Synthesis and ^{31}P Chemical Shift Identification of Tripeptide Active Site Models That Represent Human Serum Acetylcholinesterase Covalently Modified at Serine by Certain Organophosphates

Charles M. Thompson,* Alirica I. Suarez, and Oscar P. Rodriguez

Department of Chemistry, University of Montana, Missoula, Montana 59812

Received June 14, 1996[⊗]

Most organophosphorus (OP) insecticides impart their toxic action via inhibition of cholinesterases by reacting at an essential serine hydroxyl group. The inhibition process is dependent upon the reactivity, stereochemistry, leaving group, and the mechanism of phosphorylation and/or reactivation (or aging) inherent to the OP compound under consideration. Because a wide array of phosphorylated structures are possible following inhibition by an OP, a simple model system was sought to investigate the mechanistic details of these and related reactions. In the present study, the tripeptide N-CBZ-Glu-Ser(OH)-Ala-OEt (chosen as a truncated form of human serum cholinesterase) was chemically modified at the serine hydroxyl group by various *O*-methyl phosphate groups and the ^{31}P NMR chemical shift recorded. Six tripeptides, representing (a) phosphorylation by dimethyl phosphorothionates (N-CBZ-Glu-Ser[O-P(S)(OMe)$_2$]Ala-OEt; **5**), (b) phosphorylation by dimethyl phosphates (N-CBZ-Glu-Ser[O-P(O)(OMe)$_2$]Ala-OEt; **6**), (c) phosphorylation by *O*,*S*-dimethyl phosphorothiolates (N-CBZ-Glu-Ser[O-P(O)(OMe)(SMe)]Ala-OEt; **7**), (d) aging following inhibition by dimethyl phosphorothionates (N-CBZ-Glu-Ser[O-P(O)(OMe)(S$^-$)]Ala-OEt; **8**), (e) aging following inhibition by dimethyl phosphates (N-CBZ-Glu-Ser[O-P(O)(OMe)(O$^-$)]Ala-OEt; **9**), and (f) phosphorylation by (R/S)$_P$$S_C$-isomalathion stereoisomers (N-CBZ-Glu-Ser[O-P(O)(OMe)(SCH(CO$_2$CO$_2$Et)CH$_2$-CO$_2$Et)]Ala-OEt; **10**) have been synthesized. Tripeptides **5** and **6** were prepared via preliminary formation of an intermediate tripeptide phosphite followed by direct conversion to **5** using S_8 or to **6** with m-CPBA, respectively. Tripeptides **8** and **9** were prepared by dealkylation of **5** and **6**, respectively. Tripeptides **7** and **10** were prepared by reaction of **8** with dimethyl sulfate and (*R*)- or (*S*)-diethyl (trifluoromethanesulfonyl)malate, respectively.

Introduction

Most organophosphate (OP)[1] insecticides are toxic to target and nontarget organisms by virtue of covalent phosphorylation of cholinesterase(s) at an essential serine residue. Once modified at this residue by the OP, cholinesterase is unable to hydrolyze essential neurotransmitters leading to neurochemical imbalances (*1*). The mechanism of cholinesterase inactivation by OP agents occurs with the ejection of a leaving group Z synchronous with formation of a phosphoserine linkage (eq 1). To be a potent inhibitor of cholinesterase, an OP compound usually contains a phosphoryl π-bond (P═O) and Z must be a good leaving group (*2*−*4*).

Several postinhibitory outcomes are also possible for OP-inhibited cholinesterase including reactivation (eq 1), which leads to restoration of all or part of the cholinesterase activity following hydrolysis with water (as OH$^-$) or certain oxime antidotes (e.g., 2-pyridine aldoxime methiodide; 2-PAM). It is generally accepted that the restoration of cholinesterase activity is accompanied by scission of the serine−phosphate linkage. OP-inhibited cholinesterase may also undergo "aging" or cleavage of a phosphate-ester bond (P−O−R) or "nonreactivation" pathways (eq 1). Whereas an aging mechanism results from cleavage of a phosphorus ester (P−O−R) bond to give a phosphate anion (*5*), the non-reactivation mechanism(s) are less well characterized and possibly due to changes in the protein structure that lead to an irreversibly inactivated enzyme. Although the aging mechanism is responsible for the inactivation of neuropathy target esterase leading to OP-induced delayed neuropathy (*6*), cholinesterase aging and nonreactivation mechanisms both lead to sustained acetylcholine levels that may be mistaken for simple inhibition. Since the ill health effects and treatment of aging and nonreactivation of cholinesterase differ from the treatment of straightforward inhibition, recognition of the correct mechanism is important for clinical treatment. Moreover, the rate and extent to which a particular OP agent causes an irreversible, postinhibitory reaction to occur likely correlate with its delayed toxic action.

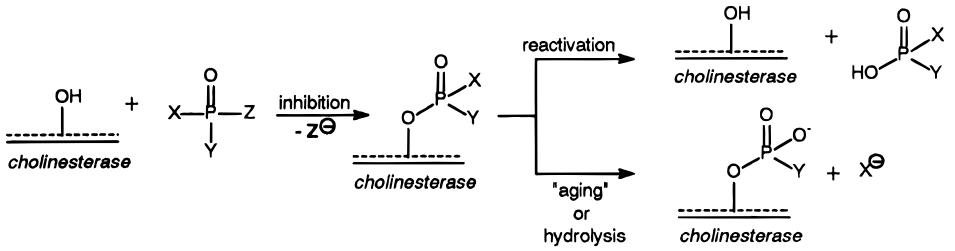
Most postinhibitory mechanisms and their toxicological consequences have been less studied than OP inhibition mechanisms, but the primary chemical alterations caused to the active site peptide structure must necessarily preface protein structural changes. The initial alterations at the active site may involve localized changes to peptide conformation, charge repulsion (e.g., with

* Corresponding author: Prof. Charles M. Thompson, Department of Chemistry, University of Montana, Missoula, MT 59812; Telephone/FAX: (406) 243-4643; e-mail: cmthomp@selway.umt.edu.

[⊗] Abstract published in *Advance ACS Abstracts*, November 1, 1996.

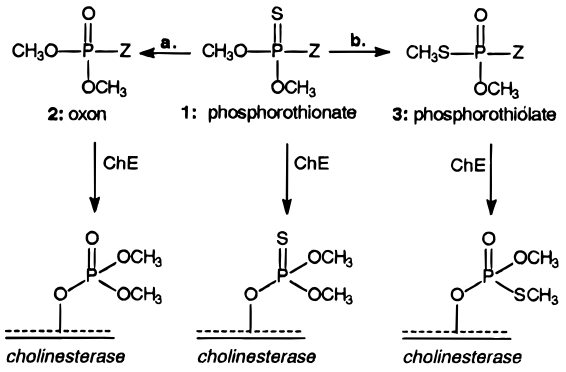
[1] Abbreviations: OP, organophosphate; 2-PAM, 2-pyridine aldoxime methiodide; TMS, tetramethylsilane; PMA, phosphomolybdic acid; DBQ, dibromoquinone 4-chloroimide; CBZ, carbobenzyloxy; m-CPBA, *m*-chloroperoxybenzoic acid; PEX, potassium ethyl xanthate; TEA, triethylamine; DCC, dicyclohexylcarbodiimide; DIPC, diisopropylcarbodiimide; ((iPr)$_2$N)$_2$P-OMe, bis-*N*,*N*-(diisopropylamino)methoxyphosphine; ((nPr)$_2$N)P(OMe)$_2$, *N*,*N*-(di-*n*-propylamino)bismethoxyphosphine; DMS, dimethyl sulfate.

