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Organophosphorus Analogues and Derivatives of the Natural L-Amino Carboxylic Acids and Peptides. II. Synthesis, Enzyme-Substrate Interactions of "Bialaphos" (SF-1293) and Its Cyclic and Phosphinic Amide Type Analogues

Ivan. A. NATCHEV
Research Centre "Konstrukcionni Polymeri", 5-003 Gara Iskar, 1528 Sofia, Bulgaria
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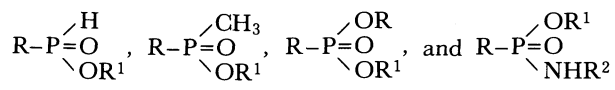
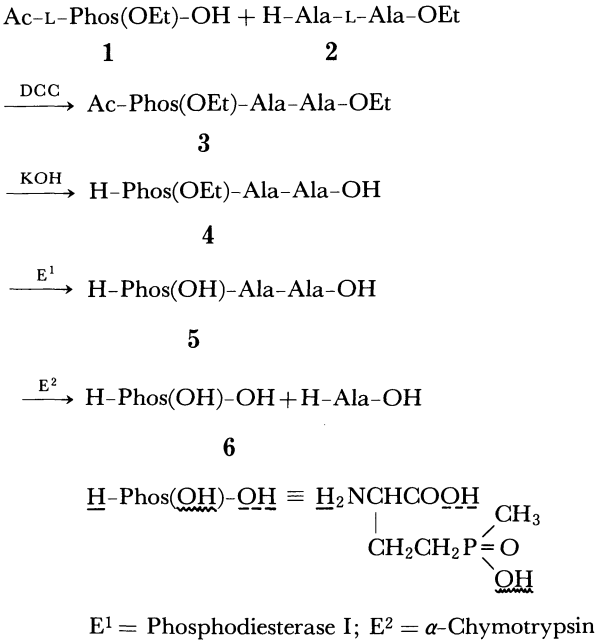
The natural tripeptide antibiotic "Bialaphos" (**5**) has been synthesized by condensation of the protected L-phosphinothricin (**1**) with the dipeptide alanylalanine ethyl ester. The dicyclohexylcarbodiimide method has been used, followed by enzyme-catalyzed hydrolysis. Analogously, a cyclic analogue and the phospha^{C}-peptide (phosphinic amide type) analogue (**14**) of the antibiotic (**5**) have been found to possess antitumour activity.

The tripeptide antibiotic γ-(hydroxymethylphosphinyl)-L-α-aminobutyryl-L-alanyl-L-alanine ("Bialaphos") (**5**) was isolated for the first time[1] from *Streptomices viridochromogenes strains* and it is known to possess herbicidal and fungicidal activity. The phosphinic acid analogue of glutamic acid: 2-amino-4-(hydroxymethylphosphinyl)butanoic acid (phosphinothricin), has a very wide application in agriculture. It should be noted that, while scientific literature abounds in methods for the synthesis of this compound, very limited information about the synthesis pathways of the tripeptide "Bialaphos" can be found. This is due to the complications that arise when attempts are made to release the blocking groups of the phosphinic function. Thus, researchers from Japan[2] synthesized the *P*-ester of "Bialaphos" (with the participation of the DL-forms of phosphinothricin and alanine), but when they tried to hydrolyze the ethoxymethylphosphinyl group to a free phosphinic acid, the "hard" hydrolysis conditions led to the hydrolysis of the peptide bonds. To solve this problem, we devised a method for enzyme-catalyzed hydrolysis[3] of esters of the type:



As starting compounds in the synthesis of "Bialaphos" we used protected phosphinothricin (**1**), prepared in our laboratory,[3] and the commercially available dipeptide L-alanyl-L-alanine ethyl ester. Condensation was carried out without complications by the dicyclohexylcarbodiimide method and the completely protected tripeptide **3** was isolated in an approx. 80% yield. Hydrolysis of the blocking *N*-acetyl and ethoxycarbonyl groups was also carried out by a standard method for peptide synthesis, i.e. mineral hydrolysis to the tripeptide **4**, obtained in an approx. 85% yield.

