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Synthesis and Application of N-Boc-L-2-amino-4-(diethylphosphono)-4,4-difluorobutanoic acid for Solid-Phase Synthesis of Nonhydrolyzable Phosphoserine Peptide Analogues

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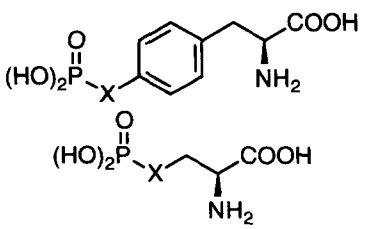
Abstract: Synthesis of N-Boc-L-2-amino-4-(diethylphosphono)-4,4-difluorobutanoic acid is reported. This analogue was utilized for the solid-phase synthesis of a peptide containing a nonhydrolyzable phosphoserine mimetic.

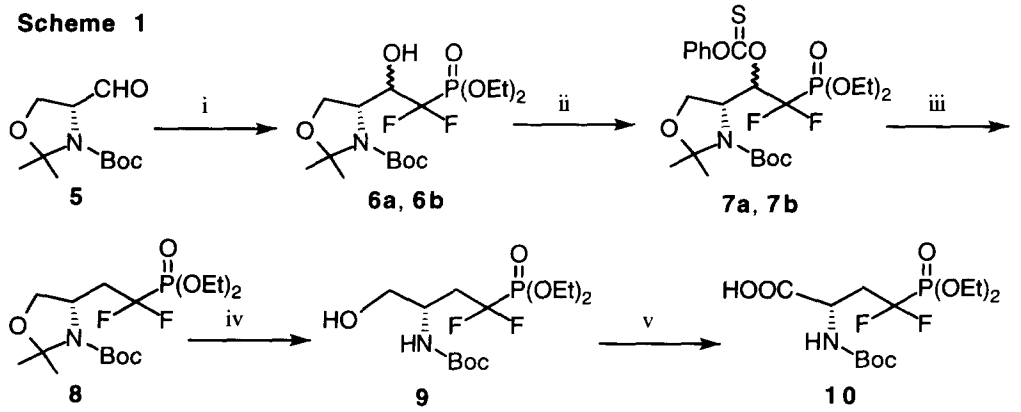
Protein phosphorylation plays an important role in intracellular signal transduction pathways[1]. Phosphoamino acid [phosphotyrosine : pTyr (**1**), phosphoserine : pSer (**3**), phosphothreonine : pThr] containing peptides provide useful tools for understanding signal transduction. Since the phosphate moiety is easily hydrolyzed by the action of protein phosphatases, phosphoamino acids isosters bearing substitution of the phosphate oxygen with a methylene have been utilized for the synthesis of phosphatases resistant phosphopeptides[2]. However, methylene substituted analogues have less activity compared to the parent phosphoamino acid containing peptides, and this may be partially attributed both to the higher second ionization constant of the phosphonate moiety and to the loss of hydrogen bonding ability at the phosphonate α-methylene[3]. Previously, we reported that 4-phosphono(difluoromethyl) phenylalanine (F_2Pmp, **2**) served as pTyr mimetic which more closely approximates pTyr[4]. Here, we report the synthesis of protected L-2-amino-4-(phosphono)-4,4-difluorobutanoic acid (L-F_2Pab, **4**) as phosphatases resistant pSer mimetic and its application to solid-phase peptide synthesis.



	X	
1	O	L-pTyr
2	CF_2	L-F_2Pmp
3	O	L-pSer
4	CF_2	L-F_2Pab

We reported earlier that ethyl (Et) groups on the F_2Pmp residue in peptides were efficiently removed with a trimethylsilyl trifluoromethanesulfonate (TMSOTf)-dimethylsulfide (DMS) reagent system[5]. By analogy to F_2Pmp$(OEt)_2$, the Et group was chosen for the side chain phosphonate protection of F_2Pab. Therefore, for preparing the protected F_2Pab derivative, we applied Martin's methodology which enable us to synthesize (α,α-difluoroalkyl)phosphonates bearing a stereogenic center at the carbon beta to the difluoromethane moiety[6].

