JOC The Journal of Organic Chemistry

Note

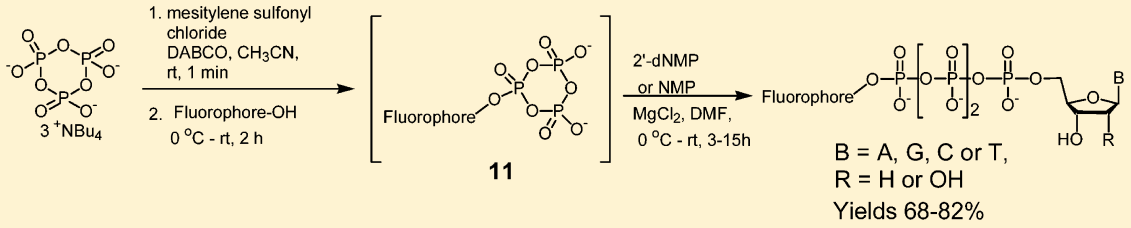
pubs.acs.org/joc

Synthesis of Nucleoside 5′-Tetraphosphates Containing Terminal Fluorescent Labels via Activated Cyclic Trimetaphosphate

Samy Mohamady and Scott D. Taylor*

Department of Chemistry, University of Waterloo, 200 University Avenue West, Waterloo, Ontario, Canada

Supporting Information

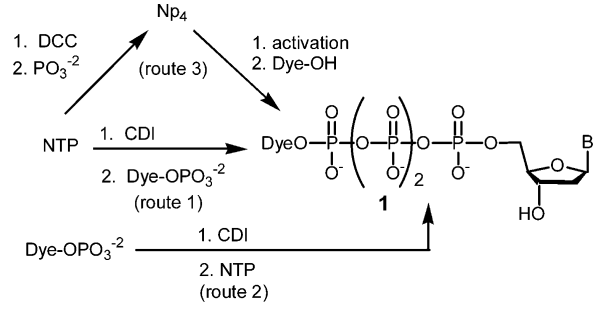


ABSTRACT: 2′-Deoxynucleotide 5′-tetraphosphates in which a fluorescent label is attached to the terminal phosphate are used as key reagents in high-throughput DNA sequencing techniques and in single nucleotide polymorphism typing assays. We demonstrate that this class of compounds can be prepared by reacting fluorophores such as 7-hydroxy-4-methylcoumarin, methylfluorescein, fluorescein and resorufin with an activated form of cyclic trimetaphosphate to give intermediate **11**. Reaction of **11** with 2′-deoxynucleoside 5′-monophosphates or a nucleoside 5′-monophosphate gave the target compounds in good yield.

Terminal phosphate-labeled nucleotides have been used as tools and probes in biochemistry and biotechnology for many years.[1−3] Very recently there has been considerable interest in 2′-deoxynucleoside 5′-polyphosphates in which a fluorescent label is attached to the terminal phosphate. Such nucleotides are used as key reagents in high-throughput DNA sequencing techniques and in single nucleotide polymorphism typing assays.[4−8] Central to the success of these methodologies is the ability of the terminally labeled nucleotides to as act as substrates for DNA polymerase. Nucleotides bearing more than three linear 5′-phosphates, such as δ-labeled nucleoside 5′-tetraphosphates (general structure **1** in Scheme 1), are used, as it has been shown that such nucleotides are much better substrates for DNA polymerases than the corresponding γ-labeled 5′-triphosphates.[4]

The synthesis of δ-labeled nucleoside 5′-tetraphosphates has been achieved by several routes (Scheme 1). These approaches are based upon routes that were developed and are widely used for preparing dinucleoside tetraphosphates (Np_4N's). In one approach (route 1), a 2′-nucleoside 5′-triphosphate (5′-dNTP) is reacted with carbonyldiimidazole (CDI) followed by reacting the resulting imidizolide with a phosphorylated fluorescent dye.[8] Alternatively, a phosphorylated dye is activated with CDI followed by reaction with a 5′-dNTP (route 2).[4] In another approach (route 3), a 5′-dNTP is reacted with DCC to give a cyclic trimetaphosphate nucleotide derivative, which is ring opened with orthophosphate to give a nucleoside 5′-tetraphosphates (Np_4).[5] This species is activated and then reacted with a dye. All of these approaches require expensive 5′-dNTP's as substrates and, in the case of routes 1 and 2, the synthesis or purchase of expensive phosphorylated dyes.[9]

Scheme 1. Literature Routes to Nucleoside 5′-Tetraphosphates Bearing Terminal Fluorescent Dyes



Besides the routes described in Scheme 1, other approaches have been developed for preparing conjugates of dinucleoside tetraphosphates, which could potentially be used for preparing δ-labeled nucleoside 5′-tetraphosphates. Methods employing 5′-chloroquinolyl esters,[10] 5′-cyclosaligenyl phosphates,[11] and 5′-salicylphosphites have been reported.[12] In each case the prior preparation of an activating agent and/or the protection of the secondary hydroxyl or amino groups on the nucleoside and/or isolation of the activated nucleoside is required. A solid phase method has recently been described; however, this method requires the multistep synthesis of a polymer with a unique linker and multistep syntheses of polyphosphites prior to the solid phase chemistry.[13] Enzymatic methods have also been used for the synthesis of Np_4N's; however, this approach is limited by the substrate specificity of the enzymes.[14] Ludwig and Eckstein developed a method for preparing nucleoside

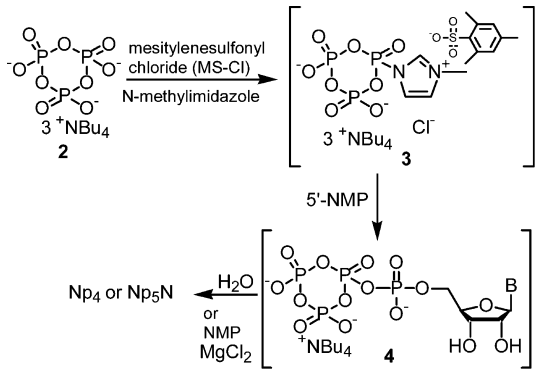
Received: January 9, 2014
Published: February 5, 2014

   dx.doi.org/10.1021/jo500051y | J. Org. Chem. 2014, 79, 2308−2313

triphosphates using salicylchlorophosphite as an activating agent.[15] To the best of our knowledge this methodology has never been used for preparing conjugates of nucleoside tetraphosphates. Although it could potentially be used for this purpose it would require the protection of the nucleoside and the use of an oxidant, which could affect the fluorophore.

