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# Plant Peptide Hormone Phytosulfokine (PSK-α): Synthesis of New Analogues and Their Biological Evaluation

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Abstract: Phytosulfokine-α (PSK-α), a sulfated growth factor (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 our studies on structure/activity relationship in PSK-α the synthesis of a series of analogues was performed: [H-D-Tyr($SO_3H$)[1]]- (**9**), [H-Phe(4-$SO_3H$)[1]]- (**10**), [H-D-Phe(4-$SO_3H$)[1]]- (**11**), [H-Phg(4-$SO_3H$)[1]]- (**12**), [H-D-Phg(4-$SO_3H$)[1]]- (**13**), H-Phe(4-$NHSO_2CH_3$)[1]]- (**14**), [H-D-Phe(4-$NHSO_2CH_3$)[1]]- (**15**), [H-Phe(4-$NO_2$)[1]]- (**16**), [H-D-Phe(4-$NO_2$)[1]]- (**17**), [H-Phg(4-$NO_2$)[1]]- (**18**), [H-D-Phg(4-$NO_2$)[1]]- (**19**), [H-Hph(4-$NO_2$)[1]]- (**20**), [H-Phg(4-$OSO_3H$)[1]]- (**21**), [Phe(4-$NO_2$)[3]]- (**22**), [Phg(4-$NO_2$)[3]]- (**23**), [Hph(4-$NO_2$)[3]]- (**24**), [H-Phe(4-$SO_3H$)[1], Phe(4-$SO_3H$)[3]]- (**25**) [H-Phe(4-$NO_2$)[1], Phe(4-$NO_2$)[3]]- (**26**), [H-Phg(4-$NO_2$)[1], Phg(4-$NO_2$)[3]]- (**27**), [H-Hph(4-$NO_2$)[1], Hph(4-$NO_2$)[3]]- (**28**) and [Val[3]]- PSK-α (**29**). For modification of the PSK-α peptide chain the novel amino acids and their derivatives were synthesized, such as: H-L-Phg(4-$SO_3H$)-OH (**1**), H-D-Phg(4-$SO_3H$)-OH (**2**), Fmoc-Phg(4-$SO_3H$)-OH (**3**), Fmoc-D-Phg(4-$SO_3H$)-OH (**4**), Boc-Phg(4-$NHSO_2CH_3$)-OH (**5**), Boc-D-Phg(4-$NHSO_2CH_3$)-OH (**6**) Boc-Phe(4-$NHSO_2CH_3$)-OH (**7**), and Boc-D-Phe(4-$NHSO_2CH_3$)-OH (**8**). Peptides were synthesized by a 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 according to the Matsubayashi *et al.* test. Copyright © 2004 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: plant peptide hormone; phytosulfokine (PSK-α)

## INTRODUCTION

The sulfated peptide phytosulfokine (PSK) is an intercellular signal 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 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]. Studies using radiolabelled PSK have provided evidence for the existence of high-affinity binding sites for PSK in plant plasma membranes [2,3]. Recently, PSK receptor has been purified from membrane fractions and cloned carrot cells [4]. The cDNA encodes a typical LRR receptor

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The symbols of the amino acids, peptides and their derivatives are in accordance with the Recommendation of the IUPAC-IUB Joint Commission on Biochemical Nomenclature (1984) [*Eur. J. Biochem.* 1984; **138**: 9–37].
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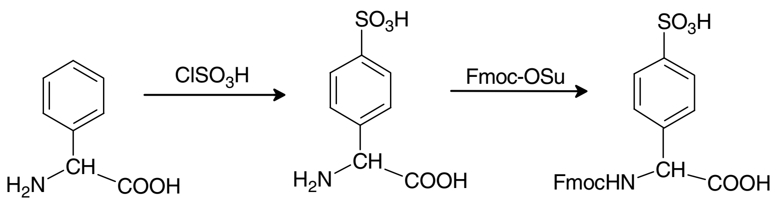
kinase that has 21 LRRs and a 36-residue island between the 17th and 18th LRRs.