S0893-228x(96)00097-5 CCC: $12.00



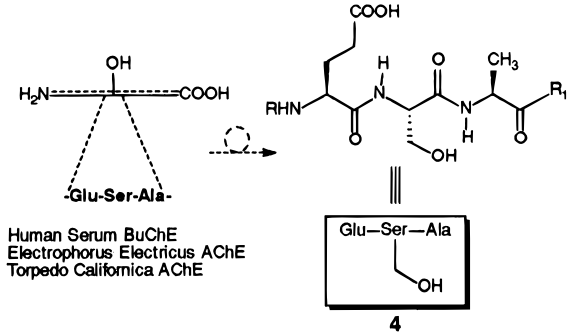
(1)

Scheme 1. Phosphorothionates, Oxons, and Phosphorothiolates and Their Mechanism of Cholinesterase Inhibition



a. oxidation, b. isomerization

Scheme 2. Expansion of the Active Site Region of Certain Cholinesterases That Adjoin the Catalytic Serine Residue



aging), or steric repulsion imposed by the phosphoryl group, all or any of which may cause disruption in the orientation of the catalytic residues. Although the cholinergic system is likely restored through protein synthesis, the toxicological role of the irreversibly OP-inhibited cholinesterase remains largely unknown. In light of the numerous delayed neuropathic maladies that cannot be explained by simple cholinesterase inhibition by OP agents, an investigation of postinhibitory mechanisms would be beneficial, particularly studies that identify OP agents that lead to irreversible inhibition.

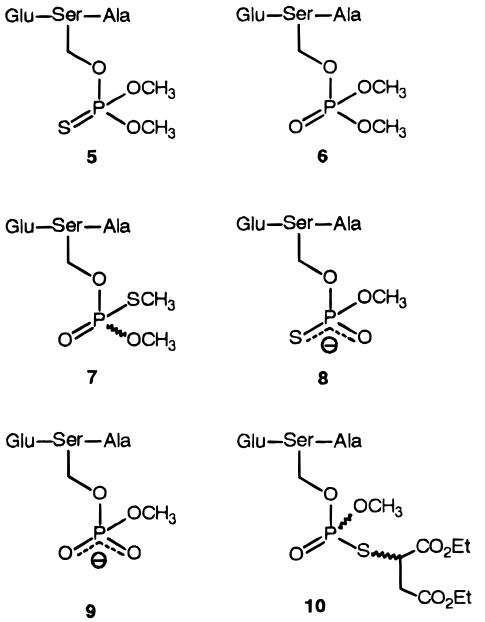
A number of commercial OP insecticides are *O,O*-dimethyl phosphorothionates $(MeO)_2P(S)Z$ (**1**), a compound class that includes malathion (**1**; Z = −SCH(CO_2-Et)CH_2CO_2Et) and parathion methyl (**1**; Z = −OPh-4-NO_2). Prior to reaction with cholinesterase, most phosphorothionates must be transformed from the relatively unreactive phosphorothionate form (**1**; P═S) into the more reactive oxon form (**2**; P═O) (Scheme 1) (*7*). Oxons react with cholinesterases to afford *O,O*-dimethyl phosphorylated enzymes via ejection of the leaving group Z to give *O,O*-dimethyl phosphate inhibited ChE representing the major mechanism of action of phosphorothionate insecticides. Alternatively, *O,O*-dimethyl phosphorothionates **1** may isomerize to form phosphorothiolates (**3**) (Scheme 1), a process that occurs during the manufacture, storage, or environmental lifetime of the OP (*8*−*10*). Because a P═O bond is formed during the isomerization, phosphorothiolates (**3**) like oxons are more potent anticholinesterase agents than thionates (*11*). Phosphorothiolates differ from oxons in their reaction with cholinesterases in that an *O,S*-dimethyl phosphorylated product is likely to result (Scheme 1) (*12*) although ejection of the thiomethyl (MeS-) rather than the Z group is a possible alternative mechanism (not shown). The inhibition of cholinesterase by the parent phosphorothionate (**1**) is also shown in Scheme 1 but is a poor reaction.

From this introduction, it is evident that a number of OP-modified cholinesterase species are possible even when a limited class of common OP inhibitors is considered. Therefore, determination of an individual phosphorylation mechanism is relatively complex and further complicated by the usual difficulties associated with the analysis of high molecular weight proteins. As such, more simplified model systems would help to decipher inhibition and reactivation pathways.

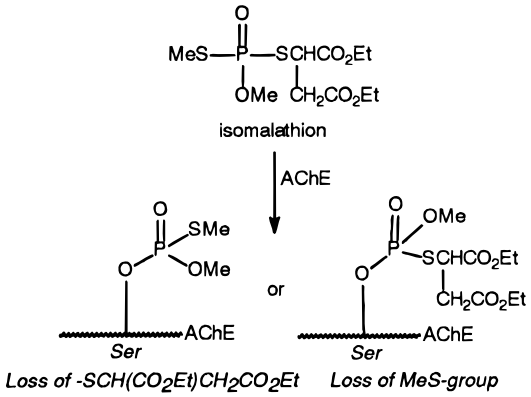
The local peptide sequence neighboring the essential serine residue at the active site of certain cholinesterases (e.g., *Torpedo californica* AChE and human serum butyrylcholinesterase) and select serine proteases show homology as a Glu-Ser-Ala tripeptide (Scheme 2) (*1*). Since human serum cholinesterase is a likely primary target in mammalian OP poisoning episodes (*4*, *13*, *14*), the Glu-Ser-Ala tripeptide sequence should be an excellent template and starting point to study mechanisms of cholinesterase inhibition and recovery. OP-modified Glu-Ser-Ala sequences could be used to evaluate local changes in peptide structure and determine inhibition and postinhibitory mechanisms that would not be possible with intact protein. In addition to their utility as mechanistic probes of cholinesterase intoxication by certain OP agents, modified Glu-Ser-Ala tripeptides could also serve as spectral and biochemical markers, as mechanistic probes, and as potential conjugates for immunochemical assays.

With this preliminary aim, we first undertook the synthesis of select Glu-Ser-Ala tripeptides that have been modified at the serine hydroxyl by phosphorus-containing groups. Six phosphorylated tripeptides **5**−**10** were selected to mimic covalently modified cholinesterase resulting from inhibition by various dimethyl phosphates and phosphorothionates (Chart 1). Tripeptides **5**, **6**, and **7** represent inhibition of cholinesterase by *O,O*-dimethyl phosphorothionates **1** (e.g., parathion methyl, malathion), *O,O*-dimethyl phosphates **2** (e.g., paraoxon, malaoxon), and *O,S*-dimethyl phosphorothiolates **3** (e.g., isoparathion methyl, isomalathion), respectively. Tripeptides **8** and **9** depict postinhibitory reactions that occur following reaction of cholinesterase with an *O,O*-dimethyl phos-

Chart 1. Phosphorylated Tripeptides Representing Cholinesterase Modified by Organophosphates



phorothionate **1** and *O,O*-dimethyl phosphate **2** (oxon), respectively. Tripeptide **10** is one of two model structures representing the inhibition of cholinesterase by the stereoisomers of isomalathion (**3**; -SCH(CO_2Et)CH_2CO_2Et). Based on comparative kinetic analyses, we previously found that the stereoisomers of isomalathion inhibit rat brain acetylcholinesterase via two different mechanisms, namely, ejection of the -SCH(CO_2Et)CH_2CO_2Et or the -SCH_3 group (eq 2), the latter leading to irreversibly



(2)

inhibited enzyme (*12*, *15*−*17*). The supposition that dual mechanisms were operative was based upon the ready reactivation of acetylcholinesterase following inhibition by either of the (R_P)-isomalathion stereoisomers yet virtual nonreactivation following inhibition by either of the (S_P)-isomalathion stereoisomers (*17*). To confirm this mechanistic anomaly, two distinct tripeptide models representing the ejection of the -S(CHCO$_2$Et)CH_2CO_2Et group (tripeptide **7**) and ejection of the -SCH_3 (tripeptide **10**) are needed.

