Fluorescent Analogues of the Insect Neuropeptide Helicokinin I: Synthesis, Photophysical Characterization and Biological Activity

Heru Chen[a], Jürgen Scherkenbeck[a,*], Tino Zdobinsky[a] and Horst Antonicek[b]

[a]*Fachgruppe Chemie, Bergische Universität Wuppertal, Gaußstraße 20, D-42119 Wuppertal, Germany;* [b]*Bayer CropScience AG, Alfred-Nobel-Straße 50, D-40789 Monheim, Germany*

Abstract: In insects numerous physiological processes are regulated by neuropeptides. Two fluorescent analogues of the amino acids tryptophan and tyrosine were synthesized and incorporated in the diuretic neuropeptide helicokinin I from the moth *Heliothis zea*. By fluorescence emission measurements it was shown that both fluorescent helicokinin I analogues react sensitive on the dielectricity of their microenvironment. A helicokinin I analogue containing the fluorescent tryptophan mimic *β*-[6'-(*N*,*N*-dimethyl)-amino-2'-naphthoyl]alanine (Ald) was shown to bind to dodecylphosphocholine (DPC) micelles by the Ald residue. A membrane binding model for helicokinin I is proposed based on data from related mammalian and insect-neuropeptides.

Keywords: Insect neuropeptides, helicokinin, fluorescent amino acids, micelles, peptides.

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 [1,2]. Peptide messengers exert their biological functions via specific signal-transduction membrane receptors [3,4]. Thus, the receptors of insect-neuropeptides represent promising targets for a novel generation of selective and environmentally beneficial insecticides. Agonists or antagonists, acting on these receptors may interfere with the development, growth, behaviour and homeostasis of insects [5,6]. The regulation of water balance is a crucial aspect of homeostasis in terrestrial insects involving excretion of excess water via the Malphigian tubules and resorption in posterior regions of the hindgut. The process of urine production has been shown to be under endocrine control, primarily by corticotrophin-releasing-factor (CRF) related peptides and kinins, also called leucokinins or myokinins [7,8]. In particular the helicokinins I-III from the moth *H. zea* have been shown to stimulate fluid secretion in isolated Malphigian tubules *in vitro* and to increase mortality *in vivo* following injection of helicokinin I (Tyr-Phe-Ser-Pro-Trp-Gly-NH_2) into the haemolymph of *Heliothis virescens* larvae [9]. Their short sequences and potent diuretic effects render the kinins prime candidates for the peptidomimetic design of metabolically stable neuropeptide based insect control agents.

Fluorescent probes positioned in the pharmacophoric domain of a peptide can provide important insights into the molecular basis of membrane and receptor binding because the fluorescence properties are influenced by the polarity of the microenvironment [10,11]. Neuropeptides react very sensitive on structural modifications at the N- and in particular at the C-terminus. Thus, the attachment of a linker coupled fluorescent dye is expected to result in a complete loss of biological activity. A more promising strategy appears to be the replacement of a natural amino acid by a structurally related fluorescent amino acid analogue [12]. Examples of small extrinsic fluorophores are the 1-dimethylamino-naphthalene-5-sulfonyl (dansyl) group, the anthraniloyl group or the 6-dimethyl-amino-2-acyl-naphthalene (DAN) group. DAN is an environmentally sensitive probe and has recently been incorporated into the side chain of L-alanine, resulting in the fluorescent amino acid aladan (Ald, **1**) [13,14]. Both the fluorescence quantum yield and the fluorescence emission maximum of aladan (**1**) are exceptionally sensitive to the polarity of its environment, ranging from 409 nm in heptane to 542 nm in water. Another fluorescent amino acid (Fig. **1**), carrying the *β*-(4'-hydroxy-2'-benzoyl)alanine (Alb, **2**) residue in the side-chain has recently been described, unfortunately without any details on its dielectric sensitivity [14]. Structure activity studies of helicokinin I revealed Tyr^1 the most flexible residue with respect to structural modifications [15]. Thus Alb (**2**) is supposed to be a valuable replacement for Tyr^1 and Ald (**1**) for the critical Trp^5 residue in helicokinin I. Following the 'Schwyzer model' lipophilic peptides interact with the cell membrane prior to receptor-binding [16-18]. Thus, dielectric sensitive fluorescence probes such as the helicokinin analogues **8** and **9** should be useful to identify the amino acids, which anchor a peptide in the cell membrane.

