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Structure-Activity Relationships for the Lipid-Mobilising Action of Locust Adipokinetic Hormone

Synthesis and Activity of a Series of Hormone Analogues

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A series of compounds structurally related to adipokinetic hormone, the decapeptide neurohormone < Glu-Leu-Asn-Phe-Thr-Pro-Asn-Trp-Gly-Thr-NH_2, have been prepared by synthesis and by enzymic cleavages of synthetic hormone. Their relative agonist activities in mobilising lipids over a fixed time interval (1 h) in locusts were assessed. The similar time courses for lipid release shown by two of the peptide analogues and adipokinetic hormone suggest that the analogues and the hormone are transported to the receptors on the fat body cells, and are also degraded, at similar rates. Consequently, the analogue activities can be correlated with the structural requirements of the locust fat body hormone receptors. The requirements for activity demonstrated in this study are as follows. Residues 1–8 from the N-terminus are necessary to elicit some activity (20%). Residues 5 and 7 in the octapeptide can be changed without affecting activity but L-pyroglutamic acid as the N-terminal residue is necessary for maximum activity both in the octapeptide and the decapeptide. Full activity is achieved only by adding the dipeptide glycyl threonine amide to the active octapeptide 'core'. In the decapeptide, residues cannot be interchanged to the same extent as in the octapeptide without reducing activity. The peptide probably has to be uncharged. Inactive analogues of seven residues or less do not interfere in the hormone-receptor interaction.

Adipokinetic hormone, extracted from the corpora cardiaca of the locust species *Schistocerca gregaria* and *Locusta migratoria* and concerned with the regulation of lipid utilisation during flight, has been identified as the blocked decapeptide, < Glu-Leu-Asn-Phe-Thr-Pro-Asn-Trp-Gly-Thr-NH_2 [1]. Confirmation of this structure has been obtained from synthesis of the hormone. The synthetic product is chemically indistinguishable from the natural hormone, as is its adipokinetic activity on a molar basis when injected into locusts [2]. Moreover, the synthetic hormone also shows other biological activities in different assay systems, namely red-pigment-concentrating activity in the prawn *Pandalus montagui*, black pigment concentrating activity in the shrimp *Crangon crangon* and hyperglycaemic activity in the cockroach *Periplaneta americana* (Mordue & Stone, unpublished observations) as does the natural hormone [3,4].

Adipokinetic hormone is the only insect neurohormone that is characterised at the present time, hence studies of its mode of action may provide the first insights into mechanisms of action of insect peptide neurohormones in general. A knowledge of the detailed workings of these hormone systems (e.g. of hormone-receptor interactions, the mechanisms of synthesis, release, transport and degradation of hormones) may eventually lead to new approaches to the problems of insect pest control. In the locust investigations of the structural requirements for biological activity of adipokinetic hormone should give some understanding of the hormone-receptor interactions involved in its regulation of lipid utilisation.

It has already been shown that the hormone has two major sites of action in locusts. It acts on the cells of the fat body [5,6], causing specific diacylglycerols [7] to be released from these cells into the haemolymph, by a mechanism involving cyclic AMP [8,9]. It also has an effect on flight muscle tissue, which

Abbreviations. Abbreviations for amino acid derivatives and peptides follow the recommendations of IUPAC-IUB Commission on Biochemical Nomenclature, see *Eur. J. Biochem. 74*, 1–6 (1977). With the exception of glycine, all amino acids are of the L-configuration unless otherwise stated. Ac, acetyl; cyclic AMP, adenosine 3′:5′-monophosphate; Boc, *t*-butoxycarbonyl; Cbz, benzyloxycarbonyl; $(cHxN)_2C$, *N,N′*-dicyclohexylcarbodiimide; dansyl chloride, 5-dimethylaminonaphthalene-1-sulphonyl chloride; ED_{50}, median effective dose; F_3AcOH, trifluoroacetic acid; < Glu, pyroglutamic acid; Me_2NOCH, *N,N′*-dimethylformamide; S.E., standard error of the mean; Xah, xanthydryl.

results in a suppression of carbohydrate oxidation and an enhancement of lipid oxidation in the muscle cells during flight [10,11]. The present study is confined to investigation of the lipid-mobilising activity of the hormone. Attempts have been made to determine what parts of the hormone molecule are necessary to interact with receptors to trigger a response ultimately leading to the release of diacylglycerols from the fat body. These receptors may be located in the membrane of the fat body cells, from analogy with other hormone systems in which cyclic AMP acts as 'second messenger' [12,13].

A number of peptides structurally related to adipokinetic hormone have been synthesised and their potencies in mobilising lipids estimated. In addition, synthetic hormone was cleaved enzymically and the resulting fragments tested for adipokinetic activity. This paper presents the results obtained from the assays of biological potency of the various compounds. Details of synthesis of the peptide analogues are also included.

MATERIALS AND METHODS

Natural adipokinetic hormone was isolated from the corpora cardiaca of *Locusta* as described earlier [1]. Synthetic red-pigment-concentrating hormone [14] was a gift from Dr P. Fernlund.

The peptide syntheses are described in the miniprint supplement at the end of this paper.

Fragmentation of Synthetic Adipokinetic Hormone

All chemicals were obtained from British Drug Houses. L-Pyroglutamic acid (grade II, approximately 95%) and dansyl chloride were from Sigma Chemical Co., ninhydrin 'Puriss' from Koch-Light Laboratories Ltd. Thermolysin, A grade, and bovine pancreatic α-chymotrypsin, A grade, were from Calbiochem Ltd.

Chymotryptic Cleavage

Synthetic adipokinetic hormone (100 nmol) in 0.06 M NH_4HCO_3 (350 µl) containing α-chymotrypsin (2 nmol) was incubated for 5 h at 37 °C, and the mixture then lyophilised. The digested peptide, redissolved in pyridine/acetate buffer, pH 6.5 [15], was applied to Whatman no. 1 paper and subjected to electrophoresis at pH 6.5 at 50 V/cm for 40 min. Staining of a portion of the electrophoretogram by the starch/iodide procedure after chlorination [16] revealed the presence of two intensely-staining charged peptides in the digest, one acidic and one basic, and minor traces of other peptides. The major acidic peptide had a mobility of +0.46 relative to aspartic acid, suggesting a molecular weight of 450 from Offord's plot [15] (assuming a single negative charge on this peptide). The peptide did not stain up with ninhydrin [17] indicating the presence of a blocked N-terminus. Following elution from the paper, a portion of the peptide was hydrolysed *in vacuo* in 6 M HCl containing 0.1% phenol for 20 h at 110 °C. Amino acid analysis: aspartic acid 1.00, glutamic acid 1.02, leucine 1.02, and phenylalanine 0.95. These data demonstrate that this peptide is the N-terminal tetrapeptide fragment of adipokinetic hormone < Glu-Leu-Asn-Phe (XXVII), molecular weight 503. The basic peptide produced by chymotryptic digestion had a mobility of −0.35 relative to aspartic acid, indicating a molecular weight of approximately 650 (assuming a single positive charge on the molecule). The presence of a free amino group at its N-terminus was demonstrated by its positive reaction to the ninhydrin reagent. The N-terminal amino acid residue was subsequently identified as threonine by reaction of a portion of the peptide with dansyl chloride [18] following elution from the paper. Analysis of the acid hydrolysate of a further portion of the peptide showed aspartic acid (1.28), threonine (2.13), proline (0.61) and glycine (0.97). Hence it may be concluded that this is the C-terminal hexapeptide fragment of adipokinetic hormone, Thr-Pro-Asn-Trp-Gly-Thr-NH_2 (XXIII), molecular weight 673.

