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Improved Synthesis of [p-Phosphono and p-Sulfo]methylphenylalanine. Resolution of [p-Phosphono-, p-Sulfo-, p-Carboxy-and p-N-Hydroxycarboxamido-]methylphenylalanine.

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Key Words : *Synthesis and resolution of p($CH_2PO_3H_2$)Phe, p(CH_2SO_3H)Phe, p(CH_2CO_2H)Phe, p($CH_2CONHOH$)Phe derivatives.*

ABSTRACT : To analyse the role of post-translational phosphorylation and sulfation of tyrosine in peptides and proteins, we recently synthesized the new amino acids, p($CH_2PO_3H_2$)Phe and p(CH_2SO_3H)Phe (D,L mixtures) as chemically and enzymatically stable analogs of (O-phospho) and (O-sulfo)-tyrosine. Here, we report improved syntheses of these amino acids and of their N-Boc protected derivatives for use in solid-phase peptide synthesis. A protocol for their enzymatic resolution by use of subtilisin Carlsberg esterase is developed and extended to the resolution of the modified amino acids p(CH_2CO_2H)-D,L-Phe and p($CH_2CONHOH$)-D,L-Phe. The optical purity of the resolved amino acids was tested by chiral phase HPLC. Physical constants of these amino acids and their N-Boc derivatized forms including their characterization under PTC form (phenylthiocarbamyl) are given.

INTRODUCTION

In a preceding paper [1], we have described the synthesis of p-phosphonomethyl-D,L-phenylalanine and p-sulfomethyl-D,L-phenylalanine, two chemically and enzymatically resistant modified amino acids mimicking (O)phosphorylated and (O)sulfated tyrosine respectively. These

compounds can be introduced into peptide or protein sequences to study the role of tyrosine phosphorylation and sulfation in essential biological processes. Indeed, tyrosine phosphorylation is involved in several important regulatory events such as cellular growth and transformation [2,3] and receptor densitization [4] and down-regulation [5] processes. On the other hand, sulfation is the most common post-translational modification of tyrosine residues in proteins [6] and the presence of sulfated tyrosine residues appears critical for the biological activity of several peptides and proteins such as fibronectin [7], heparin [8], cholecystokinin [9,10] or ceruletide [11].

However, the O-phosphate and O-sulfate bonds are chemically and enzymatically labile preventing extensive biological and pharmacological investigations on the physiological relevance of these tyrosine modifications. These problems were overcome by introducting the p-sulfomethyl-phenylalanine residue in the sequence of CCK_8 and its analogs leading to peptides as active as their parent compounds but with longer life times [12,13].

Two other new phenylalanine-derived amino acids with a carboxymethyl (CH_2CO_2H) or a N-hydroxycarboxamidomethyl ($CH_2CONHOH$) group in the para position of the phenyl ring have been recently synthesized in their racemic forms in our laboratory and incorporated in place of Tyr(SO_3H) in the CCK_8 sequence [14] in order to investigate the role of the acidic group on receptor binding and the possible occurence of a divalent cation in the binding site. Simultaneously, Tilley et al. have described another synthesis of p(CH_2CO_2H)Phe and its incorporation in the CCK_8 sequence [15,16].

In addition these modified amino acids will be interesting for the preparation of cyclic peptides through condensation of the phenyl para substituents with the amino group of either the peptide backbone or the side-chains of other residues. Such types of cyclisations have already been reported to provide molecules with receptor selectivity and resistance to peptidases [17].

In this paper, we report improved synthetic routes towards the preparation of the p-phosphonomethyl- and p-sulfomethyl-D,L-phenylalanine residues and appropriately protected forms useful for either liquid or solid phase peptide synthesis [12,13,18].

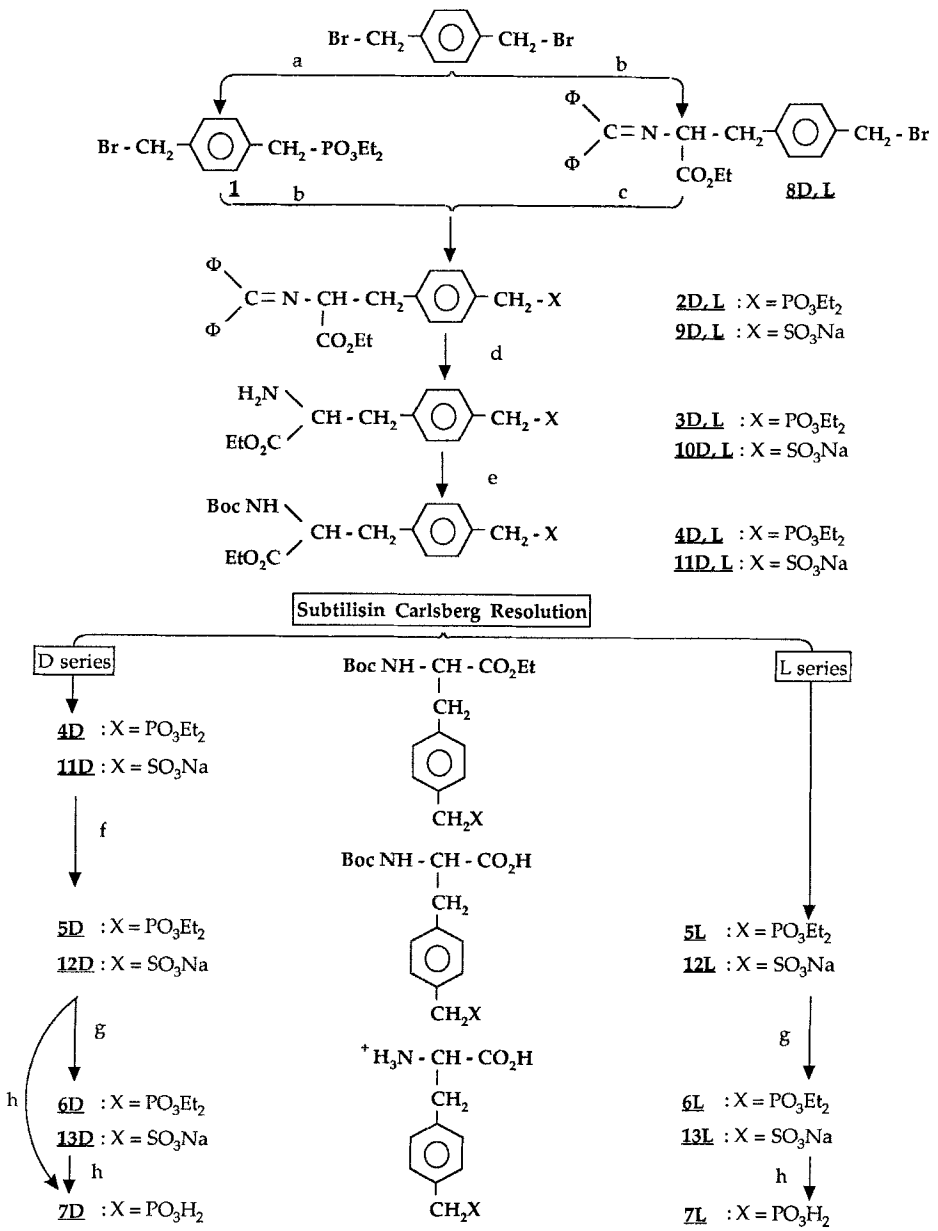
We also report the enzymatic resolution by the subtilisin Carlsberg esterase of these compounds and the p-carboxymethyl-D,L-phenylalanine and p[N-hydroxy-carboxamidomethyl]-D,L-phenylalanine, all protected under N-Boc and ethyl carboxylate forms. The optical purity of the separated enantiomers was verified by chiral phase HPLC and their optical properties determined.

