules to 5′-adenosine monophosphate (AMP), which can then be used to label the 5′-end of RNA by T7 RNA polymerase mediated *in vitro* transcription. Through the use of a boronate affinity gel, we have developed an efficient route to the preparation of folate conjugated AMP with high yields and purity. Affi-Gel boronate is an affinity resin that selectively binds nucleoside and nucleoside derivatives at pH > 7.5 and releases them at pH < 6.5. This resin is used to efficiently bind and purify ribonucleotides such as AMP. This allows for the addition of a large excess of reactants and reagents in order to drive the reaction to completion and then allow easy purification without HPLC. The synthesis can be successfully scaled up to produce large quantities of AMP conjugates.

1. Introduction

The nascent field of RNA nanotechnology makes use of the unique features of RNA, which combines the Watson–Crick base pairing attributes of DNA for simplicity in design and manipulation, with the flexibility and diversity in structure and function typical of proteins, to form versatile nanostructures [1–3]. The utility of RNA nanostructures can be enhanced by chemical modifications that improve stability or introduce useful functionalities for various applications. Modification of the 5′ terminus of RNA allows conjugation of ligands, such as folate [4], cholesterol [5,6], biotin [7,8], or fluorescent molecules [7,9,10], offering opportunities to enhance pharmacological characteristics or introduce special features to RNA nanostructures for biological/therapeutic applications.

RNA conjugates can be achieved synthetically by (i) synthesizing a phosphoramidite derivative of the conjugate group and direct incorporation during automated solid-phase RNA synthesis and (ii) incorporation of a reactive linker during solid phase RNA synthesis and post-synthetic attachment of the conjugate group to this linker. These methods work well for short RNA sequences (<50 nt), however, as the size of RNA increases, these conjugation methods are less practical due to lower yield, high levels of impurities, and high costs of chemical synthesis. RNAs of biological interest are much longer, and are either isolated from biological sources or prepared by enzymatic transcription from DNA templates using viral RNA polymerases [11,12]. Huang and coworkers [7–9,13] have reported the direct RNA labeling at the 5′ end by *in vitro* transcription with T7 RNA polymerase using the T7 φ 2.5 promoter that requires the appropriate label-linker-AMP conjugates (adenosine 5′-monophosphate, AMP) to serve as transcription initiators. This *in vitro* transcription system incorporates the modified adenosine moiety and labels the 5′ end of RNA with high efficiency during transcription initiation but not during elongation [4,7–9,13]. In order to take full advantage of this procedure, an efficient method for generating large quantities of adenosine conjugates for transcription initiation is necessary.

Synthesis of the adenosine conjugates is achieved in two synthetic steps; (i) preparation of an AMP amine terminated linker derivative and (ii) attachment of the conjugate group (typically an activated ester derivative) to the free amine of the linker (Fig. 1). The critical step for preparing these conjugates with high efficiency is the preparation of the amine terminated linker derivative. Huang and coworkers [4,7–9,13] have reported methods for preparing adenosine conjugates for transcription initiation. A range of amine terminated AMP linker conjugates have been prepared with reported coupling efficiencies ranging between 70% and 90% as determined by HPLC [7]. A subsequent report by the same authors [8] described an improved protocol for the preparation of hexanediamine-AMP (HDA–AMP) at a significantly larger scale (0.5 mmol) [8] than previously reported (0.25 μmol) [7]. The

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doi:10.1016/j.ymeth.2010.12.007

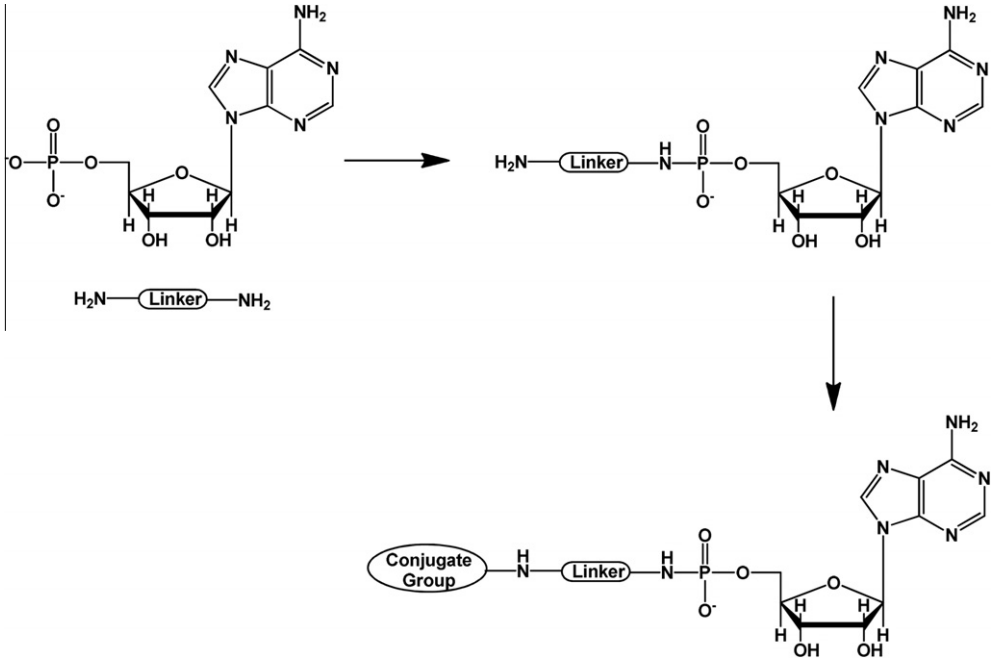

Fig. 1. General two step method for the preparation of 5′ adenosine linker conjugates for use as initiators in the synthesis of RNA conjugates by *in vitro* transcription using T7 RNA polymerase and the φ 2.5 promoter.

