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Further analogues of plant peptide hormone phytosulfokine-α (PSK-α) and their biological evaluation

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Abstract: Phytosulfokine-α (PSK-α), a sulfated growth factor of structure H-Tyr(SO_3H)-Ile-Tyr(SO_3H)-Thr-Gln-OH universally found in both monocotyledons and dicotyledons, strongly promotes proliferation of plant cells in culture. In studies on the structure/activity relationship of PSK-α the synthesis was performed of a series of a further 23 analogues modified in position 1, 3 or 4 as well as simultaneously in positions 1 and 3 of the peptide chain. Peptides were synthesized by the solid phase method according to the Fmoc procedure on a Wang-resin. Free peptides were released from the resin by 95% TFA in the presence of EDT. All peptides were tested by competitive binding assay to the carrot membrane using ^{3}H-labelled PSK-α according to the test of Matsubayashi *et al.* Among these peptide analogues, [H-Phe(4-Cl)^1]-PSK-α (**IV**), [H-Phe(4-I)^1]-PSK-α (**VII**), and [Phe(4-Cl)^3]-PSK-α (**XI**) retained 30% PSK-α activity. Analogue [Tyr(PO_3H_2)^3]-PSK-α (**IX**) showed 10% of PSK-α activity. Copyright © 2005 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: phytosulfokine-α; PSK-α; phytosulfokine-α analogues; plant peptides hormones

INTRODUCTION

The sulfated peptide phytosulfokine (PSK-α), H-Tyr(SO_3H)-Ile-Tyr(SO_3H)-Thr-Gln-OH, is an intercellular signal peptide that plays a key role in cellular de-differentiation and re-differentiation in plants [1]. Sulfated tyrosine residues are often found in secreted peptides in animals, but to date PSK-α is the only example of post-translational sulfation of tyrosine residues in plants. Several paralogous genes encoding ≈80-residue precursors of PSK-α have been identified in *Arabidopsis*. Each predicted protein has a probable secretion signal at the *N*-terminus and a single PSK-α sequence close to the *C*- terminus, similar to other peptide-hormones generally synthesized as inactive higher molecular weight precursors that must undergo a variety of post-translational processing steps to yield the active peptides [2]. Studies using radiolabelled PSK-α have provided evidence for the existence of high-affinity binding sites for PSK-α in plant plasma membranes [3,4]. Recently, PSK-α receptor has been purified from membrane fractions and cloned carrot cells [4]. The cDNA encodes a typical LRR (leucine-rich repeat) receptor kinase that has 21 LRRs and a 36-residue island between LRR 17 and LRR 18. The biological evaluation of unsulfated analogues of these peptides showed that they require the sulfate ester for the expression of their biological activity [5,6]. Moreover, Matsubayashi *et al.* [7] observed that the unsulfated PSK-α analogue was dramatically less active. Among a series of further PSK-α analogues [8] only [Phe(4-NO_2)^1]- and [Phg(4-NO_2)^1]-PSK-α showed 10% binding activity compared with that of the native peptide, whereas [D-Phg(4-NO_2)]^1-PSK-α retained only 1% biological activity. Continuing studies [8] on the structure-function relationship of the plant peptide hormone – phytosulfokine-α- and based on the above results synthesis of its further analogues **I**–**XXIII** was performed (Table 1).

Among these analogues the 4-sulfated-Tyr residue in position 1 was replaced by: (a) 4-phosphated L or D-Tyr, 4-Br, 4-Cl, 4-I Phe and 4-$NHSO_2CH_3$-Phg (**I–VIII**); (b) the amino acid in position 3 was exchanged (**IX–XII**) for 4-phosphated L or D-Tyr, 4-Br, 4-Cl, and 4-F Phe; (c) both sulfated Tyr residues in positions 1 and 3 were simultaneously replaced by 4-phosphated L- or D-Tyr (**XIII** and **XIV**), and by 4-X-Phe (where X = Cl or F) (**XV** and **XVI**). In further analogues PSK-α was modified simultaneously in position 1 by Phe(4-X) derivatives (X = Cl, Br, F, and I) and in position 3 by 3-sulfated Tyr (**XVII–XX**), by 3-sulfated Tyr in position 1 and 4-X-Phe (X = F, Cl) in position 3 (**XXI**–**XXII**). Moreover, the Thr residue in position 4 was exchanged for Ile (**XXIII**). For modification of the PSK-α structure,

