

O-Phosphorylserine

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A synthesis of the L-, D- and DL-forms of O-phosphorylserine (phosphoserine, serinephosphoric acid or 2-amino-2-carboxy-ethylphosphoric acid) is described. The L-isomer was shown to be identical with the phosphorylated serine isolated from phosphoproteins.

A large part of the phosphoprotein phosphorus is considered to be ester bound to serine hydroxyl groups. This view was first expressed by Lipmann after the isolation of O-phosphorylserine from casein and vitellin hydrolysates¹⁻⁴. Later, phosphorylated serine was obtained from several phosphoproteins⁵⁻¹², and was also found in free form¹³⁻¹⁴. The occurrence of phosphoric acid diester bound serine in phospholipids such as phosphatidyl serine is well known, and, in addition, serine-guanidoethanol¹⁵ and serine-aminoethanol¹⁶ diesters have been found. Furthermore, O-phosphorylserine has been isolated from enzymes deactivated by *diisopropylphosphoryl* fluoride and related compounds¹⁷.

Phosphoproteins and phosphopeptides have been studied for a long time¹⁸ in our laboratory. We have observed that, when cow's casein is hydrolysed by proteolytic enzymes under conditions similar to the physiological ones¹⁹ a considerable part of the casein molecule is left as an enzyme-resistant residue. This residue consists of a complex mixture of phosphorylated peptides. The amino acid composition and the phosphorus content of this peptide fraction²⁰ allow all of the serine hydroxyl groups to be bound as monophosphate esters, although other structures are possible, *e. g.* diester bridges, polyphosphate bridges, N-phosphoryl groups *etc.*²¹ The enzyme resistance of these peptides seems to be due to their phosphorylation. Another property of these phosphorylated peptides, the tendency for metal complex formation, is of great biological interest. The calcium salts are, for example well utilised in bone calcification^{22,23}. One of us (F.) has made a study of the synthesis of phosphorylated serine peptides as a link in this chain of investigation^{24,25}. Here a new synthesis of the keycompound in such peptides, O-phosphorylserine, will be described.

An esterification of serine was mentioned by Langheld in 1911²⁶, but the first synthesis of O-phosphorylserine was reported by Løvene and Schormüller²⁷. These obtained the racemic form in a yield of about 15 % by esteri-

fication of DL-serine with a mixture of phosphoric acid and phosphorus pentoxide. The barium salt of the naturally occurring L-isomer was later isolated after fractional crystallisation of the brucine salts of the racemic mixture²⁸.

Modifications of this phosphoric acid esterification were reported by Plimmer, who obtained somewhat higher yields²⁹. More recently, appreciably higher yields, 40 %, were reported by Canali *et al.*³⁰, who replaced the foregoing phosphorylation mixture with polyphosphoric acid. Unlike Canali *et al.*, Cherbuliez and Rabinowitz³¹, however, found no phosphorylation of serine with polyphosphoric acid of low degree of polymerisation. Cherbuliez and Rabinowitz obtained a yield of 15 % when serine methyl ester was used instead of the free amino acid³¹.

It has been stated several times that the direct esterifications of serine as reported by Levene and Schormüller or by Plimmer give low yields and impure compounds³². The same drawbacks seem to accompany the method of Canali *et al.* by which method we have obtained at most a 10 % yield.

These difficulties have forced us to work out a new way to synthesise O-phosphorylserine. The route we used is very similar to the one we used for synthesising phosphorylated peptides²⁴ and to that used by Baer and Stancer in their synthesis of phosphorylethanolamine³³. N-Carbobenzoxyserine benzyl esters are phosphorylated by diphenylphosphoryl chloride³⁴, and the resulting triesters are cleaved by catalytic hydrogenolysis. By this procedure, L-, D- and DL-forms of O-phosphorylserine are obtained in pure, crystalline state and in good yields.

The pure compounds were characterised by their melting points, optical rotations, elementary compositions, R_f -values in different solvents, X-ray powder diffraction patterns and by their infrared absorption spectra*.