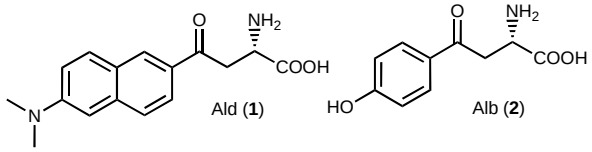

Figure 1. Fluorescent analogues of tryptophan and tyrosine.

*Address correspondence to this author at the Fachgruppe Chemie, Bergische Universität Wuppertal, Gaußstraße 20, D-42119 Wuppertal, Germany; E-mail: scherkenbeck@uni-wuppertal.de

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MATERIALS AND METHODS

General

Solvents, reagents, amino acids (Bachem) and TentaGel S RAM resin (Fluka) were purchased from commercial sources. Dodecylphosphoryl-choline was synthesized according to literature [19]. The concentrations of SDS and DPC were either 0.4 or 0.8 wt% in 20 nmol L^{-1} Hepes-HCl buffer, pH 6.5 [20]. HPLC was performed on a Varian 500 IonTrap LC-ESI-MS system (column: 5 µm RP18). For preparative HPLC separations a Gilson HPLC system with UV detection (column: 2.3x25 cm, 10 µm RP18) was used. ^{1}H and ^{13}C NMR spectra were recorded at 25°C on a Bruker ARX400 (400 MHz) spectrometer using tetramethylsilane as internal standard. Fluorescence emission spectra were recorded on a Varian Fluorescence Spectrometer (parameter: excitation slit 5 nm; emission slit 5 nm; excitation filter in auto state; emission filter open). Water was deionized and distilled prior to use. Hepes sodium salt was purchased from AppliChem (Germany) and used directly. All fluorescence measurements were performed with 1×10^{-6} mol L^{-1} of [Alb^1]helicokinin (**8**) and [Ald^5]helicokinin I (**9**). Abbreviations: Alb, β-(4'-hydroxy-2'-benzoyl)alanine; Ald, β-[6'-(*N*,*N*-dimethyl)-amino-2'-naphthoyl]alanine; CRF, corticotrophin-releasing-factor; dansyl, 1-dimethyl-amino-naphthalene-5-sulfonyl; DAN, 6-dimethyl-amino-2-acyl-naphthalene; DCM, dichloromethane; DPC, dodecylphosphoryl-choline; DIEA, diisopropylethylamine; DMF, dimethylformamide; DMSO, dimethylsulfoxide; Fmoc, fluorenylmethoxycarbonyl; PyBOP, benzotriazol-1-yl-oxytripyrrolidinophosphonium-hexafluorophosphate; THF, tetrahydrofurane; TFA, trifluoro acetic acid; TIPS, triisopropylsilane.

General Procedure for the Synthesis of Helicokinin I Derivatives

TentaGel S RAM resin (0.565 g, 0.13 mmol) was filled in a syringe equipped with a filter and a stopper. The resin was dispensed twice with 5 ml of a 30% piperidine solution in DMF, shaken for 30 min each and then washed with dry DMF. 2 equivalents amino acid and coupling reagent (PyBOP / HOBt / DIPEA) were used for Fmoc-Gly-OH, Fmoc-Trp-OH, and Fmoc-Pro-OH and 3 equivalents for Fmoc-Ser(tBu)-OH, Fmoc-Phe-OH, and Fmoc-Tyr(tBu)-OH. Quantitative removal of the Fmoc protecting group was achieved with two treatments (15 min and 5 min) of the resin with a solution of piperidine in DMF (30%). The peptides were cleaved from the resin by adding a TFA / TIPS solution and shaking for 2 h. After evaporation of the TFA / TIPS reagent the residue was purified by reversed-phase preparative HPLC (flow rate: 3.6 mL min^{-1}, gradient: 10% CH_3CN to 40% CH_3CN within 40 min). The product containing fractions were lyophilized. [Alb^1]helicokinin I (**8**): LC-MS purity: 91% (220 nm); HR-ESI-MS: calcd for $C_{40}H_{46}N_8O_9$: 782.3461; found for $[M+H]^+$: 783.3471. [Ald^5]helicokinin I (**9**): LC-MS: purity 92% (220 nm); HR-ESI-MS: calcd for $C_{44}H_{52}N_8O_9$: 836.3930; found for $[M+H]^+$: 837.3955.

