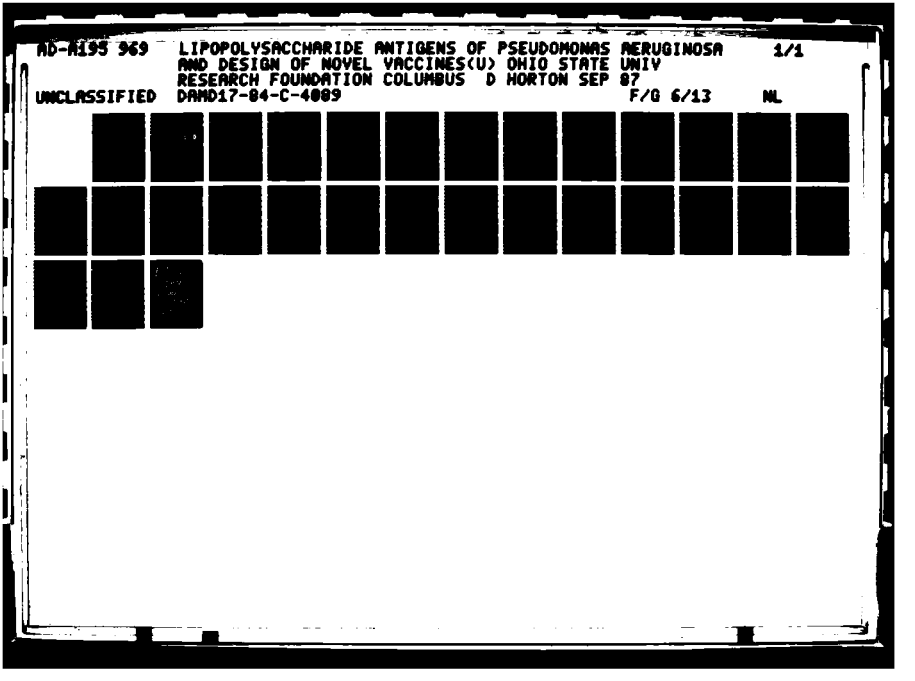
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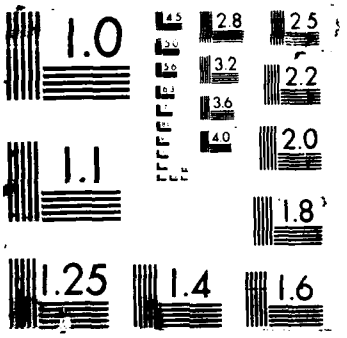
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LIPOPOLYSACCHARIDE ANTIGENS OF *Pseudomonas aeruginosa* AND DESIGN OF NOVEL VACCINES

Derek Horton

Annual/Final Report

September 1987

Supported by:

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19. ABSTRACT (Continue on reverse if necessary and identify by block number)

The antigenic lipopolysaccharides (LPS) of the cell coats of five of the seven Fisher immunotype strains of the Gram-negative pathogen Pseudomonas aeruginosa have been characterized structurally by chemical methods involving degradation techniques, and by physical methods, notably high-field NMR spectroscopy. Of the seven Fisher types, the O-Chain structures of five of the types have been characterized in our laboratories. Partial structures for the remaining two types have been elucidated.

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Table of Contents

I. ABSTRACT

The antigenic lipopolysaccharides (LPS) of the cell coats of five of the seven Fisher immunotype strains of the Gram-negative pathogen Pseudomonas aeruginosa have been characterized structurally by chemical methods involving degradation techniques, and by physical methods, notably high-field NMR spectroscopy. Of the seven Fisher types, the O-chain structures of five of the types have been characterized in our laboratories. Partial structures for the remaining two types have been elucidated.

The O-chain polysaccharides of the seven types have regular tri- or tetra-saccharide repeating units, and are considered to be immunodeterminant haptens. These oligosaccharide units are being chemically synthesized in forms suitable for covalent attachment to a carrier protein to create artificial antigens, and to solid supports, to furnish immunoadsorbent columns. Antibodies raised against LPS and the artificial antigens are to be tested for binding affinity to the synthetic material by equilibrium dialysis, fluorescence quenching, competitive-inhibition ELISA, and affinity column measurements. Once the oligosaccharide fragment that shows appropriate binding characteristics has been determined for each type, it is expected that affinity columns of these materials can be used to prepare purified antibodies from hyperimmune sera.

These investigations were supported by the U.S. Army Medical Research and Development Command, Contract No.DAMD17-84-C-4089, and are of significance in the development of new diagnostic methods for typing pathogenic Pseudomonas strains, and for therapy based on both active immunization and on use of specific antibodies for passive immunotherapy. There is a major need for effective methods for treatment of Pseudomonas infections, especially with burn victims, victims of combat injuries, cystic fibrosis patients, and individuals undergoing cancer chemotherapy. Further, correlation between the chemical structures of these macromolecular antigens and their interferon-stimulating ability may provide knowledge useful in the understanding of cancer and viral diseases.

The work was performed in collaboration with the Walter Reed Army Institute of Research in liaison with Dr. Jerald Sadoff. Agents developed for active immunization, based on hapten—protein conjugates involving the carbohydrate haptens linked to human serum albumin, tetanus toxoid, and to other proteins, are to be used in the Walter Reed laboratories to raise antibodies in animals, and the potential of these antibodies for eventual clinical use evaluated. Complementary work exploiting monoclonal antibody preparations on hand in Dr. Sadoff's laboatory, and the immunoadsorbents produced in the Ohio State University laboratories, should permit optimization of procedures for purification of these monoclonal antibody preparations to homogeneity and furnish bulk quantities of reliable, pure antibodies for passive immunotherapy.

II. STATEMENT OF THE WORK

The objectives of this project involve studies on the detailed molecular basis of action of Pseudomonas antigens through chemical structural characterization, the chemical synthesis of artificial antigens and immunoadsorbents bearing the site of antigenic specificity, and application of these materials in the development of practical therapeutic agents to combat infections by Pseudomonas aeruginosa. The chemical and biochemical phase was conducted in the laboratories of Dr. Derek Horton at The Ohio State University, Columbus, Ohio, in coordination with research at the Walter Reed Army Institute of Research under the direction of Dr. Jerald Sadoff, where complementary emphasis has been on the immunochemical and immunological aspects, with potential clinical uses in view, especially as they pertain to problems in military medicine.

