

Bacterial Lipopolysaccharides

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Structure, Synthesis, and Biological Activities

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FOREWORD

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PREFACE

LIPOPOLYSACCHARIDES (LPS), WHICH ARE BIOLOGICALLY some of the most significant components of the outer membranes of Gram-negative bacterial cells, are currently the center of considerable research activity. Three aspects of LPS have become of particular interest in biomedical investigation: the immunochemistry of O-antigenic chains as a basis for the diagnosis of and vaccination against bacterial infections; the search for inhibitors of the biosynthesis of core structures, especially those containing 3-deoxy-D-manno-2-octulosonic acid (KDO), as potential anti-infective drugs; and the study of lipid A or its derivatives as prospective pharmacological agents in the therapy of immune disorders and cancer. The emphasis of this volume, as indicated in the title of the opening chapter by Lüderitz, is on recent progress in the chemical synthesis of partial structures representing the various domains of the LPS molecule. Also, the potential of these synthetic products and of modified, natural materials in preventive medicine and therapy is examined.

Following the introductory chapter, the four chapters of the second section deal with the structure, immunochemistry, and synthesis of O-antigens. Studies of LPS from *Pseudomonas* strains that lay a basis for the development of synthetic vaccines, synthesis of the repeating units of the *Shigella* O-antigens, the total synthesis of O-antigenic chains by block polymerization, and a review of experiments with artificial antigens prepared from fragments of *Salmonella* O-polysaccharides are included.

The third section is concerned with 3-deoxy-D-manno-2-octulosonic acid (KDO), a characteristic sugar found in cell surface structures of Gram-negative bacteria. The synthesis of model oligosaccharides of KDO and the significance of KDO-containing capsular materials in the pathophysiology of infections are discussed, as is the design of potential antibacterial agents based on the essential role of KDO in the biosynthesis of LPS.

Recent developments in the elucidation of the structure and biological activity of lipid A are outlined in the fourth section. Included is a discussion of chemically modified immunostimulatory lipid A derivatives lacking many or all of the toxic properties of the natural product.

The synthesis of active compounds of the lipid A class has received considerable attention recently. This attention is reflected in the four chapters in the last section of this volume. Each chapter presents a distinctive approach to the assembly of the fundamental lipid A structure, comprising a glucosaminyldisaccharide substituted with *N*- and *O*-fatty acyl and *O*-phos-

phono groups. A particular feature of lipid A chemistry is the presence of D-3-hydroxytetradecanoyl residues, and these as well as normal fatty acyl units are incorporated into the synthetic products. Linear schemes, convergent approaches, protection-deprotection sequences, and a special glycosidation reaction are discussed in relation to the synthesis of lipid A analogs.

We are grateful to Stephen Hanessian, whose initiative led to the organization of the symposium upon which this book is based; to all speakers, especially those who undertook considerable journeys to present their papers; and to N. K. Kochetkov and A. A. Lindberg, whose splendid contributions to this volume were prepared under considerable time pressure. Our thanks also go to Suzanne Roethel, Robin Giroux, Susan Robinson, and their colleagues, who have made collaboration with the ACS Books Department a genuine pleasure.

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Structural Principles of Lipopolysaccharides and Biological Properties of Synthetic Partial Structures

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Lipopolysaccharides form a class of macromolecules unique to Gram-negative bacteria. They are of great compositional and structural diversity, and yet, they are built up according to a common architectural principle. With few exceptions, lipopolysaccharides are biologically highly active substances. Lipopolysaccharides have been called endotoxins because of their toxic properties. Due to the antigenic properties which they confer to the surface of the bacterial cell, they are also termed O antigens.

In the last decades, structural details of lipopolysaccharides derived from selected bacterial serotypes have been elucidated, and it became possible to ascribe distinct biological properties to distinct domains of the macromolecule. As a consequence, a number of organic chemists have undertaken the chemical synthesis of biologically relevant partial structures of lipopolysaccharide.

The present paper will describe the principles of lipopolysaccharide architecture, summarize analytical results concerning different regions of the molecule, and refer to synthetic approaches to partial structures.

General Architecture of Lipopolysaccharides

Figure 1 represents the general structure of Salmonella lipopolysaccharides. They contain an external polysaccharide, the O-antigenic chain, and an innermost component, termed lipid A. O-chain and lipid A are linked to each other by an oligosaccharide referred to as the core.

O-Specific Chains. As indicated in Figure 1, O chains are in general made up of repeating units of di-, tri-, or higher oligosaccharides. In rare cases the O-chain is a homopolysaccharide. The structure of the O-chain is unique to each bacterial serotype; great diversity is encountered in the structures of O-chains.

