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The Synthesis of Immunomodulating Peptide Alloferon, the Active Principle of Antiviral Drug Allokine-alpha

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Abstract—Two variants of the synthesis of tridecapeptide alloferon, the active principle of antiviral preparation allokine-alpha, were developed on the basis of fragment condensation in solution or on the Merrifield resin. The solid phase variant of the synthesis was shown to be more technological; it allows the preparation of the product at a higher total yield (40% vs. 17% for conventional synthesis in solution from the starting derivatives of the *C*-terminal dipeptide). The by-products formed during the synthesis of alloferon were identified.

Key words: *alloferon, peptide synthesis, fragment condensation*

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INTRODUCTION

Several tens of peptide preparations used in medicine for therapeutic or diagnostic purposes are now known.[2] Tridecapeptide alloferon is a rather new peptide preparation that possesses an immunomodulating activity [1]. It increases the formation of endogenous interferons and provides the development of a cascade of protective reactions mediated by cytokines. Alloferon could find application in medical practice for the correction of interferon system and the system of natural killers, as well as for the treatment of viral, fungal, and oncological diseases connected with the deficiency of interferons or cytotoxic lymphocytes. Currently, alloferon is the active principle of the antiviral preparation allokine-alpha, which is used for the treatment of diseases caused by viruses of herpes simplex of I and II types and hepatitis B.

The SPS scheme recently suggested for alloferon uses the peptide chain elongation by one amino acid residue using the Fmoc-technology and the subsequent two-step purification of product by HPLC [1]. Most likely, it is not interesting from the practical point of view, because is expensive and, besides, cannot provide the obtaining of a homogeneous product in tens gram quantities. The purification of crude SPS product by HPLC in the case of a very hydrophilic alloferon represents an independent problem that demands a careful development of the chromatographic conditions. The synthesis of alloferon at gram scale is now a topical task, because alloferon is already applied in medicine.

Our research is devoted to the development of a convenient synthetic scheme of alloferon preparation upon a 100 g scale. A summary of the results of this work has been represented on the II Russian symposium on the chemistry and biology of peptides [2] and in patent [3].

RESULTS AND DISCUSSION

Alloferon represents tridecapeptide of the following structure:

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[2] In addition to those recommended by the IUPAC–IUB commission, we used the following abbreviations: DCM, dichloromethane; DIEA, *N,N'*-diisopropylethylamine; HOBt, 1-hydroxybenzotriazole; HONb, *N*-hydroxy-5-norbornene-2,3-dicarboximide; HONp, *p*-nitrophenol; MALDI MS, matrix-assisted laser desorption ionization mass spectrometry; NMM, *N*-methylmorpholine; (*P*), styrene–1% divinylbenzene copolymer; and SPS, solid phase synthesis.

H-His-Gly-Val-Ser-Gly-His-Gly-Gln-His-Gly-Val-His-Gly-OH

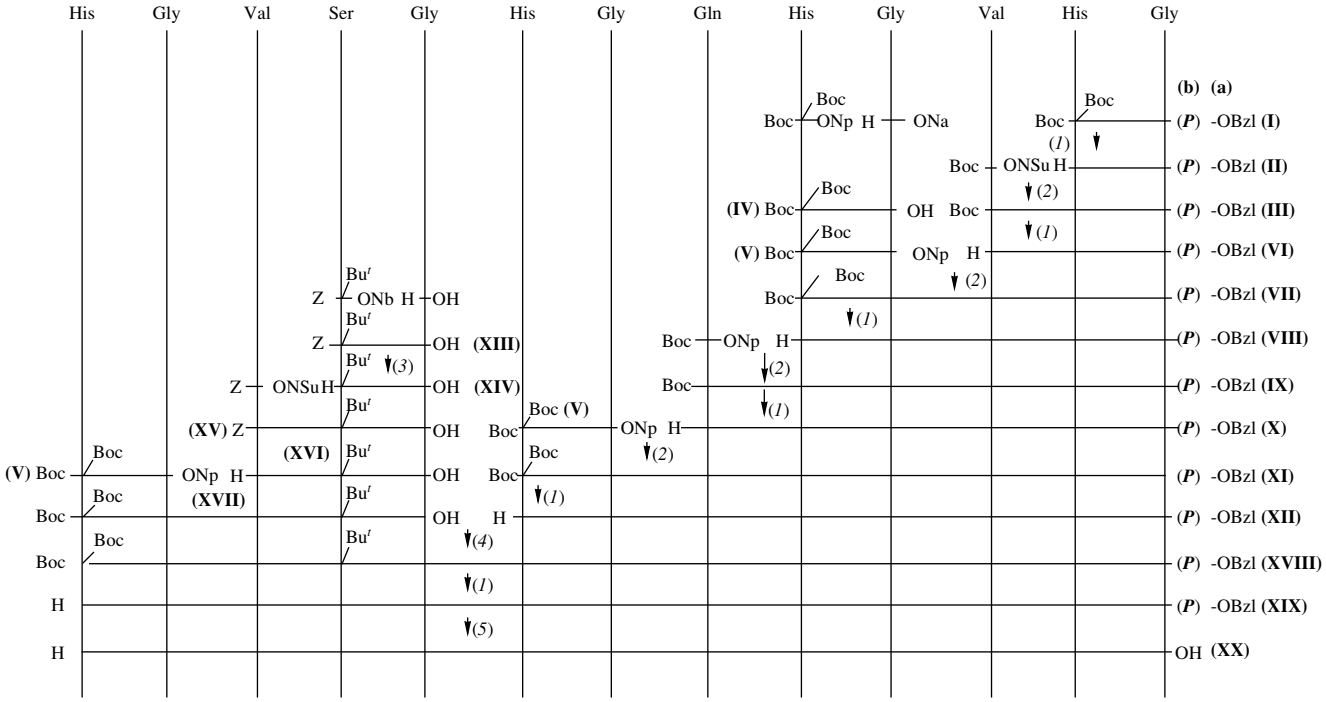
where arrows show the breakdown of its amino acid sequence to fragments during the synthesis.

