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Purification, Sequence Determination and Synthesis of Seminal Plasma Peptides and Synthesis of Some of Their Analogues

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Abstract: Three peptides were isolated from bovine seminal plasma and purified to homogeneity. The amino acid sequences, as determined by FAB mass spectrometry, are the following: *p*Glu-Ala-Glu-Ser-Asn-OH, *p*Glu-Ala-Glu-Ser(PO_3H_2-Asn-OH and *p*Glu-Val-Gly-Glu-Ser-Glu-Asn-OH. These three peptides and some of their analogues were synthesized using liquid- and solid-phase techniques. The pentapeptide *p*Glu-Ala-Glu-Ser-Asn-OH showed a remarkable affinity for kinase NII and a strong inhibiting activity in DNA transcription. These findings support the hypothesis that phosphorylated acidic domains of nuclear non-histone proteins could bind to DNA, thereby controlling transcription.

Keywords: seminal plasma peptides; phosphopeptides; synthesis of peptides; protein kinase NII; DNA-binding peptide

Low molecular weight acidic peptides have been isolated both from bovine seminal plasma and from the chromatin of various tissues such as calf thymus, trout testis and bovine spermatozoa [1,2]. The isolated peptides act as regulators of RNA synthesis by exerting inhibitory effects on transcription *in vitro* and in neoplastic and fast-growing cells and an activating effect on senescent cells [3–5].

One purified peptide fraction appears to be composed of closely related compounds with similar amino acid composition and structure. Despite the difficulty represented by the presence of a family of closely related peptides and small amounts of purified peptides, some structural features have been demonstrated: (1) a molecular weight around 1000 dalton; (2) the presence of aspartic acid, serine, glutamic acid, glycine, alanine and valine in the acid hydrolysate [1,2]; (3) a blocked N-terminus consisting of pyroglutamic acid [1]; (4) a phosphoric group bound to serine side chain [6].

In this paper we describe a further purification of the active peptide fraction, the determination of amino acid sequences by mass spectrometry and the syntheses of seminal plasma peptides and some of their analogues.

The most promising preparation of native peptides was further purified using reverse-phase HPLC. The chromatogram reported in Figure 1 revealed the presence of four major peaks. The transcription-inhibiting activity was distributed in the first, third and fourth fractions. The second fraction was neglected since it was devoid of inhibitory activity.

Abbreviations: Alloc, allyloxycarbonyl; DCC, dicycloexylcarbodimide; EtOAc, ethyl acetate; FAB-MS, fast atom bombardment mass spectrometry; HOBt, 1-hydroxybenzotriazole; HOSu, *N*-hydroxysuccinimide; ONp, *p*-nitrophenoxy; OPfp, pentafluorophenoxy; O*t*Bu, *tert*-butoxy; TLC, thin-layer chromatography; Z, benzyloxycarbonyl.

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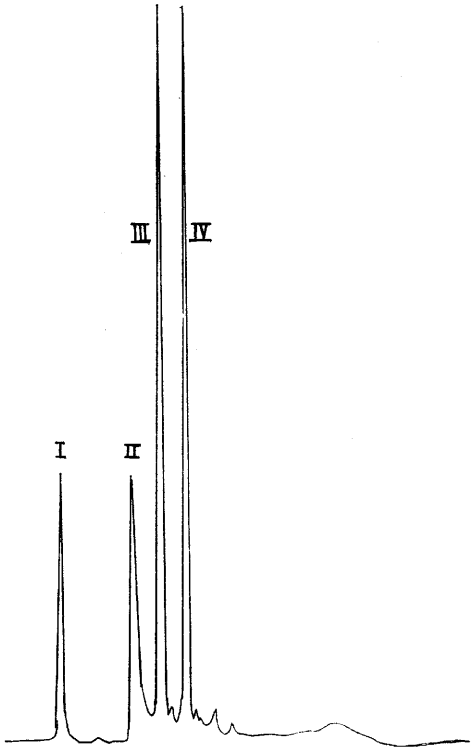

Figure 1 Reverse-phase HPLC elution profile of the purest fraction: LiChrosorb RP-18, 4 × 250 mm column; $\lambda = 224$ nm; flow rate 1 ml/min; chart speed 5 mm/min; 0.05% TFA to 0.04% TFA-80% CH_3CN in 10 min. Retention times: I, 2.32 min; III, 6.57 min; IV, 7.55 min.

On the basis of FAB mass spectra the following sequences were assigned: *p*Glu-Ala-Glu-Ser(PO_3H_2)-Asn-OH (first fraction), *p*Glu-Ala-Glu-Ser-Asn-OH (third fraction) and *p*Glu-Val-Gly-Glu-Ser-Glu-Asn-OH (fourth fraction).

