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Synthesis of Phosphopeptides

I. Peptides of DL-Serine and Glycine

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The synthesis of unphosphorylated, O-phosphorylated and O-monophenylphosphorylated DL-serylglycine, glycyl-DL-serine and glycyl-DL-serylglycine is described. Infrared absorption spectra of O-phosphorylated peptides are shown.

In 1933 Schmidt [1] as well as Levene and Hill [2] isolated a phosphorus-containing peptide from hydrolysed casein. Posternak and Pollaczek [3] proved that the structure of this peptide was O-phosphoryl-L-seryl-L-glutamic acid. Later, this and other phosphopeptides have been obtained *, particularly in recent years in connection with structural studies on phosphorus-containing proteins [4–11]. Phosphorylated peptides containing glycine have mainly been isolated from hydrolysates of phosphorylated α-chymotrypsin [5–8], but also from an enzymatically synthesized polypeptide [4]. O-Phosphorylserylglycine (Fig. 1., compound Ia) was the phosphopeptide from α-chymotrypsin first described and Shaeffer showed that this peptide was partly converted to glycyl-O-phosphorylserine (Fig. 1., compound IIa) during acid hydrolysis [7]. These two peptides, containing only glycine beside one serine residue are well adepted for model compounds in synthetic studies. As only one asymmetric centrum is present, DL-serine can be used in studies of this kind without the formation of diastereoisomers, a complication that occurs, when other amino acids than glycine are used.

We have prepared synthetic phosphorylated peptides in two different ways **. The most successful route so far has been to synthesize an N-carbobenzoxypeptide benzyl ester first, then introduce a suitable phosphoryl group and finally remove all protecting groups by catalytic hydrogenolysis [13].

* A review of earlier work is given by Perlmann, G. E. *Advances in Protein Chem.* **10** (1955) 1.

** In some cases, mixed anhydrides from N-acylamino acids [12] have been used for N-acylation of O-phosphorylserine. Such N-acylations of phosphorylated serine derivatives, including O-diphenylphosphorylserine [13] and the corresponding benzyl ester [14] will be further investigated.

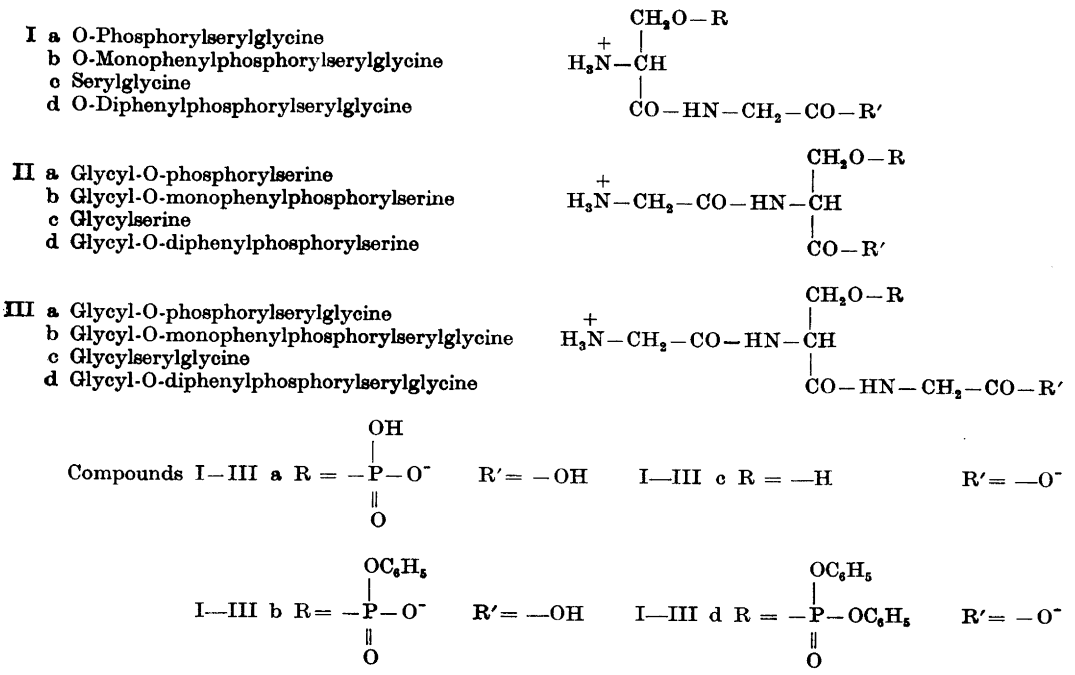

Fig. 1. Structure of serine and glycine peptides.

