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Synthesis and characterization of L-3-(pentafluorophosphato-difluoromethyl)-alanine, a structural and functional mimetic of phosphoserine

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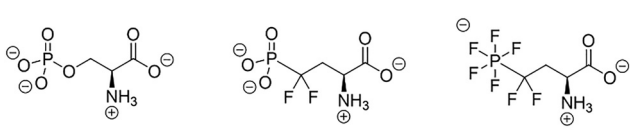
Serine phosphorylation is an essential switch for regulating the interactions and functions of many disease-related proteins. Accordingly, stable phosphoserine mimetics constitute chemical tools to study the effects of these post-translational modifications. In this work, we present the synthesis and characterization of a novel structural analog of phosphoserine, L-3-(pentafluorophosphato-difluoromethyl)-alanine. The hyper-fluorinated amino acid was synthetically accessed in six steps starting from commercially available *N*-Boc-L-serine methyl ester. The protected PF_5 amino acid and peptides derived thereof were inhibitors of the protein phosphoserine phosphatase PPP2CA, demonstrating its activity as functional phosphoserine mimetic.

Protein phosphorylation plays a crucial role in many biological processes, including signal transduction and the regulation of gene expression, cell cycle and metabolism. Aberrant activity of kinases and phosphatases are major drivers of cancer and of other diseases.[1–3] Nearly 90% of all protein phosphorylation events occur on the amino acid serine, making this post-translational modification especially important for studying associated disease states.[4] Since phosphorylation is a dynamic process, there is a need for enzymatically stable modifications mimicking the functional properties of the phosphate group. Methyl phosphonates and difluoromethyl phosphonates are important mimetics of the phosphate residue.[5] While these structures are stable toward phosphatases, they are very polar and carry two negative charges, resulting in poor cell permeability of all molecules containing these residues.[6] Recently, we have introduced 4-(pentafluorophosphato-difluoromethyl)-phenylalanine as a novel phosphotyrosine mimetic, which was shown to bind to protein tyrosine phos-phatases with more than 25-fold improved affinity in comparison to the 4-phosphono-difluoromethyl-analog. The pentafluorophosphate (PF_5) motif carries one permanent negative charge, which is distributed over a larger surface area than the corresponding phosphonates, resulting in much higher hydrophobicity, an amphiphilic character, and improved cell permeability of the amino acid.[7,8] Following these pioneering findings, we have now investigated the synthesis and biological potency of analogs of phosphoserine **1**, namely 3-(phosphono-difluoromethyl)-Ala **2** (pCF_2Ala), which has been found to effectively target pSer-binding domains, including disease-related targets like 14-3-3 σ,[9,10] Pin1,[11] and BRCA1,[12] and the novel compound, 3-(pentafluorophosphato-difluoromethyl)-alanine **3** (PF_5CF_2Ala) (Fig. 1).

Several examples in the literature describe the asymmetric synthesis[13] of L-3-(phosphono-difluoromethyl)-Ala **2**. Based on the synthetic route by Chen *et al.*,[14] we started from commercially available *N*-Boc protected L-serine methyl ester **4**, that was sulfonated and dehydrated to dehydroalanine **5**, which was then iodinated and converted into phosphonate **7** *via* a cadmium/copper promoted cross coupling step (Scheme 1). Asymmetric hydrogenation using the prochiral catalyst (*S,S*)-Et-DUPHOS-Rh with 20 bar H_2 for three days yielded phosphonate **8-(+)** ($[\alpha]_D^{20}$ = + 7.6° (*c* = 2.1, $CHCl_3$)), while hydrogenation with Pd/C and H_2 at ambient pressure yielded the racemic mixture **8-rac** ($[\alpha]_D^{20} = -0.3°$ (*c* = 1.7, $CHCl_3$)). The respective products were analyzed *via* chiral SFC-MS (Fig. S1) which showed baseline separation of the enantiomers for **8-rac** and only one enantiomer within detection limits for **8-(+)**. The Boc protection group of **8-(+)** was replaced by Fmoc to



1 **2** **3**

Fig. 1 Structures of *O*-phospho-Ser **1** (pSer) and its two mimetics 3-(phosphono-difluoromethyl)-Ala **2** (pCF_2Ala) and 3-(pentafluorophosphato-difluoromethyl)-alanine **3** (PF_5CF_2Ala).

