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The synthesis of an analogue of the locust CRF-like diuretic peptide, and the biological activities of this and some C-terminal fragments.

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The synthesis of an analogue of the locust CRF-like diuretic peptide, and the biological activities of this and some C-terminal fragments.

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Abstract

The synthesis is described of an analogue of the locust CRF-like diuretic peptide in which methionine in positions 1,3, and 13 is replaced by isosteric methyl homoserine residues. This analogue has been tested for biological activity on Malpighian tubules *in vitro*, and feeding behaviour *in vivo*. It is highly active in stimulating fluid secretion and accumulation of cAMP in tubules, and on increasing the latency to feed and reducing meal duration. A 15 residue fragment from the C-terminus of the CRF-like peptide, *Locmi*-DP_{32-46}, is fully active in the feeding assay, but has only weak ability to stimulate the accumulation of cAMP in tubules. Two smaller fragments, *Locmi*-DP_{32-37} and *Locmi*-DP_{41-46}, were tested but neither had consistent biological activity in any of the assays used here. None of the peptides tested have any substantive activity in increasing cGMP in tubules.

Keywords: Locust; Diuretic hormone; CRF-like peptide; cAMP; Feeding behaviour; Fluid secretion; Malpighian tubules; Methionine replacement

1. Introduction

In recent years, a number of diuretic hormones have been identified in insects. Although 5-hydroxytryptamine may be the major diuretic hormone in *Rhodnius* [6], the majority of insect diuretic hormones are peptides and fall into two major categories: a family of peptides that shows partial sequence identity with vertebrate corticotropin releasing factor (CRF); and a family of shorter kinins [7]. The release of the CRF-related diuretic peptide during feeding in locusts is well-established (1,2), and this study is part of an ongoing investigation of the role of the CRF-related diuretic peptide as an endogenous regulator of feeding activity in locusts [11].

One of the problems that has been encountered in studying the biology of CRF-like peptides has been a lack of stability during storage of the purified natural peptides isolated from insects. With this in mind, the synthesis was undertaken of an analogue of *Locmi-DP* in which methionine residues at positions 1, 3 and 13 were replaced by the isosteric amino acid methyl-homoserine, resulting in a product that it was hoped would retain biological activity and be less susceptible to oxidation. This methyl-homoserine analogue was tested in a range of bioassays designed to determine its activity on the Malpighian tubules, and on feeding behaviour. Because preliminary experiments suggested that a peptide with a C-terminally derived sequence from *Locmi*-DP (Cys^{31}-*Locmi*-DP_{31-46}, used in preparing antibodies against *Locmi*-DP) was active on some aspects of feeding behaviour (unpublished observations),

some short fragments from the C-terminal sequence of the natural peptide were also tested for biological activity.

2. Materials and Methods

2.1 Locusts

Locusta migratoria migratorioides (R & F) were reared in Birkbeck College at 30°C in a LD 12:12 h photocycle. They were fed with fresh grass and wheat seedlings, supplemented with dried bran flakes. To obtain *Locusta* nymphs of a known age for the behavioural assays, fourth instar nymphs were separated from the main stock and newly moulted fifth instars removed on a daily basis so that their ages were known. For measurement of fluid secretion or second messenger studies on Malpighian tubules, mature adult male *Schistocerca gregaria L.* were purchased from Peregrine Livefoods (Essex, UK).

2.2 Preparation of extracts of corpora cardiaca and of purified natural Locmi-DP

Corpora cardiaca were dissected from mature adult locusts and used immediately, or frozen in liquid nitrogen stored at -20°C. In some trials, freshly made homogenates of corpora cardiaca were made in a simple saline (7.5g NaCl, 0.375g KCl per litre). Otherwise, corpora cardiaca were extracted in acidic methanol (87% methanol, 5% glacial acetic acid) and *Locmi*-DP isolated as described previously [12] except that the diuretic peptide was detected in HPLC fractions by the use of a non-competitive ELISA using commercially produced antiserum (Sigma Genosys, Cambridge, UK) against Cys^{31}-*Locmi*-DP_{31-46} coupled to keyhole limpet haemocyanin.

2.3 Behavioural assay

Five-day-old nymphs of *Locusta* were separated from their food in the morning and kept without food for 4-5 h. They were then injected between tergites four and five, with 10 µl of one of the test solutions or the saline control. The nymphs were placed individually in 17 x 12 x 4 cm clear plastic boxes that contained an aluminium perch and some freshly cut leaves from wheat seedlings. After 10 sec, the behaviour of the locusts was recorded at intervals of 30 sec. The following categories of behaviour were recorded: feeding (which included both ingestion and chewing), contacting food (contact of wheat with at least one fore or mid tarsus orlabial or maxillary palp), resting (no ingestion or chewing) and locomoting [23]. The data were analysed to calculate the latency to the time insects took a meal, and the duration of the meal. A period of feeding of >2 min was defined as a meal and the end of a meal was defined by a period of >2 min of not feeding. The intervals used to define the start and end of a meal were based on log-survivorship analyses of feeding and non-feeding periods [21].

