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Solid-Phase Synthesis of the Aged-Nonapeptide-Nerve-Agent Adduct of Butyrylcholinesterase as Reference Materials for Analytical Verification

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Two pathways were developed and investigated for the synthesis of the 'aged'-nonapeptide nerve-agent bioadduct of human butyrylcholinesterase (BuChE). Considering the fast ageing of nerve-agent adducts of BuChE in patients and biomedical samples this target molecule is of paramount relevance for quantitative analysis with respect to the Chemical Weapons Convention. Two approaches using a precursor bearing a hydroxyl on its phosphonyl moiety and a benzyl protected precursor were considered. Several impurities were identified and circumvented during the optimization of the peptide synthesis step. The 'aged'-nonapeptide adduct was successfully synthesized by solid-phase-peptide-synthesis (SPPS).

Keywords: Chemical warfare agents, Nerve agent, Phosphonylation, Solid-phase-peptide-synthesis, Aged nonapeptide.

Introduction

Nerve agents (NAs) are among the most toxic chemical warfare agents (CWAs). Developed in the 1930's, they have been used on several occasions, *e.g.* during the Iran-Iraq war, in the Tokyo subway, in Syria (2013 & 2017), or recently in Malaysia.[1 – 6] The development, production, use, and stockpile of CWAs is prohibited by the Chemical Weapons Convention (CWC) since 1997.[7] The CWC is enforced by the Organization for the Prohibition of Chemical Weapons (OPCW) with the help of its network of designated laboratories.[8] Despite the efforts of the OPCW to destroy the stockpiled CWAs, they still pose a threat, especially for the general public in the context of terrorist attacks.

The structure of the most relevant nerve agents is given in *Figure 1*. They are organophosphorus compound (OPs) which consist of a phosphoester and a leaving group. The differentiation between G- and V-series nerve agents is of historical nature while structurally their main difference consists of the leaving groups.

Nerve agents are strong acetylcholinesterase (AChE) and butyrylcholinesterase inhibitors. The LD_{50} can be as low as 8.4 μg/kg (VX, in rats, subcutaneous). Symptoms of poisoning are miosis, nausea, cramps, loss of muscle control, paralysis, and loss of consciousness.[9] Death usually occurs through respiratory paralysis.[10] Standard therapy consists of symptomatic treatment with atropine and reactivation of inhibited acetylcholinesterase with oximes. Common reactivators are pyridinium aldoximes (2-PAM) and dipyridinium aldoximes (TMB-4, HI-6, LüH-6). However all of them are permanently charged which prevents them of passing the blood-brain-barrier and therefore they provide limited protection for the central nervous system. The reactivators show an agent-specific effectiveness and no broadband reactivator is available today. Survivors might suffer from long-term neuropathic effects.[11 – 15]

Using bioadducts to detect usage of nerve-agent is attractive, due to their relatively long half-life (several days to several weeks). On the other hand, degradation in environmental samples is fast and free metabolites in blood or urine get excreted within 48 – 72 h.[10]

The most relevant bioadducts in blood formed between nerve agents and proteins are AChE (on Ser-203), BuChE (on Ser-198) and albumin (on Tyr-411) adducts.[16][17] BuChE adducts are the most popular analytical targets, since their abundance is ten times greater than those of AChE and BuChE reacts 500 times faster with nerve agents than albumin.[16][18]

NA bioadducts of AChE and BuChE undergo an 'ageing' process which leads to the loss of the O-alkyl

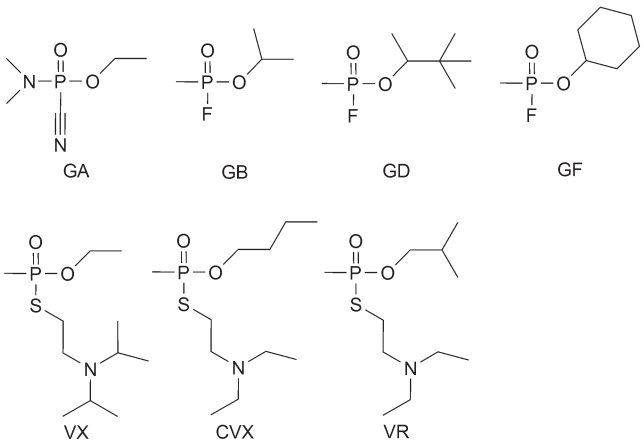
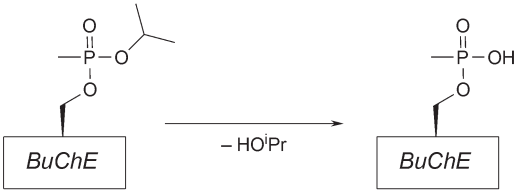


Figure 1. Structures of the nerve agents GA (Tabun), GB (Sarin), GD (Soman), GF (Cyclosarin), VX, CVX (Chinese VX), and VR (Russian VX).

chain of the phosphyl group (*Scheme 1*). This process can occur spontaneously and is due to enzymatic activity in the body.[19] The rate of ageing depends on the ester group, *e.g.*, the half-life of ageing is 2 – 6 min for soman-, 3 – 5 h for sarin-, 13 h for tabun- and 48 h for VX-adducts of AChE.[10][20] After ageing has occurred, the phosphylated enzymes cannot be reactivated since electrostatic repulsion between reactivators and the oxyanion prevent nucleophilic attacks.[21] Further the electrostatic interaction between the oxyanion and the His of the enzymatic pocket strongly stabilizes the aged structure.[22] Therefore detection methods which rely on fluoride reactivation *e.g.*, detecting reactivated OPs by GC/MS, cannot work after ageing.[23][24] Hence, the ability to detect aged bioadducts is highly relevant.

Modern detection methods rely on LC-MS/MS for the detection of nerve-agent bioadducts. BuChE is isolated from plasma with immunomagnetic beads and digested by pepsin to yield a specific nonapeptide with the sequence FGES(MPX)AGAAS, where MPX denotes the phosphyl moiety, which is then detected by tandem LC-MS/MS.[16][25 – 28] With this method, quantitative analysis is possible, but reference materials are needed, especially for the 'aged' nonapeptide adducts (*Figure 2*).



Scheme 1. Enzyme mediated 'ageing' of the sarin-butyrylcholinesterase adduct.

Posttranslational modification of proteins by phosphylation plays a key role in regulating signaling processes and protein-protein interactions in the body, therefore synthesis of phosphopeptides has been of interest for a while.[29] The use of phosphylated serines in SPPS is a challenge since the phosphyl group is decomposed by strong acids and prone to β-elimination when in contact with bases. When a building block approach is used, the phosphyl group should be protected to lower such reactivity. As an alternative approach, peptides can be phosphylated post synthetically which requires unprotected side chain hydroxyl groups to be built in.[30 – 33]

In this work, we report the first synthesis of the aged nerve-agent nonapeptide adduct using a building block approach, in which first serine is phosphonylated and then used in solid-phase-peptide-synthesis. The phosphonylation of serine is based on work carried out in our laboratory.[34] A similar approach was very recently published for the synthesis of deuterium-labelled soman- and VX-nonapeptides as reference materials.[35]

Results and Discussion

Two routes to synthesize the aged nonapeptide **2** were investigated (routes *A* and *B*). For route *A*, we chose Fmoc-Ser(MPA)-OH (**3**) as a precursor to be used in SPPS. Since it was unclear whether or not the hydroxymethylphosphonyl group would lead to side reactions during SPPS, a second route (route *B*) was investigated in parallel, using a protecting group on the phosphonyl moiety. Fmoc-Ser(MPBn)-OH (**4**) was chosen, since we expected the benzyl group to be deprotected together with the other protecting groups at the release step of the peptide from the resin after SPPS.