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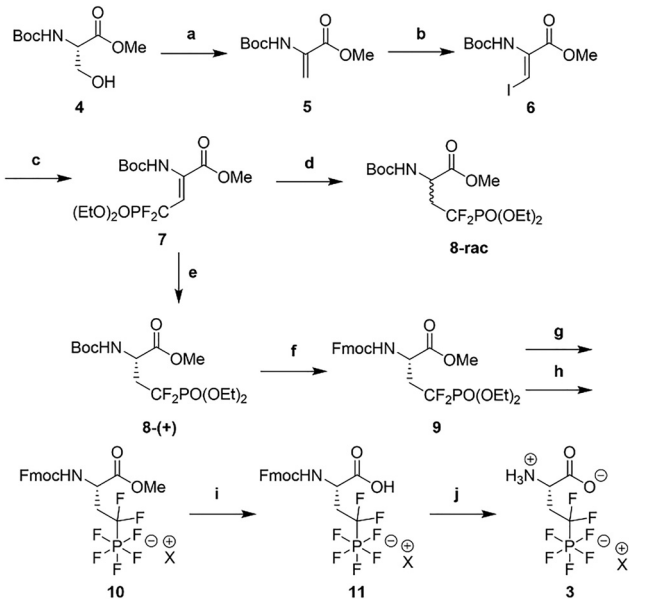


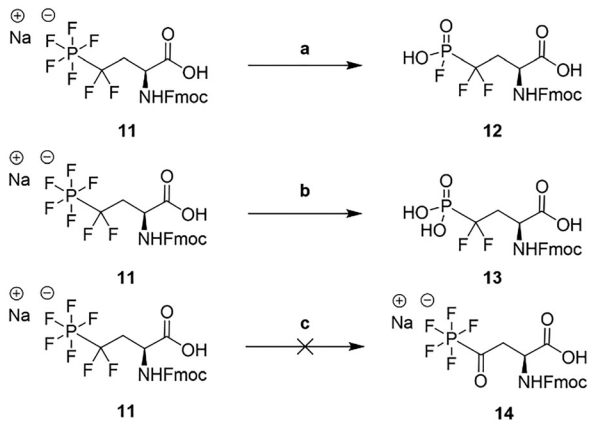
Scheme 1 Synthesis of amino acid PF_5CF_2Ala **3**. Reaction conditions: (a) MesCl (1.3 eq.), TEA (1.3 eq.), DCM, 0 °C to rt, overnight; DBU (1.2 eq.), DCM, rt, 2 h, 86%. (b) NIS (1.1 eq.), DCM, 0 °C to rt, overnight; TEA (2 eq.), 15 min; 65%. (c) Cd (6 eq.), $BrCF_2PO(OEt)_2$ (3.3 eq.), DMF, rt, 3 h; CuBr (3 eq.), DMF, rt, 18 h, 67%. (d) H_2 (ambient pressure), Pd/C (10 wt%), rt, 2 h, 87%. (e) H_2 (20 bar), (*S,S*)-Et-DUPHOS-Rh (1.5 mol%), rt, 3 d, 87%. (f) TFA : DCM (1 : 1), rt, 3 h; Fmoc-OSu (1.2 eq.), aq. $NaHCO_3$ (pH = 8–9), rt, 20 h, 90%. (g) TMSBr (5 eq.), MeCN, 60 °C, 1.5 h; $(COCl)_2$ (10 eq.), DMF (5 eq.), 40 °C, 1 h; TMAF (10 eq.), 0 °C to rt, 1.5 h, 74%. (h) HF : pyridine (70 : 30 wt%) (50 eq.), rt, 2 h, 81%; (i) protease from *B. licheniformis*, rt, 16 h, 89%; (j) 10% piperidine in MeCN, rt, 2 h, 86%. After column purification with 10 mM NH_4HCO_3 counter ion X = NH_4, after ion exchange with Amberlite™ resin IRC 120 sodium form X = Na.

