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Novel analogs of alloferon: Synthesis, conformational studies, pro-apoptotic and antiviral activity

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ABSTRACT

In this study, we report the structure-activity relationships of novel derivatives of the insect peptide alloferon (H-His-Gly-Val-Ser-Gly-His-Gly-Gln-His-Gly-Val-His-Gly-OH). The peptide structure was modified by exchanging His at position 9 or 12 for natural or non-natural amino acids. Biological properties of these peptides were determined in antiviral *in vitro* test against *Human Herpes Virus* 1 McIntrie strain (HHV-1_{MC}) using a Vero cell line. The peptides were also evaluated for the pro-apoptotic action *in vivo* on hemocytes of the *Tenebrio molitor* beetle. Additionally, the structural properties of alloferon analogs were examined by the circular dichroism in water and methanol. It was found that most of the evaluated peptides can reduce the HHV-1 titer in Vero cells. [Ala^9]-alloferon exhibits the strongest antiviral activity among the analyzed compounds. However, no cytotoxic activity against Vero cell line was observed for all the studied peptides. *In vivo* assays with hemocytes of *T. molitor* showed that [Lys^9]-, [Phg^9]-, [Lys^{12}]-, and [Phe^{12}]-alloferon exhibit a twofold increase in caspases activity in comparison with the native peptide. The CD conformational studies indicate that the investigated peptides seem to prefer the unordered conformation.

1. Introduction

Alloferon is a tridecapeptide (H-His-Gly-Val-Ser-Gly-His-Gly-Gln-His-Gly-Val-His-Gly-OH) isolated from blood of an experimentally infected larvae of the blow fly *Calliphora vicina*. Larvae were experimentally infected by pricking cuticle with a needle soaked in a suspension of heat-killed *Escherichia coli* and *Micrococcus luteus* cells [1].

It has been reported that this peptide displays the antitumor [1–4] and antiviral activities toward influenza virus, herpes viruses, and coxsackievirus [1,5,6]. Alloferon induces also the interferon (IFN) synthesis *in vivo* which was demonstrated using animal and human models. This compound also stimulates the cytotoxic activity of natural killer (NK) cells [2,3]. Thus, alloferon is interesting as a potential anticancer or antiviral drug.

Additionally, several new biological properties of alloferon have been found [7–12]. It was observed that alloferon at a dose of 10 nM strongly induces *Tenebrio molitor* hemocytes to undergo apoptosis [9] and *in vitro* studies revealed a weak cardiostimulatory activity of alloferon in *Zophobas atratus* [9]. Recently, our works also demonstrated that alloferon exhibits antinociceptive activity in rats and this effect is mediated by opioid receptors [7]. Moreover, it significantly decreased the level of tumor necrosis factor alpha (TNF-α) and vascular endothelial growth factor (VEGF), insignificantly decreased the IFN γ level, and increased the production of IL-2 (interleukin 2) in rats' plasma [10].

Recently, it has been reported that a structural analog of alloferon, referred to as allostatine (H-His-Gly-Val-Ser-Gly-Trp-Gly-Gln-His-Gly-Thr-His-Gly-OH), has antitumor properties and it is interesting as a potential anticancer drug [4].

However, in the literature not much attention has been paid to the influence of individual amino acids in the alloferon peptide chain on its structure and biological activity [5,9,13–17].

Abbreviations: CD, circular dichroism; CPE, cytopathic effect; DMF, dimethylformamide; FBS, fetal bovine serum; Fmoc, 9-fluorenylmethoxycarbonyl; HBTU, *O*-benzotriazole-N,N,N′,N′-tetramethyl-uronium-hexafluorophosphate; HOBt, N-hydroxybenzotriazole; HPLC, high performance liquid chromatography; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; NMM, N-methylmorpholine; TCID, tissue culture infected dose; TFA, trifluoroacetic acid; TIS, triisopropylsilane; UV, ultraviolet.

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The preliminary structure-activity studies show that the presence of the aromatic ring at position 1 of the alloferon peptide chain can play a role in the expression of antiviral properties *in vitro*. In addition, [Lys^1]-alloferon exhibits a strong antiviral activity against reference and clinical strains of the *Human Herpesvirus* type 1 (HHV-1) in Vero cells and Coxsackievirus B-2 (CVB-2) in HEp-2 cells [5]. It was also observed that removal of two *N*-terminal amino acids ([3-13]-alloferon) caused a greater reduction of the titer of the standard strain of 971 PT Coxsackievirus B-2 in HEp-2 cells [17]. Furthermore, the biological studies show that the *N*- and *C*-terminally truncated alloferon analogs containing the *C*-terminal sequence His-Gly strongly induce *T. molitor* hemocytes to undergo apoptosis. Moreover, [Phe(*p*-NH_2)^1]- and [Tyr^6]-alloferon exhibit a twofold increase in caspases activity in comparison with the native peptide [9]. On the basis of our results, we concluded that not only basic or aromatic character but also the volume of the side chain of amino acid at position 1 or 6 is responsible for the biologically active alloferon conformation.

The results obtained in our study have inspired us to further studies on the structure/function relationship of alloferon.