Receptor Assay

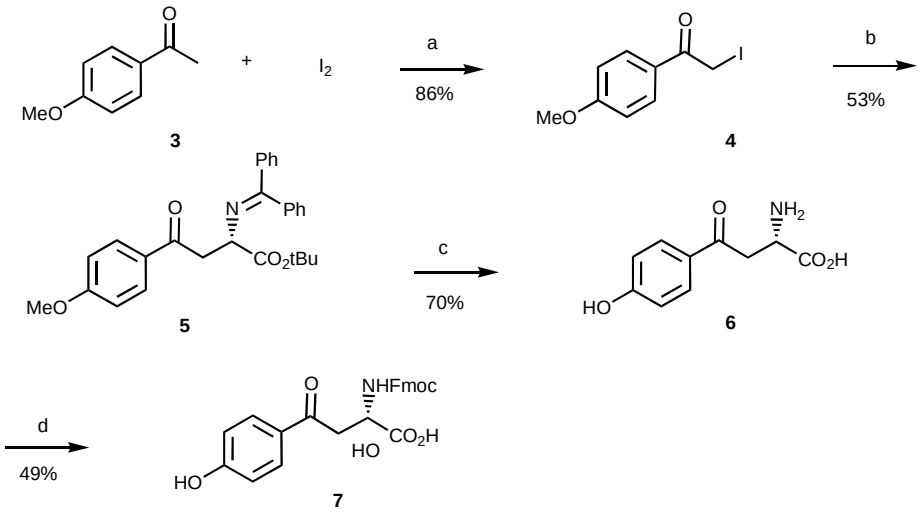
The helicokinin receptor of *Heliothis virescens* was cloned from a Malpighian tubule cDNA library and showed about 50% homology (amino acids) to the leucokinin receptor from *Lymnea stagnalis* [21, 22]. The helicokinin receptor (HKR) was functionally expressed in a CHO line. The activation of the HKR was analyzed in living cells by measuring the induced calcium ion flux in the cytosol after activation of the second messenger cascade via G-proteins [23, 24]. Calcium ion dyes were obtained from Molecular Devices and were used according to the supplier's protocol. Cells were plated in 384-well plates ($4x10^3$ 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 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 nm 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 replicates once for evaluating EC_{50} values. All test compounds were measured twice in ten dilution steps from 0.5 nM to 10 µM. EC_{50} values with test compounds and peptides were calculated after concentration-dependent induction of calcium flux analyzed in a Flexstation (Molecular Devices). Finally, EC_{50}-values were calculated as Fmax (maximal agonist signal) minus Fmin (baseline) according to Softmax-Pro 5.0 software (Molecular Devices).

RESULTS

The synthesis of Alb **2** (Scheme **1**) followed a route similar to the literature procedure used for the preparation of Ald **1** [13,14]. The overall yields and *ee* values strongly depended on the careful control of the reaction conditions. With cinchonidinium bromide as a chiral phase transfer catalyst e.e. values of 90% for Alb (**2**) and 92% for Ald (**1**) were obtained after a reaction time of 48 h at -90°C [25].

The two fluorescent peptides Alb-Phe-Ser-Pro-Trp-Gly-NH_2 **8** ([Alb^1]helicokinin I) and Tyr-Phe-Ser-Pro-Ald-Gly-NH_2 **9** ([Ald^5]helicokinin I) were prepared by solid phase synthesis based on a Fmoc/tButyl protecting group strategy. Twofold excesses of Fmoc amino acids and coupling reagents were employed for the amino acids Fmoc-Gly-OH, Fmoc-Trp-OH, and Fmoc-Pro-OH. Threefold excesses were used for the amino acids Fmoc-Ser(tBu)-OH, Fmoc-Phe-OH, and Fmoc-Tyr(tBu)-OH. Coupling times with PyBOP / DIEA were usually 2 h, depending on the monitoring results. Cleavage of the protecting groups and removal from the resin was accomplished in one step with the TFA / TIPS reagent (2 h, rt). The crude peptides were purified by preparative reverse-phase HPLC and characterized by LC-MS-ESI and HR-MS.