Scheme 1



reagents: i) $HCF_2PO(OEt)_2$-LDA / THF, ii) PhOCSCl-dimethylaminopyridine / CH_2Cl_2, iii) nBu_3SnH-AIBN / toluene, iv) HCl-EtOH, v) $RuCl_3$-$NaIO_4$ / CCl_4-CH_3CN-phosphate buffer

Synthesis of **10** began with Garner's aldehyde **5**[7] derived from D-serine, which upon addition at -78°C to 1.1 equivalents (eq.) of diethyl (lithiodifluoromethyl)phosphonate[8] [generated by the addition of diethyl (difluoromethyl)phosphonate to 1.1 eq. of LDA in THF at -78°C] provided a separable 1 : 4.5 mixture of **6a** (syn, colorless oil, minor) and **6b** (anti, white crystalline solid, major) in 58% combined yield[9]. Addition of phenyl chlorothionoformate (3 eq.) to a solution of **6a** or **6b** and dimethylaminopyridine (1 eq.) in CH_2Cl_2-pyridine, followed by stirring at rt (2 days), provided, after flash chromatography, **7a** (yellow oil) and **7b** (light yellow crystalline solid) respectively, in 86% combined yield. Radical deoxygenation[10] of **7a** and **7b** (nBu_3SnH, 2 eq., AIBN, 0.5 eq. in toluene reflux 2 hr), followed by flash chromatography, gave **8** as a colorless oil (68% yield). Treatment of **8** with 0.5 M HCl / EtOH (1.5 eq.) afforded, after purification by flash chromatography, protected amino alcohol **9** in 78% yield as a colorless oil. Ru-catalyzed oxidation[11] of **9**, followed by flash chromatographic purification, gave N-Boc-L-2-amino-4-(diethylphosphono)-4,4-difluorobutanoic acid [Boc-L-F_2Pab$(OEt)_2$-OH] **10**. Recrystallization from ether afforded **10** as a white crystalline solid in 41% yield {$[\alpha]_D^{27}$ = 9.7 (c = 1.5, $CHCl_3$), mp =112-113 °C}[12]. The optical purity of chiral protected amino acid **10** was easily demonstrated by conversion of **10** to the corresponding Mosher amide methyl ester[13], and analysis by ^{1}H NMR and by HPLC indicated that the Mosher amide was essentially optically pure.

To examine the utility of the Boc-L-F_2Pab$(OEt)_2$-OH derivative in the preparation of F_2Pab-containing peptides as nonhydrolyzable pSer peptide analogues, protected F_2Pab **10** was utilized to incorporate F_2Pab into the peptide sequence H-Arg-Arg-Val-F_2Pab-Val-Ala-Ala-Glu-OH (a partial sequence of the cAMP dependent protein kinase regulatory subunit) by Boc-based solid phase techniques. Starting from Boc-Glu(OBzl)-O-

Merrifield resin (0.67 mmol / g), protected Boc-amino acid derivatives (2.5 eq.) were coupled using 2.5 eq. of diisopropylcarbodiimide / 1-hydroxybenzotriazole (1 : 1) in dimethylformamide. The mesitylenesulfonyl (Mts) group was employed for protection of the guanidino group of Arg because the Mts group is deprotected with the TMSOTf-sulfide / trifluoroacetic acid (TFA) reagents system[14]. Removal of the Boc group was performed with 50% TFA / CH_2Cl_2 (1 x 1 min, 1 x 15 min), with 5% diisopropylethylamine / CH_2Cl_2 (2 x 1 min) being used for neutralization of the TFA salt. We previously reported that a two step deprotecting methodology consisting of S_N1/S_N2(1 M TMSOTf-thioanisole / TFA system)- and S_N2(1 M TMSOTf-thioanisole / TFA + DMS system)-type deprotecting reagents was effective for deprotection of F_2Pmp(OEt)$_2$-containing peptide resins prepared by Boc-based solid phase techniques[5,15]. This fact prompted us to utilize a two step method for deprotection and cleavage of the protected peptide resin [Boc-Arg(Mts)-Arg(Mts)-Val-F_2Pab(OEt)$_2$-Val-Ala-Ala-Glu(OBzl)-O-Merrifield resin]. Treatment of the completed resin with 1 M TMSOTf-thioanisole / TFA, *m*-cresol, ethanedithiol (EDT) (rt 2 hr), followed by addition of DMS with additional stirring for 2 hr at rt, resulted in cleavage of peptide from the resin with removal of all protecting groups including the ethyl groups on the F_2Pab residue. HPLC examination of crude material showed that the ratio of fully deprotected F_2Pab- to partly deprotected F_2Pab(OH)(OEt)-containing peptide was 95 to 5 (Figure 1)[16]. After HPLC purification, purified F_2Pab-peptide[17] was obtained in 57% yield calculated from the protected peptide resin.