2.4 Radioimmunoassays of cyclic AMP and GMP

Radioiodinated cyclic nucleotides, cAMP or cGMP, were prepared using 300 pmol of 2'O- methyl ester of each cyclic nucleotide (Sigma) and 18.5 MBq [^{125}I] NaI (Amersham), using Chloramine T [25] and were separated [^{125}I] on a C18 Sep-Pak (Waters) with 40 % isopropanol as eluant. Estimated specific activity was approximately 55.5 TBq (=1500 Ci/mmol), and aliquots were stored –20 ^{o}C (usable for about 10-12 weeks after iodination). To determine the effect of methyl-homoserine-*Locmi*-DP on cAMP production,

the Malpighian tubules (5-6/ tube) of *S. gregaria* were incubated for 30 min at room temperature with 100 µl of locust saline (140mM NaCl; 10mM KCl; 4mM $CaCl_2$; 6mM NaH_2PO4; 4mM $NaHCO_3$; Sucrose 90mM; and pH 6.8) containing an appropriate concentration of peptide (or for controls, saline alone, and extracts of corpora cardiaca for use as a positive control), in the presence of 1 mM of 3-isobuty-1-lmethylxanthine (IBMX, Sigma). At the end of incubation, the tissues were sonicated for 10 sec (MSC, UK) and centrifuged at 14000 rpm for 10 min at 4^{o}C. The whole of the supernatant was taken into a tube containing 900 µl of 0.1 M acetate buffer (pH 4.75) and acetylated [5]. Aliquots (50 µl) of acetylated standard or unknown were added to tubes containing [^{125}I] cAMP (25,000dpm /25 µl) and 25 µl of antiserum (a generous gift from Dr. J. de Vente, Maastricht, and used at a final dilution 1:7000) to give 150 µl as a final volume, and incubated overnight at 4 ^{o}C. The separation of bound from unbound cyclic nucleotide was carried out using secondary donkey anti-rabbit IgG (Sal-Cel, Immunodiagnostic Services) as described previously [25], and estimated using a gamma counter (Wallac 1270). The results were expressed as fmol of either cAMP or cGMP/ tube.

2.5 Fluid secretion assay

Malpighian tubules were dissected from adult *S. gregaria* and freed from trachea in a locust saline (168 mM NaCl, 6.4 mM KCl, 3.6 mM $MgCl_2$, 6 mM NaH_2PO_4, 2.1 mM $NaHCO_3$, 2.1 mM $CaCl_2$) to which medium 199 (GIBCO) had been added to a final concentration of 5% (v/v). Each tubule was then transferred into 20 µl of fresh medium and this was covered with water-saturated paraffin oil on a Sylgard® plate. One end of a tubule was pulled out of bathing medium and inserted into the right hand side of the Sylgard block, while the other end was inserted into the left hand side. A nick was then made by a pair of sharp forceps on the part of a tubule situated between the bathing medium and the right side of block. The fluid droplet formed in the first 20 min was discarded. The fluid droplet secreted in the subsequent 20min was put on one side and used later to determine the initial basal level. Then, 10µl of medium were removed from the bathing medium and replaced either by fresh saline or saline containing test material, and a fluid secretion droplet was collected for another 20 min. At end of the incubation, the diameters of the droplets were measured to calculate their volumes. Percentage changes in fluid secretion were calculated by dividing the volume of the second droplet by that of the first.

2.6 Statistics

Statistical significance of results was estimated at $P < 0.05$ using STATVIEW (ANOVA). Curves of dose response data were fitted using SigmaPlot 5 (SPCC Inc.). Feeding behaviour was analysed using the non-parametric Mann-Whitney U test (SPSS)

2.7 Peptide synthesis

Purity of the synthesized peptides was estimated by reverse phase high performance liquid chromatography (RP-HPLC) and retention times (t_R) were measured on Nucleosil C_{18} column 250 X 4.6mm with the following solvent systems and conditions: (S1) linear gradient from 20-100% acetonitrile containing 0.05% TFA over 30 min., (S2) linear gradient from 10-60% acetonitrile containing 0.05% TFA over 30 min., (S3) linear gradient from 5-35% acetonitrile containing 0.05% TFA over 30 min. UV detection at 214 nm, flow rate 1 ml/min. Purification of peptides was performed on a RP-HPLC Nucleosil C_{18} column 250 X 10 mm

with the following solvent system and conditions: linear gradient from 20-100% acetonitrile containing 0.05% TFA over 70 min; UV detection at 225 nm and a flow rate of 3 ml/min.

The strategy for the synthesis of [Hse(CH_3)^1, Hse(CH_3)^3, Hse(CH_3)^{13}]-*Locmi*-DP, **2**, involved the synthesis of protected peptide acid fragments (Fig. 2) in the solid phase and their successive coupling to the C-terminal octapeptide of *Locmi*-DP attached to the Rink resin (Fig. 2). Protected peptide acids were synthesized in the solid phase and on the 2-chlorotriyl-resin, OCLTR [3] using the Fmoc/t-Bu strategy. Fmoc-AA-OCLTR (1g, 0.5 mmol Fmoc-AA-OH/g resin) was used for the solid phase synthesis of the peptide acids **6-10** and **12** (Fig. 2) and peptide chain elongation was performed with a procedure described previously [17,27]. The purity of the crude peptide acids was 88-92%, as estimated by HPLC.

