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Synthesis of Suitably Protected Hydroxymethylene Phosphonate- and 'Phosphate Phosphonate'-Analogues of Phosphoserine and their Incorporation into Synthetic Peptides

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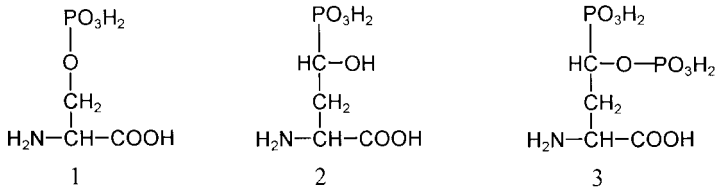
Abstract—Two suitably protected derivatives of phosphoserine **1** have been prepared in which the regular ester-oxygen is replaced by either a hydroxymethylene moiety or by a phosphorylated hydroxymethylene moiety. The second derivative termed 'phosphate phosphonate' **3** yields the hydroxymethylene phosphonate **2** upon enzymatic cleavage of the phosphate. Two peptide sequences containing regular phosphoserine or the new derivatives have been prepared by Fmoc solid phase strategy and were investigated for their potential as substrates or inhibitors of alkaline phosphatase. The peptides should be useful tools for investigations with protein phosphatases and protein kinases.

Introduction

Cell function, growth and homeostasis are regulated to a major extent by protein phosphorylation and dephosphorylation by protein kinases and protein phosphatases, respectively. It has been estimated that one out of three cellular proteins undergoes phosphorylation during its lifetime.[1] Over 99% of protein phosphorylations occur on serine or threonine residues. Protein kinases are often highly specific and phosphorylate only amino acids in the context of particular sequences, so called recognition motifs. Secondary or tertiary structures of proteins often also seem to play an important role in the recognition process. Protein phosphatases are generally much less specific, although highly specific phosphatases have also been described, especially in the field of phosphotyrosine phosphatases. Synthetic peptides have proven to be valuable tools for the investigation and characterization of protein kinases and phosphatases. Phosphatase-resistant phosphopeptide derivatives such as thiophosphates and phosphonates have applications for instance in affinity purification and inhibition of phosphatases. In our efforts to provide useful tools for such investigations, we have now prepared two analogues of phosphoserine (**1**, Scheme 1) and incorporated them in suitably protected form into synthetic peptides.

The hydroxymethylene phosphonate **2** contains a hydroxymethylene group in place of the oxygen of the regular phosphate. Derivatives of phosphoserine containing this structure have been prepared before as intermediates in the synthesis of unsubstituted methylene phosphonate analogues of phosphoserine,[2–6] but the hydroxymethylene phosphonate has not been investigated or incorporated into peptides as such. The hydroxymethylene phosphonate can be expected to be completely stable towards phosphatases. Hydroxymethylene phosphonate analogues of other phosphate esters have been prepared and have shown interesting biological activities.[7–10]



Scheme 1. Structures of phosphoserine **1**, the hydroxymethylene analogue **2** and the phosphate phosphonate **3**.

Keywords: hydroxymethylene phosphonate; phosphopeptide synthesis; phosphoserine analogues; protein phosphatase inhibitors.
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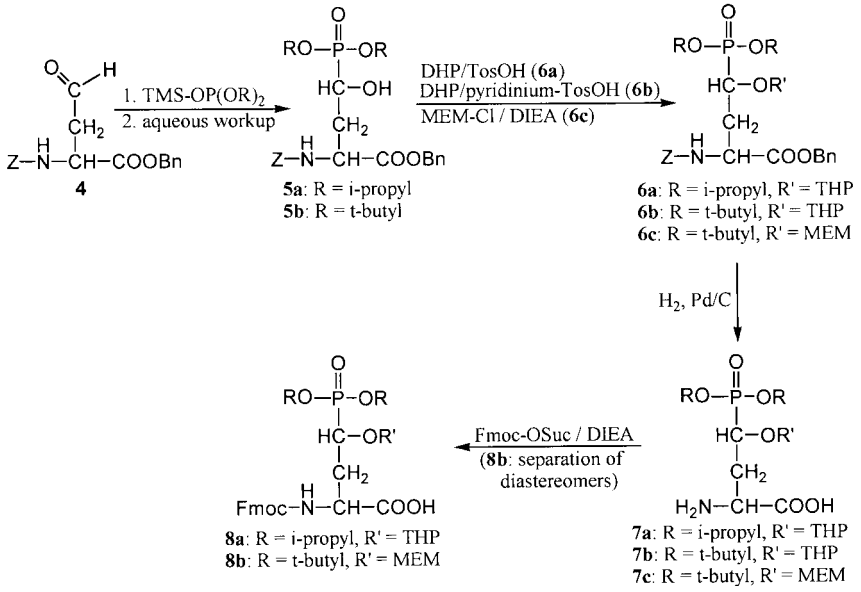


Scheme 2. Preparation of the Fmoc hydroxymethylene phosphonate derivatives.

Peptides containing C-terminal hydroxymethylene phosphonate esters have been prepared as transition state analogue renin inhibitors.[11] Peptides containing hydroxymethylene phosphonate analogues of tyrosine phosphate have been tested as tyrosine kinase inhibitors.[12,13] In the 'phosphate phosphonate' **3**, the hydroxymethylene phosphonate is esterified with phosphoric acid. By protein phosphatase catalyzed cleavage of the phosphate ester in **3** the hydroxymethylene phosphonate structure **2** is generated which might in turn inhibit the enzyme.

