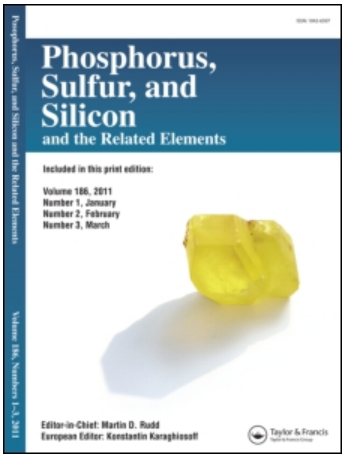

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SYNTHESIS AND ENZYME-SUBSTRATE INTERACTIONS OF *N*-PHOSPHINO-, PHOSPHONOMETHYLGLYCINE ETHYL ESTERS

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SYNTHESIS AND ENZYME-SUBSTRATE INTERACTIONS OF *N*-PHOSPHINO-, PHOSPHONOMETHYLGLYCINE ETHYL ESTERS

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Three-component condensation of glycine ethyl ester hydrochloride, formalin and hydrophosphorous compounds **1–3** was carried out to the esters **4–6**, which after acid hydrolysis led to the free *N*-phosphino-, phosphonomethylated glycines **7–9**. Strict selectivity in enzyme-catalyzed hydrolysis of phosphino-, phosphono acid esters was established. The enzyme phosphodiesterase I catalyzed the hydrolysis of the ester **4**, **5** and **6** to the corresponding **10**, **11** and **12**. Lyophilyzed bee venom hydrolyzed the ethoxycarbonyl groups of **4–6** to the corresponding free glycines **7–9**, and the enzyme alkaline phosphatase contributed to the hydrolysis only of the one from the two diethoxyphosphono groups of the substrate **6** to the monophosphono ester—**13**. Proteolytic enzymes α-chymotrypsin, protease and alkaline mesintericopeptidase catalyzed only hydrolysis of the ethoxycarbonyl groups of the substrates **5** and **6** to the free carboxylic acids **14** and **15** which after treatment with phosphodiesterase I or bee venom liberated glycines **8** and **9**, respectively. It was established that the enzymes α-chymotrypsin and alkaline mesintericopeptidase were completely inhibited by H—P(O)-group containing substrates **4** and **10**, while α-chymotrypsin was partially inhibited from substrates **11**, **12** and **13** containing —P(O)OH-group.

RESULTS AND DISCUSSION

Phosphinic acids that contain an amino group at the α-position are very interesting from a biological point of view. The analogues of glycine, valine and methionine (the COOH group is replaced with a P(O)(OH)H group) possess antimicrobial activity;[1] the DL-valine and DL-methionine analogues inhibit valyl- and methionyl-*t*-RNA-synthetases, respectively,[2] and the alanyl analogue is known to have herbicidal activity.[3]

The unsubstituted aminomethylphosphinic acid[4] was obtained for the first time by aminolysis of chloromethylphosphinic acid. Later, Khomitov and Osipova[5] carried out hypophosphorylation of oximes to some structural analogues of α-aminocarboxylic acids. Judelevich *et al.*[6] and Baylis *et al.*[7] synthesized α-aminoalkylphosphinic acids by treatment of aldehydes with amines and ammonium hypophosphite.

Notably, very few papers report on the synthesis of *N*-substituted-α-amino-phosphinic acids by addition of hypophosphorous acid to a double carbon-nitrogen bond. Starting from the work of Schmidt,[8] Maier[9] and Redmore[10] improved essentially the scope of the reaction.

Seto *et al*[25] isolated 4-hydroxyphosphinyl-2-amino-*L*-butanoic acid when cultivating bacteria *S.* hydroscopicus in the absence of Co^{2+} and showed that it was the intermediate for the biosynthesis of phosphinothricin and bialaphos. The

isolated product was the first natural one containing a H—P(O)OH-group. In contrast to phosphinothricin and bialaphos, it does not possess the biological activity characteristic for these substances.

Toy *et al.*[11] synthesized *N*-/(hydroxyphosphinyl)methyl/glycine **7** by *N*-alkylation of glycine with chloromethylphosphinic acid. We studied this compound as a ligand for the ions of some heavy metals. Oxidation with mercury dichloride afforded by *N*-phosphonomethylglycine **9**.[11]

The methylphosphinic analogue of glyphosphate-*N*-(hydroxymethylphosphinyl)methylglycine, **8**, was first synthesized by Japanese researchers[12] treating iminodiacetic acid with formalin and methylphosphonous acid in a hydrochloric acid medium and subsequent acidification with hydrogen peroxide/sulphuric acid.