The C-terminal protected octapeptide **11** and hexapeptide **13** were synthesized on the Rink-2-chlorotrityl-resin [4] starting from Fmoc-Ile-Rink-OCTLR (1g, 0.13 mmol Fmoc-Ile-OH/g of resin) and using exactly the protocol described previously [17,27].

The protected analogue of *Locmi*-DP, **2**, was synthesized by successive coupling of the protected peptide acids **10**, **9**, **8**, **7** and **6** to **11**, using the protocol described for the protected peptide acids except that the benzotriazolylester of the peptides **6-10** was prepared as follows: the protected peptide acid (0.26 mmol) and HOBt (0.42 mmol) were dissolved in DMF (4 ml) at 0 ^oC and then DIC (0.29 mmol) was added and the solution containing the corresponding benzotriazolylester was poured into the solid phase reactor containing 0.13 mmol of the amino component attached to the Rink linker-OCLTR, and the resultant suspension of the resin left to stand at room temperature for 24h. Completion of the acylation reaction was indicated by a negative Kaiser test. The final product **2** was obtained in two stages. In the first stage the protected peptide resin, after removing the Fmoc group, was stirred in 30 ml of TFE/DCM (3:7 v/v) for 3.5 h. Then the resin was filtered and washed three times with 10 ml of the above mixture. The combined filtrates were concentrated *in vacuo* and the residue solidified by addition of dry ether to yield the corresponding side chain protected peptide attached to the Rink linker. In the second stage, the linker and the side chain protecting groups were removed by treating the above peptide-Rink linker with 20 ml of TFA/DCM/anisole/EDTH/water, 70:18:4:6:2 v/v [24], for 4.5 h at room temperature. The solvent was then evaporated *in vacuo* and the residue solidified by addition of dry ether. The solid was filtered, dissolved in distilled water, and lyophilized. Purification of the crude peptide was performed by semi-preparative RP-HPLC to yield the desired product **2:** t_R 21.3 min. (S1); ESI-MS m/z: 1329.56 $[M+4H]/4^+$, 1064.42 $[M+5H]/5^+$, 887.21 $[M+6H]/6^+$; MALDI-TOF MS: actual mass 5314.98Da calc. 5315.18Da.

Following exactly the isolation procedure described for **2,** the protected fragment **13** yielded, *Locmi*-DP_{41-46}, product **5:** t_R 9.0 min. (S2); ESI-MS m/z: 763 $[M+H]^+$, 764 $[M+2H]^+$, 785 $[M+Na]^+$.

The synthesis of the decapentapeptide, *Locmi*-DP_{32-46}, was undertaken independently of the synthesis of the methyl-homoserine analogue, **2**, to allow comparison of its biological activity with that of Cys^{31}-*Locmi*-DP_{31-46}, which had been synthesized commercially by Sigma-Genosys Ltd (Cambridge, UK) as part of an earlier project to raise antibodies against *Locmi*-DP. The protected fragment *Locmi*-DP_{32-46}, **3**, was synthesized by coupling the protected peptide acid **12** to **11** as described for the fragment couplings of **2**. The resulting fully protected *Locmi*-DP_{32-46} peptide fragment was treated exactly as the corresponding fully protected peptide **2** to yield the desired product **3**: t_R 15.6 min. (S3); ESI-MS m/z: 1775 $[M+H]^+$, 1776 $[M+2H]^+$.

The final product **4** was obtained from the protected peptide acid **12** attached to the 2-chlorotrityl resin in two stages. In the first stage the Fmoc-group was removed and in the second stage the remaining peptide attached to the resin was stirred in 20 ml of TFA:H_2O:anisole (95:3:2) for 1 h at room temperature. The solvent was then evaporated *in*

vacuo and the residue solidified by addition of dry ether. The solid was filtered, dissolved in distilled water, and lyophilized. Purification of the crude peptide was performed by semi-preparative RP-HPLC to yield product **4:** t_R 11.0 min. (S3); ESI-MS m/z: 718 $[M+H]^+$, 740 $[M+Na]^+$.

3. Results

3.1 The biological activity of the full peptide analogue

For the synthesis of Hse$(CH_3)^{1,3,13}$-*Locmi*-DP, **2**, the sequence of the target peptide was divided into five parts **6-10** (Fig. 2) which were synthesized as protected fragments in the solid phase on the 2-chlorotrityl resin. These products were obtained in high yield and purity, and were used without any further purification. Fragments were condensed successively to the amino component attached on the Rink linker-2-chlorotrityl resin **11** (Fig. 2). Low substitution (0.1-0.2 mmol/g of resin) of the amino component on the Rink linker-2-chlorotrityl resin **11** was vital for completion of the acylation by the peptide acid fragments, which is why a two-molar excess was required instead of the three-molar excess used normally.

