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# New Proctolin Analogues Modified by the Novel D- or L-Phenylglycine Derivatives. Synthesis and Biological Studies

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New analogues of insect neuromodulator proctolin (H-Arg-Tyr-Leu-Pro-Thr-OH), modified in position 2 of the peptide chain by L- or D-phenylglycine and its 4-substituted derivatives were synthesized. For modification of proctolin a series of novel L- or D-phenylglycine derivatives H-Phg(4-$NO_2$)-OH (**1**), Boc-Phg(4-$NO_2$)-OH (**2**), Boc-Phg(4-$Me_2N$)-OH (**3**), H-Phg(4-OBzl)-OH (**4**), Boc-Phg(4-OBzl)-OH (**5**), H-D-Phg(4-$NO_2$)-OH (**6**), Boc-D-Phg(4-$NO_2$,)-OH (**7**), Boc-D-Phg(4-$Me_2N$)-OH (**8**), were used. The following proctolin analogues were synthesized: H-Arg-Phg-Leu-Pro-Thr-OH (**9**), H-Arg-D-Phg-Leu-Pro-Thr-OH (**10**), H-Arg-Phg(4-OH)-Leu-Pro-Thr-OH (**11**), H-Arg-D-Phg(4-OH)-Leu-Pro-Thr-OH (**12**), H-Arg-Phg(4-$NO_2$)-Leu-Pro-Thr-OH (**13**), H-Arg-D-Phg(4-$NO_2$)-Leu-Pro-Thr-OH (**14**), H-Arg-Phg(4-$NH_2$)-Leu-Pro-Thr-OH (**15**), H-Arg-D-Phg(4-$NH_2$)-Leu-Pro-Thr-OH (**16**), H-Arg-Phg(4-$NMe_2$)-Leu-Pro-Thr-OH (**17**), H-Arg-D-Phg(4-$NMe_2$)-Leu-Pro-Thr-OH (**18**). Myotropic activity of proctolin analogues **9–18** was assayed *in vitro* on the semi-isolated heart of the mealworm *Tenebrio molitor* and on the foregut of the locust *Schistocerca gregaria*. All analogues showed a weak or none activity.

**Key words:** proctolin, proctolin analogues, insect peptide proctolin, L- and D-phenylglycine derivatives

Proctolin, first structurally characterized myotropic insect neuromodulator, a pentapeptide, H-Arg-Tyr-Leu-Pro-Thr-OH, was isolated in 1975 from the whole body extracts of the American cockroach *Periplaneta americana* [1]. It was found later in six other orders of insects as well as in other invertebrates [2,3]. Since the biological effect of proctolin consists in stimulation of contractions of smooth, skeletal, heart and oviduct muscles of insects and other invertebrates [4], it is considered as an insect neuromodulator. In the course of structure-myotropic function studies on insects a hundred of proctolin analogues were synthesized, of which twenty five re-

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)].

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tained an agonistic effect in selected insect species, whereas about ten compounds showed antagonistic properties [5]. Most of proctolin analogues modified in position 2 of the peptide chain [2,5–7] showed agonistic or antagonistic activity. The known, agonistic or antagonistic analogues of proctolin with amino acid modifications at position 2, contain derivatives of phenylalanine of the Phe(4-Y) type, where Y = $NO_2$, $NH_2$ or $NMe_2$ [1,2] , α-Me-Tyr or Afb(4-$NO_2$) [5–7]. They all have one common feature, *i.e*. the benzene ring is linked to the α-C atom through the methylene bridge. To study the effect of this methylene bridge we replaced Tyr at position 2 of proctolin by L- or D-phenylglycine and its 4-substituted derivatives Phg(4-Y), where Y = –OH, –$NO_2$, –$NH_2$ or –$NMe_2$. Phenylglycine derivatives were chosen for proctolin modification in position 2 of the peptide chain, because they lack the methylene group between the C-α atom and the benzene ring.

