Design and Synthesis of Fmoc-Thr[PO(OH)(OPOM)] for the Preparation of Peptide Prodrugs Containing Phosphothreonine in Fully Protected Form

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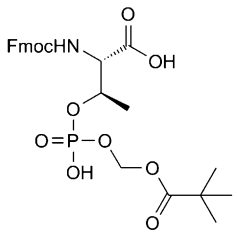
The design and efficient synthesis of *N*-Fmoc-phosphothreonine protected by a mono-(pivaloyloxy)methyl (POM) moiety at its phosphoryl group (Fmoc-Thr[PO(OH)(OPOM)]-OH, **1**, is reported. This reagent is suitable for solid-phase syntheses employing acid-labile resins and Fmoc-based protocols. It allows the preparation of phosphothreonine (pThr)-containing peptides bearing bis-POM-phosphoryl protection. The methodology allows the first reported synthesis of pThr-containing polypeptides having bioreversible prodrug protection, and as such it should be useful in a variety of biological applications.

Introduction. – Post-translational modification by phosphorylation plays central roles in cellular signaling. Phosphoryl esterification of proteins on the side-chain OH groups of threonine, serine, and tyrosine residues to yield the corresponding phosphothreonine (pThr), phosphoserine (pSer), and phosphotyrosine (pTyr) species, respectively, can introduce unique molecular features that facilitate signaling cascades, often by promoting specific protein–protein interactions (PPIs) [1–3]. The contributions that aberrations in protein phosphorylation can make to the etiology of a number of diseases, including cancers [4][5], have rendered synthetic phosphopeptides as valuable pharmacological tools for studying both normal and pathological processes [6][7]. However, in cellular systems, the bioavailability of such phosphopeptides may be limited by poor membrane transport due to the dianionic character of the phosphoryl species [8–10]. Many approaches have been investigated to enhance cellular bioavailability of organic phosphates. The most common methodologies involve masking the phosphoryl moiety with 'prodrug' protecting groups that can be removed biologically within the cell [11]. The (pivaloyloxy)methyl (POM) moiety is an esterase-cleavable group that has found wide utility in phosphoryl prodrug protection, particularly in nucleotides [12], and in phosphate and phosphonic acid functionality in peptide mimetics [13–16]. In spite of its usefulness, there have been no reports of pThr or pSer reagents that utilize phosphoryl-POM protection in forms suitable for direct solid-phase synthesis. This may be one reason why there have been no reports of pThr or pSer-containing polypeptides bearing POM phosphoryl protection. Furthermore, to the best of our knowledge, there are no reports of polypeptides containing pThr bearing bioreversible protecting groups of any kind. Given the physiological importance of pThr-containing sequences [3], there is a considerable need for a reagent that would permit the solid-phase synthesis of peptides containing POM-protected pThr residues. Accordingly, we report herein the prepara-

tion of Fmoc-Thr[PO(OH)(OPOM)]-OH (**1**) as a new reagent for the synthesis of peptides containing pThr with its phosphoryl group in fully protected bis-POM prodrug form, *i.e.*, Thr[$PO_3(POM)_2$].



Fmoc-Thr[PO(OH)(OPOM)]-OH (**1**)

Results and Discussion. – *Reagent Design*. When considering protocols for the incorporation of Thr[$PO_3(POM)_2$] into peptides, we noted that the solid-phase synthesis of pTyr-containing peptides can be achieved in the absence of phosphoryl protection using Fmoc-Tyr(PO_3H_2)-OH [17]. Accordingly, we envisioned that one potential route to the synthesis of Thr[$PO_3(POM)_2$]-containing peptides could involve using unprotected Fmoc-Thr(PO_3H_2)-OH, which would result in resin-bound peptides bearing full side-chain protection except for a free pThr phosphoryl group. In this approach, *in situ* introduction of phosphoryl POM protection ('POMylation') would be carried out immediately prior to cleavage of the peptide from the resin. However, when we investigated this approach, we found that, although initial coupling of unprotected Fmoc-Thr(PO_3H_2)-OH occurred cleanly, further peptide synthesis through the introduction of additional amino acid residues, followed by resin cleavage, led to very low yields of isolated pThr-containing peptides. This indicated that peptide-chain elongation in the presence of unprotected Thr(PO_3H_2) was problematic.