The Hse$(CH_3)^{1,3,13}$-*Locmi*-DP synthesised here was active in three of the four assays in which it was tested. It stimulated fluid secretion by isolated Malpighian tubules (Fig. 3) in a dose-dependent manner, with an EC_{50} of *c*. 5 nmol l^{-1}. In the assay that measured second messenger production by Malpighian tubules *in vitro*, the Hse(CH_3)-analogue elicited a dose-dependent accumulation of cAMP with an EC_{50} of *c.* 75 nmol l^{-1} (Fig. 4). The magnitude of the response elicited by a supramaximal concentration of the Hse(CH_3)-analogue was comparable with that caused by an extract of corpora cardiaca (data not shown): it had no significant effect on the production of cGMP production by Malpighian tubules (Fig. 4).

The methyl-homoserine analogue was also active in a behavioural bioassay. When compared with saline-injected control nymphs, the latency to the first meal (the period before locust nymphs contacted the wheat and started to feed) was significantly increased by the injection of the Hse(CH_3)-analogue. The duration of the first meal was reduced significantly when as little as 150 fmol of the Hse(CH_3)-analogue was injected (Fig.5). The reduction in meal duration brought about by the Hse(CH_3)-analogue was comparable with that brought about by a sample of natural *Locmi*-DP (Fig. 5).

*3.2 The biological activity of C-terminal fragment*s

Of the fragments tested in this study, only *Locmi*-DP_{32-46} had activity in the fluid secretion assay (Cys^{31}-*Locmi*-DP_{31-46} was not tested), but this was very low, eliciting only about a 120% increase in the rate of secretion at a high concentration of 2 x 10^{-6} M. This peptide also showed very weak but statistically significant (P<0.005 at 10^{-5} M) stimulatory activity in cAMP production by Malpighian tubules, with a maximum response of only *c*. 35 fmol cAMP/tube; *Locmi*-DP_{41-46} was inactive, while *Locmi*-DP_{32-37} had some weak activity but showed no clear dose-response relationship (Fig. 6). Two of the fragments appeared to have weak stimulatory activity on cGMP production when tested at a high dose of 800 pmol per tube (Fig. 6), but this was not investigated further because the activity was very low when compared with the effect of an extract of corpora cardiaca, and was not a property shown by the full peptide (see Fig. 4).

The Cys^{31}-*Locmi*-DP$_{31-46}$ fragment was active in the behavioural assay. As little as 150 fmol injected per nymph increased the latency to feed significantly to an extent comparable with the effect of natural *Locmi*-DP (Fig. 5). Meal duration was also reduced by this short peptide but, while the methyl-homoserine-analogue and the natural *Locmi*-DP were active at 150 fmol, a significant effect on meal duration by Cys^{31}-*Locmi*-DP$_{31-46}$ was only observed when 1 pmol was injected (Fig. 5).

The fragments *Locmi*-DP$_{32-46}$, *Locmi*-DP$_{32-37}$, and *Locmi*-DP$_{41-46}$ were tested also in the behavioural assay. Only *Locmi*-DP$_{32-46}$ showed consistent activity, increasing the latency to feed when as little as 150 fmol were injected, and decreasing the duration of meal; the latter response was significant, however, only when 1 pmol of peptide was injected (Fig. 7). The *Locmi*-DP$_{32-37}$ fragment appeared initially (at 150 fmol injected) to have some activity in this assay, but this was not substantiated by the results of injections at higher doses (Fig. 7).

4. Discussion

Locmi-DP and other diuretic peptides have been synthesized in the solid phase and on various resins with the stepwise strategy either manually or by automated peptide synthesizers [1,10,14,15]. In the latest of these syntheses which is described in detail [14], almost all of the amino acid couplings had to be performed twice followed by acetylation for end-capping uncoupled chains. Thus a very low yield of the final product was obtained after appropriate purification. These results and the experience gained from the use of two acid sensitive resins for solid phase [13,17,24,27] prompted the adoption of a different strategy for the synthesis of diuretic peptides in this study. The synthesis described here is an improvement on those reported previously because it meets the requirements of high purity, and sufficient quantity of product at low cost to provide peptide material for the study of physiological function. This is due mainly to the nature of the resins used, the completion of amino acid and peptide couplings and the protection of the side chain of glutamines during the synthesis. A 46-amino acid analogue of *Locmi*-DP was synthesized in which Met at positions 1, 3 and 13 were replaced by the isosteric amino acid methyl-homoserine. This replacement was chosen because in most cases it does not affect the activity of the parent peptide [16] and, in addition, it would provide information on the importance of sulfur in the side chains of methionines on the biological activity of *Locmi*-DP.