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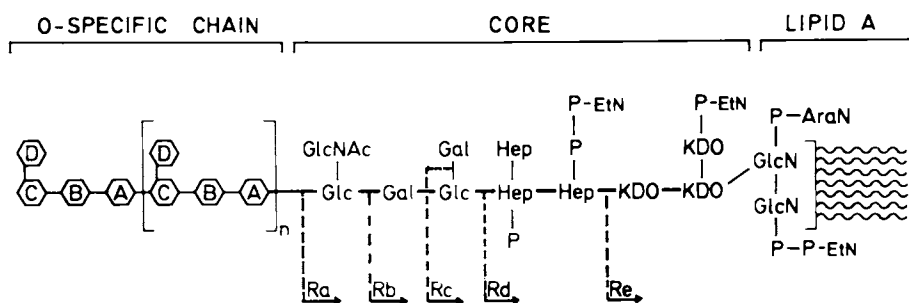


Figure 1. General structure of a Salmonella lipopolysaccharide. Key: A-D, sugar residues; Glc, D-glucose; Gal, D-galactose; GlcN, D-glucosamine; GlcNAc, N-acetyl-D-glucosamine; Hep, L-glycero-D-manno-heptose; KDO, 2-keto-3-deoxy-D-manno-octonate; AraN, 4-amino-L-arabinose; P, phosphate, EtN, ethanolamine; ~, hydroxy and nonhydroxy fatty acids; and Ra to Re indicated are incomplete R form lipopolysaccharides. The structure of the KDO oligosaccharide is presently being reinvestigated (H. Brake, unpublished results).

Table I
Constituents Identified in Lipopolysaccharides*

<u>Neutral Sugars</u>	
Pentose (5)	
4-Deoxypentose (1)	
Pentulose (1)	
Hexose (3)	
6-Deoxyhexose (7)	
3,6-Dideoxyhexose (5)	
Hexulose (1)	
Heptose (4)	
6-Deoxyheptose (1)	
Heptulose (1)	
<u>Acidic Sugars</u>	
Hexuronic acid (2)	
2-Amino-2-deoxyhexuronic acid (3)	
3-Deoxyoctulosonic acid (1)	
3-O-Lactyl-6-deoxyhexose (2)	
4-O-Lactylhexose (1)	
2,3-Diamino-2,3-dideoxyhexuronic acid (3)	
2,3-(1-Acetyl-2-methyl-2-imidazolino-5,4) D-hexuronic acid (1)	
<u>O-Methylated Sugars (30)</u>	
<u>Amino Sugars</u>	
4-Amino-4-deoxypentose (1)	
2-Amino-2-deoxyhexose (3)	
2-Amino-2,6-dideoxyhexose (4)	
3-Amino-3,6-dideoxyhexose (2)	
4-Amino-4,6-dideoxyhexose (3)	
2,3-Diamino-2,3-dideoxyhexose (1)	
2,4-Diamino-2,4,6-trideoxyhexose (2)	
2-Amino-2-deoxyheptose (1)	
<u>Non-Sugar Constituents</u>	
2,4-Dihydroxybutanoic acid	
Glycine	
Alanine	
Lysine	
Pyruvic acid	
Ethanolamine	

* (): Number of isomers identified. For references and details see Kenne and Lindberg, 1983; Rietschel et al., 1983.

Table II

Bacterial Serotypes with Known O-Chain Structures *

<u>Salmonella</u>	(19 strains)	<u>Shigella flexneri</u>	(9)
<u>E. coli</u>	(13)	<u>Sh. dysenteriae</u>	(9)
<u>Citrobacter</u>	(2)	<u>Sh. boydii</u>	(2)
<u>V. cholerae</u>	(1)	<u>Sh. sonnei</u>	(1)
<u>Proteus</u>	(1)	<u>Klebsiella</u>	(10)
<u>Pseudomonas</u>	(8)	<u>Y. pseudotuberculosis</u>	(1)
		<u>Y. enterocolitica</u>	(1)
		<u>Serratia</u>	(2)

* For structures of the O chains and for references see Kenne and Lindberg, 1983; Rietschel et al., 1983.

O-Chains (and core structures) contain many unique or unusual sugar constituents and, in recent years, many members of different sugar classes have been identified in lipopolysaccharides (Table I).

As the result of the application of new technologies in the analysis of lipopolysaccharides, the structures of an increasing number of O-chains are elucidated mainly in the laboratories of B. Lindberg, Stockholm, and N.K. Kochetkov, Moscow (Table II); presumably, in the near future, most O-chain structures of biological and clinical interest will be known. Recently, a number of O-chain partial structures have been chemically synthesized (Table III). It must be kept in mind, however, that these oligosaccharides do not necessarily represent "biological" repeating units (D-C-B-A in Figure 1), but in some cases correspond to "chemical" repeating units (A-(D-)C-B in Figure 1; Lüderitz et al., 1966). The latter ones would express antigenic specificities differing in part from those of the natural O-chain.

This discrepancy is illustrated by the results of a recent study by A.A. Lindberg and his colleagues (Svenson and Lindberg, 1981). These authors have chemically synthesized the terminal disaccharide abequose-mannose of the natural (biological) repeating unit abequose-mannose-rhamnose-galactose of the Salmonella typhimurium O-chain (expressing O-factors 4 and 12). The disaccharide was then converted into an immunogen by linking it to the protein carrier, bovine serum albumin (BSA). This artificial antigen was compared to analogous, BSA-coupled oligosaccharides which had been obtained through specific cleavage of the Salmonella typhimurium O-chain by a bacteriophage