We recently described a novel route to the synthesis of Np_4's and dinucleoside 5′-pentaphosphates (Np_5N's), which involves reacting a nucleoside 5′-monophosphate with activated trimetaphosphate (TriMP, **3**) to give intermediate **4** (Scheme 2).[16] Hydrolysis of this intermediate yielded the Np_4's, while

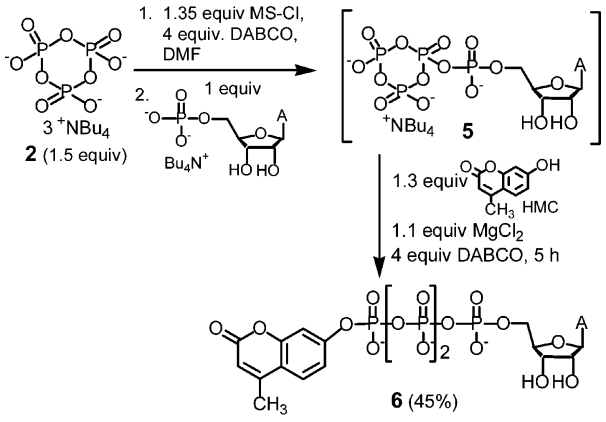
Scheme 2. Synthesis of Np_4's and Np_5N's via Activated TriMP (3)



reaction with a nucleoside 5′-monophosphate (5′-NMP) gave Np_5N's in high yield and purity. The success of these studies prompted us to examine whether intermediates of type **4** and their 2′-deoxy analogues could be reacted with other nucleophiles, such as a fluorescent dye, to give a rapid and straightforward route to δ-labeled nucleoside 5′-tetraphosphates.

7-Hydroxy-4-methylcoumarin (HMC, Scheme 3) was chosen as a model fluorescent dye, as it is relatively inexpensive and

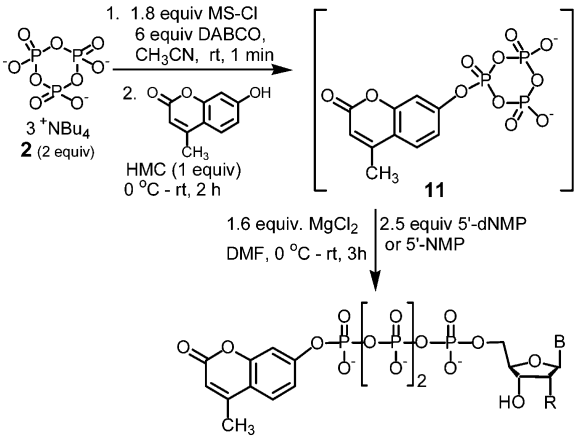
Scheme 3. Synthesis of Compound 6 via Intermediate 5



readily available. We initially attempted to prepare the adenosine derivative **6** by reacting intermediate **5**, prepared using our previously reported procedure,[16] with HMC in the presence of $MgCl_2$. This did not result in consumption of the HMC as determined by TLC. However, when an excess of sterically hindered organic base such as DABCO was present the coumarin was consumed and the formation of **6** was evident by ^{31}P NMR (characteristic peaks: −9.3 ppm (d), −15.2 ppm (d) and two apparent triplets at approximately −20.0 to −20.3 ppm) along with other unidentified impurities. Optimal conditions were developed, which consisted of 1.3 equiv of HMC, 4 equiv of DABCO and 1.1 equiv of $MgCl_2$. The HMC was consumed in about 5 h. After quenching with triethylammonium acetate and purification by reversed-phase column chromatography, compound **6** was obtained in a 45% yield.

The modest yield obtained with the approach outlined in Scheme 3 prompted us to examine the reverse procedure in which the coumarin is reacted with activated TriMP followed by the addition of NMP (Scheme 4). To ensure complete

Scheme 4. Synthesis of δ-Labeled Nucleoside 5′-Tetraphosphates via Intermediate 11