Commercially available Fmoc-Ser-OH (**5**) was protected using benzyl alcohol and thionyl chloride giving Fmoc-Ser-OBn (**6**) with 82% yield (*Scheme 2*).[36] Compound **6** was treated with methyl phosphonic dichloride (**7**; MPDC) using DABCO/DMAP as bases with two-fold and 0.2-fold excess, respectively. This base combination has proven to be crucial to prevent the loss of the phosphonyl group by β-elimination. The intermediate Fmoc-Ser(methyl phosphonic chloride)-OBn (**8**) was hydrolyzed directly on the column during purification by reversed phase flash chromatography yielding **9** (purity 87%, P-NMR). The benzyl group was then selectively deprotected by hydrogenolysis with 1,4-cyclohexadiene and Pd/C yielding **3** in mediocre purity. Further purification of **3** by flash chromatography led to considerable losses; hence it was used without further purification for SPPS.

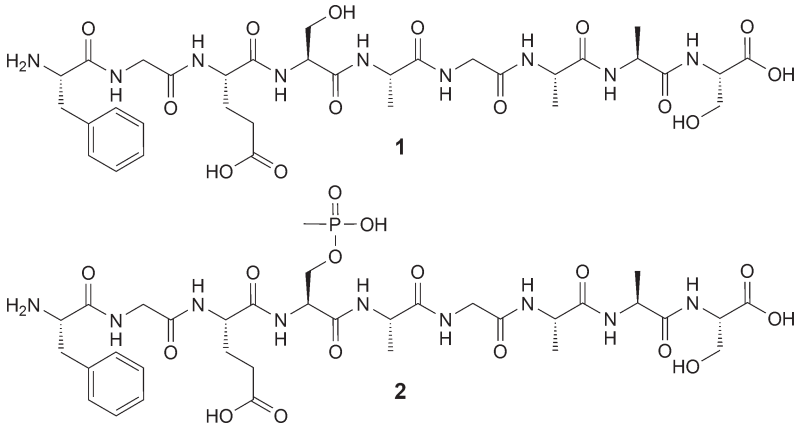
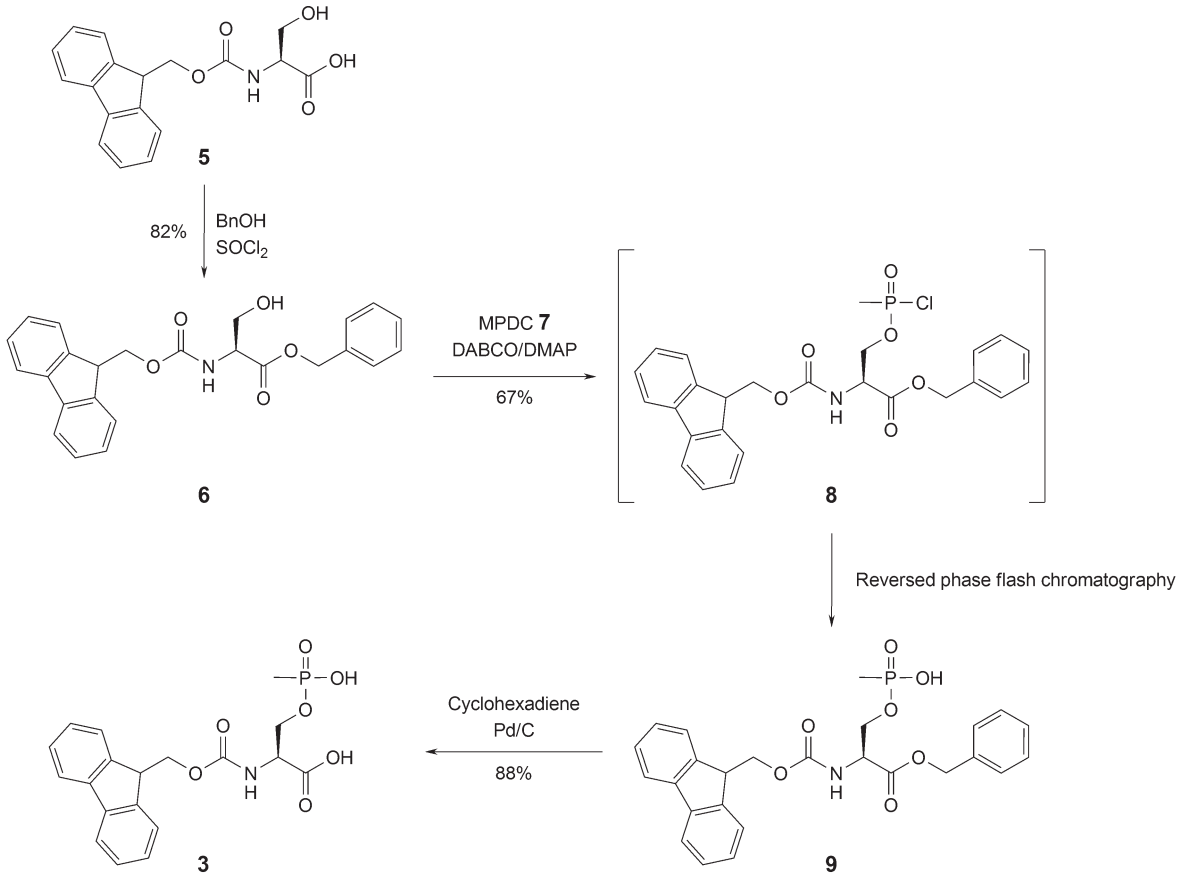
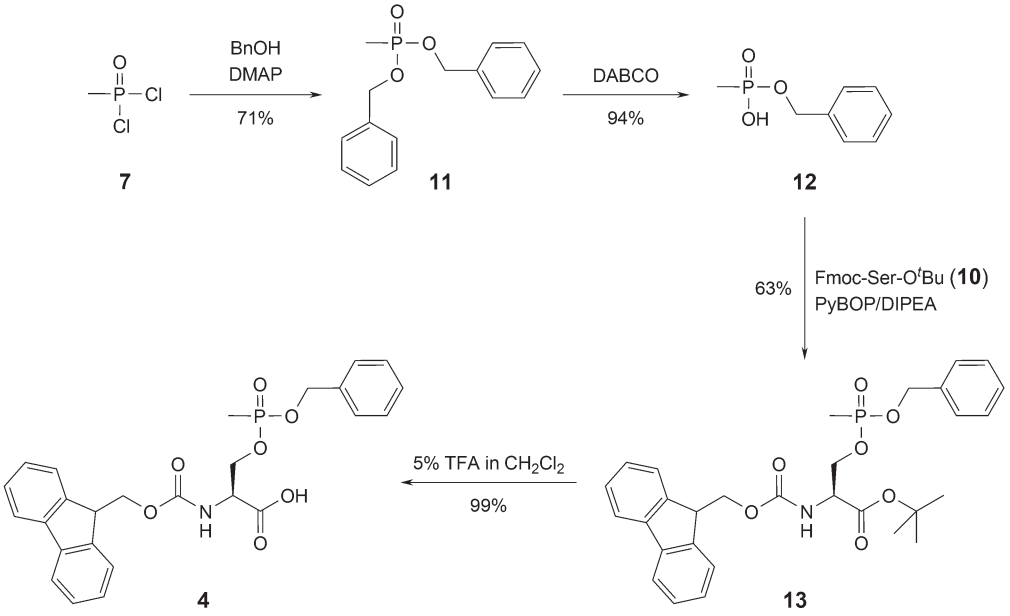


Figure 2. Non-phosphylated nonapeptide **1** and 'aged' nerve-agent bioadduct **2** after pepsin digestion of phosphylated human BuChE.