The presence of five Gly residues in alloferon molecule together with a repeating fragment His-Gly gives wide opportunities for the use in the synthesis of the fragment condensation with the conventional breakdown of the amino acid chain to blocks at the Gly residues. It is known that the fragment condensation both in conventional and in solid phase variant as a rule provides the obtaining of product of higher quality than the step-by-step synthesis [4, 5]. In addition, the purification of peptides obtained by block synthesis is substantially easier. Therefore, we have separated the peptide sequence to short fragments containing the *C*-terminal Gly residue. The Val and Gln residues were not included in the fragment structures. The fragments chosen, the repeating dipeptide block His-Gly and the *N*-terminal pentapeptide His-Gly-Val-Ser-Gly, were presumed to be synthesized in solution.

The cheaper Boc-methodology was substituted for the Fmoc-methodology [1]. The problem of optimum protection of histidine imidazole remains disputable until now [6, 7]. The majority of groups used for this purpose have some restrictions, one of which is incompleteness of the splitting off of the corresponding protective group after the end of synthesis. This factor has a decisive significance in the synthesis of alloferon that has four His residues in its sequence. Therefore, we used a temporary (only during condensation) protection of the His imidazole rings, namely, the Boc group, which was subsequently split off by trifluoroacetic acid. The *tert*-butyl group was used for the protection of Ser hydroxyl function.

The N^{α}-Boc protected fragments, peptides (**IV**) and (**XVII**) were obtained using the method of active esters (see the scheme). During the synthesis of these peptides, the glycine carboxyl group was protected by salt formation. Note that fragments (**IV**) and (**XVII**) and intermediates of their synthesis are crystal substances; they are obtained in sufficiently high yields practically on any scale.

It was interesting to us to compare the results of conventional and solid phase variants of the assembly of alloferon molecule, as each of these approaches has their own advantages and drawbacks. The coupling of fragments in solution seems to be more economic, since it does not require the application of great volumes of solvents and two- to threefold excesses of acylating agents. The coupling of fragments on polymer seems to be more technological due to the simplicity of isolation of intermediates, a possibility of the process



The scheme of alloferon synthesis according to (**a**) the conventional and (**b**) solid phase variant of fragment coupling. (***P***) the Merrifield polymer. (*1*) TFA, (*2*) HOBt, (*3*) H_2/Pd, (*4*) HONp + DCC/HOBt, (*5*) H_2/Pd in the case of synthesis in solution or trifluoromethanesulfonic acid in TFA in the case of SPS.

Table 1. Homogeneity of SPS intermediates of alloferon analyzed by HPLC*

Peptidyl-(***P***)	Amino acid sequence of the SPS product obtained from peptidyl polymer	Target peptide		Impurities		Impurity structure
		RT, min	content, %	RT, min	content, %	
(**VIb**)	H-Val-His-Gly-OH	7.5	98.8	–	<2	Not determined
(**VIIIb**)	H-His-Gly-Val-His-Gly-OH	10.6	98.7	–	<2	Not determined
(**Xb**)	H-Gln-His-Gly-Val-His-Gly-OH	12.2	95.0	12.9	2.5	Glp-His-Gly-Val-His-Gly-OH
(**XIIb**)	H-His-Gly-Gln-His-Gly-Val-His-Gly-OH	13.8	92.3	12.2	4.0	H-Gln-His-Gly-Val-His-Gly-OH
				12.9	2.2	Glp-His-Gly-Val-His-Gly-OH
(**XIXb**)	H-His-Gly-Val-Ser-Gly-His-Gly-Gln-His-Gly-Val-His-Gly-OH	15.5	64–85	13.8	24–8	H-His-Gly-Gln-His-Gly-Val-His-Gly-OH

* Conditions: Ultrasphere ODS 4.6 × 250 mm column. Buffer A, 0.1% TFA; buffer B, 80% acetonitrile in 0.1% TFA. Gradient of B in A from 0 to 40% for 40 min. Flow rate 1 ml/min, detection at 226 nm.

automation, and also a possibility of regeneration of the excessive peptide fragments.