The methyl-homoserine analogue of *Locmi*-DP is highly active in the biological assays employed in this study. Its potency in stimulating cAMP production and fluid secretion by Malpighian tubules from *Schistocerca gregaria* is comparable with that reported for the endogenous CRF-like peptide assayed in tubules from *Locusta* [15]. The lack of effect of the analogue on cGMP production by tubules is consistent with what is known of the mechanism of action of *Locmi*-DP [7]. Although the stability of the methyl-homoserine analogue during storage has not been tested in a rigorous manner, we have no reason to suspect that it loses biological activity during long-term storage as a lyophilized powder at -20 °C: it retains potent biological activity, and HPLC analysis shows that it comprises a single major peak of UV-absorbing material, even after more than 18 months of storage under these conditions.

The regulation of feeding in insects involves central excitation that is augmented positively by both food and non-food stimuli, both from within and outside the animal, and modulated by deterrent stimuli and by feedback from peripheral systems such as stretch receptors on the gut wall, hormones and blood composition [22]. Stretching of the foregut is one of the first consequences of food intake, and it is likely that this is a signal for the release of a number of chemical messengers, including diuretic factors like *Locmi*-DP [1,2] and

myotropins like locustasulfakinin [20,26], which may play a part in the regulation of food intake.

The release of *Locmi*-DP appears to play a role in satiety; signaling the end of a meal in *Locusta* by reducing taste-receptor input from the maxillary palps [9,11]. The present study shows that Hse$(CH_3)^{1,3,13}$-*Locmi*-DP has the same effects as the parent peptide in influencing some aspects of feeding behaviour. Compared with saline-injected control nymphs, the latency to the first meal (the period before locust nymphs contact the wheat and start to feed) is significantly increased by the injection of the Hse(CH_3)-analogue, and the duration of the first meal decreases. The active peptides are highly potent: injection of as little as 150 fmol is required (assuming a blood volume of *c*. 200 μl, this represents an effective concentration in the locust of *c*. 0.7×10^{-9} M). It is remarkable that this biological activity in regulating satiety in locusts [11] is retained in peptides derived from the C-terminal$_{32-46}$ sequence of the hormone, because these small peptides have only weak activity on cAMP production or fluid secretion by Malpighian tubules. Furthermore, it is reported that *Locmi*-DP$_{27-46}$ does not stimulate fluid secretion by Malpighian tubules [14]. More significantly, however, it has been reported previously that the first few residues at the N-terminus of *Locmi*-DP are important for receptor activation [8]. On the other hand, C-terminal fragments from the CRF-like diuretic peptide of *Manduca sexta* (*Manse*-DP), retain good binding affinity for the *Manduca* receptor, but do not stimulate cAMP production [18,19]. The exact mechanism by which *Locmi*-DP affects feeding behaviour is unknown, but it is thought to be a direct effect, at least in part, on the opening and closing of the apical pores on taste sensillae [11]: if this is so, then it seems that the structure-activity requirements of the relevant receptors for the diuretic peptide on these sensillae are different from those of the receptors in the Malpighian tubules. It is disappointing that the smaller fragments tested, especially *Locmi*-DP$_{41-46}$, were not active in any of the assays. Work is now in progress to determine more systematically whether (and how far) the C-terminal 15-mer can be truncated N-terminally without loss of its effects on the feeding behaviour of locusts.

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1 5 12
H-Met-Gly-Met-Gly-Pro-Ser-Leu-Ser-Ile-Val-Asn-Pro-Met-Asp-Val-Leu-Arg-Gln-Arg-Leu-

23 33 37
Leu-Leu-Glu-Ile-Ala-Arg-Arg-Arg-Leu-Arg-Asp-Ala-Glu-Glu-Gln-Ile-Lys-Ala-Asn-Lys-Asp-

46
Phe-Leu-Gln-Gln-Ile-NH_2 **1**, *Lom*-DP

[Hse$(CH_3)^1$, Hse$(CH_3)^3$, Hse$(CH_3)^{13}$]-*Lom*-DP **2**

Lom-DP_{32-46} **3** *Lom*-DP_{32-37} **4** *Lom*-DP_{41-46} **5**

Figure 1. *Locusta* Diuretic Hormone (Lom-DP) and its synthesized analogues

Fmoc-Hse(CH_3)-Gly-Hse(CH_3)-Gly-Pro-OH	**6**, Fmoc-[1-5]-OH
Fmoc-Ser(Bu-t)-Leu-Ser(Bu-t)-Ile-Val-Asn-Pro-OH	**7**, Fmoc-[6-12]-OH
Fmoc-Hse(CH_3)-Asp(OBu-t)-Val-Leu-Arg(Pbf)-Gln(Trt)-Arg(Pbf)-Leu-Leu-Leu-Glu(OBu-t)-OH	**8**, Fmoc-[13-23]-OH
Fmoc-Ile-Ala-Arg(Pbf)-Arg(Pbf)-Arg(Pbf)-Leu-Arg(Pbf)-Asp(OBu-t)-Ala-Glu(OBu-t)-OH	**9**, Fmoc-[24-33]-OH
Fmoc-Glu(OBu-t)-Gln(Trt)-Ile-Lys(Boc)-OH	**10**, Fmoc-[34-37]-OH
H-Ala-Asn-Lys(Boc)-Asp(OBu-t)-Phe-Leu-Gln(Trt)-Gln(Trt)-Ile-Rink linker-OCLTR	**11**, H-[38-46]-Rink linker-OCTLR
Fmoc-Ala-Glu(OBu-t)-Glu(OBu-t)-Gln(Trt)-Ile-Lys(Boc)-OH	**12**, Fmoc-[32-37}-OH
H-Asp(OBu-t)-Phe-Leu-Gln(Trt)-Gln(Trt)-Ile-Rink linker-OCLTR	**13**, H-[41-46]-Rink linker-OCTLR