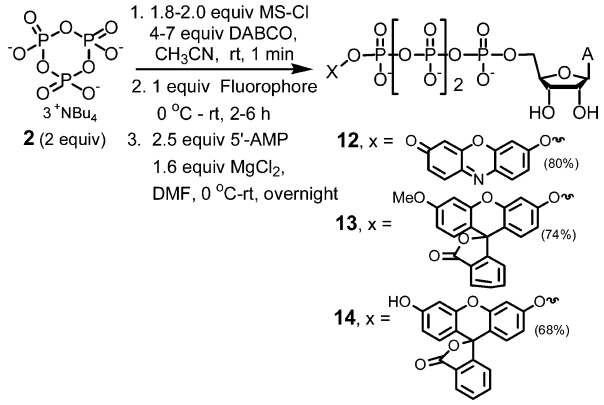
phosphorylation of the OH group of HMC and to prevent formation of the dicoumarin triphosphate, we employed a 1.8-fold excess of activated TriMP. Hence, 1.8 equiv of mesitylenesulfonyl chloride was added to a solution of 2.0 equiv of TriMP (**2**) and 6 equiv of DABCO, and the mixture was stirred for 1 min, and then 1.0 equiv of HMC was added. The reaction turns from a clear solution to a white turbid mixture after about 45 min. All of the coumarin was consumed in about 2 h as determined by TLC. No reaction occurred in the absence of DABCO, and it was found that NMI was not necessary for reaction to occur. This mixture was then added dropwise to a cooled (ice bath) solution of 1.6 equiv of $MgCl_2$ and 2.5 equiv of 5′-AMP as its tetrabutylammonium salt in DMF. The ice bath was removed, and the progress of the reaction was monitored by withdrawing samples, quenching them with a 5% solution of EDTA in triethylammonium acetate buffer (pH 7.0), and then analyzing them by ^{31}P NMR. Peaks corresponding to **6** were clearly evident. After 3 h, peaks corresponding to **6** no longer increased in intensity, and **6** was the dominant product. The reaction was quenched with triethylammonium acetate buffer (pH 7.0), and the resulting solution was washed with chloroform, and magnesium ions were removed by Chelex resin. The ^{31}P NMR of the crude material exhibited peaks corresponding to product as well as peaks corresponding to TriMP, unreacted 5′-AMP, and minor peaks that we attributed to Ap_5A[15] (for example, see Figure S1 in the Supporting Information). With the activated TriMP and 5′-AMP being in considerable excess to HMC, it was expected for a considerable amount of Ap_5A byproduct to have been

dx.doi.org/10.1021/jo500051y | J. Org. Chem. 2014, 79, 2308−2313

produced and little or no unreacted 5′-AMP or TriMP to be present. Since this was not the case, we suggest that the reaction of the 5′-AMP with any *activated* TriMP intermediates that might be formed during the reaction (vide infra) is slower than the reaction of 5′-AMP with intermediate **11** in the absence of NMI and/or in the presence of DABCO. Compound **6** was obtained in an 80% yield after purification by reversed-phase chromatography using tributylammonium acetate buffer in methanol/water and conversion to its tetraammonium salt using Dowex 50W resin (NH_4^+ form). It is worthy of note that the Ap_5A byproduct exhibited a very different retention time from **6** and so was easily removed. This procedure was then applied to the four common deoxynucleotides, which gave the labeled products **7**–**10** in yields of 80–82%. Purification of these compounds was equally straightforward.

To ascertain if this methodology can be applied to the synthesis of nucleoside tetraphosphate conjugates bearing other fluorophores, we prepared adenosine tetraphosphate conjugates in which the terminal phosphate was labeled with resorufin, methyl fluorescein and fluorescein (compounds **12**–**14**, Scheme 5). These compounds were prepared in good yield

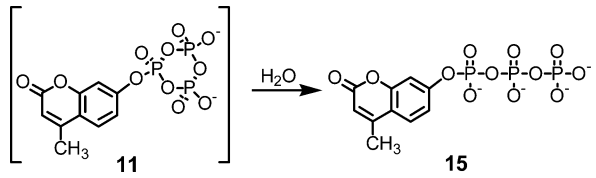
Scheme 5. Synthesis of Adenosine-5′-tetraphosphate δ-Labeled with Resorufin, Methyl Fluorescein, and Fluorescein



with compounds **13** and **14** obtained as mixtures of diastereomers and/or as a mixture of the lactone and free acid forms. The reactions of the trimetaphosphorylated derivatives of these dyes with 5′-AMP took longer (overnight as opposed to 3 h in case of HMC). For the methylfluorescein and fluorescein derivatives **13** and **14**, the yield varied significantly with the amount of DABCO present. When using 6 equiv of DABCO, compound **14** was obtained in a 54% yield together with a significant quantity of disubstituted product ($((O_3P)_3O$-fluorescein-$O(PO_3)_3)$). Adding just one more equivalent of DABCO (7 equiv) resulted in a yield of 68%, and the amount of disubstituted product was significantly reduced. On the other hand, using 6 equiv of DABCO gave a low yield of compound **13** (42%). Increasing the DABCO to 7 equiv reduced the yield to 25%, while using 4 equiv of DABCO gave a 74% yield of compound **13**. The effect of DABCO on the yields of **13** and **14** may be a result of DABCO affecting the relative amounts of lactone and free acid forms of fluorescein and methyl fluorescein substrates.

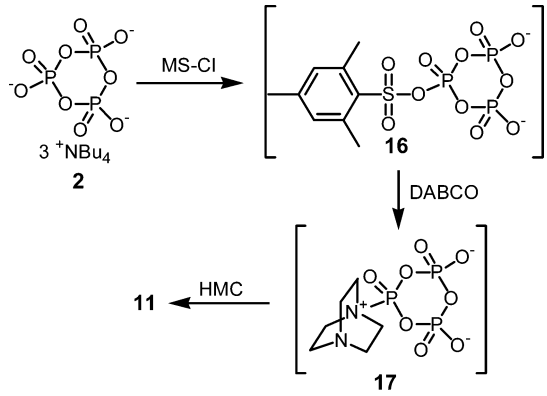
^{31}P NMR data suggests that intermediate **11** is indeed formed during the reaction. The ^{31}P NMR spectrum of a mixture of HMC, TriMP, mesitylenesulfonyl chloride, and DABCO (approximately 1:1.3:1.3:4) in CH_3CN after 2 h showed mainly a doublet at −20.7 ppm and a triplet at −24 ppm (Figure S2 in the Supporting Information). The ^{31}P NMR spectrum of this mixture after having been quenched with water showed doublets at −4.0 and −14.5 ppm and a triplet at −20 ppm (see Figure S3 in the Supporting Information), and these peaks can be attributed to compound **15** formed by hydrolysis of intermediate **11** (Scheme 6).[17]

