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A facile and effective synthesis of lamivudine 5′-diphosphate

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ABSTRACT

We report on the first solution synthesis of lamivudine 5′-diphosphate in both high yield and purity. Efficient synthesis of lamivudine 5′-monophosphate was obtained through lamivudine *H*-phosphonate oxidation by (−)-(8,8-dichlorocamphorylsulfonyl)oxaziridine.
Diphosphorylation was performed by nucleophilic substitution of the phosphorimidazolate derivative of lamivudine. HPLC coupled with UV or MS detection was found to be an invaluable tool for the follow-up of phosphorylation reactions.

Non-natural L-nucleoside analogs are increasingly used as therapeutic agents to treat cancer and viral infections.[1] To be active, nucleoside analogs need to be stepwise phosphorylated to their respective 5′-triphosphates by human enzymes. This stepwise phosphorylation relies on human enzymes ability to process L-nucleoside enantiomers. As part of our research program on the molecular interaction of L-nucleotides with nucleotide kinases,[2] we focused our attention on lamivudine 5′-diphosphate, a substrate of human 3-phosphoglycerate kinase[3] and thus a valuable tool for investigating the mechanism of this enzyme. Lamivudine (3TC, Epivir®, Zeffix®), chemically known as (−)-[2*R*,5*S*]-4-amino-1-[2-(hydroxyl-methyl)-1,3-oxathiolan-5-yl]-2(1*H*)-pyrimidin-2-one, is currently used for the treatment of human immunodeficiency types 1 and 2 and hepatitis B viral infections.[4] This L-nucleoside bearing an oxathiolane ring in place of the furanose ring,[5] acts as a chain terminator after its intracellular phosphorylation to lamivudine 5′-triphosphate.

The objective of the present study was to develop a synthetic method that could provide lamivudine 5′-diphosphate (3TCDP) with high purity and good yields, taking into account the acid- and base-sensitive oxathiolane ring.[6]

The Literature survey reveals a few methods for the synthesis of nucleoside 5′-diphosphates. Among these, two multi-step procedures are most widely used: (i) synthesis involving direct displacement of a 5′-*O*-tosyl group with tris(tetra-*n*-butylammonium) pyrophosphate;[7] (ii) synthesis via nucleophilic attack of tri-*n*-butylammonium phosphate on an activated nucleoside 5′-monophosphate, usually a phosphorimidazolate.[8]

Our initial attempts to synthesize lamivudine 5′-diphosphate via the tosyl approach were not satisfactory since low yields of tosylation were obtained. Moreover, this approach was not convenient due to the requirement of an additional step, that is, protection of the nucleobase. On the other hand, the phosphorimidazolate methodology was successfully applied (Scheme 1). This synthetic route required prior synthesis of lamivudine 5′-monophosphate **1** (3TCMP).

Yoshikawa's method is widely used for the synthesis of nucleoside 5′-monophosphates.[9] It relies on the selective phosphorylation of the 5′-hydroxyl group of nucleosides by phosphorus oxychloride ($POCl_3$) in trialkylphosphate. Treatment of nucleoside 5′-dichlorophosphate intermediates with triethylammonium bicarbonate buffer affords the corresponding 5′-monophosphorylated derivatives. Conversely, addition of tri-*n*-butylammonium phosphate with additional tributylamine to the intermediate results in the formation of a mixture of the nucleoside 5′-mono, 5′-di and 5′-triphosphates.[10] Throughout the literature $POCl_3$ is used in small excesses (1.5–3 equiv). Risbood et al., on the other hand, recently used a very large excess (43 equiv) of $POCl_3$ in the synthesis of gemcitabine 5′-triphosphate.[11]

In order to optimize the experimental conditions for 3TCMP synthesis, we investigated the dependence of the following parameters on the product yield: equivalent number of $POCl_3$, order of reagent addition, and reaction time. The progress of the phosphorylation reaction was monitored by HPLC coupled with Photo Diode Array detector, using a porous graphitic carbon column.[12]

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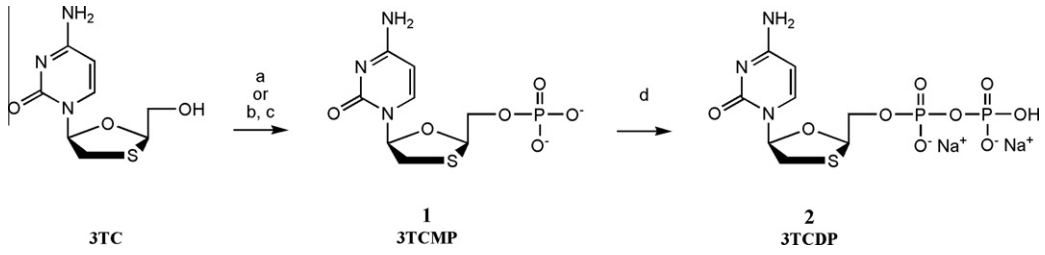