Abbreviations: The symbols of amino acids, peptides and their derivatives are in accordance with the Recommendations of the IUPAC-IUB Joint Commission on Biochemical Nomenclature (1984) [*Eur. J. Biochem.* 1984; **138**: 9] and *J.Pept.Sci.* 1999; **5**: 465, *J.Pept.Sci.* 2003; **9**: 1.

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Table 1 Physico- chemical Data of New PSK-α Analogues (**I–XXIII**)

Peptide	$[\alpha]_D^{20a}$	Rt[b] (HPLC)	MW	
			Calc.	Found.
[Tyr(PO_3H_2)^1]-PSK-α (**I**)	−16.1 c = 0.7%	13.94	846.8	845.2
[D-Tyr(PO_3H_2)^1]-PSK-α (**II**)	−25.4 c = 0.7%	16.47	846.8	845.4
[Phg(4-OPO_3H_2)^1]-PSK-α (**III**)	−27.5 c = 0.7%	14.48	832.7	830.8
[Phe(4-Cl)^1]-PSK-α (**IV**)	−18.3 c = 0.5%	21.48	784.5	783.7
[Phe(4-Br)^1]-PSK-α (**V**)	−1.8 c = 0.7%	20.82	828.8	828.1
[Phe(4-F)^1]-PSK-α (**VI**)	−13.5 c = 0.5%	21.95	768.1	768.2
[Phe(4-I)^1]-PSK-α (**VII**)	−22.4 c = 0.5%	21.42	876.0	875.3
[Phg(4-$NHSO_2CH_3$)^1]-PSK-α (**VIII**)	−12.7 c = 0.7%	8.70	829.4	828.2
[Tyr(PO_3H_2)^3]-PSK-α (**IX**)	−10.8 c = 0.7%	13.82	846.8	845.2
[D-Tyr(PO_3H_2)^3]-PSK-α (**X**)	−4.4 c = 0.7%	15.61	846.8	845.4
[Phe(4-Cl)^3]-PSK-α (**XI**)	−17.6 c = 0.5%	24.70	784.6	783.8
[Phe(4-F)^3]-PSK-α (**XII**)	−22.5 c = 0.7%	21.84	768.1	767.4
[Tyr(PO_3H_2)^1, Tyr(PO_3H_2)^3]-PSK-α (**XIII**)	−9.3 c = 0.7%	10.82	846.7	845.0
[D-Tyr(PO_3H_2)^1, D-Tyr(PO_3H_2)^3]-PSK-α (**XIV**)	−6.9 c = 0.7%	17.59	846.7	845.2
[Phe(4-Cl)^1, Phe(4-Cl)^3]-PSK-α (**XV**)	−2.5 c = 1.0%	32.95	723.7	724.0
[Phe(4-F)^1, Phe(4-F)^3]-PSK-α (**XVI**)	−13.0 c = 0.7%	27.40	690.8	688.4
[Phe(4-Cl)^1, Tyr(3-SO_3H)^3]-PSK-α (**XVII**)	−27.8 c = 0.7%	22.34	784.5	783.5
[Phe(4-Br)^1, Tyr(3-SO_3H)^3]-PSK-α (**XVIII**)	−14.2 c = 1.0%	23.82	828.8	827.5
[Phe(4-F)^1, Tyr(3-SO_3H)^3]-PSK-α (**XIX**)	−10.5 c = 0.5%	19.87	768.1	767.4
[Phe(4-I)^1, Tyr(3-SO_3H)^3]-PSK-α (**XX**)	−2.8 c = 1.0%	22.29	876.0	876.7
[Tyr(3-SO_3H)^1, Phe(4-Cl)^3]-PSK-α (**XXI**)	−7.8 c = 0.5%	26.03	784.5	783.7
[Tyr(3-SO_3H)1, Phe(4-F)^3]-PSK-α (**XXII**)	−42.8 c = 0.5%	23.91	768.1	766.6
[Ile^4]-PSK-α (**XXIII**)	−17.9 c = 0.7%	17.36	858.1	857.3