Table III**Synthetic O-Specific Structures**

<u>Salmonella anatum</u>	Trisaccharide	Kochetkov et al., 1975
<u>Salmonella senftenberg</u>	Tetrasaccharide	Kochetkov et al., 1977
<u>Salmonella muenster</u>	Tetrasaccharide	Kochetkov et al., 1980
<u>Salmonella strasbourg</u>	Pentasaccharide	Kochetkov et al., 1980
<u>Shigella flexneri</u>	Tetrasaccharide	Bundle and Josephson, 1980
<u>Salmonella newington</u>	Trisaccharide	Betaneli et al., 1980
	Di-, Tri-, Oligomers ^{a)} of Trisaccharide	Kochetkov et al., 1981 Dimitriev et al., 1982
<u>Salmonella</u> , groups A,B,D	Disaccharides ^{b)}	see: Jörbeck et al., 1979
<u>Shigella dysenteriae</u>	Pentasaccharide	Paulsen and Bünsch, 1981
<u>E. coli 075</u>	Tetrasaccharide	Paulsen and Lockhoff, 1981

a) These oligosaccharides represent multiples of biological repeating units.

b) Tetra, octa, and dodecasaccharides have been prepared from Salmonella typhimurium lipopolysaccharide by specific degradation of the O chain with phage enzyme. These oligosaccharides contain 1,2, or 3 chemical repeating units (Svenson and Lindberg, 1981).

enzyme acting as an endo-rhamnosidase. The oligosaccharides thus produced represent mono- and oligomers of the chemical repeating unit galactose-(abequose-)mannose-rhamnose.

When rabbits were immunized with these antigens and the antisera analyzed with S. typhimurium lipopolysaccharide as the test antigen, high titers were observed with the di-, octa-, and dodecasaccharide antigens, while, as expected, the tetrasaccharide antigen had induced a serum of low titer. The authors showed also that the transfer of the antisera raised by immunization with the di- and octasaccharide antigens (but not those raised against the tetrasaccharide antigen) conferred specific protection to mice challenged with a 100-fold LD50 of S. typhimurium bacteria (Svenson and Lindberg, 1981).

Artificial antigens containing immunodeterminant structures of O-antigens represent potent non-toxic immunogens that induce high-titer antisera which may be used for diagnostic purposes and which may have the capacity to protect specifically against bacterial infections.

The Core. Core oligosaccharides are structurally less diverse than O-chains. The core oligosaccharide shown in Figure 1 is common to many or all Salmonella serotypes. Distinct core types have been

identified in other enterobacterial genera, but in their structural make up they resemble each other, as well as the Salmonella core, in their main features (Jansson et al., 1981).

As shown in Figure 1, the Salmonella core contains adjacent to the O-chain a branched oligosaccharide with N-acetyl-D-glucosamine, D-glucose, and D-galactose units, and adjacent to lipid A a branched oligosaccharide with L-glycero-D-manno-heptose and 2-keto-3-deoxy-D-manno-octonate (KDO) units. Most of the linkages have been determined but some uncertainty remains about the structure of the KDO region which is presently under renewed investigation (H. Braude, unpublished results), and the anomeric configuration of the KDO units. The core oligosaccharide is substituted by phosphoryl, pyrophosphoryl, and ethanolamine residues and expresses a negative net charge.

In Figure 1, the structures of the Ra to Re lipopolysaccharides have been indicated. These incomplete lipopolysaccharides which are devoid of O chains and parts of the core are formed by R mutant strains which are defective in the synthesis of the O chain (Ra) or of the core (Rb-Re) (reviewed by Lüderitz et al., 1982). Ra to Re lipopolysaccharides represent intermediates in lipopolysaccharide biosynthesis. They express distinct serological specificities since the respective terminal sugar units act as the main immunodeterminant. The different R lipopolysaccharides and the respective R mutants, therefore, can be differentiated by serological methods.

In contrast to the O chains which are specific for individual serotypes, the R lipopolysaccharides, especially those made by mutants with deeper defects, represent structures which are common to many Gram-negative bacteria, certainly to all Enterobacteriaceae. Therefore, R antibodies recognize common structures in enterobacterial lipopolysaccharides.

Braude (Braude et al., 1977; Ziegler et al., 1981) and McCabe (McCabe, 1972; McCabe et al., 1977) and their colleagues were the first to ask the question whether such R antisera would be effective in protecting animals against experimental infections with organisms differing in O specificity, in contrast to O antisera which would protect only against the homologous organism. In fact, these authors could show that rabbit or human antisera against Rc and Re mutant lipopolysaccharides (from E. coli) are capable of cross-protecting mice infected with E. coli, Klebsiella, or Pseudomonas bacteria.

Until now, R-specific determinants or carrier linked artificial antigens of the Rc to Re types have not been synthesized, although their availability might prove most useful. In contrast to killed bacteria which are presently used for the preparation of antisera, synthetic immunogens would be non-toxic. Further, one would be able to investigate mono-specific sera and to identify those R determinants which are most effective in protection.

