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New Synthesis of D,L-Fmoc Protected 4-Phosphonomethylphenylalanine Derivatives and their Enzymatic Resolution

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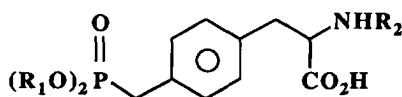
Abstract : A new synthesis of *N*-Fmoc 4-phosphonomethyl-D,L-phenylalanine protected under di-*tert*-butyl or dimethyl phosphonate forms (Fmoc-Pmp(OR)₂), suitable for solid phase peptide synthesis is described. Resolution of these hydrolytically stable analogs of *O*-phosphotyrosine was tried either by fractional recrystallization of diastereoisomeric salts or by using the subtilisin Carlsberg esterase. Only the enzymatic resolution of ethyl 4-[(dimethylphosphono)methyl]-D,L-phenylalaninate succeeded. These results are discussed by comparison with the literature data. The L and D amino acids were used to prepare separately, through solid-phase peptide synthesis, followed by deprotection of dimethylphosphonate group by trimethylsilyliodide (TMSI) in acetonitrile, the L and D isomers of Glu-Asp-Val-Pmp-Glu-Asn-Leu-His-Thr, a peptide corresponding to a potentially phosphorylated site of the phosphatase PTP 1C.

INTRODUCTION

A large number of modifications to increase the stability and activity of biological peptides have been developed during the last ten years. Several methods have been used, such as modifications of peptide bonds, cyclization of native peptides and recently, by grafting amino acid side-chains on different scaffolds. ¹⁻⁴ The design of modified amino acids constitutes an important part of this field and their incorporation into protein and peptide sequences has provided very highly active molecules allowing their mechanisms of action to be analyzed *in vitro* ⁵ and *in vivo*. ⁶

The post-transcriptional *O*-phosphorylation of proteins is a widespread enzymatic modification involved in several cellular signalling pathways with Ser, Thr and Tyr being the residues most commonly modified. ⁷⁻⁹ Synthetic *O*-phosphopeptides have been prepared in order to analyze these processes *in vitro*. ¹⁰⁻¹³ For instance, small *O*-phosphotyrosine containing peptides corresponding to phosphorylation sites of human growth factor (i.e. PDGF) or to polyoma middle T have been shown, *in vitro*, to block the association of these proteins with their protein substrates. ^{14,15}

Since the modulation of phosphorylation *in vivo* is the result of a balance between kinases and phosphatases, we designed in 1988 a non-hydrolysable phosphonate model of *O*-phosphotyrosine, phosphonomethylphenylalanine (Pmp 1). ¹⁶ This modified amino acid has since been widely used for designing biological and pharmacological probes ¹⁷⁻²⁰ such as phosphotyrosine containing peptides able to inhibit receptor recognition by SH2 domains and resistant to tyrosine phosphatase activity. ¹⁸⁻²¹ Recently, fluoro analogs of Pmp 1 with improved acidity, have been reported ²²⁻²⁴ and several syntheses have been described of phosphonomethylphenylalanine derivatives, which can be incorporated into biological peptides by solid phase synthesis. ^{19,25-29}



- | | |
|---|--|
| 1 | : R ₁ = H ; R ₂ = H |
| 2 | : R ₁ = Et ; R ₂ = Boc |
| 3 | : R ₁ = Me ; R ₂ = Boc |
| 4 | : R ₁ = <i>t</i> Bu ; R ₂ = Fmoc |
| 5 | : R ₁ = Me ; R ₂ = Fmoc |

Structure of phosphonomethyl derivatives

Thus, starting from our first synthesis of the racemic amino acid **1**,¹⁶ we have prepared the modified amino acid Boc-D,L-Pmp(OEt)₂OH (**2**) from α,α' -dibromoxylene and separated the L and D isomers with high yield through enzymatic resolution.²⁵ The L isomer was then introduced in a β_2 adrenergic receptor sequence by solid phase synthesis.¹⁷ Independently, Boc-L-Pmp(OMe)₂OH (**3L**) was enantioselectively synthesized from bis-lactim ether²⁸ but the product was not completely characterized and more recently, it was prepared by coupling an alanine-based organozinc reagent with dimethyl 4-iodophenylmethylphosphonate.²⁹ However, in Boc chemistry, the diethylphosphonate hydrolysis, requiring vigorous conditions, was shown to provide a mixture of peptides with mono and fully-deprotected Pmp residues.¹⁷

While the first syntheses of Pmp-containing peptides were accomplished by the use of Boc Pmp(OEt)₂OH in solid phase synthesis,¹⁷ later synthetic studies turned to the use of Fmoc derivatives. In Fmoc chemistry, the *t*-butyl group clearly appeared to be the best phosphonate protective group since it is readily eliminated during the acidolytic cleavage of the peptide from the resin support.^{11,18,19} Thus, Fmoc-D,L-Pmp(O*t*Bu)₂OH (**4**) was prepared via a non-stereoselective synthesis, then incorporated into different peptide sequences and the diastereoisomers were separated by RP-HPLC.^{18-20,26,27} The stereochemical assignment of the absolute configuration of Pmp in peptides was determined by treatment with aminopeptidase M¹⁹ and by comparison of their circular dichroism (CD) spectra to those of authentic linear L-Tyr-peptides and D-Tyr-peptides.¹⁸ Nevertheless, this method is time-consuming and the final assignments by spectroscopic methods are far from unambiguous. Therefore, in this paper, we report the preparation of both D and L isomers of Pmp useful for Fmoc chemistry, and we compare these methods with those recently reported in the literature. We then describe the replacement of *O*-phospho-Tyr by Pmp in a sequence corresponding to a potentially phosphorylated site of phosphatase PTP 1C.

