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Synthesis of Casein-Related Peptides and Phosphopeptides. XVI*
The Efficient Synthesis of the Casein-Related *O*-Phosphoseryl-Containing Peptide Ac-Glu-Ser*(P)*-Leu-Ser*(P)*-Ser*(P)*-Ser*(P)*-Glu-Glu-NHMe†

John W. Perich,[A–C] *R. B. Johns*[B] *and Eric C. Reynolds*[A]

[A] Biochemistry and Molecular Biology Unit, School of Dental Science, University of Melbourne, Melbourne, Vic. 3000.
[B] School of Chemistry, University of Melbourne, Parkville, Vic. 3052.
[C] Author to whom correspondence should be addressed.

Abstract

The multiple-*O*-phosphoseryl-containing peptide, Ac-Glu-Ser*(P)*-Leu-Ser*(P)*-Ser*(P)*-Ser*(P)*-Glu-Glu-NHMe, was prepared in high yield by the use of Boc-Ser(PO_3Ph_2)-OH in the Boc mode of peptide synthesis for the preparation of the protected Ser(PO_3Ph_2)-octapeptide followed by its one-step hydrogenolytic deprotection with platinum oxide. Peptide synthesis was performed by the repetitive excess mixed-anhydride coupling procedure and 40% CF_3CO_2H/CH_2Cl_2 for removal of the Boc group from intermediate peptides. The phosphopeptide was found to be greater than 99% pure by C_8 r.p.-h.p.l.c. analysis, and was readily characterized by amino acid analysis, ^{13}C n.m.r. spectroscopy and f.a.b. mass spectrometry.

Introduction

The chemical and physical properties of the α_{s1}- and β-caseins of bovine milk are attributed[2] to the heavily phosphorylated -Glu-Ser*(P)*-Ile{Leu}-Ser*(P)*-Ser*(P)*-Ser*(P)*-Glu-Glu- segments in which the phosphorylated residues are found in a clustered arrangement. Furthermore, the structural integrity of the casein micelle is believed to be due to extensive interaction of these phosphorylated segments with calcium.[3] In Part I,[4] we reported that the attempted preparation of this phosphopeptide segment by the 'global phosphorylation' of Ac-Glu-Ser-Leu-Ser-Ser-Ser-Glu(OBzl)-Glu(OBzl)-NHMe by using diphenyl phosphorochloridate in pyridine[5] was unsuccessful and gave decomposition products. Later, we described an alternative novel synthetic approach[6–8] for the efficient preparation of various

* Part XV, *Aust. J. Chem.*, 1991, **44**, 1683.
† Part of this work has appeared in preliminary form.[1]

[1] Perich, J. W., and Johns, R. B., *J. Chem. Soc., Chem. Commun.*, 1988, 664.
[2] Whitney, R. McL., in 'Fundamentals of Dairy Chemistry' (Ed. N. P. Wong) 3rd Edn, Ch. 3 (Van Nostrand–Reinhold: New York 1988).
[3] Dickson, I. R., and Perkins, D. J., *Biochem. J.*, 1971, **124**, 235.
[4] Perich, J. W., Alewood, P. F., and Johns, R. B., *Aust. J. Chem.*, 1987, **40**, 257.
[5] Folsch, G., *Sven. Kem. Tidskr.*, 1967, **79**, 38.
[6] Alewood, P. F., Perich, J. W., and Johns, R. B., *Tetrahedron Lett.*, 1984, **25**, 987.
[7] Perich, J. W., Alewood, P. F., and Johns, R. B., *Tetrahedron Lett.*, 1986, **27**, 1373.
[8] Perich, J. W., Alewood, P. F., and Johns, R. B., *Aust. J. Chem.*, 1991, **44**, 233.

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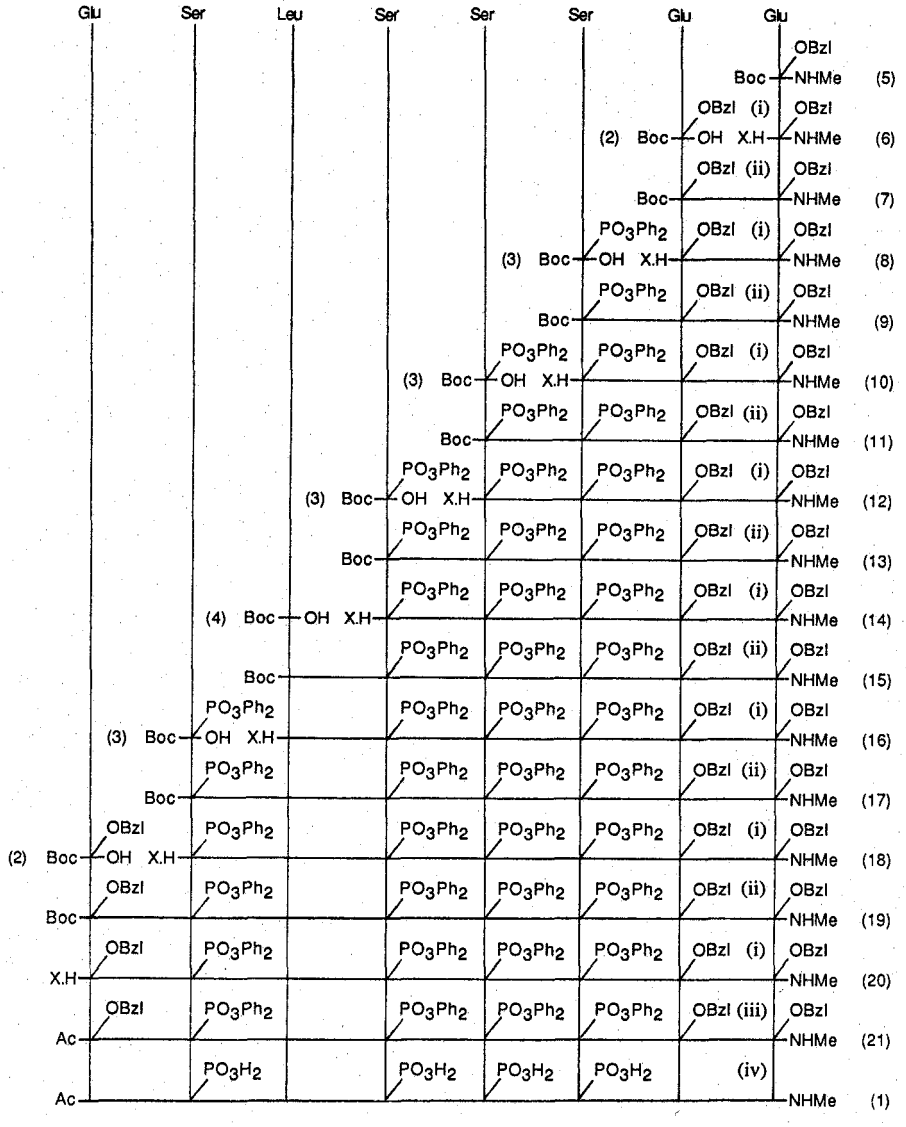
O-phosphoseryl-containing peptides[9–11] by the use of Boc-Ser(PO_3Ph_2)-OH in the Boc mode of peptide synthesis followed by platinum-mediated hydrogenolytic cleavage of the phenyl groups. In this paper, we finally describe the use of