Maier[13] treated *N*-benzylglycine with formaldehyde and methyldichlorophosphine and, after debenzylation, obtained the product **8** with an yield of about 60%. In the same work[13] the diester **5** was obtained from 1,3,5-triethoxycarbonylmethylhexahydrotriazine and the ester **2** (yield 40%), following a 4-hours heating of the components at 110°C.

N-Phosphonomethylglycine, **9**, in the form of isopropylammonium salt, is universally applied in agriculture as a weedkiller.[14] Now, more than ten years since its introduction into practice,[15] its high total herbicidal activity, fast decomposition by the soil microorganisms, and the negligible toxicity, have prompted many intensive studies on both the synthesis of the modified glycine **9**, and the discovery of its new derivatives and analogues.

With a view to clarifying the relationship between chemical structure and physiological activity, we intend, in several consecutive papers, to discuss the synthesis of *N*-phosphinylmethylated glycines, having the following common formula:

$$\begin{array}{l} \mathrm{COOH} \\ | \\ \mathrm{CH_2NHCH_2P(O)RR^1}, \end{array}$$

where R and R^1 are H and OH, CH_3 and OH, OH and OH, C_6H_5 and H, C_6H_5 and CH_2NH_2, OH and CH_2NH_2, and their esters (OC_2H_5, instead of OH). The enzyme-catalyzed hydrolysis of the ethoxycarbonyl and ethoxyphosphinyl groups has been studied.

We were not satisfied with the rather low yield of the phosphinyl-substituted glycine **7**, obtained by the method in,[11] which is rendered still less applicable considering the instability of the chloromethylphosphinic acid and the fact that it is not easily available. In our laboratory, we succeeded in developing a general method for preparation of *N*-substituted glycines, sharing the above-mentioned formula, by three-component condensation of glycine ethyl ester hydrochloride, formaldehyde (as formalin) and the corresponding hydrophosphoryl compound —H—P(O)RR^1, with R and R^1 as above. It is very typical for this condensation that, when R and R^1 are —OC_2H_5 the interaction takes place at the margin of the two phases, i.e. the water and the organic solvent, which does not mix with water. In all cases studied, the condensation product turned out to be very easily soluble in the organic phase and quite insoluble in the water phase—it went into the solvent the moment it was formed.

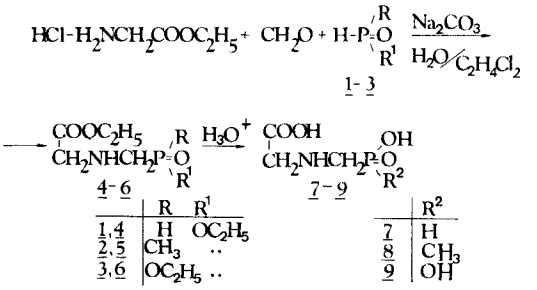
To enhance the process, it is better to use a mixture (vigorously stirred) of 1 eq. glycine ethyl ester hydrochloride, dissolved in water, and a non-water-mixing solvent, to which a solution of 1.1 eq. sodium carbonate in 1.1 eq. formalin and 1 eq. respectively, hypophosphorous acid ethyl ester,[16] —$H_2P(O)(OEt)$, $HP(O)CH_3(OEt)$ and $HP(O)(OEt)_2$, dissolved in the same solvent, is simultaneously added. Addition takes about 30 min. at room temperature and is accompanied by vigorous stirring. When addition is over, the water layer contains only sodium chloride and the excess of formalin. To complete the interaction, it is necessary to remove the water layer and boil the reaction mixture for another 60 min. The type of the solvent is not very important, but it is better if its boiling point is lower than 90°C. After the usual work-up, the diester **4** is isolated in an yield of about 55% (Scheme 1).

In the interaction of ethylglycinate, formalin and the other two selected H—P— compounds, —$HP(O)CH_3(OEt)$, and $HP(O)(OEt)_2$, the esters **5** and **6** are obtained with yields of about 75–80% respectively.