[a] 1% NH_3 in H_2O was used as solvent.
[b] HPLC on Ultrasphere ODS columns (Beckman) 4.5 × 250 mm; solvent system: S1–0.1% aqueous TFA, S2–80% acetonitrile in water; linear gradient: 0–100% of S2 in 60 min. The yield for these peptide was 30%–97%. The composition of all peptides was confirmed by amino acid analysis.

synthesis of H-Phg(4-OPO_3H_2)-OH (**IIIa**), Fmoc-Phg(4-OPO_3H_2)-OH (**IIIb)** and Fmoc-Tyr(3-SO_3H)-OH (**XVIIa**) was performed.

MATERIAL AND METHODS

Chemical Part

General procedures. Amino acid compositions were determined on an amino acid analyser Mikrotechna T339 (Czechoslovakia). The optical activity of the chiral compounds was measured with a Jasco DIP-1000 polarimeter (Jasco, Japan). The molecular weights of the peptides were determined using a Finigan Mat TSQ 700 (USA) mass spectrometer. The purity and homogeneity of all final products were checked by HPLC (Beckman Peptide Gold System), amino acid analysis and molecular weight determinations. The purity of all peptides was about 100%.

N-protected amino acid derivatives: Fmoc-Thr(Bu^t)-OH, Fmoc-Ile-OH, Fmoc-Val-OH, Fmoc-Tyr-OH, Fmoc-Phe(4-Cl)-OH, Boc-Phe(4-Br)-OH, Fmoc-Phe(4-F)-OH and Boc-Phe(4-I)-OH (Novabiochem) were used.

Fmoc-Tyr(SO_3H)-OH was obtained according to [9]. H-Tyr(4-PO_3H_2)-OH and H-D-Tyr(4-PO_3H_2)-OH were prepared by reaction of L or D-tyrosine, phosphoric acid and phosphorus pentoxide essentially as described by Alewood *et al.* [10]. The product was converted to Fmoc- L or D-Tyr(4-PO_3H_2)-OH by a procedure proposed by Ottinger *et al.* [11]. Boc-Phg(4-$NHSO_2CH_3$)-OH was synthesized according to [1]. H-Tyr(3-SO_3H)-OH was synthesized by the procedure proposed by Praviero *et al.* [12].

All peptides (**I–XXIII**) were obtained by the solid-phase method according to the Fmoc procedure. Amino acids were assembled on a Fmoc-Gln(Trt)-Wang resin (Novabiochem). *N*-terminal residues were introduced as *N*-Fmoc-derivatives, except peptides **V, VII, VIII, XVIII** and **XX**. For these peptides *N*-Boc derivatives were used. The *C*-terminal Gln residue was bound to the resin as Fmoc-Gln(Trt) (Novabiochem). HBTU in the presence of HOBt and *N*-ethylmorpholine were used as coupling reagents. The N^{α}-Fmoc group was removed with 20% piperidine in DMF according to standard methods. During the synthesis of peptides **IV**–**VII**, and **XI**–**XII** the Tyr residue was used as Fmoc-Tyr-OH. The partially protected peptide-resin in DMF–pyridine (4 : 1) was sulfated by DMF–SO_3 complex. The sulfated peptide-resin was cleaved in 95% TFA in the presence of EDT.

