solution was left at room temperature for 15 minutes and then evacuated for 10 minutes to remove as much of the hydrogen chloride as possible. Four ml. of dilute ammonium hydroxide was added to the solution and the benzyl alcohol extracted with ether (3 × 10 ml.). After removal of the excess of ammonia under vacuum the aqueous solution was applied to the top of a column (24 × 135 mm.) of Dowex 2[14] ion-exchange resin (200–325 mesh, formate form). The sample was washed in with 15 ml. of water and elution was carried out successively with 0.02 *N* formic acid (250 ml.), 0.1 *N* formic acid (750 ml.) and finally 250 ml. of 0.5 *N* formic acid. Fractions of twenty ml. each were collected, the elution being followed spectrophotometrically (optical density at 270 mμ). Cytidylic acid and cytidine-2′-benzyl phosphate emerged as separate peaks with 0.1 *N* formic acid and cytidine-3′-benzyl phosphate was eluted rapidly with the 0.5 *N* acid.[8] The proportions of the components eluted were found to be: cytidine-3′-benzyl phosphate, 65%; cytidine-2′-benzyl phosphate, 26%; unreacted cytidylic acid, 9%. The R_f values in three solvent systems are reported in Table I.

Adenosine-2′(3′)-propyl Phosphates.—Using a solution of the mixture of adenosine-2′:3′-cyclic phosphate and the adenylylureas in anhydrous *n*-propyl alcohol, the propyl esters were prepared as described above for cytidine benzy phosphates. The esters were isolated by paper chromatography on large sheets of Whatman paper No. 3MM in the solvent system A (Table I) and the eluted material further purified on an ion-exchange column as described above. The tubes containing the single broad peak eluted with 2.0 *N* formic acid were combined and lyophilized. On heating, the product decomposed above 164°, $\lambda_{max.}$ in 0.01 *N* hydrochloric acid, 257 mμ; ε 13,150.

Anal. Calcd. for $C_{13}H_{20}N_5O_7P \cdot 1H_2O$: C, 38.33; H, 5.44; P, 7.62. Found[15]: C, 38.33; H, 5.64; P, 7.63, 7.76.

By the procedure described above a number of ribonucleotide alkyl esters were prepared. The R_f values of the esters, the parent nucleotides and the cyclic phosphates are listed in Table I.

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(14) The Dow Chemical Company, Midland, Michigan.

(15) Analyses by Dr. D. R. Idler, Fisheries Experimental Station, Vancouver, B. C.

VANCOUVER 8, B.C.

[CONTRIBUTION FROM THE LABORATORY OF ORGANIC CHEMISTRY, UNIVERSITY OF ATHENS]

N-Phosphoroamino Acids and Peptides

BY LEONIDAS ZERVAS AND PANAYOTIS G. KATSOYANNIS[1]

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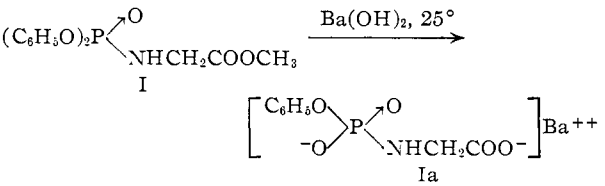
A method for the synthesis of N-phosphoroamino acids and peptides is described. Di-*p*-nitrobenzylphosphorochloridate or di-*p*-iodobenzylphosphorochloridate was coupled with esters of amino acids or peptides; this was followed by removal of the *p*-nitrobenzyl and *p*-iodobenzyl groups through catalytic hydrogenation in alcoholic alkaline medium.

