Release of Covalently Bound Metabolites of Organophosphate Pesticides from Synthetic Dialkyl Phosphoserine Peptides by Supercritical Fluid Extraction†

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Two model peptides immobilized on a solid support (Wang resin), Boc-Ser(PO_3Et_2)-Phe-Wang and Boc-Ser(PO_3Ph_2)-Phe-Wang, and two nonimmobilized compounds, Boc-Ser(PO_3Et_2)-OBzl and Boc-Ser(PO_3Et_2)-Phe-OBzl, were prepared and subjected to a supercritical fluid extraction (SFE) process. Approximately one-third of the immobilized peptides were cleaved from the resin, and the alkyl-substituted phosphoseryl moieties were identified in the distillates by means of ^1H- and ^{31}P-NMR spectroscopy. The peptides that were not immobilized were redistilled during the SFE without any changes in their structures. The results indicate that metabolites of organophosphate pesticides covalently bound to serine residues in a protein chain in plants or grains could be released by SFE.

Keywords: *Covalently bound metabolites; organophosphate pesticides; supercritical fluid extraction; model peptides; model resin bound peptides; ^1H-, ^{31}P-NMR spectra*

INTRODUCTION

It has been well established that organophosphate pesticides phosphorylate the serine residue in the active centers of esterases. This reaction represents a basis of physiological action of organophosphate pesticides (Eto, 1974a; Matsumura 1975). Covalent attachment of metabolites of organophosphate pesticides can also be expected to occur in plant proteins (Bourke et al., 1968; Rowlands, 1970), thereby resulting in the formation of bound (nonextractable) residues. The latter has been a subject of investigation in our laboratory for many years. Supercritical fluid extraction (SFE) has been used to release the nonextractable pesticides and/or metabolites from plants (Khan et al., 1990). It is suspected that in some cases covalent bonds may be cleaved by SFE. Therefore, it became of interest to determine whether or not SFE could be used to detect the covalently bound metabolites of organophosphate pesticides from plant components such as proteins. For this reason we synthesized on a solid support [aminomethylated (Wang) resin] (Stewart and Young, 1984a) dialkyl phosphoserine peptides Boc-Ser(PO_3Et_2)-Phe-Wang and Boc-Ser(PO_3Ph_2)-Phe-Wang as models of proteins containing covalently attached metabolites of organophosphate pesticides. The products (uncleaved from the resin) were subjected to the SFE process, and the released material was examined with ^{31}P- and ^1H-NMR spectroscopy for the presence of dialkyl phosphate residues. The nonimmobilized amino and carboxyl protected peptides, Boc-Ser(PO_3Et_2)-OBzl and Boc-Ser(PO_3Et_2)-Phe-OBzl, were also studied in the same manner. This paper presents results of these investigations.

MATERIALS AND METHODS

Chemicals. Amino acids and the coupling reagents BOP and EDC were obtained from Sigma Chemical Co., St. Louis, MO. Diethyl phosphochloride and Pd/C catalyst were obtained from Aldrich Chemical Co., Milwaukee, WI, and the Fmoc-Phe-Wang resin (substitution 0.62 mmol/g) was obtained from Aminotech Ltd., Nepean, ON.

NMR Spectroscopy. ^{31}P- and ^1H-NMR spectra were obtained in CH_3OD at 500.13 and 202.5 MHz, respectively, on a Bruker AM 500 NMR spectrometer. ^{31}P-NMR spectra are referenced to an external standard of 85% phosphoric acid. Chemical shifts of ^1H-NMR spectra are referenced to CH_3OD at 3.30 ppm and are reported relative to tetramethylsilane.

HPLC analyses were carried out with a Varian 5020 liquid chromatograph on a 10 μm Bondapak C_{18} column, using UV absorbency detection at 208 nm. Elution solvents were appropriate mixtures of acetonitrile with water (ratios indicated with individual experiments) containing 0.1% phosphoric acid.

Syntheses. Boc-Ser(PO_3Ph_2)-OH was prepared as described in an earlier publication (Paquet and Johns, 1990).

Boc-Ser(PO_3Et_2)-OBzl. To a solution of Boc-Ser-OBzl (2.36 g, 8.0 mmol) in diethyl ether was added diethyl phosphochloride (2.07 g, 12 mmol) and pyridine (0.949 g, 12 mmol). The reaction mixture was stirred at room temperature for 48 h, ether evaporated, and the residue taken into DCM and washed successively with an aqueous solution of citric acid (10%, three times), sodium bicarbonate (10%, three times), and water to neutrality, dried over sodium sulfate, and evaporated, affording 2.87 g (83%) of an oily residue of 95.2% HPLC purity: ^1H-NMR ($CDCl_3$) δ 7.3 (5H), 5.18 (2H), 4.41 (1H), 4.22 (2H), 4.02 (2H), 1.32 (9H), 1.27 (6H).