ensure chemical stability under acidic fluorination conditions and to obtain a product suitable for solid-phase peptide synthesis (SPPS). Next, the pentafluorination of the *N*-Fmoc-protected derivative **9** was investigated (Scheme 1). Previously, we synthesized the PF_5-group in a three-step one pot reaction using tetramethyl ammonium fluoride (TMAF) as fluorinating agent after activation with trimethylsilyl bromide and oxalyl chloride.[7] TMAF is a hygroscopic salt that required extensive drying prior to the reaction, since already small traces of water led to low yields in the reaction. Another drawback of the previous method was the use of saturated sodium hydrogen carbonate solution for quenching excess HF which led to large amounts of salt by-product. Therefore, we aimed to optimize our protocol. After testing different conditions, we successfully employed Olah's reagent, an HF : pyridine complex (70 : 30 wt%), as fluorinating agent. 50 equivalents of Olah's reagent neat reacted different phosphonate esters smoothly to their corresponding PF_5-derivatives in short reaction times.[8] Trimethylsilyl methyl ether was utilized as new quenching agent, furnishing trimethylsilyl fluoride as volatile side product that can easily be removed under reduced pressure. We tested both protocols with structure **9** and were able to obtain the pentafluorinated molecule **10** using the TMAF protocol with 74% yield and the new method using HF:pyridine with 81% yield.

The fully deprotected amino acid **3** was obtained by Fmoc-deprotection of **11** in 86% yield (Scheme 1). Due to the amphiphilic character of the pentafluorinated amino acid derivatives extraction was not possible. All PF_5-molecules were purified *via* reversed-phase column chromatography using a C18 column with a 10 mM NH_4HCO_3 buffer. Ion exchange was performed on the Amberlite™ IRC120 Na resin to yield the corresponding sodium salts. ^{19}F NMR analysis confirmed the expected octahedral structure of the PF_5-group, showing a doublet–quintet splitting of the axial fluorine and a doublet–doublet–triplet splitting of the four equatorial fluorine atoms, as was already observed for the amino acid PF_5CF_2Phe.[7] Stability studies of structure **11** showed no decomposition under physiological conditions, and in a pH range of 3–12 for 48 hours. Under strong acidic conditions, hydrolysis to monofluorophosphonate **12** and phosphonate **13** were observed (Scheme 2 and Fig. S2). Little to no decomposition was found after incubation with silicon-based compounds trimethylsilyl bromide and hexamethyldisilazane. In contrast to the amino acid PF_5CF_2Phe, no hydrolysis of the CF_2-group to the carbonyl was observed, which might be explained by the absence of the benzylic position in PF_5CF_2Ala (Scheme 2 and Table S3).

The new chemical structure was employed for SPPS after deprotection of the methyl ester **10** using a protease from *B. licheniformis* as described previously furnishing *N*-Fmoc-(3-PF_5CF_2)-Ala **11**.[7] Cleavage of synthesized PF_5CF_2Ala-peptides from the resin was performed with HF:pyridine and 10% anisole for one hour at room temperature. Under these conditions, the peptides were entirely stable, and no hydrolysis of the PF_5-moiety was observed.

