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Receptor assay guided structure-activity studies of helicokinin insect neuropeptides and peptidomimetic analogues

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Neuropeptides control numerous physiological processes in insects. The regulation of water balance is a crucial aspect of homeostasis in terrestrial insects and has been shown to be under endocrine control, primarily by corticotrophin releasing factor (CRF)-related peptides and kinins. For helicokinin I, a diuretic neuropeptide from the economically important insect pest *Heliothis virescens*, detailed structure-activity relationships have been established based on truncated structures, diverse amino acid scans and peptidomimetic analogues. The activities of selected compounds on functional expressed helicokinin receptors are compared with the results of a Malphigian tubule assay. Implications for further peptidomimetic variations are provided.

Keywords: insect neuropeptides; helicokinins; structure-activity data; peptidomimetic

Introduction

Neuropeptides comprise a versatile class of extracellular messenger molecules that function as chemical communication signals between the cells of an organism. In insects, essential physiological processes, such as the embryonal- and post-embryonal development, the ion-homeostasis, the osmoregulation and muscle activity are regulated by neuropeptides and peptide hormones [1–4]. Therefore, insect neuropeptides and their receptors present promising targets for a novel generation of insecticidal agents offering levels of selectivity and environmental compatibility, absent from conventional, neurotoxic insecticides [5,6].

The myokinins form a large group of insect neuropeptides that have been isolated from a number of insects, including species of Dictyoptera, Lepidoptera and Orthoptera. These peptides encompass 6–13 residues and have a highly conserved *C*-terminal pentapeptide sequence with the general formula FX_1X_2WGamide, where X_1 is S, H, N, Y and X_2 is S or P (Table 1) [7]. Myokinins are multifunctional neuropeptides expressing myotropic and potent diuretic activities. They stimulate Malpighian tubules, organs which are involved in the regulation of salt and water balance, with EC_{50} values in the range of 10^{-11}–10^{-12} M and act synergistically on the larger corticotrophin releasing factor (CRF)-related diuretic hormones in insects [8–11]. The helicokinins inhibit weight gain and increase mortality after injection into larvae of *Heliothis virescens*, a serious agricultural pest. Furthermore, the *in vivo* activity of helicokinin I increases significantly following addition of ACE inhibitors, such as captopril or enalapril, which suppress the proteolytic degradation of the kinins [12,13]. Their short sequences and interference with vital physiological processes render the myokinins prime candidates for the design of metabolically stable peptidomimetics.

Unfortunately, until now only a limited number of structure-activity data of economically important pest insect neuropeptides has been published [14,15]. Most of the available data were obtained from organ-assays, such as the Malphigian tubule or cockroach hindgut assay. In these test systems it remains unclear whether a neuropeptide or a derivative addresses specifically only one receptor or a family of closely related receptors. Furthermore, a general toxicity of unnatural neuropeptide-mimics may pretend a specific activity in *in vivo* assays.

We detail here for the first time a comprehensive receptor assay guided structure-activity study of helicokinin insect neuropeptides and first peptidomimetic analogues. For selected compounds the receptor assay data are compared with a *H. virescens* Malphigian tubule assay [13].

Materials and Methods

Materials

Solvents, reagents and amino acids were purchased from commercial sources. The Rink-amide and 2-chlorotrityl resin were purchased from Novabiochem. DMF, piperidine, TEA and DIPEA were dried over CaH_2 and distilled prior to use. Methanol was distilled from magnesium. Chloroform and DCM were dried by passing the solvents through a basic aluminium oxide filled column. Precoated plates (silica gel 60 F_{254}, 250 µm, Merck, Darmstadt, Germany) were used for TLC. Flash-chromatography was performed on an Argonaut Flash Master Personal system

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Table 1. Selected myokinins from various insect species