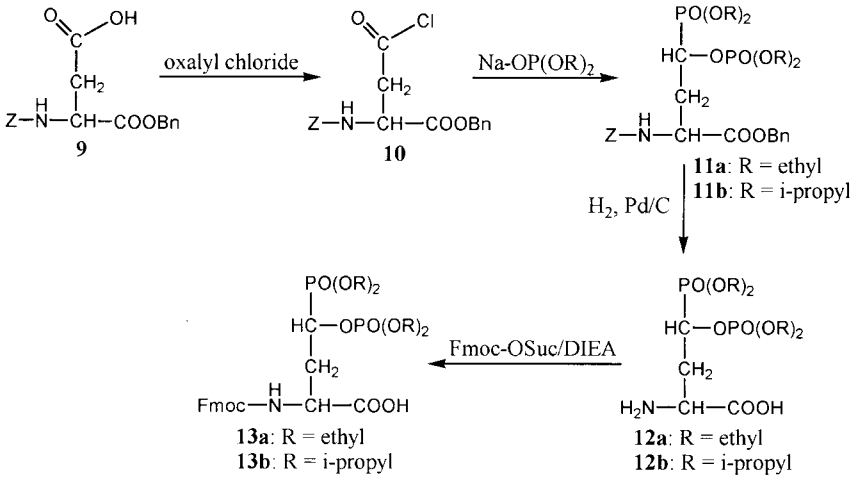
Results and Discussion

The hydroxymethylene phosphonate was prepared from commercially available N-carbobenzoxy- and Cα-benzyl-protected l-aspartic acid by reduction to the homoserine derivative followed by Swern oxidation to the aldehyde **4** (Scheme 2), similar to published procedures.[2–6,14,15] Reacting aldehyde **4** with the TMS derivatives of diisopropyl and di-*tert*-butyl phosphite similar to the procedures published for the dimethyl and diethyl phosphites[2–6] afforded the hydroxymethylene phosphonate structures **5a** and **5b** (the intermediate hydroxy-TMS derivatives were hydrolyzed under the workup conditions). In the peptide synthesis procedure described below, it was found necessary to protect the hydroxyl function in order to avoid the formation of an intramolecular lactone under the conditions of carboxyl activation.

Protection was achieved either with THP (**6a** and **6b**) or with MEM (**6c**). In the case of the isopropyl phosphonate protection, the THP group was introduced with dihydropyran with catalysis by *p*-toluenesulfonic acid. The *tert*-butyl phosphate protection proved unstable under these conditions, but the THP derivative could be obtained in good yield by using the much milder pyridinium *p*-toluenesulfonate as catalyst.[16] The derivatives **6a** and **6c** were purified by preparative HPLC under neutral conditions. A small amount of the THP group of **6a** was cleaved under these conditions. In the chromatographic purification of the THP derivative **6b**, 5 mM ammonium hydrogen carbonate was used in the eluent, which resulted in a slightly alkaline pH of 7.8 and prevented any cleavage of the THP group, but residual ammonium hydrogen carbonate in the product hampered the hydrogenolytic cleavage of the Z- and Bn-groups, which is generally best carried out under at least slightly acidic conditions. Even with the highly active Pearlman's catalyst several rounds of hydrogenation had to be carried out for **6b** in ethanol for a complete cleavage. The MEM derivative **6c** was much more suitable in this respect, allowing the hydrogenolysis to be carried out in glacial acetic acid in a short period of time. The reductive deprotection of **6a** was carried out in methanol without problems. The Fmoc group was introduced into the compounds **7a** and **7c**. The product **8b** could be separated into the two diastereomers by flash chromatography with over 90% efficiency according to HPLC and ^{31}P NMR spectroscopy. **8a** was used as the mixture of diastereomers.

The phosphate phosphonate structure was also prepared by starting with Z-Asp-OBn (**9**, Scheme 3). The acid chloride **10** was obtained quantitatively upon treatment of **9** with oxalyl chloride. Reaction of **10** with sodium diethyl phosphite or sodium diisopropyl phosphite gave the protected phosphate phosphonate structures **11a** and **11b** in moderate yields. Di-*tert*-butyl and dibenzyl phosphite were also investigated but did not lead to the corresponding products. Hydrogenolysis of **11a** and **11b** provided **12a** and **12b** into which the Fmoc group was introduced by standard methods. The product **13a** could be separated into the two diastereomers, **13b** was obtained and used as the mixture of diastereomers.

The separated diastereomers of the hydroxymethylene phosphonate **8b** were incorporated individually into the peptide sequences Ac-AAEGGS**X**NVFSK-amide (P1), which is derived from myosin light chain, and RRRRAA**X**VA-amide (P2), a protein phosphatase 2A substrate[17] (with **X** representing the new derivatives; **X** is serine in the original



Scheme 3. Preparation of the phosphate phosphonate.

sequences). The mixed diastereomers **8a** were also incorporated into sequence P1. Standard Fmoc synthesis protocols were used with activation by TBTU/diisopropylethylamine. Complete deprotection of the peptides containing **8b** (P1-hmP and P2-hmP) (including the MEM group) was achieved by a 3 h treatment with trifluoroacetic acid containing 2% H_2O and 3% triisobutylsilane. Removal of the isopropyl groups from the peptide containing **8a** was investigated under various conditions, but invariably led to cleavage of the peptide backbone at the C-terminal position of the new derivate, probably assisted by an intramolecular lactam formation between the side chain hydroxyl function and the carboxyl group.