In preliminary experiments, dibenzylphosphoryl chloride³⁶ and di-(*p*-nitrobenzyl) phosphoryl chloride³⁷ were used as phosphorylating agents. These acid chlorides reacted readily with serine hydroxyl groups** but the yields were not satisfactory in some cases. Better results were obtained with the easily available and stable diphenyl derivative³⁴. The phosphorylation step takes place practically quantitatively (*cf.* Riley *et al.*³⁹). The triesters obtained are hydrogenolyzed more slowly than the corresponding benzyl esters, but the yields are good. Two intermediates, O-diphenylphosphorylserine and O-mono-phenylphosphorylserine can easily be isolated during the hydrogenolysis. This indicates the stepwise elimination of blocking benzyl- and phenyl groups shown in the reaction scheme.

EXPERIMENTAL

When not otherwise stated, the compounds were before analysis dried at room temperature *in vacuo* over phosphorus pentoxide. The melting or decomposition points were determined by using sealed capillary tubes and a well stirred oil bath. They are uncorrected.

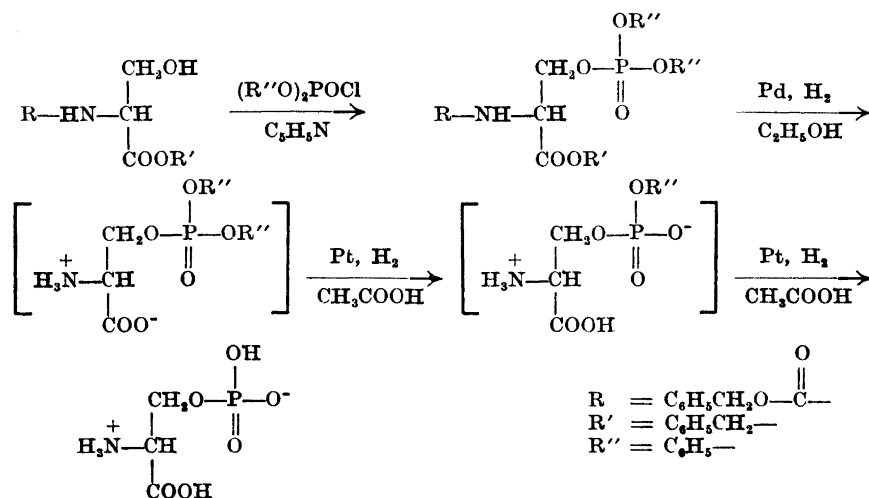
Elementary analyses were made in the Microanalytical Laboratory of the Institute of Medical Chemistry, Uppsala University.

Infrared absorption spectra were recorded with a double beam in time spectrophotometer of the Hornig type built by Dr. M. Skogh at the Institute of Medical Chemistry,

* A study of some metal complexes of the DL-compound has been reported by Österberg³⁵.

** Li and Eakin³⁸ observed no reaction between dibenzylphosphoryl chloride and DL-serine methyl ester.

Reaction scheme



Uppsala University. Sodium chloride prism was used. The compounds (1.4 mg) were pressed into freeze dried potassium bromide (300 mg) pellets.

X-Ray powder diffraction patterns were taken with a Guinier camera using quartz monochromated $\text{CuK}\alpha$ -radiation ($\lambda = 1.5418 \text{ \AA}$). The solvent mixtures used in the paper chromatography were: (I) Phenol-water (80/20 v/v), ascending, about 6 h. (II) *n*-Butanol-acetic acid-water (40/10/50 v/v), descending; 18 h.

N-Carbobenzoxyserine benzyl esters

These compounds were obtained by an azeotrope distilling procedure similar to that of Ciperia and Nicholls⁴⁰. Ten g of N-carbobenzoxyserine⁴¹ and 20 ml of benzyl alcohol were distilled with 100 ml of carbon tetrachloride in the presence of 0.5 g of *p*-toluenesulphonic acid. Carbon tetrachloride was added periodically to keep the volume constant. After a reaction time of 5 h the carbon tetrachloride solution was allowed to cool and was then washed with 50 ml of 20 % sodium bicarbonate solution and three 50 ml portions of water. It was dried over anhydrous sodium sulphate. Light petroleum (b. p. 20–40°) was added to the warmed solution. After some hours at room temperature most of the ester had crystallised and was filtered off. More material was obtained by cooling of the concentrated mother liquor to -16° . The yields varied between 70 and 78 %. The physical constants of the three isomers agreed well with the values reported by Baer and Maurukas⁴¹. Jones and Lipkin⁴² recently prepared the DL- and L-compounds, and Skinner *et al.*⁴³ the D-compound by other procedures.

