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Synthesis of Phosphopeptides

V. Further Dipeptides, Tripeptides and O-Phosphorylated Derivatives of L-Serine

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1. Di- and tripeptides, having the sequences* Asp-SerP, Glu(SerP), Ile-SerP, Lys-SerP, SerP-Ala, SerP-His, Asp-SerP-Gly, Asp-SerP-Ala, Asp-SerP-Glu, Glu-SerP-Gly, Glu-SerP-Ala, and Leu-Gly-SerP, as well as the corresponding phosphate-free peptides were synthesized *via* N-benzyloxycarbonyl peptide benzyl esters.

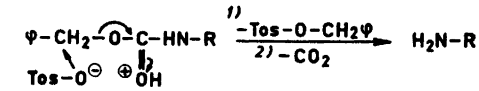
2. It was observed, that protected dipeptide and tripeptide derivatives of β-benzyl-aspartylserine all underwent a rapid cyclization to aspartimides under weakly alkaline conditions, *e.g.* when dissolved in pyridine-water, whereas the corresponding γ-benzyl-glutamylserine derivatives were stable under the same conditions.

3. Some further new derivatives of serine and threonine were prepared and studied.

A method for the synthesis of peptides containing O-phosphorylserine residues has been described and applied in earlier communications of this series.[2–5] The method consists in synthesizing a peptide derivative having the actual amino acid sequence and all its functional groups protected except those hydroxyl groups which are intended to be esterified with phosphoric acid. The phosphorylation is effected by the use of monofunctional derivatives of phosphoric acid such as dibenzylphosphoryl [6] or diphenylphosphoryl [7] chloride. In the final step, the protecting groups are removed and the phosphorylated peptide isolated. The protecting groups were choosen so that they were removed after the phosphorylation step by treatments that do not alter the structure of the peptide or phosphate groupings. N-Benzyloxycarbonyl, N-benzyl, benzyl ester and ether groups fulfill this requirement excellently, since they can all be removed by catalytic hydrogenolysis.

* Abbreviations and nomenclature: According to the recommendations [1] with the addition of SerP = O-phosphoryl-L-serine, *i.e.* $H_2N{\cdot}CH(CH_2{\cdot}O{\cdot}PO_3H_2)CO_2H$. All amino acids used as starting materials in this work were of L configuration (except glycine).

The protection of carboxyl groups as *t*-butyl or *p*-nitro-benzyl esters may be of value in the synthesis of higher and more complex phosphopeptides. Selective "decarbobenzoxylations" of N-benzyloxycarbonylserine peptide benzyl esters by the action of hydrogen bromide in nitromethane [8] or acetic acid have been reported, but the amino end groups of the corresponding *t*-butyl esters (using hydrogenolysis) or *p*-nitrobenzyl esters (using hydrogen bromide) seem to be unmasked more safely.[9,10] In the same way, *t*-butyloxycarbonyl protection of the amino groups permits selective unmasking without affecting benzyl esters groups [11] (hydrogen chloride in ethanol). The dipeptide benzyl esters used in the present work were prepared as their crystalline *p*-toluenesulphonic acid salts, either from the free dipeptides as earlier described, or by the action of *p*-toluenesulphonic acid on a benzene-benzyl alcohol solution of the benzyloxycarbonyl-peptide benzyl ester. The latter procedure seems to be an alternative to the earlier known conditions for the selective removal of N-benzyloxycarbonyl groups. In the light of the work of Homer *et al.*,[12] the reaction may involve decomposition of an ion pair formed from *p*-toluenesulphonic acid and the protected peptide as follows (Tos = $CH_3C_6H_4SO_2-$; $\varphi = C_6H_5-$):



A second equivalent of *p*-toluenesulphonic acid was added for salt formation with the amino group liberated. The benzyl alcohol was added to keep the carboxyl groups esterified.

The phosphopeptides prepared have been studied with regard to their hydrolysis catalyzed by acid,[13–15] proteolytic enzymes,[16,17] and phosphatases.[15,18,19] The acid ionization constants [5,20] and the metal complexing properties [21,22] of several of the compounds have also been investigated. These studies made the synthesis of further model compounds necessary. The results of this synthetic work will be described here.

A number of new protected serine peptides were prepared by reacting a mixture of the appropriate *N-benzyloxycarbonylamino acids* and amino acid or peptide benzyl esters with N,N′-dicyclohexylcarbodiimide. In order to minimise racemization, the solutions of the reactants were routinely cooled to −16°, and then mixed and shaken at +4°. Several control experiments shaken at +20° and −16° gave, however, in each case tested, material with the same optical rotation and in about the same yield. In two cases *N-protected dipeptides* were used as the carboxyl component. In the case of benzyloxycarbonyl-L-leucylglycine, racemization by azlactone formation at the free carboxyl group was impossible since the symmetric amino acid glycine was involved. In the second case benzyloxycarbonyl-α-L-glutamyl-L-serine γ-benzyl ester was reacted with L-alanine benzyl ester. Here at least partial racemization at the serine residue had to be considered.[10] In fact, the optical rotation in acetic acid of the compound obtained ($[\alpha]_D^{26}$ −11.2), differed considerably from that of the pure L-L-L tripeptide derivative ($[\alpha]_D^{26}$ −16.4). The latter was obtained by condensing benzyloxycarbonyl-L-glutamic acid γ-benzyl ester with L-seryl-L-alanine benzyl ester.

The protected α-aspartylseryl and α-glutamylseryl tripeptides prepared were of more general interest, since they may be regarded as simple models for a part of the active sites of esterases [23] and of alkaline phosphatase.[24] Aspartyl derivatives are known to undergo easily acid- as well as base-catalyzed α,β-

transpeptidation reactions *via* the cyclic aspartimides, [25–27] and the protection of the β-carboxyl group as benzyl (or *t*-butyl) ester is used frequently in the synthesis of pure α-peptides.[10,27] From the work of Bernhard *et al.*[28] and of Hanson and Rydon,[29] one could expect a rapid intramolecularly catalyzed hydrolysis of the β-benzyl ester groups in our aspartyl serines, because of the presence of the unusually nucleophilic [30] serine hydroxyl group, and this was actually found. When N-benzyloxycarbonyl-α-L-aspartyl-L-serylglycine dibenzyl ester, $[\alpha]_D^{26}$ −8.0, (or the corresponding compounds having L-alanine or L-glutamic acid γ-benzyl ester in the place of glycine) was dissolved in pyridine-water (70:30) at room temperature, a rapid increase in optical rotation was observed (*cf.* Fig. 1). The rotation maximum was reached within a

