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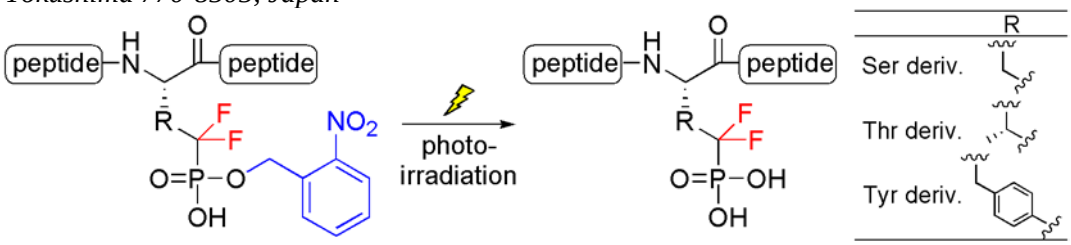

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Development of caged non-hydrolyzable phosphoamino acids and application to photo-control of binding affinity of phosphopeptide mimetic to phosphopeptide-recognizing protein

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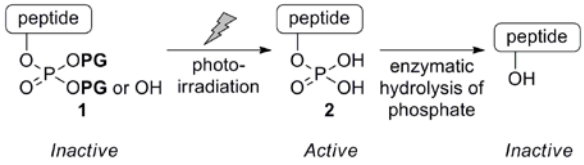
ABSTRACT

The design and synthesis of caged non-hydrolyzable phospho-serine, -threonine, and -tyrosine derivatives that generate parent non-hydrolyzable phosphoamino acids, containing a difluoromethylene unit instead of the oxygen of a phosphoester, after UV-irradiation are described. The caged non-hydrolyzable amino acids were incorporated into peptides by standard Fmoc solid-phase peptide synthesis, and the obtained peptides were successfully converted to the parent non-hydrolyzable phosphopeptides by UV-irradiation. Application of the caged non-hydrolyzable phosphoserine-containing peptide to photo-control the binding affinity of the peptide to 14-3-3β protein is also reported.

1. Introduction

Protein phosphorylation/dephosphorylation is an indispensable post-translational regulatory mechanism in a wide range of cellular processes.[1] Numerous chemical tools have been developed for controlling or monitoring phosphorylation and/or dephosphorylation events, enabling clarification of their biological significance.[2] A caged phosphopeptide is a useful tool for spatiotemporal regulation of phosphopeptide biological activity (Scheme 1).[3] Caged peptide **1** has a photo-removable protective group on a phosphate moiety to mask its key functionality. Its biological activity derived from interaction between the phosphate and a target biomolecule is therefore turned off. On photo-irradiation followed by removal of the protective group, the biologically active parent phosphopeptide **2** is generated. Although this technique is useful in the study of phosphorylation/dephosphorylation events, there is a risk of conversion of the active phosphopeptide **2** to an inactive form by hydrolysis of the phosphate by endogenous phosphatases. It is therefore desirable to avoid removal of the phosphate of phosphopeptides.



Scheme 1. Caged phosphopeptide (**PG**: protective group removable by photo-irradiation)

We and other groups have already developed non-hydrolyzable phosphopeptides in which an oxygen of a phosphoester moiety has been replaced by a difluoromethylene (CF_2) unit to prevent phosphate hydrolysis (Scheme 2).[4–6] Because the physical properties of difluoromethyl phosphonates are similar to those of the corresponding phosphates (e.g., pK_{a2}, charge under physiological conditions, and bond angle of C–X–P in $H_2NCH_2XPO_3H_2$: 5.6, –2, and 117°, respectively, for CF_2 derivative, X is CF_2; 6.5, –2, and 119°, respectively, for the monoalkyl phosphate, X is oxygen),[7] they can be successfully used to elucidate the biological functions of phosphorylated peptides and proteins.[8,9] We therefore designed a caged non-hydrolyzable phosphopeptide that should work as a caged phosphopeptide mimetic without enzymatic hydrolysis of the phosphate.[10] Although introduction of two photo-responsive

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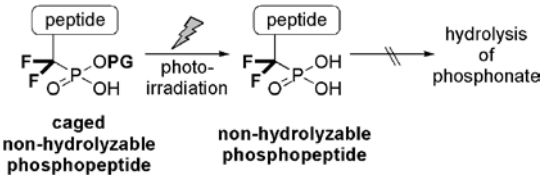
protective groups into the phosphonate is possible, monoprotection was used in this study because the bulkiness of the group was expected to be enough to prevent binding of the caged peptide to the phosphate-recognizing pockets of proteins. The mono-protected phosphonate was presumed to be compatible with solid-phase peptide synthesis (SPPS) because amino acid building blocks containing the mono-protected phosphate or non-protected phosphonate have been employed for preparation of phosphopeptides and their analogs.[11] In this paper, the synthesis and photoreactions of phosphopeptide mimetics with caged non-hydrolyzable phospho-serine, -threonine, and -tyrosine are described. Application of the serine derivative to photo-control of binding affinity to 14-3-3β protein is also reported.



Scheme 2. Design of caged non-hydrolyzable phosphopeptide (**PG**: protective group removable by photo-irradiation)

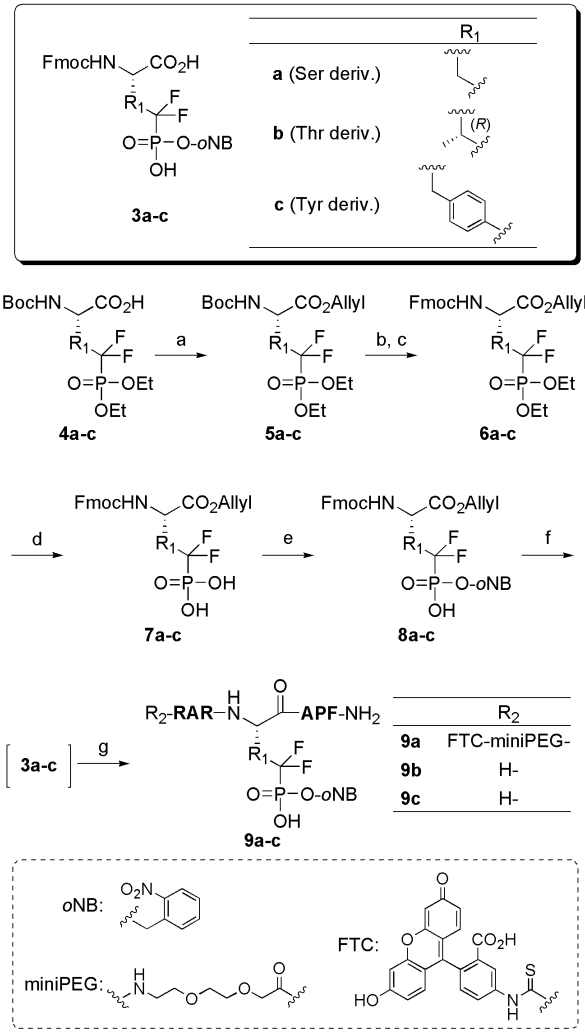
2. Results and discussion

2.1. Synthesis of caged non-hydrolyzable phosphoamino acid derivatives and their incorporation into peptides

Fmoc-protected non-hydrolyzable phosphoserine **3a** containing a photo-removable protection was first synthesized as shown in Scheme 3. In this study, an *o*-nitrobenzyl (*o*NB) group was chosen as the photo-responsive protective group because of its widespread use in a chemical biology field, simple structure without a chiral center and commercial availability.[3a,12] Carboxylic acid **4a**[4a] was treated with allyl bromide in the presence of K_2CO_3 to afford ester **5a**. The Boc group of **5a** was replaced by an Fmoc group to give **6a**. The ethyl groups of **6a** were removed by treatment with TMSBr in CH_2Cl_2 followed by aqueous MeCN. Monoesterification of phosphonate **7a** was then achieved by the use of a *p*-toluenesulfonyl chloride (TsCl)-pyridine system. Compound **8a** was used for a subsequent reaction without hesitation because of its insctability. Finally, the allyl group of **8a** was removed in the presence of palladium(0) to generate Fmoc-protected non-hydrolyzable phosphoserine **3a**. Purification of the product was highly complicated because of its high polarity, so crude **3a** without column chromatography purification was used in subsequent reactions.[13]