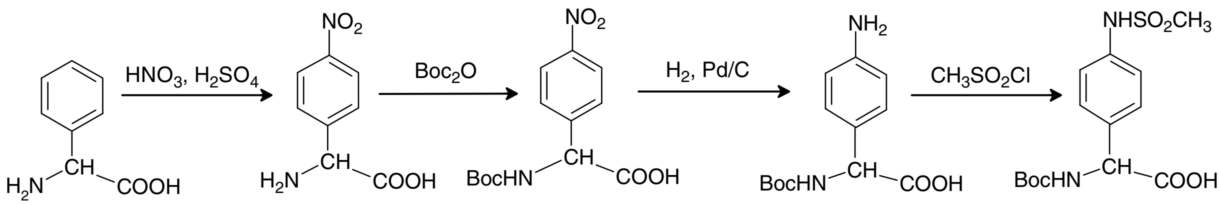
The biological evaluation of unsulfated analogues of these peptides showed that these peptides required the sulfate ester for the expression of their biological activity [5,6]. Moreover, Matsubayashi *et al.* [7] observed that the unsulfated PSK-$\alpha$ analogue was dramatically less active. Based on the above results studies were performed on the structure/activity relationship in PSK-$\alpha$ and three series of multidirectional modified analogues obtained: (1) analogues modified in position 1 by different non-protein aromatic amino acid residues (**9**–**21**): H-D-Tyr($SO_3H$)-Ile-Tyr($SO_3H$)-Thr-Gln-OH (**9**), H-Phe(4-$SO_3H$)-Ile-Tyr($SO_3H$)-Thr-Gln-OH (**10**), H-D-Phe(4-$SO_3H$)-Ile-Tyr($SO_3H$)-Thr-Gln-OH (**11**), H-Phg(4-$SO_3H$)-Ile-Tyr($SO_3H$)-Thr-Gln-OH (**12**), H-D-Phg(4-$SO_3H$)-Ile-Tyr($SO_3H$)-Thr-Gln-OH (**13**), H-Phe(4-$NHSO_2CH_3$)-Ile-Tyr($SO_3H$)-Thr-Gln-OH (**14**), H-D-Phe(4-$NHSO_2CH_3$)-Ile-Tyr($SO_3H$)-Thr-Gln-OH (**15**), H-Phe(4-$NO_2$)-Ile-Tyr($SO_3H$)-Thr-Gln-OH (**16**), H-D-Phe(4-$NO_2$)-Ile-Tyr($SO_3H$)-Thr-Gln-OH (**17**), H-Phg(4-$NO_2$)-Ile-Tyr($SO_3H$)-Thr-Gln-OH (**18**), H-D-Phg(4-$NO_2$)-Ile-Tyr($SO_3H$)-Thr-Gln-OH (**19**), H-Hph(4-$NO_2$)-Ile-Tyr($SO_3H$)-Thr-Gln-OH (**20**), H-Phg(4-$OSO_3H$)-Ile-Tyr($SO_3H$)-Thr-Gln-OH (**21**); (2) analogues modified in position 3 by 4-$NO_2$ Phe, Phg and Hph (**22**–**24**): H-Tyr($SO_3H$)-Ile-Phe(4-$NO_2$)-Thr-Gln-OH (**22**), H-Tyr($SO_3H$)-Ile-Phg(4-$NO_2$)-Thr-Gln-OH (**23**), H-Tyr($SO_3H$)-Ile-Hph(4-$NO_2$)-Thr-Gln-OH (**24**); (3) analogues modified in both positions 1 and 3 by 4-$SO_3H$ or 4-$NO_2$ aromatic amino acids (**25**–**28**), such as: H-Phe(4-$SO_3H$)-Ile-Phe(4-$SO_3H$)-Thr-Gln-OH (**25**), H-Phe(4-$NO_2$)-Ile-Phe(4-$NO_2$)-Thr-Gln-OH (**26**), H-Phg(4-$NO_2$)-Ile-Phg(4-$NO_2$)-Thr-Gln-OH (**27**), H-Hph(4-$NO_2$)-Ile-Hph(4-$NO_2$)-Thr-Gln-OH (**28**) and H-Tyr($SO_3H$)-Ile-Tyr($SO_3H$)-Val-Gln-OH (**29**).

In the first group of analogues the 4-sulfated-Tyr residue in position 1 was replaced by: (a) its D-isomer (**9**), (b) L- or D-Phe derivatives 4-substituted by $-SO_3H$, $-NO_2$ or $-NHSO_2CH_3$ groups, (c) amino acids deprived of the methylene group between the $C^\alpha$ atom and the phenyl ring (4-substituted L- or D-phenylglycine derivatives) (**10**–**19**), or (d) amino acid containing two methylene groups between the $C^\alpha$ atom and the phenyl ring (4-nitro-L-homo-phenylalanine (**20**)). In analogues modified in position 3, the sulfonated Tyr residue was replaced by 4-nitro-derivatives of Phe, Phg and Hph (**22**–**24**). In the third series of analogues of PSK-$\alpha$ modified in both position 1 and 3, the Tyr($SO_3H$) residues were replaced by Phe(4-$SO_3H$), Phe(4-$NO_2$), Phg(4-$NO_2$) and Hph(4-$NO_2$) (**25**–**28**). Moreover, the synthesis was performed of PSK analogues modified in position 4 by Val, an amino acid with the isosteric side chain relative to Thr (analogue **29**). For modification of the above peptides the novel amino acid derivatives were synthesized: H-Phg(4-$SO_3H$)-OH (**1**), H-D-Phg(4-$SO_3H$)-OH (**2**), Fmoc-Phg(4-$SO_3H$)-OH (**3**), Fmoc-D-Phg(4-$SO_3H$)-OH (**4**), Boc-Phg(4-$NHSO_2CH_3$)-OH (**5**), Boc-D-Phg(4-$NHSO_2CH_3$)-OH (**6**) Boc-Phe(4-$NHSO_2CH_3$)-OH (**7**), and Boc-D-Phe(4-$NHSO_2CH_3$)-OH (**8**) (Scheme 1 and Scheme 2).