Scheme 1. Reagent conditions: (a) (i) $POCl_3$, $(EtO)_3PO$; (ii) TEAB 1 M pH 7.5; (b) (i) diphenyl *H*-phosphonate; (ii) NEt_3/H_2O 1/1 v/v; (c) (i) BSA/DMF; (ii) (−)-(8,8-dichlorocamphorylsulfonyl)oxaziridine then methanol/DBU 97/3 v/v; (d) (i) nBu_3N, DMF; (ii) CDI, (iii) CH_3OH; (iv) tri-*n*-butylammonium phosphate, DMF; (v) DEAE-Sephadex A25, RP-18, Dowex-Na^+.

Lamivudine in triethylphosphate was first reacted at 0 °C with 2–20.0 equiv of freshly distilled phosphorus oxychloride.[13] As shown in Figure 1a, the nucleoside was poorly converted to the corresponding 5′-monophosphate when a slight excess of reagent was used. The formation of cytosine, resulting from deglycosylation, was also observed. Increasing the equivalent number of phosphorus oxychloride improved both phosphorylation rate and yield. The best results were obtained using 10 equiv of reagent (Fig. 1b). Under these conditions, the conversion was maximal after 1 h of reaction. After this time, the amount of 3TCMP decreased rapidly together with cytosine increase. The substrate was almost completely consumed after 2 h. Increasing the amount of $POCl_3$ up to 20 equiv did not improve the results. Addition of 1,8-bis(dimethylamino)naphthalene, also known as proton sponge, has been reported to mitigate side-reactions promoted by the acidity of the reaction medium and thus increase the yield.[14] However, neither addition of proton sponge, nor a change in the order of addition of the reagents, did improve phosphorylation. Implementation of the optimized protocol depicted in Figure 1b to larger quantities of substrate was performed. Under these conditions, lamivudine 5′-monophosphate could not be purified by standard ion-exchange chromatography on DEAE-Sephadex since inorganic phosphate co-eluted with the nucleotide. The purification was thus performed on preparative TLC after exchanging the triethylammonium counterions with sodium ions. Workup of the reaction afforded the monophosphate **1** in 41–52% isolated yield. This low yield combined with fastidious purification lead us to consider an alternative synthetic approach based on *H*-phosphonate oxidation.

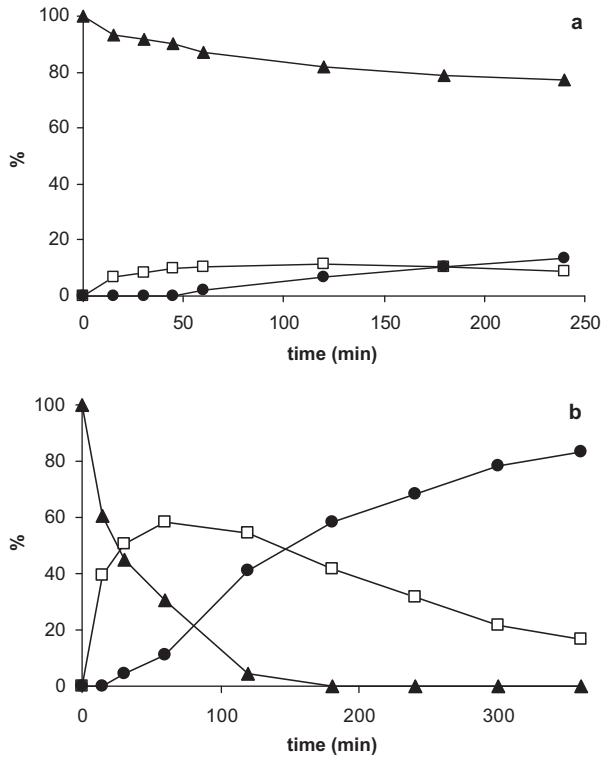


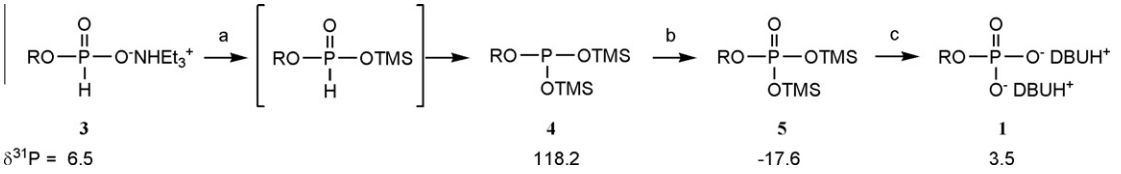
Figure 1. Kinetics of phosphorylation using (a) 1 equiv $POCl_3$; (b) 10 equiv $POCl_3$; ▲, 3TC; □, 3TCMP; ●, cytosine.