drawback to this improved scale is that the coupling efficiency was reduced to 50% as determined by HPLC.

We employed an affinity based purification method (Fig. 2) based on the use of Affi-Gel boronate affinity resin that has an affinity for adjacent *cis*-diols in ribonucleotides and other molecules [14–17]. The use of boronate affinity chromatography for separation of nucleic acid components and carbohydrates was first reported by Gilham and coworkers [17] and have since been used to separate ribonucleosides [18], ribonucleotides [19], sugars [17], catecholamines [20] and coenzymes [15,21]. This strategy is particularly useful for ribonucleotides/ribonucleosides having bases with an exocyclic NH_2 group which was recently shown to increase the retention on a boronate column [16]. Affinity based purifications methods such as Affi-Gel boronate are simple to implement, and produce near quantitative product recovery in a significantly shorter time and use fewer resources than HPLC. In contrast to the cation exchange method employed by Huang and coworkers [8] for removal of excess diamine in the first reaction, boronate affinity purification can be applied to the purification and removal of all impurities at both stages of the preparation of the AMP-linker-conjugate.

Herein, we present an optimized, scalable method for the preparation and purification of folic acid AMP conjugates which result in high yields (>95%) and high purity. Folate-AMP conjugates when incorporated into RNA sequences provide a means of delivering RNA nanoparticles via the folic acid endocytosis pathway [4]. We provide a detailed analysis of the major side products, and steps used to prevent the formation of undesired products in order to drive the reaction toward complete conversion to the desired products. This method can be applied to the synthesis of an assortment of amine linkers and conjugate groups to AMP (or other nucleoside monophosphates) in order to label RNA for a wide range of applications [3,4,9,10,22–24].

2. Description of method

2.1. General information

Reagents for organic synthesis were purchased from Sigma–Aldrich Chemical Company Inc. (Milwaukee, WI) and were used

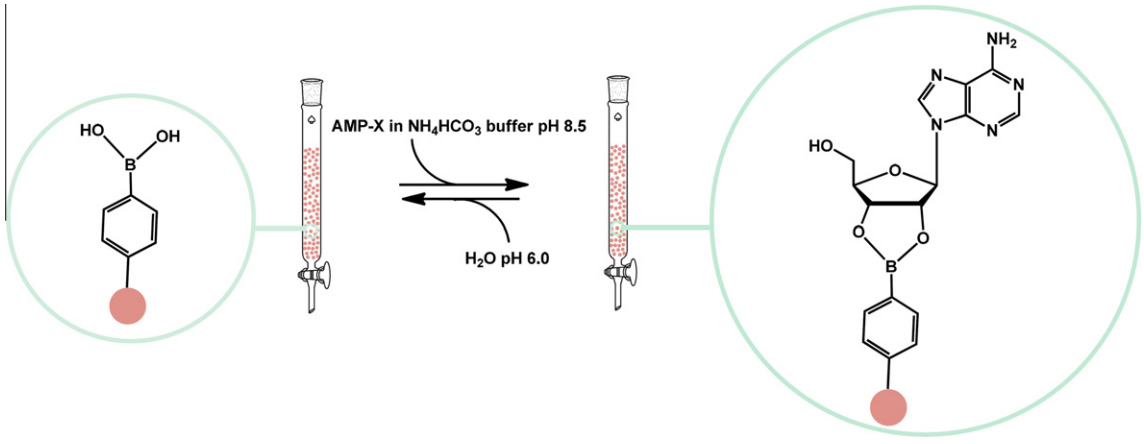

Fig. 2. Schematic description of the Affi-Gel boronate purification method. AMP conjugates are loaded onto the column at high pH (>7.5) and binds to the boronate by the 5-membered ring structure shown. The purified products are released at low pH (<6.5).

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as obtained unless otherwise stated. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride was purchased from Advanced ChemTech (Louisville, KY), and Affi-Gel boronate was purchased from Bio-Rad (Hercules, CA). Liquid Chromatography Mass Spectrometry (LCMS) was performed on an Agilent 1200 Series LCMS. Nuclear magnetic resonance spectra were obtained using a 500 MHz Bruker spectrometer. High Resolution Electrospray Ionization Mass Spectrometry (HR-ESI-MS) was performed by the Purdue University Campus Wide Mass Spectrometry facility. ^{1}H spectra were referenced to TMS while ^{31}P spectra were referenced to an 85% phosphoric acid external standard.

