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Solid Phase Synthesis of Alkylphosphonopeptides

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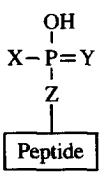
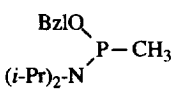
Abstract: The solid phase assembly of alkylphosphonylated peptides **3a**,**b** using the readily available new phosphonylating reagents *p*-methoxybenzyl *N*,*N*-diisopropylaminomethylphosphonamidite (**9a**) and its octyl analogue (**9c**) is described.

INTRODUCTION

Reversible phosphorylation of proteins mediated by kinases and phosphatases is one of the major posttranslational modifications by which many cellular processes are regulated.[1] In order to assess in more detail the conformational behaviour and regulatory effect of phosphoproteins, much effort has been devoted towards the preparation of well-defined synthetic phosphopeptides and analogues thereof bearing a non-hydrolyzable and/or isoelectronical modified phosphate function, e.g. phosphorothioate[2] (**1a**), phosphonate[3] (**1b**) and difluoromethylenephosphonate[4] (**1c**). A recent study from this laboratory[5] revealed that the phoshinylating reagent benzyl *N*,*N*-diisopropylmethylphosphonamidite (**2**) showed promise for the synthesis of methylphosphonylated peptides (**1d**).

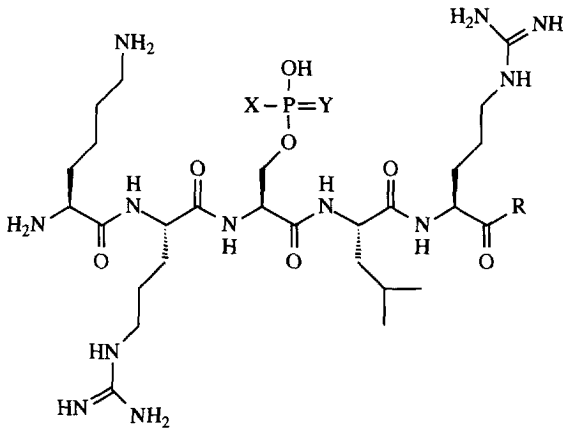




1a: X = OH, Y = S, Z = O
b: X = OH, Y = O, Z = CH_2
c: X = OH, Y = O, Z = CF_2
d: X = CH_3, Y = Z = O

2

The favourable outcome of the latter syntheses in solution was a stimulus in attuning the methylphosphonamidite approach to a solid phase synthesis of alkylphosphonopeptides. We now wish to report that the novel reagents *p*-methoxybenzyl *N,N*-diisopropylmethylphosphonamidite (**9a**) as well as its octyl analogue (**9c**) are convenient tools in a solid-supported synthesis of the respective phosphonylated peptides **3a,b**.



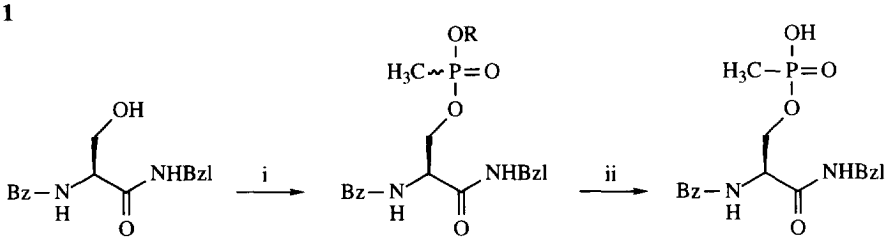
3a: X = CH_3, Y = O, R = NH_2
b: X = C_8H_{17}, Y = O, R = NH_2
c: X = OH, Y = O, R = OH
d: X = OH, Y = S, R = OH

RESULTS AND DISCUSSION

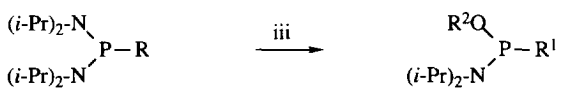
A well-established approach to the solid phase synthesis of phosphopeptides comprises a so-called global phosphorylation of hydroxyl-bearing amino acid residues incorporated into a pre-assembled polymer-bound peptide.[6] For instance, it was demonstrated[6b] that the phosphopeptide **3c** was accessible in good yield by phosphitylation of immobilized Boc-Lys(Boc)-Arg(Mtr)-Ser-Leu-Arg(Mtr)-O-polymer with di-*p*-chlorobenzyl *N,N*-diisopropylphosphoramidite in the presence of 1*H*-tetrazole followed by *tert*-butyl hydroperoxide-mediated oxidation of the intermediate phosphite triester. In addition, sulfurization of the latter phosphite intermediate with phenylacetyl disulfide and further processing led to the thiophosphorylated peptide **3d**.[2a] The latter results and the favourable phosphonylating properties of reagent **2** augur well with the intended synthesis of the methylphosphonylated peptide **3a** following a global phosphonylation strategy. However, it is not excluded that the application of reagent **2** may be hampered by an unacceptable slow removal of the benzyl protective group from an intermediate methylphosphonate function under standard acidolysis condition (*i.e.* 95% TFA/phenol)[7] for the deblocking of the Boc and Pmc amino acid side-chain protective groups. The time required for complete removal of the benzyl phosphonate protecting group was established by subjecting serine derivative **6a** (Scheme 1), prepared in 85% yield by phosphinylation of the serine derivative **5** with reagent **2** followed by *in situ* oxidation with *t*-BuOOH, to 95% TFA/phenol. ^{31}P NMR and TLC analysis revealed that the removal of the benzyl group from the phosphonate function in **6a** went to completion after 3 h at 20 °C. The relatively slow deblocking of the benzyl phosphonate protecting group, which will not be accelerated by performing the above mentioned acidolysis on similarly protected serine methylphosphonate residues embedded in an immobilized peptide, was a stimulus to prepare methylphosphonates **6b,c** containing the more acid-labile *p*-methoxybenzyl