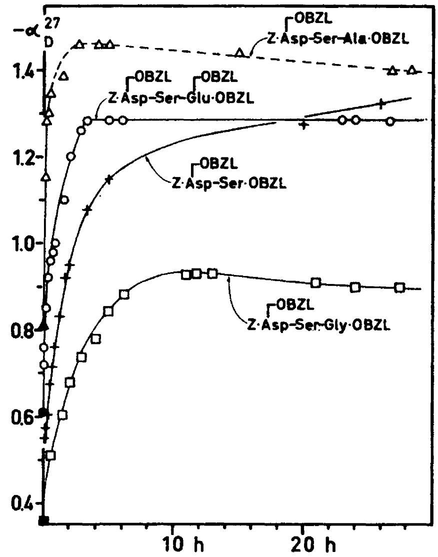


Fig. 1. Changes in optical rotation observed, when β-benzyl N-benzyloxycarbonyl-α-L-aspartyl-L-seryl tripeptide benzyl esters (2 %) were dissolved (open symbols) in pyridine-water (70:30) at 27°; $l = 2$ dm. The rotation observed in anhydrous pyridine (filled symbols) are used in the figure as zero time values; no change in rotation was observed under anhydrous conditions. Some rotation data (+), obtained as above in aqueous pyridine with a dipeptide derivative, are included in the figure. This compound had zero rotation in anhydrous pyridine. The compounds studied were:

■, □ $C_7H_7OCO{\cdot}NH{\cdot}CH(CH_2CO_2C_7H_7)CO{\cdot}NH{\cdot}CH(CH_2OH)CO{\cdot}NH{\cdot}CH_2{\cdot}CO_2C_7H_7$
▲, △ $C_7H_7OCO{\cdot}NH{\cdot}CH(CH_2CO_2C_7H_7)CO{\cdot}NH{\cdot}CH(CH_2OH)CO{\cdot}NH{\cdot}CH(CH_3)CO_2C_7H_7$
●, ○ $C_7H_7OCO{\cdot}NH{\cdot}CH(CH_2CO_2C_7H_7)CO{\cdot}NH{\cdot}CH(CH_2OH)CO{\cdot}NH{\cdot}CH{\cdot}CO_2C_7H_7$
└$CH_2CH_2CO_2C_7H_7$
+ $C_7H_7OCO{\cdot}NH{\cdot}CH(CH_2CO_2C_7H_7)CO{\cdot}NH{\cdot}CH(CH_2OH)CO_2C_7H_7$

few hours and was about twice that of the starting material. This indicates hydrolysis of the β-benzyl ester group and ring closure forming aspartimides, known to have comparatively high optical rotation values.[26,28] Further evidence for this mechanism (Fig. 2) was obtained by the isolation of some of

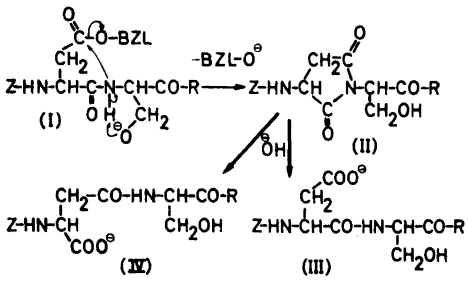


Fig. 2. A mechanism for the alkaline hydrolysis of various β-benzyl N-benzyloxycarbonyl-α-L-aspartyl-L-serine derivatives (I), indicating intramolecular nucleophilic catalysis caused by the serine β-hydroxyl group (shown ionized in (I)). A mixture of α-aspartyl (III) and β-aspartyl (IV) peptides are formed, presumably *via* the cyclic aspartimide (α,β-aspartyl peptide; II). Z = $C_6H_5CH_2OCO-$; BZL = $C_6H_5CH_2-$; R = $-O\cdot BZL$; $-NH\cdot CH_2CO_2BZL$; $-NH\cdot CH(CH_3)CO_2BZL$ and $-NH\cdot CH(CH_2CH_2CO_2BZL)\ CO_2BZL$.

the aspartimides, the structure of which was confirmed by their I. R. spectra and hydrolysis products (*cf.* Experimental).

In contrast to their behaviour in aqueous pyridine, the above aspartylserines did not change their optical rotation when dissolved in *anhydrous* pyridine and they were recovered unchanged.* The corresponding tripeptides having glutamic acid in place of aspartic acid (as in the active sites of horse liver aliesterease and of cholinesterases [23]) were resistant also in *aqueous* pyridine, as far as changes in optical rotation were concerned. Fosker *et al.*[32] found a similar stability for N-benzyloxycarbonyl-γ-benzyl-L-glutamyl-L-serine amide. There exists, therefore, a clear difference in reactivity between models having the sequences of chymotrypsin, trypsin, thrombine, elastase (Asp-Ser-Gly), and alkaline phosphatase (Asp-Ser-Ala), and those having that of cholin- and ali-esterases (Glu-Ser-Ala).

The protected serine peptides were phosphorylated in anhydrous pyridine solution using dibenzylphosphoryl chloride as described previously.[3] The phosphate triesters formed were purified by washing with aqueous acid and base, and then hydrogenolyzed in *t*-butanol solution. The phosphopeptides formed were isolated and purified by using the anion exchange chromatographic procedure of Strid [15,33] instead of simple crystallization. The potentialities of the procedure are apparent from the following facts:

(1) pyrophosphate and triphosphate peptides formed under certain conditions are separated from their monophosphate analogues;[15] (2) diastereomeric phosphopeptides have been separated;[14] (3) the procedure has been used in micro scale for the preparation of ^{14}C-marked phosphopeptides (*cf.* Experimental); (4) the aspartimido analogue of Asp-SerP-Ala, having one free carboxyl group only, was well separated from the latter (*cf.* Fig. 3).

* The carbodiimide condensation of N-benzyloxycarbonyl-aspartic acid β-benzyl ester with serylpeptide benzyl esters gave material of high optical rotation and unsharp melting point, when triethylamine was present in slight excess, or when the reaction product was washed extensively with aqueous bicarbonate. The two aspartylserine derivatives reported by Theodoropoulos and Souchleris had these properties (*cf.* Experimental), and they gave more than one product upon hydrogenolysis in ethanol.[31] It seems that their protected derivatives were more or less cyclized to aspartimides, the five-rings of which may open in ethanol forming a mixture of α- and β-ethyl esters.

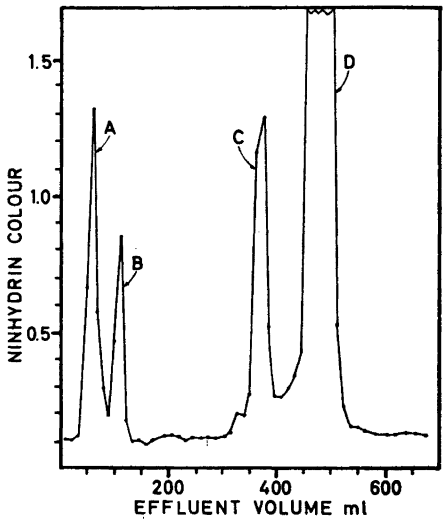


Fig. 3. Separation of the succinimide derivative, α,β-L-aspartyl-(O-phosphoryl)-L-seryl-L-alanine (fraction C, $[\alpha]_D^{26}$ −40.3° in 1 M HCl) from α-L-aspartyl-(O-phosphoryl)-L-seryl-L-alanine (fraction D, $[\alpha]_D^{26}$ −17.3°), using anion exchange chromatography. Fractions A and B consist of phosphate-free materials.