Prime targets of the work presented here, on the basis of an initial one-year contract with an extension period, involved structural elucidation of the cell-surface LPS antigens, chemical preparation of determinant ligands, and their transformation into artificial antigens (through coupling to various proteins) and immunoadsorbents. Also included was the verification of the native structure of artificial glycoconjugate antigens prepared in Dr. Sadoff's laboratory, and binding-site mapping of complementary monoclonal antibodies. The latter may be considered for use, once the proper size and sequence of the haptens are determined, to produce bulk quantities of purified homologous antibodies, both from hyperimmune plasma and from the monoclonal antibody preparations developed by Dr. Sadoff.

The project has endeavored to bring an ongoing program in The Ohio State University into broader significance through collaboration with the Walter Reed laboratories. It has brought chemical and biochemical expertise in the area into the broader context of ultimate clinical goals.

The succeeding discussion describes the major phases of the project as developed in Dr. Horton's laboratories. The description indicates the principal work accomplished under support from the U.S. Army Medical Research and Development Command, Contract No. DAMD17-84-C-4089, details of the facilities used in the work, and specifies the major targets envisaged for the project.

III. MILITARY SIGNIFICANCE

Military Significance: The availability for field use of a vaccine effective against Pseudomonas infections would be of great value in the treatment of military and civilian personnel who are victims of severe burn injuries. Direct consequences of the research proposed here could be improved and rapid methods for serological typing, and the development of vaccines in which adverse side-effects are reduced. Long-range implications of the studies are an improved understanding of the mode of action of pathogenic bacteria and methods for their control. These aspects

are significant and useful military objectives in the treatment of infections arising from to battlefield injuries, and in the peace-time work of the armed services concerned with relief of victims of natural disasters.

IV. DISCUSSION

A. Background: This work, supported by the U.S. Army Medical Research and Development Command Contract No. DAMD17-84-C-4089, is an orderly continuation of our research for the total structural characterization of the lipopolysaccharide (LPS) antigens of Pseudmonas aeruginosa, synthesis of artificial antigens of LPS, and immunological studies of these artificial antigens. Earlier work on this subject was conducted in Dr. Horton's laboratory under support from Grant No. 1 RO1 GM20181-01—04 from the National Institute of General Medical Sciences.

Specific overall targets in this study may be subdivided into the following categories: (1) detailed structural characterization of the O-specific chains of the LPS antigens, as well as the core saccharide and the lipid A components, (2) chemical synthesis of O-chain repeating units, together with derived artificial antigens and immunoadsorbents, and synthesis of lipid A analogs, (3) evaluation of the antigenicity of the artificial antigens and immunoadsorbents, (4) screening of whole O-chain polysaccharide antigens for native structure, and (5) binding-site mapping of the antibodies using the synthetic antigens.

B. Significance: General Background.

The Gram-negative bacterium Pseudomonas aeruginosa is ubiquitous in its occurrence and is characterized by a very wide variety of strains as determined by conventional serotyping methods.[1] Although healthy individuals normally have a high innate resistance to infection by Pseudmonas aeruginosa as a result of effective combat by matured antibodies, the organism is an opportunistic pathogen; virulent strains lead to dangerous, often fatal, infections in human subjects whose natural resistance has been lowered. Conventional treatment with antibiotics effective against Gram-positive organisms is of little value, as such therapeutic agents are unable to penetrate the lipopolysaccharide layer that forms a thick, hydrophobic outer coating of the cells in Gram-negative organisms.

Subjects having particular susceptibility to Pseudomonas infection, and liable to protracted deterioration with usually fatal outcome, include especially burn patients and patients suffering from cystic fibrosis, together with subjects whose immune-response capacity has been weakened as a result of aggressive therapy by chemical or radiation procedures to combat neoplasia, as well as surgical patients and individuals whose condition has become debilitated by any of a wide range of primary causes.[1,2]

In view of the limited effectiveness of conventional antibiotic agents for combat of Pseudomonas infections, immunotherapeutic methods have been explored as an alternative method, either by direct challenge with antigenic material from the organism to elicit development of homologous antibodies in the patient, or by passive therapy with antibodies raised in donors for use in patients already manifesting Pseudomonas sepsis.

A complication in this approach results from the wide variety of individual serotypes of Pseudomonas aeruginosa that have been recognized by conventional bacteriological methods. There have been various attempts to classify these serotypes into smaller, more manageable subgroups. A significant advance was made by Fisher and coworkers[3], who developed an immunotype classification scheme wherein rabbits were used to raise homologous antibodies against numerous different serotypes of Pseudomonas aeruginosa. The cross-reactivities of the antibodies corresponding to each serotype were then studied.

Among approximately 100 serotypes studied, Fisher's group found seven immunotypes whose homologous antibodies in combination offered cross-reactive protection against almost all of the serotypes. The seven strains that afforded these cross-protective antibodies were cultured on a large scale, and the antigenic lipopolysaccharide outer-coats of the trichloroacetic acid-killed cells were isolated to provide seven different lipopolysaccharide antigens. These antigenic lipopolysaccharides, from the immunotypes designated 1—7, were formulated in admixture as a vaccine (Pseudogen^{R})[4] that has proven very effective for active immunization of patients (for example, burn victims) at high risk for the development of lethal Pseudomonas infection. Detailed literature citations documenting this aspect are recorded in one of the initial papers from our research on this project.[2]

C. Objectives of this investigation: The initial purpose of our work was the chemical characterization of seven lipopolysaccharide antigens from the original strains of P. aeruginosa that form the basis of Fisher's immunotype classification. These materials were furnished by the manufacturer of the Pseudogen^{R} vaccine, Warner-Lambert/Parke-Davis and Co., Ann Arbor, Michigan, as high-molecular weight substances that can be stored in water solution at 4°C over a period of several years without decrease of biological potency.

The lipopolysaccharides of the cell coats of Gram-negative organisms are generally considered to have a three-component structure[5] comprising a lipid portion (Lipid A) that anchors the macromolecular complex to the cellular outer membrane. The Lipid A is linked to a "core" oligosaccharide through two acid labile residues of 3-deoxy-D-manno-octulosonic acid (KDO), as has been demonstrated by using a P. aeruginosa mutant deficient in O-specific polysaccharide synthesis.[6] The proposed core structure is a branched octasaccharide containing neutral, deoxy, and aminodeoxy sugars as well as KDO. This core is further linked via an unidentified acid-labile linkage to the O-specific polysaccharide. There is a correspondence between this model for P. aeruginosa and that developed for Salmonella[7,3], although this has not been rigorously proven. The antigenic O-specific region is responsible, together with the H and Vi surface antigens, for the interaction of Gram-negative bacteria with homologous, O-specific antisera.

