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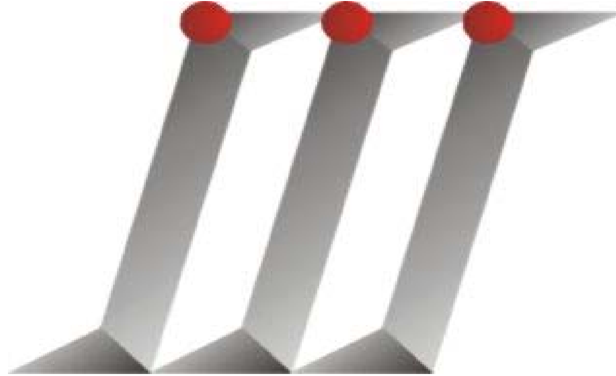

3rd German-Polish-Russian Meeting on Bacterial Carbohydrates

1st Baltic Meeting on Bacterial Carbohydrates

with INTAS Strategic Workshop
"Bacterial glycoconjugates in prevention and diagnostics of emerging pathogens"

October 6-9, 2004

Wrocław, Poland

GPR3 MEETING
ABSTRACTS OF LECTURES

Introductory lecture
THE BEGINNINGS OF MY STUDIES ON BACTERIAL O-ANTIGENS

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In the late sixties Profesor Ślopek, then the director of the Institute of Immunology and Experimental Therapy encouraged me to start immunochemical studies on *Shigella sonnei* bacteria causing dysentery. Professor Ślopek has worked on *Shigella* genus for a long time.
At the time the chemical nature of antigens isolated from various bacterial strains was weakly known, but several laboratories have jest started studies in this direction. The most advanced ones concerned *Salmonella* genus. *Salmonella* are gram-negative pathogenic organisms. In the works of Westphal, Lüderitz et al., and Staub et al. it was found that O-antigenic chain of *Salmonella* polysaccharide is built of oligosaccharide repeating units. The repeating unit may be di-, tri-, tetra- or even hexa- or heptasaccharide. It depends on the serotype of bacteria from which the antigen was isolated. The structure of antigenic chains was defined for some *Salmonella* serotypes. The base chain of the repeating unit is formed of galactose, mannose and rhamnose or of galactose, mannose, rhamnose and glucose. As the side chains of the repeating units, there occur 3,6-dideoxy-hexoses, like colitose, abequose, tyvelose and paratose, identified as terminal groups determining serological specificity of the studied *Salmonella* antigens.
As it is known, *Shigella sonnei* occurs in three forms: phase I (smooth), phase II and R (rough). The chemical studies on *Shigella sonnei* were tough and for a long time I have got no satisfactory results. The first difficulty was to obtain pure antigen preparation, it means pure lipopolysaccharide. The equipment of our laboratory in those times was really mean. We had no ultracentrifuge to obtain preparation free of cell ribonucleic acids. For this reason I worked out the simple method of purification of lipopolysaccharide using gel filtration on Sepharose 2B column. The method worked well, but it was more time-consuming than ultracentrifugation. We managed to get pure lipopolysaccharide of *Sh. sonnei* phase I and tried to analyse its O-specific polysaccharide obtained upon moderate hydrolysis of the lipopolysaccharide.
It was known from the earlier works of Lüderitz and coworkers on O-antigens of *Salmonella* and *Escherichia coli* that the mild acid hydrolysis (0.5 N sulphuric acid, 100°C, 0.5–1 h) of the lipopolysaccharide yielded a mixture of oligosaccharides suitable for structural studies. However, the hydrolysis of *Sh. sonnei* phase I lipopolysaccharide carried out under similar conditions in our laboratory released only minute amount of oligosaccharides and a polymer which showed all serological activity. In our further laborious studies 2-amino-2-deoxyhexuronic acid was identified as a main sugar component of the O-specific part of *Sh. sonnei* phase I lipopolysaccharide.
To determine the complete structure of O-specific part of *Sh. sonnei* lipopolysaccharide more modern technique had to be used. It was necessary to carry out the methylation of the examined polysaccharide and then after acid hydrolysis of the methylated polysaccharide to analyse partially methylated alditol acetates of monosugars by gas-liquid chromatography/mass spectrometry. Unfortunately at that time this kind of equipment was unavailable for us. Thus we started the collaboration with Professor Lindberg and Dr Kenne from Sweden where such experiments were successfully performed.
As shown in their structural studies, the O-specific polysaccharide of phase I *Sh. sonnei* is composed of disaccharide repeating units formed of two rare sugars: N-acetyl-L-altrosaminouronic acid as α-pyranosyl residue substituted at O-4 and linked to O-3 of N4-N-acetyl-D-fucosamine.

Basing on ^{1}H-NMR analysis (carried out in Institute of Chemistry at Wrocław University) it was calculated that phase I specific polysaccharide is formed of four repeating disaccharide units. It is highly probable that this material is a mixture of polysaccharides with 2-6 repeating units per lipopolysaccharide molecule.
In phase I specific polysaccharide N4-N-acetyl-D-fucosamine is the sugar component of the O-specific chain, which is bound to terminal β-D-glucosyl residue of the core.
In further studies on *Shigella sonnei* phase II and R-form lipopolysaccharides we determined the structure of phase II core oligosaccharide as a nonasaccharide composed of two α-D-galactosyl, two α-D- and one β-glucosyl and three α-LD-heptosyl residues, terminated at reducing end by 3-deoxy-*manno*-octulosonic acid. R-form oligosaccharide was identified as hexasaccharide composed of two α-D-glucosyl, three α-LD-heptosyl residues terminated at reducing end by 3-deoxy-manno-octulosonic acid (Fig. 1).
Our further structural studies were performed on *Shigella flexneri* lipopolysaccharides isolated from serotypes 2b, 5b and 6. My first work on *Shigella flexneri* in collaboration with Professor Lachowicz was carried out on lipopolysaccharides isolated from *Sh. flexneri* serotype 1b and its smooth mutant Z. Chemical analysis of the lipopolysaccharides of the both strains showed that the mutation is connected with de-O-acetylation of lipopolysaccharide of serotype 1b.
After *Shigella flexneri* we turned to *Citrobacter* strains and managed to obtain quite interesting results about their lipopolysaccharides. The next group of gram-negative bacteria we worked on, was *Hafnia alvei*, but it is another story. Today I wanted to present our beginnings in immunochemistry of O-antigens.

O1 – Opening lecture

NOVEL CONSIDERATIONS ON GRAM-NEGATIVE ENVELOPE STRUCTURAL ORGANIZATION AND FUNCTIONING

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The envelopes Gram-negative bacteria consist of two major structural moieties: the external part, which is composed of the outer membrane, and the periplasm, which is located between the cytoplasmic and outer membrane, i.e., within the periplasmic space. Periplasm is a cumulative definition for a dense physiological gel composed of peptidoglycan, lipoproteins and numerous proteins that perform different biological functions.

The outer membrane represents an asymmetrical lipid bilayer and perforated with numerous pores made of proteins (porins) that adopt a barrel-like conformation. The structural principles of the outer membrane organization are well understood.

The situation with structural organization of the periplasm is somewhat complicated. Indeed, the classical concept implies that the distance between outer and inner membranes does not exceed 8-12 nm and, therefore, the periplasmic space is rather compact. These small parameters of the periplasm are in conflict with dimensions of lipoprotein and TolC protein molecules, which are essential components of the periplasm. Moreover, classical concept considers bacterial cell wall to adopt a very thin network structure located just beneath the outer membrane, and this cannot be true because cell wall sacculus spans from membrane to membrane. And the last, quantitative determination of the protein content within the periplasm suggested that that the height of the periplasmic space should be about 35 nm.

Now it is recognized that techniques used in traditional electron microscopy for the sample preparation was associated with dehydration of the biological material followed by its drastic shrinking. We believe that this artifact was underestimated by the current concept.

To solve the problem of the periplasm structural organization, we revised the adopted tertiary structure of murein and found that perpendicular orientation of the peptidoglycan strands within the cell wall changes radically the overall murein architecture to convert it into thick cross-linked matrix. Our computer simulation studies demonstrated that novel murein tertiary structure (scaffold model) is in agreement with known chemical and physical properties of the Gram-negative sacculus. The most important property of the scaffold-like murein tertiary structure is the ability to function as a gel, the latter being able to shrink and swell depending on amount of the retained molecules of water.