RESULTS AND DISCUSSION

As shown in Scheme 1, *N*-Fmoc-D,L-Pmp(O*t*Bu)₂OH (**4**) was prepared through a different pathway from that described in the literature.^{26,27}

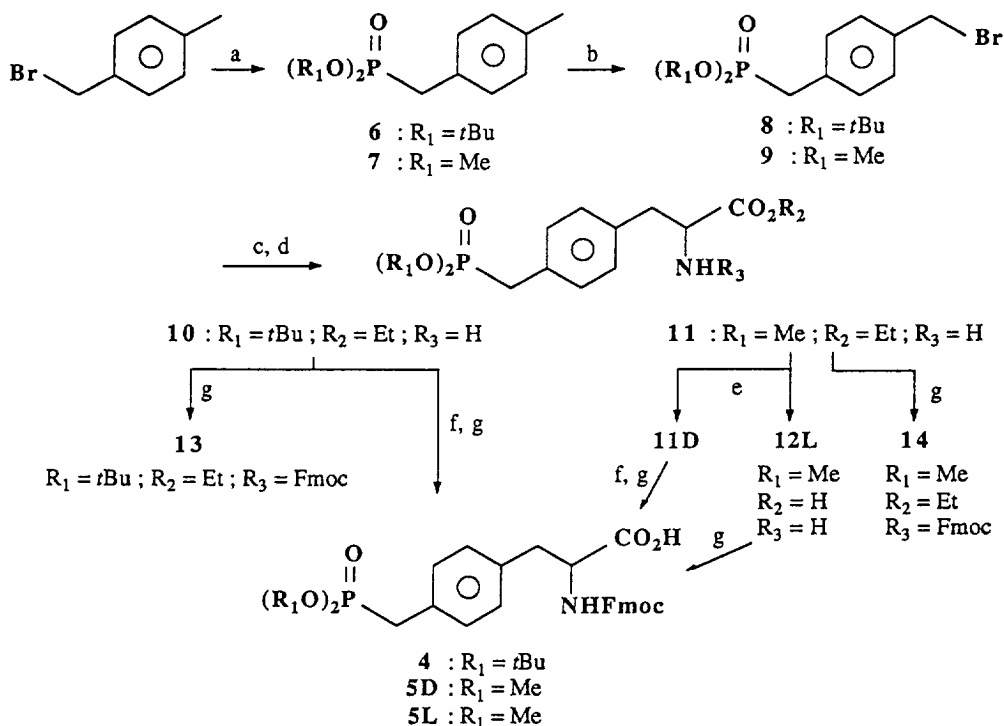
4-methylbenzylbromide was converted into phosphonate **8** by reaction with sodium di-*t*-butylphosphonate followed by bromination with NBS.³⁰ This two-step preparation improved the yield (> 62%) as compared with the previously reported one-step reaction from α,α' -dibromoxylene.³¹ The product of alkylation of ethyl *N*-(diphenylmethylene)glycinate with **8**, under phase transfer catalysis conditions was then hydrolysed without purification using 5% citric acid to provide **10** as a D,L mixture. The mixture **10**, successively saponified with 1N sodium hydroxide and condensed with FmocOSu, affording the *N*-Fmoc amino protected compound **4**.

A tentative assay to resolve Fmoc-D,L-Pmp(O*t*Bu)₂OH (**4**) through salification with optically pure enantiomers of α -methylbenzylamine, ephedrine and quinine failed. Similarly, the resolution of **10** by fractional crystallization as mandelic or tartaric acid salts was inefficient.

In addition, our attempts to resolve *N*-Fmoc-D,L-Pmp(O*t*Bu)₂OEt **13** and D,L-Pmp(O*t*Bu)₂OEt **10** (Scheme 1) using subtilisin Carlsberg also failed. This was attributed to the large size of the *t*Bu protecting groups of the phosphonate which could not fit the enzyme active site.

During our work, the deprotection of ethyl or methylphosphonates which was considered as the limiting step for the use of these protected amino acids, has been greatly optimized either by action of trimethylsilyltrifluoromethanesulfonate (TMSOTf) in dimethylsulfide (DMS)³² or by treatment with trimethylsilyliodide (TMSI) in acetonitrile.³³ Thus, we tried to develop a synthesis providing Fmoc Pmp(OMe)₂OH as a mixture of D and L isomers. In the preceding pathway (Scheme 1) the phosphonate was