In this paper, we report full experimental procedures for the synthesis of the native Glu-Ser-Ala tripeptide, OP-modified tripeptides **5**−**10**, and a brief comparative ^{31}P chemical shift analysis of the phosphorylated tripeptides. As precise representations of the cholinesterase primary structure, the spectral features of the proposed OP-modified Glu-Ser-Ala tripeptides are likely to match fragments obtained via denaturation or digestion of intact protein.

Experimental Section

Melting points were determined on a Mel-Temp melting point apparatus and are uncorrected. ^{1}H, ^{13}C, and ^{31}P were taken at 400, 100.6, and 161.9 MHz, respectively. ^{1}H and ^{13}C NMR spectra were taken in D_2O or $CDCl_3$ using 0.05% dioxane or tetramethylsilane (TMS) as internal standards, respectively. ^{31}P NMR spectra were taken in the indicated deuterated solvents using 85% phosphoric acid as an external standard.

Analytical thin-layer chromatography (TLC) was conducted on E. Merck aluminum-backed, 0.2 mm silica gel 60F_{254} plates. Visualization was accomplished with an ultraviolet (UV) lamp and/or ninhydrin, phosphomolybdic acid (PMA), dibromoquinone 4-chloroimide (DBQ), or ammonium molybdate staining reagents. All solvents and reagents were purified by standard literature methods. Air- or moisture-sensitive reactions were conducted under an argon atmosphere by utilizing standard techniques. Flash chromatography was done on Kieselgel 60, 230−400 mesh silica gel in the solvent(s) indicated. Commercially available reagents, amino acids (only L-amino acids used in this study), and solvents were purified when necessary by standard literature methods. Elemental analyses were performed by Midwest Microlab Ltd., Indianapolis, IN. Optical rotations were recorded (Na lamp) at room temperature in the specified solvents using a Perkin Elmer Model 241 polarimeter.

Caution: *The OP compounds used in this study are hazardous and should be handled in a well-ventilated hood by trained personnel. These materials may be destroyed by stirring with 1 N NaOH overnight.*

[*N*-(Benzyloxycarbonyl)glutamyl-γ-methyl ester]serylalanine Ethyl Ester (4). Step 1. To a 100 mL, two-neck flask fitted with a stir bar, septum, and gas inlet was added a solution of CBZ-serylalanine ethyl ester (**11**) (1 g, 2.9 mmol) in 20 mL of absolute ethanol followed by 10% Pd/C (25 mg). The flask was degassed while stirred (using a water aspirator) and purged with argon thrice, degassed again, and finally reacted with H_2 via a balloon. The reaction was maintained at ambient balloon H_2 pressure for 3−4 h, replacing the H_2 in the balloon at 1 h intervals. When the reaction showed no starting material remained, the reaction mixture was evacuated of hydrogen, flushed with nitrogen, and filtered through a 0.5 cm pad of Celite, and the solvent evaporated to afford serylalanine ethyl ester as a light yellow semisolid. This material was used directly in the next step but recrystallization (small scale) from absolute ethanol−diethyl ether afforded white crystals for spectral characterization. Yield = 72%; mp = 71−73 °C. ^{1}H NMR ($CDCl_3$): δ 1.25 (3 H, t), 1.47 (3 H, t), 2.0−2.3 (3 H, br s), 3.43 (1 H, t), 3.66−3.72 (2 H, q), 3.76−3.82 (1 H, q), 4.12−4.21 (2 H, q), 4.46−4.56 (1 H, m), 7.76 (1 H, d).

Step 2. Serylalanine ethyl ester (1 g, 4.23 mmol) and DCC (0.86 g, 4.23 mmol) were dissolved in 30 mL of freshly distilled CH_2Cl_2 and chilled to 0 °C. CBZ-glutamic acid 5-methyl ester (1.18 g, 4.23 mmol) was added, and the reaction mixture was stirred under argon for 4 h at 0 °C. Upon completion (as determined by TLC), the mixture was filtered through a fritted funnel containing a 1 cm layer of Celite, and the resulting solution was carefully neutralized to pH 8 by the addition of 5% sodium bicarbonate. The organic layer was separated and dried over anhydrous magnesium sulfate, the solvent was evaporated *in vacuo*, and the crude mixture was chromatographed on silica gel using a 3:1 mixture EtOAc/petroleum ether to afford **4** as a white powder. Yield = 56%; mp = 120−121 °C; $[\alpha]^{23}_D$ = −15.66° (*c* 1.10, CH_2Cl_2). ^{1}H NMR ($CDCl_3$): δ 1.20 (3 H, t, J = 7.1 Hz), 1.35 (3 H, d, J = 7.2 Hz), 1.71 (1 H, dt, J = 7.1, 6.9 Hz), 1.90−2.11 (1 H, dt, J = 7.6, 6.2 Hz), 2.15 (2 H, t, J = 7.6 Hz), 2.38−2.42 (2 H, m), 3.61 (3 H, s), 3.72 (1 H, q, J = 7.8 Hz), 4.12 (1 H, q, J = 7.6 Hz), 4.35 (1 H, m), 4.50 (2 H, m), 5.06 (2 H, m), 6.21 (1 H, d, J = 7.6 Hz), 7.29 (5 H, m), 7.43 (1 H, d). ^{13}C NMR ($CDCl_3$): δ 14.1, 17.6, 23.3, 27.9, 30.0, 33.7, 42.3,

48.4, 51.8, 54.4, 61.6, 62.7, 67.0, 127.9, 128.1, 128.4, 136.0, 156.4, 170.0, 171.9, 172.8, 173.7. Anal. Calcd for $C_{22}H_{31}N_3O_9$: C, 54.87; H, 6.49; N, 8.72. Found: C, 55.10; H, 6.55; N, 8.63.

[*N*-(Benzyloxycarbonyl)glutamyl-γ-methyl ester]seryl-*O*-[*O*,*O*-dimethylthiophosphoryl]alanine Ethyl Ester (5). Tripeptide **4** (0.3 g, 0.58 mmol) was dissolved in 50 mL of freshly distilled CH_2Cl_2 and chilled to 0 °C. Tetrazole (0.14 g, 2 mmol) was added, and the reaction was stirred for 0.5 h, whereupon dimethoxy *N*,*N*-di-*n*-propylaminophosphite (0.15 g, 0.76 mmol) was added and the ice bath removed. Stirring was continued for 0.5 h or until TLC showed consumption of the starting material. The reaction mixture was concentrated *in vacuo*, and the residue was suspended in 25 mL of dry toluene. Freshly recrystallized elemental sulfur (700 mg) was added, and the reaction was stirred under an argon atmosphere for 48 h at room temperature. The mixture was diluted with 30 mL of ether, the excess sulfur filtered through a pad of Celite, the solvent evaporated *in vacuo*, and the crude product purified by flash chromatography using ether to afford **5** as a white powder. Yield = 81%; mp = 118−119 °C; $[\alpha]^{23}_D = -13.9$ (*c* 0.24, $CHCl_3$). ^{1}H NMR ($CDCl_3$): δ 1.23 (3 H, t, J = 7.1 Hz), 1.35 (3 H, d, J = 7.2 Hz), 1.99−2.03 (1 H, m), 2.14−2.21 (1 H, m), 2.43−2.50 (2 H, m), 3.64 (3 H, s), 3.66 (3 H, d, J = 14.2 Hz), 3.71 (3 H, d, J = 14.2 Hz), 4.12−4.20 (1 H, m), 4.43−4.50 (1 H, m), 4.72−4.76 (1 H, m), 5.08 (2 H, s), 6.02 (1 H, d, J = 7.6 Hz), 7.28−7.31 (5 H, m). ^{13}C NMR ($CDCl_3$): δ 14.2, 18.0, 27.3, 30.4, 48.6, 52.1, 53.0, 53.1, 54.9, 55.0, 55.2, 61.5, 67.2, 119.3, 127.7, 127.8, 127.9, 128.2, 128.4, 128.5, 135.8, 167.4, 171.2, 172.1. ^{31}P NMR ($CDCl_3$): δ 72.8. Anal. Calcd for $C_{24}H_{36}N_3PO_{11}S$: C, 47.60; H, 5.99; N, 6.94. Found: C, 47.75; H, 6.00; N, 6.84.

