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Synthesis, cardiostimulatory, and cardioinhibitory effects of selected insect peptides on *Tenebrio molitor*

K. SZYMANOWSKA-DZIUBASIK,[a] P. MARCINIAK,[b] G. ROSIŃSKI[b] and D. KONOPIŃSKA[a]*

[a] Faculty of Chemistry, University of Wrocław, 50-383 Wrocław, 14 F. Joliot-Curie Str., Poland
[b] Department of Animal Physiology and Developmental Biology, A. Mickiewicz University, 61-701 Poznan, Poland

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Abstract: The subject of these studies was a search for proctolin antagonists among peptides originating from insect species because the proctolin antagonists constantly pose a problem. During these studies we performed the synthesis of the following peptides: a native decapeptide from *Manduca sexta* Mas-MT-I and its 11 analogs with shortened sequences at the *N*-end as well as a growth suppressor, a pentapeptide isolated from *Antheraea yamamai*, Any-GS and its 10 analogs, modified at position 1 and with a shortened peptide chain.

Biological effects were evaluated by the cardiotropic test on the semi-isolated heart of the insect species *Tenebrio molitor*. Mas-MT-I and six analogs stimulate the heartbeat frequency, especially [6–10]-Mas-MT-I, whereas the [4–10]-Mas-MT-I analog shows a strong inhibition of the heartbeat frequency, if insect. The Any-GS and the analogs [Gln[1]]- and [Gly[1]]-Any-GS also show a strong cardioinhibitory effect. Copyright © 2008 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: insect myotropins; cardiostimulatory peptides of insects; cardioinhibitory peptides of insects; myotropic peptides from *Manduca sexta*; growth-suppressing pentapeptides

INTRODUCTION

The aim of these investigations was to find potential proctolin antagonists among peptides originating from the insect species selected because proctolin antagonists still pose a current problem. Proctolin antagonists will be useful tools for examining the physiological importance of proctolin in insects as well as helping to identify receptor subtypes [1–3].

We performed the synthesis and biological evaluation of two groups of selected insect peptides: (i) myotropins isolated from *Manduca sexta* [4] and (ii) a growth suppressor from *Antheraea yamamai*, Any-GS [5].

As regards the first group of peptides, we performed the synthesis of Mas-MT-I (H-Glp-Asp-Val-Val-His-Ser-Phe-Leu-Arg-Phe-NH_2) (**1**), Mas-MT-II (H-Asp-Pro-Ser-Phe-Leu-Arg-Phe-NH_2) (**2**), Mas-MT-III (H-Gly-Asn-Ser-Phe-Leu-Arg-Phe-NH_2) (**3**), the analogs of Mas-MT-I with a shortened sequence at the *N*-end, H-Asp-Val-Val-His-Ser-Phe-Leu-Arg-Phe-NH_2 (**4**), H-Val-Val-His-Ser-Phe-Leu-Arg-Phe-NH_2 (**5**), H-Val-His-Ser-Phe-Leu-Arg-Phe-NH_2 (**6**), H-His-Ser-Phe-Leu-Arg-Phe-NH_2 (**7**) H-Ser-Phe-Leu-Arg-Phe-NH_2 (**8**), H-Phe-Leu-Arg-Phe-NH_2 (**9**), H-Leu-Arg-Phe-NH_2 (**10**), and the analogs (**11**)–(**13**) of Mas-MT-II modified at the *C*-end by Phe(p-X) (the Phe residue modified at position 4 of the aromatic ring), such as H-Asp-Pro-Ser-Phe-Leu-Arg-Phe(p-F)-NH_2 (**11**), H-Asp-Pro-Ser-Phe-Leu-Arg-Phe(p-Cl)-NH_2 (**12**), and H-Asp-Pro-Ser-Phe-Leu-Arg-Phe(p-I)-NH_2 (**13**).