Scheme 1. Reagents (X = CF_3CO_2H): (i) 40% CF_3CO_2H/CH_2Cl_2 (20°, 30 min); (ii) *N*-methylmorpholine, isobutyl chloroformate (−20°, 3 min), then peptide (6), (8), (10), (12), (14), (16) or (18) and *N*-methylmorpholine (1 equiv.) (−20°, 2 h); (iii) acetic acid, *N*-methylmorpholine, isobutyl chloroformate (−20°, 3 min), then octapeptide (20) and *N*-methylmorpholine (1 equiv.) (−20°, 2 h); (iv) H_2–80% PtO_2 (1·1 mequiv./phenyl group), 50% CF_3CO_2H/CH_3CO_2H (20°, 1 h).

[9] Perich, J. W., and Johns, R. B., *Aust. J. Chem.*, 1991, **44**, 397.
[10] Perich, J. W., and Johns, R. B., *Aust. J. Chem.*, 1991, **44**, 405.
[11] Perich, J. W., and Johns, R. B., *Aust. J. Chem.*, 1991, **44**, 1683.

Boc-Ser(PO_3Ph_2)-OH in this approach for the efficient high-yielding synthesis of the target casein phosphopeptide Ac-Glu-Ser*(P)*-Leu-Ser*(P)*-Ser*(P)*-Ser*(P)*-Glu-Glu-NHMe.

Results and Discussion

The synthesis of the fully protected Ser(PO_3Ph_2)-octapeptide (21) was accomplished in an overall yield of 61% from Boc-Glu(OBzl)-NHMe (5)[4] by successive isobutoxycarbonyl mixed-anhydride couplings with Boc-Glu(OBzl)-OH (2), Boc-Ser(PO_3Ph_2)-OH (3) and Boc-Leu-OH (4) (all couplings proceeding in over 90% yields), and the use of 40% CF_3CO_2H/CH_2Cl_2 for repetitive cleavage of the Boc group from the intermediate Boc-peptides (Scheme 1). The *N*-acetylation of octapeptide trifluoroacetate (20) was accomplished by the use of the isobutoxycarbonyl mixed anhydride of acetic acid and gave peptide (21) as a white amorphous solid in 94% yield.

Amino acid analysis of all the protected peptides gave the respective amino acids in their correct ratios, and the structure of each protected peptide was supported by its ^{13}C n.m.r. and ^{31}P n.m.r. spectra (see Experimental). The ^{13}C n.m.r. spectra of peptides (15), (17), (19) and (21) were obtained as $(CD_3)_2SO$ solutions since $CDCl_3$ solutions gave spectra which contained exceptionally broad carbon resonances. The incorporation of the four Ser(PO_3Ph_2) residues in the assembled peptide was established from its ^{31}P n.m.r. spectrum ($CDCl_3$ solution) which displayed four clearly resolved phosphorus resonances at −11·0, −12·9, −13·0 and −13·2 ppm. However, only a single phosphorus resonance at −12·4 ppm was observed in $(CD_3)_2SO$, this being the result of strong solvation of the peptide by this solvent.

A feature of the phenyl group for phosphate protection was that it imparted good solubility on the protected peptides; (9), (11) and (13) were soluble in ethyl acetate, and (15), (17), (19) and (21) were readily soluble in dichloromethane. This was in marked contrast to the corresponding Ser(PO_3Et_2)- and Ser(PO_3Me_2)-peptides which had low solubility in dichloromethane and necessitated the use of warm dimethylformamide for dissolution.[12] However, because the concentration of dichloromethane solutions of protected peptides (15), (17), (19) and (21) led to their isolation as crunchy, brown glass flakes, it was convenient to evaporate the solvent to a small volume and to precipitate the peptide as an amorphous white solid by the addition of ethyl acetate.

The phenyl and benzyl groups were cleaved from octapeptide (21) by the one-step hydrogenation procedure* described in Part XI[9] for the deprotection of peptides containing both Glu(OBzl) and Ser(PO_3Ph_2) residues. Thus, the hydrogenation of octapeptide (21) in 50% CF_3CO_2H/CH_3CO_2H with 1·1 equiv. of platinum oxide per phenyl group proceeded with rapid hydrogen uptake and ceased after 30 min. The hydrogenation was stopped after 60 min and subsequent removal of platinum by filtration, evaporation of solvent, and diethyl ether precipitation gave octapeptide (1) as a white amorphous solid. Complete cleavage

* In the preliminary description of this work,[1] peptide deprotection was effected by a two-step palladium/charcoal and platinum oxide hydrogenation procedure; octapeptide (1) was separated from three other minor peptide fractions (5·0% of total weight) by C_{18} r.p.-h.p.l.c. with 0·1% CF_3CO_2H/9% CH_3CN as the mobile phase.