N-Carbobenzoxy-L-serine benzyl ester, m. p. 83–84°, $[\alpha]_D^{25} + 5.5^\circ$ (chloroform, *c* 4.1, *l* 1); N-Carbobenzoxy-D-serine benzyl ester, m. p. 84–85°, $[\alpha]_D^{25} - 5.6^\circ$ (chloroform, *c* 4.0, *l* 1); N-Carbobenzoxy-DL-serine benzyl ester, m. p. 72–73°.

O-Diphenylphosphoryl-N-carbobenzoxy-L-serine benzyl ester

To a solution of 6.6 g (20 mmole) of N-carbobenzoxy-L-serine benzyl ester in 10 ml of anhydrous pyridine 6.4 g (24 mmole) of diphenylphosphoryl chloride³⁴ was added within 10 min. The reaction mixture was shaken and was held below 40° by occasional cooling in

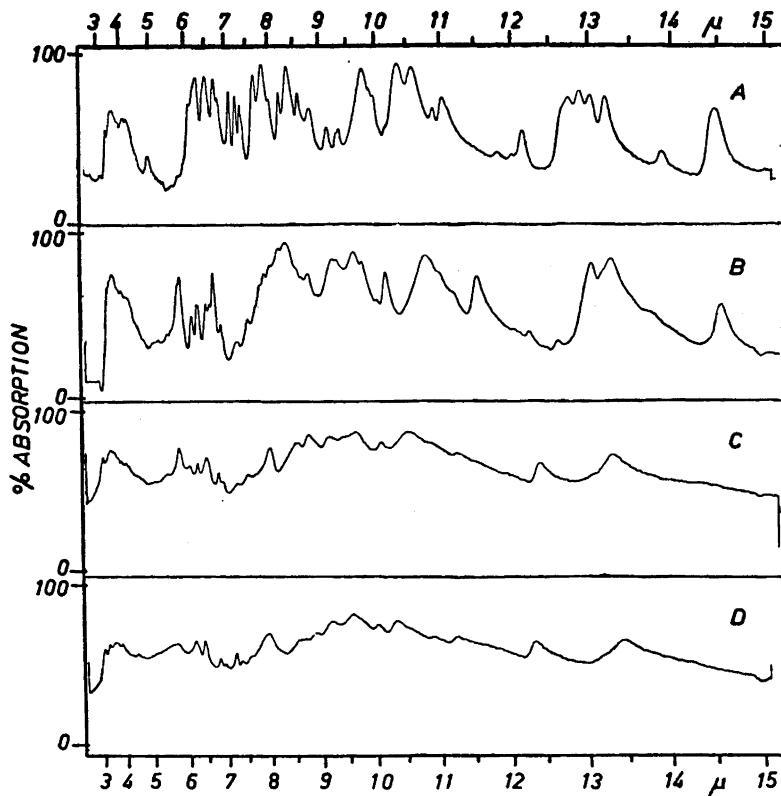


Fig. 1. Infrared absorption spectra of O-diphenylphosphoryl-DL-serine (A), O-mono-phosphoryl-DL-serine (B), O-phosphoryl-DL-serine (C) and O-phosphoryl-L-serine (D). Compounds in potassium bromide pellets.

cold water. A crystalline precipitate of pyridinium chloride was formed. After 4 h at room temperature, the excess of diphenylphosphoryl chloride was decomposed with 1 ml of water. After an additional hour the mixture was poured into 150 ml of ice water and 150 ml of ether. The ether extract was washed successively with 2×50 ml of cold 4 N sulphuric acid, 50 ml of water, 50 ml of 20 % sodium bicarbonate and 50 ml of water.

