Solid-Phase Synthesis of *O*-Phosphorothioylserine- and -threonine-Containing Peptides as Well as of *O*-Phosphoserine- and -threonine-Containing Peptides

Dries B. A. de Bont, Wilna J. Moree, Jacques H. van Boom, and Rob M. J. Liskamp*

Department of Organic Chemistry, Gorlaeus laboratories, University of Leiden, P.O. Box 9502, 2300 RA Leiden, The Netherlands

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This paper describes the synthesis of *O*-phosphorothioylserine, -threonine, and -tyrosine by sulfurization of the intermediate phosphite triester using phenylacetyl disulfide. Several deprotection procedures were investigated to find the optimal conditions necessary for obtaining the deprotected phosphorothioate amino acids. This method could then be extended to the preparation of *O*-phosphorothioylserine- and -threonine-containing pentapeptides on a solid phase. After completion of the synthesis of the peptides on the solid support, the amino acid residue containing the free hydroxyl function was phosphitylated using *N,N*-diisopropylbis(4-chlorobenzyl)phosphoramidite and subsequently sulfurized using phenylacetyl disulfide. Silylitic cleavage of the protecting groups, using thiophenol and *m*-cresol as scavengers, gave the pure deprotected phosphorothioylated peptides. The corresponding phosphopeptides were obtained when the phosphitylated peptides attached to the solid support were oxidized instead of sulfurized using *tert*-butyl hydroperoxide. To demonstrate that these methods were equally suitable for the synthesis of larger phosphopeptides or phosphorothioate peptides, we prepared the phospho- and the phosphorothioate analog of a pentadecapeptide (H-Leu-Ser-Asp-Gln-Glu-Lys-Arg-Lys-Gln-Ile-Ser-Val-Arg-Gly-Leu-OH) comprising the regulatory phosphorylation site, i.e., serine-14 of phosphorylase b. This example also illustrates that phosphorylation as well as phosphorothioylation is possible of a specified serine residue.

Introduction

Protein phosphorylation is now generally recognized as a major regulatory process mediated by protein kinases and phosphatases.[1] Serine/threonine as well as tyrosine kinases and corresponding phosphatases are involved in this process. An outstanding example that illustrates the regulatory action by phosphorylation is the conversion of inactive glycogen phosphorylase b to the enzymatically active phosphorylase a[2] by phosphorylation of serine-14. A recent intriguing example[3] is the inhibition of the potassium current in a potassium channel by direct phosphorylation of the channel protein at serine-103. Furthermore, there is evidence that protein phosphorylation is a regulatory element in carcinogenesis and that it is associated with certain diseases.[4]

Despite the importance of protein phosphorylation, the molecular basis of regulation induced by phosphorylation is virtually unknown. One has to realize that any attempt to investigate this in more detail is dependent on the availability—in sufficient quantities—of phosphopeptides and phosphoproteins. Consequently, we are engaged in the development of synthetic methods for preparation of phosphoramino acids and phosphopeptides[5-10] with the ultimate goal to investigate the influence of phosphorylation on the structure of a peptide.

To study the role of phosphatases in the process of protein phosphorylation/dephosphorylation, peptides containing a non-hydrolyzible and/or an isoelectronically modified phosphate moiety could be very useful. These peptides could act as potential selective inhibitors of phosphatases. A nice example supporting this concept is rabbit muscle phosphorylase a containing a thiophosphate on serine-14. The resulting phosphorylase a was a competitive inhibitor of phosphorylase phosphatase with a K_i of 3 μM.[11]

Phosphorothioate analogs of the phosphate moiety were originally developed for the synthesis of modified oligodesoxynucleotides.[12,13] As far as we know no attention has been paid to the introduction of a phosphorothioate moiety in a peptide by chemical synthesis.[14] The above

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cited example[11] of phosphorylase a containing a thiophosphate was prepared enzymatically[15] using a phosphorylase kinase and adenosine 5′-*O*-(3′-thiotriphosphate) (ATPγS). At present, the enzymatic introduction of a modified phosphate viz. a thiophosphate is still hampered by the limited scale on which the synthesis can be carried out and by the availability of the proper kinase. Therefore, we wish to report here the solid-phase synthesis of phosphorothioate peptides as well as phosphopeptides of which the phosphorothioate or phosphate group was introduced—on the solid phase—by *chemical* phosphorothioylation or phosphorylation, respectively.

Synthesis of phosphopeptides in solution has been applied successfully to a number of phosphopeptides,[16] but this method is rather time-consuming and only practical for synthesis of small phosphopeptides. For synthesis of phosphopeptides attached to a solid support two strategies are available: synthesis of a protected phosphoramino acid building block that can be introduced *in the course* of the solid-phase peptide synthesis, and alternatively, preparation of a peptide with an unprotected hydroxyl function, followed by phosphorylation on the solid phase. A disadvantage of the first method is that the widely used base-labile 9-fluorenylmethoxycarbonyl (Fmoc) group cannot be employed for solid-phase synthesis[17] of peptides containing a phosphorylated serine or threonine residue.[18] Furthermore, excess of the relatively difficultly accessible phosphorylated amino acid is necessary when introduced in course of the synthesis of the peptide attached to a solid support.[19]

We[10] showed that these disadvantages can be easily circumvented when phosphorylation is carried out *after* completion of the solid-phase synthesis using the phosphoramidite method. This strategy was then readily adopted by others.[19i,k,20,21] Phosphorylation on the solid phase using the phosphite method is clearly the method of choice since the phosphate triester method using, e.g., dibenzylphosphochloridate led to incomplete phosphorylation and the formation of an unknown byproduct.[22]

Using the phosphite method, phosphopeptides and -amino acids were synthesized by oxidation (i.e., with *tert*-butyl hydroperoxide or 3-chloroperoxybenzoic acid) of the intermediate phosphite triester, whereas for the synthesis of phosphorothioate analogs of peptides and amino acids, the intermediate phosphite triester was sulfurized using phenylacetyl disulfide[13a,b] via the Schönberg reaction.[23] This method is superior[13a,b] to sulfurization of phosphite esters using elemental sulfur (S_8)[13] because of its insolubility in most organic solvents.

In this paper we describe the synthesis of phosphorothioate amino acid derivatives of serine, threonine, and tyrosine. Furthermore, the solid-phase synthesis of phosphopeptides and phosphorothioate peptides is reported. After completion of the solid-phase synthesis of the peptides the phosphate or the phosphorothioate moiety can be introduced onto the peptides—still attached to the resin—employing the phosphite method using *N,N*-diisopropylbis(4-chlorobenzyl)phosphoramidite[9] followed by oxidation using *tert*-butyl hydroperoxide or sulfurization using phenylacetyl disulfide.[13a,b] To demonstrate that the methods presented in this paper are expandable to the preparation of both larger phosphorothioate peptides and phosphopeptides we have synthesized a phospho as well as a phosphorothioate analog of a pentadecapeptide (H-Leu-Ser-Asp-Gln-Glu-Lys-Arg-Lys-Gln-Ile-Ser-Val-Arg-Gly-Leu-OH) comprising the regulatory phosphorylation site, i.e., serine-14 of phosphorylase b[2]. This example also illustrates that phosphorylation as well as phosphorothioylation is possible of a specified serine residue.

Results and Discussion

The synthesis of phosphorothioate amino acids is shown in Scheme I. Phosphitylation was accomplished by adding 1.5 equiv of 1*H*-tetrazole to a mixture of the amino acid derivative **1a**, **1b**, or **1c** and 1.25 equiv of *N,N*-diisopropylbis(4-chlorobenzyl)phosphoramidite **2**[9]. The intermediate phosphite triester was not isolated but directly sulfurized using 2 equiv of phenylacetyl disulfide.[13a,b] After workup and purification the protected phosphorothioate amino acid derivatives **3a–c** were obtained in 77%–89% yield.

