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Synthesis of Casein-Related Peptides and Phosphopeptides. V* The Efficient Global 'Phosphite-Triester' Phosphorylation of Protected Serine Derivatives and Peptides by Using Dibenzyl or Di-t-butyl *N,N*-Diethylphosphoramidite†

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Abstract

Both dibenzyl *N,N*-diethylphosphoramidite and di-t-butyl *N,N*-diethylphosphoramidite are shown to be suitable reagents for the efficient 1*H*-tetrazole-catalysed 'phosphite-triester' phosphorylation of the protected serine derivative Boc-Ser-$OCH_2C_6H_4NO_2$-*p*. Both these reagents were also used for the phosphorylation of the protected serine derivative Boc-Glu(OBu^t)-Ser-Leu-OBu^t, and subsequent hydrogenolytic treatment of Boc-Glu(OBu^t)-Ser(PO_3Bzl_2)-Leu-OBu^t or acidolytic treatment of Boc-Glu(OBu^t)-Ser($PO_3Bu^t_2$)-Leu-OBu^t gave the *O*-phosphoseryl tripeptide Glu-Ser(PO_3H_2)-Leu in near-quantitative yield.

Introduction

Since 1957, the preparation of phosphorylated hydroxy-containing biomolecules has generally been approached by the 'global' phosphorylation of the hydroxy-containing biomolecule with dibenzyl or diphenyl phosphorochloridate/pyridine followed by hydrogenolytic removal of the protecting groups.[2,3] However, while the hydrogenolytic removal of benzyl groups is generally facile, the synthetic procedure suffers from the low phosphorylative reactivity of dibenzyl phosphorochloridate. In Part IV,[4] we reported the use of diethyl *N,N*-diethylphosphoramidite for the 'phosphite-triester' phosphorylation of Ac-Ser-NHMe and the isolation of Ac-Ser(PO_3Et_2)-NHMe in 96% yield. In view of the marked efficiency of this phosphorylation procedure, we realised that a benzyl or t-butyl based phosphorylation procedure would provide a ready entry to *O*-dihydrogen phosphates; benzyl and t-butyl groups being of particular synthetic use on account of their ready and quantitative removal from dibenzyl

* Part IV, *Aust. J. Chem.*, 1990, **43**, 1609.
† This work has appeared in a preliminary communication.[1]

[1] Perich, J. W., and Johns, R. B., *Tetrahedron Lett.*, 1988, **29**, 2369.
[2] Khwaja, T. A., Reese, C. B., and Stewart, J. C. M., *J. Chem. Soc. C*, 1970, 2092.
[3] Fölsch, G., *Sven. Kem. Tidskr.*, 1967, **79**, 38.
[4] Perich, J. W., and Johns, R. B., *Aust. J. Chem.*, 1990, **43**, 1609.

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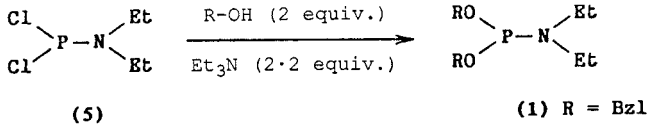
and di-t-butyl phosphorotriesters by hydrogenolysis or acidolysis respectively. We first described the use of dibenzyl *N,N*-diethylphosphoramidite (1) for the 'phosphite-triester' phosphorylation of hydroxy-containing biomolecules in 1982[5] and subsequently employed this procedure for the phosphorylation of protected serine, threonine and tyrosine derivatives[6] and simple alcohol derivatives.[7] Also, we described the first preparation of di-t-butyl *N,N*-diethylphosphoramidite[6] (2) in 1986 and later reported its use for the efficient phosphorylation of several simple alkyl and aryl alcohols,[8] the t-butyl phosphate groups being removed from the isolated di-t-butyl phosphorotriesters by very mild acidolysis. In this paper, we report on the use of dibenzyl and di-t-butyl *N,N*-diethylphosphoramidite for the efficient 'phosphite-triester' phosphorylation of Boc-Ser-$OCH_2C_6H_4NO_2$-*p* (3) and Boc-Glu(OBu^t)-Ser-Leu-OBu^t (4) and, for this latter tripeptide, describe the ready deprotection of the protected Ser(PO_3Bzl_2)- or Ser($PO_3Bu^t_2$)-peptide to the *O*-phosphoserine peptide.

Results and Discussion

The key phosphoramidite reagents, dibenzyl *N,N*-diethylphosphoramidite and di-t-butyl *N,N*-diethylphosphoramidite, were prepared in high yield by the low-temperature treatment of dichloro *N,N*-diethylphosphoroamidous acid (5) with 2 equiv. of benzyl alcohol or t-butyl alcohol respectively in the presence of excess triethylamine (Scheme 1). This procedure is superior to a previously reported preparation of dibenzyl *N,N*-diethylphosphoramidite which was performed by the less efficient thermal transesterification of tris(*N,N*-diethyl)phosphoramidite with benzyl alcohol.[9] The advantages of the former procedure are that yields are higher, the use of dichloro *N,N*-diethylphosphoroamidous acid as a common precursor permits the synthesis of a range of dialkyl *N,N*-diethylphosphoramidites, and the low-temperature esterification procedure minimizes side-product formation.