Scheme 2. Synthesis of the precursor to route *A*.

Since compound **4** already contains a benzyl group on the phosphonyl, an orthogonal protecting group for the acid group had to be found. *tert*-Butyl was assumed to be cleavable while leaving the P–OBn group intact under weak acidic conditions. A screening was made to find the mildest conditions to hydrolyze commercially available Fmoc-Ser-O^{t}Bu (**10**) with trifluoroacetic acid. The ^{t}Bu group was cleaved within five minutes when the TFA concentrations in the range from 80% to 5% were used. At concentrations below 5%, the reaction slowed down considerably, taking 30 min at 4% and > 210 min at 3%. For concentrations of 2% and 1%, full conversion was not reached after 24 h.



Scheme 3. Synthesis of the precursor to route *B*.

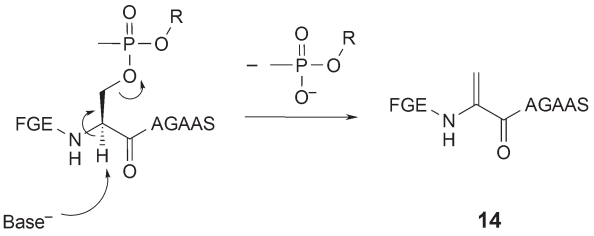
The synthesis of **4** started with MPDC **7** which was esterified with benzylic alcohol and DMAP to give methyl phosphonic acid dibenzyl ester **11** (*Scheme 3*) with excellent purity.

Under basic conditions, one benzyl group of **11** was cleaved to give monoester **12** (purity 98%, P-NMR).[37] Compound **12** was then coupled to **10** using PyBOP and DIPEA as coupling agents yielding phosphonylated serine **13** in 63% yield and 90% purity (P-NMR). Two diastereoisomers were generated due to the chiral center at the phosphorous. Compound **13** was exposed to the best cleavage conditions found in the screening which was shown to be 5% TFA in CH_2Cl_2. These were the mildest conditions in which full deprotection would occur after 5 min. Deprotection of **13** was slower than of **10**, and the reaction was extended to two days at room temperature. To our satisfaction, these conditions left the benzyl group intact and compound **4** could be obtained in 99% yield after purification. Compound **4** again was present as a mixture of two diastereoisomers due to the chiral center at the P center. In contrast to **3**, purification of **4** by flash chromatography occurred without extensive loss, and the product was gained in high purity. An alternative deprotection procedure using aqueous phosphoric acid was unsuccessfully attempted.[38]

In order to fine-tune our SPPS procedure, non-phosphylated nonapeptide **1** (which is also a helpful reference material in the development of biomedical analysis methods), was synthesized and the procedure scaled up. Pre-loaded resins were used and 20% piperidine in DMF was used for Fmoc-deprotection. For deprotection and cleavage from the resin, a cleavage mixture consisting of TFA/TIS/H_2O 95:2.5:2.5 was used. The best work-up method proved to be precipitation in cold diethyl ether and subsequent centrifugation yielding the nonapeptide in pure form, while extraction or flash chromatography gave inferior results in terms of yield and purity.

First attempts in employing **3** in solid-phase-peptide-synthesis using microwave (mw) heating (65 °C, 5 min) to speed up amino acid coupling proved unsuccessful, and led to β-elimination of the phosphonyl group and produced the dehydroalanin-containing peptide **14** as the major product (*Scheme 4*).

Therefore, all subsequent peptide syntheses were performed at room temperature. Small scale experiments (0.01 mmol) showed that the introduction of phosphonylated serine into the peptide chain was slower than the introduction of regular amino acids. In fact, deletion peptide **15** (*Figure 3*) was found to be the major product together with deletion peptide **16** in an almost 1:1 ratio. While **15** lacked the phosphylated serine, **16** lacked the last two amino acids of the sequence.



Scheme 4. β-Elimination of the phosphonyl group under mw and basic conditions.

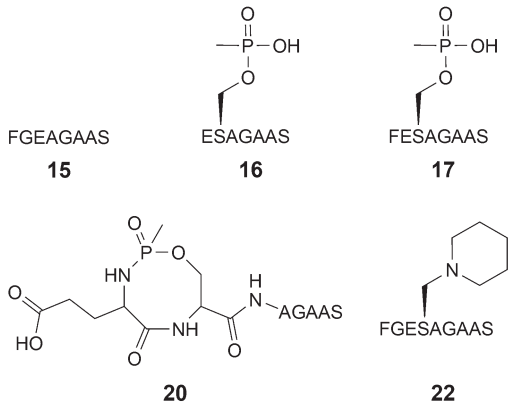


Figure 3. Side products of peptide synthesis with phosphonylated Ser and piperidine as base during the deprotection step. **15**: deletion product without Ser(MPA) incorporated. **16**: deletion product with missing Phe and Gly. **17**: deletion product with missing Gly (not observed). **20**: cyclic peptide after phosphonamidation side reaction. **22**: trapping product with the base.

The desired product **2** from route *A* was only found in traces. Prolonging the coupling time of the phosphonylated serine to 120 min prevented **15** from occurring. Switching the base for the deprotection step of SPPS from 20% piperidine in DMF to 5% piperazine in DMF completely prevented the formation of **16**. From these experiments, the absence of a deletion peptide **17** which would contain Phe but not the second Gly and the fact that Phe and Gly only missed if Ser(MPA) was incorporated we concluded that the base is crucial for the formation of side product **16** and the formation follows an unusual mechanism. The proposed mechanism is shown in *Scheme 5*.

We hypothesized that during the harsher conditions of the piperidine deprotection method, the growing peptide chain may end up as the cyclic phosphonamidate **18**, either by reaction of **19** with the coupling agents mixture, or *via* an activation pathway which might involve pyrophosphonate formation. The intramolecular cyclisation is faster than the intermolecular peptide bond formation, and the formation of the cycle prevents further elongation of the peptide chain. Under the harsh acidic cleavage and deprotection conditions after SPPS the cycle opens again and gives **16**. After scanning for cycle **20** in the HR/LC-MS data, its mass $[M + H]^+$ 652.2338 could be found as a minor byproduct (*ca.* 4%) of the crude peptide. Our proposition is further supported by the fact that if a protection group is used on P–OH, no deletion product **16** was found, because no cyclic phosphonamidate could be formed. The proposed mechanism is similar to the findings of *Amedjkoh*, who investigated the phosphonamidation reaction mechanism with standard peptide coupling agents, including DIC and Oxyma, and *Kitamura*, who used protected α-amino phosphonic acids and coupling agents to synthesize α-amino phosphonic acid oligomers by solid-phase synthesis.[39][40] However, in our system we could not confirm the formation of pyrophosphonate **21** as it was observed by *Amedjkoh*.

Using the optimized protocol from route *A* **2** could nevertheless be obtained as the major product however with low purity (79%, P-NMR).

For the SPPS following route *B*, the prolonged coupling time at room temperature for **4** was already applied and therefore byproduct **15** was not observed. Using 20% piperidine in DMF for Fmoc-deprotection gave desired product **2** as the major product but led to significant amount of piperidinyl peptide **22** and small quantities of elimination product **14** were observed as well. The formation of piperidinyl peptides is a known side reaction with phosphopeptides.[41] Switching to the optimized protocol (5% piperazine in DMF) prevented the occurrence of **22**. Interestingly the P–OBn group was cleaved with the same cleavage cocktail which was used to deprotect the ^{t}Bu protecting groups of the first Ser and Glu and to cleave the peptide from the resin (*Scheme 6*). This optimized protocol yielded **2** after a simple work-up step in very good purity (> 95% P-NMR) and as a single stereoisomer.