Scheme 6. Formation of Triphosphate 15 by Hydrolysis of Intermediate 11



Although we believe that the reaction proceeds via intermediate **11**, the route by which this intermediate is formed is not clear. The ^{31}P NMR spectrum of a mixture of TriMP, DABCO, and mesitylenesulfonyl chloride (1:3:0.9) in acetonitrile after 5 min shows a doublet at −6.3 ppm and a triplet at −20.6 ppm (see Figure S4 in the Supporting Information). However, after about one hour these peaks are no longer present. The mixture becomes turbid, and the ^{31}P NMR spectrum shows a triplet at −31 ppm and a multiplet at around −20 ppm, possibly consisting of overlapping doublets and triplets (see Figure S5 in the Supporting Information). Since it takes 2 h for the coumarin to be completely consumed, these data suggest that intermediate **11** might be formed by HMC reacting with more than one intermediate. It is possible that the initially formed mixed anhydride **16**[16] reacts rapidly with DABCO to give intermediate **17**, and this species may react with HMC to give intermediate **11** (Scheme 7).

Scheme 7. Possible Pathway for Formation of Intermediate 11



Moreover, dimeric or higher order polyphosphates might be formed, and these species may also be capable of reacting with HMC to somehow give intermediate **11**. Further studies will be required to determine this.

In summary, a novel and efficient approach to the synthesis of δ-labeled nucleoside 5′-tetraphosphates in which the terminal phosphate is labeled with a fluorescent dye was developed. The procedure is more rapid than previously reported methods,[4,5,8] it utilizes relatively inexpensive or easily

dx.doi.org/10.1021/jo500051y | J. Org. Chem. 2014, 79, 2308−2313

prepared NMP's as opposed to more expensive NTP's, and the target compounds are obtained in excellent yields. These studies also demonstrate that activated TriMP is capable of reacting with nucleophiles other than phosphates or excess water and so should prove to be a route to other types of δ-labeled nucleoside tetraphosphates and could also be a direct route to nucleoside 5′-triphosphates using nucleosides as nucleophiles.

EXPERIMENTAL SECTION

General Information. All reagents and starting nucleotides were obtained from commercial sources unless stated otherwise. Strictly anhydrous conditions are required to obtain the reported yields. Acetonitrile and DMF were distilled from calcium hydride. *N*-Methylimidazole (NMI) was distilled from sodium hydroxide and stored over 4 Å molecular sieves. Methylfluorescein was prepared according to literature procedures and crystallized from boiling ethanol.[18,19] All reactions were conducted under an inert atmosphere of Ar in oven-dried glassware. All NMR spectra were recorded using D_2O as solvent. For ^1H NMR spectra, chemical shifts are reported in ppm relative to the solvent residual peak (δ 4.79). For proton-decoupled ^{13}C NMR spectra, chemical shifts are reported in ppm relative to CH_3OH in D_2O (δ 49.5, external standard). For proton-decoupled ^{31}P NMR, chemical shifts are reported in ppm relative to aqueous 85% H_3PO_4 (δ 0 ppm, external standard). The tri(tetrabutylammonium) salt of trimetaphosphate (**2**) was prepared as previously described.[16] Preparative reversed phase chromatography was performed using a Biotage Isolera One Flash purification system equipped with a C-18 reverse phase preparative Biotage 30 g column. Negative ion high resolution electrospray mass spectra were obtained using a high resolution, accurate mass Orbitrap mass spectrometer.

Preparation of the Tetrabutylammonium Salts of 5′-AMP, 5′-dAMP, 5′-dTMP, and 5′-dCMP. The free acids or free acid monohydrates of 5′-AMP, 5′-dAMP, 5′-dTMP, and 5′-dCMP (0.5 g) were dissolved in distilled deionized water (50 mL) and titrated to pH 7.0 with a dilute solution of tetrabutylammonium hydroxide.[20] The solutions were concentrated by high vacuum rotary evaporation to approximately one-seventh the original volume and lyophilized. The lyophilized powders were stored in the freezer. An equal amount of dry acetonitrile and dry toluene was added to round-bottom flasks containing 0.675 mmol (519 mg of AMP, 508 mg of 2′-dAMP, 502 mg of 5′-dTMP, and 492 mg of 5′-dCMP) of these nucleotides, and the solutions were concentrated by rotary evaporation to dryness. This was repeated two more times. The ^1H NMR spectra of these nucleotides indicated that there was 1.7 tetrabutylammonium ions per nucleoside monophosphate. The nucleotides were subjected to a high vacuum for 3 h. The flasks were removed under Ar, the nucleotides were dissolved in dry DMF, and 4 Å molecular sieves were added. These solutions were allowed to stand for at least 3 h hour prior to the coupling reactions.

Preparation of the Tributylammonium Salt of 2′-dGMP. Using the above procedure to prepare the tetrabutylammonium salt of 2′-dGMP from commercially available 2′-dGMP disodium salt dihydrate resulted in its decomposition upon conversion to its free acid and titration with tetrabutylammonium hydroxide. Hence the tri-*n*-butylammonium salt was prepared using a procedure similar to that developed by Gibson and Leonard.[21] An aqueous solution of 2′-dGMP disodium salt dihydrate (0.5 g in 20 mL of distilled deionized water) was applied to a Dowex-50-W column (8−10 g) in its pyridinium form. The eluate was collected in a flask containing 20 mL of ethanol and 1.0 mL of tributylamine. The column was washed with water (3 × 30 mL) while collecting the eluate in the above-mentioned flask. The resulting solution was stirred for 10 min and then concentrated by high vacuum rotary evaporation to about one-seventh of the original volume and then lyophilized. The lyophilized powder was stored in the freezer. An equal amount of dry acetonitrile and dry toluene was added to a round-bottom flask containing 0.675 mmol (396 mg) of the tributylammonium salt, and the solution was concentrated by rotary evaporation to dryness. This was repeated two more times. The ^1H NMR spectrum of the residue indicated that there were 1.3 tributylammonium ions per nucleoside monophosphate. The nucleotide was subjected to a high vacuum for 3 h. The flask was removed under Ar, the nucleotide was dissolved in dry DMF, and 4 Å molecular sieves were added. This solution was allowed to stand for at least 3 h hour prior to the coupling reaction.

