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The structure, synthesis, and immunomodulating activity of bacterial lipopeptides and their analogues

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Contents

I.	Introduction	889
II.	The structure of a bacterial cell wall	889
III.	The lipopeptide composition of the bacterial cell wall	890
IV.	Effects of lipopeptides on the development of early stages of the immune response	890
V.	Lipopeptides-1	891
VI.	Lipopeptides-2	894
VII.	Possible applications of lipopeptides-2	896
VIII.	The use of lipopeptides-2 in the fight against the human immunodeficiency virus	897
IX.	The antitumour activity of lipopeptides-2	898
X.	Conclusion	898

Abstract. The current state and prospects for the use of natural lipopeptides and their synthetic analogues in the study of fine mechanisms of functioning of complex biological systems and in the solution of various problems of biochemistry and medicine are considered. The results of investigations related to the synthesis and biological activity of lipopeptides are summarised. The data on the application of lipopeptides as components of synthetic vaccines and antitumour drugs are discussed. The bibliography includes 71 references.

I. Introduction

Lipopeptides are compounds that contain a peptide chain to which one or several fatty acid residues are covalently linked. The molecules of the known lipopeptides comprise from four to sixteen amino acid residues.[1] Natural lipopeptides have been mostly detected in Gram-negative bacteria and Actinomycetes, though they are present also in some species of lower fungi. The majority of lipopeptides are characterised by strict structural specificity as regards the producing organism.

The search for the means directed against bacterial infections has stimulated studies on the structure of various bacteria. Thus, the first systematic studies on the cell wall of mycobacteria were begun in the 1970's.[2–4] It was established[4,5] that the fraction exhibiting the highest biological activity contains fragments of peptidoglycans, the minimum structural unit of which is *N*-acetylmuramyl-L-alanyl-D-isoglutamine (muramyl dipeptide, MDP). Later, the attention of numerous research groups was focussed on the study of fragments of natural peptidoglycans and their synthetic derivatives.[6–9]

These studies have shown that bacterial lipoproteins and their fragments, lipopeptides, possess biological activity. The establishment of immunostimulating and antitumour activities of bacterial lipopeptides was followed by numerous attempts directed at the synthesis of their analogues. However, the preparation of synthetic lipopeptides involved certain difficulties due to the presence in their structure of aliphatic and peptide fragments as well as the necessity to obtain optically pure compounds.

The biological activity of bacterial lipopeptides was studied in parallel with the analysis of approaches to the synthesis of their analogues. It was established that the synthetic analogues also possess immunostimulating properties and can exhibit pronounced antitumour activity. Owing to these properties, lipopeptides have received particular attention of investigators as potential components of synthetic vaccines and antitumour drugs.

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II. The structure of a bacterial cell wall

The backbone of a bacterial wall is built predominantly of a homogeneous polymer, the peptidoglycan (murein). The peptidoglycan is a heteropolymer that consists of chains with alternating residues of *N*-acetylglucosamine and *N*-acetylmuramic acid [3-*O*-(1-carboxyethyl)-*N*-acetyl-D-glucosamine] linked together with β-1,4-glycosidic bonds.[10]

These unbranched chains of a heteropolysaccharide muramine form the murein skeleton. The residues of muramic acid are linked to amino acids *via* the carboxy groups of lactic acid with peptide bonds. The typical amino acids of murein are L-alanine, D-glutamic acid, *meso*-diaminopimelic acid (for Gram-negative bacteria) or L-lysine (for Gram-positive bacteria), and D-alanine. The heteropolysaccharide chains are cross-linked by peptide bridges to form a bag-shaped giant molecule, the murein bag, which functions as a backbone of the cell wall.[10]

In Gram-negative bacteria, the murein network has only one layer and constitutes less than 10% of the dry mass of the cell wall (in *E. coli*). Murein contains only *meso*-diaminopimelic acid but not lysine; interpeptide bridges are absent. In all Gram-negative bacteria, the structure of the murein bag is identical (Fig. 1).

Above the single-layer murein bag lies the outer layer of the cell wall, the outer membrane. In ultrathin bacterial slices, it resembles the plasmatic membrane. The outer membrane is built of proteins, phospholipids, and lipopolysaccharides (Fig. 1), and is tight against the murein layer and bonded to it by lipoproteins. Lipoproteins are covalently bound to murein layers, apparently through diaminopimelic acid; they have an outside orientation of their lipophilic termini and are thus fixed in the lipophilic bilayer

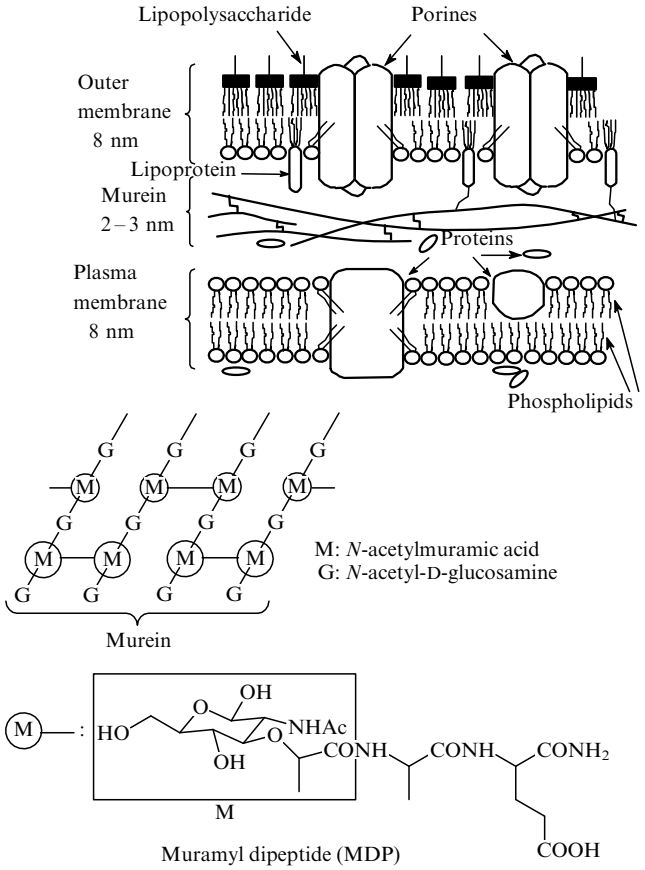


Figure 1. A structural model of the cell wall of Gram-negative bacteria. The structure of murein.[11]

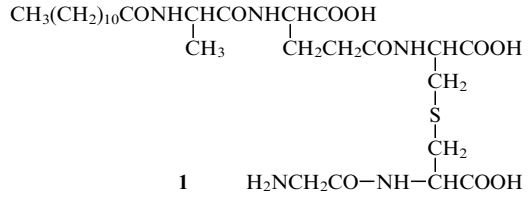
by hydrophobic interactions. This bilayer contains phospholipids and hydrophobic termini of lipopolysaccharides (lipid A); the hydrophilic termini of the latter (O-specific heteropolysaccharide side chains) are directed outside.[10]

By virtue of their capacity to stimulate the immune response, the components of bacterial membranes, such as murein, lipopolysaccharides, and lipoproteins have received particular attention of investigators.

III. The lipopeptide composition of the bacterial cell wall

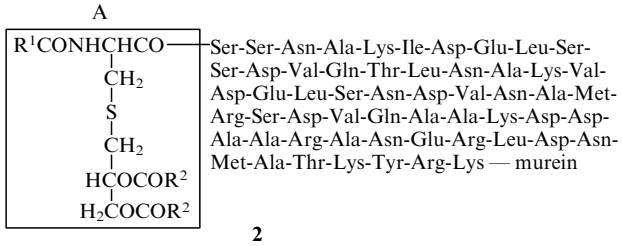
In contrast to peptidoglycans the main structural element of which is muramyl dipeptide responsible for the biological activity, lipopeptides are heterogenous as regards their composition and biological functions. Three groups of lipopeptides can be specified.

The first group includes lipopeptides the peptide component of which contains murein-specific amino acids, first of all, D-glutamic acid, as the main structural unit. It is this amino acid composition and sequence that determine largely the immunomodulating activity of lipopeptides-1 (LP-1). As a rule, the lipid component of LP-1 includes fatty acids that increase the 'lifetime' of the compound in the organism and favour its penetration into lipophilic compartments.[11] A typical representative of this group is lauroyl-L-alanyl-γ-D-glutamyl-*N*(6)-glycyl-lantionin (**1**):



1

Lipopeptides of the second group include fragments of a lipoprotein isolated from *E. coli*. This lipoprotein is a component of the outer membrane of Gram-negative bacteria. It contains 58 amino acid residues and is produced as a secretory precursor with a signal peptide at the N-terminus. A detailed description of this lipoprotein has been given by Braun.[12] The *E. coli* lipoprotein exhibits the mitogenic activity in higher organisms and stimulates the production of B-lymphocyte clones in murine cultures. The amino acid composition of the lipoprotein **2** has been established.[12]



2

R^1, R^2 — saturated and unsaturated alkyl.