14-3-3 σ is a regulatory protein binding phosphoserine- and threonine containing peptides and proteins.[15] To investigate the potency of PF_5CF_2Ala to mimic pSer in a biological context, we synthesized a 14-3-3 σ binding heptapeptide, Ac-RFR**pS**YPP-NH_2 **15**.[16] For comparison, the analogous heptapeptide **16** was prepared, in which the native phosphoserine residue (pS) was replaced by pentafluorinated amino acid PF_5CF_2Ala. A noticeable shift of retention time on a C18 column was observed for the two peptides, demonstrating higher lipophilicity of PF_5CF_2Ala compared to native pS (Fig. S4). Both peptides were tested in a fluorescence



Scheme 2 Decomposition of PF_5CF_2Ala. (a) Full hydrolysis of **11** to monofluorophosphonate **12** was observed with TFA : MeCN (50 : 50) after 1 h; (b) **11** was fully hydrolyzed to the free phosphonate **13** under strong acidic aqueous conditions (TFA : H_2O, 50 : 50) in 24 h; (c) HF:pyridine with 1% H_2O did not provide the CF_2-hydrolyzed carbonyl structure **14** as observed for benzylic PF_5CF_2 structures.[7]

polarization assay using in-house expressed 14-3-3 σ protein and the 5-carboxytetramethylrhodamine (TMR) labeled peptide 5-TMR-GGRLSH-pS-LPG-NH_2 (commercial) as fluorescent probe. While the native heptapeptide **15** exhibited an IC_{50} of 3.9 μM (Fig. S6), no binding of the PF_5CF_2Ala peptide **16** was observed up to 1 mM (Fig. S7). Looking at co-crystallized structures of 14-3-3 σ[17,18] it is apparent that the phosphoserine binding site is highly water accessible. Thus, it does not provide a positively charged, deeper amphiphilic pocket as in the protein tyrosine phosphatases, which enabled the desolvation of the pentafluorinated moiety upon binding.[7,8] These different properties might explain, why the PF_5-peptide **16** did not show interactions with 14-3-3 σ. To challenge this hypothesis, we further tested the amino acid against the catalytic site of the protein serine phosphatase PPP2CA, which is characterized by a deeper, more hydrophobic binding pocket.[19] While the free amino acid **3** (Na^+) did not show significant inhibition of PPP2CA (Fig. S8), the protected PF_5-amino acid **10** (Na^+) showed concentration-dependent inhibition of PPP2CA with full inhibition at 2 mM and an IC_{50} of 151 μM (Fig. 2A). For comparison, **10** was converted to the established pSer mimetic *N*-Fmoc-3-(phosphono-difluoromethyl)alanine methyl ester Fmoc-pCF_2Ala-OMe **17** by treatment with TFA in water/acetonitrile and tested with PPP2CA. Incubation with structure **17** also led to a concentration-dependent inhibition of the phosphatase, however, at 2 mM 18% residual enzyme activity was observed, the IC_{50} was approximated to 212 μM (Fig. 2A). To further validate the potential of these mimetics, we incorporated PF_5CF_2Ala into the peptide sequence Ac-SPQP*p*SRFQ-NH_2 obtained from the heatmap analysis of a phosphoserine-peptide library with PPP2CA[20] furnishing peptide **18**. The PO_3CF_2-analog **19** was synthesized from **18** using TFA in water/acetonitrile. The pentafluorinated peptide **18** again showed a noticeable prolongation of the retention time on the C18-column compared to the less hydrophobic PO_3CF_2-peptide **19** (Fig. S9). While previous studies suggested that difluoromethyl-based pSer peptide mimetics lack sufficient affinity for metal-dependent phosphatases in the cellular context of holoenzymes,[21] our synthesized peptides exhibited a concentration-dependent inhibition of PPP2CA with IC_{50} values of 104 and 83 μM, respectively (Fig. 2B). These results confirm the potency of PF_5CF_2Ala **3** as functional phosphoserine mimetic showing increased affinity when incorporated into peptide **18** and similar activity compared to the traditional mimetic PO_3CF_2Ala **2** and the derived peptide **19**. We further synthesized and tested analogs of the reported phosphoserine peptide substrate TPAPpSAAAK (K_M > 500 μM)[20] with PF_5CF_2Ala (**20**) and PO_3CF_2Ala (**21**), which showed similar affinities as the substrate (Fig. S10, S11) and bound substantially weaker than peptides **18** and **19**.