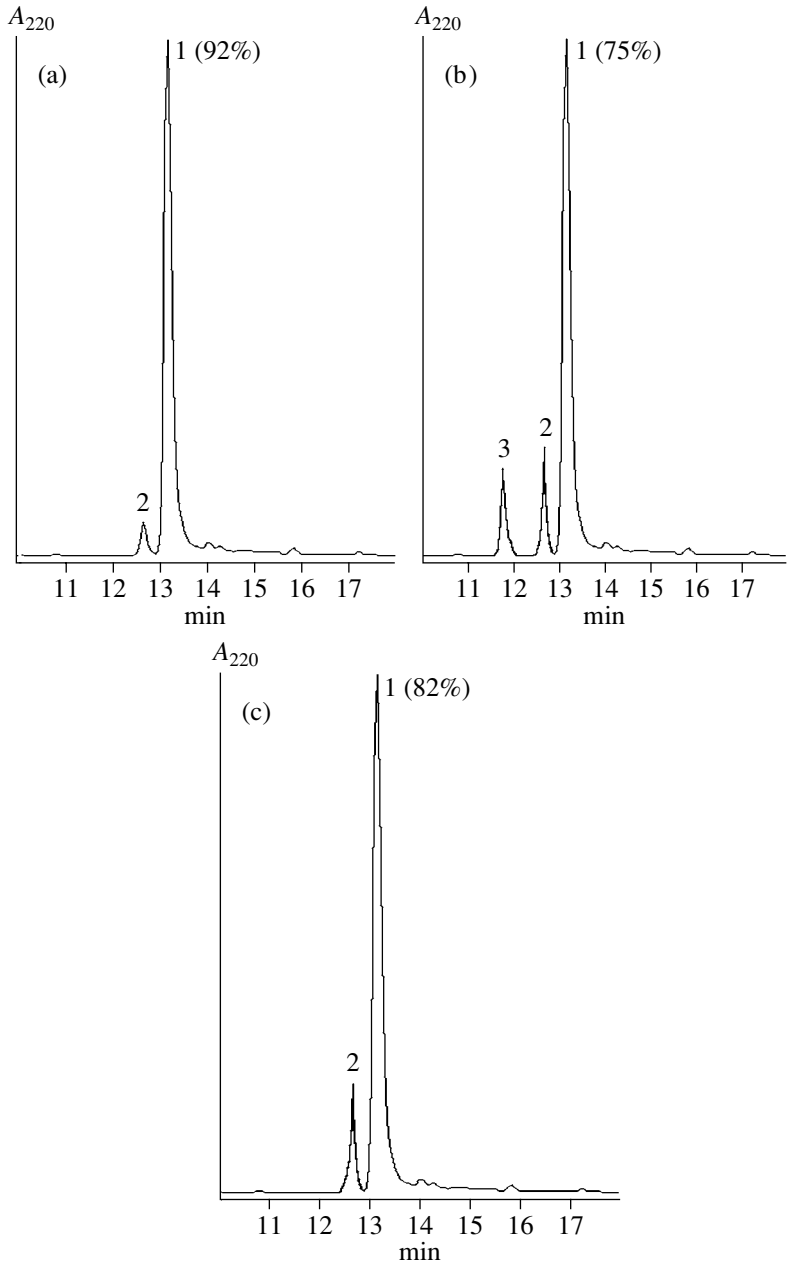
The carboxyl group of the *C*-terminal Gly residue was blocked with benzyl protection at the synthesis in solution. The use of temporary N^{im}-Boc protection and the presence unprotected (after the first treatment of the peptide with TFA) imidazole ring of His in the growing peptide chain limited the choice of the methods of fragment condensation. The method of the active *p*-nitrophenyl esters was applied to the creation of amide bond or, in the case of coupling with pentapeptide (**XVII**), the adduct of DCC and pentafluorophenol (complex F) was used, because it is known that, under these conditions, the imidazole ring of histidine and carboxamide function of glutamine do not react [8].

We have met certain difficulties at the isolation of intermediate benzyl esters of N^{α}-Boc-protected peptides at the alloferon synthesis in solution. A good solubility of these intermediates in water (beginning from the stage of hexapeptide) makes impossible their traditional isolation from aqueous reaction mixtures by extraction. All the compounds are hygroscopic and badly crystallize. Moreover, the N^{im}-Boc protection turned out to be rather labile at heating, which complicated the monitoring of the occurring transformations by TLC. Despite the fact that the reactions of formation of amide bond proceeded quickly and completely during the synthesis and the target product was enough homogeneous after the final splitting off of the protection (the content of alloferon was 92% by HPLC, see the figure), the yield of the purified alloferon was only 17% from the starting *C*-terminal dipeptide. This relatively low yield of alloferon is explained by the losses at the isolation of intermediates. The main impurity (4% by HPLC, see the figure) in the crude product of conventional synthesis was octapeptide H-His-Gly-Gln-His-Gly-Val-His-Gly-OH, *C*-deblocked (**XIIa**). In connection with the aforesaid, for preparation of greater amounts of alloferon (more than 50 g) seems to be problematic at the application of fragment condensation in solution.

A chloromethyl anchor group was used for SPS on the Merrifield polymer. The SPS scheme repeats that of the peptide preparation in solution except for the protection of Gly carboxyl group with benzyl ester connected with the polymer support. The *C*-terminal dipeptide (**IV**) was attached to the support by the method of cesium salts [9].

A trial synthesis of alloferon was carried out to determine the advantages and drawbacks of the SPS variant. We started from 11.0 g of dipeptidyl polymer Boc-His(Boc)-Gly-(P) containing 7.5 mmol of the starting dipeptide according to the standard protocol described in the Experimental section. The homogeneity of the intermediates during the trial synthesis of alloferon was monitored by HPLC. The corresponding compounds were obtained after the coupling with twofold excess of acylating agent and deblocking of an aliquot of the obtained peptidyl polymer with the use of trifluoromethanesulfonic acid. The results of these experiments are given in Table 1. The impurities whose contents exceeded 2% were isolated from the corresponding reaction mixtures by HPLC and identified with the help of ^{1}H NMR spectroscopy, amino acid analysis, or mass spectrometry.

The trial synthesis allowed us to reveal several "bottlenecks." For example, it was established that the preparation of octapeptidyl polymer (**XIIb**) by the coupling of hexapeptidyl polymer (**Xb**) with twofold excess of Boc-His(Boc)-Gly-ONp (**V**) proceeds insufficiently full: about 4% of amino component remains in the reaction mixture. Note that the qualitative test with ninhydrin [10] also shows the presence of residual amino groups in octapeptidyl polymer. After repeating of the coupling at the presence of 1 equiv of HOBt, the resid-



The HPLC of the crude products of (a) conventional and (b, c) solid phase synthesis of alloferon (a fragment with target fractions is given). (b) The reaction mixture of SPS after the splitting off of the peptide from polymer by the action of trifluoromethanesulfonic acid in TFA and (c) the subsequent treatment with 10% solution of ammonium bicarbonate (pH 8.5) for 2 h. Peak *1*, alloferon (**XX**); peak *2*, octapeptide H-His-Gly-Gln-His-Gly-Val-His-Gly-OH; and peak *3*, the product of N ⟶ O-acyl migration of Ser residue. Column: Nucleosil 100 C-18, 5 μm, 4.6 × 250 mm. Buffer A, 0.05 M KH_2PO_4, pH 3.0; buffer B, 70% CH_3CN + 30% of buffer A; a gradient elution with buffer B in buffer A from 0 to 30% for 30 min, detection at 220 nm.

ual amino groups were absent. In addition, the crude SPS product at this stage contains an impurity (about 2%) of hexapeptide with *N*-terminal pyroglutamic acid instead of glutamine (see Table 1). Most likely, this impurity is a product of an acid-catalyzed side reaction of the glutamine cyclization to the corresponding lactam, i.e., pyroglutamic acid. This side reaction was sometimes observed at the SPS of peptides with the use of Boc-methodology [11].

