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Bis-Ketol Phosphate Alkyl Triesters: Rate of Initial Ketol Group Hydrolysis

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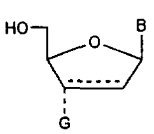
Abstract: Bis-(2-oxo-2-phenyl ethyl) tetrahydrofurfuryl phosphate (7) was synthesized by the addition of tetrahydrofurfuryl alcohol to the corresponding bis-ketol hydrogen phosphate (8) using DCC as the condensing agent. The rate of loss of a single ketol group was determined over the pH range 6.9 to 9 and at different temperatures. At pH 7.4 and 37°C compound 7 hydrolyzed with a pseudo-first rate constant of $6.2 \times 10^{-4} s^{-1}$. This hydrolysis rate is 10^8 fold faster than a simple alkyl phosphate triester. The rate of hydrolysis is similar to that observed for mono-ketol dialkyl phosphate triesters.

Nucleoside analogs of general structure **1** are used extensively as anti-viral and anti-cancer drugs[1]. These molecules effect their biological activity only after enzymatic conversion, through mono- and diphosphate intermediates **2** and **3**, to the corresponding triphosphate forms, **4** (*i.e.*, **1**→**2**→**3**→**4**)[2]. Sometimes, the initial phosphorylation (**1**→**2**) is enzymatically unfeasible and compounds which are bioactive as the triphosphate are inactive when administered as nucleosides[3]. Since the dianionic phosphates like **2** are too polar to pass through the cell membrane, many attempts have been made to produce neutral nucleoside monophosphate triesters such as **5** as pro-drugs of **2**[4]. To be suitable pro-drugs, the triesters must be sufficiently reactive so that hydrolysis can take place reasonably rapidly. They must also show a high degree of regiospecificity in the hydrolysis



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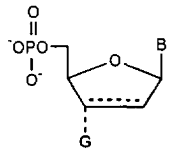

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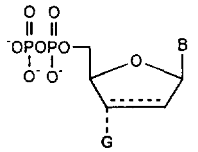

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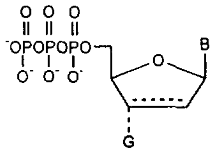

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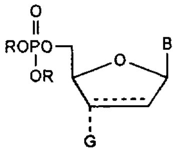

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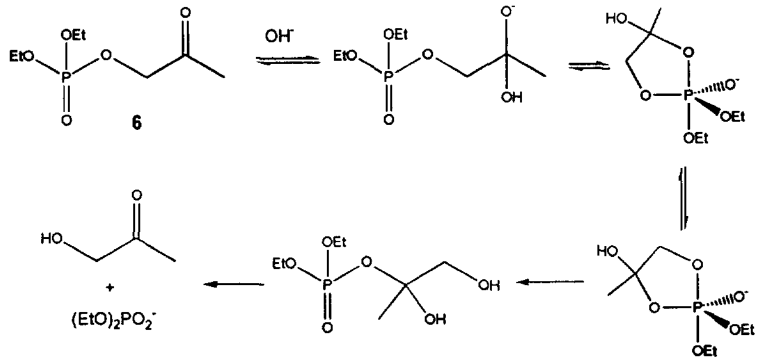
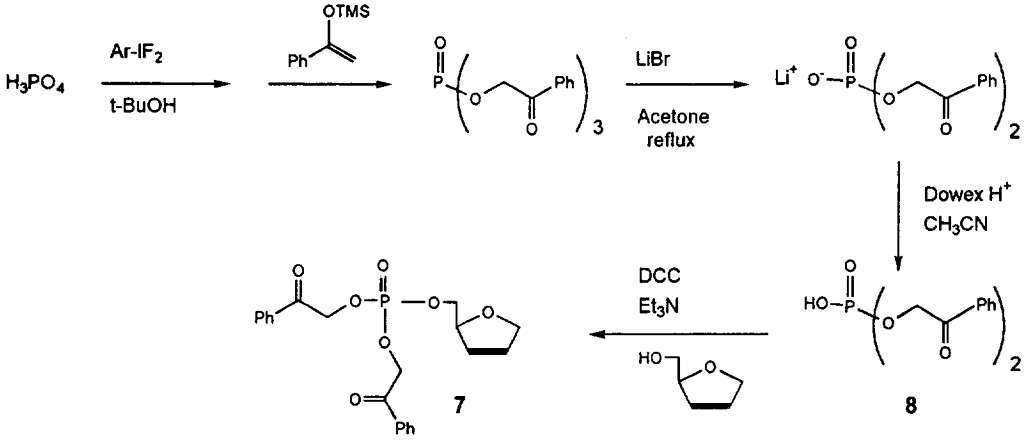
Scheme 1



reaction to avoid competitive expulsion of the free nucleoside. Since there are no known human enzymes capable of hydrolyzing phosphate triesters, the ester groups in 5 must confer the proper combination of rate and selectivity required of the hydrolysis reaction. Ketol phosphate triesters can undergo hydrolysis rapidly and regiospecifically. For example, the mechanism of acetol diethyl phosphate (6) hydrolysis allows the rapid and specific production of diethyl phosphate (scheme 1)[5]. The reaction involves an intramolecular addition to phosphorus to generate a strained five-member cyclic phosphorane. The overall rate of the hydrolysis reaction is about 10^6 fold greater than the rate of hydrolysis of triethylphosphate. In order to assess the possible use of ketol esters of nucleoside monophosphates as prodrugs, we have synthesized bis-ketol esters of tetrahydrofurfuryl alcohol and determined the rate of hydrolysis of the first ketol group.

