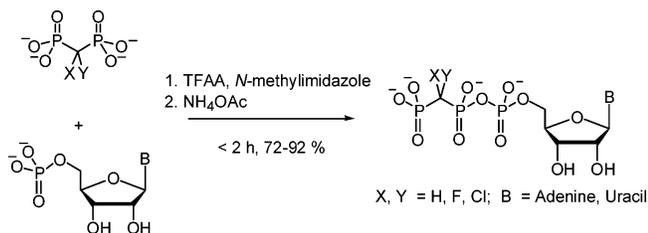# An Improved Method for the Synthesis of Nucleoside Triphosphate Analogues

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Nucleoside monophosphates, when activated by trifluoroacetic anhydride and *N*-methylimidazole, efficiently couple with a variety of electron-deficient diphosphonates in a reproducible and efficient manner (<2 h, >72% isolated yield). Unlike traditional methods for the preparation of nucleoside 5′-β,γ-methylenetriphosphate analogues, there is no requirement for predrying, or conversion to specific salt forms, of commercially available nucleoside monophosphate starting materials.

Mechanistic probes to investigate the roles of phosphate transfer in biological systems are of importance in elucidating the mechanisms of fundamental enzyme-catalyzed processes involving primary and secondary metabolism.[1] Non-hydrolyzable nucleotide analogues that are isosteric and isoelectronic with nucleoside triphosphates are examples of analogues that have been used successfully to probe phosphoryl transfer in enzyme-catalyzed processes[2−7] and receptor specificity.[8−11] Recently, such halophosphonate analogues have been shown to improve the physiological half-lives of effective HIV reverse-transcriptase inhibitors.[12] The physiochemical benefits associated with halogenation of hydrolysis-inert analogues of pyrophosphate were initially proposed independently by Blackburn and McKenna.[13,14] One of the difficulties encountered in exploiting these probes for mechanistic studies is that reported syntheses of non-hydrolyzable nucleotide analogues are lengthy and often low yielding. These reactions typically proceed by activation of a nucleoside monophosphate with subsequent addition of a nucleophile. Typically there is a requirement for a specific salt form of the nucleoside monophosphate starting material, as well as the removal of the water of crystallization, through repeated coevaporation with pyridine. Lengthy reaction times for the phosphate coupling are due to the poor nucleophilicity of (halogenated) diphosphonates (>24 h), and often lengthy purifications by ion-exchange chromatography are required to furnish pure product.[15]

Herein, we report a significantly improved synthesis and purification of substituted nucleoside 5′-β,γ-methylenetriphosphate analogues, as shown in Scheme 1. This phosphonate−phosphate coupling procedure involves the activation, and subsequent coupling, of purine and pyrimidine nucleoside monophosphate-*N*-methylimidazolides (**A**) with dichloromethylene-, difluoromethylene-, fluoromethylene-, and methylenediphosphonic acids. This reaction, which uses commercially available purine and pyrimidine nucleoside monophosphates, proceeds without the need to alter salt forms or remove moisture and is complete within 2 h. Purification is faciliated by the use of ion-pair reversed-phase chromatography that is amenable to scale-up.

The first use of nucleoside monophosphate-*N*-methylimidazolides (**A**) as electrophilic reagents for phosphate coupling was described by Bogachev for the synthesis of deoxynucleoside 5′-triphosphates using activated deoxynucleoside monophosphate-*N*-methylimidazolides and pyrophosphate.[16] Uridine monophosphate-*N*-methylimidazolide was subsequently used by Kiessling for the preparation of UDP-α-D-galactofuranose.[17]

Our coupling procedure for the synthesis of β,γ-methylenenucleoside 5′-triphosphate analogues consists of several facile steps. The electrophilic nucleoside 5′-monophosphate-*N*-methylimidazolide (**A**) is formed by the reaction of a nucleoside 5′-monophosphate, either as the free acid, the monosodium, or the disodium salt form, with an excess of trifluoroacetic anhydride in the presence of triethylamine in acetonitrile. The presence of

