# Solid Phase Synthesis of Two Phosphorylated Peptides Related to Casein using Allyl Phosphate Protection, and their CD Spectra

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Phosphoserine peptides related to the casein kinase II substrate consensus sequence ESLSSSEE have been synthesized using $N$-Boc-$O$-diallylphosphono-L-serine by solid-phase methods. The allyl groups were removed, while the peptide was still attached to the solid support, by $Pd^0$ with azide as the nucleophile. Far UV circular dichroism measurements indicate that peptides phosphorylated at Ser 4 or Ser 5 have a somewhat higher content of ordered structure than the non-phosphorylated peptide.

During studies of protective groups for the phosphate moiety in solid phase peptide synthesis we have been investigating the allyl group, which, in esters, can be removed with $Pd^0$ complexes in the presence of a nucleophile. As a test molecule in solid phase synthesis we chose a substrate for casein kinase II in which there is a triple sequence of serine residues, one of which is phosphorylated by the enzyme. The peptide ESLSSSEE-$NH_2$ was earlier prepared by solution synthesis.[1] From $^{13}C$ NMR, HPLC and CD data it has been reported to have a conformation containing a β-bend and three further hydrogen bonds keeping the ends together. As this peptide on its own can function as a substrate for the kinase, release of the peptide after phosphorylation must depend on factors existing within this relatively short sequence. Our technique of synthetic phosphorylation is ideally suited to determining whether changes in the conformation as well as polarity of the substrate are coupled to its chemical transformation. For this reason we synthesized the octapeptide in forms containing a phosphoryl group at Ser 4 and also, separately, at Ser 5.

## Results and discussion

The synthesis started with the production of $N$-Boc-diallylphosphono-L-serine. Initially we attempted to perform this synthesis via diallyl phosphorochloridite but during distillation a violent explosion occurred. Therefore phosphorus trichloride was instead reacted with diisopropylamine to form the corresponding amino dichlorophosphine that, upon reaction with allyl alcohol,

gave diallyl $N,N$-diisopropylphosphoramidite. The latter, when reacted with Boc-serine in the presence of tetrazole, gave, after oxidation, the desired building block for solid phase synthesis of phosphorylated serine. Bannwarth and Küng have reported the preparation of the phosphitylating reagent and its further reaction with a serine derivative; this was, however, not suited for solid phase peptide synthesis.[2] The peptides were then synthesized according to a standard Boc protocol.[3] When the synthesis was completed, while the peptides were still attached to the resin, the allyl groups were removed with a $Pd^0$ complex.[4] The peptides were then removed from the resin and the remaining protective groups for Ser and Glu removed with trifluoromethanesulfonic acid. The peptides showed sharp $^{31}P$ peaks in their NMR spectra and gave satisfactory amino acid analyses. In amino acid analysis phosphoserine was detected before aspartate as a distinct peak. During the synthesis of $N$-($tert$-butoxycarbonyl)-$O$-(diallylphosphono)-L-serine the intermediate phosphite was oxidized by $m$-chloroperbenzoic acid. Other oxidizing agents such as phenyliodine(III) diacetate, iodine–water, peracetic acid or $tert$-butyl hydroperoxide did not give satisfactory products.

Circular dichroism spectra of the casein kinase II substrate peptide (ESLSSSEE) in the unphosphorylated state, taken in the far UV region (190–240 nm), indicate that a certain degree of ordered structure is present, in agreement with the earlier work of Perich and Johns.[1] As seen in Fig. 1(a), the spectrum at first glance bears a rough similarity in form to those of peptides in pure extended coil conformation. That other structural com-
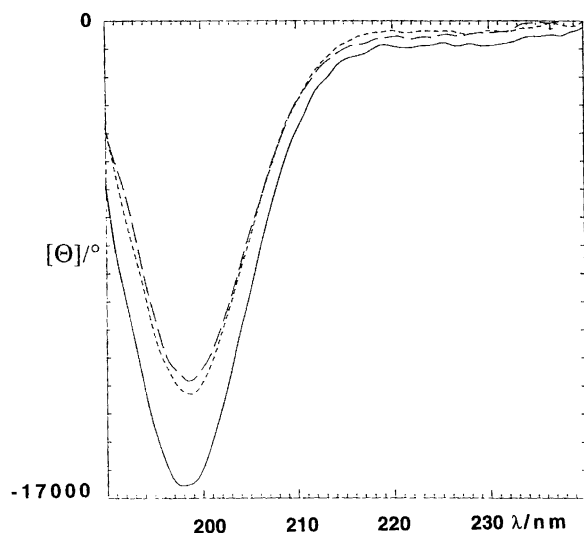
*Fig. 1.* Circular dichroism curves for peptides **2, 3** and **4**: (a) **2** (—); (b) **3** (– – –); (c) **4** (— — — —).