(5)

(1) R = Bzl

(2) R = Bu^t

Scheme 1

In phosphorylation studies, the treatment of a dry tetrahydrofuran solution of Boc-Ser-$OCH_2C_6H_4NO_2$-*p* (3) and 1*H*-tetrazole with dibenzyl *N,N*-diethylphosphoramidite followed by the low-temperature oxidation of the resultant phosphite-triester with *m*-chloroperoxybenzoic acid[10] gave the seryl

[5] Alewood, P. F., Johns, R. B., Kemp, B. E., Perich, J. W., and Valerio, R. M., presented at the Seventh National R.A.C.I. Convention, Canberra, A.C.T., 26 August 1982.
[6] Perich, J. W., Ph.D. Dissertation, University of Melbourne, 1986.
[7] Perich, J. W., and Johns, R. B., *Tetrahedron Lett.*, 1987, **28**, 101.
[8] Perich, J. W., and Johns, R. B., *Synthesis*, 1988, 142.
[9] Smirnova, L. I., Malenkovskaya, D. A., Predvoditelev, D. A., and Nifant'ev, E. E., *Zh. Org. Khim.*, 1980, **16**, 1170.
[10] Ogilvie, K. K., and Nemer. M. J., *Tetrahedron Lett.*, 1981, **22**, 2531.

dibenzyl phosphorotriester, Boc-Ser(PO_3Bzl_2)-$OCH_2C_6H_4NO_2$-*p* (6), as a light yellow oil in 98% yield.[6] Quantitative phosphorylation of the seryl hydroxy group was readily established by ^{13}C n.m.r. spectroscopy, the ^{13}C n.m.r. spectrum showing phosphorus-coupled doublet signals for the seryl carbons Cα and Cβ at 54·0 ($J_{P,C}$ 5·9 Hz) and 67·2 ($J_{P,C}$ 5·9 Hz) respectively. Likewise, the treatment of Boc-Ser-$OCH_2C_6H_4NO_2$-*p* (3) with di-t-butyl *N,N*-diethylphosphoramidite gave the seryl di-t-butyl phosphorotriester, Boc-Ser($PO_3Bu^t_2$)-$OCH_2C_6H_4NO_2$-*p* (7), as a white solid in 92% yield. In addition to phosphorus-coupled doublet signals for the seryl carbons Cα and Cβ, phosphorus-coupled doublet signals were also observed at 54·3 ($J_{P,C}$ 5·9 Hz) and 66·3 ($J_{P,C}$ 5·9 Hz) ppm for the phosphorotriester t-butyl Me and quaternary carbons respectively. The high yielding preparation of Boc-Ser($PO_3Bu^t_2$)-$OCH_2C_6H_4NO_2$-*p* by the use of reagent (2) presents a significant synthetic development in the preparation of di-t-butyl phosphorotriesters, especially in consideration that the conventional phosphorylation of alcohols with di-t-butyl phosphorobromidate is not an efficient synthetic procedure.

While iodosylbenzene, nitric oxide, nitrogen dioxide and dinitrogen tetroxide have previously been used for oxidation of various alkyl phosphite triesters,[9] *m*-chloroperoxybenzoic acid is the oxidant of choice on account of its ready commerical availability, ease of handling and high reactivity. Furthermore, the use of iodine/water is unsuitable for the oxidation of benzyl and t-butyl phosphites since this oxidation process causes extensive benzyl and t-butyl cleavage. The isolation of benzyl iodide (70%), Boc-Ser[PO_3(Bzl)H]-$OCH_2C_6H_4NO_2$-*p* (8) (65%) and Boc-Ser(PO_3Bzl_2)-$CH_2C_6H_4NO_2$-*p* (24%) from the (i) (1)/1*H*-tetrazole, (ii) iodine/water oxidation indicates that the formation of the iodo phosphonium iodide activates the benzyl group to iodide-mediated debenzylation and this process is competitive to the addition of water (Scheme 2).

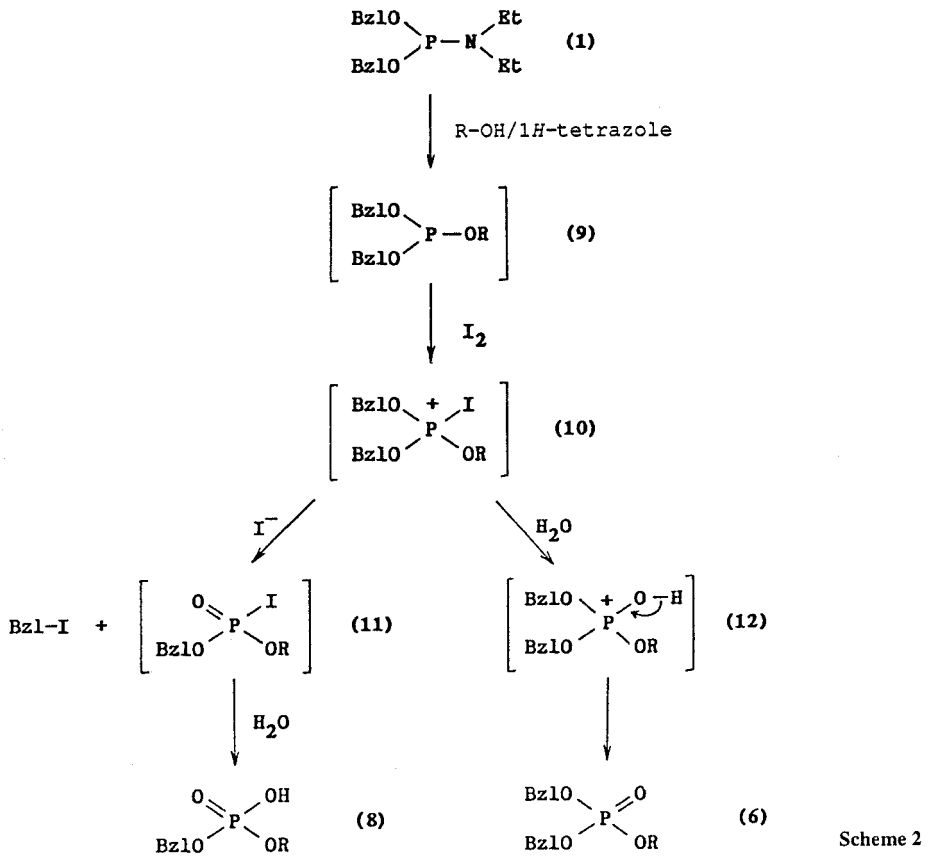
In the oxidation of di-t-butyl phosphite-triesters with iodine/water, complete de-t-butylation occurs and this is interpreted to be due to cleavage of the activated t-butyl group of the di-t-butyl iodo phosphonium iodide. In contrast to the indirect introduction of oxygen to phosphite triesters by using iodine/water, the success of *m*-chloroperoxybenzoic acid as oxidant is presumed to be due to the direct introduction of oxygen to the phosphite triester under non-nucleophilic conditions (Scheme 3).