The mechanisms of the three-component condensation is not very clear. It could be assumed that, upon treatment of the ethylglycinate, whose amino group is free, with formaldehyde, *N*-methylenethylglycinate is formed,[17] in analogy to the classical formaldehyde titration of glycine,[18] followed by addition of hydrophosphorous acid ethyl ester 1 to the double carbon–nitrogen bond according to the reaction of Kabachnik–Fields.[19,20] Unfortunately, all our attempts to obtain *N*-methylenethylglycinate by repeating the method in[17] were unsuccessful. It is quite possible that 1,3,5-triazine-1,3,5(2H, 4H, 6H)-triacetic acid triethyl ester in-situ is obtained,[21] from the interaction of ethylglycinate and formalin, which then reacts with the corresponding hydrophosphoryl compound in the organic layer. This reaction course is pointed out as the most probable one by the method of Maier[13] for the synthesis of ester **5** and of Dutra[22] for ester **6**, both using this triazine.

The hypophosphoryl group is not sufficiently stable under the conditions of mineral acid hydrolysis of the ethoxyphosphinyl group of the ester **4**. After at least 3–4 hours of boiling in 18–20% hydrochloric acid, the yield of the free acid **7** is approx. 60%. Surprisingly, alkaline hydrolysis decreases the yields considerably. By analogy 6–8 hours boiling in 18–22% hydrochloric acid of the esters **5** and **6** yields the free acids **8** and **9** with sufficient yield.

For the purposes of achieving "mild" hydrolysis of the esters of organophosphorous acids, we developed a very convenient method of enzyme-substrate



Scheme 1

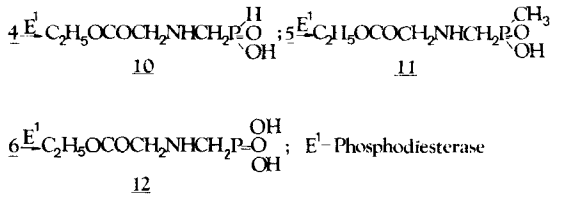
interaction with phosphodiesterase enzymes (cf. other publications by the same author, now in press). In this work, the enzyme phosphodiesterase I is applied for the first time to the hydrolysis of compounds, containing the HP(O)OR, where R is alkyl (Scheme 2).

The enzyme-substrate interaction is carried out under conditions, which we have empirically found to be the most favourable: 20 g substrate, 5 mg enzyme (or 10 mg if spread on a polymer carrier), stirring for 6 hours at the temperature and pH optimum of the enzyme used in a buffer medium. Naturally, greater or smaller quantities of the enzyme can be used, provided the ratio enzyme/substrate to be within the prescribed limits.

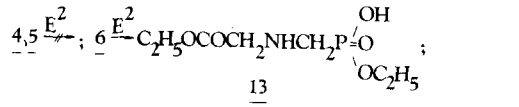
The esters **5** and **6** also participate in the enzyme-substrate interaction with the enzyme phosphodiesterase I, as a result of which the acids **11** and **12** are obtained with quantitative yields (Scheme 2).

In our laboratory a great number of experiments of enzyme-substrate interactions with phosphodiesterase I and substrates containing the groups —P(O)H(OR), —$P(O)CH_3(OR)$, —$P(O)(OR)_2$, where R is a low alkyl, are carried out and it is established, that in all the cases (more than 25 substrates tested), the liberation of the corresponding organiphosphoric acid is reached with a practical quantitative yield. The experimental technique is quite facilitated when the enzyme is spread on a polymer carrier. One and the same enzyme can be used at least in twelve consecutive experiments without losing its activity. The method is applicable very well for substrates containing asymmetric carbon (e.g. *D*-, *L*-phosphinothricin, the peptide Bialaphos, 3,4-didehydro-5-phosphono-*D*-, (*L*-)norvalin and the peptides Plumbemicin A and B, synthesized recently by us), whereas by applying mineral acid hydrolysis of phosphino- and phosphonoesters, a full racemization is observed. It is particularly valuable for synthesis of phospha C-peptides, where the easy hydrolization of the bond PO—NH does not allow acid or alkaline hydrolysis (cf. other publications by the same author, now in press). Enzyme-catalyzed hydrolysis of organophosphoric acid esters goes with strict selectivity. Ethoxycarbonyl, peptide and some other hydrolyzing groups are not hydrolyzed. The enzyme phosphodiesterase I have no effect on substrates containing tertiary butyl phosphono-, phosphino ester group, as well as on such compounds containing aryl and cyclic groups.

