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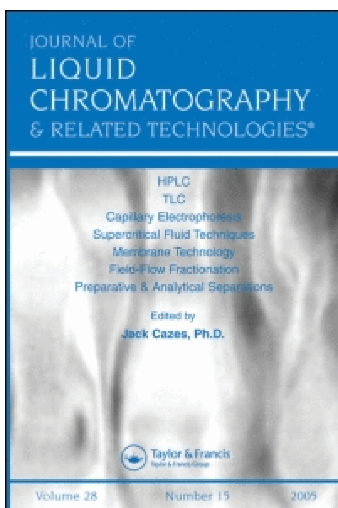

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High Performance Liquid Chromatography in Analysis of Compounds Comprising the Elements of Bacterial Peptidoglycan Structure with Immunological Activity

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Abstract: Numerous non-toxic peptidoglycan fragments of low molecular weight represent an important group of immunomodulators, and antitumor and antiviral drugs. They were isolated from natural sources or prepared by chemical synthesis. *N*-acetylmuramyl-L-alanyl-D-isoglutamine (muramyl dipeptide, MDP) was recognized as the minimal structure responsible for the biological activity. Numerous derivatives and analogues of MDP have been synthesized by modifications of the muramyl moiety or by changing the composition and configuration of the peptide portion. The promising analogs among them are murabutide, muroctasyn, adamantylpeptides, PGM, and derivatives. Isolation, purification, and characterization of peptidoglycan fragments and novel synthesized compounds were performed using the RP-HPLC. In this article, a short review of HPL-Chromatographic analyses of representative compounds comprising elements of peptidoglycan structure is given.

Keywords: HPLC, Bacterial peptidoglycan, Immunological activity

INTRODUCTION

Modern high performance liquid chromatography (HPLC) has many applications, including separation, identification, purification, and quantification of various compounds. It is customarily used in the pharmaceutical industry

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where the composition of final product and the active ingredients of the drug have to be known. Today, reverse-phase high-performance liquid chromatography, in combination with amino acid analysis and mass spectrometry, provides the best tool for analysis of the structure of both peptides and peptidoglycans.

Compounds comprising the elements of the bacterial peptidoglycan structure have been recognized as an important group of immunomodulators, and antitumor and antiviral drugs. Numerous non-toxic peptidoglycan fragments of low molecular weight were prepared and shown to possess strong immunostimulating activity. Isolation, purification, and characterization of the peptidoglycan products, following hydrolysis with specific enzymes, were performed using HPLC. This article will give a short review of HPLC analyses of some representative low molecular size compounds comprising the elements of the bacterial peptidoglycan structure that have shown promise as possible immunoadjuvants.

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PEPTIDOGLYCAN FROM THE BACTERIAL CELL WALL-STRUCTURE AND FUNCTION

The cell-wall macromolecule, called a peptidoglycan or murein, consists of linear polysaccharide chains that are cross-linked by short peptides (Fig. 1). The peptidoglycan, common to both Gramm-positive and Gram-negative bacteria, is a single, enormous, bag-shaped macromolecule, which is extensively cross linked. The peptidoglycan macromolecule confers mechanical support and prevents bacteria from bursting from their high internal osmotic pressure. Bacterial cell walls are unique in containing D-amino acids which form cross-links by a specific mechanism. Investigations of the chemical structure of peptidoglycans from numerous bacterial strains demonstrated the existence of almost 100 different peptidoglycan types.[1,2] The cell-wall polysaccharide is rather uniform and made up, mostly, of two kinds of sugars: *N*-acetylglucosamine (NAG) and *N*-acetylmuramic acid (NAM). NAM and NAG are derivatives of glucosamine in which the amino group has been acetylated. In NAM, a lactyl side chain is attached to C-3 of the sugar ring by an ether bond. In bacterial cell walls, NAM and NAG are joined by glycosidic linkages between C-1 of one sugar and C-4 of the other. All of the glycosidic bonds of the cell-wall polysaccharide have a β configuration. Thus, the cell-wall polysaccharide is an alternating polymer of NAM and NAG residues, joined by β $(1 \rightarrow 4)$ glycosidic linkages. All of peptidoglycan types emerge from this basic structure. There are numerous variations in the length and composition of the peptide side chains and differences in the way these peptides are covalently linked to one another. The peptide subunit linked to the carboxyl group of muramic acid always consists of alternating L- and D-amino acids. The number of chemically different amino acids occurring in a particular peptidoglycan is restricted to between three and six. Peptidoglycan does not contain branched amino acids, aromatic acids, cysteine, methionine, arginine, histidine, or proline.

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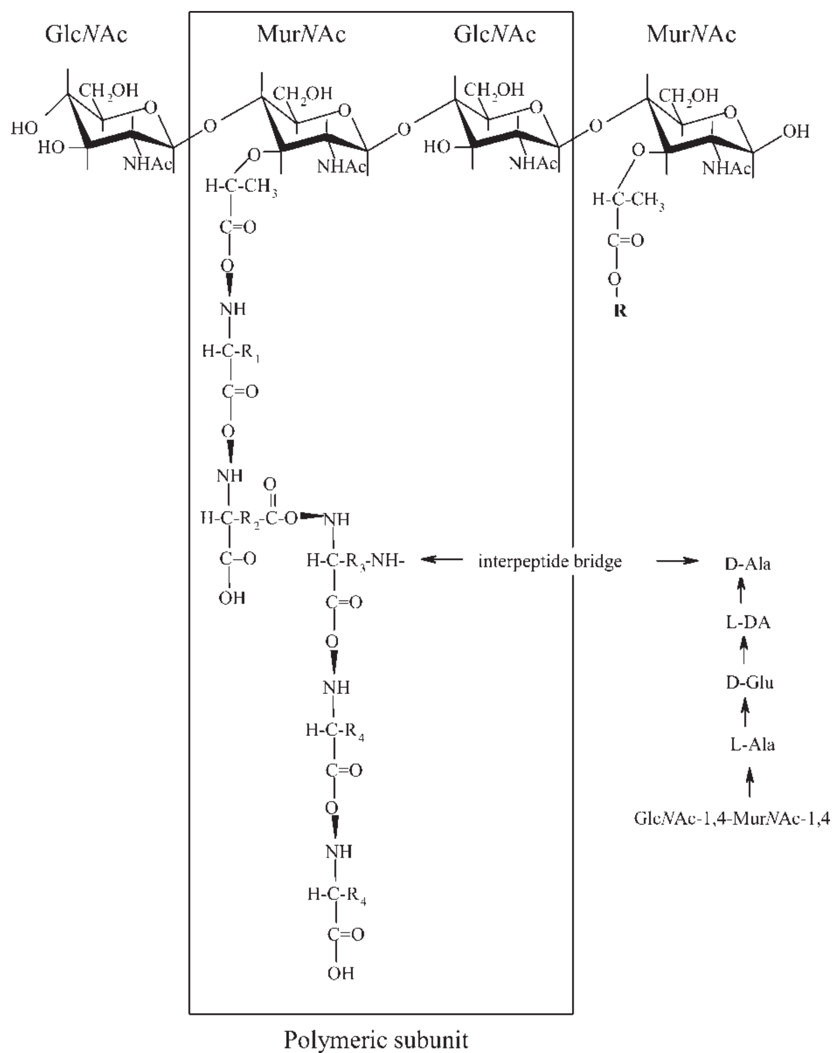

Figure 1. Chemical structure of peptidoglycan. The R_x represents the different side chains of amino acids in different bacterial strains: R_1 = L-Ala, Gly or L-Ser; R_2 = D-Glu; R_3 = *meso*-diaminopimelic acid, L,L-diaminopimelic acid, L-2,4-diaminobutyric acid; R_4 = D-Ala.

The third amino acid is always a diamino acid, necessary for the formation of the interpeptide bridge. Several specific enzymes can split the bonds in the peptidoglycan.[3] When the sacculus is digested by lysozyme or muramidases, which cleave the glycan chains, a range of subunits, muropeptides, is generated. The amidases split the linkage between muramic acid and L-Ala in position 1 of the peptide subunit; endopeptidases split the linkage in the

stem peptide or in the interpeptide bridge; D,D-carboxypeptidases split off the D-Ala in position 5 of peptide subunit; and L,D-carboxypeptidases cleave off the D-Ala in position 4.

The nature and extent of many muramyl peptide(muropeptides)-induced bioactivities depend upon their chemical structures. This observation could be important for understanding the mechanisms that govern the host's in vivo responses to bacterial challenge. The hypothesis is that, during bacterial infection, muramyl peptides released through digestion of bacteria by phagocytes will elicit structure-dependent effects; therefore, the muramyl peptide composition, unique for each bacterial species, will influence the course and outcome of the infection. Consequently, knowledge of the relationship between the biological effects and the chemical structures of the muramyl peptides of each particular bacterium is of considerable importance. Fragments of higher molecular weight are often immunogenic, toxic, and pyrogenic, while smaller fragments are mostly devoid of such undesirable properties.[4,5]

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IMMUNOADJUVANTS AND IMMUNOMODULATORY BACTERIAL CELL WALL STRUCTURES

Adjuvants have been used to augment the immune response to antigens for more than 70 years. The term adjuvant (from the Latin, adjuvare = to help) was first coined by Ramon in 1926 for a substance used in combination with a specific antigen that produces a stronger immune reaction than the antigen used alone. An immunological adjuvant is defined as any substance that accelerates, prolongs, or enhances the specific immune response to the antigens, including vaccines. The adjuvants or adjuvant formulations are also referred to as immunomodulators or immunopotentiators. Interest for vaccine adjuvants is growing rapidly for several reasons.[6] The main impetus is the development of new vaccine candidates for prevention of infectious diseases, cancer, allergic, autoimmune diseases, and application of many subunit vaccines produced by recombinant technology, which are poorly immunogenic. Many of these candidates require adjuvants. The development of experimental adjuvants has also been driven by the failure of aluminum compounds, which are the classical adjuvants and used in man for many years, to enhance the response to many new vaccines, particularly the cellular immunity. There are several classification systems of vaccine adjuvants, for example, according to the origin, according to the mechanism of action, or according to the particulate or nonparticulate form. In the classification of modern vaccine adjuvants according to the origin, several adjuvants based on bacterial cell wall structures have been studied with the purpose to find the adequate candidates for human use.