To examine the photo-reactivity and photo-control of the binding affinity of the phosphoserine mimetic-containing peptide, serine derivative **3a** was then incorporated into a model peptide. In this study, a ligand analog of a 14-3-3β protein[14] was synthesized because a phosphate moiety on the ligand is critical for binding[15,16] and the ligand sequence has been well studied.[17] The caged phosphopeptide mimetic **9a**, in which a phosphoserine of the original sequence was replaced by the caged non-hydrolyzable derivative, was prepared using standard Fmoc SPPS. For the coupling reaction, *O*-(benzotriazol-1-yl)-*N*,*N*,*N*',*N*'-tetramethyluronium hexafluorophosphate (HBTU) and *N*,*N*-diisopropylethylamine (DIEA) were used before introduction of serine derivative **3a**, whereas 2-(1*H*-7-azabenzotriaol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU), 1-hydroxyl-7-azabenzotriazole (HOAt), and DIEA were used for introduction of **3a**. An HBTU/DIEA system was used for further chain elongation.[18] After introduction of an 8-amino-3,6-dioxaoctanoyl (miniPEG) linker, a 5-fluoresceinylaminothiocarbonyl (FTC) group was incorporated by treatment with 5-fluorescein isothiocyanate (FITC) in the presence of DIEA for the subsequent fluorescence-based binding assay.

Fmoc-protected threonine derivative **3b** and tyrosine derivative **3c** were also prepared, starting from **4b**[5a,19] or **4c**[6a], similarly to the serine derivative **3a** except for the introduction of the *o*NB group on **7b**. When **7b** was treated with TsCl and pyridine, the yield of monoester **8b** was not sufficient but a mass spectrometric analysis of the crude reaction mixture gave a main peak identical to $[\mathbf{7b} - H_2O - H]^-$ (data not shown). We thought that dehydrative cyclization took place via electrophilic activation of the phosphonate by TsCl, therefore, *o*-NB bromide was employed to avoid the cyclization induced by TsCl. Crude **3b** and **3c**, without column chromatography purification, were successfully used in Fmoc SPPS to prepare model peptide **9b** and **9c**, respectively.



Scheme 3. Synthesis of caged non-hydrolyzable phosphoamino acid derivatives (*o*NB: *o*-nitrobenzyl). Reagents and conditions: a) allyl bromide, K_2CO_3 for **5a** and **5c** or Na_2CO_3 for **5b**, DMF, 97% (**5a**), 98% (**5b**), 85% (**5c**); b) TFA; c) *N*-(9*H*-fluoren-2-ylmethoxycarbonyloxy)succinimide (FmocOSu), MeCN, 10% (w/v) Na_2CO_3 aq, 60% (**6a**), 97% (**6b**), 77% (**6c**) (two steps); d) TMSBr, CH_2Cl_2, then MeCN aq, 97% (**7a**), 81% (**7b**), 51% (**7c**); e) *o*NB alcohol, TsCl, pyridine, DMF for **8a** and **8c**, or *o*NB bromide, DIEA, DMF, 50 ˚C for **8b**, 68% (**8a**), 84% (**8b**), 54% (**8c**); f) $(Ph_3P)_4Pd$, *N*-methylaniline,

THF; g) Fmoc SPPS using HBTU/DIEA or HATU/HOAt/DIEA system (**A**: alanine; **F**: phenylalanine; **P**: proline; **R**: arginine).

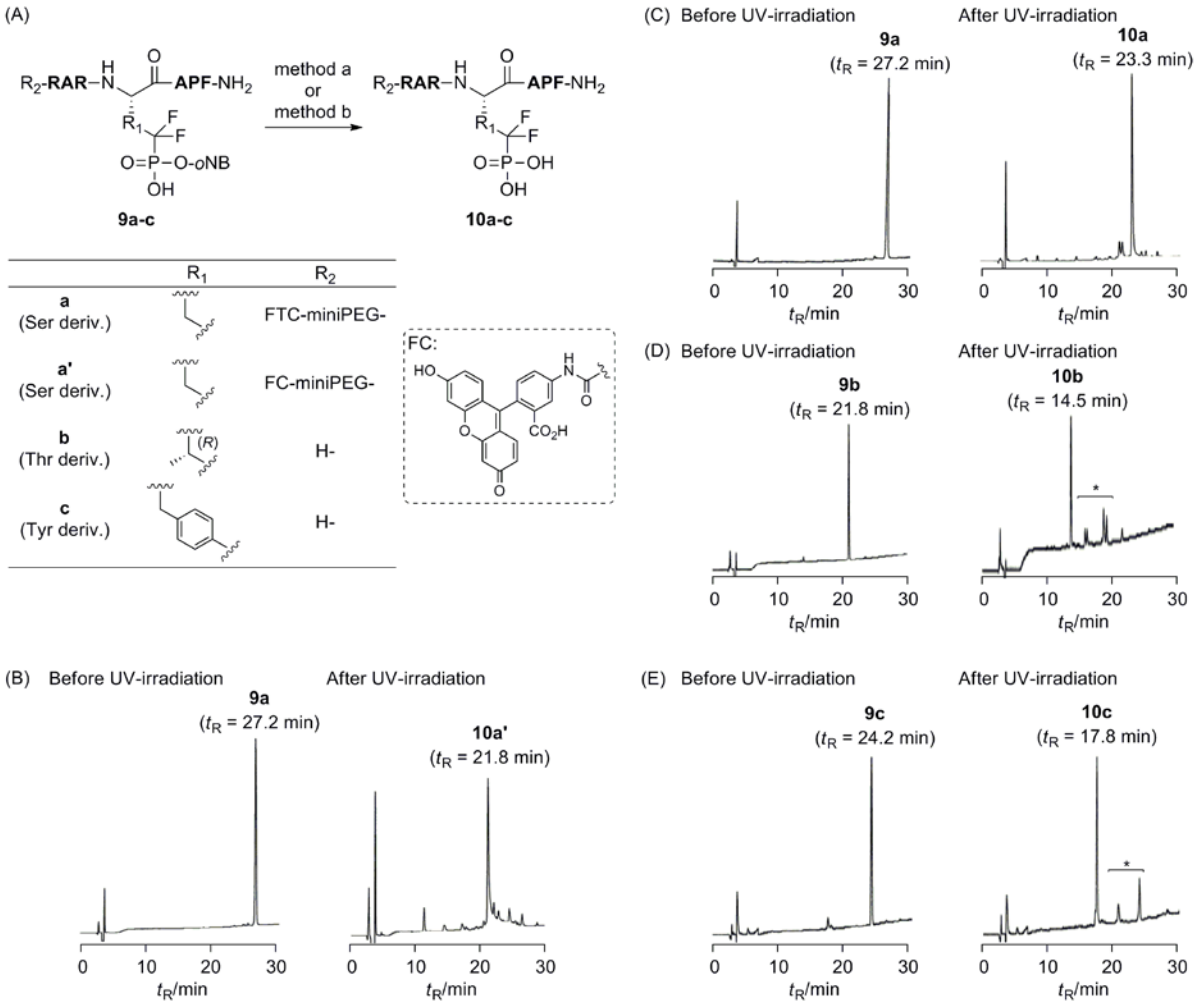


Figure 1. Photo-irradiation reactions. (A) Reagents and conditions: method (a) UV-irradiation (>365 nm, 7 min) in sodium phosphate buffer (10 mM, pH 7.6); method (b) UV irradiation (>365 nm, 7 min) in 0.1% (v/v) aqueous solution of TFA. (B)–(E) Analytical HPLC profiles of reaction of peptide **9a** (B: method a; C: method b), **9b** (D: method a), and **9c** (E: method a), before and after UV irradiation. *Non-peptidyl peaks. HPLC conditions: linear gradient of 0.1% (v/v) TFA/MeCN in 0.1% (v/v) TFA aq, 10–45% (B, C) or 5–45% (D, E) over 30 min.