The final fragment condensation (5 + 8) on solid phase also proceeded in insufficiently high yield of the product (**XVIIIb**). In dependence on the coupling conditions, the content of the target alloferon in the reaction mixture changed from 64 to 85%, and the content

Table 2. The effect of conditions of the fragment condensation of pentapeptide Boc-His(Boc)-Gly-Val-Ser(Bu^t)-Gly-OH (**XVII**) with octapeptidyl polymer H-His-Gly-Gln-His-Gly-Val-His-Gly-OCH$_2$-(***P***) (**XIIb**) on the yield of alloferon

Acylating agent	Excess, mol/mol	Solvent	Reaction time, h	Alloferon content, % (HPLC**)
1. Boc-His(Boc)-Gly-Val-Ser(Bu^t)-Gly-ONp* + HOBt	2	DMF	16	64
2.	1	DMF	16	68
	2	DMF/NMP, 1 : 1	16	75
	2	1.5 M urea in DMF	16	80
Boc-His(Boc)-Gly-Val-Ser(Bu^t)-Gly-OH (**XVII**) + complex F	2	DMF/NMP, 1 : 1	16	85

Notes: * *p*-Nitrophenyl ester of the pentapeptide was not isolated.

** Conditions: Ultrasphere ODS 4.6 × 250 mm column. Buffer A, 0.1% TFA; buffer B, 80% acetonitrile in 0.1% TFA. Gradient of B in A from 0 to 40% for 40 min. Flow rate 1 ml/min, detection at 226 nm.

of the amino component (octapeptide) not entered into the reaction, from 24 up to 8%, respectively. These data are listed in Table 2.

We did not managed to find the conditions that would provide at this stage an yield of the target peptide above 85%. Low yields of products at separate stages of SPS of peptides are frequently caused by a decreased swelling of peptidyl polymers in the used solvents and, consequently, inaccessibility of amino group. Mixtures of such solvents as DMF, NMP, and DMSO and additives of urea, salts, or other reagents [12] are frequently used to increase the swelling of peptidyl polymers. At the synthesis of alloferon, the decrease in the yield is also most likely caused by the insufficient swelling octapeptidyl polymer (**XIIb**). This presumption is confirmed by the fact that a repeated coupling of (**XVII**) with (**XIIb**) in DMF practically did not improve the yield, whereas the replacement of DMF with a DMF–NMP mixture or 1.5 M urea solution in DMF leads to a substantial increase in the yield of target product (up to 75–80%). The application at this stage of a more active condensing agent, complex F, has allowed an increase in the yield of target peptide by 5% (see Table 2).

A solution of trifluoromethanesulfonic acid in TFA was used for splitting off of alloferon from the polymeric support [13]. An N ⟶ O acyl migration of the Ser residue was observed under these rigorous acidic conditions. This side reaction was repeatedly described at the treatment of Ser- and Thr-containing peptides by hydrogen fluoride or trifluoromethanesulfonic acid [13, 14]. On can see from the figure that the crude SPS product in comparison with a product of conventional synthesis contains an additional impurity, the substance corresponding to peak with RT 11.8 min. Most likely, this is formed due to the N ⟶ O-acyl migration. This impurity disappears after the treatment of the reaction mixture with 10% solution of ammonium bicarbonate with pH 8.5 for 2 h [14].

The crude products of both conventional and solid phase synthesis were purified in two stages: first, a chromatography on ion exchanger and, second, by desalting by preparative HPLC. The chromatography on CM-Sephadex in a gradient of ionic force of pyridine-acetate buffer, pH 4.5 (from 0.1 up to 2.0 M) allowed us to completely separate the impurities of the truncated peptides and to transfer the target peptide in the acetate form. The product of ion-exchange chromatography contained 95–96% of alloferon. After HPLC, the yield of alloferon synthesized by SPS was 40% from the starting dipeptide attached to polymer. The purity of product determined with the help of analytical HPLC was 98%.

Thus, the schemes of conventional and solid phase synthesis of alloferon were developed, which allow the preparation of the substance of necessary purity on a scale of 50–100 g. An advantage of the fragment condensation on polymer for the preparation of alloferon was shown (a higher yield and a better technology). The main impurities formed during the alloferon synthesis were identified.

EXPERIMENTAL

Derivatives of *L*-amino acids (Bachem, Switzerland); and DCC, HONSu, HONp, HONb, pentafluorophenol, and NMM (Fluka, Switzerland) were used in this work. Dichloromethane, TFA, and NMP (Fluka, Switzerland) were used for synthesis. DMF was distilled over ninhydrin and barium oxide. Solvents of pure and chemically pure grade were used for extraction and crystallization of peptides. Analytical HPLC was carried out on a Gilson chromatograph (France) equipped with Ultrasphere ODS column 5 μm (4.6 × 250 mm) (Beckman, United States). Buffer A was 0.05 M KH_2PO_4, pH 3.0, and buffer B, 70% acetonitrile in buffer A; the elution with a gradient of the buffer B concentration in the buffer A from 0 to 40% for 40 min. Flow rate was 1 ml/min, detection at 220 nm.