In this work, we synthesized the following peptides:

1. Alloferon and its analogs modified at position 9 of the peptide chain

H-His-Gly-Val-Ser-Gly-His-Gly-Gln-**His**-Gly-Val-His-Gly-OH (I)
H-His-Gly-Val-Ser-Gly-His-Gly-Gln-**Ala**-Gly-Val-His-Gly-OH (II)
H-His-Gly-Val-Ser-Gly-His-Gly-Gln-**Arg**-Gly-Val-His-Gly-OH (III)
H-His-Gly-Val-Ser-Gly-His-Gly-Gln-**Lys**-Gly-Val-His-Gly-OH (IV)
H-His-Gly-Val-Ser-Gly-His-Gly-Gln-**Phe**-Gly-Val-His-Gly-OH (V)
H-His-Gly-Val-Ser-Gly-His-Gly-Gln-**Phg**-Gly-Val-His-Gly-OH (VI)
H-His-Gly-Val-Ser-Gly-His-Gly-Gln-**Tyr**-Gly-Val-His-Gly-OH (VII)
H-His-Gly-Val-Ser-Gly-His-Gly-Gln-**Trp**-Gly-Val-His-Gly-OH (VIII)
H-His-Gly-Val-Ser-Gly-His-Gly-Gln-**Phe(*p*-Cl)**-Gly-Val-His-Gly-OH (IX)
H-His-Gly-Val-Ser-Gly-His-Gly-Gln-**Phe(*p*-OMe)-**Gly-Val-His-Gly-OH (X)

2. Alloferon analogs modified at position 12 of the peptide chain

H-His-Gly-Val-Ser-Gly-His-Gly-Gln-His-Gly-Val-**Ala**-Gly-OH (XI)
H-His-Gly-Val-Ser-Gly-His-Gly-Gln-His-Gly-Val-**Lys**-Gly-OH (XII)
H-His-Gly-Val-Ser-Gly-His-Gly-Gln-His-Gly-Val-**Arg**-Gly-OH (XIII)
H-His-Gly-Val-Ser-Gly-His-Gly-Gln-His-Gly-Val-**Phe**-Gly-OH (XIV)
H-His-Gly-Val-Ser-Gly-His-Gly-Gln-His-Gly-Val-**Phg**-Gly-OH (XV)
H-His-Gly-Val-Ser-Gly-His-Gly-Gln-His-Gly-Val-**Tyr**-Gly-OH (XVI)
H-His-Gly-Val-Ser-Gly-His-Gly-Gln-His-Gly-Val-**Trp**-Gly-OH (XVII)
H-His-Gly-Val-Ser-Gly-His-Gly-Gln-His-Gly-Val-**Phe(*p*-Cl)**-Gly-OH (XVIII)
H-His-Gly-Val-Ser-Gly-His-Gly-Gln-His-Gly-Val-**Phe(*p*-OMe)-**Gly-OH (XIX)

Biological effects of these peptides were evaluated *in vitro* against *Human Herpes Virus* 1 McIntyre strain (HHV-1_{MC}) using a Vero cell line and *in vivo* in relation to hemocytes of *T. molitor*.

Furthermore, CD studies were carried out to obtain information about the role of a peptide conformation in the biological activity of alloferon.

2. Material and methods

2.1. Materials

The Wang resins preloaded with Fmoc-Gly and Fmoc-amino acids, HOBt, HBTU, and TFA were purchased from IRIS Biotech. HPLC-grade solvents were purchased from Fisher Scientific. All other reagents were purchased from Sigma-Aldrich. All solvents and reagents used for the solid-phase synthesis were of analytical quality and were used without further purification.

All the chemicals and reagents used for antimicrobial studies were of bacteriological grade.

2.2. Synthesis

Peptides were obtained by a stepwise elongation of the peptide chain according to procedures described previously by Kuczer et al. [9]. In brief, the analogs were synthesized by the classical solid phase method using the Fmoc procedure starting from an Fmoc-Gly-Wang resin. 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 Kaiser test [18].

Final cleavage of the peptides was achieved with TFA, TIS, 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 Varian Microsorb-MV 100-5 CN column (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 chemical identities of the peptides were confirmed by ESI-MS using micrOTOF-Q or Apex-Qe Ultra 7T FT-ICR instruments (Bruker Daltonic, Bremen, Germany).

2.3. 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 molar ellipticity [θ].

2.4. Cells

Vero cell line was used in this study. Cells were grown and maintained at 37 °C in Eagle's medium 1959 (Biomed, Lublin) supplemented with 10% FBS from Gibco and 1% of antibiotic antimycotic solution (100×): penicillin, streptomycin, amphotericin B (Sigma-Aldrich) at 37 °C.

2.5. Virus

The viral strain used in this study was the standard (reference) strain of Human Herpes Virus 1 (HHV-1) named McIntyre strain ($HHV\text{-}1_{MC}$). The herpesvirus stock was propagated in Vero cells. After the cytopathic effect was evident, the cells were frozen-thawed three times. The cell debris was removed by centrifugation. The supernatant was aliquoted, titrated, and kept at −70 °C. In the antiviral assay, the medium was supplemented with 2% FBS and the above mentioned antibiotics.

2.6. Insects

Studies were carried out on adults beetles, *T. molitor* L. which were maintained in laboratory cultures (Department of Animal Physiology and Development, Poznań University). The *T. molitor* was reared as described previously [9]. As the mealworm parents' age is important for the developmental features of their offspring [19–21], all insects in our experiments are derived from younger than 1 month parents.

2.7. In vitro cytotoxicity assay

Cytotoxic activity of peptides was assessed by a light inverted microscopy Olympus CK2 (Olympus Corp., Germany) and quantified by the MTT (Sigma-Aldrich) colorimetric assay *in vitro* using a *Vero* cell line. The absorbance was read spectrophotometrically at a wavelength of 405 nm on a reader (Reader 230, Organon Technica Turnhout, Belgium). Cells were inoculated in a 96-microwell plate. After incubation for 24 h, the peptides were added to the culture medium in doses ranging from 50 to 500 μg/ml and incubated for further 24 or 48 h. The control was prepared without any sample. All experiments were performed in triplicate.

2.8. Antiviral activity assay

Antiviral activity was assessed *in vitro* by phenotypic assays using a *Vero* cell line. Cell culture in flat-bottom microwell plates (2×10^4 cells/0.2 ml) was infected with an $HHV\text{-}1_{MC}$ strain (0.01 TCID50/cell) for 60 min at 37 °C. After the virus absorption, the inoculum was aspired and fresh culture medium containing a peptide was added.

After a virus infection the cells were incubated for 2 days at 37 °C with various concentrations of the respective compounds, ranging from 50 to 500 μg/ml diluted in an assay medium.

