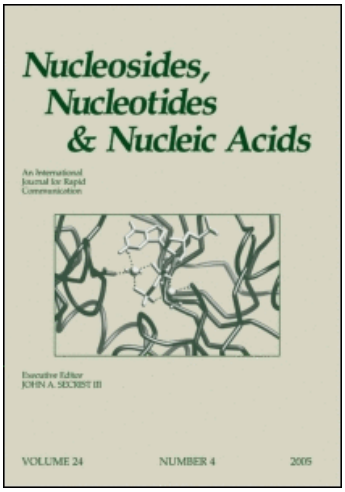

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FACILE SMALL SCALE SYNTHESIS OF NUCLEOSIDE 5′-PHOSPHATE MIXTURES

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□ *We present a facile method to phosphorylate small amounts of nucleosides (0.05 μmol) into mixtures of their 5′-mono-, di-, and triphosphates in a one-pot reaction. The nucleosides were first converted into their dichlorophosphates using a large excess (15–18 equivalents) of phosphorous oxychloride in trimethylphosphate. The large excess resulted in good dichlorophosphate yields (46–76%) for the four nucleosides tested. Upon the addition of tributylammonium-phosphate with additional tributylamine (20 equivalents both), the dichlorophosphate was converted into a mixture containing equal amounts of the mono-, di-, and triphosphate. The presented method was successfully applied to synthesize mixtures of stable isotope labeled nucleotides, which can be used as internal standards in quantitative mass spectrometric assays.*

Keywords 5′-Triphosphates; nucleoside analogs; bioanalysis and mass spectrometry

INTRODUCTION

Nucleoside analogs are used in anti-cancer, anti-(retro)viral, and immunosuppressive therapies. The analogs are phosphorylated to nucleoside mono-, di-, and triphosphates intracellularly. The triphosphate form inhibits human and viral polymerases and reverse transcriptases and is incorporated into nucleic acids. Although the mono- and diphosphates are not the main active metabolites, they can possess pharmacological activity as well.[1,2]

Monitoring of the intracellular concentration of these metabolites is pivotal in understanding the pharmacology and toxicology of nucleoside

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analogs.[3] In the past decade, liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) has become the method of choice to analyze nucleotide analogs in cells.[4] For optimal performance of LC-MS/MS assays, an internal standard should be used to correct for interferences caused by other sample constituents. Stable isotope labeled analytes are the ideal internal standards because they have identical chemical properties, but their mass difference can be distinguished in the mass spectrometer.[5]

The availability of stable isotope labeled nucleotide analogs is, however, limited. Many of their nonphosphorylated, stable isotope labeled nucleosides, on the other hand, are commercially available. We, therefore, explored the possibility to phosphorylate small quantities of nucleosides to a mixture of their mono-, di-, and triphosphate to serve as internal standards for LC-MS/MS analysis.

Many methods have been developed to synthesize nucleoside triphosphates, as reviewed by Burgess and Cook.[6] Most of these methods first convert the nucleoside into an activated monophosphate, such as a dichlorophosphate or phosphoramidate. A commonly used phosphytilating agent is phosphorous oxychloride ($POCl_3$). When $POCl_3$ is used in a trialkylphosphate solution it results in selective phosphorylation of the 5′-hydroxyl group of the nucleoside.[7,8]

Addition of tributylammonium(TBA)-pyrophosphate to the thus formed nucleoside dichlorophosphate results in the formation of the nucleoside 5′-triphosphate.[9] Reaction with water, on the other hand, deactivates the dichlorophosphate intermediate resulting in the formation of a nucleoside 5′-monophosphate.

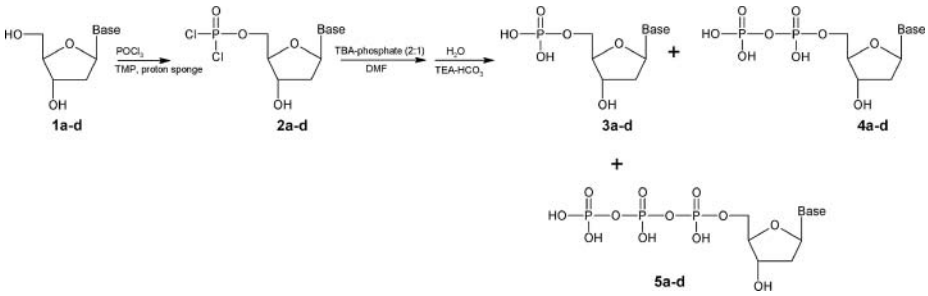
In attempt to synthesize the 5′-diphosphate, Mishra and Broom added TBA-phosphate to the nucleoside dichlorophosphate.[10] To their surprise, the 5′-triphosphate was formed instead of the 5′-diphosphate. Similarly, Hoffmann et al. added crystalline phosphoric acid in combination with different organic bases.[11] Depending on the organic base added, they obtained mixtures of 5′-mono-, di-, and triphosphates in different ratios.