The pentapeptide *p*Glu-Ala-Glu-Ser-Asn-OH proved to be a good substrate for calf thymus protein kinase N II [7]. To demonstrate the specifity of phosphorylation and to study the relationship between chemical structure and phosphorylation rate, we synthesized a series of nine peptide analogues. *p*Glu-Ala-Glu-Ser-Asp-OH, Glu-Ala-Glu-Ser-Asn-OH, *p*-Glu-Ala-Gln-Ser-Asn-OH, *p*Glu-Ala-Glu-Ser-Leu-OH, *p*Glu-Gln-Gly-Ser-Asn-OH, *p*Glu-Ala-Glu-Ser-OH, *p*Glu-Ala-Glu-Ser-Asn-Ala-OH and *p*Glu-Ala-Glu-Gly-Gly-Ser-Asn-OH are analogues of the pentapeptide *p*Glu-Ala-Glu-Ser-Asn-OH, whereas *p*Glu-Val-Gly-Glu-Ser-Glu-Asp-OH is an analogue of the heptapeptide *p*Glu-Val-Gly-Glu-Ser-Glu-Asn-OH. The analogues have been designed by changing one or more of the amino acids flanking the serine phosphorylatable by protein kinase N II, which probably represents the key point for the peptide structure–function relationship. The phosphorylation rates obtained (Table 1) show that the pentapeptide *p*Glu-Ala-Glu-Ser-Asn-OH has the highest affinity for the protein kinase N II. This property appears related to the biological activity of this phosphorylated structure, shown by the binding to DNA and the control of the transcription reaction.

The synthesis of the heptapeptide *p*Glu-Val-Gly-Glu-Ser-Glu-Asn-OH and its analogue with aspartic acid in the C-terminal position was carried out by the solid-phase method on an automated synthesizer employing a polyamide resin and the Fmoc strategy [8].

The other peptides were synthesized in solution by the stepwise elongation method, The benzyloxycarbonyl group was selected for the temporary protection of the α-amino group and the *tert*-butyl

Table 1 Physicochemical Properties and Phosphorylation Rates of Free Peptides

Compound	$[\alpha]^{20}$ (*c* 1.0, H_2O)	R_f (system)	FAB-MS ($M+1$)	Relative phosphorylation rate
*p*Glu-Ala-Glu-Ser(PO_3H_2)-Asn-OH	− 37.8	0.10(C)	611	–
*p*Glu-Ala-Glu-Ser-Asn-OH	− 66.5	0.32(C)	531	100
*p*Glu-Ala-Glu-Ser-Asp-OH	− 56.0	0.35(C)	532	85
Glu-Ala-Glu-Ser-Asn-OH	− 47.3	0.30(C)	549	66
*p*Glu-Ala-Gln-Ser-Asn-OH	− 58.9	0.14(C)	530	55
*p*Glu-Ala-Glu-Ser-Leu-OH	− 83.1	0.47(C)	530	18
*p*Glu-Gln-Gly-Ser-Asn-OH	− 32.9	0.18(C)	516	64
*p*Glu-Ala-Glu-Ser-OH	− 53.3	0.37(C)	417	not detectable
*p*Glu-Ala-Glu-Ser-Asn-Ala-OH	− 74.0	0.30(C)	602	59
*p*Glu-Ala-Glu-Gly-Gly-Ser-Asn-OH	− 50.8	0.19(C)	645	22
*p*Glu-Val-Gly-Glu-Ser-Glu-Asn-OH	− 48.2	0.11(C)	767	43
*p*Glu-Val-Gly-Glu-Ser-Glu-Asp-OH	− 47.3	0.15(C)	768	28

ester for the permanent protection of carboxyl groups. The coupling methods employed were the pentafluorophenyl ester, *p*-nitrophenyl ester, *N*-hydroxysuccinimide ester and DCC–HOBt. The choice of protecting groups and of different activation methods was suggested mainly by availability of the intermediates in our laboratory.

The dipeptide Z-Asn-Ala-OtBu had been prepared previously by König and Geiger [9], but these authors did not report the optical rotation.

The phosphorylated pentapeptide was synthesized using Alloc-Ser[PO(O*t*Bu)$_2$]-OH prepared according to Lacombe *et al.*[10]. This compound was described as a colourless oil and used by these authors without further purification. We obtained this product in a crystalline form and therefore we were able to determine its physicochemical constants. Our ^{1}H-NMR data are identical to those reported by Lacombe *et al.*. The Alloc protecting group was removed by tributyltin hydride, acetic acid and tetrakis(tri-phenylphosphine)-palladium (O) [11]. In fact, direct chemical phosphorylation of the protected pentapeptide, in which only the side-chain hydroxyl group of serine was unprotected, gave unsatisfactory results.

The protected intermediates were purified by crystallization until homogeneous in thin-layer chromatography.

All free peptides were purified by preparative reverse-phase HPLC. The mass spectra of synthetic peptides have been found to be identical with those of natural products.

The physicochemical data for the synthesized compounds are reported in Tables 1–6.

Table 1 also shows the kinetic data of peptide phosphorylation. The rates of phosphorylation indicate that three requirements are important for peptide phosphorylation: (1) an aspartic acid and especially an asparagine at the C-terminal side of the serine residue; (2) a glutamic acid at the N-terminal side of the serine residue; (3) a blocked peptide N-terminus (absence of the positive charge of the α-ammonium group).