In order to evaluate the applicability of this procedure for the phosphorylation for seryl-containing peptides, the synthesis of the peptide H-Glu-Ser(PO_3H_2)-Leu-OH (19) was undertaken. The synthesis of this peptide was approached by the initial synthesis of the protected seryl-peptide Boc-Glu(OBu^t)-Ser-Leu-OBu^t, 'phosphite-triester' phosphorylation of the seryl hydroxy group by using (1) or (2) followed by peptide deprotection.

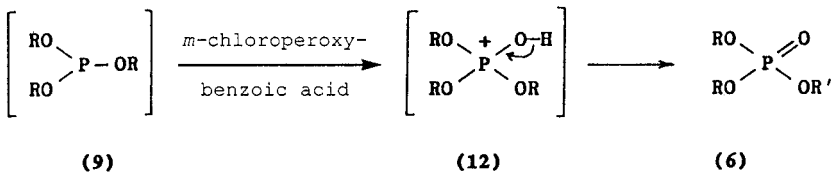
The synthesis of Boc-Glu(OBu^t)-Ser-Leu-OBu^t was accomplished by the use of Fmoc-Ser(Bzl)-OH[11]* (20) and the selection of global t-butyl protection, these groups offering facile acidolytic deprotection. Thus, the mixed anhydride coupling of Fmoc-Ser(Bzl)-OH with H-Leu-OBu^t.AcOH (13) gave the dipeptide Fmoc-Ser(Bzl)-Leu-OBu^t (14) in 86% yield. The Fmoc group was cleaved from

* Fmoc represents fluorenylmethoxycarbonyl.

[11] Lapatsanis, L., Milias, G., Proussios, K., and Kolovos, M., *Synthesis*, 1983, 671.



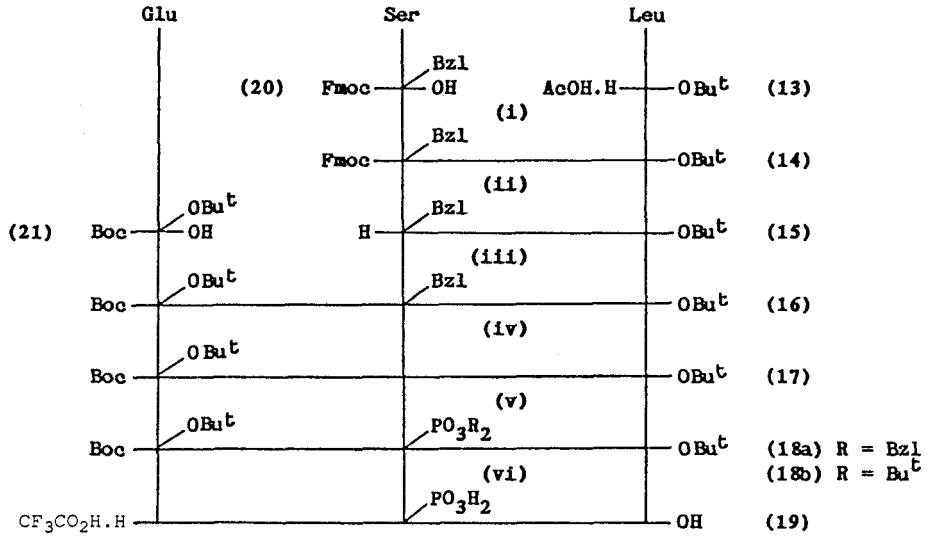
Scheme 2



Scheme 3

dipeptide (14) by the use of 10% Et_2NH/dimethylformamide[12] (2 h, 20°C), and subsequent coupling of the resultant deprotected dipeptide (15) with the mixed anhydride of Boc-Glu(OBu^t)-OH (21) gave the tripeptide Boc-Glu(OBu^t)-Ser(Bzl)-Leu-OBu^t (16) as a white solid in 82% yield. Removal of the seryl benzyl group was effected by palladium-catalysed hydrogenolysis of tripeptide (16) in acetic acid, the serine-tripeptide (17) being obtained in 99% yield (Scheme 4).

[12] Bodanszky, A., Bodanszky, M., Chandramouli, N., Kwei, J. Z., Martinez, J., and Tolle, J. C., *J. Org. Chem.*, 1980, **42**, 72.



Scheme 4. Reagents: (i) *N*-Methylmorpholine/isobutyl chloroformate (–20°C), then amino acid (13) and *N*-methylmorpholine (1 equiv.); (ii) 10% Et_2NH/dimethylformamide (20°C, 2 h); (iii) *N*-methylmorpholine/isobutyl chloroformate (–20°C), then dipeptide (15); (iv) H_2, 10% Pd/C, AcOH; (v) $(RO)_2PNEt_2$ (R = Bzl or Bu^t)/1*H*-tetrazole (15 min), then *m*-chloroperoxybenzoic acid (–40°C, 5 min); (vi) for (18a) H_2, 10% Pd/C, 40% CF_3CO_2H/AcOH, followed by CF_3CO_2H (20°C, 1 h), or for (18b) 95% CF_3CO_2H/AcOH (20°C, 1 h).