A technique for the preparation of semi-synthetic R antigens has been worked out by Galanos and Lehmann (described in Lüderitz et al., 1973; Galanos et al., 1977; Galanos and Nerkar, unpublished results). The principle of the method (Figure 2) is based on the finding that

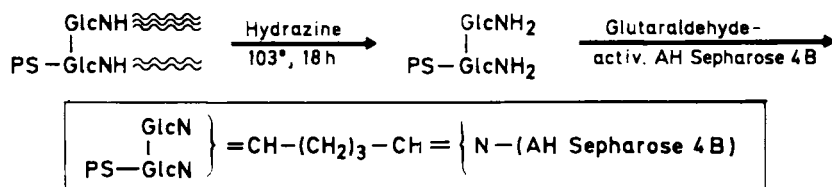


Figure 2. Specific immunoabsorbents for Salmonella O and R form antibodies. Key: PS, O-specific polysaccharide, core, core fragment; and GlcNH₂, glucosamine and fatty acids. Specific immunoabsorbents for lipid A antibodies have also been prepared (C. Galanos and D. Nerkar, unpublished results; Lüderitz et al., 1982).

extensive hydrazine treatment of an R form lipopolysaccharide will remove the long chain fatty acids from the lipid A part and yield a product which still contains the respective core fragment attached to the glucosamine disaccharide of the lipid A backbone. The free amino groups of the glucosamine residues are used to link the antigen via glutardialdehyde to a carrier (a protein for the preparation of immunogens, AH Sepharose for the preparation of immunoadsorbents). These systems have proven very useful for the preparation and purification of R-specific antibodies.

Lipid A. All R form lipopolysaccharides as well as free lipid A (obtained by mild acid cleavage of the KDO linkage) represent potent endotoxins, comparable in activity to complete lipopolysaccharides. This shows that lipid A represents the component of lipopolysaccharides which is responsible for its endotoxic properties.

Structural analyses of *Salmonella* lipid A have revealed the formula given in Figure 3. It contains a diphosphorylated, β 1,6-linked D-glucosamine disaccharide, the so-called lipid-A-backbone, which is partly substituted by 4-amino-L-arabinose and phosphorylethanolamine. In the lipopolysaccharide, the KDO of the core is linked to a hydroxyl group of the non-reducing glucosamine (whether it is that at carbon 3, as determined previously, is now uncertain; E.Th.Rietschel et al., unpublished data). The lipophilic character of lipid A is provided by 7 long chain fatty acyl residues. Two (R)-3-hydroxytetradecanoyl residues which are O-substituted by dodecanoyl and hexadecanoyl residues are linked to the aminogroups of the two glucosamine units. Two further (R)-3-hydroxytetradecanoyl residues, being O-substituted to 50% by tetradecanoic acid, are ester-linked to two, so far unidentified hydroxyl groups of the two glucosamine units. It is obvious that lipid A exhibits a high degree of heterogeneity similarly to the other regions of lipopolysaccharides (for details see the following article of Rietschel et al.).

Investigations on lipid A's of different origin have revealed structural similarities as well as dissimilarities among different lipid A's which in some cases influence certain biological activities (Rietschel et al., 1983; Lüderitz et al., 1978). Indications concerning minimal structural prerequisites for some activities could thus be evaluated. Recently, a lipid A precursor molecule which accumulates in KDO-defective *Salmonella* mutants has been isolated and its structure identified (Lehmann, 1977). This incomplete lipid A lacks 4-aminoarabinose, phosphorylethanolamine, and the non-hydroxylated fatty acids do-, tetra-, and hexadecanoic acid. Nevertheless, this molecule expresses most of the biological activities of complete lipid A (Lüderitz et al., 1978). This structure, therefore, may serve as a guide for those groups who have undertaken the chemical syntheses of lipid A (Inage et al., 1980; Kiso et al., 1981; Nashed and Anderson, 1981; van Boeckel et al., 1982).

A number of lipid A analogues that have been synthesized by Shiba and Kusumoto and their colleagues in Osaka (for details see the following article by Shiba et al.) have been tested in Freiburg for biological

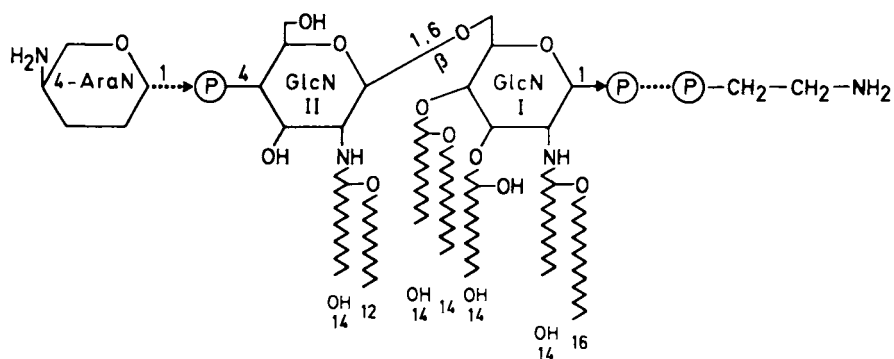


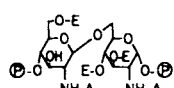
Figure 3. Proposed structure for *Salmonella* lipid A. See Key for Figure 1, and OH-14, 3-hydroxytetradecanoic acid; 12, 14, 16, dodeca-, tetradeca-, hexadecanoic acid. The distribution of tetradecanoic acid on the two 3-hydroxytetradecanoic acids, as well as the distribution and positions of the two ester-bound 3-hydroxytetradecanoic acids, is not known.

activities in comparison to natural lipid A (Tanamoto et al., to be published). These preparations represent derivatives of the β 1,6-linked glucosamine disaccharide which carries acyl and phosphoryl residues as indicated in Figure 4. Most of the preparations were insoluble in water and could not be tested directly. However, they were rendered soluble by conversion into succinyl derivatives. It had been shown previously that the introduction of carboxyl groups into Re lipopolysaccharide by succinylation would not greatly affect the biological properties (Rietschel et al., 1971).