After drying over anhydrous sodium sulphate the ether was distilled off at reduced pressure, finally at 0.1 mm Hg. The triester remained as a colourless oil, weighing 10.8 g (96 % of theory), n_D^{25} 1.5640. It was dissolved in 30 ml of 99 % ethanol, and light petroleum (b. p. 20–40°) was added to slight turbidity whereupon O-diphenyl phosphoryl-N-carbenzoxy-L-serine benzyl ester slowly crystallised out. After a night at room temperature and a few hours at 4° the crystallisation was complete: 10.5 g (95 % of theory); m. p. 53–53.5°; $[\alpha]_D^{18}$ -3.8° (abs. ethanol, c 10.1, l 1); $[\alpha]_D^{18}$ $+18.0^\circ$ (chloroform, c 5.3, l 1). (Found: C 64.29; H 5.15; N 2.58; P 5.12. Calc. for $C_{30}H_{28}O_8NP$ (561.5): C 64.17; H 5.03; N 2.49; P 5.52).

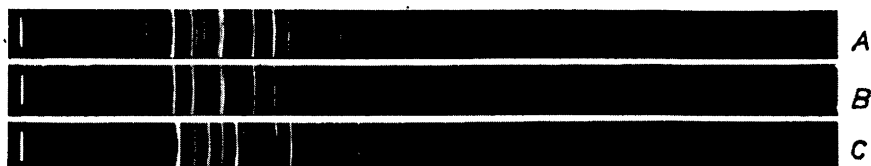


Fig. 2. X-Ray powder diffraction patterns of synthetic (A) and natural (B) O-phosphoryl-L-serine and of O-phosphoryl-DL-serine (C). The strongest reflections are listed in the following table.

Synthetic O-phosphoryl-L-serine		Reported ⁴⁵ for natural O-phosphoryl-L-serine		Synthetic O-phosphoryl-DL-serine	
intensity	Å	intensity	Å	intensity	Å
—	—	v.w.	6.82	v.s.	4.94
—	—	v.w.	6.21	m.	4.50
—	—	v.w.	5.88	m.	4.16
s.	5.10	s.	5.10	m.	3.88
s.	4.53	s.	4.53	s.	3.63
—	—	m.	4.40	m.	3.60
—	—	w.	4.26	m.	3.07
s.	3.92	s.	3.93	m.	2.91
s.	3.88	s.	3.85		
—	—	v.w.	3.56		
s.	3.36	s.	3.36		
s.	3.10	w.	3.10		

v.s. = very strong s. = strong m. = medium w. = weak v.w. = very weak

O-Diphenylphosphoryl-N-carbobenzoxy-D-serine benzyl ester

The D-isomer was prepared in the same way as the foregoing compound from 3.3 g of N-carbobenzoxy-D-serine benzyl ester. The yield was 5.3 g (95 % of theory); m. p. 52–53°; $[\alpha]_D^{25} +3.7^\circ$ (abs. ethanol, *c* 10.2, *l* 1); $[\alpha]_D^{18} -18.3^\circ$ (chloroform, *c* 5.0, *l* 1). (Found: C 64.37; H 5.10; N 2.67; P 5.06. Calc. for $C_{30}H_{28}O_8NP$ (561.5): C 64.17; H 5.03; N 2.49; P 5.52).

O-Diphenylphosphoryl-N-carbobenzoxy-DL-serine benzyl ester

The DL-compound was prepared in the same way as the enantiomers. From 16.5 g of N-carbobenzoxy-DL-serine benzyl ester was obtained 25.8 g (92 % of theory); m. p. 47–48°; Found: C 64.21; H 5.20; N 2.62; P 5.26. Calc. for $C_{30}H_{28}O_8NP$ (561.5): C 64.17; H 5.03; N 2.49; P 5.52).