Preparative HPLC was achieved on a Beckman (United States) instrument equipped with a column (50 × 250 mm) packed with Diasorb-130T-C16, 10 μm (ZAO Biokhimmak NO, Russia). Peptides were eluted at a flow rate of 50 ml/min and detected at a wavelength

of 220 nm. Buffer A was 0.01 M ammonium acetate, buffer B, 80% aqueous acetonitrile, elution was carried out with a 0.3%/min gradient of B in A beginning from 0%. Acetonitrile from Lecbiopharm (Russia) was used for HPLC.

The homogeneity of the compounds obtained was confirmed by TLC on pre-packed Kieselgel 60 plates (Merck, Germany) in the following solvent systems: (A) 15 : 4 : 1 chloroform–methanol–32% CH_3COOH, (B) 6 : 3 : 1 ethyl acetate–chloroform–methanol, (C) 60 : 45 : 20 chloroform–methanol–32% CH_3COOH, (D) 5 : 3 : 1 chloroform–methanol–32% CH_3COOH, (E) 9 : 1 : 0.5 chloroform–methanol–32%CH_3COOH, (F) 7 : 3 isopropanol–25% ammonia, and (G) 8 : 8 : 8 : 1 chloroform–methanol–benzene–water. Substance spots on chromatograms were detected by a chlorine–benzidine or a ninhydrin reagent. Mps (not corrected) were determined on Boetius hot plate. An amino acid analysis of peptides hydrolyzed with 6 N hydrochloric acid containing 2% phenol at 110°C for 24 h was carried out on a Biotronik LC 5001 analyzer (Germany). ^{1}H NMR spectra were measured on a Bruker WH-500 spectrometer (Germany) at 500 MHz in DMSO-d_6 at 300 K; the concentrations of peptides were 2–3 mg/ml. Chemical shifts (δ, ppm) were calculated relative to tetramethylsilane. Mass spectra were registered on a device PC-Kompact MALDI (Kratos, UK).

Boc- and Z-derivatives of amino acids and their active esters were obtained by the described procedures [15].

Boc-His(Boc)-Gly-OBzl (Ia). NMM (12.5 ml) and a solution of Boc-His(Boc)-ONp (54.0 g, 114 mmol) in DMF (400 ml) were added to a solution of H-Gly-OBzl · TosOH (36.4 g, 114 mmol) in DMF (200 ml). The reaction mixture was kept at 20°C for 16 h, while monitoring the course of reaction by TLC in systems B and E. DMF was evaporated, and the residue was dissolved in ethyl acetate (1200 ml) and washed with 0.1% ammonia (3 × 400 ml), water (2 × 400 ml), 2% aqueous solution of citric acid (300 ml), and water (3 × 300 ml). The organic phase was evaporated, and the residue was dissolved in a 5 : 1 ethyl acetate–benzene mixture, and evaporated. The residue was dried in a vacuum to give an oily product (**Ia**); yield 46.9 g (85%); R_f 0.80 (A), 0.32 (B), and 0.58 (E).

Boc-Val-His-Gly-OBzl (IIIa). A solution of (**Ia**) (46.9 g, 96.8 mmol) in TFA (200 ml) was kept for 1 h at 20°C and evaporated. The residue was successively triturated with fresh portions of a 1 : 1 ether–hexane mixture (2 × 250 ml) and with ether (3 × 250 ml), the solvents were decanted, and the residual oil was dried in a vacuum over alkali. Hereinafter, the completeness of splitting off of Boc protection and the homogeneity of the deblocked product were monitored by TLC in systems A and D. The resulting product (**IIa**) was dissolved in DMF (500 ml), NMM (19.8 ml, 180 mmol) and Boc-Val-ONSu (28.6 g, 90 mmol) were added, and the mixture was stirred for 16 h at 20°C. The completeness of the reaction was checked with TLC. The reaction mixture was evaporated in a vacuum, the residue was dissolved in ethyl acetate (1000 ml), and the solution was washed with water (3 × 350 ml) and evaporated. The residue was triturated with 1 : 1 ether–hexane mixture (3 × 300 ml), the solvent was decanted, and the residue was triturated with hexane. The precipitate was quickly filtered and dried in a vacuum to give peptide (**IIIa**); yield 38.8 g (89%); R_f 0.55 (A), 0.89 (D), 0.75 (E).

Boc-His(Boc)-Gly-OH (IV). A solution of Boc-His(Boc)-ONp (33.42 g, 70 mmol) in DMF (250 ml) was cooled to –10°C, and glycine (5.2 g, 70 mmol) in 2 N NaOH (35 ml) was added. After 4 h (TLC monitoring in system C), the reaction mixture was evaporated, and the oily residue was dissolved in water (400 ml) and extracted with ether (3 × 150 ml). The water phase was acidified with citric acid to pH 4, extracted with ethyl acetate (3 × 200 ml), and the combined ethyl acetate extract was washed with water (3 × 150 ml), evaporated in a vacuum, and crystallized from an ethyl acetate–ether mixture to give (**IV**), yield 28.2 g (75%); R_f 0.60 (A), 0.51 (E); mp 134–135°C.

Boc-His(Boc)-Gly-ONp (V). A solution of DCC (33.0 g, 157 mmol) in DMF (150 ml) was added to a cooled to –20°C solution of (**IV**) (53.0 g, 143 mmol) and HONp (22.0 g, 157 mmol) in DMF (500 ml). The mixture was kept for 16–24 h at 4°C. The precipitated dicyclohexylurea was filtered off, and the filtrate was evaporated. The residual oil was triturated with hexane (3 × 500 ml), hexane was decanted, and the residue was crystallized from an isopropanol–hexane mixture to give (**V**); yield 54 g (71%); R_f 0.72 (A), 0.38 (B), 0.64 (E); mp 128–130°C.

