*Articles*

# Mechanism-Based Inactivation of Phosphotriesterase by Reaction of a Critical Histidine with a Ketene Intermediate†

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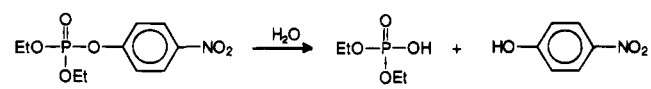
ABSTRACT: Five alkynyl phosphate esters have been synthesized as probes of the active site structure of phosphotriesterase. These compounds have the potential to be converted by the enzyme to a highly reactive ketene intermediate which can then react with an active site nucleophile causing irreversible inhibition of the enzyme by formation of an inactive covalent adduct. All five compounds completely inactivate enzyme function in less than 15 s at pH 7.0. The partition ratios of 1-hexynyl diethyl phosphate (**I**), 1-propynyl diethyl phosphate (**II**), 1-hexynyl diphenyl phosphate (**III**), 1-hexynyl dimethyl phosphate (**IV**), and ethynyl diethyl phosphate (**V**) fall in the range between 480 and 1700; thus, all five alkynyl phosphate esters work equally well as inactivators despite the differences in their structures. The rate constants for enzyme inactivation, $k_{inact}$, are 1.7 $s^{-1}$ with **I**, 1.3 $s^{-1}$ with **II**, and 0.12 $s^{-1}$ with **IV**. They compare well with the $k_{cat}$ for the Co-substituted phosphotriesterase; hence these compounds are good substrates. The stoichiometry of inhibitor bound to protein is 1:1, as determined by inactivation of the enzyme using the radiolabeled compound [3-$^{14}$C]-1-propynyl diethyl phosphate. Addition of an exogenous nucleophile, azide, did not protect phosphotriesterase from being inactivated by the alkynyl phosphate esters, suggesting that the reactive intermediate produced from the inhibitor is not released from the enzyme surface prior to covalent labeling of the protein. Chemical and spectroscopic evidence suggests that a histidine residue is modified in the inactivation reaction. The inactivated phosphotriesterase can be reactivated by increasing the pH of the protein solution. *N*-Acylimidazoles are known to be easily hydrolyzed at alkaline pH values. The difference spectrum between modified and unmodified protein shows an increase in the UV region between 230 and 270 nm, and the difference spectrum between *N*-acetylimidazole and imidazole also shows an absorption band in the same region. The similarities in the properties of the inactivated enzyme compared with *N*-acetylimidazole indicate that a histidine is the residue modified by the alkynyl phosphate ester.

The bacterial phosphotriesterase from *Pseudomonas diminuta* is a zinc metalloenzyme which catalyzes the hydrolysis of organophosphate triesters. The details of the mechanism of action are of interest because of the potential usefulness of this enzyme for detoxifying chemical wastes, chemical warfare agents, and agricultural pesticides. The reaction mechanism of this enzyme is thought to involve the nucleophilic attack by an activated water molecule resulting in the cleavage of a phosphorus−oxygen bond, as illustrated in Scheme 1 for the hydrolysis of paraoxon. The net stereochemical outcome at the phosphorus center is inversion of configuration (Lewis et al., 1988). Inactivation studies of the enzyme with diethyl pyrocarbonate and the pH rate profiles for the hydrolysis of paraoxon by the phosphotriesterase suggest that an active site histidine residue acts as the general base or proton shuttle group (Dumas & Raushel, 1990).

Scheme 1




Atomic absorption and $^{113}$Cd NMR studies have shown that two divalent metal ions are bound to the enzyme (Omburo et al., 1993). The position of the chemical shifts for the two cadmium signals indicate that the ligands to both metals are composed of a mixture of oxygen (i.e., aspartate, glutamate, or $H_2O$) and nitrogen (i.e., histidine) atoms (Omburo et al., 1993). The exact function of each of the two metal ions is unknown, although it has been demonstrated that at least one metal ion is required for catalytic activity (Dumas et al., 1989; Omburo et al., 1992). It has also been shown that the naturally occurring Zn(II) can be replaced with Cd(II), Co(II), Mn(II), and Ni(II) without any decrease in the specific activity of the enzyme (Omburo et al., 1992). EPR experiments with the Mn/Mn-substituted enzyme have also been used to probe the environment of

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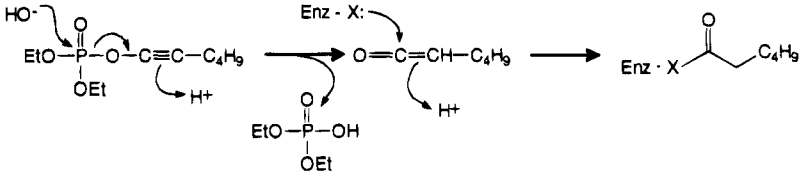
Scheme 2



the two cation binding sites. These experiments have demonstrated unequivocally that the two Mn(II) ions are present as an antiferromagnetically-coupled binuclear center. The two metals bound to phosphotriesterase are therefore in close proximity and are most likely bridged by a common ligand (Chae et al., 1993).