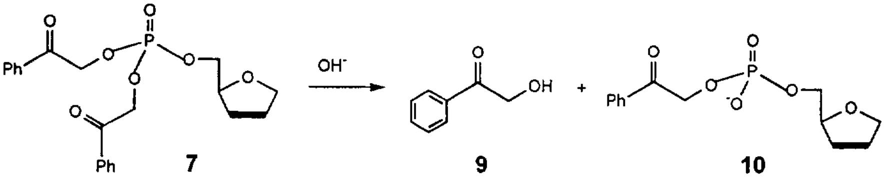
The synthesis of bis-(2-oxo-2-phenyl ethyl) tetrahydrofurfuryl phosphate (7) is accomplished as outlined in scheme 2. The tris-ketol phosphate is produced by treating crystalline phosphoric acid with 3.1 equivalents of (difluoroiodo)-p-toluene in t-butanol followed by 3.5 equivalents of the trimethylsilyl enol ether of acetophenone[6a]. Upon treating the tris-ketol phosphate with 1.1 equivalents of LiBr in refluxing acetone, the solid lithium salt of the bis-ketol phosphate is formed. This salt is converted to the acid form by treating with Dowex ion exchange resin in dry acetonitrile at 45°C[6b]. The bis-ketol hydrogen phosphate is treated with DCC and tetrahydrofurfuryl alcohol in methylene chloride. The triester 7 forms after addition of triethylamine[6c, 12].

Scheme 2



Reversed phase HPLC was used to separate and quantitate the reactants and products present at various times during the hydrolysis of 7 (scheme 3)[7]. As shown in figure 1, three major compounds were found during the hydrolysis. The peak eluting at 5.8 minutes is 7, while 9 and 10 elute at 2.1 and 1.3 minutes, respectively as determined by co-chromatography with authentic material. Rate constants for the disappearance of 7 (3.1×10^{-4} s^{-1}) and the appearance of 9 (3.0×10^{-4} s^{-1}) and 10 (3.0×10^{-4} s^{-1}) at 23°C and pH 7.65 were determined by fitting the peak area vs time data to the equation for a first order reaction. Using this same procedure, the effect of pH and temperature on the rate constant of hydrolysis of 7 was determined.

Scheme 3



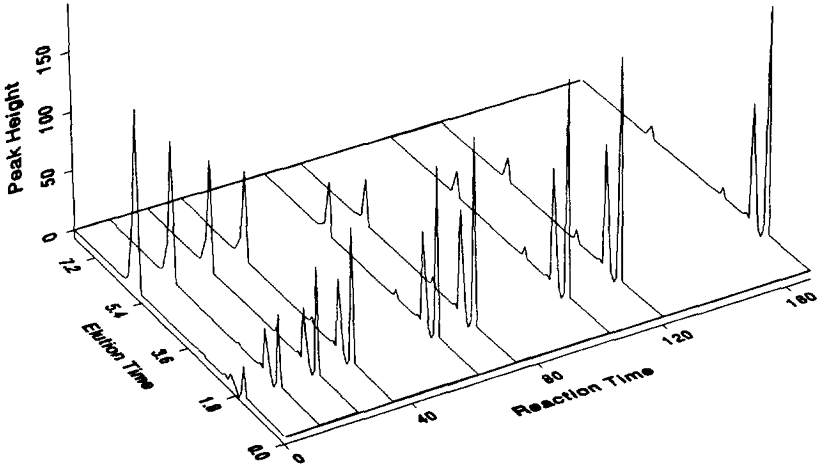


Figure 1. Stacked plot showing the chromatographic separation of the hydrolysis reaction of 7 at various times.

The temperature of the hydrolysis reaction was varied from 18.0°C to 41.2°C at pH 7.65 while the pH (at 25°C) was varied from 6.91 to 9.05 at a constant ionic strength of 0.2 M (adjusted using KCl). A phosphate buffer was used for solutions with pH between 6.91 and 8.09 while a borate buffer was used for pH 9.05. A plot of the log of the rate constant for hydrolysis vs pH gave a straight line with slope 1. A plot of the rate constant for hydrolysis vs hydroxide ion concentration gave a second order rate constant of 730 $M^{-1}s^{-1}$for hydrolysis of 7 at 25°C. From the temperature vs rate data, we calculate a $\Delta H^{\ddagger}$ of 18.4 kcal/mole and a $\Delta S^{\ddagger}$ of -12.6 eu. Using these data, we calculate a first order rate constant of 6.2×10^{-4} s^{-1} for hydrolysis of 7 at physiological conditions (pH 7.4 and 37°C).

