Synthesis of Substrates of Cyclic AMP-dependent Protein Kinase and Use of Their Protected Precursors for the Convenient Preparation of Phosphoserine Peptides [1]

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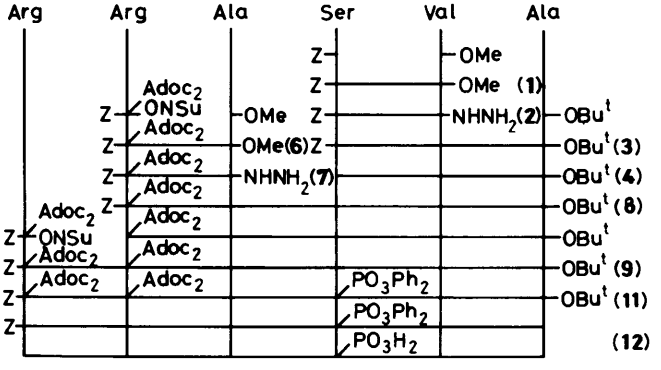
The synthesis of protected hexa- to nona-peptide precursors of substrates of cyclic AMP-dependent protein kinase, based on a partial amino acid sequence from rat liver pyruvate kinase, as well as of related phosphoserine peptides has been explored. A convenient scheme has been developed which furnishes both *N*-terminally elongated peptides of variable lengths and intermediates suitable for chemical phosphorylation. The use of adamantyloxycarbonyl as a protecting group for the two important guanidine functions involved, gave rise to the highly lipophilic intermediates (**9**), (**21**), (**22**), (**28**), and (**31**), which could easily be purified. Treatment of these with anhydrous hydrogen fluoride [or trifluoroacetic acid in the case of compound (**31**)] afforded the pure substrate peptides (**10**), (**23**), (**24**), (**29**), and (**32**) in high overall yield. All of the free peptides obtained could be phosphorylated by cyclic AMP-dependent protein kinase at a significant rate. The chemical synthesis of two phosphoserine peptides (**12**) and (**14**) and their purification by preparative reversed-phase ion-pair chromatography are also reported.

In the search for peptidases with a potential specificity for proteins phosphorylated by cyclic AMP-dependent protein kinase, we recently detected an extralysosomal tripeptidyl-peptidase by using synthetic, ^{32}P-labelled phosphopeptides, derived from the phosphorylated site of rat liver pyruvate kinase.[2] Studies on the substrate specificity of this tripeptidyl peptidase (tripeptidyl peptidase II), indicated the feasibility of synthetic peptides for representing various segments of the phosphorylated site.[2,3] Such phosphopeptides, and a number of their derivatives, would serve to further characterize this unique enzyme. Previously, phosphopeptides were prepared from adenosine triphosphate and unphosphorylated peptides using the catalytic subunit of cyclic AMP-dependent protein kinase.[2,4] For this phosphorylation to occur at a significant rate, a prerequisite is the presence of two consecutive arginine residues, spaced from the serine undergoing phosphorylation by one amino acid.[5] Shorter phosphopeptides with one or no arginine residue had to be prepared by enzymatic and Edman degradations of longer phosphopeptides.[2,4] In our recent attempts to develop specific inhibitors of tripeptidyl peptidase II, the access to peptide fragments with blocked or activated functional groups was required. Since this cannot easily be achieved by the solid phase method,[6] an efficient solution synthesis of protected peptide precursors was developed.

Here, we report the synthesis of five peptides, varying in size from the hexapeptide Arg-Arg-Ala-Ser-Val-Ala (**10**) to the nonapeptide Gly-Val-Leu-Arg-Arg-Ala-Ser-Val-Ala (**24**), as well as that of two phosphoserine peptides, Arg-Arg-Ala-Ser(P)-Val-Ala (**12**) and Arg-Ala-Ser(P)-Val-Ala (**14**). The amino acid sequence of the peptides is based on that of a phosphoserine peptide, derived from rat liver pyruvate kinase of L-type.[7] The non-phosphorylated peptides were assembled from various *N*-terminal precursors and a common *C*-terminal pentapeptide fragment, made by a [2 + 3]-condensation. As the hydroxy group of serine was left unprotected throughout the syntheses, the corresponding protected peptides could be used directly as the starting materials in the preparation of the phosphoserine peptides. After all the protecting groups had been removed the two phosphoserine peptides were purified by reversed-phase ion-pair chromatography to a high degree of purity. Chemical phosphorylation of small serine model peptides has been accomplished by several authors,[8] in particular by Fölsch.[9] To our knowledge, however, this is the first time phosphoserine peptides related to protein kinase substrates have been prepared using chemical methods.



Scheme.

Preparation of Protected Hexapeptide Z-Arg(Adoc)$_2$-Arg(Adoc)$_2$-Ala-Ser-Val-Ala-OBu^t (**9**).—The synthetic procedure outlined in the Scheme was used for the preparation of the protected peptide Z-Arg(Adoc)$_2$-Arg(Adoc)$_2$-Ala-Ser-Val-Ala-OBu^t (**9**), which could be converted both to the corresponding free peptide (**10**) and to the protected compound (**11**) and, ultimately, the free phosphopeptide (**12**). Thus, we started by preparing a *C*-terminal tripeptide fragment, Z-Ser-Val-Ala-OBu^t (**3**), which was found to be crystalline and could be obtained pure in excellent overall yield. Next, a dipeptide fragment Z-Arg(Adoc)$_2$-Ala-NHNH$_2$ (**7**) was prepared. Adamantyloxycarbonyl (Adoc) was chosen as protecting group for arginine to give maximum lipophilicity to the corresponding

Table. List of additional synthetic peptides and peptide intermediates, prepared essentially as described for compounds (**1**)—(**10**), together with selected synthetic and analytical details as well as physico-chemical characteristics