Figure 2. Protected fragments used in the synthesis of [Hse(CH_3)^1, Hse(CH_3)^3, Hse(CH_3)^{13}]-*Locmi*-DP and of *Locmi*-DP_{32-46}, *Locmi*-DP_{32-37}, *Locmi*-DP_{41-46}

Fig 3 The effect of Hse$(CH_3)^{1,3,13}$-*Locmi*-DP on fluid secretion *in vitro* by Malpighian tubules from *Schistocerca*. Points represent the mean ± SE (vertical bars): *n*=5-8, the open circle is the saline control value.

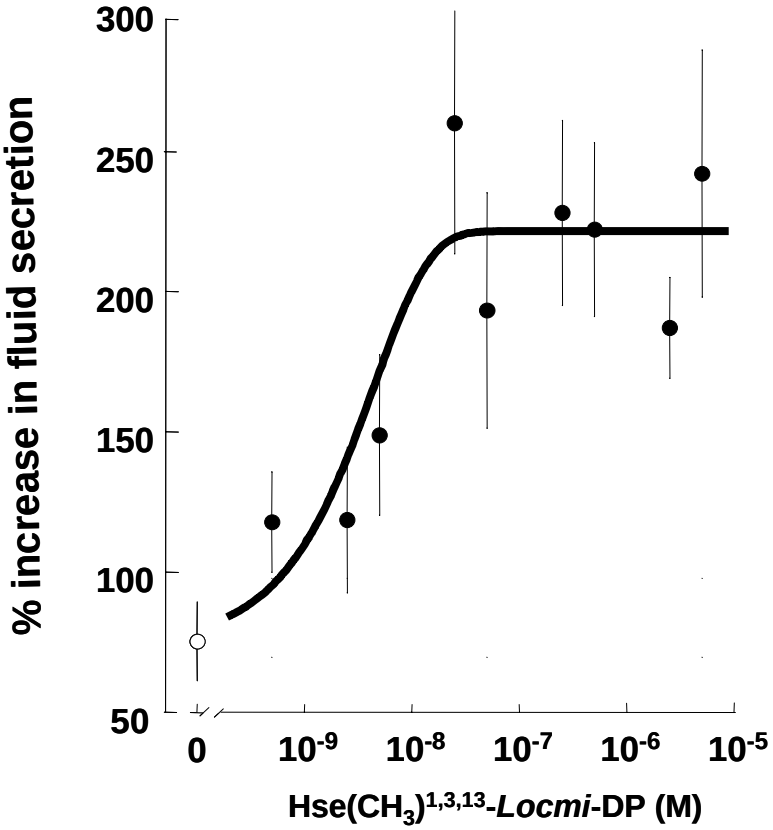

Fig. 4. The effect of Hse$(CH_3)^{1,3,13}$-*Locmi*-DP on second messenger production *in vitro* by Malpighian tubules from *Schistocerca*. Points represent the mean ± SE (vertical bars): *n*=5