[*N*-(Benzyloxycarbonyl)glutamyl-γ-methyl ester]seryl-*O*-[*O*,*O*-dimethylphosphoryl]alanine Ethyl Ester (6). To a stirred, room temperature solution of tripeptide **4** (0.25 g, 0.5 mmol) in 25 mL of dry CH_2Cl_2 were added tetrazole (0.07 g, 1 mmol) and *N*,*N*-di-*n*-propylbismethoxyphosphine (0.15 g, 0.77 mmol). The phosphitylation step was stirred for 1 h, and m-CPBA (0.25 g, 1.45 mmol) was added. Stirring was continued approximately 1 h or until TLC indicated that the oxidation step (conversion to phosphoryl) was complete. The reaction was terminated by the addition of 25 mL of ether and 25 mL of 5% NaOH, and after 0.5 h of stirring, the organic layer was separated, washed with 5% $NaHCO_3$ and brine, and dried with Na_2SO_4. The solution was filtered and evaporated *in vacuo* to yield **6** as a yellow oil. Purification by flash chromatography using 3:1 EtOAc/petroleum ether gave **6** as a white power. Yield = 52%; mp = 104−105 °C; $[\alpha]^{23}_D = -19.50$ (*c* 0.5, $CHCl_3$). ^{1}H NMR ($CDCl_3$): δ 1.24 (3 H, t, J = 7.1 Hz), 1.38 (3 H, d, J = 7.2 Hz), 1.51 (3 H, dt, J = 7.1 Hz), 2.21−2.25 (3 H, dt, J = 7.1 Hz), 2.39−2.54 (2 H, m), 3.65 (3 H, s), 3.74 (3 H, d, J = 11.2 Hz), 3.79 (3 H, d, J = 11.2 Hz), 4.15−4.16 (1 H, q, J = 7.1 Hz), 4.26−4.34 (1 H, m), 5.07−5.09 (2 H, dd, J = 12 Hz), 6.15 (1 H, d, J = 6.6 Hz), 7.35 (5 H, m), 7.72−7.78 (1 H, d, J = 7.9 Hz). ^{13}C NMR ($CDCl_3$): δ 14.5, 18.2, 25.3, 25.9, 30.6, 34.3, 48.8, 49.7, 52.3, 53.6, 55.1, 55.2, 55.3, 61.8, 67.5, 128.4, 128.5, 136.5, 158.2, 168.0, 172.0, 172.5, 173.3. ^{31}P NMR ($CDCl_3$): δ 2.2. Anal. Calcd for $C_{24}H_{36}N_3O_{12}P$: C, 48.90; H, 6.15; N, 7.12. Found: C, 49.07; H, 6.30; N, 7.06.

[*N*-(Benzyloxycarbonyl)glutamyl-γ-methyl ester]seryl-*O*-[*O*,*S*-dimethylphosphoryl]alanine Ethyl Ester (7). To a 100 mL flask, fitted with a condenser, argon inlet/outlet tube, and a stir bar was added **5** (0.2 g, 0.31 mmol) as a solution in 40 mL of anhydrous acetone. Potassium ethyl xanthate (PEX) (0.096 g, 0.6 mmol) was added and the solution heated to reflux for 5 h. The reaction was returned to room temperature, dimethyl sulfate (0.6 mmol) was added, and the reaction was brought to reflux for 1 h. The solution was again returned to room temperature, and the solvent was evaporated *in vacuo* to afford the crude product as a semisolid, which was purified using flash chromatography (100% ethyl acetate) to give **7** as a diastereomeric mixture of products in a near 1:1 ratio. Yield = 56%; mp = 61−62 °C; $[\alpha]^{23}_D = -17.80$ (*c* 0.13, $CHCl_3$). ^{1}H NMR ($CDCl_3$): δ 1.23 (3 H, t, J = 7.1 Hz), 1.36 (3 H, d, J = 7.2 Hz), 2.01 (2 H, m), 2.25 (3 H, d, J = 15.3 Hz), 2.44 (2 H, m), 3.63 (3 H, s), 3.74 (3 H, dd, J = 12.7 Hz), 4.15 (2 H, q, J = 7.1 Hz), 4.26 (2 H, m), 4.46 (2 H, m), 4.79 (1 H, m), 5.08 (2 H, s), 6.02 (1 H, m), 7.24 (1 H, s), 7.31 (5 H, m). ^{13}C NMR ($CDCl_3$): δ 14.1, 17.8, 24.9, 30.1, 30.2, 33.9, 48.5, 51.9, 53.0, 53.1, 53.2, 54.8, 54.9, 61.4, 67.1, 127.8, 127.9, 128.1, 128.5, 136.1, 167.5, 167.6, 171.0, 172.1. ^{31}P NMR: δ 33.2 and 33.6. Anal. Calcd for $C_{24}H_{36}N_3PO_{11}S$: C, 47.60; H, 5.99; N, 6.93. Found: C, 47.40; H, 6.14; N, 7.06.

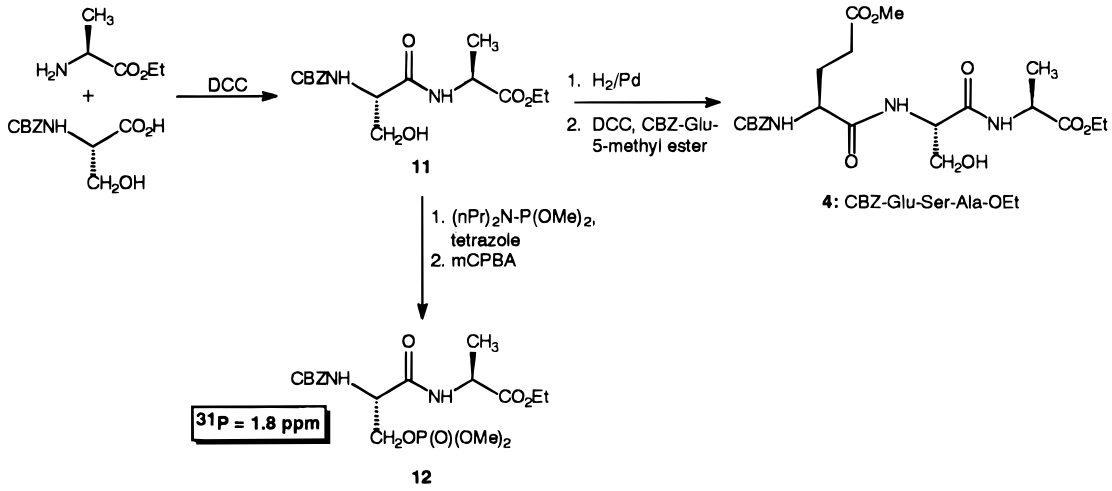
[*N*-(Benzyloxycarbonyl)glutamyl-γ-methyl ester]seryl-*O*-[hydrogen-*O*-methylthiophosphoryl]alanine Ethyl Ester (8, Salt; 13, Free Acid). A solution of tripeptide **5** (0.2 g, 0.315 mmol) and potassium ethyl xanthate (0.096 g, 0.6 mmol) in 25 mL of dry acetone was heated to reflux under argon for 4 h. The solution was cooled to 25 °C and citric acid (0.06 g, 43 mmol) was added. The mixture was stirred for 2 h and filtered, and the solvent removed *in vacuo* to afford a solid material. The product was purified by flash chromatography using 9:1 $CHCl_3$/MeOH to give a 2:1 diastereomeric mixture of **13**. Yield = 80%; mp = 144−145 °C; $[\alpha]^{23}_D = -9.62$ (*c* 0.66, $CHCl_3$). ^{1}H NMR ($CDCl_3$): δ 1.23 (3 H, t, J = 6.0 Hz), 1.39 (3 H, d, J = 6.9 Hz), 2.0−2.4 (2 H, m), 2.82−2.86 (3 H, m), 3.50 (3 H, d, J = 16.5 Hz), 3.57 (3 H, s), 4.14 (2 H, q, J = 7.0 Hz), 4.31−4.60 (4 H, m), 5.06−5.14 (3 H, m), 7.27 (5 H, m). ^{13}C NMR ($CDCl_3$): δ 14.0, 17.5, 29.7, 30.0, 48.7, 51.9, 53.6, 53.7, 54.4, 61.6, 67.5, 128.0, 128.1, 128.5, 135.9, 156.7, 172.4, 172.5, 176.3. ^{31}P NMR ($CDCl_3$): δ 57.6 and 59.5 in a 2:1 ratio, respectively. Anal. Calcd for $C_{23}H_{34}PN_3SO_{10}\cdot H_2O$: C, 45.32; H, 5.95; N, 6.89. Found: C, 45.15; H, 5.52; N, 6.79.

