# Degradation Kinetics of Oxycarbonyloxymethyl Prodrugs of Phosphonates in Solution

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## INTRODUCTION

Phosphonate analogs of nucleotides have recently received considerable attention as potential antiviral agents. The ionic character of these agents limits their permeability across the human intestinal mucosa, resulting in low bioavailability after oral administration (1,2). We have previously demonstrated the utility of the bis-isopropyloxycarbonylmethyl (bis-POC) moiety in improving the oral bioavailability of phosphonate nucleotides (3–5). The bis-POC promoiety utilizes the oxycarbonyloxymethyl spacer group. The lipophilicity of the prodrug can be adjusted by varying the chain length of the alcohol. Scheme I depicts the putative enzymatic steps involved in the bioconversion of bis-POC prodrugs of a phosphonate moiety to the corresponding phosphonate monoester. The initial enzymatic catalysis is believed to occur at the site remote from phosphorus, thus avoiding enzymatic phosphorylation.

The oxycarbonyloxymethyl promoiety has been previously applied to amines and hindered alcohols (6,7). Safadi and co-workers utilized this spacer group promoiety to enhance the water solubility of various compounds by chemically linking inorganic phosphates to hindered alcohols and amines (6). These prodrugs were chemically unstable and were not suitable as commercially viable pharmaceuticals. Alexander and co-workers also applied this spacer group to alter the lipophilicity of amine containing agents (7). To our knowledge, the degradation kinetics and hydrolytic pathway of oxycarbonyloxymethyl spacer group applied to phosphonates have not been reported.

In the present study, we have applied the bis-POC promoiety to 9-((R)-2-(phosphonomethoxy)ethyl)adenine (Adefovir, PMEA) and to 9-((R)-2-(phosphonomethoxy)propyl) adenine (Tenofovir, PMPA). The chemical stability of bis-POC PMEA and bis-POC PMPA (Scheme II) in solution were investigated. In addition, the $^{18}O$ incorporation studies were conducted to elucidate the degradation pathway(s) for the hydrolysis of the prodrug in aqueous solution.

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## MATERIALS AND METHODS

### Materials

The crystalline fumarate salts of bis-POC PMEA and bis-POC PMPA and the corresponding mono-POC esters were synthesized by the Process Chemistry Departments, Gilead Sciences, Inc. Sodium chloride was available from Mallinckrodt. All salts and solvents were either reagent or HPLC grade and used as received. $^{18}O$ heavy water (95% enriched) was obtained from Aldrich Chemical Corporation. Deionized water was used for buffer and HPLC mobile phase preparation.

### Solution Stability

Buffers were prepared and the pHs measured at 50°C. The pH 1 buffer was prepared from 0.1 N HCl; pH 2–3 buffers were prepared from $H_3PO_4$ and $KH_2PO_4$; pH 4–5 buffers were prepared from $CH_3COOH$ and $CH_3COOK$; pH 6 - 8 buffers were prepared from $KH_2PO_4$ and $K_2HPO_4$. Total buffer concentrations were 10, 50, and 100 mM, and the total ionic strength was adjusted to 0.30 M with KCl. Bis-POC PMEA fumarate and bis-POC PMPA fumarate stock solutions (20 mM) were prepared using a diluent comprised of 80% aqueous/20% acetonitrile and were stored at –20°C. Working solutions were prepared by transferring 0.5 mL of the stock solution to a 25 mL volumetric flask and adding the appropriate buffer solution. The stability samples had a final concentration of 0.40 mM. The solution stability samples were filled in two mL, 13 mm, flint type I tubing vials (West Company). These vials were capped with 4416/50 gray butyl, 13 mm, Teflon-faced stoppers (West Company) and sealed with 13 mm, flip-off aluminum seals (West Company). The stability was monitored at 50°C. Samples removed from the stability chambers were stored at –20°C until HPLC analysis. The hydrolysis of pH 8 stability samples (10, 50, and 100 mM) were quenched to pH 3.0 using 1.0 N HCl solutions and then stored at –20°C until HPLC analysis. The pseudo-first order rate constant, $k_{obs}$, was obtained by following the disappearance of the peak area of the prodrugs as a function of time for at least two half-lives. The ionization constant determined using potentiometric titration of the free base of bis-POC PMEA was determined to be $1.58 \times 10^{-4}$ (pKa = 3.8) and was used for the curve fitting of the pH-rate profile.