The antiviral activity of the peptides was determined using a cytopathic effect (CPE). The inhibition of the viral CPE was assessed by a light inverted microscopy. The yield reduction assay (YRA), which measures the inhibition of infectious *virus* in the presence of the investigated peptides was applied. Virus titers were determined according to the Reed-Muench formula [22] and expressed in $TCID_{50}$/ml. The Reed-Muench statistical method was used to determine the 50% end point (EC_{50}) which was the lowest concentration of the tested drugs that reduce the viral infections of the control to 50%. The antiviral effect was estimated according to the reduction of the virus titer in the presence of compounds investigated as compared with the control. $TCID_{50}$/ml (median tissue culture infective dose) was calculated. $TCID_{50}$/ml denotes the amount of a pathogenic agent that will produce pathological changes in 50% of cell virus-inoculated cultures. Each analysis was performed at least in triplicate.

2.9. Apoptosis assay

Bioassays with insect hemocytes were performed as described previously [9,23]. The *T. molitor* beetles were anaesthetized with CO_2, washed in distilled water, and disinfected with 70% ethanol. The peptide solution was injected to the beetles by hand (2 μl, in a dose of 10 nM of peptide per insect) through the ventral membrane between the second and the third abdominal segments toward the head with a Hamilton syringe (Hamilton Co., Bonaduz, Switzerland). The control insects were injected with the same volume of physiological saline. All solutions were sterilized by filtration through the 0.22 μm pore filter membrane (Millipore) and all injections were performed in sterile conditions. The peptide concentration (1 mM stock solution) was assessed before filtration and next a working solution, which was injected to the beetles, was prepared.

Before hemolymph collection, the beetles were anaesthetized again with CO_2, washed in distilled water, and disinfected with 70% ethanol. Hemocytes from control and insects injected with peptides were collected 1 h after injection. Upon cutting off a tarsus from a foreleg, hemolymph samples (5 μl), were collected with "end to end" microcapillaries (Drummond). They were diluted with 20 μl of ice-cold physiological saline containing anticoagulant buffer (4.5 mM citric acid and 9 mM sodium citrate) at a 5:1 v/v ratio. Hemolymph from control and peptide-injected insects was placed on alcohol-cleaned cover glasses coated with 7 μl 0.01% poly-L-lysine (Sigma P4707). After allowing hemocytes to settle (15 min, at room temperature), the remaining fluid was removed and the cells were washed twice with physiological saline. The prepared hemocytes were used for the microscopic analysis of activation of caspases.

The presence of active caspases was investigated by using a sulforhodamine derivative of valylalanylaspartic acid fluoromethyl ketone, a potent inhibitor of caspase activity (SR-VAD-FMK) (in accordance with the manufacturer's instructions of the sulforhodamine multi-caspase activity kit, AK-115, BIOMOL, PA). Hemocytes were incubated in a reaction medium (1/3x SR-VAD-FMK) for 1 h at room temperature in the dark, rinsed three times with a wash buffer for 5 min at a room temperature, and finally fixed in 3.7% paraformaldehyde for 10 min. After washing again in physiological saline, the hemocytes were stained with DAPI (4′,6-diamidino-2-phenylindole). Incubation in the dark lasted for 5 min. Thereafter, the hemocytes were washed once with distilled water and mounted using a mounting medium. The prepared hemocytes were studied with a Nikon Eclipse TE 2000-U fluorescence microscope with filters set for rhodamine (excitation 543 nm and emission 560 nm) and the intensity of fluorescence of the apoptosing cells was measured by using an NIS-Element AR 3.1 programme. The changes in fluorescence intensity were used to quantify the activity of caspases. Data are shown as mean ± SD.

3. Results and discussion

In this work, the structure-activity relation of the insect peptide alloferon was studied to understand the structural requirements for its biological activity.

In the literature, many strategies have been suggested to study structure-activity relationship of biologically active compounds

[24–28]. Structural modifications include a consecutive replacement of amino acid residues by other natural or unnatural amino acids as well as synthesis of analogs with a truncated or elongated peptide chain. Several studies have demonstrated that the presence of the basic and nucleophilic imidazole ring in the side chains of peptides may be of importance in creation of new biological activities of these molecules [24,25].

Continuing our studies on structure/function relationship of alloferon, we designed further alloferon analogs. The aim of these modifications was to explain which potential attributes of the imidazole group might be responsible for the biological activity of alloferon. Because the role of His at position 9 and 12 has not been established in the previous investigations, we performed the synthesis of two groups of alloferon analogs, where histidine at position 9 or 12 was replaced by natural or non-natural amino acid residues. Each of 9 standard and non-standard amino acids has been used in the place of His^9 or His^{12} to investigate the importance of this residue's basic, hydrophobic or aromatic properties for the biological activity of alloferon.

New alloferon analogs were synthesized by the manual solid-phase techniques. All peptides were purified by preparative HPLC. In each case, the free peptides had a purity of >95%. The analytical data of new analogs of alloferon are presented in Table 1.

In *in vivo* insect bioassay using the sulforhodamine labeled caspase inhibitor (SR-VAD-FMK), we discovered that the peptides tested showed diverse activities during the investigation of their influence on the pro-apoptotic action on hemocytes of *T. molitor* (Fig. 1 and Table 2). Fluorescence intensity of labeled caspase inhibitor-caspase complex in hemocytes was used to quantify caspase activity. Activation of caspase was our marker of pro-apoptotic activity of studied peptides, because once caspases are initially activated there seems to be an irreversible commitment toward cell death. Recently, we found that insect peptide hormones cause apoptosis in hemocytes of *T. molitor* [9,23]. However, the molecular mechanism underlying the activation of the apoptotic program in hemocytes by these hormones remains unknown.