The second group of investigated peptides comprised Any-GS [5] (H-Asp-Ile-Leu-Arg-Gly-NH_2) (**14**) and its analogs modified at position 1, or peptides with a shortened sequence, such as: H-Asp-Ile-Leu-Arg-Gly-NH_2 (**14**), H-Ile-Leu-Arg-Gly-NH_2 (**15**), H-Leu-Arg-Gly-NH_2 (**16**), H-Asp-Ile-Leu-Arg-NH_2 (**17**), H-Asp-Ile-Leu-NH_2 (**18**), H-Asn-Ile-Leu-Arg-Gly-NH_2 (**19**), H-Glp-Ile-Leu-Arg-Gly-NH_2 (**20**), H-Arg-Ile-Leu-Arg-Gly-NH_2 (**21**), H-Gln-Ile-Leu-Arg-Gly-NH_2(**22**), H-Gly-Ile-Leu-Arg-Gly-NH_2 (**23**), H-Lys-Ile-Leu-Arg-Gly-NH_2 (**24**), and H-Ala-Ile-Leu-Arg-Gly-NH_2 (**25**).

The synthesis of these peptides was performed by the solid-phase method according to the Boc-procedure. The physicochemical data of peptides are presented in Tables 1 and 2.

Biological effects were evaluated by the cardiotropic test on the semi-isolated heart of insect species, *Tenebrio molitor* according to [1,5]. The effect was compared with insect neuromodulator, proctolin (Tables 2 and 3 and Figures 1 and 2).

MATERIAL AND METHODS

Synthesis

Amino acid compositions were checked using an amino acid analyzer AAA-88a (Czechoslovakia). The optical activity of the chiral compounds was determined with a Jasco DIP-1000 polarimeter (±0.1°) (Jasco, Japan). The molecular weights of the peptides were determined with a Bruker Daltonios microTOF-Q mass spectrometer (USA). The purity and homogeneity of all final products were checked by HPLC, TLC on silica gel plates (Merck), and amino acid

*Correspondence to: D.Konopińska, Faculty of Chemistry, University of Wrocław, 50-383 Wrocław, 14 F. Joliot-Curie Str., Poland; e-mail: dk@wchuwr.chem.uni.wroc.pl

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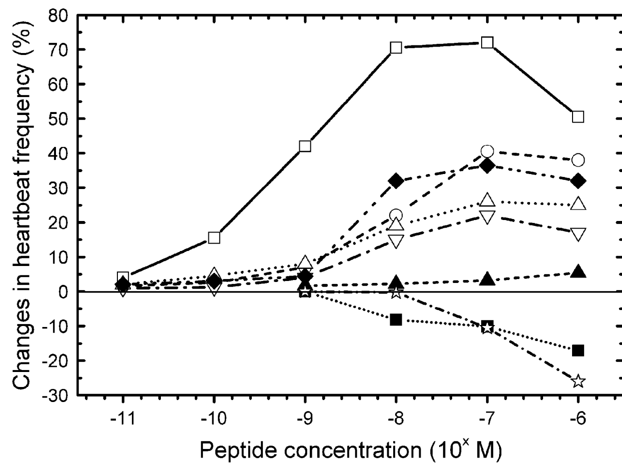

Figure 1 Cardioexcitatory effect of proctolin and Mas-MT in *Tenebrio molitor*. □ – Proctolin, ○ – peptide **9**, Δ – Mas-MT-III, ♦ –peptide **8**, ■ – peptide **6**, ▲–Mas-MT-I, ☆ – peptide **5**, ▽ – Mas-MT-II.

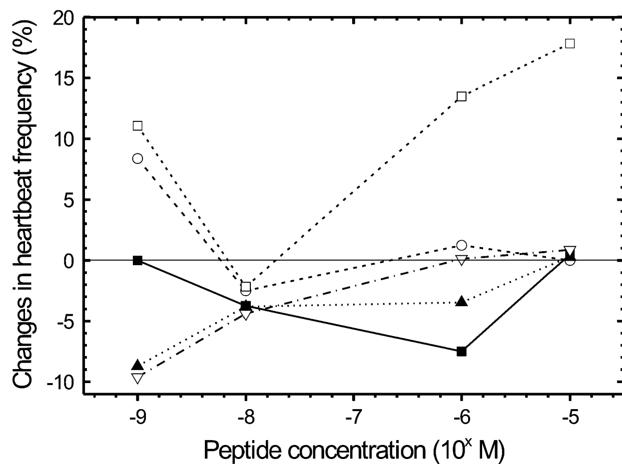

Figure 2 Cardioexcitatory effect of Any-GS and its analogs in *Tenebrio molitor*. ■–peptide Any-GS, ○–peptide **17**, ▲–peptide **21**, □- peptide **24** ▽ – peptide **22**.