The phosphate phosphonate was initially incorporated in the form of the ethyl protected derivative **13a** into sequence P1 (P1-PP) but the final cleavage of the ethyl groups under the required highly acidic conditions invariably led to the destruction of the peptide. Better results were obtained with the more acid labile isopropyl phosphate protection of **13b**. The coupling yields in each step during the synthesis of peptide P1-PP with this derivative were monitored and a sluggish coupling of the phosphate phosphonate derivative as well as the following coupling was noticed, most likely due to the sterically demanding side chain of the new building block. After double-couplings of the critical units the desired peptides were formed in good yields. Deprotection of the phosphate phosphonate again caused problems due to the lability of the peptide backbone to the strongly acidic conditions needed and had to be optimized. Also the loss of 18 mass units was observed under the deprotection conditions, most likely due to the dehydration of the asparagine residue at position 8 of peptide P1. Best results were obtained by treating the crude peptide after TFA cleavage and ether precipitation with 5% TMS triflate in TFA or 5% trifluoromethane sulfonic acid in TFA for 1 h, similar to literature procedures,[18,19] followed by HPLC purification.

Peptide P1 was also prepared with a regular serine phosphate at position 'X' by using Fmoc-Ser[OP(OBn)OH]-OH[20] during peptide assembly (P1-rP). The cleavage of the phosphate from this peptide and from the phosphate phosphonate moiety in peptide P1-PP by alkaline phosphatase was investigated kinetically. The phosphate was released with a K_m of 2.2 mM and a V_{max} of 0.59 μmol min^{-1} mg^{-1}, whereas the regular serine phosphate in this sequence was cleaved with a K_m of 1.62 mM and a V_{max} of 1.57 μmol min^{-1} mg^{-1}.

The dephosphorylation of peptide P1-rP by alkaline phosphatase was inhibited by the sequence containing the major diastereomer of the hydroxymethylene phosphonate (P1-hmP) with a K_i of 0.43 mM.

These and other peptides containing the new phosphoserine derivatives are currently investigated for their potential to inhibit protein phosphatases and protein kinases selectively. The hydroxymethylene phosphonate might also serve in affinity purifications of protein phosphatases and/or kinases from cellular extracts.

Experimental

General procedures

Analytical HPLC was performed on 4×250 mm Nucleosil 300-7 C18 columns from Macherey and Nagel (Düren, Germany) with gradients of acetonitrile in water containing 0.1% TFA or 0.1% AcOH, with detection at 214 nm. Preparative HPLC was carried out on a 40×300 mm 10 μm C18 column from Latek (Eppelheim, Germany) with the gradients specified in the particular experimental procedure. ^1H, ^{13}C and ^{31}P NMR spectra were recorded at ambient temperature on Bruker ARX 400 or DPX 300 spectrometers locked to the deuterium resonance of the solvent. The chemical shifts are reported in parts per million downfield from internal tetramethylsilane (TMS) or 85% orthophosphoric acid. All NMR spectra were measured in $CDCl_3$. MALDI-MS was performed on a Kratos Kompact Maldi III in the reflectron mode with 2,5-dihydroxybenzoic acid as matrix.

During peptide assembly the following amino acid side chain protections were used: Glu, Ser: t-Bu; Asn: trityl; Arg: Pmc; Lys: Boc. For peptide assembly TentaGel S

RAM, Rapp Polymers (Tübingen, Germany) with a loading of 250 μmol/g was used as the solid support. The peptides were obtained as the C-terminal amides. Fmoc- Ser[OP(OBn)–OH]–OH was obtained from Calbiochem-Novabiochem (Läufelfingen, Switzerland) and Z-Asp-OBn from Bachem (Bubendorf, Switzerland).

In the syntheses of the hydroxymethylene phosphonates, diastereomers were formed in a ratio of ca. 2:1. The major diastereomer was designated 'diastereomer I', the one that was formed in a smaller amount 'diastereomer II'. In the experiments with the 'phosphate phosphonate' structures, the diastereomers were formed in a ratio of ca. 1:1 according to NMR analysis.

Alkaline phosphatase (calf intestinal mucosa) was obtained from Sigma (Deisenhofen, Germany). The optimal pH for the dephosphorylation of peptide P1-rP containing the regular serine phosphate was determined to be 7.5 and the kinetic determinations with the new derivatives were carried out at this value. The released phosphate was determined colorimetrically with malachite green and ammonium molybdate.[21,22]

Preparation of the diisopropyl hydroxymethylene phosphonate 5a

To a solution of 1 mL (6 mmol) diisopropylphosphite and 835 μL (6 mmol) triethylamine in 10 mL DCM were added 760 μL (6 mmol) trimethylsilyl chloride. After a few minutes the mixture was added to 1 g (2.9 mmol) aldehyde **4** in 10 mL DCM. After 12 h at RT 30 mL water were added and the mixture was extracted with ethyl acetate. The organic layer was washed with saturated aqueous NaCl, dried with $MgSO_4$ and the solvent was removed. The crude product was stirred for 1 h in 10 mL methanol with 50 mg citric acid. 20 mL ethyl acetate were added and the mixture was extracted with saturated sodium hydrogen carbonate and water. The organic layer was dried with $MgSO_4$, the solvent was removed and the product was isolated by column chromatography on 200 g silica with 800 mL ethyl acetate/methanol 8:2 followed by 400 mL ethyl acetate/methanol 1:1. Final purification was carried out by preparative HPLC with a gradient from 50% methanol in water to 100% methanol to yield 0.94 g **5a** as the mixture of diastereomers (1.9 mmol, 66%).

