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Synthesis and Properties of the *retro*-Analogue of Myelopeptide MP-2

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Abstract—The bone marrow myelopeptide MP-2 (Leu-Val-Val-Tyr-Pro-Trp), exhibiting antitumor activity, and its *retro*-analogue (Trp-Pro-Tyr-Val-Val-Leu) were synthesized, and their properties were studied. The *in vitro* and *in vivo* activities of *retro*-MP-2 were comparable with those of MP-2. Both peptides equally restored the functional activity of T-lymphocytes inhibited by toxins released by HL-60 cells and inhibited by 70–82% the growth of various types of transplantable solid tumors: Ca-755 adenocarcinoma of the mammary gland, Lewis adenocarcinoma of the lung, and S180 sarcoma. The positions and intensities of the Cotton effects in CD spectra of the MP-2 peptide and its *retro*-analogue in various solvents are almost indistinguishable. The positions of extrema and integral intensities of the amide I and amide A bands in IR spectra of both peptides were practically identical.

Key words: myelopeptide MP-2, retro-analogue, synthesis, antitumor activity, CD-spectroscopy; IR-spectroscopy

INTRODUCTION

In recent years, an important role of peptides from the bone marrow (myelopeptides) in the immunoregulatory function of organism has become evident.[2] A comprehensive study of myelopeptides demonstrated that they exhibit a wide spectrum of biological activities: immunoregulatory, antitumor, and differentiation [1]. Myelopeptide also affect the functional activity of the mononuclear phagocyte system [1].

The structure–function study of endogenous regulatory peptides has attracted a considerable attention of chemists, biologists, and pharmacologists for a number of years. This interest can primarily be explained by the possibility of creation of therapeutic agents on the basis of short linear biologically active peptides. The identification of structural elements of a peptide molecule responsible for its biological activity provides an advanced level of creation of analogues with the preset properties.

Construction of the molecules with a topochemical similarity to the starting peptides, in particular, *retro*-analogues (isomers of the natural peptides with an opposite direction of one or several peptide bonds) is of special interest. Such modified molecules could combine biological activity intrinsic for the starting compound [2, 3] with an increased stability to biodegradation due to the reversed acylation of the peptide chain.

In this paper, we report the synthesis and studies of the biological activity of Trp-Pro-Tyr-Val-Val-Leu, *retro*-analogue of the Leu-Val-Val-Tyr-Pro-Trp myelopeptide (MP-2). This analogue is distinguished from its natural prototype by the opposite direction of the amide bonds between all the amino acid residues. Previously, we have demonstrated [4] 70–80% inhibition by the MP-2 peptide of growth of various types of murine solid transplantable tumors, in particular, the P-388 lympholeucosis, the Ca-755 adenocarcinoma of mammary gland, the Lewis adenocarcinoma of lung, the B 16 melanoma, and the S 180 sarcoma.

RESULTS AND DISCUSSION

The MP-2 peptide was synthesized by the conventional methods of peptide chemistry in solution. The α-amino functions were blocked by Z-groups. The carboxyl functions of amino acid residues were protected by the conversion into *tert*-butyl and benzyl esters. The condensations were performed by the carbodiimide

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[2] Abbreviations: CM, condition medium; DCHA, dicyclohexylamine; DMF, dimethylformamide; MP, myelopeptides; PHA, phytohemagglutinin, and TFE, trifluoroethanol.

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Table 1. Effect of MP-2 and *retro*-MP-2 on the restoration of the PHA-induced proliferation of the human T-lymphocytes decreased by the action of the HL-60 CM

Substances added to T lymphocytes			Proliferation level, %
PHA (control)			100
PHA + HL-60 CM			28 ± 9
PHA + HL-60 CM +	MP-2 (μg/ml)	100*	61 ± 15*
		10**	48 ± 9**
	retro-MP-2 (μg/ml)	100*	62 ± 13*
		10*	49 ± 10*

* $p < 0.01$; ** $p < 0.05$.

method and the method of active esters. The *retro*-MP-2 was synthesized by solid phase method on a chloromethylated copolymer of styrene and divinylbenzene. The target MP-2 and *retro*-MP-2 hexapeptides were isolated and purified by the reversed phase HPLC and were characterized by HPLC, mass spectrometry, and sequencing.