analysis. Analytical RP-HPLC was performed with a Termo Separation Product with a VYDAC C18 column (ODS 250 × 4.6 mm). Solvent systems: S1 – 0.1% aqueous TFA, S2 – 80% acetonitrile; linear gradient from 0 to 100% of S2 for 60 min, flow rate 1.0 ml/min, determined at 223 nm. Final purification was carried out by semipreparative HPLC on a Varian ProStar, column-Tosoh Biosciences ODS-120T C18 (ODS 300 × 21.5 mm), linear gradient of 0.1% aqueous TFA-acetonitrile, flow rate 7.0 ml/min, determined at 223 nm. Boc-Phe-OH was connected to the MBHA•HCl-resin (4-methylbenzhydrylamine hydrochloride) containing 0.86 mmol of NH_2/g of resin with DCC. The protected amino acid was coupled to the resin with DCC in the presence of HOBt or by the symmetrical anhydride method. The following amino acid derivatives (Bachem, USA) were used: Boc-Ala-OH, Boc-Ser(Bzl)-OH, Boc-Gly-OH, Boc-Phe-OH, Boc-Val-OH, Z-Glp-OH, Boc-Leu-OH, Boc-Arg(Tos)-OH, Boc-His(π-Bom)-OH, and Boc-Asp(Bzl)-OH. During the synthesis, Boc-Val-OH and Boc-Ile-OH were introduced to the peptide chain by the symmetrical anhydride method. Boc-blocked α-amino groups were deprotected with 30% TFA in DCM according to the general method. Neutralization was performed with 10% TEA in DCM. Free peptides were cleaved from the support resin and deprotected by TFMSA, TFA, and *m*-cresol according to the standard method. Final purification was carried out by preparative HPLC on a C18 column. Purity and homogeneity of the free peptides were established by analytical HPLC, amino acid analysis, and optical activity. The physicochemical data of peptides (**1**–**13**) and (**14**–**25**) are presented in Tables 1 and 2.

H-Glp-Asp-Val-Val-His-Ser-Phe-Leu-Arg-Phe-NH$_2$ (1). The peptide was obtained by a stepwise elongation of the chain by the method outlined above starting from 0.68 g (2.56 mmol) of Boc-Phe-OH and 1 g MBHA•HCl-resin (substitution level of 0.86 mmol of NH_2/g resin). The following amino acids (three-fold excess) were coupled to the resin by the DCC method: Boc-Arg(Tos)-OH, Boc-Leu-OH, Boc-Phe-OH, Boc-Asp(Bzl)-OH, Boc-Ser(Bzl)-OH, Boc-His(π-Bom)-OH, and Z-Glp-OH. Boc-Val-OH and Boc-Ile-OH were introduced to the peptide chain by the symmetrical anhydride method. Then the resin was dried overnight under reduced pressure over KOH. The free peptide was obtained according to the following procedure: the peptide-resin was suspended in 0.5 ml *m*-cresol, 0.5 ml TFMSA, and 4 ml TFA. The whole mixture was kept at a room temperature for 2 h and triturated with ether (70 ml). The above reaction mixture gave a precipitate which was washed with ether, dried *in vacuo* over KOH, and dissolved in water. The aqueous solution was subsequently stirred with Amberlite CG-4B (acetate form) for 30 min, filtered, and lyophilized. The peptide was dissolved in a small volume of 5% acetic acid, filtered on a column of Sephadex G-25 according to [6] and then purified by the preparative HPLC. The main fractions were pooled and lyophilized giving 1.2 g of the peptide.

Peptides (**2**–**25**) were obtained in the same manner as peptide (**1**).

BIOLOGICAL INVESTIGATION

T. molitor L. adults were obtained from a culture maintained as described previously [7]. Peptides were dissolved in saline water to yield a stock solution of 1 mM and were stored at −30 °C. Working dilutions were made from the stock saline solution.