The standard procedure of Westphal, et al.[5] for the isolation of lipopolysaccharide antigens involves treatment of the cultivated cells with hot aqueous phenol followed by isolation of the lipopolysaccharide from the aqueous phase of the phenol—water mixture. This procedure is not suitable for large-scale production, and the antigens used in the formulation of the Pseudogen^{R} vaccine were isolated by suspending the cells in aqueous trichloroacetic acid at 4°C, following the older isolation technique of Boivin. After several purification steps, including gel-permeation chromatography, the lipopolysaccharides were isolated by lyophilization from their opalescent, aqueous solutions as products having molecular weights of several millions.

Our studies on the Pseudomonas LPS antigens were aimed at elucidating some of the fundamental questions of immunology and also at providing practical information concerning the use of materials formulated for protection against Pseudomonas infection.

First of all, the vaccine Pseudogen^{R} elicits an undesirable pyrogenic response that has been attributed to the Lipid A portion of the LPS. However, it was shown that the Pseudogen^{R} admixture induced production of antibodies of the IgG class[9] having an intravascular half-life on the order of 23 days, whereas active Pseudomonas cultures induced weaker[10], IgM class antibodies having a half-life of only 5.1 days. It was hoped that our studies would help in the development of a vaccinating agent that demonstrates the positive attributes of the Pseudogen^{R} vaccine without displaying toxic effects. It was recently shown by Sadoff et al.[54], that use of synthetic glycoconjugates of the whole O-chain polysaccharides also elicits antibodies of the IgG class in mice.

Secondly, knowledge of the chemical constitutions of the LPS could provide a basis for rapid, analytical—chemical methods for typing unknown strains of Pseudomonas, as isolated from hospital subjects, by procedures more convenient than conventional bacteriological serotyping. These analytical procedures would also be effective for analyzing antigenic conjugates of O-chain material to establish rapidly the extent to which the coupling process used may have affected the chemical constitutions of the polysaccharides.

Thirdly, accurate structural attribution of the O-chains provides a firm basis for the chemical synthesis of immunodeterminant haptens based on segments of the O-chain sequences. These oligosaccharide segments may then be used to prepare affinity-adsorbent media that could permit the ready isolation of substantial quantities of pure homologous antibodies from hyperimmune sera. Affinity studies conducted with these synthetic haptens would also give binding-site mapping data for the antibodies raised against the Pseudomonas antigens. Such studies on the synthetic haptens should demonstrate which residues are important in binding and allow extrapolation, by use of suitably modified haptens, to establish which groups on the individual residues are important for binding to the antibodies. This knowledge would be a great advance in the passive treatment of established Pseudomonas infections, as would be the chemical synthesis of artificial antigens free from the side effects of the native lipopolysaccharides. Such artificial antigens would also be useful in the study of antibody production in animals when the antigens are coupled to

appropriate carrier proteins.

In addition, detailed structural characterization of these endotoxins would significantly assist efforts to elucidate the molecular basis of the antigen—antibody interaction, and could also throw light on the interferon-stimulating activity of these antigens. The latter aspect could be very rewarding, especially in the area of cancer and viral diseases.

Since our initial reports on the structures of the O-specific chains of the LPS in the Fisher immunotypes of Pseudomonas aeruginosa , there has subsequently been much activity in the field, especially by Kochetkov et al.[11-17]. These investigators have described numerous structures for the O-chains of various Lányi serotypes of Pseudomonas. In addition, there are reports of two new strains that do not fit into either the Fisher or Lányi typing schemes.[18,19] Work done by Kochetkov et al. has aided the field of structural evaluation, for example through the characterization of novel 2,3-diaminoglycuronic acids. However, extension of our work into the preparation of O-chain segments of these additional types would require an extensive further commitment of research resources. The scope of our own work was projected to establish the O-chain structures for the seven Fisher immunotypes, as these demonstrate cross-reactivity for nearly all P. aeruginosa strains; consequently the synthetic targets can be restricted to a relatively small number of O-chain segments.

There are many instances where O-chain structures reported by others have shows only small variations from the structures we have found for the Fisher immunotypes. There are problems of correlation between different classification schemes. Much work has been done in this area[20-22], but no definitive method has yet been developed to resolve this confusion. These questions could eventually be readily resolved by cross comparison on the basis of chemotypes.

D. Progress Report: This section details the work performed under U.S. Army Contract No. DAMD 17-84-C-4089. Some of this consolidated earlier work performed under support from NIGMS Grant 20181. The sequence of the Fisher Type 2 O-chain was essentially completed during this earlier support period.

1. Summary: The structures of the O-specific side chains of P. aeruginosa types 1, 5, 6, and 7 (Fisher) have been determined as follows:

Immunotype:

1. 2)-α-L-Rhap-(1→3)-α-D-QuiNFap-(1→4)-α-D-Glcp6-Ac-(1→4)-ß-D-ManImAp-(1→

2. 3)-α-L-FucpNAc-(1→3)-ß-D-FucpNAc-(1→2)-α-D-Glcp-(1→

5. 3)-α-L-Rhap2Ac-(1→4)-α-L-GalNAcAp-(1→3)-ß-D-QuiNAcp-(1→

6. 6)-ß-D-Glcp-(1→2)-ß-L-Rhap-(1→3)-ß-L-Rhap-(1→

7. 4)-ß-D-ManImAp-(1→4)-ß-D-Man2,3(NAc)$_2$Ap-(1→3)-ß-D-FucNAcp-(1→

(Fo = formyl and ManImA = 1-acetyl-2-methyl-(ß-D-mannuronic acid)[3,2-d]-imidazoline.

Types 3 and 4 remain under contiuing investigation. Type 3 has a trisaccharide repeating-unit containing one imidazoline sugar, one 2,3-diacatamido sugar, and one unit of fucosamine. Type 4 has a tetrasaccharide repeating-unit containing three amino sugars and perhaps an aminouronic acid.

Synthetic efforts have been focused on the trisaccharide repeating units of types 2, 5, and 6. Monosaccharide units for the sequential building up of the trisaccharide units have been largely completed and these will permit synthetic access to the di- and tri-saccharide haptens. It is anticipated that artificial antigens at the disaccharide level may suffice for effective immune response and immunoaffinity through the smaller, more accessible disaccharide units of several of the immunotypes.

