Polish J. Chem., **78**, 423–430 (2004)

Proctolin Analogues Modified at Position 4 of the Peptide Chain. Synthesis and Myotropic Effects in Insects*

by I. Woźnica[1], G. Rosiński[2] and D. Konopińska[1]**

[1]*Faculty of Chemistry, University of Wrocław, PL 50-383 Wrocław, ul. F. Joliot-Curie 14, Poland*
e-mail: dk@wchuwr.chem.uni.wroc.pl
[2]*Department of Animal Physiology, A. Mickiewicz University, 60-225 Poznań, Poland*

(Received October 27th, 2003; revised manuscript December 1st, 2003)

The object of these investigations was synthesis and biological evaluation of new analogues of proctolin (H-Arg-Tyr-Leu-Pro-Thr-OH) modified at position 4 of the peptide chain by natural or non-natural amino acid residues, such as: Phe (**1**), D-Phe (**2**), Phg (**3**), D-Phg (**4**), N-Me-Ala (**5**), N-Me-Val (**6**), N-Me-Leu (**7**), Tyr (**8**), Arg (**9**), Lys (**10**), Nva (**11**), Acp (**12**), Ser (**13**), γ-Abu (**14**), and $\Delta^{3,4}$-Pro (**15**). Synthesis was performed by classical solid-phase method. Myotropic activity of proctolin analogues was assayed *in vitro* on the semi-isolated heart of the yellow mealworm *Tenebrio molitor*. Analogues **1**, **9**, and **14** retained about 50% of proctolin activity. Other analogues showed about 20% activity or were inactive. The importance of the hydrophobic amino acid residues at position 4 for the myotropic activity of proctolin was inferred.

Key words: proctolin, proctolin analogues, insect peptide proctolin, new proctolin analogues

In our further studies [1–3] on the structure-function relationship of the insect neuropeptide proctolin, Arg-Tyr-Leu-Pro-Thr, we became interested in its analogues modified at position 4 of the peptide chain by natural or non-natural amino acid residues. The theoretical conformational analysis of proctolin molecule showed that the presence of the Pro moiety at position 4 of the peptide chain is necessary for stabilization of its biological conformation [3]. Moreover, the earlier structure-myotropic function studies on insects have prompted the syntheses of proctolin and many of its analogues [1,2]. Studies on the significance of Pro at position 4 of the proctolin skeleton for its myotropic function in insects may shed some light on the interaction of the proctolin molecule with its receptor site. In earlier studies, a series of proctolin analogues was synthesized by replacement of Pro^4 by the natural or non-natural amino acids. Among analogues tested only [Hyp^4]-proctolin preserved about 50% of proctolin activity. Other analogues containing Pro derivatives at position 4, such as Thz, *homo*-Pro, Hyp(4-OMe) or Ach, were inactive.

---

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

** Author for correspondence.

Basing on these results it was formulated that Pro-4 plays an essential role in the hydrophobic interaction of proctolin with its receptor and the presence of the pyrrolidine ring is esspecially important for biological activity in insects [3].

Continuing these investigations, we synthesized the following proctolin analogues modified at position 4 by such residues as: Phe (**1**), D-Phe (**2**), Phg (**3**), D-Phg (**4**), N-Me-Ala (**5**), N-Me-Val (**6**), N-Me-Leu (**7**), Tyr (**8**), Arg (**9**), Lys (**10**), Nva (**11**), Acp (**12**), Ser (**13**), γ-Abu (**14**), and $\Delta^{3,4}$-Pro (**15**). The aim of the synthesis of these analogues was explanation of the role of other amino acid residues at position 4 for myotropic activity in insects. All peptides were synthesized by classical solid-phase method. Biological activity was evaluated *in vitro* by cardiostimulating test on the heart of *Tenebrio molitor* according to Rosiński and Gäde method [5].