In our previous paper,[3] the enzyme phosphodiesterase I was successfully used in the enzyme-catalyzed hydrolysis of the *P*-ester of L-phosphinothricin. It could therefore be assumed that this enzyme would retain its catalytic activity in the case of the tripeptide **4**

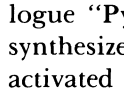


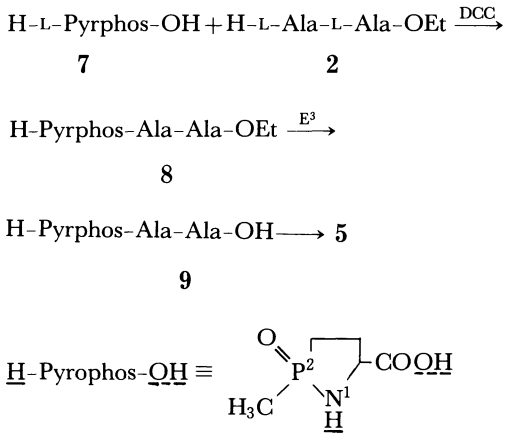
Scheme 1.

as well. Under the empirically confirmed most favorable hydrolysis conditions, i.e. 20 g substrate, 5 mg enzyme (of 15 mg enzyme, if spread on a polymer carrier), stirring for 6 h at 37 °C in 500 ml buffer (pH 8.8), the free tipeptide **5** was isolated in a yield of over 90%. Its spectral data were found to be identical with those of its natural counterpart. No additional purification of the phosphodiesterase I, supplied by "Sigma," was necessary. Its inevitable contamination did not noticeably affect the enzyme-catalyzed hydrolysis, despite the fact that, after the termination of the process, 6—8% phosphinothricin was isolated as a result of the hydrolysis of the phosphinothricin-alanyl peptide bond. It is interesting to note that when D-phosphinothricin (D-**1**) was used in an analogous synthesis, no presence of phosphinothricin was observed during the last phase of the enzyme hydrolysis. The occurence of the side hydrolysis process could be caused by some impurities of the commercial product, because phos-

phodiesterase I has never been found to possess any proteolytic activity. All our attempts to purify the phosphodiesterase I led to a marked decrease of its hydrolytic activity. When, under the same conditions, crude dried venom from *Crotalusatrox* was used, which hydrolyzes minimum 0.1 mmol bis(*p*-nitrophenyl)hydrogenphosphate per mg solid per minute at pH 8.8 and 37 °C, complete decomposition of the tripeptide **5** was observed and the only products that were isolated phosphinothricin and alanine.

The tripeptide "Bialaphos" **5** and its cyclic analogue "Pyrobialaphos" **9** (cf. below) have also been synthesized in our laboratory by the method of the activated esters and an *N*-protective group, *t*-butoxycarbonyl (*t*-BOC). This approach, though it is by no means better than the one described here, will be discussed separately.

With a view to elucidating the relationship of chemical structure-physiological activity, the cyclic analogue of phosphinothricin-2-amino-1,2-azaphospholidine-5-carboxylic acid **7**, recently synthesized by us,[3] was chosen as the N-terminal component of the tripeptide "Bialaphos". Condensation of **7** with the dipeptide alanylalanine ethyl ester was carried out by the dicyclohexylcarbodiimide method. The modified dipeptide **8** was isolated in an approx. 85% yield. When mineral hydrolysis was attempted of the ethoxycarbonyl group, the cyclic phosphinic amide group  turned out to be highly unstable and the tripeptide "Bialaphos" **5** was isolated in a very good yield. This made it clear that the enzyme approach should in this case be used. Alkaline mesintericopeptidase was chosen as the esterase enzyme, as it is known to hydrolyze the peptide C-terminal pro-group rather faster, than the peptide bonds. Nevertheless, strict control of the hydrolysis process is essential until alanine is detected chromatographically. When 20 g substrate and 15—20 mg enzyme were used in a buffer medium

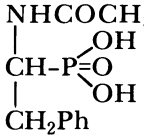


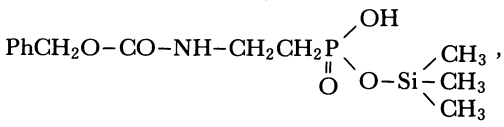
Scheme 2.

(pH 7.8), the tripeptide 9 was isolated in about 70% yield. It differs from its natural counterpart in the presence in it of an N-terminal cyclic bond between the nitrogen and the phosphorus.

This modification leads to considerable changes in the physiological activity. Laboratory tests showed a marked decrease of the herbicidal activity. Surprisingly, the modified tripeptide **9** does not appear to have any bactericidal activity, which is characteristic for "Bialaphos". This fact can probably be accounted for by the absence of a free phosphinic acid group . The *P*-esterified tripeptide **4** shows a certain decrease of its bactericidal activity.