Chromatographic isolation of the O-phosphorylated peptides obtained by hydrogenolysis of the intermediate phosphate triesters makes the crystallization or extensive purification of the latter unnecessary. We have observed, however, that our triesters separate well from benzyloxycarbonylpeptide benzyl esters and from the phosphorylating reagents on thin layer plates (Kieselgel G; *cf.* Experimental), indicating that purification may be achieved by chromatography. The use of di-*p*-nitrobenzylphosphoryl chloride, a phosphorylating reagent introduced and used with excellent results by Zervas and collaborators,[34] does not seem to be of great advantage in the synthesis of O-phosphoryl-serine derivatives, since Theodoropoulos and Souchleris had to use counter-current distribution technique to purify their triesters.[31] Phosphorylation of serine derivatives with *diphenyl*phosphoryl chloride [3,4,20,35] may be of advantage in cases where the selective removal of N-benzyloxycarbonyl and benzyl esters groups by palladium-catalyzed hydrogenolysis is desired; the removal of one or both of the phenyl groups requiring platinum catalyst.[3]

Some reactions of free O-phosphorylserine and -threonine have been studied with the aim of finding derivatives, useful for synthetic or analytical purposes. The carboxyl group, but not the phosphate group, is esterified in methanol and thionyl chloride, as the mono-methyl ester formed from O-phosphoryl-DL-serine is identical to the one we obtained [20] by catalytic hydrogenolysis of N-benzyloxycarbonyl-(O-diphenylphosphoryl)-DL-serine methyl ester. The aforementioned procedure was used for the preparation of the methyl esters of O-phosphoryl-L- and -DL-serine and of O-phosphorylated threonine. The latter compound was prepared from L-threonine in 48 % yield by the action of chlorophosphoric acid as described for serine.[36] The procedure [37] for preparation of benzyl esters failed to give a pure and crystalline compound in the case of O-phosphoryl-DL-serine. Also, N-acylation with benzyloxycarbonyl chloride of this compound in bicarbonate solution failed, whereas N-dinitrophenylation of O-phosphorylserine as well as its peptides proceeds without difficulties.

The stereochemical purity of the compounds prepared was tested by two methods. Leucine aminopeptidase hydrolyzed completely all the phosphate-

free peptides and many of the phosphorylated peptides studied forming the expected component amino acids. Therefore the synthetic methods used retained the L configuration of the starting materials. No hydrolysis was observed in the case of the two γ-glutamyl peptides; γ-peptides are regarded to be resistant to the enzyme.[38] Several of the peptides containing O-phosphorylserine or O-pyrophosphorylserine were attacked slowly or incompletely by leucine aminopeptidase. The resistance of these phosphorylated peptides was due to their hydrophilic character and negative charge rather than to an inversion of configuration, since those of them having a hydrophobic part in their molecule, *e.g.* as an amino acid side chain (leucine, isoleucine) or as a second phosphate substituent (phenyl ester) were hydrolyzed.[17]

In this connection it may be mentioned, that the bond between tyrosine and glycine in Gastrin I is hydrolyzed by chymotrypsin, whereas the same bond in Gastrin II, having the phenolic hydroxyl group esterified with sulphuric acid, is resistant to this enzyme. Subtilisin, however, hydrolyses the bond also in Gastrin II.[39]

A second method was used to verify the L configuration of the phosphorylated peptides containing aspartic or glutamic acid. They were hydrolyzed in 6 M HCl, and the optical rotation of the hydrolysate was in each case the same as that of the component L-amino acids treated in the same way. The L stereochemistry of the protected peptide derivatives follows from that they gave peptides of L configuration by catalytic hydrogenolysis.

The phosphate groups of all the O-phosphorylated peptides prepared were completely removed by alkaline phosphatases, forming the parent un-phosphorylated serine peptides.[19] These results confirm the structure of the peptides, but are irrelevant with respect to stereochemistry, since also O-phosphoryl-D-serine and its peptides seem to be hydrolyzed [18] by such un-specific phosphatases.

EXPERIMENTAL

Nitrogen analyses were made by a micro-Kjeldahl method except in the case of histidine compounds; the latter were analyzed at Mikroanalyslaboratoriet, Med. Kem.-Inst., Uppsala University, by a Dumas method. Phosphorus was determined according to Dryer *et al.*[40] after combustion with 70 % $HClO_4$ for 2 h at 150°.

Before analysis, the compounds were dried for 24 h *in vacuo* over P_2O_5 or KOH at room temperature. The melting points were determined using a Kofler bench. Solvents frequently used in the paper chromatography (Whatman No. 1; *descending*) were:

(A): 1-butanol-acetic acid-water, 4:1:1 v/v, (R_{FA});
(B): 1-butanol-methanol-formic acid-water, 2:1:1:1 v/v (R_{FB}); The chromatograms were sprayed with ninhydrin and with phosphate reagents. Solvents used for thin layer (Kieselgel G; Merck) chromatography included:
(C): light petroleum-tetrahydrofuran-acetic acid, 60:40:1 v/v, (R_{FC}). The spots were revealed by using the chlorine-starch-iodide procedure of Rydon and Smith [41] or with the phosphate reagent. All peptides and derivatives (2—5 mg) were analyzed by total hydrolysis in 6 M HCl (100 µl) for 18 h at 110°. The amino acids and phosphate present in the hydrolysate were identified paper chromatographically. Hydrolyses catalyzed by leucine aminopeptidase [17] and phosphatases [15,19] are described separately.

Dipeptide derivatives.

Benzyloxycarbonyl-α-L-aspartyl-L-serine dibenzyl ester. Benzyloxycarbonyl-L-aspartic acid β-benzyl ester [42] (3.6 g; 10 mmoles), L-serine benzyl ester benzenesulphonate [3] (3.6 g, 10 mmoles), and triethylamine (1.4 ml) were reacted [43] with N,N'-dicyclohexylcarbodiimide* (2.1 g, 10 mmoles) in tetrahydrofuran-acetonitrile 1:1 (100 ml) by shaking 4 h at +4° and then 18 h at room temperature. The precipitate of dicyclohexylurea (2.1 g; 100 %) was discarded, the solution concentrated *in vacuo* at 40°, and added to ice-water (1 l) with stirring. The precipitate obtained was crystallized twice from ethyl acetate-diethyl ether; yield 4.4 g (82 %), m.p. 129°; $[\alpha]_D^{26}$ +6.9 (acetic acid, *c* 5.8); $[\alpha]_D^{26}$ ±0 (pyridine, *c* 3.0). (Found: N 5.3. Calc. for $C_{29}H_{30}N_2O_8$ (534.6): N 5.2). The optical rotation of this compound (300 mg) increased steadily (Fig. 1) when dissolved in pyridine (10.5 ml) and water (4.5 ml). After 5 days at 26°, the solution was evaporated to dryness *in vacuo*, the residue triturated with diethyl ether, and the white solid (120 mg, m.p. 118—122°) hydrogenolyzed in *t*-butanol-water solution. Paper chromatographic analysis of the product formed showed the presence of three ninhydrin-positive compounds. One of the spots gave the normal, purple colour and had the R_{FB} = 0.50 of α-L-aspartyl-L-serine (*cf.* Table 2). A brownish spot of about the same intensity (R_{FB} 0.45) was possibly β-aspartylserine. The third spot, (R_{FB} 0.59), apparently representing the main product, had a bright yellow colour. The observations of Young and collaborators on the ninhydrin colour reactions of aspartyl peptides (Ref. 42 and earlier papers) indicate that the yellow spot corresponds to the aspartimide, α, β-aspartylserine. The I.R. spectrum of the *mixture* differed from that of α-aspartylserine mainly in the carbonyl region. The absorption band was now split into well-separated peaks at 5.80 and 6.1 μ, as is the case of succinimide.[44]