In contrast to phosphodiesterase I, the very similar alkaline phosphatase enzyme has no enzyme-catalytic influence on phosphino esters **4** and **5** (Scheme 3). Surprisingly, when it is applied upon the triester **6**, the enzyme hydrolyses only one of the two diethoxyphosphono groups to the monophosphono ester **13**. Enzyme-substrate interaction is carried out like the above described interaction



Scheme 2



Scheme 3

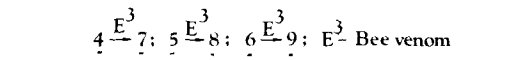
with phosphodiesterase I. The interaction is with a strict selectivity and goes with a practical quantitative yield.

It is interesting to note that the enzyme α-chymotrypsin was completely inhibited upon attempts at enzyme-catalyzed hydrolysis of the esters **4**. When this enzyme was mixed with **4** and then removed after 10 min., it was found to be inactive towards the standard substrate *N*-benzoyl-*L*-tyrosine ethyl ester (BTEE). To a lesser extent, this is also true for the enzyme protease, which is inhibited after the third consecutive application. An inhibition occurs also when those two enzymes are not dissolved, but spread on a polymer carrier. A possible explanation could be the presence of a —HP(O)-group in the substrate. Thus, when the substrate used contains HOP(O)OR-, $P(O)(OH)_2$-, $P(O)(OH)CH_3$, or $P(O)(OR)CH_3$, and the corresponding alkoxycarbonyl or amide (peptide) groups, no such inhibition is observed.

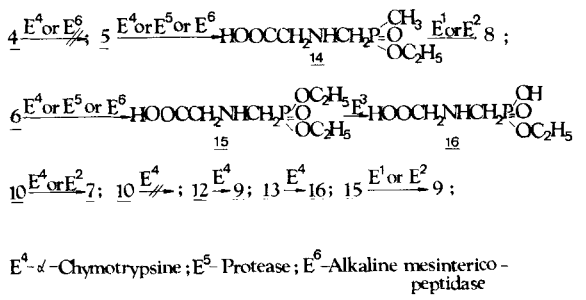
A possible approach to the catalytic hydrolysis of the esters **4**, **5** and **6** was the use of the relatively easily available bee venom. 6 hours' stirring of 20 g substrate and 25 mg raw freeze-dried bee venom in 600 ml trisbuffer at 25°C afforded the free acids **7**, **8** and **9**, respectively, in practically quantitative yields (Scheme 4). The pure enzymes, which we have isolated from the bee venom—hyaluronidase and apamin,[23] do not lead to enzyme-substrate interaction with either of the substrates **4**–**6**. Most probably the raw bee venom contains some low-molecular weight enzymes, peptides and activators, because after dialysis the phosphodiesterase activity is entirely missing. The fact is rather interesting—the bee venom we employed did not lead to any enzyme-substrate interaction with bis(*p*-nitrophenyl)phosphate, which is the substrate usually used with phosphodiesterase I.

In the present work, the enzyme-substrate interaction of the substrates **4**, **5** and **6** with the proteolytic enzymes α-chymotrypsin, protease and alkaline mesintericopeptidase also have been studied (Scheme 5). The three proteolytic enzymes show equal action towards substrates **5** and **6**—only the ethoxycarbonyl group is hydrolyzed to the carboxylic acids **14** and **15**, respectively. The enzyme-substrates interaction is carried out under conditions, which we have found empirically to be the most favourable: 20 g substrate, 5–10 mg enzyme, stirring for 6 hours in a water buffer with pH optimum for the enzyme. The hydrolysis goes with a quantitative yield practically.

The proteolytic enzymes are especially valued for separation of racemic mixtures of α-aminocarboxylic acids to optically active antipodes of substrates containing organophosphoric acids. This approach, combined with the phos-



Scheme 4



Scheme 5

phoesterase enzymes, has been used by us for the synthesis of the naturally uncommon aminoacids and peptides phosphinothricin, Bialaphos, Plumbemicin A and B.

The presence of a free —P(O)(OH) group in the substrates **11**, **12** and **13**, inhibits partially the enzyme α-chymotrypsin. After the third consecutive experiment, no activity was observed any more.

The presence of a free carboxylic group does not inhibit the effect neither of phosphodiesterase I, alkaline phosphatase, nor of bee venom. When using the substrates **14** and **15**, and phosphodiesterase or bee venom, the corresponding free acids **8** and **9** are obtained with a practically quantitative yield. The enzyme alkaline phosphatase, used with the substrate phosphono diester **15** leads to the monoester **16**, isolated with a practically quantitative yield.