and *t*-butyl group, respectively. The required methylphosphonamidite **9a** was readily accessible by the reaction of bis(diisopropylamino)methylphosphine[8] (**8a**) with *p*-methoxybenzyl alcohol in the presence of a catalytic amount of *sym*-collidine hydrochloride. On the other hand, the synthesis of *t*-butyl protected phosphonamidite **9b** proceeded sluggishly (*i.e.* 10 days) and resulted in the isolation of an impure product. Phosphonylation of compound **5** with reagents **9a,b** and subsequent *in situ* oxidation with *t*-BuOOH afforded, after work-up and purification, the respective *p*-methoxybenzyl and *tert*-butyl protected methylphosphonates **6b,c** in 70% and 76% yield. Treatment of phosphonate diesters **6b,c** with 95% TFA/phenol led to the formation of methylphosphonate monoester **7** within 2 min, as gauged by ^{31}P NMR and TLC analysis. It may therefore be expected that both the *p*-methoxybenzyl and *t*-butyl methylphosphonamidites **9a,b** are suitable reagents for the preparation of methylphosphonopeptides on a solid support. Nevertheless, the availibility of pure **9a** was a decisive factor in utilizing this reagent for the synthesis of methylphosphonate peptide **3a**.

Scheme 1



5 **6a**: R = Bzl; **b**: R = *p*-MeOBzl; **c**: R = *t*-Bu **7**



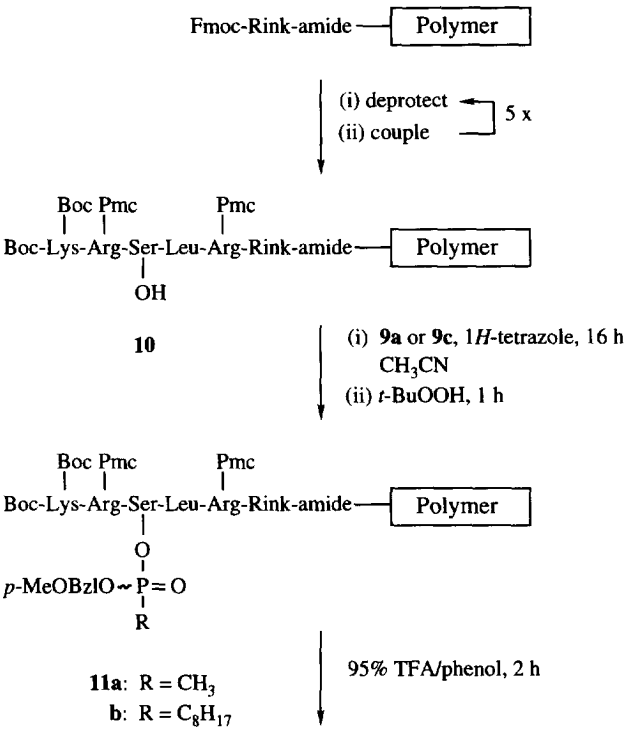
8a: R = CH_3; **b**: R = C_8H_{17} **9a**: $R^1 = CH_3$, R^2 = *p*-MeOBzl; **b**: $R^1 = CH_3$, R^2 = *t*-Bu; **c**: $R^1 = C_8H_{17}$, R^2 = *p*-MeOBzl

Conditions: (i) (a) **2** (2 eq.) or **9a,b** (2 eq.), 1*H*-tetrazole (1 eq.), CH_3CN, 30 min; (b) *t*-BuOOH, 5 min; (ii) 95% TFA/phenol; (iii) *t*-BuOH (1.4 eq.) or *p*-MeOBzlOH (1.4 eq.), *sym*-collidine hydrochloride (0.1 eq.), CH_2Cl_2, 24 h (**8a**→**9a**, 87%; **8b**→**9c**, 81%) or 10 days (**8a**→**9b**, 70%).

The viability of reagent **9a** was demonstrated in the global phosphonylation of the immobilized Ser-containing peptide **10**. The requisite peptide **10** was assembled, as depicted in Scheme 2, by Fmoc solid phase peptide synthesis on a MilliGen 9050 continuous flow synthesizer using commercially available kieselguhr-polydimethylacrylamide methyl ester resin (Macrosorb® SPR, loading 0.10 mmol/g, Novabiochem). Functionalization of the resin was effected by aminolysis with ethylene diamine and subsequent introduction of

the Rink-amide[9] linker. Repetitive Fmoc-cleavage was accomplished with 2% DBU/2% piperidine/DMA (v/v/v) for 5 min. Peptide couplings were carried out using three equivalents each of Fmoc-amino acid and BOP as the condensating reagent in the presence of DIEA for 1 h. The serine residue was incorporated without side-chain protection. In view of the final mild acidic deblocking conditions, the Boc-group was installed to protect the α- and ε-amino group of the N-terminal lysine, while the guanidinium side-chain of arginine was protected with the acid-labile Pmc-group.