Boc-Ser(PO_3Et_2)-OH. To a solution of Boc-Ser(PO_3Et_2)-OBzl (1.72 g, 4.0 mmol) in a mixture of ethyl acetate with methanol (10 mL) was added Pd/C catalyst (172 mg). The mixture was stirred in a hydrogen atmosphere at atmospheric pressure for 4 h, catalyst was filtered off, and the solvents were evaporated on a rotary evaporator. The resulting product was recrystallized from ethanol and was found to be identical (according to the identical characteristics) with the same product obtained previously by others from the *p*-nitrobenzyl ester (Alewood et al., 1982).

Synthesis of Boc-Ser(PO_3Et_2)-Phe-OBzl was carried out by coupling Boc-Ser(PO_3Et_2)-OH to Phe-OBzl in DCM and employing EDC as coupling reagent using a previously published procedure (Paquet and Johns, 1990). The product was obtained in high yield, recrystallized (99% purity, HPLC), and characterized by means of NMR and mass spectra.

Boc-Ser(PO_3Et_2)-Phe-Wang. To the Fmoc-Phe-Wang resin (483 mg, corresponding to 0.30 mmol of Phe) placed in a filtration funnel provided with a sintered glass bottom was

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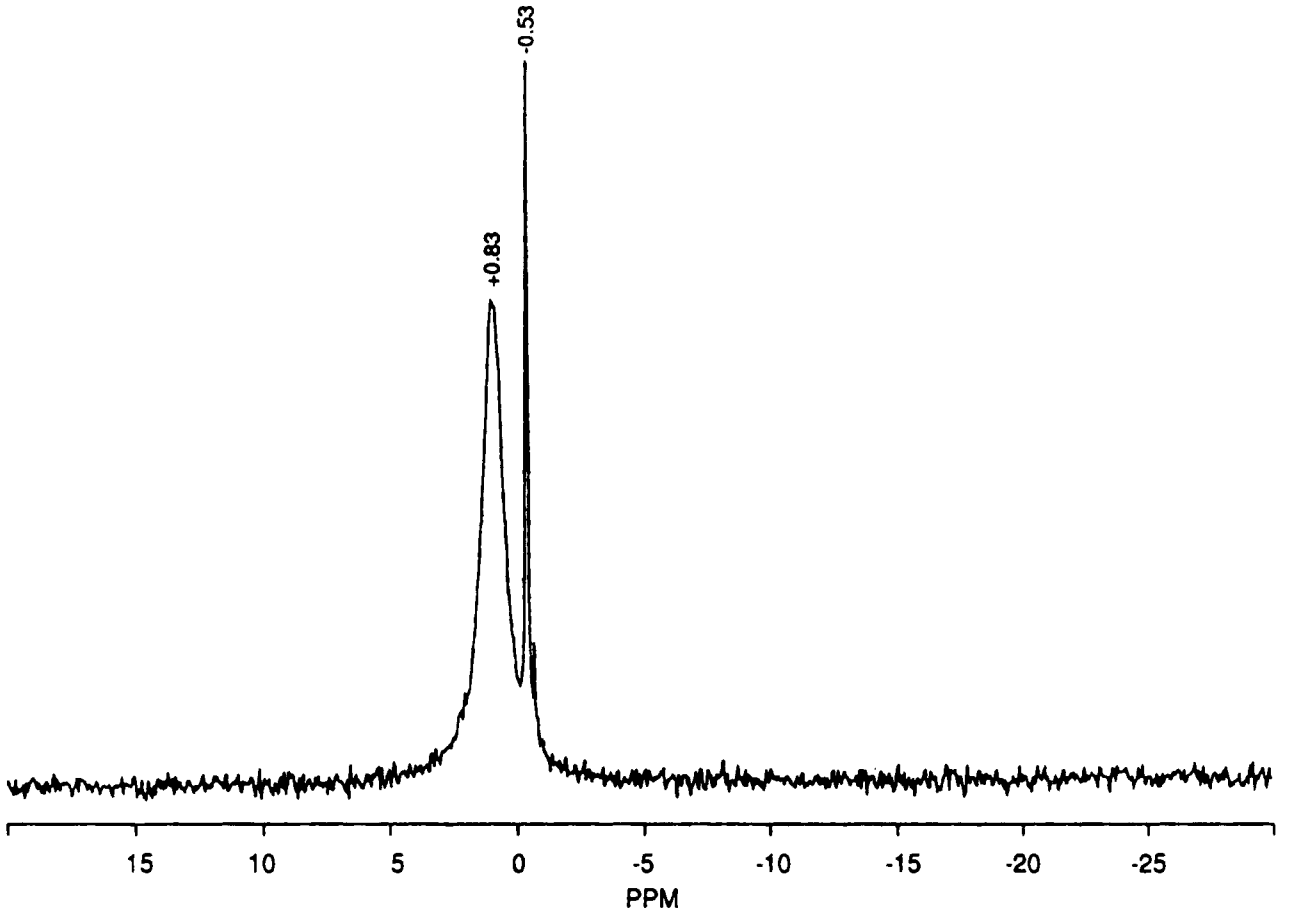


Figure 1. ^{31}P-NMR spectrum of SFE distillate of Boc-Ser(PO_3Et_2)-Phe-Wang.

