

Organophosphorus Analogues and Derivatives of the Natural L-Amino Carboxylic Acids and Peptides. VI.¹⁾ A Phospha^C-Peptide Analogue of Plumbemycin A

Ivan A. NATCHEV

Research Centre Konstrukcionni Polimeri, 5-003 Gara Iskar, 1528 Sofia, Bulgaria

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Synopsis. A phosphat^c-peptide of Plumbemycin A has been studied. Condensation of *N*-trifluoroacetyl-3,4-didehydro-5-(ethoxyhydroxyphosphinyl)norvaline (1) and L-analyl-L-aspartic acid diethyl ester (2) is carried out by the DCC method to give the entirely protected phosphat^c-tripeptide (3). Conditions for the enzyme-catalyzed hydrolysis of the ester groups with alkaline mesintericopeptidase, phosphodiesterase I, and α -chymotrypsin and the removal of the trifluoroacetyl group with an aqueous ammonia solution has been achieved.

Phospha^C-peptides (the CO-NH group is exchanged for PO-NH) were first synthesized by Imoto et al.²⁾ and Martell et al.³⁾ The protected phospha^C-peptide [[(benzyloxycarbonyl)amino]methylphosphinyl]phenylalanine⁴⁾ has been synthesized and studied as inhibitor of enzyme carboxypeptidase. A. Yamauchi et al.⁵⁾ synthesized *N*-[(2-aminoethyl)ethoxyphosphinyl]glycine ethyl ester. This compound is a phospha^C-peptide, in which a hydroxyl group in the phosphoryl group is protected as an ester. The major problem in the synthesis of "pure" phospha^C-peptides (without protected groups) is its highly sensitive nature upon hydrolysis to even mild acids and bases.³⁾ Campbell et al.,⁶⁾ recommend for removal of two *O*-ethyl groups in diethyl phosphonate ($-\text{PO}(\text{OEt})_2 \rightarrow -\text{PO}_3\text{H}_2$) to use HBr/AcOH or to carry out treatment of $-\text{PO}(\text{OSiMe}_3)_2$, which are more readily hydrolyzable with Me_3SiBr . The last possibility was explored by Issleib et al.⁷⁾ to obtain "pure" phospha^C-peptides. Our attempts to apply these methods to the synthesis of phospha^C-peptides analogues of Plumbemycin were unsuccessful due to the rather sensitive olefinic bond of 3,4-didehydronorvaline. Our studies directed toward the synthesis of methylphosphoryl analogue of Plumbemycin A,⁸⁾ methylphosphoryl- and phosphono analogues of glutathione^{9,10)} and phospha^C-peptide analogue of Bialaphos (SF-1923)¹¹⁾ showed as a most promising method for the synthesis of "pure" phospha^C-peptides, the one developed in our laboratory¹²⁾ which makes possible the use of highly selective enzymes to catalyze the hydrolysis of the blocking groups.

The condensation of *N*-trifluoroacetyl-3,4-didehydro-5-(ethoxyhydroxyphosphinyl)norvaline ethyl ester (**1**) synthesized by us (cf. later communication from the same author) and L-alanyl-L-aspartic acid diethyl ester (**2**) was carried out by the DCC method. When we used *N*-TFA-3,4-didehydro-5-phosphono-L-norvaline ethyl ester free phosphono groups, extra products were obtained and the yield of the protected phosphapeptide **3** was much lower.

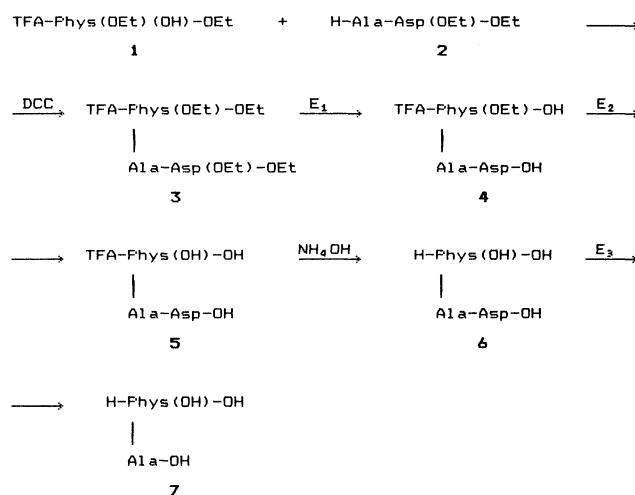
A mixture of molar equivalent amounts of components **1** and **2** and a 10% excess of DCC in dry ethyl

acetate at room temperature was stirred for 12 h and after the usual work-up, the phospho^C-tripeptide **3** was isolated in about 70% yield.