The following test systems were employed. For the determination of serological cross reactions with free lipid A from *Salmonella*, the preparations were tested either attached to erythrocytes in the passive hemolysis test with anti-lipid A antiserum or as inhibitors in the passive hemolysis test with *Salmonella* lipid A-coated erythrocytes and anti-lipid A antiserum, both in the presence of complement (Galanos et al., 1971b). Lethal toxicity was determined in galactosamine-sensitized C57BL/6 mice (Galanos et al., 1979) and pyrogenicity in rabbits (Watson and Kim, 1963). Mitogenic activity of the preparations toward mouse spleen cells was determined by thymidine incorporation (Anderssen et al., 1973), and complement inactivation was tested as previously (Galanos et al., 1971a). The preparations were also tested for hemolytic activity by incubation with sheep red blood cells at 37°C over several hours.

The results of Figure 4 show that the synthetic preparations tested exhibit serological cross reactivity with *Salmonella* lipid A antiserum. The inhibitory capacity was found to be of the same order as that of natural free lipid A. Lipid A specificity could also be demonstrated in the direct passive hemolysis test using red blood cells coated with synthetic preparations and anti-*Salmonella* lipid A antiserum. It was also found that O-deacylation of the preparations like with natural lipid A did not diminish but rather increase reactivity. The results showed that phosphate substitution of the backbone is not a prerequisite for expression of antigenicity, and that the amide-bound 3-hydroxytetradecanoic acid can be replaced by tetradecanoic acid without loss of inhibitory reactivity in the lipid A system. The results with the synthetic preparations are in good agreement with previous results obtained with degradation products of natural lipid A, which had indicated that the immunodeterminant structure of lipid A includes the amide grouping of the amide-linked acyl residue, and part of the glucosamine residues.

Figure 4 shows that some of the synthetic preparations exhibit lethal toxicity and/or pyrogenicity. Compared to lipid A, however, the effective doses of the synthetic preparations for lethality and pyrogenicity were ca. 1000 and 100 times higher. Obviously, there is a separation of activities: Preparation 302 is pyrogenic, but not lethal, while preparations 314 and 315 are lethal, but not pyrogenic. Furthermore, preparation 316, which could be directly solubilized in water, was toxic to galactosamine-treated mice in a dose of 50 µg/mouse. With this dose, a low monophasic fever curve was seen in rabbits. In contrast,



		4 304	5 305	2 302	14 316	10 311	12 312	11 314	13 315
	E:	14	14	14	14	OH-14	OH-14	OH-14	OH-14
	A:	14	14	OH-14	OH-14	OH-14	OH-14	14-O-14	14-O-14
	P:	4P	1,4P		1P		4P		4P
Antigenicity	Orig.	●	●	—					
	Succ.	●	●	●					
Lethality	Orig.	—	—	—	●	—	—	●	●
	Succ.	—	—	—	●	—	—	●	●
Pyrogenicity	Orig.	—	—	—	●	●	—	—	—
	Succ.	—	—	●	●	●	—	—	●
Mitogenicity	Orig.	—	—	—	●	—	—	—	—
	Succ.	—	—	●	—	●	—	●	●
Hemolysis	Orig.	—	—	—	●	●	●	●	—
	Succ.	—	—	●	●	●	●	●	—
Complement Activity	Orig.	—	—	—	—	—	—	—	—
	Succ.	●	—	●	—	●	—	—	—

Figure 4. Biological activities of synthetic lipid A analogues. The designations of the preparations are as in Chapter 11.

the succinylated preparation 316 was of lower toxicity, but in a dose of 50 μ g gave rise to a typical biphasic fever increase of 1.9°C.

As shown in Figure 4, some preparations exhibit mitogenic activity for mouse B lymphocytes, and anti-complementary activity. Adjuvant activity of preparation 305 has been demonstrated by Yasuda et al. (1982) using a liposomal system.

In summary, it was found that some of the synthetic preparations definitively exhibit endotoxic activities. But in all cases, except for antigenicity, the activities, on a weight basis, were much lower than those observed with natural lipid A.

Many questions can be posed, but presently no clear cut answers can be given regarding structure/activity relationships. Only for one biological activity, antigenic specificity, structural requirements could be identified. It should be noted, however, that so far none of the structures of the synthetic preparations is identical with that proposed for natural lipid A.