δ-(4-Methyl-7-coumarinyl) adenosine 5′-tetraphosphate tetraammonium salt (6). Method A (Scheme 3). To a mixture of **2** (0.405 mmol, 390 mg) in acetonitrile (4.5 mL) and *N*-methylimidazole (0.12 mmol, 97 μL) was added mesitylenesulfonyl chloride (MS-Cl, 0.36 mmol, 80 mg) at room temperature, and the reaction mixture was stirred for 15 min. This solution was withdrawn via syringe and injected dropwise over 1 min into a cooled flask (ice bath) containing tetrabutylammonium salt of AMP (0.27 mmol, 206 mg) in DMF (3 mL). 7-Hydroxy-4-methylcoumarin (0.351 mmol, 62.0 mg) and DABCO (0.81 mmol, 91 mg) were added to the reaction flask followed by anhydrous magnesium chloride (0.315 mmol, 30 mg). The ice bath was then removed, and the reaction mixture was stirred at room temperature for 5 h. The reaction mixture was then cooled in ice and quenched with 100 mM triethylammonium acetate buffer (pH 7.0, 6 mL) and then washed with chloroform (3 × 10 mL). Chelex resin (ca. 0.1 g) was added, and the mixture stirred for 1 min and then filtered through a cotton plug. The filtrate was purified by reversed-phase column chromatography using a gradient of 75% buffer A (10 mM tributylamine-30 mM acetic acid), 25% buffer B (methanol, 15 mM tributylamine) to 40% buffer A, 60% buffer B over 55 min. The flow rate was 25 mL/min and monitored at 265 and 280 nm (t_R = 40 min). Fractions containing the desired product were pooled and concentrated by high vacuum rotary evaporation, and the residue was dissolved in water and repeatedly freeze-dried until the ^1H NMR spectrum indicated that no residual buffer was present (four times). The resulting white powder was converted to its ammonium salt using a Dowex-50-W ion-exchange resin in NH_4^+ form to afford after lyophilization, 99 mg (45%) of the tetraammonium salt of compound **6**: ^1H NMR (D_2O, 300 MHz) δ 2.10 (s, 3H, CH), 4.14 (s, 2H), 4.19 (s, 1H),4.35 (s, 1H),4.46 (t, *J* = 4.5 Hz, 1H), 5.75 (d, *J* = 4.3 Hz, 1H), 5.89 (s, 1H), 6.8 (s, 1H), 6.92 (d, *J* = 8.6 Hz, 1H), 7.27 (d, *J* = 8.6 Hz, 1H), 7.88 (s, 1H), 8.15 (s, 1H); ^{13}C NMR (D_2O, 75 MHz) δ 17.8, 65.0 (d, *J* = 5.2 Hz), 70.1, 74.5, 83.5 (d, *J* = 9.2 Hz), 86.9, 107.9, 108.0, 111.5, 115.6, 117.4 (d, *J* = 4.5 Hz), 117.6, 125.9, 140.0, 147.8, 149.8, 152.8, 154.3 (d, *J* = 6.6 Hz), 155.5, 163.7; ^{31}P NMR (D_2O, 121 MHz) δ −9.02 (d, *J* = 16.5 Hz), −14.52 (d, *J* = 14.4 Hz), −21.02 (m); HRMS (ESI−) *m*/*z* = 743.99319, $C_{20}H_{22}O_{18}N_5P_4$ $[M - H]^-$, requires 743.99158.

General Method for the Preparation of δ-(4-Methyl-7-coumarinyl) nucleoside 5′-tetraphosphate tetraammonium salts (6−10), δ-(3*H*-Phenoxazin-3-one-7-yl) adenosine 5′-tetraphosphate tetraammonium salt (12), δ-(6′-Methoxy-3*H*-spiro[2-benzofuran-1,9′-xanthen]-3′-yl) adenosine 5′-tetraphosphate tetraammonium salt (13), and δ-(6′-Hydroxy-3*H*-spiro[2-benzofuran-1,9′-xanthen]-3′-yl) adenosine 5′-tetraphosphate tetraammonium salt (14). Method B (Schemes 4 and 5). To a mixture of **2** (0.54 mmol, 520 mg) and DABCO (1.62 mmol, 180 mg for compounds **6**−**12**, 1.08 mmol, 121 mg for compound **13**, 1.89 mmol, 212 mg for compound **14**) in dry acetonitrile (6 mL) was added MS-Cl (0.48 mmol, 106 mg for compounds **6**−**10**, **12**, and 0.54 mmol, 118 mg for compounds **13** and **14**). The mixture was stirred at room temperature for 1 min, and then 7-hydroxy-4-methylcoumarin (HMC), resorufin, 3′-*O*-methylfluorescein, or fluorescein (0.27 mmol, 48, 58, 93, and 90 mg, respectively) were added, and the reaction mixture was allowed to stir at room temperature for 2 h (6 h in case of **13**). For compounds **6**−**10**, a white turbidity appeared after about 45 min. For compound **12**, the turbid solution was orange-red, and for compounds **13** and **14** it was yellowish-brown. The reaction mixture was then withdrawn by a syringe and injected dropwise over 1 min into a cooled (ice bath) solution of NMP or 2′-dNMP (0.675 mmol) and anhydrous magnesium chloride (0.42 mmol, 40 mg) in DMF (7.5 mL). The ice bath was removed, and the reaction mixture was allowed to stir at room temperature for 3 or 15 h in cases of **12**−**14**. The turbidity disappears except when using 2′-dCMP or 2′-dGMP. The reaction