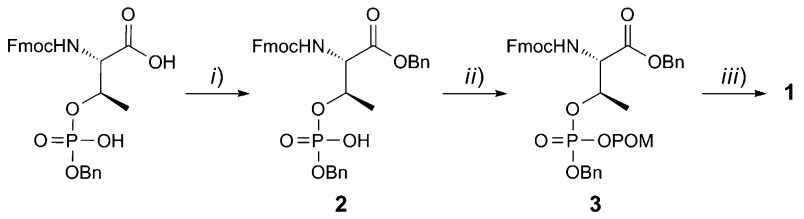
In further studies, we observed that treatment of resin-bound Fmoc-Thr(PO_3H_2) with excess iodomethyl pivalate (POMI) [18] and EtN^iPr_2, followed by resin cleavage (1% TFA in CH_2Cl_2) cleanly provided Fmoc-Thr[$PO_3(POM)_2$]-amide. This demonstrated both the ability to perform on-resin POM protection of the phosphoryl group and the stability of the resulting Thr[$PO_3(POM)_2$] residue to the conditions of resin cleavage. However, we found that piperidine-mediated Fmoc deprotection of resin-bound Fmoc-Thr[$PO_3(POM)_2$], followed by 1% TFA resin cleavage, gave the Thr[PO(OH)(OPOM)]-containing peptide, indicating that mono-POM deprotection had occurred in the presence of piperidine. This is consistent with the reported ability of piperidine to selectively cleave a single POM group from the triester $PO(OPOM)_3$ to yield $PO(OH)(POM)_2$ [19]. The instability of pThr phosphoryl bis-esters to piperidine treatment is one reason why incorporation of pThr and pSer residues on acid-labile resins is normally achieved using commercially-available Fmoc derivatives bearing mono-Bn protection of the phosphoryl group, *i.e.*, [Fmoc-Thr[PO(OH)(OBn)]-OH and Fmoc-Ser[PO(OH)(OBn)]-OH, respectively (reviewed in [20–22]). Based on this information, we concluded that synthesis of Thr[$PO_3(POM)_2$]-containing peptides could best be accomplished using Fmoc-Thr[PO(OH)(OPOM)]-OH (**1**) as a reagent,

with conversion of the Thr[PO(OH)(OPOM)] residue to the desired fully-protected Thr[$PO_3(POM)_2$] form by POMI/EtN^iPr_2 treatment after completion of peptide synthesis and Fmoc deprotection, but immediately prior to final resin cleavage. It should be noted that the current protocol provides terminal-*N*-acetylated peptides. To obtain peptides with a free terminal amines, the procedure can be altered through the use of terminal *N*-Boc protection, in which 'POMylation' is undertaken with maintenance of Boc protection. Desired peptides bearing a free terminal amine can then be obtained by cleavage under conditions that are compatible with Boc removal and retention of phosphoryl POM protection (95% TFA; unreported results).

Synthesis of Fmoc-Thr[PO(OH)(OPOM)]-OH (**1**) *from Commercially-Available Fmoc-Thr[PO(OH)(OBn)]-OH.* Benzyl esterification of the carboxylic acid of commercially available Fmoc-Thr[PO(OH)(OBn)]-OH was achieved by treatment with BnOH and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCI) in the presence of 4-(dimethylamino)pyridine (DMAP; *Scheme 1*). Reaction of the resulting Fmoc-Thr[PO(OH)(OBn)]-OBn (**2**) with excess POMI (formed from the corresponding commercially available chloride by halide exchange using NaI in MeCN [18]) and EtN^iPr_2 resulted in esterification of the free phosphoryl OH group to yield Fmoc-Thr[PO(OBn)(OPOM)]-OBn (**3**) as a 1:1 mixture of phosphoryl diastereoisomers. Subsequent hydrogenolytic cleavage of all Bn groups cleanly yielded the desired reagent **1** (*Scheme 1*).