The following L- and D-phenylglycine derivatives and peptides were synthesized: H-Phg(4-$NO_2$)-OH (**1**), Boc-Phg(4-$NO_2$)-OH (**2**), Boc-Phg(4-$NMe_2$)-OH (**3**), H-Phg(4-OBzl)-OH (**4**), Boc-Phg(4-OBzl)-OH (**5**), H-D-Phg(4-$NO_2$)-OH (**6**), Boc-D-Phg(4-$NO_2$,)-OH (**7**), Boc-D-Phg(4-$NMe_2$)-OH (**8**), H-Arg-Phg-Leu-Pro-Thr-OH (**9**), H-Arg-D-Phg-Leu-Pro-Thr-OH (**10**), H-Arg-Phg(4-OH)-Leu-Pro-Thr-OH (**11**), H-Arg-D-Phg(4-OH)-Leu-Pro-Thr-OH (**12**), H-Arg-Phg(4-$NO_2$)-Leu-Pro-Thr-OH (**13**), H-Arg-D-Phg(4-$NO_2$)-Leu-Pro-Thr-OH (**14**), H-Arg-Phg(4-$NH_2$)-Leu-Pro-Thr-OH (**15**), H-Arg-D-Phg(4-$NH_2$)-Leu-Pro-Thr-OH (**16**), H-Arg-Phg(4- $NMe_2$)-Leu-Pro-Thr-OH (**17**) and H-Arg-D-Phg(4- $NMe_2$)-Leu-Pro-Thr-OH (**18**).

## EXPERIMENTAL

**Method and materials:** General procedure: Amino acid compositions were determined on an amino acid analyzer Mikrotechna T339 (Czechoslovakia). The optical activity of the chiral compounds was determined with a Jasco DIP-1000 polarimeter (±1.2$^0$) (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. All peptides showed about 100% purity. **Synthesis methods.** Proctolin was obtained from Sigma Chemical Co. Ltd. All peptides (**9**–**18**) were synthesized by the classical solid-phase Boc procedure [8]. Dicyclohexylcarbodiimide (DCC) in the presence of HOBt as a coupling reagent was used. The C-terminal amino acid acid derivatives (Boc-Thr(OBzl) was connected to the chloromethylated classical Merrifield resin containing 0.8 mmol of Cl/g resin by standard cesium salt procedure. The protected amino acids were coupled using DCC method. The following amino acid derivatives were used: Boc-Arg(Tos)-OH, Boc-Leu-OH, Boc-Pro-OH, Boc-Thr(OBzl)-OH (Bachem). A series of new L-or D-phenylglycine derivatives: H-Phg(4-$NO_2$)-OH (**1**), Boc-Phg(4-$NO_2$)-OH (**2**), Boc-Phg(4-$NMe_2$)-OH (**3**), H-Phg(4-OBzl)-OH (**4**), Boc-Phg(4-OBzl)-OH (**5**), H-D-Phg(4-$NO_2$)-OH (**6**), Boc-D-Phg(4-$NO_2$,)-OH (**7**), Boc-D-Phg(4-$NMe_2$)-OH (**8**) were synthesized. The N-protected amino acids and DCC were used in three fold excess. $N^{\alpha}$-Boc-group was subsequently removed with 30% TFA in dichloromethane (DCM) according to standard methods.

The neutralization was made with 10% triethylamine (TEA) in DCM. Finally peptides were obtained by deprotection and cleavage from the support resin with trifluoromethanesulfonic acid ($CF_3SO_3H$) in anisole and the nitro group was reduced in presence of aqueous hydrazine with Raney nickel as a catalyst. 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 conducted on a Beckman Peptide Gold System chromatograph with C-18, 5 μm Beckman column (ODS 250 × 4.6 mm), ultrasphere plus 4.6 × 4.5 mm

precolumn. Solvent systems: S1 – 0.1% aqueous TFA, S2 – 80% acetonitrile; linear gradient from 100–0% 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 a Alltech Econsil 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.

Amino acid analysis, molecular weight and optical activity established purity and homogeneity of the free peptides. The physico-chemical data of D- and L-phenylglycine derivatives (**1–8**) are summarized in Table 1 and of free peptides (**9**–**18**) in Table 2.

**Biological tests:** Myotropic activity of the proctolin analogues (**9**–**18**) was tested by two *in vitro* bioassays: 1/ on the semi-isolated heart preparation of the yellow mealworm *Tenebrio molitor* (cardioexcitatory assay) according to the Rosiński and Gäde method [4]; and 2/ on the isolated foregut of the locust *Schistocerca gregaria* (mytropic activity) [5–7].

**Cardioexcitatory assay:** Peptides were bioassayed *in vitro* on the semi-isolated heart preparations of *T. molitor* according to the Rosiński and Gäde method [4] on adult males (7 day-old). The dose response relationship was established for each proctolin analogue (a separate determination for 6–10 insects, ±SEM), (Fig. 1).