Moreover, following phosphorylation *in vitro* by kinase N II, the synthetic pentapeptide *p*Glu-Ala-Glu-Ser-Asn-OH shows strong inhibiting activity in DNA transcription directed by prokaryotic and eukaryotic RNA polymerases. Transcription of 2 μg of phage DNA is almost completely inhibited by 5 ng of the enzymatically phosphorylated peptide. This inhibition is apparently due to a Mg^2-mediated interaction between the phosphopeptide and DNA [7].

These results support the hypothesis that phosphorylated acidic domains of nuclear non-histone proteins can bind directly to DNA, thereby controlling transcription and RNA synthesis in several cell systems.

EXPERIMENTAL PART

Peptide purification

Bovine seminal plasma, obtained by centrifugation of total semen, was ultrafiltered (M cut-off 10 kDa). After lyophilization, the powder was twice extracted with glacial acetic acid. The insoluble material was discarded by centrifugation, and the recovered supernatant was stirred with diethyl ether to remove lipids.

After centrifugation, the precipitate was lyophilized and gel-filtered on a Sephadex G-15 column in 0.1 M in acetic acid. Active material emerged from the column at 1.6 to 1.9 times the void volume. This active fraction, lyophilized, was redissolved in a 10 mM ammonium acetate buffer (pH 6), and chromatographed on a DEAE–cellulose column equilibrated with the same buffer. The column was eluted stepwise with increasing concentrations of ammonium acetate. Slightly acidic active material was eluted from DEAE–cellulose by 0.2 M ammonium acetate.

Then, three subsequent reverse-phase HPLC runs were performed under the following conditions: Lihrosorb RP-18 column (Merck), 250×4 mm; $\lambda = 224$ nm; solvent A, 0.05% TFA; solvent B, 0.04% TFA–80% CH_3CN; solvent A to solvent B in 10 min, with a flow rate of 1 ml/min.

The HPLC elution profile of the purest preparation is reported in Figure 1: the first, third and fourth peaks show a clear inhibitory activity, while the second peak is inactive.

Sequence Determination

Mass spectrometric analyses were performed on a VG/70 VSEQ instrument with FAB caesium ion gun; kinetic energy 35 keV, emission current 2 μA, resolution 1000, positive ion ode. Samples were prepared as an aqueous thioglycerol matrix.

The mass spectrum of the first fraction shows the molecular ion peak at m/z 611 and other prominent peaks at m/z 183, 312, 327, 513, 531 which may be assigned to the following breakdown products: m/z 183 = *p*Glu-Ala/; 312 = *p*Glu-Ala-Glu/; 327 = /(+CO) Ser (PO_3H_2)-Asn; 513 = $MH^+ - H_3PO_4$; 531 = MH^+

Table 2 Physicochemical Properties of Intermediates of pGlu-Ala-Glu-Ser-Asn-OH and pGlu-Ala-Glu-Ser(PO_3H_2)-Asn-OH

Compound	Method of coupling and solvent	Yield (%)	M.P. (°C) (solvent)	$[\alpha]_D^{20}$ ($c = 1.0$,EtOH)	R_F (system)	Formula	Analysis Found (calc.)		
Z-Ser-Asn-O*t*Bu	Pfp ester, DMF	85	110–112 (EtOAc–light petroleum)	−9.8	0.50(A)	$C_{19}H_{27}N_3O_7$	55.43 (55.72)	6.79 (6.64)	10.51 (10.62)
Z-Glu(O*t*Bu)-Ser-Asn-O*t*Bu	Su ester, THF	87	118–120 (EtOAc–light petroleum)	−19.3	0.53(A)	$C_{28}H_{42}N_4O_{10}$	56.79 (56.54)	7.48 (7.12)	9.11 (9.42)
Z-Ala-Glu-(O*t*Bu)-Ser-Asn-O*t*Bu	Pfp ester, THF	81	162–164 (EtOAc)	−32.6	0.41(A)	$C_{31}H_{47}N_5O_{11}$	56.18 (55.91)	6.69 (7.11	10.27 (10.51)
Boc-*p*Glu-Ala-Glu-(O*t*Bu)-Ser-Asn-O*t*Bu	Pfp ester, THF	89	199 dec. (EtOAc)	−52.6	0.15(A)	$C_{33}H_{54}N_6O_{13}$	53.01 (53.36	7.16 (7.33)	11.18 (11.31)
Alloc-Ser [PO(O*t*Bu)$_2$]-Asn-O*t*Bu	DCC (HOBt), DMF	78	Oil	–	0.61(A)	$C_{23}H_{42}N_3O_{10}P$	50.41 (50.08)	7.39 (7.67)	7.85 (7.62)
Z-Glu(O*t*BU)-Ser[PO(O*t*Bu)$_2$]-Asn-O*t*Bu	Su ester, THF	81	Amorphous	−14.2	0.57(A)	$C_{36}H_{59}N_4O_{13}P$	54.67 (54.95)	7.73 (7.56)	6.99 (7.12)
Z-Ala-Glu(O*t*Bu)-Ser[PO (O*t*Bu)$_2$]-Asn-O*t*Bu	Pfp ester, THF	83	101–103 (EtOAc–light petroleum)	−12.7	0.54(A)	$C_{39}H_{64}N_5O_{14}P$	54.38 (54.60)	7.43 (7.52)	8.31 (8.16)
Boc-*t*Glu-Ala-Glu(O*t*Bu)-Ser[PO(OtBu)$_2$]-Asn-O*t*Bu	Pfp ester, THF	79	140 dec. (EtOAc)	−37.8	0.16(A)	$C_{41}H_{71}N_6O_{16}P$	52.84 (52.66)	7.51 (7.65)	9.04 (8.99)