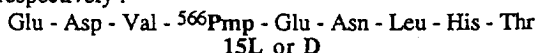
obtained by the method of Arbusov using $P(OMe)_3$. Moreover, the hydrolysis of the imino intermediate could be performed more easily by treatment with 1N HCl, since under these conditions the methyl phosphonate group is more stable than the *t*-butyl one. Our tentative assays to separate the Fmoc protected compound **14** with subtilisin Carlsberg (Scheme 1) also aborted, although *N*-Boc-D,L-Pmp(OEt)₂OEt has been resolved by this method²⁵. These results suggest that the size and therefore the steric hindrance on the *N*-terminal part of the amino acid is also critical for enzymatic resolution. However, we were able to separate D and L isomers of *N*-FmocPmp(OMe)₂OH (**5L**, **5D**) by enzymatic hydrolysis of D,L-Pmp(OMe)₂OEt **11**. The isomer **5D** was then saponified with 1N NaOH and the two enantiomers were subsequently protected as Fmoc i.e. on the amino group. To determine their optical purity, compounds **12L** and **11D** were hydrolysed in refluxing 6N HCl yielding **1L** and **1D** respectively. Optical rotations were obtained as $[\alpha]_D^{23} = +9.1$ (c 1.0, HCl 1N) and $[\alpha]_D^{23} = -9.7$ (c 1.0, HCl 1N) for **1D** and **1L** respectively in accordance with $[\alpha]_D = +10.5$ of L-Pmp previously reported.²⁵



a : NaH, HPO_3tBu_2 or $P(OMe)_3$; b : NBS, $(PhCO_2)_2$; c : $Ph_2C = NCH_2CO_2Et$, KI, $PhCH_2N^+(CH_3)_3 OH^-$; d : citric acid 5% for $R_1 = tBu$ or HCl 1N for $R_1 = Me$; e : subtilisin Carlsberg ; f : NaOH 1N ; g : FmocOSu, DIEA.

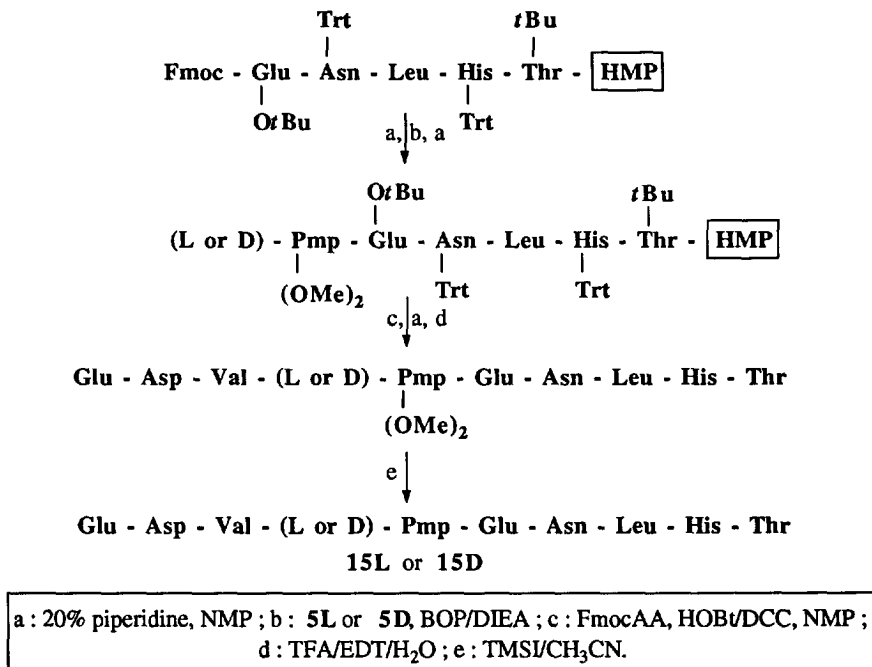
Scheme 1

In the second step of this work, Tyr⁵⁶⁶ was replaced either by **5L** or by **5D**, in the sequence, affording the peptides **15L** and **15D** respectively :



in which residue 566 corresponds to a potential phosphorylation site of protein phosphatase PTP 1C, whose association with p60c-Src seems to play an important role in platelet activation.³⁴ These peptides (**15L** and **15D**) will be tested for their ability to inhibit the interaction between PTP 1C and p60-cSrc which might occur by means of the c-Src SH2 domain.

Fmoc amino acids : Thr protected as *t*.butyl ether, His as triphenylmethylimidazole (Trt), Leu, Asn as triphenylmethylamide, and Glu as *t*.butyl ester, were successively connected by a standard stepwise solid-phase method using HMP (*p*-hydroxymethylphenoxy) resin, HOBt/DCC as coupling agents and the Fmoc group was removed by 20% piperidine/*N*-methylpyrrolidone (NMP) (Scheme 2).



Scheme 2

At this point the resin-peptide was separated into two parts, which were further coupled with either L or D *N*-Fmoc Pmp(OMe)₂ OH (**5L**, **5D**) using BOP/DIEA. Final sequence assembling was performed on each part of the resin-peptide following standard methods (HOBt/DCC). At the completion of peptide synthesis, simultaneous cleavage of the peptide from the resin and side-chain deprotection were realized by treatment with a mixture TFA/EDT/H₂O and finally, the demethylation of the dimethylphosphonate moiety was performed using a mixture of trimethylsilyliodide (TMSI)/CH₃CN for each isomer. The final phosphonopeptides **15L** and **15D** were purified by preparative C₁₈ RP-HPLC. Their capacity to inhibit the interaction between c-Src and PTP 1C is now being tested.