added a solution of piperidine in DMA (1:4), and the mixture was stirred with a stream of nitrogen for 5 min. Liquid was removed with suction, and the process was repeated (Fmoc group removal). A solution of Boc-Ser(PO_3Et_2)-OH (205 mg, 0.60 mmol) in DMA (2.5 mL), BOP (265 mg, 0.60 mmol), and diethylisopropylamine (77.5 mg, 0.60 mmol) was added to the resin, and the mixture was stirred with a stream of nitrogen for 6 h until the Kaiser test (Stewart and Young, 1984b) indicated absence of free amino groups. Solvents were removed with suction, the resin was washed with DMA (5 mL), and the coupling process was repeated using the same amounts of the reaction components. The resin was washed with DMA (three times, 10 mL), DCM (three times, 10 mL), and methanol (three times, 10 mL) and dried in vacuum to give 457 mg (69%) of the title product.

Boc-Ser(PO_3Ph_2)-Phe-Wang was prepared essentially as described for Boc-Ser(PO_3Et_2)-Phe-Wang using 484 mg of Fmoc-Phe-Wang (corresponding to 0.30 mmol of Phe), 262 mg of Boc-Ser(PO_3Ph_2)-OH (0.6 mmol), 265 mg of BOP (0.6 mmol), and 77.5 mg of diethylisopropylamine (0.6 mmol). The yield of the title product was 528 mg (71%).

To determine the purity of the immobilized peptides, part of each resin was separated and treated with TFA in DCM (50%) and the cleaved, deprotected peptide was analyzed by HPLC and NMR analysis. In both cases 98% purity was determined.

Supercritical Fluid Extraction (SFE). The SFE system (Suprex Model SFE-50, Suprex Corp., Pittsburg, PA) used consisted of a 250-mL syringe pump, a control module for the SFE system, an extraction oven, a 5-mL extraction vessel containing sample, and a four-port valve connected with the outlet restriction (fused silica tubing, 50 μm i.d.) that was vented into the first of three glass tubes containing 50 mL of methanol. The three glass tubes containing methanol were connected in series for collection of the released material. Extraction was carried out with the modified CO_2 using methanol (25% CH_3OH in CO_2) which was delivered by an HPLC pump (Varian 2510). The flow rates for CO_2 and methanol were maintained at 0.65 and 0.50 mL/min, respectively, and the extraction was carried out at 200 °C and 355 atm for 4 h after initial equilibration of the SFE system for 5 min at 120 °C and 150 atm.

The resin-bound peptides were placed in the extraction vessel and subjected to SFE under the conditions described above. The amino and carboxyl protected peptides were dissolved in DCM and dropped on a disk of filtration paper, solvent was evaporated in air, and the paper was placed in the extraction SFE vessel. SFE was carried out under the conditions described above except the extraction time was only 1.5 h.

Analysis of Distillates. The distillates were evaporated until dryness, and the residues were weighed. The yield of a residue indicated for Boc-Ser(PO_3Et_2)-Phe-Wang represents an average value of two determinations. The distillates were analyzed using ^{31}P- and ^{1}H-NMR techniques and by HPLC analysis.

SFE of Polymer-Bound Peptides. *Boc-Ser(PO_3Et_2)-Phe-Wang* (150 and 35 mg of sample) was subjected to SFE, and the distillates were evaporated to give 5.3 and 3 mg of the solids, respectively (39% of the total bound matter, an average of both experiments). These were dissolved in CH_3OD and subjected to NMR analysis: ^{31}P-NMR [of the residue from 150 mg of the resin (5.3 mg)] −0.53 (sharp singlet, serine-bound diethyl phosphate), 0.83 (broad signal, diethyl phosphoric acid cleaved from the serine residue) ppm (Figure 1) (this spectrum was identical with the ^{31}P-NMR spectrum obtained with the 3-mg residue); ^{1}H-NMR 7.27−7.23 (aromatics, Phe), 3.22−3.06 (CH_2− of the side chain, Phe), 4.0−4.3 (C-1 of Phe, C-1 of Ser, CH_2− of the side chain of phosphorylated serine, CH_2− of the ethyl groups on the phosphate moiety), 3.73−3.68 (CH_2− of the side chain of dephosphorylated serine), 1.45 (*tert*-butyl alcohol), 1.34 and 1.31 (Boc-, CH_3− from the phosphate and additional CH_3− originating possibly from the aminomethylated resin) ppm.