The biological activity of MP-2 and *retro*-MP-2 was estimated according to the degree of restoration of the PHA-induced proliferation of human T-lymphocytes *in vitro* and according to the inhibition of growth of various transplantable murine tumors *in vivo*.

T-Lymphocytes are known to be the main factor of antitumor protection of organism. The tumor cells of any nature release toxic substances that inhibit the functional activity of T-lymphocytes [5, 6]. In particular, the cells of HL-60 line produce a protein that inhibits the proliferative ability of T-lymphocytes [5]. The MP-2 peptide was shown to restore the functional activity of T-lymphocytes inhibited by the toxins of HL-60 cells [7], and it is believed that this property of the MP-2 peptide is a basis of its antitumor effect.

The functional state of lymphocytes subjected to the action of tumor toxins was determined according to their ability to the blast transformation in the presence of PHA. This model is based on the evaluation of proliferation of T-lymphocytes of human peripheral blood cultured at suboptimal concentration of the mitogen. This property of the T-lymphocytes is characteristic of their functional activity [8].

The peripheral blood lymphocytes taken from healthy donors were used in this study. CM from the leukemia cells of the HL-60 line was used for the inhibition of the T-lymphocyte functional activity. The proliferative response of T-lymphocytes to the PHA introduction was evaluated according to the incorporation of [^{3}H]thymidine into DNA as described in [8]. Results of the experiments are presented in Table 1.

One can see from the table that a significant decrease (by 72%) in the PHA-induced proliferative response of T-lymphocytes is observed in the presence of CM from HL-60 cells. Both peptides under study restore the T-lymphocyte functional activity inhibited by the products of HL-60 cell line to a practically the same degree. This degree depends on the peptide dose: maximum effect (61% level of the proliferation) is observed at the peptide dose of 100 μg/ml. A decreased dose (10 μg/ml) results in a decreased effect (48%).

Three types of transplantable mouse cancers (the Ca-755 adenocarcinoma of mammary gland, Lewis adenocarcinoma of lung, and the S 180 sarcoma) were used for the estimation of antitumor effect. MP-2 and *retro*-MP-2 were introduced using two schemes: subcutaneously two times with the 96 h interval at doses of 1 or 2 μg/ml and five times daily at doses of 0.5 mg/kg. Sizes of the tumors were measured several days after their transplantation.

The results of studying the antitumor activity are summarized in Table 2. One can see that the *retro*-analogue exhibits a pronounced inhibiting effect on the growth of all the studied tumors, similar to that of the MP-2 peptide. The percentages of inhibition of the tumor growth by the *retro*-peptide and by the MP-2 peptide over different periods after their transplantation are practically the same.

Table 2. Effect of MP-2 and *retro*-MP-2 on the growth of transplanted mice tumors

Tumor type	Introduction scheme	Days after transplantation	Tumor growth inhibition, %	
			MP-2	*retro*-MP-2
Ca 755 adenocarcinoma of mammal gland	0.5 mg/kg, five times at 24-h intervals	7	78	72
		14	69	82
		18	62	67
Lewis lung adenocarcinoma	2 mg/kg, two times at 96-h interval	7	69	59
		14	71	66
		18	74	73
S180 Sarcoma	1 mg/kg, two times at 96-h interval	7	69	76
		14	75	68
		18	65	64