The 'phosphite-triester' phosphorylation of tripeptide (17) with dibenzyl *N,N*-diethylphosphoramidite (1) and subsequent *in situ* oxidation of the resultant phosphite-triester with *m*-chloroperoxybenzoic acid gave the phosphorylated tripeptide Boc-Glu(OBu^t)-Ser(PO_3Bzl_2)-Leu-OBu^t (18a) in 96% yield. The ^{13}C n.m.r. spectrum displayed the characteristic phosphorus-coupled doublets for the carbons Cα and Cβ of the Ser(PO_3Bzl_2) residue at 53·1 and 66·7 (both $J_{P,C}$ 5·9 Hz) respectively, and showed clearly resolved Glu and Leu Cα signals at 54·8 and 51·7 ppm respectively. The ^{31}P n.m.r. spectrum contained a single peak at –0·8 ppm which is typical for alkyl dibenzyl phosphorotriesters. Palladium-catalysed hydrogenolysis of tripeptide (18a) in 40% CF_3CO_2H/AcOH readily cleaved the benzyl and t-butyl groups and gave H-Glu-Ser(PO_3H_2)-Leu-OH.CF_3CO_2H (19) as white flakes in 99·5% yield. The ^{31}P n.m.r. spectrum of tripeptide (19) gave a single peak at +0·10 ppm while its ^{13}C n.m.r. spectrum showed the phosphorus-coupled -Ser(PO_3H_2)- Cα and Cβ signals at 54·2 and 64·2 and the Glu and Leu Cα signals at 52·3 and 51·6 respectively. Structural confirmation of tripeptide (19) was established by fast atom bombardment (f.a.b.) mass spectrometry, the f.a.b. mass spectrum displaying a high intensity molecular ion at *m/z* 428 and subsequent peaks corresponding to fragments formed from successive amido cleavage.[13] In addition, the loss of 98 mass units (*m/z* 299–201) corresponds to loss of the seryl phosphate group, and this fragmentation has been observed to be a diagnostic fragmentation for several Ser(PO_3H_2)-containing peptides examined.

[13] Johns, R. B., Alewood, P. F., Perich, J. W., Chaffee, A. L., and MacLeod, J. K., *Tetrahedron Lett.*, 1986, **27**, 4791.

Complementary to the above approach, the 'phosphite-triester' phosphorylation of Boc-Glu(OBu^t)-Ser-Leu-OBu^t (17) with (2) and subsequent *m*-chloroperoxybenzoic acid oxidation gave the tripeptide Boc-Glu(OBu^t)-Ser(PO$_3$Bu$^t_2$)-Leu-OBu^t (18b) as a white solid in 95% yield. In the case of this peptide, the ^{31}P n.m.r. spectrum contained a single peak at −9·2 ppm which is consistent with chemical shifts reported for various alkyl di-t-butyl phosphorotriesters. The ^{13}C n.m.r. spectrum of the Ser(PO$_3$Bu$^t_2$)-tripeptide contained phosphorus-coupled doublet signals for the Ser carbons Cα and Cβ at 53·2 ($J_{P,C}$ 5·5 Hz) and 65·8 ($J_{P,C}$ 5·5 Hz) ppm, and showed phosphorus-coupled doublet signals for the t-butyl phosphorotriester methyl and quaternary carbons at 29·7 ($J_{P,C}$ 3·3 Hz) and 83·2 ($J_{P,C}$ 7·7 Hz) respectively. Subsequent acidolytic treatment of tripeptide (18b) with 95% CF_3CO_2H/AcOH (1 h, 20°C) readily cleaved the t-butyl groups and gave H-Glu-Ser(PO$_3$H$_2$)-Leu-OH.CF_3CO_2H (19) in quantitative yield. Tripeptide (19) was established to be homogeneous by C_{18} r.p.–h.p.l.c. with 0·1% CF_3CO_2H/10% CH_3CN as the mobile phase. A feature in the use of t-butyl phosphate protection is that these groups are particularly sensitive to mild acidolysis and that the isolation of the Ser(PO$_3$H$_2$)-peptide is simple and is performed under a metal-free environment.

The major advantages of this 'phosphite-triester' phosphorylation procedure described above are that both phosphoramidites (1) and (2) (i) are easy to prepare, (ii) are stable when stored under nitrogen at low temperature, (iii) exhibit high reactivity on 1*H*-tetrazole activation, (iv) the phosphite-triester is quantitatively converted into the phosphorotriester on oxidation with *m*-chloroperoxybenzoic acid, and (v) product yields are high. In view of the high efficiency of this phosphoramidite 'phosphite-triester' phosphorylation procedure, we consider that this procedure will provide much synthetic flexibility in the preparation of Ser(*P*)-containing peptides and other phosphorylated biomolecules.

Experimental

^{1}H and ^{13}C n.m.r. spectra were recorded on a JEOL FX-90Q Fourier transform instrument operating at 89·55 and 22·50 MHz respectively with chemical shifts reported in δ relative to external tetramethylsilane for $CDCl_3$ solutions, and internal dioxan set to δ 66·5 for D_2O solutions. ^{31}P n.m.r. spectra were recorded on a JEOL FX-100 Fourier transform instrument operating at 40·26 MHz with chemical shifts reported in δ relative to external 85% H_3PO_4. F.a.b. mass spectra were obtained on a JEOL DX-300 mass spectrometer equipped with an f.a.b. source and used argon as ionization gas. All solvents used were of AnalaR grade.

