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Efficient Chemical Synthesis of AppDNA by Adenylation of Immobilized DNA-5′-monophosphate

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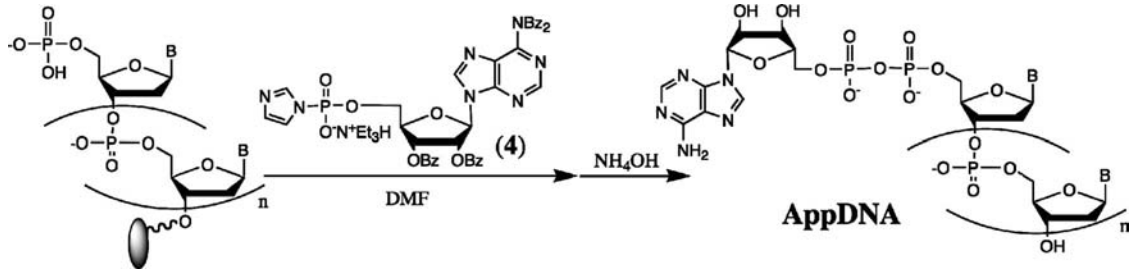
ABSTRACT



AppDNA is an intermediate in enzyme-catalyzed DNA ligation reactions, and its efficient enzymatic synthesis requires a donor-template duplex of at least 11 base pairs in length. An efficient chemical synthesis of AppDNA with the coupling of an adenosine 5′-phosphorimidazolidate to an immobilized DNA-5′-monophosphate as the key step is described. The adenylation efficiencies of DNA-5′-monophosphate were excellent for oligonucleotides containing less than 11 nucleotides and at least 50% for oligonucleotides containing 15−25 nucleotides.

DNA containing a 5′,5′-adenylpyrophosphoryl cap (5′-adenylated DNA; AppDNA) is an activated form of DNA with a high-energy pyrophosphate linkage. AppDNA occurs naturally as a reaction intermediate in the nucleic acid ligations catalyzed by DNA[1] and RNA ligase[2] but rarely accumulates under normal reaction conditions. AppDNA can serve as a substrate for various reactions catalyzed by ribozymes and deoxyribozymes identified by in vitro selection,[3] and can be used to synthesize large double-stranded DNA using DNA ligase without ATP.[4] Chiuman and Li recently reported an enzymatic preparation of AppDNA using T4 DNA ligase.[5] They used a template DNA strand along with a DNA 5′-monophosphate substrate ("donor") to be adenylated. By omitting the "acceptor" strand that normally becomes ligated to the donor by the action of T4 DNA ligase, AppDNA was obtained as the final product.

We recently reported a systematic method for quantitatively detecting 2′-OMe modifications in RNA using T4 DNA ligase,[6] and AppDNA is a known intermediate in the ligation reaction. The ligation-based assay works very

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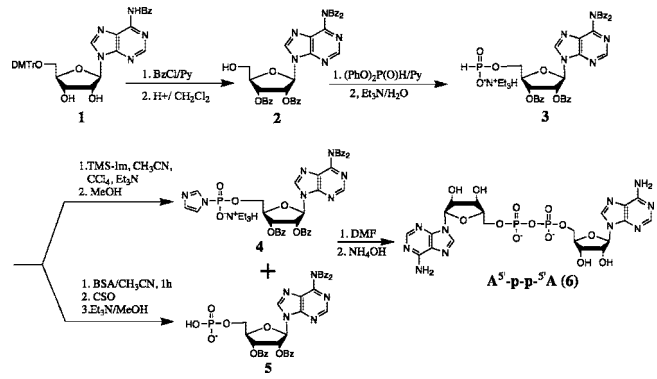
effectively in most cases but inconsistencies in the assay results became apparent during simultaneous study of multiple 2′-OMe sites in yeast rRNA. The inconsistencies were likely caused by two drawbacks associated with the step of enzymatically generating AppDNA. The first drawback is that T4 DNA ligase requires a donor-template duplex of 11-bp in length for the adenylation step to be fully efficient.[5] The requirement for a donor-template duplex was also observed in the preparation of AppRNA by T4 DNA ligase.[7] This drawback is difficult to overcome when using biological RNAs, which contain varying degrees of structure that interfere with DNA substrate hybridization. The second drawback is the dependence of adenylation efficiency on the sequence of the DNA donor substrate. Some DNA donor substrates are adenylated efficiently while others react poorly, such that the outcome for any new substrate is generally unpredictable.[8]

