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Synthesis and Properties of a New Conformational Antigen which Models an Extracellular Region of β1-Adrenoreceptor

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Abstract—Two fragments corresponding to the 125–133 and 206–218 sequences of a molecule of the β1-adrenoreceptor (autoantibodies to this protein are often found in patients with dilated cardiomyopathy) were synthesized by the solid phase method with the use of Fmoc technology. Two new conformational antigens were prepared by directed (regioselective) and undirected (spontaneous) formation of intramolecular and intermolecular disulfide bridges between the corresponding cysteine residues of the synthesized peptides. One of these antigens consisted of a mixture of disulfide isomers, and another antigen was an isomer with a natural arrangement of S–S bridges. Immunosorbents were obtained by immobilization of the synthesizes antigens on the bromocyanogen-activated sepharose and applied to the removal of autoantibodies in a β1-adrenoreceptor from the blood plasma of patients. We demonstrated that the sorbents on the basis of the conformational antigens were more effective in comparison with those containing linear peptide precursors.

Key words: peptide antigens, spontaneous and regioselective formation of disulfide bonds, β1-adrenoreceptor, dilated cardiomyopathy.

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INTRODUCTION

Dilated cardiomyopathy is one of the main reasons of heavy cardiac decompensation and the most frequent reason for heart transplantation.[2] Pathogenesis of this disease is not studied in detail. A hypotheses of chronic virus infection and genetic determination are proposed, but recent published data point to an autoimmune character of this heavy pathology [1, 2]. Clinical evidence demonstrates that procedures of therapeutic apheresis of immunoglobulins considerably improve the state of patients with DCMP [3–5]. Autoantibodies that are found in the serum of DCMP patients mainly belong to the immunoglobulins of G class (IgG) and are antibodies against self-antigens such as a β_1-adrenoreceptor, myosin, adenine nucleotide translocator, sarcolemma laminin, protein of the M2 muscarinic receptor, and others [6]. It was established that precisely autoantibodies to the second extracellular loop of the molecule of a β_1-adrenoreceptor are most often found in patients with DCMP [7].

The introduction of the peptide corresponding to the sequence of the second loop of a β_1-adrenoreceptor in rabbits has been shown to result in DCMP development [8]. Hence, the presence of autoantibodies of this specificity plays a key role in pathogenesis of this disease. Moreover, the presence of antibodies in a β_1-adrenoreceptor has been found to be one of the factors of the development of tachyarrythmia and acute cardiac decompensation [9, 10].

β_1-Adrenoreceptor has three extracellular and three intracellular polypeptide sequences (loops) with seven transmembrane α-helical domains. Two disulfide bridges (Cys^{209} – Cys^{215} in the second loop and Cys^{131} – Cys^{216} between the first and the second loop) are in the molecule (Fig. 1) [11, 12].

Peptide fragments of the second extracellular loop of a β_1-adenoreceptor are known to be antigenic determinants which can be bound to autoantibodies in this protein [7, 13]. One of the approaches to DCMP treatment is the removal of autoantibodies in a β_1-adenoreceptor from blood plasma of patients by specific immunoabsorption on columns with sorbents which contain antigenic peptide fragments of this protein. For example, Coraffin® columns [3] are proposed for this purpose. Their active component is a mechanical mixture

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[2] Abbreviations: Acm, acetamidomethyl; DCMP, dilated cardiomyopathy; DIC, *N,N'*-diisopropylcarbodiimide; Fmoc, 9-fluorenylmethyloxycarbonyl; HOBt, 1-hydroxybenzotrazole; NMP, *N*-methylpyrrolidone; TIBS, triisobutylsilane; Pmc, 2,2,5,7,8-pentamethylchromane-6-sulfonyl; SPyr, 2-pyridinesulfenyl; SPPS, solid phase peptide synthesis. Other abbreviations correspond to IUPAC-IUB: Eur. J. Biochem, 1994, vol. 183, pp. 9–37.