Table 3 Physicochemical Properties of Intermediates of *p*Glu-Ala-Glu-Ser-Asp-OH and *p*Glu-Ala-Glu-Ser-Leu-OH

Compound	Method of coupling and solvent	Yield (%)	MP (°C) (solvent)	$[\alpha]_D^{20}$($c = 1.0$, EtOH)	R_f(system)	Formula	Analysis Found (calc.)		
Z-Ser-Asp(O*t*Bu)$_2$	Pfp ester, THF	88	135–137 (EtOAc–light petroleum)	−6.8	0.83(A)	$C_{23}H_{34}N_2O_8$	58.99 (59.20)	7.51 (7.34)	6.14 (6.00)
Z-Glu(O*t*Bu)-Ser-Asp(O*t*Bu)$_2$	Su ester, THF	90	78–80 (EtOAc–light petroleum)	−16.4	0.82(A)	$C_{32}H_{49}N_3O_{11}$	58.79 (58.96)	7.72 (7.57)	6.28 (6.44)
Z-Ala-Glu(O*t*Bu)-Ser-Asp(O*t*Bu)$_2$	Pfp ester, THF	92	158–160 (EtOAc–light petroleum)	−27.2	0.74(A)	$C_{35}H_{54}N_4O_{12}$	58.27 (58.14)	7.39 (7.53)	7.61 (7.75)
Boc-*p*Glu-Ala-Glu-(O*t*Bu)-Ser-Asp(O*t*Bu)$_2$	Pfp ester, THF	85	191–193 (EtOAc–light petroleum)	−51.2	0.30(A)	$C_{37}H_{61}N_5O_{14}$	55.68 (55.55)	7.61 (7.68)	8.82 (8.75)
Z-Ser-Leu-O*t*Bu[a]	Pfp ester, THF	97	94–95 (light petroleum)	−33.2	0.82(A)	$C_{21}H_{32}N_2O_6$	61.48 (61.73)	7.72 (7.89)	6.97 (6.86)
Z-Glu(O*t*Bu)-Ser-Leu-O*t*Bu	Su ester, THF	84	96–97 (EtOAc–light petroleum)	−35.0	0.84(A)	$C_{30}H_{47}N_3O_9$	60.92 (60.67)	7.68 (7.97)	7.15 (7.07)
Z-Ala-Glu(O*t*Bu)-Ser-Leu-OtBu	Pfp ester, THF	89	175–177 (EtOAc–light petroleum)	−48.8	0.75(A)	$C_{33}H_{52}N_4O_{10}$	59.76 (59.61)	7.61 (7.88)	8.29 (8.42)
Boc-*p*Glu-Ala-Glu(O*t*Bu)-ser-Leu-O*t*Bu	Pfp ester, THF	90	200 dec. (EtOAc–light petroleum)	−54.2	0.20(A)	$C_{35}H_{59}N_5O_{12}$	56.44 (56.65)	7.93 (8.01)	9.27 (9.44)

[a]Wang and Li [12] have recorded mp 91–93°C and $[\alpha]_D = 42°$ (in methanol).

Table 4 Physicochemical Properties of Intermediates of *p*Glu-Glu-Gly-Ser-Asn-OH, *p*Glu-Ala-Glu-Ser-OH and *p*Glu-Ala-Gln-Ser-Asn-OH