RESULTS AND DISCUSSION

Synthesis of N-Boc-p($CH_2PO_3Et_2$)-D,L-Phe-OEt 4 and N-Boc-p(CH_2SO_3Na)-D,L-Phe-OEt 11 :

Previous syntheses of p($CH_2PO_3H_2$)-D,L-Phe and p(CH_2SO_3Na)-D,L-Phe derivatives were performed either from p-cyanobenzylbromide [1] or α,α'-dichloro-p-xylene [19]. In these synthetic routes, the Cα of the amino acid was provided by a malonic substitution with diethylacetamidomalonate.

Figure 1 : Synthesis and enzymatic resolution of p($CH_2PO_3H_2$)Phe and p(CH_2SO_3Na)Phe derivatives.



a) $P(OEt)_3$; b) Φ_2 - C = N - CH_2 - CO_2Et, KI, $\Phi CH_2 N^+(CH_3)_3$,OH^- dioxane ; c) Na_2SO_3 in dioxane water (1 : 1) ; d) 1) HCl 1N ; 2) aqueous $NaHCO_3$; e) Boc_2O, in THF ; f) 1) NaOH 1N ; 2) HCl 1N ; g) TFA ; h) HCl 6N or $(Me)_3SiBr$.

Thus, acetamido group obtained was not convenient for peptide synthesis especially for solid phase. In our synthesis, starting from α,α' dibromo-p-xylene, the intermediates **3** and **10** were easily obtained as free amines (see figure 1) and protected as N-tert-butyloxycarbonyl to adapt their use in solid phase synthesis while the ester functions allowed the resolution by the subtilisin Carsberg esterase. To prepare p($CH_2PO_3Et_2$)Phe-OEt **3D,L**, the α,α' dibromo-p-xylene was successively substituted by $P(OEt)_3$ under Arbusov conditions, condensed with the carbanion of ethyl-N-(diphenylmethylene)glycinate [20] and hydrolyzed in mild acidic conditions. On the other hand, p(CH_2SO_3Na)Phe-OEt **10D,L** has only been obtained when the alkylation of the Schiff base by α,α' dibromo-p-xylene was performed before the substitution using Na_2SO_3, followed by acidic hydrolysis.

Enzymatic resolution of the N-protected amino esters :