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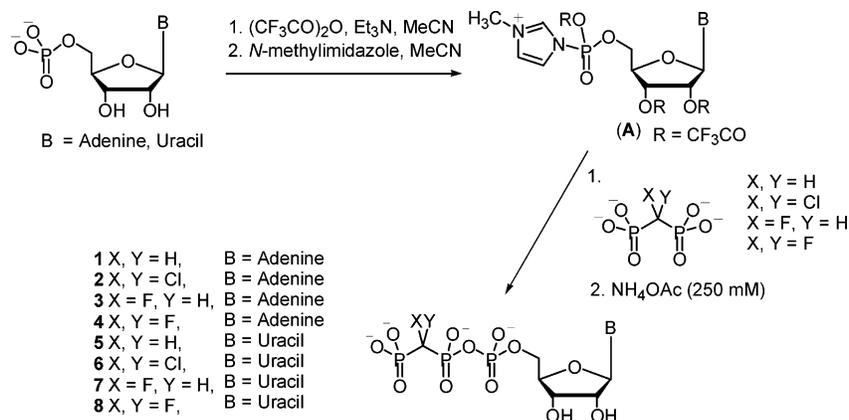
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SCHEME 1. Synthesis of Substituted β,γ-Methylenenucleoside 5′-Triphosphate Derivatives[a]


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1 X, Y = H,      B = Adenine
2 X, Y = Cl,     B = Adenine
3 X = F, Y = H,  B = Adenine
4 X, Y = F,      B = Adenine
5 X, Y = H,      B = Uracil
6 X, Y = Cl,     B = Uracil
7 X = F, Y = H,  B = Uracil
8 X, Y = F,
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[a] See Tables 1 and 2 for yields.

TABLE 1. Preparation of Substituted β,γ-Methylenenucleoside 5′-Triphosphate Derivatives via an Adenosine Monophosphate-N-methylimidazolide Intermediate

entry	nucleotide salt form	diphosphonate linker	diphosphonate counterion	diphosphonate pH (starting)	volume TE (mL)	reaction time (min)	yield (%)[a]	product
1	free acid	CH_2	$2\ ^nBu_4N$	2.5	0.2	10	89	(**1**)
2	free acid	CH_2	$2\ ^nBu_4N^+$	6	0	10	75	(**1**)
3	free acid	CH_2	$3\ ^nBu_4N^+$	10	0	10	87	(**1**)
4	Na^+	CH_2	$3\ ^nBu_4N^+$	10	0	10	89	(**1**)
5	free acid	CCl_2	$^nBu_4N^+$	2.5	0.2	40	92	(**2**)
6	free acid	CHF	$3\ ^nBu_4N^+$	8	0	60	76	(**3**)
7	free acid	CF_2	$3\ ^nBu_4N^+$	7.3	0	80	82	(**4**)

[a] Yields were determined by UV using $\lambda_{max}\ 259 = 1.54 \times 10^4\ M^{-1}\ cm^{-1}$ after chromatography.

trifluoroacetic anhydride results in acylation of the hydroxyl groups, the amino group (in case of adenosine-5′-monophosphate), and the phosphate group. After removal of the volatile components under reduced pressure, addition of N-methylimidazole results in the formation of the corresponding N-methylimidazolide, which is detected by ^{31}P NMR spectroscopy and TLC. The nucleoside 5′-monophosphate-N-methylimidazolide (**A**) is then added to 2 equiv of the diphosphonate analogue in acetonitrile, thus affording the corresponding nucleoside triphosphate derivative. The N-methylimidazolide intermediate is critical to the success of the coupling reaction, as addition of the diphosphonate directly to the mixed anhydride gave significantly lower yields. We also found that none of the steps within the entire coupling procedure is enhanced by the presence of N,N-dimethylaniline, as was previously indicated by others for phosphate couplings, and therefore this reagent was omitted.[16,17] Given the potential acid lability of the P—O—P bond during workup, aqueous ammonium acetate was used to neutralize the reaction.[17] After workup, the products were purified by automated reversed-phase flash column chromatography.