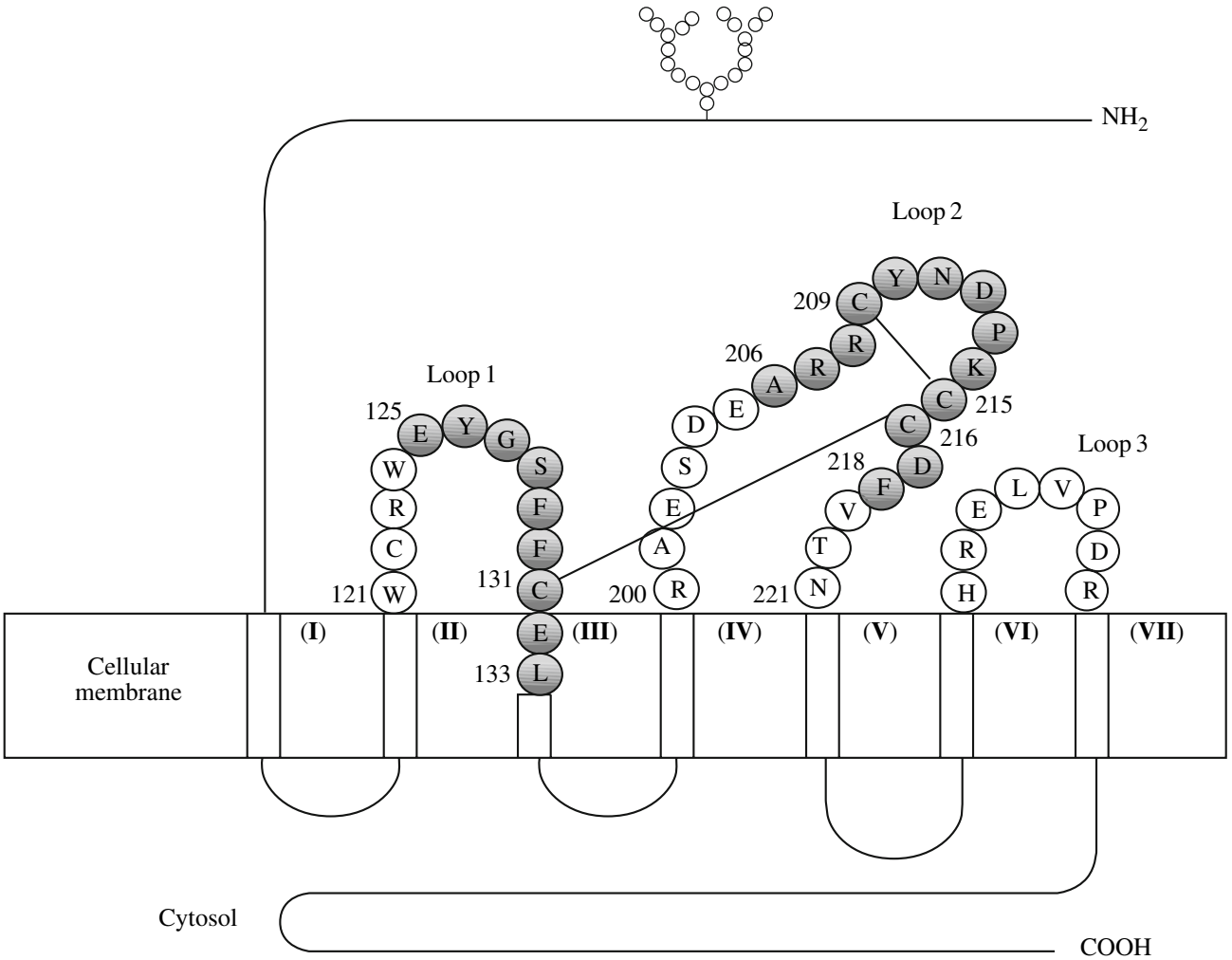
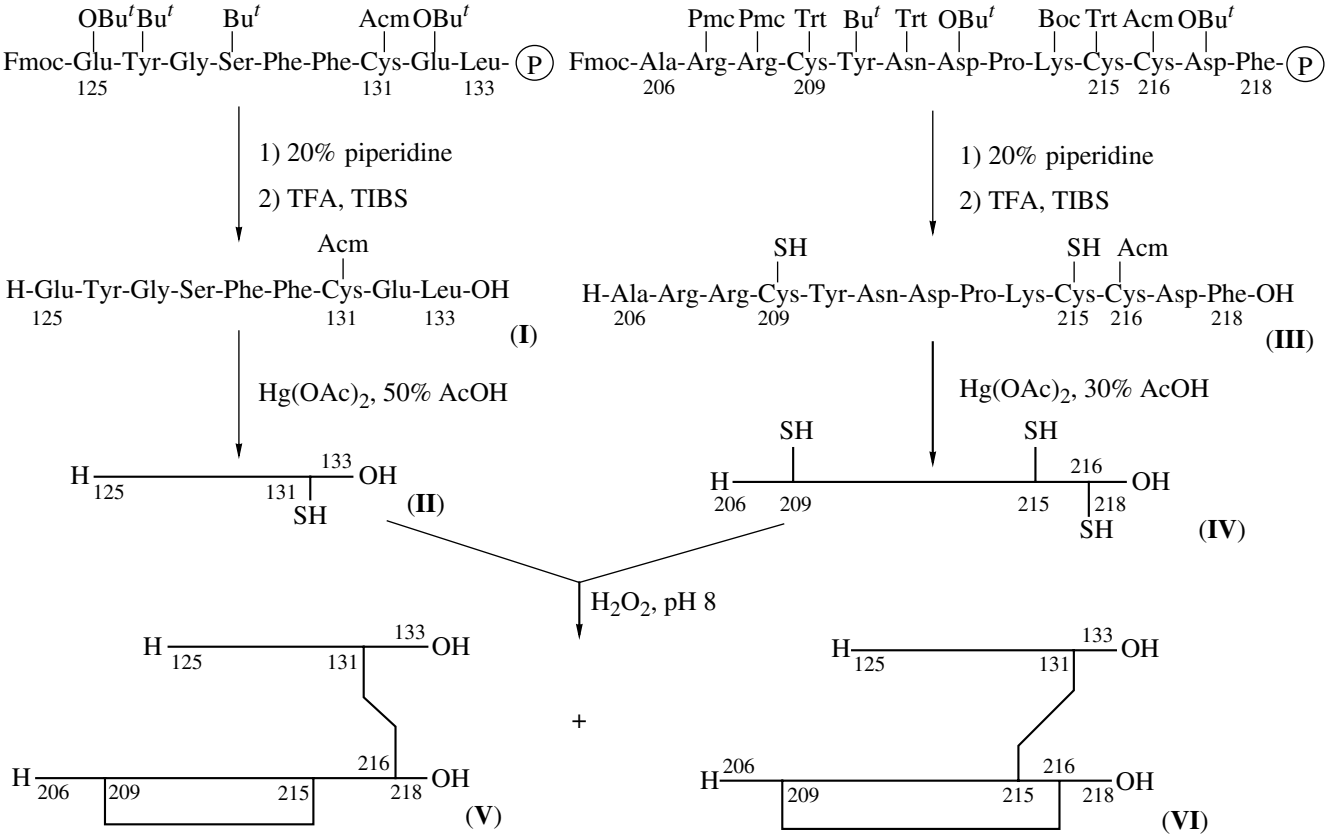


Fig. 1. Structure of a β_1-adrenoreceptor [3]. Fragments of the polypeptide chain chosen for the synthesis of conformational antigens are marked with gray color: ^{125}Glu-Tyr-Gly-Ser-Phe-Phe-^{131}Cys-Glu-^{133}Leu **(II)** and ^{206}Ala-Arg-Arg-^{209}Cys-Tyr-Asn-Asp-Pro-Lys-^{215}Cys-^{216}Cys-Asp-^{218}Phe **(IV)**.

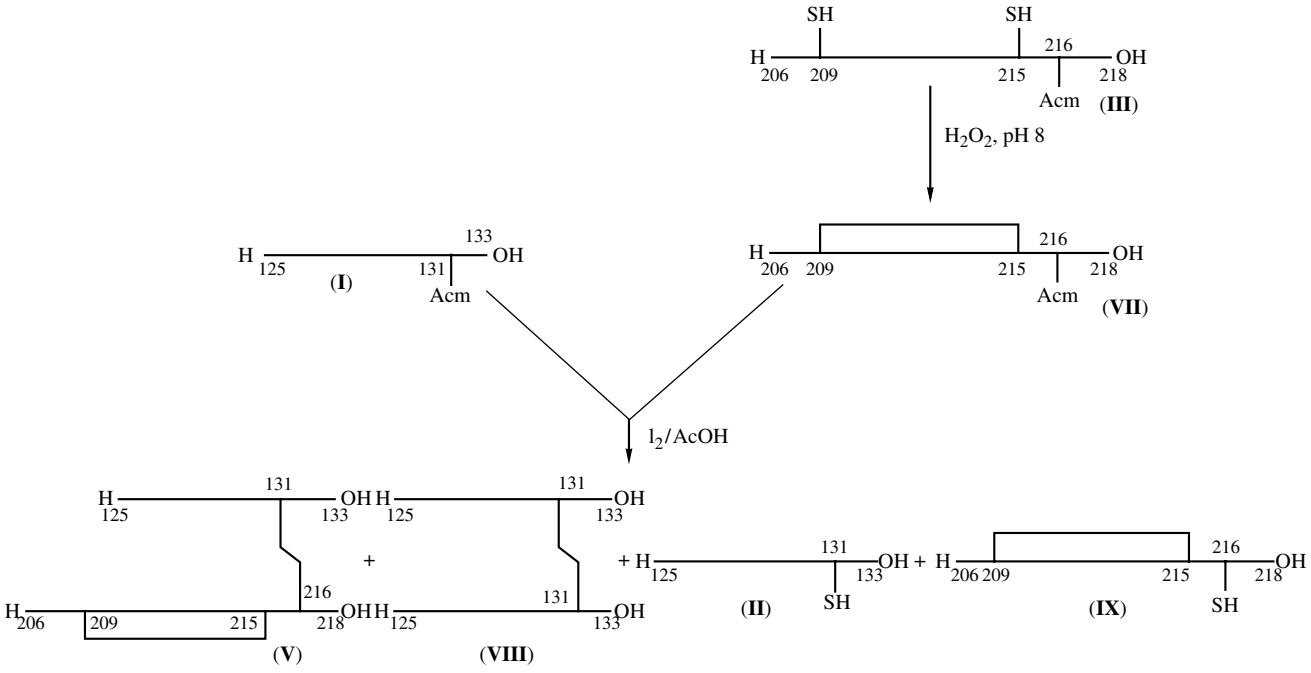
of fragments of the first and the second extracellular loops (the Glu-Tyr-Gly-Ser-Phe-Phe-Cys-Glu-Leu nonapeptide and Ala-Arg-Arg-Cys-Tyr-Asn-Asp-Pro-Lys-Cys-Cys-Asp-Phe tridecapeptide corresponding to the 125–133 and 206–218 sequences of the β_1-adenoreceptor, respectively [3]) immobilized on the bromocyanogen-activated agarose matrix. From our point of view, the Coraffin® columns have a significant disadvantage. Each of the two sorbents in the mechanical mixture contains one of the aforementioned peptides and binds autoantibodies specific to linear antigenic determinants which are located only in the first or only in the second extracellular loop of a β_1-adrenoreceptor.