**Contractile activity on the foregut:** Proctolin analogues were bioassayed *in vitro* on the isolated foregut of locust *S. gregaria* according to the method described earlier [5]. The dose-response relationship was determined for each proctolin analogue. When peptides with no obvious agonist action were tested for potential antagonistic properties, the putative antagonist was added to the tissue before adding the next dose of proctolin [5–7]. The tests were performed separately for 6–8 insects (see Fig. 2).

L-4-Nitro-phenylglycine (H-Phg(4-$NO_2$)-OH) (**1**). The title product was obtained in the manner presented for H-Phe(4-$NO_2$)-OH [9]. L-Phenylglycine (25.0 g, 160 mmol) was dissolved in concentrated sulfuric acid (40 ml) and then concentrated nitric acid (8 ml) was added dropwise slowly with stirring at 0°C. The solution was mixed for 30 min. at 0–5°C, dropped to 400 ml of cold water and pH was adjusted to 6.5 with aqueous ammonia. The yellow product was filtered and crystallized from water. 16.3 g of the product was obtained. Physico-chemical data are presented in Table 1.

L-N-(tert-Butoxycarbonyl)-4-nitro-phenylglycine (Boc-Phg(4-$NO_2$)-OH) (**2**). Compound **1** (9.0 g, 46 mmol) was dissolved in 1 M NaOH (50 ml) and then water (50 ml) was added. 11.0 g (50 mmol) of di-t-butyl-dicarbonate ($Boc_2O$) was dissolved in 50 ml of dioxane and poured into the solution of compound **1**. The reaction was carried out according to [10]. After crystallization in diethyl ether-pentane (1:3), 7.6 g of the product was obtained. Physico-chemical data are presented in Table 1.

**Table 1.** Physicochemical data of L- and D-phenylglycine derivatives.

| Compound | Yield (%) | M.p [°C] | $[\alpha]_D^{20}$ c = 0.1 $CH_3OH$ | T.L.C.[a] Rf X | Y | Z |
|---|---|---|---|---|---|---|
| **1** | 52 | >250 | –24.7* | 0.34 | 0.28 | 0.67 |
| **2** | 48 | 224 | –32.3 | 0.22 | 0.43 | 0.41 |
| **3** | 66 | 212 | –24.8 | 0.34 | 0.45 | 0.39 |
| **4** | 76 | 227 | +55.2#& | 0.22 | 0.57 | 0.63 |
| **5** | 59 | 182 | +56.6 | 0.12 | 0.52 | 0.33 |
| **6** | 46 | >250 | +24.9* | 0.33 | 0.31 | 0.67 |
| **7** | 56 | 224 | +32.2 | 0.18 | 0.38 | 0.42 |
| **8** | 64 | 214 | +24.6 | 0.36 | 0.45 | 0.40 |

[a]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), Z = n-butanol:Ac-OH:water (4:1:1), * 50% AC-OH in MeOH, #c = 0.3 in 0.1 M NaOH , & lit .–54.7° for D-Phg(4-OBzl)-OH [12]. Analyses and molecular weights calculations confirm the compositions.