The commercially available nucleoside 5′-monophosphates used in this study were in the form of either free acid, monosodium, or disodium salts. In all of these compounds, molar equivalents of water are present. Previous coupling procedures first necessitate the removal of this moisture by azeotroping with pyridine.[18]

However, by forming nucleoside monophosphate-N-methylimidazolides (**A**), the moisture is conveniently removed as it reacts with the trifluoroacetic anhydride in the first step of the method. This approach enabled us to directly employ the commercially available nucleoside 5′-monophosphates without the need to convert them into the corresponding triethylammonium salts or spend significant time drying them.[16]

The results from our phosphonate—phosphate coupling reactions are shown in Table 1. The first experiments performed (entries 1−3) assessed the effect of the counterion of the diphosphonate on the coupling step using three different forms of methylenediphosphonate: namely, the free acid form of methylenediphosphonate (**12**) (entry 1), a bis(tetra-n-butylammonium) dihydrogen methylenediphosphonate form (pH 6, entry 2), and a tris(tetra-n-butylammonium) hydrogen methylenediphosphonate form (**15**) (pH 10, entry 3). It was found that we were required to add triethylamine directly to the reaction mixture to solubilize the diphosphonate for the free-acid form of the diphosphonate; however, no significant change to the yield was observed by changing salt forms, implying that the dianionic or the trianionic form of the diphosphonate is equally effective in the coupling of the diphosphonate to the activated nucleoside 5′-monophosphate (**A**). Next, the salt form of the nucleotide was assessed as a potential starting material (Table 1, entries 3 and 4), and no significant differences in yield were observed using the free acid or sodium salt form.

The reaction time for the coupling of (**A**) and the diphosphonate is dependent on the nature of the substituent on the methylene group of the diphosphonate

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TABLE 2. Preparation of Substituted β,γ-Methylenenucleoside 5′-Triphosphate Derivatives via a Uridine Monophosphate-*N*-methylimidazolide Intermediate

entry	nucleotide salt form	diphosphonate linker	diphosphonate counterion	diphosphonate pH (starting)	reaction time (min)	volume TEA (mL)	yield (%)[a]	product
1	$2Na^+$	CH_2	$3\ ^nBu_4N^+$	10	10	0	84	(**5**)
2	$2Na^+$	CCl_2	$^nBu_4N^+$	2.5	40	2.5	86	(**6**)
3	$2Na^+$	CHF	$3\ ^nBu_4N^+$	8	60	0	84	(**7**)
4	$2Na^+$	CF_2	$3\ ^nBu_4N^+$	7.3	80	0	81	(**8**)

[a] Yields were determined by UV using $\lambda_{max}\ 262 = 1 \times 10^4\ M^{-1}\ cm^{-1}$ after chromatography.

(Tables 1, entries 5−7 and Table 2). Diphosphonates containing more electronegative substituents react more slowly. This is attributed to the reduction in the nucleophilicity of the phosphoryl oxygen atoms caused by the inductively electronegative substituent upon the bridging carbon. However, in all of these coupling reactions, the reaction time (<2 h) was significantly shorter than previous coupling methods.[18,19] We did observe that if the diphosphonate was not dried thoroughly prior to use, side reactions occurred including the formation of AppA or cAMP (or correspondingly UppU or cUMP) as indicated by the characteristic ^{31}P NMR chemical shifts of these species.[17]

The nature of the nucleoside base does not influence the reaction as similar yields were observed using UMP as starting nucleoside monophosphate (Table 2). Using the more inductively electron-withdrawing diphosphonates did not alter the reaction yields with UMP, although consistently longer reaction times were required, as observed with AMP.

