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Synthesis of a Triply Phosphorylated Pentapeptide from Human τ-Protein

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Abstract—Two different strategies for the synthesis of a triply phosphorylated pentapeptide are described. In both cases a mono-phosphorylated selectively *N*-deprotected tripeptide is employed as C-terminal fragment. Coupling of this building block with a C-terminally unmasked bis-phosphorylated seryl-dipeptide unexpectedly failed due to decomposition of this peptide upon activation with different coupling reagents. Instead stepwise N-terminal elongation of the peptide chain with serine derivatives and subsequent *O*-phosphorylation of the serine OH-groups was successful. These results indicate that assembly of multiply phosphorylated peptides from preformed multiply phosphorylated phosphopeptide building blocks in general may be problematic and that a stepwise elongation of the amino acid chain may be preferable.

Introduction

Regulated phosphorylation and dephosphorylation of proteins at serine and threonine residues belong to the key chemical processes employed by cells for the orchestration of intracellular events.[1] Among these, in particular, signal transduction and progression through the cell cycle have recently attracted widespread interest.

For the study of the events influenced by phosphorylated proteins, phosphopeptides that embody the characteristic phosphorylated partial structures of the parent proteins have proven to be invaluable reagents,[2] and the development of methods for their synthesis in solution and on solid supports[3,4] is the subject of various research enterprises. The major problems in phosphopeptide synthesis are the need for a set of orthogonally stable protecting groups and the pronounced base-sensitivity of phosphopeptides due to β-elimination of the phosphate already under weakly basic conditions (Scheme 1). To overcome these problems several solutions have been advanced, in particular the use of amino acid building blocks incorporating a phosphodiester that is less prone to β-elimination[4] and the use of enzyme-labile protecting groups that can be removed under exceptionally mild conditions.[5–7] In the overwhelming majority of these investigations phosphopeptides were targeted that embody only one phosphate group. For the synthesis of multiply phosphorylated seryl- and threonyl-peptides reliable methods have not been thoroughly addressed. This may be due to the clustering of the β-phospho carboxylic acid functions in such molecules resulting in a potentiated base-sensitivity.

Multiple protein phosphorylation, however, is of utmost importance in vivo. Numerous proteins such as the tumor suppressor p53,[1] the signal-transducing Raf kinase[1] and the cell-cycle regulating cyclins[1] are regulated by phosphorylation at multiple sites. In addition hyperphosphorylation of proteins is involved in the development of diseases, in particular neurodegenerative disorders like Alzheimer's disease.[8] An important step in the establishment of Alzheimer's disease is the hyperphosphorylation of the τ-protein in nerve cells, which results in an altered interaction of τ with the microtubuli and its association to paired helical filaments and the formation of neurofibrillary tangles.[9] However, the molecular details governing these processes are still

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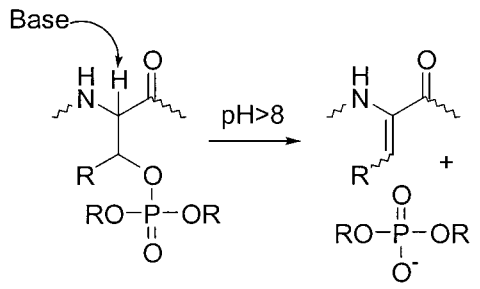


Scheme 1. Base-lability of *O*-phosphorylated peptides.

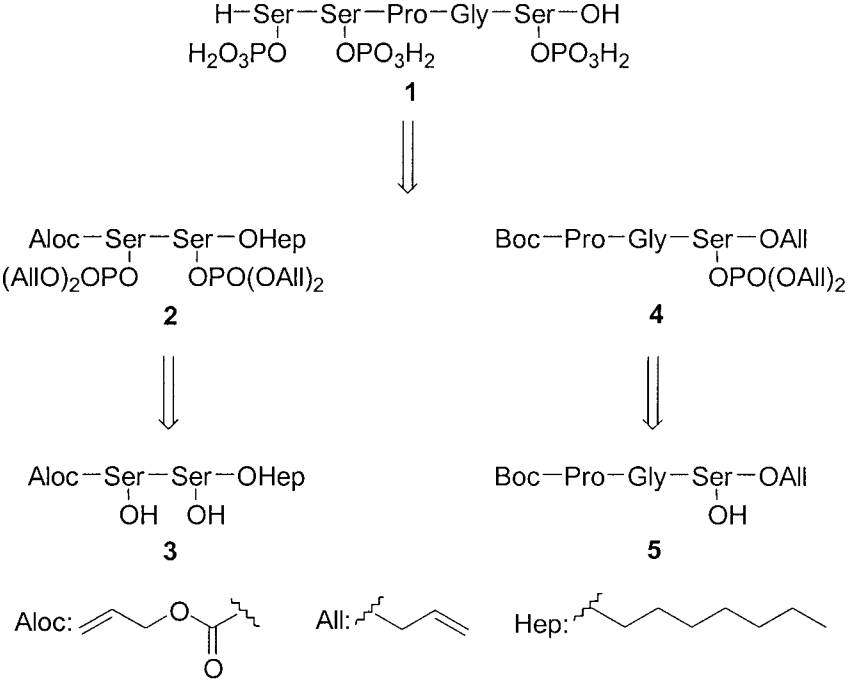
unclear and subject to an intense debate. In order to develop tools for the study of such biological phenomena and to devise a generally applicable method for the synthesis of multiphosphorylated base-sensitive seryl- and threonyl-peptides, we have embarked on a synthesis of the triply phosphorylated pentapeptide **1** from the flanking region of the microtubuli binding domain of human τ-protein (Scheme 2).