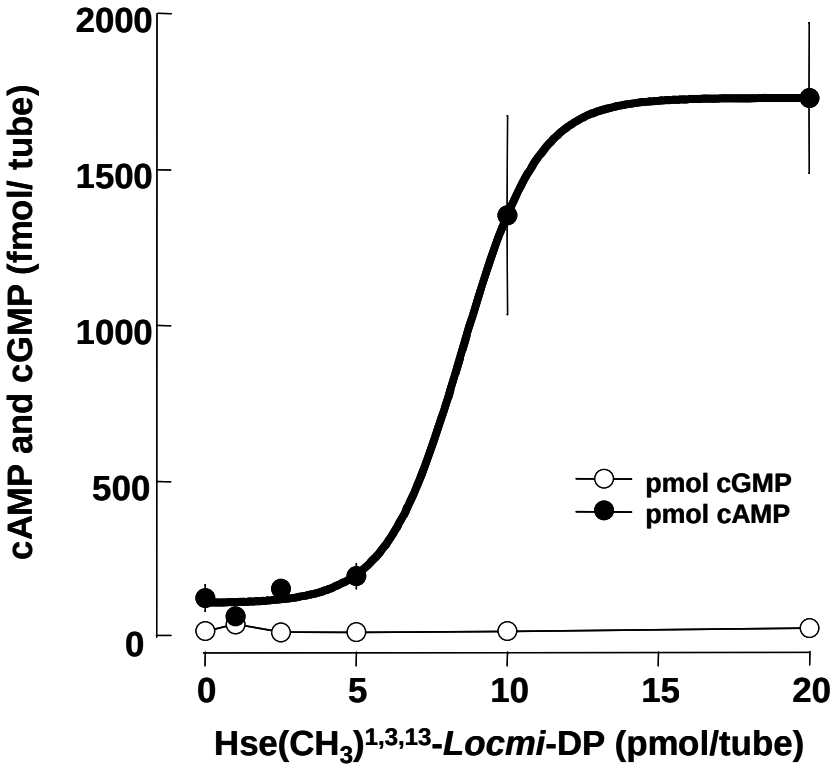

Fig. 5. The effects of Hse$(CH_3)^{1,3,13}$-*Locmi*-DP, Cys^{31}-*Locmi*-DP_{31-46}, and natural *Locmi*-DP, on latency to feed (upper bar chart) and duration of the first meal (lower bar chart) when nymphs of *Locusta* were presented with fresh wheat after 4-5 h of food deprivation. The bars represent the mean ± SE (vertical bars): n=10-15, asterisk shows significant difference between control (0 dose, saline) and test peptide at P<0.05.

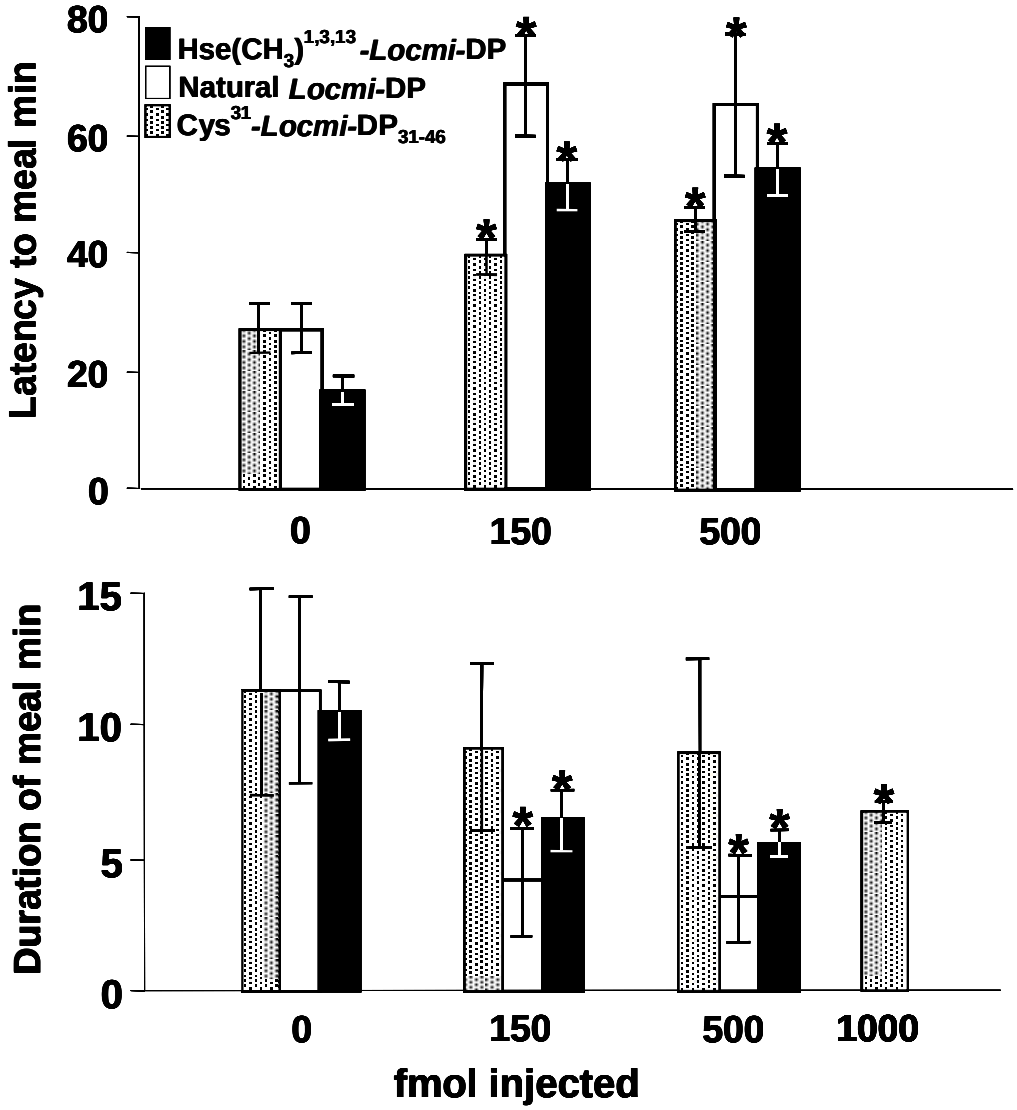

Fig. 6. The effects of *Locmi*-DP_{32-37}, *Locmi*-DP_{41-46}, and *Locmi*-DP_{32-46} on the production of cAMP and cGMP *in vitr*o by Malpighian tubules from *Schistocerca*. In the assay for cGMP production (bar chart), each of the peptides was tested at 800 pmol per tube against an extract of corpora cardiaca (0.5 pairs per tube). In the assay for cAMP production (line graph) *Locmi*-DP_{41-46} was inactive even at 800 pmol (data not shown), and addition of an extract of corpora cardiaca (0.5 pair of glands per tube) as a positive control (data not shown) resulted in a level of cAMP of 1390 ± 110 fmol per tube (*n*=5). Bars and data points represent the mean ± SE (vertical bars): *n*=4-6.