Many different approaches have been used for the design of potential immunostimulating drugs:

a) fractionation of crude natural substances already known to enhance immune functions, followed by chemical characterization;
b) chemical synthesis of a great variety of molecules which are then screened in vitro and in vivo for immunopharmacological activity;
c) chemical modification of natural substances of known chemical structure in order to potentiate or change their biological activities or reduce their toxicity, such as murabutide and lipophilic muramyl dipeptide (MDP) derivatives.[7–9]

Freund's complete adjuvant–killed mycobacterial cells emulsified in mineral oil–has been known for a long time as a powerful adjuvant of humoral and cell-mediated immune reactions in animals. The smallest biologically active unit of bacterial cell wall peptidoglycan capable of replacing whole mycobacteria in Freund's complete adjuvant is muramyl dipeptide (Fig. 2).[10] The culmination of numerous studies on the immunoadjuvant activities of bacterial peptidoglycans was the discovery of muramyl dipeptide, followed by chemical synthesis of many analogs.

The pioneering work of Lederer and his colleagues,[11] leading to the elucidation of the minimum structure of peptidoglycan units (muramyl dipeptides) responsible for adjuvant effects is a remarkable achievement. In addition to its immunoadjuvant activity, MDP has also other biological effects, including undesirable pyrogenicity and somnogenic effect.[12]

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HPLC ANALYSIS OF PEPTIDOGLYCANS

Currently, HPLC is the most accurate method for analyzing the muropeptide composition of peptidoglycan following hydrolysis of peptidoglycan polymers. An HPLC method generally applicable for the separation of

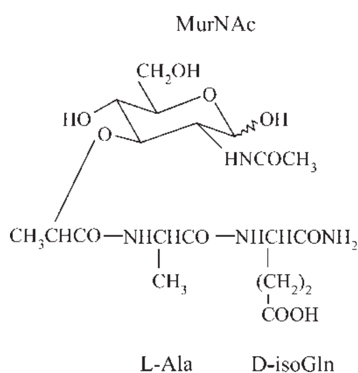

Figure 2. Chemical structure of muramyl dipeptide (MDP).

muropeptides should fulfill the following requirements: commercially available column material, simple, inexpensive eluents, good recovery of the muropeptides or their derivatives, high sensitivity of detection, and reproducibility of separation and quantification. The muropeptides are relatively hydrophilic and their behavior on reversed phase supports is highly sensitive to the pH and ionic strength of the eluent, the steepness of the organic phase gradient, and the temperature of the column. Muropeptides with a free reducing sugar exist in different anomeric forms (α and β anomers) which are in equilibrium. These anomers are separated by HPLC, thereby complicating the muropeptide pattern due to the existence of two to four peaks per compound. Furthermore, mutarotation takes place during the chromatography, causing a smear between the separated anomeric compounds. A quantification of the peak profile is impossible under these circumstances. However, the different anomeric configurations could be avoided by reduction of the muramic acid to the corresponding sugar alcohol. Thus, a single peak per compound was obtained when the samples were treated with sodium borohydride prior to chromatography.

Among the first HPLC investigations of murein was the analysis of composition of peptidoglycan in *E. coli* by Glauner and Schwarz.[13] The sample of murein from *E. coli* was digested first with muraminidase and, after that, the muropeptide solution was fractionated on the analytical reverse phase column, Hibar LiChrosorb RP-18, 250 × 4 mm, 5 μm, Merck, Darmstadt, Germany, and eluted with a linear gradient of 50 mM phosphate buffer, pH 4.69, with 0.0005% NaN_3 to 15% methanol in the same buffer. The flow was 0.5 mL/min. Analysis was performed at ambient temperature. Run time was 110 min and the compounds were detected by UV adsorption at 202 nm. After HPLC analysis of murein, the chromatogram with about 60 well separated peaks in one single run was obtained (Fig. 3). The composition of most peaks was determined by partial enzymatic hydrolysis and amino acid

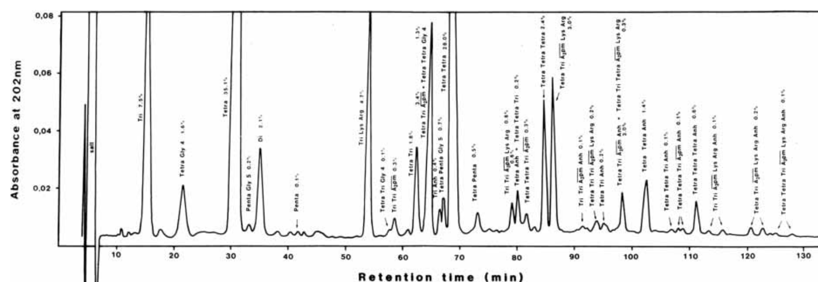

Figure 3. Separation of *E. coli* muropeptides by HPLC, strain KN 126. The muropeptides were reduced by sodium borohydride and separated on a Hibar LiChrosorb RP18 column, 250 × 4 mm, 5 mm, Merck, Darmstadt, Germany). Reproduced with permission from Glauner, B. and Schwarz, U.[13]

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analysis. The amounts of the compounds were calculated by integration of the UV-response.

Separation and quantification of about 80 different muropeptides, the subunits which comprise the polymer murein of *E. coli*, by HPLC, was described in the another paper of Glauner.[14] The effect of temperature, pH, ionic strength, and the steepness of the gradient, and of different support materials on the separation of the muropeptides was investigated. In addition, octadecyl silica (ODS) supports from four different manufactures were compared. Results revealed that the resolution was improved when the temperature was increased from 20 to 55°C. This may be explained by an accelerated exchange of the muropeptides between the stationary phase and the eluent. Besides that, most of the compounds showed a decrease of retention time. The retention of muropeptides on reversed phase supports depends on their polarity and, therefore, is strongly influenced by the dissociation equilibrium of the functional groups. By lowering the pH of the eluent buffer in the range 5.5 to 3.0, the protonization of carboxyl groups causes the increase of the retention times. This increase is positively correlated with the molecular weight of the analyzed compounds. Increased ionic strength intensifies the hydrophobic interaction between sample molecules and reversed phase support. Therefore, most of the muropeptides showed increased retention time at 75 mM compared with 50 mM ionic strength. This effect is more pronounced for the relatively hydrophobic 1,6-anhydro compounds. A constant flow rate (0.5 mL/min) and three different run times (90, 110, and 145 min) were used to change the steepness of the gradients. Essentially, steeper gradients cause a proportionally small decrease of retention times. The decrease of the retention times is positively correlated with the molecular weight of the compounds. The comparison of the ODS supports from four different manufacturers showed that, in principle, all ODS supports seem to be suitable for the separation of muropeptides. Among the materials tested, LiChrosorb RP-18, 5 μm was from Merck, Darmstadt, Germany; Spherisorb ODS, 5 μm, Nucleosil ODS, 5 μm, Hypersil ODS 5 μm and 3 μm were from Bischoff, Leonberg, FRG, Hypersil ODS 3 μm showed the best performance. It was shown that the detection limit with the equipment used was about 5 nmol per compound (Fig. 4). The obtained data have proven that the HPLC is a valuable tool for the study of different murein structures (in resolution, speed, and sensitivity). Li and coworkers[15] compared high performance liquid chromatography and fluorophore-assisted carbohydrate electrophoresis (FACE) methods for analyzing peptidoglycan composition of *E. coli*. They found that the FACE may substitute the HPLC in many cases, but the technique does have drawbacks. FACE does not detect anhydromuropeptides because they have no reducing ends for labeling. These compounds represent approximately 2–5% of the total muropeptides detected by HPLC. Although, many other minor muropeptides are detected by FACE, they are difficult to quantitate and confirm their identities. The authors concluded that the FACE technique is an accurate and biologically informative method, but

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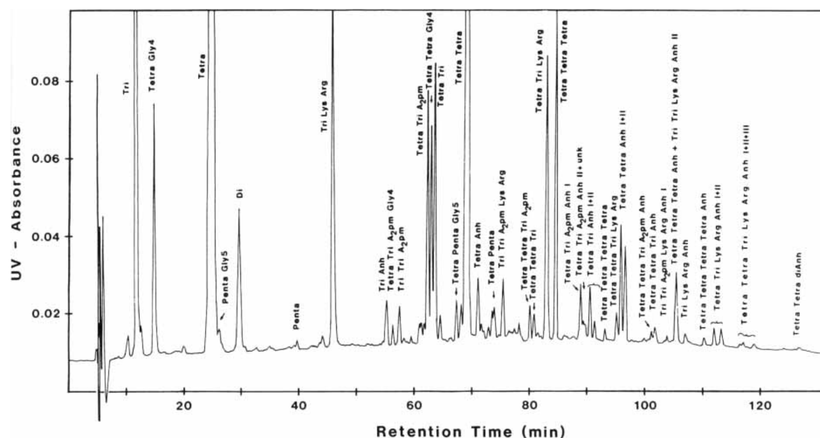

Figure 4. Separation of *E. coli* W7 muropeptides by HPLC. The muropeptides were reduced by sodium borohydride and separated on a Hypersil ODS column, 250 × 4.6 mm, 3 mm, Bischoff, Leonberg, FRG. Reproduced with permission from Glauner, B.[14]

HPLC is the method of choice for determing the types and amounts of muropeptides subunits comprising bacterial peptidoglycan.