To determine whether the inconsistencies in the ligation reaction with rRNA arise primarily from the generation of the adenylated DNA, we conducted ligation reactions using purified AppDNA substrates. First, we generated AppDNA substrates enzymatically according to Chiuman and Li's procedure. After purification by gel electrophoresis we used them in ligation reactions. Using these independently generated AppDNA substrates, we found the ligation results were reproducible. However, this procedure is extremely labor intensive and impractical for high-throughput analysis. For example, to analyze the 55 2′-OMe sites in yeast rRNA, we would have to generate enzymatically and isolate 55 AppDNA substrates, possibly at vastly different efficiencies. Another way to make AppDNA is through chemical synthesis. However, solution synthesis of AppDNA usually gives unsatisfactory yield.[9] In this work, we establish conditions for efficient 5′ adenylation during solid phase oligonucleotide synthesis, thereby making it possible to reliably and conveniently generate 5′ adenylated DNA oligonucleotides. We demonstrate that the chemically synthesized AppDNA products efficiently support ligation-based detection of RNA modifications.

The key step in the chemical synthesis of AppDNA involves pyrophosphate bond formation, which entails coupling of an activated 5′-phosphorylated adenylate derivative with an immobilized DNA-5′-monophosphate. Several types of activated phosphoester derivatives have been reported to react with monophosphates in solution to form the pyrophosphate bond, including phosphorimidazolidate,[10] phosphor-*N*-methylimidazolidate,[11] phosphoromorpholidates,[12] substituted benzotriazol-1-yl phosphorothioates,[13] and phosphate derivatives.[14] As formation of the pyrophosphate bond can be monitored conveniently by ^{31}P NMR, we first studied the coupling reactions of two activated 5′-phosphorylated adenylate derivatives with the adenosine 5′-monophosphate derivative **5** and then extended the conditions to the solid phase synthesis of AppDNA.

Phosphorimidzaolides have been used widely as phosphorylation reagents for pyrophosphate bond formation and are prepared readily from the corresponding 5′-*H*-phosphonates.[15] Therefore, we first studied the solution coupling of **5** with adenosine 5′-phosphorimidazolidate **4** (Scheme 1).

Scheme 1. Synthesis of A$^{5'}$-p-p-$^{5'}$A by Coupling of **4** and **5**



For two reasons, we chose to protect the 2′, 3′-OH, and N^6-NH_2 groups by benzoylation: First, after the coupling reaction the benzoyl groups can be removed readily by ammonia treatment. Second, benzoyl groups have greater hydrophobic character than acetyl groups, which facilitates the workup and chromatographic purification of **3** and **5** relative to the corresponding acetyl derivatives.

Thus, commercially available **1** was converted to intermediate **2** by benzoyl chloride followed by removal of the trityl group. Treatment of **2** with diphenyl phosphite followed by hydrolysis in the presence of Et_3N[16] afforded 5′-*H*-phosphonate **3** as pale foam, which is quite stable and can be stored at rt for months. We also tried to synthesize the corresponding 2′, 3′, N^6-triacetyl 5′-*H*-phosphonate derivative; however, aqueous phase solubility during extraction rendered the product difficult to purify.

5′-*H*-phosphonate **3** was then activated to phosphorimidazolidate **4** in quantitative yield (as indicated by ^{31}P NMR, δ changed from 3.3 to −9.4 ppm in DMF) by treatment with (trimethylsilyl)imidzaole in the presence of Et_3N in CH_3CN-

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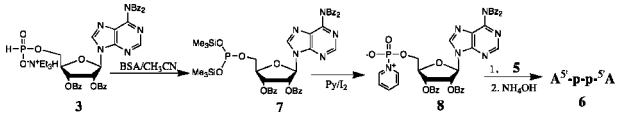
CCl_4 (v/v, 1:1) followed by addition of methanol.[15] We also obtained the 5′-AMP derivative **5**[17] by first treating **3** with *N*,*O*-bis(trimethylsilyl)acetamide (BSA, 5 equiv) followed by oxidation with (2R, 8aS)-(+)-(camphorylsulfonyl)-oxaziridine (CSO) and subsequent treatment with Et_3N in MeOH to remove trimethylsilyl groups.[18]

Considering the labile nature of phosphorimidazolidates, we used excess **4** (2.5 equiv) in coupling reactions with **5**. Reactions were carried out in DMF and monitored by ^{31}P NMR spectrum. After 1 h reaction, the ^{31}P NMR spectrum showed that the resonance corresponding to **5** (0.9 ppm) disappeared and a new resonance appeared at 0.6 ppm along with the resonance for unreacted **4** (−9.4 ppm). After overnight reaction, the ^{31}P NMR spectrum showed that 80% of the unreacted **4** remained intact with only 20% decomposing gradually to a byproduct with ^{31}P resonance at −10.5 ppm. This unexpected stability of **4** allowed us to consider longer coupling times in subsequent experiments. Removal of DMF followed by overnight debenzoylation with ammonia hydroxide gave $A^{5'}$-p-p-$^{5'}A$ (**6**) ($[MH]^+ = 677$ Dalton) quantitatively.