Results and Discussion

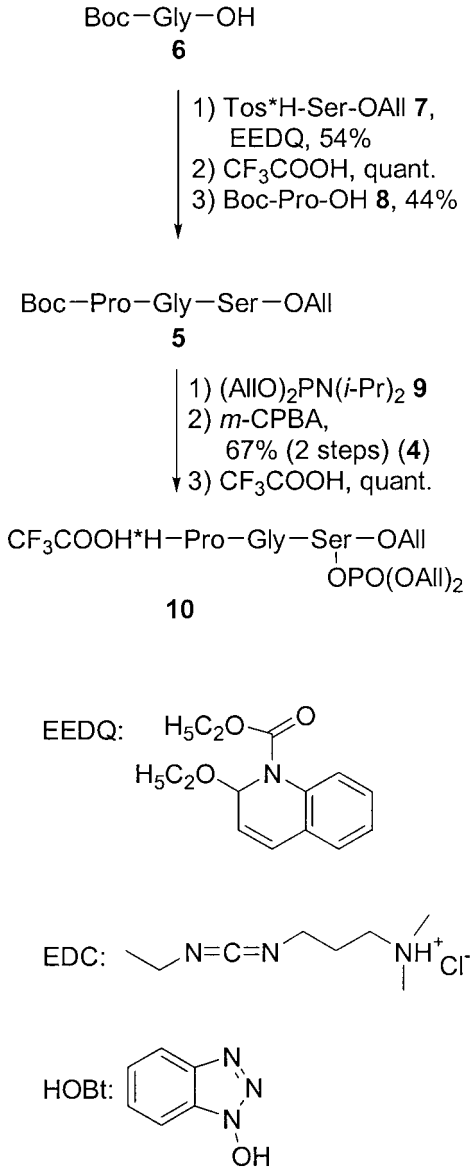
In developing a first strategy for the synthesis of covalently modified peptide **1** we have drawn from our previous experience in phospho- and glycophosphopeptide chemistry.[5–7] It was planned to deprotect the peptide completely in the final step by means of a palladium(0)-catalyzed cleavage of allyl-type protecting groups, i.e., an allyloxycarbonyl group, allyl phosphates and an allyl ester. For intermediary selective deprotection of phosphorylated building blocks the enzyme-labile heptyl ester,[6] which can be removed with lipases under very mild conditions, and the Boc group were chosen. Thus, fully protected peptides **2** and **4** were viewed as appropriately substituted intermediates. It was planned to synthesize the parent unphosphorylated peptides **3** and **5** first and to convert them into the required phosphoric acid esters.

The C-terminal tripeptide **4** and its selectively unmasked derivative **10** were synthesized as shown in Scheme 3. Boc-glycine **6** was condensed with serine allyl ester **7,** and after removal of the Boc group from the resulting dipeptide the amino acid chain was elongated by proline. Conversion of the serine hydroxyl group into the diallyl phosphate was carried out by phosphitylation with the diallylphosphoramidate **9**[10] and subsequent oxidation with *m*-CPBA. Selective removal of the Boc group yielded phosphotripeptide building block **10**. The synthesis of bis-phosphorylated dipeptide ester **2** is shown in Scheme 4. Aloc-serine **11** and serine heptyl ester **12** were condensed and then both hydroxyl groups were phosphorylated as described above. Upon treatment of the resulting diphosphopeptide **2** with lipase from *Aspergillus niger* at pH 6.5, the C-terminal heptyl ester was removed smoothly. Although the starting material had been consumed completely the desired selectively deprotected dipeptide **13** could be isolated in only 49% yield. A closer inspection of the reaction mixture revealed that the twice phosphorylated peptide **2** had also undergone a side reaction resulting in loss of one phosphate. Such an attack on a phosphate group in the course of the lipase-mediated transformation had not been observed in a related unmasking of the corresponding phosphorylated Aloc-serine heptyl ester[6] and was completely unexpected. Furthermore, peptide bis-phosphate **2** proved to lose one phosphate group even if stored neat at 4 °C. On the other hand bis-phosphorylated carboxylic acid **13** was stable under these conditions.

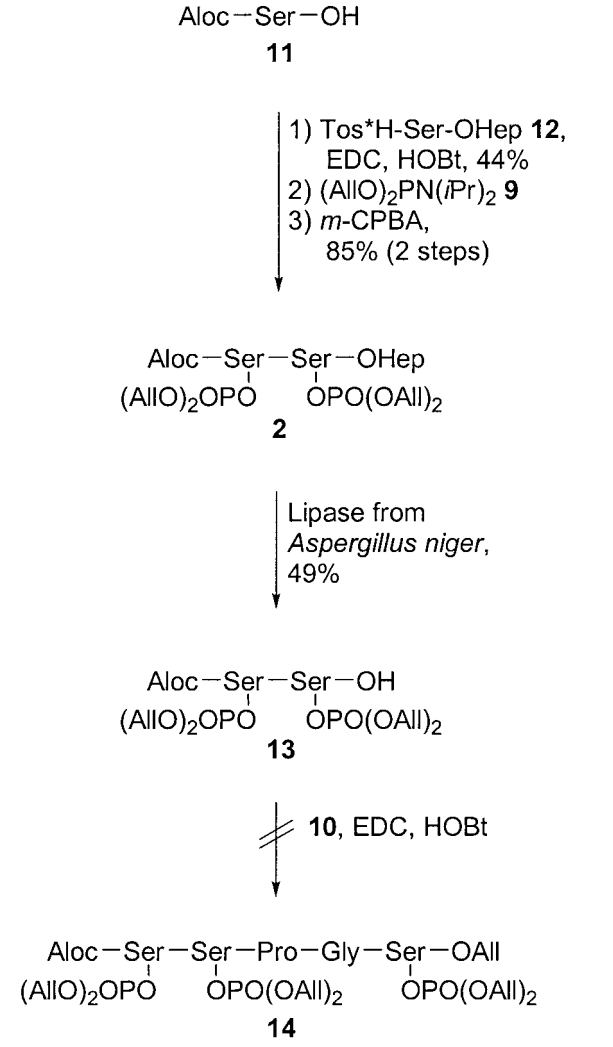
Finally, the high sensitivity of the bis-phosphorylated peptide became fully apparent upon attempted coupling of carboxylic acid **13** with N-terminally deprotected



Scheme 2. Initial strategy for the synthesis of phosphopeptide **1**.



Scheme 3. Synthesis of selectively deprotected phosphopeptide building block **10**.