Compound	Method of coupling and solvent	Yield (%)	MP (°C) (solvent)	$[\alpha]_D^{20}$ ($c = 1.0$, EtOH)	R_f (system)	Formula	Analysis Found (calc.)		
Z-Gly-Ser-Asn-O*t*Bu	Pfp ester, THF	91	151–153 (EtOAc–light petroleum)	−20.2	0.26(A)	$C_{21}H_{30}N_4O_8$	53.89 (54.06)	6.54 (6.48)	11.87 (12.01)
Z-Gln-Gly-Ser-Asn-O*t*Bu	Np ester, DMF	79	202–204 (EtOH–EtOA-c)	−17.5	0.33(B)	$C_{26}H_{38}N_6O_{10}$	52.75 (52.52)	6.32 (6.44)	14.02 (14.13)
Boc-*p*Glu-Gln-Gly-Ser-Asn-O*t*Bu	Pfp ester, DMF	83	147–149 (EtOAc)	−31.0	0.11(B)	$C_{28}H_{45}N_7O_{12}$	49.86 (50.07)	7.52 (7.34)	14.45 (14.60)
Z-Glu(O*t*Bu)-Ser(*t*Bu)-O*t*Bu	Su ester, EtOAc	85	94–96 (EtOAc–light petroleum)	−2.4	0.92(A)	$C_{28}H_{44}N_2O_8$	62.41 (62/65)	8.06 (8.26)	5.47 (5.22)
Z-Ala-Glu(O*t*Bu)-Ser(*t*Bu)-O*t*Bu	Pfp ester, EtOAc	87	154–155 (EtOAc–light petroleum)	−18.1	0.87(A)	$C_{31}H_{49}N_3O_9$	61.10 (61.25)	7.91 (8.12)	7.08 (6.91)
Boc-*p*Glu-Ala-Glu(O*t*Bu)-Ser(*t*Bu)-O*t*Bu	Su ester, DMF	82	130–132 (EtOAc–light petroleum)	−27.2	0.80(A)	$C_{33}H_{56}N_4O_{11}$	57.59 (57.86)	8.39 (8.24)	7.89 (8.18)
Z-Gln-Ser-Asn-O*t*Bu	Np ester, DMF	68	177–180 (EtOH)	−18.6	0.14(A)	$C_{24}H_{35}N_5O_9$	53.38 (53.62)	6.64 (6.56)	13.31 (13.02)
Z-Ala-Gln-Ser-Asn-O*t*Bu	Pfp ester, DMF	59	210 dec. (EtOH)	−32.5	0.73(C)	$C_{27}H_{40}N_6O_{10}$	53.03 (53.28)	6.79 (6.62)	13.68 (13.81)
Boc-*p*Glu-Ala-Gln-Ser-Asn-*t*Bu	Pfp ester, DMF	85	147–149 (EtOH)	−45.1	0.41(C)	$C_{29}H_{47}N_7O_{12}$	50.84 (50.79)	7.05 (6.91)	14.51 (14.30)

Table 5 Physicochemical Properties of Intermediates of *p*Glu-Ala-Glu-Ser-Asn-Ala-OH and Glu-Ala-Glu-Ser-Asn-OH

Compound	Method of coupling and solvent	Yield (%)	MP (°C) (solvent)	$[\alpha]_D^{20}$ ($c = 1.0$, EtOH)	R_F (system)	Formula	Analysis Found (calc.)		
Z-Asn-Ala-O*t*Bu[d]	Np ester, DMF	81	162–163 (EtOAc)	−25.7	0.64(A)	$C_{19}H_{27}N_3O_6$	57.86 (58.00)	7.21 (6.92)	10.48 (10.68)
Z-Ser-Asn-Ala-O*t*Bu	Pfp ester, DMF	85	179–180 (EtOAc)	−25.3	0.31(A)	$C_{22}H_{32}N_4O_8$	54.75 (54.98)	6.53 (6.71)	11.78 (11.66)
Z-Glu-(O*t*Bu)-Ser-Asn-Ala-O*t*Bu	Su ester, DMF	83	194–196 (EtOAc)	−27.7	0.29(A)	$C_{31}H_{47}N_5O_{11}$	56.17 (55.92)	7.33 (7.12)	10.29 (10.52)
Z-Ala-Glu(O*t*Bu)-Ser-Asn-Ala-O*t*Bu	Pfp ester, DMF	79	230 dec. (EtOH–EtOAc)	−39.4	0.16(A)	$c_{34}H_{52}N_6O_{12}$	55.26 (55.41)	7.01 (7.11)	11.57 (11.40)
Boc-*p*Glu-Ala-Glu(O*t*Bu)-Ser-Asn-Ala-O*t*BU	Pfp ester, DMF	76	220 dec. (EtOAc)	−58.2	0.11(A)	$C_{36}H_{59}N_7O_{14}$	52.89 (53.12)	7.43 (7.31)	11.85 (12.05)
Z-Glu(O*t*Bu)-Ala-Glu(O*t*Bu)-Ser-Asn-O*t*Bu	Su ester, DMF	81	191–193 (EtOAc)	−31.8	0.27(B)	$C_{40}H_{62}N_6O_{14}$	56.73 (56.46)	7.08 (7.34)	9.57 (9.88)

(a)König and Geiger [9] do not mention the optical rotation.

Table 6 Physicochemical Properties of Intermediates of *p*Glu-Ala-Glu-Gly-Gly-Ser-Asn-OH

Compound	Method of coupling and solvent	Yield (%)	MP (°C) (Solvent)	$[\alpha]_D^{20}$ ($c = 1.0$, EtOH)	R_F (system)	Formula	Analysis Found (calc.)		
Z-Gly-Gly-Ser-Asn-O*t*Bu	Pfp ester, THF	91	164–165 (EtOAc–light petroleum)	− 13.6	0.17(A)	$C_{23}H_{33}N_5O_9$	52.51 (52.78)	6.49 (6.35)	13.22 (13.38)
Z-Glu(O*t*Bu)Gly-Gly-Ser-Asn-O*t*Bu	Su ester, THF	87	137–140 (EtOAc–light petroleum)	− 14.1	0.38(B)	$C_{32}H_{48}N_6O_{12}$	54.08 (54.22)	6.61 (6.82)	11.93 (11.85)
Z-Ala-Glu(O*t*Bu)-Gly-Gly-Ser-Asn-O*t*Bu	Pfp ester, DMF	88	177–180 (EtOAc)	− 21.7	0.28(B)	$C_{35}H_{53}N_7O_{13}$	53.77 (53.90)	6.98 (6.85)	12.35 (12.57)
Boc-*p*Glu-Ala-Glu(O*t*Bu)-Gly-Gly-Ser-Asn-O*t*Bu	Pfp ester, DMF	82	185 dec. (EtOH–EtOAc)	− 41.9	0.10(B)	$C_{37}H_{60}N_8O_{15}$	52.07 (51.86)	7.24 (7.06)	12.88 (13.07)

$-HPO_3$. The spectrum was interpreted as defining the phosphoryled pentapeptide: *p*Glu-Ala-Glu-Ser-(PO_3H_2)-Asn-OH.