Among proctolin analogues modified at position 4, peptides [Phe^4]- (**1**), [Phg^4]- (**3**), [D-Phg^4]- (**4**), [N-Me-Ala^4]- (**5**), [Arg^4]- (**9**) and [γ-Abu^4]- proctolin (**14**) preserved about 60–20% of proctolin activity at the physiological concentration (10^{-8}–10^{-7} M). Other analogues were inactive. Especially interesting is that analogue **15**, containing 3,4-dehydro-L-proline at position 4 of the proctolin chain, was inactive.

EXPERIMENTAL

Chemical part: General procedures: Amino acid compositions were determined on an amino acid analyzer Mikrotechna T339 (Czechoslovakia) and Dionex AAA AS50 from Czech Republic with electrochemical detector AAA Certified Gold Cell. The optical activity of the chiral compounds was measured with a Jasco DIP-1000 polarimeter (±1.2°) (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. Purity of all peptides was about 100%.

Proctolin was obtained from Sigma Chemical Co. (Germany) Ltd. All peptides were synthesized by the classical solid-phase Boc procedure [4]. Dicyclohexylcarbodiimide (DCC) in the presence of 1-hydroxybenzotriazole (HOBt) was used as a coupling reagent. The C-terminal amino acid derivative Boc-Thr(OBzl) was connected to the chloromethylated classical Merrifield resin containing 1.08 mmol of Cl/g resin by a standard caesium salt procedure. The protected amino acids were coupled using the DCC method. The following amino acid derivatives were used: Boc-Arg(Tos)-OH, Boc-Tyr(OBzl)-OH, Boc-Leu-OH, Boc-Pro-OH, Boc-Thr(OBzl)-OH, Boc-Phe-OH, Boc-Phg-OH, Boc-Me-Ala-OH, Boc-Me-Val-OH, Boc-Me-Leu-OH, Boc-Lys-OH, Boc-D-Phe-OH, Boc-D-Phg-OH, Boc-Nva-DCHA salt, Boc-Acp-OH, Boc-Ser-OH, Boc-γ-Abu-OH (AG Switherland Bachem), and Boc-$\Delta^{3,4}$-Pro-OH (TriMen Chemicals Poland). The N-protected amino acids and DCC were used at three-fold excess. The N$^{\alpha}$-Boc-group was subsequently removed with 30% TFA in dichloromethane (DCM) according to standard methods.

The neutralization was achieved with 10% triethylamine (TEA) in DCM. Final peptides were obtained by deprotection and cleavage from the support resin with trifluoromethanesulfonic acid (TFMSA) in anisole.

All free peptides were desalted with Amberlite CG-4B and then purified on a Sephadex G-10 column, with 5% acetic acid as eluent. Analytical RP-HPLC was performed on a Beckman Peptide Gold System chromatograph with a C-18.5 μm Beckman column (ODS 250× 4.6), 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. Final purification was carried out by semi-preparative HPLC on an Alltech Econosil (Poland) C-18, 10 μm column (ODS 250×22 mm), linear gradient 23–39% S2 for 15 min., flow rate 7 ml/min., determined at 223 nm.

Table 1. Physico-chemical data of proctolin analogues modified at position 4 of the peptide chain.