Sequence	Name	Insect species	
SGAD**F**YP**WG**amide	Achetakinin I	*Acheta domesticus*	Cricket
NSKYVSKQK**F**YS**WG**amide	Aedeskinin I	*Aedes aegypti*	Mosquito
NP**F**HS**WG**amide	Culekinin I	*Culex salinarius*	Mosquito
Y**F**SP**WG**amide	Helicokinin I	*Heliothis zea*	Moth
VR**F**SP**WG**amide	Helicokinin II	–	–
KVK**F**SA**WG**amide	Helicokinin III	–	–
DPA**F**NS**WG**amide	Leucokinin I	*Leucophaea maderae*	Cockroach
A**F**SS**WG**amide	Locustakinin	*Locusta migratoria*	Locust

with silica gel 0.04–0.063 mm from Macherey-Nagel. HPLC-MS was performed on a Varian 500 IonTrap LC-ESI-MS system (column: 5 µm RP18). High-resolution MS were measured on a Bruker MicroTOF. A Gilson HPLC system with UV detection (column: 2.3 × 25 cm, 10 µm RP18) was used for preparative HPLC separations. ^{1}H and ^{13}C NMR spectra were recorded at 25 °C on a Bruker ARX400 (400 MHz) spectrometer using tetramethylsilane as internal standard.

Chemistry

Linear peptide amides

Rink-amide resin (0.68 mmol g^{-1}) was swollen in DMF (10 ml g^{-1}) for 15 min. Then the excess of solvent was removed. The Fmoc protecting group was cleaved by two treatments with 30% piperidine in DMF for 15 min each. The amino acids were introduced by double couplings (2 × 10 h) using a 2.5-fold excess of Fmoc-amino acids and PyBOP and a 5-fold excess of DIPEA in DMF. HATU (2.5 eq.) was used as coupling reagent for *N*-methyl amino acids. After each coupling step and Fmoc cleavage the resin was washed with DMF (5×), MeOH (3×) and DCM (5×). Completeness of the coupling steps were controlled by using the chloroanil-test. In the final peptide-cleavage step the dried resin was treated for 2 h with TFA : triisopropylsilane (TIPS) : H_2O 95 : 2.5 : 2.5. The resin was filtered, treated for another 2 h with the cleavage mixture and washed with DCM (5×), The filtrates were combined, evaporated, dissolved in MeCN/water and lyophilized. The crude products were purified by preparative HPLC and characterized by HPLC-MS.

Linear side-chain protected peptide-acids

2-Chlorotritylchloride resin (1.3 mmol g^{-1}) was swollen in DMF (10 ml g^{-1} resin) for 30 min. After removal of excess solvent the resin was treated with 5 equivalents of the first amino acid and 10 equivalents of DIPEA in DMF (10 ml g^{-1} resin, 3 h) followed by filtration and washing with DMF (1 × 10 ml g^{-1} resin). The coupling step was repeated once. Unreacted chlorine-atoms were quenched (30 min) with a solution of DIPEA : MeOH : DCM 1 : 1 : 5 (10 ml g^{-1} resin). The resin was thoroughly washed with DMF (5×), MeOH (3×) and DCM (5×) and dried in vacuo. The resin was swollen again in DMF (10 ml g^{-1} resin, 30 min) followed by removal of the Fmoc-group (30% piperidine in DMF, 15 min, 2×). Subsequent amino acids were introduced by double couplings (2 × 10 h) using 2.5 equivalents of the Fmoc-amino acids and PyBOP and 5 equivalents of DIPEA in DMF (10 ml g^{-1} resin). After each coupling- and Fmoc-cleavage step the resin was washed with DMF (5×), MeOH (3×) and DCM (5×). Completeness of the coupling steps were controlled by the chloroanil-test. The side-chain protected peptides were cleaved from the dried resin after swelling (DCM, 30 min) with AcOH : TFE : DCM 1 : 1 : 3 (2×, 2 h each). The cleavage- and washing-solutions (TFE : DCM 1 : 4, 5×) were combined, evaporated, resolved in water and lyophilized. The crude product was characterized by HPLC-ESI-MS. Tyr(*t*Bu)-Phe-Ser(*t*Bu)-Pro-Trp-Gly-OH: MS 868.6 $[M + H]^{+}$; 91% (HPLC).

