Pestycydy, 2008, (1-2), 109-117.
ISSN 0208-8703

Anti-pathogenic properties of plant peptide hormone phytosulfokines (PSK's) and its selected analogues

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Abstract: Phytosulfokine-α (PSK-α) (H-Tyr(SO_3H)-Ile-Tyr(SO_3H)-Thr-Gln-OH) (I), a sulfated growth factor universally found in both monocotyledons and dicotyledons, strongly promotes proliferation of plant cells in culture. The C-terminal truncated analog named PSK-β (Tyr(SO_3H)-Ile-Tyr(SO_3H)-Thr) (II) showed a 10-fold lower activity than that of the parent pentapeptide. Because PSK-α promotes proliferation and differentiation during the plant growth we undertook the studies on the influence of PSK-α on plant defense mechanisms in that period. In present studies on PSK-α (I), PSK-β (II), and its analogues, we performed a search of another biological properties.

The aim of our investigation was evaluation of PSK-α, PSK-β and their selected analogues in relation to growth and development of plant pathogens, such as *Phoma narcissi* and *Botrytis tulipae*. In these studies we elaborated the synthesis of PSK-α or PSK-β and their 22 analogues modified by natural and non-natural amino acid residues.

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.

Biological effect of these peptides was evaluated by test on the growth and development of pathogens of *P. narcissi* and *B. tulipae*.

Keywords: phytosulfokine-α, PSK-α, phytosulfokine-β analogues, plant peptide hormones, antifungal activity, *Phoma narcissi, Botrytis tulipae*, PSK-β

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., 138, 9, (1984)] and J. Pept. Sci., 5, 465 (1999), J. Pept.Sci, 9, 1 (2003).

INTRODUCTION

The sulfated peptide phytosulfokine (PSK) is an intercellular signal peptide that plays a key role in the cellular dedifferentiation and redifferentiation in plants [1]. Sulfated tyrosine residues are often found in secreted peptides in animals, but to date PSK is the only example of post-translational sulfation of tyrosine residues in plants. Several paralogous genes encoding ≈80-residue precursors of PSK have been identified in *Arabidopsis*. Each predicted protein has a probable secretion signal at the *N*-terminus and a single PSK sequence close to the *C*-terminus, similar to other peptide-hormones generally synthesized as inactive higher molecular weight precursors which must undergo a variety of post-translational processing steps to yield the active peptides [2]. The biological evaluation of unsulfated analogues of these peptides showed that they require the sulfate ester for the expression of their biological activity [3, 4]. In studies on PSK Matsubayashi *et al*. [5] observed that the unsulfated PSK-α analogue was dramatically less active. Moreover, in the same paper [5] authors observed that the truncated analog of PSK-α without the *C*-terminal amino acid (PSK-β) retained only 8% activity of the parent pentapeptide. Among a series of further analogues [6, 7] only [Phe(4-Cl)^1-PSK-α showed 30% biological activity compared with that of the native peptide, whereas [Phe(4-I)^1]-, and [Phe(4-NO_2)^1]-PSK-α retained 10% biological effect.

PSK, as described above, promotes proliferation and differentiation of dispersed cell in the culture. Additionally, PSK-α has several other biological activities. For example, PSK-α has promotive effects on chlorophyll synthesis in etiolated cotyledons of cucumber [8] as well as the growth and chlorophyll content of *Arabidopsis* seedlings under high night-time temperature conditions [9]. PSK-α has promotive effects on adventitious root formation by hypocotyls of cucumber and adventitious bud formation in *Antirrhinum majus* [10].

Furthermore, PSK-α stimulates tracheary element differentiation of isolated mesophyll cells from zinnia [11] and also reinforces the frequency of somatic embryogenesis in carrot cultures [12]. This implies that PSK is the general factor involved in plant cell growth.

Basing on these results in respect to PSK and its analogues, we performed a further search for its biological properties in plants. In our investigation we undertook the studies on the influence of PSK-α on defense mechanisms in the period of plants growth. The aim of our search was evaluation of PSK-α and selected analogues in relation to the growth and development of *Phoma narcissi* and *Botrytis tulipae*.