In the synthesis of N-carbobenzoxypeptide benzyl esters used in this work, the procedure of Sheehan [15] has given equal or better over all yields than with the azide route [16] or with the mixed anhydride technique [12]. The phosphorylation and hydrogenolysis have been performed in this work as described in the synthesis of O-phosphorylserine *, and in the case of peptides it was also possible to isolate diphenylphosphorylated and monophenylphosphorylated intermediates during the hydrogenolysis **. The structure of the compounds is shown in Fig. 1.

Infrared absorption spectra have been recorded and some of them are shown in Fig. 2. The paper chromatographic behaviour of the compounds has been studied, and R_F values are given in Table 1. As in the case of other peptides containing N-terminal glycine, also all the synthesised O-phosphorylated, mono-and diphenylphosphorylated peptides having N-terminal glycine

* For an reaction scheme, see this synthesis (Ref.[13]). Riley *et al.*[14] have recently described the synthesis of O-phosphorylated derivatives of hydroxyamino acids and peptides, for the investigation of which alkaline hydrolysis was used as an alternative for the elimination of phenylic ester groups.

** Bevan *et al.*[17] recently isolated this type of O-monophenylphosphorylated derivatives by hydrogenolytic cleavage of diphenylphosphates.

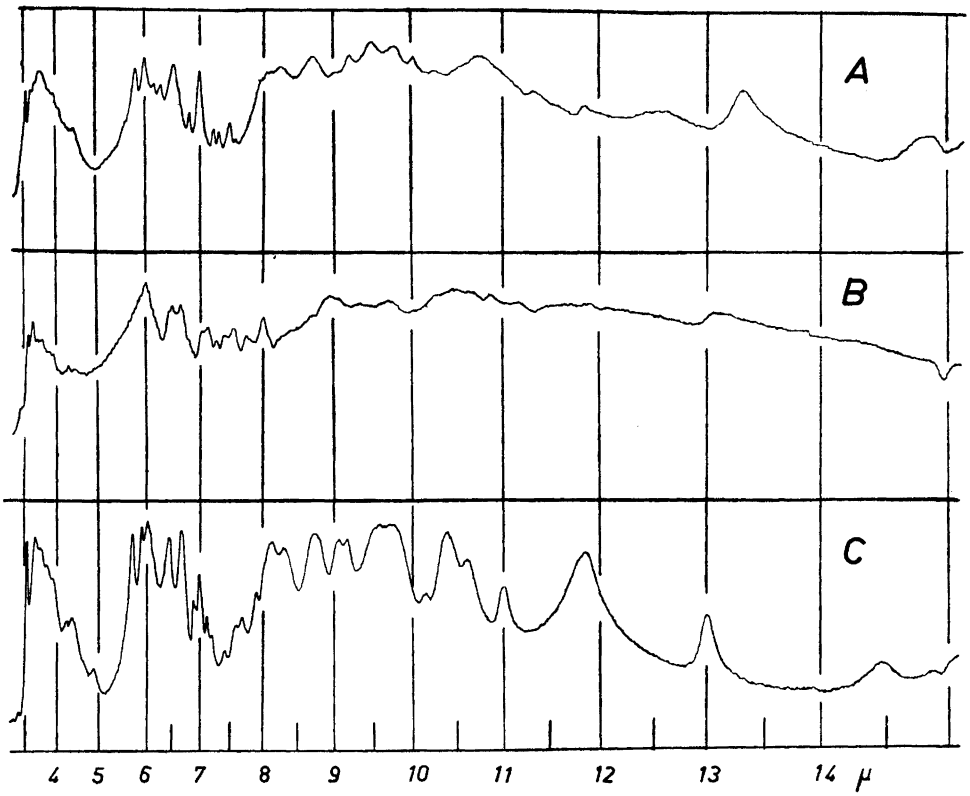

Fig. 2. Infrared absorption spectra of O-phosphoryl-DL-serylglycine (A), glycyl-O-phosphoryl-DL-serine (B) and glycyl-O-phosphoryl-DL-seryl-glycine (C). Compounds (1.4 mg) in potassium bromide (300 mg) pellets.

give an initial yellow colour with ninhydrin spray reagent. After some time (about half an hour) the colour changes slowly to the normal brown-violet tones.

EXPERIMENTAL

Melting or decomposition points, elementary analyses and infrared spectra were all determined as described previously [13]. Solvents in the paper chromatography were also as before: (I) Phenol-water (80/20 v/v), ascending, about 6 h, (II) *n*-Butanol-acetic acid-water (40/10/50 v/v), descending, about 18 h.