In the last decades, many papers on HPLC of peptidoglycans and related compounds have been published.[16–19] The muramyl peptides derived from the peptidoglycan of the oral pathogen *Streptococcus sanguis* were separated by RP-HPLC and their structures were determined by mass spectrometry and amino acid analysis.[17] The chromatographic analysis was achieved on a Varian RP-18 column, 150 × 4.6 mm, 5 μm, that was preceded by a Varian guard column, 30 × 4.6 mm, 5 μm, Varian, Analytical Instruments, Sugar Land, TX, USA. Peptides were eluted with a gradient of organic modifier acetonitrile in water, at a flow-rate of 1 mL/min. The linear gradient was from 100% water containing 0.05% TFA to 17% acetonitrile containing 0.035% TFA in water, in 68 min. The peptides were detected using UV absorption at 205 nm. The muramyl peptides were analyzed in their native form and, also, after reduction with sodium borohydride. The chromatogram obtained after analysis of reduced muramyl peptides contained eight well resolved peaks which were later identified (Fig. 5).

Hoijer and coworkers[19] used RP-HPLC for separation of peptidoglycan monomers from the reaction products after incubation with *N*-acetylmuramyl-L-alanine amidase (NAMLAA) (Fig. 6). NAMLAA hydrolyses peptidoglycan by cleaving the lactamide bond between *N*-acetyl muramic acid and L-alanine in the peptide side chain of the peptidoglycan molecule. The samples were separated using a Superfac Sephasil RP-18 column, 250 × 4 mm, 5 μm, Pharmacia, Uppsala, Sweden. 25 mM sodium phosphate pH 3.5 and 15% methanol in 25 mM sodium phosphate pH 4.7

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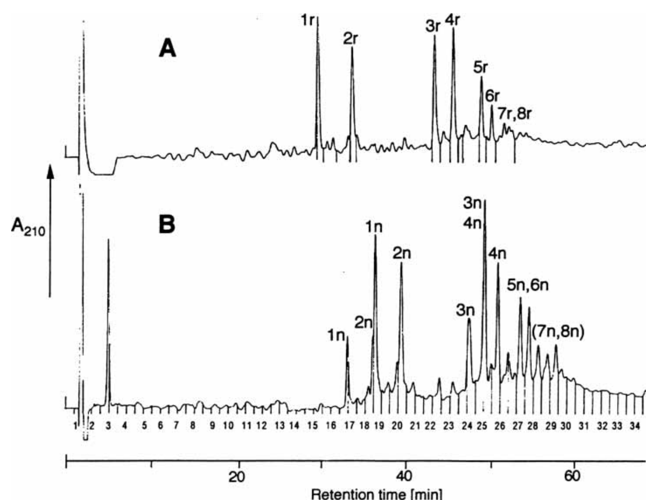

Figure 5. Separation of muramyl peptides from peptidoglycan of *S. sanguis* by reversed-phase HPLC: A) $NaBH_4$ -reduced peptides; B) native peptides. The individual muramyl peptides are labeled with numbers. The suffix r or n indicates the reduced or native form. The chromatographic analysis was achieved on a Varian RP-18 column, 150 × 4.6 mm, 5 μm, that was preceded by a Varian guard column, 30 × 4.6 mm, 5 μm, Varian, Analytical Instruments, Sugar Land, TX, USA). Reproduced with permission from Beranova–Giorgianni, S. et al.[17]

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were used for elution. Gradient elution was used and the percentage of eluent B at 0, 2, 10, 12.5, 13, and 15 min was 0, 0, 100, 100, 0, 0, respectively. The flow rate was 1 mL/min and the compounds were detected using UV detection at 205 nm.

PEPTIDOGLYCAN MONOMER AND STRUCTURALLY RELATED PEPTIDES

Various peptidoglycan fragments were isolated from bacteria, and numerous lower molecular mass compounds have been synthesized. Of particular interest are the lower molecular mass peptidoglycans devoid of toxic properties, which affect the immune system of the host.[20–22] The peptidoglycan monomer, the disaccharide pentapeptide Glc*N*Ac-Mur*N*Ac-L-Ala-D-*iso*Gln-*meso*-DAP(ωNH_2)-D-Ala-D-Ala (PGM), originates from *Brevibacterium divaricatum.* The structure of PGM was determined and its biological properties are well described.[16,23–25]

Its two, more lipophylic derivatives, *tert*-butyloxycarbonyl-L-tyrosyl-PGM (Boc-Tyr-PGM)[26] and (adamant-1-yl)-CH_2-CO-PGM [(Ada-1-yl)-

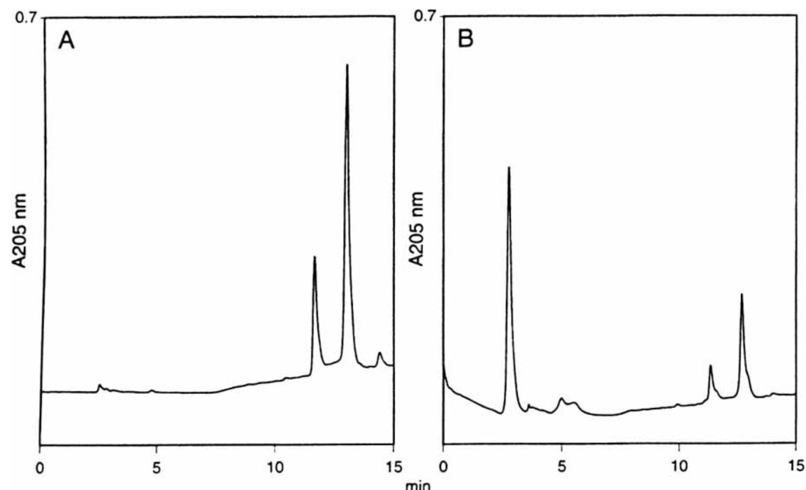

Figure 6. RP-HPLC chromatogram of *B. divaricatum* disaccharide pentapeptide before A) and after B) incubation with N-acetylmuramyl-L-Alanine amidase. The samples were separated using a Superfac Sephasil RP-18 column, 250 × 4 mm, 5 μm, Pharmacia, Upsala, Sweden. Reproduced with permission from Hoijer, M.A. et al.[19]

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CH_2-CO-PGM)][27] were obtained by semi synthetic modifications. *N*-acetylmuramyl-L-alanine amidase hydrolyses peptidoglycan monomers to the disaccharide and the respective peptide portions (Fig. 7).[28] These peptides are further substrates for an L,D-aminopeptidase, which cleaves L-alanine from the amino end of the peptide.[29]

The peptidoglycan monomer from *B. divaricatum* is composed of the disaccharide pentapeptide containing muramic acid with a reducing end (90–95%) and of the anhydromuramyl analogue (containing intramolecular 1,6-anhydromuramyl residues, 5–10%), according to the analysis by high-performance liquid chromatography and fast atom bombardment mass spectroscopy (FAB-MAS).[16] The two peptidoglycan analogues cannot be separated by simple physico-chemical procedures. *N*-acetylmuramyl-L-alanine amidase hydrolyses PGM yielding the disaccharide and pentapeptide portion. The amidase hydrolysed preferentially PGM rather than the anhydromuramyl-PGM. The experimental conditions were adjusted with respect to time and enzyme concentration. Characterization of the peptidoglycan material at various stages during the enzyme hydrolysis and subsequent isolation of products was accomplished by reversed-phase HPLC using an RP-18 column, 250 × 4.6 mm, Vydac, The Separations Group, Hesperia, CA, USA. The solvent gradient employed was linear, composed of acetonitrile containing 0.035% trifluoroacetic acid and water containing 0.05% trifluoroacetic acid. The peaks were