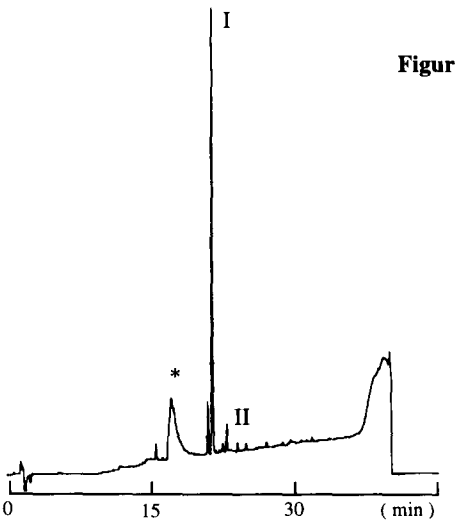


Figure 1. HPLC examination of crude F_2Pab-containing peptide

I : F_2Pab-containing peptide
II : F_2Pab(OH)(OEt)-containing peptide
* non peptidic impurities

HPLC conditions
μBondasphere $5C_{18}$ (3.9 x 150 mm) column.
A : 0.1% TFA in H_2O; B : 0.1% TFA in CH_3CN;
gradient (B%) : 0-30% over 30 min; 1.0 mL/min;
UV detection at 220 nm.

In summary, we have reported herein the stereoselective synthesis of Boc-L-F_2Pab(OEt)$_2$-OH **10** and its practical application for the synthesis of a nonhydrolyzable phosphoserine peptide analogue. Further examination of deprotection conditions for ethyl groups and evaluation of the biological activity of F_2Pab-containing peptide are now in progress.

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12. IR ($CHCl_3$) 3450, 2975, 2920, 1700, 1490, 1385, 1365, 1155, 1015 cm^{-1}; ^1H NMR (270 MHz, $CDCl_3$) δ 1.39 (t, J = 6.9 Hz, 6H), 1.45 (s, 9H), 2.50-2.88 (m, 2H), 4.24-4.35 (m, 4H), 4.60 (m, 1H), 5.43 (d, J = 8.1 Hz, 1H). Anal Calcd for $C_{13}H_{24}NO_7PF_2$: C, 41.60; H, 6.45; N, 3.73. Found: C, 41.50; H, 6.38; N, 3.62.
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15. 1 M TMSOTf-thioanisole/TFA reagents system may be operating by a mechanism which is intermediate between S_N1 and S_N2 (S_N1/S_N2). Increasing the DMS concentration has been reported to alter the reaction mechanism of strong acid catalyzed dealkylation from S_N1 to S_N2[19].
16. Peptide resins (0.01 mmol) were treated with 1 M TMSOTf-thioanisole (molar ratio, 1:1) /TFA (1 mL) in the presence of EDT (50 μL) and *m*-cresol (50 μL) for 2 hr at rt, and then DMS (200 μL) was added to this reaction mixture and the treatment was continued for 2 hr at rt. After treatment with the above reagents, addition of cold ether, followed by centrifugation, afforded crude peptides. After being washed with ether (3 times), crude deprotected peptides were analyzed and purified using HPLC.
17. Ion-spray MS (reconstructed) m/z : 1000.24 [999.99 calcd for $C_{37}H_{66}N_{14}O_{14}PF_2$ (F_2Pab-peptide)], 1028.24 [1028.05 calcd for $C_{39}H_{70}N_{14}O_{14}PF_2$ (F_2Pab(OH)(OEt)-peptide)]
18. Amino acid ratios after 6 N HCl-0.1% phenol hydrolysis [(expected) found] Glu (1) 0.97; Ala (2) 2.00; Val (2) 2.00; Arg (2) 1.84.
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