A versatile approach for introducing modifications at the phosphorus center is oxidation of phosphorus(III) precursors such as *H*-phosphonate derivatives.[15] Indeed, *H*-phosphonate monoesters can be used as substrates for oxidative transformations to provide a variety of phosphate monoester analogs.

Firstly, the nucleoside 5′-*H*-phosphonate monoester **3** (Scheme 2) was prepared through transesterification of diphenyl H-phosphonate with lamivudine in pyridine, according to the literature.[16] To this purpose, 7 equiv of diphenyl H-phosphonate were allowed to react with 3TC for 30 min in pyridine at room temperature. Addition of water/NEt_3 1/1 (v/v) to the reaction mixture resulted in rapid hydrolysis of the nucleoside phenyl *H*-phosphonate diester to **3**. The isolated yield was 95%.[17]

Since *H*-phosphonate monoesters are more resistant to oxidation than the corresponding H-phosphonate diesters, these compounds need to be converted into trivalent silyl phosphite prior to the reaction to facilitate the oxidative transformations.[15] We performed this oxidation step using mild conditions.[18] The progress of the reaction was monitored by HPLC-UV and also by ^{31}P NMR. Scheme 2 depicts the reaction pathway along with the ^{31}P NMR chemical shifts for this one pot-three step reaction. Addition of 5 equiv of *N*,*O*-bis(trimethylsilyl)acetamide to the unprotected nucleoside *H*-phosphonate monoester **3** allowed the quantitative formation of the corresponding bis(trimethylsilyl) phosphite **4** in 10 min. Subsequent addition of 2 equiv (−)-(8,8-dichlorocamphorylsulfonyl)oxaziridine (DCSO) to the reaction mixture resulted in the quantitative formation of bis(trimethylsilyl) phosphate **5**, characterized in ^{31}P NMR by a triplet at −17.6 ppm ($^3J_{P-H}$ = 6.8 Hz). Treatment with methanol/DBU 97/3 (v/v) removed the TMS groups. Compound **1** was obtained in 90% yield starting from **3**, leading to an overall yield of 85%.[19]

The synthesis of lamivudine 5′-diphosphate **2** was carried out by a modification of the Hoard-Ott procedure[7] recently described by Calleri et al.[20] Activation of the terminal phosphate group of the tri-*n*-butylammonium salt of lamivudine 5′-monophosphate was performed in the presence of 1,1′-carbonyldiimidazole (Scheme 1). Monitoring of the reaction course either by HPLC-UV or by ^{31}P NMR indicated that the conversion of nucleotide **1** into the corresponding phosphorimidazolate was complete within 90 min (δ ^{31}P ~−10.6 ppm). After hydrolyzing unreacted carbonyldiimidazole with methanol, a large excess of tri-*n*-butylammonium orthophosphate was added at room temperature.[21] Follow-up of the reaction by LC/MS indicated that the reaction was complete after one night and also revealed the formation of the N^4-(methylcarbamoyl)lamivudine 5′-diphosphate.[22,23] This side-reaction



Scheme 2. Synthesis of 3TCMP **1** starting from *H*-phosphonate **3** (ROH = 3TC). Reagents and conditions: (a) BSA/DMF, 10 min, rt; (b) DCSO, 10 min, rt; (c) methanol/DBU 97/3 v/v 30 min, rt. Measured ^{31}P NMR chemical shifts are indicated above each compound.

product could be converted to compound **2** by treatment in dilute alkali medium (data not shown). Purification of lamivudine 5′-diphosphate was performed by anion exchange chromatography followed by chromatography on reverse phase. The yield of the diphosphorylation step was 56%.

In conclusion, the present method is highly effective for the synthesis of lamivudine 5′-diphosphate. The best synthetic route to lamivudine 5′-monophosphate was through *H*-phosphonate oxidation by DCSO. Follow-up of the reaction course by HPLC was found to be more convenient than ^{31}P NMR analysis since it is a much lower time- and material-consuming technique.