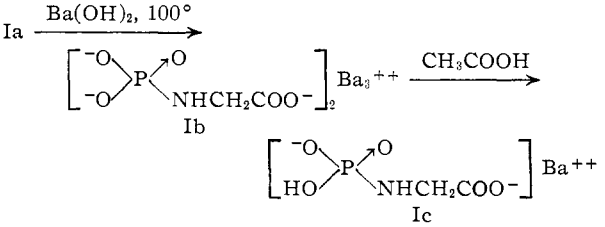
A number of phosphorylating agents have been used for the synthesis of N-phosphoroamino acids and peptides. With phosphorus oxychloride[2,3] the yields were low and the preparation of pure substances was difficult. The reaction of diphenylphosphorochloridate[4] (DPPCl) or diisopropylphosphorochloridate[5] with esters of amino acids yielded crystalline N-diphenylphosphoro or N-diisopropylphosphoro derivatives; however, it was not possible to split off the isopropyl groups without attacking the P–N bond[5] and saponification of the ester group in the case of the diphenyl compounds was not entirely successful.[4] In a few cases dibenzylphosphorochloridate[6] yielded crystalline dibenzylphosphoroamino acid esters; the benzyl groups can be removed easily by catalytic hydrogenation.[7] Non-esterified phosphoroamino acids have not been prepared by this method.

With DPPCl as the phosphorylating agent, we prepared N-phosphoroamino acids in some cases. The treatment of N-diphenylphosphoroglycine methyl ester (I), N-diphenylphosphoro-L-tyrosine ethyl ester (II) and N-diphenylphosphoroglycyl-L-tyrosine ethyl ester (III) with barium hydroxide resulted in saponification of the carboxyl ester group and splitting off of one phenyl group[8] as illustrated below



I Ia

Removal of the remaining phenyl group was accomplished without hydrolysis of the P–N bond by heating of the monophenyl derivative in a strongly alkaline solution (barium, sodium or potassium hydroxide)[9] as illustrated below for Ia



Ib Ic

In this case the insoluble barium salt (Ib) was con-

(1) This paper is based in part on the doctoral dissertation of P. G. Katsoyannis, Division of Natural Sciences (Chemistry Section), University of Athens, May, 1952. Present address: Department of Biochemistry, Cornell University Medical College, New York.

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(8) This behavior toward alkali is similar to that of triphenyl phosphate (von Glutz, *Ann.*, **143**, 192 (1867); E. Baer, THIS JOURNAL, **69**, 1253 (1947), and of tribenzyl phosphate (W. Lossen and A. Koehler, *Ann.*, **262**, 196 (1891); our conditions are milder.

(9) Considerable hydrolysis of the P–N bond accompanied removal of the phenyl group by heating Ia in an aqueous solution; some hydrolysis occurred when a buffered solution (*p*H 7.5) was warmed.

TABLE I

N-DIARYLPHOSPHOROAMINO ACID (DIPEPTIDE) ESTER

DPP = diphenylphosphoro, DIBP = di-*p*-iodobenzylphosphoro, DNBP = di-*p*-nitrobenzylphosphoro

No.	Diarylphosphoro group	Amino acid or dipeptide	Ester group	Yield, %	M.p., °C.	Formula	Analyses, % Nitrogen Calcd.	Nitrogen Found	Iodine Calcd.	Iodine Found
I	DPP	Glycine	CH_3	90	93	$C_{15}H_{16}O_5NP$[g]	4.35	4.25		
IIt[a,b,e]	DPP	L-Tyrosine	C_2H_5	70	93–94	$C_{23}H_{24}O_6NP$[h]	3.2	3.3		
IIIt[a,b,e]	DPP	Glycyl-L-tyrosine	C_2H_5	70	123–124	$C_{25}H_{27}O_7N_2P$	5.6	5.6		
IV	DIBP	Glycine	CH_3	80	124–125	$C_{17}H_{18}O_5NPI_2$	2.3	2.25	42.3	42.4
V	DIBP	Glycine	$C_6H_5CH_2$	80	89	$C_{23}H_{22}O_5NPI_2$	2.1	2.1	37.5	37.35
VI[a,c,f]	DIBP	L-Tyrosine	C_2H_5	75	143	$C_{25}H_{26}O_6NPI_2$	2.0	2.05	35.21	35.05
VII[d]	DNBP	Glycine	CH_3	70	89	$C_{17}H_{18}O_9N_3P$	9.6	9.7		
VIII[d,f]	DNBP	Glycylglycine	C_2H_5	70	112–113	$C_{20}H_{23}O_{10}N_4P$	11.0	11.2		
IX[a,c,f]	DIBP	Glycyl-L-tyrosine	C_2H_5	75	127–128	$C_{27}H_{29}O_7N_2PI_2$	3.6	3.75	32.6	32.4