Peptide	Yield (%)	$[\alpha]_D^{20}$ c = 1% CH_3OH	Rt[a] (HPLC)	Amino acids analysis	Mw		T.L.C.[b] Rf			
					Calc.	Found	X	Y	W	Z
H-Arg-Tyr-Leu-Phe-Thr-OH (**1**)	67	+27.01	16.13	Arg 1.08 Tyr 1.02 Leu 0.9 Phe 0.9 Thr 1.1	698.38	699.0	0.11	0.55	0.54	–
H-Arg-Tyr-Leu-D-Phe-Thr-OH (**2**)	48	–38.92	32.96	Arg 1.15 Tyr 1.05 Leu 1.0 D-Phe 0.9 Thr 0.9	698.38	699.2	0.43	0.61	0.53	–
H-Arg-Tyr-Leu-Phg-Thr-OH (**3**)	79	+22.22	15.14	Arg 1.2 Tyr 0.9 Leu 0.8 Thr 1.1	684.36	685.0	–	0.64	0.53	–
H-Arg-Tyr-Leu-D-Phg-Thr-OH (**4**)	51	–13.65	22.38	Arg 1.05 Tyr 0.9 Leu 1.0 Thr 1.05	684.36	685.2	0.48	0.79	0.57	0.11
H-Arg-Tyr-Leu-N-Me-Ala-Thr-OH (**5**)	49	–23.61	19.49	Arg 1.1 Tyr 0.86 Leu 0.94 Thr 1.1	637.37	638.6	0.37	0.65	0.31	0.12
H-Arg-Tyr-Leu-N-Me-Val-Thr-OH (**6**)	27	–15.80	26.73	Arg 1.1 Tyr 0.82 Leu 1.2 Thr 0.88	665.40	665.3	0.20	0.61	0.55	0.19
H-Arg-Tyr-Leu-N-Me-Leu-Thr-OH (**7**)	38	–32.34	25.64	Arg 1.07 Tyr 0.9 Leu 1.09 Thr 0.94	679.41	679.6	–	0.75	0.66	–
H-Arg-Tyr-Leu-Thr-OH (**8**)	35	–18.06	23.86	Arg 0.9 Tyr 0.9 Leu 1.1 Tyr 0.98 Thr 1.12	714.37	715.1	0.11	0.58	0.93	–
H-Arg-Tyr-Leu-Arg-Thr-OH (**9**)	35	+28.63	18.2	Arg 1.0 Tyr 0.8 Leu 1.07 Arg 1.03 Thr 1.1	707.41	708.4	0.20	0.59	0.49	–
H-Arg-Tyr-Leu-Lys-Thr-OH (**10**)	42	–42.74	15.88	Arg 1.04 Tyr 0.9 Leu 1.0 Lys 1.03 Thr 1.03	679.40	680.3	0.35	0.57	0.51	–
H-Arg-Tyr-Leu-Nva-Thr-OH (**11**)	45	–25.43	22.98	Arg 1.1 Tyr 1.1 Leu 0.9 Thr 0.9	650.38	650.9	0.54	0.72	0.68	0.49
H-Arg-Tyr-Leu-Acp-Thr-OH (**12**)	73	–11.91	26.80	Arg 1.0 Tyr 0.92 Leu 0.87 Thr 1.21	662.38	664.8	0.11	0.59	0.51	–

Table 1 (continuation)										
H-Arg-Tyr-Leu-Ser-Thr-OH (**13**)	27	–17.35	17.76	Arg 1.09 Tyr 0.82 Leu 1.1 Ser 0.81 Thr 1.18	638.34	639.5	–	0.53	0.25	–
H-Arg-Tyr-Leu-γ-Abu-Thr-OH (**14**)	19	+16.4	20.40	Arg 1.08 Tyr 0.88 Leu 1.04 Abu 1.0 Thr 1.0	636.36	637.7	0.69	0.68	0.38	0.79
H-Arg-Tyr-Leu-$\Delta^{3,4}$-Pro-Thr-OH (**15**)	38	–21.10	19.93	Arg 1.0 Tyr 0.91 Leu 1.0 Thr 1.09	646.36	646.9	0.38	0.68	0.58	0.15

[a]HPLC on Ultrasphere ODS column (Beckman) 4.5 mm×250 mm; gradient: 0–80% solvent B in 60 min. (B = 80% acetonitrile in water + 0.1% TFA).
[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:water (30:20:6:24), W = n-butanol:Ac-OH:ethyl acetate:water (1:1:1:1), Z = n-butanol:Ac-OH:water (4:1:1).

Purity and homogeneity of the free peptides was established by amino acid analysis and determination of molecular weights and optical activity. The physico-chemical data of free peptides are presented in Table 1.