2.2. UV-induced generation of non-hydrolyzable phosphopeptides

Next, UV-irradiation experiments were performed on caged peptide **9a** possessing the serine derivative (Figure 1A and 1B). Caged peptide **9a** in sodium phosphate buffer (pH 7.6) was subjected to UV irradiation (>365 nm). HPLC analysis showed that **9a** completely disappeared within 7 min on UV irradiation. The observed mass of the product, however, did not correspond to $[\mathbf{10a} + H]^+$ but to $[\mathbf{10a} + H - 16]^+$. The structure of the product was assumed to be **10a'** in which a sulfur atom of the thiocarbonyl moiety of **10a** was replaced by oxygen, presumably as a result of reaction with singlet oxygen generated via photo-sensitization by the FTC group.[20] Although the conversion of **10a** to **10a'** was not thought to be problematic for biological applications,[20c] the uncaging reaction was also examined under acidic conditions because the absorption coefficient of a fluorescein under acidic conditions is smaller than that under neutral conditions [0.1% (v/v) TFA in H_2O, pH 1.9; Figure 1C].[21] In this case, uncaged peptide **10a** instead of **10a'** was obtained in high conversion yield. UV-irradiation experiments were also performed on threonine derivative **9b** and tyrosine derivative **9c** in phosphate buffer (pH 7.6), and high-purity uncaged phosphopeptide mimetics **10b** and **10c** were generated (Figure 1D and 1E). These results encouraged us to examine whether photo-uncaging of the caged non-hydrolyzable phosphopeptide can control interactions with phosphopeptide-binding proteins.

2.3. Photo-control of phosphopeptide mimetics-protein interaction

Binding of the caged non-hydrolyzable phosphoserine derivative **9a** to 14-3-3β protein was examined (Figure 2). In this study, ΔmP values [ΔmP = (FP in the presence of 14-3-3β) – (FP in the absence of 14-3-3β), where FP is the fluorescence polarization signal in mP (milli-polarization units)] before and after UV irradiation were compared. If the peptide can bind to 14-3-3β protein, the FP increases on addition of the protein. The ΔmP value should therefore be positive. When 14-3-3β was added to a solution of caged peptide **9a** in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (pH 7.4),[22] ΔmP was close to zero, similar to that for the negative control **11** (**9a**: –0.2 ± 3.6 mP; **11**: 5.2 ± 0.7 mP). In contrast, large ΔmP values were observed in the case of UV-irradiated **9a** (>365 nm, 7 min)[23] and positive control **10a** (UV-irradiated **9a**: 18.9 ± 1.6 mP; **10a**: 39.4 ± 1.5 mP) compared with those for **9a** without UV irradiation and negative control **11**, respectively. Reason for a difference of ΔmP values of **9a (UV+)** and **10a** is not clear at present. However, in these experiments, the point is that the ΔmP

value of **9a** was increased by the UV irradiation. It was therefore demonstrated that binding of the non-hydrolyzable phosphopeptide to 14-3-3β was controlled by UV irradiation.

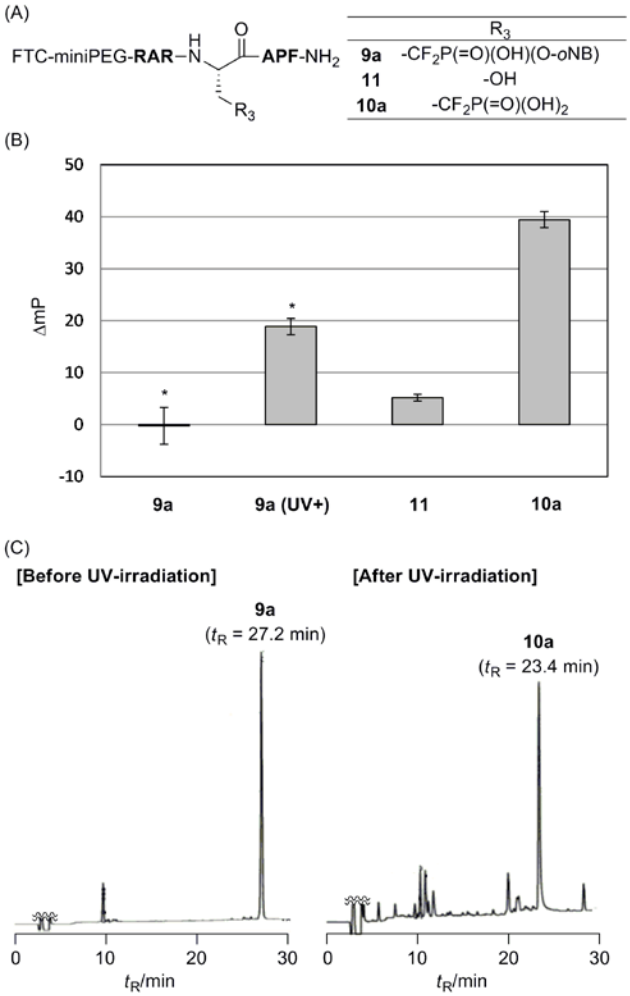


Figure 2. Binding assay based on FP. (A) Structures of caged non-hydrolyzable peptide and reference peptides and (B) binding assays of peptides to 14-3-3β. The peptide (20 nM, final) in HEPES buffer [10 mM HEPES, 150 mM NaCl, 0.05% (w/v) Tween-20, 0.5 mM dithiothreitol, pH 7.4] was incubated at 20 ˚C in the absence or presence of 14-3-3β (500 nM, final) for 1 h.[22] The FP signals were recorded in mP (milli-polarization units), and the ΔmP values were calculated as follows. ΔmP = (FP in the presence of 14-3-3β) – (FP in the absence of 14-3-3β). Averages of nine measurements are shown with the standard error of the mean. A statistical analysis was performed using the *t* test (**p*<0.005). **9a (UV+)**: UV (>365 nm) was irradiated to **9a** in the HEPES buffer for 7 min before the FP experiments. (C) HPLC monitoring of preparation of **9a (UV+)** for subsequent binding assay. HPLC charts before and after UV irradiation to caged peptide **9a** in HEPES buffer are depicted. Reaction conditions are shown above and in the experimental section. Analytical HPLC conditions: linear gradient of 0.1% (v/v) TFA/MeCN in 0.1% (v/v) TFA aq, 10 to 45% over 30 min.

3. Conclusion

Caged non-hydrolyzable phosphoserine, phosphothreonine, and phosphotyrosine derivatives were synthesized and incorporated into peptides using standard Fmoc SPPS. UV-induced deprotection of the caged peptides generated the parent non-hydrolyzable phosphopeptide mimetics in high purity. The binding assay of the non-hydrolyzable phosphoserine-containing peptide to 14-3-3β protein was also examined, and the affinity was successfully controlled by UV irradiation. Applications of the caged non-hydrolyzable phosphopeptide mimetics to tools for photo-control the phosphopeptide-protein interaction events are being investigated.