In these studies we performed the synthesis of a few groups of analogues

of PSK-α and PSK-β. Structure of analogues PSK-α (**I**) was designed by modification at position 1 of the peptide chain, at position 3, modified in both positions 1 and 3 of the peptide chain, such as: H-Tyr-Ile-Tyr(SO_3H)-Thr-Gln-OH (**III**), H-D-Tyr(SO_3H)-Ile-Tyr(SO_3H)-Thr-Gln-OH (**IV**), H-Phe(4-F)-Ile-Tyr(SO_3H)-Thr-Gln-OH (**V**), H-Phe(4-NO_2)-Ile-Tyr(SO_3H)-Thr-Gln-OH (**VI**), H-Tyr(SO_3H)-Ile-Tyr-Thr-Gln-OH (**VII**), H-Tyr(SO_3H)-Ile-D-Tyr(SO_3H)-Thr-Gln-OH (**VIII**), H-Tyr(SO_3H)-Ile-Tyr(PO_3H_2)-Thr-Gln-OH (**IX**), H-Tyr(SO_3H)-Ile-Phe(4-F)-Thr-Gln-OH (**X**), H-Tyr-Ile-Tyr-Thr-Gln-OH (**XI**), H-D-Tyr(SO_3H)-Ile-D-Tyr(SO_3H)-Thr-Gln-OH (**XII**), H-Tyr(PO_3H_2)-Ile-Tyr(PO_3H_2)-Thr-Gln-OH (**XIII**), H-Phe(4-F)-Ile-Phe(4-F)-Thr-Gln-OH (**XIV**) and H-Phe(4-NO_2)-Ile-Phe(NO_2)-Thr-Gln-OH (**XV**)

Analogues of PSK-β (**II**) was obtained by modification at 1or 3 and both 1 and 3 positions of the peptide chain, such as: H-Tyr-Ile-Tyr(SO_3H)-Thr-OH (**XVI**), H-D-Tyr(SO_3H)-Ile-Tyr(SO_3H)-Thr-OH (**XVII**), H-Phe(4-F)-Ile-Tyr(SO_3H)-Thr-OH (**XVIII**),H-Tyr(SO_3H)-Ile-Tyr-Thr-OH (**XIX**), H-Tyr(SO_3H)-Ile-D-Tyr(SO_3H)-Thr-OH (**XX**) H-Tyr(SO_3H)-Ile-Phe(4-F)-Thr-OH (**XXI**), and H-Tyr-Ile-Tyr-Thr-OH, (**XXII**), H-D-Tyr(SO_3H)-Ile-D-Tyr(SO_3H)-Thr-OH (**XXIII**), H-Phe(4-F)-Ile-Phe(4-F)-Thr-OH (**XXIV**).

In the analogues **I-VI** of PSK-α: the 4-sulfated-Tyr residue at position 1 was replaced by: Tyr (without the –SO_3H group) (**III**), its D-isomer (**IV**), and Phe derivative 4-substituted by –F and –NO_2 (**V**, **VI**). The amino acid at position 3 was exchanged for: Tyr (**VII**), D-Tyr(SO_3H) (**VIII**), Tyr(4-PO3H2) (**IX**) and Phe(4-F) (**X**). In analogues simultaneously modified in positions 1 and 3 were introduced: Tyr (**XI**), D-Tyr(SO_3H) (**XII**), Tyr(PO_3H_2) (**XIII**), the

Phe (4-F) and Phe(4-NO_2) (**XIV**). The structure of PSK-β was modified by replacement of Tyr(SO_3H) at position 1 for Tyr and D-Tyr(SO_3H) (**XVII**, **XVIII**), at position 3 for Tyr (**XVIII**), D-Tyr(SO_3H) (**XIX**), and Phe(4-F) (**XX**), and both at position 1 and 3 for Tyr (**XXI**), D-Tyr(SO_3H) (**XXII**), and Phe(4-F) (**XXIII**).

These peptides were tested *in vitro* on the growth and development of *Phoma narcissi* and *Botrytis tulipae.*

MATERIALS AND METHODS

Chemical part

General procedures:

Amino acid compositions were determined on an amino acid analyzer 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) and TLC on silica gel plates, 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 and Fmoc-Phe(4-F)-OH (Novabiochem) were used.

All peptides were obtained by the solid-phase method according to the Fmoc procedure. Amino acids were assembled on a Fmoc-Gln(Trt)-Wang or Fmoc-Thr(Trt)-Wang resin (Novabiochem). *N*-terminal residues were introduced as *N*-Fmoc-derivatives. 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 *N*,*N*-dimethylformamide (DMF) according to the standard methods. During the synthesis of peptides the Tyr residues to be sulfated were 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 with 95% trifluoroacetic acid (TFA) in the presence of ethanedithiol (EDT). All peptides were purified by semi-preparative HPLC on an Alltech Econsil C_{18}, 10 μm column (ODS 22 x 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 x 250 mm), and an ultrasphere plus 4.6 x 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 amino acid analysis and determination of molecular weights and optical activity. The physico-chemical data of PSK-α analogues are summarized in Table 1.

