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Structure–activity relationships for the cardiotropic action of the Led-NPF-I peptide in the beetles *Tenebrio molitor* and *Zophobas atratus*

PAWEŁ MARCINIAK,[a] SEBASTIAN GRODECKI,[b] DANUTA KONOPIŃSKA[b] and GRZEGORZ ROSIŃSKI[a]*

[a] Department of Animal Physiology and Development, Poznan University, Umultowska 89, 61-614 Poznan, Poland
[b] Institute of Chemistry, Wroclaw University, Joliot-Curie 14, 50-383 Wroclaw, Poland

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Abstract: We have examined the effects of the Led-NPF-I peptide (Ala-Arg-Gly-Pro-Gln-Leu-Arg-Leu-Arg-Phe-amide) and a series of ten analogues on the heart contractile activity of *Tenebrio molitor* and *Zophobas atratus*, and the structure–activity relationships for cardioactive action of Led-NPF-I were established. A video microscopy technique and computer-based method of data acquisition and analysis were used to study the action of the peptides on continuously perfused heart preparations. Cardiac activity was progressively inhibited by Led-NPF-I when the peptide concentrations were increased from 10^{-9} to 10^{-5} M. Substitution of the L-proline residue at position 4 of the native peptide with hydroxyproline, valine or D-proline caused a loss of cardioinhibitory activity. Also, replacement of arginine residues at all three positions 2, 7 and 9 with another basic amino acid histidine, reduces cardioinhibitory action of Led-NPF-I. Some modifications of the *C*-terminal residues, as the Phe(4-NO_2)-, Phe(4-NH_2)- and Phe(4-NMe_2)-analogues, resulted in agonistic peptides with biological activity similar to that of the native peptide. However, three other *C*-terminal analogues tested [Tyr^{10}]-, [D-Phe^{10}]-Led-NPF-I, and Ala-Arg-Gly-Pro-Gln-Leu-Arg-Leu-Arg-Phe-OH were inactive in the heart bioassay, which suggests that this end of the amino acid chain may play an important role in bioactivity and interaction of the native peptide with its receptor on the myocardium. Copyright © 2007 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: heart contractile activity; short NPFs; Led-NPF-I; cardioinhibitory activity; structure–activity relationships; *Tenebrio molitor*; *Zophobas atratus*

INTRODUCTION

In insects, the short peptide hormones related to neuropeptide F [short neuropeptide Fs (sNPFs)] are a growing group of structurally related peptides; these have been identified in tissue extracts of a variety of insect species. The first members of this peptide family (Aea-NPF-I, Aea-NPF-II) were isolated from the head extract of the mosquito *Aedes aegypti* [1]. Subsequently two peptides, Led-NPF-I and Led-NPF-II were isolated from Colorado potato beetle's (*Leptinotarsa decemlineata*) brain [2] and further bioanalogues were identified also in *Schistocerca gregaria* [3], *Drosophila melanogaster* [4], *Helicoverpa zea* [5], *Periplaneta americana* [6] and *Anopheles gambiae* [7]. In contrast, sNPFs were absent from nervous system extracts of diapausing *L. decemlineata*, but present in those of nondiapausing beetles which suggested that these peptides exert a role in the adult diapause [8]. All the sNPFs identified have a typical *C*-terminal R/K-X_1-R-X_2amide motif, where the first amino acid of this motif is always a basic amino acid residue such as Arg or Lys. X_1 can be Leu, Thr or Pro and X_2 is always an aromatic amino acid residue such as Phe or Trp.

Studies focusing on a few insect species implicate sNPFs in physiological processes related to reproduction and feeding. Injection of the heterologous Led-NPF-I peptide into female migratory locusts, *Locusta migratoria*, over several days stimulates ovarian development [9]. Also the *Schistocerca* NPF-related isoform, or Scg-NPF, clearly accelerates ovarian maturation in the desert locust, *S. gregaria* [3]. However, Led-NPF-I appeared to have no gonadostimulatory effect in the grey flesh fly, *Neobellieria bullata* [9]. Recent findings, which show an increase in the volume of fat body tissue of the locust after Led-NPF-I injection, suggest that the peptide may increase food intake [8]. In *D. melanogaster*, manipulation of the sNPF gene affects food consumption in both larvae and adult insects [10]. Gain-of-function sNPF mutants display increased food intake, resulting in flies larger than the wild type; whereas loss-of-function sNPF mutants exhibit reduced food intake [10]. A sNPF G-protein coupled receptor has been cloned and characterized from *D. melanogaster* [11] and *A. gambiae* [12]. In *D. melanogaster*, this receptor occurs in larval gut, fat body, brain, Malpighian tubules and ovaries of adult flies [11]. Short *Drosophila* NPFs and heterologous peptides Led-NPF-I and to a