The enzyme protease is an analogue of the bee venom concerning its action on the substrate **10**. In both cases one and the same product is obtained—the free acid **7**. The inhibition of the enzyme protease is quite less than that of α-chymotrypsin.

In this work, however, it is not our intention to study the mechanism and kinetics of these unusual enzyme-substrate interactions. We are just proposing a purely synthetic approach to solving some problems of the chemistry of organophosphorus compounds, connected with the difficulties occurring when groups of the type —P(O)OR— should be hydrolyzed to —P(O)OH—.

EXPERIMENTAL

1. *General notes.* IR-spectra, elemental analysis and HPLC—on Perkin-Elmer instruments; ^{1}H NMR-spectra—on Jeol-100 MHz and Brucker-250 MHz; mass-spectra—on LKB-900 and Varian; m.p.—measured on a Kofler apparatus; TLC—silica gel film "Merck", phosphomolybdenate detection; reagents and solvents—from "Aldrich" and "Merck"; enzymes and buffers—from "Sigma"; α-chymotrypsin—from "Pharmachim", Sofia; bee venom—from "Koopsoyuz", Sofia.

2. *Synthesis of the esters* **4–6**. Glycine ethyl ester hydrochloride (69.79 g, 0.5 M) is dissolved in water (100 ml) and mixed with dichloroethane (200 ml). A solution of sodium carbonate (29.15 g, 0.275 M) in water (250 ml) and formalin (36.5%, 45.23 ml, 0.55 M —CH_2O) is prepared separately. The formalin-carbonate solution and a solution, respectively, of hypophosphorous acid ethyl ester (47.03 g, 0.5 M), ethylmethylphosphinate (54.04 g, 0.5 M) and diethylphosphite (69.05 g, 0.5 M), in dichloroethane (250 ml) are added dropwise, simultaneously, and with an equal velocity, for the duration of 30 min. at room temperature and with continuous stirring. The layers are then separated and the water layer is extracted with dichloroethane (3 × 25 ml). The combined organic extracts are

boiled for 1 hour. After distillation in vacuum to dryness, the residue—an amber-coloured oil—is placed on a silica gel column and eluted with chloroform/methanol to give:

N-[(ethoxyphosphinyl)methyl]glycine ethyl ester, **4**: $C_7H_{16}NO_4P$; 11.55 g (55.1%); oil (decomposes at over 100°C/6.10^{-4} Torr); IR (film): 2380 (P—H), 1745 (C=O), 1200–980 (P=O, P—O—C); ^{1}H NMR ($CDCl_3$): 1.28 and 1.42 (6 H, t, $CH_3 \times 2$), 2.31 (1H, br.s, NH), 2.91 (2 H, d, $J = 16$ Hz, PCH_2), 3.80–4.20 (6 H, m, $CH_2 \times 3$), 6.92 (1 H, d, $J_{H—P} = 580$ Hz PH); Mass-spect. (M^+/e): 209.182/209 (29%); R_f: 0.66 ($CHCl_3$:MeOH:C_6H_6 = 9:1:1);

Calc'd: C 40.19 H 7.71 N 6.70%
Found: C 40.38 H 7.56 N 6.51%

N-[(ethoxymethylphosphinyl)methyl]glycine ethyl ester, **5**: $C_8H_{18}NO_4P$; 16.41 g (73.5%); spectral data are identical with that of the product, prepared according to;[13] Mass-spect. (M^+/e), calc'd/found: 223.209/223;

Calc'd: C 43.05 H 8.13 N 6.28%
Found: C 43.30 H 7.99 N 6.33%

N-[(diethoxyphosphonyl)methyl]glycine ethyl ester, **6**: $C_9H_{20}NO_5P$; 20.33 g (80.3% spectral data are identical with that of the product, prepared according to;[21] Mass-spect. (M^+/e), calc'd/found: 253.257/253;

Calc'd: C 42.69 H 7.96 N 5.53%
Found: C 42.88 H 7.59 N 5.48%

Substances **4**, **5** and **6** are colourless oils, which distil with decomposition above 100°C and 6.10^{-4} Torr; **5** is unstable at storage as described in the literature.[13]