The third peptide fraction has a protoned molecular ion at *m*/z 531 and the following breakdown products: *m*/*z* 133 = /+2HAsn; 155 = *p*Glu-Ala (-CO)/; 312 = *p*Glu-Ala-Glu/; 399 = *p*Glu-Ala-Glu-Ser/, corresponding to the pentapeptide: *p*Glu-Ala-Glu-Ser-Asn-OH. Figure 2 shows the FAB mass spectrum of this native pentapeptide.

The interpretation of the mass spectrum of the fourth fraction was the following: protonated molecular ion at *m*/*z* 767, breakdown products *m*/*z* 133 = /+2HAsn 261 = /+HGlu-Asn; 369 = *p*Glu-Val-Gly-Glu (−CO)/ 397 = *p*Glu-Val-Gly-Glu/. The assumed sequence was: *p*Glu-Val-Gly-Glu-Ser-Glu-Asn-OH.

Peptide Synthesis

Thin-layer chromatography was performed on Merck Kieselgel 60 plates in the following solvent systems (by volume): System A–benzene, ethyl acetate, acetic acid and water (10:10:2:1); System B–chloroform, methanol and acetic acid (85:10:5); System C–1-butanol, acetic acid and water (4:1:1). The chromatograms were developed with iodine vapours.

All free peptides were purified by preparative reverse-phase HPLC using an LKB instrument and a C_{18} ODS (5 μM) column (10 × 250 mm). The peptides were eluted with a 0–60% linear gradient of CH_3CN in 0.1% aqueous TFA over a period of 60 min, with a flow rate of 5 ml/min and detection at 226 nm.

Melting points were taken on a Büchi apparatus and were uncorrected.

Optical rotations were measured in a jacked 1 dm cell, with a Perkin Elmer model 141 polarimeter.

Protected amino acids, NovaSyn resins and the dye Novachrome were purchased from Calbiochem-Novabiochem AG, Läufelfingen, Switzerland.

Peptide Synthesis in Solution

The amino component was dissolved in DMF or in THF (see Tables 2–6). The concentrations were in the range 0.02–0.2 M. The solution, cooled to 0 °C, was treated with an equimolar amount of active ester of the protected amino acid. The mixture was stirred for 4 h at 0 °C and overnight at room temperature.