The maximum emission wavelength and fluorescence intensity of the peptides [Alb^1]helicokinin I (**8**) and [Ald^5]helicokinin I (**9**) were measured in different solvents (Table **1**). As expected, the fluorescence intensity was the lowest and the maximum emission wavelength (λ_{em}) was the largest for both peptides **8** and **9** in water and Hepes-buffer (Table **1**). With decreasing polarity of the solvent the fluo-



Scheme 1. Synthesis of Alb (**1**). (a) $LiN(SiMe_3)_2$ in dried THF, -78 °C, then addition of I_2. (b) $Ph_2CNCH_2CO_2{}^tBu$, cinchonidinium bromide, $CsOH{\cdot}H_2O$, DCM, -90 °C, 48 h. (c) 6 mol L^{-1} HI/HOAc, reflux, 6 h. (d) Fmoc-OSu, $NaHCO_3$, dioxane/H_2O (1:1), rt, 24 h.

Table 1. Dielectric constant (ε), Solvents, Fluorescence Intensity (a.u.) and Fluorescence Emission Maximum (λ_{em}) of [Alb[1]]helicokinin I (8) and [Ald[5]]helicokinin I (9). [Alb[1]]helicokinin I (8): λ_{ex} = 280 nm for all solvents except for toluene (240 nm). Concentration of the peptide: 1×10^{-6} mol L^{-1}. [Ald[5]]helicokinin I (9): λ_{ex} = 350 nm for all solvents. Concentration of the peptide: 1×10^{-6} mol L^{-1}.

Solvents	ε	[Alb[1]]helicokinin I (8)		[Ald[5]]helicokinin I (9)	
		λ_{em} (nm)	a.u.	λ_{em} (nm)	a.u.
toluene	2.38	295	17	457	743
ethyl acetate	6.02	344	64	463	945
1-octanol	17.5	299	37	482	755
methanol	32.7	346	28	506	515
DMF	36.7	339	65	472	840
DMSO	46.7	349	93	475	705
H_2O	80.2	358	18	532	88
20 mM Hepes-HCl (pH 6.5)	~80.2	358	8	532	36

rescence intensity increased (except toluene) and the maximum emission wavelength was blue-shifted. When the solvent was changed from DMF to DMSO a red-shift of 10 nm was observed for [Alb[1]]helicokinin (**8**) compared to only 3 nm for [Ald[5]]helicokinin I (**9**). [Alb[1]]helicokinin (**8**) showed a higher fluorescence intensity in DMSO whereas almost no differences were found for [Ald[5]]helicokinin I (**9**) in DMSO and DMF. The aprotic toluene and ethyl acetate caused a significant blue-shift of the maximum emission wavelength for both peptides **8** and **9**, respectively.

Measurements of the steady-state fluorescence of [Ald[5]]helicokinin **9** in 20 mM Hepes-HCl buffer (pH 6.5), 0.4 wt% DPC in 20 mM Hepes-HCl (pH 6.5), and 0.8 wt% DPC in 20 mM Hepes-HCl (pH 6.5) revealed a blue-shift of the fluorescence emission maximum wavelength from 533 nm in buffer solution to around 490 nm in 0.4 wt% DPC micelles and 0.8 wt% DPC micelles (Fig. **2**). Only a weak fluorescence was found for [Alb[1]]helicokinin **8** with a broad maximum emission wavelength identical to that in Hepes buffer.

All three helicokinins (Table **2**) activated the helicokinin receptor in the low nanomolar range (EC_{50}: 2-5 nM). The fluorescent analogue [Alb[1]]helicokinin **8** was found to be only slightly less active (EC_{50}: 32 nM) than helicokinin I. The replacement of the critical tryptophan residue by aladan (**9**) resulted in an almost complete loss of receptor activity.