Scheme 2



Prior to the introduction of the phosphonate moiety, the peptide anchored to the resin (**10**) was removed from the column, thoroughly washed and dried *in vacuo* over P_2O_5. Phosphinylation of **10** was performed with an excess of reagent **9a** (15 eq.) and 1*H*-tetrazole (7.5 eq.) in acetonitrile for 16 h. Subsequent *in situ* oxidation with *t*-BuOOH for 1 h afforded the immobilized methylphosphonate peptide **11a**. Removal of the protecting groups and cleavage of the peptide from the solid support with 95% TFA/phenol for 2 h gave crude **3a**, reversed-phase (RP) HPLC analysis of which revealed the presence of one major product having a different retention time than H-Lys-Arg-Ser-Leu-Arg-NH_2 (see Experimental section). Purification of the crude product by semi-preparative RP HPLC yielded homogeneous methylphosphonopeptide **3a** (51%, calculated from the initial

loading of functionalized resin), as corroborated by analytical RP HPLC as well as capillary zone electrophoresis (CZE, see Figure 1). The structure of **3a** was unambiguously ascertained by electrospray MS, ^{1}H, ^{13}C and ^{31}P NMR spectroscopy.

The successful synthesis of the methylphosphonylated peptide **3a** was a firm basis to prepare peptide **3b** containing the more lipophilic octylphosphonate function. Octylphosphonamidite **9c** was attained by reaction of bis(*N,N*-diisopropylamino)octylphosphine[8] (**8a**) with *p*-methoxybenzyl alcohol according to the same procedure described for the synthesis of **9a** (yield 81%). The phosphonylating properties of the new octyl reagent **9c** were illustrated by the smooth conversion of Z-serine benzyl ester into *O*-(*N*-benzyloxycarbonyl-L-serine benzyl ester) *p*-methoxybenzyl octylphosphonate. Next, global phosphonylation of resin-bound peptide **10** with amidite **9c** was carried out in a similar fashion as described for the synthesis of **3a**. Finally, treatment of peptide resin **11b** with 95% TFA/phenol and analysis of crude **3b** by RP chromatography revealed the presence of one main product. Subsequent purification by semi-preparative RP HPLC afforded homogeneous octylphosphonopeptide **3b** in 48% yield. The analytical (RP HPLC and CZE, Figure 1) and spectroscopic (electrospray MS, ^{13}C and ^{31}P NMR) data of **3b** were in complete accordance with the proposed structure.[10]

In conclusion, the results presented in this paper indicate that application of *p*-methoxybenzyl protected phosphonamidites may open the way to the future synthesis of alkylphosphonylated *via* a global phosphonylation approach.

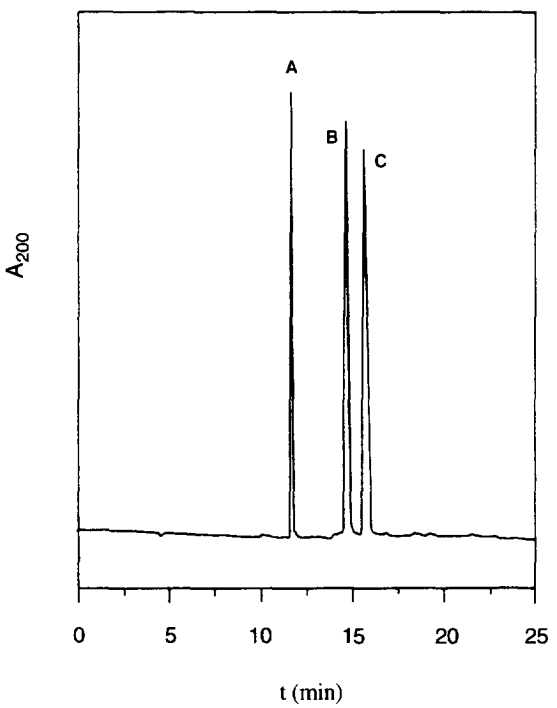


Fig. 1 CZE of purified (**A**) H-Lys-Arg-Ser-Leu-Arg-NH_2, (**B**) methylphosphonate **3a** and (**C**) octylphosphonate **3b** (for conditions see Experimental section).

EXPERIMENTAL

General methods and materials

CH_2Cl_2 and toluene were distilled from P_2O_5 and stored over molecular sieves (4Å). DMA was stirred with CaH_2 and distilled under reduced pressure. DIEA was distilled from KOH. Acetonitrile (Rathburn HPLC grade) was stored over molecular sieves (3Å). TFA was obtained from Janssen (Belgium). Ethylene diamine p.a. and *tert*-butyl hydroperoxide (80% solution in di-*tert*-butyl peroxide) were purchased from Merck-Schuchardt (Germany). Fmoc-Leu-OH and Fmoc-Arg(Pmc)-OH were obtained from SENN Chemicals (Switzerland). Macrosorb® SPR resin (substitution 0.10 mmol/g) and *p*-[(R,S)-α-[1-(9H-fluoren-9-yl)-methoxyformamido]-2,4-dimethoxybenzyl]phenoxyacetic acid were obtained from Novabiochem (Switzerland).