4. Experimental Section

4.1. General Methods

All reactions of small molecules were carried out under a positive pressure of argon. For column chromatography, Silica Gel 60 N (spherical, neutral, Kanto Chemical Co.,Inc.) was employed. NMR spectra were recorded for ^{1}H and ^{31}P (proton decoupled) and ^{13}C (signals of difluoromethylene units sometimes were not detected). For HPLC separations, a Cosmosil 5C_{18}-AR-II analytical column (Nacalai Tesque, 4.6 × 250 mm, flow rate 1.0 mL/min) or a Cosmosil 5C_{18}-AR-II preparative column (Nacalai Tesque, 20 × 250 mm, flow rate 10.0 mL/min) was employed, and eluting products were detected by UV at 220 nm. A solvent system consisting of 0.1% (v/v) TFA in H_2O (solvent A) and 0.1% (v/v) TFA in MeCN (solvent B) was used for HPLC elution. UV-irradiation was performed using a Moritex MUV-202U (3000 mW/cm^2 HgeXe lamp) with the filtered output (>365 nm) and the irradiation power was adjusted to one third of the maximum.

4.2. Preparation of caged non-hydrolyzable phosphoamino acid derivatives

4.2.1. Allyl esters 5a-c

Typical procedure for preparation of **5a**: Potassium carbonate (121 mg, 0.880 mmol) and allyl bromide (76 μL, 0.880 mmol) were added to a solution of carboxylic acid **4a**[4a] (300 mg, 0.800 mmol) in DMF (4.0 mL). The resulting mixture was stirred at room temperature overnight and was quenched by the addition of water. The mixture was extracted with Et_2O. The obtained organic layer was washed with brine, dried over $MgSO_4$, and concentrated *in vacuo*. The resulting crude product was purified by column chromatography (hexanes/EtOAc = 4/1 (v/v)) and 316 mg of allyl ester **5a** (0.760 mmol, 94%) was obtained as a pale yellow oil.

4.2.1.1. (*S*)-Allyl 2-{(tert-butoxycarbonyl)amino}-4-(diethoxyphosphoryl)-4,4-difluorobutanoate (5a)

Pale yellow oil; 316 mg; 94% yield; $[\alpha]^{19}_{D}$ 0.03 (*c* 1.0, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz) δ = 1.38 (6H, t, *J* = 7.2 Hz), 1.44 (9H, s), 2.53-2.80 (2H, m), 4.27 (4H, q, *J* = 7.2 Hz), 4.58-4.63 (1H, br m), 4.65 (2H, d, *J* = 6.0 Hz), 5.26 (1H, d, *J* = 10.4 Hz), 5.34 (1H, d, *J* = 16.4 Hz), 5.90 (1H, ddt, *J* = 16.4, 10.4 and 6.0 Hz); ^{13}C NMR ($CDCl_3$, 75 MHz) δ = 16.2 (d, *J* = 5.4 Hz), 28.2, 35.2 (td, *J* = 19.5 and 15.4 Hz), 48.4, 64.7 (d, *J* = 7.0 Hz), 66.3, 80.1, 118.8, 131.1, 154.9, 170.6; ^{31}P NMR ($CDCl_3$, 162 MHz) δ = –5.82 (t, *J* = 105.7 Hz); HRMS (ESI-TOF) *m/z* calcd for $C_{16}H_{28}F_2NNaO_7P$ ([M + Na]$^+$) 438.1469, found 438.1479.

4.2.1.2. (2*S*,3*R*)-Allyl 2-{(tert-butoxycarbonyl)amino}-4-(diethoxyphosphoryl)-4,4-difluoro-3-methylbutanoate (5b)

Preparation of substrate **4b**, see reference 5a. Na_2CO_3 was used instead of K_2CO_3; colorless oil; 11.3 mg; 98% yield; $[\alpha]^{27}_{D}$ 17.5 (*c* 2.0, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz) δ =1.30 (3H, d, *J* = 7.2 Hz), 1.37 (6H, t, *J* = 7.0 Hz), 1.45 (9H, s), 3.13 (1H, m), 4.26 (4H, m), 4.54 (1H, dd, *J* = 9.8 and 3.4 Hz), 4.63 (2H, ddd, *J* = 5.9, 1.5 and 1.5 Hz), 5.24 (1H, ddt, *J* = 11.7, 1.5 and 1.5 Hz), 5.34 (1H, ddt, *J* = 17.2, 1.5 and 1.5 Hz), 5.44 (1H, d, *J* = 9.8 Hz), 5.90 (1H, ddt, *J* = 17.2, 11.7 and 5.9 Hz); ^{13}C NMR ($CDCl_3$, 75

MHz) δ =12.0 (m), 16.3 (d, *J* = 1.2 Hz), 16.4 (d, *J* = 1.2 Hz), 28.3, 40.2 (m), 54.6 (m), 64.8 (m), 66.2, 80.1, 118.7, 131.6, 156.0, 170.6; ^{31}P NMR ($CDCl_3$, 162 MHz) δ = 5.54 (dd, *J* = 109.6 and 104.1 Hz); HRMS (ESI-TOF) *m/z* calcd for $C_{17}H_{30}F_2NNaO_7P$ ($[M + Na]^+$) 452.1626, found 452.1614.

4.2.1.3. (*S*)-Allyl 2-{(tert-butoxycarbonyl)amino}-3-[4-{(diethoxyphosphoryl)difluoromethyl}-phenyl]propanoate (5c)

Preparation of substrate **4c**, see reference 6a; Colorless oil; 263 mg; 85% yield; $[\alpha]^{27}_D$ –5.06 (*c* 1.4, MeOH); ^{1}H NMR ($CDCl_3$, 400 MHz) δ = 1.31 (6H, t, *J* = 7.2 Hz), 1.42 (9H, s), 3.10 (1H, dd, *J* = 18.2 and 7.6 Hz), 3.19 (1H, dd, *J* = 18.2 and 7.2 Hz), 4.07-4.32 (4H, m), 4.59 (2H, d, *J* = 5.7 Hz), 4.62 (1H, m), 5.02 (1H, d, *J* = 7.5 Hz), 5.25 (1H, d, *J* = 8.1 Hz), 5.30 (1H, d, *J* = 17.3 Hz), 5.85 (1H, ddt, *J* = 17.3, 8.1 and 5.7 Hz), 7.23 (2H, d, *J* = 8.1 Hz), 7.55 (2H, d, *J* = 8.1 Hz); ^{13}C NMR ($CDCl_3$, 75 MHz) δ = 16.1 (d, *J* = 5.0 Hz), 28.0, 37.8, 54.1, 64.5 (d, *J* = 6.2 Hz), 65.8, 79.7, 117.8 (td, *J* = 259.6 and 216.3 Hz), 118.8, 126.1, 129.3, 131.1 (m), 131.2, 139.1, 154.8, 171.0; ^{31}P NMR ($CDCl_3$, 162 MHz) δ = –6.32 (t, *J* = 115.6 Hz); HRMS (ESI-TOF) *m/z* calcd for $C_{22}H_{32}F_2KNO_7P$ ($[M + K]^+$) 530.1522, found 530.1526.

4.2.2. Fmoc derivatives 6a-c

Typical procedure for preparation of **6a**: To allyl ester **5a** (313 mg, 0.760 mmol) was added TFA (6.0 mL) with stirring at room temperature for 2 h. The mixture was concentrated *in vacuo*, and the residue was dissolved in MeCN/10% (w/v) Na_2CO_3 aq. (2/1 (v/v), 14 mL). To the solution was added FmocOSu (279 mg, 0.828 mmol) and the reaction mixture was stirred at room temperature overnight. After addition of 5% (w/v) $KHSO_4$ aq., the mixture was extracted with EtOAc. The organic layer was washed with brine, dried over $MgSO_4$, and concentrated *in vacuo*. The obtained crude material was purified by column chromatography (hexanes/EtOAc = 4/1 (v/v)) and 311 mg of Fmoc derivative **6a** (0.579 mmol, 77 %) was obtained as a colorless oil.