In order to evaluate which deprotection conditions are suitable for preparation of phosphorothioate amino acids and phosphorothioate peptides (vide infra), the model

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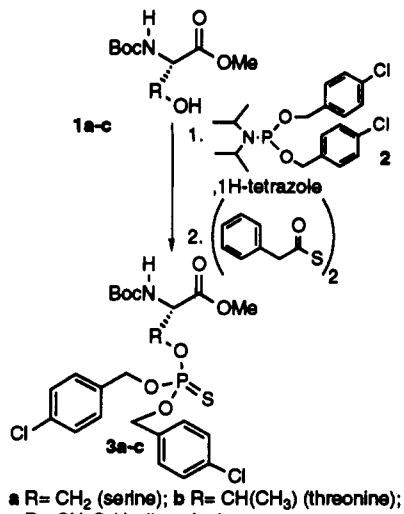
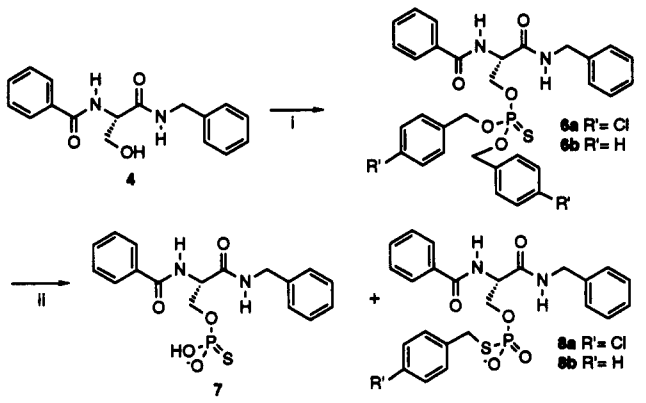
Scheme I



Scheme II[a]



[a] Key: (i) (a) 2, 1*H*-tetrazole or *N,N*-diisopropyldibenzylphosphoramidite (5), 1*H*-tetrazole; (b) phenylacetyl disulfide; (ii) deprotection using (A) TMSBr, *m*-cresol, thiophenol, TFA or (B) TMSBr, *m*-cresol, thioanisol, TFA or (C) TFMSA, *m*-cresol, thiophenol, TFA.

Table I. Deprotection of 6a and 6b

	method	deprotection of 6a ratio of 7 to 8a	deprotection of 6b ratio of 7 to 8b
TMSBr/TFA/ *m*-cresol/thioanisol	A	1:1.8[a]	1:0.7[b]
TMSBr/TFA/ *m*-cresol/thiophenol	B	1:0.0[a]	1:0.1[b]
TFMSA/TFA/ *m*-cresol/thiophenol	C	1:0.3[b]	1:0.7[b]
TMSBr/TFA/ *m*-cresol/ethane thiol		*c*	*d*

[a] Determined after isolation. [b] Determined by ^{31}P NMR. [c] Neither 7 nor 8a could be isolated. [d] Experiment not carried out.

compounds 4-chlorobenzyl-protected phosphorothioylserine derivative **6a** and benzyl-protected phosphorothioylserine derivative **6b** were chosen. Phosphitylation of *N*-benzoylserine benzylamide (**4**) was achieved by reaction with **2** or with *N,N*-diisopropyldibenzylphosphoramidite[16c] (**5**) both in the presence of 1*H*-tetrazole as depicted in Scheme II. The resulting phosphite triesters were immediately converted to, respectively, phosphorothioylserine derivative **6a** and **6b** using phenylacetyl disulfide.

However, when **6a** and **6b** were deprotected under conditions (data not shown in Table I) which were used earlier[6,10] with success both in the solution-phase and the solid-phase synthesis of phosphopeptides, i.e., a mixture of TFMSA and TFA with *m*-cresol and thioanisol as scavengers, the corresponding deprotected phosphorothioate analog **7** could not be isolated. Because we hoped that silylation[16k] of the phosphorothioate moiety would diminish side reactions, we tried TMSBr[24] (method A, Table I) instead of TFMSA. Using this method we were able to isolate a small amount of the desired compound **7** as well as a larger amount of a byproduct, i.e., **8a** or **8b**. Interestingly, more byproduct (**8a**) was formed after deprotection of the phosphorothioate containing a 4-chlorobenzyl group (**6a**) as compared to formation of the byproduct **8b** in the deprotection of the phosphorothioate containing a benzyl group (**6b**). The formation of the byproduct gave us a clue as to what was at least partly responsible for the low yield: apparently, after formation of the 4-chlorobenzyl carbocation in the deprotection, the sulfur of the phosphorothioate acts as an efficient, probably intramolecular, scavenger. This situation could be remedied by adding thiophenol instead of thioanisol as a scavenger (method B, Table I) to circumvent this unwanted side reaction. This completely abolished the formation of byproduct **8a** and greatly reduced the formation of **8b** (ratio **7**/**8b** 1/0.1). Surprisingly, replacing thiophenol by another thiol viz. ethanethiol reduced the yield to almost zero of both **7** and the unwanted product **8a**. Having uncovered the favorable effect of thiophenol on the deprotection of **6a** and **6b** leading to the phosphorothioate **7**, we went back to the original strong acid used in the deprotection in the preparation of phosphopeptides viz. TFMSA (method C, Table I). However, using this acid in the presence of *m*-cresol and thiophenol as scavengers still gave a significant amount of the unwanted product **8a** or **8b**. Thus, thiophenol as well as TMSBr are essential for the best results of the deprotection.

In conclusion, these experiments with model compounds **6a** and **6b**, which are summarized in Table I, show that the use of a 4-chlorobenzyl-protected phosphorothioate moiety in combination with deprotection with TMSBr, thiophenol, and *m*-cresol in TFA (method B) is clearly the method of choice for preparation of deprotected phosphorothioate amino acid derivatives. In our opinion it was therefore justified to use this protocol for the preparation of phosphorothioate *peptides*. This was applied first to the solid-phase synthesis of the phosphorothioate peptide **13a** which comprises the phosphorylation site of the EGF receptor.[25] Phosphorothioate peptide **13c** was synthesized to demonstrate that the method is equally well suitable for preparation of *O*-phosphorothioate-containing serine peptides. The corresponding *O*-phosphothreonine- and *O*-phosphoserine-containing peptides, **13b** and **13d**, respectively, were prepared as described before.[26]

The synthesis of **13a–d** commenced with the synthesis of **11a** and **11b** (Scheme III) having a unprotected hydroxyl function of the threonine and serine residue, respectively.

Aminolysis with ethylenediamine[17b] of the commercial available Kieselguhr dimethylacrylamide resin[27] and sub-

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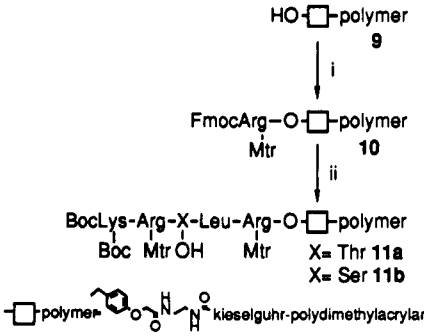
(26) The synthesis of these peptides, albeit without full experimental details, was reported in an earlier communication, see ref 10.

(27) Pepsin K resin (0.1 mequiv/g) obtained from Milligen/Biosearch (Millipore) was used.

Scheme III[a]



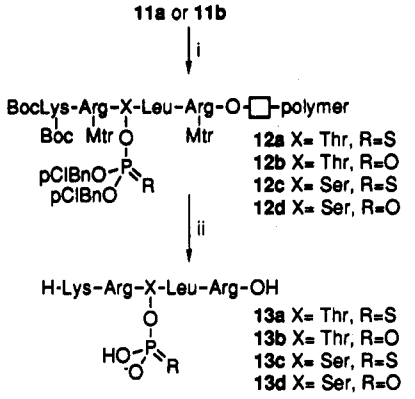
[a] Key: (i) FmocArg(Mtr)OBt, HOBt, DMAP; (ii) (a) 20% piperidine in DMA, (b) FmocLeuOPfp, HOBt; (iii) (a) 20% piperidine in DMA, (b) FmocX(OH)OPf, HOBt; (iv) (a) 20% piperidine in DMA, (b) FmocArg(Mtr)OPfp, HOBt; (v) (a) 20% piperidine in DMA, (b) BocLys(Boc)OPfp, HOBt.

sequent introduction of 4-(hydroxymethyl)phenoxyacetic acid[17b] (Wang linker[28]) afforded resin **9**. The first amino acid was introduced to the solid support as its hydroxybenzotriazole ester[29] analogous to van Nispen et al.[30] to yield **10**. Coupling and deprotection cycles were carried out on an automatic peptide synthesizer using standard procedures.[17b] After each coupling cycle the Fmoc moiety was cleaved using a 20% piperidine solution in DMA. As solvent for the elongation reactions dry DMA was used. The appropriate amino acids were introduced as their pentafluorophenyl esters[31] in the presence of 1-hydroxybenzotriazole. During the last cycle a Boc group was used as an α-amino protecting group instead of the α-amino Fmoc derivative, thus avoiding one additional step otherwise necessary for removal of the N-terminal Fmoc group in the final deprotection and cleavage from the resin of the peptide.[19i,k] As is known from the literature[32] and our own work[10] serine and threonine can be introduced during solid-phase synthesis with an unprotected hydroxyl function. As a consequence, the phosphate or phosphorothioate moiety can be introduced to the immobilized pentapeptides **11a** and **11b** immediately after the last coupling cycle as is depicted in Scheme IV.