Reactions were run at ambient temperature unless noted otherwise. TLC analysis was performed on Schleicher and Schüll DC Fertigfolien F1500 LS254 employing the following solvent systems: A (light petroleum (bp 40-60 °C)/TEA, 19/1, v/v), B (ethyl acetate). Compounds were visualized by UV (254 nm) and TDM (*N,N,N',N'*-tetramethyl-4,4'-diaminodiphenylmethane) reagent.[11] Solvents were evaporated under reduced pressure at 40 °C. Column chromatography was performed on silica gel 0.063-0.200 mm (Baker, The Netherlands).

Mass spectra were recorded on a Finnigan MAT TSQ-70 equipped with an electrospray interface. ^1H, ^{13}C and ^{31}P NMR spectra were recorded on a Jeol JNM-FX 200 spectrometer, operating at 200, 50.1 and 80.7 MHz, respectively. The ^{13}C spectra were monitored using the Attached Proton Test (APT) technique. 2D (^1H-^1H COSY, ^1H-^{13}C COSY) ^1H NMR spectra were recorded at 300 MHz on a Bruker DPX-300 spectrometer interfaced with an ASPECT Station computer. Chemical shifts (δ) are given in ppm relative to the signal for internal Me_4Si for ^1H, and to the signal for internal $CDCl_3$ (δ 77.0) CD_3OD (δ 49.0), acetone-d_6 (δ 206.0 or 29.8) or DMSO-d_6 (δ 39.5) for ^{13}C. ^{31}P chemical shifts are given relative to 85% H_3PO_4 as external standard.

Solid phase syntheses were carried out on a MilliGen 9050 continuous flow peptide synthesizer. Deprotection and coupling reactions were monitored at 306 nm. Analytical and semi-preparative HPLC was performed using a LKB Bromma apparatus consisting of a 2151 controller, low pressure gradient mixer, 2150 pump (flow rate 1 or 3 mL/min), 2155 column oven (40 °C), 2158 UVICORD SD detector (λ 206 nm) and a Lichrospher column (5C18, 250 × 4 mm or 10C18, 250 × 10 mm). Elution was effected using an appropriate gradient from 0.1% TFA/H_2O to 0.1% TFA/CH_3CN. Capillary zone electrophoresis (CZE) was carried out on an Applied Biosystems 270A Capillary Electrophoresis System using a capillary column (lenght 72 cm; lenght to detector 50 cm; diameter 50 μm) at 30 °C. A citrate buffer pH 2.5 (20 mM) was used. For detection a variable-wavelenght detector was used operating at 200 nm

p-Methoxybenzyl *N,N*-diisopropylmethylphosphonamidite (9a)

p-Methoxybenzyl alcohol (0.87 mL, 7.0 mmol) and *sym*-collidine hydrochloride (80 mg, 0.5 mmol) were dried by coevaporation with CH_3CN (3 × 5 mL) and dissolved in CH_2Cl_2 (5 mL). Next, bis(diisopropylamino)methylphosphine[8] (**8a**, 1.23 g, 5.0 mmol) was added. After 24 h, TEA (0.5 mL) was added and the mixture was concentrated. Purification of the residue by column chromatography (eluent: light petroleum (bp 40-60 °C)/TEA, 19/1, v/v; R_f 1.0) yielded **9a** (1.23 g, 87%) as a colourless liquid.

^{31}P {^1H} NMR (acteone-d_6): δ 122.8; ^1H NMR (acetone-d_6): δ 7.24, 6.87 (2 × d, each 2H, H-arom., ^3J 8.5 and 8.6 Hz, resp.), 4.56 (d, 2H, CH_2 *p*-MeOBzl, $^3J_{H,P}$ 8.3 Hz), 3.77 (s, 3H, OCH_3), 3.6 (m, 2H, CH *i*-Pr), 1.21, 1.18, 1.11 (3 × d, 15 H, CH_3 *i*-Pr, P-CH_3, J 6.7, 8.6 and 6.8 Hz, resp.); ^{13}C {^1H} NMR (acetone-d_6): δ 159.9 (C_q *p*-MeOBzl), 133.2 (d, C_q *p*-MeOBzl, $^3J_{C,P}$ 8.8 Hz), 129.2, 114.3 (CH-arom.), 68.6 (d, CH_2 *p*-MeOBzl, $^2J_{C,P}$ 17.6 Hz), 55.4 (OCH_3), 44.7 (d, CH *i*-Pr, $^2J_{C,P}$ 10.3 Hz), 25.1, 24.4 (2 × d, CH_3 *i*-Pr, $^3J_{C,P}$ 7.3 Hz), 18.2 (d, P-CH_3, $^1J_{C,P}$ 11.7 Hz).