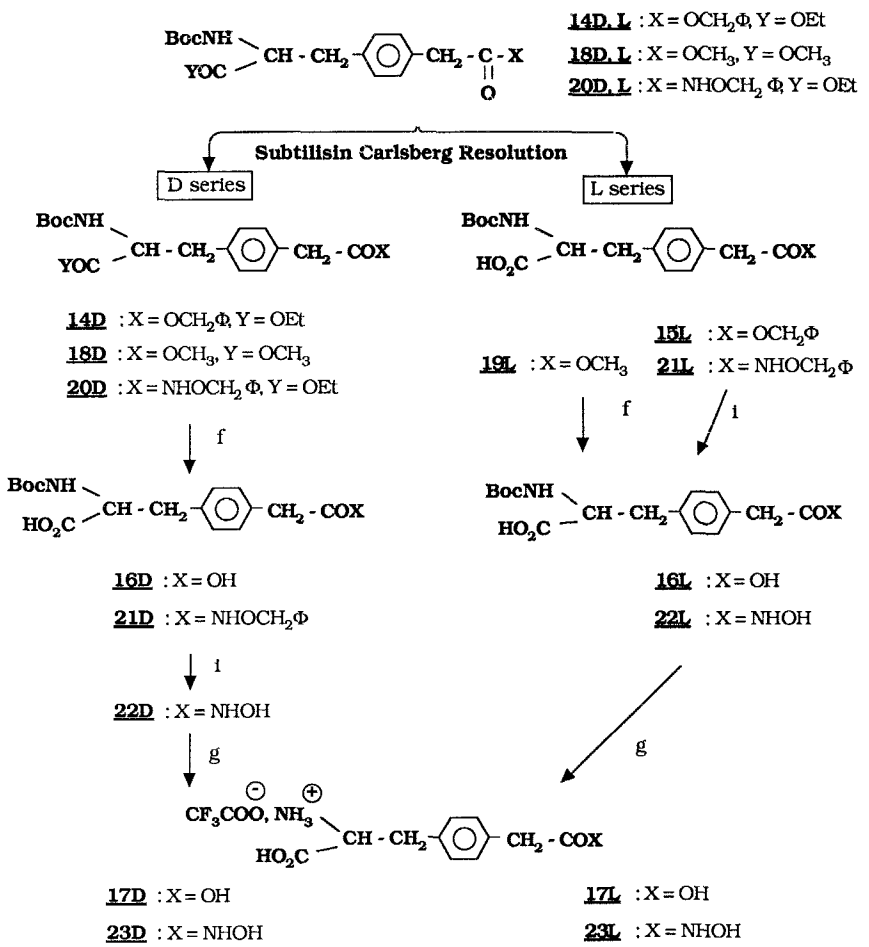
Numerous enzymatic systems have been used for amino acid derivatives resolution such as α-chymotrypsin [21,22], hog kidney acylase [22], carboxypeptidase A [23] or subtilisin Carlsberg esterase [24]. With this latter enzyme, Berger et al. [24] failed to resolve N-benzyloxycarbonyl-p-phenyl-D,L-phenylalanine ethylester. Here, we show that this enzyme is capable of accepting aromatic rings bearing a large variety of substituents such as the sterically hindered and hydrophobic p-CH_2-PO_3Et_2 and p-CH_2-$NHOCH_2\Phi$ moieties or the highly polar anionic p-CH_2-SO_3Na group. Moreover, in the case of the methyl p-(carbomethoxymethyl)-phenylalaninate **18**, subtilisin discriminates between the two methyl ester groups and hydrolyses selectively the C-terminal carboxylate (Figure 2). Due to the weak water solubility of the N-protected modified amino esters in most cases, addition of organic solvents was needed for their enzymatic hydrolysis. DMSO had already been used [25] as a co-solvent, but we have preferred dioxane which proved more efficient and easier to remove. The resolution steps were performed with a mixture of dioxane and water in a ratio depending on the hydrophobicity of the starting modified Boc-phenylalaninates.

The time needed for the asymmetric hydrolyses varied with the quantity of starting material and with the percentage of organic solvent. The nature of the substituent on the aromatic ring also seemed to modify the hydrolysis time, but there was no direct correlation with hydrophobicity or size of the substituents. The rate of the enzymatic hydrolysis was followed by reversed phase HPLC. After consumption of the expected quantity of NaOH 1N necessary for the asymmetric hydrolysis, the HPLC signals of the two species were equivalent and their isolation was performed as described in experimental section. Owing to the fact that the new amino acids are not natural, their optical purity was analyzed by HPLC on a commercially available column containing an immobilized protein stationary phase consisting of BSA (Bovine Serum Albumin) covalently bound to 10 μm silica support. This support was shown to act as a chiral discriminator for the phenyl ring containing amino acids [26].

Figure 3 shows the optical purity of N-Boc-p($CH_2PO_3Et_2$)Phe as L and D enantiomers after enzymatic resolution. On Figure 3A is reported the chiral HPLC profile of N-Boc-p($CH_2PO_3Et_2$)Phe obtained after saponification of the racemate N-Boc-p($CH_2PO_3Et_2$)Phe-OEt,

with conditions giving two well separated peaks for L and D isomers. Figure 3B, shows the optical purity of the L-isomer obtained after enzymatic hydrolysis and Figure 3C, the optical purity of the D-isomer obtained after enzymatic resolution followed by saponification.

Figure 2 : Enzymatic resolution of p(CH_2CO_2H)Phe and p($CH_2CONHOH$)Phe derivatives.



f) 1) NaOH 1N ; 2) HCl 1N ; g) TFA ; i) H_2, Pd/C.

The HPLC characteristics on the chiral column of the other modified N-Boc amino acids are reported in table 1. Even raising the pH and percentage of isopropanol did not allow the resolution of the L and D isomers of the hydroxamate **22** by chiral phase HPLC. After enzymatic resolution, the appropriate deprotections were performed on the L and D series respectively. As shown on Figure 1 and 2, Me and Et esters were saponified, benzyl-esters and O-benzyl-

hydroxamate groups were hydrogenolysed, N-Boc groups were removed by action of TFA and methyl phosphonates were hydrolysed by HCl 6N for 4 hours or with $BrSi(CH_3)_3$ [27,28].

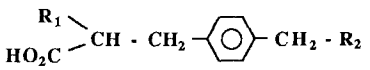


Table 1 : HPLC characteristics on a chiral stationary phase and optical rotations of the resolved p-substituted Phe residues.

n#	R_1	R_2	Vr	α	Specific rotations in degrees (c1 solvent)
5D	BocNH	PO_3Et_2	7.1	α = 1.57	-26.4(EtOAc)
5L	BocNH	PO_3Et_2	9.5		+26.2(EtOAc)
7D	NH_3^+	PO_3H_2	N.D.		+11(HCl 1N)
7L	NH_3^+	PO_3H_2	N.D.		-10.5(HCl 1N)
12D	BocNH	SO_3Na	7.5	α = 2.6	-16.3(H_2O)
12L	BocNH	SO_3Na	4.7		+16.6(H_2O)
13D	NH_3^+	SO_3Na	N.D.		+15.8(H_2O)
13L	NH_3^+	SO_3Na	N.D.		-15.4(H_2O)
16D	BocNH	COOH	5.4	α = 1.68	-13.2(AcOH)
16L from 15L	BocNH	COOH	4.9		+12.4(AcOH)
16L from 19L	BocNH	COOH	4.9		+12.6(AcOH)
17D	NH_3^+	COOH	N.D.		+18.7(H_2O)
17L	NH_3^+	COOH	N.D.		-18.7(H_2O)
21D	BocNH	$CONHOCH_2\Phi$	12.1	α = 1.4	-21.6(MeOH)
21L	BocNH	$CONHOCH_2\Phi$	15.7		+21.9(MeOH)
22D	BocNH	CONHOH	12.2	α = 1	-3.7(AcOH)
22L	BocNH	CONHOH	12.2		+3.8(AcOH)
23D	NH_3^+	CONHOH	N.D.		+13.3(H_2O)
23L	NH_3^+	CONHOH	N.D.		-13.8(H_2O)