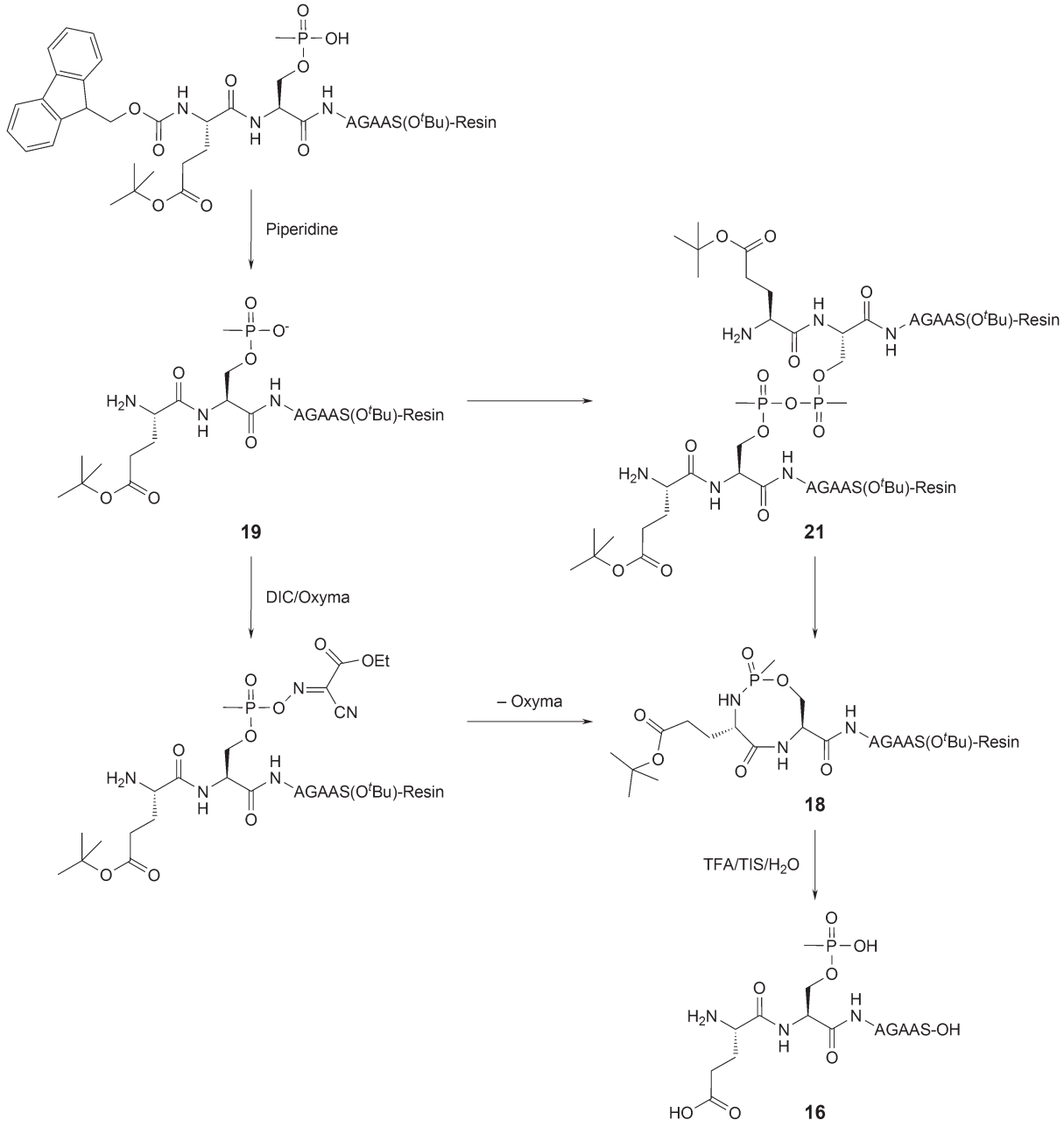
Neither side products **16** nor **17** could be found in any syntheses using **4**. This can be explained by the protection of the P–OH group and further supports our proposition of the side reaction mechanism.

HR-MS measurement of **2** obtained by route *B* clearly shows the molecular ion peak at *m/z* $[M + H]^+$ 874.3328 and the sodium adduct at *m/z* $[M + Na]^+$ 896.3138 (*Figure 4*). The first fragment in the MS/MS analysis shows the loss of methyl phosphonic acid while generating a β-elimination type peptide fragment. The molecular ion further fragmentizes by subsequent loss of Ser, Ala, Ala, Gly, and Ala from the C-terminus on. The aged nonapeptide shows the same fragmentation pattern as non-aged nonapeptide nerve-agent adducts.[42]

Conclusion

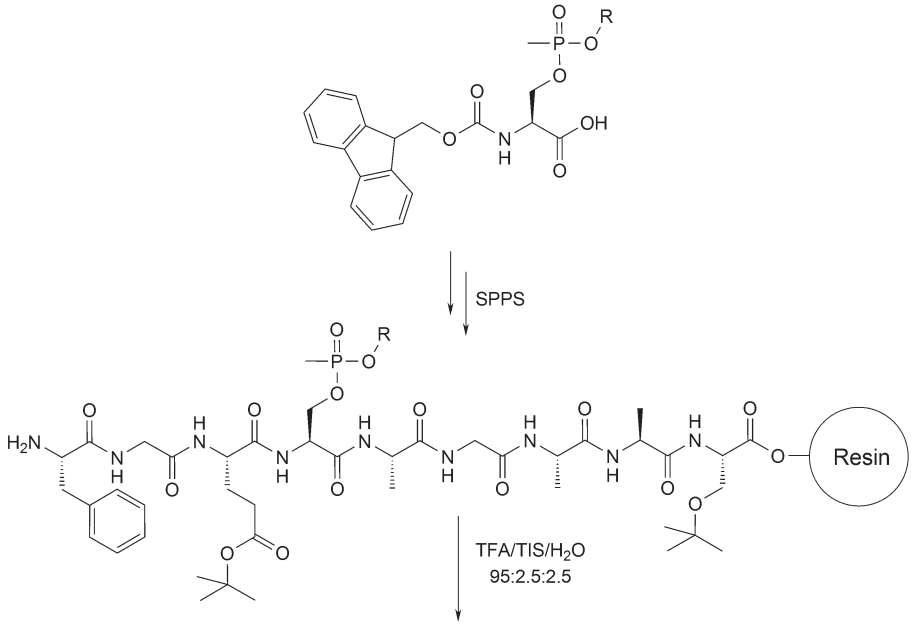
Two routes to synthesize the aged nonapeptide **2**[1] using different protecting group strategies were

[1] Reference material **2** was used for the development of analytical methods at Spiez Laboratory to detect the aged nonapeptide and led to the discovery of aged nonapeptide in sarin spiked blood samples during the 2nd biomedical proficiency test conducted by OPCW.