New vision of the periplasm structural organization allows explaining the molecular principles for the whole envelope functioning. First, the structure readily accommodates long peptide chains of all components that did not fit previous structure. Second, gel-like murein structure provides excellent possibility for the periplasm compartmentation, the latter being of great importance for solute efflux. Third, the process of murein turnover caused by action of soluble transglycosylase Slt70 could initiate thinning of the murein matrix to result in formation of the membrane adhesion zones. Fourth, the controlled variation of the murein-gel thickness allows the adjustment of different secretion systems through the periplasm, and this needs the specific connections between the proper protein components of both inner and outer membranes. Fifth, the dynamic behavior of the periplasm allows assuring the perpendicular orientation of the glycan strands in the course of murein biosynthesis.

O2

DEVELOPMENT OF THE BACTERIAL CARBOHYDRATE STRUCTURAL DATABASE

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The work is devoted to the development of a database containing data on all bacterial carbohydrates with known primary structure (currently about 8000 records). In addition to the primary structures and bibliographic data, the database contains abstracts of the publications, data on bacterial sources, methods of structure elucidation, information on the availability of data on conformation, biological activity, chemical synthesis, biosynthesis, genetics and other related data as well as cross-references to other databases containing information on bacterial carbohydrates. The key feature is the possibility to search within the database using fragments of the structure as parameter as well as using indexed tags, including bacterial source, keywords and bibliography. After beta-testing the database will be free available on the Internet.

O3

USE OF ARTIFICIAL NEURAL NETWORKS FOR THE ANALYSIS OF ^{13}C SPECTRA OF POLYSACCHARIDES

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The daily routine work of structure elucidation of polysaccharides is still one of the most important demands on chemists. Tools that assist sprectroscopists to elucidate the structure of organic molecules, or are even able to predict the structure of unknowns automatically, are therefore of general importance.

The use of artificial neural networks (NN) for this purpose in case of saccharides was suggested in work [1]. However, the common algorithm consists of predicting NMR spectra for hypothetical structures in order to obtain the best fit of the predicted and experimentally observed spectra. In our presentation we discuss the possibility of the direct structure elucidation by means of NN, using the characteristic combinations of signals in a spectrum.

The algorithm proposed in this work functions as follows: neural network simulator is employe to construct a simple feed-forward three layer NN. It is then trained to recognize characteristic sets of signals using the database of experimental NMR chemical shifts of oligosaccharides. The validation is performed by the elucidation of the structure of higher oligisaccharides and some sample polysaccharides.

In this work the applicability of this approach is shown on the example of ^{13}C spectra of several fucoidans – natural irregular polysaccharides.

[1]. J. Meiler, M. Will *J. Chem. Inf. Comput. Sci.* **2001**, 41, 1535-1546

O4

IS IT POSSIBLE TO PREDICT PATHWAY FOR BIOSYNTHESIS OF BACTERIAL POLYSACCHARIDES FROM THEIR STRUCTURE?

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Immence information is accumulated on chemical structure of bacterial polysaccharide chains, composed of repeating oligosaccharide units and located on bacterial cell surface, especially concerning O-specific chains of lipopolysaccharides and capsular polysaccharides. Extremely high structural variability of these polysaccharides is well established.

These polymers are located outside of bacterial cytoplasmic membrane but, in most cases, their biosynthesis occurs from intracellular glycosyl donors (sugar nucleotides) with participation of polyprenyl-linked intermediates. For O-specific polysaccharides, three different biosynthetic mechanisms were demonstrated: (a) “block” mechanism with assembly of the repeating unit inside the cell, followed by transfer of the unit through the membrane and formation of the polysaccharide chain on the outer side of the membrane; (b) “monomeric” mechanism with formation of the chain inside the cell by consecutive transfer of monosaccharide units followed by transport of the polymer through the membrane; (c) “synthase” mechanism when processive glycosyltransferase performs simultaneous chain elongation and transmembrane transport of the growing chain. Similar mechanisms seem to hold in most cases for capsular and exocellular bacterial polysaccharides, although definite evidence for participation of polyprenyl-linked intermediates was obtained only in the case (a).

The mechanism for biosynthesis of bacterial polysaccharides was established only in limited number of cases. Nevertheless, analysis of these examples shows that definite correlations seem to exist between the structure of the polysaccharides and the mechanism of their biosynthesis. On the basis of these correlations, empirical rules will be formulated to predict the biosynthetic mechanism and the structure of biological repeating units from the established chemical structure of polysaccharides. Application of these rules to different structures will be discussed.

The work was supported by RFBR (project 02-04-48794) and Grant for Support of Leading Scientific Schools of Russia (NSh-1557.2003.3).

O5

THE BIOSYNTHESIS OF URIDINE DIPHOSPHATE N-ACETYL-L-FUCOSAMINE IN CELL-FREE SYSTEM FROM *SALMONELLA ARIZONAE* O:59

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The conversion of uridine diphosphate N-acetyl-*D*-glucosamine into uridine diphosphate N-acetyl-*L*-fucosamine was demonstrated with enzymes from cytoplasmic fraction of *Salmonella arizonae* O:59 cells in the presence of NAD+ (NADP+) and NADPH.The reaction product was identified by ion-pair, reverse-phase HPLC with the use of synthetic nucleoside diphosphate sugar standards under conditions, specially developed for separation of uridine diphosphate-2-acetamido-2,6-dideoxyhexoses. *L*-Fucose dehydrogenase from porcine liver was shown to be applicable for determination of N-acetyl-*L*-fucosamine, this enzyme was used to confirm *L*-configuration of the amino sugar residue in the sugar nucleotide formed.

O6

ENDOGENIC LIPOPOLYSACCARIDE AS MOLECULAR CHAPERONE OF OUTER MEMBRANE NONSPECIFIC PORIN (EFFECT OF LIPOPOLYSACCHARIDE-PORIN INTERACTION ON PROTEIN CONFORMATION AND FUNCTIONAL ACTIVITY)

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Various folding catalysts are known to be implicated in the folding of some membrane proteins. However, when purified chaperone proteins were included in the refolding reaction *in vitro,* they had no effect on the refolding kinetics.

A specific interaction between lipopolysaccharide (LPS) and porin in the native bacterial membrane determines the outer membrane (OM) properties and is crucial for the life of the bacterial cell. Only interacting to LPS, porin oligomerizes and reconstitutes as a mature protein to the OM. However, it is rather the conformation of the mature porin that determines its insertion into the OM. Therefore, it is still unclear whether or not a prefolded by LPS protein intermediate really exists *in vivo*.

Changes in the structure and functional activity of the porin, resulting from the removal of LPS, normally bound with the protein were studied. According the data of SDS, PAGE, LPS-free porin retained a trimer. However, conformational changes in the spatial structure of the protein have been revealed using CD and UV spectroscopies and intrinsic protein fluorescence. LPS-free protein folded into a completely β-structured protein aggregate. The bilayer lipid technique showed that the pore-forming activity of the LPS-free porin decreased, and its concentration should be increased by two orders of magnitude to achieve the same effect. Incubation of the LPS-free porin with the LPS led to a porin-LPS complex and affected the character of the protein functional activity. The treatment of the LPS-free porin by octyl glucoside, a nonionic detergent, resulted in the restoration of the protein pore-forming activity. It was suggested that the LPS and detergent provide a definite protein conformation necessary for its function.

To elucidate the effect of LPS structure on the character and efficiency of LPS-protein complex formation, the interaction of the isolated porin with the S- and R-forms of endogenous LPS was studied by the quenching of intrinsic protein fluorescence. The samples of S-LPS differed both in the length of O-specific polysaccharide (number repeating units or polymerization degree, n= 1 and 4) and in the acylation degree (AD) of the 3-hydroxytetradecanoic acid residues of the lipid A moiety (12-66%). R-LPS binding to porin was found to occur with positive cooperativity on two integrated structural regions of the R-LPS macromolecule, namely, core oligosaccharide and lipid A. The mode of porin interaction to the with low-acylated S-LPSs (15 or 20%) coincided with a model involving three types of binding sites.The shape of Scatchard curves of binding indicates that a complex formation in the case of R-LPS and S-LPSs with low AD is cooperative. A significant increase (to 66%) in the degree of S-LPS acylation substantially changed its porin-binding character: the process become anti-cooperative with lowered affinity. Thus, the features of LPS-porin interaction significantly depend on the conformational changes in the LPS molecule due to expanding of its hydrophobic region.

The second part of the work devoted to revealing the chaperone activity of LPS on refolding recombinant porin. According the SDS, PAGE data, the addition of LPS in refolding reaction led to formation of porin trimer. The calculation of the secondary structure (from the CD data using the CONIN/LL-CONTIN method) indicated that porin gave values 4% α-helix, 41% β-sheet, 21% β-turn and 34 % random coil, that coincided with the content of secondary stricture

elemens of the porin, isolated by SDS extraction according Rosenbusch method. However, the tertiary structure of this sample of the renaturated porin determined by intrinsic protein fluorescence was similar to the structure of LPS-free porin, obtained by the treatment with 30% SDS: maxima at λ_{ex} =280 nm and λ_{ex} =296 nm are in 310 nm and 345 nm, respectively. Thus, most probably, *in vitro* renaturation of porin even in addition of LPS led to misfolded trimer of the protein with the spatial structure differ from the molecular organization of the isolated porin.