Vr are the retention volumes in mL. α is the separation factor ($\alpha = \frac{R_L - R_0}{R_D - R_0}$), R_L, R_D, the retention times of **L** and **D** isomers and Ro the void time. N.D. not determined. Optical rotations are given as $[\alpha_D]$ except for compound **16** which is refered to the 365 nm Hg absorption.

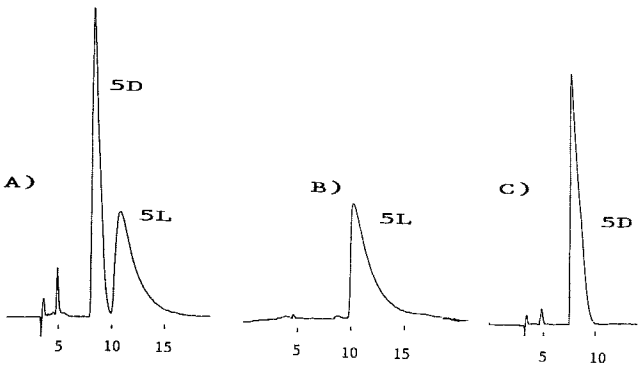


Figure 3 : HPLC profile on a chiral stationary phase of the different samples of N-Boc-p($CH_2PO_3Et_2$)Phe **5** obtained : A) as D,L after saponification of the racemate N-Boc-p ($CH_2PO_3Et_2$)-D,L-Phe-OEt, **4**. B) as L-isomer **5L** isolated from the aqueous phase of the enzymatic hydrolysis ; C) as D-isomer **5D** after saponification of **4D** isolated from the organic phase of the enzymatic hydrolysis. The purity of the resolved **5D** and **5L** isomers is checked on B) and C).

The optical rotations of the new phenylalanyl modified amino acids are reported in Table 1. There is a good correspondence between the optical rotations of compounds belonging to the D and L series showing that enzymatic resolution with subtilisin Carlsberg provides highly optically pure enantiomers in high yields.

The novel amino acids, p($CH_2PO_3H_2$)Phe (L or D), p(CH_2SO_3Na)Phe and p(CH_2CO_2H)Phe were characterized as PTC (phenylthiocarbamyl) derivatives. The residue p($CH_2CONHOH$)Phe was not derivatized since it is hydrolyzed to p(CH_2CO_2H)Phe during amino acid analysis of the peptides. Under the described conditions (see experimental section),p($CH_2PO_3H_2$)Phe derivatized under PTC form has a retention time of 4.7 min (between His and Arg), p(CH_2SO_3Na)Phe has a retention time of 5.7 min (between Thr and Ala, just before Ala) and p(CH_2CO_2H)Phe has a retention time of 7.2 min (between Pro and Tyr, just before the peak of aniline).

In conclusion, this work allows the improved preparation of the new amino acids p-phosphonomethyl- and p-sulfomethyl-phenylalanine, their enzymatic resolution as well as that of p-carboxymethyl- and p-N-hydroxycarboxamidomethyl-phenylalanine and characterization in free base and N-Boc protected forms as pure optical enantiomers. The N-protected amino acids have already been introduced into the sequences of biologically-active peptides by solid phase method [14,18].

EXPERIMENTAL SECTION

Synthesis.

^1H NMR spectra were recorded on a Bruker WH 270 spectrometer at 270 MHz. Mass spectra were recorded on a double focusing VG 7°-250 spectrometer (VG instrument) equipped with a fast atom bombardment (FAB) gun (Ion Techn.). Optical rotations were measured at 25°C on a Perkin-Elmer Model 141 polarimeter in a 1 dm microcell. Melting points were obtained on a Electrothermal apparatus and are uncorrected. Elemental analyses were performed by the Université Paris VI. Analytical HPLC was performed on an Applied Biosystem 151A apparatus using either a nucleosil C_8 column (7 µm, 4.6 x 220 mm) or a chiral column (BSA) obtained from SFCC-France (5 µm, 4.6 x 250 mm). The C_8 column was eluted with mixtures of solution A (0.1% TFA in water) and solution B (70% CH_3CN in water and 0.09% TFA). Flow rate 1.5 mL/min, (U.V. detection, 214 nm). The chiral column was eluted with phosphate buffer 0.1 M, pH 8, 3% isopropanol. Flow rate 0.9 mL/min. Derivatization of amino acids as their PTC (phenylthiocarbamyl) form was made by means of PITC (phenylisothiocyanate)in presence of DIEA and their characterization was performed on a Applied Biosystem 130A PTC amino acid analyzer equipped with a C18 column 21x220 mm (5 µm, spheric) special for PTC amino acids separation. The mobile phase consisted of solution A (50 mM sodium acetate buffer, pH 5.4) and solution B (70% acetonitrile in 32 mM sodium acetate, pH 6.1). The gradient was progressively 4% to 100% of solution B in 30 min (flow rate 0.3 ml/min, UV detection at 254 nm). 50 ng samples were derivatized.