N-Carbobenzoxy-DL-serylglycine benzyl ester

a) By the azide route [16]: A vigorously stirred mixture containing 15.6 g (0.1 mole) of DL-serine methyl ester hydrochloride, 5.2 g (0.13 moles) of magnesium oxide, 75 ml of water and 180 ml of chloroform was cooled in an ice-salt mixture, and 23 g of carbobenzoxy chloride [18] was added dropwise within 1/2 h. After continued stirring for 1/2 h,

Table 1. DL-Serine and glycine peptides.

Compounds *	Molecular formula	Decomp. p.** °C	R_F values *** in solvent I	R_F values *** in solvent II	Analysis Found C	Found H	Found N	Found P	Calc. C	Calc. H	Calc. N	Calc. P
					O-phosphorylated peptides							
I a H.SerP-Gly.OH ****	$C_5H_{11}O_7N_2P$	(242.1) 189—192	0.12	0.11	25.0	4.6	11.5	11.7	24.8	4.6	11.6	12.8
II a H.Gly-SerP.OH	$C_5H_{11}O_7N_2P$	(242.1) 201—204	0.09	0.08	25.3	4.6	11.4	12.4	24.8	4.6	11.6	12·8
III a H.Gly-SerP-Gly.OH	$C_7H_{14}O_8N_3P$	(299.2) 220—223	0.13	0.08	28.2	4.7	13.9	10.3	28.1	4.7	14.0	10.4
					O-monophenylphosphorylated peptides							
I b H.SerφP-Gly.OH	$C_{11}H_{15}O_7N_2P$	(318.2) 207—209	0.42	0.37	41.6	4.8	8.6	9.7	41.5	4.8	8.8	9.7
II b H.Gly-SerφP.OH	$C_{11}H_{15}O_7N_2P$	(318.2) 176—178	0.38	0.30	38.4	4.8	8.5	9.3	41.5	4.8	8.8	9.7
III b H.Gly-SerφP-Gly.OH	$C_{13}H_{18}O_8N_3P$	(375.3) 198—202	0.40	0.34	40.8	4.7	10.9	8.4	41.6	4.8	11.2	8.3
					Corresponding peptides							
I c H.Ser-Gly.OH	$C_5H_{10}O_4N_2$	(162.1) 203—205	0.40	0.18	35.2	5.6	16.5		37.0	6.2	17.3	
II c H.Gly-Ser.OH	$C_5H_{10}O_4N_2$	(162.1) 197—199	0.35	0.16	36.1	5.8	16.7		37.0	6.2	17.3	
III c H.Gly-Ser-Gly.OH	$C_7H_{13}O_5N_3$	(219.2) 208—210	0.40	0.16	37.7	6.2	18.7		38.4	6.0	19.2	

* For abbreviations and symbols, see Sachs, H. and Brand, E. *J. Am. Chem. Soc.* **75** (1953) 4608. The symbol SerP is here used for an O-phosphorylserine residue, $HN \cdot CH[CH_2OPO(OH)_2]CO$, SerφP for an O-monophenylphosphorylserine residue, $HN \cdot CH[CH_2OPO(OH)OC_6H_5]CO$. Compounds numbered according to Fig. 1.

** See Ref.[13], p. 1237, foot note three.

*** The R_F values are calculated from several one-dimensional chromatograms, on which serine had R_F 0.36 in solvent I and 0.18 in solvent II. The three phosphorylated peptides are well separated on two-dimensional chromatograms in solvent I and II.

**** This compound has also been obtained in a low yield by the use of dibenzylphosphoryl chloride as phosphorylating reagent [22]. Riley *et al.*[14] report a m.p. of 150—154° (decomp.) for the compound obtained by alkaline hydrolysis.