[12] Perich, J. W., Ph.D. Thesis, University of Melbourne, 1986.

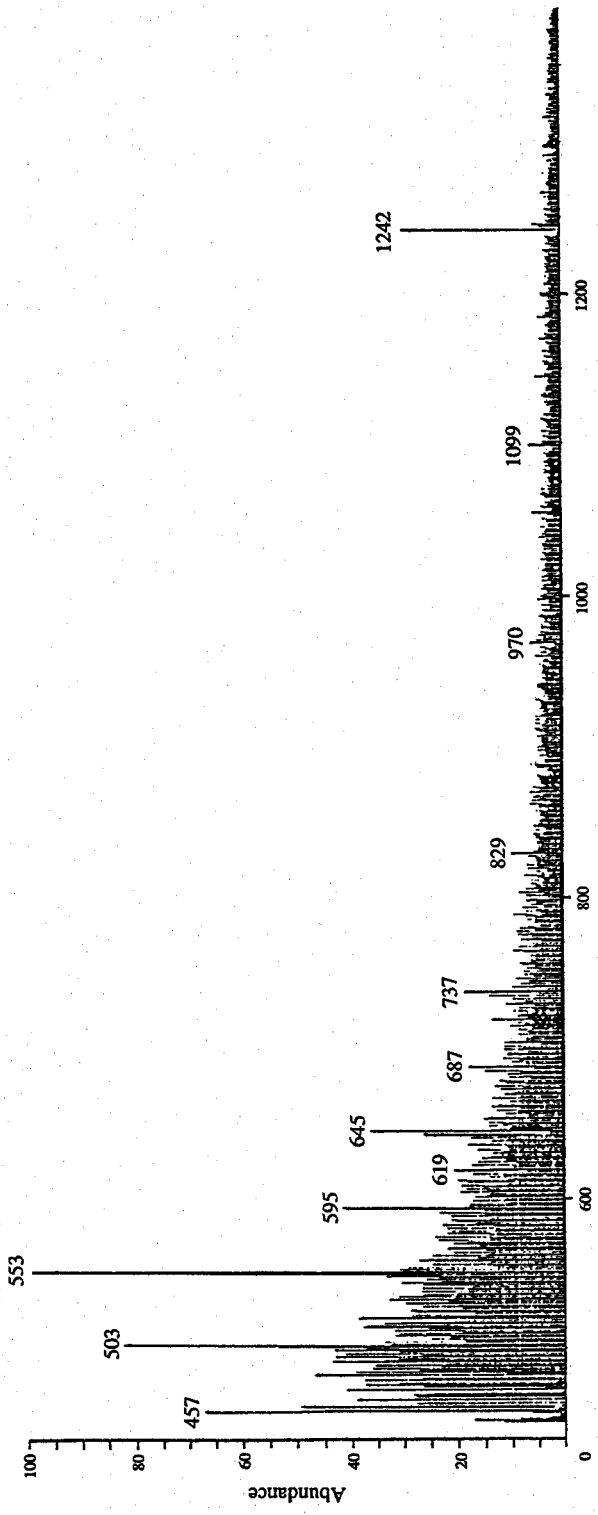


Fig. 1. F.a.b. mass spectrum of octapeptide (1).

of the benzyl and phenyl groups was established by C_8 r.p.-h.p.l.c. and f.a.b. mass spectrometry; the f.a.b. mass spectrum of the isolated peptide displayed a high-intensity [M+H] ion at *m/z* 1242 (Fig. 1) and no other higher molecular weight ions corresponding to phenyl-containing peptides. Crude octapeptide (1) was shown to be homogeneous by C_8 r.p.-h.p.l.c. by using a linear gradient elution of 0·1% CF_3CO_2H/0–80% CH_3CN (20 min) (Fig. 2) and its amino acid analysis gave the constituent amino acid residues in their correct molar ratios.

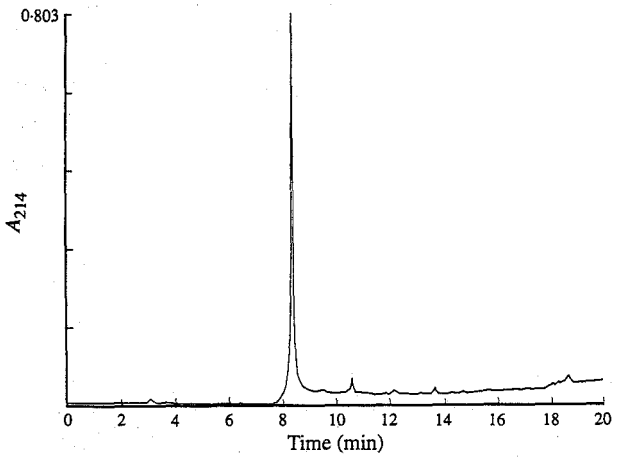


Fig. 2. C_8 r.p.-h.p.l.c. of octapeptide (1), 0·1% CF_3CO_2H/0–80% CH_3CN over 20 min.