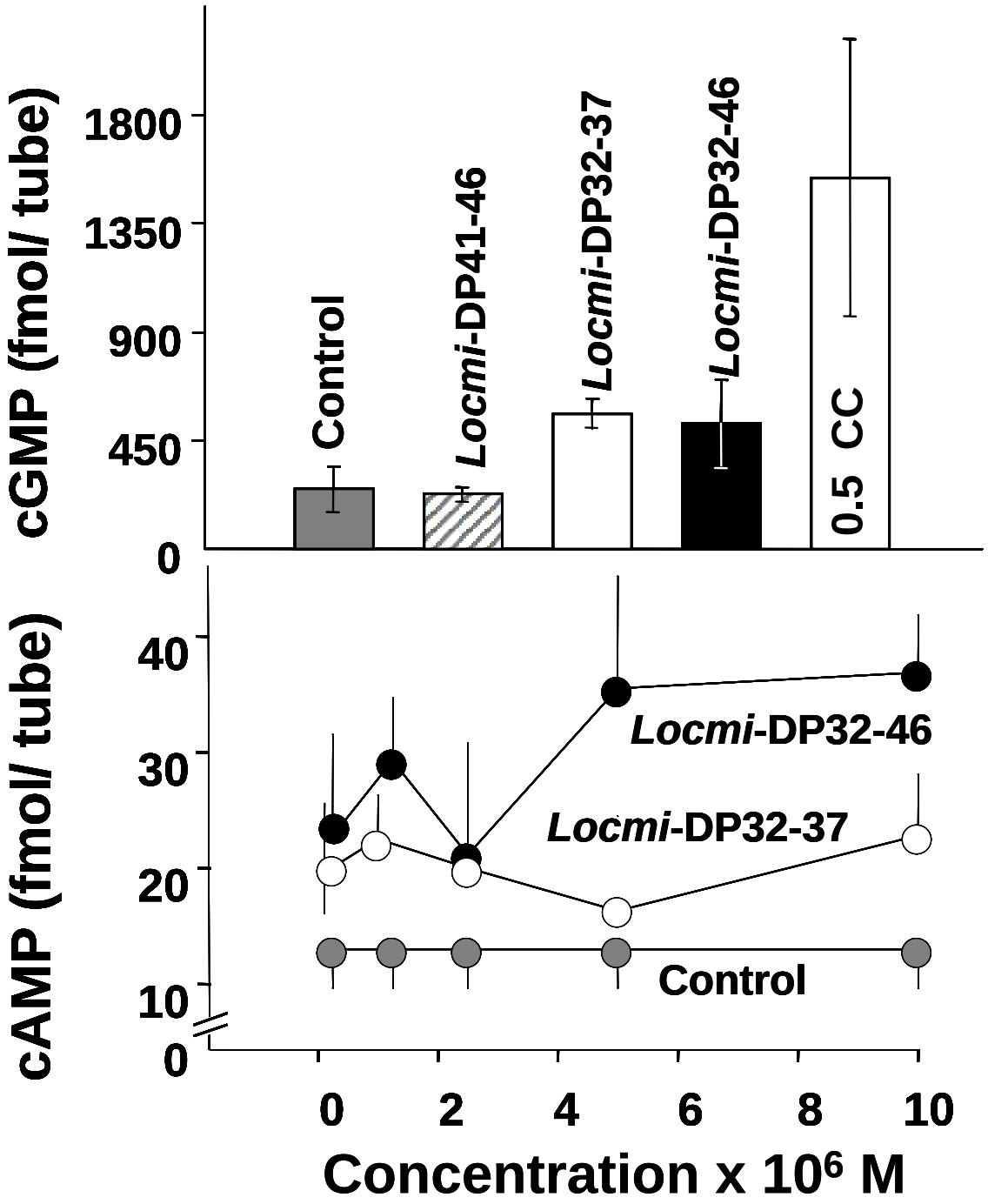

Fig. 7. The effects of *Locmi*-DP_{32-37}, *Locmi*-DP_{41-46}, and *Locmi*-DP_{32-46}, on latency to feed (upper bar chart) and duration of the first meal (lower bar chart) when nymphs of *Locusta* were presented with fresh wheat after 4-5 h of food deprivation. The bars represent the mean ± SE (vertical bars): n=10-15, asterisk shows significant difference between control and test peptide at P<0.05.