The participation of specific amino acid residues in binding and catalysis has been probed by site specific mutagenesis of the seven histidine residues to asparagine (Kuo & Raushel, 1994). The results of these experiments support a working model of the active site whereby His-201 acts as the general base or proton shuttle, His-55 and His-57 are ligands to one metal site, His-254 and His-257 are ligands to the second metal site, and His-230 acts as a bridge for the two metals. It is still unclear, however, what other residues might be required for the proper functioning of the active site.

An alternative and complementary approach to the identification of nucleophilic residues at the active site involves the synthesis of suicide or mechanism-based inhibitors. In this article, alkynyl phosphate esters have been constructed as active site probes. These compounds have the potential to be converted by the enzyme to highly reactive ketene intermediates via the hydrolysis of the phosphorus−oxygen bond, as shown in Scheme 2. The resulting ketene intermediate can then potentially react with an active site nucleophile, resulting in an irreversible inhibition of the enzyme via the formation of an inactive covalent adduct.

## MATERIALS AND METHODS

*Materials.* Paraoxon, imidazole, and all buffers were purchased from Sigma Chemical Co. Anhydrous acetonitrile and *N*-acetylimidazole were obtained from Aldrich Chemical Co. The micro-BCA protein assay reagent kit was obtained from Pierce. 1,2-Dibromoethylene was purchased from Aldrich and $^{14}CH_3I$ was purchased from Dupont NEN Research Products.

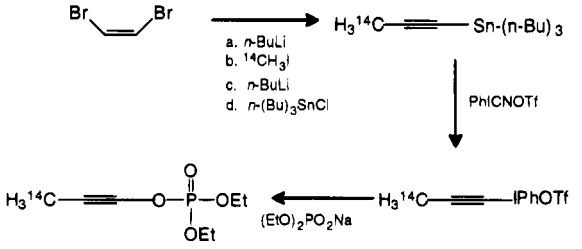
*Synthesis of Alkynyl Phosphate Esters.* The alkynyl phosphate esters 1-hexynyl diethyl phosphate (**I**), 1-propynyl diethyl phosphate (**II**), 1-hexynyl diphenyl phosphate (**III**), and 1-hexynyl dimethyl phosphate (**IV**) were prepared as previously reported (Stang et al., 1986, 1989). Ethynyl diethyl phosphate (**V**) was synthesized using a similar procedure. A solution of ethynyl(phenyl)iodonium triflate (0.50 g, 1.3 mmol) in 200 mL of $CH_2Cl_2$ was added dropwise over 1 h to a cold (−42 °C) suspension of sodium diethyl phosphate (0.26 g, 1.4 mmol) in 500 mL of $CH_2Cl_2$. The reaction mixture was stirred at −42 °C for 6 h, then allowed to warm to room temperature, and stirred for another 14 h. Silica gel was added to absorb the reaction products, and the solvent was removed *in vacuo*. Purification by flash column chromatography (Still et al., 1978) ($CH_2Cl_2$/acetone, 99:1) gave ethynyl diethyl phosphate in 16% yield (37 mg) as a red oil. IR 3312, 3262, 2988, 2937, 2916, 2174, 1299, 1166, 1029 $cm^{-1}$; $^1$H-NMR ($CDCl_3$) $\delta$ 4.26 (dq, 4H, $^3J_{H,H}$ = 7.1 Hz, $^3J_{P,H}$ = 7.1 Hz), 1.67 (d, 1H, $^4J_{P,H}$ = 4.2 Hz), 1.38 (dt, 6H, $^3J_{H,H}$ = 7.1 Hz, $^4J_{P,H}$ = 1.2 Hz); $^{13}$C NMR $\delta$ 80.61 (d, $^2J_{P,C}$ = 9.2 Hz), 66.33 (d, $^2J_{P,C}$ = 6.4 Hz), 28.92 (d, $^3J_{P,C}$ = 6.3 Hz), 15.94 (d, $^3J_{P,C}$ = 6.5 Hz); $^{31}$P NMR $\delta$ −5.26.

*Synthesis of [3-$^{14}$C]Tributyltin−1-Propyne.* *n*-Butyllithium (0.8 mL, 2.0 mmol, 2.5 mol/L in hexane) was added dropwise to a cold solution (−78 °C, dry ice/2-propanol) of 1,2-dibromoethylene (0.18 g, 1.0 mmol) in 4 mL of THF. The reaction mixture was stirred for 15 min, and then $^{14}CH_3I$ (2 mCi, 54 mCi/mmol) in 1 mL of THF was added. After allowing the reaction mixture to warm to room temperature and stir for 4 h, it was recooled to −78 °C and methyl iodide (0.14 g, 0.96 mmol) was added dropwise. The reaction mixture was allowed to warm to room temperature and was stirred for another 7 h. After recooling to −78 °C, *n*-butyllithium (0.4 mL, 1.0 mmol, 2.5 mol/L in hexane) was added dropwise and the reaction mixture was stirred for 15 min. Tributyltin chloride (0.32 g, 1.0 mmol) was added dropwise; the reaction mixture was allowed to warm to room temperature and then stirred for 10 h. While stirring, 2.5 mL of $H_2O$ was added to the ice cooled (0 °C) reaction mixture. The layers were separated, and the aqueous phase was extracted with diethyl ether (3 × 2 mL). The combined organic extracts were dried over anhydrous magnesium sulfate. The organic phase was filtered through Celite, and the solvent was removed *in vacuo*, yielding a gold colored oil which was used without further purification.