Biological part: Peptides were bioassayed *in vitro* on the semi-isolated heart preparations of *T. molitor* according to the Rosiński and Gäde method [5] on adult males (7-day-old). The dose response relationship was established for each proctolin analogue (a separate determinations for 8 insects, ±SEM) (Table 2, Fig. 1 and Fig. 2).

Table 2. Myotropic effect on the heart of *T. molitor* of proctolin analogues modified at position 4 relative to proctolin (%).

Peptide	Myotropic effect on *T. molitor* heart, at 10^{-8} M conc. (8.0 ± SEM)
H-Arg-Tyr-Lue-Pro-Thr-OH (proctolin)	100
H-Arg-Tyr-Leu-Phe-Thr-OH (**1**)	50
H-Arg-Tyr-Leu-D-Phe-Thr-OH (**2**)	0
H-Arg-Tyr-Leu-Phg-Thr-OH (**3**)	20
H-Arg-Tyr-Leu-D-Phg-Thr-OH (**4**)	25
H-Arg-Tyr-Leu-N-Me-Ala-Thr-OH (**5**)	20
H-Arg-Tyr-Leu-N-Me-Val-Thr-OH (**6**)	0
H-Arg-Tyr-Leu-N-Me-Leu-Thr-OH (**7**)	0
H-Arg-Tyr-Leu-Tyr-Thr-OH (**8**)	0
H-Arg-Tyr-Leu-Arg-Thr-OH (**9**)	60
H-Arg-Tyr-Leu-Lys-Thr-OH (**10**)	0
H-Arg-Tyr-Leu-Nva-Thr-OH (**11**)	0
H-Arg-Tyr-Leu-Acp-Thr-OH (**12**)	0
H-Arg-Tyr-Leu-Ser-Thr-OH (**13**)	0
H-Arg-Tyr-Leu-γ-Abu-Thr-OH (**14**)	50
H-Arg-Tyr-Leu-Δ-^{3,4}-Pro-Thr-OH (**XV**)	0

Synthesis methods: H-Arg-Tyr-Leu-Phe-Thr-OH (1). The peptide was obtained by a stepwise elongation of the peptide chain by the method outlined above. 1.5 g of Boc-Thr(OBzl) resin (substitution level 1.08 mmol/g) (AG Switzerland, Bachem), was suspended in 30% TFA in CH_2Cl_2. The mixture was mixed for 30 min. at room temp. Then it was filtered and washed for 10 min. with CH_2Cl_2 and $CHCl_3$, 3 times each. The solution was neutralized with 10% TEA in CH_2Cl_2 in 10 min. and washed as above with $CHCl_3$ and CH_2Cl_2. The next amino acid Boc-Phe-OH (1.09 g, 4.1 mmol) was dissolved in CH_2Cl_2 and coupled to the resin in the presence of one equivalent of DCC/HOBt for 2 h. The end of reaction was determined by the Kaiser test. The further Boc-amino acids Boc-Leu-OH, Boc-Tyr(OBzl)-OH, and Boc-Arg(Tos)-OH were connected in the same way. The protected pentapeptide resin was dried overnight over KOH under reduced pressure.