It would be interesting to try still another approach to bonding the unusual amino acid L-phosphino thricin with alanylalanine by creating a PONH bond.

Such di- and tripeptide were synthesized for the first time and quite independently from Imoto et al.[4] and Martell et al.[5] Unfortunately, no peptides with free terminal or side functional groups can be obtained, as "they are very sensitive to any mild alkaline or acidic treatment, aimed at the removal of the blocking groups".[5] Some Soviet researchers[6] were confronted with the same problem. They used the DCC-method to condense the phosphone analogue of phenylalanine, (1-acetamido-2-phenylethyl)phosphonic acid,  with the methyl ester of phenylalanine.

Recently, it was reported in the patent literature that Issleib et al.[7] have succeeded in obtaining free phospha^C-peptides by condensation of the mono (trimethylsilyl) ester of 2-(benzyloxycarbonylamino)-ethylphosphonic acid,  , with ethyl isocyanoacetoacetate C≡N-CH$_2$COCH$_2$COOEt, and subsequent hydrogenation and hydrolysis of the trimethylsilyl protective group.

To this moment, no information is available in the literature about phospha^C-peptides with phosphinic acids.

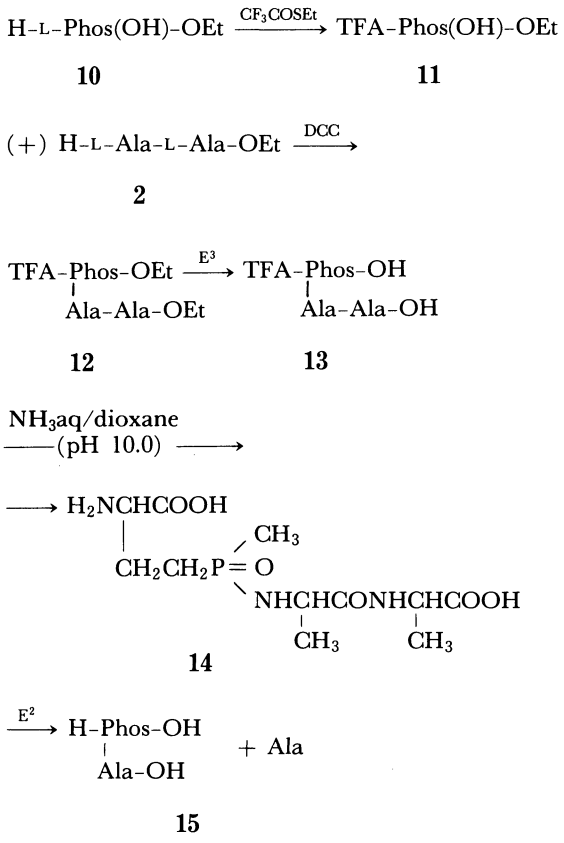
The major problem in the synthesis of a phospha^C-peptide analogue of "Bialaphos" is finding a suitable protective group, which can be removed under such conditions, as do not noticably affect the PONH bond. Urethane-type protective groups provided to be unsuitable, because these derivatives of phosphinothricin are difficult to obtain and, besides, they lead to some side reactions upon treatment with hydrogen bromide-iced acetic acid. The use of an *N*-acetyl protective group is also undesirable, due to the instability

of the phosphinic amide group under conditions of mineral hydrolysis. Enzyme-catalyzed hydrolysis with α-chymotrypsin, protease, peptidase, etc., is also to be avoided, as in this case nonselective hydrolysis of the peptide alanylalanin bond occurs. Protective groups, released by hydrogenolysis, are ineligible as well, because they cause side reactions. The only group found suitable for the protection of the amino group of phosphinothricin, and with some reservations at that, is the trifluoroacetyl group.

The synthesis of the starting material **11**, having a free phosphinic acid function, $-P\begin{matrix}=O\\ \diagdown OH\end{matrix}$, and protected amino and carboxyl groups, was carried out by the method given in Ref. 8, where *N*-trifluoroacetyl protection has been applied to amino carboxylic acids.

Treatment of L-phosphinothricin ethyl ester, synthesized by us earlier, with *N*-ethyl trifluorothioacetate was carried out at room temperature in a borate buffer. The ester amide **11** was isolated in a yield of approx. 90%.