Purity of the compounds was also checked on precoated plates of silica gel (60F-254, 0.2 mm thick, Merck) using the following solvent systems (v/v). A : EtOAc/hexane, 3/1 ; B : EtOAc/hexane, 8/2 ; C : CH_2Cl_2/MeOH, 9/1 ; D : Butanol/AcOH/H_2O : 4/1/1 ; E : Hexane/Et_2O, 9/1 ; F : $CHCl_3$/MeOH, 8/2 ; G : $CHCl_3$/MeOH/H_2O/AcOH : 5/5/1/0.5 ; H : CH_2Cl_2/MeOH, 7/3 ; I : $CHCl_3$/MeOH/H_2O/AcOH : 7/3/0.6/0.3 ; J : $CHCl_3$/MeOH/H_2O/AcOH/EtOAc : 7/3/0.6/0.3/21.8 ; K : CH_2Cl_2/MeOH : 95/5.

Column flash chromatographies were performed with silica gel 60, 60-229 mesh ASTM (Merck). Ethyl-N-(diphenylmethylene)glycinate, α,α' dibromo-p-xylene and trimethylsilylbromide were purchased from Aldrich (France), Subtilisin Carlsberg from Sigma. All other reagents and solvents were from Prolabo (France). The following abbreviations were used : Boc_2O, di-tert-butyl dicarbonate ; TFA, trifluoroacetic acid ; THF, tetrahydrofuran ; HMDSO, hexamethyldisiloxane ; DMSO, dimethyl sulfoxide ; D_2O, deuterium oxide ; mp, melting point.

Diethyl [(4-bromomethyl)phenyl]methyl phosphonate (1).

Procedure a : In a flask equipped for distillation, 34.5 g (0.13 mole) of α,α'-dibromo-p-xylene was heated with triethylphosphite (22.1 ml) at 80-90°C for 3 hours. Then volatile products were removed by distillation under normal pressure and the remaining triethylphosphite by distillation under reduced pressure. A viscous oil was obtained. Addition of a small quantity of MeOH to the oil crystallized 12.1 g of the starting α,α'-dibromoxylene. Purification of the remaining oily material by flash chromatography with EtOAc/hexane, 3/1 as eluent, provided 22 g of crude

compound **1** (80% yield, Rf_A 0.25). ^1H NMR (DMSO d6) δ ppm from HMDSO, δ 1.1 (t, 6H, $POCH_2\underline{CH_3}$), 3.15 (d, 2H, CH_2P), 3.85 (q, 4H, $PO\underline{CH_2}CH_3$), 4.6 (s, 2H, CH_2Br), 7.2 (m, 4H, Ar).

*Ethyl N(diphenylmethylene)-4-(diethylphosphonomethyl)-D,L-phenylalaninate (**2**).*

Procedure b : 11.2 g (0.035 mole) of the phosphonate **1**, 9.3 g (1 eq) of ethyl N-(diphenylmethylene) glycinate and 0.57 g (0.1 eq) of KI were dissolved in 280 ml dioxane and cooled at 10°C under stirring. 14.5 ml (1 eq) of benzyltrimethylammonium hydroxide (40% aqueous) were added dropwise over 2 hours. The reaction mixture was brought to room temperature and stirring maintained for 6 hours. 300 g ice were added to the mixture. The aqueous phase was then extensively extracted with toluene. After drying over $MgSO_4$, the solvent was evaporated and 14.3 g (80% yield) of crude oily compound **2** was obtained. A pure sample was obtained by flash chromatography with EtOAc/hexane 3/1 as eluent, (Rf_B 0.25). ^1H NMR (DMSO d6) δ ppm from HMDSO, δ 1.07 (t, 3H, $CO_2CH_2\underline{CH_3}$), 1.1 (t, 6H, $POCH_2\underline{CH_3}$) 2.7 (m, 2H, $H\beta_1$, $H\beta_2$), 3.1 (d, 2H, CH_2P), 3.5 (q, 1H, Hα), 3.8 (q, 4H, $POCH_2$), 3.9 (m, 3H, $CHCO_2CH_2$), 7.0 (m, 4H, Ar), 7.1-7.5 (m, 10H, Ar).

*Ethyl 4-(diethylphosphonomethyl)-D,L-phenylalaninate (**3**).*

Procedure d : The N-protected compound **2**, 13.9 g (27.4 mmole) was dissolved in Et_2O (110 ml) and HCl 1N (110 ml, 4eq) and stirred for 2 hours at room temperature. The ethereal phase was discarded and the aqueous layer alcalinized with $NaHCO_3$ before extracting with CH_2Cl_2. The organic phase was dried on Na_2SO_4 and evaporated to provide 5.2 g of **3** as oily material (60% yield, Rf_C 0.30). ^1H NMR (DMSO d6) δ ppm from HMDSO, δ 1.1 (t, 6H, $POCH_2\underline{CH_3}$), 1.07 (t, 3H, $CO_2CH_2\underline{CH_3}$), 2.73 and 2.78 ($H\beta_1$, $H\beta_2$), 3.1 (d, 2H, CH_2P, $^2J_{HP}$ = 24.5 Hz), 3.9 (q, 4H, $PO\underline{CH_2}CH_3$), 4.0 (m, 3H, $CHCO_2CH_2$), 7.12 (m, 4H, Ar).

*Ethyl (N-tert-butyloxycarbonyl)-(4-diethylphosphonomethyl)-D,L-phenylalaninate (**4**).*

Procedure e : 4.48 g (12.8 mmole) of amine **3** was dissolved in THF (220 ml) at 0°C with Boc_2O (5.14 g, 23 mmol, 1.8 eq). After 1 h at 0°C the solution was stirred at room temperature overnight. THF was evaporated, water was added (100 ml),and the aqueous phase extracted with CH_2Cl_2. The organic layer was then dried over Na_2SO_4 and evaporated. The crude extract was purified by flash chromatography on silica gel with EtOAc/hexane 3/1 as eluent. 2.5 g of pure **4** were obtained as oily product (43% yield, Rf_B 0.25). ^1H NMR (DMSO d6) δ ppm from HMDSO, δ 1.07(t, 3H, $CO_2CH_2\underline{CH_3}$), 1.1 (t, 6H, $POCH_2\underline{CH_3}$), 1.3 (s, 9H, $C(CH_3)_3$, 2.78 and 2.9 ($H\beta_1$, $H\beta_2$), 3.1 (d, 2H, CH_2P), 3.85 (q, 4H, $PO\underline{CH_2}CH_3$), 4.0 (m, 3H, $CHCO_2CH_2$), 7.2 (d, 1H, NH), 7.1 (s, 4H, Ar). Found C, 56.86 ; H, 7.83 ; N, 3.17. $C_{21}H_{34}NO_7P$ requires C, 56.87 ; H, 7.73 ; N, 3.16%.