The main structural element of the second group lipopeptides is that derived from L-cysteine and three fatty acid residues (the A-fragment of compound **2**). Owing to the presence of this structural unit, the lipopeptides belonging to the second group (LP-2) can be easily incorporated into biological membranes. The amino acid composition of LP-2, in contrast to that of LP-1, can vary widely. Lipopeptides of this group are characterised by a broader spectrum of biological activity.[11]

Group 3 includes lipopeptides possessing antibiotic activity. Many representatives of this group have a cyclic or mixed (cyclic-linear) structure of their peptide chains; the lipid component is a higher fatty acid. Many compounds of this group are produced by bacteria; the functions of the majority of lipopeptides-3 (LP-3) are associated with ion transport across membranes. LP-3 are characterised by strict specificity with respect to the producing organism, which makes them different from, *e.g.*, the lipophilic portion of the *E. coli* lipoprotein, which may be common for most bacteria.[11] LP-3 do not exhibit immunomodulating properties and are not considered in more detail in this article. The information about their properties can be found in a number of reviews.[13–15]

IV. Effects of lipopeptides on the development of early stages of the immune response

The penetration of a foreign infection into the organism causes a rapid increase in the blood level of coloni-stimulating factors (CSF). These factors are synthesised by various kinds of cells, including endothelial cells, fibroblasts, macrophages, and lymphocytes.[16] Their effects on pluripotent stem cells induce, after a series of differentiation stages, the formation of large amounts of various leucocytes. They include B-lymphocytes responsible for antibody synthesis and T-lymphocytes: T-helpers (T_H) manifesting helper activity, T-suppressors (T_S) manifesting suppressor activity, and cytotoxic T-lymphocytes manifesting cytotoxic activity (T_C).[16] The population of killer cells includes also natural killer cells (NK) and large granular lymphocytes (LGL).[11]

Among granulocytes, a leading role in inducing a rapid response of the organism to a foreign infection is played by neutrophils. The eosinophils (Eos) and basophils fulfil specific functions aimed at protection from parasitic organisms. Protection from bacterial and viral infections or tumour metastases is provided by monocytes (Mo) circulating in the blood and by macrophages (Mp) in tissues. The functions of these cells can be assessed using various test systems *in vivo*.

The interactions of different cells and cell systems are mediated by various factors and receptors. A prominent role in cell interactions is played by well-known factors, such as interleukins (IL-1, IL-2, IL-3, IL-4, and IL-5), colony-stimulating factors,

interferons (*e.g.*, γ-interferon, IFN_γ), and factors possessing suppressor activity. In most cases, interleukins represent secreted proteins that function as local chemical mediators.[17]

The main components of the humoral immune response are specific antibodies the titers of which are determined in mice devoid of specific pathogens. The cell-type immune response is mediated by different components: T_C-cells, macrophages, granulocytes of various types, and NK-cells. This type of immune response is assayed by skin tests (delayed-type hypersensitivity, DTH) in guinea pigs.[11]

In order to provide the development of efficient immune responses to many antigens, in particular, soluble antigens, toxins, anatoxins, and vaccines, the specific immunising substance must be administered together with the so-called adjuvant, *i.e.*, the agent that stimulates nonspecifically the immune response to specific antigens. Two main types of tests are employed in the studies of the adjuvant effects. In tests aimed at assessment of the antibody synthesis carried out on mice, different doses of adjuvants under examination are co-administered.[11] Bovine serum albumin (BSA) or human γ-globulin are used as antigens.

Delayed-type hypersensitivity tests are performed on guinea pigs to which bovine serum albumin and the substance under study (in different doses) are administered in an incomplete Freund's adjuvant. As a result, the animals develop long-lasting skin tumours.[11]

Immunostimulating effects produced by lipopeptides depend on the subsequent stages of the immune response. Lipopeptides stimulate phagocytic macrophages and monocytes in such a way that these cells begin to secrete factors stimulating the formation of colonies of differentiated blood cells as well as interleukin-1. The secretion of IL-1 is a signal for T-helper cells to begin proliferation. In turn, T-helper cells secrete IL-2, which stimulates the proliferation of all types of T-lymphocytes,[17] as well as of IL-4 and IL-5, which in turn stimulate the proliferation and maturation of B-cells.[11] Interleukin-3 secreted by certain populations of T-helper cells produces a stimulating effect on the proliferation of various types of haemopoietic cells. In addition, endothelial cells (End) and fibroblasts (Fibr) begin to synthesise colony-stimulating factors. Thus the factors secreted by all these cells result in the activation of bone marrow cells that are leucocyte progenitors (Leuc. prog).[11] As a result, different populations of leucocytes become replenished with new cells. The increase in the number of eosinophils, cytotoxic T-lymphocytes, killer cells, and polymorphonuclear leucocytes or neutrophils (PMN) provides effective stimulation of the immune response,[11] since most leucocytes are destroyed while fulfilling their protective functions. Thus, the interaction of lipopeptides with monocytes and macrophages induces an immune response, which results in effective destruction of viruses, bacterial infectious pathogens, and tumour cells (Fig. 2).

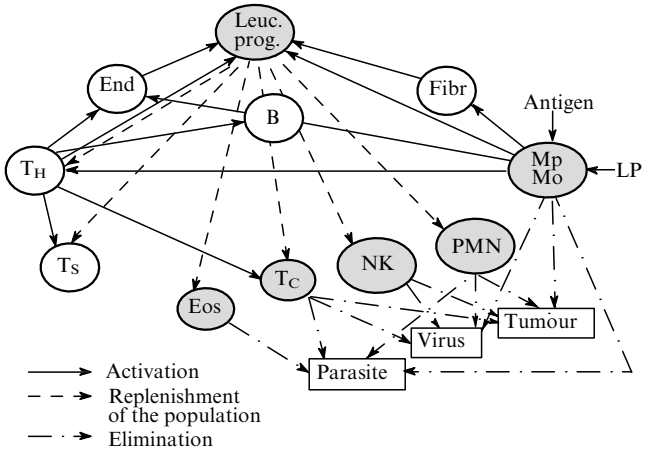


Figure 2. Stimulation of early stages of the immune response by lipopeptides.[11]

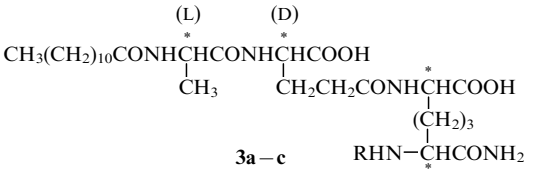
V. Lipopeptides-1

Some time ago, it was believed that the biological activity of murein isolated from bacterial cell walls was associated with the muramic acid residue.[11] It was established, however, that the attachment of peptides containing murein-specific amino acids to the lipophilic residue gives compounds that also exhibit biological activity. Thus, it was shown [18] that chemical binding of lauric acid to the biologically inactive tetrapeptide L-alanyl-γ-D-glutamyl-*N*(6)-glycyl-L,L-2,6-diaminopimelic acid isolated from a crude extract of a *Streptomyces sp*. gave a compound exhibiting immunostimulating activity both *in vitro* and *in vivo*.[18–20]

Subsequently, the total chemical synthesis of the lauroyltetrapeptide (pimelautide) **3a**,**b** was carried out and its biological properties were fully characterised.[20,21]

Experiments with synthetic isomers of the lauroyltetrapeptide **3a**,**b** containing either the L,L- (**3a**) or the D,D-diaminopimelic acid (**3b**) led to the conclusion that only compound **3a** is biologically active *in vivo*.[22]

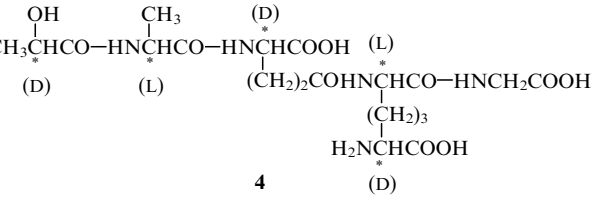
Moreover, the lauroyltripeptide **3c** was found to be as active as compound **3a**. Both compounds stimulate the production of interleukin-1 by macrophages and induce the release of CSF in the blood of mice when used in doses sufficient to protect experimental animals from bacterial infections.[23]



3a–c

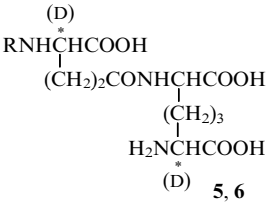
a: R = H_2NCH_2CO (L,L); **b**: R = H_2NCH_2CO (D,D); **c**: R = H (L,L).