In summary, we propose an easy and improved access to protected amino acids **4** and **5** mimicking the phosphorylated tyrosine residues. The two enantiomers L and D of Fmoc derivative **4** could not be enzymatically separated on account of the steric hindrance of *t*Bu and Fmoc groups. However, Pmp(OMe)₂OEt **11** was separated by enzymatic resolution, allowing D and L FmocPmp(OMe)₂OH (**5L**, **5D**) to be obtained. It

is interesting to note that we have recently developed an enantioselective synthesis of Fmoc-L-Pmp(O i Bu)₂OH (4L), using camphor sultam as a chiral auxiliary with high enantiomeric purity (> 97%).³⁵ The advantage of the method described here is the possibility of obtaining simultaneously two isomers for solid phase peptide synthesis. The incorporation of the protected amino acids 5L and 5D in the sequence of a potential phosphorylation site of PTP 1C has been successfully achieved by Fmoc strategy.

EXPERIMENTAL SECTION

¹H NMR spectra were recorded on a Bruker WH 270, spectrometer operating at 270 MHz. In the case of the final peptides 15L and 15D, ¹H NMR spectra were performed at 400 MHz. Chemical shifts are given in ppm relative to HMDS as internal standard. Mass spectra were recorded on a double focusing VG 7^o-250 spectrometer equipped with a FAB gun. Optical rotations were measured on a Perkin-Elmer Model 141 polarimeter. Melting points were obtained on a Electrothermal apparatus as uncorrected values. Elemental analyses were performed by the Université Paris VI. The purity of the final products was checked on analytical HPLC, using a Nucleosil C₈ column 4.6 x 250 mm (5 μ , 100Å). The mobile phase consisted of solution A (H₂O + 1% TFA) and solution B (70% acetonitrile in H₂O + 0.9% TFA). The gradient was progressively raised from 40% to 90% of solution B in 30 min (flow rate 1 ml/min, UV detection at 214 nm). Purity of the compounds and reaction progress were monitored by TLC on silica gel plates (60F₂₅₄, 0.2 mm thick, Merck). Column flash chromatographies were performed with silica gel 60, 40-60 μ (Merck) and filtration with silica gel 60, 5-35 μ (Merck). Fmoc/solid-phase peptide synthesis was conducted on a fully automated Applied Biosystem 431B peptide synthesizer using *p*-hydroxymethylphenoxy resin (HMP, Applied Biosystems Inc.). Preparative HPLC for purification of the peptides 15L and 15D, was performed using a Vidac C₁₈ column and a linear gradient (elution of 0 to 30% of solution B in 90 min at a flow rate of 2 ml/min). N-protected amino acids were obtained from Biochem (France) and peptide synthesis reagents (HOBt, DCC, EDT, NMP and piperidine) were obtained from Applied Biosystems Inc., except BOP which was supplied by Propeptide. All starting materials for organic synthesis were purchased from Aldrich except HPO₃ i Bu₂ from Lancaster and subtilisin Calsberg type VIII from Sigma.

4-(Di-*tert*-butylphosphonomethyl)toluene (6).

To a stirring suspension of NaH (80% in mineral oil, 5.0 g, 166.5 mmol) in anhydrous THF (60 ml) at 0°C was added di-*tert*-butylphosphite (31 g, 160 mmol) in anhydrous THF (90 ml) dropwise over 10 min. The stirring was continued for 0.5 hour at 0°C and then 4-methylbenzylbromide (25 g, 135 mmol) in anhydrous THF (120 ml) was added dropwise. The reaction mixture was stirred for 20 hours under reflux. Solvent was removed and the mixture was subjected to an extractive workup (EtOAc/brine). After purification by chromatography with AcOEt/cyclohexane 1/9 and AcOEt as eluent, 38.7 g (96% yield) of 6 was obtained as an oil: R_f (AcOEt/cyclohexane 1/3) 0.21; ¹H NMR (DMSO- d_6) δ : 1.3 (s, 18H, t -C₄H₉); 2.18 (s, 3H, CH₃); 2.82 (d, 2H, J_{HP} = 24Hz, CH₂P); 7.0 (d, 2H, H-2, H-6); 7.05 (d, 2H, H-3, H-5).

4-(Di-*tert*-butylphosphonomethyl)- α -bromotoluene (8).

A solution of the phosphonate ester 6 (38 g, 127 mmol), N-bromosuccinimide (25 g, 140 mmol) and benzoyl peroxide (1.2 g, 5 mmol) in anhydrous CCl₄ (350 ml) was refluxed for 3 hours, then cooled to room temperature, filtered and the filtrate evaporated to dryness. The resulting oil was dissolved in Et₂O, washed with brine, dried over Na₂SO₄ and the solvent evaporated. Silica gel chromatography (AcOEt/cyclohexane 1/1) followed by recrystallization in petroleum ether gave 31.2 g (65% yield) of 8 as a white solid (mp = 89-91°C)

$R_f(\text{AcOEt}/\text{cyclohexane } 1/1)$ 0.40 ; $^1\text{H NMR}$ ($\text{DMSO-}d_6$) δ : 1.27 (s, 18H, $t\text{-C}_4\text{H}_9$) ; 2.98 (d, 2H, $J_{\text{HP}} = 24\text{Hz}$, CH_2P) ; 4.60 (s, 2H, CH_2Br) ; 7.28 (d, 2H, H-3, H-5) ; 7.30 (d, 2H, H-2, H-6). Anal. Calcd. for $\text{C}_{16}\text{H}_{26}\text{BrO}_3\text{P}$: C, 50.94 ; H, 6.95. Found : C, 50.85 ; H, 6.95.