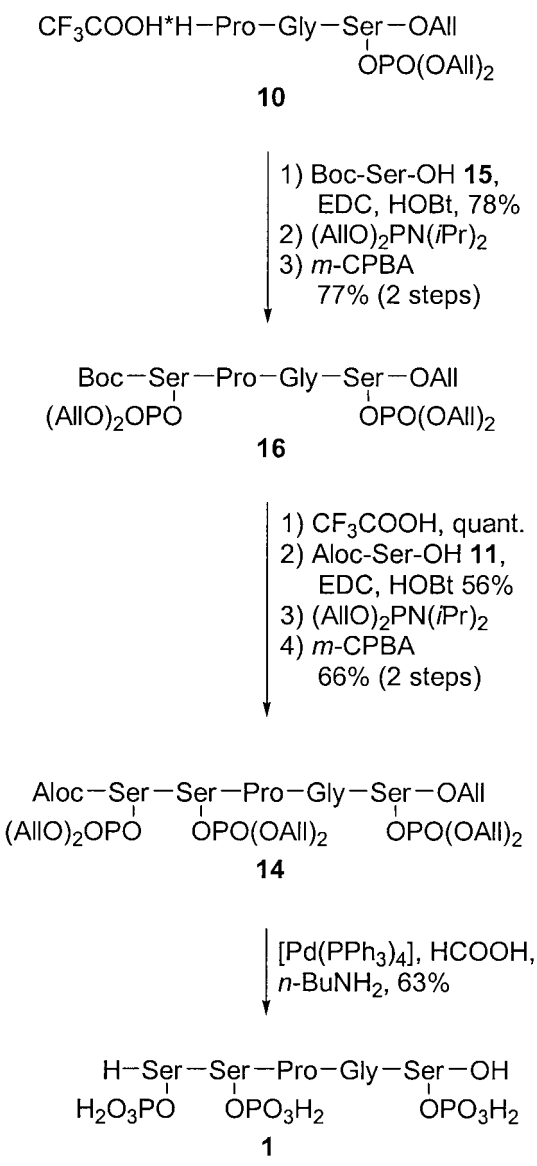
Scheme 4. Synthesis of selectively deprotected phosphopeptide **13** and attempted coupling with tripeptide **10**.

building block **10** (Scheme 4). Activation of carboxylic acid **13** with different reagents (EEDQ, carbodiimides) did not lead to formation of the desired tris-phosphorylated pentapeptide **14**. Instead, once more elimination of a phosphate occurred. Thus, the activated intermediates formed from acid **13** and the different coupling reagents are even more prone to β-elimination than the ester **2**.

The base-sensitivity of fully protected phosphopeptides is a known problem (see above). Our findings indicate, however, that this lability may be potentiated if several phosphate groups are clustered. Probably phosphopeptides **2** and **13** adopt conformations in which the phosphate group(s) assist in the β-elimination process.

Our findings suggest that assembly of multiply phosphorylated peptides from preformed multiply phosphorylated phosphopeptide building blocks in general may be problematic and that a stepwise elongation of the growing amino acid chain might be preferable. Also, it might be advantageous to activate unphosphorylated hydroxy-amino acids followed by *O*-phosphorylation, although this does not seem to be absolutely necessary since *O*-phosphorylated Aloc-serine could be activated without major problem.[6,7]

In order to test if this holds true for triply phosphorylated τ-protein fragment **1**, an alternative synthesis was investigated in which monophosphorylated peptide **10** was sequentially chain-elongated and phosphorylated. To this end, N-terminally unmasked compound **10** was condensed with Boc-serine and the resulting tetrapeptide was *O*-phosphorylated and oxidized to the phosphate **16** as described above (Scheme 5). Subsequently, the N-terminal Boc-group was cleaved under acidic conditions and the peptide chain was elongated with Aloc-protected serine **11**. Derivatization of the serine β-OH with phosphoramidite **9** and oxidation of the resulting phosphite to the phosphate yielded fully masked tris-phosphopentapeptide **14**. Finally, all allyl-type blocking groups were removed simultaneously by treatment with a Pd(0)-catalyst in the presence of formic acid and



Scheme 5. Synthesis of τ-protein peptide **1** via stepwise assembly of the peptide chain.

n-butyl amine.[7,11] The desired τ-protein peptide **1** was obtained in 63% yield.

In this paper we describe problems occurring in the synthesis of multiply phosphorylated peptides. These difficulties are caused by the intrinsic lability of these compounds even under the extremely mild conditions of an enzymatic transformation or during storage, i.e., in the absence of any further reagent. Upon activation for peptide coupling the lability is enhanced, resulting in complete failure of the desired chain elongation reaction.

Furthermore, we have shown one solution to this problem. It avoids the use of also potentially labile phosphorylated amino acid building blocks and instead employs regular masked hydroxy amino acids followed by phosphorylation of the hydroxy groups. We would like to stress that the general applicability of this approach remains to be demonstrated, although it served well in the synthesis of τ-protein peptide **1**. In particular, the use of amino acid building blocks incorporating a phosphodiester group[4] might provide a viable strategy for the efficient synthesis of multiply phosphorylated peptides in solution or in the solid phase.

Experimental

^{1}H and ^{13}C NMR spectra were recorded with Bruker AM 400 and DRX 500 spectrometers. High-resolution mass spectra were recorded with a Finnigan MAT 90 spectrometer, with a Perseptive Biosystem Voyager™ Biospectrometry™ spectrometer (MALDI-TOF, nitrogen-UV-laser $\lambda = 237$ nm, accelerating voltage = 28 kV, matrix: dihydroxy benzoic acid (DHB)) and with a Thermoquest LCQ ESI-mass spectrometer. Specific rotations were measured with a Perkin–Elmer 241 polarimeter. Flash chromatography was performed using Baker silica gel (230–400 mesh ATM). Reaction progress was monitored by TLC using Merck silica gel $60F_{254}$ aluminum sheets (Merck, Darmstadt, Germany). All reagents were obtained from Fluka (Buchs, Switzerland), Aldrich (Steinheim, Germany) or Degussa (Frankfurt, Germany).

