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The pro-apoptotic action of new analogs of the insect gonadoinhibiting peptide *Neb*-colloostatin: Synthesis and structure–activity studies

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ABSTRACT

Neb-colloostatin (SIVPLGLPVPIGPIVVGPR), an insect oostatic factor found in the ovaries of the flesh fly *Neobellieria bullata*, strongly induces apoptosis in insect haemocytes. To explain the role of Ser[1] and Pro[4] residues of *Neb*-colloostatin in the pro-apoptotic activity of this peptide, the synthesis of a series of analogs was performed, such as: [Ac-Ser[1]]- (1), [D-Ser[1]]- (2), [Thr[1]]- (3), [Asp[1]]- (4), [Glu[1]]- (5), [Gln[1]]- (6), [Ala[1]]- (7), [Val[1]]- (8), [D-Pro[4]]-(9), [Hyp[4]]- (10), [Acp[4]]- (11), [Ach[4]]- (12), [Ala[4]]- (13), [Ile[4]]- (14), and [Val[4]]- colloostatin (15). All peptides were bioassayed *in vivo* for the pro-apoptotic action on haemocytes of *Tenebrio molitor*. Additionally, the structural properties of *Neb*-colloostatin and its analogs were examined by the circular dichroism in water and methanol. Peptides 1, 4, 5, 7, 8, 10, 12, 14, and 15 strongly induce *T. molitor* haemocytes to undergo apoptosis and they show about 120–230% of the *Neb*-colloostatin activity at a dose of 1 nM. The CD conformational studies show that the investigated peptides seem to prefer the unordered conformation.

1. Introduction

Neb-colloostatin (SIVPLGLPVPIGPIVVGPR), an insect peptide hormone, has been isolated from the ovaries of the flesh fly *Neobellieria bullata* [2]. It is thought to be a degradation product of a collagen-like protein. It contains many hydrophobic amino acid residues: 3 Ile, 4 Val, 5 Pro, 2 Leu and 3 Gly. *Neb*-colloostatin exhibits a structural similarity to known vertebrate and invertebrate collagens, described in the literature [2]. In the peptide chain of *Neb*-colloostatin, like in collagen, characteristic tripeptide amino acid sequences $(Gly\text{-}X_1\text{-}X_2)_n$ are present, where X_1 and X_2 are Pro and Hyp, respectively, with Val instead of Gly at positions 3, 9, and 15. The largest structural analogy is observed for *Neb*-colloostatin and the 468–480 sequence of preprocollagen α1 (IV) present in the fruit fly *Drosophila melanogaster* [2].

The biological properties of *Neb*-colloostatin consist in inhibition of the ovarian development in the flesh fly *N. bullata* [2] and the mealworm *Tenebrio molitor* [14,16,33]. In *T. molitor*, it inhibits the oocyte growth, reduces the number of eggs and their hatchability, and delays embryonic development [16,33]. Biochemical tests show that *Neb*-colloostatin does not inhibit trypsin and ecdysone biosynthesis although it inhibits the accumulation of yolk in the insect oocytes [2]. The oostatic activity of *Neb*-colloostatin found in *N. bullata* and *T. molitor* indicates the species unspecificity of the peptide.

Additionally, *Neb*-colloostatin has several other biological activities. For example, it stimulates the growth of plant pathogens *Phoma narcissi* and *Botrytis tulipae* [13] and in *T. molitor* it increases the frequency of the heartbeat without changing the amplitude of the contractions [15]. Moreover, structure–activity studies of *Neb*-colloostatin showed that an *N*-terminal tetrapeptide fragment (Ser-Ile-Val-Pro) is important for cardiostimulatory properties of this peptide in *Tenebrio* [15].