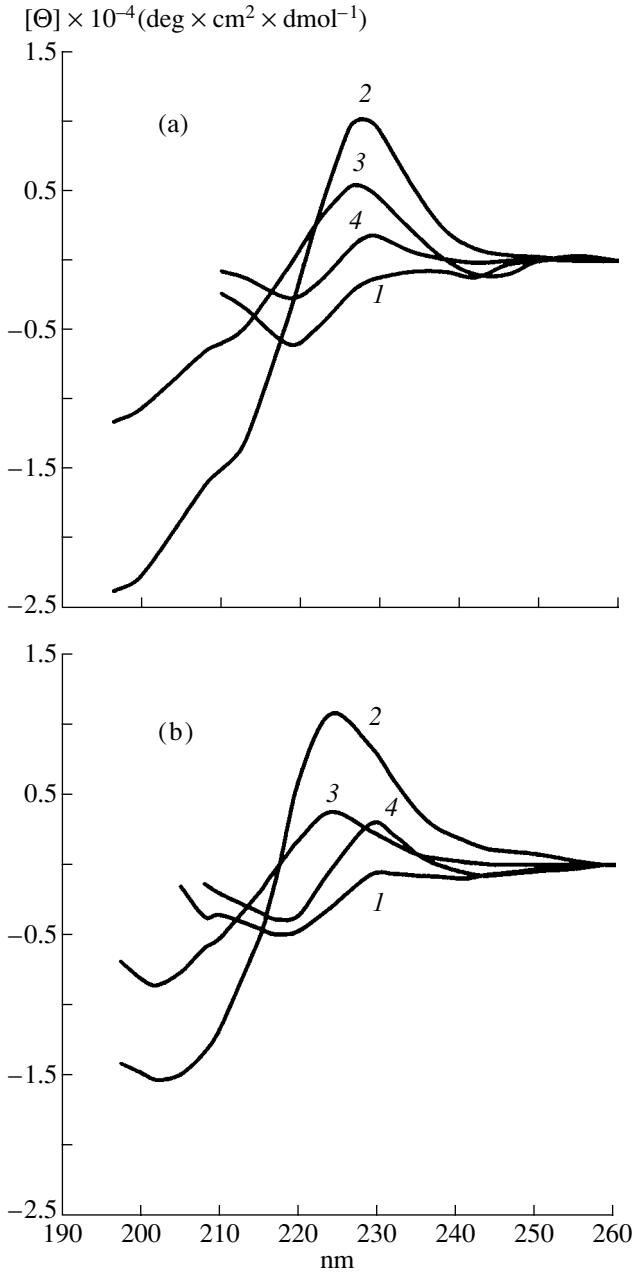

Fig. 1. CD spectra of (a) MP-2 and (b) *retro*-MP-2 hexapeptides in (*1*) THF, (*2*) water, (*3*) TFE, and (*4*) dioxane.

The results of the study of biological activity of MP-2 and *retro*-MP-2 in the *in vitro* and *in vivo* tests suggest that the converse direction of acylation of the MP-2 peptide chain has no effect on the structure of its active site; i.e., it does not disturb the spatial structure of this peptide.

This conclusion was confirmed by the studies of spatial structure of the MP-2 peptide and its *retro*-analogue. The conformational polymorphism of the peptide chains was evaluated by the method of circular dichroism (CD). The CD spectra were recorded in a number of solvents with various physicochemical parameters: water, TFE, dioxane, and THF. An analysis of the CD spectra demonstrated that the positions and the intensities of the Cotton effects for both peptides were practically the same in all of the used solvents (Fig. 1). These results confirm similar conformations of both compounds.