Synthesis of these peptides was performed by the solid phase method according to standard procedure. Amino acid derivatives were assembled

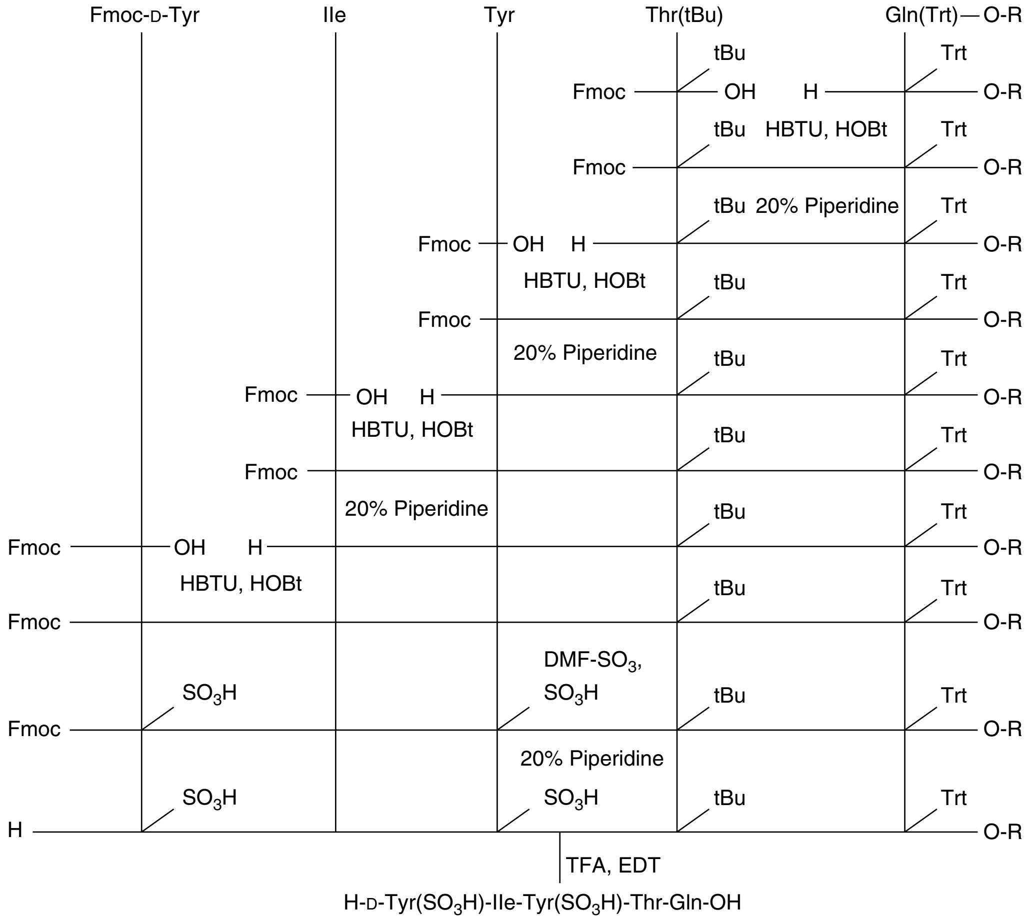



Scheme 1 Synthesis of Fmoc-Phg(4-$SO_3H$)-OH.




Scheme 2 Synthesis of Boc-Phg(4-$NHSO_2CH_3$)-OH.




Scheme 3 Synthesis of [H-D-Tyr($SO_3H$)[1]]-PSK-$\alpha$.

on a Wang-resin, according to the Fmoc-procedure. Free peptides were released from the resin by 95% TFA in the presence of EDT (Scheme 3).

All peptides were tested according to the method of Matsubayashi *et al.* [4].