Benzyloxycarbonyl-γ-L-glutamyl-L-serine dibenzyl ester. Prepared as above from benzyloxycarbonyl-L-glutamic acid α-benzyl ester [45] (3.7 g, 10 mmoles), L-serine benzyl ester benzenesulphonate (3.6 g), triethyl amine (1.4 ml) and "DCCI" (2.1 g) in tetrahydrofuran-acetonitrile (1:1, 50 ml).

The precipitate of dicyclohexyl urea was removed, the solution evaporated to dryness *in vacuo* and the residue dissolved in methylene chloride. The solution was filtered and washed successively with 1 M HCl, water, saturated $NaHCO_3$ solution, and water, and then dried (Na_2SO_4) and evaporated to dryness. The residue was crystallized from ethyl acetate-diethyl ether, yield 4.1 g (75 %), m.p. 118°, $[\alpha]_D^{27}$ +1.2 (acetic acid, *c* 4.0). (Found: N 5.1. Calc. for $C_{30}H_{32}N_2O_8$ (548.6): N 5.1).

Benzyloxycarbonyl-L-isoleucyl-L-serine benzyl ester. From benzyloxycarbonyl-L-isoleucine (8.0 g, 30 mmoles; oil, prepared as the leucine derivative [46]) as above; yield 9.8 g (74 %), m.p. 175° (crystallized from ethyl acetate); $[\alpha]_D^{25}$ −17.9° (acetic acid, *c* 6.1). (Found: N 6.3. Calc. for $C_{24}H_{30}N_2O_6$ (442.5): N 6.3).

N^{α}, N^{ε}-Dibenzyloxycarbonyl-L-lysyl-L-serine benzyl ester. From di-benzyloxycarbonyl-L-lysine [47] (8.3 g, 20 mmoles) as above; yield 9.4 g (79 %). The compound melted at 144° when crystallized from ethyl acetate-light petroleum. When crystallized from ethyl acetate-diethyl ether, the compound frequently melted at 122°, solidified and melted again at 144°. The optical rotations of the two forms were identical, $[\alpha]_D^{26}$ −4.8° (acetic acid, *c* 6.1), indicating dimorphism. (Found: N 7.2. Calc. for $C_{32}H_{37}N_3O_8$ (591.7): N 7.1).

N-Benzyloxycarbonylglycyl-L-serine benzyl ester. From benzyloxycarbonyl-glycine[46] as above; crystals from ethyl acetate m.p. 149°; $[\alpha]_D^{26}$ +4.7° (acetic acid, *c* 6.1). (Found: N 7.3. Calc for $C_{20}H_{22}N_2O_6$ (386.2): N 7.3).

N-Benzyloxycarbonyl-L-seryl-L-alanine benzyl ester. From N-benzyloxycarbonyl-L-serine [48,49] (4.8 g, 20 mmoles) and L-alanine benzyl ester *p*-toluenesulphonate [50] (7.0 g, 20 mmoles). Crystallized from ethyl acetate-ether, yield 12.7 g (80%), m.p. 117° (reported [51] m.p. 114°): $[\alpha]_D^{26}$ −22.1° (acetic acid, *c* 5.9). (Found: N 7.0. Calc for $C_{21}H_{24}N_2O_6$ (400.4): N 7.0.)

Benzyloxycarbonyl-α-L-glutamyl-L-serine γ-benzyl ester. The mixed anhydride, prepared at −5° from benzyloxycarbonyl-L-glutamic acid γ-benzyl ester [52] (3.7 g, 10 mmoles), triethylamine (1.4 ml) and ethyl chloroformate (0.95 ml, 10 mmoles) in dry dioxane-tetrahydrofurane (1:1; 40 ml) was left for 15 min at −5°. A cold (−5°) solution of L-serine (2.1 g, 20 mmoles) in 4 M NaOH (5.0 ml) was added, and the mixture shaken at room temperature for 30 min. The organic solvents were removed *in vacuo*, the water solution

* Hereafter abbreviated as "DCCI".

acidified with 3 M HCl and then extracted with ether. The ether solution was washed with water, dried (Na_2SO_4), and evaporated to dryness. The residue was crystallized from ethyl acetate-light petroleum; yield 2.4 g (53 %), m.p. 62–64°. (Found: N 5.9. Calc. for $C_{23}H_{26}N_2O_8$ (458.5): N 6.1).

To 3.8 g (8.3 mmoles) of the compound in 50 ml of acetone was added 1.8 g (10 mmoles) of dicyclohexylamine in 100 ml of ether. The dicyclohexylammonium salt of the γ-benzyl benzyloxycarbonyl peptide, 4.7 g (88 %), crystallized; m.p. 180°. (Found: N 6.6. Calc. for $C_{35}H_{49}N_3O_8$ (638.5): N 6.6).

Dipeptide benzyl esters. The esterification procedure for amino acids, described by Zervas *et al.*[37] was used. Free dipeptides (10 mmoles), benzyl alcohol (10 ml), *p*-toluenesulphonic acid monohydrate (11 mmoles) and benzene (30 ml) were refluxed for 1.5–2 h in a Dean and Stark apparatus (azeotropic removal of the water formed). Benzene (30 ml) was added, the crystalline peptide ester salts collected and recrystallized from *i*-propanol-diethyl ether. The *p*-toluenesulphonate salts of the following peptide esters (yields 85–90 %) were obtained:

L-*Serylglycine benzyl ester*, m.p. 176°; R_{FA} 0.55; $[\alpha]_D^{26}$ +8.2 (acetic acid; *c* 1.7) reported [16] m.p. 180° (capillary tube). (Found: N 6.6 Calc. for $C_{19}H_{24}N_2O_7S$ (424.5): N 6.6).

L-*Seryl*-L-*alanine benzyl ester*, m.p. 130°; R_{FA} 0.69; $[\alpha]_D^{26}$ −14.3 (acetic acid, *c* 5.5). (Found N 6.4. Calc. for $C_{20}H_{26}N_2O_7S$ (438.5): N 6.4).

L-*Seryl*-L-*glutamic acid dibenzyl ester*, m.p. 72°; R_{FA} 0.86; $[\alpha]_D^{26}$ −4.0 (acetic acid, *c* 5.7); reported as an oil.[31] (Found: N 4.8. Calc. for $C_{30}H_{34}N_2O_9S$ (586.7): N 4.8).