DISCUSSION

In addition to NMR studies peptide analogues containing dielectric sensitive fluorescent probes can provide important information on peptide-lipid interactions and may be useful tools to identify peptide-receptors in living cells. Since in-

sect-neuropeptides are very sensitive to structural modifications, the conventional coupling of a fluorescent dye to the N- or C-terminus is expected to produce inactive peptide analogues. A different and less common strategy is based on fluorescent amino acid mimics. For helicokinin I it has been shown that Tyr^1 and to some extent also Trp^5 can be modified without immediate loss of activity [15]. In sharp contrast Phe^2 and Gly^6 tolerate almost no structural modifications [15]. Thus, the fluorescent insect neuropeptide analogues [Alb^1]helicokinin I (**8**) and [Ald^5]helicokinin I (**9**) have been synthesized and their fluorescence properties were studied in solvents of different polarities as well as in phospholipid-micelles as membrane mimics.

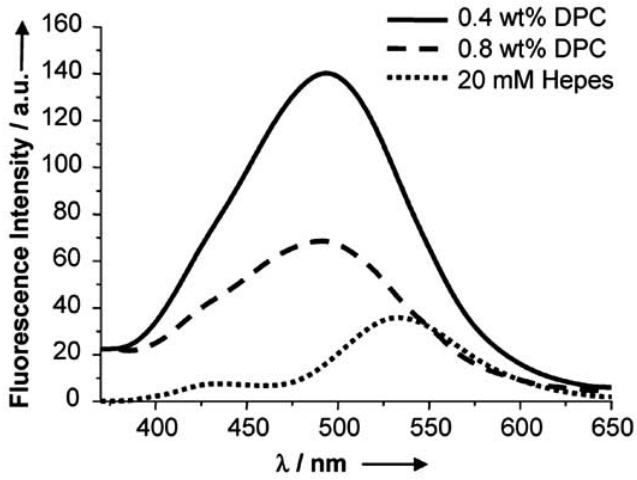


Figure 2. Fluorescence emission spectra of [Ald^5]helicokinin (**9**) in DPC micelles (λ_{ex} = 350 nm. Concentration of the peptide: 1×10^{-6} mol L^{-1}).

Table 2. Receptor Assay Data of Helicokinins I-III and Fluorescent Analogues 8 and 9

	Sequence	EC_{50} (nM)
Helicokinin I	Tyr-Phe-Ser-Pro-Trp-Gly-NH_2	2
Helicokinin II	Val-Arg-Phe-Ser-Pro-Trp-Gly-NH_2	5
Helicokinin III	Lys-Val-Lys-Phe-Ser-Ala-Trp-Gly-NH_2	5
Alb-helicokinin (**8**)	**Alb**-Phe-Ser-Pro-Trp-Gly-NH_2	32
Ald-helicokinin (**9**)	Tyr-Phe-Ser-Pro-**Ald**-Gly-NH_2	>10000

A profound blue-shift of the maximum emission wavelength and increase in fluorescence intensity (except toluene) is observed for both helicokinin analogues with increasing lipophilicity of the solvent. Noteworthy is the high dielectrical sensitivity of [Ald^5]helicokinin I (**9**) with blue-shifts of more than 100 nm between a water and a toluene environment. In case of [Ald^5]helicokinin I (**9**) the contribution of the tyrosine fluorescence emission is expected to be minimal at the wavelengths observed. Additionally the tyrosine emission maximum is relatively insensitive to solvent polarity changes. In contrast to [Ald^5]helicokinin I (**9**) the [Alb^1]helicokinin I (**8**) fluorescence reflects the contributions of both the tryptophan- and the Alb-residue, which is a considerably stronger chromophore than tyrosine. Compared to helicokinin I the fluorescence sensitivity of [Alb^1]helicokinin I (**8**) is higher and the fluorescence shifts in solvents of different polarities are more pronounced.

A significant red-shift of 10 nm for [Alb^1]helicokinin I (**8**) compared to only 3 nm for [Ald^5]helicokinin I (**9**) is observed when the solvent was changed from DMF to DMSO. For tyrosine and some of its derivatives it has been shown that a very strong interaction of DMSO with the phenolic hydroxy group causes substantial changes in the fluorescence emission wavelength as well as in the fluorescence intensity. The red-shift of the maximum emission wavelength in [Alb^1]helicokinin I (**8**) reflects the higher hydrogen-bond acceptor strength of DMSO compared to DMF. On the other side the dimethylamino group in the fluorophore **1** serves as a proton acceptor and thus no difference is expected in the interaction with the proton acceptors DMSO and DMF for [Ald^5]helicokinin I (**9**) [26].