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Lipopolysaccharide Antigens of *Pseudomonas aeruginosa*

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Our laboratory has been concerned with the structural characterization of the antigenic lipopolysaccharides associated with the outer membrane of the common Gram-negative organism *Pseudomonas aeruginosa* (1-5). This organism is of very widespread occurrence and normally presents no hazard to healthy human subjects whose immune-response capacity is high (6). However, it is an opportunistic pathogen; certain virulent strains cause progressive and frequently lethal infections that respond poorly to conventional antibiotic therapy in subjects having impaired immune-response capacity. Such individuals include especially victims of severe burns and other injuries, newborn infants, cystic fibrosis patients, patients undergoing radiation therapy or cancer chemotherapy, and individuals under deliberate immunosuppression for the purpose of organ transplants (6,7). Attempts have been made, with some measure of success, to produce vaccines (8,9) from extracts of the organism, but the use of these suffers from a number of drawbacks, in particular because of the multiplicity of different strains identifiable by conventional serotyping procedures. It is difficult to assure obtaining cross-protection by antibody raised to the antigen of one strain against the antigen of a different strain.

There have been numerous efforts to classify the various serotypes of *Pseudomonas aeruginosa*, and a significant advance was made by Fisher (10) on the basis of challenge studies in mice. He demonstrated that the antibodies raised against the antigens of seven selected serotypes of the organism afforded cross-protection, when used in combination as a heptavalent vaccine, against a wide range of strains of the organism.

The vaccine formulation comprises a mixture of the outer-membrane lipopolysaccharides from bacteria of the seven different serotypes. The lipopolysaccharides may be obtained from cultures of each of the seven types by the standard procedure of Westphal (11), involving phenol-water extraction of the cells. However, for large-scale purposes, the older procedure of Boivin, (12) involving extraction of the cells with trichloroacetic acid, was

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considered more satisfactory. The two procedures do not lead to identical lipopolysaccharide preparations and the Boivin procedure gives material containing additional fatty acid constituents not present in preparations isolated by the phenol-water procedure.

Following the well-established pattern of structural organization in the lipopolysaccharides of Gram-negative bacteria, (13) it may be expected that these materials should consist of high-molecular-weight molecules containing three discrete subunits, bound together by covalent linkages that are very labile to mild acid treatment. Thus, treatment of each of the lipopolysaccharides with hot, 1% acetic acid leads to liberation of a chloroform-extractable precipitate recognizable as lipid A, and the production of lipid-free, water-soluble materials containing products of low molecular weight, an oligosaccharide component of molecular weight ~2,000, identified as the core oligosaccharide, together with a polysaccharide having molecular weight in the range of 20,000, termed the O-polysaccharide. It is probable that the unfavorable toxic response elicited by these antigens is associated with the lipid A component, and an aspect of our study involves approaches to antigenic formulations that could elicit the production of protective antibodies without significant associated toxicity.

Work at the outset focused on physical and chemical characterization of the whole lipopolysaccharide antigens of each of the seven Fisher immunotypes. All give opalescent solutions in water and have very high apparent molecular weights. Hydrolysis by 1% acetic acid for a period of time sufficient to liberate the lipid A gave this material as 10-20% by weight of the entire lipopolysaccharide. Hydrolysis under more-vigorous conditions, followed by analysis for various constituents, as displayed in Table I, showed all of the lipopolysaccharides to

Table I. Composition of the Lipopolysaccharide Antigens of *Pseudomonas aeruginosa* (Relative Percentages)

Immunotype	Neutral Sugars	Amino Sugars	Lipid A	KDO	Protein	Phosphate
1	15	17	16	5	4	3
2	18	22	16	4	5	2
3	12	18	11	4	8	3
4	14	27	19	4	3	2
5	17	14	13	5	7	2
6	18	11	13	4	3	3
7	14	14	8	5	3	4

contain neutral sugars, amino sugars, a component reacting as 3-deoxy-D-manno-octulosonic acid, together with material reacting as protein, and inorganic phosphate. Although the individual

immunotypes show somewhat different quantitative compositions in this analysis, the observed values demonstrate no immediate and noteworthy differences that could be used as diagnostic chemical means of identifying the lipopolysaccharide of an individual immunotype.

Comparison of the whole lipopolysaccharide antigens of the seven immunotypes by phosphorus-31 n.m.r. spectroscopy (5) does provide a tool for differentiating the seven types, as shown in Figure 1, where it may be seen that each individual immunotype gives a characteristic ^{31}P -n.m.r. profile that could be used as a diagnostic reference for the lipopolysaccharide of that individual immunotype. The ^{31}P -n.m.r. spectra have also been used to glean information as to the chemical nature of the phosphate groups in the lipopolysaccharides (5,14-16).

The most noteworthy differentiation of the lipopolysaccharides of the seven antigens is evident upon comparison of the different components present in the amino sugar fraction, as shown in Table II. All of the antigens contain 2-amino-2-deoxy-D-glucose,

Table II. Amino Sugar Composition of the Lipopolysaccharide Antigens of *Pseudomonas aeruginosa* (Relative Percentages)

Immunotype	Quinovosamine	Fucosamine	Glucosamine	Galactosamine
1	-	26	49	25
2	-	81	11	8
3	4	39	28	29
4	10	55	12	23
5	60	-	25	15
6	-	60	23	17
7	-	38	38	24

and this sugar is located uniformly in the lipid A component. In addition, all of them contain 2-amino-2-deoxy-D-galactose, which is the most common constituent of the core oligosaccharide. Especially noteworthy is the incidence of the aminodideoxy sugars 2-amino-2,6-dideoxygalactose (fucosamine) and 2-amino-2,6-dideoxyglucose (quinovosamine). The analytical profiles of these aminodideoxy sugars alone provide a ready means of differentiating between the immunotypes. Thus type 5 is characterized by a high content of quinovosamine and the absence of fucosamine, whereas the reverse is true for type 2. Furthermore, certain of the immunotypes also contain amino uronic acids.