H-Tyr-Ile-Tyr(SO_3H)-Thr-OH (XVI)

The peptide was obtained by a stepwise elongation of the peptide chain by the method outlined above. 0.5 g of the Fmoc-Thr(Trt)-resin (substitution level 0.56 mmol/g), was suspended in 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 x 2 min) and CH_2Cl_2 (5 x 2 min). The next amino acid, Fmoc-Tyr-OH (g, mmol), was dissolved in DMF and coupled to the resin in the presence of one equivalent of HBTU/HOBt and two equivalents of NEM (*N*-ethylmorpholine) (198 μl) for 2 h. The end of the reaction was determined by the Kaiser test.

Table 1. Physicochemical data of PSK analogues modified in position 1, 3 and 4 of the peptide chain

Peptide		Yield (%)	$[\alpha]_D^{20}$	Rt[a] (HPLC)	Amino acids analysis	Mw Calc.	Found	T.L.C.[b] Rf		
								X	Y	Z
H-Tyr(SO_3H)-Ile-Tyr(SO_3H)-Thr-OH	(II)	56	-18.5 c=1.0	13.63	Tyr 1.8 Ile 1.2 Thr 1.0	718.2	717.1	0.10	0.36	0.33
H-Tyr-Ile-Tyr(SO_3H)-Thr-OH	(XVI)	51	-14.8 c=1.0	15.95	Tyr 1.8 Ile 1.0 Thr 1.2	638.2	637.2	0.10	0.35	0.36
H-D-Tyr(SO_3H)-Ile-Tyr(SO_3H)-Thr-OH	(XVII)	55	-8.8 c=1.0	14.74	Tyr 1.9 Ile 1.1 Thr 1.0	718.2	717.2	0.11	0.37	0.37
H-Tyr(SO_3H)-Ile-Tyr-Thr-OH	(XVIII)	56	-24.4 c=1.0	15.68	Tyr 1.8 Ile 1.2 Thr 1.0	638.2	637.5	0.10	0.36	0.36
H-Tyr(SO_3H)-Ile-D-Tyr(SO_3H)-Thr-OH	(XIX)	56	-9.5 c=1.0	15.09	Tyr 1.8 Ile 1.2 Thr 1.0	718.2	717.3	0.10	0.38	0.34
H-Phe(4-F)-Ile-Tyr(SO_3H)-Thr-OH	(XVIII)	81	-14.9 c=1.0	19.32	Ile 0.8 Tyr 1.0 Thr 1.2	641.2	641.4	0.10	0.64	0.40
H-Tyr(SO_3H)-Ile-Phe(4-F)-Thr-OH	(XX)	54	-18.7 c=1.0	18.16	Tyr 0.8 Ile 1.2 Thr 1.0	641.2	640.6	0.11	0.35	0.33
H-Tyr-Ile-Tyr-Thr-OH	(XXI)	98	-20.9 c=1.0	16.40	Tyr 1.8 Ile 1.1 Thr 1.1	558.3	557.5	0.10	0.36	0.36
H-D-Tyr(SO_3H)-Ile-D-Tyr(SO_3H)-Thr-OH	(XXII)	48	-25.0 c=1.0	13.80	Tyr 1.8 Ile 1.2 Thr 1.0	718.2	717.6	0.11	0.39	0.35
H-Phe(4-F)-Ile-Phe(4-F)-Thr-OH	(XXIII)	97	-19.2 c=1.0	21.03	Ile 0.8 Thr 1.2	564.3	564.4	0.11	0.37	0.37

[a] HPLC on Ultrasphere ODS columns (Beckman) 4.5 x 250 mm; solvent system: S1 – 0.1% aqueous TFA, S2 – 80% acetonitrile in water; linear gradient: 0-100% of S2 in 60 min.

[b] T.L.C. on silica gel plates (Merck), eluents: X = n-butanol:Ac-OH:water (4:1:5), Y= n-butanol:pyridine:Ac-OH(30:20:6:24) Z= n-butanol: Ac-OH:ethyl acetate:water (1:1:1:1),

Other Fmoc-amino acid derivatives Fmoc-Ile-OH and Fmoc-Tyr(Bu^t)-OH, were connected to the resin in the same way.

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.