In this article, we describe the optimization of nucleoside phosphorylation with $POCl_3$, and the pitfalls that we encountered. Moreover, the subsequent conversion of the nucleoside dichlorophosphate into mixtures of mono-, di-, and triphosphates is presented (Scheme 1). We applied these reactions to obtain stable isotope labeled nucleotides. These mixtures were successfully applied in quantitative LC-MS/MS analyses.

MATERIALS AND METHODS

Chemicals

2-Chloro-2′-deoxyadenosine (cladribine, 2CdA, **1a**) was obtained from Sequoia Research Products (Pangbourne, UK), 2′, 2′ -difluoro 2′-



SCHEME 1 Reagents and conditions: $POCl_3$ (15–18 equivalents), trimethylphosphate (TMP), tributylammonium(TBA)-phosphate (2:1, mole/mole, 20 equivalents), *N,N*-dimethylformamide (DMF), 1 M triethylammonium-bicarbonate (TEA-HCO_3).

deoxycytidine (gemcitabine, dFdC, **1b**)·HCl and 2′, 2′ -difluoro 2′-deoxyuridine (dFdU, **1c**) were kindly provided by Eli Lilly and company (Indianapolis, IN, USA), whereas 5-aza-2′-deoxycytidine (decitabine, aza-dC, **1d**) was purchased from Sigma (St. Louis, MO, USA). ^{13}C, $^{15}N_2$-labeled dFdC (*dFdC, ***1b**) and dFdU (*dFdU, ***1c**) were obtained from Toronto Research Chemicals (North York, ON, Canada). The structural formulas of the starting nucleosides are presented in Figure 1.

Trimethylphosphate and *N,N*-dimethylformamide (DMF) were from Aldrich (St. Louis, MO, USA), whereas 1 M triethylammonium bicarbonate pH 8.5 and 3 Å molecular sieves were from Fluka (St. Louis, MO, USA). 1,8-Bis(dimethylamino)naphthalene (proton sponge), phosphoric acid crystals, and tributylammonium (TBA)-pyrophosphate (1.5 moles TBA per mole pyrophosphate, (1.5:1, mole/mole)) were bought from Sigma. Phosphorous oxychloride ($POCl_3$) and tributylamine (TBA) were obtained from Fluka and Sigma Aldrich, respectively. All solvents used in the synthesis were purchased as anhydrous and required no further drying.

TBA dihydrogenphosphate, potassium dihydrogen phosphate, 25% ammonia, and 2-propanol were all from Merck (Darmstadt, Germany) and methanol from Biosolve Ltd. (Amsterdam, The Netherlands).

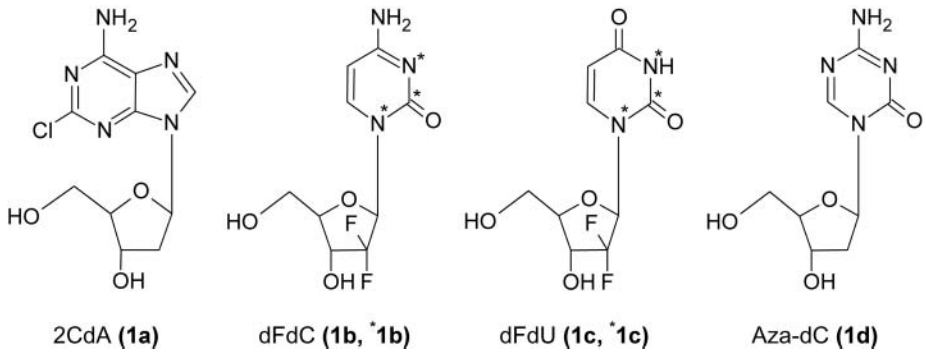


FIGURE 1 Structural formulas of the nucleosides described in the experiments. The asterisks indicate the position of ^{13}C- and ^{15}N-atoms in the stable isotope labelled compounds ***1b** and ***1c**.

TBA-phosphate (1:1. mole/mole) in DMF (250 μM) was prepared by mixing 600 μL TBA with 245 mg phosphoric acid crystals and adding DMF to 10 mL. TBA-phosphate (2:1, mole/mole) was prepared in the same way, but by adding 1200 μL TBA. Water attracted during preparation was removed by overnight storage over 3 Å molecular sieves.