**Table 2.** Physicochemical data of free peptides.

| Peptide | Yield (%) | Rf[a] (HPLC) | $[\alpha]_D^{20}$ c = 0.1 $CH_3OH$ | T.L.C.[a] Rf X | Y | Z | Amino acid analysis | Mw Calc. | Found |
|---|---|---|---|---|---|---|---|---|---|
| **9** | 87 | 17.39 | –31.2 | 0.33 | 0.56 | 0.78 | Arg 0.9 Leu 1.0 Pro 1.0 Thr 1.0 | 618.6 | 619.6 |
| **10** | 76 | 18.54 | –42.4 | 0.45 | 0.34 | 0.82 | Arg 0.98 Leu 1.04 Pro 1.02 Thr 0.96 | 618.6 | 619.8 |
| **11** | 34 | 22.14 | –27.5 | 0.34 | 0.22 | 0.67 | Arg 0.99 Leu 1.0 Pro 1.01 Thr 1.0 | 634.7 | 635.2 |
| **12** | 56 | 19.87 | –46.1 | 0.56 | 0.55 | 0.52 | Arg 0.98 Leu 1.02 Pro 1.0 Thr 1.0 | 634.7 | 635.3 |
| **13** | 68 | 21.38 | –36.2 | 0.41 | 0.22 | 0.64 | Arg 1.02 Leu 1.0 Pro 1.0 Thr 0.98 | 663.7 | 664.5 |
| **14** | 63 | 20.45 | –33.1 | 0.34 | 0.25 | 0.61 | Arg 1.0 Leu 1.0 Pro 1.0 Thr 1.0 | 663.7 | 664.8 |
| **15** | 55 | 16.23 | –28.9 | 0.44 | 0.18 | 0.28 | Arg 0.94 Leu 1.06 Pro 1.0 Thr 1.0 | 633.6 | 634.4 |
| **16** | 54 | 16.02 | –37.6 | 0.26 | 0.22 | 0.34 | Arg 0.98 Leu 1.0 Pro 1.02 Thr 1.0 | 633.6 | 634.7 |
| **17** | 46 | 18.51 | –40.3 | 0.45 | 0.32 | 0.36 | Arg 1.0 Leu 1.04 Pro 1.0 Thr 0.96 | 661.7 | 662.1 |
| **18** | 41 | 17.23 | –31.9 | 0.34 | 0.52 | 0.34 | Arg 0.98 Leu 1.0 Pro 1.01 Thr 1.01 | 661.7 | 662.9 |

[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), Z = n-butanol:Ac-OH:water (4:1:1).

L-N-(tert-Butoxycarbonyl)-4-(N,N-dimethylamino)-phenylglycine (Boc-Phg(4-$NMe_2$)-OH) (**3**). 7.0 g (24 mmol) of compound **2** was dissolved in 40 ml of methanol, 13 ml of 30% formaldehyde was added and the solution was hydrogenated in the presence of 10% Pd/C (0.1 g) for 24 h. The whole reaction was carried out according to [11]. Crystallization from diethyl ether-pentane (1:3) gave 4.6 g of the title product. Physico-chemical data are presented in Table 1.

4-Benzyloxy-phenylglycine (H-Phg(4-OBzl)-OH) (**4**).The title product was synthesized from 6 g (36 mmol) of 4-hydroxy-L-phenylglycine obtained according to [12]. 7.0 g of the product were obtained after crystallization from acetic acid-water (4:1) (Table 1).

L-N-(tert-Butoxycarbonyl)-4-benzyloxy-phenylglycine Boc-Phg(4-OBzl)-OH) (**5**). The title compound was obtained from 7.0 g (27 mmol) of Phg(4-OBzl)-OH [12] in the same manner as compound **2**. Crystallization from diethyl ether–pentane (1:3) gave 5.7 g of the product. Data in Table 1.

D-4-Nitro-phenylglycine (H-D-Phg(4-$NO_2$)-OH) (**6**). The title compound was obtained in the same manner as compound **1** from 25.0 g (160 mmol) of D-phenylglycine. Crystallization from water gave 14.4 g of the product. Physico-chemical data in Table 1.

D-N-(tert-Butoxycarbonyl)-4-nitro-phenylglycine (Boc-D-Phg(*p*-$NO_2$)-OH) (**7**). 8.0 g (41 mmol) of compound **6** was reacted with 9.1 g (42 mmol) of $Boc_2O$ in the same manner as in the case of **2**. After crystallization from diethyl ether-pentane (1:3) 6.7 g of the product was obtained (Table 1).

D-N-(tert-Butoxycarbonyl)-4-(N,N-dimethylamino)-phenylglycine (Boc-D-Phg(4-$NMe_2$)OH) (**8**). 5.0 g (17 mmol) of compound **7** was hydrogenated in the presence of formaldehyde in the same manner as in the case of **3**. After crystallization from diethyl ether-pentane (1:3) 3.1 g of the pure product was obtained. Physico-chemical data are given in Table 1.