These and some other properties of lipopeptides have been confirmed in experiments conducted in other laboratories.[24] It was found that diaminopimelic acid can be substituted by lanthionine **1** without any decrease in activity and that lipodipeptides, such as palmitoyl-L-alanyl-D-glutamic acid or behenoyl-L-alanyl-D-glutamic acid, still retain the immunostimulating activity *in vivo*. The work by Hemmi *et al*.[25] reported on the immunostimulating activity of compound (**4**) (D-lactoyl-L-alanyl-γ-D-glutamyl-L-*meso*-2,6-diaminopimeloylglycine), a metabolite of *Streptomyces olivaceogriseus*.



4

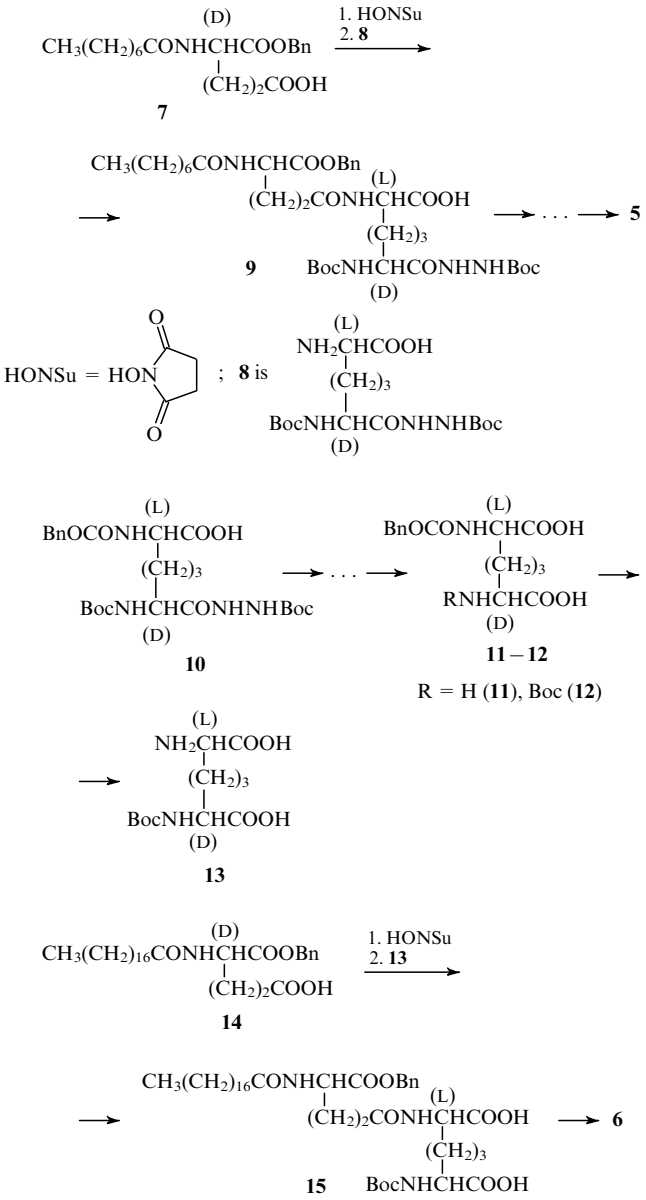
The demonstration of the biological activity of compound **4** has stimulated the synthesis of a large series of its analogues and their assay.[26–28] Shown here is the scheme of synthesis of the octanoyl and stearoyl derivatives (**5**) and (**6**) (Scheme 1), which are also biologically active.[26]



5, **6**

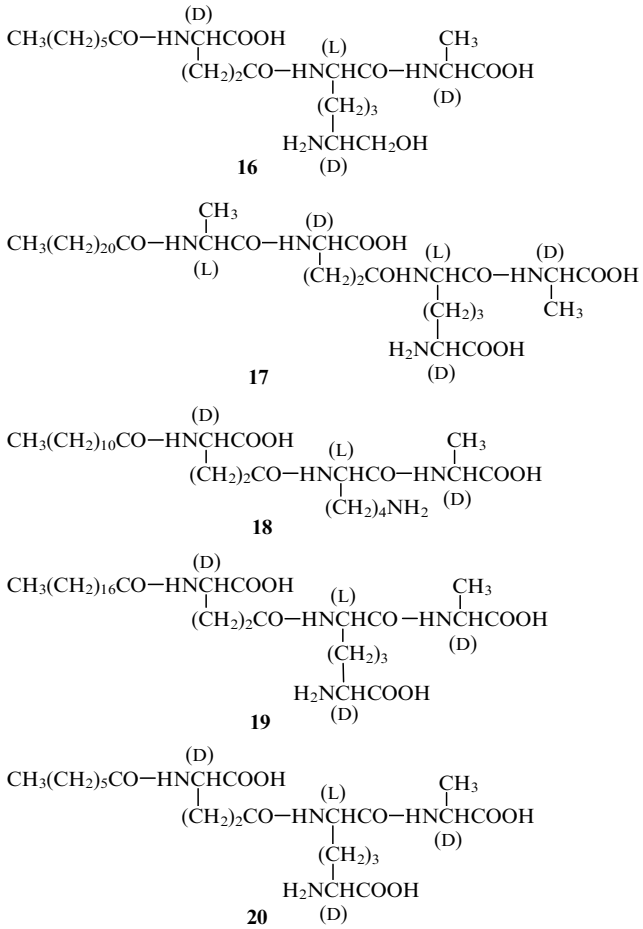
5: R = $CH_3(CH_2)_6CO$;
6: R = $CH_3(CH_2)_{16}CO$.

Scheme 1



The acylation of α-benzyl D-glutamate with octanoyl chloride gave α-benzyl *N*-octanoyl-D-glutamate (**7**), which was further converted into the *N*-hydroxysuccinimide ester and coupled with $H_2NCH(COOH)CH_2(CH_2)_2CH(NHBoc)CONHNHBoc$ (**8**). The removal of protective groups in the product **9** by hydrogenolysis and treatment with trifluoroacetic acid followed by oxidation with sodium metaperiodate gave the octanoyl derivative **5**. This procedure could not be applied for the synthesis of stearoyl derivative **6** because of low solubility of intermediate products preventing their deprotection. This problem was overcome by removing the protective hydrazide group in the *meso*-2,2 -diaminopimelic acid derivative prior to the incorporation of the fatty acid residue. To this end, BnOCONHCH(COOH). $.CH_2(CH_2)_2CH(NHBoc)CONHNHBoc$ (**10**) was treated with trifluoroacetic acid and oxidised with sodium metaperiodate to give the compound **11**. The incorporation of the protective *tert*-butyloxycarbonyl (Boc) group into this compound gave the product **12**. Its subsequent hydrogenolysis resulted in compound **13**. α-Benzyl *N*-stearoyl-D-glutamate **14** derived from α-benzyl D-glutamate by acylation with stearoyl chloride was converted into the *N*-hydroxysuccinimide ester, which was further coupled with compound **13** to give the compound **15**. This was followed by removal of the protective groups by hydrogenolysis and treatment with trifluoroacetic acid, eventually resulting in the final product **6**.

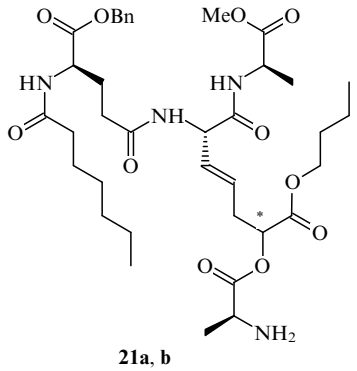
Similarly, compounds **16**–**20** were synthesised,[27] among which the lipopeptide **20** exhibited the highest activity.



Biological assay of the synthetic analogues of the lipopeptide **4** made it possible to establish that γ-D-glutamyl-*meso*-diaminopimelic acid is the minimum structural unit responsible for the immunostimulating activity of compounds of this series.[28]

It was suggested[28] that the diaminopimelic acid residue is responsible not only for the biological activity, but also for toxic effects of the lipopeptide **20** and its derivatives, which prevents the therapeutic application of these compounds.