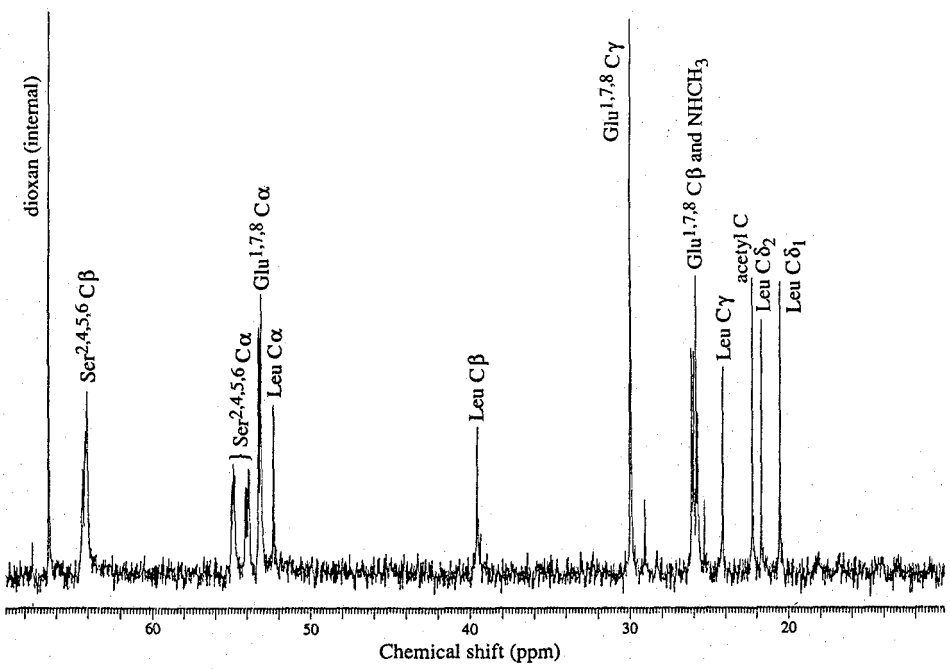


Fig. 3. ^{13}C n.m.r. spectrum of octapeptide (1), aliphatic region.

The ^{31}P n.m.r. spectrum of octapeptide (1) contained a single broad resonance at $-0{\cdot}1$ ppm and its ^{13}C n.m.r. spectrum (400 MHz) was consistent with its structure and contained well-resolved aliphatic and carbonyl regions (Figs 3 and 4). In the aliphatic region, all eight α carbons were clearly resolved, the four α carbons of the Leu and Glu residues being observed at 52·36, 53·13, 53·18 and 53·29 ppm, and the four phosphorus-coupled doublet signals of the four Ser(PO_3H_2) residues being observed at 53·90 ($J_{P,C}$ 7·3 Hz), 54·08 ($J_{P,C}$ 8·8 Hz), 54·82 ($J_{P,C}$ 7·3 Hz) and 54·95 ppm ($J_{P,C}$ 7·3 Hz). In the carbonyl region, nine distinct carbonyl resonances were observed, the four Ser*(P)* carbonyl carbons resonating at 170·65, 170·94, 171·31 and 171·37 ppm, and the acetyl, Glu (×3) and Leu carbonyl carbons resonating at 172·97, 173·35, 173·56, 174·45 and 174·59 ppm.

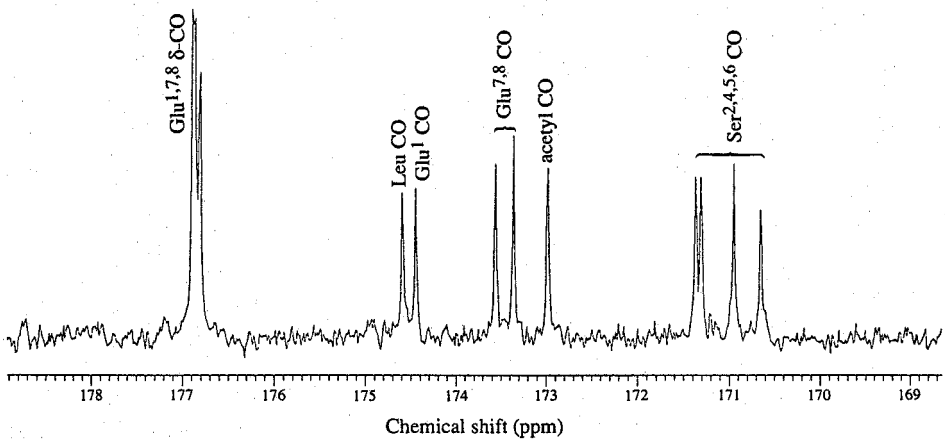


Fig. 4. ^{13}C n.m.r. spectrum of octapeptide (1), carbonyl region.

In view of the high yield and purity of this peptide, we consider that this synthetic approach will be suitable for the synthesis of a wide range of Ser*(P)*-containing peptides and, as a result, it will permit detailed biochemical studies of this special class of peptides. In addition, the preparation of *O*-phosphoseryl-containing peptides by this procedure can be increased to the gram scale as demonstrated by Paquet and Johns[13] by the preparation of 4·2 g of H-Ser*(P)*-Ser*(P)*-Ser*(P)*-Glu-OH.HCl by platinum-mediated hydrogenation (45 p.s.i.) of the protected Ser(PO_3Ph_2)-containing peptide. Although this phenyl-based approach is unsuitable for the preparation of phosphopeptides containing Met, Cys, Trp, Tyr, Phe and His (because of poisoning of the platinum or reduction of the aromatic systems), the development of other phosphate protecting groups will make this synthetic approach suitable for the general preparation of phosphopeptides containing these amino acid residues.

[13] Paquet, A., and Johns, M., *Int. J. Pept. Protein Res.*, 1990, **36**, 97.

Conclusion

To our knowledge, octapeptide (1) represents the most complex multiple-*O*-phosphoseryl-containing peptide which has been prepared. In our opinion, the simple and high-yielding synthesis of phosphopeptide (1) by the use of Boc-Ser(PO_3Ph_2)-OH in Boc-peptide synthesis confirms that this is the method of choice for the preparation of *O*-phosphoseryl-containing peptides and overcomes the many synthetic problems of a 'global phosphorylation' approach.[5]

Experimental

^{13}C n.m.r. spectra were obtained on a JEOL FX-90Q Fourier-transform instrument operating at 22·5 MHz, and were referenced to internal tetramethylsilane for $CDCl_3$ solutions and to the central resonance of the deuterium septet of $(CD_3)_2SO$ set to 39·5 ppm for $(CD_3)_2SO$ solutions. The ^{13}C n.m.r. spectrum of octapeptide (1) in D_2O was recorded on a JEOL GX-400 Fourier-transform instrument operating at 100 MHz and referenced to internal dioxan set to 66·5 ppm. ^{31}P n.m.r. spectra were obtained on a JEOL FX-100 Fourier-transform instrument operating at 40·26 MHz referenced to external 85% H_3PO_4. F.a.b. mass spectra were obtained on either a JEOL DX-300 or JEOL AX-505H mass spectrometer equipped with an f.a.b. source and used argon as the ionization gas. H.p.l.c. was performed on an Applied Biosystems instrument with a linear acetonitrile gradient provided by a 140A solvent delivery system linked to a 1000S Diode Array detector, a Brownlee C_8 Aquapore RP300 column (22·0 cm by 4·6 mm), a flow rate of 1·0 ml/min and a gradient elution of 0·1% CF_3CO_2H/0–80% CH_3CN over 20 min.