2.2. Synthesis of conjugates

2.2.1. Adenosine 5′-(13-amino-4,7,10 trioxa-tridecyl)phosphoramidate (TOTDDA–AMP) (**1**)

Adenosine 5′-monophosphoric acid (AMP) (0.183 g, 0.5 mmol) was suspended in water. *N*-methylimidazole (0.41 g, 5 mmol) and *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC) (0.25 g, 1.3 mmol) were added. The pH was adjusted to between 6.2 and 6.8 with HCl and the mixture stirred for 10 min with a magnetic stirrer. 4,7,10-Trioxa-1,13-tridecanediamine (1.1 g, 5 mmol) and EDC (0.25 g, 1.3 mmol) were added and the pH readjusted to between 6.2 and 6.8 with conc. HCl. The mixture was stirred at ambient temperature with EDC (0.25 g, 1.3 mmol) added at 1 h intervals with monitoring by LCMS for conversion of starting material to product (~4 h). The pH was adjusted to 8.5 with conc. ammonium hydroxide and loaded onto Affi-Gel boronate beads for purification. The purified product was lyophilized to yield a white solid (209 mg, 76% isolated yield) with >95% purity by RP-HPLC. ^{1}H NMR (500 MHz, D_2O, δ, ppm): 8.51 (s, 1H), 8.26 (s, 1H), 6.12 (d, J = 5.5 Hz, 1H), 4.51 (t, J = 4.5 Hz, 1H), 4.35 (s, 1H), 3.99 (m, 2H), 3.69–3.62 (m, 3H), 3.60–3.56 (m, 6H), 3.51–3.43 (m, 4H), 3.38–3.28 (m, 3H), 3.25 (d, J = 7 Hz, 1H), 3.19 (t, J = 6, 1H), 3.13–2.05 (m, 4H), 2.98 (t, J = 7.5 Hz, 1H), 2.84 (s, 2H), 2.74 (s, 1H), 2.70–2.67 (m, 3H), 1.96–1.87 (m, 4H), 1.56 (p, J = 7 Hz, 2H). ^{31}P NMR (202.46 MHz, D_2O, δ, ppm): 9.79 (referenced to external 85% H_3PO_4). HRMS: (ESI, $M + H^+$) calcd. for $C_{20}H_{37}N_7O_9P$: 550.2390, act. 550.2388.

2.2.2. Adenosine 5′-(6-aminohexyl)phosphoramidate (HDA–AMP) (**2**)

Adenosine 5′-monophosphoric acid (AMP) (0.183 g, 0.5 mmol) was suspended in water. *N*-methylimidazole (0.41 g, 5 mmol) and *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC) (0.25 g, 1.3 mmol) were added. The pH was adjusted to between 6.2 and 6.8 with HCl and the mixture stirred for 10 min with a magnetic stirrer. Hexane-1, 6-diamine (0.58 g, 5 mmol) and EDC (0.25 g, 1.3 mmol) were added and the pH readjusted to between 6.2 and 6.8 with HCl. The mixture was stirred at ambient temperature with EDC (0.25 g, 1.3 mmol) added at 1 h intervals with monitoring by LCMS for conversion of starting material to product (~4 h). The pH was adjusted to 8.5 with conc. ammonium hydroxide and loaded onto Affi-gel boronate beads for purification. The purified product was lyophilized to yield a white solid (185 mg, 83% isolated yield) with >95% purity by RP-HPLC. ^{1}H NMR (500 MHz, DMSO-d_6, δ, ppm): 8.48 (s, 1H), 8.14 (s, 1H), 7.33 (s, 2H), 5.91 (d, J = 5.5 Hz, 1H), 4.59 (t, J = 5.0 Hz, 1H), 4.21(t, J = 5.0 Hz, 1H), 4.05 (m, 1H), 3.81–3.76 (m, 2H), 2.72–2.760 (m, 4H), 1.54 (m, 2H), 1.25 (m, 6H). ^{31}P NMR (202.46 MHz, DMSO-d_6, δ, ppm): 7.29 (referenced to external 85% H_3PO_4). HRMS: (ESI, $M + H^+$) calcd. for $C_{16}H_{29}N_7O_6P$: 446.11917, act. 446.1918.