[a] Prepared with ethyl acetate as the solvent. [b] The drying step was omitted. Water was added instead of hexane and the product crystallized after several hours cooling. [c] A little warm ether was added before the hexane. [d] The reaction mixture contained 0.02 mole of triethylamine. [e] Recrystallized from ether. [f] Recrystallized from ethanol. [g] Calcd. for $C_{15}H_{16}O_5NP$: C, 56.07; H, 5.02. Found: C, 56.25; H, 5.15. [h] Calcd. for $C_{23}H_{24}O_6NP$: C, 62.58; H, 5.48. Found: C, 62.75; H, 5.57.

verted to the sparingly soluble salt (Ic) by acetic acid.

Although heating of the monophenylphosphoro-barium salts of II and III with dilute alkali resulted in the removal of the remaining phenyl group, we did not pursue this method because of possible racemization of the amino acid component along with some hydrolysis of the peptide bond. Removal of the phenyl group by catalytic hydrogenation[4] does not have these disadvantages.

A general method for the synthesis of N-phosphoroamino acids and peptides is the reaction of the new phosphorylating agents,[10] di-*p*-nitrobenzylphosphorochloridate (DNBPCl) and di-*p*-iodobenzylphosphorochloridate (DIBPCl), with esters of amino acids and peptides to form the corresponding DNBP and DIBP derivatives. Saponification of the carboxyl ester group is followed by removal of the *p*-substituted benzyl groups through catalytic hydrogenation in alcoholic alkali, a reaction which is illustrated in the preceding paper. If the benzyl ester of the amino acid or peptide is used, saponification of the coupled product prior to hydrogenation is unnecessary; in that case the hydrogenation is carried out in alcoholic triethylamine solution.

By this method N-phosphoro derivatives of glycine, L-tyrosine, glycylglycine and glycyl-L-tyrosine were prepared as the barium salts. Solutions of the sodium or potassium salts were used to study the stability of the phosphoroamino acids and peptides at various *p*H's. As expected,[3] the N-phosphoroamino acids and peptides are stable in alkali, fairly stable in a neutral medium, and particularly sensitive to acid.[11]

Experimental

The solvents used were dried and freshly distilled; the hydrogenation catalyst was freshly prepared, wet palladium black. For analysis, the barium salts were dried under vacuum at 100°, and the other compounds at 56°. Phosphorus was determined colorimetrically[12] on compounds which had been hydrolyzed with hydrochloric acid. The nitrogen content of the barium salts was determined by the Kjeldahl method and that of the other compounds by the Dumas method.

N-Diarylphosphoroamino Acid and Dipeptide Esters.—The N-diarylphosphoroamino acid and dipeptide esters listed in Table I were prepared from the corresponding diarylphosphorochloridate and amino acid (or dipeptide) ester. N-Diphenylphosphoroglycine methyl ester (I) was synthesized by procedure A. The other compounds were prepared essentially by procedure B; the slight modifications used for the individual compounds are indicated in the footnotes to the table.

A. N-Diphenylphosphoroglycine Methyl Ester (I).—To a suspension of 2.5 g. (0.02 mole) of glycine methyl ester hydrochloride in 15 ml. of anhydrous pyridine at 0°, was added 6 g. (10% excess) of DPPCl.[13] The mixture was shaken for two hours and then poured into 100 ml. of ice-water. The product, which separated as an oil, soon crystallized. It was collected, washed with water, dried and recrystallized from ether.