All solvents were of AnalaR grade and tetrahydrofuran was distilled from the potassium ketyl of benzophenone immediately prior to use. *N*-Methylmorpholine and isobutyl chloroformate were obtained from Tokyo Kasei Kogyo Co. Ltd and were used without further purification. Trifluoroacetic acid was obtained from Merck–Schuchardt and used without further purification. Acetic acid was successively dried over 4A sieves and distilled immediately prior to use. Platinum oxide (80%) was obtained from Fluka AG. All mixed-anhydride couplings were performed under dry nitrogen. Acidolytic removal of the Boc group from intermediate peptides was accomplished by treatment of the peptide with 40% CF_3CO_2H/CH_2Cl_2 for 30 min at 20° followed by evaporation of the solvent under reduced pressure and precipitation of the peptide trifluoroacetate by the addition of diethyl ether. Melting points were obtained on a Reichert melting point apparatus and are uncorrected. Amino acid analyses of the protected peptides were performed by solution hydrolysis of the peptide in 5·7 M HCl (24 h at 110°) followed by analysis of the phenylthiocarbamoyl derivatized hydrolysate on a Waters h.p.l.c. instrument, the seryl value being corrected on the basis of 10% degradation. The amino acid analysis of octapeptide (1) was performed by vapour hydrolysis with 5·7 M HCl (24 h at 110°).

Boc-Ser(PO_3Ph_2)-Glu(OBzl)-Glu(OBzl)-NHMe (9)

N-Methylmorpholine (0·364 g, 3·50 mmol) in tetrahydrofuran (1 ml) and isobutyl chloroformate (0·444 g, 3·25 mmol) in tetrahydrofuran (1 ml) were successively added to a solution of Boc-Ser(PO_3Ph_2)-OH (1·53 g, 3·50 mmol) in tetrahydrofuran (10 ml) at −20°. After an activation period of 3 min, a solution of the dipeptide trifluoroacetate (8) (2·50 mmol) and *N*-methylmorpholine (0·252 g, 2·50 mmol) in tetrahydrofuran (5 ml) was added to the reaction mixture, and the resulting solution was stirred for 2 h at −20°. A solution of 5% $NaHCO_3$ (5 ml) was then added and the solution was stirred for a further 30 min at 0°. Ethyl acetate (60 ml) was then added and the organic phase was washed with 5% $NaHCO_3$ (2×30 ml) and 1 M HCl (2×30 ml), and then dried (Na_2SO_4) and filtered. The solvent was then evaporated under reduced pressure, and the solid residue was triturated with diethyl ether (2×30 ml) and dried under high vacuum to give tripeptide (9) (2·12 g, 96%) as a white solid, m.p. 106–108°. δ (^{13}C) ($CDCl_3$) 26·1, NHMe; 26·5, 26·7, Glu^{2,3} Cβ; 28·1, Boc Me; 30·8, Glu^{2,3} Cγ; 52·8, 54·4, Glu^{2,3} Cα; 55·7, d, $J_{P,C}$ 5·9 Hz, Ser Cα; 66·4, 66·8, Glu^{2,3} Bzl CH_2; 67·8, d, $J_{P,C}$ 5·9 Hz, Ser Cβ; 81·0, Boc **C**Me$_3$; 119·9, d, $J_{P,C}$ 4·4 Hz, Ph C2; 125·6, Ph C4; 128·2, 128·4, Bzl C2,3,4; 129·8, Ph C3; 135·3, 135·7, Glu^{2,3} Bzl C1; 150·6,

d, $J_{P,C}$ 8·8 Hz, Ph C1; 155·9, urethane CO; 169·6, 170·7, 171·1, Ser CO and $Glu^{2,3}$ CO; 172·8, 173·9, $Glu^{2,3}$ δ-CO. δ (^{31}P) ($CDCl_3$)−11·5. Amino acid analysis: Glu 1·93 (2), Ser 1·07 (1).

Boc-Ser(PO_3Ph_2)-Ser(PO_3Ph_2)-Glu(OBzl)-Glu(OBzl)-NHMe (11)

N-Methylmorpholine (0·313 g, 3·10 mmol) in tetrahydrofuran (1 ml), and isobutyl chloroformate (0·390 g, 2·86 mmol) in tetrahydrofuran (1 ml) were successively added to a solution of Boc-Ser(PO_3Ph_2)-OH (1·355 g, 3·10 mmol) in tetrahydrofuran (9 ml) at −20°. After an activation period of 3 min, a solution of the tripeptide trifluoroacetate (10) (2·20 mmol) and *N*-methylmorpholine (0·222 g, 2·20 mmol) in tetrahydrofuran (5 ml) was added to the reaction mixture and the resulting solution was stirred for 2 h at −20°. A solution of 5% $NaHCO_3$ (5 ml) was then added and the solution was stirred for a further 30 min at 0°. Ethyl acetate (60 ml) was then added and the organic phase was washed with 5% $NaHCO_3$ (2×30 ml) and 1 M HCl (2×30 ml), and then dried (Na_2SO_4) and filtered. The solvent was then evaporated under reduced pressure, and the solid residue was triturated with diethyl ether (2×30 ml) and dried under high vacuum to give tetrapeptide (11) (2·43 g, 92%) as a white solid, m.p. 129–131°. δ (^{13}C) ($CDCl_3$) 25·8, NHMe; 26·3, $Glu^{3,4}$ Cβ; 28·0, Boc Me; 30·9, $Glu^{3,4}$ Cγ; 53·0, 54·2, $Glu^{3,4}$ Cα; 54·5, d, $J_{P,C}$ 5·9 Hz, Ser^2 Cα; 55·7, d, $J_{P,C}$ 5·9 Hz, Ser^1 Cα; 66·2, $Glu^{3,4}$ Bzl CH_2; 66·9, d, $J_{P,C}$ 5·9 Hz, $Ser^{1,2}$ Cβ; 81·5, Boc **C**Me_3; 120·0, d, $J_{P,C}$ 4·4 Hz, Ph C2; 125·7, Ph C4; 128·1, 128·4, Bzl C2,3,4; 129·9, Ph C3; 135·5, 135·8, $Glu^{3,4}$ Bzl C1; 150·6, d, $J_{P,C}$ 8·8 Hz, Ph C1; 155·9, urethane CO; 169·9, 170·7, 170·8, 171·3, $Ser^{1,2}$ CO and $Glu^{3,4}$ CO; 172·7, 172·9, $Glu^{3,4}$ δ-CO. δ (^{31}P) ($CDCl_3$)−10·5, 11·4. Amino acid analysis: Glu 2·03 (2), Ser 1·99 (2).