Compd.	Peptide structure[a]	Coupling method[b]	Yield[c] (%)	M.p. (°C)	$[\alpha]_D^{25}$ [*c* 1.0 (solvent)]	Remarks
(**13**)	Z-Arg(Adoc)$_2$-Ala-Ser(PO$_3$Ph$_2$)-Val-Ala-OBu^t[d]		100	140 (decomp.)	−7.8 (DMF)	Optical rotation recorded at *c* 0.50
(**14**)	Arg-Ala-Ser(P)-Val-Ala		41			Yield after preparative h.p.l.c.
(**15**)	Z-Val-Leu-OMe	M.A.	95	102—102.5	−39.5 (HOAc)	Lit.,[11] m.p. 93—96 °C, $[\alpha]_D^{22}$ −39.7
(**16**)	Z-Val-Leu-NHNH$_2$[d]		91	193—194.5	−47.7 (EtOH)	
(**17**)	Z-Gly/Val-Leu-OMe[d]	M.A.	93	118.5—119.5	−8.2 (DMF)	AAA: $Gly_{1.06}Val_{0.98}Leu_{096}$
(**18**)	Z-Gly-Val-Leu-NHNH$_2$[d]		95	199—200	−13.9 (DMF)	
(**19**)	Z-Val-Leu/Arg(Adoc)$_2$·H$_2$O[e]	Azide	95 (75)	161—163 (decomp.)	0.0 (DMF)	AAA: $Val_{1.01}Leu_{0.99}Arg_{1.00}$
(**20**)	Z-Gly-Val-Leu/Arg(Adoc)$_2$·H$_2$O[d]	Azide	95	168—169 (decomp.)	−6.6 (DMF)	AAA: $Gly_{1.00}Val_{1.01}Leu_{1.00}Arg_{1.00}$
(**21**)	Z-Val-Leu-Arg(Adoc)$_2$/Arg(Adoc)$_2$-Ala-Ser-Val-Ala-OBu^t[d]	DCC–HONSu	100 (67)	240 (decomp.)	−8.9 (DMF)	AAA: $Val_{2.01}Leu_{1.00}Arg_{2.02}Ala_{2.01}$-$Ser_{0.96}$
(**22**)	Z-Gly-Val-Leu-Arg(Adoc)$_2$/Arg-(Adoc)$_2$-Ala-Ser-Val-Ala-OBu^t[d]	DCC–HONSu	(56)	235—240 (decomp.)	−6.2 (DMF)	AAA: $Gly_{1.01}Val_{1.99}Leu_{0.98}Arg_{2.02}$-$Ala_{2.03}Ser_{0.95}$
(**23**)	Val-Leu-Arg-Arg-Ala-Ser-Val-Ala·3HOAc	HF	[84]			AAA: $Val_{1.97}Leu_{0.97}Arg_{2.14}Ala_{1.98}$-$Ser_{0.94}$; F: 0.04; (Figure 1c)
(**24**)	Gly-Val-Leu-Arg-Arg-Ala-Ser-Val-Ala·3HOAc	HF	[83]			AAA: $Gly_{1.00}Val_{1.96}Leu_{0.95}Arg_{2.14}$-$Ala_{1.99}Ser_{0.95}$; F: 0.13; (Figure 1d)
(**25**)	Isovaleryl-Leu-OMe		86	Oil		
(**26**)	Isovaleryl-Leu-NHNH$_2$[d]		88	158.5—159.5	−25.7 (DMF)	
(**27**)	Isovaleryl-Leu/Arg(Adoc)$_2$·H$_2$O[d]	Azide	93	155—160 (decomp.)	−6.2 (DMF)	AAA: $Leu_{0.99}Arg_{1.01}$
(**28**)	Isovaleryl-Leu-Arg(Adoc)$_2$/Arg(Adoc)$_2$-Ala-Ser-Val-Ala-OBu^t[f]	DCC–HONSu	(59)	*ca.* 250 (decomp.)	−10.4 (DMF)	AAA: $Leu_{1.00}Arg_{2.01}Ala_{2.02}Ser_{1.01}$-$Val_{0.96}$
(**29**)	Isovaleryl-Leu-Arg-Arg-Ala-Ser-Val-Ala·2HOAc	HF	[85]			AAA: $Leu_{0.97}Arg_{2.13}Ala_{1.98}Ser_{0.94}$-$Val_{0.98}$; F: 0.08; pure by RP-HPLC
(**30**)	Ac/Val-Leu-Arg(Adoc)$_2$	AcONSu	*ca.* 100			
(**31**)	Ac-Val-Leu-Arg(Adoc)$_2$/Arg-(Adoc)$_2$-Ala-Ser-Val-Ala-OBu^t[g]	DCC–HONSu	(71)	*ca.* 240 (decomp.)	−4.7 (DMF)	AAA: $Val_{1.98}Leu_{0.97}Arg_{2.05}Ala_{2.03}$-$Ser_{1.00}$
(**32**)	Ac-Val-Leu-Arg-Arg-Ala-Ser-Val-Ala·2HOAc	TFA	[83]			AAA: $Val_{2.01}Leu_{0.98}Arg_{2.02}Ala_{2.01}$-$Ser_{0.98}$; pure by RP-HPLC

[a] / Indicates the new peptide bond formed. [b] M.A. Indicates mixed anhydride formation in THF with isobutyloxycarbonyl chloride in the presence of *N*-methylmorpholine; azide indicates azide coupling, essentially carried out as for compound (**3**); DCC–HONSu indicates DCC coupling in DMF at −15 °C in the presence of 2 equiv. of *N*-hydroxysuccinimide, typically for 1 h, followed by 10—15 h at 0 °C and a few hours at room temperature. After filtering the reaction mixture, the DMF was evaporated below 30 °C (oil pump) and the residue dissolved in EtOAc and extracted and chromatographed as described for compound (**9**); AcONSu refers to acetylated HONSu; HF refers to HF-treatment as in the case of compound (**10**); TFA was used in a similar fashion for (**32**). [c] Yields refer to pure or essentially pure, crude products; figures in round brackets refer to products purified on silica columns; figures in square brackets for free peptides refer to peptide content and are determined by amino acid analysis (AAA) after hydrolysis. [d] Elemental analysis for C,H,N within ±0.4% of theoretical values. [e] [Found: C, 62.30. Calc. for (**19**): C, 63.06%]. [f] [Found: C, 62.24. Calc. for (**28**): C, 62.82%]. [g] [Found: N, 11.14. Calc. for (**31**): N, 11.66%].