O7

THE REACTION OF *HELICOBACTER PYLORI* AND *PROTEUS MIRABILIS* LIPOPOLYSACCHARIDES WITH HUMAN COMPLEMENT PROTEINS

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Gram-negative *Helicobacter pylori* have been reported in human chronic gastritis and cancer development and *Proteus mirabilis* rods are considered as one of the bacteria that cause the urinary tract infections (UTI), respectively. The outer membrane lipopolisaccharide (LPS), being important pathogencity factor of *Helicobacter pylori* and *Protues mirabili*s strains. The aims of ours studies were to test the human factor C3 complement deposition by chemically defined *H. pylori* and *Proteus mirabilis* LPSs.

The *H. pylori* LPSs, that differs on their Lewis X and Lewis Y epitopes expression as well as on the polysaccharide content were used. LPSs were from strains GU2, CA2 and CG10 isolated, from patients with gastric ulcer, gastric cancer and chronic gastritis, respectively. GU2 LPS expresses Le^x, Ly^y and La^a. CA LPS expresses Le^y on the polysaccharide chains.

Only rough *H. pylori* CG 10 LPS and whole CG10 cells deposited in dose-dependent manner human C3. It may indicate on the role of polysaccharide part of *H.pylori* LPS on inhibition of complement activation and C3 deposition.

The human complement activation might be strongly enhanced by presence of anti-*H.pylori* antibodies. The presence of anti-*H.pylori* antibodies in patients sera were tested with the aims of series synthetic peptides. By this method the antibodies reacting with *H.pylori* urease epitopes were detected.

Similarly to *H.pylori* LPSs the *.P. mirabilis* endotoxins deposited factor C3 complement depends on the presence of O-specific polysaccharide chains of LPS. The *P. mirabilis* R45 (deep rough Re type mutant) LPS deposited C3 molecules on the antibody-independent classical pathway. The R110 (Ra type mutant) and S1959 (smooth form) LPSs fixed the C3 molecules by alternative complement activation pathway.

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O8

THE COMPLEXES OF LPS WITH POLYSACCHARIDES. THE MODIFICATION OF BIOLOGICAL PROPERTIES OF LPS BY MARINE POLYSACCHARIDES.

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Bacterial lipopolysaccharides (LPS) or endotoxins are well known as important contributing factors to the pathogenesis of gram-negative infections. Endotoxins act on vascular cell, macrophages and other cells, producing a variety of mediators, including intereukine (IL) and tumor necrosis factor – alpha (TNF). The present paper reveals effect of marine polysaccharide – chitosan and carraggenans - upon some biological properties of LPS from *Y. pseudotuberculosis* and *E. coli*.

Chitosan (β-1,4-glucosaminoglycan) proved to be a widespread natural polycationite compound. Its application as constituent of various biochemical specimens is connected with availability and biological resistance. Chitosan with molecular mass of 140 kDa (Ch-HM) was obtained by alkaline treatment of crab shell chitin and chitosan with molecular mass of 20 kDa (Ch-LM) was obtained by further depolymerization of the chitosan.

Carrageenans - are sulfated galactans containing D-galactose and its derivatives, the residues of which are connected by regularly alternating β1-4 and α1-3 bonds. Carrageenans are used extensively in food and pharmaceutical industries. Carrageenans – kappa - and lambda –type were isolated from red algae *Chondrus armatus*, collected from the Russian Pacific coast .

Interaction of bacterial endotoxins with chitosan was investigated using sedimentation analysis, isopicnic centrifugation, spectroscopy, fluorescence and electronic microscopic. The mechanism of binding of lipopolysaccharide to chitosan was shown to be a multistage process that depended on time and reaction temperature, the macromolecular organization of endotoxin as well as on the degree of polymerization of the chitosan. Reorganization of endotoxin macromolecular structure during binding of LPS with chitosan was observed. It should be noted that process of LPS complexation with chitosan is accompanied by additional disaggregating of LPS. The effect of ionic strength on the stability of formed complexes was studied. The complex formation was shown to be a result not only of ionic binding, but also of other types of interactions.

LPS with chitosans and carrageenans were found to shown a considerably lower toxicity in a comparison with the parent LPS. The toxicity of LPS from *Y. pseudotuberculosis* as well as LPS from *E.coli* decrease tenfold and 20-fold respectively, after forming a complexes with Ch-HM. Complexing with Ch-LM results in five-fold and tenfold decrease in toxicity, respectively. The toxic effects of endotoxin of gram-negative bacterium have been abrogated by carrageenans. Pretreatment of mice with carrageenan resulted in 20-40% survival after injected of high toxic dose of endotoxin and 50-100% at low doze of endotoxin .

It is know, that LPS increase of platelet aggregation The action of chitosan and carrageenan in comparison with LPS on the aggregation of platelets was studied in experiments *in vitro.* The platelet aggregation with LPS was found to increase by an average of 11% in comparison with control tests while the Ch-HM with LPS was shown to decrease the aggregation ability of platelets by an average of 11% and carrageenan kappa- an 38% and lambda- 34%, reverse of LPS.

O9

IMMUNOMODULATING ACTIVITY OF POLYSACCHARIDES FROM MARINE FUNGUS *PHOMA GLOMERATA*

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At wide research of terrestrial fungi polysaccharides the marine fungi have not been studied as producers of these bioactive polymers. Now we study polysaccharides from marine fungus *Phoma glomerata*.

The glucan (PS-1) and glucomannogalactan (PS-2) were isolated from cells at exponential growth stage, PS-2 was composed from Glc, Man and Gal (50:25:25, in mole). PS-1 and PS-2 stimulate generation of reactive oxygen species (ROS) in mice myelomonocyte cells «Wehi-3» more than 2 times at concentration 0.1-1 μg/ml. Moreover PS-1 and PS-2 increase nitric oxide synthase (NOS) activity of these cells in 2.5-3 times at same concentration. PS-1 enhances synthesis of tumor necroses factor (TNF-α) by human peripheral mononuclear cells in 20 times, and PS-2 more then 50 times.

Thus the marine fungus *Phoma glomerata* synthesizes high immunostimulating polysaccharides which may be used as producer of promise pharmacological preparations.

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O10

GLYCOPHORIN A OF HUMAN ERYTHROCYTE MEMBRANE IS RECOGNIZED BY ANTI-*CITROBACTER BRAAKII* O37 ANTIBODIES AS A SECONDARY EPITOPE

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Molecular mimicry is defined as a structural similarity of microbial molecules to host tissue contributing to the pathogenicity. This phenomenon was found for *Citrobacter braakii* O37 which shared epitopes with human and horse erythrocytes (Gamian *et al* 1992). Band 3 and glycophorin A, the major glycoproteins of human erythrocyte membranes, were expected to carry the common epitope. Band 3 was strongly recognized by the anti-*C. braakii* O37 antibodies purified on LPS affinity column (Ebaid 2002). The present study aimed to investigate the role of GPA in this molecular mimicry. Immunochemical methods like as immunoblotting, ELISA, inhibition of hemagglutination and affinity columns were employed. The further investigation of the epitopes on the erythrocyte membrane showed that GPA when immobilized in an affinity column could purify specific antibodies (GPA-Abs) from the whole anti-*C. braakii* O37 serum. The purified antibodies in turn, recognized GPA in immunoblotting test. Treatment of human erythrocytes with sialidase significantly improved the agglutination titer. Furthermore, these antibodies agglutinated both sialidase-treated human and sheep erythrocytes. The agglutination was more inhibited by asialo-GPA than the native one. GPA^{M} and GPA^{N} could similarly inhibit the agglutination, however, the most significant inhibition was recorded by the GPA^{MN}. GPA-Abs could not recognize the LPS. Results of this work confirm that some antibodies in the anti-*C. braakii* O37 serum recognized GPA, however, did not show any reactivity with LPS, what means that it may be produced against the OMP of the bacteria.

Gamian A., Romanowska A., Romanowska E (1992) Immunochemical studies on sialic acid-containing lipopolysaccharides from enterobacterial species. FEMS Microbiol. Immunol. 89, 1992, 323-328

Ebaid H. (2002) Investigation of the serological reactivity of the antibodies produced against Citrobacter braakii O37 with human and horse erythrocytes. PhD. Institute of Immunology and Experimental Therapy, PAN, Wroclaw, Poland

O11
PARTIAL STRUCTURE OF EXOPOLYSACCHARIDE OF *MESORHIZOBIUM LOTI* NZP 2213

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High molecular weight exopolysaccharide synthesized in large amounts by *Mesorhizobium loti* strain NZP 2213 contains glucosyl, galactosyl, riburonyl and glucuronyl residues. The acidic exopolysaccharide is depolimerized by the bacteriophage A1 induced polysaccharase (endogalactosidase) to oligosaccharide fragments containing galactosyl residue in their reducing end.