As it has been mentioned above, two disulfide bonds are present in the native molecule. Disulfide bonds are known to have various functions in proteins. They stabilize folded proteins. The thiol–disulfide exchange can be a basis for regulation of enzymatic activity, and so forth. Moreover, both cysteine residues of the Cys^{215}-Cys^{216} sequence, which is located in the second extracellular loop of a β_1-adrenoreceptor, participate in the formation of S–S bridges. This Cys–Cys sequence is an important structural element of a number of proteins [14]. It creates the possibility of binding and approximation of three segments of the amino acid chain.

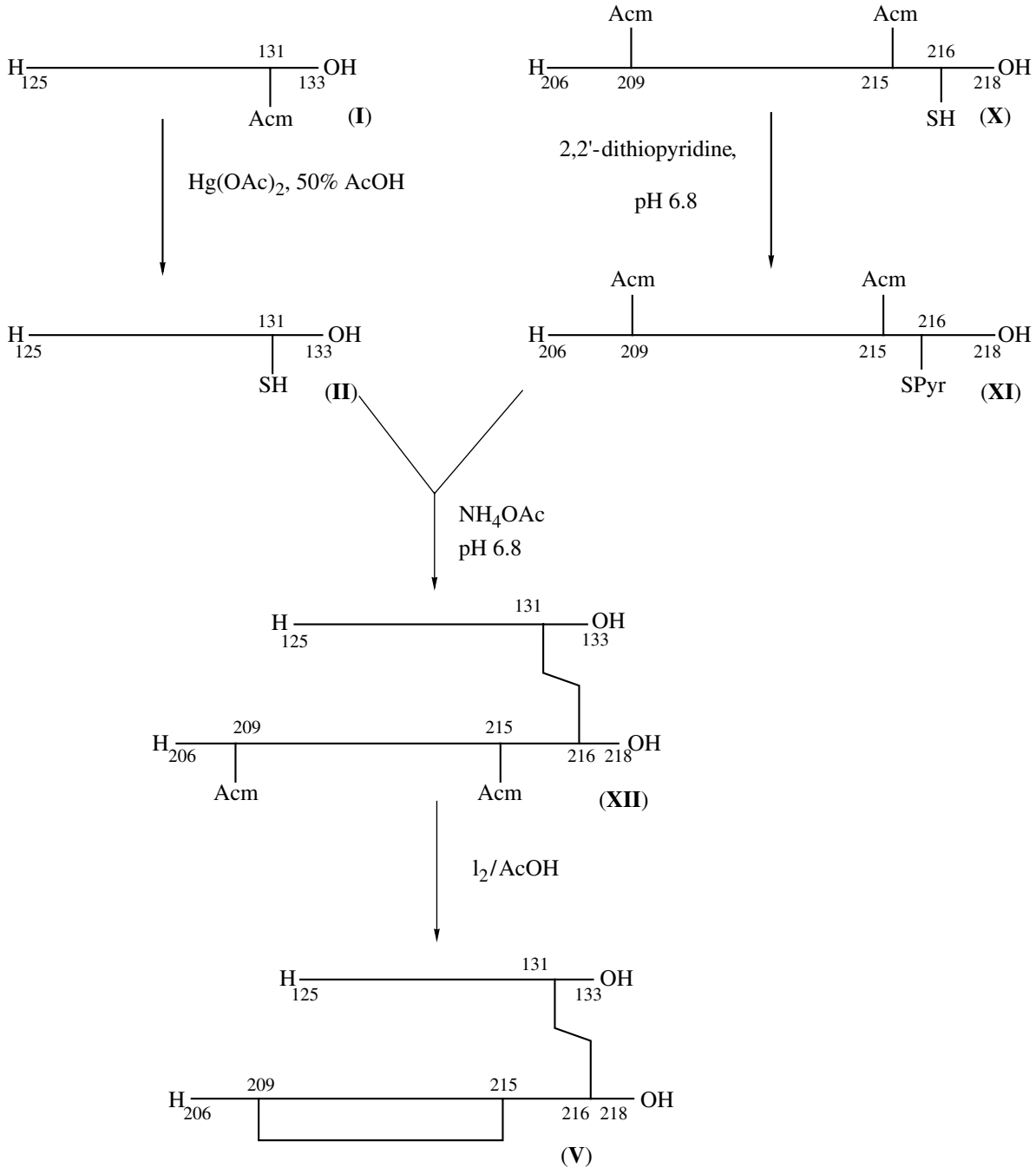
According to the published data, the disulfide bond between the Cys^{131} and Cys^{216} residues plays an important role in supporting the conformation of the ligand-binding site of the protein [15]. The presence of disulfide bridges in a molecule of a β_1-adrenoreceptor most probably contributes to the antigenic properties of this protein (the existence of a conformational antigenic determinant to which autoantibodies can form is quite possible). Thus, the affinity of such a sorbent as Coraffin® will be inevitably insufficient for removal of all autoantibodies to the β_1-adrenoreceptor. This conclusion was experimentally proven [3] and could possibly explain the fact that no new data on the application of



Scheme A. Synthesis of the conformational antigen by simultaneous undirected (spontaneous) formation of the disulfide bridges. Here and in the next schemes, numeration of the amino acid residues in peptides corresponds to the fragments of the amino acid sequence of a β_1-adrenoreceptor.