An attempt at the base- or acid-catalyzed hydrolysis of the ethoxycarbonyl groups of **3** afforded the hydrolytic decomposition of PO-NH.

When the enzyme alkaline mesintericopeptidase was applied under the most favorable conditions established by us [20 g substrate **3**, 6–7 mg enzyme in aqueous buffer (500 ml, pH 7.8), stirring at 25°C to a ninhydrin-positive detection], selective hydrolysis of the ethoxycarbonyl groups proceeded to give the *N*- and *P*-protected phospho⁶-tripeptide **4**.

The enzyme phosphodiesterase I, free or spread on a polymer carrier, contributed to the hydrolysis of the ethoxyphosphonyl group to afford phospho^C-tripeptide acid *N*-trifluoroacetyl **5**, which was isolated in almost quantitative yield. Naturally, the previously established amount of 5 mg phosphodiesterase I for 20 g substrate can be varied, but the enzyme/substrate ratio must be within the prescribed limits. The experimental technique was much simplified when the enzyme was spread on a polymer carrier. In that case 10–15



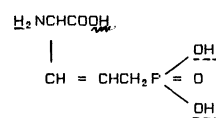
E₁: Alkaline mesentericopeptidase;

E₂: Phosphodiesterase I:

E₃: α -Chymotrypsin:

TFA: $\text{CF}_3\text{CO}_2\text{H}$

H-Phys(OH)₂-OH = 5-phosphono-3,4-didehydro-L-norvaline):



Scheme 1.

mg enzyme was used for 20 g of the substrate and continuous stirring of the reaction mixture was necessary. The enzyme spread on a polymer carrier can be used at least in twelve consecutive experiments without losing its activity towards the standard substrate bis(4-nitrophenyl) hydrogenphosphate.

It is of interest to note that none of the substrates with PO-NH bond studied in our laboratory underwent hydrolytic cleavage of PO-NH bonds with phosphodiesterase I.

On the other hand alkaline phosphatase with closely related activity has a catalytic effect towards neither ethoxyphosphinyl, nor aminophosphinyl groups.

The main problem in the synthesis of "pure" phospho^C-peptide analogue of Plumbemycin A is the removal of the *N*-protective group. Unfortunately, positive results were not obtained for the samples studied here. Thus, the test for selective hydrolysis of *N*-acetyl group with the proteolytic enzymes resulted in decomposition of the peptide bond Ala-Asp. The catalytic hydrogenation, the treatment with HBr/AcOH or the base- or acid-catalyzed hydrolysis of other *N*-protective groups were also unsuitable due to lability of the PO-NH group and the olefinic bond. The optimum conditions were found by using the *N*-trifluoroacetyl protective group, which could be removed in about 45% yield with aqueous ammonia-dioxane solution (pH 10) at 30–40 °C for 1 h to afford phospho^C-peptide analogue **6** of Plumbemycin A.

Base-catalyzed hydrolysis of **6** liberates, 3,4-didehydro-5-phosphono-L-norvaline¹²⁾ and the dipeptide alanyl-aspartic acid.

Under the optimum conditions¹²⁾ [20 g substrate **6**, 5 mg α -chymotrypsin in an aqueous buffer medium (500 ml, pH 7.8), stirring at 25 °C for 6 h] the dipeptide **7** was isolated in about 85% yield.

Studies of the physiological activity of the newly synthesized phospho^C-peptides **6** and **7** are under way and will be published in due course.

Experimental

General. IR spectra, elemental analysis, mp, Mw, HPLC, $[\alpha]_D^{25}$ on Perkin-Elmer instruments; reagents and solvents from "Fluka", "Aldrich", and "Merck"; phosphodiesterase I—"Sigma"; α -chymotrypsin—Pharmachim, Bulgaria; alkaline mesintericopeptidase—Inst. Org. Chem., Bulg. Acad. Sci.; TLC—silica-gel film "Merck"—molibdophosphate or ninhydrin detection.