2.2.3. Folate-TOTDDA–AMP (**3**)

Folic acid (99.3 mg, 0.225 mmol) and NHS (51.8 mg, 0.45 mmol) were dissolved in 1 M sodium bicarbonate buffer (pH 7.5, 10 mL) and stirred for 5 min. EDC (86.3 mg, 0.45 mmol) was added and the mixture stirred for 30 min to activate folic acid. TOTDDA–AMP (41.2 mg, 0.08 mmol) was added and the mixture stirred with addition of EDC (86.27 mg, 0.45 mmol) every 2 h over a period of 6 h. The reaction was monitored by LCMS until conversion of starting material to product was complete (4–6 h). The pH was adjusted to 8.5 with conc. ammonium hydroxide and loaded onto Affi-Gel boronate beads for purification. The purified product was lyophilized to yield a yellow solid (50 mg, 68% isolated yield) with >90% purity by RP-HPLC. (500 MHz, D_2O, δ, ppm): 8.62 (s, 1H), 8.41–8.39 (m, 1H), 7.62–7.59 (m, 2H), 6.74 (d, J = 8.0 Hz, 2H), 6.02 (t, J = 5.0 Hz, 1H), 4.56 (s, 2H), 4.51–4.46 (m, 2H), 4.35–4.31 (m, 2H), 3.99 (m, 2H), 3.56 (s, 1H), 3.49–3.42 (m, 2H), 3.39–3.32 (m, 12H), 3.09–2.93 (m, 3H), 2.71–2.67 (m, 3H), 2.37–2.21 (m, 6H), 1.73 (p, J = 6.5 Hz, 1H) 1.61–1.56 (m, 3H), 1.52–1.47 (m, 2H). ^{31}P NMR (202.46 MHz, D_2O, δ, ppm): 9.79 (referenced to external 85% H_3PO_4). HRMS: (ESI, $M + H^+$) calcd. for $C_{39}H_{54}N_{14}O_{14}P$: 973.3682, act. 973.3686.

2.2.4. Folate-HDA–AMP (**4**)

Folic acid (99.3 mg, 0.225 mmol) and NHS (51.8 mg, 0.45 mmol) were dissolved in 1 M sodium bicarbonate buffer (pH 7.5, 10 mL) and stirred for 5 min. EDC (86.3 mg, 0.45 mmol) was added and the mixture stirred for 30 min to activate folic acid. HDA–AMP (33.4 mg, .08 mmol) was added and the mixture stirred with addition of EDC (86.27 mg, 0.45 mmol) every 2 h over a period of 6 h. The reaction was monitored by LCMS until conversion of starting material to product was complete (~4 h). The pH was adjusted to 8.5 with conc. ammonium hydroxide and loaded onto Affi-Gel boronate beads for purification. The purified product was lyophilized to yield a yellow solid (65 mg, 83% isolated yield) with >92% purity by RP-HPLC. (500 MHz, DMSO-d_6, δ, ppm): 8.62 (s, 1H), 8.50–8.44 (m, 1H), 8.14 (s, 1H), 7.62–7.55 (m, 2H), 7.25 (s, 2H), 7.06 (t, J = 6 Hz, 1H), 6.59 (m, 1H), 5.91 (d, J = 5.5 Hz, 1H), 4.62–4.46 (m, 2H), 4.21 (m, 2H), 4.03 (m, 1H), 3.82–3.79 (m, 2H), 3.02–2.91 (m, 3H), 2.78–2.58 (m, 3H), 2.23–1.99 (m, 3H), 1.87 (m, 1H), 1.57 (m, 1H), 1.45–1.11 (m, 6H). ^{31}P NMR (202.46 MHz, D_2O, δ, ppm): 9.79 (referenced to external 85% H_3PO_4). HRMS: (ESI, $M + H^+$) calcd. for $C_{35}H_{46}N_{14}O_{11}P$: 869.3208, act. 869.3214.

2.3. Purification by Affi-Gel boronate

A low pressure liquid chromatography column (2.5 × 20 cm) containing Affi-Gel boronate resin (5 g) was pre-equilibrated with a buffer containing 1 M aqueous ammonium bicarbonate and 100 mM magnesium chloride adjusted to pH 8.5. The crude reaction mixture was adjusted to pH 8.5 with concentrated ammonium hydroxide, loaded onto the column and washed with 4–5 column volumes of ammonium bicarbonate buffer. The purified product was eluted with deionized water adjusted to pH 6.0 with carbon dioxide. The fractions were combined and lyophilized for analysis and further use.

2.4. Liquid chromatography mass spectrometry

Liquid chromatography mass spectrometry was performed on an Agilent 1200 Series LC/MS system. Liquid chromatography was performed on an Agilent Eclipse XDB C18, 5 μM, 4.6 × 150 mm reversed phase column and run with: Method A – A linear gradient of 15% MeOH/85% 5 mM NH_4OAc increasing to 90% MeOH/10% 5 mM NH_4OAc over 6 min at a flow rate of 1 mL/min. Method B: A stepped gradient beginning with 0% MeOH/100% 5 mM NH_4OAc increasing to 15% MeOH/85% 5 mM NH_4Oac over 5 min followed by 15% MeOH/85% 5 mM NH_4OAc increasing to 30% MeOH/70% 5 mM NH_4OAc over 3 min followed by 30% MeOH/70% 5 mM NH_4OAc increasing to 50% MeOH/50 % 5 mM NH_4OAc over 2 min and held at 50% MeOH/50% 5 mM NH_4OAc for 15 min.

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Mass spectrometry was performed with an electrospray ionization (ESI) source in positive ion mode and fragmentor set at 100 V.