Thermolysin Cleavage

Synthetic adipokinetic hormone (100 nmol) in 0.06 M NH_4HCO_3 (350 µl) containing thermolysin (0.86 nmol) and $CaCl_2$ (2 µmol) was incubated for 5 h at 37 °C and the incubation mixture lyophilised. The major products of the digestion, the N-terminal acidic tripeptide (< Glu-Leu-Asn, XXVI) and the C-terminal basic heptapeptide (Phe-Thr-Pro-Asn-Trp-Gly-Thr-NH_2, XXIV) were separated by high-voltage electrophoresis on Whatman no. 1 paper at pH 6.5 and each was subsequently eluted and identified as described previously [1] for the natural hormone.

Assay of Adipokinetic Activity

Adult male *Locusta* were taken 10−20 days after fledging from colonies maintained at 30 °C under crowded conditions with a constant photoperiod of 14 h and fed on bran supplemented with fresh grass or lettuce. Precise molar quantities of the peptides to be assayed for biological activity were determined from the amino acid contents of portions of the peptides after acid hydrolysis under the conditions already described. Amino acid analyses were performed on a Rank Hilger Chromaspek analyser.

Samples to be tested were dissolved in simple insect saline (20 µl, 7.5 g NaCl, 0.375 g KCl/l). Aliquots of haemolymph (5 µl) were withdrawn from a

puncture in the arthroidal membrane at the base of the hind leg immediately before and 1 h after injection of the sample between the abdominal sterna of the locust. The locusts were maintained at room temperature during the assay. The changes in haemolymph total lipid concentration were measured as described previously [19], using the Boehringer Mannheim test combination kit for total lipids. Each compound was tested at between three and six doses on groups of six locusts for each dose. The relative activity of each compound was obtained from comparison with the activity elicited by doses (1 – 6 pmol) of natural adipokinetic hormone assayed under identical conditions. Standard errors of the mean activity per dose of sample were of the order of ± 10%.

In the experiments where time-response curves for lipid mobilisation produced by peptides and the natural hormone were compared, 5-μl aliquots of haemolymph were taken from groups of four locusts for each time interval immediately before, and at several time intervals after, injection of the test samples.

RESULTS AND DISCUSSION

The aim of the synthetic work described in the miniprint supplement at the end of this paper was to prepare adipokinetic hormone and a series of peptide analogues and fragments in which the amino acid substitutions and deletions are in the C-terminal part of the molecule. This portion of the molecule was chosen for an initial study of structure-activity relationships in view of the cross-activity of adipokinetic hormone and the structurally related octapeptide, red-pigment-concentrating hormone (XXIX). Accordingly, a synthetic strategy was adopted based on coupling the N-terminal hexapeptide sequence of the hormone (XII), synthesised by the solid-phase method [20], to a series of peptides related to the C-terminus of adipokinetic hormone.

The solid-phase synthesis utilised well-established methods apart from the use of the xanthydryl groups for protection of the side-chain amide group of asparagine. Although this group is removed during N-deprotection, it successfully prevented the β-cyanoalanine formation that tends to accompany the coupling of *t*-butoxycarbonyl asparagine itself by means of *N,N'*-dicyclohexylcarbodiimide. However, the indole ring of tryptophan reacts with xanthydrol [21] and transxanthylation can occur during N-deprotection of peptides containing this residue. Thus, although the solid-phase synthesis of the hexapeptide proceeded smoothly, in the solution-phase synthesis of C-terminal fragments where tryptophan is adjacent to an asparagine residue some problems occurred. The tetrapeptide III gave only a modest yield of the desired product on deprotection, while the dipeptide V seemed to undergo transxanthylation almost quantitatively.

The synthesis of the decapeptide XIII corresponding to the sequence of adipokinetic hormone from III and XII using *N,N'*-dicyclohexylcarbodiimide and 1-hydroxybenzotriazole also went in rather low yield. Use of the coupling reagents in 20-fold molar excess somewhat improved the efficiency (to 38%); the bulk of the component peptides can be recovered unchanged from the reaction mixture. Experiments using XII in the preparation of analogues suggests that use of an excess of amino component is also beneficial. Although a good recovery was obtained after chromatography on Sephadex G25 of the crude reaction product, rechromatography of the purified decapeptide led to severe losses (e.g. 83%). However, a single passage through the column proved sufficient to separate completely decapeptide from other peptides. Although closely similar to natural adipokinetic hormone, particularly in its mass spectrum, and homogeneity by thin-layer chromatography, the product showed only 70% of the biological activity of the natural hormone. High-pressure liquid chromatography resolved it into two components, the faster running of which had the full adipokinetic activity of the natural hormone and was indistinguishable from it. The slower running component was of low biological activity. The presence of the latter was traced to the use of partly racemic pyroglutamic acid in the synthesis of the hexapeptide XII; the impurity is therefore the epimer [D-< Glu^1]adipokinetic hormone (XV). The sample of pyroglutamic acid used in the synthesis was prepared from *N*-benzyloxycarbonyl-L-glutamic anhydride by the method of Gibian and Klieger [22]. This synthetic scheme is well established and should be free of racemisation, but in this instance racemisation seems to have occurred during formation of the anhydride under the standard conditions previously described [22]. The proportion of [D-< Glu^1]adipokinetic hormone in the mixture of decapeptides corresponds to the proportion of D-pyroglutamic acid in the sample of pyroglutamic acid used. D-< Glu^1 analogues of peptide hormones have in fact hitherto little been studied; the sole recorded example is thyrotropin-releasing hormone, (< Glu-His-ProNH$_2$) [23], the analogue of which has little biological activity. However, the tripeptide sequence is too short to be able to make any valid comparisons. The preparation of hexapeptide XII used in the hormone synthesis was used also in the preparation of the other analogues (i.e. XVI, and XVIII – XXII). In each case the D-< Glu^1 isomer could be cleanly separated from the desired epimer by high-pressure liquid chromatography.

Four peptides were isolated by high-voltage electrophoresis after enzymic cleavages of synthetic adipokinetic hormone. Chymotrypsin primarily cleaves the

Table 1. *Adipokinetic activities of adipokinetic hormone and structurally-related compounds*
The activity of adipokinetic hormone is defined as 1. Other preparations were compared with hormone standard on a molar basis. ED_{50} of adipokinetic hormone is 3.0–4.5 pmol per locust. Activities shown as < 0.003 indicate that the maximum dose tested (200 pmol per locust) did not produce a significant adipokinetic response

Compound	Residues										Relative agonist activity
	1	2	3	4	5	6	7	8	9	10	
XIV							Asn-Trp-Gly-Thr-NH_2				< 0.003
XVII							Ac-Asn-Trp-Gly-Thr-NH_2				< 0.003
XXIII						Thr-Pro-Asn-Trp-Gly-Thr-NH_2					< 0.003
XXIV					Phe-Thr-Pro-Asn-Trp-Gly-Thr-NH_2						< 0.003
XXV	< Glu										< 0.003
XXVI	< Glu-Leu-Asn										< 0.003
XXVII	< Glu-Leu-Asn-Phe										< 0.003
XII	< Glu-Leu-Asn-Phe-Thr-Pro										< 0.003
XVI	< Glu-Leu-Asn-Phe-Thr-Pro-NH_2										< 0.003
XVIII	< Glu-Leu-Asn-Phe-Thr-Pro-Trp-NH_2										< 0.003
XIX	< Glu-Leu-Asn-Phe-Thr-Pro-Asn-Trp-NH_2										0.2
XXVIII	D- < Glu-Leu-Asn-Phe-Thr-Pro-Asn-Trp-NH_2										0.08
XXIX	< Glu-Leu-Asn-Phe-Ser-Pro-Gly-Trp-NH_2										0.2
XX	< Glu-Leu-Asn-Phe-Thr-Pro-Asn-Trp-Gly-NH_2										0.07
XXI	< Glu-Leu-Asn-Phe-Thr-Pro-Gly-Trp-Gly-Thr-NH_2										0.3
XXII	< Glu-Leu-Asn-Phe-Thr-Pro-Asn-Trp-Thr-Gly-NH_2										0.03
XV	D- < Glu-Leu-Asn-Phe-Thr-Pro-Asn-Trp-Gly-Thr-NH_2										0.05
XIII	< Glu-Leu-Asn-Phe-Thr-Pro-Asn-Trp-Gly-Thr-NH_2										1

molecule at the C-terminal side of the phenylalanine residue, giving rise to the tetrapeptide XXVII and the hexapeptide XXIII (Table 1). Thermolysin breaks the peptide chain initially after the first asparaginyl residue, liberating the tripeptide XXVI and the heptapeptide XXIV.