3. *Acid hydrolysis of the esters* **4**, **5** *and* **6**. 0.1 M from each ester is boiled during 3–6 hours in 100 ml hydrochloric acid (for the triester **6** in a mixture of 30 ml hydrochloric acid and 20 ml acetic acid). After distillation in vacuum to dryness and crystallization in ethanol, the following products are obtained:

N-[(hydroxyphosphinyl)methyl]glycine, **7**: $C_3H_8NO_4P$; 8.97 g (58.6%; m.p. 180–185°C (decomp.); spectral data are identical with that of the product, prepared according to;[11] Mass-spect. (M^+/e) calc'd/found: 153.075/153;

Calc'd: C 23.56 H 5.27 N 9.15%
Found: C 23.86 H 5.18 N 9.16%

N-[(hydroxymethylphosphinyl)methyl]glycine, **8**: $C_4H_{10}NO_4P$; 12.48 g (74.7%) m.p. 220–223°C (decomp.), 223–224°C (decomp.),[13] 207–208°C;[12] spectral data are identical with that of the product prepared according;[13] Mass-spect. (M^+/e) calc'd/found: 167.102/167;

Calc'd: C 28.75 H 6.03 N 8.38%
Found: C 29.07 H 5.91 N 8.56%

N-phosphonomethylglycine, **9**: $C_3H_8NO_5P$; 15.40 g (91.1%); m.p. 228–231°C (decomp.); spectral data are identical with that of the product prepared according to;[22] Mass-spect. (M^+/e) calc'd/found: 169.074/169; R_f: 0.55 (*n*-BuOH:25%NH_4OH – 4:1);

Calc'd: C 21.31 H 4.77 N 8.21%
Found: C 21.56 H 4.49 N 8.51%

4. *Enzyme-catalyzed hydrolysis of the diesters* **4**, **5** *and* **6**. *Method A*: Phosphodiesterase I (5 mg, or 15 mg if spread on a polymer carrier) is added to a homogeneous mixture, respectively of the diester **4** (20.91 g, 0.1 M), diester **5** (20 g), and triester **6** (20 g) and Tween-80 in a buffer medium (600 ml, pH 8.8), which has been tempered at 37°C. The mixture is stirred for 6 hours at the same temperature. The enzyme is removed by dialysis or ultrafiltration (or by centrifugation, if on a polymer carrier) and can be used again with its activity unchanged. The reaction mixture is acidified (pH 6.0–6.5) and evaporated in vacuum to dryness. The residue is extracted with boiling ethanol. After cooling, the following products crystallize slowly:

N-[(Hydroxyphosphinyl)methyl]glycine ethyl ester, **10**: $C_5H_{12}NO_4P$; 17.63 g (97.3%); m.p. 129–132°C; IR (KBr): 2320, 1750, 1185 (P=O), 1065 (P—O—); ^{1}H NMR (D_2O + NaOD); 1.19 (3H, t, $OCH_2\underline{CH_3}$), 2.99 (2 H, d, $J = 16$ Hz, PCH_2), 3.68 (2 H, q, OCH_2CH_3), 4.11 (2 H, s, CH_2), 6.70 (1H, d, $J_{H-P} = 532$ Hz, PH); Mass-spect. (M^+/e): 181.129/181; R_f: 0.73 (*n*-BuOH:AcOH:H_2O =

9:1:1 v/v);

Calc'd: C 33.16 H 6.68 N 7.73%
Found: C 33.42 H 6.46 N 7.59%

N-[(Hydroxy-methylphosphinyl)methyl]glycine-ethylester, **11**: $C_6H_{14}NO_4P$; 18.97 g (97.2%); m.p.: the product decomposes at about 200°C; Mass-spect. (M^+/e), calc'd/found: 195.156/195;

Calc'd: C 36.93 H 7.23 N 7.18%
Found: C 37.21 H 6.99 N 7.07%

N-Dihydroxyphosphonylmethylglycine ethyl ester, **12**: $C_5H_{12}NO_5P$; 15.29 g (98.2%); m.p. 204–207°C (decomp.); Mass-spect. (M^+/e) calc'd/found: 197.178/197;

Calc'd: C 30.46 H 6.14 N 7.11%
Found: C 30.28 H 6.21 N 7.30%

Method B: The ester **4**, **5** and **6** (20 g each), respectively, beforehand honogenized with Tween-80, are added to a buffer medium (600 ml, pH 8.7) and are worked up as in Method A with 25 mg raw-freeze-dried bee venom. The products **7**, **8** and **9** respectively, are isolated after acidification, evaporation in vacuum to dryness and crystallization. The spectral data for the products **7**, **8** and **9** are identical with that of the products prepared as in item **3**, and the yields are respectively: **7**—14.79 g (96.3%), **8**—14.58 g (97.3%) and **9**—12.72 g (95.3%).