*Synthesis of [3-$^{14}$C]Propynyl(phenyl)Iodium Triflate.* A solution of [3-$^{14}$C]tributyltin−1-propyne (0.3 g, 0.91 mmol) in 5 mL of $CH_2Cl_2$ was added dropwise to a cold suspension (−42 °C, dry ice/acetonitrile) of phenyl(cyano)iodonium triflate (Zhandkin et al., 1990; Stang et al., 1990, 1991; Stang & Zhdankin, 1991) (0.34 g, 0.91 mmol) in 5 mL of $CH_2Cl_2$. During addition, the suspension gave way to a homogeneous solution which was allowed to warm to room temperature and stir for 50 min. The solvent was removed *in vacuo*, yielding an oil which upon trituration with 3:1 pentane/diethyl ether solution gave a light brown solid (0.18 g, 0.47 mmol). Vacuum filtration, followed by washing three times with 3:1 pentane/diethyl ether solution, gave [3-$^{14}$C]-1-propynyl(phenyl)iodonium triflate in 77% yield (0.27 g).

*Synthesis of [3-$^{14}$C]-1-Propynyl Diethyl Phosphate.* A solution of [3-$^{14}$C]-1-propynyl(phenyl)iodonium triflate (90 mg, 0.23 mmol) in 40 mL of $CH_2Cl_2$ was added dropwise, over 35 min, to a cold (−42 °C) suspension of sodium diethyl phosphate (45 mg, 0.26 mmol) in 150 mL of $CH_2Cl_2$. After completion of the addition, the reaction mixture was stirred at −42 °C for 7 h and then allowed to warm to room temperature and stirred for 14 h. Silica gel was added to absorb the reaction products, and the solvent was removed *in vacuo*. Purification by flash column chromatography (Still et al., 1978) ($CH_2Cl_2$/acetone, 99:1) yielded [3-$^{14}$C]-1-propynyl diethyl phosphate in a 40% yield (18 mg) as a light yellow oil. A TLC plate spotted with [3-$^{14}$C]-1-propynyl

Scheme 3




diethyl phosphate and 1-propynyl diethyl phosphate (Stang et al., 1989) and eluted with 95:5 $CH_2Cl_2$/acetone showed identical $R_f$ values of 0.53 (developed using $KMnO_4$ solution). Scintillation counter analysis of a sectioned TLC plate, in which the product was eluted using 95:5 $CH_2Cl_2$/acetone, revealed excellent radiological purity. Dilution with 0.27 g (1.4 mmol) of 1-propynyl diethyl phosphate gave a product with a specific activity of 0.12 mCi/mmol (806 cpm/nmol). The synthetic protocol is summarized in Scheme 3.

*Purification of Phosphotriesterase.* The wild-type phosphotriesterase was purified according to the method of Omburo et al. (1992). The Co-substituted wild-type enzyme was used in all experiments.

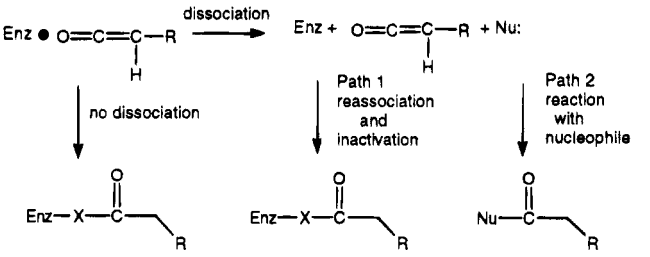
*Efficiency of Inactivation by Alkynyl Phosphate Esters.* Phosphotriesterase (60 nM) was incubated at 25 °C with various concentrations of inhibitor in 0.1 M PIPES,[1] pH 7.0, containing 3% $CH_3CN$ in a final volume of 100 $\mu$L. After 1−15 min of incubation, a 20 $\mu$L aliquot of the enzyme solution was removed and measured for catalytic activity as described by Omburo et al. (1992) using 1.0 mM paraoxon as a substrate in 0.1 M CHES, pH 9.0. The partition ratio was calculated as described by Knight and Waley (1985) by plotting the fraction of remaining enzyme activity ($[E]_r/[E]_0$) versus the initial ratio of inhibitor to enzyme ($[I]/[E]_0$).