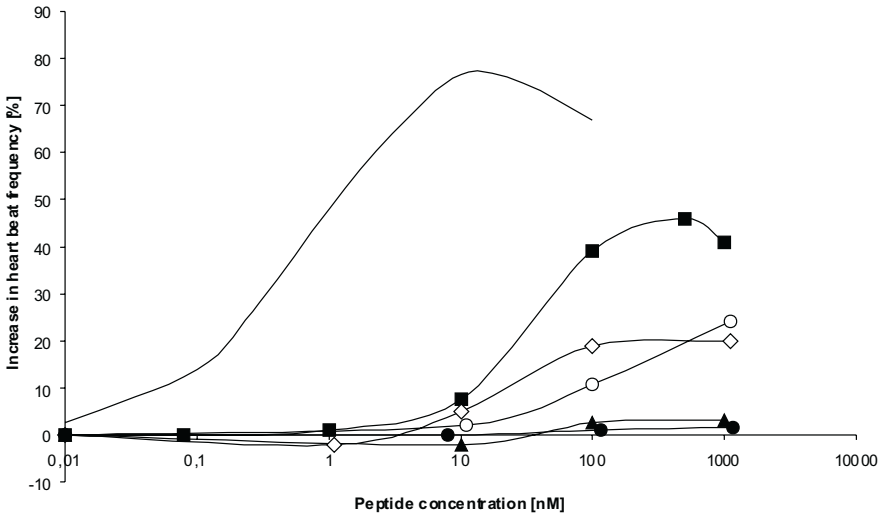

Figure 1. Cardioexcitatory effect of proctolin and its analogues modified at position 4 on *Tenebrio molitor* heart beat frequency (for 8 separate determination; ±SEM):
(–) proctolin, (■) H-Arg-Tyr-Leu-Phe-Thr-OH (**1**), (◇) H-Arg-Tyr-Leu-D-Phg-Thr-OH (**4**), (O) H-Arg-Tyr-Leu-N-Me-Ala-Thr-OH (**5**), (▲) H-Arg-Tyr-Leu-N-Me-Leu-Thr-OH (**7**), (•) H-Arg-Tyr-Leu-Lys-Thr-OH (**10**).

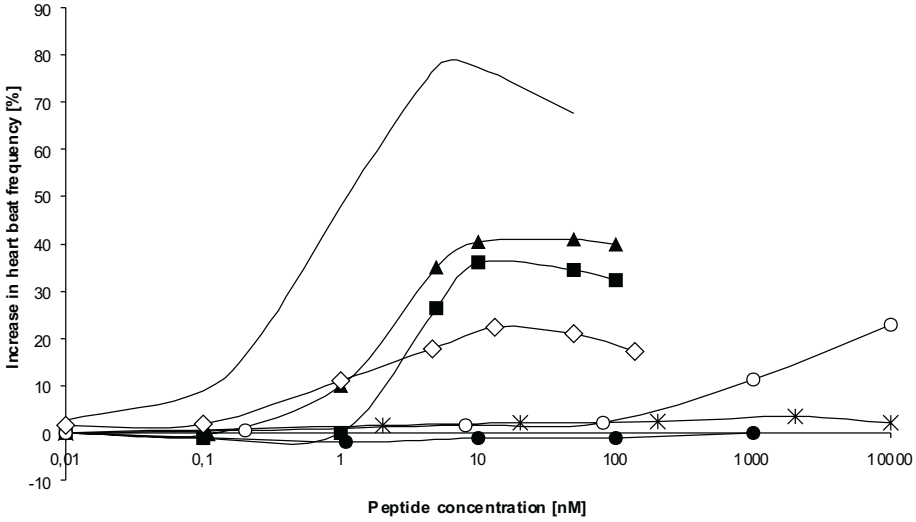

Figure 2. Cardioexcitatory effect of proctolin and its analogues modified at position 4 on *Tenebrio molitor* heart beat frequency (for 8 separate determination; ±SEM):
(–) proctolin, (▲) H-Arg-Tyr-Leu-Arg-Thr-OH (**9**), (■) H-Arg-Tyr-Leu-γ-Abu-Thr-OH (**14**), (◇) H-Arg-Tyr-Leu-Phg-Thr-OH (**3**), (O) H-Arg-Tyr-Leu-Ser-Thr-OH (**13**), (✳) H-Arg-Tyr-Leu-$\Delta^{3,4}$-Pro-Thr-OH (**15**), (•) H-Arg-Tyr-Leu-D-Phe-Thr-OH (**2**).