*Correspondence to: Grzegorz Rosiński, Department of Animal Physiology and Development, Poznan University, 89, Umultowska Street, 61-614 Poznan, Poland; e-mail: rosin@amu.edu.pl

lesser extent Led-NPF-II were able to activate the receptor [13]. Because the sNPF peptides were expressed during all developmental stages and were localized in neurosecretory structures of *D. melanogaster* adult brain [10], they may be multifunctional neurohormonal regulators in insects.

Thus far, the physiological roles of sNPFs in regulation of muscle contractile activity of gut and other visceral organs in insects remain largely unknown. Onken *et al.* observed in a specific *in vitro* assay that Aea-NPF-2 peptide inhibits peristalsis of the isolated anterior midgut of larval *Ae. aegypti* [14]. Recently we reported a cardioinhibitory action of two *Leptinotarsa* NPFs, Led-NPF-I and Led-NPF-II in *Tenebrio molitor* and *Zophobas atratus* [15]. The mechanisms underlying the action of these peptides on the heart contractile activity are not well understood. In the present article we have studied cardioinhibitory activity of Led-NPF-I and a series of analogues as a screen for major inhibitory effects, and in order to gain insight into its structure–activity relationships. We have analyzed the roles of five residues (Arg^2, Pro^4, Arg^7, Arg^9 and Phe^{10}) of the Led-NPF-I molecule in cardioinhibitory bioactivity.

MATERIALS AND METHODS

Insects

T. molitor L. adults were obtained from a culture maintained as described previously [16]. *Z. atratus* was reared according to the Quennedy procedure [17].

Peptides

Led-NPF-I peptide and Led-NPF-I analogues were synthesized by the classical solid-phase method according to the Boc procedure. As a coupling reagent, dicyclohexylcarbodiimide (DCC) or HBTU, both in the presence of HOBt, were used. The Boc protecting group was removed with 30% CF_3COOH in CH_2Cl_2. The side-chain residues of arginines were protected by Tos (tosyl) group. The Tos protecting groups were removed and peptides were released from the resin using CF_3SO_3H. All peptides were purified finally by preparative HPLC on a C-18 column (Ultrasphere ODS Beckman). The following analogues were used in bioassay: [Hyp^4]- (**I**), [D-Pro^4]- (**II**), [Val^4]- (**III**), [His^2, His^7, His^9]- (**IV**), [Tyr^{10}]- (**V**), [D-Phe^{10}]- (**VI**), [Phe(4-NO_2)^{10}]- (**VII**), [Phe(4-NH_2)^{10}]- (**VIII**), [Phe(4-NMe_2)^{10}]-Led-NPF-I (**IX**) and Ala-Arg-Gly-Pro-Gln-Leu-Arg-Leu-Arg-Phe-OH peptide (**X**). The physicochemical data of the peptides are summarized in Table 1. Peptides were dissolved in saline to yield a stock solution of 1 mM and were stored at −30 °C. Working dilutions were made from the stock solution in saline.

Heart Bioassay

Peptides were assayed *in vitro* in a semi-isolated heart prepared according to Rosinski and Gade [18]. 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 superfused in *Tenebrio* saline (274 mM NaCl, 19 mM KCl, 9 mM CaCl, 5 mM glucose, and 5 mM HEPES, pH 7.0) [19]. 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 a further 2 min.

A 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 in 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 a sampling frequency of 25 frames per s, using the Pinnacle Studio Plus 9.3 software package. We developed the edge-tracing software (AnTracker) and employed of 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.