ponents must be present, however, is clear from the fact that the rotational strength of the 198 nm negative maximum which here reaches $\approx 17\,000$ deg cm$^2$ dmol$^{-1}$, is significantly less than the 30–40 000 seen in pure extended coil spectra. Furthermore the typical positive band at 220 nm is absent, the spectrum remaining negative throughout its range. When the curve fitting program PEPFIT[5] is applied to this spectrum, it calculates an average secondary structure content of 55% extended coil and 45% reverse turn. With an octapeptide, a single reverse turn would be expected to constitute around 28% of the structure, so that the figure of 45% is consistent with the existence of one fairly persistent turn plus transient turn formation at other sites, in agreement with the 1D NMR spectral data.[1]

The introduction of a phosphoryl group at Ser 4 [Fig. 1(b)] has a significant effect on the preferred average conformation of the peptide. The amplitude of the low wavelength negative maximum is further reduced, implying the presence of further positive bands at this position, while the portion of the spectrum above 210 nm is relatively little affected. This suggests the appearance of a strong type III β-bend component, and in fact deconvolution of the spectrum gives a solution of 45% coil, 47% reverse turn and 10% β-sheet. Although attribution of a particular reverse turn through CD spectra alone is not fully reliable, it should be remarked that the reverse turn content of the Ser 4 phosphorylated peptide can be almost completely modeled with a single dominant component, type III turn comprising $\approx 30\%$ of the structure. The unphosphorylated peptide, in contrast, required multiple turn components, suggesting a less fixed structure subject to extensive fluctuation. In any case, the net effect of phosphorylation has been to increase the average amount of stabilized secondary structure within the peptide, and to alter the structure present. It is thus plausible that the introduction of a phosphoryl group is

in itself sufficient to alter the conformation of the substrate at the binding site so as to effect its release. In this respect it is interesting that insertion of a phosphoryl group at the non-physiological site [Fig. 1(c)] has virtually the same effect on the conformation of the peptide. This would seem to indicate that although structural factors external to the amino acid sequence in the immediate neighbourhood of the target residue may play a role in the specificity of substrate recognition, (the $K_m$ for consensus peptides is usually higher than that for the native protein), they are not necessary for substrate release.

## Materials and methods

*Reagents. tert*-Butoxycarbonyl (Boc)-protected amino acids were purchased from Bachem Feinchemikalien, Switzerland. 1,3-Diisopropylcarbodiimide (DIPCDI), 1-hydroxybenzotriazole (HOBt), dimethyl sulfide (DMS) and trifluoroacetic acid (TFA) were from Fluka, tetrazole, *m*-chloroperbenzoic acid and anisole were from Aldrich and trifluoromethanesulfonic acid (TFMSA) from Merck. Dichloromethane (DCM) was run through an alumina column and then dried over 4 Å molecular sieves, *N,N*-dimethylformamide (DMF) was dried over 4 Å molecular sieves, and diisopropylethylamine (DIPEA) was distilled over KOH and ninhydrin successively.

*Fast atom bombardment mass spectrometry (FAB-MS) and nuclear magnetic resonance (NMR) spectroscopy.* FAB-MS spectra were recorded on a JEOL SX102 double-focussing mass spectrometer with a FAB ion source, and a JMA DA6000 data system. The peptide was treated as follows. An aliquot of the sample was dissolved in 30% aqueous acetic acid and evaporated on the FAB-target, then a mixture of 70% aqueous formic acid and glycerol (1:1) as matrix was added, and the sample was bombarded with 10 keV Xenon atoms. Spectra of positive ions were recorded in the range $m/z$ 50–2550 with a resolution of 3000.

Carbon ($^{13}$C), phosphorus ($^{31}$P) and proton ($^{1}$H) NMR spectra were recorded on a JEOL GSX 270 spectrometer. Chemical shifts are given in parts per million (ppm) relative to tetramethylsilane (TMS $\delta_C =$ 0.00, $\delta_H = 0.00$) in C$^2$HCl$_3$ (CDCl$_3$) for $^{13}$C and $^{1}$H and 85% H$_3$PO$_4$ ($\delta_P = 0.00$) for $^{31}$P.