Reaction of the resin **11a** or **11b** with *N,N*-diisopropylbis(4-chlorobenzyl)phosphoramidite (**2**) in the presence of 1*H*-tetrazole afforded the corresponding phosphite triester, which was immediately sulfurized using phenylacetyl disulfide. After treatment of resin **12a** and **12c** using method B (TMSBr, *m*-cresol, thiophenol, and TFA, Table I, vide supra) to achieve deprotection and simultaneous cleavage from the resin, the fully deprotected pure phosphorothioate peptides **13a** and **13c** were obtained after Sephadex LH-20 gel filtration in 88% and 87% yield, respectively (Scheme IV).

The synthesis of phosphopeptides **13b** and **13d**[26] is also shown in Scheme IV. Phosphitylation of resin **11a** or **11b** was carried out in the same way as described above in the synthesis of **13a** and **13c**. The resulting phosphite triester was oxidized on the solid support using a 80% solution of *tert*-butyl hydroperoxide thus affording resin **12b** and **12d**

Scheme IV[a]



[a] Key: (i) (a) **2**, 1*H*-tetrazole, (b) phenylacetyl disulfide affording **12a** and **12c** or *tert*-butyl hydroperoxide affording **12b** and **12d**; (ii) TMSBr, *m*-cresol, thiophenol, TFA (**12a** and **12c**) or TFMSA, *m*-cresol, thioanisol, TFA (**12b** and **12d**).

containing the fully protected phosphopeptides. After treatment of the resin, using the original procedure (TFMSA, *m*-cresol, thioanisol, TFA) for deprotection and simultaneous cleavage of phosphopeptides,[10] phosphopeptides **13b** and **13d** were obtained in 75% and 84% yield, respectively.

The purity of the phosphorothioate peptides **13a** and **13c** as well as the phosphopeptides **13b** and **13d** was checked with analytical HPLC and capillary zone electrophoresis (CZE). Using HPLC as well as CZE a difference in retention time was observed between the phosphorothioate peptides and the corresponding phosphopeptides (Δt_R between **13a** and **13b** 0.2 min (HPLC), 0.3 min (CZE), Δt_R between **13c** and **13d** 0.2 min (HPLC and CZE)).

As a second application to demonstrate that the above described methodology is equally suitable for the synthesis of longer phosphorothioate and phosphopeptides we prepared **18** and **19** (Scheme VI) which are part of the subunit interface around the phosphorylated serine-14 residue present in phosphorylase a.[2,33] As is shown in Scheme V, synthesis of **14** containing the resin attached peptide with an unprotected serine hydroxyl function in position 11 is relatively straightforward. Coupling of the first amino acid to the resin was carried out as described above for **10** using leucine instead of arginine. Extension, leading to **14**, was carried out as described for the synthesis of **11a** and **11b** (vide supra). In contrast to the preparation of **11a** and **11b**, the appropriate amino acids were activated in situ using (benzotriazol-1-yloxy)tris(dimethylamino)-phosphonium hexafluorophosphate (BOP)[34] except for the serine in the position 11 which was introduced as its pentafluorophenyl ester as described for the synthesis of **13a–d** (vide supra). The serine residue in position 11 was introduced with the hydroxyl function unprotected, whereas the serine in position 2 was introduced with its hydroxyl function protected as a *tert*-butyl ether.

As a consequence, the immobilized peptide **14** could be phosphitylated followed by oxidation or sulfurization *specifically* in position 11 using the above-described methodology for the preparation of **13a–d**. In this way resins **16** and **17** containing the phosphorothioate peptides and phosphopeptides were prepared. Deprotection and

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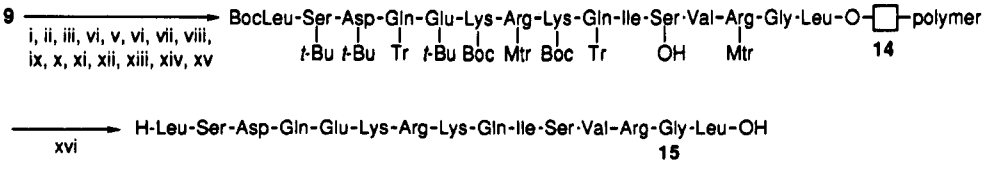
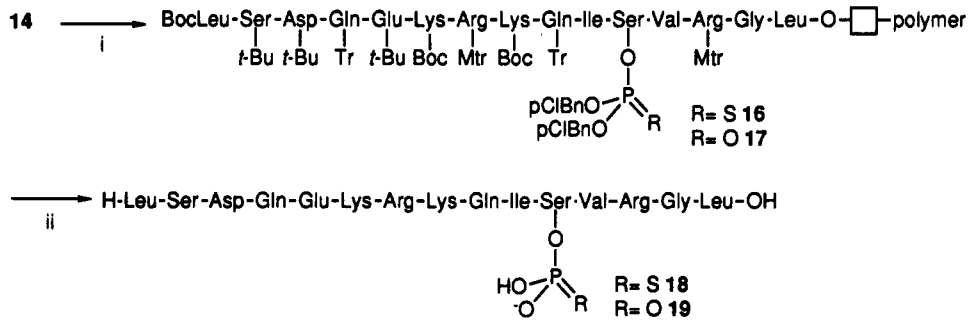
Scheme V[a]



[a] Key: (i) FmocLeuOBt, HOBt, DMAP; (ii) (a) 20% piperidine in DMA, (b) FmocGly, BOP, NMM; (iii) (a) 20% piperidine in DMA, (b) FmocArg(Mtr), BOP, NMM; (iv) (a) 20% piperidine in DMA, (b) FmocVal, BOP, NMM; (v) (a) 20% piperidine in DMA, (b) FmocSer(OH)OPFp, HOBt; (vi) (a) 20% piperidine in DMA, (b) FmocIle, BOP, NMM; (vii) (a) 20% piperidine in DMA, (b) FmocGln(Tr), BOP, NMM; (viii) (a) 20% piperidine in DMA, (b) FmocLys(Boc), BOP, NMM; (ix) (a) 20% piperidine in DMA, (b) FmocArg(Mtr), BOP, NMM; (x) (a) 20% piperidine in DMA, (b) FmocLys(Boc), BOP, NMM; (xi) 20% piperidine in DMA, (b) FmocGlu(*t*-Bu), BOP, NMM; (xii) (a) 20% piperidine in DMA, (b) FmocGln(Tr), BOP, NMM; (xiii) (a) 20% piperidine in DMA, (b) FmocAsp(*t*-Bu), BOP, NMM; (xiv) (a) 20% piperidine in DMA, (b) FmocSer(*t*-Bu), BOP, NMM; (xv) (a) 20% piperidine in DMA, (b) BocLeu, BOP, NMM; (xvi) TMSBr/*m*-cresol/thiophenol/TFA.

Scheme VI[a]



[a] Key: (i) (a) **2**, 1*H*-tetrazole, (b) phenylacetyl disulfide affording **16** or *tert*-butyl hydroperoxide affording **17**; (ii) TMSBr/*m*-cresol/thiophenol/TFA.

cleavage from the resin using TMSBr in TFA in the presence of thiophenol and *m*-cresol as scavengers (method B, Table I, vide supra) gave the phosphorothioate peptide **18** and phosphopeptide **19**. The nonphosphorylated peptide **15**, serving as a control, was obtained by direct cleavage (method B) or resin **14**.

Although **15**, **18** and **19** each display characteristic signals in their NMR-spectra, HPLC and CZE retention times are practically identical.

In conclusion, we have developed methods for the synthesis of *O*-phosphonothioylserine- and *O*-phosphoserine-containing peptides as well as *O*-phosphonothioylthreonine- and *O*-phosphothreonine-containing peptides. The methodology features the phosphitylation of peptides using *N*,*N*-diisopropylbis(4-chlorobenzyl)phosphoramidite in the presence of 1*H*-tetrazole, while they are still attached to the solid phase which was used for their synthesis. This was immediately followed by oxidation using *tert*-butyl hydroperoxide or by sulfurization using phenylacetyl disulfide. By carefully studying the deprotection of a model phosphorothioate amino acid derivative, we were able to establish the optimal deprotection conditions in order to obtain pure phosphorothioate peptides. We were able to apply this methodology to the synthesis of a pentadecaphosphorothioate peptide and a pentadecaphosphopeptide, indicating that large phosphorothioate and phosphopeptides are in principle accessible via this method. Moreover, this example also shows that by leaving the hydroxyl function of the hydroxyl amino acid to be phosphorylated unprotected, it is possible to introduce a phosphorothioate or phosphate moiety attached to a specified hydroxyl amino acid residue (serine 11, serine 2 is not phosphorylated or phosphorothioylated).

Since peptides containing a modified phosphate viz. a phosphorothioate could act as potential selective inhibitors of phosphatases and therefore might be useful for studying the role of phosphatases in the process of protein phosphorylation/dephosphorylation, we hope that we have supplied the basic tools necessary for undertaking such studies.