From linear regression analysis of the plots of $k_{obs}$ versus the total buffer concentration at a fixed pH and ionic strength, the intercepts yielded the first-order buffer independent rate constant, $k_o$. The respective slopes yielded the second-order buffer dependent rate constants, $k_{cat}$. The buffer independent pH-rate profile was curve fit to a semiempirical equation.

### HPLC Analysis

All prodrugs and their degradation products were analyzed by a reverse phase HPLC method using the modular system described in the instrumentation section. The HPLC method employed an Inertsil ODS-2, 5 μ, 4.6 × 150 mm column (Keystone Scientific). Elutions were performed at ambient temperature using mixtures of mobile phases consisting of (A) 5% acetonitrile/95% 20 mM sodium phosphate pH 6.8 v/v and (B) 65% acetonitrile/35% 20 mM sodium phosphate

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pH 6.8 v/v. The flow rate was set at 1.0 mL/min with UV detection at 260 nm. The mobile phase was set at 100% mobile phase (A) from 0 to 5 min, followed by an 18 minute linear gradient to 100% mobile phase (B) and a 7 minute re-equilibration with 100% mobile phase (A). The elution times of bis-POC PMEA, bis-POC PMPA, mono-POC PMEA, and mono-POC PMPA were 18.6 min, 19.2 min, 11.8 min, and 12.4 min respectively.

### Determination of the Site of Bond Cleavage (C-O and/or P-O)

The site of nucleophilic attack was determined by hydrolyzing bis-POC PMEA in $H_2{}^{18}O$ and $H_2{}^{16}O$ and then performing mass spectral analysis of the hydrolytic product, mono-POC PMEA. Complete hydrolysis of bis-POC PMEA, confirmed by HPLC, was performed at 37°C in 460 μL of 95% enriched $H_2{}^{18}O$, which was adjusted to pH 7.0 using 20 μL of 0.5 M ammonium acetate. The final content of $^{18}O$ in water was approximately 91%. The control samples were prepared similarly, except $H_2{}^{16}O$ was used as the solvent to estimate the uncertainties of the measurements. The mass spectra samples were analyzed using the liquid secondary ion mass spectrometry technique (LSIMS, Negative FAB-MS). The mass spectrometry samples were prepared by mixing 1 μL of matrix with 1 μL of sample solution on the LSIMS probe tip and introducing the probe into the source of the mass spectrometer. Glycerol was used as the matrix to perform all the sample analysis. The incorporation of $^{18}O$ to the phosphorous for bis-POC PMEA was determined based on the ratio of (M+1)/(M–1) of the corresponding mono-POC PMEA by negative FAB-MS, corrected for the final content of $^{18}O$ in water.

To determine if oxygen exchange between the solvent and the hydrolytic products was occurring, additional control experiments, under identical conditions, were performed using mono-POC PMEA as starting materials. The incorporation of $^{18}O$ label into monoesters was examined using negative FAB-MS.

## RESULTS AND DISCUSSION

### Solution Hydrolysis of bis-POC PMEA

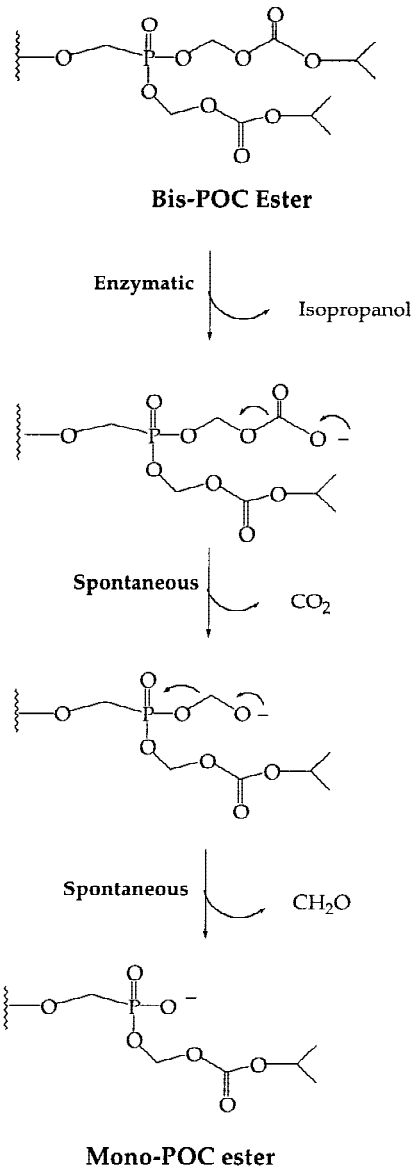
The kinetics of hydrolysis bis-POC PMEA were studied at 50°C and $\mu = 0.30$ M as functions of pH and buffer concentration. Hydrolysis of bis-POC PMEA leads to the formation of one mole of each formaldehyde, isopropanol, carbon dioxide, and mono-POC PMEA (Scheme I). Mono-POC PMEA was the only degradation product observable by HPLC. Mass balance was obtained for all pH values studied. The plausible intermediates shown in Scheme I were not chromatographically detected and are considered to be relatively unstable.