Steady-state fluorescence measurements of [Ald^5]helicokinin I (**9**) in DPC micelles revealed a significant blue-shift of 43 nm compared to a Hepes-HCl buffer solution. With regard to pure solvents this blue-shift corresponds to 1-octanol, which is another well-known membrane model. These results demonstrate that the fluorophoric side chain of [Ald^5]helicokinin **9** sticks in the lipophilic core of the DPC micelles close to the phosphate head group.

A weak fluorescence detected for [Alb^1]helicokinin I (**8**) in DPC is identical with the maximum emission wavelength of [Alb^1]helicokinin I (**8**) in water or Hepes buffer. For a membrane embedded Alb residue a significantly higher fluorescence intensity and a blue-shift to around 300 nm similar to octanol would be expected. The low fluorescence of [Alb^1]helicokinin I (**8**) in DPC micelles may be caused by a partial quenching of the Alb fluorescence by phosphate groups in close proximity to the phenolic hydroxy group, according to a mechanism well known for tyrosine [27]. These findings suggest the fluorophoric side-chain of [Alb^1]helicokinin **8** to be exposed to the aqueous phase close to the zwitterionic headgroups of the DPC micelles.

The presence of a C-terminal β-turn in the insect kinins and its significance for receptor-binding has been discussed since a couple of years. For the leucokinin active core Asp^1-Pro^2-Gly^3-Phe^4-Ser^5-Ser^6-Trp^7-Gly^8-NH_2 a β-turn encompassing the C-terminal amino acids Phe^4 to Trp^7 was observed by molecular dynamics simulations. The essential Phe^4 and Trp^7 side-chains were shown to be located in close proximity on the same side of the turn [28]. Further conclusions can be drawn from a comparison of helicokinin I with the mammalian peptide hormone bradykinin (RPPG **FSPF**Ramide), which binds to an evolutionarily related GPCR. Bradykinin and helicokinin I share the amino acids Phe, Ser, Pro in the C-terminal positions 5, 4, 3 and both carry lipophilic residues (Trp and Phe) in position 2 (counted from the C-terminus for better comparison). The backbone-structure of bradykinin has recently been elucidated in the receptor-bound state and in empty DDM micelles by solid-state NMR spectroscopy [29]. As for membrane-bound helicokinin I the torsion angles of receptor-bound bradykinin are

consistent with a type-I β-turn. Bradykinin is anchored in the micelles mainly by the two lipophilic phenylalanine residues.

Taking all these findings into account helicokinin I is supposed to adopt a C-terminal β-turn by interaction with a membrane. The lipophilic amino acids tryptophan and phenylalanine which are located on the same side of the peptide backbone anchor the peptide in the membrane, while tyrosine points to the aqueous phase (Fig. **3**).

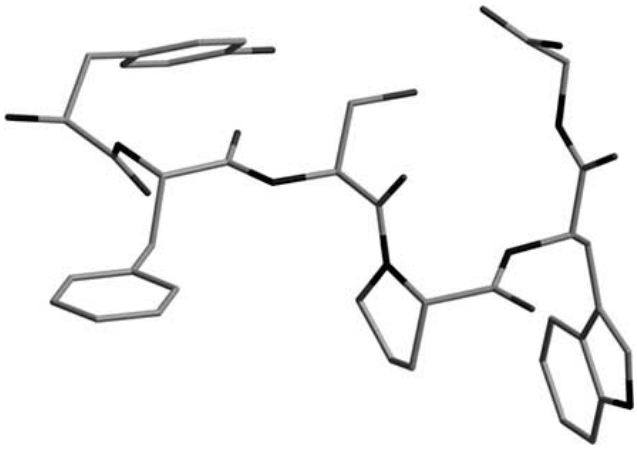


Figure 3. Model for the membrane-bound conformation of helicokinin I

In conclusion, a qualitative membrane-binding model of the insect neuropeptide helicokinin I has been suggested by fluorescence measurements of neuropeptide analogues containing dielectrically sensitive amino acid mimetics. Future work will focus on biologically highly active fluorescent tryptophan analogues as probes for the identification of helicokinin receptors in Malpighian tubule cells.

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