C-terminal monomethyl and dimethyl peptide amides 23 an 24

DIPEA (60 eq.) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC)*HCl (3 eq.) were dissolved in chloroform (2 ml mg^{-1} peptide). Methylamine hydrochloride (45 eq.) or dimethylamine hydrochloride (45 eq.) were added. The linear side-chain protected peptide-acid (Tyr(*t*Bu)-Phe-Ser(*t*Bu)-Pro-Trp-Gly-OH), dissolved in chloroform (0.8 ml mg^{-1} peptide), was added dropwise to this mixture within 2 h. After stirring at room temperature for 24 h the mixture was washed with water (1.2 ml mg^{-1} peptide) followed by evaporation of chloroform. The side-chain protecting groups were removed with TFA : TIPS : H_2O 95 : 2.5 : 2.5 (10 ml 100 mg^{-1} peptide, 2 h, 0 °C). TFA was evaporated, the residue dissolved in H_2O : MeCN 1 : 1 and lyophilized. The crude products **23** and **24** were purified by preparative HPLC and characterized by HPLC-ESI-MS.

Cycohelicokinin I (4)

A mixture of TBTU : HOBt : DIPEA 3 : 3 : 5 equivalents was dissolved in DCM (0.5 ml mg^{-1} peptide, 2% DMF). The linear side-chain protected peptide Tyr(*t*Bu)-Phe-Ser(*t*Bu)-Pro-Trp-Gly-OH dissolved in DCM (1 ml mg^{-1} peptide) was added dropwise to this mixture within 2 h. The progress of the reaction was checked by HPLC. After 4 h the reaction mixture was washed with 0.1 M HCl and evaporated. The side-chain protecting groups were removed with TFA : TIPS : H_2O 95 : 2.5 : 2.5 (10 ml 100 mg^{-1} peptide, 2 h, 0 °C). TFA was evaporated, the residue dissolved in H_2O : MeCN 1 : 1 and lyophilized. The crude product **4** was purified by preparative HPLC and characterized by HPLC-ESI-MS.

Receptor assay

The helicokinin receptor (HKR) of *H. virescens* was cloned from a Malpighian tubule cDNA library and showed about 50% homology (amino acids) to the leucokinin receptor from *Lymnea stagnalis* [16,17]. The HKR was functionally expressed in a CHO line. The activation of the HKR was analysed in living cells by measuring the induced calcium ion flux in the cytosol after activation of the second messenger cascade via G-proteins [18,19]. Calcium

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ion dyes were obtained from Molecular Devices and were used according to the supplier's protocol. Cells were plated in 384-well plates (4×10^3 cells per well) and incubated over night (37 °C, 5% CO_2). Cells were removed from the incubator, allowed to reach room temperature over the course of 10 min, and then washed with 50 µl of Hanks balanced salt solution (HBSS). Subsequently, the medium was replaced with 50 µl of calcium ion dye solution in HBSS, and cells were loaded for 1 h in the dark. Then, the plates were read on a Flexstation fluorescence plate reader (Molecular Devices). Excitation and emission wavelengths were set to 485 and 525 nm, respectively. Measurements were made every 2.7 s intervals for 100 s. Basal fluorescence was determined for 15–30 s, followed by addition of 25 µl test compounds (to assess agonist activity). Helicokinins I or II were used as standards and were tested in four independent experiments for evaluating EC_{50} values. Standard deviations were 2 nM for helicokinin I, 3 nM for helicokinin II and 3 nM for helicokinin III. EC_{50} values were calculated after concentration-dependent induction of calcium flux analysed in a Flexstation (Molecular Devices). Finally, EC_{50}-values were calculated as F_{max} (maximal agonist signal) minus F_{min} (baseline) according to Softmax-Pro 5.0 Software (Molecular Devices). All tests compounds were measured in two independent experiments in ten dilution steps (in duplicates) from 0.5 to 10 µM.

Results

All peptides were synthesized on solid phase using the Fmoc protecting group strategy and standard coupling reagents. Removals from the resins were accomplished in one step with the TFA/TIPS reagent or with AcOH/TFE. All peptides were purified by semi-preparative reverse-phase HPLC and characterized by HPLC-MS-ESI.