The pH-dependency of the buffer-independent rate constants, $k_o$, for bis-POC PMEA is shown in Figure 1. Over the pH range studied, the rates of hydrolysis of bis-POC PMEA were described by the following equation:

$$k_o = k_H f_{AH} (H^+) + k_{H2O} f_{AH} + k'_{H2O} f_A + k_{OH} f_A (OH^-) \quad \text{Eq (1)}$$

where, $f_{AH}$ is the fraction of conjugate acid and $f_A$ is the fraction of free base, $k_H$ is the microscopic second-order rate constant for the hydronium ion catalyzed hydrolysis, $k_{H2O}$ is the first-order rate constant for water catalyzed or spontaneous hydrolysis of the conjugate acid, $k'_{H2O}$ is the first-order rate constant for water catalyzed or spontaneous hydrolysis of the free base, and $k_{OH}$ is the microscopic second-order rate constant for hydroxide ion catalyzed hydrolysis. The ionization of the adenine ring appears to modestly perturb the pH-rate profile. The value of $k'_{H2O}$ (0.015 $hr^{-1}$) is only 1.5 times higher than the $k_{H2O}$ (0.01 $hr^{-1}$), indicating that the free base is slightly more reactive than the conjugate acid.

Hydrolysis of bis-POC PMEA was subjected to buffer catalysis in the pH range of 2–8. The rate of hydrolysis increased with increasing buffer concentration while maintaining constant pH and ionic strength. The extent of buffer catalysis increased with increasing the solution pH, indicating general base catalysis.




**Scheme I.** Bioconversion of bis-POC ester to the corresponding mono-POC ester.

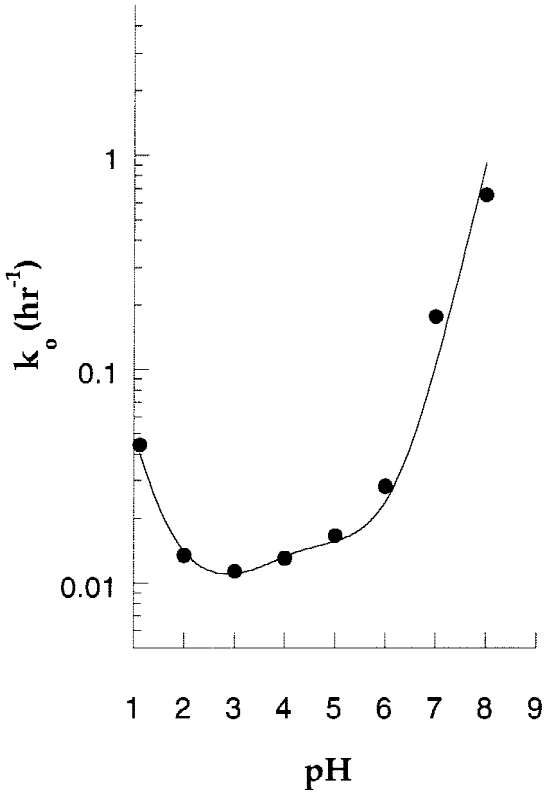
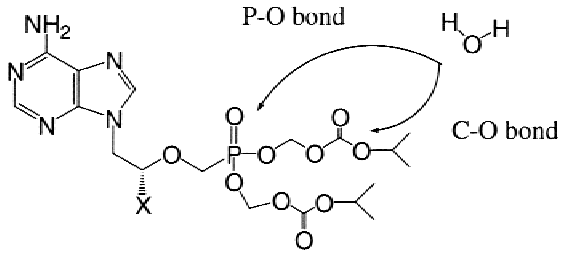



**Fig. 1.** pH-rate profile for the degradation of bis-POC PMEA at 50°C. The pH-rate profile was fitted to the following equation: $k_o = k_H f_{AH}(H^+) + k_{H2O} f_{AH} + k'_{H2O} f_A + k_{OH} f_A (OH^-)$, where $k_H = 0.40$ $M^{-1}$ $hr^{-1}$, $k_{H2O} = 0.01$ $hr^{-1}$, $k'_{H2O} = 0.015$ $hr^{-1}$, and $k_{OH} = 1.65 \times 10^{-5}$ $M^{-1}$ $hr^{-1}$.