Peptides were assayed *in vitro* on a semi-isolated heart prepared according to Rosiński and Gade [8,9]. In brief, insects were decapitated and the abdomen was removed as close to the metathorax as possible. The ventral body wall of the abdomen was trimmed away so that lateral spiracular structures remained attached to the dorsal sclerites. The fat body, digestive organs, and Malpighian tubules were removed from the abdominal dorsum. The final preparation consisted of the dorsal vessel, (i.e. the heart), alary muscles, internal body muscles, the tracheae, and the dorsal cuticle. The heart preparations were selected on the basis of regularity of beating and then they were

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Table 1 Physicochemical data of peptides (**1–13**)

Peptide	Yield (%)	$[\alpha]_D^{20}$ methanol (%)	R_t (HPLC[a])	Amino acid analysis	T.L.C[b] R_f			MW	
					x	y	z	Calculated	Found
H-Glp-Asp-Val-Val-His-Ser-Phe-Leu-Arg-Phe-NH_2	77	−45.1 $c = 0.7$	24.82	Glp 1.02 Asp 0.97 Val 1.9 His 0.96 Ser 0.95 Phe 2.0 Leu1.0 Arg 0.99	0.38	0.62	0.43	1229.6	1229.9
H-Asp-Pro-Ser-Phe-Leu-Arg-Phe-NH_2	79	−37.4 $c = 0.7$	20.12	Asp1.0 Pro 0.99 Ser1.01 Phe1.97 Leu 0.95 Arg 1.02	0.42	0.65	0.5	920.5	921.8
H-Gly-Asn-Ser-Phe-Leu-Arg-Phe-NH_2	66	−38.4 $c = 0.7$	21.15	Gly 1.0 Asn 0.99 Ser 0.97 Phe 2.02 Leu 0.96 Arg 0.98	0.34	0.63	0.58	838.4	839.2
H-Asp-Val-Val-His-Ser-Phe-Leu-Arg-Phe-NH_2	70	−36.9 $c = 0.7$	22.02	Asp 0.97 Val 1.99 His 0.96 Ser 0.95 Phe 1.98 Leu 0.96 Arg 0.99	0.47	0.76	0.59	1117.6	1116.9
H-Val-Val-His-Ser-Phe-Leu-Arg-Phe-NH_2	63	−31.7 $c = 0.7$	23.59	Val 1.98 His 0.96 Ser 0.95 Phe 2.01 Leu 0.99 Arg 1.1	0.52	0.74	0.49	1002.5	1003.1
H-Val-His-Ser-Phe-Leu-Arg-Phe-NH_2	68	−24.5 $c = 0.5$	22.41	Val 1.0 His 0.96 Ser 0.95 Phe 1.99 Leu 0.95 Arg 0.97	0.74	0.78	0.59	903.5	904.2
H-His-Ser-Phe-Leu-Arg-Phe-NH_2	59	−26.9 $c = 0.5$	19.34	His 0.96 Ser 0.95 Phe 1.96 Leu 0.97 Arg 1.0	0.66	0.74	0.51	804.4	804.9
H-Ser-Phe-Leu-Arg-Phe-NH_2	65	−21.5 $c = 0.5$	21.29	Ser 1.0 Phe 1.9 Leu 1.1 Arg 1.0	0.57	0.82	0.61	667.4	668.1
H-Phe-Leu-Arg-Phe-NH_2	56	−14.6 $c = 0.5$	20.54	Phe 1.99 Leu 0.97 Arg 0.98	0.49	0.71	0.51	580.4	580.9
H-Leu-Arg-Phe-NH_2	86	7.9 $c = 0.9$	16.9	Phe 1.1 Leu 0.99 Arg 1.0	0.62	0.75	0.73	433.3	432.9
H-Asp-Pro-Ser-Phe-Leu-Arg-Phe(p-F)-NH_2	60	−24.6 $c = 0.5$	22.82	Asp 0.98 Pro 1.02 Ser 0.97 Phe 1.99 Leu 1.0 Arg 0.98	0.39	0.69	0.48	939.5	938.9
H-Asp-Pro-Ser-Phe-Leu-Arg-Phe(p-Cl)-NH_2	62	−27.1 $c = 0.5$	23.61	Asp 1.01 Pro 0.99 Ser 1.0 Phe 0.97 Leu 0.95 Arg 1.0	0.47	0.71	0.53	955.9	956.4
H-Asp-Pro-Ser-Phe-Leu-Arg-Phe(p-I)-NH_2	66	−23.2 $c = 0.5$	24.23	Asp 0.99 Pro 1.0 Ser 0.98 Phe 1.9 Leu 0.96 Arg 0.98	0.41	0.67	0.54	1047.4	1048.2

[a] HPLC Termo Separation Products ODS column (VYDAC C18) 4.6 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 : acetic acid ethyl acetate : water (2 : 1 : 1 : 1); $y = n$-butanol : pyridine : acetic acid : water (30 : 20 : 6 : 24); $z = n$-butanol : acetic acid : ethyl acetate : water (1 : 1 : 1 : 1).