^{1}H NMR: 7.32 (m, 10 H, H_{ar}), 6.00 (dd, 1 H, NH), 5.17 (s, 2 H, CH_2Ph), 5.09 (s, 2 H, CH_2Ph), 4.63 (m, 3 H, Hα, *i*Pr), 3.91 (m, 1 H, Hγ), 2.13 (dm, 2 H, Hβ), 1.26 (m, 12 H, *i*Pr). ^{13}C NMR: 171.8 (CH*C*OOBzl), 156.8 (NH–*C*OOBzl), 136.1 (C_{ar}), 135.2 (C_{ar}), 128.1–128.6 (CH_{ar}), 71.4 (CH, *i*Pr), 67.4 (CH_2Ph), 67.2 (CH_2Ph), 64.7 (d, ^1J(C,P)=169 Hz, Cγ), 51.5 (Cα), 34.3 (Cβ), 23.9 (CH_3, *i*Pr). ^{31}P NMR: 23.1 (diastereomer I), 22.9 (diastereomer II).

Preparation of the di-*tert*-butyl hydroxymethylene phosphonate 5b

To a solution of 970 μL (5 mmol) di-*tert*-butylphosphite and 765 μL (5.5 μmol) triethylamine in 5 mL DCM were added 635 μL (5 mmol) TMS-Cl at 0°C. After a few minutes the mixture was filtered and the filtrate was added to a solution of 1.1 g (3.2 mmol) aldehyde **4** in 10 mL DCM at 0°C. After 4 h at RT 40 mL diisopropyl ethyl ether were added and the mixture was extracted three times with 100 mL 1 M phosphate buffer pH 7. The product **5b** was purified from the organic layer by preparative HPLC with a gradient from 50% methanol in water to 100% methanol, with elution at ca. 80% methanol. Yield 1.4 g (2.6 mmol, 80%).

^{1}H NMR: 7.31 (m, 10 H, CH_{ar}), 6.28 (t, 2 H, NH), 5.15 (s, 2 H, CH_2Ph), 5.08 (s, 2 H, CH_2Ph), 4.57 (m, 1 H, Hα), 4.17 (1 H, OH), 3.82 (m, 1 H, Hγ), 2.16 (m, 2 H, Hβ), 1.42 (s, 9 H, *t*Bu), 1.39 (s, 9 H, *t*Bu). ^{31}P NMR: 17.2 (diastereomer II), 16.8 (diastereomer I).

Introduction of the THP group into 5a

To a solution of 527 mg (1.04 mmol) **5a** in 15 mL toluene were added 0.46 mL (5.1 mmol) dihydropyran and 0.5 mL from a saturated anhydrous solution of *p*-toluenesulfonic acid in toluene. After 18 h at 20°C, 500 mg of dry sodium carbonate were added and the mixture was stirred for 1 h. After the addition of 20 mL ethyl acetate the organic layer was extracted with saturated sodium hydrogen carbonate and water. The product was purified from the organic layer by preparative HPLC with a gradient from 50% methanol in water to 100% methanol. Yield 469 mg **6a** (0.79 mmol, 76%). 7% **5a** could also be recovered.

^{1}H NMR: 7.33 (m, 10 H, H_{ar}), 6.52 (dd, 1 H, NH), 5.17 (m, 2 H, CH_2Ph), 5.10 (m, 2 H, CH_2Ph), 4.80 (m, 1 H, THP), 4.69 (m, 2 H, *i*Pr), 4.55 (m, 1 H, Hα), 4.00 (m, 2 H, THP), 3.41 (m, 1 H, Hγ), 2.22 (m, 2 H, Hβ), 1.77 (m, 3 H, THP), 1.46 (m, 3 H, THP), 1.28 (m, 12 H, *i*Pr). ^{13}C NMR: 171.5 (CH*C*OOBzl), 156.3 (NH*C*OOBzl), 136.6 (C_{ar}), 135.5 (C_{ar}), 128.1–128.6 (CH_{ar}), 100.9 (CH, THP), 72.0 (CH, *i*Pr), 71.1 (CH, *i*Pr), 66.9 (CH_2Ph), 66.5 (CH_2Ph), 67.4 (d, ^1J(C,P)=169 Hz, Cγ), 64.9 (CH_2, THP), 51.7 (Cα), 31.7 (Cβ), 30.9 (CH_2, THP), 24.9 (CH_2, THP), 24.0 (CH_3, *i*Pr), 21.2 (CH_2, THP). ^{31}P NMR: 21.1 (diastereomer I), 20.9 (diastereomer II).

Introduction of the THP group into 5b

To a solution of 570 mg (1.067 mmol) **5b** in 5 mL DCM were added 54 mg (0.2 mmol) pyridinium *p*-toluene sulfonate and 180 μL (2.34 mmol) dihydropyran. After stirring the solution for 18 h at 20°C, 20 mL ethyl acetate were added and the organic layer was extracted three times with 50 mL 1 M phosphate buffer pH 7. The organic layer was dried with $MgSO_4$ and the product was purified by preparative HPLC with a gradient from 50% methanol in water to 100% methanol, with 5 mM ammonium hydrogen carbonate in the water (pH 7.8). The methanol from the fraction that contained the product was evaporated and **6b** was extracted into DCM. Yield 440 mg (0.71 mmol, 67%).