*Protection from Inhibition by a Nucleophilic Scavenger.* The wild-type phosphotriesterase (50 nM) was incubated at 25 °C in 0.1 M PIPES, pH 7.0, containing 3% $CH_3CN$ in the presence of inhibitor and varying concentrations of $NaN_3$ (5−200 mM) in a total volume of 100 $\mu$L. The ratio of inhibitor/enzyme was 2000:1 using 1-hexynyl diethyl phosphate (**I**) and 7000:1 using ethynyl diethyl phosphate (**V**). The reaction was initiated with inhibitor. After 15 min of incubation, a 20 $\mu$L aliquot of enzyme solution was assayed with 1.0 mM paraoxon in 0.1 M CHES, pH 9.0, as previously described (Omburo et al., 1992). Control reactions were also performed where $CH_3CN$ replaced the inhibitor or $H_2O$ replaced $NaN_3$. Attempts were also made to use hydroxylamine as a nucleophilic scavenger. The reaction conditions were the same as described above for $NaN_3$ except that the concentration range used was 0−20 mM $NH_2OH$.

Three different experiments were performed to rule out the possibility of inactivation occurring after the release of the reactive intermediate, as shown in Scheme 4. In the initial experiment, phosphotriesterase and $NH_2OH$ were incubated for 15 s, then inhibitor was added. A 20 $\mu$L aliquot was removed and assayed. In the second experiment, phosphotriesterase and inhibitor were incubated together for 5 min in the presence of 1 mg/mL BSA. $NH_2OH$ was then

[1] Abbreviations: PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); CHES, 2-(*N*-cyclohexylamino)ethanesulfonic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; BSA, bovine serum albumin.

Scheme 4




added to a final concentration of 20 mM. A 20 $\mu$L aliquot was assayed after various times (25 s−2 h). In the third experiment, inhibitor and $NH_2OH$ (20 mM final concentration) were incubated for a specified amount of time (10−45 s), and then enzyme was added. A 20 $\mu$L aliquot was assayed after at least 15 s of incubation.

*Rate of Inactivation of Phosphotriesterase by Alkynyl Phosphate Esters.* The inactivation rate by the alkynyl phosphate esters was too fast to determine by manual removal of aliquots because incubation of enzyme and inhibitor eliminated all catalytic activity within 1 min. Therefore, the rate of inactivation of enzyme was determined using a High-Tech stopped-flow spectrophotometer (Model SF-51) and the chromogenic substrate paraoxon (diethyl *p*-nitrophenyl phosphate) as a reporter substrate to monitor the activity of enzyme as a function of time. The reaction of enzyme, inhibitor, and 0.25 mM paraoxon in 100 mM PIPES, pH 7.0, containing 5% $CH_3CN$, was continuously monitored at 400 nm with the initial ratio of inhibitor to enzyme kept constant at 3000:1 with **I**, 61 000:1 with **II**, and 50 000:1 with **IV**. The phosphotriesterase concentrations varied from 0.65 to 148 nM and the inhibitor concentrations ranged from 32 to 1500 $\mu$M.

*Stoichiometry of Inhibitor Covalently Bound to Enzyme.* Phosphotriesterase (200 $\mu$g), 0.1 M HEPES, pH 7.5, 5% acetonitrile, and [3-$^{14}$C]-1-propynyl diethyl phosphate ($I/E_0$ = 10 000) in a total volume of 1 mL were incubated at 25 °C for 30 min. The reaction mixture was diluted to 2.5 mL with 0.1 M HEPES, pH 7.5/5% $CH_3CN$. Radiolabeled phosphotriesterase was separated from excess [3-$^{14}$C]-1-propynyl diethyl phosphate using a PD-10 gel filtration column from Pharmacia equilibrated with 0.1 M HEPES pH 7.5/5% $CH_3CN$. The protein was eluted with the same buffer, and 500 $\mu$L fractions were collected. A 100 $\mu$L aliquot from the protein fraction was dissolved in 16 mL of Beckman Ready Safe Liquid scintillation cocktail and counted for radioactivity with a Beckman LS-9000 scintillation counter. The amount of protein in 100 $\mu$L of each fraction (diluted to 1 mL in deionized $H_2O$) was determined using the Pierce micro-BCA protein assay reagent. The protein assay was carried out as directed by the manufacturer using phosphotriesterase as the standard.