Dibenzyl N,N-*Diethylphosphoramidite (1)*

A solution of dichloro *N,N*-diethylphosphoramidous acid (11·57 g, 66·5 mmol) in diethyl ether (20 ml) was added to a stirred solution of benzyl alcohol (14·36 g, 133·0 mmol) and triethylamine (14·78 g, 146·3 mmol) in dry diethyl ether (40 ml) at −20° such that the reaction solution was kept below 0°. After stirring for 2 h at −20°, 5% $NaHCO_3$ (50 ml) and diethyl ether (100 ml) were added and the organic phase was washed with 5% $NaHCO_3$ (1×30 ml) and saturated NaCl (1×30 ml), dried (Na_2SO_4) and filtered. Evaporation of the solvent under reduced pressure gave a liquid residue which on distillation gave the title compound (1) as a clear, colourless liquid (18·8 g, 89%), b.p. 150–152°/0·01 mmHg (lit.[9] 131–132°/10^{-4} mmHg). δ (^{13}C) ($CDCl_3$) 14·6, d, $J_{P,C}$ 2·9 Hz, NCH_2**Me**; 36·3, d, $J_{P,C}$ 20·5 Hz, N**C**H$_2$Me; 64·7, d, $J_{P,C}$ 16·1 Bzl CH_2; 126·5, Bzl C2; 127·7, Bzl C3 and C4; 138.8, d, $J_{P,C}$ 5·9 Hz, Bzl C1. δ (^{31}P) ($CDCl_3$) +148·6.

N^{α}-(t-Butoxycarbonyl)-O-(dibenzylphosphono)serine 4-Nitrobenzyl Ester (6), Boc-Ser(PO_3Bzl_2)-$OCH_2C_6H_4NO_2$-p

1*H*-Tetrazole (1·05 g, 15·00 mmol) was added to a stirred solution of Boc-Ser-$OCH_2C_6H_4NO_2$-*p* (3) (1·70 g, 5·00 mmol) and dibenzyl *N,N*-diethylphosphoramidite (2·20 g, 7·00 mmol) in tetrahydrofuran (5 ml) and stirred at 20° for 15 min. The solution was then cooled to –40° and a solution of *m*-chloroperoxybenzoic acid (1·46 g, 7·00 mmol) in dichloromethane (7 ml) was added such that the temperature was maintained below –20°. After stirring for 15 min, 10% $Na_2S_2O_5$ (10 ml) and diethyl ether (50 ml) were added and the organic phase was washed with 10% $Na_2S_2O_5$ (25 ml), 5% $NaHCO_3$ (2×25 ml) and 1 M HCl (1×15 ml), dried (Na_2SO_4) and filtered. Evaporation of the solvent under reduced pressure followed by trituration of the residual oil with hexane (3×40 ml) gave Boc-Ser(PO_3Bzl_2)-$OCH_2C_6H_4NO_2$-*p* (6) as a light yellow oil (2·94 g, 98%). Recrystallization of a portion of the oil (0·5 g) from diethyl ether/hexane gave (6) as white solid, m.p. 50–51° (lit.[14] 50°).

N^{α}-(t-Butoxycarbonyl)-O-(di-t-butylphosphono)serine 4-Nitrobenzyl Ester (7), Boc-Ser($PO_3Bu^t_2$)-$OCH_2C_6H_4NO_2$-p

1*H*-Tetrazole (1·05 g, 15·00 mmol) was added to a stirred solution of Boc-Ser-$OCH_2C_6H_4NO_2$-*p* (3) (1·70 g, 5·00 mmol) and di-t-butyl *N,N*-diethylphosphoramidite (1·74 g, 7·00 mmol) in tetrahydrofuran (5 ml) and stirred at 20° for 15 min. The solution was then cooled to –40° and a solution of *m*-chloroperoxybenzoic acid (1·46 g, 7·00 mmol) in dichloromethane (7 ml) was added such that the temperature was maintained below –20°. After stirring for 15 min, 10% $Na_2S_2O_5$ (10 ml) and diethyl ether (50 ml) were added and the organic phase was washed with 10% $Na_2S_2O_5$ (25 ml), 5% $NaHCO_3$ (2×25 ml) and 1 M HCl (1×15 ml), dried (Na_2SO_4) and filtered. Evaporation of the solvent under reduced pressure gave an oily residue which solidified on standing. The serine derivative was separated from the phosphorylation by-products by repeated trituration of the residue with warm hexane. The hexane was evaporated under reduced pressure to give pure Boc-Ser($PO_3Bu^t_2$)-$OCH_2C_6H_4NO_2$-*p* (7) as a white solid (2·50 g, 94%). Recrystallization of the solid from hexane gave Boc-Ser($PO_3Bu^t_2$)-$OCH_2C_6H_4NO_2$-*p* (7) (2·20 g, 83%) as white needles, m.p. 70–72°, $[\alpha]_D^{14}$ –9·47° (*c*, 1 in $CHCl_3$) (Found: C, 51·9; H, 7·0; N, 5·3; P, 5·8. $C_{23}H_{37}N_2O_{10}P_1$ requires C, 51·7; H, 7·3; N, 5·7; P, 6·2%). δ (^{1}H) ($CDCl_3$) 1·44, s, 9H, Boc Me; 1·44, d, 18H, $J_{P,H}$ 0·49 Hz, $POBu^t$ Me; 4·00–4·70, m, 3H, Ser α-CH and β-CH_2; 5·25, s, 2H, $CH_2C_6H_4NO_2$-*p*; 5·52, d, 1H, $J_{P,H}$ 7·2 Hz, Ser NH; 7·45 and 8·20, AA′XX′ system, each 2H, $J_{AX'} = J_{A'X}$ 8·80 Hz, $CH_2C_6H_4NO_2$-*p* H2 and H3. δ (^{13}C) ($CDCl_3$) 28·3, Boc Me; 29·7, $POBu^t$ Me; 54·3, d, $J_{P,C}$ 5·9 Hz, Ser Cα; 65·7, $\mathbf{C}H_2C_6H_4NO_2$-*p*; 66·3; d, $J_{P,C}$ 5·9 Hz, Ser Cβ; 80·1, Boc $\mathbf{C}Me_3$; 83·2, d, $J_{P,C}$ 5·9 Hz, $POBu^t$ $\mathbf{C}Me_3$; 123·7, $CH_2C_6H_4NO_2$-*p* C2; 128·3, $CH_2C_6H_4NO_2$-*p* C3; 142·6, $CH_2C_6H_4NO_2$-*p* C1; 147·7, $CH_2C_6H_4NO_2$-*p* C4; 155·3, Boc CO; 169·4, Ser CO. δ (^{31}P) ($CDCl_3$) –10·0.