Scheme 5. Proposed mechanism for the deletion product **16**.

developed and investigated in detail. The synthesis for the precursor for route *A* consists of three steps with an overall yield of 48%. The synthesis for the precursor of route *B* consists of four steps with an overall yield of 42%. While both pathways have a similar yield in the final step, the route *B* through protected phosphonyl serine delivers better purity. When possible, the side products were identified and mechanisms for their generation proposed. Several side reactions could be identified when using the standard Fmoc-deprotection method for SPPS with 20% piperidine in DMF during the deprotection step. An optimized protocol for the use of phosphylated serine in SPPS using piperazine in the deprotection step was developed which takes into account the slower coupling to the peptide chain as well as the thermo- and base lability of the phosphonyl group. The optimizations applied in this work can also be used for the synthesis of other phosphonylated peptides which suffer from the same labilities.



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Scheme 6. Precursors **3** and **4** in SPPS (**3** R = H, **4** R = Bn). Cleavage from the resin and deprotection of the side chains and phosphonyl group in a one-pot reaction.

Experimental Part

All reactions, except the SPPS and synthesis of **4**, were carried out under Ar atmosphere in dried glassware. Chemicals were obtained from standard commercial sources like *Sigma–Aldrich*, *Roth*, *Alfa Aesar*, and *Tokyo Chemical Industry Co.* and were used without further purification. Amino acids were obtained from *Sigma–Aldrich* and *Aroz Technologies*. Resins for SPPS were obtained from *Iris Biotech*, *Sigma–Aldrich*, and *Protein Technologies*.

Solid-phase-peptide-synthesis was performed on an *Alstra* automated peptide synthesizer from *Biotage*. Flash chromatography was done on an *Isolera One* coupled to a *Dalton* mass detector from *Biotage*. ^{1}H-NMR (400 MHz), ^{13}C-NMR $\{^1H\}$ (100 MHz) and ^{31}P-NMR $\{^1H\}$ (162 MHz) were measured with a *Bruker Avance III HD 400* MHz *Nano Bay* in $CDCl_3$ or D_2O. The chemical shifts (δ) are indicated in ppm with respect to the internal standard TMS or the solvent peak. Coupling constants (*J*) are indicated in Hertz [Hz] and the spins are indicated with letters *s* = *singlet*, br. *s* = broad *singlet*, *d* = *doublet*, *t* = *triplet*, *q* = *quartet*, and *m* = *multiplet*. LC/ESI-MS measurements were performed on an *Agilent Technologies 1290 Infinity LC* coupled to a *Bruker Daltonics maXis plus UHR QTof* system. The column used in the LC was a *Sigma–Aldrich Discovery HS C18* (150 mm × 2.1 mm, particle size 5 μm). H_2O with 5 mM NH_4Ac and MeOH with 5 mM NH_4Ac were used as eluent with a flow rate of 0.6 ml/min. Melting points were measured on a *Büchi 535*. IR were measured on a *Bruker Tensor II* equipped with a *Golden Gate diamond ATR* system.

Benzyl *N*-[(9*H*-Fluoren-9-ylmethoxy)carbonyl]serinate (Fmoc-Ser-OBn; **6**). Fmoc-Ser-OH (5.5 g, 16.8 mmol, 1 equiv.) was dissolved in 100 ml CH_2Cl_2 and benzyl alcohol (8.7 ml, 84 mmol, 5 equiv.) was added. The solution was cooled to 0 °C. Thionyl chloride (1.8 ml, 25 mmol, 1.5 equiv.) was added dropwise and the solution was stirred for 1.5 h at 0 °C. Another portion of thionyl chloride (1.8 ml, 25 mmol, 1.5 equiv.) was added to the reaction. The ice bath was renewed after the second addition. Then the mixture was stirred over night while slowly warming up to r.t. The reaction was cooled to 0 °C, quenched with sat. $NaHCO_3$ solution (200 ml) and extracted with AcOEt (2 × 200 ml). The organic layer was washed with brine (3 × 200 ml) and dried over Na_2SO_4. After filtration, the solvent was removed under reduced pressure. The crude product was purified by flash chromatography (*Isolera One*, *SNAP Ultra* 100 g, 1 column volume (CV) AcOEt/hexane 1:3, gradient during 10 CV up to 100% AcOEt). The fractions containing the product were collected and the solvent removed under reduced pressure. The crude mixture was dissolved in CH_2Cl_2 (12.5 ml) and precipitated in cold hexane (600 ml). The solid was collected, dried under reduced pressure and then lyophilized. 5.77 g (82%

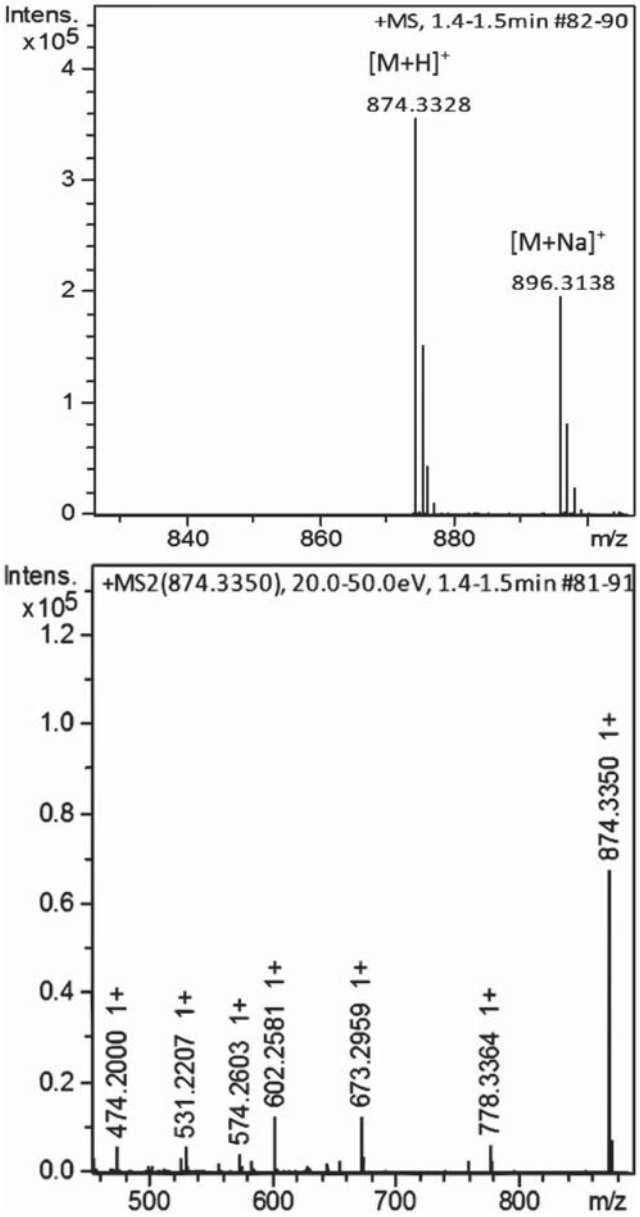

Figure 4. HR-MS and HR-MS/MS show the fragmentation pattern of **2**.

yield) of product was obtained as a white solid. M.p. 97-98 °C. FT-IR (neat): 3315, 3065, 3037, 2948, 2889, 2361, 2341, 1734, 1686, 1609, 1537, 1500, 1478, 1449, 1393, 1339, 1254, 1217, 1193, 1104, 1084, 1045, 1030, 977, 907, 757, 135, 696, 647, 621, 604, 587, 571, 542, 523, 505, 482, 470, 464, 443. ^{1}H-NMR (400 MHz, $CDCl_3$): 7.75 (*d*, *J* = 8, 2 H); 7.58 (*d*, *J* = 8, 2 H); 7.38 – 7.29 (*m*, 9 H); 5.79 (*d*, *J* = 8, 1 H); 5.20 (*s*, 2 H); 4.49 – 4.38 (*m*, 3 H); 4.21 – 4.18 (*m*, 1 H); 4.02 – 3.90 (*dm*, *J* = 18, 2 H). ^{13}C-NMR {^{1}H} (100 MHz, $CDCl_3$): 170.3; 156.2; 143.7; 141.3; 128.7;135.0 128.7; 128.5; 128.2; 127.8; 127.0; 125.0; 120.0; 67.6; 67.2; 63.4; 56.1; 47.1. HR-MS (ESI/Q-TOF): 269.132503 ([*M* + H]$^+$, $C_{25}H_{23}NO_5^+$; calc. 269.132477).