Ethyl 4-(di-*tert*-butylphosphonomethyl)-D,L-phenylalaninate (10).

7.5 g (19.9 mmol) of the phosphonate **8**, 5.3 g (19.9 mmol) of ethyl *N*-(diphenylmethylene) glycinate and 0.3 g (2.0 mmol) of KI were dissolved in dioxane (100 ml) and cooled at 10°C under stirring. Benzyltrimethylammonium hydroxide (40% in H_2O , 7.9 ml, 20.0 mmol) was added dropwise over 20 min. The reaction mixture was brought to room temperature and stirring maintained for 6 hours. The aqueous phase was then extensively extracted with toluene. After drying over Na_2SO_4 , the solvent was evaporated to yield an imine intermediate as a yellow oil (10.8 g, 97% yield), which was used without further purification in the next step.

The imine (10.6 g, 18.8 mmol) was dissolved in THF (180 ml) and 5% aqueous citric acid (360 ml, 93.8 mmol). The mixture was stirred for 2 hours at room temperature. After dilution with Et_2O , the organic phase containing benzophenone was discarded and the aqueous layer alcalinized with NaHCO_3 (pH ~ 8) before extracting with CH_2Cl_2 . The combined organic phases were washed with brine and dried on Na_2SO_4 , then evaporated to provide 5.7 g (76% yield) of the amine **10** as a light yellow oil : $R_f(\text{CH}_2\text{Cl}_2/\text{MeOH } 9/1)$ 0.55 ; $^1\text{H NMR}$ ($\text{DMSO-}d_6$) δ : 1.06 (t, 3H, CH_3) ; 1.28 (s, 18H, $t\text{-C}_4\text{H}_9$) ; 1.70 (br s, 2H, NH_2) ; 2.74 (m, 2H, $\text{CH}_2\beta$) ; 2.90 (d, 2H, $J_{\text{HP}} = 24\text{Hz}$, CH_2P) ; 3.46 (t, 1H, $\text{CH}\alpha$) ; 3.95 (q, 2H, OCH_2) ; 7.04 (d, 2H, H-2, H-6) ; 7.08 (d, 2H, H-3, H-5). Anal. Calcd. for $\text{C}_{20}\text{H}_{34}\text{NO}_5\text{P}$: C, 60.13 ; H, 8.58 ; N, 3.51. Found : C, 60.05 ; H, 8.68 ; N, 3.42.

Ethyl (N-fluoren-9-ylmethoxycarbonyl)-(4-di-*tert*-butylphosphonomethyl)-D,L-phenylalaninate (13).

To a solution of the amine **10** (0.4 g, 1 mmol) and FmocOSu (0.5 g, 1.4 mmol) in a mixture acetonitrile/ H_2O (2/1, 10 ml) was added diisopropylethylamine (0.2 ml, 1 mmol) resulting pH ~ 8. The reaction mixture was stirred for 20 hours at room temperature and acetonitrile was removed. The reaction mixture was partitioned between AcOEt and 5% solution of citric acid, then the organic layer, was washed with 5% citric acid, brine, dried (Na_2SO_4) and evaporated to dryness. The residue was filtered through silica gel with CH_2Cl_2 and $\text{CH}_2\text{Cl}_2/\text{MeOH } 100/1$ as eluent. 620 mg (81%) of a white foam were obtained after evaporation of the solvent and subsequently recrystallized from Et_2O /petroleum ether to yield **13** (mp = 136-138°C) with an HPLC ($t_R = 32.1$ min) purity > 94% ; $R_f(\text{CH}_2\text{Cl}_2/\text{MeOH } 100/1)$ 0.17 ; $^1\text{H NMR}$ ($\text{DMSO-}d_6$) δ : 1.05 (t, 3H, CH_3) ; 1.25 (s, 18H, $t\text{-C}_4\text{H}_9$) ; 2.90 (m, 4H, CH_2P , $\text{CH}_2\beta$) ; 4.0 (q, 2H, OCH_2) ; 4.15 (m, 4H, $\text{CH}\alpha$, H-9', NCO_2CH_2) ; 7.15 (m, 4H, H-2, H-3, H-5, H-6) ; 7.27 (dt, 2H, H-2', H-7') ; 7.37 (t, 2H, H-3', H-6') ; 7.62 (dd, 2H, H-4', H-5') ; 7.85 (m, 3H, H-1', H-8', NH). Anal. Calcd. for $\text{C}_{35}\text{H}_{44}\text{NO}_7\text{P}$: C, 67.62 ; H, 7.13 ; N, 2.25. Found : C, 67.57 ; H, 7.12 ; N, 2.24.

N-fluoren-9-ylmethoxycarbonyl 4-(di-*tert*-butylphosphonomethyl)-D,L-phenylalanine (4).

To a solution of the amine **10** (0.4 g, 1 mmol) in dioxane (5 ml) were added dropwise 5 equiv. of NaOH 1N (5 ml, 5 mmol). After stirring at room temperature for 0.5 hour, the reaction mixture was neutralized (pH ~ 7) with a solution of AcOEt saturated with HCl.