Oligosaccharides represented mainly by octa- and nonasaccharide molecules were composed of Glc, Gal, RibA and HexA in molar ratios 5:1:1:1: and 5:1:1:2 respectively as was proved by MALDI-TOF and ^{1}H NMR spectra. Methylation analyses of the exopolysaccharide, oligosaccharide fractions and as well as periodate oxidized exopolysaccharide considered in conjunction with data of ^{1}H and ^{13}C NMR experiments showed that high molecular polymer is composed of repeating units having structure:

→4)βGlc(1→4)Glc(1→4)Glc(1→3)Gal(1→
6
↑
1

βRibA[(1→4)GlcA]$_n$(1→4)Glc(1→6)Glc   n = 1 or 2

O12

FURTHER STRUCTURAL STUDIES ON *HAFNIA* AND *CITROBACTER* O-ANTIGENS

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Bacteria belonging to the genera *Hafnia* and *Citrobacter* are typical members of *Enterobacteriaceae* family and are commonly distributed in natural environment such as soil, water and dairy products. They are also opportunistic pathogens causing typical nosocomial infections including wounds and enteric, urinary and respiratory tract disorders. Some incidents of bacteremias, meningitis, brain abscesses and sepses, caused by *Citrobacter* strains, have also been reported. The genus *Hafnia* contains only one single species – *Hafnia alvei* and according to the serological classification is divided into 39 O-serotypes. First chemical characterization of *Hafnia alvei* lipopolysaccharides from 33 bacterial strains was reported in 1988.[1] *Citrobacter* strains are ascribed to 43 O- serogroups and divided into 20 chemotypes [2]. In 1993 the classification of *Citrobacter* was revised basing on the results of the genetic studies and 11 different genomospecies were distinguished (*C .youngae, C. braakii, C. werkmanii, C. freundii, C. farmeri, C. amalonaticus, C. sedlakii, C. rodentium, C. gillenii and C. murliniae*).
Up to date the structures of 30 different *Hafnia alvei* O antigens [3-8] and more than 25 *Citrobacter* O-polysaccharides [9] have been established. The structural and serological studies on *Citrobacter* and *Hafnia* O-specific polysaccharides are carried out to elucidate the chemical basis for their serological diversity and the mechanisms of close interrelationships with other bacterial strains. These studies can help to improve the existing classification of the genus *Citrobacter*, to determine the structures of the O-antigens from the serogroups non-examined yet and to establish the taxonomic position for the strains wrongly classified. The knowledge on the structures of O-antigens from different bacterial endotoxins (LPSs) can facilitate the construction of the universal antibacterial vaccine.
We report now on the new structures of *Citrobacter* O-antigens and on the results of our recent studies concerning the biological repeating units in *Hafnia alvei* O-polysaccharides.
For the majority of enterobacterial O-antigens examined so far the structures of so-called chemical repeating units have been determined. The structures of the biological repeating units in ten *H. alvei* O-antigens have been elucidated basing on the results of methylation analysis and ^{1}H and ^{13}C NMR spectroscopy carried out on the core fractions substituted with one repeating unit. It was established that in all oligosaccharides examined it is 3-substituted β-D-GlcNAc or 3-substituted β-D-GalNAc which forms reducing end of OPS unit linked to the the core oligosaccharide. There are three strains (PCM 1199, 1205 and 1185) in which the β-configuration of D-GlcNAc in the first repeating unit connected to the core is opposite to that found in all the following OPS repeating units. At the non-reducing termini of the biological repeating units D-Qui3NAcyl (strains 1216, 1185), D-Qui3NFo (1204), D-Qui4NAc (1205 and 1199), D-Glc (1211, 1189, 1546) D-GlcA (1188) or D-Gal (1196) have been found. The results of methylation analyses of unsubstituted core fractions show that LPSs from all strains examined have the typical for *Hafnia alvei* core region containing in the hexose part α-D-Glc*p*-(1→3)-α-D-Glc*p* or α-D-Gal*p*-(1→3)-α-D-Glc*p* disaccharide.
The knowledge on the structures of the biological repeating units in bacterial O-antigens is very important from the biological point of view, it can lead to the elucidation of the OPS

biosynthesis pathways and understanding on the molecular level the immunospecificity of the bacterial strain defined by a non-reducing end sugar of the O-polysaccharides.

The new O-antigen structures were established in *Citrobacter* strains PCM 1555 [O22], PCM 1443 [O39] and PCM 1505 [O6]. It was found that *Citrobacter* O22 antigen contains tetrasaccharide repeating units with side-chain α-abequose and resembles the O-antigen of *Salmonella* group B. *Citrobacter* O39 LPS contains O-polysaccharide consisting of two different galactans and is chemically and serologically related to *Klebsiella* O1 LPS.

The structures of the O-specific polysaccharides from the strains PCM 1504 [O6] and PCM 1573 [O2] were found to be identical with those established earlier for *Citrobacte*r O3 and O8 antigens. *Citrobacter* strains PCM 1504 and PCM 1573 should be moved from serogroups O6 and O2 to serogroups O3 and O8, respectively. Basing on the results of chemical and serological studies on *Citrobacter* lipopolysaccharides it can be concluded that the classification of the genus *Citrobacter* will need revision.

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O13

STRUCTURAL STUDIES OF ENDOTOXIN FROM PLESIOMONAS SHIGELLOIDES O37 (CNCTC 39/89)

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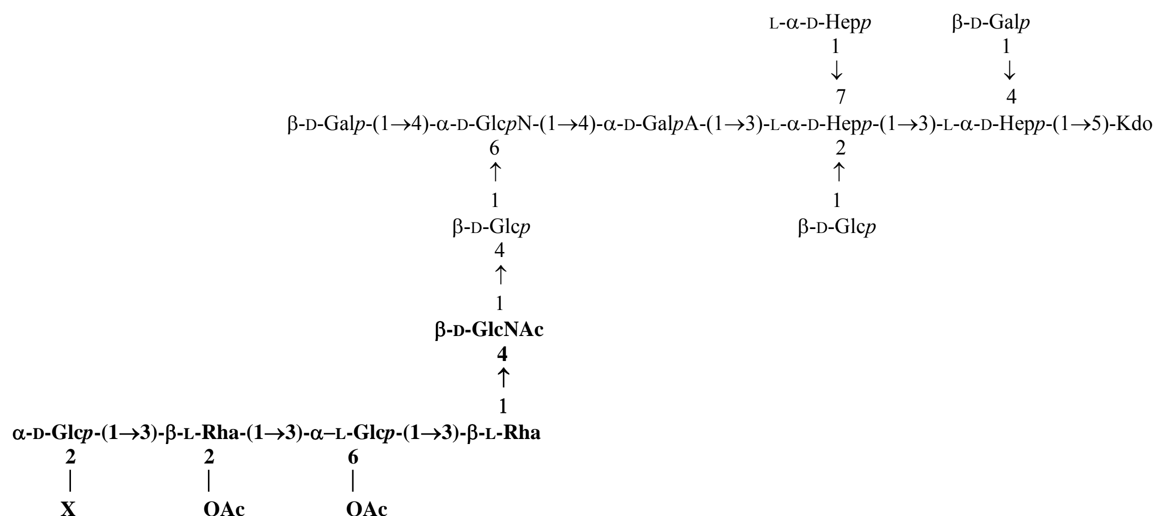
The structure of the lipopolysaccharide of *Plesiomomonas shigelloides* O37 (CNCTC 39/89) has been investigated by the ^{1}H and ^{13}C NMR, matrix-associated laser-description/ionisation time of flight MS, sugar and methylation analysis. It was concluted that the main core oligosaccharide and lipid A of this strain are identical as in *Plesiomonas shigelloides* O54 (CNCTC 113/92). The core oligosaccharide which is composed of a decasaccharide is substituted at C-4 of the outer core β–D-Glc*p* residue with a *O*-polysaccharide repeating unit –β-D-*Glcp*NAc. The *O*-polysaccharide is composed of the hexasaccharide. The polysaccharide of this strain has a following structure:



in which **X** is **3-deoxy-2-hydroxy-4-keto valeric acid**.