Among the analogs modified at position 9 of the peptide chain, the analog containing lysine (peptide **IV**), the amino acid that mimics the basic properties of the His side chain, as well as peptide **VI** ([Phg^9]-alloferon) with an amino acid which is deprived of the methylene group between the C^{α} atom and the benzene ring, showed the highest percentage activity (~220%) relative to alloferon. Moreover, analogs with phenylalanine *para*-substituted by chlorine atom ([Phe(*p*-Cl)^9]-alloferon, **IX**) and a methoxy group ([Phe(*p*-OMe)^9]-alloferon, **X**) also strongly induced caspase activity (~160%). Other derivatives evoked 100% ([Arg^9]-alloferon, **III**) and 85% ([Phe^9]-alloferon, **V**) of the native peptide activity or were practically inactive (peptides **II**, **VII**, and **VIII**).

These results indicate that the pro-apoptotic activity in insect hemocytes depends on the presence of the aromatic or basic side chain at position 9. Additionally, a 2-fold increase in the cytotoxic activity for [Lys^9]-alloferon suggests that the presence of a flexible side chain can play an important role in creation of the biological properties against hemocytes of *T. molitor*. Furthermore, it is interesting that the phenylglycine analog (peptide **VI**), in which the phenyl ring is attached directly to the α-carbon of residue 9, exhibits a higher caspase activity in comparison with alloferon. This result and similar data obtained for analogs substituted at position 9 by other aromatic amino acids, such as Phe(*p*-Cl) or Phe(*p*-OMe) point out that the spatial orientation of the phenyl ring with respect to the polypeptide backbone of alloferon appears to be crucial for the optimal pro-apoptotic activity. On the other hand, replacing of histidine by other natural aromatic amino acids, such as Trp or Tyr, greatly reduces the pro-apoptotic activity of analogs in hemocytes. It is probably a result of their lower stability against enzymatic hydrolysis as compared with the native peptide.

In the case of the second group of analogs, modified at position 12 of the alloferon peptide chain, we found that most analogs substituted by aromatic amino acids showed the pro-apoptotic effect of 184–230% relative to alloferon. The most active were [Phe^{12}]- (**XIV**), [Phg^{12}]- (**XV**), [Trp^{12}]- (**XVII**), [Phe(*p*-Cl)^{12}]- (**XVIII**), and [Phe(*p*-OMe)^{12}]-alloferon (**XIX**) at 230%, 223%, 223%, 184%, and 200%, respectively. However, the introduction of polar amino acids at position 12, such as Lys (compound **XIII**), Arg (compound **XII**), and Tyr (compound **XVI**) caused a dramatic decrease in biological activity. Moreover, we found that the alloferon analog modified at position 12 by Ala (peptide **XI**) is inactive.

In a series of analogs modified at position 12, it seems that the presence of the aromatic side chain at this place is more important for the pro-apoptotic properties of alloferon than that of the

Table 1
Physicochemical data of alloferon analogs.

Peptide	Yield (%)[a]	HPLC		$[M+H]^+$		$[M+2H]^{2+}$		$[M+3H]^{3+}$	
		Rt_1[b] (min)	Rt_2[c] (min)	Calc.[d]	Found	Calc.[d]	Found	Calc.[d]	Found
II	81	13.0	9.0	1199.567	1199.621	600.287	600.307	400.527	400.531
III	76	10.8	8.7	1284.631	1284.708	642.818	642.844	428.881	428.895
IV	77	13.7	9.0	1256.624	1256.632	628.815	628.823	419.546	419.551
V	80	15.8	14.7	1275.597	1275.669	638.302	638.321	425.870	425.880
VI	78	14.3	12.7	1261.582	1261.640	631.294	631.312	421.198	421.207
VII	78	13.7	12.3	1291.593	1291.658	646.299	646.319	431.202	431.212
VIII	76	16.5	16.0	1314.609	1314.678	657.808	657.828	438.874	–
IX	81	15.5	8.3	1309.559	1309.537	655.283	655.274	437.191	437.186
X	73	15.1	9.0	1305.608	1305.685	653.307	653.334	435.874	435.895
XI	80	12.3	9.8	1199.567	1199.573	600.287	600.295	400.527	400.533
XII	85	12.5	10.5	1284.631	1284.638	642.818	642.820	428.881	428.884
XIII	78	12.5	10.5	1256.624	1256.610	628.815	628.811	419.546	419.543
XIV	87	13.4	12.5	1275.597	1275.610	638.302	638.300	425.870	425.870
XV	80	15.0	13.4	1261.582	1261.594	631.294	631.305	421.198	421.207
XVI	77	13.5	12.7	1291.593	1291.605	646.299	646.315	431.202	439.207
XVII	75	16.2	16.0	1314.609	1314.635	657.808	657.811	438.874	438.879
XVIII	75	14.5	12.3	1309.559	1309.537	655.283	655.275	437.191	437.187
XIX	73	14.5	12.8	1305.608	1305.685	653.307	653.334	435.874	435.895

[a] Crude yield: yield after cleavage from the resin. The purity of crude product was analyzed according to HPLC peak integrals at λ 210 nm on analytical HPLC. The crude peptide had a purity of >80%.
[b] Analytical HPLC performed on Vydac C18 column.
[c] Analytical HPLC performed on Varian Microsorb-MV 100-5 CN column.
[d] Monoisotopic mass of the indicated ion formed by the peptide calculated using the mMass program.