Experimental Section

^1H and ^{13}C spectra (ppm) were measured on a 200-MHz spectrometer unless stated otherwise. The ^{13}C spectra were monitored using the attached proton test (APT) technique.[35] $CDCl_3$ was used as the solvent unless stated otherwise. The numbering of the carbon atoms of the amino acids is according to IUPAC recommendations.[36] 2D ^1H–^1H COSY and ^{13}C–^1H COSY NMR spectra were recorded at 300 K on a 300-MHz or a 400-MHz apparatus. Peptide synthesis was performed on continuous flow peptide synthesizer using the Fmoc methodology[17a,b] with as side-chain protection the 4-methoxy-2,3,6-trimethylbenzene sulfonyl (Mtr) protecting group[37] for the guanidinium side chain of arginine. The *tert*-butyl group was used for the protection of the hydroxyl function of serine and the acid function of aspartic acid, the ϵ-amino group of lysine was protected with a *tert*-butyloxycarbonyl (Boc) group, and the trityl (Tr) was used as a protective group of the amide moiety of glutamine.

Unless otherwise noted, chemicals were obtained from commercial sources and used without further purification. DMA was distilled from calcium hydride and stored over activated molecular sieves (4 Å) prior to use. Acetonitrile, CH_2Cl_2, and *N*-methylmorpholine (NMM) were distilled from CaH_2, dioxane from $LiAlH_4$, and piperidine from KOH. Thioanisol, *m*-cresol, TFMSA, TFA, and TMSBr were distilled before use. Thin-layer chromatography (TLC) was carried out on Merck precoated silica gel F 254 plates (0.25 mm). Spots were visualized with an

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UV lamp and an aqueous 10% H_2SO_4 solution (1 L) containing ammonium molybdate (25 g) and ceric ammonium sulfate (10 g) followed by charring. For chromatography Merck silica 60 (70–230 mesh) was used.

Capillary zone electrophoresis (CZE) was carried out using a capillary column (length 72 cm, length to detector 50 cm, diameter 50 μm) at 30 °C. A citrate buffer pH = 2.5 (20 mM) was used. For detection a variable-wavelength detector was used operating at 200 nm.

HPLC equipment with an analytical reversed-phase (C^{18}) column (Lichrospher, 250 mm × 4 mm, 5 μm), an UV detector operating at 206 nm, at a flow rate of 1 mL min^{-1} and at a temperature of 40 °C, was used with an appropriate gradient from 0.1% TFA in water to 0.1% TFA in acetonitrile. Preparative fast protein liquid chromatography (FPLC) was carried out using a Pro HR16/10 reversed-phase column equipped with a variable-wavelength detector and using an appropriate gradient from 0.1% TFA in water to 0.1% TFA in acetonitrile/water (2/1, v/v) at rt.

***N,N*-Diisopropylbis(4-chlorobenzyl)phosphoramidite (2).** 2 was synthesized as described before.[9] ^{13}C NMR: 24.5 (d, $CHCH_3$, J_{PC} = 7.3 Hz), 43.0 (d, $CHCH_3$, J_{PC} = 13.2 Hz), 64.7 (d, CH_2Ph, J_{PC} = 17.6 Hz), 128.2, 128.3, 132.9 and 137.8 (Ph). ^{31}P NMR: 148.7. ^1H NMR: 1.19 (d, 12 H, $CHCH_3$, J = 7.3 Hz), 3.66 (m, 2 H, $CHCH_3$), 4.62 (dd, 2 H, CH_aPh, J_{gem} = 12.6 Hz, J_{PH} = 8.7 Hz), 4.71 (dd, 2 H, CH_bPh, J_{gem} = 12.6 Hz, J_{PH} = 8.3 Hz) and 7.25 (s, 8 H, Ph).

Boc-*O*-[bis(4-chlorobenzyl)phosphonothioyl]serine Methyl Ester (3a). 0.60 g of **1a** (2 mmol) and 1.04 g of **2**[9] (2.5 mmol) were dissolved in dry dioxane and evaporated. This was repeated once. Subsequently, a 0.5 M solution of 1*H*-tetrazole in dry acetonitrile (6 mL, 3 mmol) was added and the reaction stirred for 1 h at rt under an argon atmosphere. After completion of the reaction, as was monitored by TLC, 1.60 g of phenylacetyl disulfide[13a,b] (5 mmol) was added, and the mixture was stirred for 0.5 h. Workup was carried out as follows: The solvent was evaporated, and the residue was dissolved in butanone, washed with water and brine, and dried (Na_2SO_4). Purification by silica gel chromatography (eluent: gradient of 0% ether in petroleum ether 40/60 (petroleum ether) to 30% ether in petroleum ether) afforded **3a** in 77% yield (0.84 g) R_f (ether/petroleum ether, 1/1, v/v) = 0.40. ^{13}C NMR: 27.9 (CH_3 Boc), 52.3 (Me), 53.4 (d, C^2, J_{PC} = 7.3 Hz), 67.5 (C^3), 68.7 (d, CH_2Ph, J_{PC} = 5.9 Hz), 79.8 ($C(CH_3)$ Boc), 128.4, 129.1, 133.7, 133.8 and 134.0 (Ph), 154.7 (C(O) Boc) and 169.3 (C^1). ^{31}P NMR: 69.4. ^1H NMR: 1.44 (s, 9 H, CH_3 Boc), 3.72 (s, 3 H, Me), 4.25–4.48 (m, 3 H, C^2H and C^3H_2), 4.97 (d, 2 H, CH_aPh, J_{gem} = 10.8 Hz), 5.02 (d, 2 H, CH_bPh, J_{gem} = 10.8 Hz), 5.24 (bs, 1 H, N(H)), 7.23 and 7.32 (two d, 8 H, pH, J_{AB} = 8.7 Hz).

Boc-*O*-[bis(4-chlorobenzyl)phosphorothioyl]threonine Methyl Ester (3b). **3b** was synthesized as described above for the preparation of **3a**. Yield: 84% (0.94 g). R_f (ether/petroleum ether, 1/1, v/v) = 0.52. ^{13}C NMR: 18.0 (C^4), 27.9 (CH_3 Boc), 52.1 (Me), 57.4 (d, C^2, J_{PC} = 7.3 Hz), 68.6 (d, CH_2Ph, J = 5.9 Hz), 76.0 (d, C^3, J_{PC} = 5.9 Hz), 79.7 ($C(CH_3)$ Boc), 128.3, 129.0, 133.7 and 133.9 (Ph), 155.3 (C(O) Boc) and 169.8 (C^1). ^{31}P NMR: 68.4. ^1H NMR: 1.34 (d, 3 H, C^4H_3, J = 6.4 Hz), 1.46 (s, 9 H, CH_3 Boc), 3.70 (s, 3 H, Me), 4.40 (d, 1 H, N(H), J = 9.5 Hz), 4.84–5.17 (m, 6 H, C^2H, C^3H and CH_2Ph), 7.23 and 7.31 (two d, 8 H, Ph, J_{AB} = 8.5 Hz).

Boc-*O*-[bis(4-chlorobenzyl)phosphorothioyl]tyrosine Methyl Ester (3c). **3c** was prepared as described above for **3a**. Purification by silica gel chromatography (eluent: gradient of 0% ether in petroleum ether to 50% ether in petroleum ether) afforded **3c** in 89% yield (1.13 g). R_f (ether/petroleum ether, 1/1, v/v) = 0.33. ^{13}C NMR: 27.8 (CH_3 Boc), 37.1 (C^3), 51.6 (Me), 54.0 (C^2), 68.9 (d, CH_2Ph, J_{PC} = 5.9 Hz), 79.2 ($C(CH_3)$ Boc), 120.4 (d, C^6, J_{PC} = 4.4 Hz), 128.2, 129.0, 129.9, 133.1, 133.5 and 133.8 (Ph), 148.9 (d, C^7, J_{PC} = 7.3 Hz), 154.5 (C(O) Boc) and 171.6 (C^1). ^{31}P NMR: 64.3. ^1H NMR: 1.41 (s, 9 H, CH_3 Boc), 3.04 (m, 2 H, C^3), 3.70 (s, 3 H, Me), 4.05 (bs, 1 H, N(H)), 4.90 (m, 1 H, C^2H), 5.09 (d, 4 H, CH_2Ph, J_{gem} = 10.8 Hz), 7.00 and 7.07 (two d, 4 H, C^5H and C^6H, J_{AB} = 8.7 Hz), 7.24 and 7.29 (two d, 8 H, Ph, J_{AB} = 8.5 Hz).