The reactivity of the trifluoroacyl phosphate at the phosphorus atom is in contrast to the reactivity of acyl phosphates observed by Kluger et al., where reaction occurs at the carbonyl group and thus the phosphate group is displaced.[20]

Initially, we attempted the purification of our reaction mixtures by using an ion-exchange resin (DEAE or Q-sepharose). However, our separation efficiency using this method suffered from unreacted diphosphonate coeluting along with the nucleotide triphosphate derivative. Moreover, the low flow rates made the purification runs lengthy. Mökkönen et al. have reported the use of an ion-pairing HPLC method for the analysis of AppCCl$_2$p.[21] We have adapted this method to the preparative-scale purification of our reaction mixtures using reversed-phase column chromatography. This method provided pure nucleoside triphosphate derivatives, in shorter times compared to other methods.[16−18]

After lyophilization, the purified nucleoside triphosphate derivatives (**1**−**8**) were passed through an Amberlite IR-120 H$^+$ column to convert the nucleoside triphosphate derivative into the corresponding free acid form, which was neutralized with dilute aqueous ammonium hydroxide and freeze-dried. Finally, small amounts of ammonium acetate and ammonium trifluoroacetate found in the final product were quantitatively removed by precipitation of the nucleotide salt from water by the addition of ethanol.

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SCHEME 2. Synthesis of Substituted Methylenediphosphonic Acid Derivatives

9 R^1, R^2 = H
10 R^1 = H, R^2 = F
11 R^1, R^2 = F

12 R^1, R^2 = H
13 R^1 = H, R^2 = F
14 R^1, R^2 = F

15 R^1, R^2 = H
16 R^1 = H, R^2 = F
17 R^1, R^2 = F

The diphosphonic acids (**12**−**17**) used in our coupling procedure were prepared in quantitative yield by treating the corresponding tetraisopropyl methylene-, monofluoro-, and difluoromethylenediphosphonate with refluxing hydrochloric acid and titrating the deprotected materials with tetrabutylammonium hydroxide (Scheme 2).

This deprotection was found to be more convenient than the reported deprotection using trimethylsilyl bromide in CH_2Cl_2.[22] The tetraisopropyl monofluoro- and difluoromethylenediphosphonates were prepared by fluorination of tetraisopropyl methylenediphosphonate by modifying the ratio of base and fluorinating reagent compared to a reported procedure.[22]

In conclusion, we have described an effective, fast, and reproducible method to prepare nucleoside triphosphate analogues. Our protocol involves the rapid reaction of a methylenediphosphonate analogue and an activated 5′-*N*-methyl phosphorylimidazolide nucleoside. The increased reactivity of the electrophilic phosphoryl group used here eliminates the long reaction times typically required for nucleoside triphosphate synthesis.

Experimental Section

A representative synthesis is presented below.

Uridine 5′-β,γ-Fluoromethylenetriphosphate, UppCHFp (7). A solution of trifluoroacetic anhydride (480 μL, 3.4 mmol) in acetonitrile (300 μL) was cooled in an ice-water bath and added dropwise by syringe to a similarly cooled flask containing 5′-UMP disodium salt dihydrate (110 mg, 0.27 mmol) suspended in a mixture of acetonitrile (1 mL) and triethylamine (480 μL, 3.44 mmol), under nitrogen. The reaction was stirred for 10 min at room temperature, whereby a pale orange solution was obtained and volatiles were removed in vacuo. The resulting