dx.doi.org/10.1021/jo500051y | J. Org. Chem. 2014, 79, 2308−2313

mixture was then cooled in an ice bath and quenched by adding triethylammonium acetate buffer (pH 7.0, 8 mL). The solution was washed with chloroform (3 × 10 mL). Chelex resin (ca. 0.1 g) was added, and the mixture was stirred for 1 min and then filtered through a cotton plug. The filtrate was purified by reversed-phase chromatography using a linear gradient of 75% buffer A (10 mM tributylamine-30 mM acetic acid), 25% buffer B (15 mM tributylamine in MeOH) to 40% buffer A, 60% buffer B over 55 min, except for compound **13**, where a linear gradient of 60% buffer A, 40% buffer B to 30% buffer A, 70% buffer B over 55 min was used. The flow rate was 25 mL/min, and eluate was monitored at 265 and 280 nm. Fractions containing the desired product were pooled and concentrated by high vacuum rotary evaporation, and the residue was dissolved in water and repeatedly freeze-dried until the ^{1}H NMR spectrum indicated that no residual buffer was present (four times). The resulting white powder (except for compound **12**, which was a reddish brown powder) was converted to its ammonium salt using a Dowex-50-W ion-exchange resin in NH_4^+ form.

δ-(4-Methyl-7-coumarinyl) adenosine 5′-tetraphosphate, tetraammonium salt (6). Obtained in 80% yield (176 mg). Characterization data were identical to that reported above using method A.

δ-(4-Methyl-7-coumarinyl) 2′-deoxyadenosine 5′-tetraphosphate, tetraammonium salt (7). Obtained in 80% yield (172 mg): t_R = 32 min; ^{1}H NMR (D_2O, 300 MHz) δ 2.05 (s, 3H), 2.31−2.51 (m, 2H), 4.01−4.08 (m, 3H), 4.56 (s, 1H), 5.83 (s, 1H), 6.05 (t, *J* = 6.8 Hz, 1H), 6.82 (s, 1H), 6.94 (dd, *J* = 9.0, 1.8 Hz, 1H), 7.25 (d, *J* = 9.0 Hz, 1H),7.79 (s, 1H), 8.07 (s, 1H); ^{13}C NMR (D_2O, 75 MHz) δ 17.8, 39.2, 65.3 (d, *J* = 5.4 Hz), 71.0, 83.5, 85.4 (d, *J* = 8.9 Hz), 107. 8, 107.9, 111.4, 115.5, 117.4 (d, *J* = 4.5 Hz), 125.9, 139.6, 147.4, 150.5, 152.6, 153.4, 154.2 (d, *J* = 6.8 Hz), 155.3, 163.5; ^{31}P NMR (D_2O, 121 MHz) δ −9.06 (d, *J* = 15.7 Hz), −14.77 (d, *J* = 12.8 Hz), −21.02 (s); HRMS (ESI−) *m*/*z* = 727.99745, $C_{20}H_{22}O_{17}N_5P_4$ $[M - H]^-$, requires 727.99666.

δ-(4-Methyl-7-coumarinyl) 2′-deoxy-cytosine 5′-tetraphosphate, tetraammonium salt (8). Obtained in 81% yield (169 mg): t_R = 31 min; ^{1}H NMR (D_2O, 300 MHz) δ 2.01 (m, 1H), 2.18 (m, 1H), 2.24 (s, 3H), 3.98 (br, 3H), 4.38 (s, 1H), 5.88 (br, 2H), 6.06 (s, 1H), 7.06 (br, 2H), 7.52 (d, *J* = 7.6 Hz, 1H), 7.70 (d, *J* = 6.0 Hz, 1H); ^{13}C NMR (D_2O, 75 MHz) δ 17.9, 39.4, 65.1 (d, *J* = 5.2 Hz), 70.5, 85.6 (d, *J* = 9.3 Hz), 86.0, 95.4, 108.2 (d, *J* = 5.1 Hz), 111.8, 116.1, 117.6 (d, *J* = 5.0 Hz), 126.4, 142.8, 150.6, 153.2, 154.6 (d, *J* = 6.8 Hz), 155.8, 160.6, 164.0; ^{31}P NMR (D_2O, 121 MHz) δ −8.96 (d, *J* = 9.8 Hz), −14.77 (d, *J* = 10.6 Hz), −20.94 (s); HRMS (ESI−) *m*/*z* = 703.98597, $C_{19}H_{22}O_{18}N_3P_4$ $[M - H]^-$, requires 703.98543.

δ-(4-Methyl-7-coumarinyl) 2′-deoxythymidine 5′-tetraphosphate, tetraammonium salt (9). Obtained in 82% yield (174 mg): t_R = 34 min; ^{1}H NMR (D_2O, 300 MHz) δ 1.64 (s, 3H), 2.07−2.13 (m, 2H), 2.27 (s, 3H), 3.96 (s, 1H), 4.04 (brs, 2H), 4.43 (brs, 1H), 6.01 (t, 1H), 6.09 (s, 1H), 7.12 (br, 2H), 7.38 (s, 1H), 7.55 (d, *J* = 6.0 Hz, 1H); ^{13}C NMR (D_2O, 75 MHz) δ 11.5, 17.8, 39.5, 65.3 (d, *J* = 5.5 Hz), 70.7, 84.6 85.0 (d, *J* = 9.2 Hz), 108.1 (d, *J* = 5.2 Hz), 111.2, 111.7, 116.0, 117.6 (d, *J* = 5.0 Hz), 126.3, 136.8, 151.1, 153.2, 154.6 (d, *J* = 6.9 Hz), 155.8, 163.9, 165.8; ^{31}P NMR (D_2O, 121 MHz) δ −9.26 (d, *J* = 15.9 Hz), −14.78 (d, *J* = 16.3 Hz), −21.09 (s); HRMS (ESI−) *m*/*z* = 718.98522, $C_{20}H_{23}O_{19}N_2P_4$ $[M - H]^-$, requires 718.98510.