[*N*-(Benzyloxycarbonyl)glutamyl-γ-methyl ester]seryl-*O*-[*O*-sodio,*O*-methylphosphoryl]alanine Ethyl Ester (9). A solution of *O*,*O*-dimethylphosphoryl tripeptide **6** (0.2 g, 0.34 mmol) and sodium iodide (0.05 g, 0.34 mmol) in dry butanone (5 mL) was heated to reflux for 1 h. The solution was allowed to cool to room temperature, the solvent evaporated, and the residue purified by column chromatography using 9:1 CH_2Cl_2/MeOH to give **9** as the sodium salt. Yield = 92%; mp = 112 °C; $[\alpha]^{23}_D = -2.06$ (*c* 0.8, H_2O). ^{1}H NMR ($CHCl_3$): δ 1.18−1.20 (3 H, t, J = 7.0 Hz), 1.37 (3 H, d), 1.8−2.5 (4 H, m), 3.52−3.59 (6 H, m), 4.13 (3 H, q, J = 7.0 Hz), 4.21−4.52 (4 H, m), 4.81−5.22 (3 H, m), 6.2 (1 H, br), 7.26 (5 H, s), 7.6 (1 H, br s), 7.8 (1 H, br s). ^{13}C NMR (90% $CDCl_3$/10% DMSO-d_6): δ 14.0, 17.5, 17.8, 30.1, 30.9, 48.4, 48.5, 52.9, 53.0, 53.1, 54.8, 54.9, 61.4, 67.0, 127.8, 127.9, 128.1, 128.4, 136.10, 167.5, 167.6, 171.7, 172.1. ^{31}P NMR ($CDCl_3$): δ 4.3. Anal. Calcd for $C_{23}H_{33}N_3PO_{12}Na$: C, 46.23; H, 5.57; N, 7.03. Found: C, 45.95; H, 5.72; N, 6.88.

[*N*-(Benzyloxycarbonyl)glutamyl-γ-methyl ester]seryl-*O*-[*O*-methyl-*S*-diethylsuccinylphosphoryl]alanine Ethyl Ester (Diastereomers; 10). To 20 mL of dry THF in a flask fitted with a reflux condenser and nitrogen inlet tube were added tripeptide **5** (0.3 g, 0.47 mmol) and PEX (0.144 g, 0.5 mmol). The mixture was brought to reflux for 4 h, and then cooled to 0 °C whereupon (*S*)-diethyl (trifluoromethanesulfonyl)-succinate (0.156 g, 0.47 mmol) was added as a 10 mL solution in THF and stirred for 1 h at 0 °C. Water (20 mL) was added and the mixture extracted with ether (3 × 25 mL). The ether extracts were combined, washed with brine, separated, and dried over sodium sulfate. Flash chromatography using 100% ethyl acetate affords the pure product as a light yellow wax. Recrystallization from methylene chloride−petroleum ether afforded white crystals of **10.** Yield = 65%; mp = 68−70 °C; $[\alpha]^{23}_D = +20.31$ (0.6, CH_2Cl_2). ^{1}H NMR ($CDCl_3$): δ 1.01 (3 H, d, J = 4 Hz), 1.21 (3 H, m), 1.35 (3 H, m), 2.02 (2 H, m), 2.23 (2 H, m), 2.62 (3 H, m), 2.82−3.08 (3 H, m), 3.4 (1 H, br s), 3.63 (3 H, s), 3.81 (3 H, d). 4.15 (5 H, m), 4.23 (3 H, m), 4.6−4.8 (4 H, m), 5.1 (2 H, m), 5.8 (1 H, br s), 7.25 (5 H, s). ^{13}C NMR ($CDCl_3$): δ 14.05, 14.1, 17.6, 23.4, 27.4, 30.1, 36.1, 36.3, 42.2, 48.4, 51.8, 53.1, 53.2, 54.6, 54.7, 61.4, 66.7,121.4, 127.8, 127.85, 128.0, 128.4, 136.0,167.5, 169.9, 170.0, 170.2, 171.8, 172.1, 173.55. ^{31}P NMR ($CDCl_3$): δ 35.7 and 35.8. Anal. Calcd for $C_{31}H_{46}N_3O_{15}PS$: C, 48.75; H, 6.07; N, 5.50. Found: C, 48.70; H, 6.41; N, 5.90.

***N*-(Benzyloxycarbonyl)serylalanine Ethyl Ester (11) (*18*).** CBZ-serine (3.87 g, 16.2 mmol) and dicyclohexylcarbodiimide (DCC) (3.34 g, 16.2 mmol) were dissolved in 30 mL of CH_2-

Scheme 3. Synthesis of Tripeptide Target and Model Reaction of Dipeptide with Phosphitylating Agents



Cl_2 and chilled to 0 °C. Alanine ethyl ester (16.1 mmol; formed *in situ* from equimolar reaction between alanine ethyl ester hydrochloride and triethylamine in THF) was added as a solution in 2 mL of CH_2Cl_2 and the reaction stirred for 4−5 h at 0 °C. The reaction was monitored by TLC for loss of starting material and then filtered through a 1 cm pad of Celite, and the solvent was removed *in vacuo* to give the crude product as a sticky yellow oil. Purification by flash chromatography using 100% ether afforded white crystals. Yield = 50%; mp = 103−104 °C [lit. mp = 100−101 °C]; $[\alpha]^{23}_D = -26.80$ (*c* 1.22, CH_2Cl_2). ^{1}H NMR ($CDCl_3$): δ 1.22 (3 H, t), 1.24 (3 H, d), 3.7 (2 H, q), 4.0 (1 H, d), 4.1−4.2 (2 H, m), 4.3−4.35 (1 H, m), 4.5−4.55 (1 H, m), 5.1 (2 H, s), 7.3 (5 H, s). ^{13}C NMR ($CDCl_3$): δ 14.0, 17.6, 48.4, 55.5, 61.7, 63.0, 67.2, 128.0, 128.2, 128.3, 128.4, 136.0, 156.4, 170.5, 172.9. Anal. Calcd for $C_{16}H_{22}N_2O_6$: C, 56.78; H, 6.56; N, 8.28. Found: C, 56.65; H, 6.50; N, 8.16.