protected intermediates. In this context it should be pointed out that in the preparation of Z-Arg(Adoc)$_2$ we found it highly advantageous to isolate this compound as its dicyclohexylamine (DCHA) salt (**5**). The protected pentapeptide (**8**), obtained from (**7**) and (**4**), was conveniently obtained in excellent yield and satisfactory purity. After hydrogenation, this compound was extended with the help of an *N*-hydroxysuccinimide (ONSu) ester to the protected peptide (**9**). The product was crystalline and easily soluble in ordinary organic solvents.

Treatment of (**9**) with liquid HF,[10] completely removed all protecting groups. After passing through a short ion-exchange column, pure fluoride-free peptide (**10**) was directly obtained. The detailed preparation of compounds (**1**)—(**10**) is described in the Experimental section.

Preparation of N-*Terminally Extended Additional Peptides.*—A number of additional peptides related to (**9**) but further elongated at the *N*-terminal end have been prepared using methods similar to those described for the synthesis of compounds (**1**)—(**10**). Final couplings were accomplished at the Arg-Arg bond with the hydrogenated compound (**8**) as the amino component. These peptides (**15**)—(**32**) are listed in the Table together with pertinent physical and analytical data and are not further discussed in the Experimental section. Among those compounds are the fully protected peptides (**21**), (**22**), (**28**), and (**31**), which like (**9**) dissolved easily in organic solvents and consequently could be chromatographed on silica. Treatment of the first three with HF as described for compound (**10**) furnished the corresponding free peptides (**23**), (**24**), and (**29**), respectively, whereas compound (**31**) was deprotected with trifluoroacetic acid (TFA) to give the peptide (**32**).

Preparation and Purification of Phosphoserine Peptides Arg-Arg-Ala-Ser(P)-Val-Ala (**12**) *and* Arg-Ala-Ser(P)-Val-Ala

(**14**).—The protected phosphoserine peptide (**11**) was prepared directly from (**9**) with diphenyl phosphorochloridate as outlined in the Scheme and could also be purified by chromatography on silica. Peptide (**11**) was deprotected in two steps, first with trifluoroacetic acid to remove the Adoc and t-butyl ester groups and then by catalytic hydrogenation to give the crude phosphoserine peptide (**12**). This peptide was finally completely purified by preparative ion-pair chromatography on a reversed-phase column using (+)-camphor-10-sulphonate as the counter ion and a volatile buffer, essentially as described earlier for related non-phosphorylated peptides.[12a]

Using procedures similar to those described in the previous paragraph, Z-Arg(Adoc)$_2$-Ala-Ser(PO_3Ph_2)-Val-Ala-OBu^t (**13**) was first prepared directly from the peptide (**8**) and then converted to the phosphoserine peptide (**14**) and purified. Peptides (**13**) and (**14**) are listed with their physical and analytical data in the Table and are not discussed further in the Experimental section.

Phosphorylation of the Synthetic Peptides (**10**), (**23**), (**24**), (**29**), *and* (**32**) *by Cyclic AMP-dependent Protein Kinase.*—The peptides were phosphorylated as described,[13] at near saturation concentrations, *i.e.* 0.2 mM, with 14 ng of the purified catalytic subunit of cyclic AMP-dependent protein kinase of rabbit skeletal muscle. This amount of protein kinase phosphorylated histone IIA (Sigma) at the rate of 18 pmol min^{-1}. All experiments were run in triplicate and the values given are mean values ± standard deviations. The phosphorylation rates were 43 ± 6 (**10**), 51 ± 7 (**23**), 60 ± 2 (**24**), 59 ± 2 (**29**), and 62 ± 4 (**32**) pmol min^{-1}, respectively. A previously prepared sample of (**10**)[5a] was included in these experiments and phosphorylated at a rate of 39 ± 5 pmol min^{-1}. These figures are compatible with previous results[5] and show that this type of peptide is phosphorylated at least as fast as a good protein substrate.