Analyses of the lipid A components show a general similarity of composition between the lipid A material isolated from each of the seven immunotypes. All contain a β -D-(1 \rightarrow 6)-linked 2-amino-2-deoxy-D-glucose disaccharide (17,18), substituted at both amino groups and at most of the hydroxyl groups by 3-hydroxy fatty acyl chains, and phosphate ester groups are present at O-1 and O-4'.

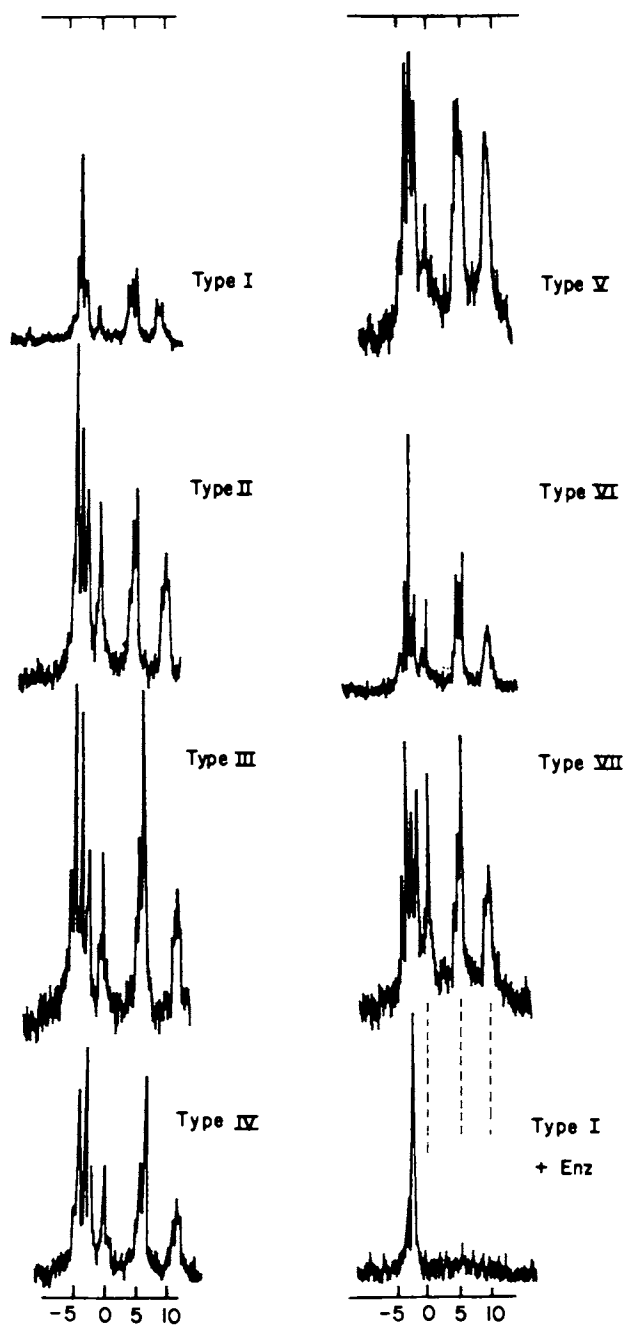


Figure 1. Phosphorus-31 NMR profiles of the seven Fisher immunotypes and that of the type I lipopolysaccharide pretreated with alkaline phosphatase. (Reproduced with permission from Ref. 5. Copyright 1981, Biochem. Biophys. Acta.)

The amino pentose identified (14) in the lipid A constituents of several other species of Gram-negative bacteria was not encountered in these *Pseudomonas* lipopolysaccharides.

The structures of the core oligosaccharides have not been worked out in detail, but the analytical compositions (Table III)

Table III. Analytical Data for the Core Oligosaccharide of *Pseudomonas aeruginosa* (Relative Percentages)

Immunotype	Yield from LPS	Alanine	Rhamnose	Glucose
1	10-16	1.7	6	42
2	16-18	1.4	3	36
3	16-17	4.6	5	59
4	20-21	6.0	6	33
5	15-17	2.1	4	33
6	25-26	1.0	4	24
7	30-33	1.7	4	50

Immunotype	Heptose	Galactosamine	Fucosamine	Quinovosamine
1	10	1	-	-
2	11	3	13	-
3	11	6	trace	trace
4	8	4	2	1
5	9	2	-	-
6	6	1	7	-
7	4	2	trace	-

indicate each to contain glucose, rhamnose, 2-amino-2-deoxy-galactose, heptose, and the amino acid L-alanine. It may be noted that aminodideoxy sugars are essentially absent from the core of all immunotypes except for type 2 and type 6. The presence of fucosamine in the core oligosaccharide of type 2 is of significance, as will be evident later.