4.2.2.1. (*S*)-Allyl 2-([{(9*H*-fluoren-9-yl)methoxy}carbonyl]amino)-4-(diethoxyphosphoryl)-4,4-difluorobutanoate (6a)

Colorless oil; 311 mg; 77% yield; $[\alpha]^{19}_D$ 3.10 (*c* 0.86, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz) δ = 1.38 (6H, td, *J* = 7.1 and 2.2 Hz), 2.58-2.82 (2H, m), 4.20-4.32 (5H, m), 4.37 (2H, d, *J* = 7.1 Hz), 4.67 (2H, d, *J* = 5.4 Hz), 4.65-4.78 (1H, m), 5.26 (1H, dd, *J* = 10.5 and 1.2 Hz), 5.35 (1H, dd, *J* = 17.3 and 1.2 Hz), 5.76 (1H, d, *J* = 8.3 Hz), 5.91 (1H, ddt, *J* = 17.3, 10.5 and 5.4 Hz), 7.31 (2H, td, *J* = 7.3 and 1.2 Hz), 7.40 (2H, t, *J* = 7.3 Hz), 7.60 (2H, m), 7.76 (2H, d, *J* = 7.3 Hz); ^{13}C NMR ($CDCl_3$, 75 MHz) δ = 16.3 (d, *J* = 5.6 Hz), 35.5 (d, *J* = 16.2 Hz), 47.0, 48.9, 64.9 (dt, *J* = 6.2 and 3.7 Hz), 66.6, 67.4, 119.1, 119.9, 125.1, 127.1, 127.7, 131.3, 141.2, 143.7, 155.6, 170.3; ^{31}P NMR ($CDCl_3$, 162 MHz) δ = –5.76 (t, *J* = 104.7 Hz); HRMS (ESI-TOF) *m/z* calcd for $C_{26}H_{30}F_2NNaO_7P$ ($[M + Na]^+$) 560.1626, found 560.1628.

4.2.2.2. (2*S*,3*R*)-Allyl 2-([{(9*H*-fluoren-9-yl)methoxy}carbonyl]amino)-4-(diethoxyphosphoryl)-4,4-difluoro-3-methylbutanoate (6b)

Colorless oil; 137 mg; 97% yield; $[\alpha]^{23}_D$ 12.0 (*c* 2.91, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz) δ = 1.35 (3H, d, *J* = 7.4 Hz), 1.42 (6H, td, *J* = 7.1 and 2.7 Hz), 3.21 (1H, m), 4.23-4.60 (7H, m), 4.61-4.74 (3H, m), 5.28 (1H, dd, *J* = 10.4 and 1.2 Hz), 5.38 (1H, dd, *J* = 17.2 and 1.2 Hz), 5.94 (1H, ddt, *J* = 17.2, 10.4 and 5.8 Hz), 6.01 (1H, d, *J* = 9.8 Hz), 7.33 (1H, t, *J* = 7.5 Hz), 7.34 (1H, t, *J* = 7.5 Hz), 7.42 (2H, t, *J* = 7.5 Hz), 7.64 (1H, d, *J* = 7.5 Hz), 7.66 (1H, d, *J* = 7.5 Hz), 7.79 (2H, d, J = 7.5 Hz); ^{13}C NMR ($CDCl_3$, 75 MHz) δ = 12.1 (t, *J* = 9.8 Hz), 16.3, 16.4, 40.5 (m), 47.1, 55.0 (m), 64.8 (d, *J* = 7.5 Hz), 65.0 (d, *J* = 6.8 Hz), 66.3, 67.4, 118.9, 119.9, 125.2, 125.2, 127.0, 127.1, 127.7, 127.7, 131.5, 141.3, 143.7, 143.9, 156.6, 170.2; ^{31}P NMR ($CDCl_3$, 162 MHz) δ = 5.51 (dd, *J* = 109.2 and 103.5 Hz); HRMS (ESI-TOF) *m/z* calcd for $C_{27}H_{32}F_2NNaO_7P$ ($[M + Na]^+$) 574.1782, found 574.1786.

4.2.2.3. (*S*)-Allyl 2-([{(9*H*-fluoren-9-yl)methoxy}carbonyl]amino)-3-[4-{(diethoxyphosphoryl)-difluoromethyl}phenyl]propanoate (6c)

Colorless oil; 518 mg; 55% yield; $[\alpha]^{27}_D$ –7.58 (*c* 1.0, MeOH); ^{1}H NMR ($CDCl_3$, 400 MHz) δ = 1.30 (6H, t, *J* = 7.1 Hz), 3.15 (1H, dd, *J* = 14.0 and 5.8 Hz), 3.20 (1H, dd, *J* = 14.0 and 5.8 Hz), 4.10-4.25 (5H, m), 4.38 (1H, dd, *J* = 10.5 and 7.1 Hz), 4.45 (1H, dd, *J* = 10.5 and 7.1 Hz), 4.64 (2H, d, *J* = 5.9 Hz), 4.70 (1H, dt, J = 8.0 and 5.8 Hz), 5.27 (1H, d, *J* = 11.2 Hz), 5.83 (1H, d, *J* = 17.6 Hz), 5.85 (1H, ddt, *J* = 17.6, 11.2 and 5.9 Hz), 7.20 (2H, d, *J* = 8.1 Hz), 7.32 (2H, t, *J* = 7.4 Hz), 7.41 (2H, t, *J* = 7.4 Hz), 7.55 (2H, d, *J* = 8.1 Hz), 7.57 (2H, d, *J* = 7.4 Hz), 7.72 (2H, d, *J* = 7.4 Hz); ^{13}C NMR ($CDCl_3$, 75 MHz) δ = 16.3 (d, *J* = 6.9 Hz), 37.9, 47.1, 54.7, 64.8 (d, *J* = 6.9 Hz), 66.2, 66.9, 119.3, 120.0, 125.0 (d, *J* = 4.4 Hz), 126.5 (td, *J* = 22.0 and 14.4 Hz), 127.1, 127.8, 129.5, 1310, 131.4 (td, *J* = 21.8 and 13.7 Hz), 139.0, 141.3, 143.7, 143.8, 155.6, 170.1; ^{31}P NMR ($CDCl_3$, 162 MHz) δ = –6.35 (t, *J* = 115.8 Hz); HRMS (ESI-TOF) *m/z* calcd for $C_{32}H_{35}F_2NO_7P$ ($[M + H]^+$) 614.2119, found 614.2128.

4.2.3. Phosphonates 7a-c

Typical procedure for preparation of **7a**: Trimethylsilyl bromide (22 mL, 163 mmol) was added to a solution of diethyl ester **6a** (4.39 g, 8.17 mmol) in CH_2Cl_2 (162 mL), and the mixture was stirred at room temperature for 14 h. The reaction progress was monitored using ^{31}P NMR. After addition of H_2O/MeCN, the mixture was concentrated *in vacuo*. The crude material was purified by reprecipitation from hexanes and 3.39 g of phosphonate **7a** (7.04 mmol, 86%) was obtained as a white solid.