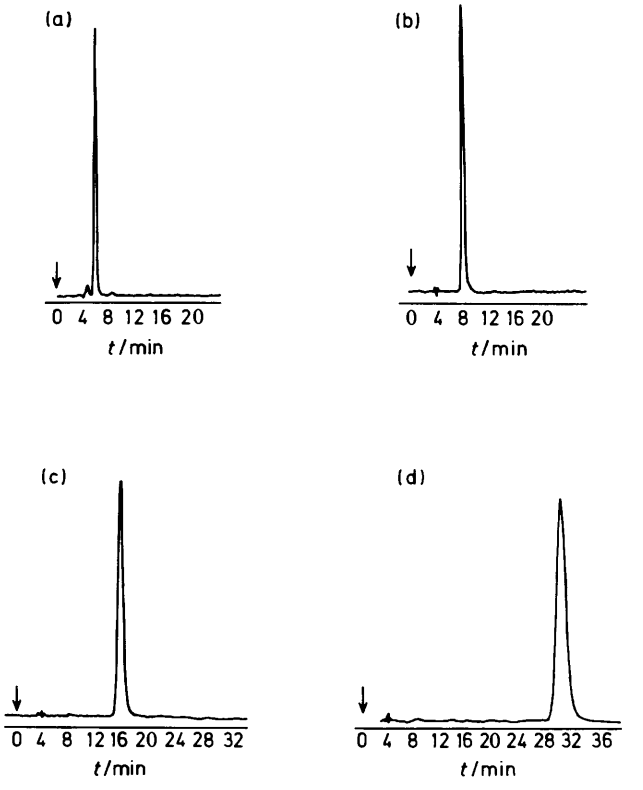


Figure 1. Chromatograms of four free peptides. Mobile phase: 0.1M-phosphate buffer (pH 2.9)–ethanol (79:21), flow rate 1.0 ml min^{-1}, counter ion: pentanesulphonate (0.015M), support: Spherisorb C_{18} (10 μm, 4.6 × 150 mm), detection: 210 nm at 0.04 AUFS. (a) refers to Arg-Ala-Ser-Val-Ala [HF-treated (**8**)], (b) to Arg-Arg-Ala-Ser-Val-Ala (**10**), (c) to Val-Leu-Arg-Arg-Ala-Ser-Val-Ala (**23**), and (d) to Gly-Val-Leu-Arg-Arg-Ala-Ser-Val-Ala (**24**)

Discussion

This work was initiated with two major aims in mind: (*i*) For on-going enzymological projects as well as for future structural studies we required considerably larger amounts of some key substrate peptides[5a] than those obtained earlier, and (*ii*) we wanted to prepare phosphoserine peptides in larger amounts than the minute quantities obtained before by the enzymatic procedure.[4,14] In addition, we wanted to establish a supply of pure precursor peptides, containing the penta- or hexa-peptide framework required to make them substrates to cyclic AMP-dependent protein kinase,[5] which could be elongated when required at their *N*-termini to give new substrates. Although our former preparations[5a] could undoubtedly have been scaled up to satisfy our first aim, our interest in preparing phosphoserine peptides led us to attempt a solution synthesis rather than the solid phase procedure, either with some selectively removable OH-protecting group or with no OH-protection on serine. Two papers on the synthesis of substrates related to ours should be mentioned in this context. Marchiori *et al.*[15] have used a solution approach involving incorporation of ornithine in place of arginine followed by guanylation. However, no experimental details are given. Kondo *et al.*[16] in their synthesis of fluorescent peptide derivatives used a strategy with protection of all functional groups. Boc, nitro, and benzyl were employed for amino groups and guanidine and serine hydroxy functions, respectively.

Here, we have chosen a rather straightforward approach with a free serine hydroxy group and benzyloxycarbonyl for temporary amino protection (see the Scheme). With this choice we could mask arginine and simultaneously suppress the overall polarity with the highly lipophilic Adoc-protecting group. Benzyloxycarbonyl was generally removed by catalytic hydrogenolyis. In most cases Adoc and *C*-terminal t-butyl ester groups were cleaved off afterwards using liquid HF. Over the years, we have treated a large number of peptides of this kind with this strong acid without noticing any deleterious effects. Coupling of the peptide fragments was accomplished by the azide and carbodi-imide–hydroxysuccinimide methods. In order to avoid racemization these methods rank among the safest available.

The phosphorylation reaction was optimized to proceed essentially quantitatively. Elevated temperature gave a lower yield of a less pure product and it was also observed that the phosphorylation proceeded more sluggishly with concomitant formation of by-products, when dichloromethane containing one equivalent of *N*-methylmorpholine was exchanged for pyridine. The protected phosphoserine peptides could be easily purified by chromatography on silica. The subsequent, final removal of the protecting groups had to be performed in two steps, as the serine-phosphate bond is not stable enough to a strong acid such as liquid HF.

A striking feature of both types of known peptide substrates of cyclic AMP-dependent protein kinase is their rather hydrophilic nature.[5,13] Therefore, a different choice of side-chain protecting group for arginine might have led to protected intermediates with poor solubility in organic solvents and caused extraction problems, resulting in impure protected peptides. Our choice of Adoc not only overcame this problem completely but made these peptides so highly lipophilic that chromatography on silica could be made a routine step in their purification. This strategy worked well for the protected