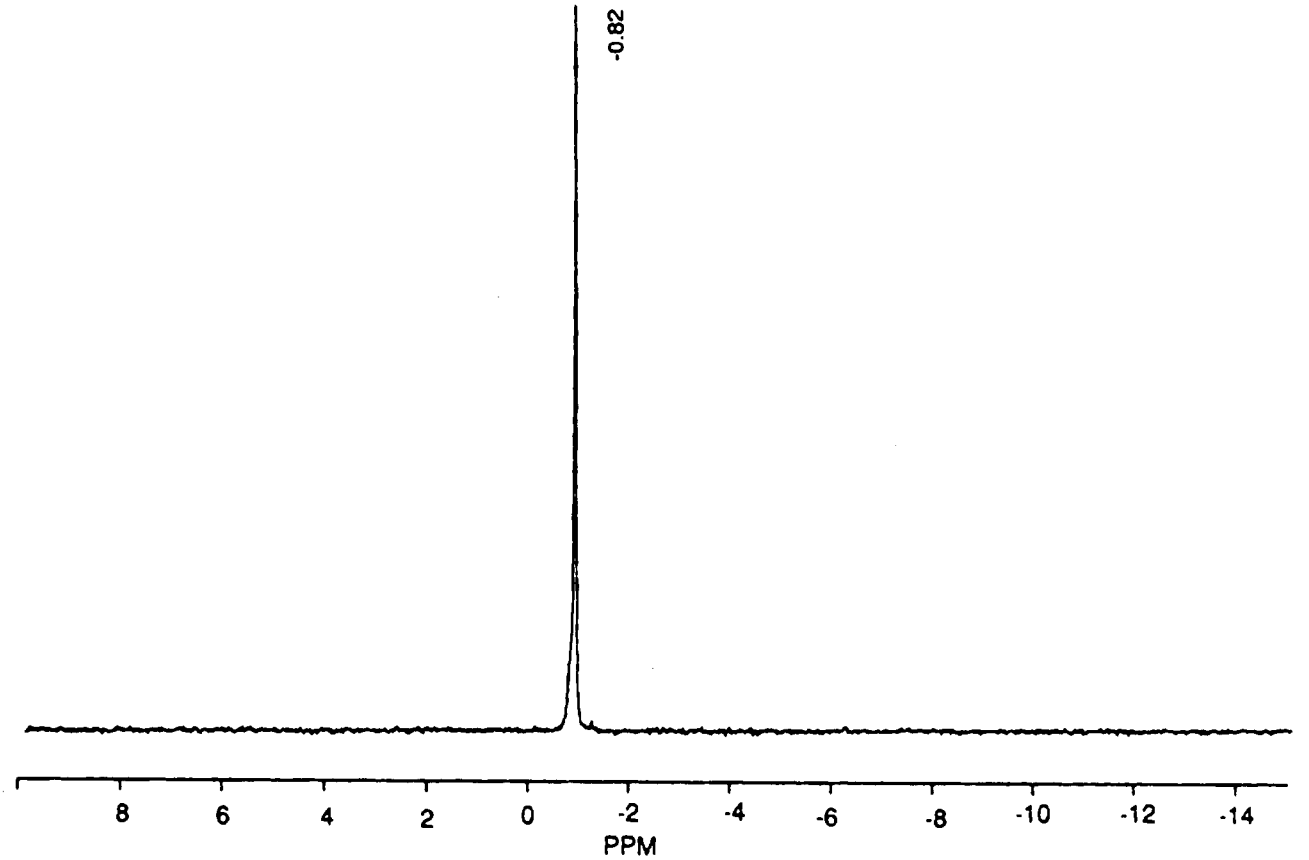


Figure 2. ^{31}P-NMR spectrum of authentic Boc-Ser(PO_3Et_2)-OBzl.

Boc-Ser(PO_3Ph_2)-Phe-Wang. This polymer-bound peptide (140 mg) was subjected to SFE, and 13.8 mg (37% of the total bound matter) was obtained upon evaporation of the distillate. It was dissolved in CH_3OD and subjected to NMR spectroscopic analysis: ^{31}P-NMR −9.10 (broad peak, diphenyl phosphoric acid cleaved from the serine residue), −11.63 (sharp singlet, serine-bound diphenyl phosphate) (a minor peak at −11.15 ppm remained unidentified) ppm (Figure 2); ^{1}H-NMR 7.31−7.06 (aromatics, Phe and phenyl groups from diphenyl phosphoric acid), 3.2−2.96 (CH_2− of the side chain of Phe), 3.72−3.67 (CH_2− of the dephosphorylated serine), 4.7−4.2 (traces, CH_2− of diphenyl phosphorylated serine) ppm. Some additional peaks in the spectrum were assigned to the presence of decomposition products possibly originated from the resin.

HPLC Analysis of the Residue. Solvent system: acetonitrile−water (10−90); relative peak area, Ser (13%), Phe (12%), Boc-Ser(PO_3Ph_2)-Phe-OH (4%). Several other peaks remained unidentified.

SFE of Amino and Carboxyl Protected Peptides. *Boc-Ser(PO_3Et_2)-OBzl* (85.0 mg) was subjected to SFE conditions, and the distillate was evaporated and dried in vacuum to give 84.5 mg of the residue (99.4%). The ^{1}H-NMR spectrum of the residue was identical with the ^{1}H-NMR spectrum of the starting material.