***N*-Benzoylserine Benzylamide (4).** 4 was prepared according to standard procedures.[38] R_f (CH_2Cl_2/MeOH, 95/5, v/v) = 0.20. ^{13}C NMR ($CDCl_3/CD_3OD$, 1/1, v/v): 42.9 (NCH_2Ph), 55.1 (C^2), 61.9 (C^3), 126.8, 126.9, 128.0, 131.5, 132.9 and 137.4 (Ph), 168.0 and 170.4 (C(O)). ^1H NMR ($CDCl_3/CD_3OD$, 9/1, v/v): 3.78 (dd, 1 H, C^3H_a, J_{vic} = 6.2 Hz, J_{gem} = 11.3 Hz), 3.99 (dd, 1 H, C^3H_b, J_{vic} = 4.7 Hz, J_{gem} = 11.2 Hz), 4.46 (s, 2 H, NCH_2Ph), 4.65 (t, 1 H, C^2H, J = 5.4 Hz), 7.25–7.87 (m, 10 H, Ph).

***N,N*-Diisopropyldibenzylphosphoramidite (5).** 5 was synthesized as described before[9] using benzyl alcohol instead of 4-chlorobenzyl alcohol. ^1H NMR data were in agreement with the published data.[16c]

***N*-Benzoyl-*O*-[bis(4-chlorobenzyl)phosphorothioyl]serine Benzylamide (6a).** 0.60 g of 4 (2.mmol) and 1.04 g of **2** (2.5 mmol) were dissolved in dry dioxane and the solvent evaporated. This was repeated once. After the mixture was redissolved in 20 mL dry dioxane, 210 mg of 1*H*-tetrazole (3 mmol) was added and the reaction stirred for 1.5 h at rt under an argon atmosphere. After completion (TLC) of the reaction, 1.60 g of phenylacetyl disulfide (5 mmol) was added and the mixture was stirred for another hour. The solvent was then evaporated, and the residue was redissolved in EtOAc, washed with water and brine, and dried (Na_2SO_4). Purification by silica gel chromatography (eluent: gradient of petroleum ether to 55% EtOAc in petroleum ether) afforded **6a** in 83% yield (1.07 g). R_f (CH_2Cl_2/MeOH, 95/5, v/v) = 0.53. ^{13}C NMR: 43.1 (NCH_2Ph), 52.9 (d, C^2, J_{PC} = 8 Hz), 67.2 (C^3), 68.6 (OCH_2Ph), 126.9, 127.1, 128.0, 128.2, 128.9, 131.5, 132.6, 133.5, 133.7, 133.8 and 137.3 (Ph), 167.2 and 168.4 (C(O)). ^{31}P NMR: 69.5. ^1H NMR: 4.34 (six lines, 1 H, C^3H_a, J_{vic} = 5.2 Hz, J_{gem} = 10.3 Hz, J_{PH} = 10.3 Hz), 4.43 (d, 2 H, NCH_2Ph, J = 5.9 Hz), 4.59 (six lines, 1 H, C^3H_b, J_{vic} = 5.2 Hz, J_{gem} = 10 Hz, J_{PH} = 10 Hz), 4.94 (d, 2 H, OCH_aPh, J_{gem} = 10.4 Hz), 4.95 (m, 1 H, C^2H), 4.97 (d, 2 H, OCH_bPh, J_{gem} = 10.4 Hz), 6.90 (bs, 1 H, N(*H*)Bn), 7.12–7.77 (m, 18 H, Ph).

***N*-Benzoyl-*O*-(dibenzylphosphorothioyl)serine Benzylamide (6b).** The same procedure was used as described above for the synthesis of **6a** using 0.77 g of 4 (2.6 mmol), 1.12 g of **5** (3.25 mmol), 0.27 g of 1*H*-tetrazole (3.9 mmol), and 1.97 g of phenylacetyl disulfide (6.5 mmol). Purification by silica gel chromatography (eluent: gradient of petroleum ether to ether) afforded **6b** in 85% yield (0.97 g). R_f (CH_2Cl_2/MeOH, 95/5, v/v) = 0.60. ^{13}C NMR: 43.2 (NCH_2Ph), 53.1 (d, C^2, J_{PC} = 7.3 Hz), 67.0 (C^3), 69.5 (OCH_2Ph), 126.6, 127.0, 127.2, 127.7, 128.1, 128.2, 129.0, 131.5, 132.7, 135.2 and 137.3 (Ph), 167.3, 168.5 (C(O)). ^{31}P NMR: 69.1. ^1H NMR: 4.26 (six lines, 1 H, C^3H_a, J_{vic} = 5.2 Hz, J_{gem} = 10.1 Hz, J_{PH} = 10.4 Hz), 4.42 (d, 2 H, NCH_2Ph, J = 5.6 Hz), 4.60 (six lines, 1 H, C^3H_b, J_{vic} = 5.1 Hz, J_{gem} = 10.1 Hz, J_{PH} = 9.7 Hz), 4.81 (q, 1 H, C^2H, J_{C^3Ha} = 5.2 Hz, J_{C^3Hb} = 5.2 Hz, $J_{N(H)}$ = 5.2 Hz), 5.00 (dd, 2 H, OCH_aPh, J_{gem} = 9.6 Hz, J_{PH} = 1.3 Hz), 5.03 (d, 2 H, OCH_bPh, J_{gem} = 9.6 Hz), 6.88 (bs, 1 H, N(*H*)Bn), 7.26–7.83 (m, 21 H, Ph and N(H)).

***N*-Benzoyl-*O*-phosphorothioylserine Benzylamide (7), *N*-Benzoyl-*O*-[[(4-chlorobenzyl)thio]phospho]serine Benzylamide (8a), and *N*-Benzoyl-*O*-[(benzylthio)phospho]serine Benzylamide (8b). Method A (TMSBr/TFA/*m*-Cresol/Thioanisol).** A cooled (0 °C) mixture of 1.3 mL of TMSBr (10 mmol), 1.2 mL of thioanisol (10 mmol), and 1 mL of *m*-cresol (10 mmol) in 6.7 mL of TFA was added to 0.32 g of **6a** (0.5 mmol). The reaction mixture was kept under argon at 0 °C. After being stirred for 1 h, the mixture was poured into 300 mL of hexanes and stirred for another hour. Subsequently, the solvent was decanted, the residue dissolved in a mixture of MeOH/H_2O (1/1, v/v) and neutralized with NH_4HCO_3, and the solvent evaporated. After purification using a Sephadex LH-20 gel filtration column (eluent MeOH/H_2O, 85/15, v/v) compounds **7** (yield 23%, 45 mg) and **8a** (yield 41%, 105 mg) were obtained, both pure according to analytical HPLC.

Treatment of **6b**, as described for **6a**, gave after workup a mixture of **7** and **8b** in a ratio of 1:0.7 (based on ^{31}P NMR).

Method B (TMSBr/TFA/*m*-Cresol/Thiophenol). **6a** or **6b** was treated as was described for method A, except that 1 mL of thiophenol (10 mmol) was used instead of thioanisol. **6a** gave **7**

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after purification using a Sephadex LH-20 gel filtration column (eluent MeOH/H_2O, 85/15, v/v) in a yield of 88% (183 mg) which was pure according to analytical HPLC.

After workup **6b** gave a mixture of **7** and **8b** in a molar ratio of 1:0.1 (based on ^{31}P NMR).

Method C (TFMSA/TFA/*m*-Cresol/Thiophenol). A cooled (0 °C) mixture of 0.9 mL of TFMSA (10 mmol), 1.1 mL of *m*-cresol (10 mmol), and 1.1 mL of thiophenol (10 mmol) in 7 mL of TFA was added to 0.32 g of **6a** (0.5 mmol) or 0.31 g of **6b** (0.5 mmol). After workup, **6a** gave a mixture of **7** and **8a** in a molar ratio of 1:0.3 (based on ^{31}P NMR), whereas **6b** gave a mixture of **7** and **8b** in a molar ratio of 1:0.7 (based on ^{31}P NMR).

7. ^{13}C NMR (CD_3OD/D_2O, 1/1, v/v): 44.0 (NCH_2Ph), 57.7 (d, C^2, J_{PC} = 5.9 Hz), 65.0 (d, C^3, J_{PC} = 2.9 Hz), 128.0, 128.2, 128.8, 129.5, 133.0, 134.4, and 139.4 (Ph), 170.4 and 173.0 (C(O)). ^{31}P NMR (CD_3OD/D_2O, 1/1, v/v): 47.0; ^1H NMR (300 MHz) (D_2O): 4.36 (d, 1 H, NCH_aPh, J_{gem} = 15.4 Hz), 4.37 (m, 2 H, C^3H_2), 4.49 (d, 1 H, NCH_bPh, J_{gem} = 15.4 Hz), 4.63 (dd, 1 H, C^2H, J = 3.74 and 5.25 Hz), 7.16–8.04 (m, 10 H, Ph). FAB Mass $(M - H)^-$ = 393.