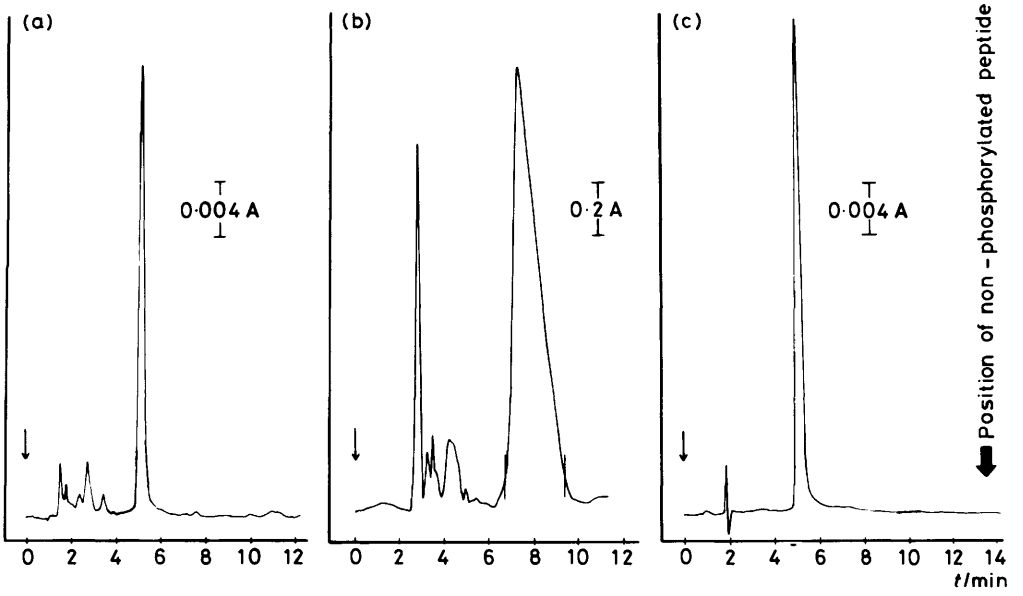


Figure 2. Chromatograms of Arg-Arg-Ala-Ser(P)-Val-Ala (**12**). (a) and (c) Analytical separations, before and after purification. Mobile phase: 0.1M-phosphate buffer (pH 3.0)–ethanol (86:14), flow rate 1.0 ml min^{-1}, counter ion: pentanesulphonate (0.015M), support: Spherisorb C_{18} (ODS-2, 5 μm, 4.6 × 150 mm), detection: 210 nm. (b) Preparative separation (40 mg), mobile phase: volatile buffer, triethylamine–0.1M-formic acid (pH 3.0)–ethanol (89.5:10^5), flow rate 4.0 ml min^{-1}, counter ion: (+)-camphor-10-sulphonate (0.05M), support: Spherisorb C_{18} (5 μm, 10 × 250 mm), detection 232 nm at 2.0 AUFS.

peptides (**9**), (**21**), (**22**), (**28**), and (**31**) described in this paper, provided they were made from the fragments indicated. When different fragments were used, such as a (3 + 6) instead of the (4 + 5) approach for peptide (**22**),[1] or a (2 + 6) instead of the (3 + 5) one for the peptide (**21**),[12a] the pure protected peptides could not be obtained by chromatography on silica. Obviously, in these cases the larger fragments were about as lipophilic as the products.

The free, non-phosphorylated peptides, obtained by treatment with liquid HF as described for compound (**10**) in the Experimental section, as well as the trifluoroacetate (**32**), were passed through a short ion-exchange column. The fluoride-free lyophilized powders were analysed by reversed-phase ion-pair chromatography[12b] and found to be satisfactorily pure [Figure 1(a)—(d)]. The fact that no further purification was required at this stage proved the effectiveness of the chromatography on silica in the previous step as well as demonstrating that HF did not cause any side-reactions in the deprotection step.

On the other hand the free phosphopeptide (**12**) did require purification [Figure 2(a)]. This was accomplished in the same way as that described previously for non-phosphorylated peptides [Figure 2(b)].[12a] After desalting, pure (**12**) was obtained (Figure 2(c)].

To summarize, our synthetic scheme furnished quantities of the very pure (**10**) and proved to be a reasonable approach to the corresponding phosphoserine peptide (**12**). Among the peptides collected in the Table several exemplify the convenient application of common precursors for the synthesis of new substrates, modified at their *N*-termini.

Experimental

All amino acids used were obtained from Ajinomoto Co., Inc., Tokyo, Japan, and were of L-configuration. Melting points were recorded in open capillaries on a Gallenkamp melting point apparatus and are uncorrected. T.l.c. was performed on precoated silica (Merck DC-Fertigplatten Kieselgel 60 F_{254}) or cellulose plates eluting with (A) CH_2Cl_2–acetone–HOAc (40:10:1), (B) CH_2Cl_2–acetone–HOAc (5:5:1), (C) $CHCl_3$–EtOH–water (100:50:4), (D) CH_2Cl_2–MeOH (9:1), (E) BuOH–pyridine–HOAc–water (15:10:3:12), and (F) toluene–MeCN (2:1). The protected peptides were visualized with iodine vapour. The h.p.l.c. instrumentation and procedures were as described earlier.[12]

Z-Ser-Val-OMe (**1**).—To a solution of Z-Ser (23.9 g, 0.100 mol) in CH_2Cl_2 (200 ml) was added Val-OMe·HCl (17.6 g, 0.105 mol) and the suspension was treated with NEt_3 (14.6 ml, 0.105 mmol) with vigorous stirring at −20 °C. DCC (21.0 g, 0.102 mol) in CH_2Cl_2 (75 ml) was added dropwise during 30 min, and the stirring was continued for 30 min at −20 °C, 1 h at 0 °C, and finally overnight at room temperature. The precipitate was filtered off, rinsed with CH_2Cl_2, the combined filtrates were evaporated to dryness and the semisolid residue was partitioned between EtOAc (1 l) and aqueous $KHSO_4$ (1M; 250 ml). Traces of insoluble material were removed and the organic extract was washed consecutively with 1M-$KHSO_4$ (3 × 250 ml), 1M-aqueous $NaHCO_3$ (3 × 250 ml), and saturated aqueous NaCl (2 × 250 ml) and then dried ($MgSO_4$). Evaporation gave an oily residue which was taken up in ether (400 ml) and refrigerated overnight. The solution was filtered before being evaporated to dryness (oil pump), to yield a pale yellow, chromatographically pure oil (A) (33.9 g, 96%), satisfactory for further work. An analytical specimen was obtained by wasteful crystallization from ether–light petroleum (1:4 v/v; 50 ml g^{-1}), after having cooled the solution to −20 °C for a few days; m.p. 56—57 °C; $[\alpha]_D^{25}$ +1.7° (*c* 1.00, DMF); t.l.c. (B) gave one spot (Found: C, 57.55; H, 6.95; N, 7.9. $C_{17}H_{24}N_2O_6$ requires C, 57.94; H, 6.87; N, 7.95%).

Z-Ser-Val-$NHNH_2$ (**2**).—To a solution of the crude dipeptide (**1**) (33.9 g, 96.1 mmol) in MeOH (250 ml) was added hydrazine hydrate (20 ml, 0.4 mol) and the resulting mixture was left at room temperature for 3 days when it was diluted with water (250 ml) the slurry was stirred for 1 h. The white fine-grained precipitate was filtered off and rinsed with MeOH–water (1:1)

followed by water. The solid was air-dried before being thoroughly triturated with dry ether, further washed with several portions of dry ether and dried (oil pump) at 50 °C overnight to yield a crude product (20.3 g, 86%), sufficiently pure for further work, m.p. >200 °C. T.l.c. (B) gave one spot (Found: C, 54.55; H, 6.95; N, 15.65. $C_{16}H_{24}N_4O_5$ requires C, 54.53; H, 6.86; N, 15.90%).