O14

LIPOPOLYSACCHARIDES OF *AZOSPIRILLA* – STRUCTURE AND INVOLVEMENT IN INTERACTION WITH THE ROOTS OF WHEAT

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The gram-negative bacteria of the genus *Azospirillum* are an intensively studied associative partner of a wide range of plants growing in diverse climatic zones. Interest in the surface polysaccharides of azospirilla is due to the important role of these macromolecules in bacterial competition in soil and also to their involvement in the key stages of the associations.
This talk will summarize recent data from studies of the structure of the O-specific polysaccharides from associative nitrogen-fixing rhizobacteria of the genus *Azospirillum*. Data will be presented to elucidate the role of *Azospirillum* lipopolysaccharides in plant-microbial interactions with the example of the endophytic strain *A. brasilense* Sp245 and wheat seedlings

O15

STRUCTURES AND SEROLOGICAL SPECIFICITY OF THE O-ANTIGENS OF *PROVIDENCIA* SP

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The genus Providencia consists 5 species: P. alcalifaciens, P. rustigianii, P. stuartii, P. retgerri and P. heimbachae. They are Gram-negative bacilli classified in Enterobacteriaceae family. P. alcalifaciens, P. rustigianii, P. stuartii, P. retgerri are recognized as facultative pathogens that under favourable conditions cause enteric disease, wound and urinary tract infections. P. alcalifaciens and P. rustigianii cause diarrhea in children, as well as diarrhea in adults who traveled abroad. P. stuartii has been recognized as a pathogen with an increasing involvement in urinary tract infections, primarily in nursing home patients with long term indwelling urinary catheters. P. rettgerii was also recognized as pathogen which causes UTI, it also causes nosocomial infections.

The serological classification scheme of Providencia strains used in serotyping of clinical isolates is based on the lipoplysaccharide (LPS) flagella (H-antigen) and capsular (K) antigen. The antigen scheme for the Providencia group has developed in 1954 Ewing et al. From a collection of 631 cultures they defined 56 somatic O-antigens, 28 types of flagella and 2 capsular antigens. Later, six more O-antigens were defined and the scheme was extended to include 62 O-antigens. The original scheme of Ewing was reconstituted by Penner et al. in 1976. This scheme includes 62 serogroups: 17 P. stuartii and 46 . alcalifaciens, as well as P. rustigianii, which formerly has belonged to P. alcalifaciens as biogroup 3. Later, another O-specificity was defined in strain untypable in 62 different O-antisera and was added to the scheme as O63.

The aim of our study is to create molecular basis for the Ewing's serological classification scheme. P. alcalifaciens, P. rustigianii and P. stuartii come from the Hungarian National Collection of Medical Bacteria. Lipoplysaccharides were isolated according to the method of Westphal and purified by treatment with trichloroacetic acid at pH 2.5-3.0 followed by separation of a precipitate and dialysis of the supernatant against distilled water. A high molecular mass PS was prepared by degradation of LPS with 2% acetic acid and fractionation by gel chromatography on a column of Sephadex G50. The OPS was hydrolyze with 2M trifluorocaetic acid. Chemical composition and structural studies were performed by use of Gas-Liquid Chromatography, methylation analysis and NMR spectroscopy. The serological studies was done by use of polyclonal O-antisera obtained after immunization of rabbit white heat killed bacterial strains, representing the particular serogroup. In this study passive hemolysis with alkali-treated LPS and ELISA with LPS as antigens, as well as inhibition of these test and SDS-PAGE and Western blot were use.

Until now the O-specific Providencia polysaccharides of the following serogroups O4, O5, O7, O14, O16, O18, O19, O21, O23, O33, O34, O35, O43, O47, O49 and O57 were studied. In some OPSs rare compounds were found, such as 3-acetamido-3,6-dideoxyglucose (Quin3Nac - O5 and O18), N-acetylmuramic acid (O16), 3-acetamido-3,6-dideoxygalactose (Fuc3NAc - O19), 3-formamido-3,6-dideoxy-D-galactose (Fuc3NFo – O21), 4-amino-4,6-dideoxy-D-glucose substituted by L-or D-aspartic acid (Qui4N[Ac-L/D-Asp] - O4 and O33), alaninolysine - AlaLys (O14 and O23). The epitope specificity of antisera against Providencia O18, O19, O21, as well as O4 and O33, O14 and O23 will be discussed.

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O16
GLYCOCONJUGATE VACCINES AGAINST BACTERIAL PATHOGENS

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Glycoconjugate vaccines, in which a cell surface carbohydrate from a bacterial or fungal pathogen is covalently attached to an appropriate carrier protein, have proven to be the most effective means to elicit protective immunity against a wide range of pathogens. Vaccines against *Haemophilus influenzae* type b (Hib), against *Neisseria meningitidis* Group C (MenC) and against multiple *Streptococcus pneumoniae* serotypes have been licensed, and many similar conjugates are being developed. These vaccines induce high levels of high avidity, complement activating antibodies, induce immunological memory and, crucially, are immunogenic in infants. The greater efficacy of glycoconjugate vaccines compared to older, purified polysaccharide vaccines arises from the different molecular mechanism by which they provoke an immune response. In addition, by exploiting a different immune mechanism, it is possible to produce glycoconjugate vaccines against saccharide structures which would not otherwise be immunogenic. This is particularly important as it allows protective immune reposnses against relatively small glycans, including lipopolysaccharide (LPS) O-chains and lipo-oligosaccharides (LOS) to be induced. Whilst the specificity of the immune response is defined by the structure of the glycan, it may be possible to target responses by choice of carrier protein, or to boost immunogenicity by optimal choice of carrier.
Three basic structural types of glycoconjugate vaccine exist, which can be referred to as "neoglycoconjugates", "cross-linked matrix" or "vesicle vaccines". These types differ in molecular weight, glycan size and the degree of crosslinking between the glycan and the carrier protein, and are produced using different combinations of activation and conjugation chemistry. The principal factor defining which of these basic types is produced as a product is the structure of the glycan. This aspect will be discussed in the talk.
Due to their relative simplicity and the lack of appropriate animal models for the Hib vaccines, glycoconjugate vaccines have been subjected to an unprecedented degree of physicochemical characterisation. NMR spectroscopy provides a means to probe the integrity of the glycan chains and the conditions under which they degrade, whilst optical spectroscopy reports on protein integrity. Size exclusion chromatography, sometimes coupled to MALLS detection, also plays an important role in conjugate characterisation. In turn, it is now becoming which factors are most important for vaccine efficacy. Quality control of these vaccines depends on such approaches, which demonstrate structural consistency between batches of products rather than consistent biological activity.
Whilst the bacterial pathogens against which glycoconjugate vaccines protect are particular problems in developing countries, these products are relative expensive compared to most vaccines used in mass immunisation programmes. This limits their availability to those countries which most need them, and limits the commercial development of novel vaccines. Existing vaccine prices, and target prices for some novel vaccines, will be discussed briefly. There is a clear need for product development to be funded outside of commercial constraints and for new, high production, low cost manufacturers to enter the market if the benefits of these vaccines are to be made available to all who need them.
The scientific literature contains many reports of the early development and pre-clinical and clinical trials of novel glycoconjugate vaccines, produced from capsular polysaccharides, LPS O-chains and LOSs. These will be listed and briefly discussed, but represent only a small fraction of the opportunities which exist.

O17

APPROACHES TO THE SYNTHESIS OF CANDIDATE GLYCOCONJUGATE VACCINES AGAINST BACTERIAL AND FUNGI INFECTIONS

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Carbohydrate structures, in the form of capsular polysaccharides (CPSs) or lipopolysaccharides (LPSs), are important surface antigens of bacteria and accordingly of interest for serotyping of and as vaccines against bacteria. The successful introduction of glycoconjugate vaccines, i.e. saccharide structures conjugated to a carrier protein, has dramatically increased the interest in this type of vaccines. There are now three efficacious commercial glycoconjugate vaccines, against *Haemophilus influenzae* type b (Hib), *Neisseria menigitidis* type c and *Streptococcus pneumoniae* (seven serogroups), all based on partly hydrolyzed native capsular polysaccharide structures. However, it is sometimes most difficult to use native bacterial polysaccharides due to, e.g., heterogeneity, instability, toxicity or molecular mimicry of these structures. An interesting alternative is then synthetic part structures or analogues. Owing to the fast progress in oligosaccharide synthesis during the last years the synthesis of these often most complex structures has become feasible. For the Hib vaccine there is now already a commercial vaccine based on chemically synthesized oligosaccharide structures. We will present our approach towards glycoconjugate vaccine candidates against infections caused by the fungi *Cryptococcus neoformans*, a major cause of death in AIDS patients. Chemical syntheses of CPS structures will be presented, as well as their conjugation to carrier proteins and use in immunization experiments.