The relative potencies, with respect to mobilisation of lipids in locusts, of the 17 peptide analogues and fragments of adipokinetic hormone, compared with that of the hormone itself, are shown in Table 1. It is apparent that only larger peptides, with a minimum of the first eight residues (starting from the N-terminus of the molecule), possess any significant adipokinetic activity.

Compounds XII, XIV, and XXIII–XXVII, all small fragments of the hormone containing up to seven residues and with unblocked (and thus charged) groups at either the N-terminus or the C-terminus, are all inactive at the doses tested. As adipokinetic hormone itself is uncharged, the fact that all of these molecules possess some charge at neutral pH might be thought to account for their inactivity. However, this cannot be the sole reason. Compound XIV is identical to compound XVII except that the free amino group at the N-terminus is acetylated in the latter. Similarly, compounds XII and XVI are identical except that the free carboxyl group at the C-terminus of compound XII is blocked with an amide in compound XVI. All of the octapeptide, nonapeptide and decapeptide analogues of adipokinetic hormone tested are uncharged and so at this stage one cannot say definitely that no charged analogues possess activity.

No antagonistic activity was demonstrated by any of the inactive compounds when present in 15-fold molar excess over the hormone, suggesting that none of these molecules interferes or competes with the hormone to any significant extent in its action on the fat body.

Compound XIX, an octapeptide fragment of adipokinetic hormone with an amide blocking the C-terminal carboxyl group of tryptophan, is 20% as active as the hormone on a molar basis, whereas a heptapeptide analogue of this molecule, XVIII, in which the penultimate asparagine residue is deleted, is inactive. This suggests that the presence of tryptophan amide at the C-terminus in a peptide of approximately the same size as compound XIX is insufficient to produce activity. The necessity of a minimum of eight residues for activity is further demonstrated by the activity (20%) of compound XXIX, the naturally occurring analogue, red-pigment-concentrating hormone from prawns [24]. Although this molecule differs from compound XIX in possessing a serine residue instead of threonine as residue 5 and glycine instead of asparagine as residue 7, the fact that the two octapeptides are equally potent in eliciting an adipokinetic response implies that their activities may be accounted for by certain structural features only. These features are presumably the particular length (eight residues) of the peptide, also perhaps the pyroglutamic acid and C-terminal tryptophan amide residues and possibly certain other residues.

Evidence that for maximum biological activity the N-terminus of the molecule must be the L-enantiomer

of pyroglutamic acid is provided from a comparison of the activities of compounds XIX and XXVIII and of compounds XV and XIII (adipokinetic hormone). For both epimer pairs, when the D-form replaces the L-form of pyroglutamic acid the activity of the molecule is greatly reduced (by 60% in the octapeptides and 95% in the decapeptides).

The effects of adding further residues to the partially active eight-residue peptide were also investigated. Addition of the ninth residue of the hormone, glycine as an amide, to the octapeptide fragment of the hormone (compound XX) reduces its activity to only 35% of that of the octapeptide, whereas addition of glycyl-threonine amide to produce the hormone itself dramatically increases the biological activity of the molecule to 500% of that of the octapeptide. Compound XXII, a decapeptide differing in structure from adipokinetic hormone by the reversed order of residues 9 and 10 (-Thr-Gly- instead of -Gly-Thr-), has only 3% of the activity of the hormone. It would therefore appear that if the structure of the part of the molecule extending beyond residues 1 – 8 is not exactly that of the hormone (i.e. -Gly-Thr-NH_2) then the biological activity is reduced to a level below that of the octapeptide molecules.

A decapeptide analogue of the hormone, compound XXI, with glycine in place of asparagine as residue 7 (as in red-pigment-concentrating hormone) has only 30% of the activity of the natural hormone. This is unexpected as this substitution in position 7 does not affect the (albeit lower) biological activity of the octapeptide molecule. The result implies that with the decapeptide there is a much more precise structural requirement for maximum adipokinetic activity.

The relative activities of compounds XV, XIX to XXII, XXVIII and XXIX shown in Table 1 demonstrate that these peptides cause increases in haemolymph lipid concentration after 1 h lower than those produced by similar doses of adipokinetic hormone. There are several possible explanations for the lower activities observed. The activities may reflect the structural requirements of the receptor, hence the molecules with the higher relative activities are those which more effectively combine with the receptor to trigger the response leading to lipid release. It is also conceivable that the different activities reflect differences in rates of transport of peptides to the fat body or in degradation rates of the peptides and adipokinetic hormone. Therefore the time courses for lipid mobilisation were investigated, to give a measure of the speed of response and an estimate of the half-life in the haemolymph of the various peptides compared with the natural hormone. The lipids released into the haemolymph reach a maximum level 1 – 2 h after injection of extracts of corpora cardiaca containing adipokinetic hormone [19]. Here the time-response

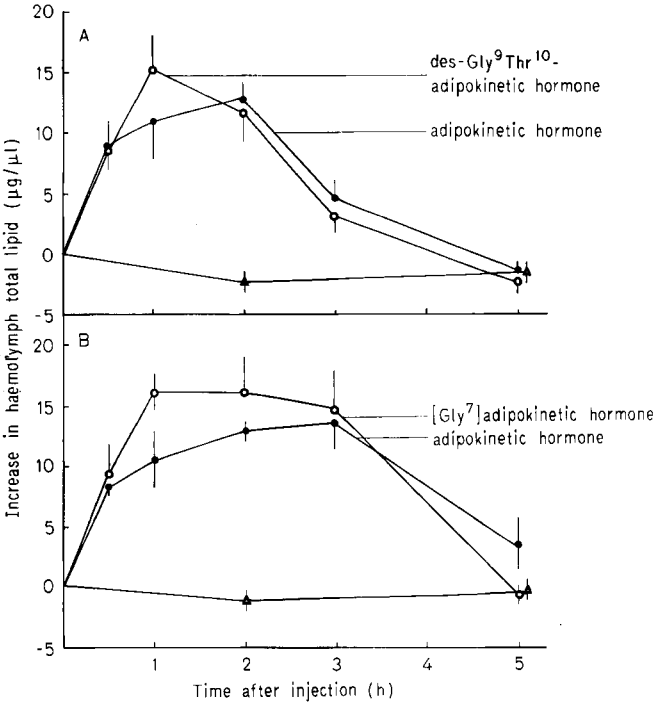

Fig. 1. *Comparison of the time-response curves for lipid mobilisation in* Locusta *elicited by hormone analogues and adipokinetic hormone.* (A) The increases in haemolymph total lipid with time after injection of 10 pmol of the octapeptide analogue (des-Gly^9,Thr^{10}-adipokinetic hormone, XIX, O——O) and (B) the increase from 7.4 pmol of the decapeptide analogue ([Gly^7]adipokinetic hormone, XXI, O——O) each compared to those from 2 pmol adipokinetic hormone (●——●) assayed under identical conditions. Different batches of locusts were used in A and B to produce the data shown. In each case the responses from injecting saline (Δ——Δ) alone are included. Samples were dissolved in 20 µl simple insect saline. The mean response ± S.E. from four locusts for each time interval after injection is shown

curves for the octapeptide, compound XIX, and the decapeptide analogue, compound XXI, were each compared with that of the hormone (Fig. 1). In each case, the doses used were known to produce similar responses 1 h after injection. All three peptides promote maximum lipid release 1 – 2 h after injection, wih the lipid levels returning to the initial level after 5 h. This indicates that the analogues and adipokinetic hormone are transported to their site of action, and are degraded, over similar time periods. Consequently, the relative activities in Table 1 most probably reflect the different abilities of the analogues to activate the receptors.