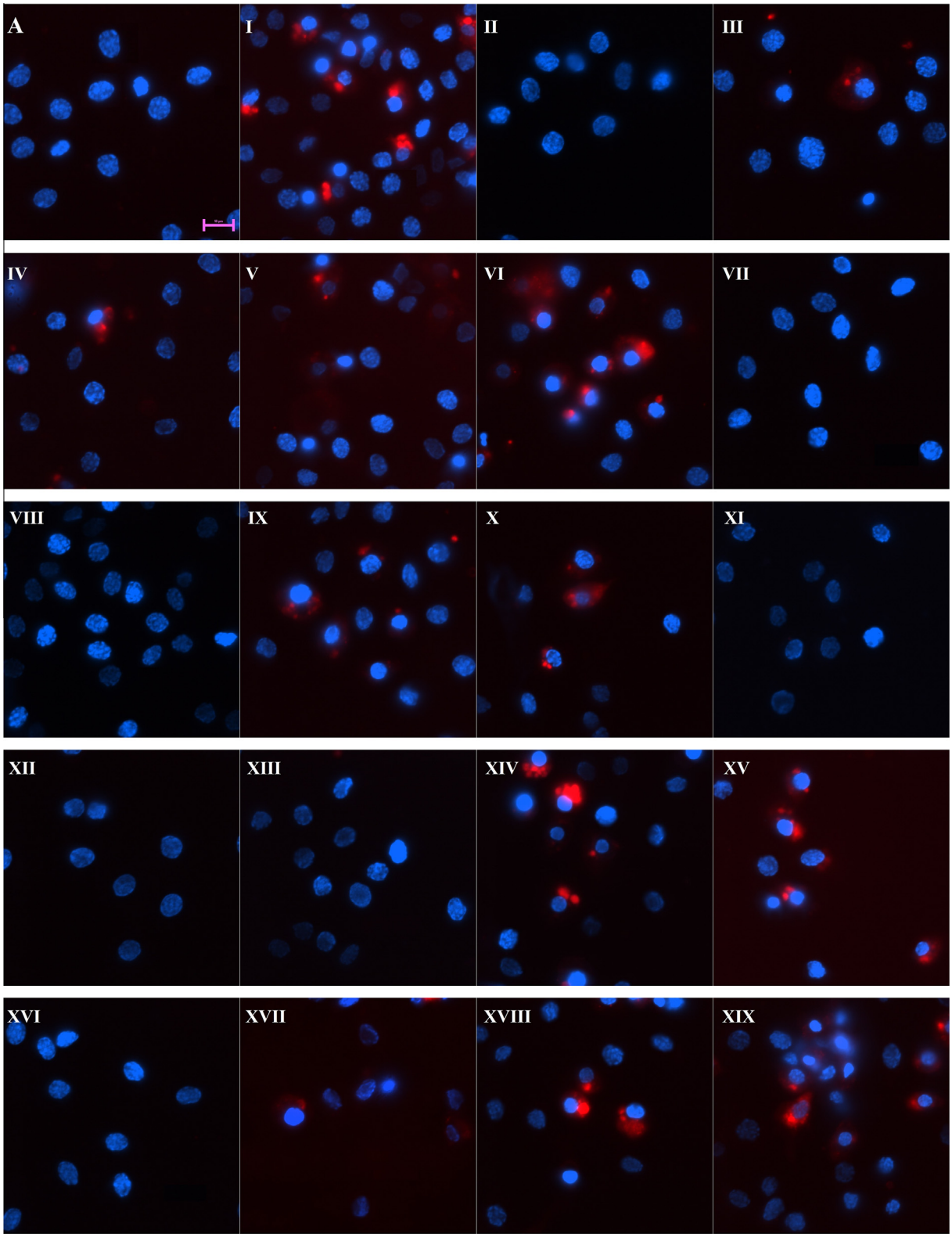

Fig. 1. Fluorescence microscopy images showing induced apoptosis in *T. molitor* hemocytes following saline (A), alloferon (I) and its analogs (II-XIX) injections. All hemocytes were stained with SR-VAD-FMK reagent for caspase activity detection (red color) and DAPI for cell nuclei detection (blue color). Scale bars indicate 20 μm.

positive charge. Moreover, data obtained for five analogs tested (peptides **XIV-XV, XVII-XIX**) indicate that aromatic residue His^{12} can be replaced by another aromatic residues. These studies suggest that a high biological activity of [Trp^{12}]-, [Phe^{12}]-, [Phg^{12}]-, [Phe(*p*-Cl^{12})]-, and [Phe(*p*-OMe^{12})]-alloferon may be a result of the aromatic-aromatic stacking interactions of side chains of phenylalanine or tryptophan residues with other aromatic amino acids, possibly with His^{1}, His^{6} or His^{9}.

Table 2
Hemocytotoxicity of alloferon analogs in *T. molitor* adults.

Sample	Number of cells	MFI ± SD[a]	Effect on the caspases activity relative to alloferon (%)[b]
Control	50	3.80 ± 0.86	0
Alloferon (**I**)	50	17.71 ± 3.15	100
[Ala^{9}]-alloferon (**II**)	50	4.34 ± 3.61	NA
[Arg^{9}]-alloferon (**III**)	50	17.34 ± 3.42	100
[Lys^{9}]-alloferon (**IV**)	50	31.50 ± 17.48	207
[Phe^{9}]-alloferon (**V**)	50	15.19 ± 9.65	84
[Phg^{9}]-alloferon (**VI**)	50	43.97 ± 8.27	285
[Tyr^{9}]-alloferon (**VII**)	50	4.45 ± 1.58	NA
[Trp^{9}]-alloferon (**VIII**)	50	3.86 ± 0.58	NA
[Pe(*p*-Cl)^{9}]-alloferon (**IX**)	50	26.45 ± 7.58	170
[Phe(*p*-OMe)^{9}]-alloferon (**X**)	50	24.94 ± 6.98	161
[Ala^{12}]-alloferon (**XI**)	50	3.43 ± 0.22	NA
[Arg^{12}]-alloferon (**XII**)	50	2.99 ± 0.78	NA
[Lys^{12}]-alloferon (**XIII**)	50	3.78 ± 1.03	NA
[Phe^{12}]-alloferon (**XIV**)	50	34.22 ± 2.03	230
[Phg^{12}]-alloferon (**XV**)	50	33.52 ± 2.75	223
[Tyr^{12}]-alloferon (**XVI**)	50	3.54 ± 0.67	NA
[Trp^{12}]-alloferon (**XVII**)	50	33.24 ± 3.99	223
[Phe(*p*-Cl)^{12}]-alloferon (**XVIII**)	50	28.03 ± 5.61	184
[Phe(*p*-OMe)^{12}]-alloferon (**XIX**)	50	30.23 ± 3.56	200

MFI – mean fluorescence intensity, SD – standard deviation, NA – not active.
[a] Mean values are given from 3 to 4 separate determinations.
[b] Activity (%) = [(MFI analog − MFI control)/MFI alloferon − MFI control] × 100%.