Z-Ser-Val-Ala-OBu^t (**3**).—A solution of compound (**2**) (28.2 g, 80.0 mmol) in dry DMF (250 ml) was cooled to −20 °C and treated with 3.76M-HCl in dioxane (75.0 ml; 282 mmol) with rapid stirring, followed by isopentyl nitrite (10.3 g, 88.0 mmol). The clear solution was left for 1 h at −20 °C, then NEt_3 (28.5 g, 282 mmol) was added at −40 °C, followed by a solution of Ala-OBu^t (12.8 g, 88 mmol) in DMF (20 ml). The reaction mixture was stirred at −20 °C for an additional 6 h, while maintaining the pH 7.5 ± 0.5 by the dropwise addition of NEt_3 (moist indicator strip); the reaction was completed at 0 °C for 24 h. The mixture was diluted with ice-cold 1M-$KHSO_4$, and the resulting precipitate was collected, rinsed consecutively with 1M-$KHSO_4$, 1M-$NaHCO_3$, and water and allowed to dry in air to yield the crude tripeptide (**3**) (31.6 g, 85%), t.l.c. (A,B,D), one spot. Recrystallization from EtOAc (20 ml g^{-1}; decolourizing carbon) afforded a fluffy substance with m.p. 163—164 °C (recovery 80%). Further recrystallization from the same solvent gave an analytical specimen, m.p. 165—166 °C; $[\alpha]_D^{25}$ −7.3° (*c* 1.00, DMF); amino acid analysis: $Ser_{0.96}Val_{1.04}Ala_{1.01}$ (Found: C, 59.25; H, 7.6; N, 9.0. $C_{23}H_{35}N_3O_7$ requires C, 59.34; H, 7.58; N, 9.03%).

Ser-Val-Ala-OBu^t (**4**).—To a solution of the tripeptide (**3**) (4.65 g, 10.0 mmol) in MeOH (100 ml g^{-1}) was added Pd–C (5%; 0.50 g) and the mixture was hydrogenated at room temperature and normal pressure for 4 h. The catalyst was removed by filtration and the colourless solution taken to complete dryness below 30 °C (oil pump). The residual glassy foam was thoroughly triturated with light petroleum, collected, and dried at 40 °C overnight (oil pump) to yield crude product, obtained as a pale, yellow powder (3.14 g, 95%), t.l.c. (B,C) gave one spot with ninhydrin (Found: C, 54.35; H, 8.9; N, 12.2. $C_{15}H_{29}N_3O_5$ requires C, 54.36; H, 8.82; N, 12.68%).

Z-Arg(Adoc)$_2$: Modified Procedure, Including Isolation of Z-Arg(Adoc)$_2$ DCHA Salt (**5**).—Z-Arg (17.1 g, 55.5 mmol) was dissolved in a mixture of dioxane (33 ml) and 2M-NaOH (111 ml) and the resulting clear solution was cooled in ice. A solution of crude Adoc-Cl[17a] (47.4 g, 221 mmol) in dry dioxane (45 ml) was added dropwise together with 2M-NaOH (165 ml) with vigorous stirring over a period of 1 h, whilst maintaining the reaction temperature at 5 ± 2 °C, and the resulting suspension was stirred for a further 3 h; ice–water (150 ml) was added when necessary to facilitate the stirring. The mixture was then partitioned between ether (600 ml) and 1M-$KHSO_4$ (600 ml), the organic extract collected and the aqueous phase, together with some insoluble material, was extracted with ether (400 ml). The combined ethereal extracts were filtered to remove insoluble material and washed consecutively with 1M-$KHSO_4$ (3 × 200 ml), water (200 ml), and saturated aqueous NaCl (2 × 200 ml). The dried ($MgSO_4$) and filtered solution was treated with dicyclohexylamine (DCHA, 10.0 g, 55.5 mmol) in one portion. The solution was seeded and allowed to stand at room temperature and the precipitated DCHA salt was collected and rinsed with several portions of dry ether. The vacuum-dried DCHA-salt (**5**) (30.3 g, 65%), had m.p. 137—138 °C; $[\alpha]_D^{25}$ +9.0° (*c* 0.50, DMF) (Found: C, 68.15; H, 8.45; N, 8.3. $C_{48}H_{71}N_5O_8$ requires C, 68.13; H, 8.45; N, 8.22%).

The acid was conveniently liberated from its DCHA salt by partitioning compound (**5**) between ether (20 ml g^{-1} salt) and 1M-$KHSO_4$ (10 ml g^{-1} salt). The ether extract was washed with 1M-$KHSO_4$ (5 × 10 ml g^{-1} salt), water (2 × 10 ml g^{-1} salt), and saturated NaCl (2 × 20 ml g^{-1}). After drying ($MgSO_4$), the extract was evaporated to dryness, to afford Z-Arg(Adoc)$_2$ in 88% yield as calculated from the salt. T.l.c. (A) gave one spot. An analytical specimen was obtained by dissolving the crude product, obtained as a foam, in dry ether (10 ml g^{-1}). After a few minutes at room temperature, crystallization started and after 24 h the fine-grained crystals were collected, rinsed with several portions of dry ether and dried, m.p. 121.5—122 °C; $[\alpha]_D^{25}$ +20.1° (*c* 1, $CHCl_3$) (lit.,[17b] 120—122 °C; $[\alpha]_D^{22}$ +20.8° (*c* 1, $CHCl_3$).