^{1}H NMR: 7.33 (m, 10 H, H_{ar}), 6.60 (dd, 1 H, NH), 5.17 (m, 2 H, CH_2Ph), 5.11 (m, 2 H, CH_2Ph), 4.84 (m, 1 H, THP), 4.59 (m, 1 H, Hα), 3.90 (m, 2 H, THP), 3.42 (m, 1 H, Hγ), 2.28 (m, 2 H, Hβ), 1.71 (m, 2 H, THP), 1.51 (m, 4 H, THP), 1.48 (m, 9 H, *t*Bu), 1.43 (m, 9 H, *t*Bu).

Introduction of the MEM group into 5b

620 mg (1.16 mmol) **5b**, 500 μL (2.87 μmol) DIEA and 1 mL (8.1 mmol) methoxyethoxymethyl chloride in DCM were kept for 18 h at 20°C. 10 mL diisopropyl ethyl ether were added and the mixture was extracted three times with 50 mL 1 M phosphate buffer pH 7. The product **6c** was isolated from the organic layer by preparative HPLC with a gradient from 50% methanol in water to 100% methanol, with elution at 85–90%. Yield 430 mg (0.69 mmol, 59%).

^{1}H NMR: 7.25 (m, 10 H, H_{ar}), 6.31 (m, 1 H, NH), 5.10 (m, 2 H, CH_2Ph), 5.04 (m, 2 H, CH_2Ph), 4.58 (dm, 1 H, Hα), 4.36 (dm, 1 H, Hγ), 3.77 (m, 2 H, MEM), 3.45 (m, 4 H, MEM), 3.23 (m, 3 H, MEM), 2.26 (dm, 2 H, Hβ), 1.38 (m, 18 H, *t*Bu). ^{31}P NMR: 15.1 (diastereomer I), 15.0 (diastereomer II).

Removal of the Z- and Bn-groups from 6a–c

Removal of the Z- and Bn-groups was carried out with palladium hydroxide on carbon. For **6a** methanol and for **6c** glacial acetic acid were used as the solvents, respectively, and the reductions were complete after 2 h. For **6b** absolute ethanol was used as the solvent and the reduction had to be carried out for two days with the exchange of the catalyst after one day for a complete deprotection. The catalyst and the solvents were removed and the products were lyophilized from dioxane. Quantitative yields of **7a** to **7c**.

Compound **7a** (both diastereomers): ^{1}H NMR: 4.69 (m, 3 H, *i*Pr, THP), 4.16 (m, 1 H, Hα), 3.95 (m, 2 H, THP), 3.45 (m, 1 H, Hγ), 2.26 (m, 2 H, Hβ), 1.78 (m, 2 H, THP), 1.44 (m, 4 H, THP), 1.29 (m, 12 H, *i*Pr). ^{13}C NMR: 173.7 (COOH), 102.0 (CH, THP), 73.9 (d, ^1J(C,P)=169 Hz, Cγ, one diastereomer), 72.0 (CH, *i*Pr), 69.9 (d, ^1J(C,P)=168 Hz, Cγ, one diastereomer), 66.1 (CH_2, THP), 51.6 (Cα), 31.9 (CH_2, THP), 31.3 (Cβ), 25.3 (CH_2, THP), 24.5 (CH_3, *i*Pr), 21.7 (CH_2, THP). ^{31}P NMR: 21.3 (diastereomer II), 20.2 (diastereomer I).

Compound **7c** (both diastereomers): ^{1}H NMR: 4.90 (m, 1 H, Hγ), 4.77 (m, 1 H, Hα), 3.90 (m, 2 H, MEM), 3.72 (m, 2 H, MEM), 3.47 (s, 2 H, MEM), 3.28 (m, 3 H, MEM), 2.25 (m, 2 H, Hβ), 1.43 (m, 18 H, *t*Bu). ^{31}P NMR: 14.7 (diastereomer II), 14.4 (diastereomer I).

Introduction of the Fmoc group into 7a

To a solution of 755 mg (2.05 mmol) **7a** and 1.074 mL (6.17 mmol) DIEA in 50 mL DCM was added 1.586 g (6.17 mmol) Fmoc chloride. After 2 h at 20°C the solvent was evaporated and the product was isolated by column chromatography on 200 mL silica with elution by 800 mL ethyl acetate/cyclohexane 1:4 followed by 400 mL ethyl acetate/cyclohexane 1:1 (both mixtures containing 5% acetic acid) and by 200 mL methanol. Yield 685 mg **8a** (1.16 mmol, 57%). A separation into the two diastereomers was not achieved.

^{1}H NMR: 7.74 (d, 2 H, Fmoc), 7.61 (dd, 2 H, Fmoc), 7.37 (dd, 2 H, Fmoc), 7.29 (d, 2 H, Fmoc), 6.57 (d, 1 H, NH), 4.75 (m, 3 H, *i*Pr, THP), 4.56 (m, 1 H, Hα), 4.35 (d, 2 H, Fmoc), 4.21 (m, 1 H, Fmoc), 4.15 (m, 1 H, THP), 3.92 (m, 1 H, THP), 3.46 (m, 1 H, Hγ), 2.29 (dm, 2 H, Hβ), 1.80 (m, 2 H, THP), 1.48 (m, 4 H, THP), 1.31 (m, 12 H, *i*Pr). ^{13}C NMR: 174.6 (COOH), 156.1 (Fmo*c*), 143.7 (Fmoc), 140.7 (Fmoc), 127.2 (CH_{ar}, Fmoc), 126.6 (CH_{ar}, Fmoc), 124.9 (CH_{ar}, Fmoc), 119.4 (CH_{ar}, Fmoc), 99.9 (CH, THP), 71.1 (CH, *i*Pr), 68.5 (d, ^1J(C,P)=170 Hz, Cγ), 66.2 (F*m*oc), 61.9 (CH_2, THP), 49.5 (Cα), 46.4 (Fmoc), 33.8 (Cβ), 30.2 (CH_2, THP), 25.0 (CH_2, THP), 24.8 (CH_2, THP), 23.6 (CH_3, *i*Pr). ^{31}P NMR: 21.1 (both diastereomers).