δ-(4-Methyl-7-coumarinyl) 2′-deoxy-guanosine 5′-tetraphosphate tetraammonium salt (10). Obtained in 82% yield (180 mg): t_R = 36 min; ^{1}H NMR (D_2O, 300 MHz) δ 2.10 (s, 3H), 2.34 (m, 1H), 2.42 (m, 1H), 4.06 (m, 3H), 4.54 (s, 1H), 5.90 (brs, 2H), 6.90 (s, 1H), 7.00 (d, *J* = 8.6 Hz, 1H), 7.33 (d, *J* = 8.6 Hz, 1H), 7.99 (s, 1H); ^{13}C NMR (D_2O, 75 MHz) δ 17.7, 39.0, 65.2 (d, *J* = 5.4 Hz), 70.8, 83.9, 85.4 (d, *J* = 9.0 Hz), 107. Eight (d, *J* = 5.5 Hz), 111.4, 113.0, 117.3 (d, *J* = 4.5 Hz), 126.0, 136.3, 149.7, 152.8, 153.5, 154.3 (d, *J* = 6.8 Hz), 155.4, 156.6, 163.5; ^{31}P NMR (D_2O, 121 MHz) δ −9.05 (d, *J* = 15.3 Hz), −14.77 (d, *J* = 13.9 Hz), −21.03 (s); HRMS (ESI−) *m*/*z* = 743.99260, $C_{20}H_{22}O_{18}N_5P_4$ $[M - H]^-$, requires 743.99158.

δ-(3*H*-Phenoxazin-3-one-7-yl) adenosine 5′-tetraphosphate tetraammonium salts (12). Obtained in 80% yield (184 mg): t_R = 43 min; ^{1}H NMR (D_2O, 300 MHz) δ 4.14 (br, 3H), 4.37 (brs, 1H), 4.42 (t, *J* = 5.0 Hz, 1 H), 5.75 (*J* = 5.0 Hz, 1 H), 6.16 (s, 1H), 6.96 (d, *J* = 9.7 Hz, 1 H 1H), 7.15 (s, 1H), 7.24 (t, *J* = 10.0 Hz, 2H), 7.52 (d, *J* = 8.9 Hz, 1H), 7.88 (s, 1H), 8.08 (s, 1H); ^{13}C NMR (D_2O, 75 MHz) δ 65.1 (d, *J* = 5.4 Hz), 70.2, 74.8, 83.5 (d, *J* = 8.7 Hz), 86.8, 105.6, 107.8 (d, *J* = 5.4 Hz), 117.9, 119.2 (d, *J* = 4.3 Hz), 129.2 130.7, 133.6, 134.4, 139.4, 144.6, 145.2, 148.2, 150.3, 151.8 154.3, 156.2 (d, *J* = 7.6 Hz), 188.2; ^{31}P NMR (D_2O, 121 MHz) δ −8.77 (d, *J* = 10.9 Hz), −14.72 (d, *J* = 14.2 Hz), −20.61 (s); HRMS (ESI−) *m*/*z* = 780.98726, $C_{22}H_{21}O_{18}N_6P_4$ $[M - H]^-$, requires 780.98683.

δ-(6′-Methoxy-3*H*-spiro[2-benzofuran-1,9′-xanthen]-3′-yl) adenosine 5′-tetraphosphate tetraammonium salts (13). Obtained as a mixture of diastereomers in 74% yield (196 mg): t_R = 38 min; ^{1}H NMR (D_2O, 300 MHz) δ 3.53 (s, 1H), 4.07 (br, 3H), 4.35 (s, 2H), 5.73 (dd, *J* = 3.7, 3.6 Hz, 1H), 6.10 (brs, 2H), 6.46 (s, 1H), 6.57 (brm, 1H), 6.67 (brs, 1H), 6.83 (d, *J* = 8.1 Hz, 1H), 7.07 (s, 1H), 7.39 (brt, *J* = 8.4 Hz, 3H), 7.79 (d, *J* = 5.7 Hz, 1H), 7.90 (s, 1H), 8.26 (s, 1H); ^{13}C NMR (D_2O, 75 MHz) δ. 55.5, 65.0, 70.0 (d, *J* = 4.2 Hz), 74.9, 83.5 (overlapping doublets), 84.1 (d, *J* = 4.6 Hz), 87.4, 100.69, 100.75, 108.8, 109.8, 111.2, 113.8, 116.6, 117.9, 123.5, 124.8, 124.9, 125.0, 128.3, 128.8, 128.9, 130.2, 135.9, 140.5, 147.9, 148.3, 151.3, 151.5, 152.1, 152.2, 152.3, 153.5 (d, *J* = 6.5 Hz), 160.7, 171.56, 171.63; ^{31}P NMR (D_2O, 121 MHz) δ −9.05 (d, *J* = 15.2 Hz), −14.67 (d, *J* = 15.5 Hz), −20.95 (s); HRMS (ESI−) *m*/*z* = 914.02907, $C_{31}H_{28}O_{20}N_5P_4$ $[M - H]^-$, requires 914.02836.