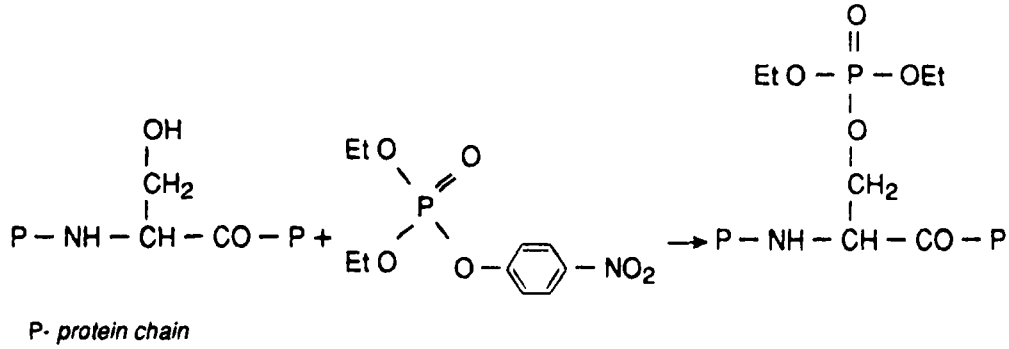
Boc-Ser(PO_3Et_2)-Phe-OBzl (100 mg) was subjected to SFE, giving 67.5 mg of the residue (67.5%). The ^{1}H-NMR spectrum was identical with the ^{1}H-NMR spectrum of the starting material, suggesting that most of the compound remained unaffected by SFE conditions.

RESULTS AND DISCUSSION

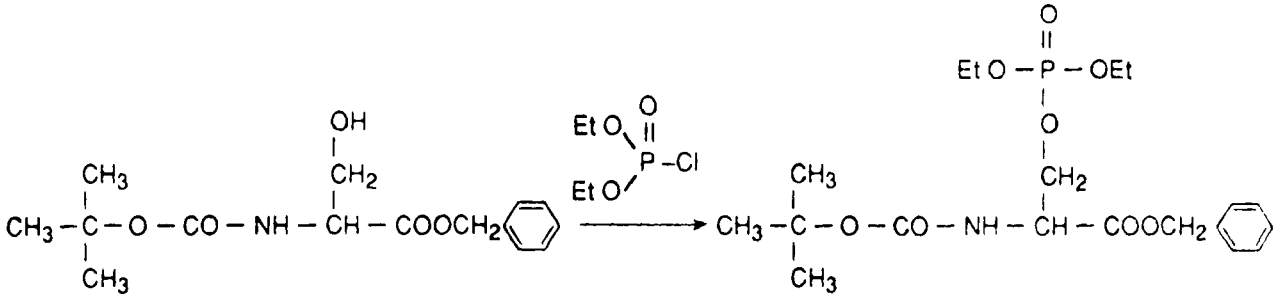
Synthesis of peptides on a solid support Boc-Ser(PO_3Et_2)-Phe-Wang and Boc-Ser(PO_3Ph_2)-Phe-Wang, proceeded without difficulties using a modified process described previously (Stewart and Young, 1984c). The structure was proven by cleaving the peptide from a small amount of the resin and subjecting it to ^{1}H- and ^{31}P-NMR spectroscopy and to HPLC analysis (over 98% purity was detected in both cases).

Boc-Ser(PO_3Et_2)-Phe-OBzl was easily synthesized in solution in high yield using a process described previously (Paquet and Johns, 1990) and was recrystallized and characterized (Schemes 1 and 2). When Boc-Ser(PO_3Et_2)-Phe-Wang was subjected to the SFE process, the ^{31}P-NMR spectrum of the distillate showed a sharp singlet at −0.53 ppm and a broad signal at 0.83 ppm (Figure 1). The sharp signal was assigned to a diethyl phosphate moiety bound to serine because the ^{31}P-NMR spectra of trisubstituted phosphate esters typically exhibit a sharp singlet (Paquet and Johns, 1990, and references cited therein), and the ^{31}P-NMR spectrum of authentic Boc-Ser(PO_3Et_2)-OBzl gave a signal at −0.82 ppm (Figure 2). Phosphoric acid esters with one or two free hydroxy groups exhibit broad signals in ^{31}P-NMR spectra. The broad peak at 0.83 ppm in the spectrum of this distillate was therefore ascribed to diethyl phosphoric acid ester cleaved from the serine residue in Boc-Ser(PO_3Et_2)-Phe-Wang. Broadness of this peak might also suggest the presence of another species of phosphate ester—the monoethyl phosphoric acid—resulting potentially from the loss of one of the ethyl groups during the SFE process. Integration of this spectrum revealed that there was 4.4 times as much of the species cleaved off the serine residue (0.83 ppm) than of one bound to serine (−0.53 ppm). This ratio suggests that the peptide that remained bound to the Wang resin was to a large extent dephosphorylated. Furthermore, the ^{1}H-NMR spectrum showed the presence of phosphorylated serine (CH_2− of the side chain at 4.0−4.2 ppm, appearing together with the CH_2− of the ethyl phosphate moiety) as well as dephosphorylated serine (CH_2− of the side chain at 3.73−3.68 ppm).