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 the standard procedure. Then the peptide was purified by preparative HPLC. The main fractions were pooled and lyophilized. Peptides **I-XXIII** were obtained and purified in the same manner as peptide **XVI**. Their data are presented in Table 1.

Biological part

The anti-pathogenic test was performed according to earlier described method [14].

The stock culture of *Botrytis tulipae* (Lib.) Lind and *Phoma narcissi* Aderh. [syn. *Stagonospora curtisii* (Berk.) Sacc.] was maintained on potato-dextrose-agar (PDA-Merck) slants at 25 °C in the dark.

Five mm diameter plugs taken from 7-day-old culture of tested fungus were placed in the middle of 90 mm Petri dishes containing PDA medium with PSK-α or its analogues at the concentration between 10^{-4} to 10^{-5} M/dcm^3 Control plates contained the culture growing on PDA without any amendments. The diameter of fungi colony was measured within 4- or 5-day incubation at 25 °C in the dark. The data were subjected to analysis of variance and Duncans multiple range test.

RESULTS AND DISCUSSION

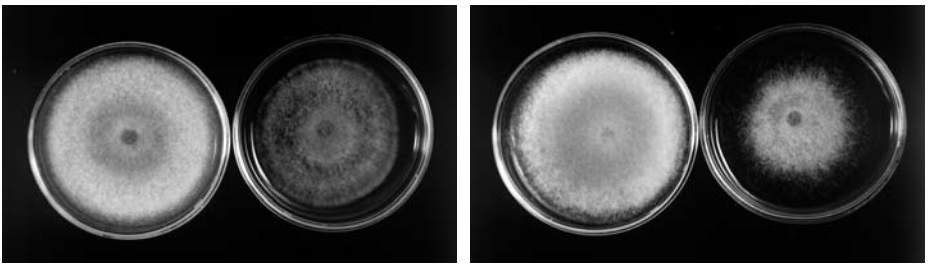
It was found that the PSK-α has antifungal properties, inhibits *in vitro* the mycelium growth of *P. narcissi* and *B. tulipae* in a dose-dependent manner (Table 2 an Figure 1 and 2**)**). PSK-α at the concentration of 10^{-4} M, after 4 days incubation, inhibits development of *P. narcissi* and *B. tulipae*. Among of PSK-α analogues, the [Phe(4-F)^1]-(**V**) and Phe(4-F)^{1,4}]- PSK-α (**XIV)** at the concentration of 10^{-5} M inhibits the growth of mycelium *P. narcissi* and *B. tulipae* (Table 2), after 3 days of incubation. Other analogues such as [D-Tyr(SO_3H)^1]- (**XII**) show a weak inhibitory activity at the some range of concentration. Whereas, peptides

IV or **XXII**. are practically inactive. In contrast to above shown activity peptide [Tyr^3]-PSK-α (**III**) stimulate *in vitro* the mycelium growth of *P. narcissi* and *B. tulipae.*

Table 2. Influence of PSK analogues on the colony growth of *Phoma narcissi* and *Botrytis tulipae.*
(Control: plates contained the culture growing on PDA without any amendments)