Acknowledgments

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13. 0.5 mmol (50 mg) of 3TC was dissolved in 1 mL triethylphosphate under argon. The solution was cooled at 0 °C before addition of freshly distilled $POCl_3$ and allowed to proceed at the same temperature. At time intervals, an aliquot of the reaction media was submitted to hydrolysis with triethylammonium bicarbonate 1 M pH 7.5 prior to analysis by HPLC-UV.
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17. Excess of phosphonylating agent was also rapidly hydrolyzed to phenyl *H*-phosphonate monoester. Compound **3** was separated from the phenyl *H*-phosphonate monoester on a silica gel column. Compound **3** (3TC*H*P): ^{31}P NMR (300 MHz, D_2O): δ 6.5 ($^1J_{P-H}$ = 642.4 Hz); HPLC: t_R = 19.3 min (λ_{max} 272 nm).
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19. The mixture was diluted with water–pyridine (1:1, v/v) and washed with dichloromethane. The aqueous layers were concentrated to dryness to give compound **1** as a colorless foam. The nucleotide was purified by Sephadex DEAE A-25 resin ion exchange column chromatography with a linear gradient (0–0.5 M) of 0.5 M ammonium bicarbonate. Compound **1** (3TCMP): ^{31}P NMR (300 MHz, D_2O): δ 3.5 ($^3J_{P-H}$ = 5.5 Hz). HRMS (*m*/*z*) calcd for $C_8H_{11}N_3O_6PS$ $(M-Na)^-$, 308.0130; found 308.0106. HPLC: t_R = 13.9 min (λ_{max} 272 nm).
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21. To a solution of the tri-*n*-butylammonium salt of lamivudine 5′-monophosphate (0.15 mmol) dissolved in 1 mL of dry DMF was added 36 μL of tri-*n*-butylamine (0.15 mmol). The solution was stirred for 20 min at room temperature. After evaporation of the solvent under anhydrous conditions, the residue was suspended in 1.4 mL of dry DMF, then *N*,*N*′-carbonyldiimidazole (122 mg, 0.75 mmol) was added and the mixture was stirred for 3 h at room temperature. Excess of CDI was eliminated by addition of methanol (49 μL, 1.2 mmol) and the mixture was stirred for 30 min. Then 6 mL (3 mmol) of a 0.5 M solution of tri-*n*-butylammonium phosphate in dry DMF were added. The mixture was stirred for another 17 h. The solvent was removed under high vacuum. ^{31}P NMR analysis of the crude reaction product in D_2O showed a characteristic doublet (δ ~−10.5 and −11.9 ppm, J_{P-P} = 17 Hz) assigned to the diphosphate product along with a singulet signal at ~0 ppm corresponding to excess tri-*n*-butylammonium phosphate. The progress of the reaction was also monitored by HPLC-UV. The mixture dissolved in water was purified by Sephadex DEAE A-25 resin ion exchange column chromatography with a linear gradient 0–0.5 M of ammonium bicarbonate, followed by chromatography on RP-18. The triethylammonium counter ions were exchanged to sodium by passing the nucleotide solution through a Dowex-AG 50WX2-400 column. Compound **2** (3TCDP) was obtained as a white solid after lyophilization (56% yield). HPLC: t_R = 18.4 min (λ_{max} 272 nm). ^{1}H NMR (300 MHz, D_2O): δ 8.04 (d, $J_{6,5}$ = 7.6 Hz, 1H, H-6), 6.30 (pseudo, t, $J_{1',2'a}$ = 5.4 Hz, $J_{1',2'b}$ = 4.4 Hz, 1H, H-1'), 5.73 (d, $J_{5,6}$ = 7.6 Hz, 1H, H-5), 5.45 (t, $J_{4',5'}$ = 3.5 Hz, 1H, H-4'), 4.35 (m, 1H, H_a-5'), 4.22 (m, 1H, H_b-5'), 3.52 (dd, $J_{1',2'a}$ = 5.4 Hz, $J_{2'a,2'b}$ = 12.2 Hz, 1H, H_a-2'), 3.20 (dd, $J_{1',2'b}$ = 4.4 Hz, $J_{2'a,2'b}$ = 12.2 Hz, 1H, H_b-2'). ^{13}C NMR (D_2O): δ 166.1 (C-4), 157.0 (C-2), 141.9 (C-6), 95.9 (C-5), 87.5 (C-1'), 84.3 (C-4'), 66.1 (C-5'), 36.7 (C-2'). ^{31}P NMR (D_2O): δ −8.8 (d, J_{P-P} = 20.7 Hz), −11.1 (d, J_{P-P} = 20.7 Hz). HRMS (*m*/*z*) calcd for $C_8H_{11}N_3O_9P_2SNa$ $(M-Na)^-$, 409.9589; found 409.9588.
22. In HPLC-UV, the retention time of N^4-(methylcarbamoyl)lamivudine is 26.3 min (λ_{max} 292 nm). Mass spectroscopy was carried out on a triple quadrupole mass spectrometer TSQ Quantum Ultra (Thermo Fisher Scientific Inc). The molecular formula of N^4-(methylcarbamoyl)lamivudine was determined to be $C_{10}H_{15}N_3O_{11}P_2S$ by negative-ion ESI-MS ($[M-H]^-$ at *m*/*z* 446).
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