All the synthesized compounds were also screened for *in vitro* antiviral activity against Herpesvirus using a *mammalian* cell line.

To determine the cytotoxic effect of these compounds on cells, the MTT assay was performed using the Vero cell line.

Microscopic observations showed that no changes occurred in the Vero cells growth or morphology in the presence of the peptides tested.

The MTT assay also proved that they have no effect on cell proliferation (Table 3). The viability of cells in the presence of tested compounds was higher than 95%. The compounds at all concentrations tested were nontoxic against a Vero cell line. For this reason the TC_{50} (the toxic drug concentration which caused the reduction of viable cell numbers by 50%) and SI (Selectivity Index; CC_{50} to IC_{50} value) were not calculated. In the antiviral test, it was found that most of the investigated peptides can inhibit the titer of the virus in Vero cells (Table 3) at a higher or equal dose as compared with the native peptide.

Table 3
Cytotoxicity and antiviral activity of alloferon and its analogs against HHV-1_{MC}.

Sample	EC_{50} (μg/ml)	Toxicity[a] (μg/ml)
Alloferon (**I**)	305.00	>500
[Ala^{9}]-alloferon (**II**)	194.80	>500
[Arg^{9}]-alloferon (**III**)	205.58	>500
[Lys^{9}]-alloferon (**IV**)	238.45	>500
[Phe^{9}]-alloferon (**V**)	495.74	>500
[Phg^{9}]-alloferon (**VI**)	478.05	>500
[Tyr^{9}]-alloferon (**VII**)	201.87	>500
[Trp^{9}]-alloferon (**VIII**)	292.45	>500
[Pe(*p*-Cl)^{9}]-alloferon (**IX**)	222.89	>500
[Phe(*p*-OMe)^{9}]-alloferon (**X**)	242.81	>500
[Ala^{12}]-alloferon (**XI**)	442.45	>500
[Arg^{12}]-alloferon (**XII**)	1538.4	>500
[Lys^{12}]-alloferon (**XIII**)	463.30	>500
[Phe^{12}]-alloferon (**XIV**)	395.38	>500
[Phg^{12}]-alloferon (**XV**)	395.27	>500
[Tyr^{12}]-alloferon (**XVI**)	582.23	>500
[Trp^{12}]-alloferon (**XVII**)	656.86	>500
[Phe(*p*-Cl)^{12}]-alloferon (**XVIII**)	376.17	>500
[Phe(*p*-OMe)^{12}]-alloferon (**XIX**)	395.33	>500
Acyclovir	1.0	>250[b]

[a] Maximal non-cytotoxic concentration for target cells.
[b] The 50% cytotoxic concentration for target cells in μg/ml.

Among the analogs modified at position 9, the highest antiviral effect was observed for [Ala^{9}]-alloferon (peptide **II**, EC_{50} = 194.80 μg/ml). The antiviral bioassay also demonstrated that analogs of alloferon substituted at the position 9 by the aromatic amino acid showed modified activities. As shown in Table 3, peptide **VIII** ([Trp^{9}]-alloferon) and analogs containing derivatives of phenylalanine *para*-substituted by hydroxyl group ([Tyr^{9}]-alloferon (peptide **VII**)), methoxy group ([Phe(*p*-OMe)^{9}]-alloferon (peptide **X**) as well as chlorine atom ([Phe(*p*-Cl)^{9}]-alloferon (peptide **IX**)) display the antiviral activity at lower concentration (EC_{50} = 292.45 μg/ml, EC_{50} = 201.87 μg/ml, EC_{50} = 242.81 μg/ml, EC_{50} = 222.89 μg/ml, respectively) than alloferon. However, other analogs modified by aromatic acids, such as [Phe^{9}]-(peptide **V**) and [Phg^{9}]-alloferon (peptide **VI**) show a weak inhibitory activity.

Interestingly, the substitution of histidine at position 9 by basic amino acids ([Arg^{9}]- (peptide **III)** and [Lys^{9}]-alloferon (peptide **IV**) resulted in an increase activity against HHV-1.

Data obtained from analysis of the antiviral bioassay of analogs modified at position 12 (Table 3) indicate that the peptides tested display the antiviral activity at higher concentrations (EC_{50} from 376.17–656.86 μg/ml) than alloferon (EC_{50} = 305.00 μg/ml). Furthermore, the substitution of His at position 12 by another basic amino acid as Arg (peptide **XII**) causes a complete loss of the antiviral activity.

Based on the antiviral activities obtained here it is difficult to discuss the structure/function relationship. Tests for the antiviral activity show that all structural changes do not appear to affect the activity of the peptides tested. Among all modified analogs, only peptides containing Phg and Phe at position 9 showed a slightly reduced antiviral activity relative to the native peptide. This effect might be explained by more hydrophobic side chains of Phe and Phg as compared to His. In contrast, a higher antiviral activity toward HHV-1 observed for [Ala^{9}]-alloferon may be explained by the small size of the Ala residue. Our results indicate that histidine at position 9 can be replaced with another basic residues or aromatic residues with a large surface area without causing a significant change in reduction of the HHV-1 titer in Vero cells.

Studies on the analogs modified at position 12 demonstrate that most of the evaluated peptides display the antiviral activity against HHV-1 standard strain; however, this effect was somewhat lower

in comparison with alloferon. These results show that the antiviral activity of alloferon against HHV-1 seems to be more sensitive to structural changes at position 12 than at position 9. Also, in our previous study we suggested that the *C*-terminal amino acid residues of alloferon are more important for antiviral activity than the *N*-terminal ones [17].