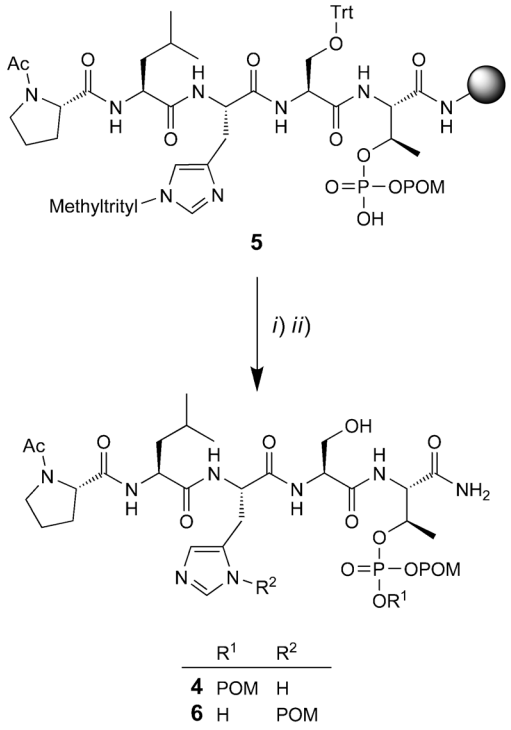
Utilization of Fmoc-Thr[PO(OH)(OPOM)]-OH (**1**) *for the Synthesis of Ac-Pro-Leu-His-Ser-Thr[$PO_3(POM)_2$]-amide* (**4**). To demonstrate the applicability of **1** for the synthesis of Thr[$PO_3(POM)_2$]-containing peptides, we chose as a target the pentapeptide 'Pro-Leu-His-Ser-pThr', which has been reported as a high affinity ligand of the polo-like kinase 1 (Plk 1) polo box domain (PBD) [23–25]. PBD-Binding ligands may potentially serve as anticancer agents by blocking the spatial organization required for Plk 1 to function in oncogenic processes [26]. The synthesis of Ac-Pro-Leu-His-Ser-Thr[$PO_3(POM)_2$]-amide (**4**) was accomplished on *NovaSyn TG Sieber* resin using reagent **1** according to standard Fmoc protocols. Histidine and serine were employed in their methyltrityl (Mtt) and trityl side chain-protected forms, respectively, to allow selective on-resin cleavage using 1% TFA (*Scheme 2*). Following peptide formation and terminal amino acetylation of the Pro residue, the resin **5** was treated with POMI (10 equiv.) and EtN^iPr_2 (10 equiv.) in DMF (4 h). Reaction products were then cleaved

Scheme 1



i) BnOH (1.5 equiv.), 4-(Dimethylamino)pyridine (DMAP; 1.0 equiv.), 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide·HCl (EDCI; 2.5 equiv.), CH_2Cl_2, 0° to r.t., 2 h; 70%. *ii*) Iodomethyl pivalate (POMI; 2.0 equiv.), EtN^iPr_2 (2.0 equiv.), DMF, r.t., 12 h; 60%. *iii*) 10% Pd/C, MeOH, r.t., 1 h; 68%.

Scheme 2



NovaSyn®TG Sieber resin; *i*) POMI, EtN^iPr_2, DMF, 4 h; *ii*) 1% CF_3COOH (TFA), CH_2Cl_2.

from the resin (1% TFA) and purified by reversed-phase (RP) HPLC to provide two peptides with retention times (t_R) of 18.8 (peptide **6**) and 20.9 min (peptide **4**), respectively. Both peptides exhibited molecular weights and elemental compositions (HR-MS) corresponding to the desired product, *i.e.*, Ac-Pro-Leu-His-Ser-Thr(PO_3POM_2)-amide (**4**).

MS/MS Analysis of Peptides **4** *and* **6**. High-resolution (HR) MS/MS analysis of the $[M+H]^+$ ions (*m*/*z* 903.4) of peptides **4** and **6** indicated that, while both peptides contained two POM groups, the locations of one of the POM moieties differed for each. The MS/MS fragmentation pattern of the slower-eluting peptide (*Fig.*, *a*) was consistent with product **4**, in which both POM groups are on the phosphoryl moiety. With this peptide, the primary MS/MS product-ion peak at *m*/*z* 559.2997 resulted from concerted loss of the elements of $(HO)PO_3(POM)_2$ and H_2O from the $[M+H]^+$ ion, which can only occur if the phosphate is 'di-POMylated'. In addition, b_3 and b_4 product-ions peaks were also observed, providing strong evidence for an unaltered Ac-Pro-Leu-His-Ser-sequence (*Fig.*). The MS/MS of the faster-eluting peptide (*Fig.*, *b*) strongly suggested that both the phosphoryl group and histidine residue each contained a single POM adduct (*i.e.*, **6**; *Scheme 2*). In this spectrum, the most abundant MS/MS product-ion peak at *m*/*z* 771.3430 resulted from loss of *t*-$C_4H_9CO_2H$ and CH_2O from the $[M+H]^+$