N^{α}-(Fluorenylmethoxycarbonyl)-O-(benzyl)serylleucine t-Butyl Ester (14), Fmoc-Ser(Bzl)-Leu-OBu^t

N-Methylmorpholine (0·354 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 Fmoc-Ser(Bzl)-OH (20) (1·460 g, 3·50 mmol) in tetrahydrofuran (7 ml) at –20°. After an activation period of 3 min, a solution of H-Leu-OBu^t.AcOH (13) (0·578 g, 2·50 mmol) and *N*-methylmorpholine (0·250 g, 2·50 mmol) in tetrahydrofuran (4 ml) was added to the reaction mixture at –20° and then stirred for 2 h at –20° prior to the addition of 5% $NaHCO_3$ (2×30 ml) and 1 M HCl (2×30 ml), dried (Na_2SO_4) and filtered. Evaporation of the solvent under reduced pressure gave dipeptide (14) as a white solid (1·26 g, 86%), m.p. 128–130°, $[\alpha]_D^{27}$ +14·8° (*c*, 1 in $CHCl_3$). δ (^{1}H) ($CDCl_3$) 0·90, br d, 6H, Leu δ-Me; 1·45, s, 9H, Bu^t Me; 1·40–1·80, m, 3H, Leu β-CH_2 and γ-CH; 3·40–4·14, m, 2H, Ser β-CH_2; 4·20–4·60, m, 5H, Ser α-CH, Leu α-CH, Fmoc CH and CH_2; 4·58, s, 2H, Bzl CH_2; 5·70, d, 1H, $J_{H,H}$ 7·47 Hz, Ser NH; 6·94, d, 1H, $J_{H,H}$ 7·69 Hz, Leu NH; 7·33, s, 5H, Bzl Ar H; 7·20–7·85, m, 8H, Fmoc Ar H.

[14] Alewood, P. F., Perich, J. W., and Johns, R. B., *Aust. J. Chem.*, 1984, **37**, 429.

δ (^{13}C) ($CDCl_3$) 21·88 and 22·66, Leu Cδ; 24·71, Leu Cγ; 27·82, Bu^t Me; 41·42, Leu Cβ; 46·93, Fmoc CH_2; 51·51, Leu Cα; 53·80, Ser Cα; 67·11, Fmoc CH; 67·79, Ser Cβ; 73·39, Ser Bzl CH_2; 81·63, **C**Me_3; 119·84, 124·96, 126·91 and 128·32, Fmoc Ar C; 127·54 and 127·73, Bzl Ar C; 137·19, Bzl Ar C1; 141·09 and 143·57, Fmoc Ar C1 and C2; 155·86, Boc CO; 169·50, Leu CO; 171·40, Ser CO.

N$^\alpha$-(t-Butoxycarbonyl)-O-(t-butyl)glutamyl-O-(benzyl)serylleucine t-Butyl Ester (16), Boc-Glu(OBu^t)-Ser(Bzl)-Leu-OBu^t

Dipeptide (14) (1·17 g, 2·00 mmol) was dissolved in 10% diethylamine/dimethylformamide (2 ml) and stirred for 2 h at 20°. The solvent was evaporated under reduced pressure and the resultant residue then dried under high vacuum.

N-Methylmorpholine (0·280 g, 2·80 mmol) in tetrahydrofuran (1 ml) and isobutyl chloroformate (0·355 g, 2·60 mmol) in tetrahydrofuran (1 ml) were successively added to a solution of Boc-Glu(OBu^t)-OH (21) (0·848 g, 2·80 mmol) in tetrahydrofuran (6 ml) at –20°. After an activation period of 3 min, a solution of the above deprotected dipeptide (15) (2·00 mmol) in dimethylformamide (3 ml) was added to the coupling solution at –20°. After stirring for 2 h at –20°, 10% Na_2CO_3 (2 ml) was added, the solution stirred for a further 30 min and then transferred to a separating funnel by using diethyl ether (50 ml). The organic phase was washed with 5% $NaHCO_3$ (2×25 ml) and 1 M HCl (2×25 ml), dried (Na_2SO_4) and filtered. Evaporation of the solvent under reduced pressure gave tripeptide (16) as a white solid (1·06 g, 82%), m.p. 80–83°, $[\alpha]_D^{27}$ –5·1° (*c*, 1 in $CHCl_3$). δ (^{1}H) ($CDCl_3$) 0·89, br d, 6H, Leu δ-Me; 1·40, s, 9H, Boc Me; 1·45, s, 18H, Leu and Glu Bu^t Me; 1·50–2·50, m, 3H, Leu β-CH_2 and γ-CH; 3·43–4·23, m, 2H, Ser β-CH_2; 4·40–4·60, m, 3H, Glu α-CH, Ser α-CH, Leu α-CH; 4·56, s, 2H, Ser Bzl CH_2; 5·39, d, 1H, $J_{H,H}$ 6·59 Hz, Glu NH; 7·02, d, 2H, $J_{H,H}$ 7·69 Hz, Ser NH and Leu NH; 7·32, s, 5H, Bzl Ar H. δ (^{13}C) ($CDCl_3$) 21·64 and 22·51, Leu Cδ; 24·36, Leu Cγ; 27·00, Glu Cβ; 27·73, Bu^t Me; 31·72, Glu Cγ; 41·08, Leu Cβ; 51·32, Leu Cα; 52·44, Ser Cα; 54·53, Glu Cα; 69·30 Ser Cβ; 73·10, Bzl CH_2; 79·73, 80·56 and 81·14, Boc **C**Me_3; Glu Bu^t **C**Me_3 and Leu Bu^t **C**Me_3; 127·49 and 128·08, Bzl Ar C; 137·24, Bzl Ar C1; 155·51, Boc CO; 169·11 and 171·21 (×2), Glu CO, Ser CO, Leu CO; 172·52, Glu δ-CO.