## 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) 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 and Fmoc-Tyr-OH (Novabiochem) were used. Other amino acid derivatives, such as H-Phg(4-$SO_3H$)-OH, H-D-Phg(4-$SO_3H$)-OH, and *N*-Boc- or *N*-Fmoc-derivatives H-Phg(4-$SO_3H$)-OH, H-Phg(4-OH)-OH, H-D-Phg(4-$SO_3H$)-OH, H-Phe(4-$SO_3H$)-OH, H-D-Phe(4-$SO_3H$)-OH, H-Phg(4-$NHSO_2CH_3$)-OH, H-D-Phg(4-$NHSO_2CH_3$)-OH, H-Phe(4-$NHSO_2CH_3$)-OH, H-D-Phe(4-$NHSO_2CH_3$)-OH, H-Phe(4-$NO_2$)-OH, H-D-Phe(4-$NO_2$)-OH, H-Phg(4-$NO_2$)-OH, H-D-Phg(4-$NO_2$)-OH, and H-Hph(4-$NO_2$)-OH were synthesized in our laboratory, according to [8–13] (Table 1). All peptides (**9**–**29**) 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*-Boc-derivatives, except peptides **9,**

Table 1 Physicochemical Data of Phg and Phe Derivatives

| Compound | Yield (%) | M.p (°C) | $[\alpha]_D^{20}$ | Analysis %C Calc. | %C Found | %H Calc. | %H Found | %N Calc. | %N Found | %S Calc. | %S Found | Mw Calc. | Mw Found | TLC[a] $R_f$ X | Y | Z |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| H-Phg(4-$SO_3H$)-OH (**1**) | 82.2 | >250 | +62.2[b] | 41.5 | 41.3 | 3.9 | 3.7 | 6.0 | 6.1 | 13.9 | 13.8 | 231.1 | 231.2 | 0.125 | 0.13 | 0.73 |
| H-D-Phg(4-$SO_3H$)-OH (**2**) | 87.9 | >250 | −62.2[b] | 41.5 | 41.5 | 3.9 | 3.6 | 6.0 | 6.2 | 13.9 | 13.7 | 231.1 | 231.6 | 0.13 | 0.11 | 0.76 |
| Fmoc-Phg(4-$SO_3H$)-OH (**3**) | 58.4 | 134–137 | +3.1[c] | 61.1 | 61.3 | 4.0 | 3.9 | 3.1 | 3.1 | 7.1 | 7.1 | 452.4 | 452.2 | 0.51 | 0.56 | 0.84 |
| Fmoc-D-Phg(4-$SO_3H$)-OH (**4**) | 60.5 | 138–140 | −3.1[b] | 61.1 | 61.1 | 4.0 | 3.9 | 3.1 | 3.0 | 7.1 | 7.1 | 452.4 | 453.4 | 0.52 | 0.58 | 0.82 |
| Boc-Phg(4-$NHSO_2CH_3$)-OH (**5**) | 58.4 | 118–120 | +97.5[d] | 48.9 | 48.4 | 5.8 | 5.9 | 8.1 | 8.7 | 9.3 | 9.2 | 343.4 | 344.5 | 0.84 | 0.81 | 0.79 |
| Boc-D-Phg(4-$NHSO_2CH_3$)-OH (**6**) | 80.1 | 119–121 | −97.5[d] | 48.9 | 48.3 | 5.8 | 5.4 | 8.1 | 8.5 | 9.3 | 9.2 | 343.4 | 344.6 | 0.82 | 0.80 | 0.76 |
| Boc-Phe(4-$NHSO_2CH_3$)-OH (**7**) | 60.4 | 130–133 | +38.4[d] | 50.3 | 50.5 | 6.1 | 5.9 | 7.8 | 7.7 | 8.9 | 9.0 | 358.1 | 357.3 | 0.24 | 0.57 | 0.43 |
| Boc-D-Phe(4-$NHSO_2CH_3$)-OH (**8**) | 65.1 | 129–132 | −38.1[d] | 50.3 | 50.2 | 6.1 | 6.0 | 7.8 | 7.9 | 8.9 | 8.8 | 358.1 | 358.7 | 0.27 | 0.53 | 0.41 |

[a] TLC on silica gel plates (Merck), eluents: X = *n*-butanol : Ac-OH : water (4 : 1 : 5), Y = *n*-butanol : pyridine : Ac-OH : water (30 : 20 : 6 : 24), Z = *n*-butanol : Ac-OH : water (4 : 1 : 1), [b] c = 1% in water, [c] c = 1% in DMF, [d] c = 1% in $CH_3OH$.

**12** and **13**. For these peptides *N*-Fmoc 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. During the synthesis the Tyr residue was used as Fmoc-Tyr-OH. The $N^{\alpha}$-Fmoc group was removed with 20% piperidine in *N*,*N*-dimethylformamide (DMF) according to standard methods. 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% trifluoroacetic acid (TFA) in the presence of ethanedithiol (EDT).

