Solid-Phase Synthesis of Phosphonylated Peptides

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Abstract: We report the solid-phase syntheses of two series of phosphonylated peptides using Fmoc-protected amino acids. The peptides corresponded to regions containing phosphonylated Ser195 in the active site of butyrylcholinesterase and Tyr411 of human serum albumin. The phosphonylated Fmoc-protected amino acids were used in solid-phase peptide synthesis to prepare the peptides. Fmoc-serine and Fmoc-tyrosine with benzyl ester protection were treated with alkyl methylphosphonic monochloridates to phosphonylate the side-chain hydroxy groups. The phosphonylated peptides were designed to mimic the protein region after exposure of the proteins to organophosphorus agents.

Key words: organophosphonates, nerve agent, phosphonylated peptides, peptide synthesis, solid phase

Organophosphate (OP) moieties are the core structure of many nerve agents.[1] Their mechanism of action is the phosphonylation of hydroxy side chains of serine, threonine, and tyrosine residues in proteins thereby interfering with protein function. OPs are well known to inhibit acetylcholinesterase (AChE) and produce cholinergic toxicity. However, additional noncholinergic targets have also been identified.[2] Four commonly known OP nerve agents are shown in Figure 1.

Because of OPs toxicity and its potential use in future chemical warfare and terrorist attacks, a reliable and efficient method to detect OPs would be of considerable interest. Current methods of nerve-agent detection utilize gas chromatography protocols that require lengthy sample preparation and heavy equipment. Furthermore, nerve agents and their metabolites are not stable indefinitely, and this type of analysis is therefore problematic.[3] There is an obvious need for a quick and easy method to detect OP while in the field.

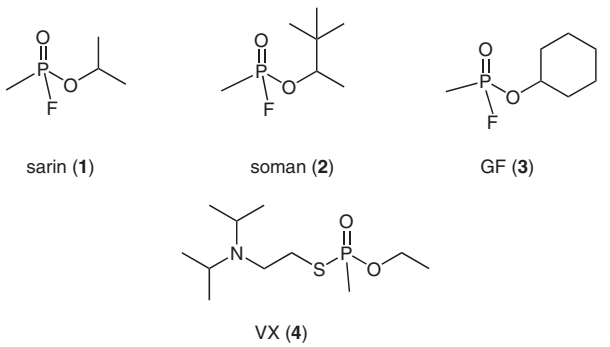

Figure 1 Structures of prominent organophosphorus nerve agents

Monoclonal antibodies against hydrolyzed agent (methylphosphonic acid) have been developed that display comparable sensitivity to previously reported spectrometric techniques.[4] An alternative method of detection is to analyze for OP-adducted proteins in physiological fluids using monoclonal antibodies targeting adducted regions of those proteins. Serum proteins that bind OPs include butyrylcholinesterase (BuChE) and human serum albumin (HSA).[5] OPs covalently modify BuChE at the active site serine 195 and they modify HSA at tyrosine 411 located on the protein surface. Other surface tyrosines are affected, but tyrosine 411 is predominantly phosphonylated.[6] OP-modified proteins retain the alkyl group that distinguishes the agent used. Ideally, monoclonal antibodies against BuChE and HSA adducted to each OP nerve agent would provide information about which agents were present. Haptens used to elicit antibody production are phosphonylated peptides mimicking affected regions of BuChE and HSA. Peptide sequences for each protein region are shown in Table 1.

Table 1 Peptide Sequences with C-Terminal Aminocaproic Acid for Human BuChE and Serum Albumin

Protein	Peptide sequence[a]
BuChE	H_2N-Lys-Ser-Val-Thr-Leu-Phe-Gly-Glu-**Ser**-Ala-Gly-Ala-Ala-Aca-COOH
HSA	H_2N-Leu-Val-Arg-**Tyr**-Thr-Lys-Lys-Val-Pro-Gln-Aca-COOH

[a] Aca = aminocaproic acid.

While there are many syntheses of phosphorylated peptides reported in the literature,[7] there are relatively few syntheses of phosphonylated peptides. In these accounts, the unmodified peptide was first assembled by solid-phase synthesis followed by phosphonylation of the serine, threonine, or tyrosine side chain by treatment with a phosphorus(III) reagent–oxidation step while the peptide was still on the resin.[8] This method required differential side-chain protection of other serine, threonine, and tyrosine residues if present in the sequence because they would also be modified by phosphorus reagents. To our knowledge, there are no reported syntheses that utilize

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phosphonylated amino acids in solid-phase peptide syntheses to produce a phosphonylated peptide. An advantage of this method is that the number of side-chain protecting groups is decreased thereby decreasing possible side reactions during their removal. Herein we describe the use of phosphonylated amino acids that are incorporated into peptides corresponding to selected regions of BuChE and HSA adducted by sarin, soman, GF, and VX at serine 195 and tyrosine 411, respectively.