The structure-activity correlations recorded here may therefore more confidently be taken as indications of the structural requirements of the locust fat body adipokinetic hormone receptors. These may be summarised in the following manner. The first eight residues starting from the N-terminus are necessary to produce any activity. Two of the eight residues can be interchanged without affecting the activity, but

L-pyroglutamic acid is an absolute requirement as the N-terminus. Maximum activity is achieved only by adding both of the residues as found in the natural hormone to this eight-residue 'core'. In the decapeptide, residues cannot be interchanged to the same extent as in the octapeptide without reducing activity. The molecule probably has to be uncharged.

There are few structural features of the adipokinetic hormone molecule which put any obvious constraints on the configuration it might adopt in solution, so it seems likely that the three-dimensional shape of the hormone is a rather flexible one. However, application of the secondary structure predictive model of Chou and Fasman [25] to the hormone indicates a highly favoured conformation in which residues 5–8 form a β-bend. Three of the amino acids involved in such a β-turn of the hormone (proline, asparagine and tryptophan) occur at the positions in which they occur in the β-turns of proteins with greatest frequency (2nd, 3rd and 4th positions respectively). The averaged probability of the β-bend occurring here (7.4×10^{-4}) is well above the value below which the conformation is unlikely. The model of Chou and Fasman was developed for globular proteins, but it emphasises the importance of relatively short-range interactions in the molecule and may therefore be applied to peptides of modest lengths such as adipokinetic hormone. Although at present there is no direct evidence to suggest that the peptide must adopt this conformation to bind to the receptor, if the conformation were necessary for the hormone to elicit a response, this might explain why a minimum of eight residues are necessary for activity.

Considering the evolution of peptide hormones in arthropods, it may be relevant that the active eight-residue 'core' of adipokinetic hormone closely resembles the structure of the other arthropod neurohormone assayed here, prawn red-pigment-concentrating hormone (XXIX). This strongly suggests that both hormones have evolved from a common ancestral molecule. The receptors on the target tissues of the locust and prawn (fat body and eythrophore cells respectively) appear to have evolved in parallel with the hormone structure, as each is most responsive to its own hormone [3].

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Supplemental Material

to

Structure-Activity Relationships for the Lipid- Mobilising Action of Locust Adipokinetic Hormone. Synthesis and Activity of a Series of Hormone Analogues.

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This supplemental material consists of the experimental details of the peptide syntheses.

Materials and methods

Dichloromethane was distilled from calcium hydride. Me_2NOCH was purified by shaking over anhydrous copper(II) sulphate overnight, filtering, and fractionally distilling under vacuum from calcium hydride. Before use dry nitrogen was bubbled through for 15 min. Xanthydrol was prepared from xanthone [26]. All amino acid derivatives were obtained by standard procedures and their characteristic properties were in accord with previous reports. Mixed anhydride couplings [27] were carried out at -15°C; 5 min. was allowed for formation of the anhydride before addition of the amino-component unless otherwise stated. The solutions of amino-components were precooled before addition; after addition the reaction mixture was stirred at -15°C for a further 1h before allowing to warm to 20°C. In $(CHxN)_2C$ couplings [28] both components were dissolved in solvent (in the presence of 1-hydroxybenzotriazole if used) and the solution cooled to -10°C before addition of $(CHxN)_2C$. The reaction mixture was kept at -10°C for 1h before allowing to warm to 20°C.

Melting points are uncorrected. Optical rotations were recorded on a Thorn Automation - MPL Automatic Polarimeter Type 245 (0.1dm path length cell). Elemental analyses were performed on a Carlo Erba Elemental Analyser Model 1102 and amino-acid analyses on a Jeol JLC - 5AH Amino-acid analyser. Peptides were hydrolysed with 6M HCl in vacuo at 110°C for 18-20h. Peptide resins were hydrolysed with 6M HCl:propionic acid (1:1, v/v) in vacuo at 130°C for 3h [29]. R_F values refer to t.l.c. on Merck silica gel plates in the following systems: A, chloroform:methanol 9:1 v/v; B, 1-butanol:water: acetic acid 3:1:1 v/v; C, propan-2-ol:water:acetic acid 25:10:1 v/v; D, ethanol:water 7:3 v/v. Protected peptides were visualised by the chlorine - starch - iodide method [16]. Peptides with free α-amino groups were detected by spraying with 0.3% ninhydrin in 1-butanol and heating to 100°C. Tryptophan containing peptides were located by spraying with xanthydrol in ethanol (100mg/90ml; acidified immediately before spraying with concentrated HCl until a yellow colour persisted) followed by heating [21]. Gel filtration was carried out on Pharmacia columns using a Varioperpex 12000 peristaltic pump and an LKB Uvicord Type 8303 A detector monitoring at 255nm. High pressure liquid chromatography (h.p.l.c.) was performed on a Waters Associates ALC/GPC-301 Liquid chromatograph fitted with an M6000 Solvent Delivery System using a μBondapak C_{18} column (3.9mm x 30cm) fitted with a small precolumn; eluate was monitored at 254nm. Aqueous methanol was used for elution; analytical samples of peptids (20-50ug) were loaded on the column in either water or Me_2NOCH (ca.50μl) while for preparative purposes samples were dissolved in the minimum volume of these solvents and injected in 90μl aliquots.

N^{α}-t-Butyloxycarbonyl-N^{γ}-xanthydryl-L-asparagine

Boc-L-asparagine (10.0g, 43mmol) and xanthydrol (8.6g, 43mol) were dissolved in glacial acetic acid (200ml) and the solution stirred at 20°C for 4 days. The voluminous white precipitate was filtered off and dried over potassium hydroxide to give an amorphous white powder (10.9g, 62%), m.p. 170-172°C. Recrystallisation from ethanol (ca. 60 ml) gave the xanthydryl derivative as white feathery crystals (6.72g, 38%), m.p. 183-184°C, $[\alpha]_D^{24} = +2.1°$ (c = 6, Me_2NOCH) R_F 0.11(A), 0.85(B). For $C_{28}H_{28}N_2O_5$ (412.45) calcd: 64.6% C, 5.9% H, 6.8% N; found: 64.2% C, 5.7% H, 6.7% N.

Cbz-Gly-Thr-NH_2 (I)

N-Benzyloxycarbonylglycine (6.27g, 30mmol) and L-threonine amide (3.54g, 30mmol) were coupled by the mixed anhydride procedure [27] using isobutyl chloroformate (4.2ml, 30mol) and triethylamine (4.2ml, 30mmol) in dichloromethane (total volume 110ml). The mixed anhydride was stirred at -15°C for 30 min before addition of the threonine amide suspension. After stirring at 20°C overnight the precipitate was filtered off, triturated under distilled water (65ml), refiltered and recrystallised from ethanol (ca. 60ml) to give the dipeptide (5.7g, 61%), m.p. 175.5-177°C, $\alpha_D^{25} = +16.5°$ (c = 1.92, Me_2NOCH), R_F 0.20(A), 0.64(B). For $C_{14}H_{19}N_3O_5 \cdot \frac{1}{2}H_2O$ (309.33) calcd: 52.9%C, 6.3%H, 13.2%N; found: 52.9%C, 6.3%H, 13.2%N.