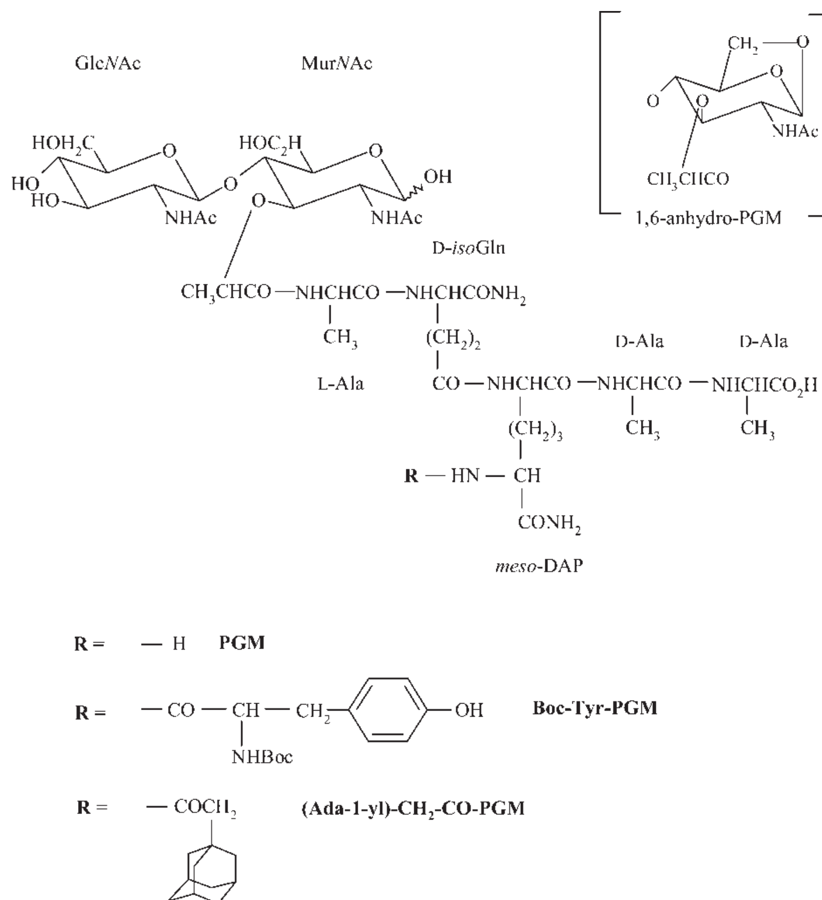

Figure 7. Chemical structures of peptidoglycan monomer (PGM) and its derivatives, Boc-Tyr-PGM and (Ada-1-yl)-CH_2-CO-PGM.

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detected using UV absorption at 214 nm. The chromatogram of PGM revealed three peaks which were separated in the base; they were identified as the β- and α-anomer of PGM and anhydromuramyl-PGM. Each HPLC peak was collected as a single fraction and further characterized. All chromatographic analyses were performed at ambient temperature.

The reversed-phase HPLC method was used for the study of the structure of PGM derivatives.[18] Chromatographic separations were carried out using the RP-18 column, 244×4 mm, 5 μm, Hibar Lichrosorb, Merck, Darmstadt, Germany. Analyses were run at ambient temperature and the eluate was monitored at 214 nm. The gradient solvents used consisted of acetonitrile containing 0.035% TFA and water containing 0.05% TFA. Three gradient solvent programs were used for the tested compounds. Each

program contained a different percentage of acetonitrile and the amount of acetonitrile was changed at the indicated running times. The chromatograms obtained for PGM and their derivatives, Boc-Tyr-PGM and (Ada-1-yl)-CH_2-CO-PGM, always contained three peaks: β- and α-anomers, respectively, and the anhydro-component as identified earlier[16] (Fig. 8). The analysis of the more lipophilic Boc-Tyr-PGM and (Ada-1-yl)-CH_2-CO-PGM required a longer time of analysis and different gradient compositions (starting with 10% of acetonitrile). Due to the increased lipophilicity, (Ada-1-yl)-CH_2-CO-PGM exhibited even higher retention times than Boc-Tyr-PGM. The resolution of α-, β-anomer and the anhydro-component of both PGM derivatives was not as distinct as for the parent PGM molecule. The method was also modified and used for the detection of the products of enzyme hydrolysis with two enzymes: the *N*-acetylmuramyl-L-alanine amidase and the L,D-aminopeptidase (Fig. 9). The isocratic chromatographic programs of water containing 0.05% TFA and the chromatographic program of water containing 0.05% TFA: acetonitrile containing 0.035% TFA (v/v 75/25) were used for separation of the products of enzyme hydrolysis. Peptides containing the adamantyl residue exhibited higher retention times due to their increased lipophilicities; better resolution was achieved between pentapeptide and tetrapeptide than for the Boc-Tyr-substituted peptides.

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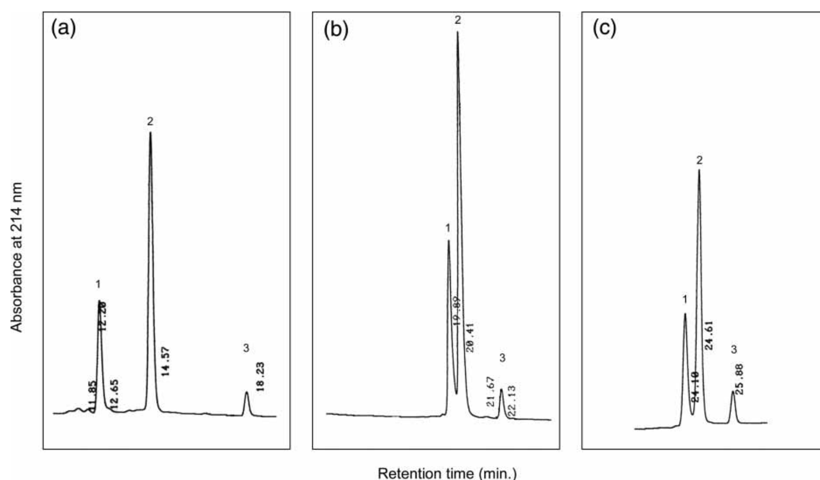

Figure 8. Chromatogram of: (a) PGM; peak 1, β-anomer; peak 2, α-anomer; peak 3, anhydro-PGM; (b) Boc-Tyr-PGM; peak 1, β-anomer; peak 2, α-anomer; peak 3, anhydro-component; (c) (Ada-1-yl)-CH_2-CO-PGM; peak 1, β-anomer; peak 2, α-anomer; peak 3, anhydro-component. Chromatographic separations were carried out using the RP-18 column, 244 × 4 mm, 5 μm, Hibar Lichrosorb, Merck, Darmstadt, Germany. Reproduced with permission from Krstanović, M. et al.[18]

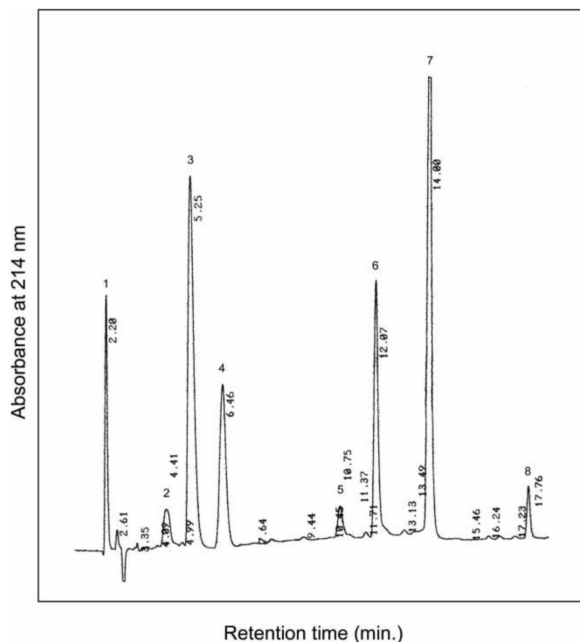

Figure 9. Chromatogram of reaction mixture following hydrolysis of PGM with *N*-acetylmuramyl-L-alanine amidase: peak 1, TRIS-buffer; peak 2, disaccharide β-anomer; peak 3, pentapeptide; peak 4, disaccharide α-anomer; peak 5, disaccharide anhydro-component; peak 6, PGM β-anomer; peak 7, PGM α-anomer; peak 8, anhydro-PGM. Reproduced with permission from Krstanović, M. et al.[18]

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MURAMYL PEPTIDES

Muramyl Dipeptide (MDP)

Since the *N*-acetylmuramyl-L-alanyl-D-*iso*glutamine (muramyl dipeptide, MDP, Fig. 2) was recognized as the minimal structure responsible for the activity exhibited by peptidoglycan of the bacterial cell walls, in 1974,[20] many research groups have undertaken syntheses of this highly active glycopeptide and its derivatives. Numerous reviews on muramyl peptides have been published.[30,31] The muramyl peptides belong to the well recognized adjuvants and immunostimulators.[6,9,21] Although the different fragments of the bacterial cell wall could be obtained by enzymatic hydrolysis of the peptidoglycan macromolecule, MDP can be prepared only by chemical synthesis. MDP is usually synthesized by the procedure of Jeanloz and Flowers[32] with later modifications.[33] The starting material, D-glucosamine hydrochloride, is converted into protected muramic acid in several steps. In the last step, protected muramic acid or nor-muramic acid is coupled to the dipeptide

(L-Ala-D-*iso*Gln). D-isoglutamine or D-glutamine is an essential component of all biologically active muramyl peptides.

Amino acid analysis, ^{1}H-NMR, ^{13}C-NMR, HPLC, and MALDI-mass spectrometry have been used in the structural studies of MDP and related derivatives.[34–36] The high-performance liquid chromatography of MDP[33] was performed on a reverse phase column, Spherisorb ODS, 250 × 3 mm, 5 μm, Spectra-Physics, Santa Clara, CA, USA, with the use of 199:1 (v/v) 5 mM ammonium acetate (pH 2.5)–acetonitrile as eluent. α- and β-D anomers were separated as two peaks showing retention times of 3.5 and 5.5 min (Fig. 10). All chromatographic runs were achieved isocratically at room temperature and the flow rate was 1 mL/min. The peaks were detected at 200 nm. Because of the rapidity of this analytical process, it was possible to study, with fairly good accuracy, the ratio of the two anomers and its variation with time as the result of the mutarotation. The areas of the two peaks were measured at various time-intervals and their ratio calculated. The ratio of α- to β-D anomer decreased slowly, reaching 2:1 through three hours. The solvents used in analyses of MDP differs from the systems used for PGM analysis. The ammonium acetate buffer with low pH and acetonitrile was used and the elution was isocratic But, in both systems, α- and β-anomers could be distinctly separated.