Scheme B. Synthesis of the conformational antigen by the subsequent formation of the intramolecular Cys^{209}–Cys^{215} bridge by the action of hydrogen peroxide and intermolecular Cys^{131}–Cys^{216} bridge by the action of iodine.



Scheme C. Synthesis of the conformational antigen by subsequent regioselective formation of the intramolecular Cys^{131}–Cys^{216} bridge in the reaction of thiol–disulfide exchange and intramolecular Cys^{209}–Cys^{215} bridge by the action of iodine.

these columns in clinical practice has appeared for the last five years.

On this basis, the problem of creation of a high-affinity synthetic antigen whose structure and antigenic properties will be close to those of the natural antigen, a β_1-adrenoreceptor, is urgent. To solve this problem, we chose the already known peptides that corresponded to the 125–133 and 206–218 sequences of both loops of a β_1-adrenoreceptor and were earlier used for the Coraffin® system. We made attempts to create new conformational synthetic antigens on the basis of theses peptides in which the peptide chains were connected with disulfide bonds.

RESULTS AND DISCUSSION

The chosen nonapeptide and tridecapeptide were prepared by automatic solid phase peptide synthesis

using Fmoc technology. The syntheses were performed on a Wang polymer with a hydroxymethylphenyloxymethyl anchoring group. The following protection groups were used for blocking of the side functions of amino acids: Bu^t for hydroxyl functions of serine and tyrosine; Bu^t for carboxyl functions of glutamic and aspartic acids; Boc for ε-amino function of lysine; Trt for carboxylamide group of asparagine, and Pmc for guanidine function of arginine. A combination of Trt and Acm groups was used for protection of sulfhydryl functions of cysteine residues in SPPS depending on the chosen scheme of the subsequent formation of the disulfide bridges (see Schemes **A–C**). The acid-labile trityl protections were removed by the treatment with trifluoroacetic acid simultaneously with the cleavage of the peptide from the polymeric support upon the completion of SPPS. Acm protections are stable under these conditions [16]. These groups were cleaved by treatment with mercury acetate (for the preparation of peptide thiols) or with iodine (for simultaneous cleavage of Acm groups and formation of S–S bridges) [16]. An amino acid chain was elongated stepwise starting from the *C*-terminus by the carbodiimide method in the presence of 1-hydroxybenzotriazole. Upon the completion of SPPS, the peptides were cleaved from the carrier with the simultaneous removal of the acid-labile protecting groups by treatment with trifluoroacetic acid with the addition of H_2O and TIBS for binding of trityl carbcations. The synthesized peptides were purified by preparative HPLC to a homogeneity of 95–97% and used for the preparation of peptide antigens with intramolecular and intermolecular disulfide bridges according to Schemes A–C.

The simplest method for preparation of peptide antigens with disulfide bonds is the simultaneous undirected (spontaneous) formation of all disulfide bridges by the action of a mild oxidant (for example, air oxygen or hydrogen peroxide). A mixture of disulfide isomers, symmetrical and asymmetrical dimers, or oligomers is usually obtained by this method. However, spontaneous cyclization sometimes occurs with high regioselectivity, especially in the case of rather large molecules with thermodynamically profitable physiologically active conformation [17]. Linear precursors of the corresponding peptides with completely protected cysteine residues are necessary for spontaneous formation of disulfide bridges. Therefore, Acm derivatives of nonapeptide (**I**) and tridecapeptide (**III**) were synthesized on the solid phase and treated with mercury acetate in aqueous acetic acid for the removal of Acm protection [16]. Peptide (**I**) was deprotected in 50% acetic acid because it was poorly soluble in 30% acetic acid. Nonapeptide (**II**) and tridecapeptide (**IV**) contained completely deprotected cysteine residues (see Scheme A). The mixture of dimers was prepared by the undirected (spontaneous) formation of S–S bridges in an equimolar mixture of nonapeptide (125–133 **(II)** and tridecapeptide (206–218 **(IV)** by the action of hydrogen peroxide [18] (Scheme **A**). The reaction was monitored by analytical HPLC and by determination of free SH groups in the oxidation reaction mixture by the Ellman reaction [19].

Mass spectrometry of the reaction mixture demonstrated that the product or products with *M* 2680 were mainly formed after treatment with hydrogen peroxide. This molecular mass corresponded to the target asymmetrical dimer (**V**). Minor peaks with molecular masses of 2184 and 3174 corresponded to dimers of nonapeptide and tridecapeptide, respectively. The minor products were separated from the main dimer by HPLC. According to analytical HPLC, this dimer consisted of two substances with the same molecular mass and close retention times. We proposed on the basis of mass spectrometry that these substances were disulfide isomers (**V**) and (**VI**) (Scheme **A**). Note that the formation of a third isomer with a disulfide bond between Cys^{215} and Cys^{216} is theoretically possible. However, it is known from published data that the formation of a disulfide bridge between neighboring cysteine residues is usually impossible [20]. Dimers are the main reaction products in this case. In addition, the geometry of a peptide chain in natural peptides prevents the formation of a disulfide bridge between neighboring cysteine residues [14]. We found that the product of spontaneous cyclization contained 28.5% of isomer (**V**) with the natural location of S–S bridges. Its retention time was identical to that of product (**V**) of the regioselective synthesis according to HPLC (Scheme **B**, see below). The content of the second isomer in the reaction mixture was 61.0%, and it can probably be identified as isomer (**VI**) according to the aforementioned published data (Scheme **A**).

The next stage was the preparation of a peptide antigen with a natural arrangement of intramolecular and intermolecular disulfide bonds (Schemes **B** and **C**). According to Scheme **B**, the 206–218 sequence was synthesized by SPPS and cleaved from the polymer with the simultaneous removal of trityl protection groups with the formation of peptide (**III**). The intramolecular S–S bridge between Cys^{209} and Cys^{215} was formed by the treatment of tridecapeptide (**III**) with hydrogen peroxide at pH 8 with a high yield. Then, we tried to form an intramolecular disulfide bond between Cys^{131} and Cys^{216} by the treatment of the corresponding Cys(Acm) derivatives of nonapeptide (**I**) and tridecapeptide (**VII**) with iodine in acetic acid [16]. This reaction gave the target asymmetrical dimer (**V**) with a yield of 14.5%, a symmetrical dimer of nonapeptide (**VIII**) with a yield of 17.7%, and nonapeptide (**II**) and tridecapeptide (**IX**) with free Cys^{216} residues (the yields were 15.0 and 52.2%, respectively).