Our findings show that the retention of the antiviral activity against HHV in Vero cells of alloferon analogs requires a higher concentration of peptides, comparable to the reference drug such as acyclovir which has an EC_{50} value of 1.0 μg/ml. Nevertheless, no toxicity of the investigated compounds at high concentrations constitutes their considerable advantage.

The CD measurements were performed to obtain information what kind of conformational changes is brought about by various substitutions in the alloferon peptide chain. They were carried out in water and methanol.

The analysis of the CD spectra of alloferon analogs revealed, that none of peptides **I-X** give a CD spectrum typical of any of the ordered structures (helix, β structure, and turns). The CD spectra of peptides **I**, **III**, and **V-X** in both water and methanol (Figs. 2–5) indicate that their conformational equilibria are dominated by the unordered structures. They show a negative band at 200 nm which is indicative of an unordered or open conformation [29]. In the case of analogs **II** and **IV** their conformations differ from those of other peptides modified at position 9. It is interesting that the CD spectra of peptides **II** and **IV** in both water and methanol are very similar to each other. It shows that their conformations are solvent-independent and hence quite stable and that substitution of His^9 by Ala or Lys results in very similar effects. Differences between the CD spectra of analogs **II** and **IV** and the spectra of alloferon and peptides **III** and **V-X** show that the conformational equilibria of peptides **II** and **IV** are shifted to some extent toward ordered structures of the helix or the turn type. This shift is more visible in methanol than in water.

In the case of analogs modified at position 12, the CD spectra show that substitution of His^{12} by the aliphatic amino acid residues (analogs **XI-XIII**) has almost no effects on the conformation of alloferon in water (Fig. 6). The presence of the negative bands at about 230 nm in the spectra of those peptides in methanol (Fig. 8) suggests a small shift of their conformational equilibria toward the ordered structures in comparison with alloferon. In both solvents, however, they seem dominated by the unordered structures which is evidenced by negative bands at or below 200 nm. Such structures dominate probably also the conformational equilibria of peptides **XIV-XIX**, in both water and methanol

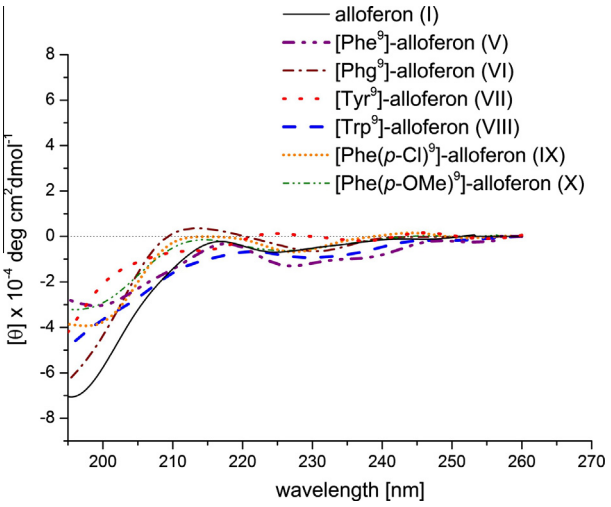

Fig. 3. CD spectra of alloferon (I) and its analogs (V-X) modified at position 9 in water.

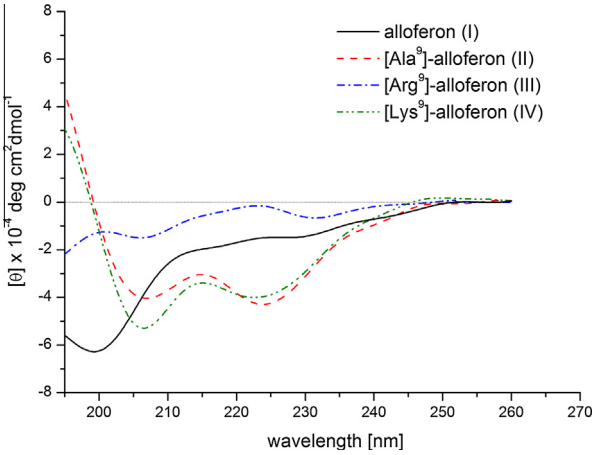

Fig. 4. CD spectra of alloferon (I) and its analogs (II-IV) modified at position 9 in methanol.

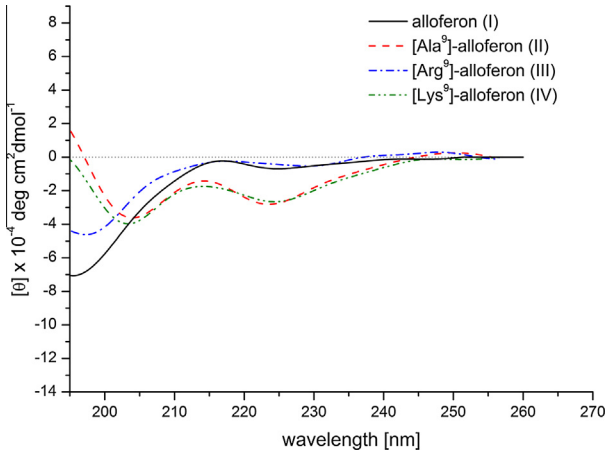

Fig. 2. CD spectra of alloferon (I) and its analogs (II-IV) modified at position 9 in water.