Benzyl *N*-[(9*H*-Fluoren-9-ylmethoxy)carbonyl]-*O*-[hydroxy(methyl)phosphoryl]-L-serinate (Fmoc-Ser(MPA)-OBn; **9**). Methyl phosphonic dichloride (1.14 g, 4.3 mmol, 2 equiv., 0.6 ml) in CH_2Cl_2 (52 ml) was given in a three-neck round bottom flask equipped with a magnetic stirrer and a dropping funnel. After cooling to 0 °C, a solution of Fmoc-Ser-OBn (**4**; 1.8 g, 4.3 mmol, 1 equiv.), DABCO (0.96 g, 8.6 mmol, 2 equiv.), and DMAP (0.1 g, 0.86 mmol, 0.2 equiv.) in CH_2Cl_2 (45 ml) was added dropwise. After the addition, the ice bath was removed, and the mixture was warmed to r.t. The reaction process was monitored by TLC (AcOEt/hexane 1:1). After 2.5 h, the mixture was filtered and the filtrate concentrated. It was further purified by reverse phase flash chromatography (*SNAP Ultra C-18* 60 g, MeCN/H_2O 1:9, gradient up to 100% MeCN). The fractions containing the product were collected and the solvent removed under reduced pressure. 1.44 g product (67% yield) was obtained as a white solid. M.p. 112 – 114 °C. FT-IR (neat): 3432, 3062, 3038, 2959, 2352, 1728, 1513, 1506, 1450, 1392, 1338, 1313, 1250, 1211, 1192, 1167, 1081, 1057, 1033, 998, 935, 897, 878, 830, 813, 788, 758, 728, 696, 655, 645, 621. ^{1}H-NMR (400 MHz, $CDCl_3$): 9.16 (br. *s*, 1 H); 7.74 (*d*, *J* = 8, 2 H); 7.57 (*d*, *J* = 8, 2 H); 7.38 – 7.26 (*m*, 9 H); 6.03 (*dd*, *J* = 64, 8, 1 H); 5.13 – 4.97 (*m*, 2 H); 4.60 – 4.48 (*m*, 2 H); 4.41 – 4.25 (*m*, 2 H); 4.21 – 4.13 (*m*, 2 H); 1.54 – 1.46 (*m*, 3 H). ^{13}C-NMR {^{1}H} (100 MHz, $CDCl_3$): 170.4; 155.9; 143.8; 141.3; 136.2; 135.6; 128.8; 128.8; 128.5; 127.9; 127.7; 127.0; 125.2; 119.9; 68.0; 67.3 54.3; 47.0; 11.7; 11.5; 11.0; 10.2. ^{31}P-NMR {^{1}H} (162 MHz, $CDCl_3$): 35.05 (87%); 34.27 (13%). HR-MS (ESI/Q-TOF): 496.151811 ([*M* + H]$^+$, $C_{26}H_{26}NO_7P^+$; calc. 496.151965).

***N*-[(9*H*-Fluoren-9-ylmethoxy)carbonyl]-*O*-[hydroxy-(methyl)phosphoryl]-L-serine** (Fmoc-Ser(MPA)-OH; **3**). Fmoc-Ser(MPA)-OBn (**9**; 1.5 g, 3 mmol, 1 equiv.) was dissolved in THF (16 ml) and MeOH (16 ml) was added. Pd/C 10% (1.9 g, 18 mmol, 6 equiv.) and 1,4-cyclohexadiene (4.2 ml, 45 mmol, 15 equiv.) were added, and the mixture was stirred for 2 h under Ar. The reaction was monitored by TLC (THF/MeOH 2:1). Another portion of 1,4-cyclohexadiene (3.4 ml, 40 mmol, 10 equiv.) was added after 2 h. After another hour of stirring, the reaction mixture was filtered through *Celite*, and the *Celite* was rinsed with MeOH (100 ml). The solvent was removed under reduced pressure and then lyophilized. The product was used without further purification. 1.07 g (88%) product was obtained. To obtain better spectral data the product was further purified by flash (*SNAP Ultra* 100 g, 100% CH_2Cl_2 (1CV), to 5% MeOH (1CV), to 30% MeOH (1CV), to 50% MeOH (10CV)). The fractions

containing the product were collected and the solvent removed under reduced pressure. 0.59 mg (48%) product was obtained after purification as a white foam. M.p. 57 – 58 °C. FT-IR (neat): 3306, 3063, 2929, 2359, 2341, 1714, 1518, 1449, 1337, 1314, 1247, 1209, 1193, 1079, 1056, 1031, 985, 902, 821, 775, 758, 737, 697, 668, 645, 620. ^{1}H-NMR (400 MHz, $CDCl_3$): 10.34 (br. *s*, 1 H); 7.77 – 7.69 (*m*, 2 H); 7.57 – 7.45 (*m*, 2 H); 7.38 – 7.25 (*m*, 4 H); 6.20 (*s*, 1 H); 4.70 – 4.14 (*m*, 5 H); 1.59 – 1.27 (*m*, 3 H). ^{13}C-NMR $\{^{1}H\}$ (100 MHz, $CDCl_3$): 172.6; 156.2; 143.8; 141.3; 127.8; 127.1; 125.2; 119.9; 67.5; 65.5; 54.6; 46.9;11.8; 10.3 ^{31}P-NMR $\{^{1}H\}$ (162 MHz): 34.17 (87%); 32.57 (13%). HR-MS (ESI/Q-TOF): 406.105056 ($[M + H]^+$, $C_{19}H_{20}NO_7P^+$; calc. 406.105015).

Dibenzyl Methylphosphonate (MPDBn; **11**). Methyl phosphonic dichloride (10 g, 71.8 mmol, 1 equiv.) and benzene (12 ml) were mixed and poured in a dry 250 ml three-neck round-bottom flask immersed in an ice bath which was equipped with a dropping funnel. In an *Erlenmeyer* flask, DMAP (19.3 g, 158 mmol, 2 equiv.), BnOH (16.4 ml, 158 mmol, 2 equiv.), and benzene (98 ml) were mixed. The mixture was added dropwise to the MPDC solution at 0 °C. After the addition the ice bath was removed and the mixture was stirred at r.t. for 1 h. Hexane (50 ml) was added and stirring was continued for 1 h. The product was separated by suction filtration through Al_2O_3 which was thoroughly washed with hexane and the solvent was removed under reduced pressure. 14.8 g (71% yield) was obtained. FT-IR (neat): 3037, 3032, 2953, 2926, 2890, 2359, 2325, 1716, 1700, 1650, 1606, 1568, 1497, 1455, 1418, 1379, 1311, 1237, 1213, 1081, 1035, 993, 918, 897, 828, 729, 695, 649, 638, 592, 523, 507, 494, 475, 467, 462, 440, 432 413. ^{1}H-NMR (400 MHz, $CDCl_3$): 7.37 – 7.34 (*m*, 10 H); 5.08 – 4.94 (*dm*, *J* = 40, 4 H); 1.47 (*d*, *J* = 16, 3 H). ^{13}C-NMR $\{^{1}H\}$ (100 MHz, $CDCl_3$): 136.4; 129.7 – 127.0; 67.1; 12.4; 11.0 ^{31}P-NMR $\{^{1}H\}$ (162 MHz, $CDCl_3$): 31.74 (99%). HR-MS (ESI/Q-TOF): 277.098585 ($[M + H]^+$, $C_{15}H_{17}O_3P^+$; calc. 277.098807).