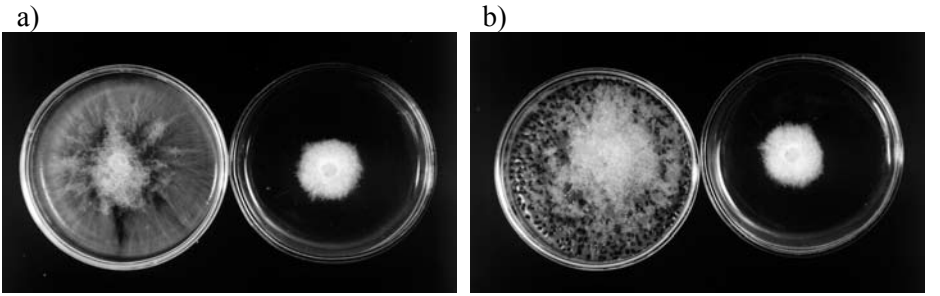
Peptides	Concentration mol/dm^3	Inhibiotory or stimulatory (+) effect on *in vitro* growth of mycelium (% of control)			
		Phoma narcissi		Botrytis tulipae	
		after days of incubation			
		3	4	3	4
PSK-α (**I**)	$4.73*10^{-4}$	41.3	42.2	51.0	57.2
[Tyr^1]-PSK-α **(III)**	$9.55*10^{-5}$	+5.8	0	+16.6	+21.9
[Phe(4-F)^1]-PSK-α (**V**)	$6.04*10^{-5}$	13.5	10.2	+9.6	38.1
[Tyr^3]-PSK-α **(VII)**	$7.83*10^{-5}$	+5.8	0	+8.3	19.2
[D-Tyr(SO$_3$H)^3]-PSK-α **(VIII)**	$7.15*10^{-5}$	14.2	13.2	9.8l	14.8
[Tyr$^{1.3}$]-PSK-α **(XI)**	$8.78*10^{-5}$	+5.8	5.7	+19.4	+37
[Phe(4-F)$^{1.3}$]-PSK-α (**XIV**)	$1.97*10^{-4}$	31.8	34.8	89.3	92.2
[D-Tyr(SO$_3$H)$^{1.3}$]-PSK-α (**XII**)	$7.17*10^{-5}$	16.2	7.5	17.2	19.7
PSK-β (**II**)	$1.42*10^{-4}$	55.6	nd	0	0
[Tyr^1]-PSK-β (**XVI**)	$1.15*10^{-4}$	14.6	21.5	+85.4	42.3
[Phe(4-F)^1]-PSK-β (**XVIII**)	$6.11*10^{-5}$	10.4	7.9	+51.2	24.3
[Tyr^3]-PSK-β (**XIX**)	$8.46*10^{-5}$	22.9	11.9	9.8	42.3
[D-Tyr(SO$_3$H)^3]-PSK-β (**XX**)	$1.08*10^{-5}$	50.0	29.9	+24.4	33.3
[D-Tyr(SO$_3$H)$^{1.3}$]-PSK-β (**XXIII**)	$5.56*10^{-5}$	19.3	17.2	10.8	11.3
[Phe(4-F)$^{1.3}$]-PSK-β (**XXIV**)	$1.65*10^{-4}$	49.0	51.7	95.7	93.6

In the studies on PSK-β (**II**) we found an inhibitory effect of the development of *P. narcissi* (62%). The PSK-β analogue (**XXIII**), the peptide modified at position 1 and 3 by 4-F-Phe residue shows anti-pathogenic activity in respect to two species of pathogen, *P. narcissi* and *B. tulipae* respectively (Figure 1). Moreover, [D-Tyr(SO$_3$H)^3]-PSK-β inhibits growth of mycelium *P. narcissi* at 30% after 4 days incubation (Figure 2).



Control Peptide XX (1.08×10^{-5} M) Control Peptide XXIV (1.65×10^{-4})

Figure 1. Influence of peptide XIX and XXIV on the growth mycelium of *P. narcissi in vitro*; after 4 days of incubation.



Control Peptide XXIV Control Peptide XXIV

Figure 2. Influence of peptide XXIV on the growth mycelium of *Botrytis tulipae in vitro*; after 4 (a), and 10 (b) days of incubation (at concentration 1.65 x 10-4) *Botrytis tulipae*.

These results pointed out that the native PSK-α inhibited a growth of both pathogens *P. narcissi* and *B. tulipae*, whereas PSK- β shows inhibitory activity against *P. narcissi* only.

Markedly inhibitory effect of both case of pathogens was observed, where Tyr(4-OSO_3H) residue in both 1 and 3 position of PSK- α and PSK- β peptide chain was replaced by Phe(4-F) PSK- β.

CONCLUSION

These results pointed out that the:

- PSK-α and PSK-β as well a some analogues shows antifungal properties;
- analogues where Tyr(SO_3H) residues was replaced by Tyr preserve weak

antufungal activity;

- exchange $-OSO_3H$ system at the para position at the aromatic ring at the 1 or 3 amino acid residues of the PSK-α and PSK- β peptide chain on –F lead to peptides with anti-pathogen properties;
- other analogues, such as **III, V, XVI** and **XX** show weak stimulatory effect of *B*. *tulipae* growth;

This problem will be a subject of further studies.

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Abbreviations:

Fmoc, 9-fluorenylmethoxycarbonyl; Boc, *tert*-butoxycarbonyl; Bu^t, *tert*-buthyl; Trt, trityl; HBTU 2(1H-benzotriazole-1-yl)-1,1,3,3,-tetramethyluronium hexafluorophosphate; HOBt, 1-hydroxybenzotriazole; NEM, *N*-ethylmorpholine; TFA, trifluoroacetic acid; EDT, 1,2-ethanedithiol, DMF, dimethylformamide; HPLC, high performance liquid chromatography.