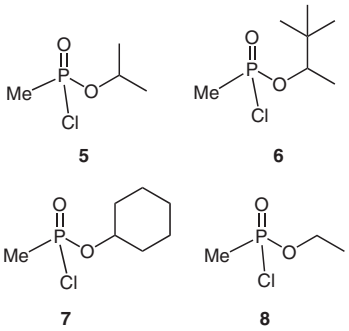
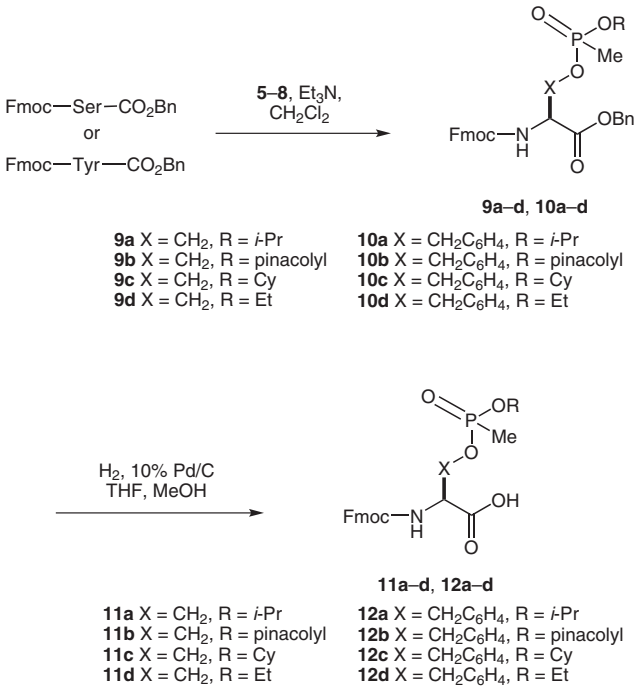


Figure 2 Monochloridate analogues of nerve agents used to modify serine and tyrosine side chains



Scheme 1

Phosphonylated Fmoc-serine and Fmoc-tyrosine were prepared and then incorporated into the solid-phase synthesis of the requisite peptides as described in Scheme 1. Protected serine and tyrosine were treated with alkyl methylphosphonic monochloridates **5**–**8** (Figure 2) to afford phosphonylated protected amino acids **9a**–**d** and **10a**–**d**. All compounds were characterized by ^{1}H NMR and ^{31}P NMR spectroscopy. Analysis of amino acid intermediates included low resolution mass spectrometry. Final amino acid products were also characterized by high-resolution mass spectrometry. In accord with a literature procedure, compounds **5**–**8** were prepared using a one-step process from methylphosphonic dichloride, and crude products were purified by vacuum distillation.[9] These products were characterized by ^{1}H NMR and ^{31}P NMR spectroscopy. Experimental procedures with compound characterization are available in the Supporting Information. **Caution:** While these compounds are not classified as nerve agents, proper precautions must be taken when handling them.

The synthesis of Fmoc-protected phosphonylated serine and Fmoc-protected phosphonylated tyrosine is depicted in Scheme 1. Fmoc-serine and Fmoc-tyrosine benzyl esters without side-chain protection were treated with phosphorus reagents **5**–**8** in the presence of DMAP and triethylamine.[10,11] Unreacted phosphorus monochloridate was first quenched by addition of isopropanol, then the reaction mixture was concentrated in vacuo. After purification by chromatography on silica gel, the benzyl ester was removed by hydrogenolysis to form the carboxylic acid. This preparation produced a mixture of stereoisomers at the phosphorus atom. The starting amino acids were enantiopure so the phosphonylated products were isolated as diastereomeric mixtures and incorporated into peptide synthesis.

There was some concern about the lability of Fmoc groups and phosphonylated serine side chains to hydrogenolysis. The hydrogenation was done following a literature procedure that described the preparation of side-chain-modified serine residues.[10] Product yields ranged from 41–87% after the removal of benzyl esters. We did not explore other ester protection such as *tert*-butyl esterification because we did not encounter any problem with the hydrogenation of the benzyl ester. There was undoubtedly some elimination of the phosphonate from serine to form dehydroalanine during the reaction based on TLC of the crude reaction mixture. However, the corresponding dehydroalanine was not the major product. Yields from hydrogenation of the serine analogs ranged from 47–87%.