8a. ^{13}C NMR (CD_3OD/D_2O): 34.8 (d, SCH_2Ph, J_{PC} = 2.9 Hz), 44.1 (NCH_2Ph), 56.2 (d, C^2, J_{PC} = 7.3 Hz), 66.0 (d, C^3, J_{PC} = 5.9 Hz), 128.2, 128.5, 129.4, 129.5, 129.7, 131.4, 133.3 and 139.1 (Ph), 171.8 (C(O)). ^{31}P NMR (CD_3OD/D_2O): 21.3. ^1H NMR (300 MHz) (D_2O): 3.89 (d, SCH_2Ph, J = 10.9 Hz), 4.18 (dd, 2 H, C^3H_2, J = 4.9 and 9.5 Hz), 4.38 (d, 1 H, NCH_aPh, J_{gem} = 15.2 Hz), 4.45 (d, 1 H, NCH_bPh, J_{gem} = 15.2 Hz), 4.71 (m, 1 H, C^2H), 7.17–7.95 (m, 14 H, Ph). FAB Mass $(M - H)^-$ = 517.

H-Lys-Arg-(*O*-phosphorothioyl)Thr-Leu-Arg-OH (13a). For coupling of the first amino acid to the resin, 340 mg of Fmoc-Arg(Mtr)-OH (0.44 mmol) and 120 mg of 1-hydroxybenzotriazole (0.88 mmol) were dissolved in DMA (2 mL). After the mixture was cooled to 0 °C 82 mg of dicyclohexylcarbodiimide (0.40 mmol) was added and the mixture was stirred for 2.5 h at 0 °C. The thus formed hydroxybenzotriazole ester of FmocArg(Mtr)-OH was filtered—to remove dicyclohexylurea—in a flask containing 1.0 g of resin **9** (0.1 mmol g^{-1}) and 50 mg of DMAP (0.40 mmol). After the reaction flask was gently rotated overnight at 4 °C, the reaction mixture was filtered and the resin was washed three times with DMA, *tert*-amyl alcohol, and ether, respectively. The loading was estimated by taking a aliquot of the resin (10 mg), cleaving the Fmoc group using 0.5 mL of 20% piperidine in DMA, diluting the mixture with MeOH to 5 mL, and measuring the absorption at 300 nm. The loading per gram of resin **10** was 73 μmol of FmocArg(Mtr). Synthesis of pentapeptide **11a** immobilized on the solid support was carried out using standard procedures. Deprotection of the α-NH_2 group was carried out using 20% piperidine in DMA. After the resin was washed with DMA, 4 equiv of the appropriate amino acid as its pentafluorophenyl ester in the presence of 4 equiv of 1-hydroxybenzotriazole (39 mg, 0.29 mmol) was dissolved in DMA (1.2 mL) and circulated over the resin for 1 h. Subsequently, the immobilized peptide was washed with DMA. The deprotection and coupling reactions were followed by monitoring the Fmoc moiety at 313 nm. After completion of the last cycle, the resin was removed from the synthesizer, washed with, respectively, *tert*-amyl alcohol, acetic acid/*tert*-amyl alcohol (1/4, v/v), *tert*-amyl alcohol, and ether (each washing was carried out three times), and dried over P_2O_5 in vacuo.

Phosphorothioylation of **11a** was carried out as follows: 160 mg of **2** (0.38 mmol) and 133 mg of 1*H*-tetrazole (1.9 mmol) were dissolved under an argon atmosphere in 3.5 mL of dry DMA (the use of dry DMA is essential, otherwise incomplete phosphitylation will occur) and added to 0.52 g of resin **11a** (38 μmol) with the immobilized peptide containing the side-chain unprotected threonine. The whole reaction was kept under an argon atmosphere. After 1.5 h at rt, the solvent was filtered and the resin washed twice with 2.5 mL of dry DMA. After addition of 230 mg of phenylacetyl disulfide (0.76 mmol), dissolved in 2 mL of dry DMA, the reaction mixture turned purple. When *dry* acetonitrile instead of DMA was used as a solvent, the purple color did not appear. The solvent was filtered after 1 h, and the resin **12a** was washed with DMA, *tert*-amyl alcohol, acetic acid/*tert*-amyl alcohol (1/4, v/v), *tert*-amyl alcohol, and ether, respectively (each washing was carried out three times), and dried over P_2O_5 in vacuo.

Deprotection and cleavage from the resin, according to method B, were performed as follows: A cooled (0 °C) mixture of 0.62 mL of TMSBr (5.3 mmol), 0.56 mL of thiophenol (5.3 mmol), and 0.56 mL of *m*-cresol (5.3 mmol) in 3.6 mL TFA was added to the immobilized phosphorothioate pentapeptide attached to the resin. The reaction mixture was kept under argon at 0 °C for 1 h. Subsequently, the mixture was filtered into a rapidly stirred mixture of hexanes (160 mL) and ether (40 mL). After being stirred for another hour, the mixture was centrifuged for 20 min at 3000 rpm and the pellet collected, dissolved in water, and neutralized with NH_4HCO_3. Purification using a Sephadex LH-20 gel filtration column (eluent MeOH/H_2O, 85/15, v/v) gave **13a** (100%, 30.5 mg) which was pure according to analytical HPLC and CZE (20 kV, t_R = 9.5 min).

^{13}C NMR (D_2O): 18.9 (C^4 Thr), 21.5 (C^5 Leu), 21.8 (C^4 Lys), 22.8 (C^6 Leu), 24.8 (C^4 Leu), 25.0 and 25.2 (C^4 Arg), 27.0 (C^5 Lys), 28.3 and 29.6 (C^3 Arg), 31.3 (C^3 Lys), 39.6 (C^6 Lys), 39.9 (C^3 Leu), 41.1 and 41.2 (C^3 Arg), 53.2, 53.6, 54.4 and 55.2 (C^2 Arg, Lys and Leu), 61.2 (d, C^2 Thr, J_{PC} = 4.4 Hz), 69.9 (d, C^3 Thr, J_{PC} = 4.4 Hz), 157.3 (C^6 Arg), 171.0, 171.8, 173.4, 173.6 and 178.7 (C(O)). ^{31}P NMR (D_2O): 43.7. ^1H NMR (300 MHz) (D_2O): 0.87 (d, 3 H, C^5H_3 Leu, J = 6.0 Hz), 0.93 (d, 3 H, C^6H_3 Leu, J = 6.0 Hz), 1.27 (d, 3 H, C^4H_3 Thr, J = 6.3 Hz), 1.43–1.97 (m, 17 H, C^4H_2 and C^3H_2 Arg, C^3H_2, C^4H_2 and C^5H_2 Lys, C^3H_2 and C^4H Leu), 3.02 (t, 2 H, C^6H_2 Lys, J = 7.5 Hz), 3.18 (t, 2 H, C^5H_2 Arg, J = 7.0 Hz), 3.21 (t, 2 H, C^5H_2 Arg, J = 6.9 Hz), 4.07 (t, 1 H, C^2H Lys, J = 6.4 Hz), 4.12 (d, 1 H, C^2H Thr, J = 9.1 Hz), 4.18 (dd, 1 H, C^2H Leu, J = 7.9, 4.9 Hz), 4.30 (t, 1 H, C^2H Arg, J = 7.2 Hz), 4.45 (m, 2 H, C^2H Arg and C^3H Thr). Exact mass *m/z*: calcd 769.3908, found 769.3875 ($C_{28}H_{58}O_9N_{12}S_1P_1$).

H-Lys-Arg-(*O*-phospho)Thr-Leu-Arg-OH (13b). Phosphorylation of **11a** was carried out as described before[26] using a slightly modified procedure.[39] 232 mg of **2** (0.56 mmol) and 196 mg of 1*H*-tetrazole (2.8 mmol) were dissolved in 4 mL of dry DMA under an argon atmosphere and transferred to 1.0 g of resin **11a** (see preparation of **13a**, loading 56 μmol g^{-1}) also under an argon atmosphere. After 1 h, the resin was washed with dry DMA (twice, 2 mL). Oxidation was carried out by adding 0.14 mL of 80% *tert*-butyl hydroperoxide (1.1 mmol), diluted with 2 mL dry DMA, to the resin. The reaction was kept under argon for 1 h at rt. Washing was carried out as described for **12a**. After drying over P_2O_5 in vacuo, deprotection and cleavage from the resin was carried out as follows: A mixture of 0.35 mL of TFMSA (3.9 mmol), 0.92 mL of thioanisol (7.8 mmol), and 0.82 mL of *m*-cresol (7.8 mmol) in 5.7 mL of TFA was added to the immobilized and protected peptide **12b**. After 1 h at 0 °C, the reaction mixture was poured in a rapidly stirred mixture of hexanes (200 mL) and ether (100 mL) and stirred for 1 h. Subsequently, the solvent was decanted and the residue purified by Sephadex G-15 gel filtration (eluent 0.1 M acetic acid). Further purification by preparative reversed-phase FPLC afforded **13b** in a yield of 85% (36 mg) pure according to HPLC and CZE (20 kV, t_R = 9.2 min).