***tert*-Butyl *N,N*-diisopropylmethylphosphonamidite (9b)**

Treatment of **8a** (1.23 g, 5.0 mmol) with *t*-BuOH (0.66 mL, 7.0 mmol) in the presence of *sym*-collidine hydrochloride (80 mg, 0.5 mmol) for 10 days, followed by work-up as described for the preparation of **9a**, gave amidite **9b** (0.77 g, 70%, purity ±85%) as a colourless liquid. When using a ten-fold excess of *t*-BuOH no acceleration of the rate of formation of **9b** was observed.

R_f 1.0 (system A); ^{31}P {^1H} NMR (acetone-d_6): δ 103.2; ^1H NMR (acetone-d_6): δ 3.6 - 3.5 (m, 2H, CH *i*-Pr), 1.29 (s, 9H, CH_3 *t*-Bu), 1.21, 1.11, 1.07 (3 × d, 15 H, CH_3 *i*-Pr, P-CH_3, *J* 6.7, 6.9 and 9.3 Hz, resp.); ^{13}C {^1H} NMR (acetone-d_6): δ 74.5 (d, C_q *t*-Bu, $^2J_{C,P}$ 13.2 Hz), 44.9 (d, CH *i*-Pr, $^2J_{C,P}$ 11.7 Hz), 30.9 (d, CH_3 *t*-Bu, $^3J_{C,P}$ 8.8 Hz), 25.2, 23.8 (2 × d, CH_3 *i*-Pr, $^3J_{C,P}$ 7.3 Hz), 21.2 (d, P-CH_3, $^1J_{C,P}$ 14.7 Hz).

***p*-Methoxybenzyl *N,N*-diisopropyloctylphosphonamidite (9c)**

Treatment of bis(diisopropylamino)octylphosphine[8] (**8b**, 1.57 g, 4.6 mmol) with *p*-methoxybenzyl alcohol, under the same conditions as described for the synthesis of **9a**, gave amidite **9c** (1.46 g, 81%) as a colourless liquid.

^{31}P {^1H} NMR (acetone-d_6): δ 129.8; ^1H NMR (acetone-d_6): δ 7.27, 6.89 (2 × d, each 2H, H-arom., ^3J 8.6 and 8.7 Hz, resp.), 4.61 (d, 2H, CH_2 *p*-MeOBzl, $^3J_{H,P}$ 7.9 Hz), 3.77 (s, 3H, OCH_3), 3.6 (m, 2H, CH *i*-Pr), 1.6 - 1.7, 1.5 - 1.6, 1.4 - 1.5, 1.3 - 1.4 (4 × m, 1, 1, 4 and 8H, resp., CH_2 octyl), 1.26, 1.15 (2 × d, 12H, CH_3 *i*-Pr, *J* 6.6 and 6.7 Hz, resp.), 0.92 (t, 3H, CH_3 octyl, *J* 7.0 Hz); ^{13}C {^1H} NMR (acetone-d_6): δ 160.1 (C_q *p*-MeOBzl), 133.4 (d, C_q *p*-MeOBzl, $^3J_{C,P}$ 8.8 Hz), 129.4, 114.5 (CH-arom.), 69.3 (d, CH_2 *p*-MeOBzl, $^2J_{C,P}$ 19.0 Hz), 55.6 (OCH_3), 45.1 (d, CH *i*-Pr, $^2J_{C,P}$ 8.8 Hz), 32.8, 30.4, 30.1, 25.0, 23.5 (CH_2 octyl), 32.6, 32.1 (2 × d, CH_2 octyl, $J_{C,P}$ 8.8 and 14.7 Hz, resp.), 25.4, 24.8 (2 × d, CH_3 *i*-Pr, $^3J_{C,P}$ 4.4 and 8.8 Hz, resp.), 14.6 (CH_3 octyl).

***O*-(*N*-benzoyl-L-serine benzylamide) benzyl methylphosphonate, Bz-Ser(P(O)CH_3OBzl)-NHBzl (6a)**

A mixture of Bz-Ser-NHBzl (**5**, 150 mg, 0.50 mmol) and phosphonamidite **2** (250 mg, 1.0 mmol) was dried by coevaporation with CH_3CN (2 × 5 mL) and dissolved in CH_3CN (10 mL). Next, a solution of dry 1*H*-tetrazole (35 mg, 0.50 mmol) in CH_3CN (2 mL) was added. After stirring for 30 min, the mixture was treated with *tert*-butyl hydroperoxide (0.5 mL) for 5 min and concentrated. The resulting residue was purified by column chromatography (eluent: 50%→100% ethyl acetate/light petroleum (bp 40-60 °C), v/v) to furnish **6a** (213 mg, 89%) as a solid.

R_f 0.5 (system B); ^{31}P {^1H} NMR ($CDCl_3$): δ 34.2, 33.8 (ratio 3/4); ^1H NMR (CD_3OD): δ 7.9, 7.5, 7.4, 7.3 - 7.2 (4 × m, 1, 2, 2 and 10H, resp., H-arom.), 5.0 (m, 2H, CH_2 OBzl), 4.9 (m, 1H, Hα), 4.4-4.3 (m, 4H, Hβ, CH_2 NHBzl), 1.46 (d, 3H, P-CH_3, $^2J_{H,P}$ 17.8 Hz); ^{13}C {^1H} NMR ($CDCl_3$): δ 168.5, 167.4 (C=O Bz, Ser), 137.8, 135.6 (C_q Bz, NHBzl), 132.9 (d, OBzl, $^3J_{C,P}$ 4.4 Hz), 131.7, 128.3-127.1 (CH-arom.), 67.5, 67.2 (2 × d, CH_2 OBzl, $^2J_{C,P}$ 5.9 Hz), 65.5, 65.0 (2 × d, Cβ, $^2J_{C,P}$ 5.9 Hz), 53.8 (d, Cα, $^3J_{C,P}$ 5.9 Hz), 43.2 (CH_2 NHBzl), 10.8, 10.7 (2 × d, PCH_3, $^1J_{C,P}$ 143.5 Hz).