All three helicokinins stimulated fluid secretion (Ramsay assay) in a dose-dependent manner over a range from 10^{-12} to 10^{-7} M with EC_{50} concentrations of 2.9×10^{-11} M (helicokinin I, **1**), 2.0×10^{-11} M (helicokinin II, **2**) and 3.6×10^{-10} M (helicokinin III, **3**). At 10^{-9} M the kinins increased fluid secretion by 410% (helicokinin I), 354% (helicokinin II) and 207% (helicokinin III). The highest rates of secretion were induced with concentrations of 10^{-8} M with increases of 6.1 times for helicokinin I, 5.3 times for helicokinin II and 4.2 times for helicokinin III [13]. In good accordance with the Ramsay assay the receptor data proved helicokinin I the most active peptide, followed by helicokinin II and helicokinin III which were slightly less active. Remarkably, cyclohelicokinin I (**4**) did not show any receptor activation but significantly increased fluid secretion in Malpighian tubules comparable to helicokinin III (Table 2).

As already established for other *C*-terminal kinin pentapeptides, helicokinin pentapeptide **5** showed only a slightly reduced secretion rate in the Malphigian tubule assay [20]. A significantly lower activity (factor 120) was detected in the receptor assay. Shorter sequences such as the tetrapeptide **11** or deletion sequences (peptides **6**–**11**) were found to be completely inactive.

Alanine scans of biologically active peptides provide evidence on the significance of the amino acid side chains without destroying the chirality or increasing the flexibility of the peptide. Again, the Ramsay assay and receptor assay data were in good to reasonable agreement. In both biological tests the amino acids Gly, Trp and Phe, shared by most members of the kinins, were shown to be essential for maintaining high levels of activity, in contrast to the side chains of Pro, Ser and Tyr which are uncritical for diuretic activity as well as receptor binding (Table 2). The significance of the Trp^5 is underlined by the inactive phenylalanine analogue **36**.

A D-amino acid scan was performed in order to deduce the influence of the amino acid chirality, which besides receptor binding also determines the formation of potential secondary structures. Except **22**, the D-Tyr analogue of helicokinin I, all other D-amino acid analogues showed a considerably reduced receptor activation by at least a factor of 200 (D-Trp analogue **18**). The D-Pro analogue **19**, the D-Ser analogue **20** and in particular the D-Phe analogue **21** were almost inactive (Table 2).

A *N*-methyl amino acid scan revealed the importance of the *C*-terminal amide function as hydrogen bond donor. Mono-methylation reduced the receptor activity already by a factor of around 300, bis-methylation caused a complete loss of activity (Table 2). Methylation of the glycine- (**25**) and tryptophan-residue (**26**) yielded inactive compounds. In contrast, the *N*-methyl serine- (**27**) and in particular the phenylalanine analogue (**28**) showed a distinct receptor activation albeit on a level reduced by a factor of 200 compared to helicokinin I (**2**). Mono-methylation of the *N*-terminal tyrosine amino group (**29**) did not show any effect on receptor activation which again confirms the *N*-terminal tyrosine as the most uncritical amino acid in helicokinin I.

As a first step towards neuropeptide mimetics two sets of compounds were synthesized. In the first set the lipophilic *N*-terminal residues was replaced by Dppa (1,2-diphenylpropanoic acid, peptide **30**) as well as the fluorescent tyrosine analogues Alb (β-(4′-hydroxy-2′-benzoyl)alanine, peptide **31**) and the tryptophan analogue Ald (Aladan, peptide **32**) [21]. In the second set the central dipeptide Pro–Ser was exchanged either by the more flexible spacers Acapa (aminocaproic acid, peptide **33**) and Gly–Gly (peptide **34**) or by the conformationally restricted Acyca (aminocyclohexanoic acid, peptide **35**) residue. Helicokinin I analogue **30** containing the branched 1,2-diphenylpropanoic acid expressed a weak but significant receptor activation (EC_{50} = 10 µM) whereas simple linear lipophilic carboxylic acids at this position turned out to be inactive. As expected, strong receptor activation (EC_{50} = 32 nM) was found for the helicokinin I analogue **31** in which tyrosine was replaced by the structurally closely related Alb residue. Replacement of tyrosine by the fluorescent tryptophan analogue Ald (**32**, EC_{50} = 390 nM) reduced the activity further by a factor of more than ten compared with the ketotyrosine **31**.