Boc-Trp-Gly-Thr-NH_2 (II)

The protected dipeptide (I) (0.93g, 3.0mmol) was dissolved in methanol (50ml) and 5% palladium on charcoal (300mg) was added. Hydrogen was bubbled through the suspension for 2.5h after which no protected dipeptide was detectable by t.l.c. The reaction mixture was filtered through kieselguhr and solvent removed from the filtrate in vacuo to give the free dipeptide amide as a clear oil which quickly solidified (0.44g, 2.5mmol, 83%). This material was dissolved in Me_2NOCH (25ml) and Boc-L-tryptophan 2,4,5-trichlorophenyl ester [30] (1.10g, 2.3mmol) added. The solution was stirred at 20°C overnight before removing the solvent in vacuo to leave a pale brown oil. This material precipitated from ethyl acetate-petroleum ether (35ml-25ml) as an amorphous solid which crystallised from ethanol-ether (ca. 20ml-50ml) to give the protected tripeptide (0.64g, 60%) m.p. 148-151°C, $\alpha_D^{24} = +0.5°$ (c = 2, MeOH), R_F 0.15(A), 0.7(B). For $C_{23}H_{31}N_5O_5 \cdot H_2O$ (461.52) calcd: 55.1%C, 6.8%H, 14.8%N; found: 55.45%C, 6.8%H, 14.8%N.

Boc-Asn(Xah)-Trp-Gly-Thr-NH_2 (III)

The protected tripeptide (II) (920mg, 2mmol) was N-deprotected using 75% aqueous F_3AcOH containing 4% mercaptoethanol (2.5ml) at 20°C for 35 min. The excess reagent was removed in vacuo to leave a pale oil which was high vacuum dried over potassium hydroxide for 5h. The free tripeptide amide (475mg, 1.32mmol) was obtained from this trifluoroacetate by passage through an anion exchanger (Dowex AG1 X4 in the OH^- form, 1cm x 9cm column). This material was then coupled to N^{α}-Boc-N^{γ}-Xah-L-asparagine (542mg, 1.32mmol) in Me_2NOCH (15ml) using $(CHxN)_2C$ [28] (300mg, 1.45mmol). After the reaction mixture had been stirred at 20°C overnight the precipitated N,N'-dicyclohexylurea was filtered off and the solution concentrated to 2-3ml in vacuo then poured into distilled water (50ml). The gelatinous precipitate was filtered off, dried and reprecipitated from aqueous Me_2NOCH to give the protected tetrapeptide (393mg, 39%, m.p. 210-214°C, $\alpha_D^{24} = -3.5°$ (c = 2, Me_2NOCH), R_F 0.29(A), 0.84(B). For $C_{53}H_{54}N_8O_8 \cdot 2H_2O$ (755.85) calcd: 58.9%C, 6.1%H, 12.3%N; found: 58.8%C, 5.9%H, 11.8%N.

Boc-Gly-Trp-Gly-Thr-NH_2 (IV)

The protected tripeptide (II) (230mg, 0.5mmol) was deprotected with F_3AcOH as previously described for III and the tripeptide amide liberated as its free base by treatment with triethylamine in methanol. The tripeptide amide was coupled by the mixed anhydride procedure to Boc-glycine (88mg, 0.5mmol) using isobutyl chloroformate (70μl, 0.55mol) and triethylamine (70μl, 0.5mmol) in Me_2NOCH (total volume 5ml). The reaction mixture was stirred overnight at 20°C, then cooled to 0°C and filtered. The solvent was removed in vacuo to leave a pale oil which was taken up in ethyl acetate (15ml) and water (15ml). The aqueous layer was adjusted to pH 10 with saturated sodium carbonate solution and extracted with ethyl acetate (3 x 10ml - 1 x 50ml). The combined organic phases were washed with water (25ml) and dried over magnesium sulphate. The solution was filtered, and the solvent removed in vacuo to leave an amorphous solid which was re-crystallised from ethyl acetate to give the protected tetrapeptide (62mg,24%) as a white powder, m.p. 190-191.5°C, R_F 0.12(A), 0.72(B). For $C_{29}H_{34}N_6O_7H_2O$ (518.57) calcd: 53.7%C, 6.6%H, 15.7%N; found: 53.8%C, 6.6%H, 15.2%N.

Boc-Asn(Xah)-Trp-NH_2 (V)

Tryptophan amide hydrochloride (240mg, 1mmol) was dissolved in Me_2NOCH (5ml) and liberated as the free base by addition of triethylamine (140μl, 1mmol). This base was coupled by the mixed anhydride procedure to N^{α}-Boc-N^{γ}-Xah-L-asparagine (412mg, 100mol) using isobutyl chloroformate (140μl, 1mmol) and triethylamine (140μl, 1mmol) in Me_2NOCH (10ml). The reaction mixture was stirred overnight at 20°C then poured into water (100ml). The aqueous mixture was adjusted to pH 8 with 2M potassium bicarbonate before the feathery white precipitate was filtered off and then high vacuum dried over calcium chloride for 18h. The amorphous solid obtained was recrystallised from methanol to give the protected dipeptide (470mg, 79%) as a white powder, m.p. 216°C, $\alpha_D^{24} = -12.3°$ (c = 2.88, Me_2NOCH) R_F 0.42(A), For $C_{33}H_{33}N_5O_6$ (597.67) calcd: 66.3%C, 5.7%H, 11.7%N; found: 66.2%C, 5.7%H, 11.4%N.

Boc-Asn-Trp-NH_2 (VI)

Boc-L-asparagine (230mg, 1mmol) and L-tryptophan amide (liberated from 240mg(1mmol) of the hydrochloride by the addition of 140ul (1mmol) of triethylamine) were coupled using $(CHxN)_2C$ (210mg, 1mmol) and 1-hydroxybenzotriazole (300mg, 2mmol) in Me_2NOCH (10ml). After the reaction mixture had been stirred at 20°C overnight the precipitated N,N'dicyclohexylurea was filtered off and solvent removed from the filtrate in vacuo to leave a pale oil. This was dissolved in ethyl acetate (50ml) and washed with equal portions (40ml) of water, saturated sodium bicarbonate, water, 2M citric acid and water. T.l.c. indicated that the product (VI) had been extracted into the citric acid whereas Boc-β-cyanoalanyl-Trp-NH_2 had remained in the organic layer. The citric acid extract was adjusted to pH 8.5 with solid sodium carbonate and extracted with ethyl acetate (3 x 50ml). The combined organic layers were dried over magnesium sulphate, filtered, and solvent evaporated in vacuo to leave an amorphous residue. This was crystallised from ethyl acetate-petroleum ether (total volume 40ml) to give the dipeptide (395mg, 46%) as a white powder, m.p. 144.5-147°C, $\alpha_D^{23} = -21.8°$ (c = 1.74, Me_2NOCH), R_F 0.15(A), 0.7(B). For $C_{25}H_{29}N_5O_5 \cdot \frac{1}{2}H_2O$ (417.46) calcd: 56.3%C, 6.6%H, 16.8%N; found: 56.1%C, 6.55%H, 15.7%N.