The resulting acid was reacted with FmocOSu (0.4 g, 1.2 mmol) and DIEA (0.34 ml, 2.0 mmol) as described for **13** and after purification by filtration through silica gel with CH₂Cl₂, CH₂Cl₂/MeOH 20/1, 522 mg (88% yield) of compound **4** were obtained as a white powder (mp = 97-99°C) (lit.²⁷ mp = 65-70°C) with an HPLC (t_R = 24.5 min) purity > 96% ; R_f(CH₂Cl₂/MeOH 20/1) 0.26 ; ¹H NMR (DMSO-d₆) δ : 1.30 (s, 18H, t-C₄H₉) ; 2.95 (m, 4H, CH₂P, CH₂β) ; 4.19 (m, 4H, CHα, H-9', NCO₂CH₂) ; 7.16 (m, 4H, H-2, H-3, H-5, H-6) ; 7.30 (dt, 2H, H-2', H-7') ; 7.40 (t, 2H, H-3'), H-6') ; 7.55 (d, 1H, NH) ; 7.67 (dd, 2H, H-4', H-5') ; 7.87 (d, 2H, H-1', H-8') ; 12.70 (br s1, 1H, OH). Anal. Calcd. for C₃₃H₄₀NO₇P : C, 66.77 ; H, 6.79 ; N, 2.36. Found : C, 66.43 ; H, 6.86 ; N, 2.29. FABMS : m/z = 538 (M⁺+H), 555 (M⁺+NH₃).

4-(dimethylphosphonomethyl)toluene (**7**).

10.0 g of 4-methylbenzylbromide (54 mmol) and 19.2 ml of trimethylphosphite (162 mmol) were dissolved in toluene (20 ml). The reaction mixture was stirred under reflux for 6 hours. Solvent and excess of trimethylphosphite were removed *in vacuo*. The residue was purified by chromatography with AcOEt/cyclohexane in mixture 1/1 to 4/1 as eluent to yield compound **7** as colorless oil (10.5 g, 95% yield) : R_f(AcOEt/cyclohexane 4/1) 0.37 ; ¹H NMR (DMSO-d₆) δ : 2.20 (d, 3H, CH₃) ; 3.15 (d, 2H, J_{HP} = 24Hz, CH₂P) ; 3.52 (d, 6H, OCH₃) ; 7.05 (d, 2H, H-2, H-6) ; 7.10 (d, 2H, H-3, H-5). Anal. Calcd. for C₁₀H₁₅O₃P : C, 56.07 ; H, 7.05. Found : C, 56.18 ; H, 7.27.

4-(dimethylphosphonomethyl)-α-bromotoluene (**9**).

As described for **8**, the phosphonate **7** (10.0 g, 46.6 mmol) provided after 3 hours reaction time under reflux, standard workup and purification by chromatography (AcOEt/cyclohexane 3/2, then AcOEt), 7.0 g (55% yield) of **9** as an oil : R_f(AcOEt/cyclohexane 1/1) 0.28 ; ¹H NMR (DMSO-d₆) δ : 3.20 (d, 2H, J_{HP} = 24Hz, CH₂P) ; 3.02 (d, 6H, OCH₃) ; 4.62 (s, 2H, CH₂Br) ; 7.16 (d, 2H, H-3, H-5) ; 7.30 (d, 2H, H-2, H-6). Anal. Calcd. for C₁₀H₁₄BrO₃P : C, 40.97 ; H, 4.81. Found : C, 40.91 ; H, 4.93.

Ethyl 4-(dimethylphosphonomethyl)-D,L-phenylalaninate (**11**).

From compound **9** (7.0 g, 23.8 mmol) were obtained after standard workup following the first step of **10**, 10.6 g as yellow oil of the intermediate imine, which was used without purification. Hydrolysis of the imine was achieved by stirring at room temperature in a biphasic system Et₂O (60 ml)/HCl 1N (60 ml) for 2 hours. After standard workup, (see second step of **10**) 4.6 g of product **11** were obtained (64% yield from **9**) as a light yellow oil : R_f(CH₂Cl₂/MeOH 20/1) 0.25 ; ¹H NMR (DMSO-d₆) δ : 1.07 (t, 3H, CH₃) ; 1.75 (br s, 2H, NH₂) ; 2.73 (m, 2H, CH₂β) ; 3.15 (d, 2H, J_{HP} = 24Hz, CH₂P) ; 3.45 (t, 1H, CHα) ; 3.50 (d, 6H, OCH₃) ; 3.95 (q, 2H, OCH₂) ; 7.05 (d, 2H, H-2, H-6) ; 7.10 (d, 2H, H-3, H-5). Anal. Calcd. for C₁₄H₂₂NO₅P, 0.5 H₂O : C, 51.85 ; H, 7.14 ; N, 4.32. Found : C, 52.00 ; H, 7.13 ; N, 4.34.

Ethyl 4-(dimethylphosphonomethyl)-L-phenylalanine (**12L**) and ethyl 4-(dimethylphosphonomethyl)-D-phenylalaninate (**11D**).