As another possible strategy for 5′-adenylation, we considered 5′-pyridinium phosphoramidates, electrophilic reagents that react with various nucleophiles, enabling synthetic access to nucleoside phosphorofluoridates, phosphorofluorido(di)thioates,[19] phosphoramidates[20] and nucleoside triphosphates.[21] We tested whether analogous reaction using nucleoside 5′-monophosphate as nucleophile could provide access to the pyrophosphate linkage. Sun et al. generated pyridinium phosphor-amidates from the corresponding nucleoside *H*-phosphonates via *in situ* silylation with TMSCl and subsequent I_2-mediated oxidation in the presence of pyridine. To convert phosphonate **3** to the corresponding disilyl phosphite, we used BSA rather than TMSCl to avoid potential complications both from its moisture sensitivity and from possible silylation of the nucleoside 5′-monophosphate, which would block its nucleophilicity.

Thus, **3** was dissolved in CH_3CN and converted to phosphite **7** (^{31}P NMR, $\delta = 120$ ppm) by treatment with BSA (Scheme 2). **7** was then oxidized to pyridinium

Scheme 2. Synthesis of $A^{5'}$-p-p-$^{5'}A$ by Coupling of **8** and **5**



phosphoramidate **8** (^{31}P NMR, $\delta = -1$ ppm) by addition of iodine in the presence of pyridine. The CH_3CN solvent is crucial for this transformation: when pyridine or DMF was used as solvent, the addition of iodine to **7** resulted in the formation of nucleoside disilyl phosphate (^{31}P NMR, $\delta =$ 14 ppm) instead of **8**.

To complete the synthesis of AppA, **5** (0.4 equiv) was added to the solution of **8** (1 equiv). After 0.5 h, the ^{31}P NMR spectrum showed that **5** had reacted quantitatively to generate a new peak at 0.6 ppm. The excess phosphoramidate **8** was decomposed completely, in contrast to phosphorimidazolidate **4**, which remained stable during the analogous reaction with **5**. Although phosphoramidate **8** reacts with **5** much faster than does the phosphorimidazolidate **4**, the more rapid decomposition of **8** potentially precludes its use in reactions that require longer time. After treatment with NH_4OH, $A^{5'}$-p-p-$^{5'}A$ **6** was obtained quantitatively.

To adapt these adenylation reactions to a solid phase platform, we tested **4** and **8** in reactions with immobilized CPG-thymidine 5′-monophosphate (**9a**). After deprotection, HPLC indicated that pT was converted to $A^{5'}pp^{5'}T$ ($MH^+ =$ 652) quantitatively in both cases.

Next, we tested these conditions for adenylation of a series of 5′-pT_{n+1}-CPG (**9b**−**e**, $n = 2, 5, 9, 14$). We generated CPG-T_{n+1} using standard DNA synthesis and coupled them to the chemical phosphorylation reagent, $DMTrO(CH_2)_2$-$SO_2(CH_2)_2OP(NPr_2^{-i})(OCH_2CH_2CN)$, followed by oxidation with I_2 to install the 5′-phosphate. Subsequent treatment with DBU (10%) in BSA/pyridine (v/v, 1:1) removed the sulfonylethyl and cyanoethyl protecting groups without affecting the linkage to the solid support.[22] We carried out adenylation reactions using **4** and **8** and used Maldi-TOF MS to determine adenylation efficiency after 16 and 1 h, respectively (Table 1).

Table 1. Adenylation Efficiency of 5′-p-DNA-polymers

entry	p-DNA-CPG	**4**	**8**	MS
1	pT-CPG **9a**	>95%[a]	>95%[b]	652
2	pT_3-CPG **9b**	>95%[a]	89%[b]	1260
3	pT_6-CPG **9c**	>95%[a]	75%[b]	2172
4	pT_{10}-CPG **9d**	83%[a]	64%[b]	3388
5	pT_{15}-CPG **9e**	35%[a]	<20%[b]	4908
6	pT_{10}-CPG **9d**	>95%[c]	–	3388
7	pT_{15}-CPG **9e**	60%[c]	–	4908

[a] Treated with **4** (0.1 M, 0.5 mL) for 16 h at room temperature. [b] Treated with **9** (0.1 M, 0.5 mL) for 1 h at room temperature. [c] Treated with **4** (0.1 M, 0.5 mL) for 48 h at 40 °C.