*Circular dichroism spectroscopy.* Circular dichroism spectra of the peptides were measured on a Jasco J-710 spectral polarimeter at a sensitivity of 10 mdeg with a 2.0 s response time at a scanning speed of 5 nm min$^{-1}$. The band width was held at 1.0 nm and the step resolution was 0.1 nm per data point. Samples were measured at room temperature at a concentration of 100 μg ml$^{-1}$ in a 1 mm dichroically neutral quartz cuvette. Curves represent the spectral range 190–240 nm after noise reduction through signal-averanging followed by Fourier

transform with a low band-pass filter. CD spectra expressed in millidegrees were converted into mean residue ellipticity [$\theta_{mr}$] for purposes of curve fitting and analysis.

*Secondary structure analysis.* The most advanced systems available for secondary structure analysis of protein far UV CD spectra[6,7] are based on globular proteins of known structure. For just this reason they are not suitable for the deconvolution of peptide CD, where many of the long range effects, β-sheet torsions helical bends, etc., typical of larger proteins, do not occur. Analysis of peptide CD is best done working from the spectra of model peptides in defined conformation. However, the model peptides used for type spectra of α-helix, β-sheet and extended coil found in the literature tend to be either totally (poly-L-Lys) or highly (poly-Lys-Leu-Lys-Leu) homogeneous. The band intensity and, to a certain extent, position associated with the major classes of secondary structure is known to vary with the side chain[8] and the CD spectrum of any single homogeneous model peptide in defined structure will thus introduce a bias when used to analyse the spectrum of a natural, more heterogeneous peptide. In an attempt to minimize this effect of side chain bias, we have collected the available model peptide spectra from the literature for each of the three main structure types: α helix, β-sheet and extended coil. These spectra have been used to compile a weighted average for each type, the contribution of a given model peptide being proportional to the relative frequency of its side chain(s) in proteins in general. While not ideal, as only a subset of the 20 possible side chains is represented (many potential homopolymers, i.e., poly-L-Phe, are not easily adapted to all classes of structure, or, indeed, to measurement in aqueous solution), such a weighted average introduces less bias than the use of any single model peptide spectrum for deconvolution. The resulting type spectra and their incorporation in the operator/intervention curve-fitting program PEPFIT will be described in a forthcoming paper.[5]

*Synthesis of N-(tert-butoxycarbonyl)-O-(diallylphosphono)-L-serine* 1. Boc-L-Ser-OH (500 mg, 2.44 mmol) and tetrazole (510 mg, 7.31 mmol) were dissolved in dry DCM (25 ml) and the solution was cooled to 0 °C. Diallyl N,N-diisopropylphosphoramidite[2] (0.897 ml, 3.66 mmol) was added to the stirred solution. The mixture was stirred for 2 h at room temperature and then cooled to −40 °C. A solution of m-chloroperbenzoic acid (841 mg, 4.87 mmol) in DCM (20 ml) was added. The mixture was stirred 1 h at 0 °C, treated with ethyl acetate (100 ml) and extracted first with 10% sodium disulfite (3 × 50 ml), then with 10% aqueous sodium hydrogencarbonate (3 × 50 ml). The carbonate solution was acidified with 1 M HCl to pH = 2–3 and extracted four times with ethyl acetate. The combined organic layer was dried with sodium sulfate and, after evaporation, the product was purified by column chromatography on silica gel [hept-

ane–ethyl acetate–acetic acid (30:30:5)] to give 1 as an oil (0.66 g, 74%), [$\alpha$]$_D$ + 34.9°. $^{13}$C NMR (67.9 MHz, CDCl$_3$): δ 28.5 (Boc-CH$_3$), 53.9 (α-CH), 67.9 (β-CH$_2$, $J_{POC}$ = 5.5 Hz), 68.9 [(OCH$_2$CH = CH$_2$)$_2$, $J_{POC}$ = 5.5 Hz], 80.5 (Boc-C), 119.1 [(OCH$_2$CH = CH$_2$)$_2$], 132.2 [(OCH$_2$CH = CH$_2$)$_2$], 155.7 (NHCOO), 171.0 (COOH). $^1$H NMR (270 MHz, CDCl$_3$): δ 1.50 (s, Boc-CH$_3$), 4.30 (m, α-CH), 4.50 (m, β-CH$_2$), 4.60 [m, (OCH$_2$CH = CH$_2$)$_2$], 5.25, 5.35 [dm, (OCH$_2$CH = CH$_2$)$_2$], 5.66 (d, NHCOO), 5.92 [m, (OCH$_2$CH = CH$_2$)$_2$]. $^{31}$P NMR (109.4 CDCl$_3$): δ − 1.4.