5 ml of pyridine was added and the mixture acidified with cold 5 N hydrochloric acid (Congo red). The N-carbobenzoxy-DL-serine methyl ester was taken up in chloroform. The solution was washed with hydrochloric acid, bicarbonate and water, and was finally dried over sodium sulphate. The chloroform was distilled off *in vacuo*, the residue evaporated three times with ethanol and finally dissolved in 100 ml of absolute ethanol. Seven ml of hydrazin hydrate (b.p. 120—121°) was added and the solution left over night. N-carbobenzoxy-DL-seryl hydrazide, 17.2 g (66 %), thus crystallised. M. p. 158—160° (Reported [14] 162—163°). To a solution of 13.2 g (0.05 moles) of the hydrazide in 60 ml of acetic acid, 25 ml of 5 N hydrochloric acid and 250 ml of water was added at −5° a concentrated solution of 3.3 g (0.053 moles) of sodium nitrite. The azide formed, was taken up in 300 ml of cold ether, and the solution was washed and dried in the cold. A cold dry etheral solution of free glycine benzyl ester, freshly prepared from 15.2 g (0.075 moles) of the hydrochloride [19] was added to this solution. The mixture was left for 24 h, whereby a large part of the peptide derivative crystallised out from the solution. The crystals were filtered off, washed with ether, the combined ether solutions were washed with acid, bicarbonate, and water and after drying over sodium sulphate, evaporated to dryness. The residue was crystallised from ethyl acetate and light petroleum (b.p. 20—40°). The total yield of crystalline N-carbobenzoxy-DL-serylglycine benzyl ester was 13.2 g (68 %). M. p. 100—101°. (Found: C 62.7; H 5.8; N 7.3. Calc. for $C_{20}H_{22}O_6N_2$ (386.2): C 62.1; H 5.7; N 7.3).

b) By the mixed anhydride route [12]: 1.95 ml (20 mmoles) of ethyl chlorocarbonate was added to a solution of 4.8 g (20 mmoles) of N-carbobenzoxy-DL-serine [20] and 2.8 ml, (20 mmoles) of triethylamine in 25 ml of anhydrous dimethylformamide at a temperature of 0—5°. Ten minutes later a solution of 4.0 g (20 mmoles) of glycine benzylester hydrochloride [19] and 2.8 ml of triethylamine in 25 ml of dimethylformamide was added, causing a vigorous evolution of carbon dioxide. Next day the mixture was evaporated to dryness *in vacuo* and the residue was extracted in ethyl acetate. The solution was washed and worked up as above, yielding 3.8 g (50 %) crystals from ethyl acetate and light petroleum, m. p. 100—101°.

c) By the carbodiimide procedure [15]: To a solution of 2.4 g (10 mmoles) of N-carbobenzoxy-DL-serine in 25 ml of dimethylformamide was added 1.8 g (15 mmoles) of free glycine benzyl ester [19] and 2.1 g (10 mmoles) of N,N′-di*cyclo*hexylcarbodiimide. The reaction mixture was filtered after 5 h, yielding 2.0 g of N,N′-di*cyclo*hexylurea (m.p. 237—240° decomp.). The solution was evaporated to dryness *in vacuo*, and the residue was dissolved in 75 ml of ethyl acetate. This solution was worked up as above, yielding 2.8 g (72 %) of N-carbobenzoxy-DL-serylglycine benzyl ester m.p. 100—101°.

Carbobenzoxyglycyl-DL-serine benzyl ester

a) By the mixed anhydride route: To the mixed anhydride, prepared as above from 8.4 g (40 mmoles) of carbobenzoxyglycine, 5.6 ml of triethylamine and 3.84 ml of ethyl chlorocarbonate in 40 ml anhydrous dimethylformamide was added a cold mixture of 10.2 g (44 mmoles) of DL-serine benzyl ester hydrochloride [21], 6.2 ml of triethylamine and 40 ml of dimethylformamide. After 5 h at room temperature, the reaction mixture was filtered and evaporated to dryness, and the residue shaken with 100 ml of ethyl acetate and 50 ml of water. The ethyl acetate solution was worked up as in the case of the aforegoing compound, yielding 11.2 g (72 %) of carbobenzoxyglycyl-DL-serine benzyl ester, m. p. 141—142° (from ethanol-ether). (Found: C 62.1; H 5.8; N 7.3. Calc. for $C_{20}H_{22}O_6N_2$ (386.2) C 62.1; H 5.7; N 7.3).

b) By the carbodiimide procedure: From 8.4 g (40 mmoles) of carbobenzoxyglycine, 10.2 g (44 mmoles) of DL-serine benzyl ester hydrochloride, 5.6 ml of triethylamine and 8.4 g of N,N′-dicyclohexylcarbodiimide, a yield of 10.7 g (69 %) m. p. 141—142° was obtained by the same procedure as already described.