In molecular docking studies, structures **10** and **17** shared similar binding modes in the catalytic center of PPP2CA (Fig. 3) in accordance with assay data. The phosphonate and PF_5-group

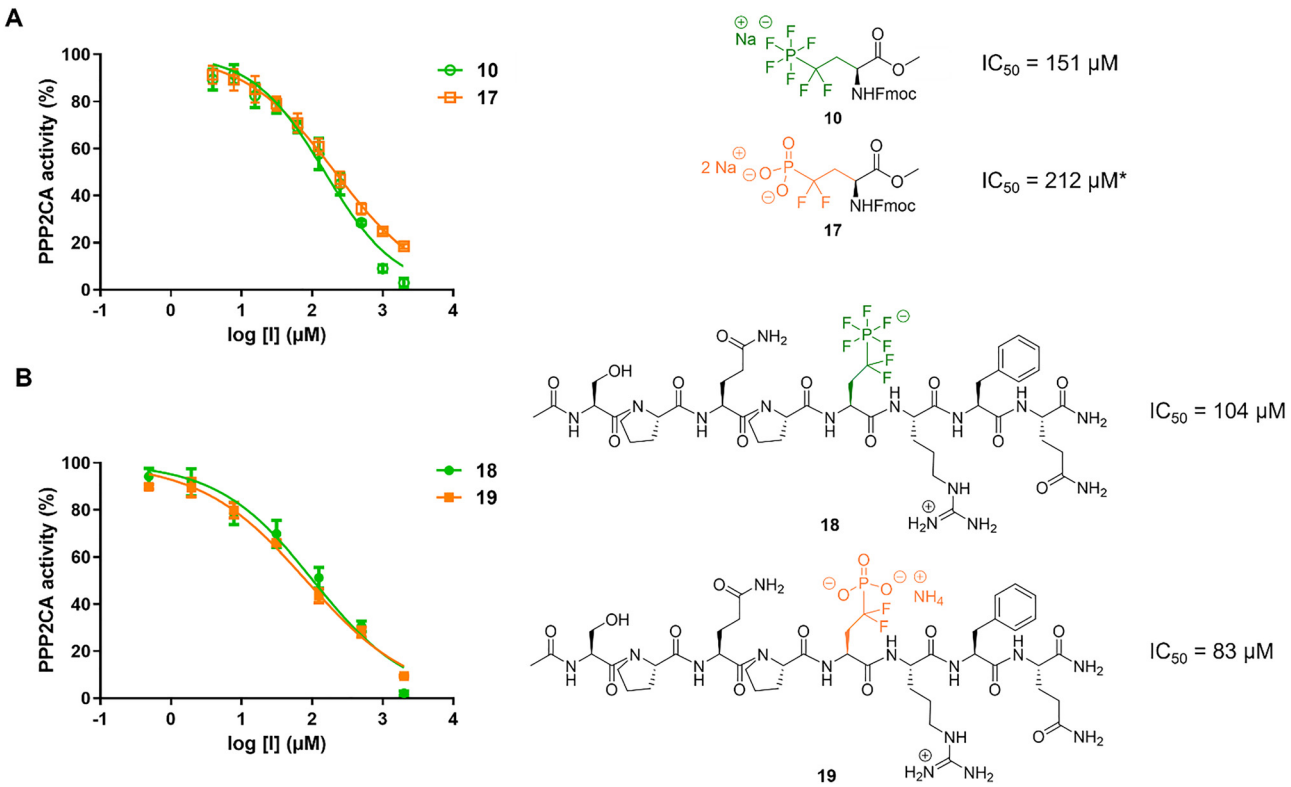


Fig. 2 Biological activity against protein serine phosphatase PPP2CA. (A) Pentafluorinated amino acid **10** and free phosphonate **17** exhibited concentration-dependent inhibition of the phosphatase, with **10** leading to full inhibition at 2 mM concentration and an IC_{50} of 151 μM. Incubation with the free phosphonate **17** led to 18% residual enzyme activity at 2 mM and showed an IC_{50} of 212 μM (* approximated with a normalized response function). (B) Pentafluorinated peptide **18** and phosphonate peptide **19** showed improved activity compared to the protected amino acids, with IC_{50} values of 104 and 83 μM, respectively.