Discussion

Short sequences and high myotropic as well as diuretic activities render the kinins promising lead structures for the development of neuropeptide based insecticides. In general, biological activities measured in a Malphigian tubule assay and receptor assay were in good accordance and demonstrate helicokinin I (**1**) the most active peptide, followed by helicokinin II (**2**) and helicokinin III (**3**). Remarkable discrepancies were only found for the truncated pentapeptide **5** and cyclohelicokinin **4**.

Pentapeptide Phe-Ser-Pro-Trp-Gly-$CONH_2$ (**5**) induced only a slightly reduced fluid secretion in the Malphigian tubules in agreement with published data on truncated kinins from other insect species [20]. However, the activity in the receptor assay was reduced by a factor of more than 100 indicating that the *N*-terminal tyrosine and thus the complete hexapeptide is essential for full receptor activation. An even more distinct discrepancy was found for cyclohelicokinin I (**4**) which was completely inactive at the receptor but showed activity in the tubule assay in the

Table 2. Receptor and Ramsay assay data for helicokinins and analogues

Compound	Sequence	Ramsay assay Secretion (nl min^{-1}, 10^{-8} M)	Receptor assay EC_{50} (nM)	MS $[M + H]^+$	Purity HPLC (%)
Helicokinin I (**1**)	Tyr-Phe-Ser-Pro-Trp-Gly-$CONH_2$	7.3	2	755.0	99
Helicokinin II (**2**)	Val-Arg-Phe-Ser-Pro-Trp-Gly-$CONH_2$	6.2	5	848.0	99
Helicokinin III (**3**)	Lys-Val-Lys-Phe-Ser-Ala-Trp-Gly-$CONH_2$	4.8	5	921.0	99
Cyclohelicokinin I (**4**)	cyclo[Tyr-Phe-Ser-Pro-Trp-Gly]	4.2	Inactive	738.0	97
Truncated analogues					
5	Phe-Ser-Pro-Trp-Gly-$CONH_2$	5.2	240	592.4	99
6	Tyr-Ser-Pro-Trp-Gly-$CONH_2$	n.d.	Inactive	608.3	96
7	Tyr-Phe-Pro-Trp-Gly-$CONH_2$	n.d.	Inactive	668.5	99
8	Tyr-Phe-Ser-Trp-Gly-$CONH_2$	n.d.	Inactive	658.4	95
9	Tyr-Phe-Ser-Pro-Gly-$CONH_2$	n.d.	Inactive	569.3	95
10	Tyr-Phe-Ser-Pro-Trp-$CONH_2$	n.d.	Inactive	698.5	96
11	Tyr-Phe-Ser-Pro-$CONH_2$	1.2	Inactive	512.3	99
Alanine scan					
12	Tyr-Phe-Ser-Pro-Trp-***Ala***-$CONH_2$	0.8	Inactive	769.0	100
13	Tyr-Phe-Ser-Pro-***Ala***-Gly-$CONH_2$	2.1	50	641.0	99
14	Tyr-Phe-Ser-***Ala***-Trp-Gly-$CONH_2$	6.0	3	730.0	99
15	Tyr-Phe-***Ala***-Pro-Trp-Gly-$CONH_2$	4.6	2	740.0	100
16	Tyr-***Ala***-Ser-Pro-Trp-Gly-$CONH_2$	1.3	Inactive	679.0	99
17	***Ala***-Phe-Ser-Pro-Trp-Gly-$CONH_2$	5.5	3	664.0	100
D-*Amino acid scan*					
18	Tyr-Phe-Ser-Pro-***D-Trp***-Gly-$CONH_2$	n.d.	490	755.1	96
19	Tyr-Phe-Ser-***D-Pro***-Trp-Gly-$CONH_2$	n.d.	2000	755.3	98
20	Tyr-Phe-***D-Ser***-Pro-Trp-Gly-$CONH_2$	n.d.	1400	755.3	96
21	Tyr-***D-Phe***-Ser-Pro-Trp-Gly-$CONH_2$	n.d.	3200	755.3	98
22	***D-Tyr***-Phe-Ser-Pro-Trp-Gly-$CONH_2$	n.d.	5	755.3	97
N-methyl amino acid scan					
23	Tyr-Phe-Ser-Pro-Trp-Gly-***CONHMe***	n.d.	630	769.6	96
24	Tyr-Phe-Ser-Pro-Trp-Gly-***CONMe***$_2$	n.d.	Inactive	783.6	94
25	Tyr-Phe-Ser-Pro-Trp-***NMeGly***-$CONH_2$	n.d.	Inactive	769.3	96
26	Tyr-Phe-Ser-Pro-***NMeTrp***-Gly-$CONH_2$	n.d.	Inactive	769.5	88
27	Tyr-Phe-***NMeSer***-Pro-Trp-Gly-$CONH_2$	n.d.	425	769.3	88
28	Tyr-***NMePhe***-Ser-Pro-Trp-Gly-$CONH_2$	n.d.	380	769.4	92
29	***MeHNTyr***-Phe-Ser-Pro-Trp-Gly-$CONH_2$	n.d.	3	769.4	85
Miscellaneous replacements					
30	***Dppa***-Tyr-Phe-Ser-Pro-$CONH_2$	n.d.	10.000	703.5	96
31	***Alb***-Phe-Ser-Pro-Trp-Gly-$CONH_2$	n.d.	32	783.3	91
32	***Ald***-Phe-Ser-Pro-Trp-Gly-$CONH_2$	n.d.	390	837.4	92
33	Tyr-Phe-***Acapa***-Trp-Gly-$CONH_2$	n.d.	Inactive	670.4	96
34	Tyr-Phe-***Gly-Gly***-Trp-Gly-$CONH_2$	n.d.	1300	685.4	86
35	Tyr-Phe-***Acyca***-Trp-Gly-$CONH_2$	n.d.	Inactive	696.3	92
36	Tyr-Phe-Ser-Pro-***Phe***-Gly-$CONH_2$	n.d.	Inactive	716.3	90