4.2.3.1. (*S*)-{3-([{(9*H*-Fluoren-9-yl)methoxy}carbonyl]amino)-4-(allyloxy)-1,1-difluoro-4-oxobutyl}phosphonic acid (7a)

White solid; 3.39 g; 86% yield; $[\alpha]^{27}_D$ –16.3 (*c* 0.90, MeOH); ^{1}H NMR (methanol-d_4, 400 MHz) δ = 2.46-2.85 (2H, m), 4.23 (1H, t, *J* = 7.1 Hz), 4.31 (2H, d, *J* = 7.1 Hz), 4.64 (2H, d, *J* = 5.6 Hz), 4.63-4.81 (1H, m), 5.20 (2H, dd, *J* = 10.5 and 1.3 Hz), 5.32 (2H, dd, *J* = 17.9 and 1.3 Hz), 5.93 (1H, ddt, *J* = 17.9, 10.5 and 5.6 Hz), 7.30 (2H, td, *J* = 7.3 and 1.2 Hz), 7.38 (2H, t, *J* = 7.3 Hz), 7.66 (2H, d, *J* = 7.3 Hz), 7.79 (2H, d, *J* = 7.3 Hz); ^{13}C NMR (methanol-d_4, 75 MHz) δ = 34.3 (m), 48.2, 48.8, 65.7, 66.7, 117.1, 119.4, 124.8, 126.7, 127.3, 131.6, 141.0, 143.7, 156.7, 170.9; ^{31}P NMR (methanol-d_4, 162 MHz) δ = 4.81 (t, *J* = 102.7 Hz); HRMS (ESI-TOF) *m/z* calcd for $C_{22}H_{22}F_2NNaO_7P$ ($[M + Na]^+$) 504.1000, found 504.0994.

4.2.3.2. {(2*R*,3*S*)-3-([{(9*H*-Fluoren-9-yl)methoxy}carbonyl]amino)-4-(allyloxy)-1,1-difluoro-2-methyl-4-oxobutyl}phosphonic acid (7b)

For purification, column chromatography ($CHCl_3$/methanol = 99/1 (v/v)) was employed instead of reprecipitation; colorless oil ; 29 mg; 81% yield; $[\alpha]^{22}_D$ 10.1 (*c* 2.53, MeOH); ^{1}H NMR (methanol-d_4, 400 MHz) δ = 1.12 (3H, d, *J* = 6.5 Hz), 2.93 (1H, m), 4.15 (1H, m), 4.24 (2H, m), 4.50 (1H, m), 4.54 (2H, d, *J* = 5.6 Hz), 5.11 (1H, dd, *J* = 10.6 and 1.4 Hz), 5.25 (1H, dd, *J* = 17.3 and 1.4 Hz), 5.85 (1H, ddt, *J* = 17.3, 10.6 and 5.9 Hz), 7.21 (2H, t, *J* = 7.4 Hz), 7.29 (2H, t, *J* = 7.4 Hz), 7.58 (1H, d, *J* = 7.4 Hz), 7.60 (1H, d, *J* = 7.4 Hz), 7.70 (2H, d, *J* = 7.4 Hz); ^{13}C NMR

(methanol-d_4, 75 MHz) δ = 12.4 (t, J = 8.6 Hz), 41.6 (m), 48.3, 56.0 (t, J = 12.4 Hz), 67.0, 68.3, 118.8, 120.9, 126.3, 126.3, 128.2, 128.8, 133.2, 142.5, 145.1, 145.2, 158.6, 171.9; ^{31}P NMR (methanol-d_4, 162 MHz) δ = 4.50 (d, J = 103.2 Hz); HRMS (ESI-TOF) *m/z* calcd for $C_{23}H_{23}F_2NO_7P$ ([M – H]$^-$) 494.1180, found 494.1180.

4.2.3.3. (*S*)-([4-{2-([{(9*H*-Fluoren-9-yl)methoxy}carbonyl]amino)-3-(allyloxy)-3-oxopropyl}-phenyl]difluoromethyl)phosphonic acid (7c)

White powder; 465 mg; 52% yield; $[\alpha]^{27}_D$ –4.30 (*c* 1.6, MeOH); ^{1}H NMR (methanol-d_4, 400 MHz) δ = 3.02 (1H, dd, J = 13.7 and 9.3 Hz), 3.21 (1H, dd, J = 13.7 and 5.0 Hz), 4.14 (1H, t, J = 6.5 Hz), 4.29 (2H, t, J = 6.5 Hz), 4.49 (1H, dd, J = 9.3 and 5.0 Hz), 4.60 (2H, d, J = 5.5 Hz), 5.20 (1H, d, J =10.7 Hz), 5.29 (1H, d, J = 17.3 Hz), 5.88 (1H, ddt, J = 17.3, 10.7 and 5.5 Hz), 7.25-7.40 (6H, m), 7.57 (2H, t, J = 8.2 Hz), 7.59 (2H, d, J = 7.5 Hz), 7.76 (2H, d, J = 7.5 Hz); ^{13}C NMR (methanol-d_4, 75 MHz) δ = 38.1, 48.2, 56.7, 66.8, 67.9, 118.8, 119.8 (td, J = 260.3 and 211.8 Hz), 120.9, 126.1, 127.5 (t, J = 6.2 Hz), 128.1, 128.7, 130.2, 133.0, 133.6 (td, J = 22.4 and 13.1 Hz), 140.1, 142.5, 1450, 145.0, 158.2, 172.8; ^{31}P NMR (methanol-d_4, 162 MHz) δ = -4.9 (t, J = 113.7 Hz); HRMS (ESI-TOF) *m/z* calcd for $C_{28}H_{26}F_2KNO_7P$ ([M + K]$^+$) 596.1052, found 596.1051.

4.2.4. *o*-Nitrobenzyl esters 8a and 8c

Typical procedure for preparation of **8a**: To a solution of phosphonate **7a** (50.0 mg, 104 μmmol) in DMF (520 μL), pyridine (20 μL) and *p*-toluenesulfonyl chloride (99.2 mg, 520 μmol) were added. After stirring at room temperature for 15 min followed by addition of *o*-nitrobenzyl alcohol (79.5 mg, 519 μmol), the reaction mixture was stirred at room temperature for additional 26 h. The crude material was concentrated *in vacuo*, and the obtained mixture was purified by column chromatography ($CHCl_3$/MeOH = 49/1 (v/v)). *o*-Nitrobenzyl ester **8a** (43.7 mg, 70.9 μmol, 68%) was obtained as a colorless oil.

4.2.4.1. (2*S*)-Allyl 2-([{(9*H*-fluoren-9-yl)methoxy}carbonyl]amino)-4,4-difluoro-4-[hydroxy-{(2-nitrobenzyl)oxy}phosphoryl]butanoate (8a)

Colorless oil; 43.7 mg; 68% yield; $[\alpha]^{27}_D$ –7.43 (*c* 0.92, MeOH); ^{1}H NMR (methanol-d_4, 400 MHz) δ = 2.35-2.77 (2H, m), 4.10 (1H, t, J = 7.1 Hz), 4.18-4.21 (2H, m), 4.50-4.59 (3H, m), 5.14 (1H, dt, J = 10.5 and 1.4 Hz), 5.23 (1H, dt, J = 17.2 and 1.4 Hz), 5.37 (2H, d, J = 7.0 Hz), 5.82 (1H, ddt, J = 17.2, 10.5 and 5.6 Hz), 7.20 (2H, t, J = 7.5 Hz), 7.28 (2H, t, J = 7.5 Hz), 7.38 (1H, t, J = 7.8 Hz), 7.57 (2H, d, J = 7.5 Hz), 7.61 (1H, dd, J = 7.8 and 7.5 Hz), 7.68 (2H, d, J = 7.5 Hz), 7.89 (1H, d, J = 7.8 Hz), 8.00 (1H, d, J = 7.5 Hz); ^{31}P NMR (methanol-d_4, 162 MHz) δ = 3.58 (t, J = 90.7 Hz); HRMS (ESI-TOF) *m/z* calcd for $C_{29}H_{26}F_2N_2O_9P$ ([M – H]$^-$) 615.1344, found 615.1346. Because time dependent decomposition of **8a** was observed, it was used without hesitation for a subsequent reaction after measurement of ^{1}H and ^{31}P NMR followed by HRMS and optical rotation. In this experiment, time-consuming ^{13}C NMR was not recorded.