Synthesis of the *N*-[(*S*)-(4-Ethoxycarbonyl-4-trifluoroacetamido-2-butenyl)ethoxyphosphinyl]alanylaspatic Acid α,β -Diethyl Ester (3**).** A mixture of *N*-trifluoroacetyl-L-L-3,4-didehydro-(*S*)-(ethoxyhydroxyphosphinyl)norvaline ethyl ester **1** (34.72 g, 0.1 mol), alanylaspatic acid diethyl ester **2** (26.03 g, 0.1 mol) and DCC (22.69 g, 0.11 mol) in dry ethyl acetate (300 ml) is stirred at room temperature for 12 h. After filtration, the reaction mixture is washed consecutively with water, 5% aqueous sodium carbonate solution, water, 5% hydrochloric acid, water, and dried over anhydrous MgSO₄, and concentrated under vacuum to dryness. The light yellow viscous oil is loaded on a silica-gel column, which is eluted with chloroform:methanol (9:1).

Compound 3: Yield, 41.38 g (70.2%); mp 73–75 °C (after a continuous stay of the obtained oil in EtOAc/*n*-C₆H₁₄ at

–5 °C in refrigerator); IR (KBr) cm^{–1}: 1750–1720, 1670–1620, 1550, 1310, 1210, 1000, 960, 855, 730, 635; *R*_f: 0.75 (CHCl₃: MeOH=9:1); $[\alpha]_D^{20}$ –96.3° (*c* 1, MeOH).

Found: C, 44.61; H, 6.11; N, 6.96%. Calcd for C₂₂H₃₅F₃N₃O₁₀P: C, 44.82; H, 5.98; N, 7.13%. Mw, Found/Calcd, 587/589.503.

The substance is soluble in most organic solvents and is insoluble in water. Heating of **3** (5.88 g, 0.01 mol) in 0.2 M HCl (25 ml) (1M=1 mol dm^{–3}) at 50 °C for 30 min gives the starting norvaline (**1**) (2.99 g, 86.3%) and the dipeptide (**2**) (1.93 g, 73.4%).

***N*-[(*S*)-(4-Carboxy-4-trifluoroacetamido-2-butenyl)ethoxyphosphinyl]alanylaspatic Acid (**4**).** A mixture of the tripeptide **3** (20 g, 0.034 mol), alkaline mesintericopeptidase (8 mg), 3–4 drops of "Tween-80" (beforehand homogenized with **3** in 200 ml of water) in an aqueous buffer (500 ml, pH 7.8) is stirred at 25 °C to a ninhydrin-positive detection. After acidification (pH 6.5) and evaporation to dryness, the amorphous residue is extracted with boiling ethanol. After cooling, the tripeptide **4** is filtered.

Compound 4: Yield, 15.19 g (88.6%); mp 182–186 °C (EtOH); IR (KBr) cm^{–1}: 3750–2840, 1760, 1640, 1520, 1370, 1250, 1100–940, 870, 630; *R*_f: 0.62 (*n*-BuOH: 25% NH₃aq=6:1); $[\alpha]_D^{20}$ –83.6° (*c* 0.1, 0.1 M NaOH);

Found: C, 44.61; H, 6.11; N, 6.96%. Calcd for C₂₂H₃₅F₃N₃O₁₀P: C, 44.82; H, 5.98; N, 7.13%. Mw, Found/Calcd, 587/589.503.

***N*-[(*S*)-(4-Carboxy-4-trifluoroacetamido-2-butenyl)hydroxyphosphinyl]alanylaspatic Acid (**5**).** A mixture of the tripeptide **4** (20 g, 0.039 mol) and phosphodiesterase I (5 mg or 10–15 mg, if spread on a polymer carrier) is stirred at 37 °C for 6 h. After removal of the enzyme by ultrafiltration or centrifugation (in case the enzyme is spread on a polymer carrier), the reaction mixture is acidified (pH 6.0) and evaporated under vacuum to dryness. The amorphous residue is extracted with boiling ethanol and after cooling, the product **5** is filtered.

Compound 5: Yield, 18.38 g, (97.3%); mp about 200 °C (decomp); IR (KBr) cm^{–1}: 3480–2860, 2840–2460, 1760, 1640, 1520, 1320, 1250, 980–840, 630; *R*_f: 0.39 (*n*-BuOH: 25% NH₃aq=6:1); $[\alpha]_D^{20}$ –83.6° (*c* 0.1, 0.1 M NaOH).