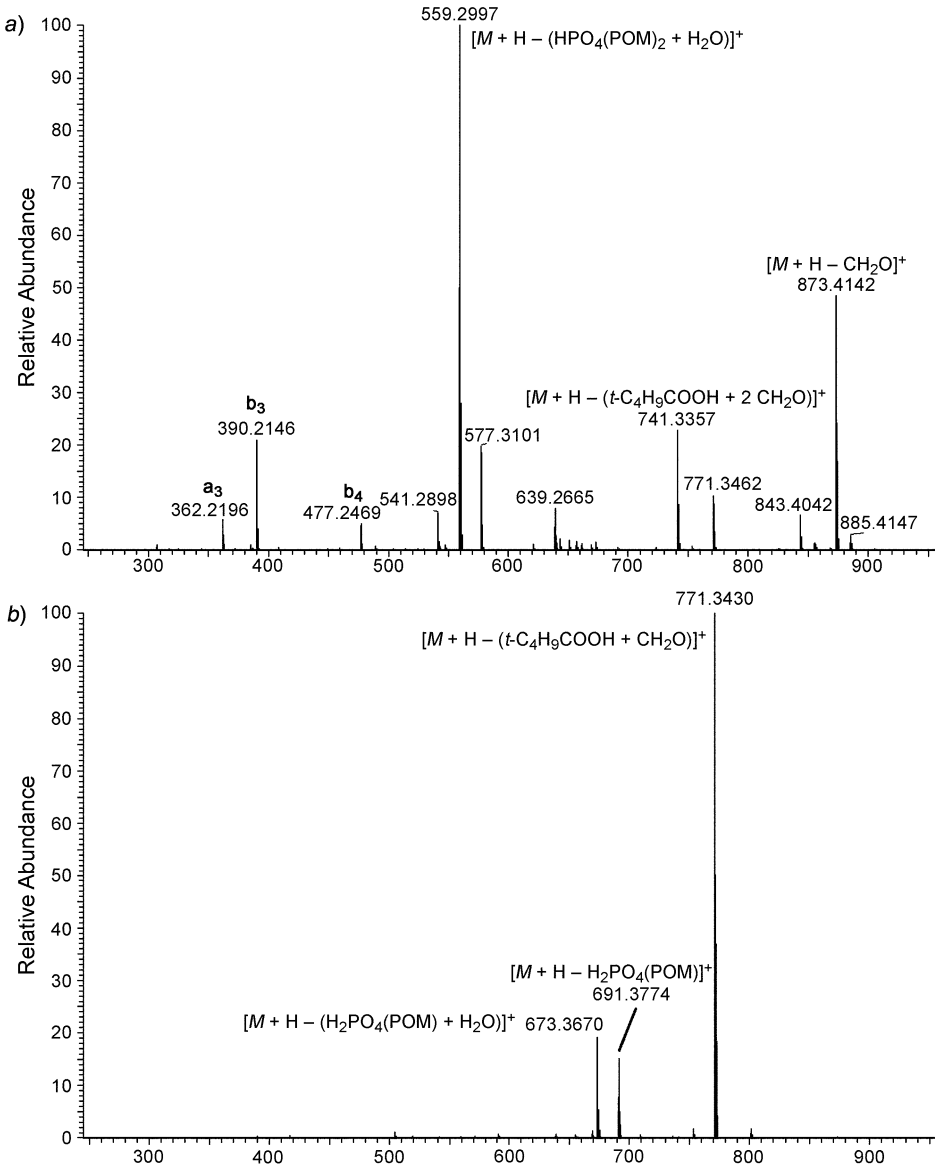


Figure. *MS/MS of peptides* a) *of* **4** *and* b) *of* **6**, *obtained by collision-induced dissociation* (CID) *of the* $[M+H]^+$ *ion* (*m*/*z* 903.4, 3.0-Da selection window) *of each peptide*

ion, which can only occur for a 'POMylated' phosphate and *not* for a 'POMylated' histidine. The subsequent loss of HPO_3 (*m*/*z* 691.3774), and of HPO_3 and H_2O (*m*/*z* 673.3670) further corroborated the presence of only one POM moiety on the phosphate (*Fig.*). The absence of b_3 and b_4 product-ion peaks, expected for an unaltered Ac-Pro-

Leu-His-Ser sequence, was additional evidence that the histidine residue had been modified.