O18

LIPOPOLYSACCHARIDES OF *HELICOBACTER* SPP.: CHARACTERISTICS, PATHOGENIC ROLES AND IMMUNODIAGNOSTIC POTENTIAL

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Of the genus *Helicobacter*, the lipopolysaccharides (LPSs) of *H. pylori* have been the most intensively studied, particularly as virulence factors. This bacterium is a prevalent human gastroduodenal pathogen – a causal agent of chronic gastritis and peptic ulcers and an important co-factor in gastric cancer development. *H. pylori* LPS displays low inflammatory and immunological activities which contributes to the ability of the bacterium to chronically colonize the gastric mucosa. These low activities have been attributed largely to its unique lipid A structure (predominantly, a tetra-acyl variety with long acyl chains and underphosohorylation) which influences the interaction of LPS with serum proteins (e.g., LPS-binding protein and CD14) and cell surface receptors. On the other hand, the precise mechanisms by which *H. pylori* induces inflammatory responses in the mucosa that contribute to disease development are only now becoming clear. Importantly, although Toll-like receptor-(TLR-) 2 is a dominant innate immune receptor for recognition of *H. pylori* and other *Helicobacter* spp., *H. pylori* LPS has recently been shown to be solely a TLR-4 agonist of low activity. Moreover, products of certain genes of the *H. pylori cag* pathogenicity island (PAI), which encodes a type IV secretion system and is associated with severe inflammation, may modulate the important TLR-2 activity of *H. pylori*. Furthermore, NOD1 – a cytosolic pathogen recognition molecule – can mediate pro-inflammatory responses to *H. pylori* by recognition of peptidoglycan delivered to host cells by the *cag* PAI-encoded secretion apparatus.

Particularly of relevance to duodenal ulcer-associated strains, core oligosaccharide structures of LPS have been implicated in induction of pepsinogen and laminin interaction. Nevertheless, serological studies have shown that, compared to enteric bacteria, there are epitopes in the core oligosaccharide that are conserved and unique to *H. pylori* LPS, which may represent candidate epitopes for immunodiagnostic or vaccine development.

Most attention has focused on the O-polysaccharide chains of *H. pylori* LPSs that exhibit mimicry of Lewis (Le) antigens. Structural studies and serological investigations have shown that the O-chains of *H. pylori* strains express partially fucosylated, glucosylated , or galactosylated *N*-acetyllactsamine (LacNAc) chains of various lengths that may or may not be terminated at the non-reducing end by Le^x and Le^y units, in mimicry of human cell surface glycoconjugates. In addition, although less widespread, certain strains express fucosylated LacNAc chains terminated with Le^a, Le^b, sialyl-Le^x and blood group A determinant. The mimicry of Le^x and Le^y has been debated to influence bacterial colonization by (i) Le^x/Le^y aiding camouflage in the gastric mucosa and (ii) Le^x acting as a bacterial adhesin. More controversially, LPS-expressed Le^x/Le^y have been implicated in the development of gastric autoimmunity by inducing autoreactive antibodies against the gastric proton pump, thereby of relevance to gastric atrophy, a precursor state in gastric cancer development. From a practical aspect, knowledge of the glycosylation in *H. pylori* strains has allowed the formulation of a lectin typing system for strain differentiation which has been applied successfully in a number of geographical populations.

Comparative analyses have been performed on the LPSs of other gastric helicobacters, e.g., *H. felis*, *H. canis*, *H. mustelae* (used in vaccine and animal studies) and *H. bizzozeronii* (an emerging zoonotic cause of human gastritis). Studies have also included enterohepatic helicobacters such as *H. bilis* and *H. hepaticus* (causing hepatitis and cholecystitis), *H.*

pullorum (implicated in human gastroenteritis), *H. rappini* (associated with chronic diarrhoea and bacteraemia). *H. mustelae* and *H. hepaticus* produce low-, whereas the other helicobacters (like *H. pylori)* produce high-molecular-mass LPS. Also, unlike *H. pylori* (expressing Le antigens) and *H. mustelae* (expressing blood group A), no similar mimicry was observed in the other LPSs. A general absence of serological cross-reactivity with the core of *H. pylori* LPS was observed indicating the occurrence of different cores which could be exploited in immunodiagnostics. Moreover, detailed chemical analysis of the LPSs revealed the occurrence of novel sugars and distinctive compositions which may prove of diagnostic value.

O19

THE LIPOPOLYSACCHARIDE OF A *PLESIOMONAS SHIGELLOIDES*

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The structure of the complete lipopolysaccharide of *Plesiomonas shigelloides* strain CNTC144/92 serogroup O75:H5 has been investigated. The analysis involved structural studies of the O-antigen, its biological repeating unit, the core oligosaccharide, the linkage between O-polysaccharide and core and the lipid A part. The presentation will emphasize on the methods and procedures used in order to obtain the structures of the different structural elements.

O20

GLYCOLIPIDS OF *ACTINOBACTERIA:* THE DIAGNOSTIC TOOL IN OPPORTUNISTIC INFECTIONS

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Actinobacteria class is a large group of gram-positive bacteria of diverse morphological properties and complex cell wall. These microorganisms are considered as clinically important because of their growing potential in infections, often connected with immune system dysfunctions.

Amphipatic polar lipids of *Actinobacteria* are essential structural constituents of cell plasma membranes, they have been also found as specific major lipids of bacterial cell envelopes. Two general structures of bacterial glycolipids are known: glycosyl glycerides and acylated sugars. Both are used in bacterial systematic as useful chemotaxonomic markers, i.e. major glycolipids from *Nocardiopsis dassonvillei* were applied for rapid recognition of actinomycetal pulmonary infection.

The structure of major glycolipids and their localisation within the cell envelope were established for *Saccharopolysopora*, *Rothia*, *Nocardiopsis* as well as *Propionibacterium* genera.

O21

GLYCOSIDIC DRUGS – HINTS FOR MEDICINAL CHEMISTS (?)

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Recognition of carbohydrates as information-rich molecules has recently initiated a new field of studies – glycobiology, dedicated to activities of functional biopolymers, which involve specific interactions of oligosaccharides or polysaccharides. There is also a considerable interest in role of sugars in biologically active small molecules containing a carbohydrate moiety, since many drugs of natural origin belong to this category. Remarkably, relatively few synthetic drugs have been designed *de novo* based on glycosidic motif as a part of pharmacophore.

Survey of glycosidic natural products, which are applied as human medicines or are being studied as drug candidates, lead to conclusion that the role of sugar moiety in biological activity of low molecular weight glycoconjugates can differ considerably from case to case. Extensive studies on structural variations of sugar residues in antibiotics serve as a good example, how far one can get from prototype natural compound without loosing activity. Flavonoid glycosides, glycolipids and saponins are less studied in this respect, moreover unlike in case of antibiotics, their aglycones are known as active principles for many biological targets.

Plant polyphenols, abundant in human diet, occur in their native biological matrix exclusively as glycosides. Corresponding aglycons, which are known to exert a number of health-related effects in mammalian physiology (estrogenic, antioxidant, etc.) undergo *in vivo* rapid, extensive metabolism, facilitating their clearance.

We have demonstrated in our recent research, that synthetically modified phenolic glycosides differ dramatically from their natural counterparts in activity, indicating a new approach for enhancement of selectivity and efficacy in some categories of medicinally useful natural products.

O22

ANTIBIOTIC AMINOSUGARS L-DAUNOSAMINE AND L-ACOSAMINE INCREASE TOPOISOMERASE II INHIBITION ABILITY OF DERIVATIVES OF 6,11–DIMETHYL–6H–INDOLO[2,3–B]QUINOLINE

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O23
SYNTHESIS OF COMPLEX GLYCOFURANOSIDES

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Glycoconjugates are one of the most functionally and structurally diverse molecules in nature; it is now well established that protein- and lipid-bounded saccharides play essential role in many molecular processes in living organisms. The notion that carbohydrates are biologically important molecules and that many human diseases are associated with alrerations of cellular carbohydrates resulted in great interest in synthetic carbohydrate chemistry, especially in glycosylation reaction. There is number of effective methods of chemical synthesis of glycopyranosides.Remarkably, relative few methods of the synthesis of furanosides have been developed.In our program synthesis of complex derivatives of natural biologically active compounds we focused our attention on glycofuranosides as a drug carriers, due to its stability in human tissues.
We present the simple synthesis of benzyl-protected glycofuranoses, starting metarial for preparation of glycosyl donors. The method of synthesis of glycofuranosyl thioglycosides will be described. Condensation of glycosyl donors , prepared, under the agency of thiophilic compounds proceeds with high yields ond 1,2-cis stereoselectivity.
The mechanism of the glycosylation will be presented.