RESULTS AND DISCUSSION

The heart rhythm of *T. molitor* and *Z. atratus* remained regular during superfusion with physiological saline for 5 h and showed on an average 72 ± 5 and 30 ± 4 beats per min, respectively. In both species, the application of Led-NPF-I caused fast, dose-dependent and reversible decrease of the heart contractile activity; a stronger effect of this peptide was marked in cardiac arrest of the activity in diastolic phase of the contraction cycle (Figures 1 and 2). A comparison of dose-dependent curves indicates species-specific action of the peptide (Figure 2). More sensitive for cardioinhibitory action of Led-NPF-I is the heart of *Zophobas*. Possibly, the peptide is structurally more related to a native sNPF-peptide of *Z. atratus*. The cardioinhibitory factors were isolated from the corpus cardiacum – corpus allatum complex of *T. molitor* [18] and *Z. atratus* [20] but their structures have not been elucidated. Led-NPF-I decreased the frequency of the heart contractions with a threshold for observable effect between $10^{-9} - 10^{-7}$ M

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Table 1 Physicochemical data of the Led-NPF-I analogues

Peptide	Yield (%)	$[\alpha]^{20}c = 0.97\%$ CH_3OH	HPLC Rt[a]	Amino acid analysis	MW		TLC[b] Rf		
					Calc.	Found	X	Y	Z
Led-NPF-I	74.4	−21.7	28.6	Ala 1.0 Arg 3.1 Gly 1.04 Pro 1.1 Gln 0.97 Leu 2.1 Phe 0.97	1212.4	1212.2	0.11	0.51	0.06
[Val^4]-Led-NPF-I	58.5	−17.0	19.2	Ala 0.98 Arg 3.1 Gly 0.98 Gln 0.99 Leu 2.0 Phe 1.1	1227.4	1227.8	0.10	0.48	0.04
[Hyp^4]-Led-NPF-I	66.9	−9.5	20.3	Ala 1.0 Arg 2.9 Gly 0.99 Pro 1.0 Gln 0.98 Leu 1.99 Phe 0.99	1212.4	1211.8	0.11	0.48	0.03
[D-Pro^4]-Led-NPF-I	65.3	−15.5	27.9	Ala 1.0 Arg 2.95 Gly 0.99 Val 1.0 Gln 0.99 Leu 2.1 Phe 0.95	1214.4	1213.8	0.09	0.54	0.08
[His^2, His^7, His^9]-Led-NPF-I	64.2	−14.9	28.1	Ala 1.0 His 3.1 Gly 0.99 Pro 1.0 Gln 0.95 Leu 1.99 Phe 1.05	1155.3	1154.6	0.12	0.52	0.10
[Tyr^{10}]-Led-NPF-I	68.6	−19.9	23.2	Ala 1.0 Arg 2.9 Gly 1.08 Pro 1.1 Gln 0.99 Leu 1.99 Tyr 1.01	1228.4	1227.3	0.11	0.39	0.12
[D-Phe^{10}]-Led-NPF-I	79.4	−18.8	25.6	Ala 1.1 Arg 3.0 Gly 0.99 Pro 0.9 Gln 0.99 Leu 2.0 Phe 1.08	1212.4	1213.8	0.10	0.49	0.08
[Phe(4-NO_2)^{10}]-Led-NPF-I	63.1	−18.8	26.8	Ala 1.1 Arg 3.0 Gly 0.94 Pro 1.1 Gln 1.1 Leu 2.05	1257.5	1256.8	0.09	0.42	0.13
[Phe(4-NH_2)^{10}]-Led-NPF-I	58.2	−18.2	27.1	Ala 0.98 Arg 2.99 Gly 1.05 Pro 1.0 Gln 1.1 Leu 2.0	1227.5	1226.7	0.13	0.38	0.56
[Phe(4-NMe_2)^{10}]-Led-NPF-I	56.5	−19.1	27.5	Ala 1.05 Arg 3.0 Gly 0.98 Pro 1.0 Gln 1.1 Leu 1.99	1255.7	1256.7	0.09	0.46	0.10
H-Ala-Arg-Gly-Pro-Gln-Leu-Arg-Leu-Arg-Phe-OH	56.7	−23.0	21.1	Ala 1.0 Arg 3.1 Gly 1.04 Pro 1.1 Gln 0.97 Leu 2.1 Phe 0.97	1213.4	1213.8	0.12	0.41	0.06

[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] 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).