3. Results

Our initial attempts to prepare 5′ amine linker conjugated AMP using the methods described by Huang and co-workers [7] produced relatively low yields and the products were difficult to purify due to the large excess (20 fold) of reagents and reactants used during the synthesis. A more efficient method for preparation of these conjugates is highly desirable due to the requirement of an excess of initiator AMP conjugate for successful *in vitro* transcription. Using the method described by Chu et al. [25] for derivatization of nucleotides and polynucleotides with various diamine linkers we observed TOTDDA AMP as 70% of the product by HPLC (Fig. 3). Upon identification of the byproducts of this reaction we discovered that a major side product is the AMP pyrophosphate dimer which prevents complete conversion to the desired amine linker product (Fig. 3). From the work of Chu et al., it was clear that the imidazolide is an important intermediate to drive the desired reaction to completion, but also drives the formation of the undesired pyrophosphate dimer.

We performed a series of reactions aimed at optimizing the transformation toward complete conversion of the AMP to the desired product and elimination of the undesired pyrophosphate (Table 1). We discovered that the pyrophosphate dimer is formed more readily at higher pH (>7.0) where the imidazolide intermediate is more stable. Chu et al. [25] reported that the imidazolide is hydrolyzed to the corresponding nucleoside monophosphate at low pH. Analysis of the imidazolide formation at pH 6.0 revealed that an equilibrium of ~60%: 40% AMP imidazolide/AMP is achieved within 3 h which is hydrolyzed almost completely to AMP when left standing for 12 h with minimal pyrophosphate dimer formation (Fig. 4). Addition of 4,7,10-trioxa-1,13-tridecanediamine (TOTDDA) and additional EDC to this reaction mixture produced ~60% conversion to TOTDDA–AMP within 1 h. It is well known that EDC is consumed rapidly in aqueous solution so we hypothesized correctly that timely addition of aliquots of EDC to the reaction mixture while maintaining the pH at between 6.0 and 7.0 would produce complete conversion to product (Table 1). Using this strategy, AMP was quantitatively converted to the amine terminated linker derivatives (**1** and **2**, Scheme 1) with high yields (76–83% isolated yield) and purity (>95%) after purification by Affi-Gel boronate (Fig. 5).

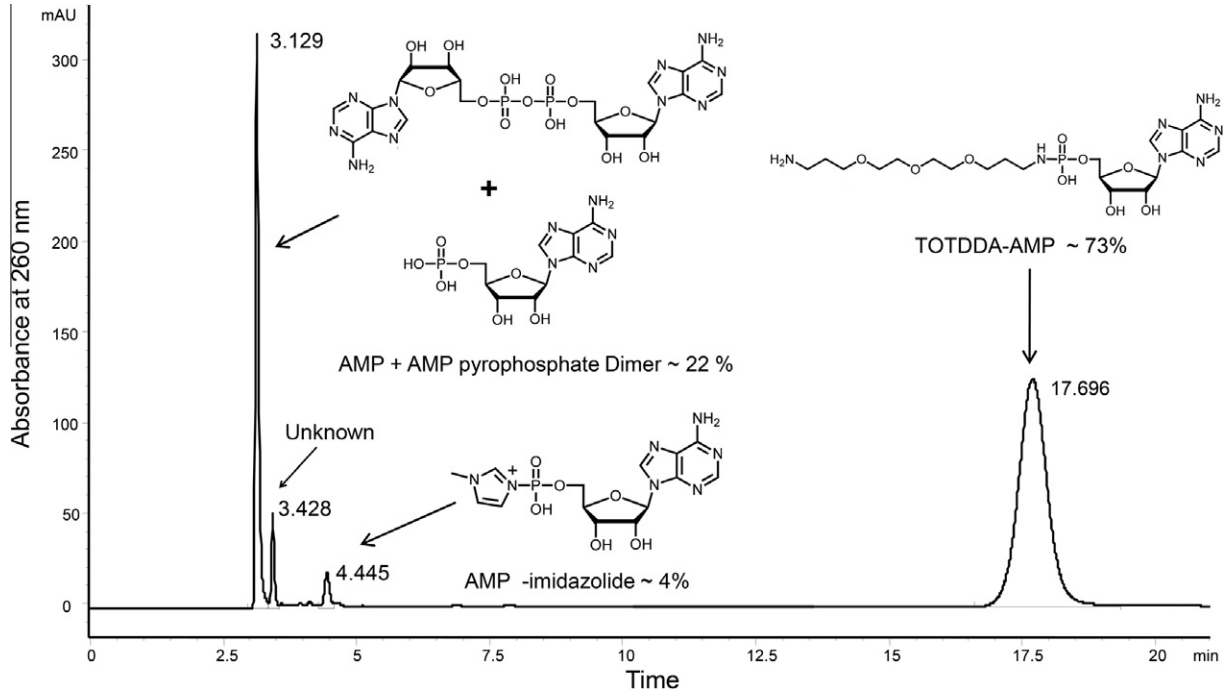

Fig. 3. HPLC profile of reaction 2 (Table 1) indicating the identity of the major peaks as determined by LCMS (HPLC method B).

Table 1
Optimization of 5′-TOTDDA AMP reaction.