In addition to the foregoing, we have also purified antibodies for immunotype 5 by passing murine ascites serum over a column derivatized with whole type 5 O-chain. The major portion of the protein passed through the column in the void volume, and a single protein peak, presumably type 5 monoclonal antibody was eluted with a KCN buffer gradient. We have not yet received a report from Dr. Sadoff concerning the nature or activity of this fraction. Instruments for performing assays to test the activity of these purified antibodies are now available to us. The entire foregoing experiment should be repeated and the activities tested.

2. Project Period: August 1, 1984 through April 30, 1986 (last six months as a no-cost extension).

3. Staffing: The following personnel were engaged in research work on the project during the report period. Not all of the individuals worked full time on the project during this period.

Derek Horton (Supervisor) 08/01/1984 - present
Mark Glen Schweitzer, GRA 08/01/1984 - 12/31/1984
Abul Anisuzzaman, Postdoctoral Research Associate - 07/01/1985 - present
Steven Warner, Postdoctoral Research Associate - 02/01/1985 - present
Sunetary Chanapai Berlin, GRA 08/01/1984 - present
William Berlin, GRA 08/01/1984 - present

4. Publications: The following list gives work from our laboratory related to the lipopolysaccharide antigens of Pseudomonas aeruginosa.

1. Derek Horton and Avraham Liav, A Synthesis of 2-Amino-2,6-dideoxy-D-allose and -D-altrose Hydrochlorides and Their Tetraacetyl Derivatives, Carbohydr. Res., 47 (1976) 326—331.

2. Derek Horton, Günther Rodemeyer, and Theordore H. Haskell, Analytical Characterization of Lipopolysaccharides from Seven Strains of Pseudomonas aeruginosa, Carbohydr. Res., 55 (1977) 35—47.

3. Derek Horton, Günther Rodemeyer, and Roswitha Rodemeyer, Characterization of 2-Amino-2,6-dideoxy-D-glucose as a Constitutent of the Lipopolysaccharide Antigens of Pseudomonas aeruginosa Immunotype 4, Carbohydr. Res., 56 (1977) 129—138.

4. Derek Horton, Günther Rodemeyer, and Hiromichi Saeki, A Synthesis of 2-Acetamido-2,6-dideoxy-D-galactose (N-Acetyl-D-fucosamine), Carbohydr. Res., 59 (1977) 607—611.

5. Derek Horton and Hiromichi Saeki, Conversion of 2-Acetamido-2-deoxy-D-glucose Into 2-Acetamido-2,6-dideoxy-D-galactose (N-Acetyl-D-fucosamine) and Its Benzyl 3-O-Benzyl Glycosides, Carbohydr. Res., 63 (1978) 270—276.

6. Derek Horton, Studies on the Lipopolysaccharide Antigens of Seven Immunotypes of Pseudomonas aeruginosa, SELAQ, Proc. VIII Seminario Latinoamericano de Quimica, pp. 143—159 (1979).

7. Derek Horton, David A. Riley, and Poul M. T. Hansen, Sedimentation-Equilibrium Studies of the Polysaccharide Components of Pseudomonas aeruginosa, Biopolymers, 19 (1980) 1801—1814.

8. Derek Horton and David A. Riley, Phosphorus-31 Nuclear Magnetic Resonance Spectroscopy of Lipopolysaccharides from Pseudomonas aeruginosa, Bioc im. Biophys. Acta, 640 (1981) 727—733.

Presentations at Meetings

9. Derek Horton and Günther Rodemeyer, Structural Studies on Seven Serotype Antigens of Pseudomonas aeruginosa, Abstracts Papers Am. Chem. Soc. Meet., 170 (1975) CARB-63.

10. Derek Horton and Günther Rodemeyer, Structural Studies on Lipopolysaccharides of Pseudomonas aeruginosa, Abstr. Pap. VIIIth Intern. Symp Carbohydr. Chem., Kyoto, Japan, August 16—20, 1976, No. B243.

11. Derek Horton and Günther Rodemeyer, Linkage Sequences in Lipopolysaccharide Antigens of Pseudomonas aeruginosa, Abstr. Pap. Am. Chem. Soc. Meet., 172 (1976) CARB-97.

12. Derek Horton, Structural Studies on Lipopolysaccharide Antigens of Pseudmonas aeruginosa, Perspectives in Carbohydrate Chemistry: Symposium Honoring Professor J. K. N. Jones, Kingston, Ontario, May 25—27, 1977.

13. Derek Horton, Judith R. Lubbers, David Riley, Günther Rodemeyer, and

Hiromichi Saeki, Compositions of the Lipopolysaccharide Antigens of the Seven Fisher Immunotypes of Pseudomonas aeruginosa, Abstr. Pap. Am. Chem. Soc./Chem. Soc. Jpn. Chem. Congr., April 1—6, 1979, CARB-49.

14. Poul M. T. Hansen, Derek Horton, and David Riley, Sedimentation-Equilibrium Studies of the Polysaccharide Components of Pseudomonas aeruginosa, Abstr. Pap. Am. Chem. Soc. Meet., 178 (1979) CARB-65.

15. Derek Horton, Studies on the Lipopolysaccharide Antigens of Seven Immunotypes of Pseudomonas aeruginosa, SELAQ, Buenos Aires, Argentina, Nov. 19—23, 1979.

16. Derek Horton, Chemical Structures of Pseudomonas Antigens, Joint Workshop on Pseudomonas aeruginosa, Walter Reed Army Institute of Research, December, 7, 1979.

17. Derek Horton and David A. Riley, Structural and Physical Studies on Lipopolysaccharides of Pseudomonas aeruginosa, Abstr. Pap. Am. Chem. Soc. Meet., 181 (1981) CARB-50.

18. Derek Horton, David A. Riley, and Günther Rodemeyer, The Repeating-Unit Sequence of the O-Antigenic Chain of Pseudomonas aeruginosa Immunotype 5.

19. Derek Horton and Hiromichi Saeki, Analytical Compositions of the Core Oligosaccharides of the Fisher Immunotype Antigens of Pseudomonas aeruginosa.

20. Derek Horton, David A. Riley, and Günther Rodemeyer, Sequence of the Repeating Unit in the O-Antigenic Chain of Pseudomonas aeruginosa Immunotype 2.

21. Judith R. Lubbers (Derek Horton, preceptor), Thesis, An Examination of the Lipid of Pseudomonas aeruginosa Bovin Lipopolysaccharides of Fisher Immunotypes 1—7, The Ohio State University, 1978.

22. David A. Riley (Derek Horton, preceptor), Ph.D. Dissertation

23. Mark G. Schweitzer (Derek Horton, preceptor)

5. Report: The specific targets of the U.S. Army Medical Research & Development Command Contract No. DAMD17-84-C-4089 were as follows:

(a) Total structural elucidation of the O-specific polysaccharides of the seven Fisher immunotypes.