All peptides (**I–XXIII**) were purified by semi-preparative HPLC on an Alltech Econsil C_{18}, 10 μm column (ODS 22 × 250 mm), linear gradient 23%–39% S2 for 15 min, flow rate 7 ml/min, determined at 223 nm.

Analytical RP-HPLC was conducted on a Beckman Peptide Gold System chromatograph with a C_{18}, 5 μm Beckman column (ODS 4.6 × 250 mm), and an ultrasphere plus 4.6 × 4.5 mm precolumn. Solvent systems: S1–0.1% aqueous TFA, S2–80% acetonitrile; linear gradient from 0–100% of S2 for 60 min, flow rate 1.0 ml/min, determined at 223 nm. An isocratic system (18% acetonitrile) was also applied to check the purity.

Purity and homogeneity of the free peptides were established by HPLC, amino acid analysis and determination of molecular weights. The physico-chemical data of PSK-α analogues are summarized in Table 1.

H-Phg(4-OPO$_3$H$_2$)-OH (IIIa). In a two-necked round bottom 100 ml flask equipped with a magnetic stirrer/heater and a nitrogen inlet, fresh phosphorus pentoxide (10 g, 70.4 mmol) and 85% phosphoric acid (13.0 g) were placed. 4-hydroxy-L-phenylglycine (2.97 g, 17.88 mmol) was added and the reaction mixture was heated and stirred at 80 °C for 24 h. Then water (30 ml) was added and the mixture was stirred for 30 min. The reaction mixture was cooled to room temperature, diluted with *n*-butanol and kept at 0 °C for 3 h. The product was filtered, washed with ice/water (2 × 20 ml), ethanol (2 × 20 ml) and ether (4 × 20 ml). 3.8 g (yield 81%) of the white precipitate were obtained. m.p. 224°–225 °C; $[\alpha]_D^{20} = +83.3$ (c = 1% in 10% NH_4OH); $m/z = 245.8$ [$(M - H)^-$]; analysis calc. for $C_8H_{10}NO_6P$: 39.0%C, 4.0%, %H, 5.7%N, 12.5%P; found: 38.7%C, 3.9%H, 5.6%N, 12.6 %P.

Fmoc-Phg(4-OPO$_3$H$_2$)-OH (IIIb). H-Phg(4-OPO_3H_2)-OH (3.8 g, 8.1 mmol), Fmoc-OSu (2.7 g, 8.1 mol) and Et_3N (1.1 ml, 8.1 mol) were suspended in a mixture of H_2O (50 ml) and acetonitrile (25 ml). This heterogeneous mixture was stirred at 25 °C for 2 h, while the pH was adjusted to 8.5–9.0 with additional Et_3N until the pH was constant. The resultant homogeneous solution was concentrated *in vacuo*, and both EtOAc (20 ml) and H_2O (20 ml) were added. The mixture was acidified to pH 2.0 with 1 M HCl and the organic phase was washed with 5% citric acid (2 × 40 ml), H_2O (2 × 40 ml), saturated aqueous NaCl (2 × 40 ml) and dried over $MgSO_4$. After concentration *in vacuo* the product was crystallized from ethyl acetate: pentane. 5.2 g (72%) of product was obtained. m.p. 120°–121 °C; $[\alpha]_D^{20} = +65.4$ (c = 1% in methanol); $m/z =$ 467.2 [$(M - H)^-$]; analysis calc. for $C_{23}H_{20}NO_8P$: 58.9%C, 4.3%H, 3.0%N, 6.6%P; found: 58.8%C, 4.2%H, 2.9%N, 6.5%P.