Z-Arg(Adoc)$_2$-Ala-OMe (**6**).—A solution of Z-Arg(Adoc)$_2$-ONSu[17b] (9.58 g, 12.6 mmol) and Ala-OMe·HCl (2.10 g, 15.1 mmol) in DMF (42 ml) was cooled in ice, NEt_3 (2.10 ml, 15.1 mmol) added and the resulting turbid mixture stirred with ice-cooling for 3 h and overnight at room temperature. Most of the solvent was removed below 30 °C using an oil pump, the semi-solid residue partitioned between ether (500 ml) and 1M-$KHSO_4$ (200 ml), the extract was washed successively with 1M-$KHSO_4$, 1M-$NaHCO_3$, and saturated aqueous NaCl (3 × 200 ml each) and dried ($MgSO_4$). Evaporation gave a crisp foam, (9.23 g, 98%). T.l.c. (A) indicated that this crude product was pure enough for further work. Two crystallizations from MeOH (20 ml g^{-1}) afforded an analytical specimen as a voluminous microcrystalline solid, m.p. 96—97 °C; $[\alpha]_D^{25}$ −0.5° (*c* 1.00, DMF) (Found: C, 63.6; H, 7.5; N, 9.25. $C_{40}H_{55}N_5O_9$ requires C, 64.06; H, 7.39; N, 9.34%).

Z-Arg(Adoc)$_2$-Ala-NHNH$_2$ (**7**).—A solution of the dipeptide (**6**) (9.70 g, 12.9 mmol) in MeOH (15 ml g^{-1}) was treated with hydrazine hydrate (6.50 ml; *ca.* 130 mmol) in one portion at 40 °C with thorough mixing and was then left at room temperature for 2 days. The resulting precipitate was collected, rinsed with small portions of cold MeOH, and dried. Cooling of the combined filtrate and washings to −25 °C for a few days afforded a second (pure) crop of the product (**7**) (8.11 g, 84% total) essentially pure by t.l.c. (A), m.p. 133—134 °C; $[\alpha]_D^{25}$ +1.6° (*c* 1.00, DMF). Attempted recrystallization of the crude product from MeOH did not improve its quality.

Z-Arg(Adoc)$_2$-Ala-Ser-Val-Ala-OBu^t (**8**).—A solution of the crude hydrazide (**7**) (7.88 g, 10.5 mmol) in dry DMF (10 ml g^{-1}) was cooled to −30 °C and treated with precooled 4.16M-HCl in dioxane (10.0 ml; 41.6 mmol) with vigorous stirring. Isopentyl nitrite (1.55 ml; 11.6 mmol), was then added and the reaction mixture was stirred at −25 °C for 45 min, before NEt_3 (5.78 ml; 41.6 mmol) was added, followed by a solution of the tripeptide (**4**) (3.83 g, 11.6 mmol) in dry DMF (10 ml g^{-1}). The resulting mixture was stirred at −20 ± 2 °C for 3 h, during which time the pH was maintained at 7.5 ± 0.5 with NEt_3 as for (**3**). After a further 4 h at room temperature the turbid mixture was left at 4 °C overnight. Dilution with 1% aqueous HOAc (1.5 l) gave a fine-grained precipitate which was collected, washed with plenty of water and dried at 40 °C (oil pump). This material was triturated with dry ether (*ca.* 100 ml) and filtered off, rinsed with several small portions of ether and again meticulously dried to give an essentially pure product (**8**), [t.l.c. (A)], (9.55 g, 87%). This compound showed surprisingly poor solubility in the usual organic solvents. An analytical specimen was obtained by repeated wasteful recrystallizations from EtOAc (12.0 ml g^{-1}, decolourizing carbon), m.p. 230—235 °C (decomp.); $[\alpha]_D^{25}$ −6.8° (*c*. 1.00, DMF): amino acid analysis: $Arg_{1.00}Ala_{1.99}$-$Ser_{0.97}Val_{1.04}$ (Found: C, 61.2; H, 7.7; N, 10.55. $C_{54}H_{80}N_8O_{13}$ requires C, 61.80; H, 7.68; N, 10.68%).

Z-Arg(Adoc)$_2$-Arg(Adoc)$_2$-Ala-Ser-Val-Ala-OBu^t (**9**).—(*a*) *Deprotection Step.* The protected peptide (**8**) (4.85 g, 4.62

mmol), suspended in MeOH (125 ml g^{-1}), was hydrogenated at atmospheric pressure in the presence of Pd–C (5%; *ca.* 0.5 g). After 5 h t.l.c. (B,C) indicated that all starting material had been consumed and a new ninhydrin-positive spot appeared at lower R_F on the chromatogram. After removal of the catalyst, the colourless filtrate was evaporated to dryness. The solid residue was thoroughly dried at 30 °C for a few hours (oil pump), to yield the deprotected pentapeptide (3.93 g, 93%).

(*b*) *Coupling Step.* The crude product (3.84 g, 4.20 mmol) freshly prepared as described above and dissolved in dry DMF (20 ml) was cooled in an ice-bath and treated with Z-Arg(Adoc)$_2$-ONSu (3.20 g, 4.20 mmol) in DMF (11 ml) for a period of 15 min. The reaction mixture was stirred for 1 h at 0 °C and overnight at room temperature. Most of the DMF was stripped off below 30 °C (oil pump), the oily residue partitioned between EtOAc (500 ml) and 1M-$KHSO_4$ (200 ml), and the extract was washed consecutively with 1M-$KHSO_4$, 1M-$NaHCO_3$, and brine (3 × 100 ml each) and dried ($MgSO_4$). Evaporation left a solid residue which was dissolved in CH_2Cl_2 (100 ml). The solution was filtered to remove traces of insoluble impurities and then taken to dryness. Careful drying gave the essentially pure hexapeptide (**9**) [t.l.c. (A,B,C)] (6.32 g, 96%). From batches of up to 10 g the last traces of impurities could be removed by column chromatography on silica, eluting with CH_2Cl_2–acetone (4:1) in 80—90% yield. From this product an analytical sample was crystallized with acetone–ether (2:1; 70 ml g^{-1}) to give a white microcrystalline product, m.p. 225—230 °C (decomp.); $[\alpha]_D^{25}$ −4.5° (*c* 1.00, DMF); amino acid analysis: $Arg_{2.04}Ala_{1.98}Ser_{0.98}Val_{1.01}$ (Found: C, 62.9: H, 7.8; N, 10.65. $C_{82}H_{120}N_{12}H_{18}$ requires C, 63.05; H, 7.74; N, 10.76%).