δ-(6′-Hydroxy-3*H*-spiro[2-benzofuran-1,9′-xanthen]-3′-yl) adenosine 5′-tetraphosphate tetraammonium salts (14). Obtained as mixture of isomers (lactone and free acid forms) in 68% yield (178 mg): t_R = 47 min; ^{1}H NMR (D_2O, 300 MHz) δ 4.09 (s, 3H), 4.29 (s, 2H), 5.76 (brd, 1H), 6.28 (m, 3H), 6.56 (m, 1H), 6.76 (brs, 2H), 6.99 (d, *J* = 9.0 Hz, 1H), 749 (brs, 2H), 7.82 (brs, 1H), 7.95 (s, 1H), 8.24 (d, *J* = 5.9 Hz, 1H); ^{13}C NMR (D_2O, 75 MHz) δ 64.8, 69.8, 74.9, 83.3 (overlapping doublets), 87.6, 102.4, 108.4, 109.6, 113.1, 113.2, 113.8, 113.9, 116.7, 118.0, 124.0, 125.0, 125.06, 125.7, 125.8, 128.9, 129.9, 130.1, 135.6, 140.8, 147.3, 147.6, 151.0, 151.2, 151.3. 151.85, 151.94, 153.48, 153.53, 159.4, 159.6, 171.9, 172.0; ^{31}P NMR (D_2O, 121 MHz) δ −8.80 (d, *J* = 14.3 Hz), −14.08 (d, *J* = 12.8 Hz), −20.66 (s); HRMS (ESI−) *m*/*z* = 900.01384, $C_{30}H_{26}O_{20}N_5P_4$ $[M - H]^-$, requires 900.01271.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Figures S1−S5. Copies of ^{1}H, ^{13}C, and ^{31}P NMR spectra for compounds **6**−**10** and **12**−**14**. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: s5taylor@sciborg.uwaterloo.ca.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

S.D.T. is grateful to the Natural Sciences and Engineering Research Council (NSERC) of Canada for support of this work through a Discovery Grant.

■ REFERENCES

(1) Ankilova, V. N.; Knorre, D.G .; Kravchenko, V. V.; Lavrik, O. I.; Nevinsky, G. A. *FEBS Lett.* **1975**, *60*, 172.

(2) Yarbrough, L. R. *Biochem. Biophys. Res. Commun.* **1978**, *81*, 35.

(3) Grachev, M. A.; Zaychikov, E. F. *FEBS Lett.* **1974**, *49*, 163.

(4) Sood, A.; Kumar, S.; Nampalli, S.; Nelson, J. R.; Macklin, J.; Fuller, C. W. *J. Am. Chem. Soc.* **2005**, *127*, 2394.

(5) Kumar, S.; Sood, A.; Wegener, J.; Finn, P. J.; Nampalli, S.; Nelson, J. R.; Sekher, A.; Mitsis, P.; Macklin, J.; Fuller, C. W. *Nucleosides, Nucleotides Nucleic Acids* **2005**, *24*, 401.

(6) Korlach, J.; Bibillo, A.; Wegener, J.; Peluso, P.; Pham, T. T.; Park, I.; ClarK, S.; Otto, G. A.; Turner, S. W. *Nucleosides, Nucleotides Nucleic Acids* **2008**, *27*, 1072.
(7) Eid, J.; Fehr, A.; Gray, J.; Luong, K.; Lyle, J.; Otto, G.; Peluso, P.; Rank, D.; Baybayan, P.; Bettman, B. *Science* **2009**, *323*, 133.
(8) Sims, P. A.; Greenleaf, W. J.; Duan, H.; Xie, S. *Nat. Methods* **2011**, *8*, 575.
(9) Specific yields for any of these routes were not reported.
(10) Fukuoka, K.; Suda, F.; Ishikawa, M.; Hata, T. *Nucleosides Nucleotides* **1995**, *14*, 693.
(11) Warnecke, S.; Meier, C. *J. Org. Chem.* **2009**, *74*, 3024.
(12) Han, Q.; Gaffney, B. L.; Jones, R. A. *Org. Lett.* **2006**, *8*, 2075.
(13) Ahmadibeni, Y.; Parang, K. *Org. Lett.* **2007**, *9*, 4483.
(14) (a) Theoclitou, M. E.; El-Thaher, T. S. H.; Miller, A. D. J. *J. Chem. Soc., Chem. Commun.* **1994**, *5*, 659. (b) Theoclitou, M. E.; Wittung, E. P. L.; Hindley, A. D.; El-Thaher, T. S. H.; Miller, A. D. J. *J. Chem. Soc., Perkin Trans. 1* **1996**, *16*, 2009. (c) Huang, K.; Frey, P. A. *J. Am. Chem. Soc.* **2004**, *126*, 9548.
(15) Ludwig, J.; Eckstein, F. *J. Org. Chem.* **1989**, *54*, 631.
(16) Mohamady, S.; Taylor, S. D. *Org. Lett.* **2013**, *15*, 2612.
(17) Inoue, H.; Baba, Y.; Miyajima, T.; TsuHako, M. *Chem. Pharm. Bull.* **1992**, *40*, 3127.
(18) Zaikova, T.; Rukavishnikov, A. V.; Birrell, B.; Griffith, O. H.; Keana, J. F. W. *Bioconjugate Chem.* **2001**, *12*, 307.
(19) Mugherli, L.; Burchak, O. N.; Chatelain, F.; Balakirev, M. Y. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 4488.
(20) 2′-dTMP was purchased as its sodium salt and was converted into its free acid using a Dowex-50W ion exchange column (H^+ form) column before conversion to its tetrabutylammonium salt. 2′-AMP, 2′-dAMP, and 2′-dCMP were purchased as their monoacid hydrates.
(21) Gibson, K. J.; Leonard, N. J. *Biochemistry* **1984**, *23*, 78.

dx.doi.org/10.1021/jo500051y | *J. Org. Chem.* 2014, 79, 2308−2313