Recently, we have found that *Neb*-colloostatin exerts a pro-apoptotic action on haemocytes of *T. molitor* adults [4]. These results inspired us to perform structure–activity studies to estimate what determines such an action of the peptide. The subject of these studies was the synthesis of new *Neb*-colloostatin analogs modified at position 1 and 4 of the peptide chain, such as:

(1) X^1IVPLGLPVPIGPIVVGPR where X^1 = Ac-Ser (**1**), D-Ser (**2**), Thr (**3**), Asp (**4**), Glu (**5**), Gln (**6**), Ala (**7**), Val (**8**);
(2) SIVX^2LGLPVPIGPIVVGPR where X^2 = D-Pro (**9**), Hyp (**10**), Acp (**11**), Ach (**12**), Ala (**13**), Ile (**14**), Val (**15**).

Abbreviations: Ach, 1-aminocyclohexane-1-carboxylic acid; Acp, 1-aminocyclopentane-1-carboxylic acid; CD, circular dichroism; DMF, dimethylformamide; EDT, 1,2-ethanedithiol; Fmoc, 9-fluorenylmethoxycarbonyl; HBTU, *O*-benzotriazole-*N*,*N*,*N*′,*N*′-tetramethyl-uronium-hexafluorophosphate; HOBt, *N*-hydroxybenzotriazole; HPLC, high performance liquid chromatography; Hyp, 4-hydroxyproline; NMM, *N*-methylmorpholine; TFA, trifluoroacetic acid.

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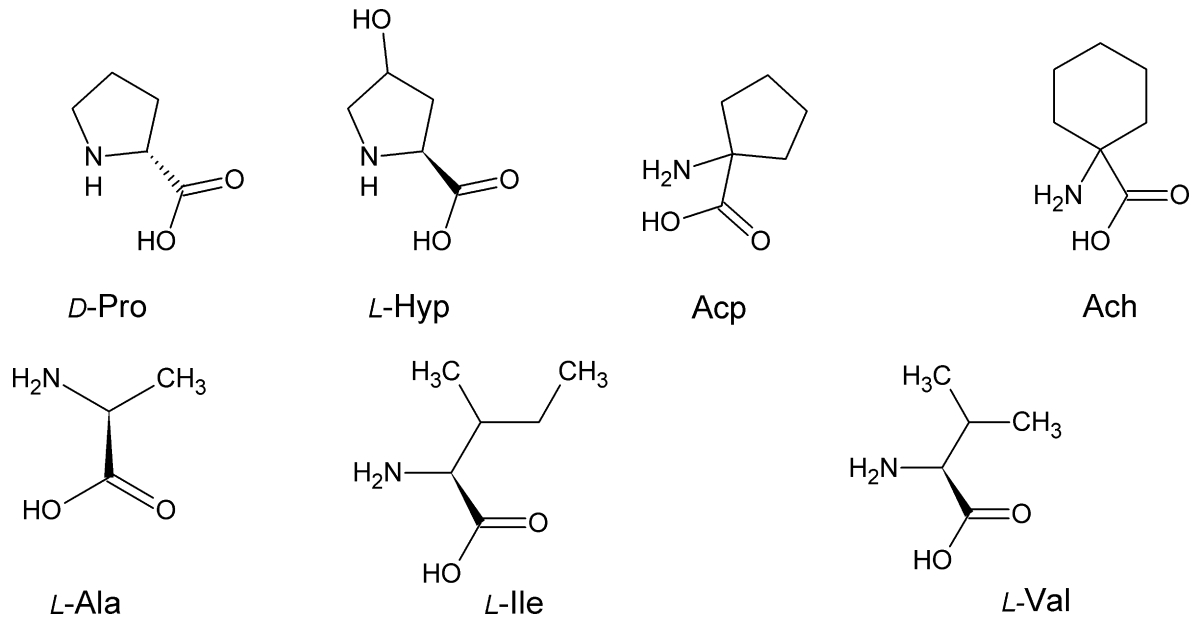

Fig. 1. Structure of amino acids incorporated at position 4 of *Neb*-colloostatin analogs.

The first group of analogs was synthesized to investigate the role of a polar side chain of the residue at position 1 of *Neb*-colloostatin on the pro-apoptotic activity in insect cells. The Ser[1] residue was replaced in the investigated analogs by polar amino acids (D-Ser, Thr, Asp, Glu, Gln) and non-polar ones (Ala, Val).