Boc-His(Boc)-Gly-Val-His-Gly-OBzl (VIIa). A solution of 38.8 g (80 mmol) of protected tripeptide (**IIIa**) in TFA (200 ml) was kept for 1 h at 20°C and evaporated. Dry ether (1000 ml) was added to the residue, and the resulting precipitate was filtered and dried in a vacuum over alkali to give 50 g of deblocked tripeptide (**VIa**) (99%). A solution of (**VIa**) (25.2 g, 40 mmol) in DMF (300 ml) was successively treated with NMM (8.8 ml, 80.0 mmol) and a solution of active ester (**V**) (21.4 g, 40.0 mmol) in DMF (200 ml). The reaction mixture was stirred for 16 h at 20°C and evaporated in a vacuum, and the residue was dissolved in ethyl acetate (700 ml) and washed with water (3 × 350 ml). Ethyl acetate was evaporated, and the residue was triturated with dry ether (4 × 150 ml). The solvent was decanted, the residue was triturated with hexane, and the precipitated solid was filtered and dried in a vacuum. The yield of (**VIIa**) was 28.6 g (90%); R_f 0.52 (A), 0.75 (D); mp 94–98°C; amino acid analysis: Gly 2.00 (2), Val 0.98 (1), His 2.05 (2).

Boc-Gln-His-Gly-Val-His-Gly-OBzl (IXa). Protected pentapeptide (**VIIa**) (28.6 g, 36 mmol) was deblocked with TFA as described above for (**IIa**).

NMM (10.0 ml, 99.0 mmol) and a solution of Boc-Gln-ONp (12.4 g, 32.8 mmol) in DMF (200 ml) were successively added to a solution of pentapeptide (**VIIIa**) trifluoroacetate (27.0 g, 32.1 mmol) in DMF (300 ml). The mixture was stirred for 16 h at 20°C and evaporated in a vacuum. Ethyl acetate (1500 ml) was added to the residue, the precipitated solid was filtered, washed with ethyl acetate (3 × 150 ml) and ether (3 × 150 ml), and dried. The product was precipitated from isopropanol with hexane and dried, to yield (**IXa**) (19.6 g, 72%); R_f 0.12 (A), 0.59 (D), 0.48 (G); amino acid analysis: Glx 1.02 (1), Gly 2.00 (2), Val 0.96 (1), His 2.00 (2). For ^{1}H NMR spectrum, see Table 3.

Boc-His(Boc)-Gly-Gln-His-Gly-Val-His-Gly-OBzl (XIa). NMM (7.90 ml, 72.0 mmol) and a solution of active ester (**V**) (13.36 g, 25.0 mmol) in DMF (200 ml) were successively added to a solution of hexapeptide trifluoroacetate (**Xa**), prepared from 19.5 g (23.8 mmol) of hexapeptide (**IXa**) in DMF (300 ml). The mixture was kept for 16 h at 20°C and evaporated, and the residue was triturated with 8 : 2 ether–ethyl acetate mixture (500 ml). The precipitate was filtered, washed with 1 : 1 ethyl acetate–ether mixture (2 × 150 ml), ethyl acetate (3 × 150 ml), and ether (3 × 150 ml), and dried. The product was precipitated with hexane from isopropanol and dried to yield (**XIa**) (20.5 g, 78%); R_f 0.2 (A), 0.65 (C), 0.57 (D); amino acid analysis: Glx 1.05 (1), Gly 3.00 (3), Val 0.99 (1), His 3.10 (3).

H-His-Gly-Gln-His-Gly-Val-His-Gly-OBzl trifluoroacetate (XIIa). Protected octapeptide (**XIa**) (20.5 g, 18.4 mmol) was deblocked with TFA as described above to give (**XIIa**); yield 25.1 g (99%); purity by HPLC (elution with 10 to 70% gradient of buffer B in buffer A for 30 min) no less than 90%; R_f 0.13 (C), 0.08 (D), 0.51 (F); amino acid analysis: Glx 1.00 (1), Gly 3.00 (3), Val 0.98 (1), His 3.05 (3). For ^{1}H NMR spectrum, see Table 3.

Z-Ser(Bu^t)-Gly-OH (XIII). A solution of glycine (9.3 g, 0.124 mol) in 2 N NaOH (61.5 ml) was added to a solution of Z-Ser(Bu^t)-ONb (56.2 g, 0.123 mol) in DMF (400 ml) cooled to –10°C. The reaction mixture was kept for 18 h and evaporated. Distilled water (500 ml) was added to the residue, and it was extracted with ether (3 × 200 ml). The water layer was acidified with 20% H_2SO_4 to pH 4 and the target product was extracted with ethyl acetate (300 ml). The organic layer was washed with water to neutral reaction, evaporated, and the residue was crystallized from ether (300 ml). The precipitate was filtered, washed with ether (3 × 50 ml), and dried. The yield of (**XIII**) was 41.3 g (95%); R_f 0.64 (A), 0.87 (D), 0.52 (G); mp 98–100°C.

Z-Val-Ser(Bu^t)-Gly-OH (XV). Compound (**XIII**) (41.3 g, 0.117 mol) was hydrogenated in 100 ml of methanol in the presence of 35%Triton B (66.7 ml) and 10% PdO/C catalyst (4.0 g). The catalyst was filtered off, and the filtrate was evaporated. The residue was dissolved in DMF (300 ml), cooled to –10°C, and a solution of Z-Val-ONSu (40.7 g, 0.117 mol) in DMF (100 ml) was added. After the end of reaction, the solution was evaporated, ethyl acetate (500 ml) was added to the residue, and the mixture was acidified with 1 N H_2SO_4. The organic layer was separated, washed with water (3 × 200 ml), and evaporated. Ether (200 ml) was added to the residue; the precipitated solid was filtered, washed with ether (3 × 50 ml), and dried. The yield of peptide (**XV**) was 40.9 g (98%); mp 105–107°C. R_f 0.71 (A), 0.28 (B), 0.88 (C).