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Lipophilic MDP Derivatives

Numerous derivatives and analogues of MDP have been synthesized, mainly by researchers of pharmaceutical companies in Europe, Japan, and the United

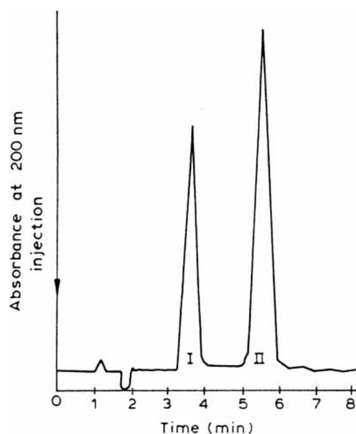

Figure 10. The HPLC chromatogram of MDP. Peak I, α anomer; peak II, β anomer. The analyses was performed on a reverse phase column, Spherisorb ODS, 250 × 3 mm, 5 μm, Spectra-Physics, Santa Clara, CA, USA. Reproduced with permission from Halls, T.D.J. et al.[33]

States. The intensive studies of the dependence of the biological activity of MDP on the chemical structure have shown that chemical structure and chemical modifications of the glucopyranose ring and/or of the peptide chain influence, considerably, the biological properties. The acylamido substituent at C-2 in the pyranose ring is required for biological activity. In the side chain, the methyl groups of the lactyl and alanyl residues are not essential. The L-Alanyl residue may be replaced by other L-amino acids, e.g., L-valine, L-serine, etc. Studies also have revealed the importance of the amino acid composition of the peptide moiety for their immunomodulatory activity. It was found that the presence of *N*-acetyl-D-glucosamine residue linked to the muramyl moiety, similar to natural sequence, is not essential for immunostimulating properties of analogues. Crucial prerequisites for immunostimulatory activity are L-configuration of the first amino acid and D-configuration of the C-terminal amino acid of the dipeptide unit. Replacement of the *N*-acetylmuramyl moiety with various acyl groups thus represents an important approach to the design and synthesis of new immunologically active MDP analogues–desmuramylpeptides.[37,38] The lipophilic MDP derivatives, *N*-acetyl-muramyl-L-alanyl-D-glutamine n-butyl ester, (murabutide, Mur*N*Ac-L-Ala-D-Gln-*On*Bu), N^2-[*N*-(acetyl-muramyl)-L-alanyl-D-*iso*glutaminyl]-N^6-stearoyl-L-lysine (MDP-Lys(L-18), muroctasyn, romurtide) and B30-MDP (Fig. 11) are under intensive clinical trials and the obtained results are promising. It is generally acknowledged that the synthetic

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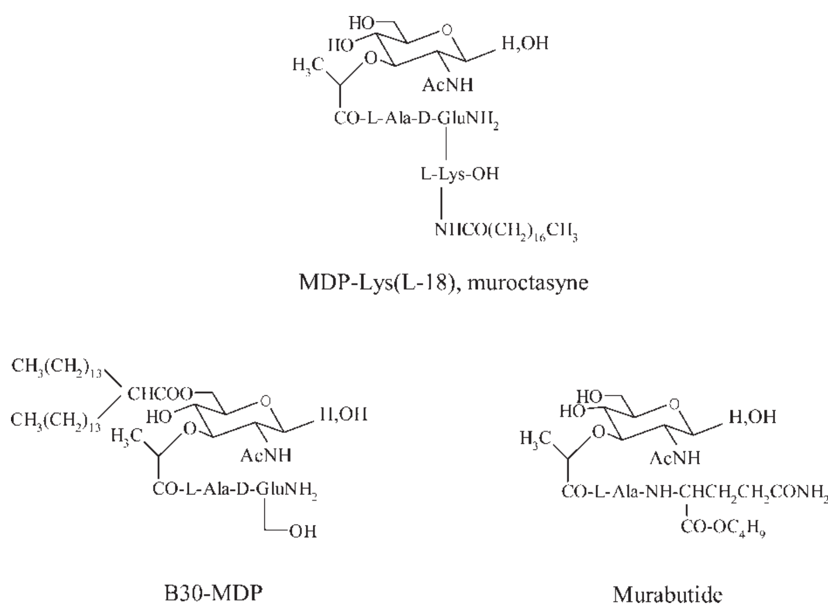

Figure 11. Chemical structure of some lipophilic MDP derivatives.

muramyl peptides are a very interesting group of immunological adjuvants, which are safe and free of contaminants of biological origin.

It has been found that the murabutide enhances the host's nonspecific resistance to bacterial and viral infections and induces colony-stimulating activity. It is apyrogenic, is well tolerated in humans, and does not induce inflammatory reactions. HPLC of murabutide demonstrated that the compound, after chemical synthesis, is homogeneous. For HPLC analysis, the Spherisorb 5-ODS column, 250 × 4.7 mm, 5 μm, was used with 0.005 M ammonium acetate pH 5.0 (pH was adjusted with HCl):acetonitrile (85:15, v/v) solvents as a mobile phase.[39] It should be noted that the α- and β-anomers were not separated using this chromatographic system.

Muroctasyn, MDP-Lys (L18), is an analog of the muramyl dipeptide in which stearic acid, a linear fatty acid with 18 carbons (L18), is combined with the carboxylic acid portion of the D-*iso*glutamine of MDP via L-lysine. HPLC of muroctasyn, and related degradation products was performed on an octadecyl-silica column, 250 × 4.6 mm, 5 μm. The eluents were acetonitrile: 0.02 M ammonium acetate: methanol (33:20:2). Flow rate was 1.5 mL/min. Analysis was performed at ambient temperature, at 25°C and detection was done at 220 nm (Fig. 12). The retention time of MDP-Lys

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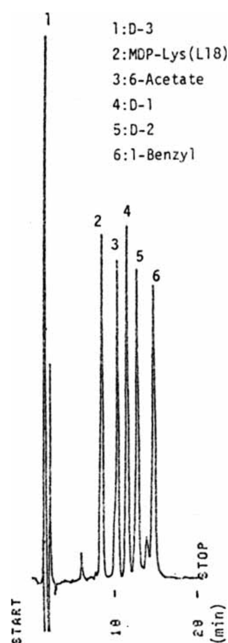

Figure 12. RP-HPLC chromatogram of muroctasyne and its related compounds. The analyses were performed on an ODS column, 250 × 4.6 mm, 5 μm. Reproduced with permission from Azuma, I. et al.[40]

(L18) was 8.6 min.[40] The aim of this work was to investigate the stability of muroctasyn and related degradation products; the HPLC method was not optimized for separation of the anomers of muroctasyn.

MDP requires a water-in-mineral oil (w/o) emulsion as a specified vehicle to exert its full adjuvant capacity, particularly the ability to induce cellular immunity. A group of authors tried to overcome these problems by using 6-*O*-acyl-MDPs that possess various acyl groups at the carbon-6 position of the muramic acids residue. The compound B30-MDP (6-*O*-(2-tetradecylhexadecanoyl)-)Mur*N*Ac-L-Ala-D-*iso*Gln is more lipophilic than the parent molecule; it showed definite adjuvant activities to stimulate cellular, as well as humoral, immune responses and, also, it was practically nonpyrogenic by intravenous injection.[41,42] In an aqueous environment, B30-MDP has amphiphatic properties resulting in vesicle formation, as has a phospholipid. RP-HPLC was used in evaluation the relationship between its physicochemical properties and chemical stability for use as a vaccine adjuvant. The chromatographic separation was carried out using a Shodex ODSpak F-511A column, 250 × 4.6 mm, 5 μm, Shodex, Tokyo, Japan, with the mobile phase: methanol-water (19:1) containing 0.1% perchloric acid. The flow rate was 1.2 mL/min. The column temperature was kept at 313 K (40°C) by a variable temperature control unit. The detection was done at 215 nm.[43]

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Nesmeyanov and coworkers studied another type of peptidoglycan analogue, the disaccharide dipeptides. They prepared compounds such as Glc*N*Ac-*β*-1 → 4-Mur*N*Ac-Ala-D-*iso*Gln (GMDP) and Glc*N*Ac-*β*-1 → 4-Mur*N*Ac-Ala-D-*iso*Gln-Lys (GMDP-Lys) (Fig. 13).[44] Their adjuvant and antitumor effects and ability to induce resistance to bacterial

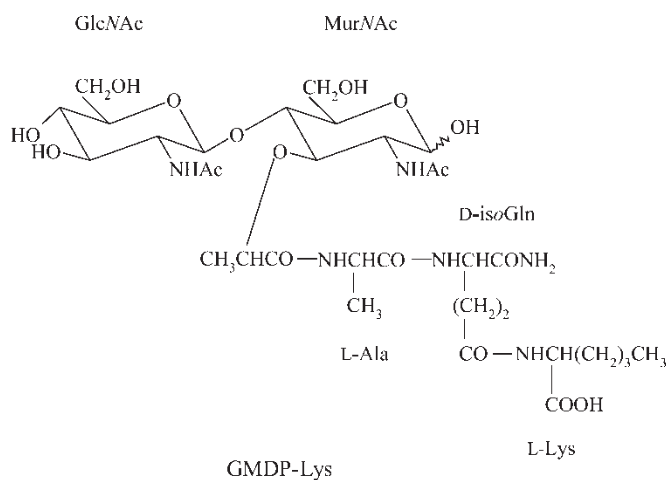

Figure 13. Chemical structure of Glc*N*Ac-*β*-1 → 4-Mur*N*Ac-Ala-D-*iso*Gln-Lys (GMDP-Lys).

and viral infections are well documented.[45] This group of authors also investigated the molecular mechanism of the MPs biological activity. They demonstrated that the muramyl peptide-binding sites are located inside target cells and that, for effective binding, an intact glycopeptide molecule and D-configuration of isoglutamine residue are important. GMDP-binding sites of murine macrophages and were biochemically characterized.[46] The compounds used in this study were isolated by reverse-phase HPLC with a Zorbax ODS column, 240 × 4 mm, 5 μm, DuPont, USA, using an acetonitrile gradient (0–60%) in 0.05 M ammonium acetate buffer, pH 6.0. Although these compounds comprise reducing sugar (*N*-acetylmuramic acid) in their structure and, therefore, exist in two anomeric forms, the anomers were not separated using the HPLC procedure described.