Introduction of the Fmoc group into 7c

To 260 mg (0.65 mmol) **7c** in 2 mL acetonitrile/methanol 2:1 containing 570 μL (3.3 mmol) DIEA were added 653 mg (1.95 mmol) Fmoc-OSuc in portions over a period of 18 h at 20°C. After the addition of 30 mL 1 M phosphate buffer pH 7 the product was extracted with DCM and the two diastereomers were separated by column chromatography on 100 g silica with 500 mL ethyl acetate/cyclohexane 4:6, followed by 500 mL of the same solvents in a ratio of 6:4 and 500 mL 7:3 (all mixtures containing 5% acetic acid). Yield 180 mg (0.29 mmol) and 120 mg (0.19 mmol) of the two diastereomers of **8b** (in the order of elution), combined yield 76%.

^{1}H NMR (for both diastereomers): 7.72 (d, 2 H, Fmoc), 7.60 (m, 2 H, Fmoc), 7.32 (m, 2 H, Fmoc), 7.25 (m, 2 H, Fmoc), 6.32 (d, 1 H, NH), 5.00 (s, 4 H), 4.74 (m, 1 H, Hα), 4.40 (m, 4 H), 4.14 (m, 5 H), 3.89 (m, 3 H), 3.5–3.8 (m, Dioxan), 3.31 (s, 3 H, MEM), 2.27 (dm, 2 H, Hβ), 1.48 (m, 9 H, *t*Bu), 1.44 (m, 9 H, *t*Bu). ^{31}P NMR: 15.1 (diastereomer I); 15.0 (diastereomer II).

Preparation of *N*-(benzyloxycarbonyl)-l-aspartic acid-4-chloride-1-benzyl ester (10)

To a solution of 5 g (14 mmol) Z-Asp-OBn **9** in 30 mL DCM were added 7 mL (14 mmol) oxalyl chloride (2 M solution in DCM) and one drop of DMF at 0°C. After stirring at 20°C until gas development had stopped, the solvent was removed to yield the acid chloride **10** quantitatively (5.2 g).

Preparation of the ethyl-protected phosphate phosphonate 11a

To a stirred suspension of 720 mg sodium hydride (60% in mineral oil, 18 mmol) in 15 mL THF were added 2.3 mL (18 mmol) diethyl phosphite at 0°C. After gas development had ceased, the mixture was added in portions of 3 mL to a solution of 5.2 g (14 mmol) acid chloride **10** in 30 mL THF at 20°C until the reaction mixture turned slightly reddish, when the addition was terminated. After stirring for 90 min at 20°C, 50 mL 1 M phosphate buffer pH 7 were added and the aqueous layer was extracted with ethyl acetate. The product was isolated from the organic phase by preparative HPLC with a gradient from 50% methanol in water to 100% methanol, with elution at 85–95% methanol. In addition to 1.26 g **11a** (2.05 mmol, 13%), 0.83 g **9** (2.32 mmol, 29%) could be recovered.

^{1}H NMR: 7.3 (m, 10 H, H_{ar}), 6.20 (d, 1 H, NH), 5.18 (s, 2 H,

CH_2Ph), 5.11 (s, 2 H, CH_2Ph), 4.74 (m, 1 H, Hα), 4.67 (m, 1 H, Hγ), 4.1 (m, 8 H, Et), 2.43 (m, 2 H, Hβ), 1.3 (m, 12 H, Et). ^{13}C NMR: 171.1 (CH*C*OOBzl), 156.1 (NH*C*OOBzl), 136.4 (C_{ar}), 135.4 (C_{ar}), 128–129 (CH_{ar}), 69.5 (d, $^1J(C,P)$=172 Hz, Cγ), 67.3 (CH_2, Et), 66.9 (CH_2, Et), 64.5 (*C*H_2Ph), 63.3 (*C*H_2Ph), 51.9 (Cα), 33.4 (Cβ), 16.4 (CH_3, Et), 16.0 (CH_3, Et). ^{31}P NMR: 20.2 (d, $^1J(P,P)$=22 Hz, phosphonate), 0.3 (d, $^1J(P,P)$=22 Hz, phosphate).

Preparation of the isopropyl protected phosphate phosphonate 11b

To a suspension of 600 mg sodium hydride (60% in mineral oil, 15 mmol) in 30 mL THF were added 2.5 mL (15 mmol) diisopropyl phosphite at 0°C. The mixture was stirred at 20°C until gas development had stopped. The solution was added in portions of 3 mL to 1.13 g (2.9 mmol) acid chloride **10** in 30 mL THF at 20°C. When the solution turned slightly reddish, 30 mL of 1 M phosphate buffer pH 7 was added and the mixture was extracted with ethyl acetate. The product **11b** was purified from the organic layer by preparative HPLC with a gradient from 50% methanol in water to 100% methanol, with elution at 85–90% methanol. Yield 549 mg (0.82 mmol, 28%); 383 mg of **9** (37%) were also recovered.