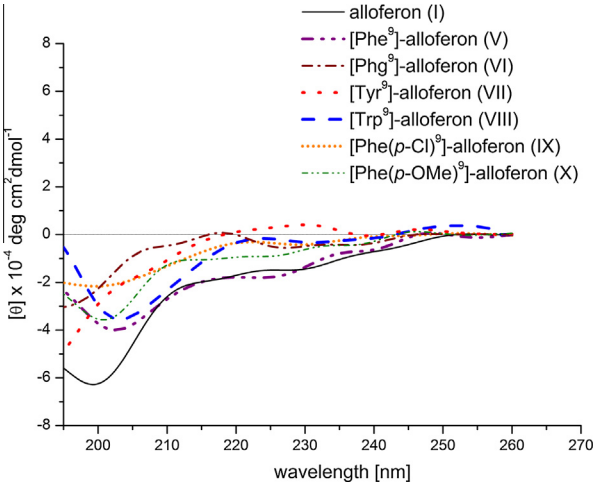

Fig. 5. CD spectra of alloferon (I) and its analogs (V-X) modified at position 9 in methanol.

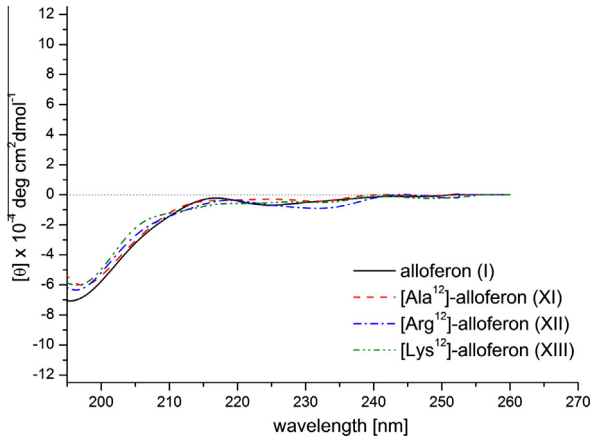

Fig. 6. CD spectra of alloferon (I) and its analogs (XI-XIII) modified at position 12 in water.

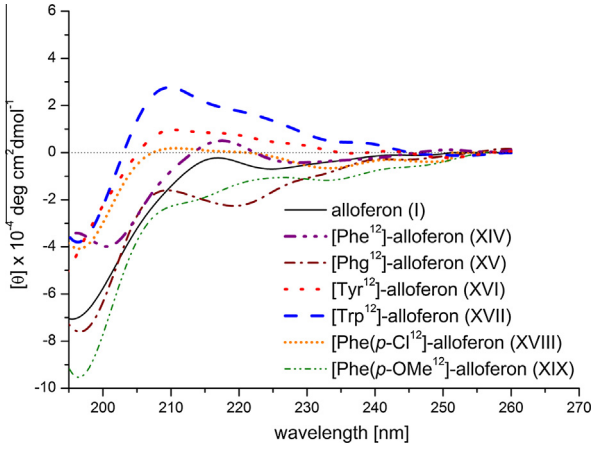

Fig. 7. CD spectra of alloferon (I) and its analogs (XIV-XIX) modified at position 12 in water.

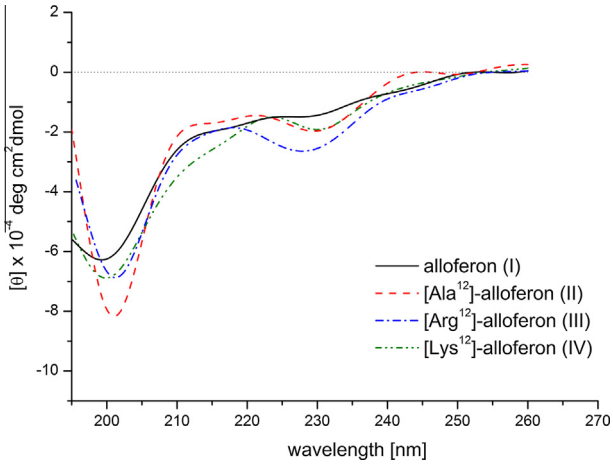

Fig. 8. CD spectra of alloferon (I) and its analogs (XI-XIII) modified at position 12 in methanol.

(Fig. 7 and Fig. 9). The conformational conclusions concerning analogs **V-X** and **XIV-XIX**, however, should be treated with caution since it is known that aromatic amino acid residues give contribution to a CD spectrum which overlaps the bands of the peptide chromophores which makes the conformational analysis difficult [30].

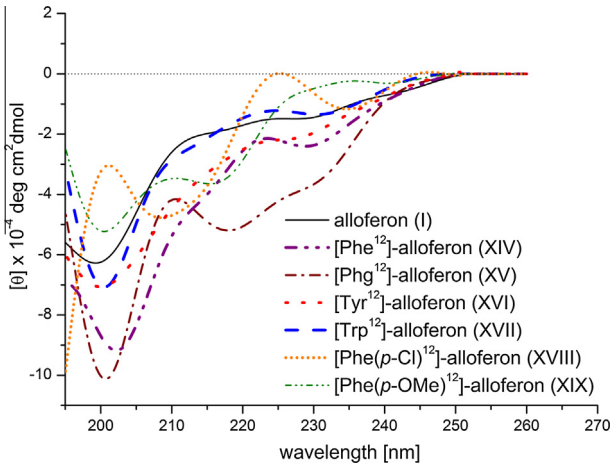

Fig. 9. CD spectra of alloferon (I) and its analogs (XIV-XIX) modified at position 12 in methanol.

It follows from the above results that there is no direct correlation between the CD spectra and biological activity of alloferon and its analogs. This result suggests that secondary structure probably cannot be the crucial factor for determination of pro-apoptotic or antiviral activity of alloferon.

4. Conclusion

Based on our results, we think that further studies toward the design and synthesis of new analogs of alloferon are required.

This could allow the development of nontoxic peptide derivatives with the antiviral activity. Unfortunately, we do not have data on the antiviral mechanism of action of that peptide and only the results of the structure-activity relationship studies could be used in the design of potential new antiviral analogs of alloferon.

The high biological activity and specificity of some new alloferon analogs indicate that these compounds are a promising avenue for development of a new generation of bioinsecticides or pharmaceutics used in medicine. A further improvement of activity should be accomplishable utilizing our structure/function data.

Conflict of interest

The authors declare no financial or commercial conflict of interest.

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