*N-tert-Butyloxycarbonyl-(4-diethylphosphonomethyl)-L-phenylalanine (**5L**) and Ethyl (N-tert-butyloxycarbonyl)-(4-diethylphosphonomethyl)-D-phenylalaninate (**4D**).*

Resolution procedure : The racemic sample **4D,L** 1.1g (2.5 mmole) was dissolved in dioxane (10 ml) and distilled H_2O (8 ml). 1.7 ml of a solution 10^{-2}M KCl, 10^{-4}M KH_2PO_4 was added and the

pH adjusted to 7 with NaOH 1N. Protease type VIII subtilisin Carlsberg (6.5 mg) was added and the reaction mixture stirred at room temperature while the pH was maintained around 7 with addition of NaOH 1N. Dioxane was added when necessary to avoid precipitation (~20 ml). When the theoretical quantity of NaOH needed for the hydrolysis of L-isomer had been consumed, the solution was diluted in water (10 ml). The aqueous layer was extracted with EtOAc (3 x 50 ml). The organic phase dried on Na_2SO_4 and evaporated provided **4D** (0.51 g, 46% yield, Rf_C 0.95). The aqueous phase was acidified with HCl 1N until pH 2 and extracted with EtOAc. After drying on Na_2SO_4 and evaporation of the organic layer, **5L** was obtained (0.41 g, 40% yield, Rf_C 0.65).

N-tert-Butyloxycarbonyl-4-(diethylphosphonomethyl)-D-phenylalanine (5D).
Procedure f : 200 mg (4.5 mmol) of compound **4D** was dissolved in EtOH (2 ml) and H_2O (0.5 ml) at 5°C and NaOH 1N (1 ml, 2.2 eq) was added dropwise under stirring at this temperature for 1 hour. Stirring was maintained for 4 hours, ethanol was then evaporated and water (5 ml) added. The aqueous phase was washed with EtOAc. Aqueous HCl 1N was added until the solution became pH 2 and the aqueous phase was extracted with EtOAc. The organic layer was dried on Na_2SO_4 and evaporated to give **5D** (140 mg, 75% yield, Rf_C 0.65). ^1H NMR (DMSO d6) δ ppm from HMDSO, δ 1.1 (t, 6H, $POCH_2\underline{CH_3}$), 1.25 (s, 9H, $(CH_3)_3$), 2.75 and 2.90 (m, 2H, $H\beta_1$, $H\beta_2$), 3.08 (d, 2H, CH_2P), 3.85 (q, 4H, $PO\underline{CH_2}CH_3$), 4.0 (m, 1H, Hα), 6.97 (d, 1H, NH), 7.15 (s, 4H, Ar). Found C, 54.86 ; H, 7.31 ; N, 3.49. $C_{19}H_{30}NO_7P$ requires C, 54.93 ; H, 7.28 ; N, 3.37%.

4-(Diethylphosphonomethyl)-(L or D)-phenylalanine, TFA salt (6L) or (6D).
Procedure g : 150 mg (0.36 mmole) of compound **5L** (or **D**) was stirred with CH_2Cl_2 (1 ml), TFA (0.5 ml, 20 eq) at 0°C for 1 h and at room temperature for 3 hours. After evaporation 132 mg of compound **6L** (or **D**) were obtained (85% yield, Rf_D 0.90). ^1H NMR (DMSO d6) δ from HMDSO, δ 1.1 (t, 6H, $POCH_2\underline{CH_3}$), 2.95 and 3.0 (q, 1H, 1H, $H\beta_1$, $H\beta_2$), 3.15 (d, 2H, CH_2P), 3.2 (t, 1H, Hα), 3.85 (q, 4H, $PO\underline{CH_2}CH_3$), 7.1 (s, 4H, Ar). FAB-MS (M^+) 316.

4-(Phosphonomethyl)-(L or D)-phenylalanine, (7L) or (7D).
Procedure i : 330 mg (0.81 mmole) of the N-protected compound **5L** (or **D**) was heated 4 hours in HCl 6N (3 ml). After evaporation under vacuo, the residue was washed with toluene/ether (1/1), a H_2O/THF mixture (5 ml) was added and the mixture was treated with propylene oxide. After evaporation the residue(160 mg, 84% yield) was cristallized from EtOH/H_2O mixture to provide pure **7** (Rf_D 0.24, mp 298°C). ^1H NMR (D_2O + TFA) δ ppm from HMDS, δ 3.1 and 3.22 (q, 2H, $H\beta_1$, $H\beta_2$), 3.1 (d, 2H, CH_2P), 4.15 (t, 1H, Hα), 7.14 (m, 4H, Ar). FAB-Mass spectrum, mp, in accordance with literature [1, 19]. Treatment of **5L** (or **5D**) with $(Me)_3SiBr$ following ref. 27 provided **7L** (or **7D**) with lower yields.

Ethyl N-(diphenylmethylene)-4-(bromomethyl)-D,L-phenylalaninate (8) : This compound was obtained from α,α'-dibromo-p-xylene (30 g ; 0.11mmol ; 1.4 eq) following *procedure b*. After purification by flash chromatography on silica gel with hexane/Et_2O 8/2 as eluent, 12.6 g of **8**

was obtained as oily material (35% yield, Rf_E 0.17). ^1H NMR (DMSO d6) δ ppm from HMDSO, δ 1.15 (t, 3H, $CO_2CH_2\underline{CH_3}$), 2.95 and 3.05 (dd, 2H,$H\beta_1$, $H\beta_2$), 4.05 (m, 3H, $CHCO_2CH_2$), 4.65 (s, 2H, CH_2Br), 6.50-7.50 (m, 14H, Ar).