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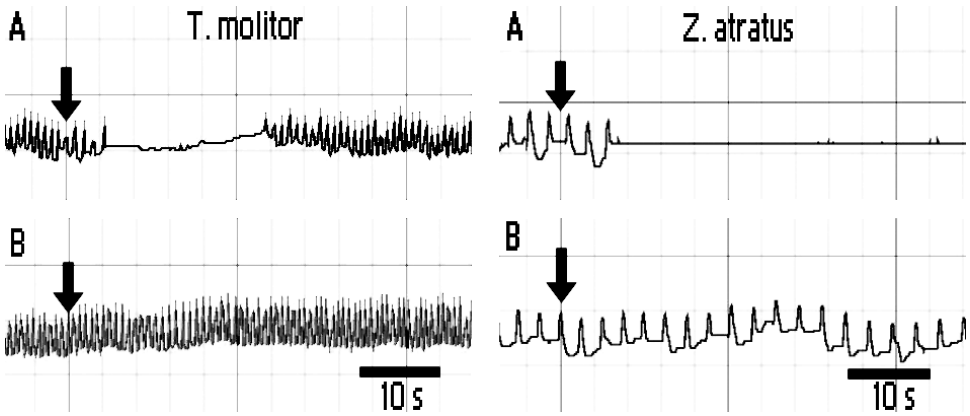

Figure 1 Myograms displaying typical responses on the spontaneous activity of the *T. molitor* and *Z. atratus* heart to 0.8×10^{-5} M Led-NPF-I (A) and 0.8×10^{-5} M Ala-Arg-Gly-Pro-Gln-Leu-Arg-Leu-Arg-Phe-OH peptide (B). Peptide application is indicated by an arrow.

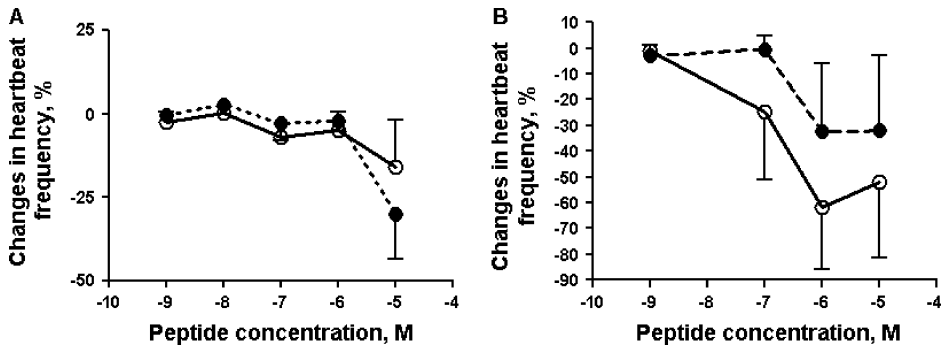

Figure 2 Dose–response curves for the effect of the Led-NPF-I (○) and [Phe(4-NMe_2)^{10}]-Led-NPF-I (●) on the frequency contraction of the *T. molitor* (A) and *Z. atratus* (B) hearts. Mean ± SEM are given from at least three determinations.

for *Zophobas*, and at about 10^{-7} M for *Tenebrio*. At lower range concentrations ($10^{-9} - 10^{-7}$ M) Led-NPF-I decreased progressively the heart frequency contractions (a negative chronotropic effect) as the peptide concentration increased but cardiac arrest was not observed in any preparation. At the two highest concentrations of 10^{-6} and 10^{-5} M the peptide induced cardiac arrest in both preparations, however, the effect in *Zophobas* was stronger than in *Tenebrio* (Figures 1 and 3). In all cases, cardiac arrest was followed by the resumption of contractility. In *Zophobas* preparations applied with the 10^{-5} M Led-NPF-I solution contractions resumed after a period of cardiac arrest lasting an average of 59 ± 10 s.