Boc-Ser(PO_3Ph_2)-Ser(PO_3Ph_2)-Ser(PO_3Ph_2)-Glu(OBzl)-Glu(OBzl)-NHMe (13)

N-Methylmorpholine (0·267 g, 2·65 mmol) in tetrahydrofuran (1 ml), and isobutyl chloroformate (0·355 g, 2·46 mmol) in tetrahydrofuran (1 ml) were successively added to a solution of Boc-Ser(PO_3Ph_2)-OH (1·16 g, 2·65 mmol) in tetrahydrofuran (8 ml) at −20°. After an activation period of 3 min, a solution of the tetrapeptide trifluoroacetate (12) (1·89 mmol) and *N*-methylmorpholine (0·191 g, 1·89 mmol) in tetrahydrofuran (5 ml) was added to the reaction mixture and the resulting solution was stirred for 2 h at −20°. A solution of 5% $NaHCO_3$ (5 ml) was added and the solution was stirred for a further 30 min at 0°. Warm ethyl acetate (60 ml) was then added and the organic phase was washed with 5% $NaHCO_3$ (2×30 ml) and 1 M HCl (2×30 ml), and then dried (Na_2SO_4) and filtered. The solvent was then evaporated under reduced pressure, and the solid residue was triturated with diethyl ether (2×30 ml) and dried under high vacuum to give pentapeptide (13) (2·75 g, 96%) as a white solid, m.p. 120–123°. δ (^{13}C) ($CDCl_3$) 25·5, NHMe; 27·2, 27·3, $Glu^{4,5}$ Cβ; 28·0, Boc Me; 30·0, $Glu^{4,5}$ Cγ; 52·0, 52·1, $Glu^{4,5}$ Cα; 52·8, d, $J_{P,C}$ 8·8 Hz, $Ser^{2,3}$ Cα; 54·5, d, $J_{P,C}$ 5·5 Hz, Ser^1 Cα; 65·4, $Glu^{4,5}$ Bzl CH_2; 67·8, br d, $Ser^{1,2,3}$ Cβ; 78·2, Boc **C**Me_3; 119·9, d, $J_{P,C}$ 4·9 Hz, $Ser^{1,2,3}$ Ph C2; 125·5, Ph C4; 127·9, 128·3, Bzl C2,3,4; 129·9, Ph C3; 136·1, $Glu^{4,5}$ Bzl C1; 149·9, d, $J_{P,C}$ 7·3 Hz, Ph C1; 155·9, urethane CO; 167·4, 167·8, 168·5, $Ser^{1,2,3}$ CO; 170·4, 171·0, $Glu^{4,5}$ CO; 172·0, 172·1, $Glu^{4,5}$ δ-CO. δ (^{31}P) ($CDCl_3$)−10·7, −11·1, −11·6. δ (^{31}P) [$(CD_3)_2SO$]−12·2. Amino acid analysis: Glu 1·96 (2), Ser 3·04 (3).

Boc-Leu-Ser(PO_3Ph_2)-Ser(PO_3Ph_2)-Ser(PO_3Ph_2)-Glu(OBzl)-Glu(OBzl)-NHMe (15)

N-Methylmorpholine (0·404 g, 4·00 mmol) in tetrahydrofuran (1 ml), and isobutyl chloroformate (0·501 g, 3·67 mmol) in tetrahydrofuran (1 ml) were successively added to a solution of Boc-Leu-OH.H_2O (0·996 g, 4·00 mmol) in tetrahydrofuran (7 ml) at −20°. After an activation period of 3 min, a solution of the pentapeptide trifluoroacetate (0·734 mmol) and *N*-methylmorpholine (0·074 g, 0·734 mmol) in dichloromethane (5 ml) was added to the reaction mixture and the resulting solution was stirred for 2 h at −20°. A solution of 5% $NaHCO_3$ (5 ml) was added and the solution was stirred for a further 30 min at 0°. Dichloromethane (60 ml) was then added and the organic phase was washed with 5% $NaHCO_3$ (2×30 ml) and 1 M HCl (2×30 ml), and then dried (Na_2SO_4) and filtered. The solution was

then concentrated under reduced pressure to a small volume and the peptide precipitated by the addition of ethyl acetate (10 ml). The solvent was then evaporated under reduced pressure and the solid residue triturated with diethyl ether (2×30 ml) and dried under high vacuum to give hexapeptide (15) (1·13 g, 94%) as a white solid, m.p. 115–117°. δ (^{13}C) [$(CD_3)_2SO$] 21·3, 22·9, Leu Cδ; 24·2, Leu Cγ; 25·5, NHMe; 27·2, 27·3, Glu^{5,6} Cβ; 28·1, Boc Me; 29·9, 30·0, Glu^{5,6} Cγ; 51·9, 52·2, Glu^{5,6} Cα; 52·5–53·2, Ser^{2,3,4} and Leu Cα; 65·4, Glu^{5,6} Bzl CH_2; 67·2–68·2, Ser^{2,3,4} Cβ; 78·1, Boc **C**Me$_3$; 119·9, d, $J_{P,C}$ 4·4 Hz, Ph C 2; 125·5, Ph C 4; 127·9, 128·3, Bzl C 2,3,4; 129·9, Ph C 3; 136·1, Glu^{5,6} Bzl C 1; 149·9, d, $J_{P,C}$ 6·6 Hz, Ph C 1; 155·3, urethane CO; 167·4, 167·7, 168·1, Ser^{2,3,4} CO; 170·4, 171·0, Glu^{5,6} CO; 172·0, 172·1, Glu^{5,6} δ-CO; 173·0, Leu CO. δ (^{31}P) [$(CD_3)_2SO$] −12·1. Amino acid analysis: Glu 1·95 (2), Ser 3·07 (3), Leu 0·98 (1).