TBA-pyrophosphate (2.4:1, mole/mole) in DMF (250 μM) was prepared by dissolving 0.163 mmol TBA-pyrophosphate (1.5:1, mole/mole) in 620 μL DMF and adding 33 μL TBA.

Monitoring of Reactions

The reactions were monitored using ion-pairing high performance liquid chromatography with ultraviolet detection (HPLC-UV). In-process samples of 10 μL were hydrolyzed in 90 μL 1 M triethylammonium-bicarbonate on ice. The nucleoside dichlorophosphate intermediates **2a–d** were thus converted into nucleoside monophosphates **3a–d**.

HPLC-UV experiments were executed on an Agilent 1100 series liquid chromatograph system (Agilent Technologies, Palo Alto, CA, USA) consisting of a binary pump, an in-line degasser, autosampler, and ultraviolet (UV) detector. Data were acquired using Chromeleon 6.50 software (Dionex Corp., Sunnyvale, CA, USA). The mobile phase consisted of 10 mM tetrabutylammonium dihydrogenphosphate with 70 mM potassium dihydrogen phosphate in methanol-water (16:84, v/v) and was delivered isocratically to a Synergi hydro-RP column (150 × 2.0 mm ID, 4 μm particles; Phenomenex, Torrance, CA, USA) with a flow of 0.25 mL/min. Injections of 1 μL were carried out with the autosampler thermostated at 4°C. Absorption was measured at 268 nm (**1b–5b** and *__1b–*5b__), 264 nm (**1a–5a**), 258 nm (**1c–5c** and *__1c–*5c__), and 243 nm (**1d–5d**).

Alternatively, reactions could be monitored semiquantitatively using thin layer chromatography. Using a capillary, 1 μL of the hydrolyzed in-process samples was spotted onto a HPTLC silica gel F_{254} plate (Merck, Darmstadt, Germany). The reaction products were separated using 2-propanol, 25% ammonia and water (55:35:10, v/v/v). Formation of the nucleoside dichlorophosphate (**2)** was observed under UV light (254 nm) as the appearance of a spot with an R_f lower (~0.7) than that of the starting nucleoside ($R_f \sim 0.9$).

Optimization of the Phosphorylation of Nucleoside Dichlorophosphates

A quantity of 0.05 mmole dFdC·HCl (**1b)** was treated with $POCl_3$ as described in the section "Final Phosphorylation Procedure." After 150 minutes, 150 μL aliquots of the reaction mixture were transferred to

1.5 mL tubes on ice with 240 μL DMF containing TBA-pyrophosphate (2.4:1, mole/mole), TBA-phosphate (1:1, mole/mole), TBA-phosphate (2:1, mole/mole) or no reagent. The reagents were tested in an excess of 5, 10, 15, and 20 equivalents. In-process samples were taken after 1 and 5 minutes, and the reaction was quenched after 10 minutes by the addition of 880 μL 1 M triethylammonium-bicarbonate.

Final Phosphorylation Procedure

The glassware and, if the melting point allowed it, nucleosides, were dried overnight at 85°C. In a 10 mL round flask, 0.05 mmole nucleoside (1 equivalent) was dissolved in 2.5 mL trimethylphosphate together with 0.1 mmol (2 equivalents) proton sponge. dFdC·HCl (**1b)** and aza-dC (**1d)** required gentle heating over a flame to dissolve. $POCl_3$ (0.8 mmol, 16 equivalents) was added dropwise to the stirred solution on ice in about 10 minutes. After addition of all the $POCl_3$, the reaction was continued at room temperature until maximal nucleoside dichlorophosphate **2a–d** was formed (2.5–6.5 hours). Then, the reaction mixture was again cooled on ice, and 4 mL cold 250 μM TBA-phosphate (2:1, mole/mole) in DMF was added under vigorous stirring. After 10 minutes, the reaction mixture was quenched by pouring it into 20 mL 1 M triethylammonium-bicarbonate buffer. Finally, the reaction product was lyophilized overnight to remove the volatile reactants, resulting in a solid white residue.

Purification

Although not required for their use as internal standards, further purification could be performed after reconstitution of the crude lyophilized reaction mixture in water. Volumes of 100 μL product (reconstituted in 500 μL water) were injected onto a Biosep DEAE-PEI column (75 × 7.8 mm ID, 7μm particles; Phenomenex) and separated using an ammonium-bicarbonate gradient (0–900 mM) at 0.5 mL/min. The UV-absorption of the eluate was monitored and the fractions containing the products were lyophilized twice to remove all mobile phase components.