***O*-(*N*-Benzoyl-L-serine benzyl amide) *p*-methoxybenzyl methylphosphonate, Bz-Ser(P(O)CH_3O*p*-MeOBzl)-NHBzl (6b)**

Compound **6b** was prepared as described for the preparation of **6a** starting from 90 mg **5** (0.30 mmol) and 170 mg **9a** (0.60 mmol).

Yield: 104 mg (70%); R_f 0.2 - 0.3 (system B); ^{31}P {^1H} NMR (acetone-d_6): δ 35.2, 34.9 (ratio 1/1); ^1H NMR (CD_3OD): δ 7.9, 7.5 - 7.4, 7.3 - 7.2, 6.9 - 6.8 (4 × m, 2, 3, 6 and 2H, resp., H-arom.), 5.0-4.9 (m, 3H, Hα, CH_2 *p*-MeOBzl), 4.4-4.3 (m, 4H, Hβ, CH_2 NHBzl), 3.7 (s, 3H, OCH_3), 1.43 (d, 3H, P-CH_3, $^2J_{H,P}$ 17.7 Hz); ^{13}C {^1H} NMR (acetone-d_6): δ 169.5, 167.4, 167.3 (C=O Bz, Ser), 160.8, 159.7 (C_q *p*-MeOBzl), 143.8,

140.3, 140.2, 135.4, 134,9 (C_q Bz, NHBzl, *p*-MeOBzl), 132.3 - 127.6, 114.7, 114.3 (CH-arom.), 68.2, 67.6 (2 × d, CH_2 *p*-MeOBzl, $^2J_{C,P}$ 5.8 Hz), 66.6, 66.5, 66.4, 64.3 (Cβ), 55.8, 55.5, 55.4 (Cα, OCH_3), 43.5, 43.4 (CH_2 NHBzl), 11.1, 10.9 (2 × d, P-CH_3, $^1J_{C,P}$ 143.8 and 142.0 Hz, resp.).

***O*-(*N*-Benzoyl-L-serine benzyl amide) *tert*-butyl methylphosphonate, Bz-Ser(P(O)CH_3O*t*-Bu)-NHBzl (6c)**

Compound **6c** was synthesized as described for the preparation of **6a** starting from 90 mg **5** (0.30 mmol) and 130 mg **9b** (0.60 mmol).

Yield: 99 mg (76%); ^{31}P {^1H} NMR (acetone-d_6): δ 30.9, 30.6 (ratio 2/3); ^{13}C {^1H} NMR (acetone-d_6): δ 169.7, 169.6, 167.5, 167.4 (C=O Bz, Ser), 140.1, 140.0, 134.8, 134.7 (C_q Bz, Bzl), 132.3, 129.0 - 127.6 (CH-arom.), 83.6 (d, C_q *t*-Bu, $^2J_{C,P}$ 8.8 Hz), 66.0, 65.9, 65.7 (Cβ), 55.6, 55.5 (2 × d, Cα, $^3J_{C,P}$ 4.4 Hz), 43.6, 43.4 (CH_2 Bzl), 30.5 (d, CH_3 *t*-Bu, $^3J_{C,P}$ 4.4 Hz).

***O*-(*N*-Benzyloxycarbonyl-L-serine benzyl ester) *p*-methoxybenzyl octylphosphonate, Z-Ser(P(O)C_8H_{17}O*p*-MeOBzl)-OBzl**

Z-Ser-OBzl (168 mg, 0.51 mmol) was phosphinylated with amidite **9c** (395 mg, 0.10) and oxidized with *t*-BuOOH as described for the preparation of **6a** to afford Z-Ser(P(O)C_8H_{17}O*p*-MeOBzl)-OBzl (177 mg, 54%) as an oil.

^{31}P {^1H} NMR ($CDCl_3$): δ 35.0, 34.8; ^{13}C {^1H} NMR ($CDCl_3$): δ 169.0 (C=O Ser), 159.8 (C_q *p*-MeOBzl), 155.8 (C=O Z), 136.1, 135.0 (C_q Bzl, *p*-MeOBzl, Z), 129.9, 128.5 - 128.0, 113.9 (CH-arom.), 67.6 - 67.1 (CH_2 Bzl, *p*-MeOBzl, Z), 65.1 - 64.9 (Cβ Ser), 55.2 (OCH_3), 54.6 (Cα Ser, $^3J_{C,P}$ 5.9 Hz), 31.7, 30.6, 30.2, 28.9, 27.0, 24.2, 22.5, 22.1, 22.0 (CH_2 octyl), 14.0 (CH_3 octyl).