***N*-(Benzyloxycarbonyl)seryl-*O*-[*O*,*O*-dimethylphosphoryl]alanine Ethyl Ester (12).** To a stirred solution of dipeptide **11** (0.17 g, 0.5 mmol) in 25 mL of dry CH_2Cl_2 were added tetrazole (0.07 g, 1 mmol) and *N*,*N*-di-*n*-propylbismethoxyphosphine (0.15 g, 0.77 mmol). The phosphitylation step was stirred for 1 h and m-CPBA (0.25 g, 1.45 mmol) was added. Stirring was continued approximately 1 h or until TLC indicated that the oxidation step (conversion to phosphoryl) was complete. The reaction was terminated by addition of 25 mL of ether and 25 mL of 5% NaOH, and after 0.5 h of stirring, the organic layer was separated, washed with 5% $NaHCO_3$ and brine, and dried with Na_2SO_4. The solution was filtered and evaporated *in vacuo* to yield **12** as a semisolid. Purification by flash chromatography using EtOAc gave a white power. Yield = 54%. ^{1}H NMR ($CDCl_3$): δ 1.29 (3 H, t, J = 7.1 Hz), 1.41 (3 H, d, J = 7.2 Hz), 3.74 (3 H, d, J = 11.0 Hz), 3.79 (3 H, d, J = 11.1 Hz), 4.1−4.26 (4 H, m), 4.42−4.64 (4 H, m), 5.15 (2 H, s), 6.2 (1 H, brd, J = 7.7 Hz), 7.25 (1 H, brd, J = 6.9 Hz), 7.35 (5 H, s). ^{13}C NMR ($CDCl_3$): δ 14.1, 18.2, 48.4, 54.6, 54.7, 61.4, 67.1, 127.9, 128.0, 128.4, 135.8, 155.9, 167.7, 172.1. ^{31}P NMR ($CDCl_3$): δ 1.8. Anal. Calcd for $C_{18}H_{27}N_2O_9P$: C, 48.43; H, 6.10; N, 6.28. Found: C, 48.14; H, 5.96; N, 6.28.

Results and Discussion

Our synthetic strategy to prepare phosphorylated tripeptides **5**−**10** was partitioned into three stages: (a) preparation of a protected Glu-Ser-Ala tripeptide sequence using traditional carbodiimide-based coupling, (b) chemoselective phosphitylation of the serine hydroxyl group by phosphoramidites (*19*−*21*), and (c) transformation of the resulting phosphitylated tripeptides into the target phosphate and phosphorothioate moieties.

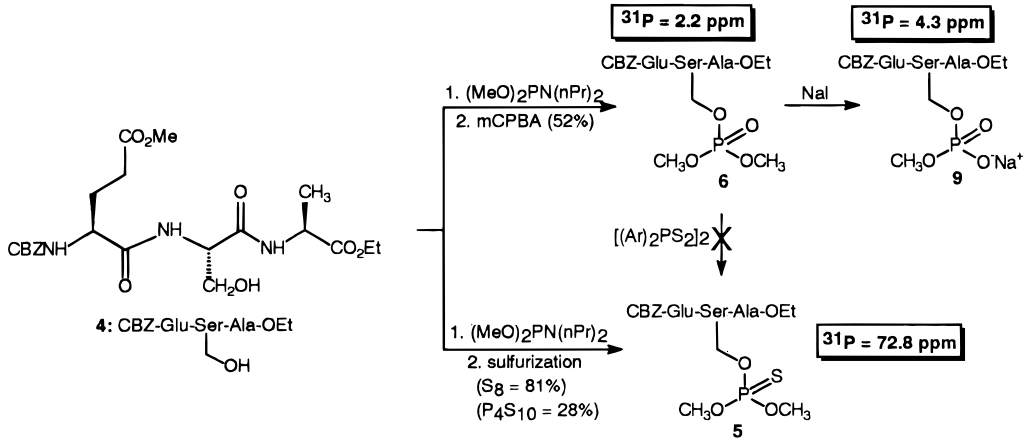
The native N-CBZ-Glu-Ser-Ala-OEt tripeptide (**4;** R = CBZ, R_1 = OEt) (*18*) was prepared according to Scheme 3. Alanine ethyl ester hydrochloride was converted to its free base using triethylamine (TEA) in THF and immediately coupled to N-CBZ-serine with dicyclohexylcarbodiimide (DCC) to form N-CBZ-serylalanine ethyl ester (**11**) in 50% yield. The CBZ group was removed by hydrogenolysis (10% Pd/H_2) to afford serylalanine ethyl ester as the primary amine. Because serylalanine ethyl ester undergoes intramolecular cyclization at room temperature, it was immediately reacted with DCC and N-CBZ-glutamic acid 5-methyl ester to form tripeptide **4** in 56% yield for the two steps. The use of 1,3-diisopropylcarbodiimide (DIPC) as coupling reagent for formation of **4** and **11** gave slightly lower yields but with a noticeable reduction of side products.

In model studies intended to optimize phosphitylation conditions, we first attempted to phosphorylate dipeptide **11** using the commercially available diamidite reagent bis-*N*,*N*-(diisopropylamino)methoxyphosphine [$(iPr_2N)_2$-POMe], which, following sequential reaction with methanol and oxidant, would provide the [dimethyl]phosphorylated dipeptide. Despite apparent clean reaction to phosphitylated dipeptide (by TLC), the exchange of the second amidite group with methanol was relatively slow and poor yielding. Clean phosphitylation of the dipeptide was achieved by use of (*N*,*N*-di-*n*-propylamino)bismethoxyphosphine [$(nPr)_2NP(OMe)_2$] (*22*) and eliminated the need for the second exchange reaction with methanol (Scheme 3). Oxidation was accomplished with *m*-chloroperoxybenzoic acid (m-CPBA; >95% purity) or magnesium monoperoxyphthalate (MMPP; *23*) to give the dimethylphosphorylated dipeptide **12** in 54% overall yield.

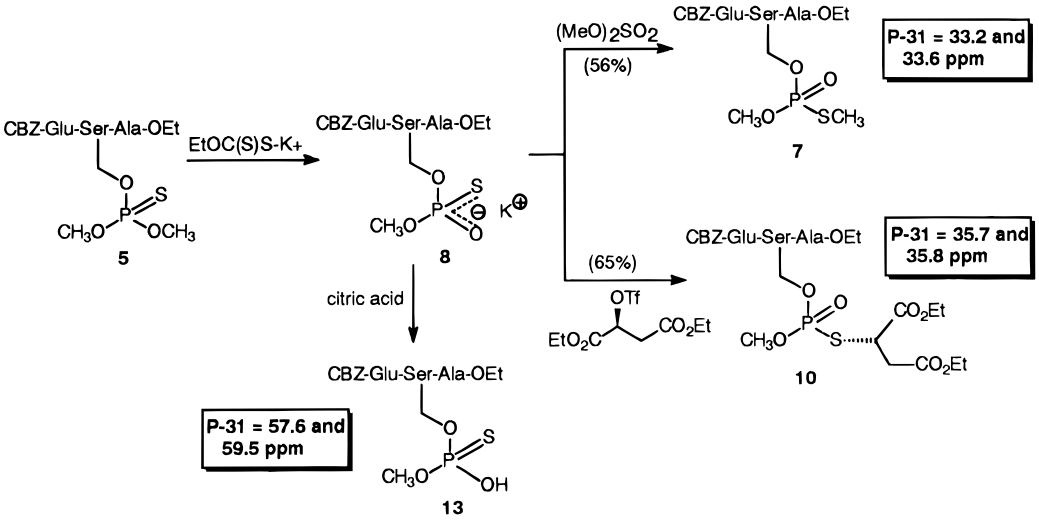
Based on this success, we began to explore the synthesis of the target phosphorylated tripeptides **5**−**10**. Using the identical phosphitylation conditions performed using the dipeptide **11**, tripeptide **4** was reacted sequentially with $(nPr)_2$N-P$(OMe)_2$/tetrazole and m-CPBA to afford the dimethylphosphorylated tripeptide **6** in 52% yield (Scheme 4). The formation of **6** was monitored by the appearance of a ^{31}P chemical shift at δ = 2.2. This chemical shift correlates well with known dimethyl alkyl phosphates (^{31}P; δ = −3.0 to 3.7) (*24*) and the model *O*,*O*-dimethylphosphorylated dipeptide **12** (^{31}P; δ = 1.8).