L-Serylglycine benzyl ester *p*-toluenesulphonate salt was also obtained directly from N-benzyloxycarbonyl-L-serylglycine benzyl ester [48] (3.9 g, 10 mmoles) by refluxing for 2 h with *p*-toluenesulphonic acid (3.8 g, 20 mmoles) in benzene (35 ml) containing benzyl alcohol (5 ml), as above; yield 2.6 g (61 %) m.p. 176°; R_{FA} 0.55.

Tripeptide derivatives

Benzyloxycarbonyl-α-L-*aspartyl*-L-*serylglycine dibenzyl ester.* To the solution of L-serylglycine benzyl ester *p*-toluenesulphonate (4.2 g, 10 mmoles) in 30 ml acetonitrile, 30 ml tetrahydrofuran and 1.4 ml triethylamine was added benzyloxycarbonyl-L-aspartic acid β-benzyl ester (3.6 g). The solution was cooled to −15°, "DCCI" (2.1 g) was added and the mixture shaken for 10 h at +4° and then for 4 h at room temperature. The product was worked up as described above for the aspartylserine derivative. The protected tripeptide was crystallized from ethyl acetate, yield 4.3 g (74 %), m.p. 120° (sharply); $[\alpha]_D^{26}$ −8.9 (dried pyridine, *c* 1.8) $[\alpha]_D^{26}$ −8.0 (acetic acid, *c* 6.0); reported [31] m.p. 112–116°; $[\alpha]_D^{25}$ −24.5 ± 1° (acetic acid, *c* 7). (Found N 7.1. Calc. for $C_{31}H_{33}N_3O_9$ (591.6): N 7.1).

Material having unsharp m.p. (105–115°) and higher optical rotation ($[\alpha]_D^{26}$ ranging between −12.9° and −19.4°) was obtained in cases, where a slight excess (10 %) of triethylamine was used in the condensation step, or when the reaction product was washed extensively with aqueous bicarbonate solution.

The optical rotation of the compound (2 %) in pyridine-water (70:30) increased in 10 h to a value of $[\alpha]_D^{27}$ −23.3° (± 1°, *i.e.* the rotation in acetic acid, reported [31] for a compound isolated after bicarbonate washings) and decreased thereafter slowly (Fig. 1).

Benzyloxycarbonyl-α-L-*aspartyl*-L-*seryl*-L-*alanine dibenzyl ester.* From benzyloxycarbonyl-L-aspartic acid β-benzyl ester (1.4 g, 3.9 mmoles), L-seryl-L-alanine benzyl ester *p*-toluenesulphonate (1.7 g, 3.9 mmoles), triethylamine (0.66 ml) and "DCCI" (0.85 g) in acetonitrile-tetrahydrofuran (50 ml) as above, yielding 1.9 g (80 %), m.p. 131°, $[\alpha]_D^{26}$ −17.1° (acetic acid, *c* 6.0), $[\alpha]_D^{27}$ −20.2 (pyridin, *c* 2.0). (Found: N 7.0. Calc. for $C_{32}H_{35}N_3O_9$ (605.7): N 6.9).

The optical rotation of this compound reached a value of $[\alpha]_D^{27}$ −36.5 within 3 h at 27° in pyridine-water 70:30 (Fig. 1).

A sample of the protected tripeptide, prepared by the isolation procedure involving washing with bicarbonate solution, had m.p. 116–118° and $[\alpha]_D^{26,5}$ −21.2 (acetic acid; *c* 5.9), indicating some aspartimide formation. Strong evidence for this was obtained as follows: phosphorylation of the material (1.8 g, 3 mmoles), followed by hydrogenolysis (*cf.* below) gave a white solid product (1.05 g), that was resolved by anion exchange

Table 1. Peptides of O-phosphoryl-L-serine.

Peptide	Paper chromatography [a] R_{FA}	R_{FB}	Optical rotation in 1 M HCl $[\alpha]_D$	t	c	Formula	M.W.	Analysis Found N	P	Calc. N	P
SerP-Ala[b]	0.09	0.48	−16.5	26	4.2	$C_6H_{13}N_2O_7P\cdot H_2O$	274.2	10.1	11.0	10.2	11.3
SerP-His	0.0	0.27	+ 9.4	27	1.9	$C_9H_{15}N_4O_7P\cdot H_2O$	340.2	16.6	9.1	16.4	9.1
Asp-SerP	0.03	0.32	+21.6	27	4.2	$C_7H_{13}N_2O_9P\cdot 2H_2O$	336.2	8.3	9.2	8.3	9.2
Glu(SerP)	0.03	0.29	+24.7	27	4.0	$C_8H_{15}N_2O_9P\cdot 3H_2O$	368.2	7.7	7.9	7.6	8.4
Ile-SerP[b,c]	0.18	0.62	+30.5	27	4.0	$C_9H_{19}N_2O_7P\cdot 2H_2O$	334.2	8.6	9.2	8.4	9.2
Lys-SerP	0.03	0.30	+25.2	25	4.0	$C_9H_{20}N_3O_7P\cdot 2H_2O$	349.3	12.2	8.2	12.0	8.9
Asp-SerP-Gly	0.02	0.30	− 4.3	26	3.9	$C_9H_{16}N_3O_{10}P\cdot 2H_2O$	393.2	10.6	7.8	10.6	7.9
Asp-SerP-Ala	0.03	0.38	−17.3	26	4.0	$C_{10}H_{18}N_3O_{10}P\cdot 2H_2O$	407.3	10.5	7.5	10.3	7.6
Asp-SerP-Glu	0.02	0.33	−11.2	26	4.2	$C_{12}H_{20}N_3O_{12}P\cdot 4H_2O$	501.3	8.3	6.1	8.3	6.2
Glu-SerP-Gly	0.02	0.35	+11.5	27	4.0	$C_{10}H_{18}N_3O_{10}P\cdot 3H_2O$	425.3	10.0	7.0	9.9	7.3
Glu-SerP-Ala	0.04	0.40	− 4.5	25	4.3	$C_{11}H_{20}N_3O_{10}P\cdot 3H_2O$	439.3	9.4	6.9	9.5	7.1
Leu-Gly-SerP[b]	0.24	0.62	+40.6	27	4.1	$C_{11}H_{22}N_3O_8P\cdot 2H_2O$	391.3	10.8	7.9	10.7	7.9

[a] Whatman No 1, descending; solvent system A = Butanol-acetic acid-water (4:1:1; volumes); B = Butanol-methanol-formic acid-water (2:1:1:1; volumes).

[b] Crystallized from water-ethanol. All the other phosphopeptides were purified chromatographically and freeze-dried.