Identification

Identity of the synthesized nucleotides was assessed using weak anion exchange chromatography (**3a** and **3d–5d**) and porous graphitic carbon chromatography (**3b–5b,** *__3b–*__5b, 3c–5c, and *__3c–*__5c**) coupled with tandem mass spectrometric detection (HPLC-MS/MS).[12,13] The identity was confirmed based on retention time and specific mass transitions.

RESULTS

Hydrolyzation Buffer

In our initial experiments with 2CdA, we used 2 equivalents of $POCl_3$, and 2 mL 0.2 M triethylammonium-bicarbonate buffer (4 equivalents) to hydrolyze the nucleoside dichlorophosphate **2a** to the nucleoside monophosphate **3a**. The desired product 2CdA monophosphate (2CdAMP, **3a**) was, however, not observed in the final reaction mixture. We did, on the other hand, mainly observe a peak that we identified as 2-chloro-adenine, the base of 2CdA, Identification was performed based on the retention time on a previously developed HPLV-UV system.[13,14] This base is formed after cleavage of the acid-labile N-glycosidic bond between the sugar and the base of 2CdA (**1a**). Although the final pH was around 8.5 we hypothesized that HCl, formed in the reaction between $POCl_3$ and water, caused local acidity resulting in the cleavage of this glycosidic bond. Indeed, 2-chloro-adenine was no longer found in reaction products that were hydrolyzed with 10 mL 1 M triethylammonium-bicarbonate (200 equivalents). A buffer with a sufficient capacity, thus, prevented acid hydrolysis of the reaction products.

Drying of Nucleosides

Still, the obtained 2CdAMP (**3a)** yields (∼4%) were far below the yields commonly reported for other nucleosides (60–90%).[15] HPLC-UV analysis of the final reaction product showed that it consisted mainly of unreacted nucleoside. Any water present in the nucleoside starting material will react with $POCl_3$ to form HCl and phosphate, thereby inactivating the phosphytilating agent. We argued that traces of water present in the starting material reacted with the $POCl_3$ instead of the nucleosides. We, therefore, 1) dried 2CdA (**1a)** before use (85°C, overnight) and 2) added extra $POCl_3$. Both approaches resulted in a marked increase in nucleoside dichlorophosphate **2a** formation (as shown in Figure 2). Using the dried 2CdA (**1a)** starting material, 2CdAMP (**3a)** was already observed after the addition of 1 $POCl_3$ equivalent. In contrast, similar 2CdAMP (**3a)** formation was only observed after the addition of 3 $POCl_3$ equivalents to the non-dried material. Drying of the nucleoside starting material, thus, improves the phosphorylation reaction. Considerable yields could, however, still be obtained with the non-dried material if sufficient amounts of $POCl_3$ were added.

Excess of Phosphorous Oxychloride

Drying of the nucleosides at elevated temperatures (as described in the section "Final Phosphorylation Procedure") was, however, not feasible for compounds with a low melting point such as dFdU (**1c)** (51–53°C)

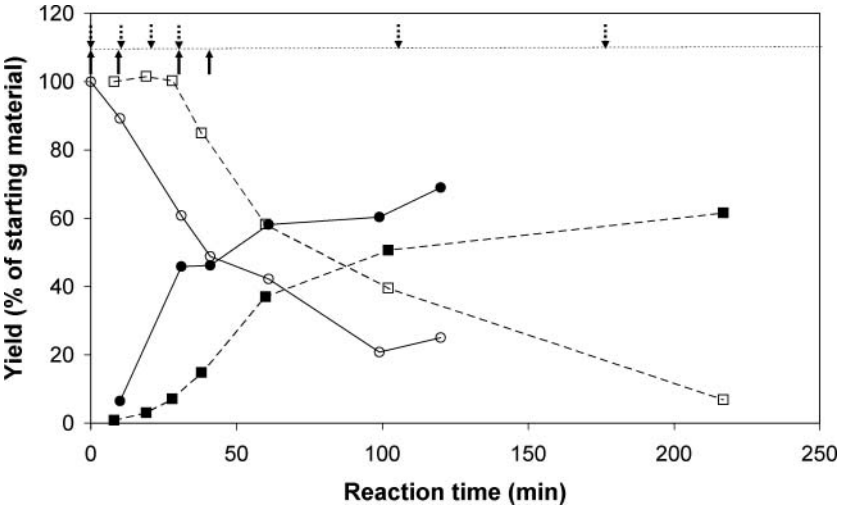


FIGURE 2 Formation of 2CdA dichlorophosphate (**2a**) and the decrease of 2CdA (**1a**) using dried (• and ○, respectively) and non-dried (■ and □, respectively) 2CdA (**1a**) starting material. The arrows represent the addition of $POCl_3$ equivalents to dried (solid arrow) and non-dried (dashed arrow) 2CdA (**1a**).

and nucleosides might still contain water after drying overnight. Moreover, water can originate from solvents, glassware, and the air. Others have dried nucleosides in vacuum, used distilled $POCl_3$, and carried out reactions under nitrogen to perform the reaction under anhydrous conditions.[16,17] We hypothesized that further increasing the $POCl_3$ excess was another, easier approach.