Desmuramyl Peptides: Adamantylamide Dipeptide and Adamantyl Tripeptides

It is well known, that introducing an adamantyl moiety into substances with known biological activity improves their pharmacological properties.[47] A novel type of immunostimulatory compounds was obtained by introducing a residue of adamantine, bound in its number one position, into the gamma-carboxamide group of the isoglutamine moiety (Fig. 14). The resulting L-alanyl-D-*iso*glutamine adamantylamide (ADP), has been found to possess remarkable immunostimulatory activity and does not evidence pyrogenity or other undesirable side effects.[48,49] The ADP may be conveniently prepared by reacting a protected L-alanyl-D-*iso*glutamine derivative with 1-aminoadamantane. Suitable protective groups for this purpose, including *tert*-butyloxycarbonyl and benzyloxycarbonyl, were used. Product purity was verified by high performance liquid chromatography. L-Alanyl-D-*iso*glutamine adamantylamide was analyzed with the ODS RP-18 column, 150 × 3 mm, 5 μm. Elution was isocratic and a mobile phase containing 60–80%, (v/v) of methanol and 40–20%, (v/v) of 0.2%

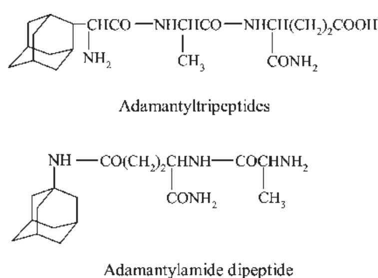

Figure 14. Chemical structures of desmuramyl peptides: D-and L-(Adamant-2-yl)-Gly-L-Ala-D-*iso*Gln (AdTP1 and AdTP2) and adamantylamide dipeptide (ADP).

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aqueous trifluoroacetic acid. Detection was done at 210 nm. The flow rate of the mobile phase was 0.5 mL/min.

Another type of compound containing the adamantyl molecule coupled to peptides characteristic for peptidoglycan structure was synthesized. Two adamantyl tripeptides, diasteroisomers L-and D-(Ada-2-yl)-Gly-L-Ala-D-*iso*Gln (Fig. 14), containing the dipeptide L-Ala-D-*iso*Gln characteristic for peptidoglycan structures, (peptide portion of muramyl dipeptide) and adamantyl moiety were obtained.[24,50] The adamantane is coupled to the peptide portion, L-alanyl-D-*iso*glutamine, through a C-C bond at position 2 of the adamantane molecule, starting from D,L-(adamant-2-yl)glycine. The products of synthesis were two diastereoisomers, L- and D-(Ada-2-yl)-Gly-L-Ala-D-*iso*Gln. As expected, these compounds exhibit versatile biological activity. The purities of all prepared compounds were checked by HPLC. Synthetic diastereoisomers of adamantyltripeptides D- and L-(adamant-2-yl)-Gly-L-Ala-D-*iso*Gln were analyzed by RP-HPLC on a Hibar LiChrosorb RP-18 column, 244 × 4 mm, 5 μm, Merck, Darmstadt, Germany. Analysis was run at ambient temperature and eluate was monitored at 214 nm. A linear gradient of acetonitrile containing 0.035% TFA and water containing 0.05% TFA was used. In the beginning, the percentage of acetonitrile was 10% and, after 15 min, it was 30%. The separation of diastereoisomer of adamantyltripeptides was complete. The chromatogram with two sharp peaks was obtained with a retention time of 13.19 min for L-diastereisomer and 17.70 min for D-diastereoisomer (Fig. 15). The results revealed that the adamantyltripeptides require a shorter time of analysis than the peptidoglycan monomers and their derivatives. HPLC analysis was used in chemical characterization of novel compounds and in the study of the structure-activity relationship between the different diastereoisomers.

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DISCUSSION

Reversed-phase high-performance liquid chromatography (RP-HPLC) involves the separation of molecules on the basis of hydrophobicity. The separation depends on the hydrophobic binding of the solute molecule from the mobile phase to the immobilized hydrophobic ligands attached to the stationary phase. The separation of solute molecules could be achieved using isocratic or gradient chromatographic procedures. The solutes are, therefore, eluted in order of increasing molecular hydrophobicity. RP-HPLC is a very powerful technique for the analysis of peptides and proteins because of a number of factors that include:

1. the excellent resolution which can be achieved under a wide range of chromatographic conditions for very closely related molecules, as well as structurally quite distinct molecules;

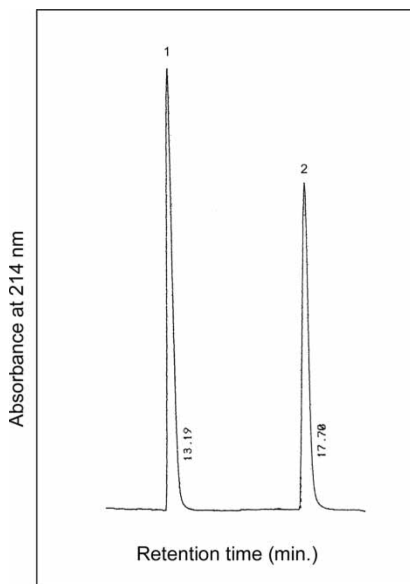

Figure 15. Chromatogram of the diastereoisomeric mixture of adamantyltripeptides: peak 1, L-(adamant-2-yl)-Gly-L-Ala-D-*iso*Gln; peak 2, D-(adamant-2-yl)-Gly-L-Ala-D-*iso*Gln. The analyses was performed on Hibar LiChrosorb RP-18 column, 244 × 4 mm, 5 μm, Merck, Darmstadt, Germany. Reproduced with permission from Krstanović, M. et al.[18]

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2. the experimental ease with which chromatographic selectivity can be manipulated through changes in mobile phase characteristics;
3. the generally high recoveries and, hence, high productivity; and
4. the excellent reproducibility of repetitive separations carried out over a long period of time, which is caused partly by the stability of the sorbent materials under a wide range of mobile phase conditions.[51,52]

The most important part of the HPLC equipment is the column. The column performance features are important; they depend on the type of column. The main goals for a column are column efficiency, symmetrical peaks, stability, and reproducibility of the analysis. The octadecyl silica adsorbents are the most popular adsorbents for RP-HPLC separation[53] ODS columns have been explored for high-performance liquid chromatography of all examined glycopeptides and products of enzymatic hydrolysis described in this paper. The analyzed compounds showed good retention and separation behavior on ODS columns. Several commercial adsorbents are used in analysis of peptidoglycans and related compounds. They are slightly different in their general properties, such as pore size, surface area, and carbon load. Spherisorb ODS was used

for MDP and murabutide, it has an 80 Å pore size, and its surface area is 220 m^2/g and 12% carbon load. LiChrosorb has a 100 Å pore size, surface area is 350 m^2/g, and 21.4% carbon load. LiChrosorb columns were used for the separation the peptidoglycan of *E. coli*, peptidoglycan monomer from *B. divaricatum* and their derivatives, Boc-Tyr-PGM, Ad-PGM, and the diastereoisomers of adamantyltripeptides. Zorbax ODS adsorbent was used for separation of the GMDP; it has a 70 Å pore size, surface area is 330 m^2/g and 20% carbon load. Shodex ODSpak with commercial name F-511A was used for analyses of the B30-MDP; it is also a silica based adsorbent with 100 Å pore size and 14% carbon load. For adamantylamide dipeptide (ADP) and muroctasyn, the type of adsorbent was not specified, but any of the ODS adsorbents could probably be used. All ODS supports used seem to be suitable for the separation of the examined compounds.

In the past, chromatographic separation of charged analytes has been achieved by ion suppression (the careful adjustment of the mobile phase pH to result in a nonionized analyte). Determining the optimum mobile phase pH in ion suppression, however, often requires extensive method development. Samples containing more than one ionizable component were often unusable. The limitations of ion suppression led to the development of a new, more generally applicable approach to separation of ionized components, i.e., ion pair chromatography. TFA is the most commonly used ion pairing agent in reversed phase HPLC because it is volatile and easily removed and it has low absorption within detection wavelengths. TFA has a different absorbance in water than in ACN. Selecting a wavelength where the absorbences converge and cause the least amount of baseline shift and using a differing amounts of TFA in buffer used could compensate some of the changes. However, small adjustments in reagent concentration may increase retention slightly and optimize the separation. Phosphate and ammonium ion, and perchloric acid have been also used as counter ions in reversed-phase ion-pair HPLC. Once the reagent has been selected, adjusting the pH of the mobile phase maximizes the resolution. Slight modifications of pH can profoundly affect retention and selectivity.