Benzyl Hydrogen Phosphonate (MPBnA; **12**). MPDBn (**11**; 1 g, 3.62 mmol, 1 equiv.) was given in toluene (12.5 ml) under Ar, and DABCO (400 mg, 3.62 mmol, 1 equiv.) was added. The mixture was stirred under reflux for 2 d. The solvent was evaporated, the residue retaken in 5% HCl (40 ml) and extracted with AcOEt (2 × 40 ml). The organic phases were collected and the solvent removed under reduced pressure. 640 mg (94% yield) product was obtained as a yellowish oil. FT-IR (neat): 3091, 3065, 3033, 2891, 2659, 2357, 2326, 1738, 1498, 1417, 1381, 1363, 1313, 1213, 1081, 995, 904, 824, 734, 698, 631. ^{1}H-NMR (400 MHz, $CDCl_3$): 7.39 – 7.32 (*m*, 5 H); 5.04 (*d*, *J* = 4, 2 H); 1.52 (*d*, *J* = 20, 3 H). ^{13}C-NMR $\{^{1}H\}$ (100 MHz, $CDCl_3$): 136.2; 128.6; 128.4; 127.8; 127.7; 127.0; 66.5; 12.6; 11.2 ^{31}P-NMR $\{^{1}H\}$ (162 MHz, $CDCl_3$): 34.65 (98%). HR-MS (ESI/Q-TOF): 187.051974 ($[M + H]^+$, $C_8H_{11}O_3P^+$; calc. 187.051857).

***tert*-Butyl *O*-[(Benzyloxy)(methyl)phosphoryl]-*N*-[(9*H*-fluoren-9-ylmethoxy)carbonyl]-L-serinate** (Fmoc-Ser(MPBn)-O^{t}Bu; **13**). To a solution of Fmoc-Ser-O^{t}Bu (1 g, 2.6 mmol, 1 equiv.) was added MPBnA (**12**; 0.48 g, 3 mmol, 1 equiv.) and PyBoB (1.63 g, 3.2 mmol, 1.2 equiv.) in CH_2Cl_2 (16 ml). DIPEA (1.8 ml, 1.35 g, 10.4 mmol, 4 equiv.) was added slowly, while the mixture was immersed in an ice bath. The mixture was stirred at r.t. for 24 h. The solvent was removed under reduced pressure, and the crude further purified by flash chromatography (*SNAP Ultra* 100 g, AcOEt/hexane, gradient from 10% AcOEt up to 100% AcOEt). The fractions containing the product were collected and the solvent removed under reduced pressure. 903 mg (63% yield) of product was obtained as a yellow oil. FT-IR (neat): 3270, 3065, 3037, 2979, 2951, 2896, 2358, 2337, 1720, 1537, 1478, 1451, 1393, 1369, 1343, 1311, 1240, 1155, 1081, 1047, 999, 920, 843, 786, 760, 740, 697, 631, 622. ^{1}H-NMR (400 MHz, $CDCl_3$): 7.76 (*d*, *J* = 8, 2 H); 7.61 (*d*, *J* = 8, 2 H); 7.36 – 7.29 (*m*, 9 H); 5.87 (*dd*, *J* = 8, 40, 1 H); 5.09 – 4.94 (*m*, 2 H); 4.45 – 4.35 (*m*, 2 H); 4.23 – 4.22 (*m*, 1 H); 4.13 – 4.11 (*m*, 2 H); 1.50 – 1.46 (*m*, 12 H). ^{13}C-NMR $\{^{1}H\}$ (100 MHz, $CDCl_3$): 171.1; 155.8; 143.8; 141.2; 136.0; 128.4; 128.6; 128.6; 127.9; 127.1; 125.1; 119.9; 83.1; 67.4; 67.1; 65.5; 54.8; 47.0; 27.9; 12.0; 10.5 (*d*, *J* = 48) ^{31}P-NMR $\{^{1}H\}$ (162 MHz, $CDCl_3$): 32.27 (39%); 32.18 (51%). HR-MS (ESI/Q-TOF): 552.214766 ($[M + H]^+$, $C_{30}H_{34}NO_7P^+$; calc. 552.214566).

***O*-[(Benzyloxy)(methyl)phosphoryl]-*N*-[(9*H*-fluoren-9-ylmethoxy)carbonyl]-L-serine** (Fmoc-Ser(MPBn)-OH; **4**). Fmoc-Ser(MPBn)-O^{t}Bu (**13**; 1.5 g, 2.7 mmol, 1 equiv.) was added to a 5% TFA solution in CH_2Cl_2 (125 ml) and stirred for 48 h. The solvent was removed under reduced pressure. The crude mixture was diluted with CH_2Cl_2 (3 ml) and further purified by flash chromatography (*SNAP Ultra C-18*, *60 g*, MeCN/H_2O, gradient from 12% – 100% MeCN). The fractions containing the product were collected and the solvent removed under reduced pressure. 1.33 g (99%) product was obtained as a light brownish solid. M.p. 50 – 52 °C. FT-IR (neat): 3413, 3316, 3064, 3036, 2953, 2927, 2895, 2539, 2359, 2341, 1715, 1609, 1516, 1478, 1450, 1414, 1381, 1313, 1294, 1194, 1079, 997, 918, 830, 759, 736, 696, 645, 621. ^{1}H-NMR (400 MHz, $CDCl_3$): 10.48 (br. *s*, 1 H); 7.72 (*d*, *J* = 8, 2 H); 7.57 (*d*, *J* = 8, 2 H); 7.36 – 7.25 (*m*, 9 H); 6.07 (*dt*, *J* = 8, 40, 1 H); 5.09 – 5.00 (*m*, 2 H); 4.56 – 4.52 (*m*, 2 H);

4.39 – 4.27 (*m*, 2 H); 4.19 – 4.16 (*m*, 1 H); 1.49 (*d*, *J* = 16, 3 H). ^{13}C-NMR {^{1}H} (100 MHz): 168.8; 154.2; 141.9; 139.4; 133.7; 126.9; 126.9; 126.3; 126.2; 125.9; 123.3; 118.1; 66.2; 66.2; 65.9; 52.5; 45.2; 9.7; 8.2. ^{31}P-NMR {^{1}H} (162 MHz, $CDCl_3$): 33.57 (54%); 41.01 (41%). HR-MS (ESI/Q-TOF): 496.151628 ([*M* + H]$^+$, $C_{26}H_{26}NO_7P^+$; calc. 496.151965).

Screening of the ^{t}*Bu Group Hydrolysis*

5 ml of the cleavage cocktail was added to Fmoc-Ser-O^{t}Bu (0.1 g, 0.26 mmol, 1 equiv.) and the time until full conversion was monitored by mass spectrometry (direct injection in the Dalton mass detector).