Throughout the literature, $POCl_3$ is used in small excesses (1.5–3 equivalents).[10,18–20] Risbood et al., on the other hand, recently used a very large excess (43 equivalents).[21]

We have increased the excess of $POCl_3$ up to 43 equivalents and found that increasing the amount of $POCl_3$ resulted in improved nucleoside dichlorophosphate **2b** formation. To decrease the risk of interference from unreacted $POCl_3$ in the subsequent reaction step, we used 15–18 $POCl_3$ equivalents in further experiments. The reaction with this excess of $POCl_3$ was monitored for all experiments. The results are shown in Figure 3. The phosphorylation of the same dFdU (**1c**) starting material was repeated and was found to be reproducible (49.7 and 57.3% after 4 hours). The reaction was, on the other hand, dependent on the type of nucleoside and their origin, as illustrated by the difference between dFdC-dFdU (**1b-1c**) and between dFdU-*dFdU (**1c–*1c**) phosphorylation. Still, acceptable nucleoside dichlorophosphate **2b, *2b, 2c, and *2c** yields (46–76%) were obtained if the reaction time was adjusted for each nucleoside. Monitoring of the reaction is, however, indispensable to obtain optimal yields.

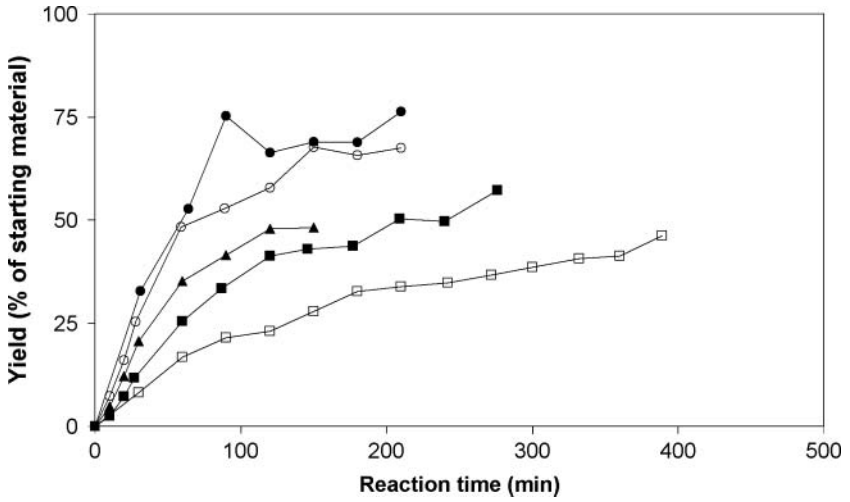


FIGURE 3 Formation of nucleoside dichlorophosphates of dFdC (**2b**) (●), ^{13}C, $^{15}N_2$-dFdC (*__2b__) (○), dFdU (**2c**) (■),^{13}C, $^{15}N_2$-dFdU (*__2c__) (□) and aza-dC (**2d**) (▲) with 15–18 equivalents $POCl_3$ added.

Conversion of Nucleoside Dichlorophosphate to Nucleotide Mixture

Hoffmann et al. showed that the addition of phosphoric acid to a nucleoside dichlorophosphate (**2**) in the presence of an organic base, resulted in the formation of nucleoside mono-, di, and triphosphates (**3–5**) in different ratios.[11] Of the organic bases tested, TBA resulted in the most balanced nucleotide formation. We, therefore, used TBA as organic base in our experiments.

We performed the reaction with several equivalents of TBA-phosphate (1:1, mole/mole and 2:1, mole/mole) and TBA-pyrophosphate (2.4:1, mole/mole). We added extra TBA because Ludwig et al. reported that this increased the yield in a similar triphosphate synthesis.[9]

The reaction mixtures were assayed after 1, 5, and 10 minutes. The reaction time of the second reaction step was, however, of minor importance as the reaction was already complete after 1 minute (data not shown).