Deprotection of model compounds 6a-c

Methylphosphonate diesters **6a-c** (0.10 mmol) were treated with 95% TFA/phenol (2 mL). The rate of formation of phosphonate monoester **7** was determined by ^{31}P NMR and TLC analysis. In case of **6b**, a red colour developed immediately upon addition of the TFA mixture.

Selective analytical data: **6a**: ^{31}P {^1H} NMR (95% TFA/phenol): δ 38.9, 38.5; **7**: ^{31}P {^1H} NMR (95% TFA/phenol): δ 39.3.

Functionalization of the resin

To 25 g Macrosorb® SPR resin (0.1 mmol/g) was added ethylene diamine (200 mL) and the reaction vessel was gently rotated for 16 h. The resin was thoroughly washed with DMA until the filtrate contained no more diamine as indicated by a negative ninhydrin test. After additional washings with *tert*-amyl alcohol (3 ×) and diethyl ether (3 ×), the resin was dried *in vacuo* over P_2O_5. Next, a minimal amount of DMA was added and after swelling, Rink amide linker *p*-[(R,S)-α-[1-(9H-fluoren-9-yl)-methoxyformamido]-2,4-dimethoxybenzyl]-phenoxyacetic acid[9] (2.02 g, 3.3 mmol), BOP (1.66 g, 3.8 mmol) and DIEA (1.3 mL, 7.5 mmol) were added. The mixture was shaken until no more free amino groups could be detected as indicated by a negative Kaiser test.[12] Finally, the resin was washed with DMA, *tert*-amyl alcohol, diethyl ether and dried *in vacuo* over P_2O_5. The loading per gram resin was estimated to be 97 μmol following a procedure described by de Bont.[13]

H-Lys-Arg-Ser(OP(O)CH_3OH)-Leu-Arg-NH_2 (3a)

Immobilized pentapeptide **10** was assembled using the Fmoc strategy. The synthesis was carried out on 1.0 g Fmoc-Rink amide-Macrosorb® SPR resin. The arginine and serine residues were incorporated using Fmoc-Arg(Pmc)-OH and Fmoc-Ser-OH, respectively. The assembly of the peptide was terminated with the coupling of Boc-Lys(Boc)-OH. Condensations were performed with BOP in the presence of DIEA. The protocol for one

deprotection/coupling cycle consisted of (flow rate 3 mL/min): (1) wash, DMF, 0.5 min; (2) Fmoc-cleavage, 2% DBU/2% piperidine/DMA (v/v/v), 5 min; (3) wash, DMA, 10 min; (4) coupling, *N*-protected amino acid (3 eq.), BOP (3 eq.), DIEA in DMA, 60 min; (5) wash, DMA, 8 min.

After completion of the synthesis, peptide resin **10** was removed from the column, washed successively with DMA, *tert*-amyl alcohol, 20% AcOH/*tert*-amyl alcohol (v/v), *tert*-amyl alcohol, diethyl ether and dried *in vacuo* over P_2O_5. To a mixture of **10** and amidite **9a** (415 mg, 1.50 mmol) in CH_3CN (8 mL) was added a solution of dry 1*H*-tetrazole (53 mg, 0.75 mmol) in CH_3CN (2 mL). After shaking for 16 h, *t*-BuOOH (0.5 mL) was added and after another 1 h, the resin was filtered and washed as described before. The thus obtained anchored phosphonopeptide **11a** was deprotected and cleaved from the solid support by treatment with 95% TFA/phenol for 2 h. The mixture was filtered and the residue was washed thoroughly with TFA. The total filtrate was concentrated, redissolved in TFA (2 mL) and poured into a vigorously stirred mixture of hexane (200 mL) and diethyl ether (100 mL). After stirring for 1 h, the precipitate was collected by centrifugation (3000 rpm, 15 min), dissolved in H_2O (15 mL) and lyophilized to obtain crude phosphonopeptide amide **3a** (69 mg) as a white fluffy solid. A 20 mg aliquot of the obtained product was purified by semi-preparative RP HPLC to obtain pure **3a** (10.4 mg, 51%) according to HPLC and CZE.

CZE: t_R 14.8 min; MS (*m/z*): 368.7 $[M+2H]^{2+}$, 736.3 $[M+H]^+$; ^{31}P NMR (D_2O): δ 28.6; ^1H NMR (D_2O): δ 4.57 (t, 1H, Hα Ser, $J_{\alpha,\beta}$ 5.1 Hz), 4.4 (m, 2H, Hα Arg, Hα Leu), 4.30 (dd, 1H, Hα Arg, *J* 5.5 and 8.4 Hz), 4.1 - 4.0 (m, 3H, Hα Lys, Hβ Ser), 3.21 (t, 4H, Hδ Arg, $J_{\gamma,\delta}$ 5.8 Hz), 3.01 (t, 2H, Hε Lys, $J_{\delta,\varepsilon}$ 7.2 Hz), 2.0 - 1.6, 1.5 - 1.4 (2 × m, 15H and 2H, resp., Hβ Arg, Hγ Arg, Hβ Leu, Hγ Leu, Hβ Lys, Hγ Lys, Hδ Lys), 1.29 (d, 3H, P-CH_3, $^2J_{H,P}$ 16.5 Hz), 0.94, 0.89 (2 × d, each 2H, Hδ Leu, $J_{\gamma,\delta}$ 4.9 Hz); ^{13}C {^1H} NMR (D_2O): δ 176.5, 174.8, 173.6, 171.3, 170.2 (C=O Arg, Leu, Lys, Ser), 157.3 (Cε Arg), 63.4 (Cβ Ser), 54.4 (d, Cα Ser, $^3J_{C,P}$ 5.9 Hz), 54.1, 53.1 (Cα Arg, Cα Leu), 53.6 (Cα Arg), 53.3 (Cα Lys), 41.1 (Cδ Arg), 40.3 (Cβ Leu), 39.6 (Cε Lys), 31.0 (Cβ Lys), 28.7, 28.6 (Cβ Arg), 26.9 (Cδ Lys), 25.1 (Cγ Arg), 24.9 (Cγ Leu), 22.7, 21.4 (Cδ Leu), 21.7 (Cγ Lys), 11.6 (d, P-CH_3, $^1J_{C,P}$ 136.3 Hz).