***N*-Allyloxycarbonyl-L-seryl-L-serine heptyl ester (3).** A solution of allyloxycarbonyl-L-serine **11** (2.31 g, 12.2 mmol), L-serine heptyl ester hydrotosylate **12** (4.58 g, 12.2 mmol), diisopropylcarbodiimide (1.54 g, 12.2 mmol) and 1-hydroxybenzotriazole (1.67 g, 12.2 mmol) in dry THF at 0 °C was added dropwise over 1 h into a solution of triethylamine in dry THF. After stirring overnight at rt, the reaction mixture was cooled to −20 °C and the urea was removed by filtration. The solvent was removed under reduced pressure and the residue was purified by flash chromatography (100% chloroform to chloroform:methanol 10:1) to yield **3** (2 g, 44%) as a yellow amorphous product. ^{1}H NMR (500 MHz, $CDCl_3$/TMS): $\delta = 0.88$ (t, $J = 7$ Hz, 3H), 1.29 (m, 8H), 1.64 (m, 2H), 3.73 (m, 1H), 3.93 (m, 3H), 4.14 (t, $J = 7$ Hz, 2H), 4.24 (s, 2H), 4.35 (s, 1H), 4.56 (d, $J = 5$ Hz, 2H), 4.63 (d, $J = 4$ Hz, 1H), 5.25 (m, 2H), 5.89 (m, 1H), 6.34 (d, $J = 8$ Hz, 1H), 7.7 (d, $J = 7$ Hz, 1H); ^{13}C NMR (125 MHz, $CDCl_3$/TMS): $\delta = 14.0$, 22.5, 25.7, 28.4, 28.8, 31.6, 55.1, 56.3, 62.3, 62.9, 66.1 (2C), 96.1, 118.0, 132.4, 156.5, 170.5, 171.2; MS *m/z*: calcd for $[M]^+$ $C_{17}H_{30}N_2O_7$ 374.2053, found 374.2041.

***N*-Allyloxycarbonyl-(*O*)-diallylphosphato-L-seryl-(*O*)-diallylphosphato-L-serine heptyl ester (2).** To a solution of allyloxycarbonyl-L-seryl-L-serine heptyl ester **7** (0.75 g, 2 mmol) and tetrazole (280 mg, 4 mmol) at rt was added bis-allyloxy-*N,N*-diisopropylphosphoramidite **9** (1.8 ml) over 2 min. After 90 min stirring, 50% *m*-chloroperbenzoic acid (2.1 g, 6 mmol) was added and the reaction mixture was stirred for 1 h at rt. Ether (200 ml) was added and the mixture was extracted with 5% $NaHSO_3$ and satd $NaHCO_3$ (three times each). The organic layer was dried over $MgSO_4$, the solvent was removed under reduced pressure and the residue was purified by flash chromatography (ethyl acetate:*n*-hexane 3:1) to yield **2** (1.18 g, 85%) as a yellow oil. R_f: 0.55

(ethyl acetate:*n*-hexane 3:1); ^{1}H NMR (500 MHz, $CDCl_3$/TMS): δ = 0.88 (t, *J* = 7 Hz, 3H), 1.3 (m, 8H), 1.64 (m, 2H), 4.17 (t, *J* = 7 Hz, 2H), 4.34 (m, 2H), 4.43 (m, 1H), 4.55 (m, 11H), 4.75 (m, 1H), 5.3 (m, 10H), 5.91 (m, 5H), 6.25 (d, *J* = 8 Hz, 1H), 7.63 (d, *J* = 7 Hz, 1H); ^{13}C NMR (125 MHz, $CDCl_3$/TMS): δ = 14.0, 22.5, 25.6, 28.4, 28.8, 31.6, 53.0, 53.1, 54.7, 62.5, 66.1, 66.2, 66.4, 66.7, 66.8, 68.5, 68.6, 117.9, 118.5, 118.6, 118.7, 131.9, 132.0, 132.1, 132.1, 132.2, 132.2, 132.3, 132.3, 132.4, 156.0, 168.2, 168.5; ^{31}P NMR (202 MHz, $CDCl_3$/TMS): δ = −0.07, 0.18.

N-Allyloxycarbonyl-(*O*)-diallylphosphato-L-seryl-(*O*)-diallylphosphato-L-serine (13). To a solution of *N*-allyloxycarbonyl-(*O*)-diallylphosphato-L-seryl-(*O*)-diallylphosphato-L-serine heptyl ester **2** (98 mg, 0.141 mmol) in acetone (1 mL) was added phosphate buffer (50 ml, pH 6.5). After 2 min in an ultrasonic bath, lipase A, Amano 6″ from *Aspergillus niger* (1 g), was added and the suspension was shaken gently for 24 h at 37 °C. The aqueous layer was extracted with ethyl acetate, and after adjusting the pH to 4, the aqueous layer was further extracted three times with ethyl acetate. The combined organic layers were dried over $MgSO_4$, the solvent was removed under reduced pressure and the residue was purified by flash chromatography (ethyl acetate:acetic acid 7:1) to yield **13** (41 mg, 49%) as a yellow oil. ^{1}H NMR (500 MHz, $CDCl_3$/TMS): δ = 4.32 (m, 2H), 4.38 (s, 1H), 4.46 (m, 1H), 4.52 (m, 10H), 4.66 (s, 1H), 4.76 (d, *J* = 4 Hz, 1H), 5.3 (m, 10H), 5.9 (m, 5H), 6.19 (d, *J* = 8 Hz, 1H), 7.98 (d, *J* = 7 Hz, 1H); ^{13}C NMR (125 MHz, $CDCl_3$/TMS): δ = 29.7, 43.6, 53.1, 54.5, 63.8, 66.0, 67.3, 67.4, 68.6, 68.7, 68.7, 117.9, 118.7, 118.7, 118.8, 119.3, 119.5, 132.0, 132.1, 132.1, 132.5, 149.3, 155.9, 168.5, 170.4; ^{31}P NMR (202 MHz, $CDCl_3$/TMS): δ = −0.94, −0.86.