The racemic amine **11** (3.0 g, 9.5 mmol) was dissolved in a solution of dioxane/H₂O (15 ml/12 ml) and 6.5 ml of a buffer (10⁻²M KCl, 10⁻⁴M KH₂PO₄). The pH was adjusted to 7.4 with HCl 1N. Protease type VIII subtilisin Carlsberg (25 mg) was added and the reaction mixture stirred at room temperature while the pH was maintained around 7.4 with addition of NaOH 1N for overnight using pH stat. Solvent was removed and the residue was subjected to an extractive workup (AcOEt/H₂O). The organic phase, evaporated and purified by flash chromatography with CH₂Cl₂/MeOH 20/1 as eluent, provided **11D** (0.98 g, 33% yield) with an HPLC

($t_R = 11.4$ min) purity > 95% * : $[\alpha]_D^{23} = -14.6^\circ$ (c 0.1 ; MeOH). The aqueous layer was lyophilized, then dissolved in MeOH, filtered and purified by filtration through silica gel with $\text{CH}_2\text{Cl}_2/\text{MeOH}$ 4/1 and $\text{CH}_2\text{Cl}_2/\text{MeOH}$ 4/1 containing 5% NH_4OH as eluent. **12L** was obtained as a white powder (1.36 g, 47% yield) (mp = 161-166°C) with an HPLC ($t_R = 11.6$ min) purity > 97% * ; $R_f(\text{CH}_2\text{Cl}_2/\text{MeOH}/\text{NH}_4\text{OH}$ 16/4/1) 0.28 ; $^1\text{H NMR}$ ($\text{DMSO}-d_6$) δ : 2.90 (m, 2H, $\text{CH}_2\beta$) ; 3.16 (d, 2H, CH_2P) ; 3.38 (m, 1H, $\text{CH}\alpha$) ; 3.55 (d, 6H, OCH_3) ; 7.14 (m, 4H, Harom). $[\alpha]_D^{23} = -22.3^\circ$ (c 0.1 ; MeOH). Anal. Calcd. for $\text{C}_{12}\text{H}_{18}\text{NO}_5\text{P}$: C, 50.17 ; H, 6.32 ; N, 4.88. Found : C, 50.14 ; H, 6.37 ; N, 4.82.

Ethyl (*N*-fluoren-9-ylmethoxycarbonyl)-(4-dimethylphosphonomethyl)-D,L-phenyl alaninate (14).

As described for **13**, 1.0 g (3.2 mmol) of **11**, provided, after standard workup and crystallization from $\text{AcOEt}/\text{cyclohexane}$ 1.55 g (91% yield) of **14** as a white solid (mp = 34-38°C) with an HPLC ($t_R = 28.6$ min) purity > 97% ; $R_f(\text{CH}_2\text{Cl}_2/\text{MeOH}$ 20/1) 0.55 ; $^1\text{H NMR}$ ($\text{DMSO}-d_6$) δ : 1.12 (t, 3H, CH_3) ; 2.95 (m, 2H, $\text{CH}_2\beta$) ; 3.20 (d, 2H, CH_2P) ; 3.56 (m, 6H, OCH_3) ; 4.05 (q, 2H, OCH_2) ; 4.20 (m, 4H, $\text{CH}\alpha$, H-9, NCO_2CH_2) ; 7.18 (m, 4H, H-2, H-3, H-5, H-6) ; 7.30 (dt, 2H, H-2', H-7') ; 7.40 (t, 2H, H-3', H-6') ; 7.52 (d, 1H, NH) ; 7.63 (d, 2H, H-4', H-5') ; 7.88 (d, 2H, H-1', H-8'). Anal. Calcd. for $\text{C}_{29}\text{H}_{32}\text{NO}_7\text{P}$: C, 64.79 ; H, 6.00 ; N, 2.61. Found : C, 64.72 ; H, 5.93 ; N, 2.55.

N-fluoren-9-ylmethoxycarbonyl 4-(dimethylphosphonomethyl)-L-phenylalanine (5L).

1.3 g (4.5 mmol) of **12L** were subjected to the same reaction conditions as described for **13** affording **5L** (1.55 g, 67% yield) (mp = 194-195°C) after purification by chromatography with $\text{CH}_2\text{Cl}_2/\text{MeOH}/\text{AcOH}$ 95/5/1. The resulting white solid was recrystallized from MeOH giving white needles with an HPLC ($t_R = 21.8$ min) purity ~ 98% ; $R_f(\text{CH}_2\text{Cl}_2/\text{MeOH}/\text{AcOH}$ -95/5/1) 0.29 ; $^1\text{H NMR}$ ($\text{DMSO}-d_6$) δ : 2.90 (m, 2H, $\text{CH}_2\beta$) ; 3.15 (d, 2H, $J_{\text{HP}} = 24\text{Hz}$, CH_2P) ; 3.50 (m, 6H, OCH_3) ; 4.10 (m, 4H, $\text{CH}\alpha$, H-9', OCH_2) ; 7.12 (m, 4H, H-2, H-3, H-5, H-6) ; 7.25 (dt, 2H, H-2', H-7') ; 7.35 (t, 2H, H-3', H-6') ; 7.50 (d, 1H, NH) ; 7.60 (m, 2H, H-4', H-5') ; 7.80 (d, 2H, H-4', H-5') ; 12.70 (br s, 1H, OH). $[\alpha]_D^{23} = -2.0$ (c 1.0 ; MeOH/DMF 10/1). Anal. Calcd. for $\text{C}_{27}\text{H}_{28}\text{NO}_7\text{P}$: C, 63.65 ; H, 5.54 ; N, 2.75. Found : C, 63.68 ; H, 5.54 ; N, 2.70. FABMS : $m/z = 510$ ($\text{M}^+\text{+H}$), 527 ($\text{M}^+\text{+NH}_3$).