### Site of Nucleophilic Attack for bis-POC PMEA

Hydrolysis of bis-POC PMEA may proceed via two distinct pathways as shown in Scheme II. The first involves the nucleophilic attack of water at the carbonyl center to form the tetrahedral intermediate (C-O bond cleavage) and the second involves the nucleophilic attack of water on the phosphorus atom (P-O bond cleavage). Both pathways lead to the formation of the mono-POC PMEA. As shown in Scheme II, the nucleophilic attack of water at phosphorus results in $^{18}O$ incorporation into the phosphonate monoester, whereas, the




Bis-POC PMEA X= H

Bis-POC PMPA X = $CH_3$

**Scheme II.** Chemical structures of bis-POC PMEA and bis-POC PMPA and the proposed scheme for hydrolysis via carbon-oxygen and/or phosphorus-oxygen bond fission.

**Table I.** Pseudo First Order Rate Constants of POC-PMPA ($k_{PMPA}$) and POC-PMEA ($k_{PMEA}$) as a Function of pH at 50°C

| pH[a] | $k_{PMEA}$ ($hr^{-1}$) × $10^2$ | $k_{PMPA}$ ($hr^{-1}$) × $10^2$ | $k_{PMEA}/k_{PMPA}$ |
|---|---|---|---|
| 1.1 | 4.42 | 2.67 | 1.7 |
| 2.0 | 1.72 | 1.30 | 1.3 |
| 3.0 | 1.55 | 1.21 | 1.3 |
| 4.0 | 1.61 | 1.65 | 1.0 |
| 5.0 | 2.7 | 2.11 | 1.3 |
| 6.0 | 9.15 | 3.95 | 2.3 |
| 7.0 | 39.17 | 17.91 | 2.2 |
| 8.0 | 243.5 | 109.0 | 2.2 |

[a] Total buffer concentration was 100 mM, and total ionic strength was adjusted to 0.30 M.

nucleophilic attack of water on the carbonyl moiety leads to $^{18}O$ incorporation into carbon dioxide. Hydrolysis of bis-POC PMEA was carried out in $H_2{}^{18}O$ and $H_2{}^{16}O$ at pH 7.0, 37°C. Mono-POC PMEA was analyzed for isotopic composition using negative FAB mass spectrometry. In addition, the mono-POC PMEA was also incubated with $H_2{}^{18}O$ under the identical condition as bis-POC PMEA. The extent of P-O bond cleavage was estimated to be 85% ± 5% for bis-POC PMEA at pH 7.0, 37°C.

These observations suggest that hydrolysis of bis-POC PMEA primarily proceeds via P-O bond cleavage with an additional minor pathway involving C-O bond cleavage. There was minimal incorporation of $^{18}O$ into mono-POC PMEA during incubation with the $H_2{}^{18}O$ enriched buffer system.

### Solution Hydrolysis of bis-POC PMPA

The kinetics of bis-POC PMPA hydrolysis were also studied at 50°C and $\mu = 0.30$ M as functions of pH. Mono-POC PMPA was the only degradation product observable by HPLC and mass balance was obtained for all the pH values. Table I compares the rate of hydrolysis of bis-POC PMPA to bis-POC PMEA. Interestingly, bis-POC PMPA demonstrated better chemical stability than bis-POC PMEA. At pH values above 6.0, the rate of hydrolysis of bis-POC PMPA is about 2 times slower than bis-POC PMEA. The side chain methyl group may stericly hinder the nucleophilic attack of water at the phosphorus center. This is consistent with the $^{18}O$ incorporation studies indicating that the attack at the phosphorus is the primary degradation pathway for bis-POC PMEA. In addition, the methyl moiety may reduce the extent of intramolecular hydrogen bonding of nucleophilic water to the side chain β-oxygen.

In summary, the degradation kinetics and the hydrolytic pathway of bis-POC PMEA and bis-POC PMPA were studied in solution. The $H_2{}^{18}O$ incorporation studies indicated that chemical hydrolysis of bis-POC PMEA predominately occurs via P-O bond cleavage with an additional minor pathway involving the C-O bond cleavage. The data suggest that the oxycarbonyloxymethyl spacer group can be successfully applied to phosphonates resulting in prodrugs with adequate chemical stability to allow for formulation development and delivery. Bis-POC PMPA is currently in Phase III clinical testing for the treatment of AIDS.

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