N$^\alpha$-(t-Butoxycarbonyl)-O-(t-butyl)glutamylserylleucine t-Butyl Ester (17), Boc-Glu(OBu^t)-Ser-Leu-OBu^t

A solution of Boc-Glu(OBu^t)-Ser(Bzl)-Leu-OBu^t (16) (0·93 g, 1·50 mmol) in 5% acetic acid/methanol (5 ml) containing 10% palladium on charcoal (0·15 g) was charged with hydrogen and hydrogenated until cessation of hydrogen uptake. The catalyst was removed by gravity filtration and the solvent removed by evaporation under reduced pressure. The residue was triturated with diethyl ether (3×30 ml) and then dried under high vacuum (0·1 mmHg) to give tripeptide (17) as a white solid (0·78 g, 99%), m.p. 60–63°, $[\alpha]_D^{27}$ –22·6° (*c*, 1 in $CHCl_3$). δ (^{1}H) ($CDCl_3$) 0·90, br d, 6H, Leu δ-Me; 1·43, s, 9H, Boc Me; 1·45, s, 18H, Leu and Glu Bu^t Me; 1·56–1·80, m, 3H, Leu β-CH_2 and γ-CH; 1·80–2·56, m, 4H, Glu β-CH_2 and γ-CH; 3·47–4·28, m, 2H, Ser β-CH_2; 4·28–4·63, m, 3H, Glu α-CH, Ser α-CH, Leu α-CH; 5·35, d, 1H, $J_{H,H}$ 6·81 Hz, Glu NH; 6·80 and 7·12, each d, 1H, $J_{H,H}$ 7·91 and 7·47 Hz respectively, Ser NH and Leu NH. δ (^{13}C) ($CDCl_3$) 20·44 and 21·52, Leu Cδ; 22·44 Leu Cγ; 24·49 Glu Cβ; 27·61, 27·71 and 27·95, Boc Me; Leu Bu^t Me and Glu Bu^t Me; 31·46, Glu Cγ; 40·48, Leu Cβ; 51·65, Leu Cα; 54·04, Ser Cα; 54·33, Glu Cα; 62·42, Ser Cβ; 79·68, 80·32 and 81·68, Boc **C**Me_3; Leu Bu^t **C**Me_3 and Glu Bu^t **C**Me_3; 155·65, Boc CO; 170·27, 171·64, 172·17 and 172·52, Glu CO, Ser CO, Leu CO and Glu δ-CO. Amino acid analysis: Glu 1·03 (1), Ser 1·00 (1), Leu 0·98 (1).

N$^\alpha$-(t-Butoxycarbonyl)-O-(t-butyl)glutamyl-O-(dibenzylphosphono)serylleucine t-Butyl Ester (18a), Boc-Glu(OBu^t)-Ser(PO$_3$Bzl$_2$)-Leu-OBu^t

1*H*-Tetrazole (0·25 g, 3·60 mmol) was added to a stirred solution of tripeptide (17) (0·56 g, 1·00 mmol) and dibenzyl *N,N*-diethylphosphoramidite (0·38 g, 1·20 mmol) in tetrahydrofuran (2 ml) and stirred at 20° for 15 min. The solution was then cooled to –40° and a solution of

m-chloroperoxybenzoic acid (0·30 g, 1·50 mmol) in dichloromethane (2 ml) was added such that the temperature was maintained below −20°. After stirring for 15 min, 10% $Na_2S_2O_5$ (10 ml) and diethyl ether (50 ml) were added and the organic phase was washed with 10% $Na_2S_2O_5$ (25 ml), 5% $NaHCO_3$ (2×25 ml), and 1 M HCl (1×15 ml), dried (Na_2SO_4) and filtered. Evaporation of the solvent under reduced pressure gave tripeptide (18a) as a clear oil (0·79 g, 96%). δ (^{1}H) ($CDCl_3$) 0·89, br d, 6H, Leu δ-Me; 1·41, s, 27H, Boc Me; Leu and Glu Bu^t Me; 1·80–2·60, m, 7H, Leu β-CH_2 and γ-CH, Glu β-CH_2 and γ-CH_2; 3·90–4·80, m, 5H, Glu α-CH, Ser β-CH_2 and α-CH, Leu α-CH; 5·00 and 5·02, each d, 2H, $J_{P,H}$ 8·18 Hz, POBzl CH_2; 5·46, d, 1H, $J_{H,H}$ 6·71 Hz, Glu NH; 7·30, br s, 10H, Ar H; 7·10 and 7·60, each br d, 1H, Ser NH and Leu NH. δ (^{13}C) ($CDCl_3$) 21·8 and 22·7, Leu Cδ; 24·7, Leu Cγ; 27·4, Glu Cβ; 27·8, 27·9 and 28·2, Boc Me, Leu and Glu Bu^t Me; 31·9, Glu Cγ; 41·2, Leu Cβ; 51·7, Leu Cα; 53.1, d, $J_{P,C}$ 5·9 Hz, Ser Cα; 54·8, Glu Cα; 66·7, d, $J_{P,C}$ 5·9 Hz, Ser Cβ; 69·8, d, $J_{P,C}$ 5·9 Hz, POBzl CH_2; 79·0, 80·7 and 81·4, Boc **C**Me$_3$, Leu Bu^t **C**Me$_3$ and Glu Bu^t **C**Me$_3$; 135·5, d, $J_{P,C}$ 5·9 Hz, POBzl C1; 155·5, Boc CO; 168·1, 171·0, 172·0 and 172·4, Glu CO, Ser CO, Leu CO and Glu δ-CO. δ (^{31}P) ($CDCl_3$) −0·8. Amino acid analysis: 1·03 (1), Ser 1·00 (1), Leu 0·98 (1).