In Table 1, the conditions for RP-HPLC analysis of compounds that are discussed in the text are presented. The data revealed that the TFA, ammonium and phosphate ions were used as ion pair reagents in analysis of examined compounds. Only in analyses of B30-MDP, the perchloric acid as ion pair reagent was used. For analysis of MDP, murabutide, murostasyne, and GMDP, ammonium acetate buffer was used and acetonitrile was used as eluent. The concentrations of ammonium acetate buffer were 5 mM, 20 mM, and 50 mM, and the pH values were 2.5, 5, and 6, respectively. The isocratic chromatographic procedures were used for the analysis of MDP, murabutide, and muroctasyne. The employed mobile phase contained different volume ratios of ammonium acetate buffer and acetonitrile (see

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Table 1. The condition of RP-HPLC analysis of different muropeptides

Compound	Column	Eluent system	Type of elution	Ref.
MDP	Spherisorb ODS, Spectra-Physics, USA	5 mM ammonium acetate (pH 2.5):acetonitrile 199:1 v/v	Isocratic	[33]
PGM	LiChrosorb C18, Merck, Germany	Acetonitrile with 0.035% TFA:water with 0.05% TFA	Linear gradient	[16, 18]
Boc-Tyr-PGM	LiChrosorb C18, Merck, Germany	Acetonitrile with 0.035% TFA:water with 0.05% TFA	Linear gradient	[18]
Ad-PGM	LiChrosorb C18, Merck, Germany	Acetonitrile with 0.035% TFA:water with 0.05% TFA	Linear gradient	[18]
Products of enzymatic hydrolysis of PGM	LiChrosorb C18, Merck, Germany	Water with 0.05% TFA or water with 0.05% TFA: acetonitrile with 0.035% TFA	Isocratic	[18]
AdTPs	LiChrosorb C18, Merck, Germany	Acetonitrile with 0.035% TFA:water with 0.05% TFA	Linear gradient	[18]
ADP	ODS C18	Methanol:water 0.2% TFA	Isocratic	[51]
Murabutide	Spherisorb ODS,	Acetonitrile: 5 mM ammonium acetate (pH 5) 15:85 v/v	Isocratic	[39]
Muroctasyne	ODS C18	Acetonitrile: 20 mM ammonium acetate:methanol 33:20:2	Isocratic	[40]
GMDP	Zorbax ODS C18, DuPont, USA	Acetonitrile: 50 mM ammonium acetate (pH 6.0)	Linear gradient	[46]
B30-MDP	Shodex ODSpak F-511 A, Tokyo, Japan	Methanol:water (19:1) containing 0.1% perchloric acid	Isocratic	[43]
Peptidoglycan from *E. coli*	LiChrosorb C18, Merck, Germany	Methanol:phosphate buffer	Linear gradient	[14]
Peptidoglycan from *S. sanguis*	Varian C18, Analytical Instruments, USA	Acetonitrile with 0.035% TFA:water with 0.05% TFA	Linear gradient	[17]

Table 1.). In the analysis of GMDP, the linear gradient of ammonium acetate buffer and acetonitrile was used. The phosphate buffer was used only in analyses of muropeptides of peptidoglycans from *E. coli*. It is important to note that precipitation problems might occur if the concentrated buffers blend with more than 25–30% ACN. During the gradient elution, the concentration of buffer decreases as the concentration of solvent increases, and there will be no precipitation problems. The increased ionic strength intensifies the hydrophobic interactions between sample molecules and reversed phase support. Glauner has shown that the retention times of examined muropeptides were increased at 75 mM, compared with 50 mM phosphate buffer.[14] In the comparison of methanol and acetonitrile, methanol may be preferred to acetonitrile because of its greater solubility for buffers and ion pair reagents. Acetonitrile is the best choice when water was used instead of buffer because it is a strong organic solvent, chemically unreactive, it has low-UV detection and low viscosity, and it is water miscible. Acetonitrile was used in the analysis of MDP, PGM and derivatives, diastereoisomers of adamantyltripeptides, murabutide, muroctasyne, and GMDP.

The peptide bonds exhibit absorption in UV spectra near 200 nm and all compounds have been detected by UV absorbance. All analyses were performed at ambient temperature, except for the analyses of B30-MDP. Concerning the high lipophilicity of B30-MDP, this analysis was performed at 40°C. It is known that the increase in the temperature causes a decrease of the retention time. Also, at the elevated temperature, the viscosities of liquids decrease and efficiency of the column increases. Volatile solvents do not allow the use of higher temperature, and the stability of the attached bonded ligands on the adsorbent surface may also be influenced by the elevated temperature.

In conclusion, HPLC is a convenient and efficient method for the analysis of peptidoglycan fragments and related compounds comprising elements of peptidoglycan structure. The method of choice is reversed phase ion pair chromatography using octadecyl silica columns. Various types of compounds, with respect to their structures and physicochemical properties (size, lipopilicity), could be analyzed and the solvent systems should be adjusted accordingly. In this paper, the analyses of several representative types of such compounds have been described and discussed. A short summary of experimental conditions used in these analyses is presented in Table 1.

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REFERENCES

1. Schleifer, K.H.; Kandler, O. Peptidoglycan types of bacterial cell walls and their taxonomic implications. Bacteriol. Rev. **1972**, *36*, 407–477.
2. Labischinski, H.; Maidhof, H. Bacterial peptidoglycan: Overview evolving concepts. In *Bacterial Cell Wall*; Ghuysen, J.-M., Hakenbeck, R., Eds.; Elsevier Science B.V: Amsterdam, 1994, 23–38.
3. Rogers, H.J.; Perkins, H.R.; Ward, J.B. *Microbial Cell Walls and Membranes*; Chapman and Hall: London, U.K, 1980.
4. Heymer, B.; Seidl, P.H.; Schleifer, K.H. Immunochemistry and biological activity of peptidoglycan. In *Immunology of the Bacterial Cell Envelope*; Stewart-Tull, D.E.S., Ed.; John Wiley: Chichester, U.K., 1985, 11–46.
5. Seidl, P.H.; Schleifer, K.H. Structure and immunochemistry of peptidoglycan. In *Biological Properties of Peptidoglycan*; Seidl, P.H., Schleifer, K.H., Eds.; Walter de Gruyter: Berlin, 1986, 1–2.
6. O'Hagan, D.T. *Vaccine Adjuvants, Preparation Methods and Research Protocols*; Humana Press: Totowa, New Jersey, 1984.
7. Werner, G.H. Synthetic immunostimulants (Excluding sulfur-containing compounds). Comm. Immun. Microbiol. Infect. Dis. **1986**, *9* (2/3), 131–136.
8. Lise, L.D.; Audibert, F. Immunoadjuvants and analogs of immunomodulatory bacterial structures. Curr. Opin. Immunol. **1989**, *2*, 269–274.
9. Georgiev, V.S. Immunomodulatory activity of small peptides. Trends Pharm. Sci. **1990**, *11*, 373–378.
10. Lefrancier, P.; Lederer, E. Muramyl-peptides. Pure Appl. Chem. **1987**, *59* (3), 449–454.
11. Adam, A.; Ciobaru, R.; Ellouz, F.; Petit, J.F.; Lederer, E. Adjuvant activity of monomeric bacterial cell wall peptidoglycans. Biochem. Biophys. Res. Comm. **1974**, *56* (3), 561–567.
12. Krueger, J.M.; Pappenheimer, J.R.; Karnovsky, M.L. Sleep-promoting effects of muramyl peptides. Proc. Natl. Acad. Sci. USA **1982**, *79* (19), 6102–6106.
13. Glauner, B.; Schwarz, U. The analysis of murein composition with high-pressure--liquid chromatography. In *The Target of Penicillin*; Hakenbeck, R., Holtje, J.-V., Labischinski, H., Eds.; Walter de Gruyter: New York, 1983, 29–34.
14. Glauner, B. Separation and quantification of muropeptides with high-performance liquid chromatography. Anal. Biochem. **1988**, *172*, 451–464.
15. Li, S.Y.; Holtje, J.V.; Young, K.D. Comparison of high performance liquid chromatography and fluorophore-assisted carbohydrate electrophoresis methods for analyzing peptidoglycan composition of *Escherichia coli*. Anal. Biochem. **2004**, *326*, 1–12.
16. Tomašić, J.; Sesartić, Lj.; Martin, S.A.; Valinger, Z.; Ladešić, B. Comparative susceptibility of a peptidiglycan monomer from *Brevibacterium divaricatum* and its anhydrmuramyl analogue to hydrolysis with *N*-acetylmuramyl-L-alanine amidase, isolation and characterization of anhydrmuramyl-peptidoglycan monomer. J. Chromatogr. **1988**, *440*, 405–414.
17. Beranova-Giorgianni, S.; Desiderio, D.M.; Pabst, M.J. Structures of biologically active muramyl peptides from Peptidoglycan of *Streptococcus sanguis*. J. Mass Spectrom. **1988**, *33*, 1182–1191.