range of helicokinin III. Impurities of helicokinin I from the solid phase synthesis can be excluded, since the linear precursor was cleaved from 2-chlorotrityl resin as the free carboxylic acid which was then cyclized in solution. For both mammalian and insect neuropeptides it is well known that the free *C*-terminal acids are inactive [22,23]. An enzymatic cleavage of cyclohelicokinin I (**4**) in the Malpighian tubules to helicokinin I appears unlikely, too. A plausible explanation for this effect might be that cyclohelicokinin I addresses different diuretic receptors in the Malpighian tubules.

In agreement with already published structure-activity data of kinins [20] an alanine scan established Phe^2 and Gly^6CONH_2 as the most important residues in helicokinin I followed by Trp^5, which shows a reduced receptor activation by a factor of 25–30. The significance of the indole side-chain of tryptophan is underlined by the inactive phenylalanine analogue **36**. Noteworthy, the side chains of Ser^3 and Pro^4 are unimportant for receptor activation and thus appear to function as a kind of spacer between the critical *C*-terminal dipeptide Trp^5–Gly^6CONH_2 and Phe [2].

A D-amino acid scan produced a more distinct picture, revealing that the stereochemistry of the amino acids, except Tyr^1, is more critical for receptor activation. A D-Trp in position 5 (**18**) reduced receptor activation by a factor of 160 while the D-Phe^2