*Ethyl N-(diphenylmethylene)-4-(sulfomethyl)-D,L-phenylalaninate (**9**).*
Procedure c : To 2.26 g (5 mmol, 1eq) of ethyl-N-(diphenylmethylene)-4-(bromomethyl)-D,L-phenylalaninate dissolved in dioxane (20 ml) was added 3.15 g of Na_2SO_3 (25 mmol, 5eq) dissolved in water (20 ml). The resulting solution was heated at 110°C for 1 hour and then evaporated in vacuo. EtOH (50 ml) was added and the precipitated salts were filtered and discarded. After evaporation of the solvent, 2.3 g of compound **9D,L** were obtained as oily material (90% yield, Rf_F 0.16). ^1H NMR (DMSO d6) δ ppm from HMDSO, δ 1.15 (t, 3H, $CO_2CH_2\underline{CH_3}$), 2.9 and 3.00 (dd, 2H, $H\beta_1$, $H\beta_2$), 3.60 (s, 2H, CH_2SO_3), 4.05 (m, 3H, $CHCO_2CH_2$), 6.50-7.50 (m, 14H, Ar).

*Ethyl 4-(sulfomethyl)-D,L-phenylalaninate (**10**) :* Starting from 5.37 g (11.9 mmol) of **9**, 3.5 g of compound **10 D,L** was obtained following *procedure d* (yield 100%, Rf_G 0.55, mp>400°C). ^1H NMR (DMSO d6) δ ppm from HMDSO, δ 1.10 (t, 3H, $CO_2CH_2\underline{CH_3}$), 3.00 (m, 2H, $H\beta_1$, $H\beta_2$), 3.75 (s, 2H, CH_2SO_3), 4.05 (m, 2H, CO_2CH_2), 4.15 (m, 1H, Hα), 7.00-7.30 (m, 4H, Ar).

*Ethyl (N-tert-butyloxycarbonyl)-4-(sulfomethyl)-D,L-phenylalaninate (**11**) :* Following *procedure e*, 2 g (6.46 mmol) of compound **10** provided 1.85 g of **11** as white crystals (70% yield, Rf_H 0.30, mp>400°C). ^1H NMR (DMSO d6) δ ppm from HMDSO, δ 1.10 (t, 3H, $CO_2CH_2\underline{CH_3}$), 1.30 (s, 9H, $(CH_3)_3$), 2.85 (m, 2H, $H\beta_1$, $H\beta_2$), 3.60 (s, 2H, CH_2SO_3), 4.05 (m, 3H, $CHCO_2CH_2$), 7.00-7.25 (m, 5H, NH, Ar).

*Enzymatic resolution of Ethyl (N-tert-butyloxycarbonyl)-4-(sulfomethyl)-D-phenylalaninate (**11D**) and the corresponding acid (**12L**) :* The two preceding compounds were separated from 395 mg (1 mmol) of the racemic **11D,L** following the enzymatic procedure already described except that it was performed in water without co-solvent in 1 hour. 190 mg of **11D** were obtained (48% yield, Rf_H 0.30, mp>400°C) and 185 mg of **12L** (47% yield, Rf_H 0.10, mp>400°C). FAB-MS (M^+) 381.

*N-tert-Butyloxycarbonyl-4-(sulfomethyl)-D-phenylalanine (**12D**) :* Following *procedure f*, 395 mg (1 mmol) of **11D** provided after 5 hours reaction time, isolation and flash chromatography (CH_2Cl_2/MeOH 8/2), 320 mg of pure compound **12D** (84% yield, Rf_H 0.10, mp>400°C).

*4-(Sulfomethyl)-(D or L)-phenylalanine, as TFA salt (**13D** or **13L**) :* Following *procedure g*, 160 mg (0.4 mmol) of **12D** or (**L**) provided 160 mg of **13D** or (**L**) (quantitative yield, Rf_I 0.05, mp>400°C). ^1H NMR in accordance with literature [13].

*Enzymatic resolution of Ethyl (N-tert-butyloxycarbonyl)-4-(carboxybenzyloxymethyl)-D-phenylalaninate (**14D**) and its corresponding acid (**15L**) :* Following the usual procedure, 1.14g

(2.6 mmol) of **14 D,L** (mp 78°C) dissolved in dioxane (10 ml) and H_2O (10 ml) provided after 24 hours, 455 mg of **14D** (40% yield, Rf_C 0.57, mp 78°C) and 400 mg of **15L** (37% yield, Rf_C 0.38, mp 121°C), which were extracted by CH_2Cl_2 and purified on flash chromatography in the preceding Rf mixtures. **14D** : found C, 68.14 ; H, 7.24 ; N, 3.19. $C_{25}H_{31}NO_6$ requires C, 68.00 ; H, 7.08 ; N, 3.17. ^1H NMR in accordance with literature [14]. **15L**, ^1H NMR (DMSO d6) δ ppm from HMDSO, δ 1.25 (s, 9H, $(CH_3)_3$), 2.82 and 3.0 (dd, 2H, $H\beta_1$, $H\beta_2$), 3.60 (s, 2H, CH_2CO_2), 3.87 (m, 1H, Hα), 5.04 (s, 2H, CO_2CH_2), 6.15 (s, 1H, NH), 7.07 (s, 4H, Ar), 7.27 (m, 5H, Ar).