N$^{\alpha}$-(t-Butoxycarbonyl)-O-(t-butyl)glutamyl-O-(di-t-butylphosphono)serylleucine t-Butyl Ester (18b), Boc-Glu(OBu^t)-Ser(PO$_3$Bu$^t_2$)-Leu-OBu^t

1*H*-Tetrazole (0·25 g, 3·60 mmol) was added to a stirred solution of tripeptide (17) (0·53 g, 0·94 mmol) and di-t-butyl *N,N*-diethylphosphoramidite (0·30 g, 1·20 mmol) in tetrahydrofuran (2 ml) and stirred at 20° for 15 min. The solution was then cooled to −40° and a solution of *m*-chloroperoxybenzoic acid (0·30 g, 1·50 mmol) in dichloromethane (2 ml) was added such that the temperature was maintained below −20°. After stirring for 15 min, 10% $Na_2S_2O_5$ (10 ml) and diethyl ether (50 ml) were added and the organic phase was washed with 10% $Na_2S_2O_5$ (25 ml), 5% $NaHCO_3$ (2×25 ml) and 1 M HCl (1×15 ml), dried (Na_2SO_4) and filtered. Evaporation of the solvent under reduced pressure gave an oily residue which solidified on standing. Repeated recrystallization of the oily residue from diethyl ether/pentane gave tripeptide (18b) as a white solid (0·67 g, 95%), m.p. 114–116°, $[\alpha]_D^{27}$ −18·7° (*c*, 1 in $CHCl_3$). δ (^{1}H) ($CDCl_3$) 0·92, br d, 6H, $J_{H,H}$ 5·05 Hz, Leu δ-Me; 1·42, s, 9H, Boc Me; 1·435, s, 18H, Leu and Glu Bu^t Me; 1·44, d, 18H, $J_{P,H}$ 3·28 Hz, POBu^t Me; 1·84–2·56, m, 7H, Leu β-CH_2 and γ-CH, Glu β-CH_2 and γ-CH; 3·90–4·80, m, 5H, Glu α-CH, Ser β-CH_2 and α-CH, Leu α-CH; 5·57, d, 1H, $J_{H,H}$ 6·81 Hz, Glu NH; 7·06 and 7·65, each d, 1H, $J_{H,H}$ 7·47 and 7·25 Hz respectively, Ser NH and Leu NH. δ (^{13}C) ($CDCl_3$) 21·95 and 22·59, Leu Cδ; 24·64, Leu Cγ; 27·56, Glu Cβ; 27·80, 27·90 and 28·19, Boc Me; Leu Bu^t Me and Glu Bu^t Me; 29·68, d, $J_{P,C}$ 3·30 Hz, POBu^t Me; 31·85, Glu Cγ; 41·26, Leu Cβ; 51·70, Leu Cα; 53·23, d, $J_{P,C}$ 5·49 Hz, Ser Cα; 54·82, Glu Cα; 65·76, d, $J_{P,C}$ 5·49 Hz, Ser Cβ; 79·93, 80·66 and 81·44, Boc **C**Me$_3$; Leu Bu^t **C**Me$_3$ and Glu Bu^t **C**Me$_3$; 83·17, d, $J_{P,C}$ 7·69 Hz, POBu^t **C**Me; 155·55, Boc CO; 168·13, Ser CO; 171·00, 171·98 and 172·42, Glu CO, Leu CO and Glu δ-CO. δ (^{31}P) ($CDCl_3$) −9·2. Amino acid analysis: Glu 1·02 (1), Ser 1·01 (1), Leu 0·97 (1).

Glutamyl-O-phosphoserylleucine Trifluoroacetate (19), H-Glu-Ser(PO$_3$H$_2$)-Leu-OH.CF$_3$CO$_2$H

Method A.—Tripeptide (18a) (0·41 g, 0·05 mmol) was dissolved in 40% trifluoroacetic acid/acetic acid (4 ml) containing 10% palladium on charcoal (100 mg) and hydrogenated until cessation of hydrogen uptake. The catalyst was removed by gravity filtration and the solvent evaporated under reduced pressure. The residue was then dissolved in CF_3CO_2H (1 ml), let stand for 1 h at 20° and the solvent then evaporated under reduced pressure. The residue was triturated with diethyl ether (3×30 ml) and then dried under high vacuum to give tripeptide (19) as white flakes (0·265 g, 99·5%).

Method B.—Tripeptide (18b) (0·38 g, 0·05 mmol) was dissolved in 95% trifluoroacetic acid/acetic acid (2 ml) and stood at 20° for 1 h. The solvent was then evaporated under reduced pressure, the residue triturated with diethyl ether (3×30 ml) and then dried under high vacuum to give tripeptide (19) as white flakes (0·27 g, 100%), $[\alpha]_D^{21}$ −14·1° (*c*, 4 in 1 M HCl). δ (^{13}C) (D_2O) 20·6 and 22·2, Leu Cδ; 24·4, Leu Cγ; 25·8, Glu Cβ; 29·0, Glu Cγ;

39·3, Leu Cβ; 51·6, Leu Cα; 52·3, Glu Cα; 54·2, d, $J_{P,C}$ 7·3 Hz, Ser Cα; 64·2, d, $J_{P,C}$ 7·3 Hz, Ser Cβ; 169·2, 170·3, 176·1 (×2), Glu CO and δ-CO, Ser CO and Leu CO. δ (^{31}P) (D_2O) −0·2. F.a.b. mass spectrum (argon, positive mode) *m/z* 428 (MH, 83%), 299 (17), 201 (25), 140 (13), 132 (58), 115 (42), 102 (92), 86 (100). Amino acid analysis: Glu 1·01 (1), Ser 1·02 (1), Leu 0·97 (1).

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