H-Lys-Arg-Ser(OP(O)C_8H_{17}OH)-Leu-Arg-NH_2 (3b)

Octylphosphonate peptide **3b** was assembled according to the procedure described for methylphosphonate **3a** using reagent **9c** instead of **9a**. A 20 mg aliquot of the crude product (yield 82 mg) was purified by semi-preparative RP HPLC to furnish homogeneous **3b** (9.4 mg, 48%) as ascertained by HPLC and CZE.

CZE: t_R 15.9 min; MS (*m/z*): 835 $[M+H]^+$; ^{31}P NMR (D_2O): δ 30.8; ^{13}C {^1H} NMR (D_2O): δ 176.4, 174.6, 173.6, 171.5, 170.3 (C=O Arg, Leu, Lys, Ser), 157.3 (Cε Arg), 63.2 (Cβ Ser), 54.1, 54.2, 53.9, 53.7, 53.4, 53.2 (2 × Cα Arg, Cα Leu, Cα Lys, Cα Ser), 41.2 (Cδ Arg), 40.5 (Cβ Leu), 39.6 (Cε Lys), 31.9 (CH_2 octyl), 31.0 (Cβ Lys), 29.2, 28.8 (Cβ Arg), 26.9 (Cδ Lys), 25.0 (Cγ Arg), 23.4 (CH_2 octyl), 21.9 (Cγ Leu), 22.9, 21.8 (Cδ Leu), 21.6 (Cγ Lys), (CH_2 octyl), 14.2 (CH_3 octyl).

H-Lys-Arg-Ser-Leu-Arg-NH_2

Immobilized peptide **10**, prepared as described above starting from 1.0 g Fmoc-Rink amide-Macrosorb® SPR (loading 0.097 mmol/g), was treated with 95% TFA/phenol for 2 h. Work-up as described before yielded the crude pentapeptide (60 mg) as a white fluffy solid, part of which (*i.e.* 20 mg) was purified by semi-preparative HPLC to furnish homogeneous H-Lys-Arg-Ser-Leu-Arg-NH_2 (11.2 mg, 53%) according to HPLC and CZE.

CZE: t_R 11.8 min; MS (*m/z*): 329.8 $[M+2H]^{2+}$, 658.3 $[M+H]^+$; ^1H NMR (D_2O): δ 4.49 (t, 1H, Hα Ser, $J_{\alpha,\beta}$ 6.2 Hz), 4.4 (m, 2H, Hα Arg, Hα Leu), 4.30 (dd, 1H, Hα Arg, *J* 5.8 and 8.5 Hz), 4.04 (t, 1H, Hα Lys, $J_{\alpha,\beta}$ 6.6 Hz), 3.86 (d, 2H, Hβ Ser, $J_{\alpha,\beta}$ 6.3 Hz), 3.21 (t, 4H, Hδ Arg, $J_{\gamma,\delta}$ 6.8 Hz), 3.01 (t, 2H, Hε Lys, $J_{\delta,\varepsilon}$ 7.7 Hz), 2.0 - 1.6, 1.5 - 1.4 (2 × m, 15H and 2H, resp., Hβ Arg, Hγ Arg, Hβ Leu, Hγ Leu, Hβ Lys, Hγ Lys, Hδ Lys), 0.94, 0.89 (2 × d, each 2H, Hδ Leu, $J_{\gamma,\delta}$ 6.1 and 6.2 Hz, resp.); ^{13}C {^1H} NMR (D_2O): δ 176.5, 175.1, 173.7, 172.2, 171.0 (C=O Arg, Leu, Lys, Ser), 157.2 (Cε Arg), 61.5 (Cβ Ser), 55.7, 54.0, 53.6, 53.1 (Cα

Arg, Cα Leu, Cα Lys, Cα Ser), 41.1, 40.9 (Cδ Arg), 40.1 (Cβ Leu), 39.5 (Cε Lys), 31.2 (Cβ Lys), 28.7, 28.6 (Cβ Arg), 26.9 (Cδ Lys), 25.0 (Cγ Arg), 24.9 (Cγ Leu), 22.6, 21.2 (Cδ Leu), 21.7 (Cγ Lys).

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