O-Phosphoryl-L-serine

To a solution of 5.6 g (10 mmole) of O-diphenylphosphoryl-N-carbobenzoxy-L-serine benzyl ester in 50 ml of 99 % ethanol was added 1.5 g of palladium oxide catalyst ⁴⁴, and the mixture was shaken in an atmosphere of pure hydrogen. Eight hours later,

practically no more hydrogen was being consumed and a precipitate, largely consisting of O-diphenylphosphoryl-L-serine, had been formed. This material was dissolved by adding 50 ml of a warm water and acetic acid (1:1) mixture. The catalyst was filtered off and washed with more hot water. The combined solutions were then evaporated to dryness at reduced pressure (bath temperature 40°). The solid residue was redissolved in 50 ml of glacial acetic acid and again shaken in a hydrogen atmosphere, now together with 1 g of platinum oxide catalyst⁴⁴. After about 20 h no more hydrogen was absorbed*. Twenty-five ml of hot water was added to dissolve some precipitated material and the catalyst filtered off. The filtrate and water washings were evaporated to dryness at reduced pressure and the residue dissolved in 100 ml of water. Ten g of cation exchange resin Amberlite IR-120 (H⁺-form) was added and the mixture was shaken for 1.5 h. The resin was filtered off and washed with water. The combined solutions were again evaporated to dryness *in vacuo*. The solid residue was finally dissolved in 8 ml of boiling water, and 99 % ethanol was added to faint turbidity. The solution was left to slowly attain room temperature**, when crystals of O-phosphoryl-L-serine were formed. These were washed with ethanol-water (2:1) and dried *in vacuo* at room temperature over phosphorus pentoxide. The yield was 1.55 g (84 % of theory); m. p.*** 168–172° (decomp.); reported 167° (decomp.)⁹; $[\alpha]_D^{19} + 7.4^\circ$ (water, *c* 3.4, *l* 1); $[\alpha]_D^{23} + 16.2^\circ$ (2 N HCl, *c* 3.0, *l* 1); reported $[\alpha]_D^{23} + 7.2^\circ$ (water, *c* 4.2)⁹; $[\alpha]_D^{25} + 16.3^\circ$ (10 % HCl, *c* 6). (Solution prepared from the *barium* salt)²⁸. (Found: C 19.81; H 4.45; N 7.57; P 16.73. Calc. for C₃H₈O₆NP (185.1): C 19.47; H 4.36; N 7.57; P 16.74). *R_F*-values****: In solvent I 0.08, in II 0.05.

The infrared absorption spectrum (Fig. 1) and the X-ray powder diffraction pattern (Fig. 2) of this compound were both identical with those obtained from O-phosphoryl-L-serine isolated from casein by Ågren and de Verdier⁹ (a sample was kindly given us by Dr. Carl-Henric de Verdier).

O-Phosphoryl-D-serine

This isomer, which has not been reported before, was prepared from 2.80 g of the corresponding triester by hydrogenolysis in exactly the same way as the preceding isomer. The yield was 0.76 g (80 % of theory); m. p. 170–173° (decomp.); $[\alpha]_D^{21} - 7.0^\circ$ (water, *c* 3.0, *l* 1); $[\alpha]_D^{21} - 15.6^\circ$ (2 N HCl, *c* 3.2, *l* 1). (Found: C 19.62; H 4.40; N 7.55; P 16.81. Calc. for C₃H₈O₆NP (185.1): C 19.47; H 4.36; N 7.57; P 16.74).

The infrared absorption spectrum and X-ray powder diffraction pattern were identical with those of the L-derivative.

O-Phosphoryl-DL-serine

The racemic compound was prepared from the corresponding racemic triester. 22.4 g of this gave a yield of 5.8 g (72 % of theory) of the well crystallising and hitherto unreported *monohydrate*; m. p. 153–156° (decomp.) after sintering at 135°. (Found: C 17.62; H 5.00; N 6.79; P 15.16. Calc. for C₃H₈O₆NP, H₂O (203.1): C 17.73; H 4.96; N 6.90; P 15.25).

* The progress of the hydrogenolysis after different times can easily be determined by paper chromatography in solvent II. At the end of the reaction only traces of O-diphenylphosphorylserine (*R_F* 0.59) and O-monophenylphosphorylserine (*R_F* 0.18) should be present beside O-phosphorylserine (*R_F* 0.05).

** To obtain complete crystallisation, it was often necessary to gradually add several volumes of absolute ethanol and to store the solution in the icebox for some days.