^1H NMR: 7.33 (m, 10 H, H_{ar}), 6.34 (d, 1 H, NH), 5.18 (s, 2 H, CH_2Ph), 5.09 (s, 2 H, CH_2Ph), 4.67 (m, 6 H, Hα, Hβ, *i*Pr), 2.43 (m, 2 H, Hβ), 1.32 (m, 24 H, *i*Pr). ^{13}C NMR: 171.1 (CH*C*OOBzl), 156.1 (NH*C*OOBzl), 136.3 (C_{ar}), 135.3 (C_{ar}), 128-129 (CH_{ar}), 73.2 (CH, *i*Pr), 72.1 (CH, *i*Pr), 69.7 (d, $^1J(C,P)$=174 Hz, Cγ), 67.0 (*C*H_2Ph), 66.6 (*C*H_2Ph), 51.2 (Cα), 33.3 (Cβ), 23.3–23.7 (CH_3, *i*Pr). ^{31}P NMR: 18.4 (d, $^1J(P,P)$=29 Hz, phosphonate), −1.2 (d, $^1J(P,P)$=29 Hz, phosphate).

Removal of the Z- and Bn-groups from 11a and 11b

Hydrogenations were carried out in glacial acetic acid with palladium hydroxide on carbon for 3 h. After filtering off the catalyst the solvent was removed by lyophylization and traces of acetic acid were removed by repeated (at least three times) lyophylizations from dioxane to yield **12a** and **12b** quantitatively.

Compound **12a**: ^1H NMR: 7.7 (br. s), 5.14 (m, 1 H, Hα), 4.13 (m, 8 H, Et), 3.85 (m, 1 H, Hγ), 2.42 (dm, 2 H, Hβ), 1.27 (m, 12 H, Et). ^{13}C NMR: 172.7 (COOH), 70.3 (d, $^1J(C,P)$=169 Hz, Cγ), 64.6 (CH_2, Et), 63.4 (CH_2, Et), 50.8 (Cα), 32.7 (Cβ), 16.3 (CH_3, Et), 15.9 (CH_3, Et). ^{31}P NMR: 20.7 (d, $^1J(P,P)$=22 Hz, phosphonate, first diastereomer), 20.3 (d, $^1J(P,P)$=20 Hz, phosphonate, second diastereomer), 0.6 (d, $^1J(P,P)$=20 Hz, phosphate, first diastereomer), −0.6 (d, $^1J(P,P)$=22 Hz, phosphonate, second diastereomer).

Compound **12b**: ^1H NMR: 4.93 (m, 1 H, Hα), 4.68 (m, 4 H, *i*Pr), 4.10 (m, 1 H, Hγ), 2.50 (br. d, 2 H, Hβ), 1.31 (m, 24 H, *i*Pr). ^{13}C NMR: 172.1 (COOH), 70.2 (d, $^1J(C,P)$=169 Hz, Cγ), 74.1 (CH, *i*Pr), 72.8 (CH, *i*Pr), 50.3 (Cα), 32.1 (Cβ), 23–24 (CH_3, *i*Pr). ^{31}P NMR: 18.1 (d, $^1J(P,P)$=25 Hz, phosphonate, first diastereomer), 17.6 (d, $^1J(P,P)$=25 Hz, phosphonate, second diastereomer), −1.2 (d, $^1J(P,P)$=25 Hz, phosphate, second diastereomer), −2.4 (d, $^1J(P,P)$=22 Hz, phosphate, first diastereomer).

Introduction of the Fmoc group into 12a and 12b

The Fmoc group was introduced into 590 mg of **12a** and into 191 mg of **12b** as described for **7c** (see above). The products were isolated by preparative HPLC with gradients from 50% methanol in water to 100% methanol (containing 0.5% TFA), with elution of the compounds at 90–95% methanol. Yields: 280 mg (30%) and 57 mg (6%) of the separated diastereomers of **13a** and 173 mg (60%) of the mixture of diastereomers of **13b**.

Compound **13a**: ^1H NMR: 7.73 (d, 2 H, Fmoc), 7.60 (dd, 2 H, Fmoc), 7.38 (dd, 2 H, Fmoc), 7.29 (dd, 2 H, Fmoc), 6.20 (d, 1 H, NH), 4.92 (m, 1 H, Hγ), 4.60 (m, 1 H, Hα), 4.34 (m, 2 H, Fmoc), 4.20 (m, 9 H, Fmoc, Et), 2.42 (m, 2 H, Hβ), 1.3 (m, 12 H, Et). ^{13}C NMR: 172.4 (COOH), 155.7 (Fmo*c*), 143.8 (Fmoc), 141.3 (Fmoc), 127.7 (CH_{ar}, Fmoc), 127.1 (CH_{ar}, Fmoc), 125.3 (CH_{ar}, Fmoc), 120.0 (CH_{ar}, Fmoc), 70.0 (d, $^1J(C,P)$=172 Hz, Cγ), 67.2 (CH_2, F*m*oc), 65.2 (CH_2, Et), 63.7 (CH_2, Et), 50.5 (CH, Fmoc), 47.1 (Cα), 34.9 (Cβ), 16.4 (CH_3, Et), 16.0 (CH_3, Et). ^{31}P NMR ($CDCl_3$, 162 MHz): 20.3 (d, $^1J(P,P)$=20 Hz, phosphonate, first diastereomer), 19.9 (d, $^1J(P,P)$=23 Hz, phosphonate, second diastereomer), 0.1 (d, $^1J(P,P)$=23 Hz, phosphate, second diastereomer), −0.2 (d, $^1J(P,P)$=20 Hz, phosphate, first diastereomer).