The dicyclohexylcarbodiimide method was used to condense the protected L-phosphinothricin **11** with the dipeptide L-alanyl-L-alanine ethyl ester. Conditions were analogous to those for the conventional peptide synthesis. The phospha^C-tripeptide **12** was isolated in an yield of about 75%. One equiv phosphinothricin **11**, 1 equiv dipeptide, and 1.1 equiv dicyclohexylcarbodiimide were kept for 12 h in an ambience of dry ethyl acetate and then the separated *N,N'*-dicyclohexylurea was filtered off. The filtrate was consecutively washed with 5% aqueous solution of sodium carbonate-water-1M HCl-water (1M=1 mol dm^{-3}), dried over anhydrous magnesium sulfate, evaporated to dryness in vacuum, and purified by column chromatography on silica gel.



Scheme 3.

The carboxyl groups were released by the above-described method of enzyme-catalyzed hydrolysis with mesintericopeptidase to the protected phospha^C-peptide **13**, isolated in an approx. 80% yield. This yield, which is somewhat low for an enzyme-catalyzed process, is due to the fact that certain hydrolysis of the peptide bonds is observed as well.

The *N*-trifluoroacetyl protective group is removed by treatment with aqueous ammonia at pH 10.0. At lower pH values no hydrolysis occurs, whereas higher pH values lead to hydrolysis of the phosphinic amide bond. Despite the numerous experiments performed, the free phospha^C-peptide **14** was never isolated in a yield of over 40%.

In spite of the fact that the tripeptide **14** readily undergoes mineral hydrolysis, we failed in our attempts to find an enzyme that catalyzes the hydrolysis of the PO-NH bond. When alkaline phosphatase was used, the presence of about 10% phosphinothricin was established in the hydrolysate, but this was probably due to the alkalinity of the medium (pH 10.4), and not to the action of the enzyme.

The proteolytic enzymes α-chymotrypsin and protease catalyze the hydrolysis of the alanylalanine bond and as a result the phospha^C-dipeptide **15** is isolated in approx. 90% yield.

The tripeptide **14** has been statistically proved to possess antitumour activity. When applied to an experimental tumour L1210 in mice in doses of 20 (mg/kg) d^{-1} for the duration of 5 d, T/C of 168% was established (T/C%=ratio of median survival time, expressed as percent of untreated controls). The T/C ratio of the dipeptide **15** is 172%. When the tripeptide "Bialaphos" and phosphinothricin were subjected to parallel tests, no antitumour activity was registered. The relatively low toxicity of **14** (LD_{50}=428 mg/kg) and **15** (LD_{50}=523 mg/kg) promises very good results, should further studies in this direction be undertaken.

Experimental

General Notes: IR spectra, elemental analysis, and HPLC—on a Perkin-Elmer instruments; mp's—on a Kofler apparatus; TLC—silica-gel film "Merck". Solvents and reagents—"Aldrich", "Merck"; enzymes and buffers—"Sigma". The compounds obtained have been also proved by measuring the total and enzyme hydrolysate content. The IR-spectra and mp's of the known natural products have been compared with those of authentic samples.

Synthesis of Bialaphos *O,P*-Diethyl Ester (3). 2-Acet-

amido-4-(ethoxymethylphosphinyl)-L-butanoic acid (**1**) (23.52 g, 0.1 mol), L-alanyl-L-alanine ethyl ester (**2**) (19.02 g, 0.1 mol), and dicyclohexylcarbodiimide (22.69 g, 0.11 mol) are mixed in dry ethyl acetate (250 ml). The mixture is then stirred on a magnetic stirrer for 24 h in a closed Erlenmeier flask at room temperature. The *N,N'*-dicyclohexylurea is filtered out, 5—6 drops of 50% aqueous acetic acid are added and the filtrate is kept for 6 h at room temperature. After another filtration, the filtrate is washed with 5% aqueous solution of sodium carbonate-water-5% hydrochloric acid-water. It is then dried over anhydrous magnesium sulfate and distilled in vacuum to dryness. The obtained oil is dissolved in dry ethyl acetate and hexane is added until the solution becomes dull. After a prolonged storage in a refrigerator, the product is filtered out: γ-(Ethoxymethylphosphinyl)-L-α-acetamidobutyryl-L-alanyl-L-alanine ethyl ester (**3**): $C_{17}H_{32}N_3O_6P$; 33.69 g (83.1%); IR (KBr): 1745, 1650, 1520, 1305, 1200, 1110—970 cm^{-1}. Elemental analysis, % C, H, N, Calcd/Found: 50.36/50.48, 7.96/7.77, 10.36/10.77; mp 96—98 °C; total hydrolysate (after 12 hours' heating at 110 °C in 6 M HCl in a sealed ampule): phosphinothricin and alanine; enzyme hydrolysate (after 6 hours' stirring of 2 g substrate and 1 mg protease at pH 7.5 and 37 °C: phosphinothricin *P*-ethyl ester and alanine.