Fmoc-Tyr(3-SO$_3$H)-OH (XVIIa). Fmoc-OSu (9.0 g, 26.7 mmol) was dissolved in THF (25 ml) and added to a solution of H-Tyr(3-SO_3H)-OH (10.5, 39.9 mmol) in H_2O (50 ml) containing Et_3N (9.75 ml, 42 mmol) and the reaction mixture was stirred for 2 h at room temperature. The solvent was evaporated *in vacuo*, and the residue was washed with AcOEt (50 ml). The aqueous layer was acidified with 1 N HCl and extracted with AcOEt (100 ml). The organic layer was washed with 5% citric acid (20 ml) and H_2O (2 × 20 ml), and dried over $MgSO_4$. The AcOEt was evaporated, and the residue was triturated with *n*-hexane to obtain a solid, which was recrystallized from AcOEt–ether to give crystals; yield 10.5 (82%), m.p. 185°–187 °C; $[\alpha]_D^{20} = -7,9$ (c = 1% in DMF); $m/z = 482.2$ [$(M - H)^-$]; analysis calc. for $C_{24}H_{21}NO_8S$: 59.8%C, 4.3%H, 2.9%N; 6.6%S; found: 59.5%C, 4.1%H, 2.7%N, 6.5%S.

H-Tyr(PO$_3$H$_2$)-Ile-Tyr(SO$_3$H)-Thr-Gln-OH (I). The peptide was obtained by a stepwise elongation of the peptide chain by the method outlined above. 0.5 g of the Fmoc-Gln(Trt)-resin (substitution level 0.56 mmol/g), was suspended in a 20% solution of piperidine in DMF. The mixture was stirred for 20 min at room temperature. Then it was filtered and washed with DMF (5 × 2 min) and CH_2Cl_2 (5 × 2 min). The next amino acid, Fmoc-Thr(Bu^t)-OH (0.33 g, 0.84 mmol), was dissolved in DMF and coupled to the resin in the presence of 1 equiv. of HBTU/HOBt and 2 equiv. of NEM (*N*-ethylmorpholine) (198 μl) for 2 h. The end of the reaction was determined by the Kaiser test. Other Fmoc-amino acid derivatives: Fmoc-Tyr(SO_3H)-OH, Fmoc-Ile-OH and Fmoc-Tyr(PO_3H_2)-OH, were connected to the resin in the same way. The N^{α}-Fmoc group was subsequently removed with 20% piperidine in DMF. The free peptide was obtained by deprotection with 4.75 ml of TFA in the presence of 0.125 ml of ethanedithiol and 0.125 ml of water at room temperature according to standard procedure. Then the peptide was purified by preparative HPLC. The main fractions were pooled and lyophilized. Peptides **II, III, IX, X, XIII** and **XIV** were obtained and purified in the same manner as peptide **I**. Their data are presented in Table 1.

H-Phe(4-Cl)-Ile-Tyr(SO$_3$H)-Thr-Gln-OH (IV). Peptide was obtained and purified in the same manner as peptide **I** (Table 1) except that the Tyr residue was introduced as Fmoc-Tyr-OH. After synthesis of the peptide on the resin the partially protected peptide-resin was sulfated with DMF-SO_3 (2.6 g, 30 equiv.) in DMF-pyridine (4 : 1, 8 ml) at room temperature for 16 h.

The sulfated pentapeptide-resin was collected by filtration, washed with water, and dried overnight over KOH under reduced pressure. Peptides **V–VIII, XI, XII, XV–XVI** and **XXIII** were obtained and purified in the same manner as peptide **IV**. Their data are presented in Table 1.

H-Phe(4-Cl)-Ile-Tyr(3-SO$_3$H)-Thr-Gln-OH (XVII). The peptide was obtained and purified in the same manner as peptide **I** (Table 1) except that the Tyr residue was introduced as Fmoc-Tyr(3-SO_3H)-OH. Peptides **XVIII–XXII** were obtained and purified in the same manner as peptide **XVII**. Their data are presented in Table 1.