Both reagents **4** and **8** adenylated short DNA-5′-monophosphates (containing six nucleotides or less) effectively; however, adenylation yields decreased as oligonucleotide length increased, with **4** giving better results than **8** in every case (Table 1). Further advantages of using **4** as adenylation reagent are that it is easy to work with and gives reproducible

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reaction kinetics while phosphoramidate **8** is much more reactive and highly sensitive to moisture.

The decreased adenylation efficiency for longer oligonucleotides could reflect formation of internal adenylated byproducts (DNA-App-DNA) or their degradation products due to the removal of cyanoethyl groups from all the phosphates by DBU. To test this, we prepared another 5′-pT_{15}-polymer **10** with cyanoethyl groups still present (SI). When treated with **4** under the same conditions, **10** became adenylated with slightly lower efficiency than did **8**, suggesting that the internal adenylation side reaction does not underlie the observed trend. Additionally, MS data of $AppT_3$ and $AppT_6$ gave expected peaks only at 1260 and 2171, respectively. We observed no peaks from internal adenylated byproducts or their corresponding degradation byproducts. This is also consistent with Sekine's report that phosphodiester compounds have been generally recognized to be inert to conventional phosphorylating reagents such as phosphorimidazolidates and phosphoromorpholidates.[14a]

To improve the coupling efficiency between **9e** and **4**, we varied reaction conditions. Neither addition of catalyst such as $ZnCl_2$,[15] 6-(trifluoromethyl)-1-hydroxyl-benzotriazole,[13b] or 1*H*-tetrazole[12a] etc., nor use of other solvents such as pyridine or DMSO gave improvement. However, longer reaction time and higher temperature increased the adenylation efficiency. When **9d** was treated with **4** at 40 °C for 48 h, it reacted quantitatively (entry 6, Table 1; no peak of pT10 at 3058 Dalton was observed). Under the same conditions, the adenylation efficiency of **9e** improved to 60% (entry 7, compare entry 5 in Table 1). We then used these improved reaction conditions to adenylate several DNA oligonucleotides of varying length and sequence (**11a**−**h**, Supporting Information), which were designed for ligation-based detection of 2′-OMe modifications in yeast rRNA.

Oligonucleotides **11a**−**d** have the same length (15mer) and similar sequences, except that the 5′-terminal nucleotide is A, G, C and T, respectively. Their MS data showed that **11a**−**d** have similar adenylation efficiencies (Supporting Information, Table S1), indicating that the identity of the nucleobase that neighbors the modification site has minimal effect on adenylation efficiency. Oligonucleotides **11e**−**h**, which have longer and different sequences (18−25 mer), also consistently gave adenylation efficiencies of 52%−75%. The AppDNA oligos were purified by denaturing gel electrophoresis (Supporting Information, Figure S1A), and the adenylated efficiencies obtained from gel electrophoresis are consistent with those obtained by MS analysis. When used in the ligation reaction to study 2′-OMe modifications in rRNA[6], the chemically synthesized AppDNA worked very well, both in yield and in the degree of discrimination between unmodified and modified RNA (as shown in Supporting Information, Figures S1B and S1C).

In summary, we have developed a convenient and efficient chemical synthesis of AppDNA. The key step involves coupling of an adenosine 5′-phosphorimidazolidate derivative **4** with an immobilized DNA-5′-monophosphate. Adenylation occurs with moderate to good efficiency for oligonucleotides containing more than 11 residues but occurs quantitatively for oligonucleotides containing fewer than 11 residues. The chemical synthesis of AppDNA described here nicely complements the enzymatic synthesis of AppDNA, which has a strong sequence dependence and become inefficient for DNA substrates containing fewer than 11 nucleotides. These chemically synthesized AppDNA substrates function well in ligation reactions to study 2′-OMe modifications in RNA. Potentially, this method may also be extended to chemical synthesis of AppRNA.

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Supporting Information Available: Full experimental and analytical data and the ^1H and ^{13}C NMR spectra for compounds **2**−**3**; general procedure for adenylation; a table for adenylatiton rate and gel electrophoresis analysis. This material is available free of charge via the Internet at http://pubs.acs.org.

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