5. *Enzyme-catalyzed hydrolysis of the ester* **6**: In a buffer medium (600 ml, pH 10.4), the substrate **6** (20 g) is homogenized in Tween-80, and then 5 mg alkaline phosphatase (or 10–15 mg if spread on a polymer carrier) added and the mixture stirred during 6 hours at 37°C. After removing the enzyme, acidification, concentration, cooling and crystallization, the following product is obtained:

N-[Ethoxyhydroxyphosphonyl)methyl]glycine ethyl ester, **13**: $C_7H_{16}NO_5P$; 17.27 g (97.1%); m.p. about 200°C (decomp.); Mass-spect. (M^+/e) calc'd/found: 225.181/225;

Calc'd: C 37.34 H 7.16 N 6.22%
Found: C 37.70 H 7.11 N 6.31%

6. *Enzyme-catalyzed hydrolysis of the carboxylic esters* **5**, **6**, **10**, **11**, **12** *and* **13** *with proteolytic enzymes*. In a buffer medium (pH 7.8 for α-chymotrypsin, pH 7.5 for protease and pH 8.0 for alkaline mesintericopeptidase) the corresponding substrate (20 g each) and the proteolytic enzyme (5 mg) are stirred during 6 hours at 25°C. After enzyme separation, acidification, concentration and cooling the corresponding products are isolated. The substrates of the enzyme α-chymotrypsin are the esters **5**, **6**, **11**, **12** and **13**, from which the products **14**, **15**, **8**, **9** and **16** are obtained respectively. The substrates of the protease and alkaline mesintericopeptidase are the esters **5** and **6** and the products of hydrolysis are **14** and **15**. The substrate of the protease is the ester **10**, and the product of hydrolysis is **7**.

N-[(Ethoxymethylphosphinyl)methyl]glycine, **14**: $C_6H_{14}NO_4P$; 18.79 g (96.3%) (from 22.32 g, 0.1 M of substrate **5**); m.p. 175–180°C (decomp.); Mass-spect. (M^+/e) calc'd/found: 195.156/195;

Calc'd: C 36.93 H 7.23 N 7.18%
Found: C 37.11 H 7.01 N 7.26%

16.32 g (91.8%); m.p. 135–137°C, 132–134 °C;[24] Mass-spect. (M^+/e) calc'd/found: 225.181/225;

Calc'd: C 37.34 H 7.16 N 6.22%
Found: C 37.68 H 7.01 N 6.18%

N-[(Ethoxyhydroxyphosphonyl)methyl]glycine, **16**: $C_5H_{12}NO_5P$; 19.88 g (76.3%); m.p. about 200 °C (decomp.); Mass-spect. (M^+/e) calc'd/found: 197.128/197;

Calc'd: C 30.46 H 6.14 N 7.11%
Found: C 30.72 H 6.01 N 7.26%

The product **16** is also obtained by the method, described in item **5** from the substrate **15** and the enzyme alkaline phosphatase with an yield of 94.3%.

The products **8** and **9** (the spectral data are identical with that of the ones obtained in item **3**) are prepared with yields of 12.54 g (73.2%) and 13.76 g (80.2%) respectively, from the substrates **11** and **12** and the enzyme α-chymotrypsin.

The same products **8** and **9** are obtained after an enzyme-substrate interaction of the substrates **14**

and **15** with the enzyme phosphodiesterase I, by the method described in 4A and with bee venom 4B. The yields are 16.63 g (97.1%) and 16.42 g (96.1) respectively.

The products **14** and **15** are isolated after enzyme-substrate interaction of the substrates **5** and **6** with the enzyme-substrate interaction of the substrates **5** and **6** with the enzyme protease and alkaline mesintericopeptidase. The yields of **14** and **15** are 17.96 g (92.1%) and 16.37 g (92.6%), respectively.

The product **7** is obtained with an yield of 13.3 g (87.3%) from 18.11 g, 0.1 M substrate **10** and the enzyme protease. The same product **7** is prepared with an yield of 14.57 g (95.2%) by the method described in 4B, from the same substrate **10** and bee venom.

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