The synthesis of the second group of *Neb*-colloostatin analogs was performed to check the role of Pro at position 4 on the biological activity in insects. In this group of analogs, the proline was substituted by: 5-membered rings of D-Pro, Hyp and Acp; a 6-membered ring of Ach or hydrophobic amino acids, such as Ala, Ile, and Val (Fig. 1).

The biological effects of the synthetic analogs of *Neb*-colloostatin were estimated by the cytotoxic test *in vivo* and compared with the activity of the native peptide.

In addition, in this work we present the results of CD studies on *Neb*-colloostatin and its analogs performed for their water and methanol solutions.

2. Materials and methods

2.1. Chemical methods

2.1.1. Peptides synthesis

The synthesis of peptides was performed using a standard Fmoc procedure on Wang resins [4]. The Wang resin preloaded with Fmoc-Arg(Pmc), HOBt, HBTU, and TFA were purchased from IRIS Biotech GmbH. NMM was purchased from Fluka. HPLC-grade solvents were purchased from Fisher Scientific. All other reagents were purchased from Aldrich. All solvents and reagents used for the solid-phase synthesis were of analytical grade and used without further purification.

Synthesis was performed in disposable plastic reactors (Intavis AG). Fmoc protecting groups were removed using 20% piperidine in DMF. Subsequently, Fmoc-protected amino acids (3 equiv.) were attached by using 3 equiv. of HBTU as the coupling agent in the presence of HOBt (3 equiv.) and NMM (6 equiv.) for 2 h at room temperature.

The completeness of each coupling reaction was monitored by the chloranil [32] or Kaiser test [8].

Final cleavage of the peptides was achieved with TFA, EDT, and water (95:2.5:2.5, v/v) for 2 h at room temperature. The crude peptides were precipitated from cold diethyl ether, washed with diethyl ether, dissolved in water, and lyophilized. The peptides were purified by semipreparative HPLC using a Varian ProStar chromatograph equipped with a TOSOH Bioscience C18 column (21.5 mm × 300 mm) (Tosoh, Tokyo, Japan) and a 210/254 nm dual-wavelength UV detector. Water–acetonitrile gradients containing 0.1% TFA at a flow rate of 7 ml/min were used for purification. The final purity of the lyophilized peptides was >95% according to analytical HPLC (Thermo Separation Product; column: Vydac Protein RP C18 (4.6 mm × 250 mm) (Grace, Deerfield, IL, USA); linear gradient 0–100% B in 60 min, solvent A = 0.1% TFA in water, solvent B = 0.1% TFA in 80% acetonitrile/water, UV detection at 210 nm). Additional HPLC analyses were performed, using a column Varian Microsorb-MV 100-5 CN (4.6 mm × 250 mm) (Varian, Palo Alto, CA, USA) with a linear gradient from 0% to 100% B for 40 min, flow rate 1.0 ml/min, solvent A = 0.1% TFA in water, solvent B = 0.1% TFA in 80% acetonitrile/water.

Finally, the peptides were re-dissolved in 50% acetic acid in water and then re-lyophilized. The physicochemical data of new analogs of *Neb*-colloostatin are presented in Table 1.

The molecular weights and structures of the synthesized compounds were confirmed by ESI-MS and ESI-MS/MS analysis (Tables 1–3) using micrOTOF-Q or Apex-Qe Ultra 7T FT-ICR instruments (Bruker Daltonic, Bremen, Germany). The representative ESI-MS/MS spectrum is presented in Fig. 2.

2.1.2. CD spectroscopy

CD measurements were performed on a Jasco J-720 spectropolarimeter, at room temperature. A pathlength of 1 mm was used. Peptides were dissolved in water or methanol at the concentration of 0.07 mg/ml. Each spectrum represents the average of four scans. The data are presented as the mean residue ellipticity [θ].

2.2. Bioassay methods

2.2.1. Insects

Studies were carried out on adults of two beetle species, *T. molitor* L. and *Zophobas atratus* Fab. which were maintained in laboratory cultures (Department of Animal Physiology and Development, Poznań University). *T. molitor* was reared as described previously [24]. As the mealworm parents' age is important for the