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syrup was then cooled in an ice-water bath to which was added a solution of *N*-methylimidazole (72 μL, 0.9 mmol) in acetonitrile (300 μL) and triethylamine (315 μL, 2.26 mmol) cooled in an ice-water bath. The reaction was stirred for 10 min, after which time a bright yellow solution was obtained. Preparation of the activated UMP-*N*-methylimidazolide was confirmed by TLC (10:10:1 CHCl$_3$/MeOH/1 M ammonium acetate pH 7, R_f 0.45) and δ_P −10.35 (s)).[17] The UMP-*N*-methylimidazolide was then added dropwise by syringe to an ice-water cooled flask containing **16** (503 mg, 0.55 mmol), 4 Å molecular sieves (10−15), and acetonitrile (1 mL). Reaction progress was monitored by TLC (4:4:1 EtOH/NH$_3$/H$_2$O) or by ^{31}P NMR spectroscopy by removing 100-μL aliquots of the reaction mixture every 15 min [δ_P UMP 4.3; UppCHFp −10.43 (d), 5.15 (ddd), 7.56 (dd); **16** 10.88 (d)]. The reaction was complete after 1 h, then quenched with cold aqueous ammonium acetate (3 mL, 250 mM, pH 7), washed with chloroform (3 × 5 mL), freeze-dried, diluted with water/tributylamine (10 mM))/acetic acid (30 mM), and purified on a C18 reversed-phase silica column, eluting with a linear gradient (0−100%, 30 column volumes) of buffer A (water/10 mM tributylamine/30 mM acetic acid) to buffer B (methanol/10 mM tributylamine). The flow rate was 25 mL min^{-1}. The appropriate fractions were pooled and adjusted to pH 7 using dilute ammonia solution, and the yield was determined by absorbance (84%). The following procedure was used to remove any excess salts: The solution was then concentrated, lyophilized, and then dissolved in water (10 mL) and passed through an Amberlite IR-120 H$^+$ column. The fractions possessing UV absorbance were combined, adjusted to pH 9.5 with dilute ammonia, and lyophilized. The product was then dissolved in water (0.5 mL) and precipitated by the dropwise addition of ethanol (3 mL) where it was left in a freezer for 20 min and then centrifuged for 10 min at 4 °C.

The aqueous ethanolic solution was decanted out, and the residue was dried under vacuum. A few drops of water were added to dissolve the residue, which was lyophilized for a final time to yield **7** as a white solid, 120 mg (82%). δ_P (D$_2$O) −11.04 (d, P$_\alpha$, $^2J_{\alpha\beta}$ 26.9 Hz), 1.39 (ddd, P$_\beta$, $^2J_{P\beta F}$ 62.8 Hz, $^2J_{\alpha\beta}$ 26.9 Hz, $^2J_{\beta\gamma}$ 15 Hz), 8.17 (dd, P$_\gamma$, $^2J_{P\gamma F}$ 59.8 Hz, $^2J_{\beta\gamma}$ 15 Hz); δ_F (D$_2$O) 213.33 (dd, $J_{F,P\beta}$ 85.56, $J_{F,P\alpha}$ 62.14); δ_H (D$_2$O) 4.17 (br, 2H, H$_{5'}$), 4.22 (br, 1H, H$_{4'}$), 4.33 (br, 2H, H$_{2'}$ H$_{3'}$), 4.8 (ddd, C*H*F $_{\beta\gamma}$, $J_{H,F}$ 46.23 Hz, $^2J_{H,P\beta}$ 12.4 Hz, $^2J_{H,P\alpha}$ 12.1 Hz) 5.92 (br, 2H, H$_{1'}$, H$_5$), 7.9 (d, 1H, H$_6$, J6,5 7.93 Hz); δ_C (D$_2$O) 64.5 (d, C$_{5'}$, $^2J_{C,P\alpha}$ 5 Hz), 69.5 (C$_{3'}$), 73.7 (C$_{2'}$), 83.3 (d, C$_{4'}$, $^3J_{C,P\alpha}$ 8.8 Hz), 88.3 (C$_{1'}$), 89.5 (ddd, C*H*F$_{\beta\gamma}$, $J_{C,F}$ 46.1, $J_{C,P\beta}$ 25.1, $J_{C,P\gamma}$ 13.8), 102.6 (C$_5$), 141.6 (C$_6$), 151.8 (C$_4$), 166.2 (C$_2$). Negative ion ESI-MS, *m/z* 499 [M − H]$^-$.

Supporting Information Available: Full experimental details for the preparation of compounds **1**−**8** and **10**−**17** and characterization data. This material is available free of charge via the Internet at http://pubs.acs.org.

JO0518598