The type of reagent and the excess in which it was used were, on the other hand, pivotal to obtain equimolar nucleotide mixtures. The effect of the type of reagent is shown in Figure 4. When no reagent was added the overall yield was the highest, but the product mainly consisted of nucleoside monophosphate (**3b**). The amount of di- and triphosphate (**4b** and **5b**)was higher when TBA-phosphate (1:1, mole/mole) was added and increased even further with extra TBA (2:1, mole/mole). TBA most likely increases the activation for the nucleophilic phosphate attack.[11] With TBA-pyrophosphate (2.4:1, mole/mole), the main product formed

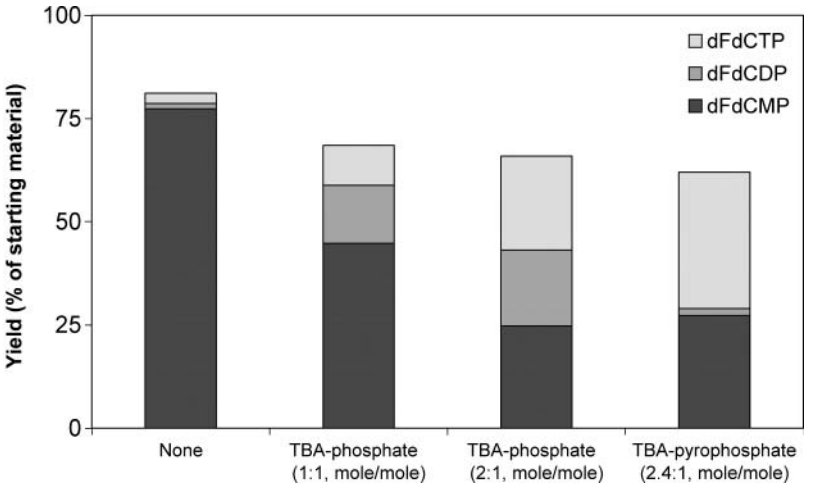


FIGURE 4 Effect of the reagent on the formation of dFdC nucleotides **3b–5b** (20 equivalents, 10 minute reaction).

was the triphosphate **5b**, but the monophosphate **3b** was only slightly less abundant.

All reagents showed an increased formation of higher phosphates when applied in a larger excess. The effect of the excess of TBA-phosphate (2:1, mole/mole) on the formation of the dFdC nucleotides **3b–5b** is depicted in Figure 5.

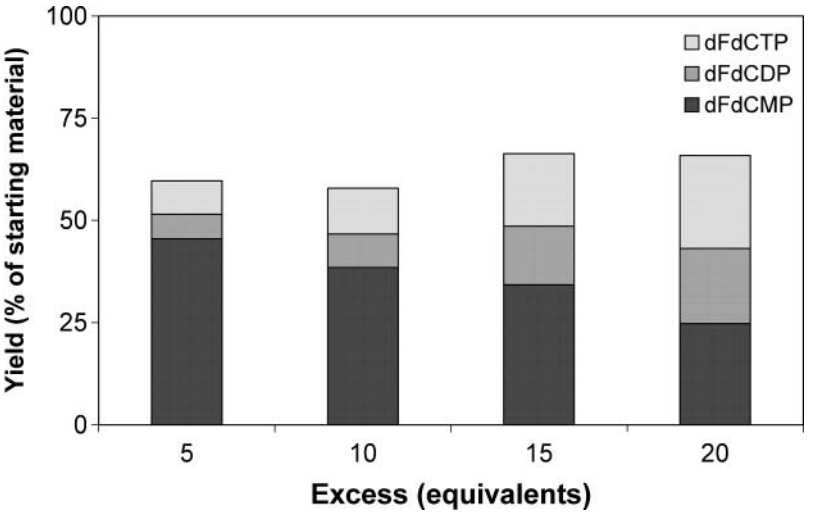


FIGURE 5 Effect of the excess of TBA-phosphate (2:1, mole/mole) on the formation of dFdC nucleotides **3b–5b** (10 minute reaction).

Based on these results, we concluded that 20 equivalents of TBA-phosphate (2:1, mole/mole) were optimal to obtain an equimolar mixture of nucleoside mono- di-, and triphosphates (**3–5**).

Identification

The synthesized 2CdAMP (**3a**) showed LC-MS/MS characteristics identical to 2CdAMP reference material[13] (retention time: 3.75 minutes; mass transition (positive ionization mode): m/z 366 → 170). Using the same chromatographic system, the aza-dC nucleotides (**3d–5d**) were detected (retention time: aza-dCMP: 3.90 minutes, aza-dCDP: 4.77 minutes, aza-dCTP: 5.10 minutes; mass transition (positive ionization mode): aza-dCMP: m/z 309 → 113, aza-dCDP: m/z 389 → 113, aza-dCTP: m/z 469 → 113).