All peptides (**9**–**29**) 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 $C_{18}$, 5 µm Beckman column (ODS 4.6 × 250 mm), 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 amino acid analysis and determination of molecular weights and optical activity. The physico-chemical data PSK-$\alpha$ analogues are summarized in Table 2.

***4-Sulfo-L-phenylglycine. H-Phg(4-$SO_3H$)-OH (1).*** The title compound was synthesized according to [8]. Phg (15.1 g, 0.1 mol) was dissolved in concentrated chlorosulfonic acid (45 ml) at 15 °C. The solution was heated to 36 °C, mixed for 30 h, and finally dropped to ice. The white product was crystallized from isopropanol: water (1 : 1). 19 g of the product was obtained. Physico-chemical data are presented in Table 1.

***4-Sulfo-D-phenylglycine. H-D-Phg(4-$SO_3H$)-OH (2).*** The title compound was obtained in the same manner as compound **1** from 15.1 g (0.1 mol) of D-phenylglycine. Crystallization from isopropanol: water gave 20.3 g of the product. Physico-chemical data are presented in Table 1.

***$N^{\alpha}$-(9-Fluorenylmethoxycarbonyl)-4-sulfo-L-phenylglycine. Fmoc-Phg(4-$SO_3H$)-OH (3).*** To introduce the Fmoc group sulfo-L-phenylglycine (19.5 g, 0.08 mol), Fmoc-OSu (27.0 g, 0.08 mol) and $Et_3N$ (11 ml, 0.08 mol) were suspended in a mixture of $H_2O$ (100 ml) and THF (50 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 (100 ml) and $H_2O$ (100 ml) were added. The mixture was acidified to pH 2.0 with 1M 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. 21.7 g of product was obtained (Scheme 1).

***$N^{\alpha}$-(9-Fluorenylmethoxycarbonyl)-4-sulfo-D-phenylglycine. Fmoc-D-Phg($SO_3H$)-OH (4).*** 19.7 g (0.085 mol) of compound **2** was reacted with 29 g (0.085 mol) of Fmoc-OSu in the same manner as in the case of **3**. 22.5 g of the product was obtained (Table 1).

***$N^{\alpha}$-(tert-Butoxycarbonyl)-4-aminosulfomethyl-L-phenylglycine. Boc-Phg(4-$NHSO_2CH_3$)-OH (5).*** Boc-Phg(4-$NO_2$)-OH [10] was dissolved in 40 ml of methanol and the solution was hydrogenated in the presence of 10% Pd/C (0.1 g) for 48 h. Boc-Phg(4-$NH_2$)-OH (16.5 g, 0.062 mol) was dissolved in 1N NaOH (50 ml) at 0 °C, and 10 ml of $CH_3SO_2Cl$ (in 100 ml acetone) was added. The reaction was carried out for 2 h. Then the solvent was evaporated *in vacuo*, the aqueous phase was acidified to pH ~3 with 1M HCl and extracted with ethyl acetate. The organic phase was washed three times with water, dried over anhydrous $MgSO_4$ and evaporated *in vacuo*. After crystallization from ethyl acetate: hexane 12.5 g of the product was obtained (Scheme 2).

***$N^{\alpha}$-(tert-Butoxycarbonyl)-4-aminosulfomethyl-D-phenylglycine. Boc-D-Phg(4-$NHSO_2CH_3$)-OH (6).*** The title compound was obtained in the same manner as compound **5**. The reaction was carried out as described above. 15.6 g of the product was obtained (Table 1).

***$N^{\alpha}$-(tert-Butoxycarbonyl)-4-aminosulfomethyl-L-phenylalanine. Boc-Phe(4-$NHSO_2CH_3$)-OH (7).*** Boc-Phe(4-$NO_2$)-OH [13] (22 g, 0.08 mol) was dissolved in 40 ml of methanol and the solution was hydrogenated in the presence of 10% Pd/C (0.1g) for 48 h. 15.0 g (0.054 mol) of Boc-Phe(4-$NH_2$)-OH was obtained. The product was dissolved in 1N NaOH (50 ml) at 0 °C, and then 8.7 ml of $CH_3SO_2Cl$ (in

