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

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(Received October 3, 1987)

Synopsis. A phospha^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 phospha^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]phenylalanine[4] 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 ($-PO(OEt)_2 \rightarrow -PO_3H_2$) to use HBr/AcOH or to carry out treatment of $-PO(OSiMe_3)_2$, 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 gluthathion[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 laboratory[12] which makes possible the use of highly selective enzymes to catalyze the hydrolysis of the blocking groups.

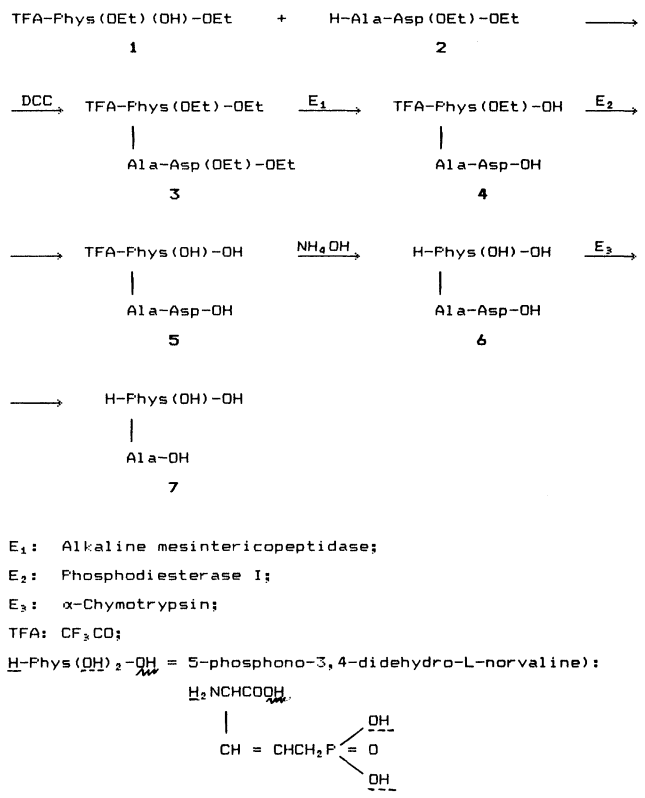
The condensation of *N*-trifluoroacetyl-3,4-didehydro-5-(ethoxyhydroxyphosphinyl)norvaline ethyl ester (**1**) synthesized by us (cf. later communication from the same author) and L-alanyl-L-aspartic acid diethyl ester (**2**) was carried out by the DCC method. When we used *N*-TFA-3,4-didehydro-5-phosphono-L-norvaline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

The enzyme phosphodiesterase I, free or spread on a polymer carrier, contributed to the hydrolysis of the ethoxyphosphonyl group to afford phospha^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



E_1: Alkaline mesintericopeptidase;
E_2: Phosphodiesterase I;
E_3: α-Chymotrypsin;
TFA: CF_3CO;
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" phospha^{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 phospha^{C}-peptide analogue **6** of Plumbemycin A.

Base-catalyzed hydrolysis of **6** liberates, 3,4-didehydro-5-phosphono-L-norvaline[12] and the dipeptide alanyl-aspartic acid.

Under the optimum conditions[12] [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 snythesized phospha^{C}-peptides **6** and **7** are under way and will be published in due course.

Experimental

General. IR spectra, elemental analysis, mp, Mw, HPLC, $[\alpha]_D^{22}$ 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]alanylaspartic Acid α,β-Diethyl Ester (3). A mixture of *N*-trifluoroacetyl-1-L-3,4-didehydro-(*S*)-(ethoxyhydroxyphosphinyl)norvaline ethyl ester **1** (34.72 g, 0.1 mol), alanylaspartic 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 $MgSO_4$, 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-C_6H_{14} 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 ($CHCl_3$: MeOH=9 : 1); $[\alpha]_D^{20}$ −96.3° (*c* 1, MeOH).

Found: C, 44.61; H, 6.11; N, 6.96%. Calcd for $C_{22}H_{35}F_3N_3O_{10}P$: 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]alanylaspartic 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% NH_3aq= 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 $C_{22}H_{35}F_3N_3O_{10}P$: C, 44.82; H, 5.98; N, 7.13%. Mw, Found/Calcd, 587/589.503.

***N*-[(*S*)-(4-Carboxy-4-trifluoroacetamido-2-butenyl)hydroxyphosphinyl]alanylaspartic 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% NH_3aq=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 $C_{14}H_{19}F_3N_3O_{10}P$: C, 35.27; H, 4.01; H, 8.80%. Mw, Found/Calcd, 481/477.287.

***N*-[(*S*)-(4-Carboxy-4-amino-2-butenyl)hydroxyphosphinyl]-alanylaspartic 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 additon 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 : $CHCl_3$: 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 $C_{12}H_{20}N_3O_9P$: 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 (D_2O+NaOD, δ, ppm): 2.75 (2H, dd, $J_{P\text{-}CH_2}$=8.3, and 23 Hz), 4.81 (1H, d, J=9 Hz), 5.66 (1H, m, C<u>H</u>C<u>H</u>), 6.12 (1H, m, CH=C<u>H</u>CH_2)

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 $C_5H_{10}NO_5P$: 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 $C_8H_{15}N_2O_6P$: C, 36.09; H, 5.68; N, 10.52%. Mw, Found/Calcd, 263/266.206.

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