Scheme 1. Phosphorylation of Protein-Bound Serine by Parathion



Scheme 2. Phosphorylation of Amino and Carboxyl Protected Serine with Diethyl Phosphochloride



Thus, the ^{1}H-NMR spectrum confirms that the sharp singlet at −0.53 ppm and the broad resonance at 0.83 ppm in the ^{31}P-NMR spectrum were correctly assigned to serine-bound and serine-cleaved phosphate moieties, respectively.

Since the NH-group resonance (Boc-Ser- around 6.9) was missing, we assumed that the Boc- group was cleaved from the peptide. It was not possible to determine whether serine and phenylalanine were cleaved from each other because the NH− involved appears in the same region as proton resonances of other amino acids. Thus, from this experiment, it can be seen that the disubstituted phosphoric acid was partially cleaved from the serine residue and that the ^{31}P- and ^{1}H-NMR spectra detected both the serine-bound phosphate moiety and the phosphate cleaved from serine. Thus, immobilization of a diethyl phosphoseryl peptide on Wang resin prevented its complete distillation during SFE.

The ^{31}P-NMR spectrum of the distillate of Boc-Ser(PO_3Ph_2)-Phe-Wang exhibited a sharp singlet at −11.63 ppm and an intense broad signal at −9.10 ppm (Figure 3). The singlet at −11.63 ppm was assigned to diphenyl phosphoserine moiety upon comparison with the ^{31}P-NMR spectrum of authentic Boc-Ser(PO_3Ph_2)-OBzl (singlet at −11.68 ppm, Figure 4). The intense signal at −9.10 ppm can be assigned to diphenyl phosphoric acid moiety cleaved off the serine residue. Monophenyl phosphoric acid is not expected to be formed during this experiment due to the pronounced stability of the phenyl esters of phosphoric acid (Paquet and Johns, 1990, and references cited therein). Integration showed that the ratio of the diphenyl phosphoric acid cleaved off the serine residue (−9.10 ppm) to diphenyl phosphate bound to serine residue was 9.8 to 1. This suggests that also in this experiment the peptide that remained bound to the resin was to a large extent dephosphorylated. The ^{1}H-NMR spectrum of the distillate exhibited the presence of a typical four-line pattern of geminal coupling of the CH_2− group of dephosphorylated serine at 3.72−3.67 ppm, which confirms that the large broad signal in the ^{31}P-NMR spectrum at −9.10 ppm was correctly assigned to diphenyl phosphoric acid cleaved off serine. Signals of diphenyl phosphorylated serine were detected in the ^{1}H-NMR spectrum at 4.2−4.7 ppm, confirming that the assignment of the singlet at −11.63 ppm in the ^{31}P-NMR spectrum to diphenyl phosphoserine moiety was correct. The HPLC analysis indicated the presence of free serine and phenylalanine (13 and 12%, respectively) and a small amount of a whole peptide the Boc-Ser(PO_3Ph_2)-Phe-OH (comparison with authentic standard, 4%). This result suggests that serine and phenylalanine were to some extent cleaved from each other during the SFE process.

The nonimmobilized amino and carboxyl protected peptides, Boc-Ser(PO_3Et_2)-OBzl and Boc-Ser(PO_3Et_2)-Phe-OBzl, were redistilled, as expected, during SFE without any changes in their structures in high yields (99.7 and 67.5%, respectively).

Most of the frequently used pesticides contain either a dimethyl phosphate group, such as dichlorvos, chlorpyrifos-methyl, and malathion, or a diethyl phosphate group, such as parathion-ethyl, acethion, or fensulfothion. Since the nature of the phosphorus protecting groups does not have a pronounced effect on the biological activity of organophosphate pesticides (Eto, 1974b), we have chosen to use the ethyl phosphate protecting group in our experiments (Scheme 2). The phosphorylating agent, diethyl phosphochloride, is inexpensive and commercially available. The peptides with the phenyl protecting phospho groups were used in this study despite the fact that the diphenyl phosphate does not occur in organophosphate pesticides. The ^{31}P-NMR chemical shift of the diphenyl protected phosphoric acid moiety (around −11 ppm) (Figures 3 and 4) is substantially different from the ^{31}P-NMR chemical shifts obtained with the diethylphospho derivatives (around −1 ppm) (Figures 1 and 2). For this reason the diphenyl protected compounds served conveniently in this study for confirmation of the results obtained with the diethyl protected derivatives.