The 'POMylation' of histidine to yield peptide **6** was not unexpected, since we had previously observed alkylation of the imidazole N(π) atom in histidine residues bearing standard trityl protection of the N(τ) atom [24][27]. While alkylation of histidine does represent an unwanted source of side-product formation, a search in the literature reveals that, in general, alkyl halide-mediated modification of side chain-protected peptides on solid support is not a significant concern. Of greater consideration regarding the chemistry reported herein is that it presents the first and so far the only method of preparing polypeptides with pThr residues in fully-protected biocleavable form. This uniqueness overcomes limitation associated with the methodology.

Conclusions. – We report the design, synthesis, and application of Fmoc-Thr[PO(OH)(OPOM)]-OH (**1**) as a reagent for the facile solid-phase preparation of peptides bearing pThr residues in the fully-protected Thr[PO_3(POM)$_2$] form. To the best of our knowledge, this represents the first methodology for preparing biolabile-protected pThr residues within a polypeptide platform. Our approach should have a significant utility in a variety of biological applications.

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Experimental Part

General. All experiments involving moisture-sensitive compounds were conducted under anh. conditions (positive Ar pressure) using standard syringe, cannula, and septa apparatus. Fmoc-Ser(Trt)-OH, Fmoc-His(Mtt)-OH, and Fmoc-Thr[PO(OH)((OBn)]-OH were purchased from *Novabiochem*. All solvents were purchased in anhy. form (*Aldrich*) and used directly. HPLC-Grade hexanes, AcOEt, CH_2Cl_2, MeCN, and MeOH were used for both column chromatography (CC) and prep. high-performance liquid chromatograhy (HPLC). LC/MS-Grade solvents (*Burdick* and *Jackson* Brand, Honeywell), ultra-high-purity acids (HCO_2H, AcOH), and buffers (NH_4HCO_3, NH_4OAc) were employed for all LC/MS analyses. Anal. TLC: *Analtech* precoated plates (*Uniplate*, silica gel *GHLF*, 250 nm) containing a fluorescence indicator. NMR Spectra: *Varian Inova* 400 MHz spectrometer; coupling constants, *J*, reported in Hz, and chemical shifts δ in ppm rel. to TMS. Low-resolution, electrospray ionization mass spectra (LR-ESI-MS): *Agilent* 1200/1100 LC/MSD single quadrupole system, equipped with an in-line UV diode-array detector (DAD), to assess compound identity and homogeneity. HR-MS: *Thermo-Fisher LTQ-Orbitrap-XL* hybrid mass spectrometer system operated at a resolution of 30,0000 (FWHM) in either positive- or negative-ion mode, depending on which mode was the most suitable based on previous LR analyses. For LC/MS analyses on the *Orbitrap*, a narrow-bore (50 × 2.1 mm), *Zorbax Rapid-Resolution* RP C_{18} column coupled with a C_{18} guard column (12.5 × 2.1 mm) was eluted at 250 µl/min with a 5–90% gradient of MeCN/H_2O containing either 0.1% HCOOH or 1 mM NH_4HCO_3. The resulting accurate mass measurement of a molecular species ($[M+H]^+$ or $[M-H]^-$) was then used to determine a unique elemental composition for each particular compound. Where appropriate, ^{1}H- and ^{13}C-NMR data were used to set elemental constraints for this calculation. For peptides **4** and **6**, MS/MS of $[M+H]^+$ ions were obtained at a resolution of 15,000 (FWHM) by both CID and HCD fragmentation. The resulting HR product-ion spectra were used to confirm peptide sequences and determine structural assignments.