Boc-Trp-Gly-NH_2 (VII)

Boc-L-tryptophan 2,4,5-trichlorophenyl ester (1.4g, 2.8mmol) [30] and glycine amide hydrochloride (0.32g, 2.9 mol) were dissolved in Me_2NOCH (20ml). Triethylamine (410μl, 3.9mmol) was added and the reaction mixture stirred at 20°C overnight. The solvent was removed in vacuo to leave a colourless oil. The protected dipeptide was purified by silica gel chromatography (50g, Merck Kieselgel under 0.08mm) using chloroform:methanol (9:1 v/v) to separate it from 2,4,5 trichlorophenol. After removal of the solvent in vacuo the protected dipeptide was obtained (0.87g, 83%) as an amorphous solid, m.p. 97-100°C, $\alpha_D^{23} = -17.1°$ (c = 2.40, Me_2NOCH), R_F 0.32(A). For $C_{18}H_{24}N_4O_5 \cdot CH_3OH$ (360.32) calcd: 58.15%C, 7.2%H, 14.3%N; found: 58.35%C, 6.9%H, 13.9.

Boc-Asn(Xah)-Trp-Gly-NH_2 (VIII)

The protected dipeptide (VII) (360mg, 1mmol) was deprotected with F_3AcOH and obtained as the free dipeptide amide by treatment with triethylamine as described previously. The free dipeptide amide was coupled by the mixed anhydride procedure to N^{α}-Boc-N^{γ} -Xah-L-asparagine (412g, 1mmol) using isobutyl chloroformate (140μl, 1mol) and triethylamine (140μl, 1mmol) in Me_2NOCH (total volume 15ml). The reaction mixture was stirred at 20°C overnight then poured into distilled water (30ml). The aqueous mixture was adjusted to pH8 with 2M potassium bicarbonate and the voluminous precipitate filtered off and high vacuum dried over calcium chloride for 18h. The white solid obtained was recrystallised from methanol to give the protected tripeptide (370mg, 56%), m.p. 191-195°C (dec), $\alpha_D^{25} = 15.1°$ (c = 1.8, Me_2NOCH, R_F 0.42(A). For $C_{39}H_{38}N_6O_7 \cdot CH_3OH$ (654.74) calcd: 63.0%C, 6.2%H, 12.3%N; found: 62.9%C, 5.8%H, 11.9%N.

Cbz-Thr-Gly-NH_2 (IX)

N-Benzyloxycarbonyl-L-threonine pentachlorophenyl ester [31] (1.80g, 3.6mmol) and glycine amide hydrochloride (0.4g, 3.6mmol) were dissolved in Me_2NOCH (25 ml) and triethylamine (510μl, 3.6mmol) added. The reaction mixture was stirred at 20°C overnight and the solvent removed in vacuo to leave a pale oil. This oil was triturated under ethyl acetate (50 ml), filtered and washed with saturated sodium bicarbonate and (25ml). The aqueous phase was extracted with ethyl acetate (3 x 25ml) and the combined organic phases were dried over magnesium sulphate, filtered, and solvent removed in vacuo to leave a pale oil. The protected dipeptide (IX) was purified by silica gel column chromatography (80g, Merck Kieselgel under 0.08mm) in chloroform-methanol (9:1 v/v) and was obtained as an amorphous white solid (720mg, 65%) after removal of the solvent in vacuo, m.p. 162-165°C, R_F 0.16(A). For $C_{14}H_{19}N_3O_5$ (309.33) calcd: 54.4%C, 6.2%H, 13.6%N; found: 54.4%C, 6.2%H, 13.6%N.

Boc-Trp-Thr-Gly-NH_2 (X)

The protected dipeptide (IX) (700mg, 2.2mmol) was N-deprotected by hydrogenolysis in methanol (25ml) using 5% palladium on charcoal (200mg) at 20°C for 2½h. The catalyst was filtered off using kieselguhr and solvent removed from the filtrate in vacuo. The residual oil was taken up in Me_2NOCH (25ml) and Boc-L-tryptophan 2,4,5-trichlorophenyl ester (1.05g, 2.2mmol) was added. The solution was stirred at 20°C overnight after which solvent was removed in vacuo to leave a pale oil, which crystallised from ethyl acetate-petroleum ether. Unreacted dipeptide was removed by trituration under distilled water to give the protected tripeptide (810mg, 88%) m.p. 200-205°C. For $C_{25}H_{31}N_5O_6$ (461.52) calcd: 57.3%C, 6.8%H, 15.2%N; found: 57.4%C, 7.1%H, 15.4%N.

Boc-Asn-(Xah)-Trp-Thr-Gly-NH_2 (XI)

The protected tripeptide (X) (790mg, 1.7mmol) was deprotected and liberated as the free base as described previously. The free tripeptide amide was coupled by the mixed anhydride procedure to N^{α}-Boc-N^{γ}-Xah-L-asparagine (71mg, 1.7mmol) using isobutyl chloroformate (245μl, 1.7mmol) and triethylamine (245ul, 1.7mmol) in Me_2NOCH (total volume 15ml). The reaction mixture was stirred at 20°C overnight, the volume reduced to ca. 7ml in vacuo and the resulting clear liquid poured into distilled water (50ml). The voluminous precipitate was filtered off, high vacuum dried over calcium chloride overnight and recrystallised from methanol (ca. 200ml) to give the protected tetrapeptide (620mg, 47%) as a white powder, m.p. 210-214.5°C (dec. and foams), $\alpha_D^{24} = -7.3°$ (c = 1.68, Me_2NOCH) R_F 0.74(B). For $C_{39}H_{41}N_7O_8 \cdot 1\frac{1}{2}H_2O$ (755.85) calcd: 59.8%C, 6.2%H, 12.5%N; found: 60.0%C, 6.0%H, 12.5%N.

<Glu-Leu-Asn-Phe-Thr-Pro-OH (Adipokinetic hormone 1-6; XII)

Solid phase resin [chloromethylated Bio-Beads SX2, 200-400 mesh, 0.9mmol Cl/g] was esterified with Boc-L-proline by refluxing in ethyl acetate in the presence of triethylamine (0.9 equiv) for 60h [32]. After shrink-swell washing with Me_2NOCH and ethanol [33], amino-acid analysis indicated a loading of 0.5 mmol of proline per g of resin. After removal of the resin fines by decantation of a dichloromethane suspension, 2.0g were placed in a solid phase synthesis vessel (Xel-F body with sintered glass end pieces; 1.8 x 12.5cm internal dimensions). Addition of solutions and solvents and their removal were carried out by application of a partial vacuum (water pump). Solution volumes of 20ml were used, and the apparatus was shaken as in the original Merrifield method [25]. After each filtration step, the vessel was inverted for further liquid addition. Fine bore PTFE tubing was attached to each end of the vessel for liquid transfer. The cycle of operations used is outlined in Table 2. For couplings, 2.5mmol (2.5 equiv.) of Boc-amino acids were used. Two couplings were found necessary before an acceptable degree of incorporation was indicated by the ninhydrin test of Kaiser [34].

TABLE 2. Sequence of Steps used for solid-phase synthesis

	Reagent[a]	Duration	Operation
1	F_3AcOH/CH_2Cl_2 1:1	2 x 2 + 30 min	Deprotection
2	CH_2Cl_2	3 x 5 min	Wash
3	EtOH	3 x 5 min	Wash
4	$CHCl_3$	3 x 5 min	Wash
5	$NEt_3/CHCl_3$ 1:10	2 x 5 + 10 min	Neutralisation
6	$CHCl_3$	3 x 5 min	Wash
7	CH_2Cl_2	3 x 5 min	Wash
8	Me_2NOCH[b]	1 x 5 min	Wash
9	Boc-AA.OH/coupling solvent[c]	1 x 2 min	Pre-equilibration[c]
10	$(CHxN)_2C$/coupling solvent[c]	1 x 120 min	Coupling
11	Me_2NOCH[b]	1 x 5 min	Wash
12	CH_2Cl_2	3 x 5 min	Wash
13	EtOH	3 x 5 min	Wash
14	CH_2Cl_2	3 x 5 min	Wash

a - Solvent volume normally 20ml; b - not included unless coupling solvent is Me_2NOCH; c - Boc.AA.OH added in 15ml coupling solvent; d - $(CHxN)_2C$ added in 5ml coupling solvent; e - coupling solvent: CH_2Cl_2 normally, but Me_2NOCH for Boc.Asn.OH and pyroglutamic acid.