(b) Synthesis of selected O-chain repeating-units as glycosides capable of being conjugated to solid supports as immunoaffinity material, and to suitable carrier proteins as artificial antigens.

(c) Studies of the artificial-antigen materials for affinity-binding characteristics and antibody characterization.

Most of the work done during the period of support was concentrated in section (a), as documented in the publications listed here. Synthetic work in section (b) was initiated and synthesis of the repeating units for types 5 and 6 are under continued study. Work with immunoaffinity columns incorporating whole O-chain of type 5 was initated, but not completed during the period of support.

The following is a detailed overview of the data obtained during the project period. The complete structural studies are condensed from the dissertations cited in the list of publications. The remaining work on the type 3 and 4 structures and the synthetic work is still in progress and is largely unpublished.

Structural Studies

Immunotype 1:— The O-chain was isolated by standard methods (detailed in the original contract proposal) and was studied by high-field ^{1}H and ^{13}C NMR spectroscopy. The ^{1}H NMR spectrum showed signals in the region of 1.25 ppm, indicating the presence of 6-deoxy sugars. Also evident were signals in the region of 2.0 ppm characteristic of N-acetate and O-acetate. A third, unusual signal was a singlet at 2.21 ppm, which was shown to be that of the 2-methyl group of an imidazoline-type ring system present in some of the O-chain structures. This signal disappeared when the sample was treated with aqueous alkali, as this procedure caused hydrolysis to give the 2,3-diacetamido sugar structure.

The anomeric-proton region of the spectrum showed four signals, one β signal and 3 α signals. A characteristic singlet at 8.21 ppm was indicative of the formyl proton of a formamido group. The ^{13}C NMR spectrum confirmed many of these assignments. The anomeric region, 97—100 ppm, showed four distinct signals. Off-resonance (one-bond-coupled ^{13}C NMR) decoupled spectra confirmed the assignment of one β linkage (JC_1H_1 = 154 Hz) and three α linkages ($J_{C-1,H-1}$ >170 Hz).

The broad-band-decoupled ^{13}C spectrum showed three signals in the region of 50—58 ppm, indicative of carbon atoms bearing a nitrogen substituent. Also present were two signals at 17.5 and 18.0 ppm characteristic of 6-deoxy sugars, and several acetyl methyl signals at 23 ppm. The corresponding carbonyl signals were evident at 174 ppm.

Significant additional ^{13}C were signals at 31.1 and 168.8 ppm. These have been shown[11—16] to arise respectively from the methyl, and alkenic carbons of the aforementioned imidazoline structure.

Classical sugar analysis, involving total hydrolysis followed by borohydride reduction to the alditols and subsequent acetylation, showed the presence, by g.l.c.—m.s. procedures, of three sugars, namely rhamnose, glucose, and quinovosamine, in roughly equal proportions. . Preparative paper chromatography of the O-chain hydrolyzates allowed isolation of these three sugars, and optical rotation measurements confirmed their enantiomeric identities.

The next step was methylation analysis of the polysaccharide. The following components were identified by their fragmentation patterns of the methylated alditol acetates by g.l.c.—m.s.; 1,5-di-O-acetyl-6-deoxy-2,3,4-

tri-O-methylmannitol, 1,2,5-tri-O-acetyl-6-deoxy-3,4-di-O-methylmannitol, 1,4,5-tri-O-acetyl-2,3,6-tri-O-methylglucitol, and 2-acetamido-1,3,5-tri-O-acetyl-4-O-methyl-2,6-dideoxyglucitol. These results indicated that the glucose residues were (1→4)-linked, the rhamnose (1→2)-linked, and the quinovosamine (1→3)-linked. An unknown component remained at that point unidentified.

Treatment of the intact O-chain with anhydrous HF, evaporation of the HF, and purification of the product on Bio-gel P-2 gave a disaccharide fragment whose NMR spectra showed 6-deoxy hexose, two amino-substituted carbon atoms, and a uronic acid. Smith degradation of the alditol obtained by borohydride reduction of the disaccharide, by periodate oxidation, and further reduction with borohydride, gave a sample which was examined in two ways. First, methylation with dimsylate ion and methyl iodide gave a product whose c.i. mass spectrum showed a molecular ion at m/z 452. Major fragment-ions were observed at m/z 77, 350, and 375. These results confirmed the presence of a diacetamido uronic acid component (appearing as its methyl glycuronate[38,39].

Complementary information on the diacetamidouronic acid was provided by acetylation of the Smith-degraded fraction. The 200 MHz ^{1}H NMR spectrum of this product, with homonuclear decoupling, allowed assignment of all ring protons. Use of these data in conjunction with the $J_{1,2}$ coupling constants allowed the configuration of the sugar to be established as manno.

The order of linkage of the two remaining components was determined as follows. Sequential Smith degradation of the original polysaccharide, through successive periodate oxidation, reduction, dialysis, and mild acid hydrolysis, gave two glycosides separable by ion-exchange chromatography. The neutral glycoside showed (after hydrolysis and acetylation) erythritol tetraacetate and N-acetylquinovosamine tetraacetate. The ^{1}H NMR spectrum of the fraction obtained by washing of the ion-exchange resin with acid and subsequent acetylation, was identical to that of the peracetate of the Smith-degraded glycoside of the diamino mannouronate obtained by HF solvolysis of the polysaccharide. This result showed the linkage sequence to be: diaminomannouronate→rhamnose→quinovosamine→glucose.

One final feature of the structure was established by non-alkaline methylation methods. Normal Hakomori methylation, using dimethylsulfinyl carbanion, removes O-acetyl groups from the polysaccharide. The use of methyl trifluoromethanesulfonate leads to methylation, but leaves OAc groups intact. By using this procedure, the type 1 polysaccharide gave 1,4,5,6-tri-O-acetyl-2,4-di-O-methylhexitol as the methylation product from glucose, showing that the glucose residues of the type 1 polysaccharide are 6-O-acetylated. This data allowed the following structure to be proposed for the Immunotype 1 O-chain polysaccharide:

→2)-α-L-Rha*p*-(1→3)-α-D-QuiNFA*c*p-(1→4)-α-D-Glc6Ac*p*-(1→4)-β-D-ManImp[3̄,2d]-(1→ .