[c] Ninhydrin colour value only 9 % of that of leucine (determined according to Moore, S. and Stein, W. H. *J. Biol. Chem.* **211** (1954) 907).

chromatography into *two* peptides with the composition of Asp-SerP-Ala·$(H_2O)_n$. The main product (Fig. 3, fraction D, 0.53 g) appeared at a place in the chromatogram normal (effluent volume 400—500 ml with the column used) for α-aspartyl-(O-phosphoryl)-serines, and gave the normal purple ninhydrin reaction on paper chromatograms (R_{FB} 0.38). The optical rotation was $[\alpha]_D^{26}$ −17.3 (1 M HCl, *c* 4.0), and the analyses were correct for Asp-SerP-Ala. 2 H_2O (Table 1). The minor component (Fig. 3; fraction C, 166 mg) appeared earlier in the chromatogram, indicating that it is "less acidic" than the α-aspartyl peptide, as could be expected from an aspartimide analogue (missing the β-carboxyl group). After isolation, it gave one single bright yellow spot (R_{FB} 0.44) with ninhydrin; after a year at room temperature, one or two new components appeared (R_{FB} 0.38—0.40, violet colour), indicating appreciable decomposition. The carbonyl I. R. absorption band had two well-separated peaks at 5.77 μ (v.s.) and 6.0 μ (s); indicating succinimide ring[44] (the carbonyl absorption band in fraction D was broad and had the peak at 6.05 μ). The high optical rotation of the compound, $[\alpha]_D^{26}$ −40.3 (1 M HCl, *c* 3.8) was a further indication for the compound to be N-L-aspartimido-(O-phosphoryl)-L-seryl-L-alanine *monohydrate*. (Found: N 11.2; P 8.2. Calc. for $C_{10}H_{16}N_3O_9P \cdot H_2O$ (371.3): N 11.3; P 8.3). The decomposition was possibly caused by the water of hydration in opening the succinimide ring and forming a mixture of α- and β-aspartyl-(O-phosphoryl)-serylalanine.

Alkaline phosphatase from swine kidney liberated one equivalent of inorganic phosphate from fraction C as well as from D, and with about the same rate.

Benzyloxycarbonyl-α-L-aspartyl-L-seryl-L-glutamic acid tribenzyl ester. From benzyloxycarbonyl-L-aspartic acid β-benzyl ester (1.8 g), L-seryl-L-glutamic acid dibenzyl ester *p*-toluenesulphonate (2.94 g; 5 mmoles), triethylamine (0.7 ml) and "DCCI" (1.1 g) as above. Crystals from ethyl acetate-light petroleum, yield 3.0 g (80 %), m.p. 80—82°; $[\alpha]_D^{27}$ −15.2 (dry pyridine, *c* 1.8); $[\alpha]_D^{26}$ −15.2 (acetic acid, *c* 5.9); (reported m.p. 64—70°; $[\alpha]_D^{25}$ −45.4° (acetic acid, *c* 1.1). (Found: N 5.6. Calc. for $C_{41}H_{43}N_3O_{11}$ (753.8): N 5.6).

The optical rotation of this compound reached a value of $[\alpha]_D^{27}$ −32.0 within 3.5 h (Fig. 1) in pyridine-water (70:30).

Benzyloxycarbonyl-α-L-glutamyl-L-serylglycine dibenzyl ester. From benzyloxycarbonyl-L-glutamic acid γ-benzyl ester dicyclohexylamine salt [53] (2.8 g), L-serylglycine benzyl ester *p*-toluenesulphonate (2.1 g) and "DCCI" (1.1 g) in tetrahydrofuran-acetonitrile (80 ml) as above. Crystallized from ethyl acetate, 2.0 g (66 %), m.p. 121°; $[\alpha]_D^{26}$ −7.7° (acetic acid, *c* 3.3). (Found: N 6.9. Calc for $C_{32}H_{35}N_3O_9$ (605.7): N 6.9).

Benzyloxycarbonyl-α-L-glutamyl-L-seryl-L-alanine dibenzyl ester. (a): Benzyloxycarbonyl-L-glutamic acid γ-benzylester dicyclohexylamine salt (2.76 g, 5 mmoles), L-seryl-L-alanine benzyl ester *p*-toluenesulphonate (2.2 g, 5 mmoles) and "DCCI" (1.1 g) were reacted as above, yielding 2.4 g (78 %) of the protected tripeptide. Crystallized from ethyl acetate-diethyl ether, m.p. 104°; $[\alpha]_D^{26}$ −16.4° (acetic acid, *c* 6.1). (Found: N 6.8. Calc. for $C_{33}H_{37}N_3O_9$ (619.7): N 6.8).

(b): Benzyloxycarbonyl-α-L-glutamyl-L-serine γ-benzyl ester dicyclohexylamine salt (3.2 g, 5 mmoles), L-alanine benzyl ester *p*-toluenesulphonate salt (1.8 g) and "DCCI" (1.1 g) were shaken (4 h at +4° and 18 h at room temperature) in 50 ml tetrahydrofuran-acetonitrile (1:1). The precipitate (1.0 g) of urea derivative was removed and the solution evaporated to dryness. Ethyl acetate (150 ml) was added, and undissolved dicyclohexylamine *p*-toluenesulphonate salt (1.55 g, m.p. 183°) was filtred off. The solution was washed successively with water, 2 M HCl, water, $NaHCO_3$ solution and water; dried over Na_2SO_4 and evaporated to dryness. The product was crystallized from ethyl acetate-ether; yield 2.9 g (93 %), m.p. 96—98°; $[\alpha]_D^{26.5}$ −11.2 (acetic acid, *c* 6.0).

Benzyloxycarbonyl-L-leucylglycyl-L-serine benzyl ester. From benzyloxycarbonyl-L-leucylglycine [54] (6.4 g, 20 mmoles), L-serine benzyl ester benzenesulphonate (7.0 g), triethylamine (2.8 ml) and "DCCI" (4.2 g) in tetrahydrofuran-acetonitrile (1:1, 100 ml). Crystallized from ethyl acetate-ether, yield 8.0 g (80 %); m.p. 128°; $[\alpha]_D^{26}$ −2.3 (acetic acid, *c* 6.5). (Found: N 8.4. Calc. for $C_{26}H_{33}N_3O_7$ (499.6): N 8.4).

Free peptides

The protected peptides (5 mmoles) were hydrogenolyzed in *t*-butanol-water solution using 10 % palladium on charcoal (0.5 g) as the catalyst. One equivalent HCl was added in the case of the histidine and two in the case of the lysine peptides.

Table 2. Peptides of L-serine.