SPPS General Procedure

The synthesis was performed on an *Alstra* automated peptide synthesizer from *Biotage*. For 0.01 mmol scale the 5 ml reactor vial was used, for 0.1 – 0.25 mmol scale the 10 mmol vial and for 1.2 mmol scale the 30 ml reactor vial was used. Amino acids and coupling agents were used in fourfold excess for the synthesis of the unlabeled nonapeptide and fivefold excess for the phosphonylated peptides. Their stock solutions were prepared with 0.5M concentration in DMF. Phosphonylated serines were used in five-and-a-half-fold excess and in 0.5M solutions in DMF. DIC and Oxyma were used as coupling agents in fourfold or fivefold excess respectively. The following protected amino acids were used: Fmoc-Ala-OH, Fmoc-Gly-OH, Fmoc-Ser(O^{t}Bu)-OH, Fmoc-Glu(O^{t}Bu)-OH, and Fmoc-Phe-OH. Preloaded Ser(O^{t}Bu)-HMPB or Ser(O^{t}Bu)-2-CT resin was swollen in CH_2Cl_2 at r.t. for 60 min. The couplings were performed at r.t. for 60 min (120 min for phosphonylated serines), followed by four washing steps with DMF. The deprotection step was done twice (3 min and 15 min) with 5% piperazine in DMF at r.t. followed by four washing steps with DMF. At the end of the synthesis the resin was washed ten times (6 × CH_2Cl_2, then MeOH, CH_2Cl_2, MeOH, CH_2Cl_2). The peptide was cleaved from the resin and deprotected using TFA/TIS/H_2O 95:2.5:2.5 as cleavage cocktail for 1 h. The resin was filtered off and washed twice with TFA. The filtrate was concentrated and precipitated in ice cold diethyl ether. After centrifugation and decantation, another portion of cold diethyl ether was added. The centrifuge tube was agitated to wash the peptide and centrifuged again. This last washing step was repeated a total of three times. After decantation, MeCN (1 ml) was added to the residue and it was agitated. The peptide was solved in H_2O (1 ml) and lyophilized.

Phe-Gly-Glu-Ser-Ala-Gly-Ala-Ala-Ser (**1**). The synthesis was performed according to the *General Procedure for SPPS*. Fmoc-Ser(O^{t}Bu) was used. The scale was 1.2 mmol and the 30 ml reactor vial was used. Ser(O^{t}Bu)-2-CT-resin (1.76 g, 1.2 mmol, 0.68 mmol/g loading, *Iris Biotech*) was used. 20% piperidine instead of 5% piperazine was used in the deprotection step. Fourfold excess of amino acids and coupling agents were used. 1 g (99%) product was obtained as a white fluffy solid. M.p. 172 °C (dec.). FT-IR (neat): 3284, 3070, 2933, 2636, 1696, 1661, 1622, 1518, 1450, 1416, 1339, 1312, 1195, 1059, 1032, 1000, 981, 904, 834, 798, 758, 738, 726, 698, 642, 621. ^{1}H-NMR (400 MHz, D_2O): 7.34 – 7.22 (*m*, 5 H); 4.38 – 4.21 (*m*, 6 H); 3.94 – 3.77 (*m*, 8 H); 3.62 (*s*, 2 H); 3.12 – 3.09 (*m*, 2 H); 2.42 – 2.36 (*m*, 2 H); 1.94 – 1.88 (*dm*, *J* = 48, 2 H); 1.33 – 1.31 (*m*, 9 H). ^{13}C-NMR {^{1}H} (100 MHz, D_2O): 177.0; 175.4; 174.8-170.7; 169.7; 133.7; 129.3; 128.0; 61.0; 60.6; 54.7; 54.4; 52.9; 49.9; 49.5; 49.5; 42.3; 42.2; 36.7; 29.9; 26.1; 16.9-16.3. HR-MS (ESI/Q-TOF): 796.347951 ([*M* + H]$^+$, $C_{33}H_{49}N_9O_{14}^+$; calc. 796.347174).

Phe-Gly-Glu-Ser-*O*-(hydroxy(methyl)phosphoryl)-Ala-Gly-Ala-Ala-Ser *synthesized by route* A (**2**). The synthesis was performed according to the *General Procedure for SPPS*. Ser(O^{t}Bu)-HMPB-resin (222 mg, 0.3 – 0.6 mmol/g loading, *Sigma–Aldrich*) and Fmoc-Ser(MPA)-OH (**3**) (223 mg, 0.55 mmol, 5.5 equiv.) were used. The scale of the synthesis was 0.1 mmol and the 10 ml reactor vial was used. 47 mg product was obtained as a white fluffy solid. ^{1}H-NMR (400 MHz, D_2O): 7.34 – 7.22 (*m*, 5 H); 4.45 – 4.21 (*m*, 7 H); 4.08 – 3.80 (*m*, 9 H); 3.50 (*s*, 2 H); 3.18 – 3.12 (*m*, 2 H); 2.18 – 1.80 (*m*, 2 H); 1.34 – 1.32 (*m*, 9 H); 1.21 (*d*, *J* = 20, 3 H). ^{31}P-NMR {^{1}H} (162 MHz, D_2O): 27.78 (76%). HR-MS (ESI/Q-TOF): 874.3337 ([*M* + H]$^+$, $C_{34}H_{52}N_9O_{16}P^+$; calc. 874.334240).

Phe-Gly-Glu-Ser-*O*-(hydroxy(methyl)phosphoryl)-Ala-Gly-Ala-Ala-Ser *synthesized by route* B (**2**). The synthesis was performed according to the *General Procedure for SPPS*. Ser(O^{t}Bu)-HMPB-resin (223 mg, 0.3-0.6 mmol/g loading, *Sigma–Aldrich*) and Fmoc-Ser(MPBn)-OH (**4**) (273 mg, 0.55 mmol, 5.5 equiv.) were used. The scale of the synthesis was 0.1 mmol, and the 10 ml reactor vial was used. 43.4 mg product was obtained as a white fluffy solid. M.p. 161 – 164 °C (dec.). FT-IR (neat): 3427, 326, 3074, 3947, 2450, 2287, 2163, 1728, 1714, 1697, 1682, 1651, 1623, 1520, 1504, 1452, 1417, 1394, 1343, 1311, 1255, 1198, 1183, 1089, 1060, 1032, 1005, 966, 929, 885, 834, 813, 795, 760, 74, 728, 699, 661, 622, 618. ^{1}H-NMR (400 MHz, D_2O): 7.37 – 7.22 (*m*, 5 H); 4.46 – 4.21 (*m*, 7 H); 4.08 – 3.78 (*m*, 7 H); 3.22 – 3.08 (*m*, 2 H); 2.44 – 2.40 (*m*, 2 H); 2.10 – 1.90 (*dm*, *J* = 60, 2 H); 1.34 – 1.31 (*m*, 9 H); 1.20 (*d*, *J* = 16,

3 H). ^{13}C-NMR $\{^1H\}$ (100 MHz, D_2O): 177.0; 175.4; 174.8; 174.7; 173.5; 173.2; 171.2; 171.0; 170.9; 169.7; 133.7; 129.4; 128.0; 69.6; 62.8; 61.0; 54.8; 54.4; 53.1; 50.2; 49.8; 49.5; 42.4; 42.2; 36.8; 29.9; 25.9; 16.9-16.3; 11.6; 10.3. ^{31}P-NMR $\{^1H\}$ (162 MHz, D_2O): 27.66 (95.5%). HR-MS (ESI/Q-TOF): 874.332770 ($[M + H]^+$, $C_{34}H_{52}N_9O_{16}P^+$; calc. 874.334240).

Supplementary Material

Supporting information for this article is available on the WWW under https://doi.org/10.1002/hlca.201700198.

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Author Contribution Statement

A. B. performed the experiments, and analyzed the data. All the authors contributed to the design of the experiments and writing the paper.

Competing Interests

The authors declare no competing financial interests.

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