Reaction #	AMP (mmol)	TOTDDA (mmol)	1-methylimidazole (mmol)	EDC (mmol)	pH	Total rxn time	Conversion
1	0.1	2	0	2	6	12 h	25%
2	0.1	2	1	2	6	12 h	73%
3	0.1	4	2	4	6	12 h	58%
4	0.1	4	2	4	6.8	12 h	62%
5	0.1	2	1	2[a]	6.2	12 h	92%
6	0.1	2	1	2[a]	6.8	12 h	93%
7	0.1	1	1	2[a]	6.2	6 h	>95%
8	0.1	1	1	2[a]	6.8	6 h	>95%
9	0.5	5	5	10[a]	6.2	6 h	>95%

[a] EDC added incrementally over indicated reaction times.

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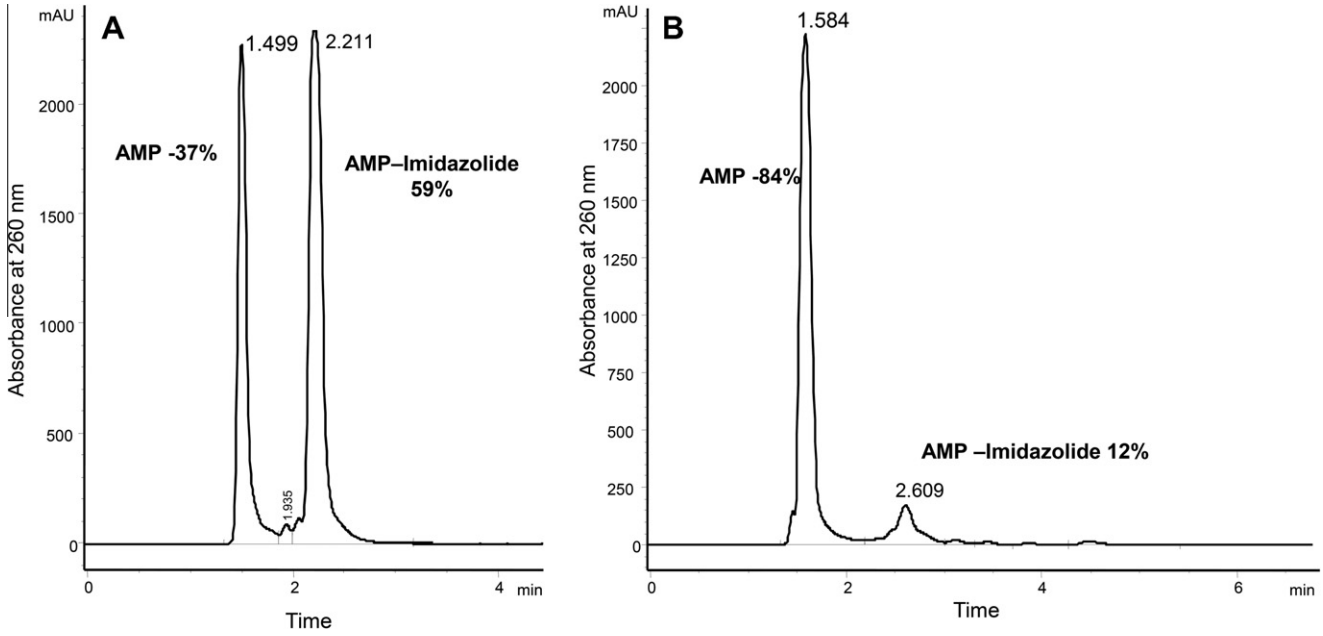
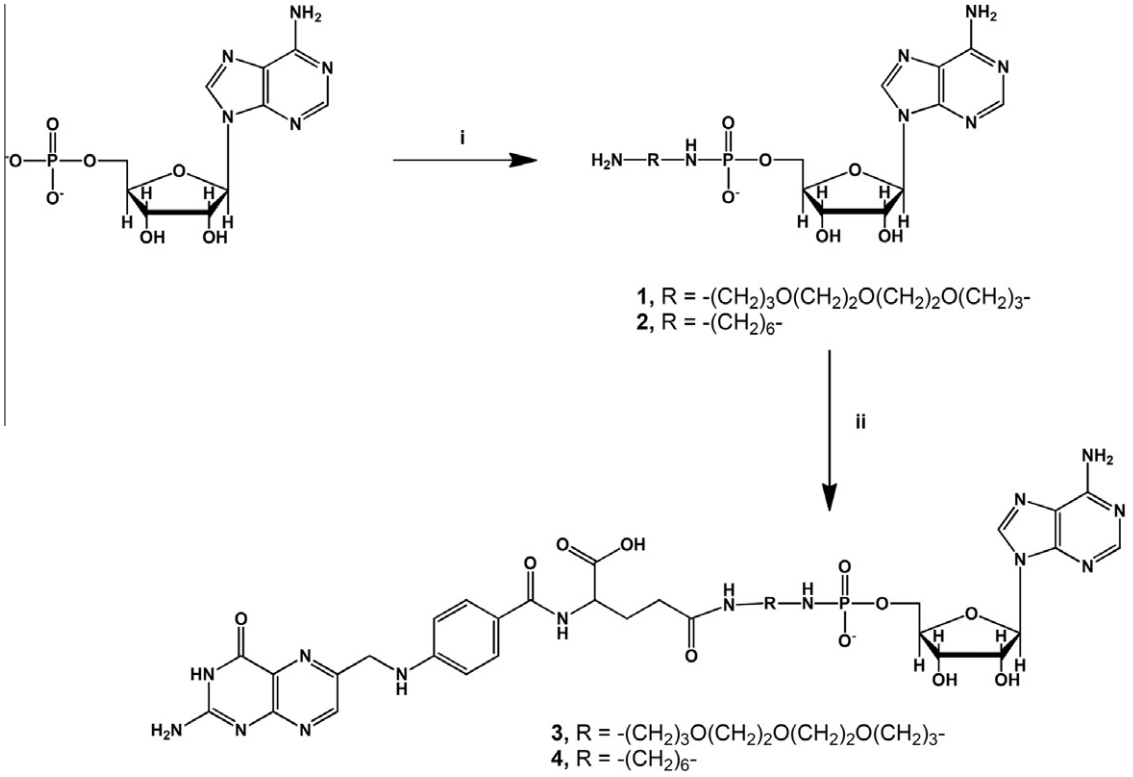