The free peptide was obtained according to the following procedure: The peptidyl resin was mixed with 0.9 ml of anisole, 0.45 ml of ethane-1,2-dithiol, 7.5 ml of TFA, and 1.2 ml of CF_3SO_3H. The mixture was kept at room temperature for 2 hours. The resin was filtered off and the filtrate was triturated with diethyl ether (200 ml). The above reaction mixture gave a precipitate, which was washed with diethyl ether, dried *in vacuo* over KOH and then dissolved in water. The aqueous solution was subsequently stirred with Amberlite CG-4B (acetate form) for 30 min., filtered, and lyophilized. The peptide was desalted on a Sephadex G-10 column eluted with 5% acetic acid. The peptide was then purified by preparative HPLC. The main fractions were pooled and lyophilized.

The peptides **2**–**15** were obtained in the same manner as above. The data are presented in Table 1.

RESULTS AND DISCUSSION

Structural modifications of proctolin at position 4 of the peptide skeleton change markedly the potency and the intrinsic cardiotropic activity of the native peptide. Analogues **2**, **6**–**8**, **10**–**13**, and **15** applied at the physiological concentration (ranging from 10^{-8} to 10^{-7} M) lost their cardiostimulatory activity against mealworm (Figs. 1 and 2). Among the analogues tested, six peptides (**1**, **3**, **4**, **5**, **9**, and **14**) retained about 20–60% of proctolin activity (Figs. 1 and 2) when applied to the heart of the insect.

Proctolin analogues modified at position 4 by the $\Delta^{3,4}$-Pro residue were practically inactive. The myotropic effects, observed in insects, depend probably on the structural requirements of the amino acid residue at position 4 of the proctolin molecule. It is interesting that the largest cardiostimulatory activity in the series of analogues was observed for compounds containing aromatic or hydrophobic amino acids residues such as Phe (**1**), Phg (**2**), D-Phg (**4**), N-Me-Ala (**5**) or γ-Abu (**14**). Biological responses suggest some specific requirements of the myocardium proctolin receptor on the *T. molitor* heart. The presence of hydrophobic residues at position 4 of the proctolin peptide chain is important for myotropic properties in insects. In our earlier paper we postulated [3] that the presence of pyrrolidine ring in the Pro moiety is required for entire cardiotropic proctolin bioactivity in the tested insect. Lack of activity for [$\Delta^{3,4}$-Pro^4]-proctolin suggests that the exchange of the pyrrolidine ring for 3–4 unsatureted pyrrolidine ring led to peptides without cardiostimulatory effect in mealworm.

CONCLUSIONS

From analysis of the myotropic effects of proctolin analogues **1**–**15** on *T. molitor* the following conclusions can be drawn:

1/ the presence of amino acids residues with a hydrophobic side chain at position 4 is important for cardiotropic activity in yellow mealworm;

2/ lower or none myotropic activity of the mentioned analogues in insects, as compared to proctolin, is probably the result of the change in the shape of the proctolin molecule;

3/ exchange of the pyrrolidine ring for the 3–4 unsaturated pyrrolidine system leads to peptides without cardiostimulatory effect in mealworm;

4/ the Arg basic amino acid residues at position 4 of the peptide chain of proctolin analogue **9** do not destroy the myotropic activity on the insect heart.

Acknowledgments

This work was supported by grants from the Wrocław University Scientific Foundation.

REFERENCES

1. Konopińska D., *J. Peptide Res*., **49**, 457 (1997).
2. Konopińska D. and Rosiński G., *J. Peptide Sci*., **5**, 533 (1999).
3. Konopińska D., Bartosz-Bechowski H., Rosiński G. and Sobótka W., *Bull. Pol. Acad. Sci. Chem*., **41**, 27 (1993).
4. Kuczer M., Rosiński G., Lisowski M., Picur B. and Konopińska D., *Int. J. Peptide Protein Res*., **48**, 286 (1996).
5. Rosiński G. and Gäde G., *J. Insect Physiol*., **33**, 451 (1988).