Another method that confirms the structural similarity of the studied peptides is IR spectroscopy. This method provides information about the presence of hydrogen bonds, because the stretching vibration frequency of NH and C=O groups of the amide bonds is sensitive to the participation of these groups in the hydrogen bonds. The quantity of free NH groups and NH groups involved into hydrogen bonding can be estimated according to integral intensities of the corresponding bands. The IR spectra of the MP-2 peptide and its *retro*-analogue are rather similar in the area of the amide I band. The bands of the stretching vibrations of C=O groups of the amide bonds (amide I) contain two components: at 1688 and 1634 cm^{-1} (MP-2) and at 1688 and 1636 cm^{-1} (*retro*-MP-2). We assigned the first component to the free (exposed to solvent) and the second, to that participating in hydrogen bonding. This assignment of the bands was based on the literature data for the model linear [9] and cyclic [10] peptides of similar structures. The ratios of integral intensities of the bands corresponding to the free and bound C=O groups in both peptides were ~3 : 2, which suggests the formation of β-turns stabilized by two intramolecular hydrogen bonds [9, 10]. The bands of stretching vibrations of NH groups of the amide bonds (amide A) in the spectra of both peptides (not given in the figure) have maxima at 3320 and 3330 cm^{-1}, respectively, which suggests the formation of strong hydrogen bonds in both peptides. Weak bands at 1735 (MP-2) and 1741 cm^{-1} (*retro*-MP-2) correspond to the band of the frequency of stretching vibrations of COOH groups.

The results of the studies of the MP-2 immunoregulatory peptide and its *retro*-analogue definitely demonstrated that both peptides exhibit practically the same *in vitro* and *in vivo* biological activities. The investigation of spatial structures of both peptides by the spectral methods revealed a similarity between the spatial organization of their peptide chains.

Thus, we experimentally demonstrated that the converse direction of acylation of all the amino acid residues in the molecule of endogenous MP-2 peptide had practically no effect on its specific biological activity and its spatial structure.

EXPERIMENTAL

Commercially available amino acids and their derivatives (Reanal, Hungary and Fluka, Switzerland) or the amino acid derivatives prepared according to the standard procedures were used in this study. Homogeneity of the obtained compounds at the intermediate stages of

the synthesis was determined by TLC on silica gel plates (Merck, Germany) in the following chromatographic systems: (A) 60 : 45 : 20 chloroform–methanol–32% acetic acid, (B) 5 : 3 : 1 chloroform–methanol–32% acetic acid, (C) 15 : 4 : 1 chloroform–methanol–32% acetic acid, (D) 3 : 1 : 1 *n*-butanol–acetic acid–water, and (E) 9 : 1 : 0.5 chloroform–methanol–acetic acid. The substances were detected by ninhydrin or *o*-tolidine.

Analytical HPLC was carried out on an LC-10ADvp chromatographic system (Shimadzu, Japan) equipped with an Ultrasphere C18 column (4.6 × 250 mm) in the concentration gradient of acetonitrile in 0.1% trifluoroacetic acid (from 0 to 80% for 32 min) at a flow rate of 1.6 ml/min; detection at 214 and 280 nm.

Preparative HPLC of the final and the intermediate peptides was carried out on a Diasorb C 16-T column (50 × 250 mm) eluted with a gradient of 80% acetonitrile/0.01 N ammonium acetate in 0.01 N aqueous ammonium acetate (from 10 to 50% for 120 min) at a flow rate of 50 ml/min; detection at 226 nm. Fractions corresponding to the main peak were combined and acetonitrile was evaporated. The resulting solution was diluted with water and lyophilized.

Synthesis of Leu-Val-Val-Tyr-Pro-Trp

Z-Val-Val-OH. Z-Val-ONp (7.45 g, 20 mmol) and 1 N NaOH (22 ml) were added to a solution of valine (2.56 g, 22 mmol) in DMF (70 ml). The reaction mixture was stirred for 12 h at 18°C and evaporated. The residue was dissolved in water (100 ml), washed with ether (3 × 30 ml), and acidified with 10% H_2SO_4 to pH 2.0. The oily precipitate was extracted with ethyl acetate (3 × 50 ml), washed with water (3 × 50 ml), dried with $MgSO_4$, and evaporated. The oily residue was triturated with anhydrous ether, the precipitated solid was filtered, washed with cool ether, and dried in a vacuum desiccator; yield 6.2 g (88%); R_f 0.71 (C), 0.54 (D)