^{13}C NMR (400 MHz) (D_2O): 18.3 (C^4 Thr), 21.5 (C^5 Leu), 21.7 (C^4 Lys), 22.5 (C^6 Leu), 24.8 (C^4 Leu), 25.0 (C^4 Arg), 26.9 (C^5 Lys), 28.2 and 28.6 (C^3 Arg), 31.0 (C^3 Lys), 39.6 (C^6 Lys), 40.4 (C^3 Leu), 40.9 and 41.1 (C^3 Arg), 52.9, 53.0, 53.3 and 54.1 (C^2 Arg, Lys and Leu), 59.4 (d, C^2 Thr, J_{PC} = 4.4 Hz), 72.2 (d, C^3 Thr, J_{PC} = 4.4 Hz), 157.3 (C^6 Arg), 170.1, 170.8, 173.5, 174.7 and 175.5 (C(O)). ^{31}P NMR (D_2O): 1.7. ^1H NMR (400 MHz) (D_2O): 0.93 (d, 3 H, C^6H_3 Leu, J = 6.0 Hz), 1.05 (d, 3 H, C^5H_3 Leu, J = 6.0 Hz), 1.31 (d, 3 H, C^4H_3 Thr, J = 6.5 Hz), 1.53 (m, 2 H, C^4H_2 Lys), 1.63–2.01 (m, 15 H, C^4H_2 and C^3H_2 Arg, C^3H_2 and C^5H_2 Lys, C^3H_2 and C^4H Leu), 3.09 (t, 2 H, C^6H_2 Lys, J = 7.5 Hz), 3.27 (t, 2 H, C^5H_2 Arg, J = 6.5 Hz), 3.29 (t, 2 H, C^5H_2 Arg, J = 7.5 Hz), 4.09 (t, 1 H, C^2H Lys, J = 6.3 Hz), 4.26 (d, 1 H, C^2H Thr, J = 9.5 Hz), 4.38–4.51 (m, 4 H, C^2H Leu, C^2H Arg and C^3H Thr). FAB mass $(M + H)^+$ = 753.

(39) Synthesis of phosphopeptides and phosphorothioate peptides on the solid support can be carried out in two ways. The phosphorylation steps can be performed in the solid-phase synthesizer or the resin—with the peptide still attached to it—can be taken out of the apparatus and then phosphorylated. The latter procedure is the method of choice, since it is hen easier to carry the phosphorylation reaction out under dry conditions. Otherwise, incomplete or no phosphorylation at all will take place.

H-Lys-Arg-(*O*-phosphorothioyl)Ser-Leu-Arg-OH (13c). The same procedure was used for the synthesis of **13c** as described above for the synthesis of **13a**, except that 0.63 g of resin **11b** (45 μmol) was used. Phosphorothioylation was carried out in the same way as described for the synthesis of **12a** using 186 mg of **2** (0.45 mmol), 157 mg of 1*H*-tetrazole (2.25 mmol), and 270 mg of phenylacetyl disulfide (0.90 mmol). For deprotection the same method was used as described for **13a**, yield (**13c**) 87%, (29.7 mg) which was pure according to analytical HPLC and CZE (20 kV, t_R = 9.8 min).

^{13}C NMR (D_2O): 21.4 (C^5 Leu), 21.4 (C^4 Lys), 22.7 (C^6 Leu), 24.8 (C^4 Leu), 25.0 (C^4 Arg), 26.9 (C^5 Lys), 28.6 and 29.6 (C^3 Arg), 30.8 (C^3 Lys), 39.5 (C^6 Lys), 40.1 (C^3 Leu), 40.9 and 41.1 (C^3 Arg), 53.1, 53.3, 54.2 and 55.0 (C^2 Ser, Arg, Lys and Leu), 63.9 (C^3 Ser), 157.2 (C^6 Arg), 170.1, 171.5, 173.6, 173.9 and 178.6 (C(O)). ^{31}P NMR (D_2O): 47.4. ^{1}H NMR (300 MHz) (D_2O): 0.84 (d, 3 H, C^5H_3 Leu, J = 6.0 Hz), 0.90 (d, 3 H, C^6H_3 Leu, J = 6.1 Hz), 1.40–1.96 (m, 17 H, C^4H_2 and C^3H_2 Arg, C^3H_2, C^4H_2 and C^5H_2 Lys, C^3H_2 and C^4H Leu), 2.97 (t, 2 H, C^6H_2 Lys, J = 7.5 Hz), 3.16 (t, 2 H, C^5H_2 Arg, J = 6.9 Hz), 3.19 (t, 2 H, C^5H_2 Arg, J = 6.7 Hz), 4.03 (t, 1 H, C^2H Lys, J = 6.3 Hz), 4.09 (m, 2 H, C^3H_2 Ser), 4.14 (dd, 1 H, C^2H Leu, J = 8.5, 4.7 Hz), 4.36 (m, 2 H, C^2H Arg) and 4.45 (dd, 1 H, C^2H Ser, J = 6.1, 4.3 Hz). Exact mass m/z: calcd 755.3752, found 755.3698 ($C_{27}H_{56}O_9N_{12}S_1P_1$).

H-Lys-Arg-(*O*-phospho)Ser-Leu-Arg-OH (13d). **13d** was prepared in the same way as described above the synthesis of **13b** starting from resin **11b** (loading 54 μmol), yield 98% (39 mg) pure according to analytical HPLC and CZE (20 kV, t_R = 9.6 min).

^{13}C NMR (400 MHz) (D_2O): 21.6 (C^5 Leu), 21.8 (C^4 Lys), 22.7 (C^6 Leu), 25.0 (C^4 Leu), 25.1 (C^4 Arg), 27.1 (C^5 Lys), 28.4 and 28.9 (C^3 Arg), 31.1 (C^3 Lys), 39.7 (C^6 Lys), 40.5 (C^3 Leu), 41.1 and 41.3 (C^3 Arg), 52.9, 53.3, 53.5 and 54.2 (C^2 Arg, Lys and Leu), 54.8 (d, C^2 Ser, J_{PC} = 7.2 Hz), 64.8 (d, C^3 Ser, J_{PC} = 4.1 Hz), 157.5 (C^6 Arg), 170.4, 171.2, 173.7, 175.1 and 175.7 (C(O)). ^{31}P NMR (D_2O): 1.4. ^{1}H NMR (400 MHz) (D_2O): 0.87 (d, 3 H, C^5H_3 Leu, J = 6.1 Hz), 0.92 (d, 3 H, C^6H_3 Leu, J = 6.1 Hz), 1.45 (m, 2 H, C^4H_2 Lys), 1.60–1.92 (m, 15 H, C^4H_2 and C^3H_2 Arg, C^3H_2 and C^5H_2 Lys, C^3H_2 and C^4H Leu), 2.99 (t, 2 H, C^6H_2 Lys, J = 7.6 Hz), 3.22 (t, 4 H, C^5H_2 Arg, J = 6.9 Hz), 4.03 (t, 1 H, C^2H Lys, J = 6.5 Hz), 4.11 (m, 2 H, C^3H_2 Ser), 4.40 (m, 3 H, C^2H Leu and C^2H Arg) and 4.58 (t, 1 H, C^2H Ser, J = 5.5 Hz). FAB mass $(M + H)^+$ = 739.

H-Leu-Ser-Asp-Gln-Glu-Lys-Arg-Lys-Gln-Ile-Ser-Val-Arg-Gly-Leu-OH (15). Attachment of the first amino acid was carried out as described above for the synthesis of **10** except that leucine was used instead of arginine. The amount of esterification of leucine was 100 μmol per gram of resin. Synthesis of peptide **14** immobilized on the solid support was carried out using standard procedures.[17a,b] Deprotection of the α-NH_2 group was carried out using 20% piperidine in DMA. After the resin was washed with DMA, 3 equiv of the appropriate amino acid derivative and 3 equiv of (benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate (BOP) (132 mg, 0.30 mmol) in the presence of 6 equiv of *N*-methylmorpholine (NMM) were dissolved in DMA (1.5 mL) and circulated over the resin for 1 h except for the valine which was circulated for 1.5 h. Although serine in the position 11 can be coupled with an unprotected side chain using the BOP methodology, Fmoc-Ser(OH)-OPfp (199 mg, 0.40 mmol) and 54 mg of 1-hydroxybenzotriazole (0.40 mmol) were used because the former was readily available. The serine in the second position was coupled with the hydroxyl function protected as a *tert*-butyl ether. Subsequently, the immobilized peptide was washed with DMA. The deprotection and coupling reactions were followed by monitoring the Fmoc moiety at 313 nm. After the last coupling cycle, the immobilized peptide **14** attached to the resin was wahsed as described above for the synthesis of **11a** and dried over P_2O_5 in vacuo. The resin **14** was divided in four portions. One portion was used to prepare **15** and deprotected in the same way as described above for the synthesis of **13a**. A mixture of 0.73 mL of TMSBr (5.5 mmol), 0.59 mL of thiophenol (5.5 mmol), and 0.57 mL of *m*-cresol (5.5 mmol) in 3.6 mL of TFA was added to the immobilized peptide **14**. The reaction mixture was kept under argon at 0 °C for 1 h. Workup was carried out as described for **13a**. After purification using a Sephadex LH-20 gel filtration column (eluent MeOH/H_2O, 85/15, v/v) and preparative FPLC **15** was obtained in a yield of 51% (22 mg) which was pure according to analytical HPLC and CZE (20 kV, t_R = 10.4 min).