Carbobenzoxy glycyl-DL-seryl glycine benzyl ester

To the mixed anhydride prepared as above from 8.4 g of carbobenzoxyglycine, was added a cold solution of 4.4 g (40 mmoles) of DL-serine in 20 ml of 2 N sodium hydroxide. Carbon dioxide was vigorously evolved. After 6 h at room temperature the mixture

was evaporated to dryness *in vacuo*, the residue dissolved in 50 ml of water, washed with ether, and acidified with cold 5 N hydrochloric acid. The oil which separated was taken up with three 50 ml portions of ethyl acetate, the ethyl acetate distilled off at reduced pressure and the residue treated with ether and scratched, resulting in crystals of carbobenzoxyglycyl-DL-serine, 10.5 g (88 %) separating. M. p. 153—154° (recryst. from ethanol-ether). (Found C 52.6; H 5.6; N 9.3. Calc. for $C_{13}H_{16}O_6N_2$ (296.3): C 52.7; H 5.4; N 9.5). To a solution of 5.9 g (20 mmoles) of this compound in 25 ml of dimethylformamide was added 4.4 g (20 mmoles) of glycin benzyl ester hydrochloride, 2.8 ml (20 mmoles) of triethylamine, followed by 4.2 g (20 mmoles) of N,N'-di*cyclo*hexylcarbodiimide. After 6 h, the precipitate of di*cyclo*hexylurea (4.0 g) was filtered off and the solution was evaporated ty dryness *in vacuo*. The remaining oil was brought to crystallisation by shaking and scratching in water and ether. After recrystallisation from ethanol, carbobenzoxyglycyl-DL-serylglycine benzyl ester, 5.8 g (65 %) was obtained. M. p. 169—170°. (Found: C 59.9; H 5.7; N 9.7. Calc. for $C_{22}H_{25}O_7N_3$ (443.4): C 59.6; H 5.7; N 9.5).

Phosphorylation

The procedure previously described [13] was used throughout. To 20 mmoles of carbobenzoxypeptide benzyl ester in 10 to 15 ml of anhydrous pyridine was added 6.4 g (24 mmoles) of diphenylphosphoryl chloride with shaking and moderate cooling. After 4 h 1 ml of water was added, 1/2 h later the reaction mixture was poured into 150 ml of water and 100 ml of chloroform *. The chloroform solution was washed with cold 4 N sulphuric acid, water, 20 % bicarbonate solution, and again with water. After drying over sodium sulphate, the solvent was evaporated *in vacuo*, the residue being the expected diphenylphosphate ester in about 90 % yield. This procedure resulted in O-diphenylphosphoryl-N-carbobenzoxy-DL-serylglycine benzyl ester, colourless oil, $n_D^{21°}$ 1.5642. (Found: N 4.7. Calc. for $C_{32}H_{31}O_9N_3P$ (618.6): N 4.5), O-diphenylphosphoryl-N-(carbobenzoxyglycyl)-DL-serine benzyl ester, colourless oil, $n_D^{20°}$ 1.5620, (Found: N 4.8. Calc. for $C_{32}H_{31}O_9N_3P$ (618.6): N 4.5) and O-diphenylphosphoryl-N-(carbobenzoxyglycyl)-DL-serylglycine benzyl ester, colourless crystals, m. p. 85—86° (from ethanol-light petroleum). (Found: C 60.3; H 5.1; N 6.2; P 4.5. Calc. for $C_{34}H_{34}O_{10}N_3P$ (675.6): C 60.4; H 5.1; N 6.2; P 4.6).

Hydrogenolysis

When monophosphate esters (free phosphorylated peptides) were wanted, the hydrogenation was performed in glacial acetic acid with platinum oxide as catalyst. Water was added after some time to dissolve precipitated material. The time required for complete hydrogenolysis varied between 8 h and 15 h. Paper chromatography was useful for checking the amount of partly hydrogenated derivatives [13]. The residue obtained after filtration and evaporation of the solvent was treated in water solution with Amberlite IR 120 cation exchange resin (H^+ form) to eliminate metal cations, the filtered solutions was evaporated again and the phosphopeptide crystallised from hot water — ethanol.

Diphenylphosphoryl derivatives of the peptides were easily obtained when the hydrogenolysis was performed in ethanol solution with palladium oxide catalyst. Monophenylphosphoryl derivatives were obtained when hydrogenolysis in glacial acetic acid with platinum oxide catalyst was discontinued after a short time. The yields varied with the solubility of the diester **.

The carbobenzoxy peptide benzyl esters and carbobenzoxyglycyl-DL-serine were also hydrogenolysed with palladium catalyst to the free peptides. Analytical data, decomposition points and R_F values of the peptides are shown in Table 1.

* The glycylserine and glycylserylglycine derivatives are only sparingly soluble in ether.

** In a synthesis of O-phosphorylserylleucine (to be published) it vas difficult to isolate the monophenylphosphoryl derivative by hydrogenolysis in acetic acid. This compound, however, crystallised out when an acetic acid-ethanol (1:2) mixture was used.

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