O24
RHIZOBIAL LIPIDS A

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Lipopolysaccharides (LPS) are components of the outer leaflet of the outer membranes of Gram-negative bacteria. Those glycoconjugates have a common general architecture. They contain three distinct regions: lipid A, a non-repeating oligosaccharide core, and an O-polysaccharide composed of a varying number of repeating units. Lipid A moiety anchors molecules of LPS into the outer leaflet of bacterial cell. Lipid A is responsible for the endotoxic properties of lipopolysaccharide. The structure of lipid A seems to be essential in maintaining outer membrane integrity and its flexibility and is crucial for bacterial cell viability.
Lipids A in many Gram-negative bacteria (especially in animal pathogens) have a conserved structure. Their backbones are composed of a β-1,6-D-glucosamine disaccharide with two phosphate residues attached at positions 1 and 4'. Up to four fatty acids are bound by ester or amide linkages to the backbone glucosamines.
Rhizobium lipids A indicate great variation in the glycosyl components of backbones as well as in the acylation patterns. The lipid A backbone of *Sinorhizobium* is similar to that from enteric bacteria. The lipids A from *R. etli* and biovars of *R. leguminosarum* are identical and have unusual structures. Those lipids are devoid of phosphate groups: (1) a galacturonic acid residue replaced the 4'-linked phosphate in the lipid A backbone, (2) the reducing end glucosamine is partly oxidised to 2-aminogluconate. Moreover, a specific deacylase removes the ester-linked fatty acids from the C-3 position of the lipid A precursor, thus this hydroxyl is only partially substituted by acyl residue in the matured lipid A. The symbiont of *Sesbsnia*, *Rhizobium* sp. Sin-1 (the bacterium closely related to *R. galegaea*), has lipid A composed of β-D-glucosamine attached to 2-aminogluconate by β-1,6 glycoside linkage. When compared with *R. etli* this lipid A lacks galacturonic acid at position 4'.
Lipid A isolated from *Mesorhizobium huakuii* belongs to DAG type lipid A (i.e. lipid A containing 2,3-diamino-2,3-dideoxyglucose). Its backbone is double decorated: (1) nonstoichiometrically, with phosphate at 4' position of the distal DAG, and (2) with α-linked galacturonic acid at position 1 of the proximal unit. Phosphorylated and non-phosphorylated lipid A preparations are a mixture of three subfractions differing in acylation patterns.
In contrast to the above mentioned lipid A structures, the mesorhizobial (except for *M. huakuii*) and bradyrhizobial lipids A have not been fully chemically characterised to date. *Bradyrhizobium* lipid A backbones are composed exclusively of 2,3-diamino-2,3-dideoxyglucose with mannose as a subsituent in some of them. *Mesorhizobium loti* lipids A contain DAG and phosphate residues. *Mesorhizobium* lipids A are known to carry a number of β-hydroxyl fatty acids accompanied by small amounts of 4-oxo fatty acids. Numerous ester linked non-polar and (ω-1) hydroxyl as well as (ω-1) oxo long chain fatty residues were found in those preparations.
No data about *Allorhizobium* (presently reclassified to *Rhizobium*) and scanty information about *Azorhizobium* lipopolysaccharides and lipids A are available.
At present, lipid A from *Azorhizobium caulinodans* is investigated in our laboratory. The lipopolysaccharide is important in the process of symbiotic interaction between *Rhizobium* and the host plant. The environmental conditions of life (*in planta* and *ex planta*) as well as plant derived molecular signals induce entire LPS (including lipid A) modifications in *Rhizobium*.

O25

STRUCTURAL ANALYSIS OF *HAFNIA ALVEI* 32 LIPID A MOLECULE USING MALDI-TOF AND ESI MASS SPECTROMETRY

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Biological activity of endotoxins depends mainly on lipid A structure and is modulated by the core oligosaccharide and O-specific chain. Chemical analysis of endotoxins, especially lipid A part, is an important step in the studies on the biological activity-structure relationship. *Hafnia alvei*, a member of *Enterobacteriaceae*, is a rather rare but important opportunistic pathogen, which causes nosocomial infections. Recently, the structures of the O-specific polysaccharides from a number of serologically different *H. alvei* strains have been elucidated [1-7]. In contrary to the intensive structural analyses of O-specific chains and core oligosaccharides of *H. alvei* LPS, lipid A part of these LPS seems to be poorly characterised. As previously shown, it is possible to determine distribution of fatty acids in lipid A by combining data from negative and positive ion mode MS analysis and considering rules of fragmentation dependent on the ionisation technique [8-11]. The strategy based on ESI MS^n and MALDI-TOF MS was used in structural analysis of intact lipid A isolated from *H. alvei* 32. Results of these studies showed that lipid A of *H. alvei* 32 LPS is built of glucosamine backbone substituted with two phosphate groups at 1 and 4’ positions. The disaccharide backbone is substituted by 14:0(3-OH) at positions 2 and 3. Positions 2’ and 3’ are substituted by 14:0(3-O-12:0) and 14:0(3-O-14:0) respectively. Comparative analysis using ESI-MS was also done for three other strains of *H. alvei* (PCM 1192, 1206 and 1207).

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O26

UNCOMMON *ESCHERICHIA COLI* LIPID X-LIKE LIPID A FROM THE MARINE BACTERIUM *CHRYSEOBACTERIU INDOLTHETICUM* CIP 103168^T

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Using compositional analysis, mass spectrometry, and NMR spectroscopy, the structure of lipid A from marine bacterium *Chryseobacterium indoltheticum* CIP 103168^T was determined. It was shown to have an uncommon monosaccharide nature, 1 phosphate of D-glucosamine acylated with (*R*)-3-hydroxy-15-methyl-hexadecanoic and (*R*)-3-hydroxy-13-methyltetradecanoic acids at C2 and C3 positions, respectively, similar to the *Escherichia coli* lipid X. A phospholipid analysis revealed an absence of phosphatidylglycerol (PG) in *C. indoltheticum*. This also correlates well with data on lipid X, lipid A biosynthesis precursor, which accumulates in *E. coli* mutants when PG levels are low, and excellently confirms earlier received data on ways of biosynthesis of lipid A.

There are some differences between lipid X and *C. indoltheticum* lipid A. Full similarity between these molecules is absent because the *E. coli* lipid X does not contain (*R*)-3-hydroxyalkanoic acids of *iso*-series. Unlike lipid X that is easily released by the acidic Bligh-Dyer procedure the *C. indoltheticum* lipid A could be isolated from defatted cells only after acid hydrolysis followed by extraction with chloroform/methanol (2:1, v/v) mixture. It can be assumed that this lipid X-like lipid A is chemically combined with other components of outer membrane but it is not lipopolysaccharide (LPS) because although the phenol/water extracts were obtained with high yields (7-8 %) and gave one band (which disappeared after hydrolysis of the samples with acetic acid) at the silver staining SDS-PAGE, they contained 3-hydroxyalkanoic acids and lipid A only in trace amounts. Taken together, these results show an early block in lipid A biosynthesis and possible loss of LPS in *C. indoltheticum* which nevertheless saves its viability (at room temperature, 0.86 g of dry bacteria on culture L was obtained for stationary growth phase).

C. indoltheticum is the only wild-type Gram-negative bacterium described so far in which biosynthesis of lipid A is stopped on one of the earliest stage. However, it seems likely that this phenomenon has a wider distribution since our experiments with other bacterium of this genus, *C. scophthalmum*, showed that it is also PG-deficient and contains 1 phosphate of D-glucosamine acylated with two residues of fatty acids. Taking into account that *C. scophthalmum* and *C. indoltheticum* are marine bacteria, it would be possible to assume that uncommon structural type of lipid A from these bacteria is generated by conditions of their habitat. However data on *Chryseobacterium defluvii* sp. nov (Kämpfer et al. (2003) *Int. J. Syst. Evol. Microbiol.* **53**, 93-97) that is phylogenetically closely related to *C. indoltheticum* but is isolated from wastewater are in contrast with such proposal. It does not contain PG that indirectly point to incomplete lipid A structure in this microorganism and implies that arrest of lipid A biosynthesis may occur not only in marine bacteria.

An existence of bacteria similar to *C. indoltheticum* raises a number of issues that remain to be resolved. Since LPS determines the asymmetry of the outer membrane and many important functions in membrane biogenesis have been assigned to this molecule there is a question why this bacterium has the unique ability to survive without LPS, what component(s) take the role of LPS, and whether can lipid X-like lipid A to carry out this function. Biological properties of the *C. indoltheticum* lipid A that showed weak toxicity (600 μg/kg) comparable to that of *E.*

coli lipid X (1,000 μg/kg) and agonistic activity towards to induction of cytokines also deserve further study.