Boc-Ser(PO_3Ph_2)-Leu-Ser(PO_3Ph_2)-Ser(PO_3Ph_2)-Ser(PO_3Ph_2)-Glu(OBzl)-Glu(OBzl)-NHMe (17)

N-Methylmorpholine (0·101 g, 1·00 mmol) in tetrahydrofuran (1 ml), and isobutyl chloroformate (0·127 g, 0·93 mmol) in tetrahydrofuran (1 ml) were successively added to a solution of Boc-Ser(PO_3Ph_2)-OH (0·437 g, 1·00 mmol) in tetrahydrofuran (8 ml) at −20°. After an activation period of 3 min, a solution of the hexapeptide trifluoroacetate (16) (0·65 mmol) and *N*-methylmorpholine (0·066 g, 0·65 mmol) in dichloromethane (5 ml) was added to the reaction mixture and the resulting solution was stirred for 2 h at −20°. A solution of 5% $NaHCO_3$ (5 ml) was added and the solution was stirred for a further 30 min at 0°. Dichloromethane (60 ml) was then added and the organic phase was washed with 5% $NaHCO_3$ (2×30 ml) and 1 M HCl (2×30 ml), and then dried (Na_2SO_4) and filtered. The solution was then concentrated under reduced pressure to a small volume and the peptide precipitated by the addition of ethyl acetate (10 ml). The solvent was then evaporated under reduced pressure and the solid residue was triturated with diethyl ether (2×30 ml) and then dried under high vacuum to give heptapeptide (17) (1·20 g, 95%) as a white solid, m.p. 123–125°. δ (^{13}C) [$(CD_3)_2SO$] 21·5, 23·0, Leu Cδ; 24·0, Leu Cγ; 25·5, NHMe; 27·2, 27·4, Glu^{6,7} Cβ; 28·0, Boc Me; 30·0, 30·1, Glu^{6,7} Cγ; 51·1, Leu Cα; 52·0, Glu^{6,7} Cα; 52·9, $J_{P,C}$ 8·8 Hz, Ser^{3,4,5} Cα; 54·5, $J_{P,C}$ 8·8 Hz, Ser^{1} Cα; 65·5, Glu^{6,7} Bzl CH_2; 67·5–68·3, Ser^{1,3,4,5} Cβ; 78·7, Boc **C**Me$_3$; 120·0, d, $J_{P,C}$ 4·4 Hz, Ph C 2; 125·5, Ph C 4; 127·9, 128·4, Bzl C 2,3,4; 130·0, Ph C 3; 136·1, Glu^{6,7} Bzl C 1; 149·9, d, $J_{P,C}$ 7·7 Hz, Ph C 1; 155·2, urethane CO; 167·4, 167·7, 168·0, 168·0, Ser^{1,3,4,5} CO; 170·4, 171·0, Glu^{6,7} CO; 172·0, Leu CO; 172·2, Glu^{6,7} δ-CO. δ (^{31}P) [$(CD_3)_2SO$] −12·1. Amino acid analysis: Glu 1·96 (2), Ser 4·06 (4), Leu 0·98 (1).

Boc-Glu(OBzl)-Ser(PO_3Ph_2)-Leu-Ser(PO_3Ph_2)-Ser(PO_3Ph_2)-Ser(PO_3Ph_2)-Glu(OBzl)-Glu(OBzl)-NHMe (19)

N-Methylmorpholine (0·277 g, 0·822 mmol) in tetrahydrofuran (1 ml), and isobutyl chloroformate (0·104 g, 0·703 mmol) in tetrahydrofuran (1 ml) were successively added to a solution of Boc-Glu(OBzl)-OH (0·277 g, 0·822 mmol) in tetrahydrofuran (8 ml) at −20°. After an activation period of 3 min, a solution of the heptapeptide trifluoroacetate (18) (0·587 mmol) and *N*-methylmorpholine (0·060 g, 0·587 mmol) in dichloromethane (5 ml) was added to the reaction mixture and the resulting solution was stirred for 2 h at −20°. $NaHCO_3$ (5%, 5 ml) was added and the solution was stirred for a further 30 min at 0°. Dichloromethane (60 ml) was then added and the organic phase was washed with 5% $NaHCO_3$ (2×30 ml) and 1 M HCl (2×30 ml), and then dried (Na_2SO_4) and filtered. The solution was then concentrated under reduced pressure to a small volume and the peptide precipitated by the addition of ethyl acetate (10 ml). The solvent was then evaporated under reduced pressure and the white solid was triturated with diethyl ether (2×30 ml) and then dried under high vacuum to give octapeptide (19) (1·265 g, 99%) as a white solid, m.p. 129–131°. δ (^{13}C) [$(CD_3)_2SO$] 21·3, 23·0, Leu Cδ; 24·0, Leu Cγ; 25·5, NHMe; 27·2, Glu^{1,7,8} Cβ; 28·1, Boc Me; 30·0, Glu^{1,7,8} Cγ; 51·2, Leu Cα; 52·0, 52·3, Glu^{7,8} Cα; 52·0–53·3, Ser^{2,4,5,6} Cα; 53·6, Glu^{1} Cα; 65·4, Glu^{1,7,8} Bzl CH_2; 67·3–68·1, Ser^{2,4,5,6} Cβ; 78·4, Boc **C**Me$_3$; 119·9, d, $J_{P,C}$ 4·4 Hz, Ph C 2; 125·5, Ph C 4; 127·9, 128·3, Bzl C 2,3,4; 129·9, Ph C 3; 136·1, Glu^{1,7,8} Bzl C 1; 149·9, d, $J_{P,C}$ 7·7 Hz, Ph C 1; 155·3, urethane CO; 167·4, 167·6, 167·7, 168·1, Ser^{2,4,5,6} CO; 170·4, 171·0, Glu^{7,8} CO; 171·8, Glu^{1} CO; 172·0, Leu CO; 172·1, Glu^{1,7,8} δ-CO. δ (^{31}P) [$(CD_3)_2SO$] −12·1. Amino acid analysis: Glu 3·01 (3), Ser 3·99 (4), Leu 1·00 (1).