Benzyl O-*[(Benzyloxy)(hydroxy)phosphoryl]*-N-*{[(9*H*-fluoren-9-yl)methoxy]carbonyl}*-L-*threoninate* (**2**). Commercially available Fmoc-Thr[PO(OH)((OBn)]-OH (1.0 g, 1.96 mmol), BnOH (0.30 ml, 2.93 mmol), and DMAP (0.24 g, 1.96 mmol) were dissolved in CH_2Cl_2 (20 ml), and the mixture was

cooled to 0°. To the cold soln. was added EDCI (0.94 g, 4.89 mmol), and the mixture was warmed to r.t. and stirred (2 h). The reaction was quenched by the addition of 1N aq. HCl soln. (10 ml), and the mixture was extracted with CH_2Cl_2. The combined org. layer was washed with 1N HCl and brine, dried ($MgSO_4$), and concentrated, and the residue was purified by CC (SiO_2; CH_2Cl_2/MeOH 20:1 → 4:1) to afford **2** (0.82 g, 70%). White semi-solid. $[\alpha]_D = 24.8$ ($c = 1.65$, $CHCl_3$). UV (DAD, MeCN/H_2O): 264, 289, 299 (Fmoc). ^{1}H-NMR (400 MHz, $CDCl_3$): 8.81 (br. *s*, 1 H); 7.78 (*d*, *J* = 8.0, 2 H); 7.62 (*dd*, *J* = 12.0, 8.0, 2 H); 7.41 (*t*, *J* = 8.0, 2 H); 7.38–7.27 (*m*, 12 H); 5.91 (*d*, *J* = 8.0, 1 H); 5.26 (*d*, *J* = 12.0, 1 H); 5.11–4.94 (*m*, 4 H); 4.54 (*d*, *J* = 12.0, 1 H); 4.44–4.33 (*m*, 2 H); 4.22 (*t*, *J* = 8.0, 1 H); 1.38 (*d*, *J* = 8.0, 3 H). ^{13}C-NMR (100 MHz, $CDCl_3$): 169.7; 156.9; 144.1; 143.7; 141.4; 135.74; 135.68; 135.1; 128.8; 128.7; 128.0; 127.9; 127.3; 125.3; 120.1; 75.6; 69.5; 68.1; 67.6; 58.7; 47.2; 18.7. HR-ESI-MS: 600.1782 ($[M-H]^-$, $C_{33}H_{31}NO_8P^-$; calc. 600.1787).

Benzyl O-*[[[(2,2-Dimethylpropanoyl)oxy]methoxy](hydroxy)phosphoryl]*-N-*{[(9*H*-fluoren-9-yl)methoxy]carbonyl}*-L-*threoninate* (**3**). Compound **2** (0.3 g, 0. 5 mmol) in DMF (5.0 ml) under Ar was treated with EtN^iPr_2 (0.174 ml, 1.00 mmol), followed by iodomethyl pivalate (POMI [18]; 0.241 g, 1.00 mmol), and the mixture was stirred at r.t. (overnight). The mixture was partitioned (H_2O/AcOEt), and the org. layer was washed with H_2O and brine, dried ($MgSO_4$), and concentrated, and the residue was purified by CC (SiO_2; AcOEt/hexanes 1:2 → 2:1) to afford **3** (mixture of two diastereoisomers (ratio 1:1 as determined by ^{1}H-NMR); 0.21 g, 60%). White semi-solid.

O-*[[[(2,2-Dimethylpropanoyl)oxy]methoxy](hydroxy)phosphoryl]*-N-*{[(9*H*-fluoren-9-yl)methoxy]carbonyl}*-L-*threonine* (= *[Fmoc-Thr[PO(OH)(OPOM)]-OH]*; **1**). A soln. of **3** (45 mg, 0.063 mmol) in MeOH (1.25 ml) was hydrogenated over 10% Pd/C (5 mg, 1 h), then filtered, and concentrated, and the residue was purified by CC (SiO_2; CH_2Cl_2/MeOH 10:1 → 4:1) to afford **1** (23 mg, 68%). Colorless oil. $[\alpha]_D = 4.98$ ($c = 0.34$, MeOH). UV (DAD, MeCN/H_2O): 264, 289, 299 (Fmoc). ^{1}H-NMR (400 MHz, CD_3OD): 7.80 (*d*, *J* = 8.0, 2 H); 7.70 (*t*, *J* = 8.0, 2 H); 7.39 (*t*, *J* = 8.0, 2 H); 7.31 (*tt*, *J* = 8.0, 4.0, 2 H); 5.58 (*d*, *J* = 16.0, 2 H); 5.04–4.96 (*m*, 1 H); 4.43–4.35 (*m*, 3 H); 4.26 (*t*, *J* = 4.0, 1 H); 1.39 (*d*, *J* = 8.0, 3 H); 1.23 (*s*, 9 H). ^{13}C-NMR (100 MHz, CD_3OD): 178.4; 172.7; 159.1; 145.4; 142.7; 128.9; 128.3; 126.4; 121.1; 84.0; 76.4; 68.4; 60.1; 60.0; 39.9; 27.4; 19.0. HR-ESI-MS: 534.1524 ($[M-H]^-$, $C_{25}H_{29}NO_{10}P^-$; calc. 534.1529).