Table 2 Physicochemical Data of PSK Analogues Modified in Position 1, 3 and 4 of the Peptide Chain

| Peptide | Yield (%) | $[\alpha]_D^{20}$ 1.0% $NH_4OH$ | Rt[a] (HPLC) | Amino acid analysis | Mw Calc. | Mw Found | TLC[b] $R_f$ X | TLC[b] $R_f$ Y | TLC[b] $R_f$ Z |
|---|---|---|---|---|---|---|---|---|---|
| H-D-Tyr($SO_3H$)-Ile-Tyr($SO_3H$)-Thr-Gln-OH (**9**) | 56 | −26.3 c = 0.25 | 18.16 | Tyr 1.8 Ile 1.2 Thr 1.0 Gln 1.0 | 846.2 | 845.6 | 0.22 | 0.67 | 0.49 |
| H-Phe($SO_3H$)-Ile-Tyr($SO_3H$)-Thr-Gln-OH (**10**) | 71 | −2.4 c = 0.5 | 7.17 | Ile 0.8 Tyr 1.0 Thr 1.2 Gln 1.0 | 830.2 | 829.4 | 0.10 | 0.64 | 0.40 |
| H-D-Phe(4-$SO_3H$)-Ile-Tyr($SO_3H$)-Thr-Gln-OH (**11**) | 58 | −22.5 c = 0.5 | 14.05 | Ile 0.8 Tyr 1.1 Thr 0.9 Gln 0.9 | 830.2 | 829.7 | 0.13 | 0.44 | 0.48 |
| H-Phg(4-$SO_3H$)-Ile-Tyr($SO_3H$)-Thr-Gln-OH (**12**) | 61 | −1.1 c = 1.0 | 11.85 | Ile 1.09 Tyr 0.9 Thr 0.8 Gln 1.0 | 816.2 | 815.1 | 0.12 | 0.63 | 0.42 |
| H-D-Phg(4-$SO_3H$)-Ile-Tyr($SO_3H$)-Thr-Gln-OH (**13**) | 60 | −1.1 c = 1.0 | 12.11 | Ile 1.2 Tyr 0.9 Thr 1.0 Gln 0.98 | 816.2 | 815.9 | 0.11 | 0.62 | 0.41 |
| H-Phe(4-$NHSO_2CH_3$)-Ile-Tyr($SO_3H$)-Thr-Gln-OH (**14**) | 48 | −14.7 c = 0.5 | 17.29 | Ile 1.05 Tyr 095 Thr 0.98 Gln 0.9 | 843.4 | 842.9 | 0.10 | 0.44 | 0.57 |
| H-D-Phe(4-$NHSO_2CH_3$)-Ile-Tyr($SO_3H$)-Thr-Gln-OH (**15**) | 45 | −5.8 c = 0.5 | 19.305 | Ile 1.1 Tyr 1.0 Thr 1.0 Gln 0.99 | 843.4 | 843.2 | 0.09 | 0.60 | 0.53 |
| H-Phe(4-$NO_2$)-Ile-Tyr($SO_3H$)-Thr-Gln-OH (**16**) | 50 | −24.7 c = 0.5 | 20.62 | Ile 1.06 Tyr 1.1 Thr 0.99 Gln 0.98 | 795.2 | 794.7 | 0.15 | 5.54 | 0.55 |
| H-D-Phe(4-$NO_2$)-Ile-Tyr($SO_3H$)-Thr-Gln-OH (**17**) | 48 | −37.4 c = 0.5 | 18.94 | Ile 0.96 Tyr 1.0 Thr 0.97 Gln 1.0 | 795.2 | 795.0 | 0.16 | 0.66 | 0.69 |
| H-Phg(4-$NO_2$)-Ile-Tyr($SO_3H$)-Thr-Gln-OH (**18**) | 54 | −16.4 c = 0.5 | 22.36 | Ile 1.2 Tyr 1.11 Thr 0.9 Gln 1.0 | 781.2 | 780.6 | 0.16 | 0.68 | 0.70 |
| H-D-Phg(4-$NO_2$)-Ile-Tyr($SO_3H$)-Thr-Gln-OH (**19**) | 46 | −5.1 c = 0.5 | 18.82 | Ile 1.0 Tyr 0.9 Thr 0.98 Gln 1.0 | 781.2 | 780.7 | 0.11 | 0.61 | 0.54 |
| H-Hph(4-$NO_2$)-Ile-Tyr($SO_3H$)-Thr-Gln-OH (**20**) | 47 | −9.4 c = 0.5 | 18.10 | Ile 1.2 Tyr 1.0 Thr 0.9 Gln 0.9 | 809.2 | 808.9 | 0.10 | 0.64 | 0.46 |
| H-Phg(4-$OSO_3H$)-Ile-Tyr($SO_3H$)-Thr-Gln-OH (**21**) | 56 | −16.1 c = 0.5 | 13.81 | Tyr 0.96 Ile 1.16 Thr 1.0 Gln 0.9 | 832.2 | 831.9 | 0.12 | 0.48 | 0.41 |
| H-Tyr($SO_3H$)-Ile-Phe(4-$NO_2$)-Thr-Gln-OH (**22**) | 49 | −1.8 c = 1.0 | 19.30 | Ile 1.0 Tyr 0.9 Thr 0.9 Gln 0.98 | 795.2 | 796.0 | 0.11 | 0.51 | 0.43 |
| H-Tyr($SO_3H$)-Ile-Phg(4-$NO_2$)-Thr-Gln-OH (**23**) | 51 | −16.8 c = 0.5 | 20.57 | Tyr 1.0 Ile 1.2 Thr 1.0 Gln 0.9 | 781.2 | 782.0 | 0.10 | 0.61 | 0.22 |
| H-Tyr($SO_3H$)-Ile-Hph(4-$NO_2$)-Thr-Gln-OH (**24**) | 48 | −15.8 c = 0.5 | 18.82 | Tyr 1.1 Ile 1.0 Thr 0.9 Gln 0.88 | 809.2 | 810.1 | 0.14 | 0.67 | 0.57 |
| H-Phe(4-$SO_3H$)-Ile-Phe(4-$SO_3H$)-Thr-Gln-OH (**25**) | 61 | −24.0 c = 1.0 | 10.76 | Ile 0.99 Tyr 1.0 Thr 1.0 Gln 0.9 | 814.2 | 813.3 | 0.10 | 0.36 | 0.22 |
| H-Phe(4-$NO_2$)-Ile-Phe(4-$NO_2$)-Thr-Gln-OH (**26**) | 90 | −3.9 c = 1.0 | 23.52 | Ile 1.0 Thr 0.9 Gln 0.98 | 744.2 | 742.9 | 0.31 | 0.79 | 0.69 |
| H-Phg(4-$NO_2$)-Ile-Phg(4-$NO_2$)-Thr-Gln-OH (**27**) | 87 | −12.9 c = 0.5 | 20.72 | Ile 1.2 Thr 1.0 Gln 1.0 | 716.2 | 717.0 | 0.30 | 0.68 | 0.68 |
| H-Hph(4-$NO_2$)-Ile- Hph(4-$NO_2$)-Thr-Gln-OH (**28**) | 89 | −11.9 c = 0.5 | 25.42 | Ile 1.0 Thr 0.9 Gln 0.98 | 772.2 | 773.1 | 0.26 | 0.72 | 0.66 |
| H-Tyr($SO_3H$)-Ile-Tyr($SO_3H$)-Val-Gln-OH (**29**) | 48 | −11.0 c = 0.5 | 15.95 | Tyr 1.98 Ile1.02 Thr 0.99 Val 1.01 | 844.2 | 845.4 | 0.09 | 0.53 | 0.58 |