The cardioinhibitory effects of Led-NPF-I in *T. molitor* and *Z. atratus* are similar to the action of the second *Leptinotarsa* Led-NPF-II neuropeptide and to the peptide leucomyosuppressin (Lem-MS) from the cockroach *Leucophaea maderae* [15,21]; however, Led-NPF-I is a less potent cardioinhibitor than Lem-MS. In both beetles, Lem-MS induces a threshold decrease in the heart contractile activity at concentrations of about $10^{-10} - 10^{-7}$ M. The sequence similarity of the *Leptionotarsa* peptides, Led-NPF-I (Ala-Arg-Gly-Pro-Gln-Leu-Arg-**Leu-Arg-Phe-amide**) and Led-NPF-II (Ala-Pro-Ser-Leu-Arg-**Leu-Arg-Phe-amide**) and cockroach Lem-MS (pGln-Asp-Val-Asp-His-Val-Phe-**Leu-Arg-Phe-amide**) is evident. The *C*-terminal Leu-Arg-Phe-amide fragment of both *Leptinotarsa* peptides is identical with that of Lem-MS. In previous studies [15] we observed that *C*-terminal pentapeptide fragment Leu-Arg-Leu-Arg-Phe-amide of Led-NPF-I, which is also common for the second *Leptinotarsa* peptide Led-NPF-II, is essential for cardioinhibitory activity of the peptide in *T. molitor* and *Z. atratus*. In *D. melanogaster*, Martens *et al.* [11] showed that a sNPF G-coupled receptor is preferentially activated by sNPFs. Other peptides, even FMRFamide-related peptides, and those with an Arg-Phe-amide carboxyterminus were not able to elicit a calcium response in receptor expressing mammalian Chinese hamster ovary cells. Garczynski *et al.* [22] found, that the sNPFs of *D. melanogaster* differ in their interactions with the sNPF receptor Drm-NPFR76R, as analyzed directly by radioreceptor assay. Binding assays revealed that longer Drm-sNPF-2 (Arg-Ser-Pro-Ser-Leu-Arg-Leu-Arg-Phe-amide) comprising of nine amino acids was clearly more potent than shorter

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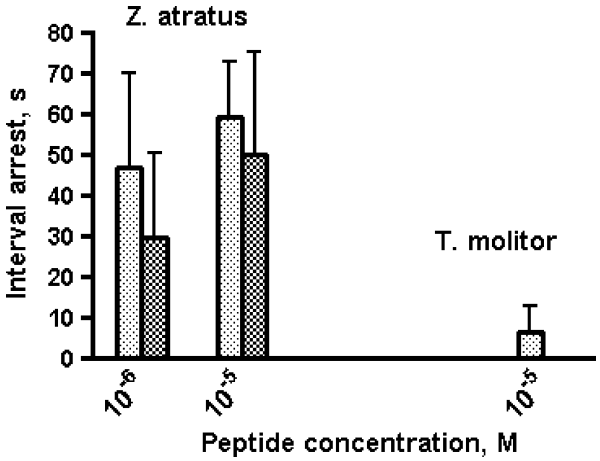

Figure 3 Intervals of cardiac arrest after application of the Led-NPF-I and [Phe(4-NMe_2)^{10}]-Led-NPF-I on the hearts of the two beetles. Mean ± SEM are given from at least three determinations.

Table 2 Cardioinhibitory activity of Led-NPF-I analogues at 10^{-5} M concentration in the *Tenebrio* heart bioassay

Analogue	Changes in heartbeat frequency (%)
[Val^4]-Led-NPF-I	3 ± 1
[Hyp^4]-Led-NPF-I	−7 ± 2
[D-Pro^4]-Led-NPF-I	−1 ± 2
[His^2, His^7, His^9]-Led-NPF-I	4 ± 2
[Tyr^{10}]-Led-NPF-I	−1 ± 2
[D-Phe^{10}]-Led-NPF-I	−3 ± 1
[Phe(4-NO_2)^{10}]-Led-NPF-I	−15 ± 5
[Phe(4-NH_2)^{10}]-Led-NPF-I	−14 ± 8
[Phe(4-NMe_2)^{10}]-Led-NPF-I	−30 ± 14
Ala-Arg-Gly-Pro-Gln-Leu-Arg-Leu-Arg-Phe-OH	4 ± 10

Mean values ± SEM are given from at least three determinations.

ones of eight (Drm-sNPF-1; Ser-Pro-Ser-Leu-Arg-Leu-Arg-Phe-amide) or six amino acids (Drm-sNPF-4; Pro-Gln-Arg-Leu-Arg-Trp-amide). It is consistent with the pattern of structural activity found for binding of *A. gambiae* sNPF peptides to Ang-sNPF receptor [12].