Fig. 4. Time course for formation and hydrolysis of the key imidazolide intermediate. (A) HPLC profile after 3 h reaction shows ~60% conversion to the imidazolide intermediate. (B) HPLC profile after 12 h reaction shows near complete hydrolysis to starting material. (HPLC method B).



Scheme 1. Preparation of Folate -5′ AMP conjugates via TOTDDA (**3**) and HDA (**4**) linkers.

With the amine terminated linker AMP derivatives in hand, conjugation of any desired molecule can be achieved by a condensation reaction with the activated ester derivative of the molecule. We sought to prepare folate-AMP conjugates (**3** and **4**, Scheme 1) for incorporation into RNA as a means of delivering RNA nanoparticles via the folic acid endocytosis pathway as described previously (**4**). Preparation of folate-AMP conjugates poses two unique issues; (i) folic acid has limited solubility in most organic solvents

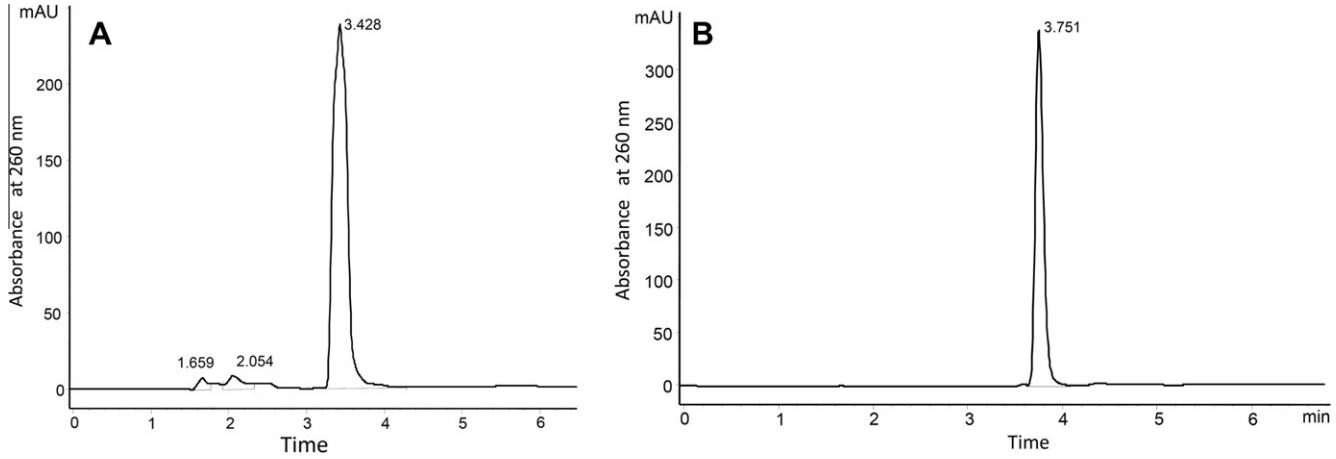


Fig. 5. HPLC profile (260 nm) for the optimized reactions of AMP with (A) TOTDDA linker (B) HDA linker. Conversion is almost quantitative and results in high yields after Affi-Gel boronate purification. Peak identities were determined by LCMS. (HPLC method A).