[a] 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.

[b] TLC 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).

100 ml acetone) was added. The reaction was carried out for 2 h. Then the solvent was evaporated *in vacuo*, the aqueous phase was acidified to pH ~3 with 1M HCl and extracted with ethyl acetate. The organic phase was washed three times with water, dried over anhydrous $MgSO_4$ and evaporated *in vacuo*. After crystallization from ethyl acetate: hexane 11.6 g of the product was obtained.

***N$^\alpha$-(tert-Butoxycarbonyl)-4-aminosulfomethyl-D-phenylalanine. Boc-D-Phe(4-NHSO$_2$CH$_3$)-OH (8).*** The title compound was obtained in the same manner as compound **7**. The reaction was carried out as described above. 12.5 g of the product was obtained (Table 1).

***H-D-Tyr(SO$_3$H)-Ile-Tyr(SO$_3$H)-Thr-Gln-OH (9).*** 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 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 equivalent of HBTU/HOBt and 2 equivalents 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-OH, Fmoc-Ile-OH and Fmoc-D-Tyr-OH, were connected to the resin in the same way. Then the partially protected peptide-resin was sulfated by 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 $N^\alpha$-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. The data are presented in Table 2.

Peptides **12–13** were obtained and purified in the same manner as peptide **9** (Table 2). Peptides **10–11** and **14–29** were obtained and purified in the same manner as peptide **9** except that the *N*-terminal amino acids were successively introduced as Boc amino acids. Their data are presented in Table 2.

### Biological Part

Competition binding of PSK analogues to PSK receptors in carrot microsome fractions was conducted in the presence of 3.2 nM of [$^3$H]PSK and varying concentrations of the PSK analogues as previously described [4]. Error bars indicate ± SE from three independent experiments.