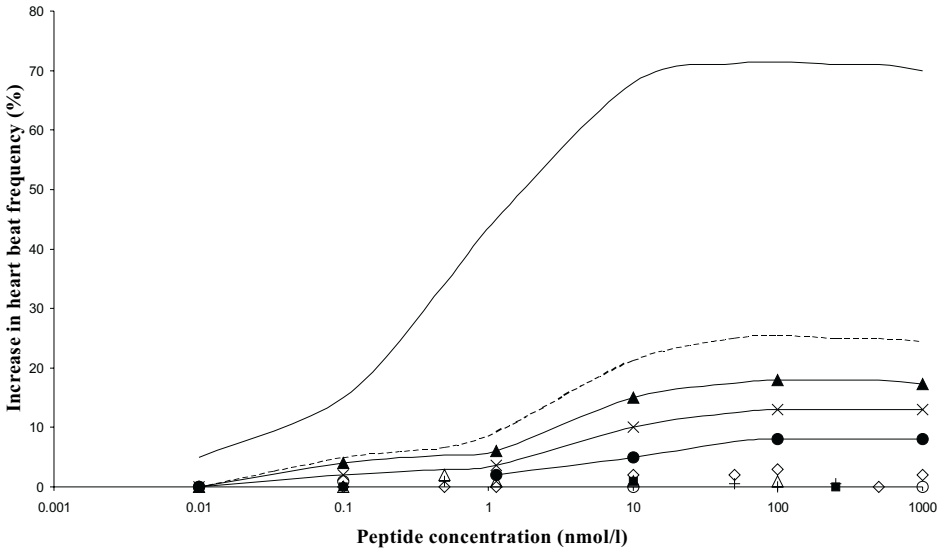


**Figure 1.** Cardioexitatory effect of proctolin and its analogues on *Tenebrio molitor* heart beat frequency (for 6–10 separate determination; ±SEM): (—) proctolin, (- -) peptide **9**, (+) peptide **10**, (❍) peptide **11**, (❑) peptide **12**, (▲) peptide **13**, (Δ) peptide **14**, (◇) peptide **15**, (■) peptide **16**, (x) peptide **17**, (●) peptide **18**.

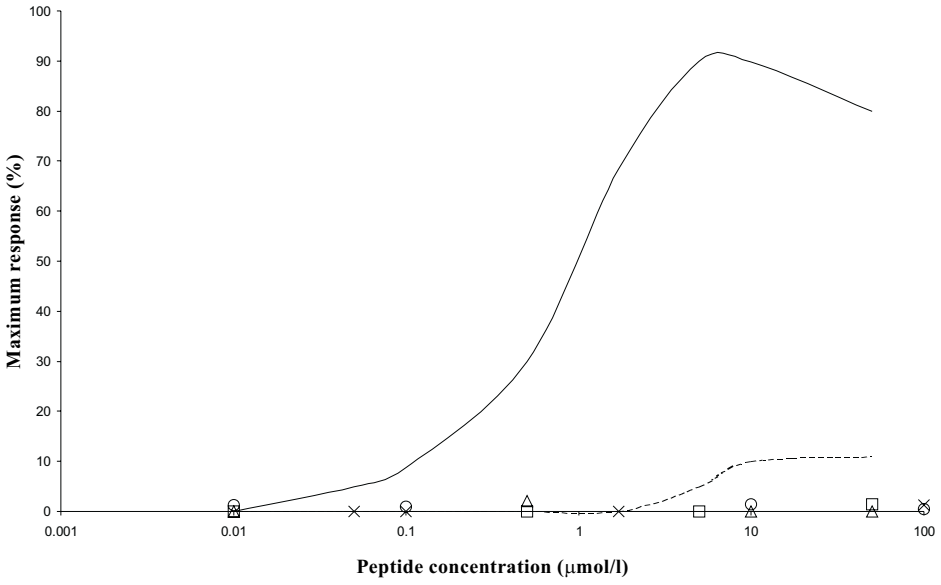


**Figure 2.** Myotropic effect of proctolin and its analogues on the isolated foregut of *Schistocerca gregaria* (for 6 to 8 separate determination; ±SEM): (—) proctolin, (- -) peptide **9**, (+) peptide **10**, (❍) peptide **11**, (x) peptide **13**, (Δ) peptide **14**.

H-Arg-Phg-Leu-Pro-Thr-OH (**9**). The peptide was obtained by a stepwise elongation of the peptide chain by the method outlined above. 1.0 g of Boc-Thr(OBzl)-resin (substitution level 0.8 mmol/g), was suspended in solution of 30% TFA in $CH_2Cl_2$. The mixture was mixed for the 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 reaction was neutralized with 10% triethylamine in $CH_2Cl_2$ in 10 min. and washed as above with $CHCl_3$ and then $CH_2Cl_2$. The next amino acid Boc-Pro-OH (0.52g, 2.4 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-Phg-OH, and Boc-Arg(Tos)-OH were connected in the same way. The protected pentapeptide resin was dried overnight over KOH under reduced pressure.