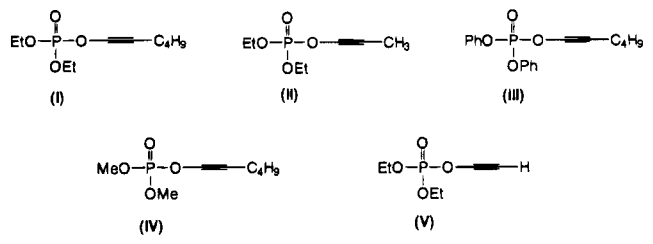
*Reactivation of Inactivated Phosphotriesterase.* Phosphotriesterase (50 nM) was incubated at 25 °C in 0.1 M PIPES, pH 7.0, containing 3% $CH_3CN$/1 mg/mL BSA in the presence of 0.042 mM 1-hexynyl diethyl phosphate ($I/E_0$ = 840:1), for 5 min. A 50 $\mu$L aliquot was diluted into 50 $\mu$L of buffer at a different pH to a final concentration of 0.25 M with 100 $\mu$M $CoCl_2$ and 1 mg/mL BSA. The buffers used were PIPES (pH 7.0), HEPES (pH 8.0), CHES (pH 9.0), and CHES (pH 10.0). A 20 $\mu$L aliquot was removed at various times (from 10 s to 20 h) and assayed. The

recovered activity was measured by comparing the activity of an enzyme solution containing no inhibitor with the activity of an enzyme solution containing inhibitor at the same time point.

*UV Difference Spectroscopy.* Phosphotriesterase (24 $\mu$M) was incubated with 0.024 M 1-hexynyl diethyl phosphate ($I/E_0$ = 1000:1) in 50 mM PIPES, pH 7.0/3% $CH_3CN$ in a total volume of 1 mL at 25 °C for 15 min to ensure complete inactivation of the enzyme. After incubation, the solution was centrifuged for 10 min at 14 000 rpm to remove any precipitate. The reaction solution (1 mL) was then loaded onto a PD-10 gel filtration column (Pharmacia) equilibrated with 50 mM PIPES, pH 7.0/3% $CH_3CN$, and the protein fraction was eluted with the same buffer. The protein fraction was loaded and eluted on the PD-10 column once more to ensure that any unbound small molecules were removed from the protein fraction. A control reaction was run in which the inhibitor was omitted and was treated identically to the inactivation reaction. UV spectra of the inactivation and control reactions were recorded on a Cary 2200 UV−visible spectrophotometer between 220 and 340 nm and stored digitally. Difference spectra were obtained by normalizing the two spectra at 280 nm and then subtracting the control spectrum from the spectrum of the inactivated enzyme.

## RESULTS

*Partition Ratio Determination.* Incubation of the phosphotriesterase with the alkynyl phosphate esters **I**−**V** at an inhibitor:enzyme ratio greater than 5000:1 resulted in the loss of >99% of all enzyme activity within 1 min. The efficiency of enzyme inactivation by the alkynyl phosphate esters was determined by incubating a fixed concentration of enzyme with increasing amounts of inhibitor. The relative activity ($A/A_0$) remaining after 15 min of incubation was plotted versus the ratio of inhibitor to enzyme ($I/E_0$). Extrapolation of the data in these plots to the horizontal axis gave the partition ratio, or the number of inactivator molecules that are hydrolyzed and released as product before the enzyme inactivation is complete (Knight & Waley, 1985). A representative plot for the inactivation of the phosphotriesterase by the hexynyl diethyl phosphate ester (**I**) is presented in Figure 1. The partition ratios for 1-hexynyl diethyl phosphate (**I**), 1-propynyl diethyl phosphate (**II**), 1-hexynyl diphenyl phosphate (**III**), 1-hexynyl dimethyl phosphate (**IV**), and ethynyl diethyl phosphate (**V**) were determined to be 500, 1700, 480, 1700, and 1200, respectively.




*Protection from Inhibition by a Nucleophilic Scavenger.* Azide was added to the inhibition reaction mixture in an attempt to intercept any reactive intermediate generated by the enzymatic hydrolysis of the alkynyl phosphate esters that was released directly into solution. If the extent of inactivation is reduced in the presence of the added nucleophile, then it is likely that the reactive electrophile produced from the inhibitor is released from the enzyme surface prior to covalent labeling of the protein (see Scheme 4). In the case of two phosphotriesterase inactivators [1-hexynyl diethyl phosphate (**I**) and ethynyl diethyl phosphate (**V**)] there was no protection against inactivation up to 200 mM $NaN_3$ (data not shown).

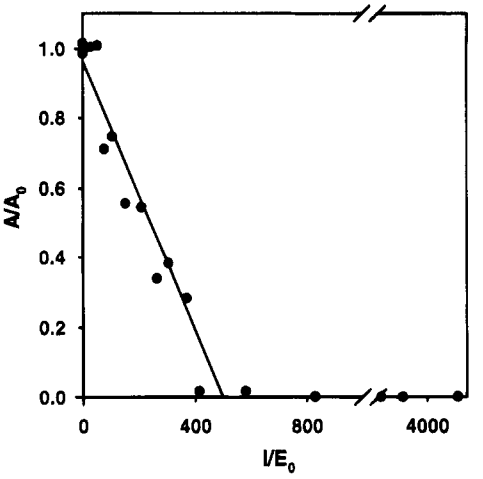



FIGURE 1: Inactivation of phosphotriesterase with variable amounts of 1-hexynyl diethyl phosphate. The partition ratio is 500.