In the coupling of Boc-Phe-OH, $(CHxN)_2C$ was added before the protected amino acid to minimise 2,5-dioxopiperazine formation [35]. On completion of the synthesis the peptide was cleaved from the resin with HBr - F_3AcOH in dichloromethane for 105 min at room temperature. The resin suspension was filtered and the resin washed with F_3AcOH. Evaporation of the combined filtrates left a pale brown oil. This was subjected to gel filtration (as its ammonium salt) on superfine G25 Sephadex (2.5 x 85cm; 35ml h^{-1}) using 0.05M ammonium bicarbonate as eluant. The major peak in the elution trace (248-335ml) was evaporated in vacuo and buffer salt removed (0.1mm Hg at 60°C to constant weight) to give the hexapeptide ammonium salt (466mg) as an amorphous solid. This material was converted to the free acid by percolation of an aqueous solution through Dowex AG50W X 4 (H^+ form, 1 x 8cm). Evaporation gave the hexapeptide (XII) (379mg, 56% based on Boc-Pro-resin), R_F 0.7(C), amino-acid analysis: Proline 1.06, threonine 0.99, phenylalanine 0.93, aspartic acid 1.02, leucine 1.00, glutamic acid 0.97.

<Glu-Leu-Asn-Phe-Thr-Pro-Asn-Trp-Gly-Thr-NH_2 (XIII; adipokinetic hormone)

Boc-Asn-(Xah)-Trp-Gly-Thr-NH_2 (III; 115mg, 154mol) was treated with 75% aqueous F_3AcOH containing 4% mercaptoethanol (1ml) at 20°C for 35 min during which the solution went deep purple. The solvent was removed in vacuo to leave a purple-brown oil which was triturated under distilled water (10ml) producing a white emulsion. This emulsion was loaded onto a column of Dowex AG1 x 4 in the OH^- form (1 x 9cm, distilled water as eluting solvent). The free tetrapeptide (XIV) eluted as an unretarded peak and was obtained as a colourless oil (34mg, 47%) after removal of the solvent in vacuo.

The free tetrapeptide was quantitatively transferred in DMF (5 x 100μl) to a flask containing adipokinetic hormone-(1-6) free acid (XII; 50mg. 73μmol) and 1-hydroxybenzotriazole (22mg, 144μmol). The solution was cooled to -5°C and $(CHxN)_2C$ (29mg, 144μmol) in Me_2NOCH (200ul) was added.

After 5 min a white precipitate was evident. The reaction was allowed to come to 20°C slowly (1.5h) then stirred overnight. The reaction mixture was transferred to another flask using Me_2NOCH (5 x 500µl) after which the solvent was removed in vacuo to leave an amorphous white solid. This was triturated with 0.05M NH_4HCO_3 (10ml), filtered, and the aqueous solution extracted with ether (10ml), degassed for 5 min (14mm Hg) and loaded onto a G25 Sephadex column (2.5 x 85cm). The fractions comprising the 3 major components were pooled and solvent and buffer salt removed in vacuo to leave amorphous solids: 245-270ml, 23.0mg (unreacted hexapeptide); 335-375ml, 14.0mg (decapeptide, 40% based on tetrapeptide added less that recovered); 420-475ml, 23.5mg (unreacted tetrapeptide)(1-hydroxybenzotriazole eluates after 490ml). The tentatively identified decapeptide was rechromatographed on G25 Sephadex and eluted as a single peak. Subsequent removal of the solvent and buffer salt in vacuo gave the decapeptide (2.4mg, 7% based on recovered tetrapeptide), R_F 0.33(B), 0.7(C), elution volume 325-380ml, amino acid analysis: aspartic acid 1.60, threonine 1.90, glutamic acid 1.06, proline 1.03, glycine 1.00, leucine 1.22, phenylalanine 1.12. This peptide was found to have approximately 70% of the biological activity of natural adipokinetic hormone. Repetition of this coupling using a larger excess of $(CH_2N)_2C$ and 1-hydroxybenzotriazole (20 equiv. of each) gave the decapeptide in 38% yield (87% based on tetrapeptide added less that recovered) after one passage through the Sephadex column (41.2mg from 94µmol of tetrapeptide) R_F (B) 0.35, R_F (C) 0.69. H.p.l.c. (47% aqueous methanol, 1ml min^{-1}) showed this material to contain two components, elution times 30 min (60%) and 36.5 min (40%). Although both components were of closely similar amino acid composition and mass spectrum, the faster running component showed the full biological activity of adipokinetic hormone while the slower component (now known to be [D-<Glu^1] adipokinetic hormone; XV) had only a low activity (5%). The faster running component could not be further resolved on extended h.p.l.c., either alone or when mixed with natural hormone. Its mass spectrum (after permethylation) was also closely similar to that of natural adipokinetic hormone.

Thermolysin digestion of natural and synthetic hormone produced peptides with the following electrophoretic mobilities:- pH 6.5, relative to aspartic acid, natural hormone: +0.56, 0, -0.29, -0.83; synthetic hormone: +0.56, 0, -0.22, -0.84; pH 2.1, relative to dansyl arginine, natural hormone: 0, +0.39, +0.68, +0.96; synthetic hormone: 0, +0.39, +0.72, +0.96.

<Glu-Leu-Asn-Phe-Thr-Pro-NH$_2$ (XVI)

Adipokinetic hormone (1-6) free acid (XII; 10mg, 15µmol) was dissolved in ME_2NOCH (500µl) and the solution cooled to -10°C before addition of isobutyl chloroformate (2µl, 15µmol) and triethylamine (2µl, 15µmol). The reaction mixture was stirred at -10°C for 30 min then a solution of ammonia in chloroform (1.5M, 0.5ml, 750µmol) was added. The reaction mixture was stirred at -10°C for 1h, then overnight at 20°C and the solvent was removed in vacuo. The residual oil was dissolved in distilled water (4ml) and eluted with water from a Dowex AG1 x 4 (OH^- form, 1 x 8 cm) column. Evaporation of the eluate gave the hexapeptide amide (1.3mg, 13%) as an amorphous solid, R_F 0.5 (C).

Ac-Asn-Trp-Gly-Thr-NH$_2$ (XVII)

H-Asn-Trp-Gly-Thr-NH$_2$ (5mg, 10µmol) and p-nitrophenyl acetate (8mg, 44µmol) were dissolved in Me_2NOCH (200µl) and the solution kept at 20°C overnight. Removal of the solvent in vacuo left a pale yellow solid which was triturated under distilled water (4ml). The aqueous solution was filtered, loaded onto a Sephadex column (1.6 x 85 cm, superfine G25) and eluted with distilled water (11ml h^{-1}). The protected tetrapeptide (ca. 4mg, 80%) was eluted separately from p-nitrophenol and was obtained as a white powder after lyophilisation of the pooled column fractions 174-200ml, R_F 0.28(B) (free tetrapeptide amide R_F 0.14(B)).