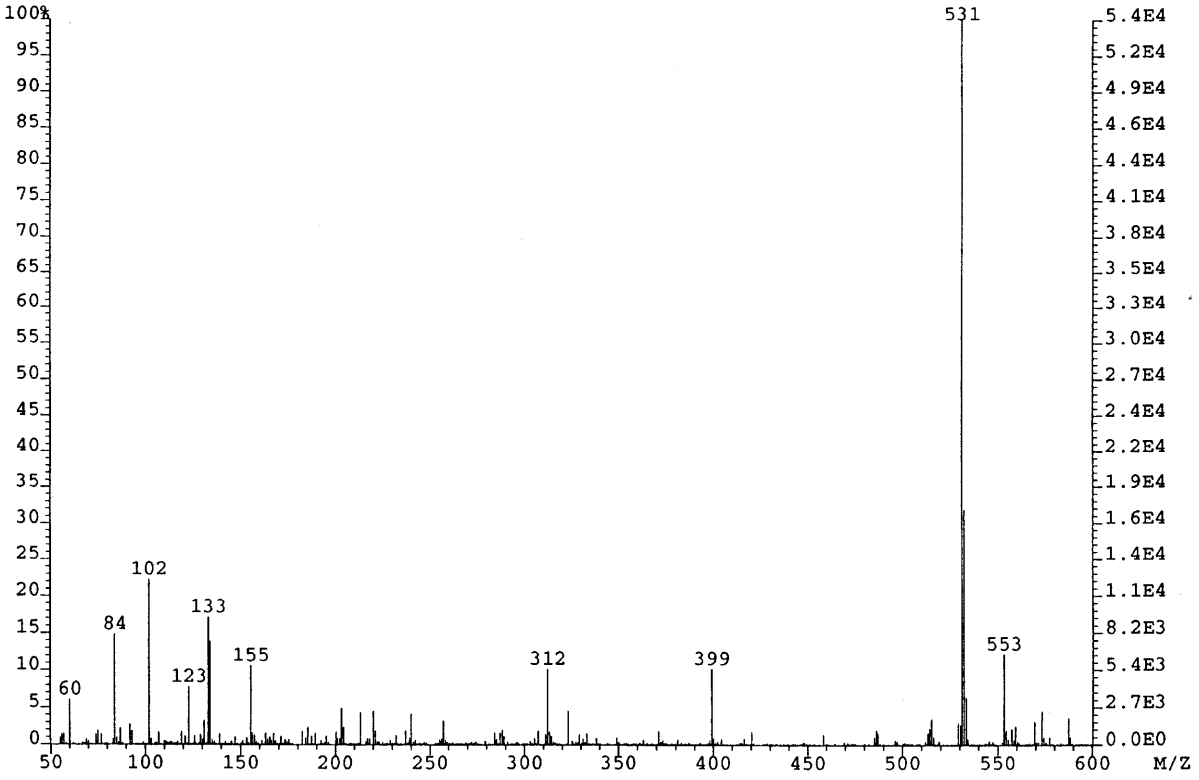

Figure 2 FAB^+ mass spectrum of the native pentapeptide pGlu-Ala-Glu-Ser-Asn-OH.

The solvent was evaporated *in vacuo*. The residue was dissolved in ethyl acetate and the solution was then washed with 0.1 N HCl, water, saturated $NaHCO_3$, water, dried over anhydrous Na_2So_4. and evaporated to dryness *in vacuo*. The protected peptides were recrystallized from suitable solvents (see Tables 2–6) until homogeneous in TLC.

The benzyloxycarbonyl group was removed by hydrogenation with 10% palladium on charcoal at atmospheric pressure.

The final removal of acid-labile protecting groups was carried out using trifluoroacetic acid at room temperature for 1 h.

N-α(allyloxycarbonyl)-O-(di-*tert*-butylphosphono)-L-serine

This compound was prepared according to Lacombe *et al.* [10]. The oily product was crystallized from EtOAc–light petroleum, mp 76–77 °C, $[\alpha]_D^{20} + 7.1°$ ($c = 1$, EtOAc). TLC: $R_F = 0.36$ (system A). ^{1}H-NMR δ: 1.47(s, 18 H, *t*-Bu), 4.23 (m, 1 H, Hβ-Ser), 4.40 (m, 1 H, Hβ-Ser), 4.49 (m, 1 H Hα-Ser), 4.58 (d, 2 H, Alloc), 5.18–5.34 (m, 2 H, Alloc), 5.84–5.99 (m, 1 H, Alloc). This product underwent spontaneous degradation after three or four months despite storage at −20°C.

Cleavage of Allyloxycarbonyl Group

Alloc-Ser[PO(O*t*Bu)$_2$]-Asn-O*t*Bu, weighing 3.6 g (6.5 mmol), 88 mg of tetrakis(triphenyl-phosphine)-palladium (0) and 0.85 ml of acetic acid were dissolved in 120 ml of ethyl acetate. Then, 1.9 ml (6.5 mmol) of tribulytin hydride were added in one portion under stirring. After 15 min, the reaction mixture was washed with saturated $NaHCO_3$ and water; the solution was dried over Na_2SO_4 and evaporated to dryness *in vacuo*. The crude material was purified by column chromatography on silica gel. Various tin impurities were first eluted with cyclohexane/diethyl ether (8:2); then H-Ser[PO(O*t*-Bu)$_2$)]-Asn-O*t*Bu was collected upon elution with ethyl acetate as a oily product (2.1 g, 70% yield). No signal characteristic of the allyl group could be detected by ^{1}H-NMR analysis.

Solid-phase Synthesis

The syntheses of the heptapeptides *p*Glu-Val-Gly-Glu-Ser-Glu-Asn-OH and *p*Glu-Val-Gly-Glu-Ser-Glu-Asp-OH were carried out on a automated peptide synthesizer (Biolynx plus, mod. 4170, Novabiochem, Nottingham, UK), employing the continuous flow techniques, a polyamide resin and the Fmoc strategy [8].

The functional groups of amino acid side chains were protected as follows: Glu(O*t*Bu), Ser(*t*Bu). The resins used were: Fmoc-Asn(Trt)-NovaSyn KA 125 (substitution 0.18 mMol/g) and Fmoc-Asp(O*t*Bu)-NovaSyn KA 125 (substitution 0.21 mMol/g).

The couplings were carried out in DMF with the appropriate Fmoc–amino acid-OPfp (5 equivalents) and HOBt (5 equivalents) in the presence of the anionic dye Novachrome for counter-ion distribution monitoring (pGlu was coupled using Boc-*p*Glu-OPfp).

Deprotection and cleavage of peptides from the resin were performed at room temperature with anhydrous TFA containing 5% water for 2 h.

Kinase N II-induced Peptide Phosphorylation

The assay mixture for nuclear kinase N II activity contained 50 mM Tris–HCl (pH 8), 10 mM $MgCL_2$, 50 mM NaCl, 10–20 μg of peptide, 50 μCi of [γ-^{32}P]ATP and 0.1 μg of enzyme in a final volume of 0.1 ml. Following incubation of 37°C for 20 min, aliquots were applied directly onto electrophoretic cellulose plates in order to isolate the phosphorylated compound. The electrophoresis was performed for 2 h at 600 V in acetic acid:formic acid:water:-acetone (8:2:75:15 by vol, pH 1.9). The sport corresponding to the phosphorylated peptide was visualized by autoradiography and scraped from cellulose plate. ^{32}P-peptide was subsequently eluted by water and lyophilized.