Immunotype 5.—Shortly after our previous report[23] of the structure of the type 5 O-specific side chain, Kochetkov *et al.*[12], reported a significantly different structure for the Lányi 0:7 type O-specific side chain. This

serotype was thought to be identical to the Fisher immunotype 5, and hence, the difference in the reported structures prompted reinvestigation of the type 5 O-chain by our group.

The ^{1}H NMR spectrum of the type 5 O-chain showed only two resolved anomeric resonances, together with one 6-deoxy signal, one O-acetyl, and 2 N-acetyl signals. The ^{13}C spectrum clearly showed the presence of three distinct anomeric resonances, indicating a trisaccharide repeating-unit. Also present were signals for one 6-deoxy sugar (17.7 ppm), N-acetyl groups (23.3 and 175.5 for the methyl and carbonyl carbons respectively), and O-acetyl groups (at 21.6 and 174.5 ppm). In addition, there were clearly two signals of carbon atoms bearing nitrogen (50.4 and 52.6 ppm).

Classical sugar analysis revealed two components, rhamnose and quinovosamine, identified as their alditol acetates. Paper chromatography of the polysaccharide hydrolyzate showed three components, suggesting that the third component was an aminouronic acid. Carboxyl reduction by diimide and sodium borohydride under pH-controlled conditions, followed by sugar analysis, gave galactosamine as the third component. Isolation of the three components from the carboxyl-reduced polysaccharide hydrolyzate by preparative paper chromatography confirmed the identity of the three components as well as their absolute configurations. The comonents were thus: L-rhamnose, L-galactosamine, and D-quinovosamine.

Methylation analysis of the carboxyl-reduced polysaccharide gave the following products: 1,5-di-O-acetyl-6-deoxy-2,3,4-tri-O-methylmannitol, 1,3,5-tri-O-acetyl-6-deoxy-2,4-di-O-methylmannitol, 2-acetamido-1,3,5-tri-O-acetyl-2,6-dideoxy-4-O-methylglucitol, and 2-acetamido-1,4,5-tri-O-acetyl-2-deoxy-3,6-di-O-methylgalactitol. These data demonstrate that rhamnose is at the reducing terminus, and is 3-O-linked in the repeating unit, as is the quinovosamine, and the galactosaminuronic acid residue is (1→4)-linked.

To demonstrate the order of linkages in the polysaccharide, a partial acid hydrolysis was performed. As aminuronic acids are hydrolyzed several orders of magnitude more slowly than amino sugars or, especially, neutral sugars, it was expected that the acid residue would be retained. Partial acid hydrolysis and subsequent gel-permeation chromatography indeed gave a disaccharide. By carboxyl reduction and sugar analysis, the two components of the disaccharide were shown to be L-galactosamine and D-quinovosamine. Acetylation of the disaccharide and ^{1}H NMR sectroscopy showed the galactosamine to be α-linked. From these data it was possible to assign the quinovosamine as having a ß linkage ($J_{1,2}$ low) and the rhamnose as having an α-linkage ($J_{1,2}$ low) from the ^{1}H NMR spectrum of the original polysaccharide.

Additional data obtained by non-alkaline methylation analysis showed that the rhamnose of the polysaccharide is 2-O-acetylated. The total structure of the type 5 O-specific polysaccharide may be written as: 3)-α-L-Rhap-2Ac-(1→4)-α-L-GalNAcAp-(1→3)-ß-D-QuiNAcp-(1→, which is identical to the structure reported by the Russian workers. These results lend credence to the suggestion that chemotyping may be the most accurate method for cross-typing between the various classification schemes.

Immunotype 6.— Analysis of the type 6 O-chain presented several problems, beginning with the content of O-chain in the LPS. Hydrolysis of the whole LPS in refluxing 1% acetic acid normally yields about 30 mg of O-chain per 100 mg of LPS. The type 6 LPS yielded only a maximum of 10% (often as low as 5%) of O-chain during the initial isolation. The conditions of acidic hydrolysis were varied over a wide range of pH, time, and concentration, and yet no additional O-chain material yield could be obtained.

The ^{1}H NMR analysis of type 6 polysaccharide showed the presence of three anomeric protons and overlapping peaks for 6-deoxy sugars. The ^{13}C NMR spectrum showed 3 anomeric resonances and overlapping signals for 6-deoxy sugars, in addition to signals for ring carbon atoms. Unexpectedly, there was no indication of nitrogenous sugars, nor, apparently, any O-acetylation.

Classical sugar analysis showed the presence of glucose and rhamnose. Separation of the hydrolyzate of the polysaccharide by preparative paper chromatography followed by optical rotation measurements showed D-glucose and L-rhamnose to be the component sugars. Methylation analysis, by the standard procedure, gave the following four components: 1,5-di-O-acetyl-2,3,4,6-tetra-O-methylglucitol; 1,5,6-tri-O-acetyl-2,3,4-tri-O-methylglucitol; 1,2,5-tri-O-acetyl-6-deoxy-3,4-di-O-methylmannitol; and 1,3,5-tri-O-acetyl-6-deoxy-2,4-di-O-methylmannitol. These results showed that the glucose is at the non-reducing terminus and is (1→6)-linked in the main chain (possibly evident by the absence of a signal at ~62 ppm in the ^{13}C NMR spectrum that would have been expected for C-6 of a non-6-substituted neutral hexose), and that there are two rhamnose units differing in their linkage, one being (1→2)-linked, the other being (1→3)-linked.

The remaining item of structural information, namely the order of the constituent sugars in the O-chain, was provided by Smith degradation. A (1→3)-linked rhamnose unit would not be cleaved by periodate, hence a glycoside of such a component would be expected. Indeed, a glycoside of rhamnose was obtained, mol. wt. ~200, from the Smith-degradation product after purification on Bio-gel P-2. Methylation analysis of this glycoside gave 2-O-acetyl-1,3-di-O-methylglycerol and 1,5-di-O-acetyl-6-deoxy-2,3,4-tri-O-methylmannitol. The anomeric configurations were deduced from the ^{13}C and ^{1}H NMR data as for the other O-chains. From these results, the structure of the type 6 O-chain repeating unit may be written as: 6)-β-D-Glcp-(1→2)-β-L-Rhap-(1→3)-β-L-Rhap-(1→.

Immunotype 7. — The type 7 O-chain, isolated conventionally, proved to be highly acid-resistant. The ^{1}H NMR spectrum showed three distinct anomeric resonances, two having very low $J_{1,2}$ values and one having a $J_{1,2}$ value of 6.9 Hz. Also observed was the signal at 2.31 ppm, indicative of the 2,3-imidazoline structure, as well as a signal for a 6-deoxy sugar and three acetyl-group signals. The ^{13}C NMR spectrum confirmed the presence of three anomeric centers, amino-substituted carbon atoms, acetamido groups, and 6-deoxyhexoses, as well as the imidazoline structure.