The total chemical synthesis of stereoisomeric compounds (*R*)-**21** and (*S*)-**21** was carried out on the basis of molecular modelling data (Scheme 2).[29]



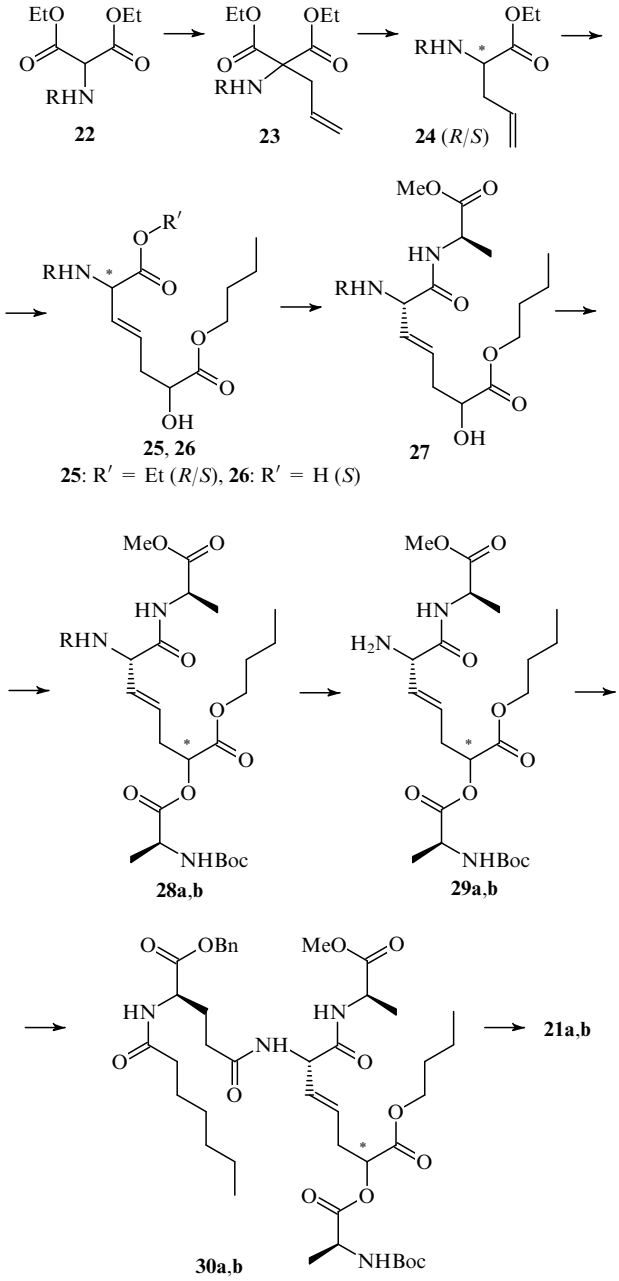
21a, **b**

21a (*R*-isomer), **21b** (*S*-isomer).

In these peptides, the residue of diaminopimelic acid was substituted by residues of unsaturated hydroxy amino dicarboxylic acids in which the hydroxy group was esterified by L-alanine.

Several pathways for the synthesis of compounds **21a**,**b** were considered.[29] All these synthetic schemes were based on the

Scheme 2



reaction of a glyoxylic acid ester with *N*-protected 2-aminopent-4-enoic acid; this reaction is catalysed by strong Lewis acids. The 2,2,2-trichloroethyloxycarbonyl group, which is stable in the presence of strong Lewis acids, was used for *N*-protection. Of the synthetic schemes proposed, that presented here (Scheme 2) was the most effective one, since it permitted the suppression of undesirable side reactions and gave the highest yields of the final products.

The protected diethyl aminomalonate **22** was treated with allyl bromide in EtOH/EtONa to give compound **23**, which was further converted into the racemic derivative **24**. The reaction of compound **24** with butyl glyoxylate gave a mixture of diastereomers **25**. Compound **25** was then hydrolysed with a-chymotrypsin to give stereospecifically the (*S*)-acid **26**, which, in turn, was coupled with the methyl ester of D-alanine to give a mixture of diastereomers **27**. The esterification of the secondary hydroxyl of compound **27** with Boc-L-alanine in the presence of dicyclohexylcarbodiimide (DCC) and dimethylaminopyridine (DMAP) gave a mixture of diastereomers **28 a**,**b**, which could be easily separated by crystallisation or chromatography. After removal of the *N*-trichloroethoxy-carbonyl protective group at pH 4.0 and 25 °C, the corresponding amines **29a**,**b** were coupled with γ-carboxy group of α-benzyl *N*-heptanoyl-D-glutamate following its activation with isobutyl chloroformate. After removal of Boc--protection with trifluoroacetic acid and treatment with hydrochloric acid in ether, the lipopeptides **30a**,**b** were converted into the hydrochlorides of the target products **21a**,**b**.

Compound **21a** induced high titers of colony-stimulating factors without exhibiting toxic effects.[30] Unfortunately, its activity strongly declined with time, since it was rapidly metabolised after oral administration.

The hydrochloride **21c** decomposed to give mainly the inactive ester **32** (Scheme 3) and a small amount of the hydroxy compound **31**, which still exhibited immunostimulating activity.

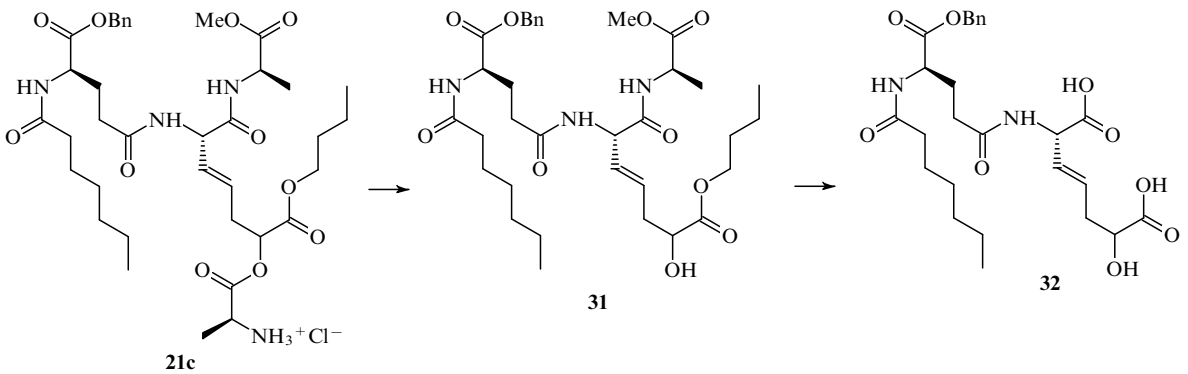
In order to prevent the rapid metabolic process inherent in compound **21**, its analogue with an additional methyl group in the α-position of the pimelic acid residue has been synthesised.[30]

All the lipopeptides mentioned above (**3a**, **4**, **14** – **20**) are highly potent immunostimulators. The former representative of lipopeptides that contains the murein-specific amino acids, the lauroyltetrapeptide **3a**, stimulates *in vivo* the phagocytic activity of macrophages in mice and of human blood monocytes and enhances the activity of the reticulo-endothelial system in mice.[20, 21] In mice, this compound induces cytotoxic lymphocytes against allogenic tumour cells and stimulates the activity of NK-cells in spleen and peripheral blood.

These activities indicate that the lauroyltetrapeptide can interact (directly or indirectly) with phagocytic cells, T-lymphocytes, and NK-cells. It was shown also that the lauroyl tetrapeptide stimulates *in vitro* the helper activity of human blood lymphocytes in the course of B-lymphocyte differentiation into plasmocytes.

In mice, the lauroyltetrapeptide manifests the adjuvant effect on DTH reactions and on the production of antibodies against

Scheme 3



various antigens, being more active than MDP in the former case and less active in the latter case.

The lauroyltetrapeptide **3a** induces the resistance of mice to various microbial infections (*Klebsiella pneumoniae, Listeria monocytogenes*) and inhibits the production of pulmonary metastases in mice inoculated intravenously with B16 melanoma cells or intradermally with Lewis lung carcinoma cells. It allows one to reduce the optimum dose of compounds exhibiting anticancer activity, *e.g.*, cyclophosphamide. In all *in vivo* systems, the lauroyltetrapeptide **3a** exhibited activity at concentrations ranging from 0.1 to 3 mg kg^{-1}, whereas in mice intravenously immunised with this agent its LD_{50} was 410 mg kg^{-1}.

The lipopeptide **20** and some other compounds increase the resistance of mice with normal and weakened immune systems against bacterial and fungal infections and enhance the immunotherapeutic activity in mice grafted with syngenic tumour cells.[31,32] At present, these compounds are subject to clinical trials aimed at testing of their therapeutic utility, especially in those cases where the patient's immune system needs to be reinforced.

Owing to their immune activity, the lipopeptides can not only protect against bacterial infections but also interfere with other processes (*e.g.*, metabolism of xenobiotics in liver) and thus prevent toxic effects of certain compounds on the organism.