Boc-His(Boc)-Gly-Val-Ser(Bu^t)-Gly-OH (XVII). Compound (**XV**) (40.9 g, 91 mmol) was hydrogenated in ethanol (500 ml) over 10% Pd/C (4 g) in the presence of 1 N NaOH (91 ml, 1 equiv). The catalyst was filtered off, and the filtrate was evaporated. The residue was dissolved in DMF (150 ml), cooled to –10°C and a solution of active ester (**V**) (48.5 g, 40.9 mmol) in DMF (100 ml) was added. After the end of reaction, DMF was evaporated, and the residue was treated as described above for (**IV**). The product was crystallized from ether (500 ml). The yield of (**XVII**) was 56.5 g (87%); R_f 0.59 (A), 0.94 (D), 0.57 (G); amino acid analysis: Ser 0.95 (1), Gly 2.00 (2), Val 1.00 (1), His 1.02 (1).

Boc-His(Boc)-Gly-Val-Ser(Bu^t)-Gly-His-Gly-Gln-His-Gly-Val-His-Gly-OBzl (XVIIIa). A solution of pentapeptide (**XVII**) (14.4 g, 20.2 mmol) in DMF (250 ml) was added to a solution of octapeptide (**XIIa**) (21.5 g, 18.3 mmol) in DMF (250 ml). The mixture was cooled to –10°C, and complex F (15.3 g, 20.2 mmol) was added. The reaction mixture was stirred for 2 h at 0°C and 20–30 h at 20°C. The dicyclohexylurea precipitate was filtered off, the filtrate was evaporated to the volume of 100 ml, and ethyl acetate (500 ml) was added. The precipitated solid was filtered, washed with ethyl acetate (3 × 150 ml) and ether (3 × 150 ml), and dried. The yield of (**XVIIIa**) was 27.5 g (92%); R_f 0.52 (C), 0.38 (D); amino acid analysis: Ser 0.97 (1), Glx 1.02 (1), Gly 5.00 (5), Val 1.96 (2), His 4.00 (4).

H-His-Gly-Val-Ser-Gly-His-Gly-Gln-His-Gly-Val-His-Gly-OH acetate (XX). The protected tridecapeptide (**XVIIIa**) (27.5 g, 16.9 mmol) was deblocked with TFA as described above. The product was dissolved in 80% AcOH (500 ml), 10% Pd/C (3.0 g) in 50 ml of water was added, and the reaction mixture was hydrogenated until the disappearance of starting compounds. The catalyst was filtered off, the filtrate was evaporated to dryness, and the residue was dissolved in 98% AcOH (100 ml) and evaporated to the consistence of liquid oil. Isopropanol (800 ml) was added, and the precipitated solid was filtered, washed with isopropanol and ether and dried. The yield of crude (**XX**) trifluoroacetate was 22.4 g. The content of target substance according to analytical HPLC was 90%. The product was dissolved in 0.1 M pyridine–acetate buffer (pH 4.5, 100 ml) and applied onto a column (2.6 × 60 cm) filled with CM-Sephadex C-25 equilibrated with the same buffer. The peptides were eluted by a linear gradient of ionic

Table 3. Chemical shifts of protons in ^{1}H NMR spectra of alloferon and its fragments in DMSO-d_6 (δ, ppm)

	Alloferon (**XX**)					Peptide (**XIIa**)					Peptide (**IX**)				
	H_N	H_α	H_β	H_γ	other	H_N	H_α	H_β	H_γ	other	H_N	H_α	H_β	H_γ	other
His^1		4.19	3.21 d		9.02 H2 7.49 H4 s (imidazole)										
Gly^2	8.78 d	3.86; 4.01													
Val^3	8.25 d	4.32	1.96 m	0.823 d; 0.858 d											
Ser^4	8.14 d	4.30	3.58 m												
Gly^5	8.13 t	3.73; 3.80													
His^6	8.20 d	4.61	2.95; 3.12				4.21	3.21 d		9.02 H2 s 7.48 H4 s (imidazole)					
Gly^7	8.32 t	3.72; 3.81				8.81 t	3.80; 3.95								
Gln^8	8.16 d	4.25	1.73 m; 1.88 m	2.08 t	6.78 s, 7.28 s (amide)	8.37 d	4.30 m	1.73 m; 1.88 m	2.10 t	6.82 s; 7.28 s (amide)	7.00 d	3.87	1.65 m; 1.80 m	2.07 m	6.78; 7.28 s (amide)
His^9	8.28 d	4.61	2.96; 3.10			8.32 d	4.62	3.00; 3.11 m		8.94 H2; 7.36 H4 (imidazole)	8.10 d	4.63	2.97; 3.01 m		8.95 H2 s 7.37 H4 s (imidazole)
Gly^{10}	8.27 t	3.76; 3.87				8.28 t	3.77; 3.89				8.14 t	3.77; 3.85			
Val^{11}	8.01 d	4.17	1.96 m	0.778 d; 0.803 d		8.01 d	4.18 d	1.95 m	0.801 d; 0.778 d		7.99 d	4.16 t	1.95 m		
His^{12}	8.35 d	4.65	2.95; 3.10			8.34 d	4.66	2.96; 3.10 m			8.34 d	4.66	2.96 m; 3.10		
Gly^{13}	8.39 t	2.89; 3.96				8.39 t	3.89; 3.97				8.37 t	3.97; 3.89			
					1.90 s CH_3 (AcOH)					5.11 s (CH_2–Ph) 7.35 (arom.)					1.36 s (Boc); 5.11s (CH_2–Ph); 7.35 (arom.)