All of these compounds were isolated from the reaction mixture by HPLC and identified by mass spectrometry. Thus, the main product of the synthesis according to Scheme **B** was tridecapeptide (**IX**) instead of the target dimer (**V**). We proposed that the presence of the intramolecular S–S bridge in the tridecapeptide

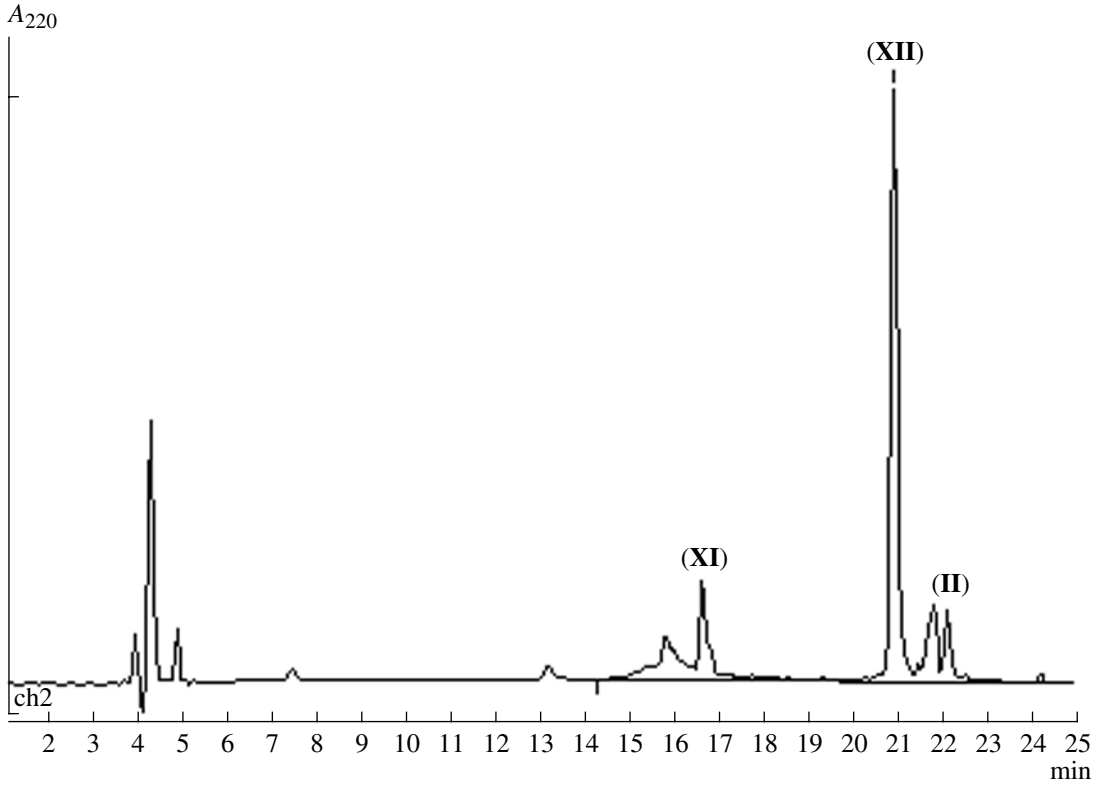


Fig. 2. Analytical HPLC of the reaction mixture of the preparation of dimer (**XII**) by formation of the intermolecular disulfide bridge between Cys^{131} and Cys^{216} via the reaction of thiol–disulfide exchange. Peaks were designated by figures of peptides in Scheme **C**. The contents of target dimer (**XII**), thiol of nonapeptide (**II**), and the SPyr derivative of tridecapeptide (**XI**) in the reaction mixture were 76, 7, and 5%, respectively.

made steric hindrance for formation of the intermolecular disulfide bond.

Therefore, we decided to form the intramolecular disulfide bond between Cys^{131} and Cys^{216} at the first and the internal disulfide bridge after that in the course of the preparation of dimer (**V**) according to Scheme **C**. In addition, we used a regioselective variation of the formation of disulfide bridges in order to avoid side symmetric dimers of the nonapeptide and tridecapeptide. For this reason, we prepared the linear precursor of tridecapeptide (**X**) by SPPS. Cys^{209} and Cys^{215} of this peptide were protected by Acm groups, and Cys^{216} remained unprotected. The crude SPPS product was treated with 2,2'-dithiopyridine for the introduction of temporary 2-pyridinesulfenyl protection (SPyr) into the Cys^{216} residue. This protection was stable in acidic conditions and the activated sulfhydryl group of cysteine for reactions of thiol–disulfide exchange [16]. In particular, SPyr protection was removed by the action of peptide thiols with the formation of disulfides. Earlier, SPyr protection was successfully used for regioselective formation of disulfide bonds in syntheses of complex peptides with several S–S bridges [16]. We succeeded in the synthesis of the target peptide antigen (**V**) according to Scheme **C**. All the reactions proceeded with high yields of the target products. The chromatogram of the reaction mixture of the preparation of dimer (**XII**) from the Cys^{216}(SPyr) derivative of tridecapeptide (**XI**) and nonapeptide thiol (**II**) by the method of thiol–disulfide exchange at pH 6.8 in an aqueous–alcohol medium is given in Fig. 2. Dimer (**XII**) was isolated from the reaction mixture by HPLC on a column with a Vydac sorbent. On the final stage of the synthesis, the internal S–S bridge between Cys^{209} and Cys^{215} of the $[Cys(Acm)]_2$ derivative of dimer (**XII**) was formed by the treatment with iodine in acetic acid. The yield of target dimer (**V**) was 64.0%. Homogeneity of the product was confirmed by analytical HPLC (Fig. 3). The product was characterized by MALDI-TOF mass spectrometry. Note that peaks of molecular ions of monomers (nonapeptide (**II**) and tridecapeptide (**IX**)) were present in the mass spectra of the peptides with intermolecular disulfide bridges, along with peaks of molecular ions of the dimer products. It is known from the published data that several protective groups can be cleaved [21] and S–S bonds can be reduced [22] during mass spectrometry of peptides.

Thus, two conformational peptide antigens in which linear antigenic determinants were connected with disulfide bonds were prepared on the basis of nonapeptide and tridecapeptide fragments of a β_1-adrenoreceptor. A comparison of the products of undirected and regiose-

lective formation of S–S bridges by analytical HPLC demonstrated that the undirected synthesis (Scheme **A**) gave a mixture of isomer (**V**) (28.5%) with a natural arrangement of S–S bridges and an isomer with the proposed structure (**VI**) (61.4%), whereas the product of the regioselective synthesis (Scheme **C**) is an individual compound (**V**) with natural arrangements of S–S bridges.

During the next stage, we prepared sorbents with immobilized peptide antigens and studied the binding of autoantibodies to a β_1-adrenoreceptor by these sorbents.