The immunomodulating lauroylpeptides can protect mice against the toxic effects of paracetamol.[33] Equal doses of compounds **3a**,**c** manifest the immune activity , prevent the toxic effects of paracetamol (80% survival in mice), and cause a decrease in ethane exhalation, which is an index of lipid oxidation *in vivo*. Also, these lipopeptides reduced the microsomal cytochrome P-450 content in liver and suppressed CCl_4-induced lipid oxidation. The mechanism of their protective effects can be explained by the presence of factors secreted under the action of lipopeptides **3a**,**c**, such as interleukin-1 inhibiting the effect of cytochrome P-450 [34] or other factors secreted by macrophages [35] or Kupffer cells.[36]

VI. Lipopeptides-2

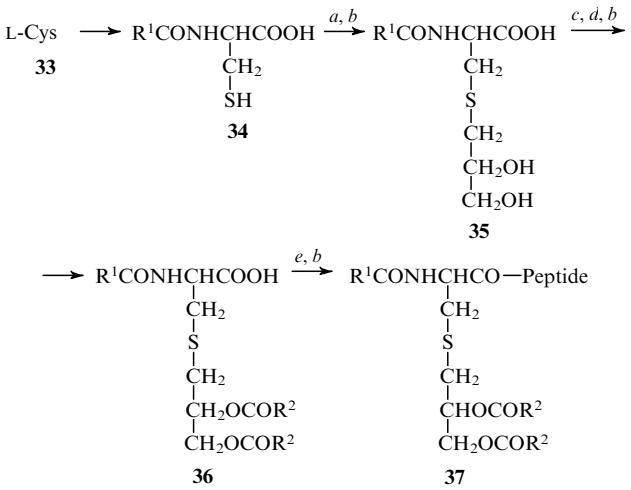
Studies on the biological activity of bacterial membrane components revealed that natural lipoproteins, particularly the *E. coli* lipoprotein and its fragments (natural lipopeptides), like the bacterial polysaccharide and muramyl dipeptide, exhibit the mitogenic activity and stimulate cells provoking the augmented release of interleukin-1, the production of prostaglandins E_2, $F_{2\alpha}$, and interferon.[37–39] In addition, the membrane lipoproteins are potent activators of polyclonal B-lymphocytes.

Synthetic lipopeptides, the analogues of natural lipopeptides, also exhibit immunostimulating activity. N-Terminal lipopeptides of the *E. coli* outer membrane lipoprotein, such as tripalmitoyl-*S*-glyceryl-L-Cys-Ser (Pam_3Cys-Ser) and its analogues with longer amino acid sequences [40] that stimulate the production of B-lymphocyte clones,[41] were synthesised (Schemes 4 and 5).

The first step in the synthesis of *E. coli* lipoprotein analogues according to Scheme 4 was the preparation of a series of *N*-acylcysteines (**34**) from the original L-cysteine (**33**). Thus it was converted into *N*-behenoyl-L-cysteine (**34**) with 1,2-*O*-isopropylidene-3-*O*-tosyl-*sn*-glycerol and acid hydrolysis yielded *N*-behenoyl-*S*-[(2*R*)-2,3-dihydroxypropyl]-L-cysteine (**35**). If (*R*,*S*)-glycidol was used instead, a mixture of *N*-behenoyl-*S*-[(2*R*,*S*)-2,3-dihydroxypropyl]-L-cysteines was obtained. The es-terification of the carboxyl group of cysteine into the benzhydryl ester and acylation of hydroxy groups were followed by deprotection using acidic hydrolysis (pH 5) yielding compound **36**.

The stage (*e*) requires special comment. The conventional peptide synthesis with dicyclohexylcarbodiimide and *N*-hydroxybenzotriazole resulted in a practically complete racemisation. The use of *N*-hydroxynorbornanedicarboximide instead of *N*-hydroxybenzotriazole gave compound **37**, which contained less than 3% of the (*S*,*R*)-diastereoisomer.[11]

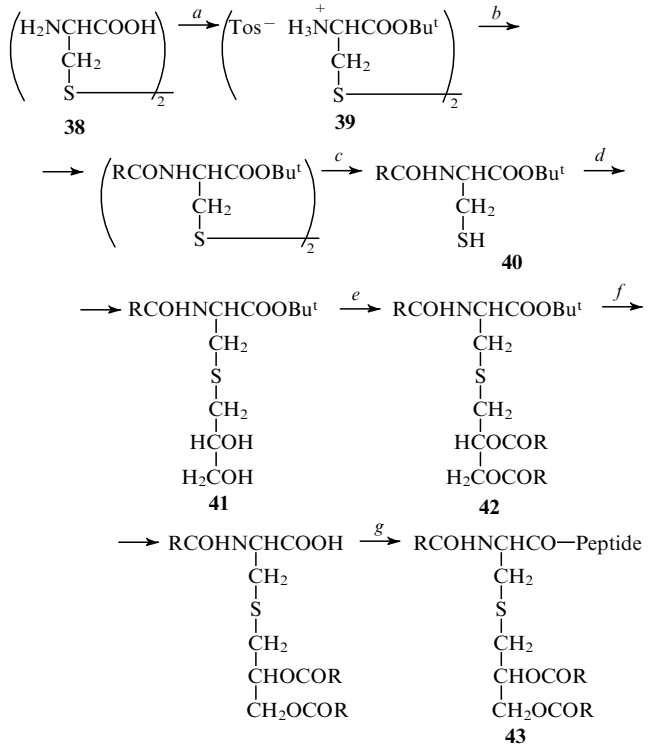
Scheme 4



34–**37**: $R^1 = CH_3(CH_2)_{20}$;
36, **37**: $R^2 = CH_3(CH_2)_{23}$.

(*a*) 1,2-*O*-isopropylidene-3-*O*-tosyl-*sn*-glycerol, K_2CO_3, EtOH; (*b*) H_3O^+; (*c*) Ph_2CN_2; (*d*): R^2COCl, Py; (*e*) DCC, *N*-hydroxynorbornanedicarboximide, C-protected peptide.

Scheme 5

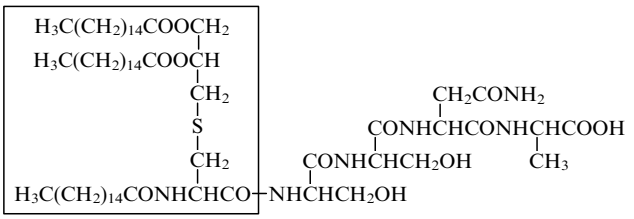


(*a*) $MeCOOBu^t$, $HClO_4$; (*b*) RCOCl, *N*-ethylmorpholine; (*c*) Zn, citric acid; (*d*) 1-*O*-tosylglycerol, DBU, DMF; (*e*) RCOCl, Py, 4-dimethylaminopyridine; (*f*) CF_3COOH; (*g*) DCC, *N*-hydroxynorbornanedicarboximide, peptide.

At the first step of the synthesis depicted in Scheme 5, the ester **39** was obtained from the original compound, L-cystine (**38**). Acylation of the amino group in **39** with palmitoyl chloride and subsequent reduction of the disulfide bond with zinc in acidic medium led to the *tert*-butyl ester of *N*-palmitoylcysteine (**40**). This compound was alkylated at the thiol group with 1-*O*-tosyl-

sn-glycerol, which reacted faster than 1,2-*O*-isopropylidene-3-*O*-tosyl-*sn*-glycerol, to give compound **41**, which was acylated with palmitoyl chloride to give compound **42**. Removal of the *tert*-butyl group with trifluoroacetic acid and attachment of the peptide fragment yielded the final product **43**.

In studies[40,42–46] on lipopeptides with various lengths of peptide chains, number of fatty acid residues and protective groups, the objects of investigation were lipopeptide monolayers. In an effort to gain information about the interaction of these compounds with a typical lipid membrane, the monolayers of lipopeptides with three fatty acid residues were mixed with those of dipalmitoylphosphatidylcholine and cholesterol. (These studies used synthetic lipopeptides that contained only the palmitic acid residues, whereas their natural counterparts contained a mixture of higher fatty acids.[47])



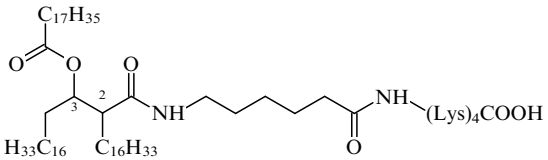
Pam$_3$Cys

44

It was shown that the synthetic lipopentapeptide, *N*-palmitoyl-*S*-[(2*R*,*S*)-2,3-dipalmitoyloxypropyl]-L-cysteinyl-D-seryl-D-seryl-D-asparaginyl-D-alanine (**44**) exhibited the same mitogenic properties as did the natural lipoprotein.[45,46,48–50]