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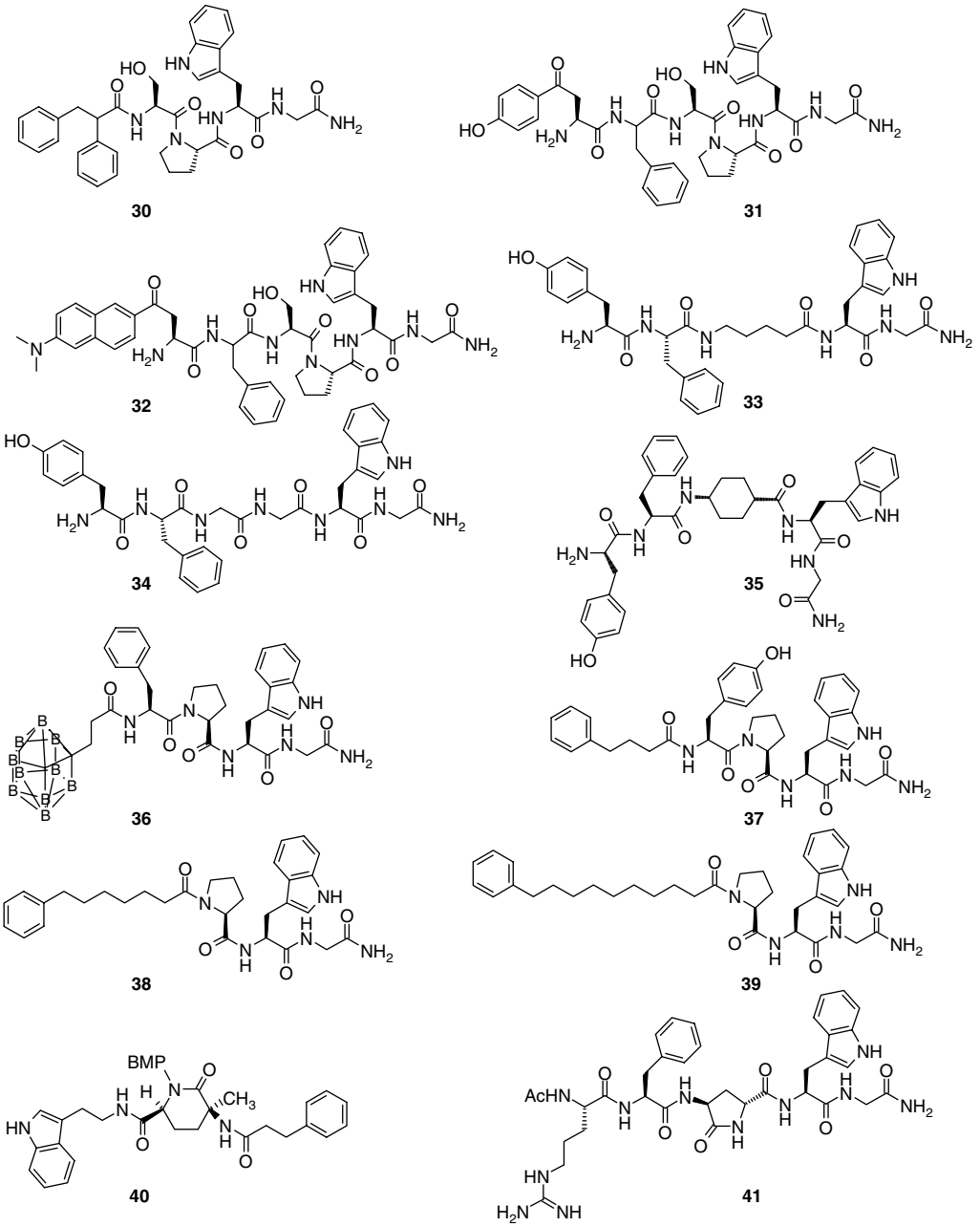

Figure 1. Kinin analogues with peptidomimetic replacements.

analogue lost activity by a factor of around 1000. Noteworthy, the stereochemistry of Ser[3] and Pro[4] turned out to be much more important for receptor activation than the nature of the side-chain. Based on NMR measurements in aqueous solutions a *C*-terminal β-turn was found for several kinins [20] and has been suggested to be essential for receptor binding. D-amino acids are well known for their turn-inducing effects and are expected to shift or to destabilize the relevant *C*-terminal β-turn in positions 3 and 4 resulting in a considerable loss of activity. Further evidence for a specific *C*-terminal conformation in helicokinin I arises from the N-methylamino acid scan. N-methylation of those amino acids (Gly[6], Trp[5] and Ser[4]) involved in the formation of the postulated β-turn resulted either in a complete or at least a major loss of receptor activity by preventing the formation of β-turn stabilizing hydrogen bonds. Presumably, the *C*-terminal β-turn formed in water is preserved when helicokinin I is recognized and bound to its receptor. Unfortunately, until now no receptor-bound conformation of an insect neuropeptide is available which would finally prove the *C*-terminal β-turn hypothesis.

According to the Ala- and D-amino acid scans neither the stereochemistry nor the side chain of Tyr[1] are essential. However for full receptor activation Tyr[1] needs to be present in the peptide. Obviously, the *N*-terminal amino group must be located in an appropriate position and distance to the *C*-terminus to be able to interact with the receptor. Further insight into the functional role of tyrosine came from derivatives **31** and **32**, containing the amino acids Alb and Ald instead of tyrosine, respectively. Only a slightly reduced receptor activity was observed for the structurally similar keto tyrosine derivative **31** whereas the keto tryptophan analogue **32** was found to be less active by a factor of more than

100, indicating limited space in the binding-pocket of the tyrosine side-chain (Figure 1).