N-fluoren-9-ylmethoxycarbonyl 4-(dimethylphosphonomethyl)-D-phenylalanine (5D).

As described for **4**, **11D** (1.0 g, 3.2 mmol) was saponified and transformed with Fmoc OSu (1.5 g, 4.4 mmol) in the presence of DIEA (0.6 ml, 3.2 mmol) to **5D** (0.72 g, 46% yield). $[\alpha]_D^{23} = +2.4$ (c 1.0 ; MeOH/DMF 10/1) with an HPLC ($t_R = 21.0$ min) purity > 99%. Anal. Calcd. for $\text{C}_{27}\text{H}_{28}\text{NO}_7\text{P}$: C, 63.65 ; H, 5.54 ; N, 2.75. Found : C, 63.73 ; H, 5.42 ; N, 2.69. FABMS : $m/z = 510$ ($\text{M}^+\text{+H}$), 527 ($\text{M}^+\text{+NH}_3$).

Glu-Asp-Val-D.Pmp-Glu-Asn-Leu-His-Thr (15D).

The peptide synthesis was performed using HMP resin (0.096 mmol). Except in the case of Fmoc-Pmp(OMe)₂OH which was coupled with BOP/DIEA (3 equiv/6 equiv.), amino acid couplings were performed by the addition HOBt/DCC/NMP (3 equiv.) and the *N*-protected amino acids (0.5 mmol) to the peptide-resin.

* in these cases the gradient was progressively raised from 10 to 100% of solution B in 30 min.

Coupling efficiency was controlled by the ninhydrin qualitative Kaiser test³⁶ after each step. The Fmoc group was cleaved from the intermediate peptide-resin by a treatment with 20% piperidine/NMP. Fmoc-Thr(*t*Bu), Fmoc-His(Trt), Fmoc-Leu and Fmoc-Asn(Trt) (each 0.5 mmol) were successively coupled to the resin. Then, the resin was separated in two parts. **5D** and **5L** were separately coupled to one part of the peptide-resin with BOP/DIEA and subsequent couplings with Fmoc-Val, Fmoc-Asp(O*t*Bu), Fmoc-Glu(O*t*Bu) and HOBt/DCC/NMP were performed on each part of the resin. After *N*-Fmoc deprotection, the two parts of the dried peptide-resin were cleaved in a solution of TFA/EDT/H₂O (9.5/0.25/0.25).

In the case of peptide **15D**, crude dimethyl phosphonomethyl protected peptide (33 mg) was suspended in MeCN (0.7 ml), and TMSI (0.7 ml) was added dropwise over 3 hours at room temperature. After evaporation of MeCN, the mixture was hydrolysed with 10% aqueous AcOH (10 ml) and submitted to an extractive workup with Et₂O. Lyophilization of the aqueous layer gave a pale yellow powder, which was purified by HPLC to give 20 mg (72% yield-last step) of **15D**. With an HPLC (gradient elution from 10% to 50% of solution B in 20 min) (*t_R* = 15.7 min) purity > 99% ; ¹H NMR (D₂O) δ* : 0.80 (m, 12H, Leu-CH₃, Val-CH₃) ; 1.15 (d, 3H, Thr-CH₃) ; 1.53 (m, 3H, Leu-CH_γ and CH₂β) ; 1.90-2.10 (m, 3H, Val-CH_β, Glu5-CH₂β) ; 2.15 (m, 2H, Glu1-CH₂β) ; 2.40 (t, 2H, Glu5-CH₂γ) ; 2.50 (t, 2H, Glu1-CH₂γ) ; 2.65-2.90 (m, 4H, Asn-CH₂β and Asp-CH₂β) ; 3.0 (d, 2H, CH₂P) ; 3.15 (m, 2H, Pmp-CH₂β) ; 3.30 (m, 2H, His-CH₂β) ; 4.05 (m, 2H, Val-CH_α and Glu1-CH_α) ; 4.28 (m, 2H, Leu-CH_α and Glu5-CH_α) ; 4.35 (m, 1H, Thr-CH_β) ; 4.60-4.90 (m, 5H, Pmp-CH_α, Asn-CH_α, Thr-CH_α, Asp-CH_α, His-CH_α) ; 6.95 (br s, 2H, NH₂) ; 7.15 (d, 2H, Pmp H-2, H-6) ; 7.25 (dd, 2H, Pmp H-3, H-5) ; 7.28 (s, 1H, His H-4) ; 7.65 (br s, 2H, NH₂) ; 8.15 (d, 1H, Thr-NH) ; 8.20 (d, 1H, Leu-NH) ; 8.25 (d, 1H, Glu5-NH) ; 8.28 (m, 2H, Pmp-NH and Val-NH) ; 8.40 (d, 1H, Asn-NH) ; 8.50 (d, 1H, His-NH) ; 8.58 (s, 1H, His H-2) ; 9.00 (d, 1H, Asp NH). FABMS : *m/z* = 1214 (M⁺+NH₃).

Following a similar procedure 16.2 mg (58% yield-last step) of **15L** were obtained with an HPLC purity > 98% (*t_R* = 15.5 min). FABMS : *m/z* = 1214 (M⁺+NH₃).

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