***N*-*tert*-Butyloxycarbonyl-glycyl-L-serine allyl ester (17).** To a solution of *N*-*tert*-butyloxycarbonyl glycine **6** (1.4 g, 8.1 mmol), L-serine allyl ester hydrotosylate **7** (2.6 g, 8.1 mmol) and triethylamine (1.1 ml, 8.1 mmol) in dry dichloromethane at 0 °C was added dropwise to a solution of EEDQ (4 g, 16.2 mmol) in dry dichloromethane. After stirring for 30 min, the mixture was allowed to warm up to rt and was stirred overnight. The reaction mixture was extracted three times with 0.5 M HCl, satd $NaHCO_3$ and water. The organic layer was dried over Na_2SO_4 and the solvent was removed under reduced pressure. The residue was purified by flash chromatography (ethyl acetate:*n*-hexane 3:1) to yield **17** (1.3 g, 54%) as a yellow oil. R_f: 0.2 (ethyl acetate:*n*-hexane 3:1); $[\alpha]_D^{20}$: −5.9 (c = 0.9, methanol); ^{1}H NMR (250 MHz, $CDCl_3$/TMS): δ = 1.45 (s, 9H), 3.3 (s, 1H), 3.85 (m, 2H,), 4.0 (m, 2H), 4.68 (m, 3H), 5.3 (m, 2H), 5.5 (s, 1H), 5.9 (m, 1H), 7.22 (m, 1H); ^{13}C NMR (100.6 MHz, $CDCl_3$/TMS): δ = 28.3, 44.1, 54.8, 62.6, 66.3, 80.4, 118.8, 131.4, 156.4, 170.1 170.1; MS *m*/*z*: calcd for $[M+H]^+$ $C_{13}H_{23}N_2O_6$ 303.1556, found 303.1551.

Glycyl-L-serine allyl ester trifluoroacetate (18). *N*-*tert*-Butyloxycarbonyl-glycyl-L-serine allyl ester **17** (1.3 g, 4.4 mmol) was dissolved in dichloromethane (5 ml). TFA (5 ml) was added and the reaction mixture was stirred for 1 h. Dichloromethane was evaporated under reduced pressure and the TFA was removed by coevaporation with toluene to yield **18** (1.38 g, quant.) as a yellow oil. R_f: 0.07 (chloroform:ethanol 10:1 + 2% NEt_3); $[\alpha]_D^{20}$: −8.2 (c = 1.1, methanol). ^{1}H NMR (500 MHz, $CDCl_3$/CD_3OD/TMS): δ = 3.91 (m, 2H), 4.13 (s, 2H), 4.64 (m, 3H), 5.31 (m, 2H), 5.89 (m, 1H). ^{13}C NMR (125 MHz, $CDCl_3$/MeOD/TMS): δ = 40.6, 55.2, 61.8, 66.5, 118.9, 131.5, 166.7, 170.2; MS *m*/*z*: calcd for $[M]^+$ $C_8H_{14}N_2O_4$ 202.0954, found 202.0968.

***N*-*tert*-Butyloxycarbonyl-L-prolyl-glycyl-L-serine allyl ester (5).** To a solution of glycyl-L-serine allyl ester trifluoroacetate **18** (500 mg, 1.58 mmol),) *N*-*tert*-butyloxycarbonyl-L-proline **8** (317 mg, 1.47 mmol) and triethylamine (0.22 ml, 1.6 mmol) in dry dichloromethane at 0 °C was added dropwise a solution of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (350 mg, 1.8 mmol) and 1-hydroxybenzotriazole (400 mg, 3 mmol) in dry dichloromethane. The reaction mixture was stirred for 30 min at 0 °C and warmed overnight at rt. The solution was washed with 1 M HCl, 1 M $NaHCO_3$ and water and dried over $MgSO_4$. The dichloromethane was removed under reduced pressure and the residue was purified by flash chromatography (ethyl acetate:*n*-hexane 4:1) to yield **5** (260 mg, 44%) as a colourless oil. R_f: 0.13 (ethyl acetate:*n*-hexane 6:1); $[\alpha]_D^{20}$: −45.3 (c = 1.1, methanol); ^{1}H NMR (500 MHz, $CDCl_3$/TMS): δ = 1.44 (s, 9H), 1.87 (m, 1H), 1.98 (s, 1H), 2.11 (m, 2H), 3.43 (s, 1H), 3.51 (m, 2H), 3.91 (dd, J_1 = 12 Hz J_2 = 2 Hz, 2H), 4.03 (m, 1H), 4.20 (m, 2H), 4.58 (s, 1H), 4.65 (d, *J* = 6 Hz, 2H), 5.29 (m, 2H), 5.89 (m, 1H), 7.20 (s, 1H), 7.37 (s, 1H); ^{13}C NMR (125 MHz, $CDCl_3$/TMS): δ = 24.6, 28.4, 29.8, 43.1, 47.6, 55.0, 60.5, 62.5, 66.2, 80.9, 118.6, 131.6, 155.5, 169.4, 170.1, 172; MS *m*/*z*: calcd for $[M]^+$ $C_{18}H_{29}N_3O_7$ 399.2005, found 399.2019.