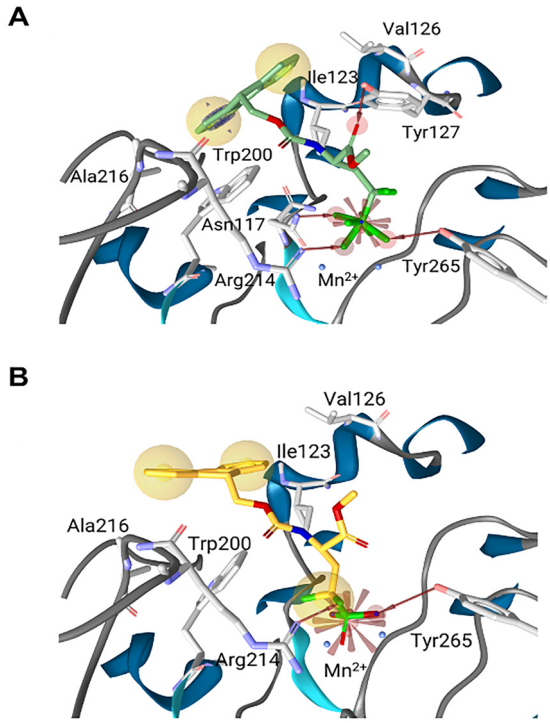


Fig. 3 Docking poses of structures **10** (A) and **17** (B) in the catalytic center of PPP2CA (PDB: 2IE4), respectively. The amino acids showed similar binding modes with the charged phosphonate group interacting with amino acids Arg214, Asn117 and Tyr265 in close proximity to the two Mn^{2+} ions, suggesting potential charge interactions with the metals. The lipophilic Fmoc group bound in a hydrophobic subpocket showing interactions with Ile123, Val126 and, in the case of structure 10, π-stacking with Trp200.

bound close to the two catalytic Mn^{2+} ions suggesting potential charge interactions. Further interactions were observed with the surrounding hydrophilic amino acids Tyr265, Arg214 and Asn117. The Fmoc group was placed in a hydrophobic subarea of the binding pocket, potentially stabilizing the overall structures in this favorable position and contributing to the binding. Structure **10** formed two additional interactions and displayed an overall better fit in the binding pocket, potentially explaining the slightly enhanced activity compared to structure **17**.

In summary, we have established a synthetic protocol to access a novel, hyperfluorinated structural mimetic of phosphoserine, L-3-(pentafluorophosphato-difluoromethyl)-alanine (PF_5CF_2Ala) **3**, and incorporated it successfully in several phosphopeptide mimetics. We have demonstrated that PF_5CF_2Ala can serve as a functional biomimetic of pSer depending on the structure and accessibility of the phosphoserine binding pocket. Presumably due to the amphiphilic character of the PF_5-residue, the deeper, less solvent-exposed and more hydrophobic pocket of PPP2CA was preferred over the water-accessible binding site of 14-3-3 σ. This hypothesis will be challenged in further studies of alternative target proteins.

Conflicts of interest

There are no conflicts to declare.

Data availability

The data supporting this article have been included as part of the supplementary information (SI), which contains descriptions of all chemical, biochemical, and computational methods, results of chiral SFC-MS, stability testing, protein binding assay and NMR spectroscopy. The authors have cited additional references within the supplementary information. See DOI: https://doi.org/10.1039/d5cc06914a.

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