Compound **13b**: ^1H NMR: 7.74 (d, 2 H, Fmoc), 7.59 (m, 2 H, Fmoc), 7.39 (dd, 2 H, Fmoc), 7.29 (dd, 2 H, Fmoc), 6.10 (d, 1 H, NH), 4.73 (dm, 6 H, Hα, Hγ, *i*Pr), 4.38 (d, 2 H, Fmoc), 4.21 (t, 1 H, Fmoc), 2.45 (m, 2 H, Hβ), 1.34 (m, 24 H, *i*Pr). ^{13}C NMR: 174.4 (COOH), 156.6 (Fmo*c*), 143.7 (Fmoc), 141.3 (Fmoc), 127.8 (CH_{ar}, Fmoc), 127.1 (CH_{ar}, Fmoc), 125.1 (CH_{ar}, Fmoc), 120.0 (CH_{ar}, Fmoc), 75.2 (CH, *i*Pr), 74.2 (CH, *i*Pr), 70.2 (d, $^1J(C,P)$=185 Hz, Cγ), 67.7 (Fmo*c*), 50.7 (Cα), 47.0 (CH, Fmoc), 33.7 (Cβ), 23.4 (CH_3, *i*Pr). ^{31}P NMR: 16.9 (d, $^1J(P,P)$=26 Hz, phosphonate), −3.3 (d, $^1J(P,P)$=27 Hz, phosphate).

Peptide assembly

The peptide sequences Ac-AAEGGS**X**NVFSK-amide (P1) and RRRRAA**X**VA-amide (P2) (with **X** representing the new derivatives or regular serine phosphate) were synthesized manually according to the Fmoc/*t*Bu method in scales of 10–20 μmol. The amino acids were coupled in DMF according to standard protocols in a three-fold excess with activation by 1 equiv. of TBTU and 2 equiv. of diisopropylethylamine. Coupling time was 1 h. After each coupling and before the removal of the Fmoc group the amount of free amino groups in the synthesis resin was determined according to the bromophenol blue method as described.[23] The new derivatives and the following amino acid were double-coupled before the removal of the Fmoc group. Cleavage of the Fmoc group was carried out with 20% piperidine in DMF for 10 min. Cleavage of the peptides from the resin and deprotection of the side chains (except for the isopropyl phosphate protection) was carried out by a 3 h treatment with TFA/triisobutylsilane/water in a ratio of 95:3:2. Crude peptides were obtained by precipitation with

Table 1.

Substrate [mM]	20.0	10.0	6.0	3.5	2.0	1.0	0.5
v [μmol min^{-1} mg^{-1}] for P1-rP	1.46	1.45	1.35	1.20	0.98	0.63	0.40
v [μmol min^{-1} mg^{-1}] for P1-PP	0.52	0.52	0.43	0.33	0.16	0.03	–

tert-butyl methyl ether. Cleavage of the isopropyl groups from peptide P1 containing the phosphate phosphonate moiety (P1-PP) was achieved by a treatment of portions of 1 mg crude peptide with 300 μL trifluoromethane sulfonic acid (5% in TFA) or 300 μL TMS triflate (5% in TFA) for 45–90 min. The optimal time point for quenching the reaction by precipitating the peptide with 3 mL *tert*-butyl methyl ether was determined by MALDI-MS. HPLC analyses of the crude peptides revealed that they contained the desired sequences as the major component. The crude peptides were purified by preparative HPLC with gradients of acetonitrile in water containing 0.5% TFA and characterized by MALDI-MS and amino acid analysis, which also served for the determination of concentrations of stock solutions of the peptides for kinetic measurements.

Kinetic determination of the dephosphorylation of peptide P1 containing the regular phosphate (P1-rP) and peptide P1 containing the phosphate phosphonate (P1-PP) with alkaline phosphatase

The assay was carried out at 30°C. Samples of 87.5 μL of the peptides in buffer A (0.1 M Tris, 1 mM $MgCl_2$, 1 mM $ZnCl_2$, 1 mg/mL BSA, pH 7.5) at the concentrations according to Table 1 were preincubated at 30°C. 10 μL of a stock solution of alkaline phosphatase (200 U/mL in buffer A), corresponding to 2 U, were added. Before the addition and after 1, 2, 3 and 5 min samples of 17.5 μL were taken from the individual assays and quenched by the addition of 17.5 μL 1 M HCl. The content of free phosphate was determined and the initial velocity was obtained by interpolation from the values that were obtained in the first 2 or 3 min. From the Hanes plots and replots the K_m and V_{max} values were determined.

Kinetic determination of the inhibition of the alkaline phosphatase catalyzed dephosphorylation of peptide P1 containing the regular phosphate (P1-rP) by peptide P1 containing the major diastereomer of the hydroxymethylene phosphonate (P1-hmP)

The assay was carried out similar to the procedure described above at 30°C in buffer A according to Table 2. The inhibitory constant was determined from the Hanes plot and replot of the initial velocities ν [μmol min^{-1} mg^{-1}] given in the table.

Table 2.

P1-hmP (inhibitor) [mM]	P1-rP (substrate) [mM]					
	17.00	10.00	6.00	3.50	2.00	1.00
0.0	1.46	1.45	1.35	1.20	0.98	0.63
0.3	1.42	1.22	1.26	1.09	–	0.45
0.6	1.34	1.08	0.95	0.77	0.74	0.49
1.0	–	1.01	0.92	0.78	0.43	0.47

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