^{13}C NMR (400 MHz) (D_2O): 11.0 (C^5 Ile,), 15.5 (C^6 Ile), 18.4 (C^4 Val), 19.2 (C^5 Val), 21.6 and 21.8 (C^5 Leu), 22.8 and 23.3 (C^6 Leu), 23.0 and 23.3 (C^4 Lys), 24.6 and 25.3 (C^4 Leu), 25.2 (C^4 Arg), 25.5 (C^4 Ile), 26.8, 27.1, 27.2 and 27.3 (C^3 Gln and C^5 Lys), 28.2 (C^3 Glu), 28.5 and 28.9 (C^3 Arg), 30.7 (C^3 Val), 30.9 (C^4 Gln), 31.8 and 31.9 (C^3 Lys), 34.8 (C^4 Glu), 36.9 (C^3 Ile), 38.7 (C^3 Asp), 39.9 (C^6 Lys), 40.9 and 41.3 (C^3 Leu), 41.3 and 41.4 (C^5 Arg), 43.5 (C^2 Gly), 52.3, 54.2, 54.3, 54.6, 54.9, 55.0, 55.7, 55.8, 55.9 and 56.3 (C^2 Leu, Ser, Asp, Gln, Glu, Lys and Arg), 59.5 (C^2 Ile), 60.4 (C^2 Val), 61.8 and 62.4 (C^3 Ser), 157.5 (C^6 Arg), 171.0, 171.6, 172.4, 172.6, 174.1, 174.3, 174.6, 174.8, 174.9, 175.4, 175.5, 175.8, 177.8, 178.3, 178.4, 180.6 and 181.7 (C(O)). PDMS: m/z = 1756.2. FAB mass $(M + H)^+$ = 1756. Protein sequencing confirmed the sequence.

H-Leu-Ser-Asp-Gln-Glu-Lys-Arg-Lys-Gln-Ile-(*O*-phosphorothioyl)Ser-Val-Arg-Gly-Leu-OH (18). A second quarter of resin **14** (containing a serine with a free hydroxyl function in position 11) was phosphorothioylated as described above for the synthesis of **13a** using 104 mg of **2** (0.25 mmol), 88 mg of 1*H*-tetrazole (1.25 mmol), and 151 mg of phenylacetyl disulfide (0.50 mmol). Deprotection and cleavage from the resin were carried out as described above for the deprotection of **13a** using a mixture of 0.86 mL of TMSBr (6.5 mmol), 0.69 mL of thiophenol (6.5 mmol), and 0.68 mL of *m*-cresol (6.5 mmol) in 4.3 mL of TFA (method B). Workup and purification were carried out as described for the synthesis of **15**, yield 28% (13 mg) which was pure according to analytical HPLC and CZE (20 kV, t_R = 12.4 min).

^{13}C NMR (400 MHz) (D_2O): 11.0 (C^5 Ile), 15.6 (C^6 Ile), 18.7 (C^4 Val), 19.2 (C^5 Val), 21.5 and 21.8 (C^5 Leu), 22.7 and 23.2 (C^6 Leu), 22.8 and 23.1 (C^4 Lys), 24.6 and 25.3 (C^4 Leu), 25.2 (C^4 Arg), 25.4 (C^4 Ile), 27.0 and 27.1 (C^3 Gln), 27.4 and 27.8 (C^5 Lys), 28.7 and 29.0 (C^3 Glu and C^3 Arg), 30.7 (C^3 Val), 30.8 and 31.0 (C^4 Gln), 31.9 (C^3 Lys), 33.7 (C^4 Glu), 36.9 (C^3 Ile), 38.5 (C^3 Asp), 39.9 (C^6 Lys), 40.7 and 41.3 (C^3 Leu), 41.3 (C^5 Arg), 43.3 (C^2 Gly), 52.4, 52.7, 53.4, 53.8, 54.2, 54.4, 54.5, 55.0, 55.1 and 55.9 (C^2 Leu, Ser, Asp, Gln, Glu, Lys and Arg), 59.4 (C^2 Ile), 60.6 (C^2 Val), 62.1 (C^3 Ser(2)), 64.5 (d, C^3 Ser(11), J_{PC} = 11.9 Hz), 157.5 (C^6 Arg), 171.2, 171.4, 172.1, 172.2, 173.8, 174.1, 174.2, 174.4, 174.5, 174.6, 174.8, 174.9, 175.0, 177.6, 178.5, 180.3 and 180.7 (C(O)). ^{31}P NMR (400 MHz) (D_2O, pD = 4.7): 47.3. PDMS: m/z = 1851.6. FAB mass $(M + H)^+$ = 1852. Protein sequencing confirmed the sequence, except for a blank cycle for the phosphorothioyl serine in position 11.

H-Leu-Ser-Asp-Gln-Glu-Lys-Arg-Lys-Gln-Ile-(*O*-phospho)Ser-Val-Arg-Gly-Leu-OH (19). For the synthesis of **19** the same procedure was used as described for the preparation of **18**, except that 62 μL of a 80% solution of *tert*-butyl hydroperoxide (0.50 mmol) was used instead of phenylacetyl disulfide, yield 46% (21 mg) which was pure according to analytical HPLC and CZE (20 kV, t_R = 12.4 min).

^{13}C NMR (400 MHz) (D_2O): 10.9 (C^5 Ile), 15.5 (C^6 Ile), 18.6 (C^4 Val), 19.2 (C^5 Val), 21.5 and 21.9 (C^5 Leu), 22.9 and 23.2 (C^6 Leu), 22.7 and 23.1 (C^4 Lys), 24.7 and 25.3 (C^4 Leu), 25.1 (C^4 Arg), 25.3 (C^4 Ile), 27.0, 27.1 and 27.5 (C^3 Gln and C^5 Lys), 28.2 (C^3 Glu), 28.7 and 29.2 (C^3 Arg), 30.7 (C^3 Val), 30.8 and 31.0 (C^4 Gln), 31.8 and 31.9 (C^3 Lys), 34.6 (C^4 Glu), 36.9 (C^3 Ile), 38.8 (C^3 Asp), 39.8 and 39.9 (C^6 Lys), 41.2 and 41.3 (C^3 Leu), 41.4 (C^5 Arg), 43.2 (C^2 Gly), 53.0, 53.5, 53.7, 54.0, 54.3, 54.5, 54.6, 54.9, 55.0, 55.3, 55.6, 56.0 and 56.4 (C^2 Leu, Ser, Asp, Gln, Glu, Lys, Arg, Val and Gly), 59.2 (C^2 Ile), 60.5 (C^2 Val), 62.2 (C^3 Ser(2)), 63.8 (C^3, phosphoSer(11)), 157.5 (C^6 Arg), 171.1, 172.4, 172.6, 173.6, 174.1, 174.3, 174.4, 174.6, 174.9, 175.1, 175.5, 176.0, 178.0, 178.4, 178.5, 180.6 and 181.8 (C(O)). ^{31}P NMR (400 MHz) (D_2O, pD = 8.1): 4.4. FAB mass $(M + H)^+$ = 1840. Protein sequencing confirmed the sequence, except for a blank cycle for the phosphoserine in position 11 which is in agreement with literature.[16k,19b]

Abbreviations: ATPγS = adenosine 5′-*O*-(3′-thiotriphosphate); Boc = *tert*-butyloxycarbonyl; BOP = (benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate; CZE = capillary zone electrophoresis; DMA = *N*,*N*-dimethylacetamide; Fmoc = 9-fluorenylmethoxy-

carbonyl; FPLC = fast protein liquid chromatography; HOBt = 1-hydroxybenzotriazole; HPLC = high-performance liquid chromatography; Mtr = 4-methoxy-2,3,6-trimethylbenzenesulfonyl; NMM = *N*-methylmorpholine; PDMS = plasma desorbtion mass spectrometry; petroleum ether = petroleum ether 40/60; Pfp = pentafluorophenol; rt = room temperature; TFA = trifluoroacetic acid; TFMSA = trifluoromethanesulfonic acid; TMSBr = bromotrimethylsilane.

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