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18. Krstanović, M.; Frkanec, R.; Vranešić, B.; Ljevaković, Đ.; Šporec, V.; Tomašić, J. Reversed-phase high-performance liquid chromatographic method for the determination of peptidoglycan monomers and structurally related peptides and adamantyltripeptides. J. Chromatogr. B **2002**, *773*, 167–174.
19. Hoijer, M.A.; Melief, M.J.; Keck, W.; Hazenberg, M.P. Purification and characterization of *N*-acetylmuramyl-L-alanine amidase from human plasma using monoclonal antibodies. Biochim. Biophys. Acta **1996**, *1289*, 57–64.
20. Ellouz, F.; Adam, A.; Ciorbaru, R.; Lederer, E. Minimal structural requirements for adjuvant activity of bacterial peptidoglycan derivatives. Biochem. Biophys. Res. Comm. **1974**, *59* (4), 1317–1325.
21. Bahr, G.M.; Chedid, L. Immunological activities of muramyl peptides. FASEB J. **1986**, *45*, 2541–2544.
22. Azuma, I. Synthetic Immunoadjuvants: application to non-specific host stimulation and potentiation of vaccine immunogeneticity. Vaccine **1992**, *10* (14), 1000–1006.
23. Tomašić, J.; Hršak, I. Peptidoglycan monomer originating from Brevibacterium divaricatum — its metabolism biological activities in the host. In *Surface Structures of Microorganisms and Their Interactions with the Mammalian Host*; Schrinner, E., Richmond, M.H., Seibert, G., Schwarz, U., Eds.; VCH, 1987, 113–121.
24. Keglević, D.; Ladešić, B.; Tomašić, J.; Valinger, Z.; Naumski, R. Isolation procedure and properties of monomer unit from lysozyme digest of peptidoglycan complex excreted into the medium by penicilin-treated *Brevibacterium divaricatum* mutant. Biochim. Biophys. Acta **1979**, *585*, 273–281.
25. Tomašić, J.; Hanzl-Dujmović, I.; Špoljar, B.; Vranešić, B.; Šantak, M.; Jovičić, A. Comparative study of the effects of peptidoglycan monomer and structurally related adamantyltripeptides on humoral immune response to ovalbumin in mouse. Vaccine **2000**, *18*, 1236–1243.
26. Hršak, I.; Ljevaković, Đ.; Tomašić, J.; Vranešić, B.. Preparation properties and biological activities of *tert*-butyloxycarbonyl-L-tyrosyl peptidoglycan monomer. In *Immunotherapy of Infection*; Masihi, N., Ed.; Marcel Dekker: New York Basel, Hong Kong, 1994, 249–257.
27. Ljevaković, Đ.; Tomašić, J.; Šporec, V.; Halassy Špoljar, B.; Hanzl-Dujmović, I. Synthesis of novel adamantylacetyl derivative of peptidoglycan monomer-biological evaluation of immunomodulatory peptidoglycan monomer and respective derivatives with lipophilic substituents on amino group. Bioorg. Med. Chem. **2000**, *8*, 2441–2449.
28. Valinger, Z.; Ladešić, B.; Tomašić, J. Partial purification and characterization of *N*-acetylmuramyl-L-alanine amidase from human and mouse serum. Biochim. Biophys. Acta **1982**, *701*, 63–71.
29. Krstanović, M.; Brgles, M.; Halassy, B.; Frkanec, R.; Vrdoljak, A.; Branović, K.; Benedetti, F.; Tomašić, J. Purification and characterization of LD-aminopeptidase (murein hydrolase) from guinea pig serum. Prep. Biochem. Biotechnol. **2006**, *36*, 175–195.
30. Devlin, J.P.; Hargrave, K.D. The design and synthesis of immune regulatory agents-targets and approaches. Tetrahedron **1989**, *45* (14), 4327–4369.
31. Dzierzbicka, K.; Kolodziejczyk, A.M. Muramyl peptides – synthesis and biological activity. Polish J. Chem. **2003**, *77*, 373–395.
32. Flowers, H.M.; Jeanloz, R.W. The synthesis of 2-acetamido-3-*O*-(D-1-carboxyethyl)-2-deoxy-α-D-glucose-(*N*-acetylmuramic acid) and of Benzyl glycoside

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derivatives of 2-amino-3-*O*-(D-1-carboxyethyl)-2-deoxy-D-glucose (Muramic acid). J. Org. Chem. **1963**, *28* (11), 2983–2986.
33. Schwartzman, S.M.; Ribi, E. A shortened synthesis of adjuvant dipeptide (MDP). Prep. Biochem. **1980**, *10* (3), 255–267.
34. Halls, T.D.J.; Muppala, S.R.; Wenkert, E. The anomeric configuration of the immunostimulant *N*-acetylmuramoyl-dipeptide and some of its derivatives. Carbohydr. Res. **1980**, *81*, 173–176.
35. Fermandjian, S.; Perly, B. A Comparative ^{1}H-N.M.R. Study of Mur*N*Ac-L-Ala-D-*i*soGln (MDP) and its analogue murabutide: Evidence for a structure involving two successive β-turns in MDP. Carbohydr. Res. **1987**, *162*, 23–32.
36. Sizun, P.; Perly, B.; Level, M.; Lefrancier, P.; Fermandjian, S. Solution conformations of the immunomodulator muramyl peptides. Tetrahedron **1988**, *44* (3), 991–997.
37. Lederer, E. Synthetic immunostimulants derived from the bacterial cell wall. J. Med. Chem. **1980**, *23* (8), 819–830.
38. Lefrancier, P.; Derrien, M.; Jamet, X.; Choay, J.; Lederer, E.; Audibert, F.; Parant, M.; Parant, F.; Chedid, L. Apyrogenic, adjuvant-active *N*-acetylmuramyl-dipeptides. J. Med. Chem. **1982**, *25*, 87–90.
39. Chedid, L.A.; Parant, M.A.; Audibert, F.M.; Riveau, G.J.; Parant, F.J.; Lederer, E.; Choay, J.P.; Lefrancier, P.L. Biological activity of a new synthetic muramyl peptide adjuvant devoid of pyrogenicity. Infect. Immun. **1982**, *35* (2), 417–424.
40. Azuma, I. Verlag fur medizin und naturwissenschaften. In *Arzneim. Forsch./Drug Res*; Classen, H.G., Schramm, V., Eds.; GmbH: Aulendorf, Germany, 1988, 951.
41. Tsujimoto, M.; Kotani, S.; Kinoshita, F.; Kanoh, S.; Shiba, T.; Kusumoto, S. Adjuvant activity of 6-*O*-acyl-muramyldipeptides to enhance primary cellular and humoral immune responses in Guinea Pigs: Adaptability and various vehicles and pyrogenicity. Infect. Immun. **1986**, *53* (3), 511–516.
42. Pabst, J.M.; Cummings, P.; Shiba, T.; Kusumoto, S.; Kotani, S. Lipophilic derivative of muramyl dipeptide is more active than muramyl dipeptide in priming macrophages to release superoxide anion. Infect. Immun. **1980**, *29* (2), 617–622.
43. Ando, S.; Tsuge, H.; Miwa, A. Relationship between physicochemical properties and chemical stability of muramyldipeptide derivative B30-MDP in liposomal solutions. Colloid Polym. Sci **1995**, *273*, 399–404.
44. Ivanov, V.T.; Andronova, T.M.; Bezrukov, M.V.; Rar, V.A.; Makarov, E.A.; Kozmin, S.A.; Astapova, M.V.; Barkova, T.I.; Nesmeyanov, V.A. Structure, design and synthesis of immunoactive peptides. Pure Appl. Chem. **1987**, *59*, 317–324.
45. Sumaroka, M.V.; Litvinov, I.S.; Khaidukov, S.V.; Golovina, T.N.; Kamraz, M.V.; Komaleva, R.L.; Andronova, T.M.; Makarov, E.A.; Nesmeyanov, V.A.; Ivanov, V.T. Muramyl peptide-binding sites are located inside target cells. FEBS Lett. **1991**, *295* (1,2,3), 48–50.
46. Golovina, T.N.; Sumaroka, M.V.; Samokhvalova, L.V.; Andronova, T.M.; Nesmeyanov, V.A. Biochemical characterization of glucosaminyl-muramyldipeptide binding sites of murine macrophages. FEBS Lett. **1994**, *356*, 9–12.
47. Gerzon, K.; Krumarkalns, E.V.; Brindle, R.L.; Marshall, F.J.; Root, M.A. The adamantyl group in medicinal agents. J. Med. Chem. **1963**, *6* (11), 760–763.
48. Masihi, K.N.; Lange, W.; Rohde-Schulz, B.; Mašek, K. Antiviral activity of immunomodulator adamantylamide dipeptide. Intl. J. Immunother. **1987**, *3*, 89–94.
49. Masihi, K.N.; Lange, W.; Schwenke, S.; Gast, G.; Huchshorn, P.; Palache, A.; Mašek, K. Effect of immunomodulator adamantylamide dipeptide on antibody

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response to influenza subunit vaccines and protection against aerosol influenza infection. Vaccine **1990**, *8* (2), 159–163.
50. Vranešić, B.; Tomašić, J.; Smerdel, S.; Kantoci, D.; Benedetti, F. Synthesis and antiviral activity of novel adamantyltripeptides. Helv. Chim. Acta **1993**, *76*, 1752–1758.
51. Engelhardt, H. *Practice of High Performance Liquid Chromatography*; Engelhardt, H., Ed.; Springer-Verlag: Berlin Heidelberg, New York, Tokyo, 1986.
52. Lim, C.K. *HPLC of Small Molecules, a Practical Approach*; Lim, C.K., Ed.; IRL Press: Oxford, Washington, 1986.
53. Gooding, K.M.; Reginer, F.E. *HPLC of Biological Macromolecules*; Gooding, K.M., Reginer, F.E., Eds.; Marcel Dekker: New York, 1990.

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