B. N-Di-*p*-iodobenzylphosphoroglycine Methyl Ester (IV).—To a chloroform solution containing at least 1.9 g. (0.02 mole) of glycine methyl ester was added 5.5 g. (0.01 mole) of DIBPCl.[10] After 12 hours the precipitated glycine methyl ester hydrochloride was filtered and the filtrate shaken successively with dilute hydrochloric acid, aqueous potassium bicarbonate and water. It was dried over sodium sulfate and the solvent removed *in vacuo*. The addition of hexane gave a crystalline product which was recrystallized from methanol.

N-Monophenylphosphoroamino Acids and Dipeptides. A. Barium Salts of N-Monophenylphosphoroglycine (Ia) and N-Monophenylphosphoroglycyl-L-tyrosine (IIIa).—A suspension of 0.01 mole of I (0.003 mole of III) and 6.7 g. (3 g.) of barium hydroxide octahydrate in 60 ml. (30 ml.) of water was shaken for four hours. Carbon dioxide was then bubbled through the mixture and the precipitated barium carbonate filtered. Ia crystallized as the dihydrate upon the addition of 60 ml. of methanol to the filtrate; yield 70%.

Anal. Calcd. for $C_8H_9O_5NPBa$: N, 3.82; P, 8.46; Ba, 37.48. Found: N, 3.68; P, 8.35; Ba, 37.60.

IIIa precipitated upon the addition of acetone; yield 63%.

Anal. Calcd. for $C_{17}H_{17}O_7N_2PBa$: N, 5.28; Ba, 25.95. Found: N, 5.05; Ba, 26.10.

B. N-Monophenylphosphoro-L-tyrosine Barium Salt (IIa).—II (4.3 g., 0.01 mole) was shaken with 30 ml. of 2 *N* sodium hydroxide and 10 ml. of water for three hours. The mixture was filtered and 12 ml. of 5 *N* hydrochloric acid and an aqueous solution containing 5.9 g. of barium acetate were added to the filtrate. The product which crystallized as the dihydrate (small prisms) was washed with cold water; yield 3.3 g. (65%).

Anal. Calcd. for $C_{15}H_{14}O_6NPBa$: N, 2.96; P, 6.55; Ba, 29.07. Found: N, 2.80; P, 6.48; Ba, 29.30.

(10) L. Zervas and I. Dilaris, THIS JOURNAL, **77**, 5354 (1955).

(11) At *p*H 7.5 and room temperature N-phosphoroglycine does not give a positive ninhydrin reaction, even after several minutes, while under the same conditions, the non-phosphorylated amino acid gives an intense brownish red color after a few seconds. At *p*H 4 and 25–30° the N-phosphorylated amino acids and peptides release phosphoric acid almost quantitatively within 5–10 minutes.

(12) C. H. Fiske and Y. SubbaRow, *J. Biol. Chem.*, **66**, 375 (1925).

(13) P. Brigl and H. Mueller, *Ber.*, **72**, 2121 (1939).

Removal of the Carboxyl Ester Group: Di-*p*-iodobenzylphosphoroglycine (IVa), N-Di-*p*-iodobenzylphosphoro-L-tyrosine (VIa) and N-Di-*p*-nitrobenzylphosphoroglycine (VIIa).—IV or V was shaken for 10–15 minutes with a slight excess of 0.2 *N* sodium hydroxide; a mixture of VI, dissolved in warm ethanol, and 2 equivalents of alkali was allowed to stand for 30 minutes; and VII was shaken for 10 minutes with an alcohol–water solution of sodium hydroxide (10% excess). The products precipitated upon acidification with hydrochloric acid. The yields were IVa and VIIa 80%, and VIa 85%. IVa and VIIa were recrystallized from ethanol. IVa which was dried *in vacuo* began melting at 115° and decomposed at 175–178°.