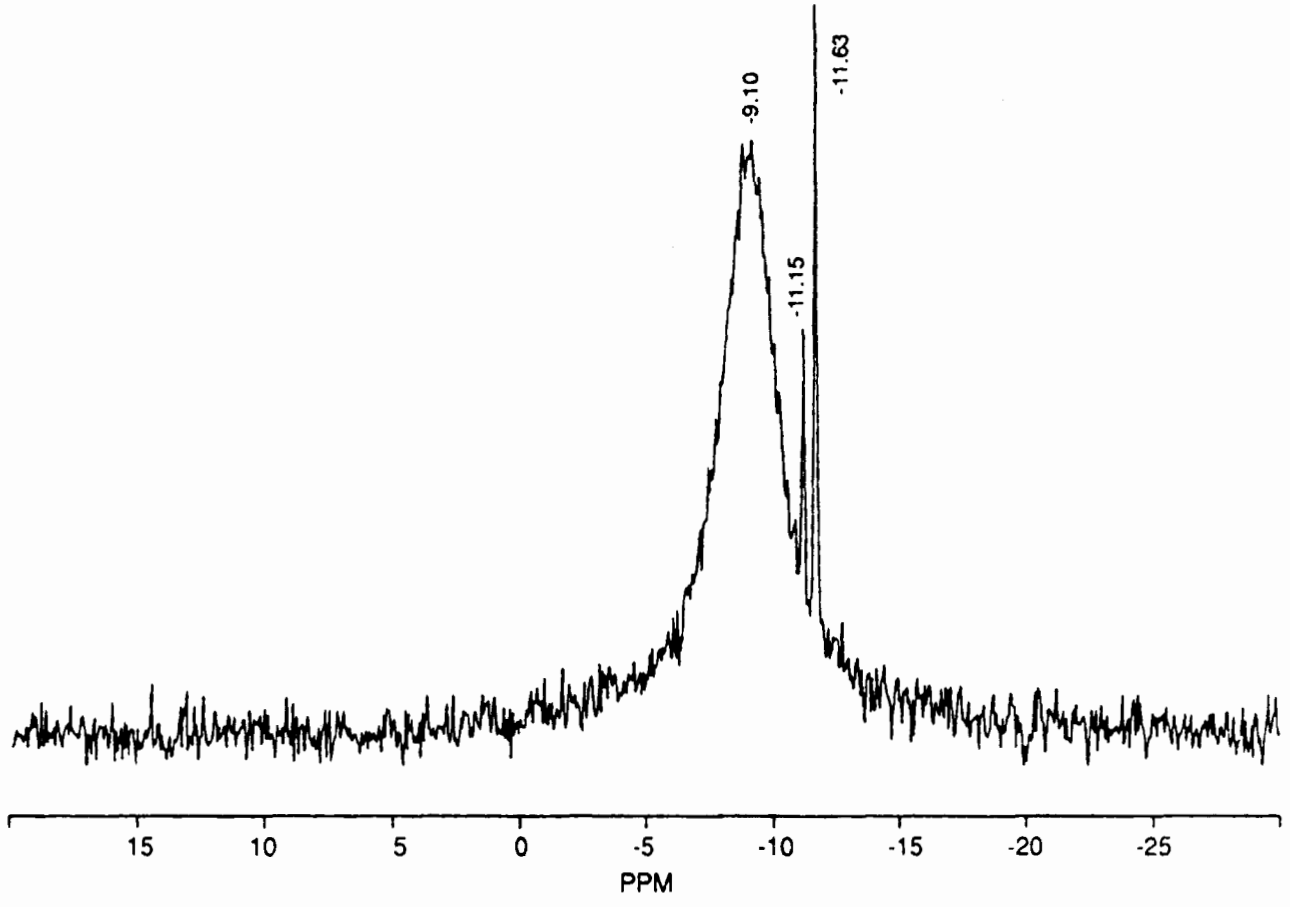


Figure 3. ^{31}P-NMR spectrum of SFE distillate of Boc-Ser(PO_3Ph_2)-Phe-Wang.