The formation of the "aged" phosphorylated tripeptide **9** was next undertaken. The "aged" tripeptide represents loss of one of the phosphate methyl esters leading to the phosphate monoester that likely exists as the monoanion

Scheme 4. Synthesis of Phosphorylate Tripeptide Targets 5, 6, and 9



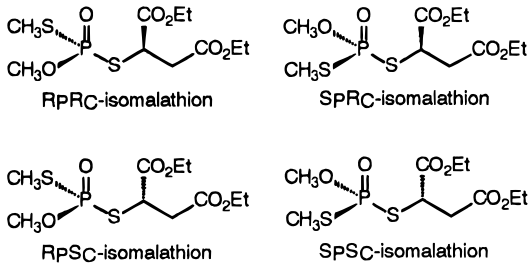
Scheme 5. Synthesis of Phosphorylated Tripeptides 7, 8, 10, and 13



at physiologic pH (*25*). Several hydrolysis reactions were attempted to selectively cleave one of the phosphorus methyl esters in the presence of the alanine ethyl ester and glutamate 5-methyl ester, but most conditions that removed the P−OMe group also gave hydrolysis of either or both of the carboxylic esters. Interestingly, selective cleavage at the Glu 5-methyl carboxy ester was realized using LiOH (1 M, room temperature, 48 h), TMS-I (1 equiv; 50 °C, $CHCl_3$), or bis-tributyltin oxide (1 equiv; toluene, reflux). Hydrolysis using NaOH gave a low yield of a mixture of ethyl and methyl carboxy ester hydrolysis products. The removal of one P−OMe group was successfully conducted in 80% yield by dealkylation using NaI in acetone or butanone at reflux. The product was isolated and characterized as the sodium salt in an effort to better simulate ionization of the phosphoric acid at physiologic pH. The "aged" tripeptide **9** shows a ^{31}P NMR chemical shift of 4.3 ppm approximately 2 ppm downfield from the *O,O*-dimethylphosphorylated tripeptide **6**.

Synthesis of the tripeptide phosphorothionate target **5** was envisioned to proceed by thionation (P=O → P=S) of phosphate **6** with P_4S_{10} or Lawesson's reagent (*26*). Unfortunately, reaction of **6** with P_4S_{10} showed no conversion to the thionate after 3 days. Reaction with Lawesson's reagent in either toluene or benzene at reflux yielded only a poor yield of dehydroalanine elimination side products preliminarily identified by a lack of a ^{31}P signal and the appearance of geminal alkene protons in the ^1H NMR. After attempting the thionation of **6** with several other reagents, we eventually found that the target phosphorothionate **5** (^{31}P δ = 72.2) could be prepared in 81% yield by phosphitylation of **4** with $[(nPr)_2NP(OMe)_2]$ followed by reaction of the intermediate phosphite with S_8 in toluene (*27*) at room temperature (Scheme 4). Substituting phosphorus pentasulfide (P_4S_{10}) for S_8 gave a 28% yield. The next phosphorylated tripeptide we sought to prepare was a model that represents reaction of cholinesterase with *O,S*-dimethyl phosphorothiolates (e.g., isoparathion methyl, isomalathion) (*11, 15*). We first intended to use (MeO)(MeS)-$PN(iPr)_2$, a novel phosphoramidate reagent that following oxidation would give **7** directly. However, we were unable to find a suitable preparation of this reagent. Since thionate **5** should be amenable to the thionate−thiolate isomerization used in the preparation of simple phosphorothiolates (*8, 11*), we subjected **5** to dealkylation with potassium ethyl xanthate (PEX) to form the thioic acid salt **8**. Realkylation with dimethyl sulfate (DMS) formed the *O,S*-dimethylphosphorothiolated tripeptide **7** in 56% yield (Scheme 5). Because tripeptide **7** contains an asymmetric phosphorus atom, diastereomers at phosphorus are formed giving ^{31}P peaks at δ = 33.2 and 33.6 in a near 1:1 ratio. It is likely that no stereoselectivity was observed because the first step occurred by a nondiscriminate dealkylation of the two phosphorus methyl esters leading to a near equal mixture of thioic acid diastereomers that subsequently react with dimethyl sulfate to give **7**. Since we wished to obtain chemical

Chart 2. Stereoisomers of Isomalathion



shifts for both stereoisomers, the nonselective procedure worked to advantage.

The intermediate tripeptide phosphorothioic acid **8** formed enroute to the *O,S*-dimethylphosphorothiolated tripeptide **7** represents the second "aged" target. Although phosphorothioic acids have not been formally postulated as "aged" intermediates, they may arise from dealkylation of a thioalkyl ligand or other related process. Because the ionization constant of a thioic acid is expected to be smaller than that of a phosphoric acid, it may exist, in part, as the protonated form. Surprisingly, the simple neutralization of **8** using a variety of dilute acids (HCl, HOAc, H_3PO_4, NaH_2PO_4, H_2SO_4, etc.) did not give **13** and led mostly to decomposition. Phosphorothioic acid tripeptide **13** was eventually isolated in 80% yield from **8** following neutralization of the reaction mixture with aqueous citric acid. The ^{31}P NMR chemical shift for this tripeptide showed a 1:1 ratio of two peaks at 57.6 and 59.5 ppm corresponding to diastereomers at phosphorus.

The last target represents cholinesterase inhibited by the (S_P)-isomalathion stereoisomers. In a previous report (*17*), we found that isomalathion stereoisomers (Chart 2) inhibit cholinesterase via two predominant mechanisms. During the inhibition mechanism, ejection of either the thiomethyl group ((S_P)-isomalathion stereoisomers) group or the diethyl thiosuccinyl group ((R_P)-isomalathion stereoisomers) occurs to furnish the *O,S*-dimethylphosphorothiolated enzyme (tripeptide **7**) or the *O*-methyl,*S*-diethylthiosuccinylphosphorylated tripeptide **10** (ejection of the thiomethyl group), respectively (eq 2).

In an effort to resolve this mechanistic anomaly, the preparation of the phosphorylated tripeptide representing inhibition by the (S_P)-isomalathion stereoisomers was warranted. Because an additional stereocenter exists at the succinyl carbon center and a complex mixture of stereoisomers could be expected, a synthesis was planned that would enable the formation of a single C-chiral isomer. S-Alkylation of thioic acid tripeptide **8** with the (S)-triflate of diethyl malate (*28*) was conducted to meet this objective. The thiophosphoric acid tripeptide **8** was reacted with diethyl (*S*)-(trifluormethanesulfonyl)malate to give a 65% yield of **10** as a diastereomeric mixture. The ^{31}P NMR chemical shifts for the diastereomers at phosphorus occur at 35.7 and 35.8 ppm in a 2:1 ratio, respectively. The slight stereochemical induction presumably results from the asymmetry imposed by the incoming electrophile since the alkylation of the identical species with dimethyl sulfate did not show any stereochemical preference.