Biological Part

Procedures for the preparation of [^{3}H]PSK-α and ligand binding assay were previously described [5]. In short, aliquots of carrot plasma membrane fraction (250 μg protein) was incubated for 30 min at 4 °C in a total volume of 250 μl of binding buffer containing 3.2 nM of [^{3}H]PSK-α and various concentrations of PSK-α analogues as a competitor. Incubations were terminated by layering the reaction mixture onto 900 μl of buffer containing 0.5 M sucrose and centrifuged for 10 min at 100 000 × g at 4 °C. After discarding the supernatant, the radioactivity contained in the pellet was determined with a liquid scintillation counter. Error bars indicate ±SE from three independent experiments.

RESULTS AND DISCUSSION

Binding affinities of PSK-α analogues to its receptor were studied by a competition assay in which the

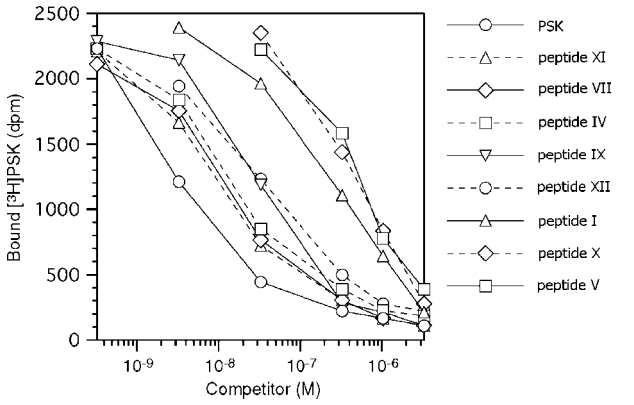

Figure 1 Competitive binding assay of PSK-α analogues to PSK-α receptor in carrot microsome fractions. The carrot microsome fraction (250 μg) was suspended in binding buffer containing 3.2 nM of [3H] PSK-α in the presence of various concentrations of PSK-α analogues as competitors. Total binding in the absence of competitors was 2480 dpm. The IC_{50} values for inhibition of binding for analogues were as follows; 1, 2 and 3 = 10 nM, 4 and 5 = 32 nM, 6 = 320 nM, 7 and 8 = 640 nM. The IC_{50} values for PSK-α standard was 3.2 nM in this condition.

binding of [^{3}H]labelled PSK-α to carrot microsomal fractions containing the PSK-α receptor was competed for increasing concentrations of the PSK-α analogues. As shown in Figure 1, compounds **IV** and **VI** inhibited the [^{3}H] PSK-α binding in a dose-dependent manner with a high binding affinity (30% binding activity compared with that of the native peptide). Compound **XI** showed a similar binding affinity, while compound **IX** preserved 10% activity. In contrast, compounds **V** and **X** bound extremely poorly to PSK-α receptor in carrot microsomal fractions. Other peptides were practically inactive.

Based on the preliminary biological activities obtained here it is difficult to discuss the structure/function relationship. Exchange of the *N*-terminal residue for Phe(4-Cl) (**IV**) and Phe(4-I) (**VII**) or the amino acid residue in position 3 for Phe(4-Cl) (**XI**) led to analogues with 30% biological effect. The analogue containing l Tyr(PO_3H_2) (**IX**) instead of Tyr(SO_3H) in position 1 retained 10% PSK-α activity. However, replacing the -OSO_3 group at position 4′ of the aromatic ring of the *N*-terminal amino acid residue by -OPO_3H_2, -Br, -F, and $NHSO_2CH_3$, and exchange of the same group in position 3 of the peptide chain for -OPO_3H_2 and F, as well as of both these groups in positions 1 and 3 (analogues **XIII**–**XXIII**) led to derivatives with no biological effect in the carrot membrane competitive binding test.

CONCLUSION

These results indicate that the presence of the -OSO_3H system at the aromatic ring in position 1 or 3 of the PSK-α peptide chain or other substituent with the electro-acceptor character (such as -Cl, -I or -OPO_3H_2 in the aromatic ring) plays an important role in the creation of biological properties of PSK-α. This problem will be the subject of further studies.

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