N-*tert*-Butyloxycarbonyl-L-prolyl-glycyl-(*O*)-diallylphosphato-L-serine allyl ester (4). To a solution of *N*-*tert*-butyloxycarbonyl-L-prolyl-glycyl-L-serine allyl ester **5** (500 mg, 1.25 mmol) and tetrazole (175 mg, 2.5 mmol) in dry acetonitrile at 0 °C was added bis-allyloxy-*N,N*-diisopropylphosphoramidite **9** (460 mg, 1.87 mmol). After 2 h the reaction was monitored by TLC. If there was still unreacted starting material, additional phosphorylation reagent was added. Otherwise, 70% *m*-chloroperbenzoic acid (388 mg, 2.25 mmol) was added and the reaction mixture was stirred overnight at rt. The solvent was removed under reduced pressure, the residue was dissolved in diethyl ether and extracted with 5% $NaHSO_4$-solution, satd $NaHCO_3$-solution and water (3 times each). The organic layer was dried over Na_2SO_4, and the solvent was removed under reduced pressure to give the crude product, which was purified by flash chromatography (ethyl acetate: *n*-hexane 4:1) to give **4** (467 mg, 67%) as a yellow oil. R_f: 0.24 (ethyl acetate:*n*-hexane 10:1); $[\alpha]_D^{20}$: −34.8 (c = 1.3, methanol); ^{1}H NMR (400 MHz, $CDCl_3$/TMS): δ = 1.46 (s, 9H), 1.9 (m, 3H), 2.18 (m, 2H), 3.43 (m, 3H), 4.05 (m, 2H), 4.32 (m, 2H), 4.45 (m, 1H), 4.55 (m, 4H), 4.65 (d, *J* = 6 Hz, 2H), 4.79 (s, 1H), 5.33 (m, 6H), 5.9 (m, 3H); ^{13}C NMR (100.6 MHz, $CDCl_3$/TMS): δ = 28.3, 42.8, 47.2, 52.7, 52.8, 60.2, 66.4, 68.4, 68.5, 80.3, 118.5, 118.9, 131.2, 132.1, 155.5, 168.2, 169.3, 172.8; ^{31}P NMR (202 MHz, $CDCl_3$/TMS):

δ = −0.58; MS *m/z*: calcd for $[M]^+$ $C_{24}H_{38}N_3O_{10}P$ 559.2295, found 559.2278.

L-Prolyl-glycyl-(*O*)-diallylphosphato-L-serine allyl ester trifluoroacetate (10). To a solution of *N-tert*-butyloxycarbonyl-L-prolyl-glycyl-(*O*)-diallylphosphato-L-serine allyl ester **4** (467 mg, 0.8 mmol) in dichloromethane (3 ml) at rt was added TFA (3 mL). The reaction mixture was stirred for 1 h and then the solvent was removed by coevaporation with toluene to give **10** (479 mg, quant.) as a yellow oil. R_f: 0.07 (chloroform:methanol 10:1 + 2% NEt_3); $[\alpha]_D^{20}$: −11 (c = 1, methanol); ^{1}H NMR (500 MHz, $CDCl_3$/TMS): δ = 1.95–2.03 (m, 3H), 2.34–2.38 (m, 1H), 3.35 (m, 2H), 3.85 (dd, J_1 = 17 Hz, J_2 = 5 Hz, 1H), 4.06 (dd, J_1 = 17 Hz, J_2 = 5 Hz, 1H), 4.26–4.37 (m, 2H), 4.42–4.47 (m, 4H), 4.52 (s, 1H), 4.53–4.55 (m, 2H), 4.74 (s, 1H), 5.16–5.30 (m, 6H), 5.78–5.88 (m, 3H), 7.91 (d, J = 7.6 Hz, 1H), 8.03 (s, 1H), 8.65 (s, 1H), 10.19 (s, 1H); ^{13}C NMR (100.6 MHz, $CDCl_3$/CD_3OD/TMS): δ = 24.4, 29.8, 43.0, 46.4, 52.9, 53.0, 59.5, 66.8, 66.9, 118.9, 119.0, 119.2, 131.3, 132.0, 132.0, 168.4, 169.4, 169.6; ^{31}P NMR (202 MHz, $CDCl_3$/TMS): δ = −1.08 (1P); MALDI-TOF-MS (DHB): *m/z*: calcd for $[M^+]$ $C_{19}H_{30}N_3O_8P_1$ 459.43, found 459.7 $[M^+]$, 481.7 $[M + Na^+]$, 497.7 $[M + K]$.

***N-tert*-Butyloxycarbonyl-L-seryl-L-prolyl-glycyl-(*O*)-diallyl-phosphato-L-serine allyl ester (19).** To a solution of *N-tert*-butyloxycarbonyl-L-serine **15** (160 mg, 0.78 mmol), L-prolyl-glycyl-(*O*)-diallyl-phosphato-L-serine allyl ester **10** (372 mg, 0.65 mmol) and triethylamine (0.09 ml, 0.65 mol) in dry dichloromethane at 0 °C was added dropwise a solution of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (174 mg, 0.91 mmol) and 1-hydroxybenzotriazole (175 mg, 1.3 mmol) in dry dichloromethane. The reaction mixture was stirred 1 h at 0 °C and warmed overnight at rt. The reaction mixture was washed with 1 M HCl, 1 M $NaHCO_3$ and water, dried over $MgSO_4$, and the solvent was removed under reduced pressure. The residue was purified by flash chromatography (ethyl acetate:hexane 10:1) to yield **19** (250 mg, 78%) as a yellow oil. R_f: 0.04 (ethyl acetate:*n*-hexane 10:1); $[\alpha]_D^{20}$: −36 (c = 0.9, methanol); ^{1}H NMR (500 MHz, $CDCl_3$/TMS): δ = 1.42 (s, 9H), 1.82–2.19 (m, 4H), 3.6–3.7 (m, 2H), 3.7–3.8 (m, 2H), 3.85–4.04 (m, 2H), 4.2–4.32 (m, 2H), 4.39–4.49 (m, 4H), 4.49 (m, 1H), 4.52–4.54 (m, 2H), 4.65–4.8 (m, 2H), 5.12–5.3 (m, 6H), 5.75–5.9 (m, 3H); ^{13}C NMR (125 MHz, $CDCl_3$/TMS): δ = 24.8, 28.2, 28.7, 42.5, 47.5, 52.6, 53.6, 60.6, 63.4, 66.3, 66.7, 68.5, 79.5, 118.6, 131.2, 131.9, 155.4, 168.0, 169.7, 171.3, 171.7; ^{31}P NMR (202 MHz, $CDCl_3$/TMS): δ = −0.77; MALDI-TOF-MS (DHB): *m/z*: cacld for $[M]^+$ $C_{27}H_{43}N_4O_{12}P$ 646.62, found 670.49 $[M + Na]^+$, 686.35 $[M + K]^+$.