Synthesis of Ac-Pro-Leu-His-Ser-Thr(PO$_3$(POM)$_2$)-amide (=*1-Acetyl-*L*-prolyl-*L*-leucyl-*L*-histidyl-*L*-seryl-*O*-(bis{[(2,2-dimethylpropanoyl)oxy]methoxy}phosphoryl)-*L*-threoninamide*; **4**). Peptide **4** was synthesized on *NovaSyn®TG Sieber* resin (*Novabiochem*, cat. No. 01-64-0092) using **1** according to standard Fmoc-based protocols employing *N*-methylpyrrolidin-2-one (NMP) as solvent, and *O*-1*H*-benzotriazol-1-yl)-*N,N,N′,N′*-tetramethyluronium hexafluorophosphate (HBTU; 5.0 equiv.), 1-hydroxybenzotriazole (HOBt; 5.0 equiv.), and (10.0 equiv.) as coupling reagents. Terminal amine acetylation was achieved using 1-acetyl-1*H*-imidazole. Following acetylation, the resin (0.1 mmol) was treated during 4 h with POMI (242 mg, 1.0 mmol) and EtN^iPr_2 (0.174 ml, 1.0 ml) in DMF (3 ml). The resulting resin (0.1 mmol) was washed with DMF, MeOH, CH_2Cl_2, and Et_2O, and then dried under vacuum (overnight). The peptide was cleaved from the resin using 1% TFA in CH_2Cl_2 (10 min; repeated five times). Resin was removed by filtration, the filtrate was concentrated under vacuum, precipitated with Et_2O, and the precipitate was washed with cold Et_2O. The resulting solid was dissolved in 50% aq. MeCN (5 ml) and purified by RP prep. HPLC with a *Phenomenex C$_{18}$* column (250 × 21 mm) with a linear gradient from 20% aq. MeCN (0.1% TFA) to 90% MeCN (0.1% TFA) over 30 min at a flow rate of 10.0 ml/min. Lyophilization provided **4** as white powder (4.8 mg; >99% pure by anal. HPLC). HR-ESI-MS: 903.4227 ($[M+H]^+$, $C_{38}H_{64}N_8O_{15}P^+$; calc. 903.4229. By-product **6** was also isolated as white powder (7.0 mg; > 99% purity by anal.). HR-ESI-MS: 903.4227 ($[M+H]^+$, $C_{38}H_{64}N_8O_{15}P^+$; calc. 903.4229).

MS/MS Analysis of Peptides **4** *and* **6**. Collision-induced dissociation (CID) product-ion mass spectra for the $[M+H]^+$ ion (*m/z* 903.4, 3.0-Da selection window) of **4** and **6** were generated by fragmentation in the LTQ at an energy of 30 and analysis in the *Orbitrap* at a resolution of 15,000 (FWHM) over the maximum allowable mass range. Each spectrum shown in the *Figure* was the average of ten or more scans and resulted from an independent LC/MS/MS analysis of individual, HPLC-purified pentapeptide. A shallow linear MeCN/H_2O gradient (2–47% MeCN in 20 min), followed by a steeper linear gradient (47–90% in 10 min), was employed on a 3-μm, 150 × 2.0 mm *Cadenza CD-C18* HPLC column (*Imtakt*, USA) at a flow rate of 250 μl/min for the separation and elution of these POM-peptide derivatives.

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