At 25°C, the second order rate constant for hydrolysis of the bis-ketol tetrahydrofurfuryl phosphate is 730 $M^{-1}s^{-1}$. The corresponding rate constant for hydrolysis of acetol diethyl phosphate[5] (6) is 58 $M^{-1}s^{-1}$. If the mechanism for hydrolysis is the same as presented in scheme 1 for 6, then we can quantitatively account for the difference in hydrolysis rate. As argued by Kluger and Taylor[5], the attack of the conjugate base of the carbonyl hydrate at phosphorus is rate limiting. Using the free energy relationship for ketone hydration[8] and the free energy relationship for the pK_a of ketone hydrates[9] we can estimate the amount of the conjugate base of the carbonyl hydrate for 7 relative to 6. Using Taft substituent constants for 6 (Me) and 7 (Ph), we estimate a 66 fold greater amount of anionic carbonyl hydrate for 7 than for 6. Using the data of Charton and Charton[10], we estimate that steric effects on the reaction will cause a 10 fold decrease in rate of hydrolysis of 7 relative to 6. We must also consider the statistical effect of two ketol groups in 7 relative to one in 6. Overall, these estimates lead to a prediction that 7 should hydrolyze about 3 fold faster than 6. The ratio of rate constants for hydrolysis of 7 compared to 6 is 13. The correspondence between the estimated and actual rates of hydrolysis based on the mechanism of scheme 1 means that the bis-ketol monoalkyl phosphate triesters must behave similarly to the mono-ketol bis-alkyl triesters in terms of hydrolysis.

The rate constant for hydrolysis of the bis-ketol phosphate triesters reported here indicates that bis-ketol phosphate derivatives of nucleosides will spontaneously hydrolyze to mono-ketol phosphate nucleosides under physiological conditions. A series of AZT derivatives containing various bis-ketol phosphate groups has been synthesized and proven effective in inhibition of HIV proliferation in cell culture[11]. The ketol group may prove optimal for temporarily masking the charge associated with a nucleoside monophosphate.

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6. a) The reaction mixture was concentrated and the residue dissolved in CH_2Cl_2 and passed through a silica gel column, eluting first with pentane then CH_2Cl_2. After concentration, the product was crystallized from methylene chloride/pentane and isolated in 66% yield. (mp 139-140°C)
   b) The lithium salt was filtered and dried under vacuum. A solution of 0.50 g of the salt in 25 mL of acetonitrile was heated at 45°C for 60 min in the presence of 3.8 g of Dowex 50W x 8 (H^+ form). The resin was filtered off and washed with acetonitrile. The solvent was removed to give clean bis-ketol hydrogen phosphate in 94% yield. (mp 128-130°C)
   c) The bis-ketol hydrogen phosphate (1.50 mmol) was mixed with 0.75 mmol of tetrahydrofurfuryl alcohol in CH_2Cl_2. DCC (1.79 mmol) was added as a solution in CH_2Cl_2. After 5 min, triethylamine (1.10 mmol) was added dropwise. The reaction ran for 18 hr and was then filtered and concentrated. The residual oil was purified by flash chromatography eluting with ethylacetate/hexanes. The product was obtained in 61% yield. ^1H NMR ($CDCl_3$) δ 1.64-2.06 (m, 4H), 3.69-3.88 (m, 2H), 4.10-4.40 (m, 3H), 5.35-5.58 (m, 4H), 7.42-7.63 (m, 6H), 7.85-7.95 (m, 4H). ^{13}C NMR ($CDCl_3$) δ 25.63, 27.44, 68.42, 69.17 (d, J_{PC}=4.5 Hz), 70.12 (d, J_{PC}=6.3 Hz), 77.08, 127.71, 128.75, 128.84, 133.90, 133.90, 192.12 (d, J_{PC}=4.8 Hz). ^{31}P NMR ($CDCl_3$) δ 0.060.
7. A methanol solution of 10 mM **7** was diluted 20 fold with a 0.020 M buffer at the appropriate pH. This solution was incubated in a sealed vial at the appropriate temperature. A 10 µL aliquot was removed at various times and injected on an Alltech Econosphere C18 column connected to a Perkin-Elmer LC4 HPLC system. Elution was accomplished at a flow rate of 1.8 mL/min using a methanol/water (2.6/1 v/v) solution. The column effluent was monitored at 248 nm and the detector connected to a Spectraphysics integrator. The peak height vs time data were fit to the equation for a first order reaction using a non-linear regression procedure contained in the program Axum. In all determinations, the standard errors of the rate constants were less than 8% of the value of the rate constants. Linear regression analysis of the data gave correlation coefficients of 0.995 or greater in each case. During the study of the pH dependence of the reaction, the pH of a control solution was checked using a glass pH electrode. Although some **9** and **10** were present in the starting **7**, this did not interfere with the rate determinations.
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