*** The melting points of O-phosphorylserines and O-phosphorylated serine peptides vary appreciably with the conditions during the determination. When heated very slowly, they decompose at temperatures below those reported here. Much more characteristic are the infrared spectra and the X-ray diffraction patterns.

**** Practically the same *R_F* values were obtained for the L-, D, and DL-forms.

The water of hydration was not removed by drying *in vacuo* at 100°. When the compound was dried at 120°, considerable decomposition occurred.

The anhydrous compound could be prepared by precipitation with ethanol and ether from the 5% solution of the *monohydrate* in water; m. p. 167–170° (decomp.); reported 165–166° (decomp.)²⁹, 166–167°³². (Found: C 19.45; H 4.52; N 7.52; P 16.50. Calc. for C₉H₉O₆NP (185.1): C 19.47; H 4.36; N 7.57; P 16.74).

The infrared absorption spectrum of the racemic product could be distinguished from the spectra of the optically active isomers, although they were very similar (Fig. 1). The X-ray powder diffraction pattern of the racemic compound was quite different from the pattern of the L- and D-isomers (Fig. 2). However, its infrared spectrum and X-ray diffraction pattern were identical with those obtained from O-phosphoryl-DL-serine prepared according to Plimmer²⁹ and Canali *et al.*³⁰.

O-Diphenylphosphoryl-DL-serine

O-Diphenylphosphoryl-N-carbobenzoxy-DL-serine benzyl ester in ethanol solution was shaken as usual in a hydrogen atmosphere together with palladium oxide catalyst. The precipitate formed during the hydrogenolysis was filtered off and dissolved in a minimum amount of a hot mixture of acetic acid and water (2:1). The solution was filtered to remove the palladium catalyst and was then allowed to cool. After some hours at +4° the crystals of O-diphenylphosphoryl-DL-serine were collected and dried *in vacuo* over potassium hydroxide; m. p. 129–130° (decomp.). (Found: C 53.39; H 4.76; N 4.14; P 9.16. Calc. for C₁₅H₁₆O₆NP (337.3): C 53.41; H 4.78; N 4.15; P 9.19).

Further hydrogenolysis of this compound in glacial acetic acid solution using platinum oxide catalyst, gave O-phosphorylserine, identified by paper chromatography.

Free amino group was indicated by the colour reaction of the compound with ninhydrin. R_F value in solvent I 0.9, in II 0.59. The infrared absorption spectrum is shown in Fig. 1. In contradistinction to the O-phosphorylserines and to O-monophenylphosphorylserine, there was no carbonyl absorption band near 5.75 μ . Instead, the strong band at 6.04 μ indicated the presence of an ionised carboxyl group.

O-Monophenylphosphoryl-DL-serine

This phosphoric diester was isolated in another hydrogenolytic experiment. O-Diphenylphosphoryl-N-carbobenzoxy-DL-serine benzyl ester was hydrogenated as usual using palladium as a catalyst and, after evaporation of the ethanol, the residue was dissolved in glacial acetic acid. The hydrogenolysis was started again, now with platinum oxide catalyst. After an hour, during which the hydrogen was rapidly consumed, the reaction slowed down and was interrupted. The catalyst was removed and the solvent distilled off *in vacuo*. The solid residue was then dissolved in a minimum amount of boiling water, and the filtered solution was left at room temperature for four hours. Crystals of O-monophenylphosphoryl-DL-serine were formed. After one more recrystallization from hot water, the crystals were collected, washed with ice-cold distilled water and dried (the mother liquor and washings contained O-phosphorylserine); m. p. 163–165° (decomp.). (Found: C 41.62; H 4.69; N 5.38; P 12.01. Calc. for C₉H₁₂O₆NP (261.2): C 41.39; H 4.33; N 5.36; P 11.86).

The compound was ninhydrin positive. The R_F value in solvent I was 0.36, in II 0.18. Hydrogenolysis in glacial acetic acid solution and with platinum oxide catalyst gave O-phosphorylserine, identified by paper chromatography. The infrared absorption spectrum is shown in Fig. 1.

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