*Solid phase synthesis of H-Glu-Ser-Leu-Ser-Ser-Ser-Glu-Glu-OH* 2, *H-Glu-Ser-Leu-Ser(P)-Ser-Ser-Glu-Glu-OH* 3, *H-Glu-Ser-Leu-Ser-Ser(P)-Ser-Glu-Glu-OH* 4. The peptides were synthesized manually using Merrifield's solid phase technique with Boc-protected amino acids.[3] The side-chain functional groups were protected as follows: benzyl for unphosphorylated serine and glutamic acid, and diallyl triester for phosphorylated serine. The first Boc-amino acid was covalently linked to a Biorad® chloromethylated polystyrene resin (1.25 mequiv. g$^{-1}$ substitution) according to the KF method[9] in DMF. Subsequent amino acids were incorporated using a twofold excess of the appropriate N-Boc protected amino acids in the presence of 2 equivs. DIPCDI and three equivalents HOBt. The coupling reaction was monitored for completion with a ninhydrin test.[10] The synthesis was performed on a 0.6–0.97 mmol scale in a 100 ml fritted glass bottomed reaction vessel, using nitrogen for stirring and filtration.[11] A general synthesis protocol for 2, 3 and 4 is as follows: (1) wash with DCM 2 × 1 min; (2) deblock with 45% TFA–5% anisole–50% DCM 1 × 1 min, then 1 × 20 min; (3) wash with DCM 2 × 1 min, DMF 1 × 1 min, EtOH 2 × 1 min, DMF 1 × 1 min, DCM 2 × 1 min; (4) neutralize with 10% DIPEA–DCM 2 × 1 min; (5) wash with DCM 2 × 1 min, DMF 2 × 1 min; (6) add Boc-amino acid 2 × molar excess + HOBT 3 × molar excess + DIPCI 2 × molar excess; (7) couple 2 h in DCM–DMF (1:2); (8) wash with DMF 2 × 1 min, DCM 2 × 1 min; (9) check for free amino groups using ninhydrin. (Repeat steps 4–9 if necessary).

*Removal of allyl blocking groups from the peptide phosphotriester.* Trimethylsilyl azide (0.631 ml, 4.8 mmol), tetrabutylammonium fluoride (567 mg, 1.8 mmol) and tetrakis(triphenylphosphine)palladium(0) (139 mg, 0.12 mmol) were premixed in 6 ml DCM under argon.[4] The mixture was added to the swelled peptide-resin 3 (500 mg, 0.6 mmol) under nitrogen. The reaction was allowed to take place under nitrogen stirring at room temperature for 30 min. After filtration, the resin was washed carefully with DCM and DMF alternately. The peptide-resin 4 was treated the same way.

*TFMSA cleavage of 2 from resin and removal of all blocking groups.* TFMSA (0.5 ml) and TFA (2.5 ml) were mixed and cooled to 0 °C, followed by addition of

DMS (1.5 ml) and *m*-cresol (0.5 ml). The mixture was stirred for 30 min after which the protected peptide-resin (300 mg) was added. The reaction mixture was stirred for 4 h at 0 °C. The peptide was precipitated from the acid with diethyl ether at −70 °C and taken up in 10% aqueous acetic acid. The aqueous phase was evaporated to dryness and the residue desalted on Sephadex® G15 in water, and lyophilized to yield the peptide as a white fluffy powder (yield 126.8 mg, 64%). Check for homogeneity was performed by reversed-phase liquid chromatography on a Waters Bondapak C18 10 μm column in a water–acetonitrile 0–100% gradient in 0.05% TFA, detection at 214 nm. The amino acid analyses were within 0.04 from expected values. FAB-MS: $(M+1)^+$ $m/z$ 866.3, calc. $m/z$ 867.1.

*TFMSA cleavage of* **3** *and* **4** *from resin and removal of all blocking groups.* TFMSA (0.2 ml), TFA (1.8 ml) and *p*-cresol (500 mg) were mixed and cooled to 0 °C, followed by addition of the protected peptide-resin (300 mg). The reaction mixture was stirred for 4 h at 0 °C. The peptide was precipitated from the acid with diethyl ether at −70 °C and taken up in 10% aqueous acetic acid. The aqueous phase was evaporated to dryness and the residue desalted on Sephadex® G15 in water, and lyophilized to yield the peptide as a white fluffy powder (**3**: 21%, **4**: 38%). Check for homogeneity was done by reversed-phase liquid chromatography on a Waters Bondapak C18 10 μm column in a water–aceto-

nitrile 0–100% gradient in 0.05% TFA, detection at 214 nm. The amino acid analyses were within 0.04 from expected values. FAB-MS: $(M+1)^+$ for **3** $m/z$ 947.0, calc. $m/z$ 946.3 and for **4** $m/z$ 947.1, calc. $m/z$ 946.3.

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