Even after the shortening of the peptide chain from the lipopentapeptide Pam$_3$Cys-Ser-Ser-Asn-Ala to the lipodipeptide Pam$_3$Cys-Ser, the immunostimulating properties were still retained. However, tripalmitoyl-*S*-glyceryl-L-cysteine as such exhibited only a weak stimulating activity.[41] The lipophilic, fully protected lipopeptide Pam$_3$Cys-Ser(Bu^t)OBu^t manifested a weak mitogenic activity. It was therefore concluded that the crucial role in the specificity of activation is played by polar head groups, in particular by hydroxy and carboxy groups of serine. Owing to these features, the synthetic mitogens described above are highly suitable objects for the study of early events of the immune response, for the isolation of specific binding proteins, and for the analysis of structure-activity relationships.[51]

However, these synthetic analogues of the *E. coli* N-terminal lipopeptide have one serious disadvantage, namely, they are poorly soluble in water, which prevents their application as adjuvants. To overcome this problem, the lipopeptide tripalmitoyl-*S*-glyceryl-cysteinyl-seryl-(lysyl)$_3$-lysine (Pam$_3$Cys-Ser-Lys$_4$), was synthesised,[52] which is both water-soluble and, as has been demonstrated in different tests, biologically active. Compounds **45a–d**, the derivatives of 3-*O*-stearoylcorinomycolinic acid, were also synthesised.[53]



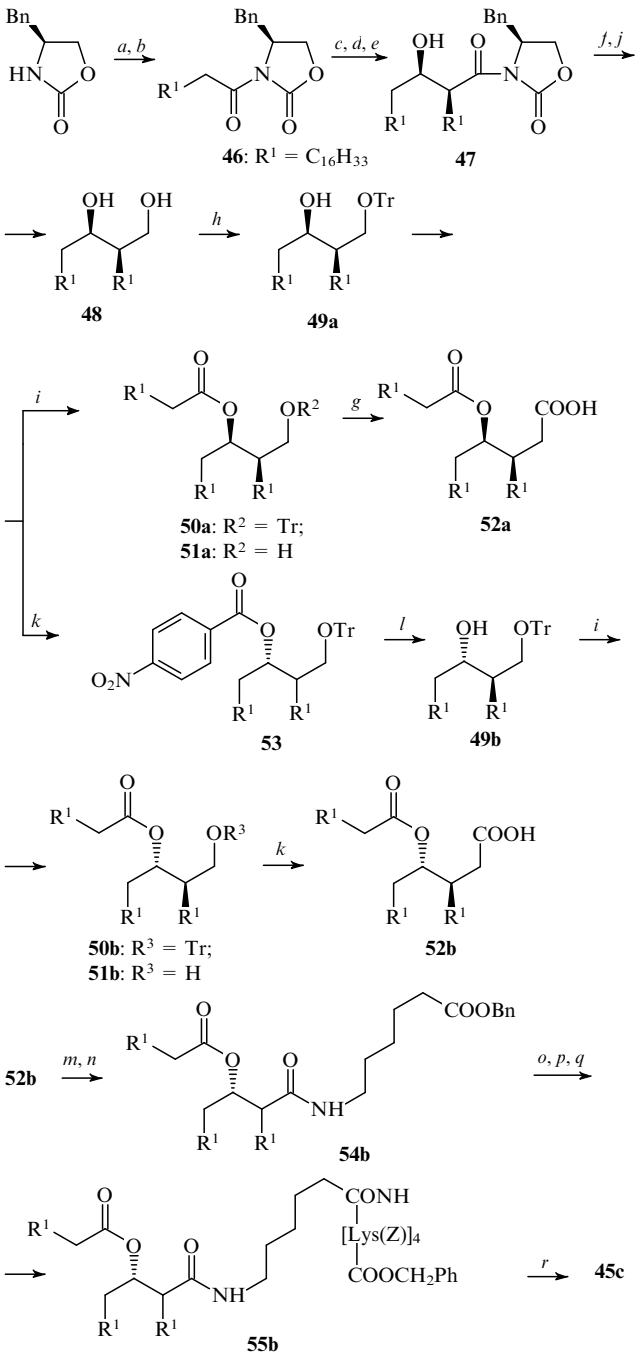
45a–d

45a: (2*S*,3*R*)-isomer; **45b**: (2*S*,3*S*)-isomer;
45c: (2*R*,3*S*)-isomer; **45d: (2***R*,3*R*)-isomer.

The main problem in the synthesis of these compounds was the elaboration of a scheme that would give all the four stereoisomers. Jacques and Grob[53] proposed the following paths for their synthesis (Scheme 6).

S-*N*-Stearoyl-2-oxazolidone (**46**) was obtained by sequential treatment of *S*-4-benzyl-2-oxazolidone with butyllithium and stearoyl chloride. Treatment of compound **46** with 9-borabicyclo[3.3.1]nonyl trifluoromethanesulfonate (9-BBN-OTf) and condensation of the reaction product with octadecanal gave compound **47**. It was further converted into the diol **48** by

Scheme 6



46: R^1 = $C_{16}H_{33}$ **47** **48** **49a** **50a**: R^2 = Tr; **51a**: R^2 = H **52a** **53** **49b** **50b**: R^3 = Tr; **51b**: R^3 = H **52b** **54b** **55b** **45c**

R′ = $CH_3(CH_2)_{15}$;
(*a*) BuLi, THF, −78 °C; (*b*) $C_{17}H_{35}COCl$, −78 °C; (*c*) 9-BBN-OTf, Pr^i_2NH, CH_2Cl_2, 0 °C; (*d*) $C_{17}H_{35}CHO$, −78 °C; (*e*) MeOH, 30% H_2O_2, buffer pH 7.0, 0 °C; (*f*) $LiBH_4$, ether; (*j*) NH_4Cl, 0 °C; (*h*) Ph_3CCl, Py; (*i*) $C_{17}H_{35}COCl$, Py/CH_2Cl_2, 0 °C; (*k*) $KMnO_4$, C_6H_{14}/AcOH/H_2O; (*g*) DEAD, PPh_3, *p*-$NO_2C_6H_4COOH$, C_6H_6; (*l*) 2 M NaOMe/MeOH; (*m*) DCC, HONSu; (*n*) $Cl^-NH_3^+(CH_2)_5COOCH_2Ph$, Pr^i_2NH, DMF/CH_2Cl_2; (*o*) 10% Pd/C, H_2, AcOEt; (*p*) DCC, HONSu, CH_2Cl_2, 0 °C; (*q*) Cl^- NH_3^+ [Lys(Z)$_4$]$COOCH_2Ph$, CH_2Cl_2; (*r*) 10% Pd/C, H_2, THF/0.1 N HCl.

treatment with $LiBH_4$ in moist ether. The introduction of a trityl protection at the primary hydroxy group and acylation of the secondary hydroxy group in the diol **48** with stearoyl chloride gave compound **50a**. Removal of trityl protection to give the ester **51a** was complicated by intramolecular acyl migration. However, subsequent oxidation of the isomer mixture gave the acid **52a**.

The acid **52b** was obtained by inversion of the stereochemistry in compound **49a** at C(3). This compound was converted into the ester **53** by interaction with *p*-nitro-(or *o*-nitro-)benzoic acid in the presence of PPh_3 and diethyl azodicarboxylate (DEAD). Its de-esterification required drastic conditions [stage (*l*) in Scheme 6] and resulted in compound **49b**, which was then converted into the acid **52b** by analogy with compound **49a** → 52a. The same reaction sequence, which began with (*R*)-4-benzyl-2-oxazolidone, gave the enantiomers of compounds **46**, **47**, **48**, and **53**, respectively, and the final products **52c** (2*R*,3*S*) and **52d** (2*R*,3*R*). Further, the peptide fragment was attached to acids **52a**–**d** with the use of the active esters method to give the lipopeptides **45a**–**d** [stages (*m*–*r*) in the Scheme 6].

However, these lipopeptides have four lysine residues, which cause certain difficulties due to the formation of high positive charges.

In the study by Kleine *et al.*,[54] two lipopeptide analogues of the *E. coli* lipoprotein, Pam_3CysOH and Pam_3Cys-SerOH, were conjugated with polyoxyethylene 3000 (POE) to increase the solubility of lipoproteins. The conjugates obtained possessed high solubility in water and were devoid of the formation of high positive charges. The increase in the solubility did not bring about any significant changes in the biological properties of the lipopeptide fragment. It was concluded that the polyoxyethylene component, which is inactive itself, enhances the activity of lipopeptides by increasing the percentage of lipopeptide molecules accessible to the cells.[54]

VII. Possible applications of lipopeptides-2

The lipopeptide fragments of the *E. coli* membrane lipoprotein are highly effective stimulators of antibody production and activators of B-cells. Synthetic lipopeptides enhance primary and secondary immune responses to corpuscular antigens *in vitro* and *in vivo*.[55] The addition of synthetic lipopeptide fragments to acetone-killed cells of *Salmonella typhimurium* makes it possible to significantly reduce the vaccine dose. It is believed that the first step in the mechanism of action of lipopeptides is their interaction with the cell plasma membrane through incorporation of lipophilic groups into the lipid bilayer and their binding to membrane proteins. Hence, the mitogenic activation of cells can be achieved through the use of conditions of a serumless culture and the use of a mitogen that is optimally dissolved in detergent-containing solutions. Under these conditions, the mitogenic activity of compound **44** *in vitro* can be observed at as low concentrations as *e.g.* 10 ng ml^{-1}. The optimum stimulating activity was observed at concentrations around 30 mg ml^{-1}.