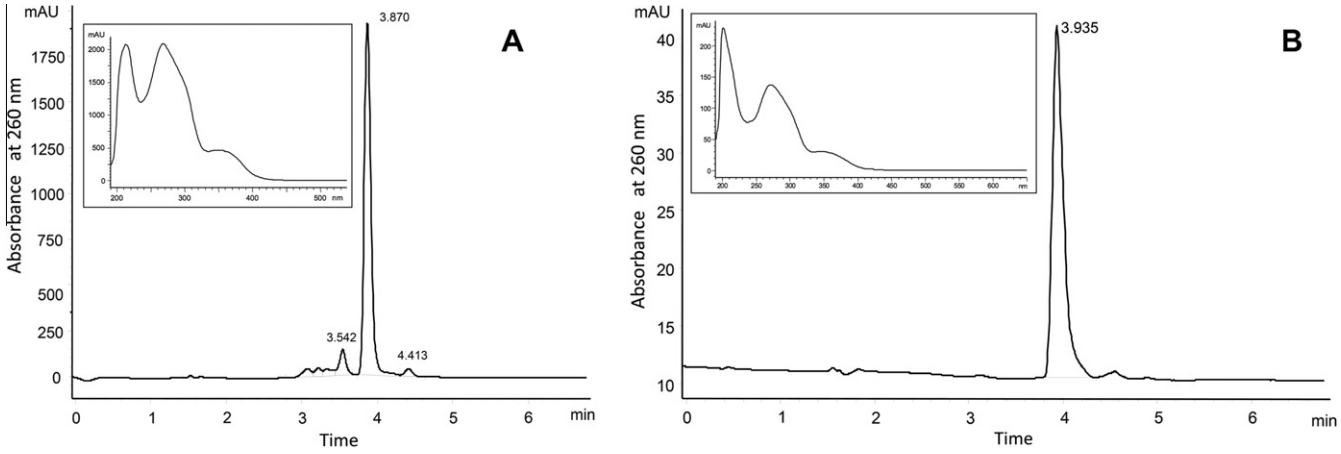


Fig. 6. HPLC profile (260 nm) and UV-Vis spectra (inset) for the optimized reactions of folic acid with: (A) TOTDDA-AMP (B) HDA–AMP. Conversion is almost quantitative and results in high yields after Affi-Gel boronate purification. Peak identities were determined by LCMS. (HPLC method A).

while aqueous solubility is pH dependent and (ii) folic acid contains two carboxyl groups (α and γ) to which the AMP linker can be attached. Several methods for regioselective conjugation at the γ carboxyl group of folic acid have been developed [26–28] as it has been shown that conjugates attached at the α carboxyl group are significantly less efficient at binding the cell surface folate receptors [28,29]. Because of the limited solubility of folic acid in most solvents, the most common solvent used for reactions involving folic acid is dimethylsulfoxide (DMSO). We found that DMSO adversely affected binding to the Affi-Gel resin; hence we sought to develop a method of conjugation in completely aqueous conditions. We decided to forgo the regioselective methods described and attempt condensation in aqueous buffer and rely on the reported higher reactivity of the γ carboxyl vs. the α carboxyl [29,30] for enrichment of the desired γ conjugate.

Synthesis of Folate-TOTDDA–AMP and Folate-HDA–AMP was achieved by the *in situ* formation of the Folate-NHS activated ester in sodium bicarbonate buffer using EDC followed by the addition of TOTDDA-AMP or HDA–AMP respectively. Formation of the final product proceeded in near quantitative yields (based on the disappearance of starting material) and high purity (>90%) after Affi-Gel boronate purification as measured by HPLC monitored at 260 nm (Fig. 6). HPLC was also monitored at 350 nm, a wavelength specific for folic acid and not AMP, and a single peak (100%) at the same retention times indicated in Fig. 6 were observed (data not shown). Based on the complexity of the NMR spectra in the region between 1.8 and 2.5 ppm we speculate that a mixture of both α and γ conjugates are present. We did not attempt to separate these isomers as we have found sufficient activity using this method in previous studies where our conjugates were much less characterized (**4**). Furthermore, it has been shown that the glutamate portion of the folic acid is not essential for activity as long as there is sufficient linker spacing between the pteroyl portion of the molecule and the conjugate group [31]. This synthesis can be successfully scaled up to synthesize large quantities of AMP conjugates by preparing amine terminated 5′ AMP linker conjugates as described above and attachment of NHS derivatives of the desired conjugate groups.

4. Concluding remarks

We have described an improved method for the preparation of adenosine conjugates for use as transcription initiators in the *in vitro* synthesis of modified 5′ modified RNA by T7 RNA polymerase employing the φ 2.5 promoter. Our method enables the speedy preparation of large quantities of conjugates with high yields and purity. This optimized method can be readily adapted for the

preparation of adenosine conjugates with a variety of diamine linkers giving the flexibility to choose a linker with the desired length, hydrophobicity/hydrophilicity, flexibility/rigidity, or other properties that may be desirable for the intended application. An assortment of conjugate molecules can be attached to the linker by preparing an activated ester derivative in the lab or purchasing the activated ester from commercial sources.

Acknowledgment

We thank Professor Eric Long and The IUPUI Department of Chemistry for use of lab space, LCMS, and NMR instrumentation. This work was supported by the NIH Nanomedicine Development Center on Phi29 DNA-packaging Motor for Nanomedicine, through the NIH Roadmap for Medical Research (PN2 EY 018230). Assistance from the National Cancer Institute Grant (P30 CA23168) awarded to Purdue University is also gratefully acknowledged.

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