Ac-Glu(OBzl)-Ser(PO_3Ph_2)-Leu-Ser(PO_3Ph_2)-Ser(PO_3Ph_2)-Ser(PO_3Ph_2)-Glu(OBzl)-Glu(OBzl)-NHMe (21)

N-Methylmorpholine (0·303 g, 3·00 mmol) in tetrahydrofuran (1 ml), and isobutyl chloroformate (0·381 g, 2·79 mmol) in tetrahydrofuran (1 ml) were successively added to a solution of acetic acid (0·180 g, 3·00 mmol) in tetrahydrofuran (8 ml) at −20°. After an activation period of 3 min, a solution of the octapeptide trifluoroacetate (20) (0·558 mmol) and *N*-methylmorpholine (0·056 g, 0·558 mmol) in dichloromethane (5 ml) was added to the reaction mixture and the resulting solution was stirred for 2 h at −20°. $NaHCO_3$ (5%, 5 ml) was added and the solution was stirred for a further 30 min at 0°. Dichloromethane (60 ml) was then added and the organic phase was washed with 5% $NaHCO_3$ (2×30 ml) and 1 M HCl (2×30 ml), and then dried (Na_2SO_4) and filtered. The solution was then concentrated under reduced pressure to a small volume and the peptide precipitated by the addition of ethyl acetate (10 ml). The solvent was then evaporated under reduced pressure and the solid residue was triturated with diethyl ether (2×30 ml) and then dried under high vacuum to give *octapeptide* (21) (1·11 g, 94%) as a white solid, m.p. 140–142° (Found: P, 6·5. $C_{105}H_{113}N_9O_{31}P_4$ requires P, 6·0%). δ (^{13}C) [$(CD_3)_2SO$] 21·3, 22·4, Leu Cδ; 23·1, acetyl Me; 24·0, Leu Cγ; 25·6, NHMe; 27·2, $Glu^{1,7,8}$ Cβ; 30·1, $Glu^{1,7,8}$ Cγ; 51·2, Leu Cα; 52·1, $Glu^{1,7,8}$ Cα; 52·9, $J_{P,C}$ 7·7 Hz, $Ser^{2,4,5,6}$ Cα; 65·5, $Glu^{1,7,8}$ Bzl CH_2; 67·4–68·0, $Ser^{2,4,5,6}$ Cβ; 120·0, d, $J_{P,C}$ 4·4 Hz, Ph C2; 125·5, Ph C4; 127·9, 128·4, Bzl C2,3,4; 130·0, Ph C3; 136·1, $Glu^{1,7,8}$ Bzl C1; 149·9, d, $J_{P,C}$ 7·7 Hz, Ph C1; 167·4, 167·68, 167·73, 168·2, $Ser^{2,4,5,6}$ CO; 169·7, acetyl CO; 170·4, 171·1, $Glu^{7,8}$ CO; 171·7, Glu^1 CO; 172·1, Leu CO; 172·2, $Glu^{1,7,8}$ δ-CO. δ (^{31}P) ($CDCl_3$) −11·0, −12·9, −13·0, −13·2. δ (^{31}P) [$(CD_3)_2SO$] −12·4. Amino acid analysis: Glu 3·01 (3), Ser 3·98 (4), Leu 1·00 (1).

Ac-Glu-Ser(P)*-Leu-Ser*(P)*-Ser*(P)*-Ser*(P)*-Glu-Glu-NHMe (1)*

A rapidly stirred solution of octapeptide (0·212 g, 0·1 mmol) (21) in 50% CF_3CO_2H/CH_3CO_2H (4 ml) containing 80% platinum oxide (0·25 g, 0·88 mmol) was hydrogenated at atmospheric pressure until hydrogen uptake ceased (60 min). The platinum was removed by gravity filtration, washed with 50% CF_3CO_2H/CH_3CO_2H (4 ml), and the solvent was evaporated under reduced pressure. The residue was triturated with diethyl ether (3×20 ml) and the white solid was lyophilized from water to give pure octapeptide (1) (0·123 g, 99%) as a fluffy white solid. δ (^{13}C) (D_2O) (400 MHz) 20·50, 22·25, Leu Cδ; 21·69, acetyl Me; 24·15, Leu Cγ; 25·74, 25·84, 25·98, 26·13, NHMe and $Glu^{1,7,8}$ Cβ; 29·94, $Glu^{1,7,8}$ Cγ; 39·54, Leu Cβ; 52·36, Leu Cα; 53·13, 53·18, 53·29, $Glu^{1,7,8}$ Cα; 53·90, 54·08, 54·82, 54·95, doublets, $J_{P,C}$ 7·3, 8·8, 7·3, 7·3 Hz, respectively, $Ser^{2,4,5,6}$ Cα; 64·05–64·20, m, $Ser^{4,5,6}$ Cβ; 64·27, d, $J_{P,C}$ 8·8 Hz, Ser^2 Cβ; 170·65, 170·94, 171·31, 171·37, $Ser^{2,4,5,6}$ CO; 172·97, acetyl CO; 173·35, 173·56, $Glu^{7,8}$ CO; 174·45, Glu^1 CO; 174·59, Leu CO; 176·80, 176·86, 176·88, $Glu^{1,7,8}$ δ-CO. δ (^{31}P) (D_2O) −0·1. Amino acid analysis: Glu 2·99 (3), Ser 3·99 (4), Leu 1·03 (1).

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