Peptide antigens, including the mixture of dimers (**V**) and (**VI**), dimer (**V**), nonapeptide (**I**), and tridecapeptide (**III**), were immobilized on the bromocyanogen-activated sepharose according to the standard procedure [23] of immobilization of protein ligands on an agarose matrix. The content of immobilized ligand was evaluated by spectrophotometry (λ 280 nm) from the difference of the amounts of the peptide used for the reaction and the peptide remaining in the solution after the completion of incubation with the matrix. The sorption properties of the prepared sorbents were examined by the method of affinity chromatography with blood plasma of the DCMP patients (patients 1, 2, 4, 5, and 6), a patient with tachyarrythmia (patient 3), and healthy donors (Fig. 4). As one can see from the figure, the conformational antigen prepared from the mixture of disulfide isomers according to Scheme **A** bound a significantly larger amount of the autoantibodies to the β_1-adrenoreceptor from the blood plasma of the patients in comparison with linear peptides (**I**) and (**III**). The sorbent with an immobilized ligand (**V**) more effectively bound the antibodies than that with the same content of ligand (**V**) + (**VI**).

Thus, the content of the recognized structures in the plasma of patients is considerably higher if antigen (**V**) with the natural arrangement of disulfide bonds is used. The efficacy of the sorbent with ligand (**V**) + (**VI**) is also comparable with that of the sorbent with ligand (**V**) for the diagnosis and treatment of DCMP.

EXPERIMENTAL

Derivatives of *L*-amino acids (Bachem, Switzerland), DIC, HOBt, TIBS, mercury acetate, iodine (Fluka, Switzerland), *L*-ascorbic acid (Sigma, United States), and 50% hydrogen peroxide (Solvay Interox, Belgium) were used in this study. Hydrogen peroxide was diluted with deionized water to the desired concentration and titrated with a 1 M solution of $KMnO_4$ before performing the reactions. *N*-Methylpyrrolidone, dichloromethane, piperidine, methanol, and trifluoroacetic acid (Applied Biosystems GmbH, Germany) were used for the peptide synthesis. Sepharose 4Fast Flow (GE Healthcare Bio-Science AB, Sweden) was used for preparation of the affinity sorbents. Acetonitrile (Technopharm, Russia) was used for HPLC.

Fig. 3. Analytical HPLC of the conformational peptide antigen (**V**) with a natural arrangement of disulfide bonds.

The analytical HPLC was performed on a Gilson chromatograph (France) equipped with a Vydac C18 column (4.6 × 250 mm, 5 μm, pore size of 300 Angstroem, Sigma, United States). The column was eluted with a gradient from 10 to 70% of buffer **B** (80% acetonitrile in 0.1% trifluoroacetic acid) in buffer A (0.1% trifluoroacetic acid) within 30 min at a flow rate of 1 ml/min at 226 nm. The preparative HPLC was performed on a Beckman chromatograph (United States) equipped with a Diasorb-C16 130T column (25 × 250 mm, 10 μm) at 220 nm or a Vydac Protein & Peptide C18 column (10 × 250 mm, 10 μm) at 226 nm. Buffers A and B were used as eluents. Mass spectra were recorded on a mass spectrometer with a time-off-flight VISION 2000 base (Thermobioanalysis Corp., Finnigan, United States) and an impulse nitrogen laser of class 3B with an irradiation length of 337 nm by the MALDI method using 2,4,6-trihydroxyacetophenone matrix. ^{1}H NMR spectra were recorded on a WH-500 Bruker spectrometer (500 MHz, Germany) in DMSO-d_6 at 300 K at a peptide concentration of 2–3 mg/ml. Chemical shifts were measured relative to tetramethylsilane. Nitrogen in cylinders of the "os. ch." (especially pure) quality (Russia) was used in this study. The IgG concentration was determined using a reagent kit from Vektor-Best (Russia). Optical absorption of solutions was evaluated on an Akvilon SF-103 spectrophotometer and a Uniplan analyzer (Pikon, Russia).

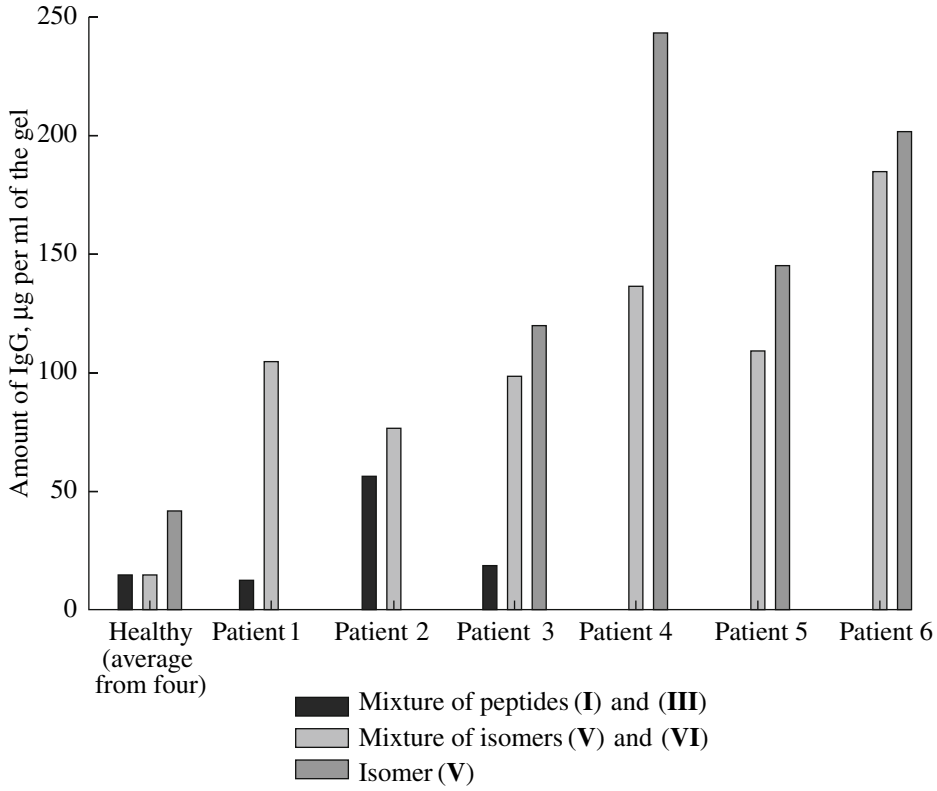


Fig. 4. Amount of bound autoantibodies to the β_1-adrenoreceptor from the plasma of patients 1, 2, 4, 5, and 6 with dilated cardiomyopathy, patient 3 with tachyarrythmia, and healthy donors on the sorbents with the following immobilized synthetic antigens: the mixture of linear peptides (**I**) and (**III**) (black columns) and the conformational antigens prepared by spontaneous (light gray columns) and regioselective (dark gray columns) formation of the disulfide bridges.