The adjuvant properties of the tripalmitoylpentapeptide with respect to the immune response was studied *in vitro*.[56] The immune response to sheep erythrocytes (both native and nitrophenol-treated) was strongly enhanced after the addition of various amounts of the tripalmitoylpentapeptide **44** to cell cultures. This effect was dose-dependent, the optimum adjuvant effect being manifested at the concentration range of 10–30 mg ml^{-1}.

A sharply pronounced adjuvant effect was observed in only those cases when the tripalmitoylpentapeptide and the antigen acted simultaneously; the addition of the lipopeptide either 24 h prior to, or 24 h after, the addition of the antigen had no effect. Hence, the adjuvant effect of the tripalmitoylpentapeptide can be associated with the formation of an adjuvant-antigen complex.

The use of lipopeptides as adjuvants is not confined to cell antigens *in vitro*. It was shown that the *in vivo* immune response to native and nitrophenol-treated sheep erythrocytes was also enhanced significantly in the presence of the lipopeptide; the *in vivo* immune response to proteins, like ovalbumin, can also be increased after the addition of the lipopeptide.

Of special interest were the studies on the mitogenic and adjuvant activities of water-soluble lipopeptides, since in the case of high immunostimulating activity these compounds might be used commercially. Pam_3Cys-Ser-POE and Pam_3Cys-POE were assayed for mitogenic activity towards human B-lymphocytes *in vitro* and for adjuvant activity in mice *in vivo*. It was established that polyoxyethylene-lipopeptide conjugates exhibit the same mitogenic activity as does the water-soluble cationic lipopeptide Pam_3Cys-Ser-Lys_4. They exhibited biological activity even at concentrations around 30 mg ml^{-1}.

In order to investigate possible areas of the application of lipopeptide-polyoxyethylene conjugates as adjuvants for the construction of vaccines, the adjuvant activities of these compounds were compared to those of three commercially available adjuvants: Freund's adjuvant, Hunter's Titermax, a nonionic block copolymer,[57] and vegetan extracted from potatoes. Ovalbumin and synthetic melittin (a 26-membered bee venom polypeptide) were used as the antigens. The polyoxyethylene-lipopeptide conjugates were less active than Pam_3Cys-Ser-Lys_4 or Freund's adjuvant, while their activities were comparable with that of the Hunter's block copolymer or vegetan. In mice immunised with the antigen (ovalbumin or melittin) and Pam_3Cys-Ser-POE or Pam_3Cys-POE, the antibody titers against both melittin and ovalbumin were similar. However, the maximum immune response (compared to that raised with the Freund's adjuvant) required repeated immunisations. In most cases, three injections were sufficient for this purpose.

The design of synthetic vaccines requires that the demand of safety of polyclonal activators used as adjuvants for routine immunisations be met. The lipopeptides tested did not exhibit any toxic or other undesirable effects in animal studies, they evoked only a 7-days-lasting inflammation at injection sites with a complete recovery of the injured area after several weeks. By contrast, animals immunised with the Freund's adjuvant developed long-lasting granulomas at injection sites.

It was thus concluded [54] that the new polyoxyethylene compounds are capable of polyclonal activation of human B-lymphocytes in culture systems and exhibit a sufficiently high adjuvant activity. They can be easily synthesised and yield stable solutions. In addition, they are non-toxic. It was therefore decided to continue the use of Pam_3Cys-Ser-POE and Pam_3Cys-POE as adjuvants for immunisation of animals and, after conducting appropriate tests, of human beings as well.

The synthetic mitogen tripalmitoylpentapeptide **44** and some of its analogues can non-specifically enhance the resistance of a host organism to bacterial infections. This property is particularly often exploited in studies aimed at a search for new effective antigens in the design of synthetic vaccines when a low-immunogenic peptide or a fragment of a natural protein are to be used as antigens. Since the procedure of covalent binding of a peptide and the lipopeptide adjuvant increases immunogenicity and can be easily performed, it can be used in studies of epitope specificity of various natural proteins. An example is the study by Beck-Sickinger *et al.*,[58] which is dedicated to the discovery of effective antigens against *Haemophilus influenzae* (Hi).

The *Haemophilus influenzae* type b (Hib) is the main causative of endemic bacterial meningites in newborns and children; the non-typeable Hi is also a potent pathogen. The most severe bacterial infections in humans (meningites, arthrites, *etc.*) are caused by Hib. The antibodies against the Hib capsular polysaccharide protect humans from diseases caused by Hi. However, this way of protection is ineffective in children under one year of age who are at the highest risk of being infected with these diseases. These facts have stimulated interest in the search for other antigens.

The P6 protein (16 600 Da), which is present in the outer membrane of two typeable and one non-typeable Hi strains, is the most important antigen in raising the immunity against Hi.

Antibodies against P6 possess protective and bactericidal activities. The progenitor of P6 is a bacterial lipoprotein containing 134 amino acid residues and having at its N-terminus fatty acids linked by an amide and ester bonds to *S*-glycerylcysteine. Since this structure resembles the *E. coli* lipoprotein, lipopeptides were synthesised [58] in which different fragments of the amino acid sequence of P6 were covalently linked to *N*-palmitoyl-*S*-[(2*R*,**S**)-2,3-dipalmitoyloxypropyl]-L-cysteinyl-D-serine. This study has made it possible to establish the epitope specificity of antibodies. Lipopeptides containing the sequence Gln-Ile-Leu-Asp-Ala-His-Ala-Ala (P6 47-54) and the murine B-cell epitopes Gly-Glu-Tyr-Val (P6 43-46) were able to induce T-helper activity in BALB/c mice. The antibodies raised in the immunisation could adequately neutralise the Hi bacteria. Thus the use of a mixture of lipopeptides containing the above segments of the P6 protein as synthetic vaccines holds much promise for *in vivo* studies of model infection systems.

Another example of the use of adjuvants covalently bound to antigens can be found in the study carried out by Deres *et al.*[59] The covalent binding of lipopeptide adjuvants to antigens is especially advantageous for compounds that exhibit low immunogenic activity by themselves. Lipopeptides used as adjuvants enhance the immunogenic activity of antigens in synthetic vaccines.

Cytotoxic T-lymphocytes make an appreciable contribution to immunity against intracellular pathogens. This refers also to viral infections, where cytotoxic T-lymphocytes recognise viral peptides presented by molecules of the major histocompatibility complex (MHC) class 1 on the surface of infected cells.[60] The induction of virus-specific cytotoxic T-lymphocytes usually requires *in vivo* priming with infectious viruses. Inactivated viruses, like viral proteins or peptides, are mostly ineffective as inducers of the cell-type immune response *in vivo*, although these antigens can initiate antibody production against viral antigens.

The absence of the anticipated effect following the use of peptides as antigens can be assigned to the 'non-physiological' type of the antigen presentation (the lack of conjugation with MHC glycoproteins), since T_C can usually recognise the fragments that are derived from proteins endogenously produced by target cells but are incapable of recognising the free peptides that are not bound to the glycoproteins of the major histocompatibility complex.[61] The second reason is the lack of secondary signals, such as cytokins, released by antigen-presenting cells.

Unlike native peptides, synthetic compounds attached to a lipophilic anchor (lipopeptides) can activate virus-specific cytotoxic T-lymphocytes *in vivo*.[59] The structure of one such lipopeptide, the epitope of the influenza virus nucleoprotein, which is the most adequately recognised by H-2d-restrictase [peptide NP 147-158(*R*-)], conjugated with Pam_3Cys-Ser-Ser, is given below (see formula **56**).

Pam_2Cys-Ser-Ser—Thr-Tyr-Gln-Arg-Thr-Arg-Leu-Val-Thr-Gly

NP 147-158(*R*-)

56

The precise mechanism of the *in vivo* activation of cytotoxic T-lymphocytes by lipopeptides is still unknown. A possible reason for this effect is that the anchor molecule of Pam_3Cys possesses the ability to activate accessory cells that secrete factors needed for the differentiation of cytotoxic T-lymphocytes.[59] If this is true, a mixture of Pam_3Cys and a native peptide can also be used for the activation of cytotoxic T-lymphocytes.