Isolation of Bialaphos *P*-Ethyl Ester 4.[2] The tripeptide **3** (20.54 g, 0.05 mol) is suspended in 200 ml 5% aqueous potassium hydroxide and the mixture is heated for 1 h at 40—50 °C. After cooling, acidification and extraction with diisopropyl ether, the organic extract is distilled in vacuum and the amorphous mass crystallized from dioxane/ethanol. The tripeptide **4** is obtained in an 84.6% yield (14.86 g).

Total hydrolysate-phosphinothricin, alanine; enzyme hydrolysate (protease)-phosphinothricin *P*-ethyl ester and alanine; satisfactory elemental analysis.

Isolation of Bialaphos (5).[1] The enzyme phosphodiesterase I (5 mg) is added to a buffer medium (500 ml, pH 8.8), containing the tripeptide **4** (20 g), at 37 °C. The mixture is stirred for 6 h at the same temperature. After dialysis, acidification and evaporation in vacuum (40—50 °C) to dryness, the solid mass is extracted with hot ethanol. The tripeptide **5** is isolated after cooling in a 90.6% yield. Its spectral data are identical with those of an authentic sample. Enzyme hydrolysis of Bialaphos with α-chymotrypsin: phosphinothricin (**6**) and alanine.

Synthesis of Pyrrobialaphos (9). The method described in Item 2 is employed, only now L-2-methyl-1,2-azaphospholidine-5-carboxylic acid 2-oxide (**7**) is used. The following product is isolated: *N*-/L-(2-methyl-1,2-azaphospholidine-5-carbonyl 2-oxide)/-L-alanyl-L-alanine ethyl ester (**8**): $C_{13}H_{24}N_3O_5P$; 29.10 g (87.3%); IR 1320 cm^{-1} (P-N); Calcd/Found (%): 46.84/46.59, 7.26/7.40, 12.61/12.55; mp 173—176 °C; total hydrolysate—phosphinothricin; enzyme hydrolysate (protease and α-chymotrypsin)-1,2-azaphospholidine analogue (pyrrophosphoshricin) (**7**) 20 g of the substrate, i.e. the protected tripeptide **8**, are well homogenized with 4—5 drops of "Tween-80" and added to a water-buffer medium (500 ml, pH 7.8), containing alkaline mesintericopeptidase (20 mg). Stirring is kept up for 3 h. The mixture is extracted with diisopropyl ether and the water layer is acidified and evaporated in vacuum to dryness. The residue is extracted with hot methanol to the product: *N*-/L-(2-methyl-1,2-azaphospholidine-5-carboxyl 2-oxide)/-L-alanyl-L-alanine (**9**): $C_{11}H_{20}N_3O_5P$; 21.16 g (69.3%); IR 3580—2920 cm^{-1} (COOH); Calcd/Found (%): 43.28/43.44, 6.60/6.51, 13.76/13.39; total hydrolysate—phosphinothricin, alanine; "mild" mineral hydrolysis (1 M HCl, 30 min., 50 °C)—Bialaphos (**5**); enzyme hydrolysis (α-chymotrypsin)-1,2-azaphospholidine (**7**), alanine; the product melts with decomposition at about 220 °C.

Synthesis of the Ester Amide 11. A mixture of L-phosphinothricin ethyl ester (**10**) (20.92 g, 0.1 mol) and *S*-ethyl trifluorothioacetate (24.71 g, 0.15 mol) in 100 ml 1 M NaOH and 400 ml borate buffer solution is shaken for 8 h on a mechanical shaker at room temperature. It is then acidified with 1M HCl, extracted with diisopropyl ether, dried over anhydrous magnesium sulfate, and distilled in vacuum to dryness. The following product is recrystallized from ethanol: L-2-trifluoroacetamido-4-(hydroxymethyl)butanoic acid ethyl ester **11**: $C_9H_{15}F_3NO_5P$; 27.56 g (90.3%); IR (KBr): 2860—2540, 2340—2100, 1740, 1650, 1520, 1310, 1170, 930, 840 cm^{-1}; Calcd/Found (%): 35.42/35.27, 4.95/5.01, 4.59/4.67; mp 163—165 °C; $[\alpha]_D^{22}$ +43.7°, (*c* 0.1, MeOH), R_f 0.43 (DMF: dioxane: MeOH=9:2:1).