Despite of its critical role it has been shown that a carboranyl-alanine is able to replace the *N*-terminal phenylalanine in the achetakinin (*Acheta domesticus*) ''superagonist'' Phe-Phe-Pro-Trp-Gly-$CONH_2$. The carboranyl analogue **36** had an EC_{50} of 0.2 nM in a cricket Malpighian tubule fluid secretion assay, which corresponds to 60% of the full diuretic activity [24]. A considerable myotropic activity was also reported for the achetakinin (Phe-Tyr-Pro-Trp-Gly-$CONH_2$) analogue **37** in a cockroach (*Leucophaea maderae*) hindgut assay [25]. Helicokinin I analogue **30** with a simple 2,3-diphenylpropanoic residue as a lipophilic substitute for the *N*-terminal Tyr–Phe still expressed a significant HKR activation in the micromolar range indicating that the replacement of the *N*-terminal dipeptide is possible, too. More elaborated Tyr–Phe mimics are expected to retain a considerably higher level of receptor activity.

To further elaborate the function of the central amino acids, Ser^3 and Pro^4 were replaced by residues with different conformational flexibility (peptides **33**, **34** and**35**). Only the Gly-Gly-derivative **34** caused a weak receptor activation ($EC_{50} = 1300$ nM), emphasizing the importance of chirality in the α-positions of the residues in positions 3 and 4. Additional removal of the central amide bond in **33** resulted in a complete loss of activity. In contrast, achetakinin analogues **38** and **39** in which the inner amino acids were replaced by 7-phenylheptanoic acid and 10-phenyldecanoic acid still had a considerable myotropic activity in a cockroach hindgut assay [25]. The inactivity of the aminocyclohexanoic derivative **35** can be explained by the *cis*-arrangement of the amino- and carboxy-function which induces a bow-shaped conformation that is considerably different from the proposed *C*-terminal β-turn structure of helicokinin I. Recently, a complete non-peptidic kinin-analogue (**40**) containing a *cis*-piperidinone scaffold and mimics for the side chains of the critical phenylalanine and tryptophan was synthesized by Borggraeve [26]. All analogues showed only modest activities in a diuretic Malpighian tubule assay. Together with our results it can be concluded that scaffolds with a *cis*-arrangement of substituents are in principle unsuited to mimic the inner amino acids Ser–Pro in the kinins. This hypothesis is corroborated by achetakinin analogue **41** which has a trans-substitued pyroglutamate as a mimic for proline. Compared to the parent pentapeptide ($EC_{50} = 0.57$ µM) the kinin-analogue **41** had almost full activity both on a recombinant kinin receptor of the southern cattle tick *Boophilus microplus* and in a cricket diuretic assay ($EC_{50} = 0.7 \times 10^{-8}$ M) [27].

A frequently used peptidomimetic strategy comprises the synthesis of cyclic peptide analogues as scaffolds for the design of non-peptidic drugs [28,29]. This approach appears problematic for the helicokinins, since the *N*-methylamino acid scan clearly revealed the significance of the unsubstituted *C*-terminal amide group as well as the internal amide hydrogens. Even mono-methylation of the *C*-terminal amide (peptide **23**) caused a reduction in receptor activation by a factor of 200. Corresponding results were reported by Roberts [20] for the cyclic *C*-terminal analogue cyclo-(Phe-Phe-Pro-Trp-Gly-Ala) of the cricket neuropeptide achetakinin. The activities found in a diuretic cricket Malphigian tubule assay and in a myotropic assay on isolated cockroach (*Leucophaea maderae*) hindguts were drastically reduced compared to the linear *C*-terminal pentapeptide. As a consequence, any type of end-to-end cyclizations or backbone-to-end cylizations of helicokinins are expected to afford weak or inactive derivatives. According to our structure-activity data it becomes evident that the only attachment points for cyclic helicokinin analogues are the serine and tyrosine hydroxy groups as well as the *N*-terminal amino group.

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