Solid-phase peptide synthesis was done using an Advanced ChemTech ACT-3926 peptide synthesizer. The peptides were synthesized using Fmoc chemistry on a 2-chlorotrityl resin and HBTU/HOBt activation. Protected amino acids used in the peptide synthesis included Fmoc-glutamic acid with the isopropyl phenyl ester on the side-chain carboxylic acid and Fmoc-lysine with Boc protection of the amine side chain. Nonadducted serine and threonine residues were used without side-chain protection. Peptide synthesis was done on an automated synthesizer up to the residue preceding the phosphonylated amino acid. After this point, couplings were done manually to ensure the highest coupling efficiency. Cleavage from the resin was achieved by treating the peptide resin with 1% TFA–CH_2Cl_2 containing 2% triisopropylsilane overnight at room temperature. Crude phosphonylated peptide products were purified by reversed-phase HPLC on a C18 column (0.1% TFA water–acetonitrile). Peptide purity was

>90% and was verified by analytical HPLC and mass spectrometry.

Serine is especially susceptible to elimination of phosphonates followed by addition of water to regenerate the side-chain hydroxy group.[12] Retention of the phosphonate throughout synthesis, cleavage from the resin, and purification was verified by electrospray mass spectrometry. Calculated masses and confirmed masses for each peptide are shown in Tables 2 and 3. Peptides mimicking the BuChE active site are shown in Table 2 while peptides mimicking the sequence around tyrosine 411 of HSA are shown in Table 3. The observed masses agree with the calculated masses leading us to conclude that the correct peptides were synthesized and isolated.

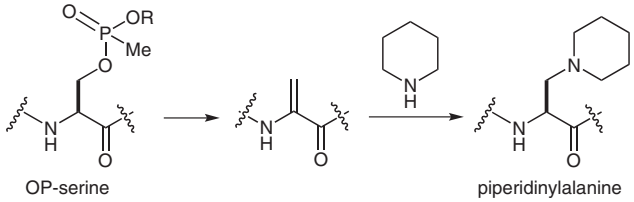
Table 2 Phosphonylated Peptides with C-Terminal Aminocaproic Acid Corresponding to Active Site Phosphonylated BuChE

Peptide #	Name	Phosphonate ester (*R*)	MW obs.	MW calcd
13	BuChE-sarin	isopropyl	1470.8	1470.6
14	BuChE-soman	pinacolyl	1513.4	1512.7
15	BuChE-GF	cyclohexyl	1511.3	1510.7
16	BuChE-VX	ethyl	1457.2	1456.6

Table 3 Phosphonylated Peptides with C-Terminal Aminocaproic Acid Corresponding to Phosphonylated HSA

Peptide #	Name	Phosphonate ester (*R*)	MW obs.	MW calcd
17	HSA-sarin	isopropyl	1465.4	1465.7
18	HSA-soman	pinacolyl	1507.4	1507.8
19	HSA-GF	cyclohexyl	1505.3	1505.8
20	HSA-VX	ethyl	1451.3	1451.7

During the synthesis of the BuChE-OP peptides **14** and **15**, a peptide byproduct was also formed. Because this byproduct consistently gave the same mass of 1418 mass units and same HPLC retention time from syntheses of different OP-BuChE peptides, we assumed that it was the same byproduct from each synthesis. A recent account described a survey of alternate Fmoc deprotection strategies to suppress elimination of phosphorylated serine side chains.[13] Based on this report we surmised that the byproduct arose from elimination of the phosphonyl portion of the serine residue during Fmoc deprotection using 20% piperidine–DMF to give a dehydroalanine-containing peptide. Addition of piperidine to dehydroalanine formed piperidinylalanine in place of the phosphonylated serine (Scheme 2). The calculated mass of the BuChE peptide with piperinylalanine replacement was also 1418 mass units supporting our belief. Phosphorylated serine is prone to elimination in the presence of 20% piperidine–DMF especially when in the N-terminal position of a peptide. This was resolved by using piperazine in place of piperidine for Fmoc deprotection of the OP-modified serine since piperizine was listed as an alternative base for Fmoc deprotection.[13] Similar byproducts were not isolated from the synthesis of the HSA peptides because tyrosine does not undergo a dehydration reaction.



Scheme 2 Elimination of OP-serine and addition of piperidine to form piperidinylalanine

In summary, we developed a new method to prepare phosphonylated peptides by solid-phase synthesis. This method incorporates serine and tyrosine amino acids with phosphonylated side chains into the synthesis. This eliminates the use of orthogonal protecting groups if multiple amino acids with hydroxy side chains are in the sequence. This can be the case with procedures to phosphonylate peptides while they are still on the resin. We also showed that peptide phosphonates can also withstand acidic conditions to cleave peptides from the resin. In conclusion, the synthesis of phosphonylated peptides is efficient and applicable to the elaboration of serine-, threonine-, or tyrosine-containing peptides.

Supporting Information for this article is available online at http://www.thieme-connect.com/ejournals/toc/synlett. It includes the syntheses and analytical data for all the compounds described as well as the HPLC chromatograms for peptides **13**–**20**.

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