Hydroxylamine was also used as a nucleophilic scavenger. When phosphotriesterase, $NH_2OH$, and alkynyl phosphate ester (**I** or **V**) were incubated together, an increase in the residual enzyme activity was observed as the concentration of $NH_2OH$ was raised. The observed protection against inactivation could be caused by $NH_2OH$ reacting with the putative ketene intermediate that dissociates from the enzyme surface; the nucleophile could also reactivate the inhibited enzyme by attacking the covalent bond that is formed after reaction with the putative ketene intermediate; alternatively, $NH_2OH$ may react chemically with the alkynyl phosphate esters themselves and reduce the effective concentration of the inhibitor. In order to distinguish among these possibilities, two additional experiments were conducted. First, phosphotriesterase and inhibitor (**I**) were incubated together for 15 s, and then $NH_2OH$ was added. Aliquots from the mixture were assayed over a 2 h span. The phosphotriesterase activity increased over time, indicating that $NH_2OH$ does reactivate the dead enzyme. In the second experiment, $NH_2OH$ and inhibitor (**I** or **V**) were preincubated together for a fixed period of time and enzyme was added. As the time interval increased before the addition of enzyme (10−45 s), the inactivation of phosphotriesterase diminished. Therefore, it appears that $NH_2OH$ reacts with both the covalently labeled enzyme and the inhibitor, resulting in net protection of the enzyme from inactivation.

*Rate of Inactivation.* The rate of inactivation of phosphotriesterase by three of the alkynyl phosphate esters was measured with a High-Tech stopped-flow spectrophotometer. In each of these experiments, the ratio of the initial inhibitor concentration to the initial enzyme concentration ($I/E_0$) was kept constant. A solution of enzyme was mixed with an equal volume of a solution containing the alkynyl phosphate ester and paraoxon. The change in absorbance was monitored as a function of time and used as a measure of the loss of catalytic activity. The apparent first order rate

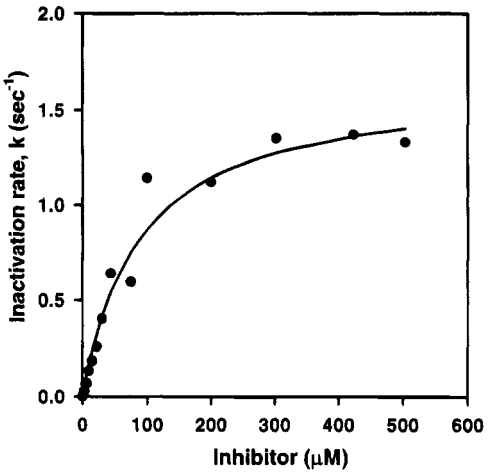



FIGURE 2: Variation in the rate of inactivation versus initial inhibitor concentration (1-hexynyl diethyl phosphate). The data were fit to eq 1. The values calculated for $k_{inact}$ and $K_i$ are 1.7 $s^{-1}$ and 12 $\mu$M, respectively.

constant for enzyme inactivation was plotted versus the initial concentration of inhibitor. The data were fit the following equation (Knight & Waley, 1985):

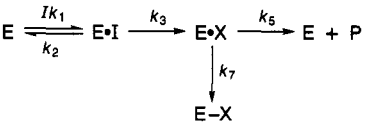
$$k_{obs} = (k_{inact}[I])/([I] + K_i(1 + [A]K_a)) \quad (1)$$

where $k_{obs}$ is the observed first order rate constant for inactivation, $k_{inact}$ is the maximal rate of inactivation at saturating inhibitor, [I] is the initial inhibitor concentration, $K_i$ is the Michaelis constant for *I*, [A] is the paraoxon concentration, and $K_a$ is the Michaelis constant for paraoxon. A representative plot for the inactivation of the enzyme with the hexynyl phosphate ester **I** is shown in Figure 2. The calculated values for $k_{inact}$ and $K_i$ are 1.7 $s^{-1}$ and 12 $\mu$M, respectively. Similar experiments were conducted with the alkynyl phosphate esters **II** and **IV** (data not shown). The $k_{inact}$ and $K_i$ values for **II** are 1.3 $s^{-1}$ and 34 $\mu$M, respectively, while values of 0.12 $s^{-1}$ and 49 $\mu$M were obtained with compound **IV**.

*Stoichiometry of Inhibitor Bound to Enzyme.* In order to determine the number of inhibitor molecules bound per inactivated enzyme molecule, a radiolabeled inhibitor, [3-$^{14}$C]-1-propynyl diethyl phosphate, was synthesized. The radiolabeled inhibitor was incubated with phosphotriesterase at a 2000:1 inhibitor:enzyme ratio for 30 min to allow for complete inactivation. The excess inhibitor was removed from the protein by gel filtration. A protein assay and radioactivity determination showed that the protein fraction from the gel filtration elution contained 1.59 nmol (58 $\mu$g) of phosphotriesterase and 1.79 nmol (1443 cpm) of $^{14}$C radiolabel, respectively, giving a label to enzyme ratio of 1.1:1.0. Attempts to isolate a radioactive peptide from the inactivated enzyme were unsuccessful.