Both the dFdC and dFdU nucleotides (**3b–5b**, ***3b–*5b**, **3c–5c**, and ***3c–*5c**) showed LC-MS/MS characteristics conform to reference compounds and their structure [12] (retention time: dFdCMP/*dFdCMP: 5.73 minutes, dFdCDP/*dFdCDP: 7.99 minutes, dFdCTP/*dFdCTP: 9.12 minutes, dFdUMP/*dFdUMP: 6.89 minutes, dFdUDP/*dFdUDP: 10.22 minutes, dFdUTP/*dFdUTP: 11.67 minutes; mass transitions (positive ionization mode): dFdCMP m/z 344 → 246, *dFdCMP 347 → 249, dFdCDP m/z 424 → 326, *dFdCDP 427 → 329, dFdCTP m/z 504 → 326, *dFdCTP 507 → 329, dFdUMP m/z 345 → 247, *dFdUMP 348 → 250, dFdUDP m/z 425 → 327, *dFdUDP 428 → 330, dFdUTP m/z 505 → 247, *dFdUTP 508 → 250).

Application to an LC-MS/MS Assay

The final method was successfully used to synthesize mixtures of *dFdC- and *dFdU nucleotides (***3b–*5b** and ***3c–*5c**). The reaction mixtures could be used as internal standards for an LC-MS/MS assay without further purification. Figure 6 shows a typical chromatogram of dFdC and *dFdC and their mono-, di-, and triphosphate using porous graphitic carbon chromatography, under previously described conditions.[12] dFdCMP and its internal standard *dFdCMP.

Because the synthesized *dFdC internal standards **(*3b–*5b)** elute at the same time as their unlabeled variants, compounds that interfere with the signal intensity will affect the signal of the dFdC analytes as much as that of the *dFdC analytes. The ratio of the two signals will, therefore, remain constant under the variable conditions in biological matrices, such as number of cells per sample. The impact of the use of an internal standard is shown in Figure 7, with dFdCMP as example. Whereas the signals of dFdCMP and *dFdCMP are lower in samples containing a high number of cells, the ratio of the two signals remains relatively unaffected. At 15 million cells, for example, the use of the internal standard improves the accuracy of the assay result from 68% to 90%.