Ribonucleotide Polymerization to RNA in Rat Liver Nuclei

Ribonucleotide polymerization was assayed in 0.125 ml reaction mixtures containing: 10^7 nuclei (isolated from rat hepatocytes in stepwise sucrose gradients), 40 mM Tris–HCl (pH 8.3), 2 mM $MgCl_2$, 0.36 mM $MnCl_2$, 0.06 mM EDTA, 3 mM dithiothreitol, 10% glycerol (v/v), 12 μg of bovine serum albumin, 1mM each ATP, CTP, GTP and [^{14}C]UTP to a total of 0.6 μCi and variable amounts of inhibiting peptide fractions.

Incubations were at 25 °C for 30 min and the reactions were stopped by adding 1 ml of cold 10%trichloroacetic acid supplemented with 3% sodium pyrophosphate. The acid-treated nuclei were collected on glass fibre filters that were extensively washed with the cold trichloroacetic acid solution.

The filters were dried at 60 °C and then counted for the retained macromolecular radioactivity with 10 ml of Pico-Fluor (Packard, USA) in a liquid scintillation spectrometer. The synthesized RNA was expressed as the pmol of [^{14}C] UMP residues incorporated into acid-precipitable nuclear material.

REFERENCES

1. G. Lugaro, F. Campagnari, R. Moretti and M. M. Casellato (1988). Inhibition of DNA polymerization and DNA transcription to RNA by seminal plasma peptides. *Biochem. Biophys. Acta 950*, 420–428.
2. G. L. Gianfranceschi, D. Barra, F. Bossa, S. Coderoni, M. Papparelli, F. Venanzi, F. Cicconi and D. Amici (1982). Small peptides controlling transcription *in vitro* are bound to chromatin DNA. *Biochim. Biophys. Acta 699*, 138–148.
3. D. Amici, G. B. Rossi, L. Cioé, G. P. Matarese, A. Dolei, L. Guglielmi and G.L. Gianfranschi (1977). Low-molecular weight peptide inhibits RNA synthesis in human leukemic and phytohemaglutinin-stimulated leukocytes and globin mRNA transcription in differentiating Friend cells. *Proc. Natl. Acad. Sci. USA 74*, 3869–3873.
4. G. Lugaro, E. Manera, M. M. Casellato and L. Riboni (1982). A non-steroidal gametic factor linked to DNA modulates δ-aminolevulinic acid synthase inducibility acting on liver transcriptional and translational process. *Exper. Gerontol. 17*, 365–373.
5. G. Lugaro, E. Manera, M. M. Casellato, E. Maselli, C. Talatin and G. Facchini (1982). Effects of nonsteroidal gametic factor linked to DNA on senile cataract in man. *J. Ophalm. 66*, 442–445.
6. S. Coderoni, A. Miano, D. Barro, M. Bramucci, F. Felici, M. Paparelli, D. Amici and G.L. Gianfranceschi (1988). Isolation and characterization of small phosphorylated peptides controlling transcription *in vitro* from trout testis chromatin DNA. *Physiol. Chem. Phys. Medical NMR 20*, 91–108.
7. F. Chillemi, G. Lugaro, D. Boari, E. Cardellini, M. Bramucci, A. Miano, D. Amici, G. F. Gianfranceschi and E. Durban (1991). Acid pentapeptide phosphorylated *in vitro* by calf thymus protein kinase N II binds to DNA in presence of Mg^{++} cations. *FEBS Lett. 291*, 67–70.
8. E. Atherton, C. J. Logan and R. C. Sheppard (1981). Procedures for solid-phase synthesis using α-*N*-fluorenylmethoxycarbonyl-amino-acid on polyamide supports. *J. Chem. Soc. Perkin Trans. I*, 538–546.
9. W. König and R. Geiger (1970). Eine neue methode zur synthese von peptiden: activierung der carboxylgruppe mit dicyclohexyl-carbodiimid unte zusatz van 1-hydroxy-benzotriazolen. *Chem. Ber. 103*, 788–802.
10. J. M. Lacombe, F. Andriamanampisoa and A. A. Pavia (1990). Solid-phase synthesis of peptides containing phosphoserine using phosphate *tert*-butyl protecting group. *Int. J. Pept. Protein Res. 36*, 275–280.
11. O. Dangles, F. Guibè, G. Balavoine, S. Lavielle and A. Marquet (1987). Selective cleavage of allyl and allyloxycarbonyl groups through palladium-catalysed hydrostannolysis with tributyltin hydride. *J. Org.Chem. 52*, 4984–4993.
12. K. T. Wang and C. H. Li (1971). Human pituitary growth hormone. XXXI. The synthesis of two protected peptide fragments occurring in the region residues 53–67. *J. Org. Chem. 36*, 2419–2422.