With the successful preparation of tripeptide **4** and modified tripeptides **5**−**10** as models of covalent modification of cholinesterase, we now can initiate NMR studies of the mechanisms of phosphorylation, dephosphorylation, and nonreactivation. In this report, significantly different ^{31}P chemical shifts were found between the sulfur-containing and non-sulfur-containing phosphate groups attached to the serine residue; for example, thionate (P═S) **5** and phosphate **6** (P═O)-modified tripeptides differ by over 70 ppm in the ^{31}P NMR. Even the modest substitution of a methyl thiolester (MeS−P) for methyl ester (MeO−P) caused a 30 ppm chemical shift difference. Importantly, a 2 ppm chemical shift difference has been established between tripeptides **7** and **10**, which represents the two truncated forms of human serum cholinesterase inhibition by the isomalathion stereoisomers. Since formation of **10** precludes a mechanism to nonreactivation and possibly delayed toxicity, the 2 ppm chemical shift difference may help identify the rate and extent to which this insidious postinhibitory reaction occurs. Although the ^{31}P chemical shifts reported in this work may not precisely correlate with chemical shifts with intact protein, the relative chemical shift differences should be conserved and provide useful standards for the spectroscopic analysis of OP inhibition mechanisms.

Acknowledgment. Financial support for this work was provided by the National Institute of Environmental Health Sciences (Grant ESO4434). We also wish to thank the National Science Foundation for an instrumentation grant (CHE9302468) used to purchase of the 400 MHz NMR instrument used in this study.

References

(1) Quinn, D. (1987) Acetylcholinesterase: Enzyme structure, reaction dynamics, and virtual transition states. *Chem. Rev.* **87**, 955−979.

(2) Eto, M. (1974) *Organophosphorus Pesticides; Organic and Biological Chemistry*, CRC Press, Florida.

(3) Fukuto, T. R. (1971) Relationship between the structure of organophosphorus compounds and their activity as acetylcholinesterase inhibitors. *Bull. World Health Org.* **44**, 31−42.

(4) Taylor, P. (1985) Anticholinesterase Agents. In *Goodman and Gilman's; The Pharmacological Basis of Therapeutics* (Goodman Gilman, A., Goodman, L. S., Rall T. W., and Murad, F., Eds.) 7th ed., pp 110−129, McMillan Press, New York.

(5) Berends, F., and Posthumus, C. H. (1959) The chemical basis of the aging process of DFP inhibited pseudocholinesterase. *Biochim. Biophys. Acta* **34**, 576−578.

(6) Richardson, R. J. (1992) Interactions of organophosphorus compounds with neurotoxic esterase. In *Organophosphates: Chemistry, Fate and Effects* (Chambers, J., and Levi, P., Eds.) pp 299−323, Academic Press, San Diego.

(7) O'Brien, R. D. (1960) *Toxic Phosphorus Esters; Chemistry, Metabolism and Biological Effects*, pp 63−67, Academic Press, New York.

(8) Metcalf, R. L., and March, R. B. (1953) The isomerization of organic thionophosphate insecticides. *J. Econ. Entomol.* **46**, 288−294.

(9) Chukwudebe, A., March, R. B., Othman, M., and Fukuto, T. R. (1989) Formation of trialkyl phosphorothioate esters from organophosphorus insecticides after exposure to either ultraviolet light or sunlight. *J. Agric. Food Chem.* **37**, 539.

(10) Rengasamy, S., and Parmar, B. S. (1988) Investigation of some factors influencing isomalathion formation in malathion products. *J. Agric. Food Chem.* **36**, 1025−1030.

(11) Thompson, C. M., Frick, J. A., Natke, B. E., and Hansen, L. K. (1989) Preparation, analysis and anticholinesterase properties of *O,O*-dimethyl phosphorothiolate isomerides. *Chem. Res. Toxicol.* **2**, 386−391.

(12) Thompson, C. M., Ryu, S., and Berkman, C. E. (1992) Consequence of phosphorus stereochemistry upon the postinhibitory reaction kinetics of acetylcholinesterase poisoned by phosphorothiolates. *J. Am. Chem. Soc.* **114**, 10710−10715.

(13) Cohen, S. D. (1984) Mechanisms of toxicological interactions involving organophosphate insecticides. *Fundam. Appl. Toxicol.* **4**, 315−324.

(14) Gallo, M. A., and Lawryk, N. J. (1991) Organic Phosphorus Pesticides. In *Handbook of Pesticide Toxicology, Vol. 2, Classes*

of Pesticides (Hayes, W. J., Jr., and Laws, E. R., Jr., Eds) pp 917−1123, Academic Press, New York.

(15) Thompson, C. M., Berkman C. E., and Suarez, A. I. (1995) Enantioenriched phosphorothiolates: Synthesis, analysis and interaction with acetylcholinesterase. In *Progress and Prospects of Organophosphorus Agrochemicals* (Eto, M., and Casida, J. E., Eds.) pp 71−88, Kyushu University Press, Japan.

(16) Ryu, S., Lin, J., and Thompson, C. M. (1991) Comparative anticholinesterase potency of chiral isoparathion methyl. *Chem. Res. Toxicol.* **4**, 517−520.

(17) Berkman, C. E., Quinn, D. A., and Thompson, C. M. (1993) Interaction of AChE with the enantiomers of isomalathion and malaoxon. *Chem. Res. Toxicol.* **6**, 724−730.

(18) Rocchi, R., Moroder, L., Marchiori, F., Ferrarese, E., and Scoffone, E. (1968) Synthesis of peptide analogs of the N-terminal eicosapeptide sequence of ribonuclease A. VIII. Synthesis of [Ser^4,Orn^{10}]- and [Ser^5,Orn^{10}]-eicosapeptides. *J. Am. Chem. Soc.* **90**, 5885−5889.

(19) Perich, J. W. (1990) Modern methods of O-phosphoserine- and O-phosphotyrosine-containing peptide synthesis. In *Protein and Peptide Phosphorylation* (Kemp, B. E., Ed.) pp 289−314, CRC Press, Boca Raton, FL.

(20) Beaucage, S. L., and Iyer, R. P. (1993) The synthesis of modified oligonucleotides by the phosphoramidite approach and their applications. *Tetrahedron* **49**, 6123−6194.

(21) Beaucage, S. L., and Iyer, R. P. (1993) The synthesis of specific ribonucleotides and unrelated phosphorylated biomolecules by the phosphoramidate method. *Tetrahedron* **49**, 10441−10488.

(22) Thompson, C. M., Suarez, A. I., Lin, J., and Jackson, J. A. (1993) Synthesis of phosphorylated tripeptides representing poisoned cholinesterase. *Tetrahedron Lett.* **34**, 6529−6532.

(23) Jackson, J. A., Berkman, C. E. and Thompson, C. M. (1992) Stereoselective and chemoselective oxidation of phosphorothionates using MMPP. *Tetrahedron Lett.* **33**, 6061−6064.

(24) Edmundson, R. S. (1991) ^{31}P NMR data of four coordinate phosphorus compounds containing a P═Ch bond but no bonds to H or group IV atoms. In *Handbook of Phosphorus-31 Nuclear Magnetic Resonance Data (*Tebby, J. C., Ed.) pp 227−286, CRC Press, Boca Raton, FL.

(25) Bender, M. L., and Wedler, F. C. (1972) Phosphate and carbonate ester "aging" reactions with α-chymotrypsin. Kinetics and mechanism. *J. Am. Chem. Soc.* **94**, 2101−2109.

(26) Cava, M. P., and Levinson, M. I. (1985) Thionation reactions of Lawesson's reagents. *Tetrahedron* **41**, 5061−5087.

(27) Burgers, P. M. J., and Eckstein, F. (1978) Synthesis of dinucleoside phosphorothioates via addition of sulphur to phosphite triesters. *Tetrahedron Lett.* 3835−3838.

(28) Berkman, C. E., Perrin, S., and Thompson, C. M. (1993) Synthesis, absolute configuration and analysis of isomalathion, malaoxon and malathion enantiomers. *Chem. Res. Toxicol.* **6**, 718−723.

TX960097W