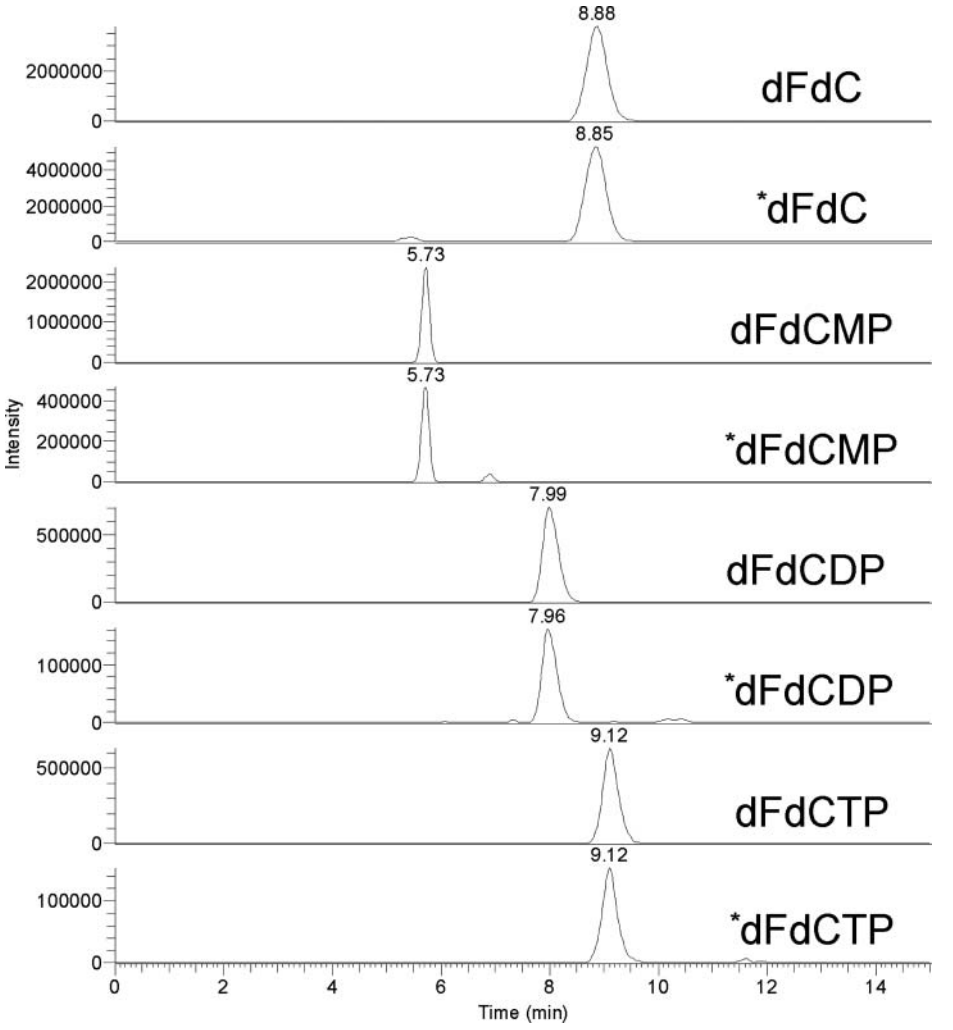


FIGURE 6 HPLC-MS/MS chromatograms of a mixture of dFdC, dFdCMP, dFdCDP, and dFdCTP (**1b, 3b–5b**) and their stable isotope labeled internal standards (*__1b__, *__3b__–*__5b__). Conditions were as previously described,[12] with the following mass transitions: m/z 264 → 112 and 267 → 115 for dFdC and *dFdC, m/z 344 → 246 and 347 → 249 for dFdCMP and *dFdCMP, m/z 424 → 326 and 427 → 329 for dFdCDP and *dFdCDP, and m/z 504 → 326 and 507 → 329 for dFdCTP and *dFdCTP.

We have experienced several problems in the general phosphorylation methods described by others. Likewise, Risbood et al. were not able to synthesize dFdCTP using these methods. They, therefore, first synthesized, isolated, and purified dFdCMP (**3b**), and then used a second reaction to phosphorylate dFdCMP to dFdCTP (**5b**). We found that an $POCl_3$ excess of 15–18 equivalents resulted in increased nucleoside phosphorylation, and that the formed nucleoside dichlorophosphate intermediate could readily be converted into a well balanced mixture of mono-, di-, and triphosphates.

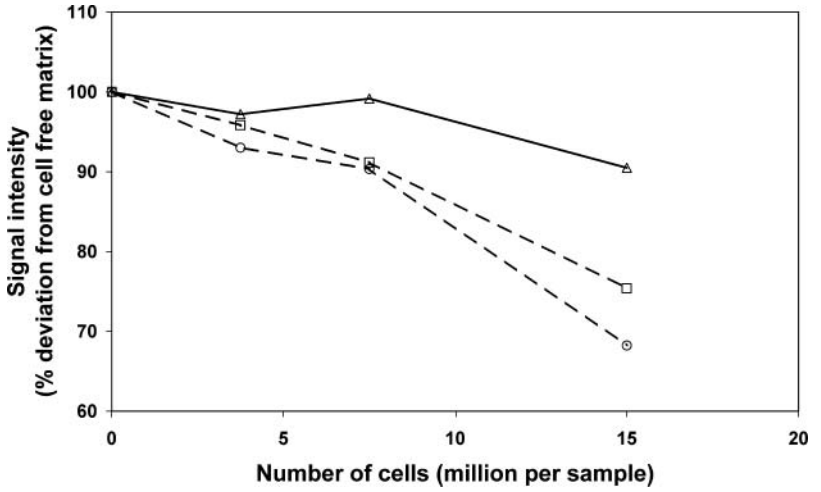


FIGURE 7 Effect of the number of cells on the MS/MS signal intensity of dFdCMP (**3b**) (○, dashed line), *dFdCMP (***3b**) (□, dashed line), and the dFdCMP/*dFdCMP ratio (Δ, solid line).

CONCLUSION

We identified residual water in nucleoside starting material and cleavage of the N-glycosylic bond as pitfalls in the reaction of nucleosides with $POCl_3$. These problems could be avoided by using a large excess of $POCl_3$ and a hydrolyzation buffer with sufficient capacity. By adding TBA-phosphate (2:1, mole/mole), the formed nucleoside dichlorophosphate can subsequently be converted into a well-balanced mixture of the mono-, di-, and triphosphate. This mixture can readily be used as internal standard for quantitative tandem mass spectrometric assays, resulting in improved assay performance.

The described reaction can easily be executed in laboratories with limited experience in organic chemistry, without extensive drying procedures.

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