H-Tyr(Bu^t)-Pro-OBu^t. Z-Tyr(Bu^t)OH DCHA salt (16.58 g, 30 mmol) was suspended in ethyl acetate (200 ml), washed with 5% H_2SO_4 (2 × 50 ml), water (3 × 50 ml) to pH 5.0, dried with $MgSO_4$, and evaporated. The residue was dissolved in anhydrous ethyl acetate (160 ml), and *N*-methylmorpholine (3.33 ml) was added at stirring. The mixture was cooled to –25°C, and isobutyl chloroformate (30 ml) was added. The reaction mixture was stirred for 10 min at –25°C, and a solution of H-Pro-OBu^t hydrochloride (6.85 g, 33 mmol) and *N*-methylmorpholine (50 ml) in ethyl acetate (70 ml) cooled to –20°C was added. The reaction mixture was stirred for 1 h with a gradual increase in temperature to 18°C, washed with water, 5% $NaHCO_3$ (2 × 50 ml), water (1 × 50 ml), 5% H_2SO_4 (2 × 50 ml), and, finally, with water (2 × 50 ml). The organic layer was dried with $MgSO_4$, filtered, and evaporated. The oily residue was dissolved in methanol (150 ml) and hydrogenated over 10% Pd/C (1 g) at 18°C until the disappearance of starting substance (TLC monitoring in 9 : 1 chloroform–methanol mixture). The residue of the oily target product was dried in a vacuum desiccator; yield 7.63 g (65%); R_f 0.77 (C), 0.64 (D).

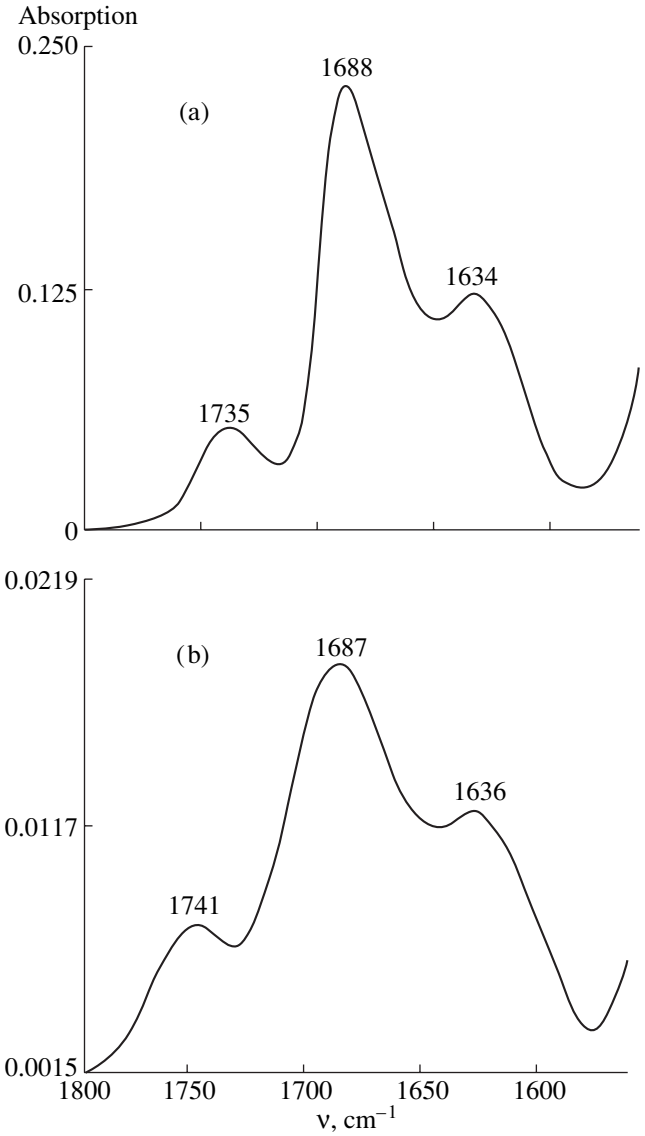

Fig. 2. IR spectra of (a) MP-2 and (b) *retro*-MP-2 hexapeptides in THF. The area of stretching vibration of C=O groups (amide I) is given.