***N-tert*-Butyloxycarbonyl-(*O*)-diallylphosphato-L-seryl-L-prolyl-glycyl-(*O*)-diallylphosphato-L-serine allyl ester (16).** To a solution of *N-tert*-butyloxycarbonyl-L-seryl-prolyl-glycyl-(*O*)-diallylphosphato-L-serine allyl ester **19** (157 mg, 0.24 mmol) and tetrazole (175 mg, 2.5 mmol) in dry acetonitrile at 0 °C was added bis-allyloxy-*N,N*-diisopropylphosphoramidite **9** (400 mg, 1.44 mmol). If after 2 h of stirring TLC indicated unreacted starting material, additional phosphorylation reagent was added. Otherwise 70% *m*-chloroperbenzoic acid (500 mg, 2.25 mmol) was added and the reaction mixture was stirred overnight at rt. The solvent was removed under reduced pressure, the residue was dissolved in diethyl ether and washed with 5% $NaHSO_4$-solution, satd $NaHCO_3$-solution and water (3 times each). The organic layer was dried over Na_2SO_4, and the solvent was removed under reduced pressure to give the crude product, which was purified by flash chromatography (ethyl acetate:*n*-hexane 4:1 to 10:1) to yield **16** (152 mg, 77%) as a colourless oil. R_f: 0.03 (ethyl acetate:*n*-hexane 10:1); $[\alpha]_D^{20}$: −10 (c = 1, methanol); ^{1}H NMR (500 MHz, $CDCl_3$/TMS): δ = 1.43 (s, 9H), 1.98–2.22 (m, 4H), 3.75–3.79 (m, 2H), 3.89–3.93 (m, 1H), 4.00–4.02 (m, 1H), 4.26–4.31 (m, 2H), 4.46–4.5 (m, 2H), 4.51 (s, 1H), 4.52–4.56 (m, 8H), 4.66 (m, 2H), 4.79 (m, 2H), 5.24–5.38 (m, 10H), 5.87–5.96 (m, 5H); ^{13}C NMR (125 MHz, $CDCl_3$/TMS): δ = 25.0, 28.3, 29.0, 43.1, 47.9, 52.1, 52.8, 61.5, 64.8, 66.4, 66.7, 68.4, 68.5, 68.6, 68.6, 68.7, 68.7, 68.8 80.2, 118.6, 118.8, 118.9 131.4, 132.1, 132.1, 132.2, 132.3, 132.3, 132.4, 155.3, 168.4, 169.7, 169.8, 171.5; ^{31}P NMR (202 MHz, $CDCl_3$/TMS): δ = −0.24 (1P), −0.87 (1P); MALDI-TOF-MS (DHB): *m/z*: calcd for $[M]^+$ $C_{33}H_{52}N_4O_{15}P_2$ 806.73, found 831.65 $[M + Na]^+$, 847.88 $[M + K]^+$.

(*O*)-Diallylphosphato-L-seryl-L-prolyl-glycyl-(*O*)-diallyl-phosphato-L-serine allyl ester trifluoroacetate (20). To a solution of *N-tert*-butyloxycarbonyl-(*O*)-diallylphosphato-L-seryl-L-prolyl-glycyl-(*O*)-diallylphosphato-L-serine allyl ester **16** (100 mg, 0.136 mmol) in dichloromethane (3 mL) at rt was added TFA (3 ml). After 1 h stirring, the solvent was removed by coevaporation with toluene to yield **20** (112 mg, quant.) as a colourless oil. R_f: 0.1 (chloroform:ethanol 10:1 + 2% TEA); $[\alpha]_D^{20}$: −7 (c = 1, methanol); ^{1}H NMR (500 MHz, $CDCl_3$/TMS): δ = 1.97–2.05 (m, 3H), 2.06–2.09 (m, 1H), 3.62 (m, 1H), 3.77 (m, 1H), 3.83–3.87 (m, 1H), 4.05–4.10 (m, 1H), 4.29–4.32 (m, 2H), 4.43–4.57 (m, 11H), 4.64–4.68 (m, 3H), 4.83 (m, 1H), 5.24–5.38 (m, 10H), 5.86–5.94 (m, 5H); ^{13}C NMR (125 MHz, $CDCl_3$/TMS): δ = 25.1, 29.3, 42.8, 47.9, 52.8, 52.9, 61.8, 64.1, 66.6, 66.9, 68.7, 68.8, 68.8, 68.9, 69.0, 69.1, 69.2, 118.8, 118.8, 119.0, 119.0, 119.1, 131.3, 131.8, 131.9, 132.0, 132.0, 165.2, 168.6, 170.1, 172.0; ^{31}P NMR (202 MHz, $CDCl_3$/TMS): δ = −0.72, −1.55; MALDI-TOF-MS (DHB): *m/z*: calcd for $[M]^+$ $C_{28}H_{44}N_4O_{13}P_2$ 706.62, found 708.58 $[M + H]^+$, 730.53 $[M + Na]^+$, 746.58 $[M + K]^+$.

***N*-Allyloxycarbonyl-L-seryl-(*O*)-diallylphosphato-L-seryl-L-prolyl-glycyl-(*O*)-diallylphosphato-L-serine allyl ester (21).** To a solution of *N*-allyloxycarbonyl-L-serine **11** (42 mg, 0.220 mmol), (*O*)-diallylphosphato-L-seryl-L-prolyl-glycyl-(*O*)-diallylphosphato-L-serine allyl ester **20** (159 mg, 0.186 mmol) and triethylamine (0.028 ml, 0.186 mmol) in dry dichloromethane at 0 °C was added dropwise a solution of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (42 mg, 0.220 mmol) and 1-hydroxybenzotriazole (45 mg, 0.3 mmol) in dry dichloromethane. The solution was stirred 1 h at 0 °C and warmed overnight to rt. The organic layer was washed with 1 M HCl, 1 M $NaHCO_3$ and water, dried over $MgSO_4$ and the solvent was removed under