## RESULTS, DISCUSSION AND CONCLUSION

Among the analogues modified in position 1 of the peptide chain, only [Phe (4-NO$_2$)$^1$]- (**16**) and [Phg(4-NO$_2$)$^1$]-PSK-$\alpha$ (**18**) showed 10% binding activity compared with that of the native peptide, whereas [D-Phg(4-NO$_2$)]$^1$-PSK$\alpha$ (**19**) showed 1% binding activity. Other peptides were practically inactive. Basing on the preliminary biological activities obtained here it is difficult to discuss the structure/function relationship. Exchange of the oxygen atom at position 4′ of the aromatic ring of the *N*-terminal amino acid residue for the sulfur atom (analogues **10–13**) or for the –NH system (peptides **14** and **15**), as well as introducing the amino acid with configuration D (**9**) lead to derivatives with no biological effect in the carrot membrane competitive binding test. However, replacing of the *N*-terminal residue by aromatic amino acids, such as Phe(4-NO$_2$) (peptide **16**), Phg(4-NO$_2$) (**18**) and D-Phg(4-NO$_2$) (**19**) lead to analogues with weak biological effects. These results point out that the presence of the –OSO$_3$H system at the aromatic ring in position 1 of the PSK-$\alpha$ peptide chain or other substituent with the electro-acceptor character (such as the nitro group in the aromatic ring) plays an important role in creation of biological properties of PSK-$\alpha$. This problem will be a subject of further studies.

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## REFERENCES

1. Matsubayashi Y, Sakagami Y. Phytosulfokine, sulfated peptides that induce the proliferation of single mesophyll cells of *Asparagus officinalis* L. *Proc. Natl Acad. Sci. USA* 1996; **93**: 7623–7627.

 

2. Matsubayashi Y, Takagi L, Sakagami Y. Phytosulfokine-α, a sulfated pentapeptide, stimulates the proliferation of rice cells by means of specific high- and low-affinity binding sites. *Proc. Natl Acad. Sci. USA* 1997; **94**: 13 357–13 362.
3. Matsubayashi Y, Sakagami Y. Characterization of specific binding sites for a miotogenic sulfated peptide, phytosulfokine-alpha, in the plasma-membrane fraction derived from *Oryza sativa* L. *Eur. J. Biochem.* 1999; **262**: 666–671.
4. Matsubayashi Y, Ogawa M, Morita A, Sakagami Y. An LRR receptor kinase involved in perception of peptide plant hormone, phytosulfokine. *Science* 2002; **296**: 1470–1472.
5. Nachman RJ, Holman GM, Haddon WF, Ling N. Leucosulfakin a sulfated insect neuropeptide with homology to gastrin and cholecystokinin. *Science* 1986; **234**: 71–73.
6. Jensen RT, Lemp GF, Gardner JD. Interaction of carboxy-terminal fragments of cholecystokinin with receptors on dispersed acini from guinea pig pancreas. *J. Biol. Chem.* 1982; **257**: 5554–5559.
7. Matsubayashi Y, Hanai H, Hara O, Sakagami Y. Active fragments and analogs of the plant growth factor, phytosulfokine: structure–activity relationship. *Biochem. Biophys. Res. Commun.* 1996; **225**: 209–214.
8. Escher E, Bernier M, Parent P. Angiotensin II analogues. Part II. Synthesis and incorporation of the sulfur-containing aromatic amino acid: L-(4′-SH)Phe, L-(4′-$SO_2NH_2$)Phe, L-(4′-$SO_3$)Phe and L-(4′-S-$CH_3$)Phe. *Helv. Chim. Acta* 1983; **66**: 1355–1365.
9. Gonzalez- Muniz R, Cornille F, Bergeron F, Ficheux D, Pothier J, Durieux C, Rogues BP. Solid phase synthesis of fully active analogues of cholecystokinin using the acid-stable Boc-Phe(*p*-$CH_2SO_3$) as a substitute for Boc-Tyr($SO_3$) in $CCK_8$. *Int. J. Peptide Protein Res.* 1991; **37**: 331–340.
10. Szeszel-Fedorowicz W, Lisowski M, Rosiński G, Isserner J, Osborne R, Konopińska D. New proctolin analogues modified by the novel D- or L-phenylglycine derivatives. Synthesis and biological studies. *Polish J. Chem.* 2001; **75**: 411–417.
11. Moroder L, Hallet A, Wünsch E, Keller O, Wersin G. Di-tert.-butyldicarbonat- ein vorteilhaftes Reagenz zur Einführung der tert.-Butyloxycarbonyl-Schutzgruppe. *Hoppe-Seyler's, Z. Physiol. Chem.* 1976; **357**: 1651–1653.
12. Woźnica I, Rosiński G, Konopińska D. New proctolin analogues modified in position 2 or 3 of the peptide chain and their myotropic effect in insects. *Polish J. Chem.* 2002; **76**: 1425–1431.
13. Schwyzer R, Cawiezel M. p-Azido-L-phenylalanine. Photoaffinity probe related to tyrosine. *Helv. Chim. Acta* 1971; **54**: 1395–1400.