Peptide	Paper chromatography[a] R_{FA}	R_{FB}	Optical Rotation in 1 M HCl $[\alpha]_D$	t	c	Formula	M.W.	Analysis Found N	Calc. N
Ser-Gly[b]	0.12	0.60	+30.9	26	1.2	$C_5H_{10}N_2O_4$	162.1	17.2	17.3
Ser-Ala[c]	0.25	0.66	−29.5	27	1.2	$C_6H_{12}N_2O_4$	176.1	16.0	15.9
Ser-Glu[d]	0.15	0.62	−11.4	26.5	4.2	$C_8H_{14}N_2O_6$	234.2	12.0	12.0
Ser-His	0.03	0.44	+14.0	26	3.9	$C_9H_{15}N_4O_4 \cdot HCl$	279.7	20.0	20.0
Ile-Ser	0.54	0.72	+30.6	26	4.2	$C_9H_{18}N_2O_4$	218.3	12.8	12.8
Asp-Ser	0.07	0.50	+24.0	27	1.7	$C_7H_{12}N_2O_6 \cdot 2H_2O$	256.2	11.0	11.0
Glu(Ser)	0.08	0.46	+19.5	26.5	4.0	$C_8H_{14}N_2O_6$	234.2	12.1	12.0
Lys-Ser	0.06	0.42	+27.9	27	3.8	$C_9H_{19}N_3O_4 \cdot 2HCl$	306.9	13.7	13.7
Asp-Ser-Gly[e]	0.06	0.47	− 5.0	26.5	4.1	$C_9H_{15}N_3O_7 \cdot 2H_2O$	313.3	13.4	13.4
Asp-Ser-Ala	0.08	0.58	−34.0	26	1.6	$C_{10}H_{17}N_3O_7 \cdot 2H_2O$	327.3	12.8	12.8
Asp-Ser-Glu[f]	0.07	0.48	−19.5	26.5	4.0	$C_{12}H_{19}N_3O_9 \cdot 4H_2O$	421.3	10.0	10.0
Glu-Ser-Gly	0.09	0.45	+ 8.3	27	4.4	$C_{10}H_{17}N_3O_7 \cdot 2H_2O$	327.3	12.9	12.8
Glu-Ser-Ala	0.14	0.58	+16.2	26.5	1.6	$C_{11}H_{19}N_3O_7 \cdot 2H_2O$	341.3	12.3	12.3
Leu-Gly-Ser	0.34	0.75	+36.5	26	3.9	$C_{11}H_{21}N_3O_5$	275.3	15.2	15.3

[a] Whatman No. 1, descending; solvent systems A and B as in Table 1.
[b] Reported[48] $[\alpha]_D^{25}$ +30.2° (1 M HCl, *c* 6).
[c] Reported [48] $[\alpha]_D^{25}$ −30.4° (1 M HCl, *c* 6).
[d] Reported [48] $[\alpha]_D^{25}$ −9.4° (1 M HCl, *c* 6); reported [3] $[\alpha]_D^{21}$ −10.8° (1 M HCl, *c* 4.4).
[e] Found: $[\alpha]_D^{26}$ −8.2° (water, *c* 3.2); reported [31] $[\alpha]_D^{25}$ −8.7° (water, *c* 3.2).
[f] Found: $[\alpha]_D^{26}$ −15.5° (water, *c* 3.2); reported [31] $[\alpha]_D^{25}$ −10° (water, *c* 3.5) for the dihydrate.

When no more hydrogen was consumed (after 0.5—2 h), the solution was filtered, the catalyst washed with water, and the combined solutions evaporated to dryness in *vacuo*. The peptides were crystallized from water-ethanol. Some of their properties are given in Table 2. They gave one spot on chromatograms in all solvent systems tested (20—100 μg peptide applied), and had the normal purple ninhydrin colour.

They were all tested by total hydrolysis by leucin aminopeptidase [17] or by 6 M HCl. In the latter case, the optical rotation of the hydrolysate was compared with that of the component amino acids, treated in the same way. These hydrolysates were, also, analysed paper chromatographically on Whatman No. 1 paper using the solvent system A; or pyridine-water (65:35 v/v; separates glycine and serine), or butanol-acetone-water-pyridine (10:10:5:2, v/v, separates aspartic or glutamic acid, glycine and serine).

Phosphorylated derivatives

Phosphorylation of N-benzyloxycarbonyl peptide benzyl esters. The protected peptide (5 mmoles) was dissolved in pyridine (10 ml, dried over barium oxide) and the solution cooled to just above the freezing point. Dibenzylphosphoryl chloride, freshly prepared [6] from dibenzyl phosphite (1.9 g, 7 mmoles) and N-chlorosuccinimide, was added. Then the mixture was shaken and left over night at +4°. Cold ethyl acetate (75 ml) and cold water (75 ml) were added, and the upper phase washed successively with cold water, 1 M H_2SO_4, water, saturated $NaHCO_3$ and water, and then dried over Na_2SO_4. The, phosphate triesters (O-dibenzylphosphorylated protected serine peptides) were obtained as solids or semisolids by evaporation of the solvent *in vacuo*.

When dibenzylphosphoryl chloride is used in excess, it is possible, after long reaction times, to detect the formation of small amounts of pyrophosphate and triphosphate analogues of the O-phosphorylated peptides.[15]

The triesters were, in most cases, hydrogenolyzed without further purification, although some of them may by crystallized.[3] Thus, N-benzyloxycarbonylglycyl-(O-dibenzylphosphoryl)-L-serine benzyl ester (V) crystallized from the ethyl acetate solution in 87 % yield during the aqueous washings; m.p. 79—80°, R_{FC} 0.42. (Found: N 4.3; P 4.7. Calc. for $C_{34}H_{35}N_2O_9P$ (646.6): N 4.3; P 4.8). Since benzyloxycarbonylglycyl-L-serine benzyl ester had R_{FC} 0.25, chromatography on silicic acid may be an alternative purification procedure for triesters like (V). A third method for purification is the monodebenzylation procedure of Zervas and Dilaris:[34] Compound (V), (6.5 g, 10 mmoles) in dry acetone (20 ml) was refluxed with dried NaI (1.5 g, 10 mmoles) for 45 min; the diester sodium salt crystallized during this time. It was filtered off and washed with cold acetone; yield 4.3 g (74 %) m.p. 178°; R_{FC} 0.0. (Found: N 4.8. Calc. for $C_{27}H_{28}N_2O_9PNa$ (578.5): N 4.8). This salt (3.5 g, 6 mmoles) was converted to the free acid by dissolving in water (25 ml) and adding 1 M HCl (7 ml). N-Benzyloxycarbonylglycyl-L-serine benzyl ester O-benzyl hydrogen phosphate (VI), 3.3 g (100 %) crystallized immediately; m.p. 72°. Recrystallization from ethyl acetate-diethyl ether raised the m.p. to 129°; R_{FC} 0.0. (Found: N 4.9; P 5.6. Calc. for $C_{27}H_{29}N_2O_9P$ (556.5): N 5.0; P 5.6). A sample (1.5 g, 2.6 mmoles) of (VI) was treated with "DCCI" (0.28 g, 1.3 mmoles) in dried methylene chloride (15 ml). Dicyclohexylurea (0.3 g, 100 %) precipitated immediately and was filtered off after 20 min at room temperature. The solution was evaporated to dryness *in vacuo*. A white, semisolid residue (VII), 1.5 g, was obtained, having a high mobility (R_{FC} 0.63) on thin layer plates. The compound was transformed into (VI) (m.p. 127—128°, R_{FC} 0) when left for a few days in an open vessel. These facts, together with the method of synthesis, indicate the compound to be a protected symmetrical diester-pyrophosphate: $[C_7H_7O{\cdot}CO{\cdot}NH{\cdot}CH_2{\cdot}CO{\cdot}NH{\cdot}CH(CO_2C_7H_7)CH_2O{\cdot}P(O)(OC_7H_7)\text{-}]_2O$, *i.e.* P^1, P^2-dibenzyl-P^1, P^2-di(N-benzyloxycarbonylglycyl-L-serine benzyl ester O-)pyrophosphate. (Found: N 5.1; P 5.7. Calc. for $C_{54}H_{56}N_4O_{17}P_2$ (1095.0): N 5.1; P 5.7). Compounds of this type have been synthesized and studied by Avaeva *et al.*[55]