The polysaccharide proved to be so highly acid-resistant that solvolysis with anhydrous HF was performed at this point. After solvolysis by HF and purification on Bio-gel P-2, a high yield of a trisaccharide was

obtained which showed all of the NMR resonances observed for the polysaccharide. The most notable difference was in the peak for a 6-deoxyhexose, suggesting that this sugar was the reducing terminus. Reduction of the trisaccharide followed by periodate oxidation and subsequent reduction eliminated the 6-deoxy peak, but left (^{13}C-NMR) all of the signals for C-N groups. D-Fucosamine was isolated from the native polysaccharide by hydrolysis, paper chromatography, and optical-rotation measurement, and hence it was concluded that fucosamine was at the reducing terminus of this trisaccharide as a 3-O-linked species.

The alkaline reduction (during the Smith degradation) removed the imidazoline signals, suggesting that the imidazoline sugar component had been hydrolyzed to a 2,3-diacetamido sugar. The ^{13}C NMR spectrum of this product showed two anomeric resonances, and from the ^{1}H NMR spectrum of the carboxyl-reduced, peracetylated derivative of the disaccharide it was possible to conclude that both of the aldose residues had the ß configuration. Selective homonuclear decoupling allowed the relative configurations of both diamino sugars to be determined by using the proton assignments from the decoupling experiments together with the $J_{H,H}$, values. Both components proved to have the manno configuration. Hydrolysis, paper chromatography, and optical rotation measurements allowed the identification of D-fucosamine and 2,3-diacetamido-2,3-dideoxy-D-mannose as components; it was assumed that both manno residues present had the D configuration.

We were not able to determine precisely which of the two manno residues carried the imidazoline ring, or whether both residues were partially sustituted (which is unlikely from the clean anomeric signals observed). It may be possible, by using a series of phase-shifted trisaccharides in affinity-binding experiments with artificial trisaccharides, and antibodies raised against native polysaccharides, to determine which of the two manno species has the imidazoline ring, as the synthetic trisaccharide exhibiting the strongest binding should reflect the actual structure of the native O-chains.

Synthetic Studies

The synthetic studies were directed toward the total trisaccharide repeating-units of the immunotypes 2, 5, and 6 O-specific polysaccharides. We targeted the mono-, di-, and tri-saccharide level repeating units in all combinations for a complete study of the immunoaffinity and antibody-eliciting ability of these oligosaccharides. We hope to show that a disaccharide repeating-unit might be sufficient for the desired activities[24], so that synthesis of the remaining artificial antigens may be targeted at these smaller, more accessible structures.

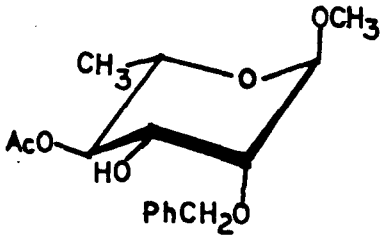
All of the oligosaccharides were targeted as their 8-methoxycarbonyl-octyl glycosides which, when converted into the corresponding carbonyl azides, may be readily coupled to proteins and to solid supports, as has been well documented.[24]

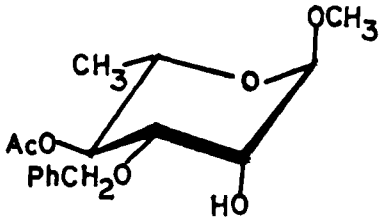
By the end of the report period we had finished syntheses of monosaccharide components for types 5 and 6. The availability of such a

set of selectively blocked monosaccharide derivatives will allow the rapid and efficient sequential coupling of the units to afford a set of all three corresponding trisaccharides, disaccharides, and monosaccharides, as their methoxycarbonyloctyl glycosides.

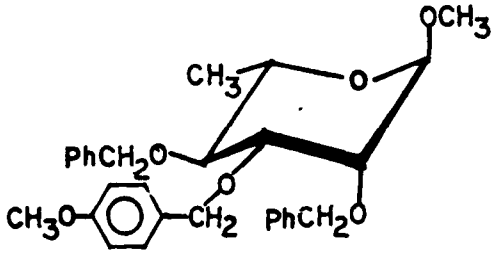
The methoxycarbonyloctyl glycoside not only allows for coupling of the artificial haptens to protein acceptors and solid-media supports, but also provides the spacer-arm requirement, which is an important part of the artificial antigen methodology.[26]

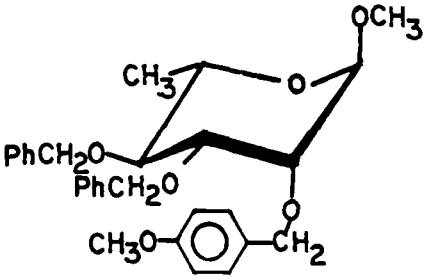
For the type 6 repeating sequence, three monosaccharide derivatives are necessary. The two protected rhamnose units are accessed by 2,3-benzylidenation of methyl α-L-rhamnopyranoside with α,α-dimethoxytoluene in DMF, followed by 4-O-acetylation and fractional crystallization of the exo- and endo-benzylidene isomers. Treatment of the exo- and endo-benzylidene acetals with HCl and sodium cyanoborohydride yields the illustrated 3-O-benzyl and 2-O-benzyl isomers, respectively.[27] Suitable choice of a third protecting group furnishes derivatives capable of being glycosidically coupled at either O-3 or O-2 as desired.





Ongoing work involves use of the corresponding p-methoxybenzylidene acetal[28], which has been shown to undergo the same acetal reductive ring-opening reaction as does the benzylidene acetal, affording the p-methoxybenzyl ether at position 2 or 3. Conventional benzylation with benzyl bromide and DMF then leads to the following derivatives:

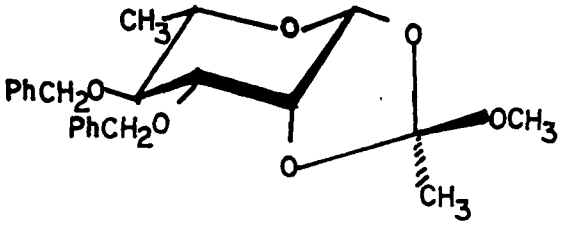




Following acetolysis, conversion into the pyranosyl halides, and coupling under conditions to give the desired β glycoside[29], the p-methoxybenzyl ethers may be cleaved selectively by the action of ceric ammonium nitrate.[30]

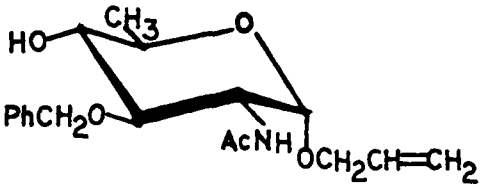
The 6-O-linked D-glucose component is introduced via the derivative 1,2,3,4-tetra-O-acetyl-6-O-triphenylmethyl-β-D-glucose[31], prepared in high yield and in a two-step, one-flask reaction. A certain proportion of the anomer is also obtained. The 6-O-triphenylmethyl group may be selectively cleaved by use of HBr--HOAc for 15 min, permitting selective coupling of the glucose residue through O-6.