The free peptide was obtained according to the following procedure: The peptide resin was mixed with 0.6 ml of anisole, 0.3 ml of ethane-1,2-ditiol, 5 ml of TFA, and 0.8 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 data are presented in Table 2.

Peptides **9**–**14**, **17** and **18** were obtained and purified in the same manner as peptide **9**. Their data are presented in Table 2. H-Arg–Phg(4-$NH_2$)-Leu-Pro-Thr-OH (**15**). 0.5 g (0.75 mmol) of peptide **13** was dissolved in methanol (20 ml), containing 1 ml of 5% aqueous hydrazine hydrate. Then Raney nickel (0.05 g) was added and the whole mixture was stirred at 38°C for 40 min. Then the catalyst was filtered off and the filtrate was evaporated *in vacuo.* The residue was triturated with diethyl ether and the obtained precipitate was filtered and dried *in vacuo* over KOH. The peptide was purified by desalting on a Sephadex G-10 column eluted with 5% acetic acid and then by preparative HPLC. The main fractions were pooled and lyophilized. The peptide was purified by the same procedure as compound **9** (Table 2). Peptide (**16** ) was obtained in the same manner as analogue (**15**) (Table 2).

## RESULTS AND DISCUSSION

The bioassay data reported here reveal that structural modification of proctolin in position 2 results in analogues some of which are biologically active. Thus, peptides **9**, **13**, **17**, and **18** retained a weak (5–20%) proctolin activity (Fig. 1), when applied to the heart of *T. molitor* at physiological concentrations ranging from $10^{-9}$ to $10^{-7}$ M. The other peptides were practically inactive. Analogues **13** and **17**, containing Phg(4-$NO_2$) and Phg(4-$NMe_2$) in position 2, show the activity which is markedly decreased in comparison to the effects found for proctolin analogues containing the Phe(4-$NO_2$) and Phe(4-$NMe_2$) residues in position 2 [8]. We found that only analogue **9** retained a weak myotropic activity at the $10^{-6}$ M concentration in the myotropic test on the foregut of *S. gregaria* (Fig. 2). Other peptides had neither agonistic nor antagonistic activity. The biological results showed the species specificity of peptides **13**, **17**, and **18**, which are active in the test on the *T. molitor* heart and not active in the test on the *S. gregaria* foregut.

The myotropic effects, observed in two insects, depend probably on the structure requirement of the amino acid residue in position 2 of the proctolin molecule. In the phenylglycine analogues of proctolin there is a smaller distance between the side chain aromatic ring in position 2 and the peptide chain than in unmodified proctolin. It seems that the conformational change resulting from the lack of the methylene group between the C-$\alpha$ atom and the aromatic ring in these analogues is so drastic that

binding of the phenylglycine analogues to the proctolin receptor is no longer possible. Thus, the presence of the methylene group next to the benzene ring of the amino acid residue in position 2 of proctolin is essential for myotropic activity.

## CONCLUSIONS

From analysis of the myotropic effects of proctolin and analogues **9**–**18** on two insect preparation studies the following conclusions can be drawn:

1) the biological results pointed out that the presence of methylene group between the C-a atom and the aromatic ring of the side chain of an amino acid residue at position 2 of the peptide chain is important for myotropic activity in insects;
2) the shortening of the side chain at position 2 and bringing the aromatic ring closer to the peptide chain changes the peptide conformation;
3) the lower or none myotropic activity of mentioned analogues in insects, as compared to proctolin, is probably a result of a change in the shape of the proctolin molecule.

Acknowledgments

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Abbreviations used: Phe(4-$NH_2$), L-4-amino-phenylalanine; Phg, L-phenylglycine; Phg(4-OH), L-4-hydroxy-phenylalanine; Phg(4-$NO_2$), L-4-nitro-phenylglycine; D-Phg(4-$NO_2$), D-4-nitro-phenylglycine; Phg(4-$NH_2$), L-4-amino-phenylglycine; D-Phg(4-$NH_2$), D-4-amino-phenylglycine; Phg(4-$NMe_2$), L-4-N,N-dimethylamino-phenylglycine; D-Phg(4-$NMe_2$), D-4-N,N-dimethylamino-phenylglycine; Me, methyl; TFA, trifluoroacetic acid. BOC – tert-Butyloxycarbonyl, Bzl – benzyl.

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