Synthesis of the Phospha^C-peptide 14. A mixture of L-phosphinothricin (**11**) (30.52g, 0.1 mol), L-alanyl-L-alanine ethyl ester (**2**) (19.02 g, 0.1 mol) and dicyclohexylcarbodiimide (22.69 g, 0.11 mol) is stirred for 12 h in dry ethyl acetate (250 ml) at room temperature. This mixture is then filtered and the filtrate is washed with 5% aqueous solution of sodium carbonate, then with water, 0.5% aqueous solution of oxalic acid, again with water, and followed by drying over anhydrous magnesium sulfate and distillation in vacuum to dryness. The residue is dissolved in dry ethyl acetate and cooled. Hexane is added until the solution becomes dull. Crystallization is initiated by rubbing the flask walls with a glass stick and then leaving the solution in a refrigerator at −5 °C for prolonged period the following product is then filtered out: *N*-/L-3-ethoxycarbonyl-3-(trifluoroacetamido)-propyl/methylphosphinyl/-L-alanyl-L-alanine ethyl ester (**12**): $C_{17}H_{29}F_3N_3O_7P$; 35.56 g (74.8%); IR 1750, 1640, 1520, 1320—1270, 1189—940, 810, 720, 640, 560 cm^{-1}; Calcd/Found (%): 42.95/43.18, 6.18/6.01, 8.84/8.20; total hydrolysate—phosphinothricin, alanine; mp 93—95 °C.

A well homogenized mixture of the tripeptide **12** (47.54 g, 0.1 mol) and 5—6 drops of "Tween-80" is added to an aqueous buffer medium (800 ml, pH 7.8), which has been previously tempered at 30 °C and which contains 20 mg alkaline mesintericopeptidase. Stirring is kept up for 3 h at the same temperature (until a ninhydrin-positive detection occurs), the mixture is extracted with diisopropyl ether, acidified to pH 6.0, and evaporated in vacuum to dryness. The solid residue is extracted with boiling ethanol to the product: *N*-/L-3-carboxy-3-(trifluoroacetamido)propyl/methylphosphinyl/-L-alanyl-L-alanine (**13**): $C_{13}H_{21}F_3N_3O_7P$; 34.09 g (81.3%); IR 3200—2600 cm^{-1}; Calcd/Found (%): 34.24/37.56, 5.05/4.88, 10.02/10.13; total hydrolysate—phosphinothricin, alanine; mp 188—191 °C.

The tripeptide **13** (18.63 g, 0.05 mol) is stirred for 12 h at room temperature in a mixture of dioxane (200 ml) and aqueous ammonia, pH 9.6. After evaporation in vacuum to dryness (50 °C/18 mmHg) the reaction residue is passed through a silica-gel column, which results in the separation of phosphinothricin, the dipeptide alanylalanine and the tripeptide: *N*-/(L-3-carboxy-3-aminopropyl)methylphosphinyl/-L-alanyl-L-alanine (**14**): $C_{11}H_{22}N_3O_6P$; 6.24 g (38.6%); IR 3400—2860, 2340—2100, 1750, 1650, 1520, 1340, 1305, 1170, 930, 845, 620, 510 cm^{-1}; Calcd/Found (%): 40.87/40.90

6.86/6.49, 13.00/13.11; total hydrolysate—phosphinothricin, alanine; mp 216—219 °C (decomp).

Synthesis of the Phospha^{C}-dipeptide 15. The tripeptide **14** (3233 g, 0.1 mol) is stirred for 6 h at 25 °C in 800 ml aqueous buffer medium (pH 7.8), containing α-chymotrypsin (10 mg). After acidification (pH 6.0), the reaction mixture is evaporated in vacuum to dryness and the resultant organic mass is treated with hot dioxane to give: *N*-/(L-3-carboxy-3-aminopropyl)methylphosphinyl/-L-alanine (**15**): $C_8H_{17}N_2O_5P$; 24.01 g (95.2%); IR 3360—2840, 2320—2100, 1750, 1650, 1525, 1340, 11300,1170, 920, 840, 620, 515 cm^{-1}; Calcd/Found (%): 38.10/38.43, 6.79/6.62, 11.11/11.17; mp approx. 200 °C (decomp).

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