4.2.4.2. (2*S*)-Allyl 2-([{(9*H*-fluoren-9-yl)methoxy}carbonyl]amino)-3-{4-(difluoro[hydroxy{(2-nitrobenzyl)oxy}phosphoryl]methyl)phenyl}propanoate (8c)

White powder; 42.0 mg; 54% yield; $[\alpha]^{27}_D$ –6.10 (*c* 0.76, MeOH); ^{1}H NMR (methanol-d_4, 400 MHz) δ = 3.01 (1H, dd, J = 14.0 and 9.2 Hz), 3.20 (1H, dd, J = 14.0 and 5.1 Hz), 4.15 (1H, t, J = 6.9 Hz), 4.29 (2H, d, J = 6.9 Hz), 4.49 (1H, dd, J = 9.2 and 5.1 Hz), 4.62 (2H, d, J = 5.6 Hz), 5.21 (1H, ddt, J = 10.5, 1.4 and 1.1 Hz), 5.28-5.37 (3H, m), 5.91 (1H, ddt, J = 16.9, 10.5 and 5.6 Hz), 7.22-7.34 (4H, m), 7.47 (1H, t, J = 7.9 Hz), 7.54-7.64 (4H, m), 7.68 (1H, t, J = 7.7 Hz), 7.78 (2H, d, J = 7.6 Hz), 7.96 (1H, d, J = 7.7 Hz), 8.06 (1H, d, J = 7.9 Hz); ^{13}C NMR (pyridine-d_5, 75 MHz) δ =37.4, 47.3, 55.9, 65.3 (d, J = 9.1 Hz), 65.4, 66.3, 118.0, 120.0, 124.4, 125.2, 125.2, 126.8 (br m), 127.1, 127.7, 127.8, 128.7, 129.0, 132.1, 133.7, 139.1, 141.3, 114.0, 144.1, 146.7, 156.7, 171.7; ^{31}P NMR (pyridine-d_5, 162 MHz) δ = 4.44 (t, J =98.1 Hz); HRMS (ESI-TOF) *m/z* calcd for $C_{35}H_{30}F_2N_2O_9P$ ([M – H]$^-$) 691.1657, found 691.1656.

4.2.5. *o*-Nitrobenzyl esters 8b

To a solution of phosphonate **7b** (24.8 mg, 50.1 μmmol) in DMF (500 μL) were added *o*-nitrobenzyl bromide (43.0 mg, 200 μmol) and DIEA (19.1 μL, 110 μmol). After stirring at 50 ˚C for 3 h, the mixture was concentrated *in vacuo*. Following to purification of the obtained crude material by column chromatography ($CHCl_3$/MeOH = 49/1 (v/v)), *o*-nitrobenzyl ester **8b** (26.6 mg, 42.2 μmol, 84%) was obtained as a white powder.

4.2.5.1. (2*S*,3*R*)-Allyl 2-([{(9*H*-fluoren-9-yl)methoxy}carbonyl]amino)-4,4-difluoro-4-[hydroxy{(2-nitro-benzyl)oxy}phosphoryl]-3-methylbutanoate (8b)

$[\alpha]^{16}_D$ 4.22 (*c* 1.2, MeOH); ^{1}H NMR (methanol-d_4, 400 MHz) δ = 1.24 (3H, d, J = 7.2 Hz), 4.08-4.18 (1H, m), 4.19-4.23 (2H, m), 4.44 (1H, m), 4.53 (2H, m), 5.09 (1H, ddt, J = 10.6, 1.4 and 1.4 Hz), 5.24 (1H, ddt, J = 17.2, 1.4 and 1.4 Hz), 5.37 (2H, d, J = 7.1 Hz), 5.83 (1H, ddt, J = 17.2, 10.6 and 5.8 Hz), 7.20 (2H, t, J = 7.6 Hz), 7.28 (2H, t, J = 7.6 Hz), 7.40 (1H, t, J = 7.7 Hz), 7.56-7.66 (3H, m), 7.69 (2H, d, J = 7.6 Hz), 7.92 (1H, d, J = 7.7 Hz), 8.02 (1H, d, J = 8.1 Hz); ^{31}P NMR (methanol-d_4, 162 MHz) δ = 2.91 (t, J =91.5 Hz); HRMS (ESI-TOF) *m/z* calcd for $C_{30}H_{28}F_2N_2O_9P$ ([M – H]$^-$) 629.1501, found 629.1491. Because time dependent decomposition of **8b** was observed, it was used without hesitation for a subsequent reaction after measurement of ^{1}H and ^{31}P NMR followed by HRMS and optical rotation. In this experiment, time-consuming ^{13}C NMR was not recorded.

4.2.6. Non-hydrolyzable amino acid derivatives 3a-c

Typical procedure for preparation of **3a**: *N*-Methylaniline (241 mg, 2.24 mmol) and $(Ph_3P)_4Pd$ (25.9 mg, 22.4 μmol) were added to a solution of allyl ester **8a** (138 mg, 224 μmol) in THF (4.48 mL), and the resulting mixture was stirred at room temperature for 4 h. After addition of 1 M HCl aq., the mixture was extracted with CH_2Cl_2. The organic layer was washed with water followed by brine, dried over Na_2SO_4, and concentrated *in vacuo*. The obtained crude material was used to a subsequent reaction without further purification.

4.2.6.1. (2*S*)-2-([{(9*H*-Fluoren-9-yl)methoxy}carbonyl]amino)-4,4-difluoro-4-[hydroxy{(2-nitrobenzyl)oxy}phosphoryl]butanoic acid (3a)

White powder; 123 mg; HRMS (ESI-TOF) *m/z* calcd for $C_{26}H_{23}F_2N_2NaO_9P$ ([M + Na]$^+$) 599.1007, found 599.0990.

4.2.6.2. (2*S*,3*R*)-2-([{(9*H*-Fluoren-9-yl)methoxy}carbonyl]amino)-4,4-difluoro-4-[hydroxy{(2-nitrobenzyl)oxy}phosphoryl]-3-methylbutanoic acid (3b)

White powder; 26.2 mg; HRMS (ESI-TOF) *m/z* calcd for $C_{27}H_{24}F_2N_2O_9P$ ([M – H]$^-$) 589.1188, found 589.1184.

4.2.6.3. (2*S*)-2-([{(9*H*-Fluoren-9-yl)methoxy}carbonyl]amino)-3-{4-(difluoro[hydroxy{(2-nitro-

benzyl)oxy}phosphoryl]methyl)phenyl}propanoic acid (3c)

White powder; 53.0 mg; HRMS (ESI-TOF) *m/z* calcd for $C_{32}H_{28}F_2N_2O_9P$ ([M + H]$^+$) 653.1501, found 653.1487.