In this study we have evaluated the role of the selected amino acid residues (Arg^2, Pro^4, Arg^7, Arg^9 and Phe^{10}) in the Led-NPF-I molecule for its cardioactivity. Two of position-4 modified analogues of Led-NPF-I with hydroxyproline (**I**) or D-proline (**II**) replacements were inactive when applied at a concentration of 10^{-5} M in the *Tenebrio* heart bioassay (Table 2). Also, replacement of a proline residue at this position with hydrophobic valine, which has the aliphatic side chain resulted in an inactive analogue (**III**). It is possible that the Pro^4 residue plays a role in stabilization of conformation of the native peptide. Substitution all of three basic residues of arginine at positions 2, 7 and 9 in the Led-NPF-I molecule with histidine caused a loss of cardioinhibitory bioactivity of analogue (**IV**) on the *Tenebrio* preparation. In *D. melanogaster*, Martens *et al.* [11] suggested that the Arg residue at position 4 from the amidated *C*-terminus, which is typical for the sNPFs, determined a sNPF G-coupled receptor specificity. They observed that an aromatic residues, such as Tyr or Phe, in this position, which is typical for the FMRFamide-related peptides, may prevent the peptide from receptor binding and hence from eliciting a response.

The structure–activity studies revealed that in addition to Arg^2-, Pro^4-, Arg^7- and Arg^9-residues in the primary structure of the amino acid chain of the Led-NPF-I molecule, *C*-terminal amino acid amidation is essential for its cardioinhibitory activity (Table 2). Removal of the amide, as in Ala-Arg-Gly-Pro-Gln-Leu-Arg-Leu-Arg-Phe-OH, abolishes the inhibitory activity of the peptide (**X**). Inactive analogues were also obtained when *C*-terminal hydrophobic phenylalanine in the native peptide was replaced with a more polar tyrosine (**V**) or D-phenylalanine isomer (**VI**).

Interestingly, only three of peptides with a *C*-terminal modified phenylalanine residue (**VII, VIII** and **IX**) retained cardioinhibitory activity. Analogues differing in the substituent (NO_2, NH_2, NMe_2) located at the *para*-position of the aromatic ring of phenylalanine showed agonistic activity but they did not cause cardiac arrest in the *Tenebrio* preparation like the native peptide did. The [Phe(4-NMe_2)^{10}]-Led-NPF-I analogue induced dose-response cardioinhibitory effects similar to that of the native peptide on the heart preparations of both beetles, although the analogue in *Zophobas* was slightly less potent than that of Led-NPF-I (Figure 1). Data obtained for the four analogues tested (**V, VII, VIII** and **IX**) indicate that the bioactivity of the peptide can be maintained when three substituents NO_2, NH_2, NMe_2 are introduced in the *para*-position of the aromatic ring of *C*-terminal phenylalanine but the peptide is inactive after replacing Phe residue with aromatic Tyr, indicating a sensitivity to the introduction of a phenolic OH group.

In conclusion, it is clear from this study that five amino acid residues (Arg^2, Pro^4, Arg^7, Arg^9 and Phe^{10}) in the Led-NPF-I molecule are essential for cardioinhibitory bioactivity of the peptide on the *Tenebrio* and *Zophobas* hearts. This structure–activity relationship data can aid in the design of agonist/antagonist analogues of this cardioactive peptide, as tools for insect endocrinologists.

The question is whether the action of Led-NPF-I on the hearts of the two insects studied here is physiologically relevant. The inhibitory action of the peptide on this organ may constitute a complex mechanism to decrease the circulation in the abdominal cavity. Further studies on the peptide action on the insect heart contractions *in vivo* are needed.

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