Anal. Calcd. for $C_{16}H_{16}O_5NPI_2$: N, 2.38; I, 43.22; Found: N, 2.47; I, 42.95. VIa began melting at 80–85° and decomposed upon further heating.

Anal. Calcd. for $C_{23}H_{22}O_6NPI_2$: I, 36.61. Found: I, 36.55. VIIa melted at 149°.

Anal. Calcd. for $C_{16}H_{16}O_9N_3P$: N, 9.88. Found: N, 10.00.

N-Carbobenzoxyglycyl-L-tyrosine Ethyl Ester.—A solution of 4.2 g. (0.02 mole) of carbobenzoxyglycine[14] and 2.8 ml. (0.02 mole) of triethylamine in 20 ml. of chloroform was cooled at 0° and 2.8 ml. (0.02 mole) of ethyl chlorocarbonate[15] was added. After storage at 0° for 10 minutes, this solution was added to a chloroform solution containing 4.9 g. (0.02 mole) of L-tyrosine ethyl ester hydrochloride and 2.8 ml. of triethylamine. After the evolution of carbon dioxide was complete, the solution was shaken successively with water, dilute hydrochloric acid, aqueous potassium bicarbonate and water, dried with sodium sulfate and concentrated *in vacuo*. Ethanol was added to the residue and the solution was concentrated *in vacuo*. The product was crystallized from ethanol as needles; yield 69–70%, m.p. 124–125°, reported m.p. 118°.[16]

Anal. Calcd. for $C_{21}H_{24}O_6N_2$: N, 7.00. Found: N, 7.15.

Glycyl-L-tyrosine Ethyl Ester Hydrochloride.—This dipeptide ester was obtained by hydrogenolysis[14] of the above dipeptide in ethanol containing hydrochloric acid; yield quantitative, m.p. 245°, reported 245°.[17]

N-Phosphoroglycine Barium Salt (Ib). A. From Monophenylphosphoroglycine Barium Salt (Ia).—A solution of 4 g. (0.01 mole) of the dihydrate (Ia) and 2 g. of barium hydroxide octahydrate in 70 ml. of water was heated at 100° for 30 minutes under nitrogen. After the mixture had been cooled, the precipitate was collected and washed with dilute aqueous barium hydroxide and methanol. It was stored *in vacuo;* yield 3.2 g. (88%).

Anal. Calcd. for $C_4H_6O_{10}N_2P_2Ba_3$: N, 3.91; P, 8.65; Ba, 57.55. Found: N, 3.80; P, 8.60; Ba, 57.25.

B. From the Methyl and Benzyl Esters of N-Di-*p*-iodobenzylphosphoroglycine (IV and V) and N-Di-*p*-nitrobenzylphosphoroglycine Methyl Ester (VII).—A mixture of 0.005 mole of IV (VII) and 25 ml. (15 ml.) of *N* ethanolic sodium hydroxide was allowed to stand for 10 minutes[18] and then hydrogenated catalytically; a suspension of 0.005 mole of V in 50 ml. of methanol containing 4 ml. of triethylamine (10% excess) was hydrogenated directly. The catalyst was filtered and washed repeatedly, first with small quantities of water and then with methanol. Ib precipitated upon the addition of a solution of 3.7 g. of barium iodide in 70 ml. of methanol saturated with barium hydroxide to the combined filtrate. Saturation of the methanol with barium hydroxide was necessary to prevent the partial hydrolysis of Ib to Ic. The yield from IV was 93%.

Anal. Found: N, 3.70; P, 8.55; Ba, 57.30. The yield of Ib from V was 93%.

Anal. Found: N, 3.78; P, 8.60; Ba, 57.67. The yield from VII was 90%.

Anal. Found: N, 3.75; P, 8.58; Ba, 57.26.