H-Val-Val-Tyr(Bu^t)-Pro-OBu^t. *N*-methylmorpholine (2.22 ml) was added to a solution of Z-Val-Val-OH (6.2 g, 17.7 mmol) in DMF (100 ml). The reaction mixture was cooled to –25°C and gradually treated with isobutyl chloroformate (2.34 ml) at stirring. The reaction mixture was stirred for 10 min at –25°C, and a cooled (–20°C) solution of H-Tyr(Bu^t)-Pro-OBu^t (7.93 g, 19.5 mmol) in DMF (100 ml) was added. The reaction mixture was stirred for 2 h with a gradual temperature increase to 20°C and evaporated in a vacuum at 40°C. The residue was dissolved in ethyl acetate

(200 ml), washed with 5% $NaHCO_3$ (2 × 50 ml), water (1 × 50 ml), 5% H_2SO_4 (2 × 50 ml), and, finally, with water (2 × 50 ml). The ethyl acetate solution was dried with $MgSO_4$ and evaporated in a vacuum. The oily residue was dissolved in 3 : 7 ethyl acetate–hexane mixture (20 ml) and applied onto a column with silica gel 60 (90–130 μm) equilibrated with the same mixture. The target substance was eluted with the same solvent mixture (500 ml). The fractions containing the target product were combined and evaporated in a vacuum. The oily residue was dissolved in ethanol (100 ml) and hydrogenated over10% Pd/C (1 g) at 18°C until the disappearance of starting substance (TLC monitoring in 9 : 1 chloroform–methanol mixture). The catalyst was filtered off, and the filtrate was evaporated on a rotary evaporator at the temperature no higher than 30°C. The oily residue was triturated with anhydrous ether (2 × 30 ml). The solvent was filtered off, and the resulting amorphous powder was washed with anhydrous ether and dried in a vacuum desiccator; yield 7.62 g (73%); R_f 0.61 (C), 0.32 (D).

Z-Leu-Val-Val-Tyr-Pro. Z-Leu-ONSu (5.8 g, 16 mmol) was added to a stirred solution of H-Val-Val-Tyr(Bu^t)-Pro-OBu^t (7.62 g) in DMF (150 ml). The reaction mixture was stirred for 12 h at 18°C and evaporated. The oily residue was dissolved in ethyl acetate (200 ml); successively washed with water (1 × 50 ml), saturated solution of $NaHCO_3$ (2 × 50 ml), water, 2% H_2SO_4 (2 × 50 ml), water (2 × 50 ml); dried with $MgSO_4$; and evaporated. The oily residue was dissolved in TFA (70 ml) and stirred for 1 h at 18°C. TFA was removed on a rotary evaporator at 18°C. The oily residue was triturated with anhydrous ether (2 × 50 ml), and the amorphous powder was filtered and dried in a vacuum desiccator over NaOH. The product was dissolved in a 9 : 1 chloroform–methanol mixture and applied onto a column (30 × 200 mm) filled with silica gel 60 (90–130 μm) and equilibrated with the same solvent mixture. The column was successively eluted with the following chloroform–methanol mixtures: 9 : 1, 9 : 2, and 9 : 3 (200 ml of the each mixture). The fractions containing the target Z-Leu-Val-Val-Tyr-Pro were combined and evaporated; yield 6.55 g (70%); R_f 0.45 (A), 0.31 (B).