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Table 2 Physicochemical data of peptides (**14 – 25**)

Peptide	Yield (%)	$[\alpha]_D^{20}$ methanol (%)	R_t (HPLC[a])	Amino acid analysis	T.L.C[b] R_f			MW	
					x	y	z	Calculated	Found
H-Asp-Ile-Leu-Arg-Gly-NH_2	74	−35.2 $c = 0.9$	14.79	Asp 1.0 Ile 0.99 Leu 1.01 Arg 1.0 Gly 0.98	0.31	0.58	0.49	571.4	571.7
H-Ile-Leu-Arg-Gly-NH_2	86	−34.4 $c = 0.9$	12.14	Ile 1.0 Leu 0.96 Arg 0.99 Gly 1.01	0.42	0.69	0.48	456.3	457.1
H-Leu-Arg-Gly-NH_2	66	−31.6 $c = 0.7$	14.52	Leu 0.99 Arg 1.01 Gly 0.95	0.38	0.57	0.44	343.2	343.9
H-Asp-Ile-Leu-Arg-NH_2	50	−38.6 $c = 0.9$	14.81	Asp 0.97 Ile 1.0 Leu 0.99 Arg 1.0	0.43	0.61	0.52	514.3	514.7
H-Asp-Ile-Leu-NH_2	52	−35.8 $c = 0.9$	15.87	Asp 0.97 Ile 1.02 Leu 1.0	0.29	0.64	0.49	358.2	357.8
H-Asn-Ile-Leu-Arg-Gly-NH_2	56	−34.8 $c = 0.9$	14.15	Asn 1.01 Asp 0.99 Ile 1.0 Leu 1.0 Arg 0.99 Gly 0.98	0.27	0.65	0.48	579.4	579.7
H-Glp-Ile-Leu-Arg-Gly-NH_2	66	−37.1 $c = 0.9$	15.12	Glp 1.0 Asp 0.99 Ile 0.98 Leu 1.0 Arg 0.97 Gly 0.96	0.35	0.59	0.46	568.4	567.9
H-Arg-Ile-Leu-Arg-Gly-NH_2	88	−26.2 $c = 0.5$	14.96	Arg 1.0 Asp 0.99 Ile 1.0 Leu 0.98 Arg 0.96 Gly 1.0	0.31	0.51	0.45	612.4	612.8
H-Gln-Ile-Leu-Arg-Gly-NH_2	56	−24.7 $c = 0.5$	15.38	Gln 0,99 Asp 1.0 Ile 0.98 Leu 1.02 Arg 0.97 Gly 1.0	0.28	0.63	0.37	584.4	583.9
H-Gly-Ile-Leu-Arg-Gly-NH_2	58	−38.4 $c = 0.9$	14.75	Gly 0.97 Asp 1.0 Ile 0.95 Leu 1.02 Arg 0.99 Gly 1.0	0.44	0.62	0.55	513.4	514.2
H-Lys-Ile-Leu-Arg-Gly-NH_2	60	−31.4 $c = 0.9$	14.91	Lys 1.0 Asp 0.99 Ile 0.98 Leu 1.0 Arg 1.01 Gly 0.99	0.49	0.37	0.62	584.4	585.1
H-Ala-Ile-Leu-Arg-Gly-NH_2	62	−44.8 $c = 0.9$	15.11	Ala 0.99 Asp 0.98 Ile 1.0 Leu 1.01Arg 0.99 Gly 0.96	0.47	0.67	0.52	527.4	527.9

[a] HPLC Termo Separation Products ODS column (VYDAC C18) 4.6 mm × 250 mm; gradient: 0–80% solvent B in 60 min (B = 80% acetonitrile in water + 0.1% TFA).
[b] TLC. on silica gel plates (Merck), eluents: $x = n$-butanol : acetic acid : ethyl acetate : water (2 : 1 : 1 : 1); $y = n$-butanol : pyridine : acetic acid : water (30 : 20 : 6 : 24); $z = n$-butanol : acetic acid : ethyl acetate : water (1 : 1 : 1 : 1).

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superfused in *Tenebrio* saline (274 mM NaCl, 19 mM KCl, 9 mM $CaCl_2$, 5 mM glucose, and 5 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), pH 7.0) [9]. The incubation chamber with the heart preparation was mounted on the stage of an Olympus SZX12 stereomicroscope equipped with a CCD camera (Camidia C-3030).