The structures of the type 5 O-antigenic polysaccharides provided greater synthetic challenge. The 2-O-acetyl, 3-O-linked rhamnose unit is readily available from the 3,4-dibenzyl ether of L-rhamnose 1,2-(methyl orthoacetate).[32]



This derivative is readily converted into the corresponding 2-O-acetyl pyranosyl halide by the action of bromo- or chloro-trimethylsilane.[33] After coupling to give the desired α glycoside, the benzyl ethers are removed by the action of hydrogen and palladium. Coupling of the next unit can be expected to strongly favor O-3, as O-4 of rhamnose is known to be only weakly reactive.

The second component, N-acetylquinovosamine, is obtainable as follows. The allyl α-glycoside of 2-acetamido-2-deoxy-D-glucose is prepared and 4,6-benzylidenated. Subsequent benzylation of the free 3-OH group, followed by sequential hydrolysis of the benzylidene acetal, selective 6-O-mesylation, and reduction of the mesylate to the 6-deoxy sugar gives the indicated derivative, allyl-2-acetamido-3-O-benzyl-2-6-dideoxy-α-D-glucopyranose in high yield.[34]

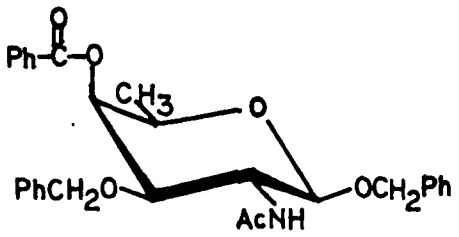


Choice of a suitable protecting group (which, because of the O-acetyl group required in the rhamnose moiety, may not be an ester) for O-4, and conversion of the allyl glycoside into a 1,2-oxazoline derivative, yields a quinovosamine derivative that can be coupled to furnish a β glycoside. Removal of the benzyl ether at O-3 will allow coupling to the next unit. Ongoing work is concerned with use of the p-methoxybenzyl ether as a protecting group for O-3 which, as previously mentioned, may be cleaved selectively by an oxidant; this methodology will allow us to use the normal benzyl ether at O-4 just prior to oxazoline formation.

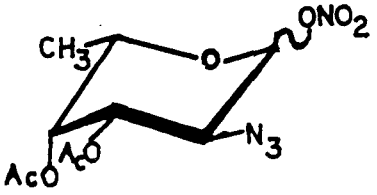
The third component of the type 5 O-chain is an L-galactose derivative. As yet we have no suitable source of L-galactose and have not yet been able to develop a practical route of access to this component. The general method of Lemieux[35], azidonitration of D-galactal, permits the preparation of an α-linked 2-amino galactose unit; we plan to use this route for the L-galactose enantiomer if a source of L-galactose can be

developed. Selective protection of this unit, and, finally, selective O-6 oxidation to the uronic acid[36], should permit preparation of the L-galactosaminuronic acid residue.

We have also synthesized appropriate derivatized monosaccharides for the type 2 O-chain. The illustrated derivative of D-fucosamine, selectively protected to allow coupling at O-3, has been prepared by a method developed in this laboratory.[37] This derivative of D-galactosamine was prepared in good net yield from the much less expensive D-glucosamine.

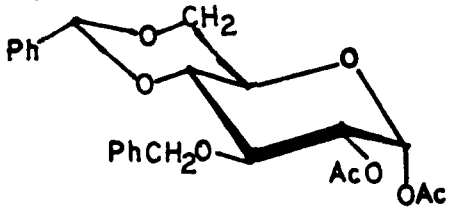


The next component, the L-fucosamine enantiomer, is available by a method already suggested for the L-galactose residue of type 5, namely by azidonitration of a glycal precursor.[35] Thus, on a small scale, we have taken 2,3-di-O-acetyl-L-fucal and treated it with ceric ammonium nitrate, and obtained the indicated 2-azido derivative in good yield.



This derivative, convertible by the action of lithium chloride into the 2-azidopyranosyl halide, will undergo coupling to yield only the α-linked product.

The final component for the type 2 O-chain is approached from 1,2;4,6-di-O-isopropylidene-α-D-glucofuranose. Benzylation of the free 3-OH group, followed by successive hydrolysis, benzylidenation, and acetylation gives the derivative shown, namely 1,2-di-O-acetyl-4,6-O-benzylidene-3-O-benzyl-β-D-glucopyranose in good yield.



The derived glycopyranosyl halide gives glycosides having the β configuration and these may be selectively deprotected to allow coupling at O-2.

V. COLLABORATIVE ARRANGEMENTS

Collaborative Arrangements: The arrangements with Warner-Lambert/Parke Davis set up under the earlier NIGMS grant support continued in effect during the work reported here; we were furnished at no cost with gram quantitites of the purified lipopolysaccharide antigens of the seven Fisher immunotypes. There were no obligations to Warner-Lambert/Parke Davis in respect to any results of research on these antigens, except for courtesy acknowledgment of the source in any publications.

The major collaboration was that established with Dr. Jerald Sadoff of the Walter Reed Army Institute of Research. Monoclonal antibody preparations for several of the Fisher immunotypes of *Pseudomonas* were produced in Dr. Sadoff's laboratory and were used in the preliminary binding studies to O-chain materials performed in our laboratory. A significant proposal emerged in Dr. Sadoff's suggestion that the protein—saccharide conjugates for the artificial antigens should include not only those with human serum albumin but also tetanus toxoid, and more recently *Pseudomonas* exotoxin A.[60]. This commercially available toxoid, in covalent conjugation with the *Pseudomonas* antigenic vaccine for simultaneous protection against both tetanus and *Pseudomonas* infections.[53] This would be a most welcome general advance in therapy, and one of particular relevance for military medicine.

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