N-Phosphoroglycine Barium Acid Salt (Ic). A. From Ia.—A solution of 2 g. (0.005 mole) of the dihydrate of Ia in 10 ml. of *N* sodium hydroxide and 30 ml. of water was heated at 100° for 20 minutes. Acetic acid (2.5 ml.) was added to the cooled mixture which was filtered quickly; 40 ml. of ethanol was then added dropwise to the filtrate with cooling and stirring. The crystals were collected, washed first with 50% and then with anhydrous ethanol and stored in a desiccator; yield 0.8 g. (55%).

Anal. Calcd. for $C_2H_4O_5NPBa$: N, 4.82; P, 10.67; Ba, 47.31. Found: N, 4.90; P, 10.55; Ba, 47.10.

Heating of a solution of 1 g. of Ia in 12.5 ml. of water at 100° for 10 minutes gave 0.6 g. of a mixed product containing 2.1% nitrogen and apparently consisting of approximately 43% Ic and 57% inorganic phosphate. The latter was removed by shaking the mixture with dilute acetic acid for 5 minutes, and 0.15 g. of Ic was thus obtained. When phosphate buffer (*p*H 7.5) was used for the hydrolysis, 0.25 g. of Ic was obtained.

B. From Ib.—When the amorphous Ib (0.71 g., 0.001 mole) was shaken either for 2 hours at 1–2° with 20 ml. of 0.1 *N* hydrochloric acid or for 10 minutes with 7 ml. of 30% acetic acid, crystalline Ic was obtained. It was washed successively with cold water, 50% ethanol and anhydrous ethanol. The yield from the hydrochloric acid hydrolysis was 60% and from the acetic acid hydrolysis 84%.

Anal. Found: N, 4.96; P, 10.77; Ba, 47.50.

N-Phosphoro-L-tyrosine Barium Salt.—A mixture of 3.6 g. (0.005 mole) of DIBP-L-tyrosine ethyl ester (VI) in 60 ml. of 0.1 *N* ethanolic sodium hydroxide was allowed to stand for 15 minutes and then hydrogenated catalytically. The hydrogenation was complete in 45 minutes with the absorption of 500 ml. of hydrogen (23°, 756 mm.). The catalyst was filtered and washed repeatedly with 90% ethanol. To the combined filtrate was added 2 g. of barium iodide dissolved in ethanol. The precipitate was centrifuged and washed with ethanol until the washings gave a negative test for iodide ions; yield 2 g. (85%).

Anal. Calcd. for $C_{18}H_{18}O_{12}N_2P_2Ba_3$: N, 3.02; P, 6.67; Ba, 44.38. Found: N, 2.95; P, 6.55; Ba, 44.15.

N-Phosphoroglycylglycine Barium Salt.—DNBP-glycylglycine ethyl ester (VIII) (2.5 g., 0.005 mole) was shaken with 30 ml. of 0.5 *N* ethanolic sodium hydroxide for 15 minutes, hydrogenated and worked up as described above. The hydrogenation was complete in 30 minutes with the absorption of 1000 ml. of hydrogen (24°, 754 mm.). The barium reagent was 3 g. of barium iodide in methanol saturated with barium hydroxide; yield 2.1 g. (80%).

Anal. Calcd. for $C_8H_{12}O_{12}N_4P_2Ba_3$: N, 6.74; P, 7.46; Ba, 49.60. Found: N, 6.55; P, 7.35; Ba, 49.30.

N-Phosphoroglycyl-L-tyrosine Barium Salt.—To 3.9 g. of DIBP-glycyl-L-tyrosine ethyl ester (IX) dissolved in warm methanol was added 25 ml. of *N* ethanolic sodium hydroxide. The mixture was shaken for 10 minutes, hydrogenated and worked up as described above. The hydrogenation was complete in 15 minutes with the absorption of 520 ml. of hydrogen (26°, 758 mm.); yield 2.5 g. (95%).

Anal. Calcd. for $C_{22}H_{24}O_{14}N_4P_2Ba_3$: N, 5.37; P, 5.95; Ba, 39.53. Found: N, 5.35; P, 6.17; Ba, 39.20.

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