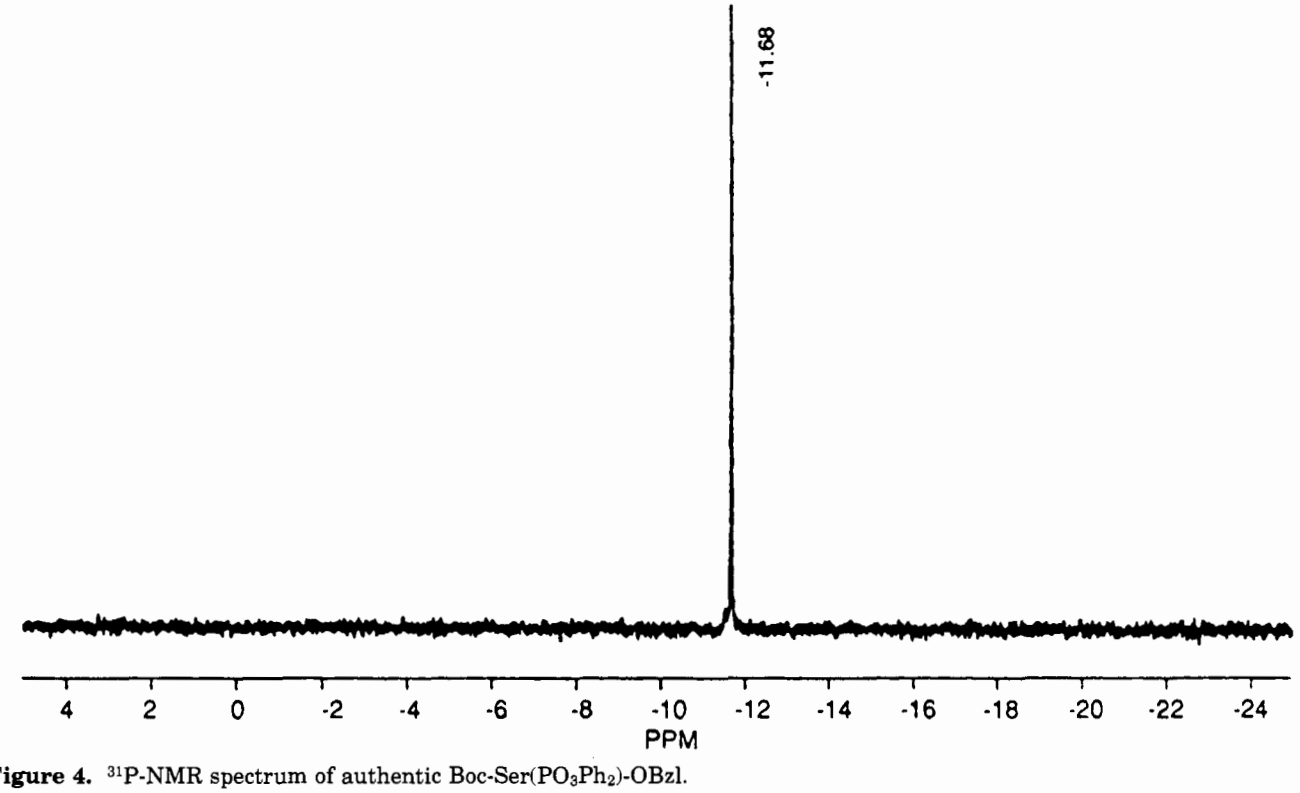


Figure 4. ^{31}P-NMR spectrum of authentic Boc-Ser(PO_3Ph_2)-OBzl.

Phosphorylation of serine residues in a protein chain by organophosphate pesticides has been very well established (Eto, 1974a; Matsumura, 1975) (Scheme 1), and the physiological effect of this reaction in a mammalian system—inhibition of the enzyme cholinesterase—is well-known. Phosphorylation of amino acids in plant proteins can also occur. Interaction between pesticides and/or metabolites and cereal proteins can be expected to occur mainly during storage conditions (Rowlands, 1970; Qureshi et al., 1992), often resulting

in the formation of bound residues via "encapsulation" or "covalent linkage". Matthews (1992) reported that the bound residues of chlorpyrifos-methyl in wheat accounted for 29% of the applied dose after 14 months of storage. Furthermore, it was observed that 59% of these bound residues was present as the hydrolytic metabolite of chlorpyrifos-methyl, 3,5,6-trichloro-2-pyridinol and that 26% was a polar material. The remaining material could not be released by the chemical solubilization procedure. The highest concentration of these residues was found in the germ layer. Since the germ layer is the physiologically most active part of cereal grains, the author assumed that this is where much of the metabolism and formation of bound residues occur. Thus, covalent binding involving phosphorylation of proteins with pesticides in the germ layer would affect the biologically most important proteins (Paquet et al., 1994). It would be, therefore, of importance to be able to detect the presence of covalently attached metabolites of pesticides in grains. Our experiments showed that approximately one-third of immobilized peptides containing a dialkyl-substituted phosphoserine residue were cleaved from the resin, and the alkyl-substituted phosphoseryl moieties were identified in the distillates by means of ^{31}P- and ^{1}H-NMR spectroscopy. This suggests that a combination of SFE and NMR techniques may be used to detect a single alkyl-substituted phosphate in polymeric material.

The peptides, which were not immobilized, but merely amino and carboxyl protected [(Boc-Ser(PO_3Et_2)-OBzl and Boc-Ser(PO_3Et_2)-Phe-OBzl] were, as expected, redistilled during the SFE process. Their structures remained unchanged, and it is noteworthy to mention that even protecting groups (Boc- and OBzl-) remained uncleaved. These experiments may support the previously described observation that the (nonextractable residues of the organophosphate pesticides, chlorpyrifos-methyl and malathion, in stored grains were released by means of SFE without any changes in their structures (Singh et al., 1993; Bitsi et al., 1994). Thus, our study with authentic synthetic models provides information that may have significant bearing when working with the natural material.

ABBREVIATIONS USED

NMR, nuclear magnetic resonance; EDC, *N*-ethyl-*N'*-[3-(dimethylamino)propyl]carbodiimide; Boc, *tert*-butyloxycarbonyl; FMOC, 9-fluorenylmethyloxycarbonyl; Et, ethyl; Ph, phenyl; TFA, trifluoroacetic acid; BOP, benzotriazolyltris(dimethylamino)phosphonium hexafluorophosphate; DMA, *N*,*N*-dimethylacetamide; DCM, dichloromethane; Pd/C, palladium catalyst on charcoal (10%). Amino acid symbols represent L-forms and are those recommended by the IUPAC–IUB Commission on Biochemical Nomenclature: *J. Biol. Chem.* **1972**, *247*, 977: Ser, serine; Phe, phenylalanine; -OBzl, benzyl ester.

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