*Reactivation of Inactivated Phosphotriesterase.* Attempts were made to reactivate the wild-type phosphotriesterase after it had been inactivated with 1-hexynyl diethyl phosphate by changing the pH of the enzyme solution. The inactivated enzyme was diluted into buffers at pH values of 7.0, 8.0, 9.0 and 10.0. Aliquots were removed and assayed to determine if any activity had been recovered. At pH 7.0, no significant activity was regained after 10 h. However, over 80% of the activity was regained after 7 h at pH 8.0, after 3 h at pH 9.0, and after 1.5 h at pH 10.0.

Scheme 5




*UV Difference Spectroscopy.* The UV spectrum of phosphotriesterase inactivated by 1-hexynyl diethyl phosphate shows an increase in absorbance in the region between 235 and 270 nm compared to unmodified phosphotriesterase. Any unbound small molecules were removed from the protein by gel filtration, and thus, the resulting spectrum of the inactivated enzyme is due to covalent modification of phosphotriesterase. The difference spectrum of the unmodified phosphotriesterase and modified phosphotriesterase has an absorption band centered at 245 nm ($\epsilon$ = 4400 $M^{-1}$ $cm^{-1}$).

## DISCUSSION

*Efficiency of Inactivation of Wild-Type Phosphotriesterase by Alkynyl Phosphate Esters.* The partition ratio of an inhibitor describes how efficiently it inactivates an enzyme. The kinetics of this inactivation reaction can be described by a mechanism that is illustrated in Scheme 5, where E is the enzyme, I is the inhibitor, E•I is the initial noncovalent enzyme-inhibitor complex, and E•X is the noncovalent enzyme-activated inhibitor species. The activated inhibitor species can either react with the enzyme to produce irreversibly inhibited enzyme or dissociate to yield free enzyme and product, P. The value of $k_5/k_7$ is equal to the partition ratio. The partition ratio depends on the reactivity of the activated intermediate, its rate of diffusion from the active site, and the proximity of the electrophile on the enzyme for covalent bond formation (Silverman, 1988).

The partition ratios of **I**–**V** fall in the range between 480 and 1700. It appears that all five alkynyl phosphate esters work equally well as inactivators of phosphotriesterase despite the difference in their structures. However, it should be noted that the larger compounds (i.e., **I** and **III**) are slightly more efficient inactivators, showing partition ratios at the lower end of the range compared to the smaller ones.

The rate expression for enzyme inactivation, derived for the mechanism shown in Scheme 5, is $k_{inact} = (k_3k_7)/(k_3 + k_5 + k_7)$. Since $k_5 \gg k_7$ for **I**, **II**, and **IV**, the minimum value of the rate constant for the reaction of the electrophilic ketene intermediate with the enzyme is 1.7 $s^{-1}$ for **I**, 1.3 $s^{-1}$ for **II**, and 0.12 $s^{-1}$ for **IV**. Thus, the minimum values of the rate constants for either P–O bond cleavage or product release ($k_3$ and $k_5$) for **I**, **II**, and **IV** are 850 $s^{-1}$, 2210 $s^{-1}$, and 204 $s^{-1}$, respectively. The $k_{cat}$ for paraoxon, one of the fastest substrates of phosphotriesterase, is 7800 $s^{-1}$ for the Co-substituted enzyme (Kuo & Raushel, 1994). The rate constants determined for $k_3$ and $k_5$ compare quite well with the $k_{cat}$; hence these alkynyl phosphate esters are good substrates for phosphotriesterase.

*Alkynyl Phosphate Esters Are Excellent Mechanism-Based Inhibitors of Phosphotriesterase.* It has been reported in a preliminary communication that the alkynyl phosphate ester **I** is a mechanism-based inhibitor of bacterial phosphotriesterase (Blankenship et al., 1991). Compound **I** acts to irreversibly inactivate phosphotriesterase by formation of a covalent adduct with the enzyme, which results from reaction

of an active site nucleophile with the putative reactive ketene generated when the P−O bond of the compound is hydrolyzed. The stoichiometry of inhibitor bound to the enzyme is 1:1, as determined by using the radiolabeled compound [3-$^{14}$C]-1-propynyl diethyl phosphate. Therefore, the modification of the protein with a single label is sufficient to completely inactivate the enzyme. However, it cannot be determined, with the available data, whether a single unique site on the protein is labeled or whether two, or more, mutually exclusive sites react with the intermediate.