An open perfusion system was used, with an injection port (for peptides) 70 mm above the superfusion chamber. The heart was subjected to a constant perfusion with fresh saline at the rate of about 140 µl/ min. All tested samples were applied at the injection port with a Hamilton syringe. Many pulse applications of samples could be sequentially assayed in a single preparation. The open system was designed to enable samples to be added without causing a change in pressure. After the initial 15 min stabilization, the activity of the isolated heart was recorded for 2 min. Next the peptide was applied and the heart activity was recorded for 2 min more.

The video microscopy technique and computer-based method of data acquisition and analysis were used to determine heart contractions. We developed a semiautomated digital-processing method to determine heart contractions directly from video signals recorded for a preparation. Video recordings were captured directly to a 3.2 MHz Pentium IV-based microcomputer equipped with a Pinnacle Studio DeLux 2 capture card, at the sampling frequency of 25 frames/s, using the Pinnacle Studio Plus 9.3 software package. We developed the edge-tracing software (AnTracker) and employed it to create a trace of the movement of the side edge of the heart. Some examples of the records of the heart contractile activity are presented in Figure 1. The activities of tested peptides are presented as percentage changes in the control frequency of the heart contractions.

Table 3 Myotropic effect of Mas-MT analogs on the heart of *T. molitor* relative to proctolin

Peptide	Effect on the heart of *T. molitor* relative to proctolin (%) (±SEM 7–10) at concentration 10^{-8}M
Proctolin	100
1	30
2	21
3	27
4	nd
5	−20
6	−60
7	nd
8	46
9	31
10	1
11	nd
12	nd
13	nd

nd,not defined.

Table 4 Myotropic effect of Any-GS analogs on the heart of *T. molitor* relative to proctolin and Any-GS

Peptide	Effect on the heart of *T. molitor* relative to proctolin (%) (±SEM 7–10) at concentration 10^{-8}M
Proctolin	100
Any-GS	−100
15	−14
16	11
17	−67
18	nd
19	7
20	5
21	2
22	−102
23	−117
24	−58
25	−35

nd,not defined.

RESULTS AND DISCUSSION

The biological effects of studied compounds at the 10^{-8} M concentration are assembled in Tables 3 and 4. In Figures 1 and 2, the biological investigations of the heartbeat frequency in relationship to the concentration are presented.

As we can see in Table 3, Mas-MT-I (**1**) and the six analogs (**1–4, 8**, and **9**) preserved the stimulatory heartbeat frequency (from 20 to 46%) relative to proctolin itself. This weak stimulatory effect is probably the result of species specificity of the myotropin originated in *M. sexta* in respect to heart of *T. molitor*. In our studies on myotropin from *M. sexta* we found that two other analogs of Mas-MT-I, [3–10]- (**5**) and [4–10]-MTS-I (**6**), showed the inhibitory activity in *T. molitor*. Especially strong inhibition of the heartbeat frequency was shown by [4–10]-Mas-MT-I analogs (Table 3) without the *N*-terminal tripeptide: Glp-Asp-Ala. Basing on the above, we can postulate that a shortened sequence at the *N*-end, like in peptides without Glp-Asp and Glp-As-Val fragments, is enough for a change of the stimulatory effect for the inhibition. These results need a further separate investigation that is in progress.

Interesting results in the cardiotropic test on *T. molitor* were observed in the case of the second group of peptides originating from insects (Table 4). Any-GS (**14**) and its six analogs (**15, 18**, and **21–25**)

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show a cardioinhibitory effect of 14–102% relative to proctolin. Especially active were Any-GS, [Gln^1]-, [Gly^1]- [Lys^1]-Any-GS, and the fragment of [1–3]-Any-GS (Table 4). Other peptides were practically inactive. From the analysis of Figure 2 one can also observe that for peptide (**20**) the effects depended on its concentration. Any-GS and its analogs, in which the *N*-terminal Asp residue was exchanged by Gln, Gly, Lys, or Ala, preserved the cardioinhibitory properties similar to native Any-GS. It is interesting that a tripeptide fragment of this peptide inhibited the heartbeat as well. It indicates that the structure of the peptide is not as conservative as in myotropin from *M. sexta.*

CONCLUSIONS

In accordance to the above data, we can postulate that the peptides with the inhibitory heartbeat frequency could be good candidates for proctolin antagonists.

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