4.3. Preparation of caged peptides 9a-c

Typical procedure for preparation of **9a**: On NovaSyn® TGR resin (loading: 0.22 mmol/g, 30 mg, 6.6 μmol) were coupled Fmoc protected amino acids (3.0 eq.) in the presence of *O*-(benzotriazol-1-yl)-*N*,*N*,*N*',*N*'-tetramethyluronium hexafluorophosphate (HBTU, 2.9 eq.) and *N*,*N*-diisopropylethyamine (DIEA, 2.9 eq.) in DMF at room temperature for 0.5 h before coupling of the caged amino acid. Incorporation of crude **3a** (7.6 mg, <2.0 eq.) was achieved by the use of 2-(1*H*-7-azabenzotriaol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU, 1.95 eq.) in the presence of DIEA (4.0 eq.) and 1-hydroxyl-7-azabenzotriazole (HOAt, 2.2 eq.) in DMF at room temperature for 4 h. After acetyl capping (10 eq. of acetic anhydride and 10 eq. of pyridine in DMF, 0.5 h at room temperature), following couplings were performed for elongation of a peptide using an Fmoc protected amino acid (5.0 eq.), HBTU (4.5 eq.) and DIEA (10 eq.) in DMF at room temperature for 1 h. Then Fmoc-8-amino-3,6-dioxaoctanoic acid (Fmoc-miniPEG-OH, 3.0 eq.) was incorporated using HBTU (2.9 eq.) and DIEA (2.9 eq.) in DMF for 1 h at room temperature. After removal of *N*-terminal Fmoc group of the miniPEG, the resin was reacted with fluorescein isomer I (FITC, 2.5 eq.) and DIEA (2.5 eq.) in DMF for 1 h twice. The resulted complete resin was treated with TFA/*m*-cresol/thioanisole/triethylsilane/H_2O (80/5/5/5/5 (v/v)) at room temperature for 2 h. After the resin was filtered off, cooled diethyl ether was added to the filtrate, and the resulting precipitate was collected by centrifugation. The obtained precipitate was washed with diethyl ether and purified by preparative HPLC to give caged peptide **9a** (4.6 mg, 44%) as a yellowish lyophilized powder. Analytical HPLC conditions: a linear gradient of solvent B in solvent A over 30 min. Preparative HPLC conditions: a linear gradient of solvent B in solvent A over 30 min.

4.3.1. Peptide 9a

Yellow lyophilized powder; 4.60 mg; 44% yield; Analytical HPLC conditions: 10 to 45% over 30 min. Retention time = 27.2 min; Preparative HPLC conditions: 10 to 35% over 50 min; LRMS (ESI-TOF) *m/z* calcd for [M + H]$^+$ 1586.6, found; 1586.7.

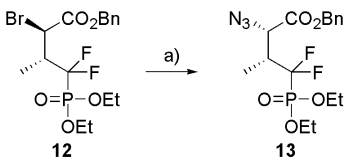
4.3.2. Peptide 9b

White lyophilized powder; 0.21 mg; 3% yield; Analytical HPLC conditions: 5 to 45% over 30 min. Retention time = 21.8 min; Preparative HPLC conditions: 15 to 35% over 30 min; LRMS (ESI-TOF) *m/z* calcd for [M + H]$^+$ 1066.5, found; 1066.1.

4.3.3. Peptide 9c

white lyophilized powder; 1.12 mg; 15% yield; Analytical HPLC conditions: 5 to 45% over 30 min. Retention time = 24.2 min; Preparative HPLC conditions: 15 to 35% over 30 min; LRMS (ESI-TOF) *m/z* calcd for [M + H]$^+$ 1128.5, found; 1128.1.

4.4. Modified preparation of threonine derivative 4b



12 **13**

Scheme 4. Preparation of synthetic intermediate **13.** Reagents and conditions: a) tetrabutylammonium azide, MeCN, 0 °C, 51%.

To a solution of substrate **12**[5a] (230 mg, 0.51 mmol) in acetonitrile (1.7 mL) was added tetrabutylammonium azide (440 mg, 1.6 mmol), and the reaction mixture was stirred at 0 °C for 12 h. After addition of water, the mixture was extracted with Et_2O. The obtained organic layer was washed with brine, dried over $MgSO_4$, and concentrated *in vacuo*. The resulting crude product was purified by column chromatography (hexanes/EtOAc = 4/1 (v/v)) and 110 mg of azide **13** (0.26 mmol, 51%) was obtained as a pale yellow oil. ^{1}H NMR spectrum was identical with that reported previously.[5a]

4.5. UV-irradiation experiment of peptide 9a-c

Typical procedure for caged peptide **9a**: Peptide **9a** (50 μg, 0.032 μmol) in sodium phosphate buffer (10 mM phosphate, pH 7.6, 500 μL) was irradiated by UV (>365 nm; irradiation power: one third of the maximum) for 7 min. Progress of the reaction was monitored by analytical HPLC and uncaged product **10a'** was characterized using ESI-MS. Analytical HPLC conditions: a linear gradient of solvent B in solvent A over 30 min.

4.5.1. Peptide 10a'

Analytical HPLC conditions: 10 to 45% over 30 min. Retention time = 21.8 min; LRMS (ESI-TOF) *m/z* calcd for [M + H]$^+$ 1435.6, found; 1434.8.

4.5.2. Peptide 10a

Analytical HPLC conditions: 10 to 45% over 30 min. Retention time = 23.3 min; LRMS (ESI-TOF) *m/z* calcd for [M + 2H]$^{2+}$ 726.3, found; 726.1. As the reaction solvent, 1% (v/v) TFA in water was used instead of the phosphate buffer.

4.5.3. Peptide 10b

Analytical HPLC conditions: 5 to 45% over 30 min. Retention time = 14.5 min; LRMS (ESI-TOF) *m/z* calcd for [M + H]$^+$ 931.4, found; 931.2.

4.5.4. Peptide 10c

Analytical HPLC conditions: 5 to 45% over 30 min. Retention time = 17.8 min; LRMS (ESI-TOF) *m/z* calcd for [M + H]$^+$ 993.5, found; 993.2.

4.6. Preparation of control peptides 10a and 11

Peptides **10a** and **11** were prepared as similar to the caged peptides. For construction of peptide **11** and a synthetic intermediate of **10a** before introduction of a non-hydrolyzable phosphoserine unit, *N*,*N*'-diisopropylcarbodiimide (DIC)/HOBt system was employed (3.0 eq. amino acid, 3.2 eq. DIC, 3.0 eq. HOBt·H_2O in DMF, 2 h at room temperature). Coupling of phosphoserine mimic **14**[4a,16b] was achieved using HATU/HOAt/DIEA system (3 eq. **14**, 2.9 eq. HATU, 2.9 eq. HOAt, 8.7 eq. DIEA in DMF, 1 h at room temperature). Elongation of following amino acids was performed using the DIC/HOBt and/or the HATU/HOAt/DIEA system.

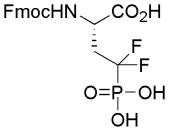


Figure 3. Structure of phosphoserine mimic **14**.

4.6.1. Peptide 10a

Yellow lyophilized powder; 2.39 mg; 14% yield; Analytical HPLC conditions: 20 to 40% over 30 min. Retention time = 18.9 min; Preparative HPLC conditions: 10 to 35% over 45 min; LRMS (ESI-TOF) *m/z* calcd for $[M + 2H]^{2+}$ 726.3, found; 726.2.

4.6.2. Peptide 11

Yellow lyophilized powder; 2.20 mg; 8% yield; Analytical HPLC conditions: 20 to 40% over 30 min. Retention time = 18.6 min; Preparative HPLC conditions: 10 to 35% over 45 min; LRMS (ESI-TOF) *m/z* calcd for $[M + H]^{+}$ 1337.6, found; 1336.9.

4.7. Binding assay using FP

Typical procedure: caged peptide **9a** (20 nM final) in HEPES buffer [10 mM HEPES, 150 mM NaCl, 0.05% (w/v) Tween-20, 0.5 mM dithiothreitol, pH 7.4, 50 μL][22] was incubated at 20 ˚C in the absence or presence of 14-3-3β protein (500 nM, final) for 1 h.[17] After recording the FP response in mP (milli-polarization units), ΔmP values were calculated as follows: ΔmP = (FP in the presence of 14-3-3β) – (FP in the absence of 14-3-3β). Averages of nine measurements with the standard error of the mean are shown in Figure 2. For the photo-irradiation experiments, peptide **9a** in HEPES buffer was subjected to UV irradiation (>365 nm; irradiation power: one third of the maximum) for 7 min before the FP experiments. Completion of the uncaging reaction was confirmed by HPLC monitoring.

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