Table 4. The standard protocol of the alloferon SPS (for 11 g, 7.5 mmol, of the starting dipeptidyl polymer)

Stage	Operation	Reagent	Time of single treatment, min*	Volume of single treatment, ml
1	Washing	$3 \times CH_2Cl_2$	3	100
2	Deprotection of α-amino groups	50% TFA/CH_2Cl_2	10	100
			30	100
3	Washing	$3 \times CH_2Cl_2$	3	100
		$3 \times$ DMF	3	100
4	Neutralization	10% DIEA/DMF	5	100
			10	100
5	Washing	$3 \times$ DMF	3	100
6	Coupling	15 mmol of active derivative of Boc-amino acid or Boc-peptide in DMF	120	60
7	Washing	$3 \times$ DMF	3	100
		$3 \times CH_2Cl_2$	3	100
8	Determination of residual amino groups	Ninhydrin solution [10, 14]		
9	Repeated coupling	7.5 mmol of active derivative of Boc-amino acid or Boc-peptide in DMF	120	60

* In the case of coupling of the active derivative of *N*-terminal pentapeptide (**XVII**) with octapeptidyl polymer (**XIIb**), the reaction time was increased to 16 h.

strength of the same buffer [from 0.1 (1 l) to 2 M (1 l)]. The fractions containing the target product (by HPLC) were combined, evaporated to dryness, and then twice coevaporated with water. The final purification of the product was achieved with the help of preparative HPLC under the conditions described above. The yield of alloferon (**XX**) was 11.5 g [46%, or 17% from the starting dipeptide (**I**)]. The purity of the product determined by analytical HPLC was 98%; R_f 0.42 (F); amino acid analysis: Ser 0.98 (1), Glx 1.02 (1), Gly 5.00 (5), Val 1.98 (2), His 4.00 (4); MS, *m/z:* 1264.7 (calculated 1265.3). Elemental analysis: found, %: C 44.90, I 6.72, N 20.59. $C_{52}H_{76}N_{22}O_{16} \cdot 2AcOH \cdot 6H_2O$. Calculated, %: C 45.04, H 6.48, N 20.63. According to the elemental analysis, the content of water in the alloferon preparation was 7.2%; the content of acetic acid, 8.0%; and the content of peptide material, 84.8%. For ^1H NMR spectrum, see Table 3.

Solid phase synthesis of alloferon. A chloromethylated styrene copolymer with 1% divinylbenzene (the Merrifield polymer) containing 1.07 equiv of chlorine per g with the particle size of 200–400 mesh (Sigma, United States) was used for SPS. Dipeptide (**IV**) was attached to the polymer support with the use of the corresponding cesium salt [9]. The cesium salt was obtained by the addition of a solution of Cs_2CO_3 (1.95 g, 6 mmol) in 6 ml of water to a solution of dipeptide (**IV**) (4.95 g, 12 mmol) in 10 ml of ethanol. After 15 min, the mixture was evaporated to dryness; the residual water was removed by coevaporation with benzene (2×20 ml) and drying in a vacuum over P_2O_5. The dried dipeptide salt was dissolved in DMF (30 ml), and the Merrifield polymer (10 g) was added. The suspension was slowly stirred for 24 h at 50°C. The polymer was filtered and successively washed with DMF (3×50 ml), a 1 : 1 DMF–water mixture (5×50 ml), DMF (3×50 ml), EtOH (3×50 ml), and ether (3×50 ml) and dried in a vacuum. The content of peptide in the polymer determined by the increase in its mass and with the help of spectrophotometric titration with picric acid [14] was 0.6–0.7 mmol/g.

SPS was carried out from 11.0 g of dipeptidyl polymer containing 7.5 mmol of starting dipeptide according to the standard protocol outlined in Table 4. After the end of SPS, the tridecapeptidyl polymer (**XVIIIb**) was deblocked by the action of 50% TFA in CH_2Cl_2 (100 ml) for 30 min. The peptidyl polymer (**XVIIIb**) was washed with CH_2Cl_2 (3×100 ml) and dried in a vacuum over P_2O_5. The mass of tridecapeptidyl polymer (**XIXb**) was 21.6 g; its additional mass after the termination of synthesis was 11.6 g.

H-His-Gly-Val-Ser-Gly-His-Gly-Gln-His-Gly-Val-His-Gly-OH (XX). Trifluoromethanesulfonic acid (20 ml) was added in portions to a cooled to 0°C suspension of tridecapeptidyl polymer (**XIXb**) (21.6 g) in TFA (200 ml) containing 2 ml of anisole. The stirring was continued for 10 min at 0°C and 1.5 h at 25°C. The reaction mixture was carefully poured out in 1 l of dry ether cooled to 0°C. The precipitated solid was filtered and washed on filter with ether (5×100 ml). The precipitate on the filter, consisting of a mixture of the polymeric support and the target peptide split off from it, was treated with three portions of TFA (50 ml each), and the solution peptide in TFA was filtered in a flask

containing 1 l of dry ether. The precipitate was separated, washed with ether (5 × 100 ml), and dried in a vacuum over alkali. The resulting crude product (12.1 g) was kept for 2 h in 10% solution of ammonium bicarbonate (pH 8.5). The purification of the peptide was carried out by ion exchange chromatography and preparative HPLC under the above-described conditions. The yield of (**XX**) was 4.2 g (40% from the starting dipeptide, attached to the polymeric support). The purity of the product determined by analytical HPLC was 98%; R_f 0.42 (F); amino acid analysis: Ser 0.98 (1), Glx 1.02 (1), Gly 5.00 (5), Val 1.98 (2), His 4.00 (4); MS, *m/z:* 1266.2 (1265.3 as calculated for $C_{52}H_{76}N_{22}O_{16}$); ^{1}H NMR spectrum was completely identical to that of the above-mentioned product of conventional synthesis.

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