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O27

THE STRUCTURE AND SOME BIOLOGICAL PROPERTIES OF LIPID A FROM MARINE PROTEOBACTERIUM *MARINOMONAS COMMUNIS* ATCC 27118^T

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The mortality rate for patients with gram-negative infections including endotoxemia and septic shock continues to be unacceptably high, in spite of therapeutic intervention and rigorous supportive care. Bacterial endotoxin, the lipopolysaccharide (LPS) component of the outer membranes of gram-negative bacteria, has been implicated as a major factor in the pathogenesis of such diseases, and its lipid domain (lipid A) represents the active center responsible for most of the pathophysiological effects of LPS.

Modern medicine has excellent antibacterial means against Gram-negatives as such but effective therapy against endotoxemia is lacking. Some lipid A structural variants having one phosphate group and/or low acylation degree were shown to be medically useful in competing with LPS by blocking possible receptor sites and/or leading to endotoxin tolerance. Therefore intensive searches for potential endotoxin antagonists on the basis of lipid A are now being carried out.

Marine proteobacteria whose habitat is characterized by a specific environment such as a generally low temperature, high hydrostatic pressure, and increased salt concentration, may provide lipids A of pharmaceutical interest. Therefore we elucidated the chemical structure of lipid A from of the marine gamma-proteobacterium *Marinomonas communis* ATCC 27118^T. Lipid A was obtained by hydrolysis of LPS with 1% AcOH and purified by Sephadex LH-20 gel-permeation chromatography. Using chemical analysis, 1D and 2D NMR spectroscopic and FAB-MS spectrometric methods, it was shown to be pentaacylated β-(1′→6)-linked D-glucosaminobiose 1-phosphate. (*R*)-3(dodecanoyl)- or (*R*)-3(decanoyl)oxydecanoic acids and (*R*)-3{(*R*)-3-hydroxydecanoyloxy)}decanoic one had amide linkages and were located at C2 and C2′ positions, respectively. C3 position of the disaccharide reducing end was substituted with ester linked (*R*)-3-hydroxydecanoic acid. Hydroxyl groups at the positions 4, 3′, 4′ and 6′ were shown to be free. Lipid A with such uncommon structural peculiarities (acyl- and phosphate-deficiency) may be useful as potential endotoxin antagonist.

During gram-negative infections LPS stimulates monocytes/macrophages to release cytokines such as tumor necrosis factor alpha (TNF-α), interleukins 1-8, and others. Most of these immunological mediators play a critical role in inflammation and immune responses, and have been recognized as key mediators in the pathogenesis of infectious diseases and, more particularly, the pathophysiological alterations observed in endotoxic shock. We analyzed the capacity of purified *M. communis* lipid A to induce the release of TNF-α in the human whole blood after incubation for 20 h at 37 °C in the presence of 5% CO_2. According to the data obtained TNF-α release induced by 1000 ng/ml lipid A was 30 times lower than that induced by LPS of *Escherichia coli* at concentration 0.1 ng/ml. The similar data were obtained in the same experiments with lipid A's from some other marine bacteria (*Marinomonas vaga*, and *Chryseobacterium indoltheticum*). These results show that lipid A from *M. communis* (and those above) has no agonistic activity and might appear to be a potential inhibitor of

lipopolysaccharide-induced release of TNF-α and an effective therapeutic agent for human septic shock.

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O28

FULL STRUCTURE OF THE *PSEUDOMONAS AERUGINOSA* LIPOPOLYSACCHARIDE

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The lipopolysaccharide (LPS) is the major surface antigen of *Pseudomonas aeruginosa*, which plays an important role in interaction of the bacterium with its host. The LPS has the structure common for Gram-negative bacteria and is composed of lipid A, a core oligosaccharide, and an O-chain polysaccharide built up of oligosaccharide repeating units. To know the LPS structure is necessary for understanding pathogenesis of bacterial diseases on the molecular level and the mechanisms of the host defence. Now we present the structure and discuss conserved and variable structural features of the LPS in 20 *P. aeruginosa* strains, including reference strains of representative serotypes of all O-serogroups and clinical isolates.

The *P. aeruginosa* LPS is characterized by high degree of heterogeneity. The main approach to the structure elucidation was degradation of the LPS by two different ways, namely, by mild acid hydrolysis or strong alkaline degradation following mild hydrazinolysis. The oligosaccharide products were isolated by gel chromatography and, when necessary, purified by high-performance anion-exchange chromatography on CarboPac PA1 and studied by NMR spectroscopy, Fourier transform-ion cyclotron resonance ESI MS and MALDI-TOF MS.

Degradation of each kind preserved some information but some other information was lost. Combining data of the different degradations together enabled determination of the full structure of the LPS. The structures of the lipid A and core moieties of the *P. aeruginosa* LPS are mostly conserved among serotypes. Lipid A is heterogeneous owing to the presence of molecules with three to six acyl groups and a non-stoichiometruc 2-hydroxylation of the secondary acyl groups.

The core is distinguished by the existence of two glycoforms differing in the position of a terminal rhamnose residue in the outer region. Only one of the glycoforms can accept the O-polysaccharide (O-antigen), which is attached to the rhamnose residue. This glycoform has the same structure in all serotypes, whereas the other glycoform is characterised by the presence of three glucose residues in some serotypes and four residues in the others. N-Acylation of galactosamine with alanine, 7-O-carbamoylation of one of the heptose residues and phosphorylation of the inner core region at three major sites are common features of the LPS of all serotypes. In most serotypes, ethanolamine phosphate is attached to one of the phosphate groups, and diphosphate groups may occupy the other phosphorylation sites. The outer core region is non-stoichiometrically O-acetylated at multiple sites.

Although the O-polysaccharides possess diverse structures in various serotypes, they share some structural features. Particularly, in all but two *P. aeruginosa* serogroups the first monosaccharide of the repeating is a 2-acetamido-2,6-dideoxy-D-hexose. Whether α- or β-linked in the interior O-units, the first monosaccharide is β-linked to the core, a result of involvement of two different enzymes with polymerisation of the O-unit and ligation of the OPS to the core. From them, O-antigen polymerase is serogroup- or subgroup-specific, whereas ligase seems to be common in all strains. The data obtained reveal a similarity of the LPS biosynthesis pathway in 11 *P. aeruginosa* O-serogroups and various mechanisms of the OPS structure diversification.

Identification of the first monosaccharide that is attached to the core enabled determination of the terminal upstream saccharide in the O-polysaccharide, which has the same or a similar structure in all serotypes of one serogroup but substantially different structures in strains of different serogroups. This terminal region of the O-polysaccharide contributes most significantly to the

immunospecifity of strains, and, therefore, the structural data obtained represent the chemical basis for the serological classification of *P. aeruginosa*.
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O29

DIFFERENTIATION OF *SALMONELLA* GALINARUM FROM *SALMONELLA* PALLORUM BY RFLP PCR OF *fim*H GENE

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Salmonella Gallinarum and *Salmonella* Pullorum are considered important pathogens, causing respectively, pullorum disease and fowl typhoid in poultry. Both diseases were eradicated in many developed country, however, they are still one of leading causes of poultry mortality in many African and Asian countries. Therefore, due to the high economic losses their accurate and fast diagnosis is of primary importance.

It is generally accepted that S. Gallinarum infects mostly adult and older chickens, however, it was as well isolated from diseases chicks. The same is also true for *S*. Pullorum, infecting mostly chicks, but also isolated from older birds. Because *S*. Gallinarum and *S*. Pullorum represent the same serovar but various biotypes, their identification and differentiation is based mostly on biochemical characteristics. Such methods are the most widely used and officially recognized, although they are time-consuming, cumbersome and costly, in particular when many samples have to be analyzed in a short period of time. Therefore, DNA-based methods, especially those based on PCR, were used for differentiation of *S*. Gallinarum from *S*. Pullorum. Of those, analysis of phase 1 flagellin C gene (*fliC*) by RFLP-PCR (Kwon et al., 2000) seems to be the most promising because of its sensitivity, specificity and speed. In our study on FimH adhesins of different *Salmonella* serovars we have cloned and sequenced *fimH* genes from *S*. Gallinarum and *S*. Pullorum. The comparison of nucleotide sequences revealed the presence of single-nucleotide polymorphism (SNP) at the position 545 bp of *fimH* gene. Further analysis of restriction enzyme sites in *fimH* genes showed that SNP at this position is responsible for a sequence specifically recognized by *Sac*I in *S*. Gallinarum. Digestion of PCR-amplicons of *fimH* gene from *S*. Gallinarum with *Sac*I gave two fragments of 555 bp and 471 bp, allowing for clear differentiation between these two biotypes.

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