Found: C, 35.46; H, 3.88; N, 8.91%. Calcd for C₁₄H₁₉F₃N₃O₁₀P: C, 35.27; H, 4.01; N, 8.80%. Mw, Found/Calcd, 481/477.287.

***N*-[(*S*)-(4-Carboxy-4-amino-2-butenyl)hydroxyphosphinyl]alanylaspatic Acid (**6**).** A solution of the tripeptide **5** (23.86 g, 0.05 mol) in a mixture of dioxane: 25% aq. ammonia (150 ml, pH 10.0) is heated at 35–40 °C for 1 h. After evaporation under vacuum to dryness and addition of 200 ml of water, the solution is once again evaporated under vacuum. The product **6** is isolated by fractional crystallization.

Compound 6: Yield, 9.38 g (49.2%); mp 163–165 °C (decomp); IR (KBr) cm^{–1}: 3480–2860, 2460–2120, 1750–1720, 1640, 1520, 1310, 1200; *R*_f: 0.66 (DMF: CHCl₃: MeOH=9:2:3); $[\alpha]_D^{20}$ –93.3° (*c* 0.1, 0.1 M NaOH).

Found: C, 38.01; H, 5.11; N, 10.93%. Calcd for C₁₂H₂₀N₃O₉P: C, 37.80; H, 5.29; N, 11.02%. Mw, Found/Calcd, 380/381.279.

The substance is quite soluble in DMF, DMSO, hexamethylphosphoric triamide, less in dioxane, and is insoluble in ethanol, ethyl acetate, ether, chloroform, and hexane. When **6** (3.81 g, 0.01 mol) is heated in 0.1 M NaOH (20 ml) at 50 °C for 30 min, the dipeptide H-Ala-Asp-OH (1.74 g, 85.3%) and 3,4-didehydro-5-phosphono-L-norvaline is isolated: yield 1.48 g (76.1%); mp 188–190 °C (decomp) [for the *D*-form 183–185 °C (decomp)];¹²⁾ IR (KBr) cm^{–1}: 1735, 1630, 1520, 1240, 1150, 1040, 930, 860, 775, 630; ¹H NMR (D₂O+NaOD, δ , ppm): 2.75 (2H, dd, *J*_{P-CH₂}=8.3, and 23 Hz), 4.81 (1H, d, *J*=9 Hz), 5.66 (1H, m, CHCH), 6.12 (1H, m, CH=CHCH₂)

and five exchangeable protons NH_2 , COOH , PO_3H_2 ; R_f : 0.24 (n -BuOH: Pyr: AcOH: H_2O = 15: 12: 3: 10) and 0.10 (n -BuOH: AcOH: H_2O = 3: 1: 1); $[\alpha]_D^{20}$ -53.2° (c 1, H_2O).

Found: C, 30.87; H, 4.93; N, 7.33%. Calcd for $\text{C}_5\text{H}_{10}\text{NO}_5\text{P}$: C, 30.78; H, 5.17; N, 7.18%. Mw, Found/Calcd, 197/195.112.

***N*-[*(S)*-(4-Carboxy-4-amino-2-butenyl)hydroxyphosphinyl]-alanine (7).** A mixture of the tripeptide **6** (20 g, 0.071 mol) and α -chymotrypsin (5 mg) in an aqueous buffer (500 ml, pH 7.8) is stirred at 25°C for 6 h. After acidification and evaporation under vacuum to dryness, the amorphous residue is extracted with hot dioxane. After cooling, the dipeptide **7** is filtered.

Compound 7: Yield, 12.01 g (86.1%); mp about 200°C (decomp); IR (KBr) cm^{-1} : 3450—2920, 2840—2460, 1750, 1640, 1520, 1320, 980—740, 635; R_f : 0.62 (n -BuOH: AcOH: H_2O = 9: 1: 1); $[\alpha]_D^{20}$ -63.4° (c 0.1, 0.1 M NaOH).

Found: C, 36.18; H, 5.41; N, 10.41%. Calcd for $\text{C}_8\text{H}_{15}\text{N}_2\text{O}_6\text{P}$: C, 36.09; H, 5.68; N, 10.52%. Mw, Found/Calcd, 263/266.206.

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