Solid phase peptide synthesis was carried out on a 431A automatic peptide synthesizer (Applied Biosystems, United States) according to Fmoc technology starting from 0.25 mmol of commercial N^{α}-Fmoc-aminoacylpolymers (Bachem, Switzerland). A copolymer of styrene with 1% divinyl benzene with a hydroxymethylphenyloxymethyl anchoring group (the Wang polymer) was used as a carrier. The contents of starting Fmoc-Leu and Fmoc-Phe were 0.73 and 0.67 mmol per 1 g of the protected polymer, respectively. The amino acid chains were elongated according to the standard programs for a one-step condensation of Fmoc-amino acids. A synthetic cycle involved activation of the attached N^{α}-Fmoc-amino acid (1 mmol, fourfold excess in relation to amino groups of the resin) in the presence of equivalent amounts of DIC and HOBt in NMP for 20 min, deprotection of α-amino groups with 20% solution of piperidine in NMP for 20 min, condensation of the activated derivative of N^{α}–Fmoc–amino acid with amino groups in NMP for 70 min, and all the necessary intermediate washings of the peptidylpolymer. Upon the completion of SPPS, N^{α}-deprotected peptidylpolymers were suspended in the mixture of TFA (10 ml), water (0.5 ml), and TIBS (0.5 ml) and stirred for 1 h at 25°C. The polymer was filtered and washed with TFA (3×1 ml). The filtrate was evaporated to a volume of approximately 1 ml and mixed with anhydrous ether. The precipitate was filtered, washed with ether, and dried.

H-Glu-Tyr-Gly-Ser-Phe-Phe-Cys(Acm)-Glu-Leu-OH (I) (the 125–133 sequence of β_1-adrenoreceptor). Crude product (**I**) of SPPS was fractionated on a column with Diasorb eluted with a concentration gradient of buffer B in buffer A (from 100% of buffer A, 0.5%/min) at a flow rate of 10 ml/min. Fractions containing the target products were evaporated for the removal of acetonitrile and lyophilized. The yield of peptide (**I**) was 0.148 g (51% relatively to the starting amino acid attached to the polymer); R_t 21.65 min; 99% purity; mass spectrum: M 1165.5 ($M_{\text{calculated}}$ for $C_{54}H_{72}N_{10}O_{17}S$ was 1164).

H-Glu-Tyr-Gly-Ser-Phe-Phe-Cys-Glu-Leu-OH (II). The solution of mercury acetate (0.03 g, 0.090 mmol) in 50% acetic acid (1.5 ml) was added to the solution of nonapeptide (**I**) (0.050 g, 0.043 mmol) in 50% acetic acid (5 ml). The reaction mixture was stirred for 1.5 h at 20°C, and hydrogen sulphide was bubbled through the solution for 30 min. The precipitate of mercury sulphide was filtered and washed with 50% acetic acid (2×5 ml). The filtrate was evaporated to a volume of approximately 2 ml and fractionated on the column with Diasorb. The column was eluted with a concentra-

tion gradient of buffer B in buffer A (from 20 to 80%, 0.5%/min) at a flow rate of 10 ml/min. Fractions containing the target product were joined, evaporated for the removal of acetonitrile, and lyophilized. The yield of compound (**II**) was 0.038 g (80.0%); R_t 22.12 min; purity of 97.2%; mass spectrum: M 1095.2 ($M_{calculated}$ for $C_{51}H_{67}N_9O_{16}S$ was 1094.2).

H-Ala-Arg-Arg-Cys-Tyr-Asn-Asp-Pro-Lys-Cys-Cys(Acm)-Asp-Phe-OH (III) (the 206–218 sequence of β_1-adrenoreceptor). Raw product (**III**) of SPPS was purified as described for compound (**I**). The yield of peptide (**III**) was 0.093 g (32.0% relatively to the starting amino acid attached to the polymer); R_t 13.98 min; purity of 97.2%; Mass spectrum: M 1161.3 ($M_{calculated}$ for $C_{68}H_{104}N_{22}O_{21}S_3$ was 1162).

H-Ala-Arg-Arg-Cys-Tyr-Asn-Asp-Pro-Lys-Cys-Cys-Asp-Phe-OH (IV) was prepared from compound (**III**) (0.032 g, 0.019 mmol) by cleavage of the Acm group according to the procedure described for peptide (**II**) in 30% acetic acid. The yield of compound (**IV**) was 0.027 g (90.0%); R_t 14.59 min; purity of 95.2%; mass spectrum: M 1591.9 ($M_{calculated}$ for $C_{65}H_{99}N_{21}O_{20}S_3$ was 1590.8).

Scheme A of the synthesis of the conformational antigen. The solution of nonapeptide (**II**) (7 mg, 6 μmol) and tridecapeptide (**IV**) (9 mg, 6 μmol) in 10% aqueous dioxane (20 ml) was brought to pH 8 by the addition of 5% aqueous ammonium hydroxide (1 ml) in portions of 0.1 ml and treated with 3% aqueous hydrogen peroxide for 10 min at 20°C. The formation of the S–S bond was monitored by the reaction with the Ellman reagent. Upon completion of the reaction, the mixture was acidified to pH 4–5, the dioxane was evaporated, and the residue was fractionated on the column with Diasorb. The column was eluted as described for compound (**I**). Fractions containing the main peak were joined, evaporated for the removal of acetonitrile, and lyophilized. The yield of the mixture of products (**V**) and (**VI**) was 0.013 g (81%); R_t 19.60 min (28.5%) and 20.34 (61.4%); mass spectrum: M 2680.7, 1588.8*, 1094.1* ($M_{calculated}$ for $C_{116}H_{162}N_{30}O_{36}S_4$ was 2680).

The **H-Ala-Arg-Arg-Cys-Tyr-Asn-Asp-Pro-Lys-Cys-Cys(Acm)-Asp Phe-OH (VII)** was prepard from compound (**III**) (0.032 g, 0.019 mmol) by formation of the intramolecular S–S bond by the procedure described for the preparation of the mixture of products (**V**) and (**VI**). The yield of compound (**VII**) was 0.030 g (94.7%); R_t 3.48 min; purity of 97.0%; mass spectrum: M 1660.1 ($M_{calculated}$ for $C_{68}H_{102}N_{22}O_{21}S_3$ was 1660).

* Hereafter, peaks corresponding to the products with the disulfide bonds which are reduced in the course of mass spectrometry are marked with asterisks.

H-Ala-Arg-Arg-Cys-Tyr-Asn-Asp-Pro-Lys-Cys-Cys-Asp-Phe-OH H-Glu-Tyr-Gly-Ser-Phe-Phe-Cys-Glu-Leu-OH(V) according to Scheme B. The solution of compound (**VII**) (30 mg, 0.018 mmol) and nonapeptide (**I**) (21 mg, 0.018 mmol) in 70% aqueous acetic acid (18 ml) was treated with a solution of iodine (45 mg, 0.18 mmol) in 70% acetic acid (60 ml) for 1 h at 20°C on stirring. The 5% solution of *L*-ascorbic acid in deionized water was added to the reaction mixture to complete decoloration. The reaction mixture was evaporated to a volume of approximately 2 ml, diluted with water (20 ml), and fractionated on the column with Diasorb. The elution was performed under the conditions described for compound (**I**). Acetonitrile was evaporated from the fractions containing the target product and byproducts, and the residue was lyophilized. The yield of dimer (**V**) was 7 mg (14.4%); R_t 19.60 min; mass spectrum: M 2680.3, 1587.6*, and 1093.0* ($M_{calculated}$ for $C_{116}H_{162}N_{30}O_{36}S_4$ was 2680). The yield of dimer (**VIII**) was 7 mg (17.7%); R_t 25.15 min; mass spectrum: M 2185.2 ($M_{calculated}$ for $C_{102}H_{132}N_{18}O_{32}S_2$ was 2184). The yield of compound (**II**) was 3 mg (15.0%); R_t 22.12 min; mass spectrum: M 1098.0 ($M_{calculated}$ for $C_{51}H_{67}N_9O_{16}S_1$ was 1094.2. The yield of compound (**IX**) was 15 mg (52.2%); R_t 14.20 min; mass spectrum: M 1589.0 $M_{calculated}$ for $C_{65}H_{99}N_{21}O_{20}S_3$ was 1588.0).