If the effect of lipopeptides is not determined by the non-specific action of accessory cells and/or T-helper cells, other possibilities may be offered. Probably, the lipid anchor prolongs the lifetime of the antigen molecule *in vivo* relative to native peptide molecules. At the same time, it is not excluded that the lipid anchor attaches to the cell membrane and thus favours the binding of the antigen with glycoproteins of the major histocompatibility complex class 1, thus offering a more 'physiological' path of antigen presentation.

The major problem, which restricts the use of peptide vaccines in natural populations, is that T-cells in individuals with different MHC always recognise different peptides from viral proteins together with their own corresponding MHC. Thus, universal synthetic vaccines stimulating the growth of populations providing the cell-type immune response to a certain viral antigen should contain all of the relevant epitopes recognised by T-cells. The fulfilment of this condition is not a very difficult task because of the relatively good availability of synthetic Pam_3Cys-Ser-Ser-peptide conjugates. Pam_3Cys-Ser-Ser covalently linked to peptides is particularly suitable for the design of vaccines, since the production of neutralising antibodies, another important element of the antiviral immunity, can also be achieved through the conjugation of Pam_3Cys-Ser-Ser to the corresponding epitopes.

VIII. The use of lipopeptides-2 in the fight against the human immunodeficiency virus

The problem of infection of humans with the immunodeficiency virus presently becomes a formidable challenge; therefore, many recent studies deal with the development of vaccines against the immunodeficiency virus, which can induce antibody synthesis and simultaneously elicit associated cytotoxic responses.

Vaccinations against the human immunodeficiency virus (HIV) with the use of inactivated viruses raises the problem of their therapeutic safety for human beings. The use of viral genes in the composition of recombinant vectors also requires further investigation. Peptide vaccination is a very promising approach; however, free peptides are often little immunogenic. The interest in lipopeptide vaccines is not incidental for these reasons. Their use is devoid of side effects, and this vaccination can be applied to humans. Moreover, the immune response induced will be free from that against the vector molecule, as is the case with recombinant vaccines. Therefore, the combination of lipopeptides inducing the cell-type response with lipopeptides stimulating antibody production will provide effective protection against the immunodeficiency virus.

The study carried out by Loleit *et al.*[62] is an example of the use of lipopeptides for immunisation against HIV. These authors found that the C-terminal segment of the reverse transcriptase (RT) of HIV 1 contains a highly immunogenic determinant that causes the proliferation of T-cells in different mouse strains and partly overlaps with the B-cell epitope. The lipopeptide *N*-palmitoyl-*S*-[(2*R*,*S*)-2,3-dipaltimoyloxypropyl]-L-cysteinyl-D-seryl-D-serine was used as the adjuvant, for it does not exhibit toxic effects characteristic of Freund's adjuvant and increases the immunogenicity of free peptides.[52]

In order to assess the ability of the lipotripeptide Pam_3Cys-Ser-Ser to enhance peptide-specific immune responses *in vivo*, Loleit *et al.*[62] have immunised mice of different strains (BALB/c, C3H/Hej, C57BL/6) with the 22-meric conjugates Pam_3Cys-Ser-Ser-[RT-(522-543)] and Pam_3Cys-Ser-Ser-[RT-(528-549)] of the reverse transcriptase of HIV-1, which contain the T-helper epitope [RT-(528-543)] of the reverse transcriptase. The results of this study [62] indicate that Pam_3Cys-Ser-Ser is an effective adjuvant capable of inducing T-helper and B-cellular responses to peptide sequences that represent the fragments of the C-terminal region of the reverse transcriptase of HIV-1.

The procedures used for the attachment of lipids or lipopeptides to HIV-derived peptides were also described by other groups of investigators.[63,64] Thus, Martinon *et al.*[63] used the C_{14}-fatty acid peptide (HDA) as well as C-terminal fragments of peptides from the V3 region of a nucleoprotein epitope corresponding to the outer envelope proteins of HIV-1 (Env). Defoort *et al.*[64] synthesised the lipopeptide Pam_3Cys and modified it through a lysine spacer with four molecules of the HIV-1 Env-peptide to obtain the so-called multiplet antigenic system (MAPS). Both

products could induce antibody production and a cytotoxic response to HIV.

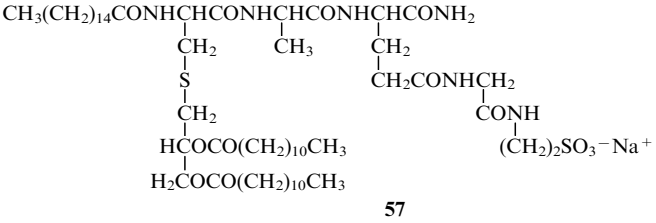
According to the most recent data, some peptides can be recognised in both mice and humans.[65] In order to assay the epitope of the reverse transcriptase RT-(528-549) for immunogenicity towards human T-lymphocytes, Loleit *et al.*[62] have studied T-cell responses in HIV-infected individuals. The first analyses of cytotoxic responses to the C-terminal region of reverse transcriptase of HIV-1 revealed that the lipopeptide Pam_3Cys-Ser-Ser-[RT-(528-549)] could be recognised by cytotoxic T-lymphocytes of patients at different stages of the disease.

Thus, the ability of lipopeptide conjugates to induce efficient humoral and T-cell responses and the lack of toxic effects makes them probable candidates for the components of synthetic vaccines designed for use in humans.

IX. The antitumour activity of lipopeptides-2

Another important aspect of the biological action of *E. coli* cell wall lipopeptides is their antitumour activity.[66, 67]

The modification of their peptide residues with taurine yielded compounds that were highly active *in vivo* and could destroy pulmonary metastases. One of these compounds is the sodium salt of *N*-hexadecanoyl-*S*-[(2*R*)-2,3-didodecanoyloxypropyl]-L-cysteinyl-L-alanyl-D-isoglutamyl-glycyl-taurine **57** (CGP 31362).



57

Its synthesis is similar to that of compound **44** and its analogues (Schemes 4 and 5). The peptide fragment of compound **57** was modified with taurine, which imparted to it a greater polarity.[68, 69]

Compound **57** can be incorporated into membranes of phospholipid liposomes.[69] Liposomes are unique carriers for targeted delivery of biologically active substances to phagocytosis-active cells *in vivo*. This delivery results in the activation of phagocytes and, correspondingly, in the destruction of established lung, lymphoid, and liver metastases. Like other circulating particles, liposomes are rapidly ingested by free and fixed phagocytic cells. The advantage of such physiological 'death' of liposomes is that they deliver immunomodulators to cells of the reticulo-endothelial system.

Compound **57** incorporated into liposomes can activate human blood monocytes as well as mouse peritoneal and bone marrow macrophages.[70] Periodical injections of this compound caused *in situ* activation of macrophages.[71] Noteworthy, macrophages that phagocytised liposomes with incorporated compound **57**, retained their antitumour activity over a period of several days, thus favouring the destruction of established cancer metastases.

Compound **57** is a more potent anticancer agent in comparison with muramyl-peptide derivatives, since it stimulates the antitumour activity at lower doses and activates monocytes in the absence of γ-interferon. Monocytes stimulated by compound **57** exhibit both the activity related to tumour necrosis and interleukin-1 activity, whereas monocytes stimulated by muramyl-peptides manifest only tumour necrosis activity.

On the basis of numerous experiments, Utsugi *et al.*[71] concluded that compound **57** can efficiently stimulate macrophages responsible for the lysis of tumour cells; this compound is a better activator of monocytes and macrophages than muramyl-peptides.

X. Conclusion

The data considered in this review show the increasing interest in the components of bacterial membranes, specifically, in lipopeptides.

The synthesis of analogues of bacterial lipopeptides is related to the fine organic synthesis of complex conjugates. This can be exemplified by the above-described methods of synthesis of compound **44** and its analogues containing a peptide fragment and three fatty acid residues. The synthesis of compounds of a mixed composition makes a contribution to the development of organic synthetic chemistry.

Since lipopeptides are optically active compounds, their synthesis brings about a number of specific problems associated with their preparation. Sofar the synthesis of the majority of lipopeptides is marked by complexity and multiphasity. Unfortunately, these difficulties are an obstacle on the way to the elaboration of a large set of modified analogues of natural compounds for research purposes.

A study of the biological activity of lipopeptides that are the components of bacterial membranes permits to expand the view of both the structural organisation of membranes and the activity of compounds of a mixed composition in the fight against various infections.

The pharmacological effects of natural lipopeptides and their synthetic analogues have been demonstrated in animal models *in vivo*. In addition to immunomodulating properties, lipopeptides exhibit antitumour activity and have been extensively used in recent years in the fight against the human immunodeficiency virus.

As lipopeptides do not possess toxic properties, it may be assumed that compounds of this class will find wide use in the design of contemporary synthetic vaccines as well as in the treatment of malignant tumours and autoimmune diseases.

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