Leu-Val-Val-Tyr-Pro-Trp-OH. H-Trp-OBzl hydrochloride (3.63 g, 11 mmol), *N*-hydroxybenzotriazole (1.35 g, 10 mmol), and *N*-methylmorpholine (1.70 ml) were added to a solution of Z-Leu-Val-Val-Tyr-Pro-OH (6.55 g, 9 mmol) in DMF (80 ml). The reaction mixture was cooled to –20°C, and dicyclohexylcarbodiimide (2.06 g, 10 mmol) was added at stirring. The reaction mixture was stirred for 1 h at –20°C and for 12 h at 20°C and evaporated in a vacuum. The residue was dissolved in 1 : 1 butanol–ethyl acetate mixture; washed with water (1 × 30 ml), saturated solution of $NaHCO_3$ (2 × 50 ml), water, 2% H_2SO_4 (2 × 50 ml), and, finally, with water to pH 7. The organic layer was evaporated. The residue was dissolved in methanol (100 ml), treated with 1 N NaOH (9 ml), and hydrogenated over 10% Pd/C until the disappearance of starting substance (TLC monitoring). The catalyst was filtered off, and 1 N H_2SO_4 (9 ml) was added to the filtrate. The precipitate was filtered, washed with water, and dried in a vacuum desiccator. The amorphous powder containing the target product was dissolved in 0.01 N CH_3COONH_4 (10 ml) and subjected to HPLC on a Diasorb C 16-T column. The fractions containing the target product were combined and concentrated on a rotary evaporator to final volume of 10 ml. This solution was diluted with water to the volume of 50 ml and lyophilized; yield 3.2 g (46%); R_f 0.61 (A), 0.48 (B), 0.64 (D).

Synthesis of *retro*-MP-2 was carried out by the solid phase method using the chloromethylated copolymer of styrene and 1% divinylbenzene with the chlorine content of 1 mmol per g. Boc-amino acids were attached by the carbodiimide method with the addition of equimolar amounts of *N*-hydroxybenzotriazole for the inhibition of the racemization. The resulting peptide was cleaved from the polymer by the treatment with 70% trifluoroacetic acid.

Amino acid sequences of the MP-2 and *retro*-MP-2 peptides were determined on a gas phase sequencer (Applied Biosystem 477A, United States) connected with an automatic analyzer of phenylthiohydantoins of amino acids.

Molecular masses of the hexapeptides determined by mass spectrometry on a Thermo Bioanalysis Vision 2000 device (England) were equal to 776.

CD spectra were registered on a Jasco 500 C dichrograph (Japan) at the temperature of 20°C in demountable quartz cuvettes (Hellma) 10^{-2} cm in thickness. Concentration of the peptide solution was 1 mg/ml. The presented spectra are averaged after three scans.

IR spectra were measured on a Perkin-Elmer 1725 X spectrometer at a 4 cm^{-1} resolution and 20°C. The spectrometer was preliminary blown off with dry nitrogen for the removal of water vapour. The measurements were done in cuvettes of 0.2 mm in thickness made from CaF_2. The number of scans was 300.

The proliferative response of T-lymphocytes to the action of the phytohemagglutinin mitogen was estimated according to the technique described in [8].

Antitumor activities of the peptides were determined on various types of mouse tumors (Lewis lung carcinoma, Ca 755 adenocarcinoma of mammal gland, and S 180 sarcoma). The tumors were obtained from the Tumor Strain Bank of the Blokhin Cancer Research Center and passed on linear mice two times. The linear and hybrid female mice (18–20 g of the body mass) were purchased from the Stolbovaya breeding nursery of the Russian Academy of Medical Sciences. The solid tumors were transplanted by the introduction of the cell suspension in the 199 medium at a dose of 50 mg per

mouse. The MP-2 or *retro*-MP-2 peptide was introduced starting from the third day after the transplantation. The peptides were dissolved in isotonic solution and subcutaneously injected according to the following schemes: 0.5 mg/kg five times at 24-h intervals or 1 or 2 mg/kg two times at 96-h interval. An isotonic solution was injected to the control group of mice. The antitumor effect was determined according to the dynamics of inhibition of the growth of tumor node (DGT) on the 7th, 14th, and 18th day after the tumor transplantation. DGT was calculated as the ratio of difference in the average volumes of tumors in the control and the experimental groups to the tumor volume in the control group.

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