H-Ala-Arg-Arg-Cys(Acm)-Tyr-Asn-Asp-Pro-Lys-Cys(Acm)-Cys(SPyr)-Asp-Phe-OH (XI). The solution of 2,2'-dithiopyridine (0.100 g, 0.454 mmol) in the mixture of phosphate buffer (pH 6.8, 5 ml) and ethanol (5 ml) was added to the solution of raw tridecapeptide (**X**) (0.470 g, 0.271 mmol) cleaved from the polymer in the mixture of the same buffer (20 ml) and ethanol (10 ml). The reaction mixture was stirred for 1 h at 20°C. The formation of the S–S bond was monitored by HPLC and with the use of the Ellman reagent. Upon completion of the reaction, acetic acid (3.5 ml) was added to the reaction mixture to pH 4, the alcohol was evaporated, and the residue was fractionated on the column with the Vydac sorbent. The column was eluted with a concentration gradient of buffer B in buffer A (from 0 to 10% within 5 min and from 10 to 60% within 100 min) at a flow rate of 3 ml/min. Fractions containing the target product were joined, and acetonitrile was evaporated. The residue was diluted with water and lyophilized. The yield of compound (**XI**) was 0.361 g (78.0%); R_t 16.52 min; purity of 95.0%; mass spectrum: M 1839, 1732.9* ($M_{calculated}$ for $C_{75}H_{112}N_{24}O_{22}S_4$ was 1842).

H-Ala-Arg-Arg-Cys(Acm)-Tyr-Asn-Asp-Pro-Lys-Cys(Acm)-Cys-Asp-Phe-OH H-Glu-Tyr-Gly-Ser-Phe-Phe-Cys-Glu-Leu-OH (XII). The solution of nonapeptide (**II**) (0.015 g, 0.014 mmol) in an ammonium acetate buffer (pH 6.8, 5 ml) was added to the

solution of tridecapeptide (**XI**) (0.025 g, 0.014 mmol) in the same buffer (7 ml). The reaction mixture was stirred at 20°C. The formation of the S–S bond was monitored by HPLC. Acetic acid (pH 4, 1.2 ml) was added 30–40 min later, and the reaction mixture was fractionated on the column with the Vydac sorbent. The column was eluted under the conditions described for compound (**XI**). Fractions containing the target product were joined, evaporated for the removal of acetonitrile, and lyophilized. The yield of compound (**XII**) was 0.023 g (57.1%); R_t 20.83 min; purity of 95.6%; mass spectrum: M 2824.8, 1733.0* ($M_{calculated}$ for $C_{122}H_{174}N_{32}O_{38}S_4$ was 2826).

Peptide (**V**) was prepared according to **Scheme C** by cleavage of Acm groups from dimer (**XII**) (0.02 g, 0.007 mmol) with the simultaneous formation of the intramolecular S–S bridge by the procedure described above. The formation of the S–S bond was monitored by HPLC. The purification was carried out on the column with the Vydac sorbent. Elution was performed under the conditions described for compound (**XI**). Fractions containing the target product were joined, evaporated for the removal of acetonitrile, diluted with water, and lyophilized. The yield of compound **(V)** was 0.012 g (64%); R_t 19.60 min; purity of 99.5%; mass spectrum: M 2680.6, 1588.9*, 1093.0* ($M_{calculated}$ for $C_{116}H_{162}N_{30}O_{36}S_4$ was 2680).

Immobilization of the peptides. A settled sepharose gel (5 ml) was washed with the solution of KOH (4.48 g) and KH_2PO_4 (4.28 g) in water (20 ml), and a suspension in the same solution (10 ml) was prepared. The solution of BrCN (1 ml) in dioxane (0.6 g/ml) was added to the suspension at 5°C, and the reaction mixture was shaken for 10 min at intervals of 25–30 s. The gel was washed with water (50 ml) at 5°C and with the buffer mixture of 0.2 M H_3BO_3 n-dash NaOH with pH 8.0 (10 ml) and suspended in the same buffer mixture (total volume was 10 ml). Solutions of the peptides in the mixture of dimethylsulfoxide and water (3 : 7) with concentration of 2.7 mg/ml were used for the immobilization. In each case, 1.5 ml of the solution of the corresponding peptide was added to 2 ml of the suspension of activated gel. The reaction mixture was stirred for 10 h at 20°C, mixed with a 1 M solution of ethanolamine in water (0.5 ml, pH 8.0), and stirred for 1 h. The gel was washed with water (50 ml) and a buffer mixture (25 ml) of 0.1 M KH_2PO_4 n-dash NaOH (pH 7.0). The amount of immobilized ligand was evaluated from the difference between its starting amount and its amount in the first washings from the sorbent after the covalent immobilization. The final content of the immobilized ligand proved to be 1.1 and 1.0 μmol per 1 ml of the settled gel for the mixture of (**V**) and (**VI**) and for compound (**V**), respectively.

Binding of immunoglobulins to the sorbents was examined by the method of volume sorption at 20°C. A sample of blood plasma of a patient (500 μl) was diluted two times with the physiological solution, added to the settled gel of the examined sorbent (100 μl), and stirred for 30 min. Plasma after the chromatography was separated by centrifugation at 12000 g for 1 min. The supernatant was collected, and the sorbent was washed with 0.01 M KH_2PO_4–NaOH (pH 7.0) with 0.15 M NaCl (3 × 500 μl). Immunoglobulins were removed from the sorbent by two subsequent incubations with the mixture of 0.2 M glycine–HCl (pH 2.5) (250μl) for 5 min and pouring of the solution. The obtained solutions were joined and brought to pH 4.5 by the addition of a 10% solution of NaOH, and the immunoglobulin content was determined in the solutions.

The concentration of the ligands in the solutions was determined on a spectrophotometer according to the optical absorption of the solutions at 280 nm, taking into account the extinction coefficients which were measured for the standard (1 mg/ml) solutions of every peptide. The protein concentration was determined by the Bradford method [24] at 595 nm.

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