Arg-Arg-Ala-Ser-Val-Ala·3HOAc (**10**).—To the chromatographically purified hexapeptide (**9**) (781 mg, 0.50 mmol) and anisole (0.5 ml; added in order to bind the fluffy powder!) in an HF-resistant Kel-F tube of a special apparatus (Toho Kasei, Type 1) was condensed liquid HF (*ca.* 5 ml). After the reaction had been stirred for 1 h at 0 °C, the HF was evaporated and the sticky residue partitioned between ether (15 ml) and 10% HOAc (30 ml). The clear colourless water phase was extracted with ether (5 × 10 ml) and then flushed with N_2 to remove dissolved ether. Freeze-drying afforded a white solid. To remove the fluoride ions, the solid was dissolved in a minimum quantity of water and run through a short column, packed with IRA 500 (acetate form). Eluted fractions were checked by t.l.c. on a cellulose plate (E). Spraying with phenanthrenequinone[18] gave blue fluorescent spots for the first fractions; these were pooled and freeze-dried twice to give the product (**10**) as a fluffy powder which was dried at 50 °C *in vacuo* for 48 h (386 mg, 92%). T.l.c. (as above) gave one spot and h.p.l.c. gave a single peak see Figure 1(b). Amino acid analysis: $Arg_{2.11}Ala_{1.97}Ser_{0.94}Val_{0.98}$; peptide content: 77% (theory requires 79%); F^- content: 0.16%.

Z-Arg(Adoc)$_2$-Arg(*Adoc*)$_2$-*Ala-Ser*(PO_3Ph_2)-*Val-Ala*-OBu^t (**11**).—A solution of compound (**9**) (781 mg, 0.50 mmol) and DMAP (6 mg, 0.05 mmol) in dry pyridine (5 ml) was cooled in ice. To this well-stirred mixture, was added dropwise diphenyl phosphorochloridate (155 μl; 0.75 mmol) during 10 min. The reaction was monitored by t.l.c. (A) which indicated only minor amounts of starting material remaining in the mixture after 10 h. An additional portion of diphenyl phosphorochloridate (78 μl; 0.38 mmol) was slowly introduced and the reaction mixture was agitated for a further 5 h at 0 °C and at ambient temperature overnight. Only traces of starting material were detected by t.l.c. and most of the solvent was removed under reduced pressure at room temperature. The semi-solid brownish residue was partitioned between CH_2Cl_2 (100 ml) and 1M-$KHSO_4$–brine (1:1; 50 ml). The organic extract was washed consecutively with 1M-$KHSO_4$–brine (1:1), 1M-$NaHCO_3$–brine (1:1), and brine (3 × 25 ml each), and dried ($MgSO_4$). After treatment with decolourizing carbon, the extract was evaporated to complete dryness, to afford a white solid essentially pure by t.l.c. (A,F) (898 mg, quantitative). The analytical specimen was obtained by column chromatography on silica, eluting with CH_2Cl_2–acetone (4:1). A pure fraction was collected and the chromatographed material was triturated with dry ether (*ca.* 15 ml g^{-1}). The resulting fine-grained powder was rinsed with small portions of dry ether and thoroughly dried *in vacuo* over paraffin chips. T.l.c. (F) revealed a trace of impurity at slightly higher R_F, m.p. 138—139 °C; $[\alpha]_D^{25}$ −12.4 (*c*, 0.25, $CHCl_3$). $\delta_P(CDCl_3)$ −12.47 (δ H_3PO_4 0 p.p.m.) (Found: C, 62.9; H, 7.1; N, 9.5. $C_{94}N_{129}N_{12}O_{21}P$ requires C, 62.9; H, 7.2; N, 9.4%).

Arg-Arg-Ala-Ser(P)-Val-Ala (**12**).—A solution of the crude above phosphorylated peptide (1.17 g, 0.652 mmol) in CH_2Cl_2 (12 ml) was treated with trifluoroacetic acid (6 ml) and the mixture was left in a sealed vessel for 1 h. The solution was evaporated to dryness at room temperature and the sticky residue dissolved in CH_2Cl_2 (20 ml) and again taken to dryness. This procedure was repeated with fresh CH_2Cl_2. The solid was thoroughly triturated with dry ether (25 ml) and the insoluble residue was collected and rinsed with several portions of dry ether and dried *in vacuo* overnight. This product was dissolved in 80% HOAc (60 ml) and hydrogenated for 50 h at 400 kPa over PtO_2 (0.8 g). The catalyst was removed by filtration and the colourless filtrate evaporated to dryness. The residual sticky mass was dissolved in water (100 ml), and the solution was filtered and extracted with ether (3 × 50 ml). The aqueous solution was degassed and freeze-dried to afford the crude title compound as a fluffy solid (464 mg, 89% calculated as monoacetate). T.l.c. (E; cellulose plate) showed, after development with phenanthrenequinone spray, that only minute traces of unphosphorylated hexapeptide were present in the crude product. A small spot at higher R_F was the only detectable impurity. The crude product was purified on a preparative scale (up to 40 mg per injection) by reversed-phase ion-pair chromatography in a volatile buffer,[13a] using (+)-camphor-10-sulphonate as the counter-ion. Chromatographic details are given in Figure 2(b). The counter-ion was in this case removed on a strong anion exchanger, QAE-Sephadex A-25, regenerated with acetic acid. Lyophilization of the crude product afforded the phosphoserine peptide (**12**) (18 mg) which had a peptide content of 84%, as determined by amino-acid analysis (theory requires 92% for the corresponding mono- and 86% for the di-acetate). N.m.r. spectroscopy confirmed that the product was free from sulphonate impurities.

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