reduced pressure. The crude product was purified by flash chromatography (ethyl acetate:*n*-hexane 10:1) to yield **21** (91 mg, 56%) as a colourless oil. R_f: 0.13 (ethyl acetate:ethanol 10:1); $[\alpha]_D^{20}$: −37 (c = 1, methanol); ^{1}H NMR (500 MHz, $CDCl_3$/TMS): δ = 1.99–2.09 (m, 3H), 2.11 (m, 1H), 3.67 (m, 2H), 3.75 (m, 2H), 3.98 (m, 1H), 4.17 (m, 1H), 4.32–4.35 (m, 4H), 4.44 (m, 1H), 4.47–4.64 (m, 12H), 4.66 (m, 1H), 4.80 (m, 1H), 5.01 (m, 1H), 5.18–5.39 (m, 12H), 5.85–5.95 (m, 6H); ^{13}C NMR (125 MHz, $CDCl_3$/TMS): δ = 25.2, 29.2, 42.8, 48.1,52.0, 52.6, 56.6, 62.2, 63.4, 65.8, 65.9, 66.4, 66.8, 68.7, 68.9, 117.9, 118.6, 118.9, 119.0, 119.1, 119.2, 131.5, 131.8, 131.9, 132.0, 132.1, 132.7, 156.0, 168.4, 168.9, 169.9, 171.0; ^{31}P NMR (202 MHz, $CDCl_3$/TMS): δ = −1.55, 0.20; MALDI-TOF-MS (DHB): *m*/*z*: cacld for $[M]^+$ $C_{35}H_{53}N_5O_{17}P_2$ 877.77, found 879.15 $[M+H]^+$, 901.35 $[M+Na]^+$, 918.53 $[M+K]^+$.

***N*-Allyloxycarbonyl-(*O*)-diallylphosphato-L-seryl-(*O*)-diallylphosphato-L-seryl-L-prolyl-glycyl-(*O*)-diallylphosphato-L-serine allyl ester (14).** To a solution of *N*-allyloxycarbonyl-L-seryl-(*O*)-diallylphosphato-L-seryl-L-prolyl-glycyl-(*O*)-diallylphosphato-L-serine allyl ester **21** (87 mg, 0.1 mmol) and tetrazole (73 mg, 1 mmol) in dry acetonitrile at 0 °C was added bis-allyloxy-*N,N*-diisopropylphosphoramidite **9** (170 mg, 0.26 mmol). If after 2 h of stirring TLC indicated unreacted starting material additional phosphorylation reagent was added. Otherwise 70% *m*-chloroperbenzoic acid (210 mg, 1.21 mmol) was added and the reaction mixture was stirred overnight at rt. The solvent was removed under reduced pressure, the residue was dissolved in chloroform and washed with 5% $NaHSO_4$-solution, satd $NaHCO_3$-solution and water (3 times each). The organic layer was dried over Na_2SO_4 and the solvent was removed under reduced pressure to give the crude product, which was purified by flash chromatography (ethyl acetate) to yield **2** (68 mg, 66%) as a colourless oil. R_f: 0.18 (ethyl acetate: ethanol 10:1); $[\alpha]_D^{20}$: −21 (c = 1, methanol); ^{1}H NMR (500 MHz, $CDCl_3$/TMS): δ = 2.01–2.14 (m, 3H), 2.26–2.29 (m, 1H), 3.71–3.8 (m, 3H), 4.23–4.3 (m, 5H), 4.41–4.44 (m, 2H), 4.47–4.64 (m, 16H), 4.65 (m, 2H), 4.77 (m, 1H), 5.09 (m, 1H), 5.19–5.40 (m, 16H), 5.86–5.96 (m, 8H); ^{13}C NMR (125 MHz, $CDCl_3$/TMS): δ = 25.1, 29.4, 42.8, 48.2, 51.1, 52.9, 55.0, 62.3, 62.3, 66.0, 66.2, 66.3, 66.8, 68.3, 68.6, 68.9, 117.9, 118.4, 118.45, 118.5, 118.77, 118.8, 119.1, 119.2, 131.5, 131.85, 131.9, 132.15, 132.2, 132.3, 132.4, 132.6, 155.9, 168.3, 168.7, 170.2, 170.9; ^{31}P NMR (202 MHz, $CDCl_3$/TMS): δ = −0.04, −0.36, −2.05; MALDI-TOF-MS (DHB): *m*/*z*: calcd for $[M]^+$ $C_{41}H_{62}N_5O_{20}P_3$ 1037.87, found 1039.9 $[M+H]^+$, 1062.16 $[M+Na]^+$, 1077.73 $[M+K]^+$.

(*O*)-Phosphato-L-seryl-(*O*)-phosphato-L-seryl-L-prolyl-glycyl-(*O*)-phosphato-L-serine (1). *N*-Allyloxycarbonyl-(*O*)-diallylphosphato-L-seryl-(*O*)-diallylphosphato-L-seryl-L-prolyl-glycyl-(*O*)-diallylphosphato-L-serine allyl ester **14** (7 mg, 6.8 μmol) was dissolved in a deoxygenated mixture of 45% DMSO, 45% DMF and 10% water at rt. *n*-Butylamine (5.1 mg, 70 μmol) and formic acid (4.6 mg, 100 μmol) were added simultaneously. Lastly, $Pd(PPh_3)_4$ (5 mg) was added and the reaction mixture was stirred overnight at rt in the dark. The solvent was removed under reduced pressure and the residue was dissolved in water and purified over a SepPak® C18-cartridge (100% water to water:acetonitrile 1:1) to yield **1** (2.9 mg, 63%) as a white foam. $[\alpha]_D^{20}$: −18 (c = 0.1, water); ^{1}H NMR (500 MHz, D_2O): δ = 2.01–2.14 (m, 3H), 2.34–2.40 (m, 1H), 3.83–3.88 (m, 2H), 4.07–4.25 (m, 7H), 4.30–4.34 (m, 1H), 4.41–4.51 (m, 3H), 5.08 (m, 1H); ^{31}P NMR (202 MHz, D_2O): δ = 0.41 (1P), 0.72 (1P), 0.84 (1P); Electrospray mass spectrum (H_2O/MeOH/HOAc): cacld for $[M+H^+]$ $C_{16}H_{30}N_5O_{18}P_3$ 674.1, found 674.2, $[M+Na^+]$ calcd 696.1, found 696.2, $[M+K^+]$ calcd 712.1, found 712.1.

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