Addition of an exogenous nucleophile, azide, did not protect the phosphotriesterase from being inactivated by the alkynyl phosphate esters. If protection had been observed, it would have been due to the reactive intermediate escaping into solution and being intercepted by the scavenging nucleophile (see Scheme 4). In the present case, the inactivation event occurs prior to the release of the reactive ketene into solution. Hydroxylamine was also tested as a scavenging nucleophile, and protection from inactivation was observed. However, subsequent experiments demonstrated that the protection was due to hydroxylamine reacting with the covalently modified enzyme and also with the inhibitor. On the basis of all of these criteria, the alkynyl phosphate esters are, by definition, mechanism-based inactivators of bacterial phosphotriesterase (Silverman, 1988).

*Evidence for the Modification of a Histidine Residue by Alkynyl Phosphate Esters.* Of the twenty natural amino acids found in proteins, only nine are potentially nucleophilic and can be considered as candidates for acylation by the mechanism-based inactivator. It has been shown that the inactivated phosphotriesterase can be reactivated by increasing the pH of the protein solution from 7.0 to 10.0, implying that the covalent bond formed with the inhibitor can be hydrolyzed at alkaline pH values. *N*-Acylimidazoles are known to be easily hydrolyzed at alkaline pH (Fife, 1965), suggesting that a histidine is a likely candidate for modification in the inactivation reaction. Changes are observed in the UV spectrum of phosphotriesterase after it is inactivated by 1-hexynyl diethyl phosphate. The difference spectrum between unmodified and modified phosphotriesterase shows an absorption band at 245 nm ($\epsilon = 4400\ M^{-1}\ cm^{-1}$), Figure 3. Modification of only three nucleophilic amino acid residues, cysteine, tyrosine, and histidine, can result in changes in the UV spectrum. When the thiol group of cysteine is ionized in a protein, an increase in absorption is observed in the UV region of the protein spectrum between 235 and 240 nm ($\epsilon_{239\ nm} = 4500\ M^{-1}\ cm^{-1}$) (Graminski et al., 1989). Thus if the cysteine is modified, forming a thioester, the absorption in this range should decrease. If the phenol side chain of a tyrosine residue in a protein is acetylated to form *O*-acetyltyrosine, a decrease in absorption between 250 and 300 nm is seen ($\epsilon_{278\ nm} = 1160\ M^{-1}\ cm^{-1}$) (Riordan & Vallee, 1972). The possibility of either a cysteine or tyrosine residue being modified in phosphotriesterase can thus be ruled out since the difference spectrum clearly shows an increase in absorption between 230 and 270 nm. The acylation of the imidazole side chain of a histidine residue should also produce a change in the UV spectrum, by comparison with *N*-acetylimidazole, the corresponding model compound of an acylated histidine residue. The difference spectrum between *N*-acetylimidazole and imidazole has an absorption band at 243 nm ($\epsilon = 2300\ M^{-1}\ cm^{-1}$). The difference spectrum between modified phosphotriesterase and unmodified phosphotriesterase shows an absorption band centered in the same region (245 nm). The similarities in the properties of the inactivated enzyme compared with *N*-acetylimidazole indicate that a histidine is the residue modified in the protein.

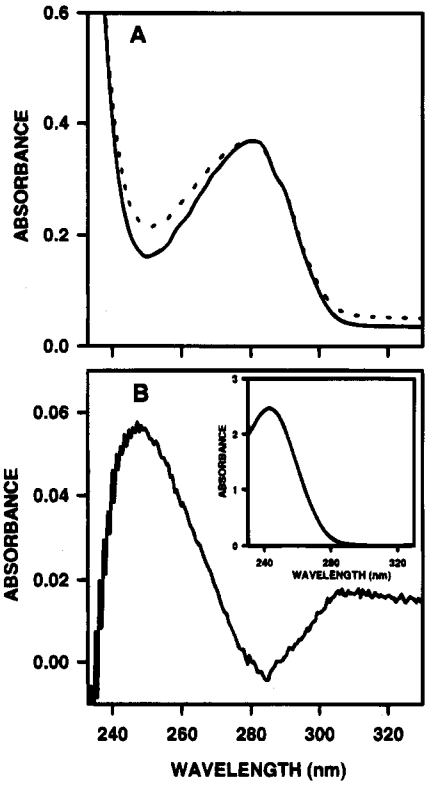



FIGURE 3: (A) Normalized spectra of unmodified phosphotriesterase (—) and phosphotriesterase inactivated by 1-hexynyl diethyl phosphate (•••). (B) Difference spectrum between modified and unmodified phosphotriesterase. The inset shows the UV spectrum of *N*-acetylimidazole.

*Summary.* Various alkynyl phosphate esters were synthesized and tested for their efficacy as mechanism-based inhibitors of phosphotriesterase and were shown to be highly reactive as such. This is one of the first examples of a mechanism-based inhibitor for a phosphatase of any type to be reported.[2] The enzyme is covalently labeled in a 1:1 stoichiometry with inhibitor, resulting in complete inactivation. Chemical and spectroscopic evidence is presented that is consistent with the modified residue being a histidine.

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[2] A mechanism-based inhibitor of prostatic acid phosphatase has been reported (Myers et al., 1993).

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