Hydrogenolysis of phosphate triesters and isolation of O-phosphorylated peptides. The triesters were dissolved in *t*-butanol-water and hydrogenolyzed as described above for the preparation of free peptides. Palladium (10 % on charcoal) was again used as the catalyst (0.3—0.5 g per gram triester), and 1—4 h shaking in a hydrogen atmosphere was required. Filtration and evaporation to dryness *in vacuo* gave the phosphorylated peptides

as white solids. Several such peptides crystallize well from water-ethanol [2-4] (*cf.* Table 1), but the majority of those prepared here were expected to be hygroscopic, and were purified by chromatography. A column (2.0 × 40 cm) of Dowex 1−X2 (200−400 mesh) anion exchange resin (formate form) was used as described previously.[15,33] The freeze-dried phosphopeptide fractions (*cf.* Fig. 3) were mostly, according to analysis, in the form of mono-pyridinium salts. They were, therefore, triturated in water with a small amount of cation exchange resin (Dowex 50, H^+ form), and the filtered solution again freeze-dried. Paper chromatographically pure peptides (Table 1) were obtained in this way in yields of 51−81 %, calculated from the amount of benzyloxycarbonyl peptide benzyl ester used.

The peptides Lys-SerP and SerP-His were pyridine-free after the first freeze-drying. The latter peptide was obtained by phosphorylation of N^{α}-(N-benzyloxycarbonyl-L-seryl)-N^{im}-benzyl-L-histidine benzyl ester.[56] The hydrogenolysis of this compound as well as the triester obtained required 12 h to ensure complete removal of the *im*-benzyl group.

The procedure was used without difficulties also in the preparation of O-phosphoryl-L-serylglycine-2-^{14}C and glycyl-(O-phosphoryl)-L-serine-3-^{14}C from glycine-2-^{14}C (37.5 mg, 100 μC) and from L-serine-3-^{14}C (50.3 mg, 100 μC); a somewhat smaller column (1.4 × 25 cm) was used in the final purification. Studies with these peptides will be reported elsewhere.[57]

O-Phosphorylserine methyl ester. (a): O-Phosphoryl-DL-serine [36] (6.8 g, 36.8 mmoles) was added to a solution (prepared at −10°) of thionyl chloride (26 ml, 335 mmoles) in methanol (100 ml). The material dissolved completely within 1 h. The solution was evaporated to dryness *in vacuo*, and the white crystalline residue washed with diethyl ether and dried; yield 7.3 g (100 %, calculated as the mono-methyl ester). After recrystallization from water-methanol, the m.p. of the compound, (VIII) was 198° (decomp., capillary tube); R_{FA} 0.19; R_F 0.55 in phenol-water (80:20). The methyl ester (IX), prepared [20] by hydrogenolysis of N-benzyloxycarbonyl-(O-diphenylphosphoryl)-DL-serine methyl ester had R_{FA} 0.19, R_F 0.55 in phenol-water and m.p. 198° (decomp.), although reported[20] m.p. 173−176° for the same sample (the m.p. of derivatives of O-phosphorylserine have been found to vary considerably with the conditions of determination;[58] a mixture of (VIII) and (IX) had m.p. 194°). The I.R. spectra of the two samples in KBr dispersion were identical. Since the structure of (IX), owing to the method of synthesis and the results of acid-base titration,[20] had the structure $H_3N^+{\cdot}CH(CO_2CH_3)CH_2O{\cdot}P(OH)O^-$, the thionyl chloride method [59] esterifies the carboxyl group only. A further proof for the presence of two free acid functions at the phosphate was given by the rapid release of one equivalent of phosphoric acid by alkaline phosphatases from swine kidney and calf bone, which attacks mono-phosphate esters only.[19] (Found: N 7.0; P 15.6. Calc. for $C_4H_{10}NO_6P$ (199.1): N 7.0; P 15.6).

(b): O-Phosphoryl-L-serine [36] (4.7 g, 25 mmoles), treated as above, gave 3.7 g (74 %) of the corresponding optically active ester, m.p. 167° (decomp.), R_{FA} 0.19; $[\alpha]_D^{25}$ +12.0 (1 M HCl, *c* 4.3). (Found: N 7.0).

O-Phosphoryl-L-threonine. L-Threonine (29 g, 243 mmoles; $[\alpha]_D^{26}$ −14.4° (5 N HCl, *c* 2.1)) was treated with chlorophosphoric acid exactly as described for serine.[36] After the acid hydrolysis, ethanol and ether precipitated 28.5 g (60 %) of practically pure O-phosphorylated threonine, m.p. (capillary tube) 181° (decomp.); $[\alpha]_D^{26}$ −8.2 (H_2O, *c* 2.6); $[\alpha]_D^{26}$ −2.0 (1 M HCl, *c* 4.0). Recrystallization from water (250 ml)-ethanol gave 22.8 g (48 %) of paper chromatographically pure material, m.p. (capillary tube) 189° (decomp.); $[\alpha]_D^{27}$ −7.9° (H_2O, *c* 2.5); R_{FA} 0.11; R_{FB} 0.46; R_F 0.03 in pyridine-isoamyl alcohol-water 35:35:30 (volumes; Whatman No. 1 paper). (Found: N 7.0; P 15.4. Calc. for $C_4H_{10}NO_6P$ (199.1): N 7.0; P 15.6).

The m.p. and optical rotation in water are well in accord with those of O-phosphoryl-L-threonine, isolated by de Verdier [60] from an acid hydrolyzate (2 M HCl, 20 h, 100°) of bovine casein: m.p. 194° (decomp.); $[\alpha]_D^{24}$ −7.4° (H_2O, *c* 2.58). Surprisingly, the acid hydrolyzate (20 h, 110°, sealed tubes) of the synthetic compound (38.7 mg in 2200.1 mg 5 M HCl) had a negligible optical rotation, $[\alpha]_D^{26}$ −1.5°. Paper chromatographic analysis of the hydrolyzate indicated threonine (R_{FA} 0.28;R_{FB}0.54; R_F 0.20 in pyridine-*i*-amyl alcoholwater 35:35:30) to be the main product present. In addition, there was a small amount of unhydrolyzed O-phosphorylthreonine. Little loss of optical activity was observed, when L-threonine was heated similarly in 5 M HCl (41.4 mg in 2167.0 mg): $[\alpha]_D^{26}$ −13.2° (*c* 1.7). When phosphoric acid (24.5 mg 85 % H_3PO_4) was present during the "hydrolysis"

of L-threonine (40.0 mg in 1987.6 mg 5 M HCl), the rotation became $[\alpha]_D^{26}$ −12.8° (*c* 1.8).

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