*N-tert-Butyloxycarbonyl-4-(carboxymethyl)-D-phenylalanine (**16D**) :* 280 mg (0.63 mmol) of **14D** were saponified in 5 hours with 2.2 eq NaOH following *procedure f*. After purification by flash chromatography (eluent J). 162 mg of **16D** was obtained as a pure white solid (80% yield, Rf_J 0.43, mp>350°C). ^1H NMR (DMSO d6) δ ppm from HMDSO, δ 1.24 (s, 9H, $(CH_3)_3$), 2.75 and 2.90 (dd, 2H, $H\beta_1$, $H\beta_2$), 3.02 (s, 2H, $\underline{CH_2}CO_2H$), 3.98 (m, 1H, Hα), 6.97 (d, 1H, NH), 7.10 (s, 4H, Ar). FAB-MS (MH^+) 324.

*N-tert-Butyloxycarbonyl-4-(carboxymethyl)-L-phenylalanine (**16L**).*
Procedure i : 124 mg (0.3 mmol) of **15L** in MeOH (2 ml) was added to a suspension of 10% Pd on charcoal (18 mg) in MeOH (2 ml) saturated with hydrogen. The mixture was stirred under hydrogen for 2 hours at room temperature. After filtration of the catalyst the solution was evaporated in vacuo to provide 90 mg of **16L** as a white solid (93% yield, Rf_J 0.43, mp>350°C). **16L** was also obtained from **19L** following *procedure f*.

*4-(Carboxymethyl)-(D or L)-phenylalanine, as TFA salt (**17D** or **17L**) :* Starting from compound **16D** (97 mg, 0.3 mmol) or compound **16L** (65 mg, 0.2 mmol), white crystals of **17D** or **17L** (86% yield, Rf_I 0.12, mp>350°C) were obtained via *procedure g*. ^1H NMR (DMSO d6) δ ppm from HMDSO, δ 2.82 and 3.07 (dd, 2H, $H\beta_1$, $H\beta_2$), 3.45 (m, 3H, Hα, $\underline{CH_2}CO_2H$), 7.14 (s, 4H, Ar).

*Enzymatic separation of Methyl (N-tert-butyloxycarbonyl)-4-(carbomethoxymethyl)-D-phenylalaninate (**18D**) and its corresponding acid (**19L**):* 640 mg (1.82 mmol) of the racemic mixture **18D, L** dissolved in dioxane (7 ml) and H_2O (7 ml) were treated following the *resolution procedure* with 4.8 mg of the enzyme in two hours and provided after purification by flash chromatography (eluent F) 300 mg of **18D** (47% yield, Rf_F 0.16, amorphous) and 306 mg of **19L** (49% yield, Rf_C 0.20) further purified for $[\alpha]_D$ measurements by flash chromatography (eluent C). **18D**, ^1H NMR (DMSO d6) δ ppm from HMDSO, δ 1.3 (s, 9H, $(CH_3)_3$), 2.78 and 2.98 (dd, 2H, $H\beta_1$, $H\beta_2$), 3.5 (s, 6H, OCH_3), 3.55 (s, 2H, CH_2CO). FAB-MS (MH^+) 352. Physical data of **19L** are in accordance with literature [14]. $[\alpha]_D$ = + 23 (c = 1.03, MeOH) (lit.[15] $[\alpha]_D$ = + 22.39 (c = 1.0, EtOH)).

*Enzymatic separation of Ethyl (N-tert-butyloxycarbonyl)-4-[(N'-benzyloxy)carboxamidomethyl]-D-phenylaninate (**20D**) and the corresponding acid (**21L**) :* 945 mg (2.07 mmol) of **20D,L**

hydrolyzed in presence of 5.5 mg of the enzyme provided in 8 hours 435 mg of **20D** purified by flash chromatography (eluent CH_2Cl_2/MeOH/95/5) (46% yield, Rf_K 0.36, mp 117°C) and 386 mg of **21L** purified by flash chromatography with eluent H (43% yield, Rf_H 0.38, mp 200-202°C). **20D** : found C, 65.74 ; H, 7.14 ; N, 6.13. $C_{25}H_{32}N_2O_6$ requires C, 65.77 ; H, 7.06 ; N, 6.14. Physical data of **21L** are in accordance with literature [14].

*N(tert-Butyloxycarbonyl)-4[(N'-benzyloxy)carboxamidomethyl]-D-phenylalanine (**21D**) :* 315 mg (0.7 mmol) of **20D** provided via *procedure f*, 280 mg of compound **21D** purified by flash chromatography (eluent H) (90% yield, Rf_H 0.38, mp 199-201°C). Physical data are in accordance with literature [14].

*N(tert-Butyloxycarbonyl)-4-[N'-hydroxycarboxamidomethyl]-(D or L)-phenylalanine (**22D** or **22L**)* : Following *procedure i*, **21D** (180 mg, 0.42 mmol) provided **22D** as a white solid (170 mg, 91% yield, Rf_H 0.11, mp>350°C). Similarly **21L** (236 mg, 0.55 mmol) provided **22L** (220 mg, 90% yield), with similar characteristics as **22D**. ^1H NMR (DMSO d6) δ ppm from HMDSO, δ 1.25 (s, 9H, $(CH_3)_3$), 2.83 and 2.99 (dd, 2H, $H\beta_1$, $H\beta_2$), 3.16 (s, 2H, $\underline{CH_2}CO_2H$), 3.85 (m, 1H, Hα), 6.07 (s, 1H, BocNH), 7.02 (s, 4H, Ar), 8.80 (s, 1H, CONH), 10.15 (s, 1H, OH) FAB-MS (MH^+) 339.

*4-[(N'-Hydroxy)carboxamidomethyl]-(D or L)-phenylalanine as TFA salt, (**23D** or **23L**) :* From compound **22D** (101 mg, 0.3 mmol) or compound **22L** (0.101 g, 0.3 mmol) white solids **23D** or **23L** were obtained (85% yield, Rf_I 0.20, mp>350°C). ^1H NMR data are in accordance with literature [14].

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