<Glu-Leu-Asn-Phe-Thr-Pro-Trp-NH$_2$ (XVIII)

Tryptophan amide was liberated from the hydrochloride (20mg, 88µmol) by treatment with triethylamine (11.7µl, 88µmol) in Me_2NOCH (1ml) and coupled to adipokinetic hormone (1-6) free acid (XII; 31mg, 44µmol) using $(CH_2N)_2C$ (18Omg, 88µmol) and 1-hydroxybenzotriazole (134mg, 880µmol). The reaction mixture was stirred at 20°C overnight and the solvent removed in vacuo to leave a white amorphous residue. This was triturated under column buffer (0.05M NH_4HCO_3; 10ml) and the aqueous mixture filtered, extracted with ether (15ml) degassed for 5min and loaded onto the superfine G25 Sephadex column. The fractions containing heptapeptide contaminated with 1-hydroxybenzotriazole were pooled and lyophilised to give a white powder. This was dissolved in column buffer (10ml) and loaded onto a G10 Sephadex column (2.5 x 135cm, 60ml h^{-1}). The heptapeptide-containing fractions, still heavily contaminated with 1-hydroxybenzotriazole,were pooled and lyophilised to give a white powder. This was dissolved in column buffer (2ml) and loaded onto a G25 Sephadex column (2.5 x 85cm). After 11h (~400ml) the column was recycled for 11h. The heptapeptide finally eluted was free of 1-hydroxybenzotriazole and was obtained as a white fluffy powder after lyophilisation of the pooled fractions. The buffer salt was removed at 60°C/0.1mm Hg to constant weight to give the heptapeptide (12mg, 31%), R_F 0.61(B), 0.77(C).

<Glu-Leu-Asn-Phe-Thr-Pro-Asn-Trp-NH$_2$ (XIX)

Removal of the N-protecting group from Boc-Asn(Xah)-Trp-NH$_2$ (V) with 75% aqueous F_3ACOH was accompanied by quantitative transxanthylation. Boc-Asn-Trp-NH$_2$, however, is cleanly deprotected under these conditions in the presence of 4% mercaptoethanol; the free dipeptide amide was obtained from the trifluoroacetate salt by flushing with excess triethylamine in methanol. Adipokinetic hormone-(1-6) acid (XII; 10.5mg, 15µmol) and asparaginyltryptophan amide (5mg, 15µmol) were coupled using $(CH_2N)_2C$ (60mg; 300µmol) and 1-hydroxybenzotriazole (50mg, 320µmol) in Me_2NOCH (600µl); the reaction mixture was stirred at 20°C overnight and the solvent removed in vacuo to leave an amorphous white solid. This was triturated under column buffer (0.05M NH_4HCO_3; 10ml) and the aqueous mixture was filtered, extracted with ether (10ml) and degassed for 5 min before loading onto a G25 Sephadex column 2.5 x 85cm, elution rate 35ml h^{-1}). The octapeptide (6mg, 40%) eluted separately from 1-hydroxybenzotriazole and was obtained as a white fluffy solid after lyophilisation of the pooled column fractions 340-395ml, R_F 0.77(C). Amino acid analysis: aspartic acid 1.57, threonine 0.84, glutamic acid 0.91, proline 1.00, leucine 1.01, phenylalanine 0.91.

<Glu-Leu-Asn-Phe-Thr-Pro-Asn-Trp-Gly-NH$_2$ (XX; [des-Thr^{10}] adipokinetic hormone}

Boc-Asn(Xah)-Trp-Gly-NH$_2$ (VIII) was deprotected with 75% aqueous F_3ACOH containing 4% mercaptoethanol (1.5ml) at room temperature for 40 min. Evaporation gave a purple oil. This oil was taken up in distilled water (15ml) and the free tripeptide amide was obtained by ion exchange chromatography (Dowex AG1 x 4 in the OH^- form) as previously described. This tripeptide amide (55mg, 88µmol) was coupled to adipokinetic hormone-(1-6) free acid (XII; 41mg, 59µmol) using $(CH_2N)_2C$ (243mg, 1180µmol) and 1-hydroxybenzotriazole (177mg, 1180µmol) in Me_2NOCH (800µl). The reaction mixture was stirred overnight at 20°C and the solvent removed in vacuo to leave an amorphous residue. This was triturated under column buffer (0.05M NH_4HCO_3; 15ml) and the aqueous mixture was filtered, extracted with ether (15ml) degassed for 5min and loaded onto a superfine G25 Sephadex column (2.5 x 85 cm, eluted at 35ml h^{-1}). The nonapeptide (40mg, 66%) eluted separately from the starting materials was obtained as a fluffy white powder by lyophilisation of the appropriate fractions (335-405ml), R_F 0.42(B), 0.73(C), Amino acid analysis: aspartic acid 1.71, threonine 0.97, glutamic acid 0.98, proline 1.00, glycine 0.96, leucine 1.00, phenylalanine 0.93.

<Glu-Leu-Asn-Phe-Thr-Pro-Gly-Trp-Gly-Thr-NH$_2$ (XXI; [Gly^7]-adipokinetic hormone}

Protected tetrapeptide (II) (50mg, 100µmol) was deprotected and the free base liberated as before. The free tetrapeptide amide was coupled to adipokinetic hormone-(1-6) free acid (XII; 35mg, 50µmol) using $(CH_2N)_2C$ 210mg, 1mmol) and 1-hydroxybenzotriazole (150mg, 1mmol) in Me_2NOCH (700µl). The reaction mixture was stirred at 20°C overnight and the solvent removed in vacuo to leave an amorphous white solid which was triturated under column buffer (15ml). The aqueous mixture was filtered, extracted with ether (15ml), degassed for 5 min and loaded onto a G25 Sephadex column (2.5 x 85cm, eluted with column buffer at 36ml h^{-1}). The decapeptide (35.8mg, 68%) eluted separately from starting materials and 1-hydroxybenzotriazole (320-395ml) and was obtained as a white powder by lyophilisation. R_F 0.46(B) 0.72(C), amino-acid analysis: aspartic acid 1.03, threonine 1.03, glutamic acid 1.07, proline 1.00, glycine 1.89, leucine 1.16, phenylalanine 0.97.

<Glu-Leu-Asn-Phe-Thr-Pro-Asn-Trp-Thr-Gly-NH$_2$ (XXII; [Thr^9, Gly^{10}]-adipokinetic hormone)

The protected tetrapeptide (XI) (200mg, 264µmol) was treated with F_3ACOH and the free tetrapeptide amide obtained by ion exchange chromatography as previously described. The tetrapeptide amide (147µmol) was coupled to adipokinetic hormone-(1-6) free acid (XII; 35mg, 50µmol) using $(CH_2N)_2C$ (210mg, 1000µmol) and 1-hydroxybenzotriazole (155mg, 1000µmol) in Me_2NOCH (65µl). The reaction mixture was stirred overnight at 20°C and the solvent removed in vacuo to leave an amorphous white solid which was triturated under column buffer (15ml). The aqueous mixture was filtered, extracted with ether (15ml), degassed and loaded onto a G25 Sephadex column (2.5 x 85cm, eluted with column buffer at 38ml h^{-1}). The decapeptide (56mg) (265-330ml) contaminated with some unreacted hexapeptide (subsequently shown by h.p.l.c.) was obtained as a white powder by lyophilisation of the pooled fractions. R_F 0.42(B), 0.73(C); amino-acid analysis: aspartic acid 1.66, threonine 1.81, glutamic acid 0.95, proline 1.05, glycine 0.98, leucine 1.00, phenylalanine 0.97.

Peptides XVI and XVIII to XXII were all prepared from peptide XII containing partly racemic pyroglutamic acid. In all cases the epimers were separated by h.p.l.c. before bioassay, and showed two major peaks in the characteristic ratio as described for XIII. All these peptides gave, after permethylation, mass spectra in accord with the required sequences. All peptides containing tryptophan gave U.V. spectra in solution in accord with the molar proportion of this amino acid.

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