The lipid material precipitated upon mild acid treatment of the Boivin-extracted lipopolysaccharide is here termed a lipoidal precipitate. The fatty-acid profile (Table IV) of hydrolyzates of this material shows little variation between the seven immunotype strains. If the original lipopolysaccharides are first treated by phenol-water extraction, and the resultant materials then subjected to hydrolysis to release the lipid, the composition of the latter is significantly different; it corresponds closely to the classic composition expected for lipid A. It is noteworthy that material extracted by the phenol-water (Westphal) method is rich in the C₁₂ saturated acid and in the hydroxy fatty acids having ten and twelve carbon atoms, whereas the C₁₆ and C₁₈ saturated acids present in the lipoidal precipitate, as prepared by the Boivin procedure, are absent or present at much lower levels in the lipid prepared by the Westphal procedure (19). It

Table IV. Analytical Composition of the lipoidal precipitates of the lipopolysaccharides of Pseudomonas aeruginosa (19)

% Composition of the lipoidal precipitate, by weight	Type 2 Lipid A only ^a							
	1	2	3	4	5	6	7	
Amino sugar	9.2	9.5	9.7	9.5	9.4	9.6	9.7	18.6
Total fatty acids	71.3	68.5	61.8	72.9	70.0	65.3	69.1	65.0
Hydroxy fatty acids	21.5	19.4	16.5	25.8	22.3	20.0	22.6	41.4
Phosphorus (as PO ₄)	5.2	5.3	5.7	5.0	4.9	6.0	5.8	--
Protein	8.6	7.2	6.7	12.3	9.4	7.3	10.7	--
Individual fatty acids								
dodecanoic	8.6	10.7	6.9	11.9	10.6	13.2	12.6	16.9
hexadecanoic	15.1	13.6	14.1	13.3	15.1	11.0	13.7	5.3
octadecanoic	11.0	11.4	9.1	5.4	8.2	7.5	6.7	trace
3-hydroxydecanoic	5.6	3.9	3.5	6.5	5.1	4.2	5.4	8.5
3-hydroxydodecanoic	9.2	9.7	8.1	12.1	11.1	10.0	10.4	20.2
2-hydroxydodecanoic	6.7	5.8	4.9	7.2	6.5	8.4	6.8	12.8

^aAs obtained by phenol-water extraction of the lipoidal precipitate.

is probable, although the point has not been established unequivocally, that the lipoidal precipitate contains additional lipid material from the cells, physically entrapped in the lipopolysaccharide preparations.

O-Chain Sequence Studies

Structural studies have been conducted in some detail on the antigens of three of the immunotypes and these results are reported here.

Immunotype 2. Initial work on characterization of the amino sugar isolated from the whole antigen of Fisher immunotype 2 led to a product identifiable as fucosamine, but having an anomalous optical rotation; this was positive, but not sufficiently high as to correlate with the D enantiomer known in the literature. This behavior was subsequently shown to arise from the fact that the fucosamine present in the core oligosaccharide is the D enantiomer but that present in the O-chain component is an equal mixture of the D and L forms. The O-chain material was isolated from the intact lipopolysaccharide in 25% yield as a strongly levorotatory polysaccharide having molecular weight 14,000, as measured by ultracentrifugation, and it contained glucose and fucosamine in 1:2 proportion (Table V). As expected, the glucose was the D

Table V. Physical Properties of *Pseudomonas aeruginosa* Immunotype 2 O-Antigenic Polysaccharide

Yield from LPS	25%
Specific rotation	-70° (H ₂ O)
Molecular weight	14,000 ±500
Sugar Content	
Glucose	29%
Fucosamine	71%

enantiomer, but the fucosamine (N-acetylated in the polysaccharide) was optically inactive and constituted an equimolar mixture of the D and L forms. Methylation linkage analysis gave rise to 3,4,6-tri-O-methylglucose and the 4-methyl ether of fucosamine as the principal fragments, indicating that the glucose residues are linked at their 2-positions, and the fucosamine residues through their 3-positions (Figure 2). These data point to a trisaccharide repeating unit for the type 2 O-polysaccharide, but it was necessary to perform a series of successive Smith degradations to establish the specific order of the units. One cycle of periodate oxidation followed by borohydride reduction and subsequent hydrolysis by very mild acid led to loss of the glucose residues, substantiating the methylation data that had established linkage through O-2 of D-glucose (Figure 3). A disaccharide glycoside containing one residue of D-fucosamine, 1 of

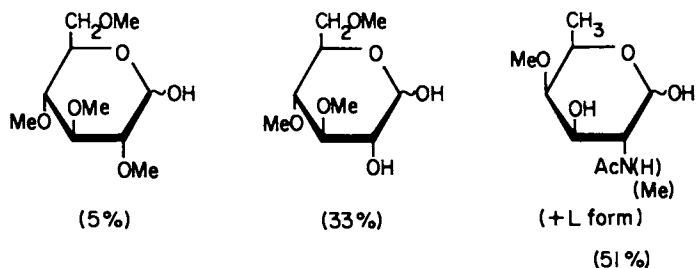


Figure 2. Methylation fragments from *Pseudomonas aeruginosa* type 2 O-chain.

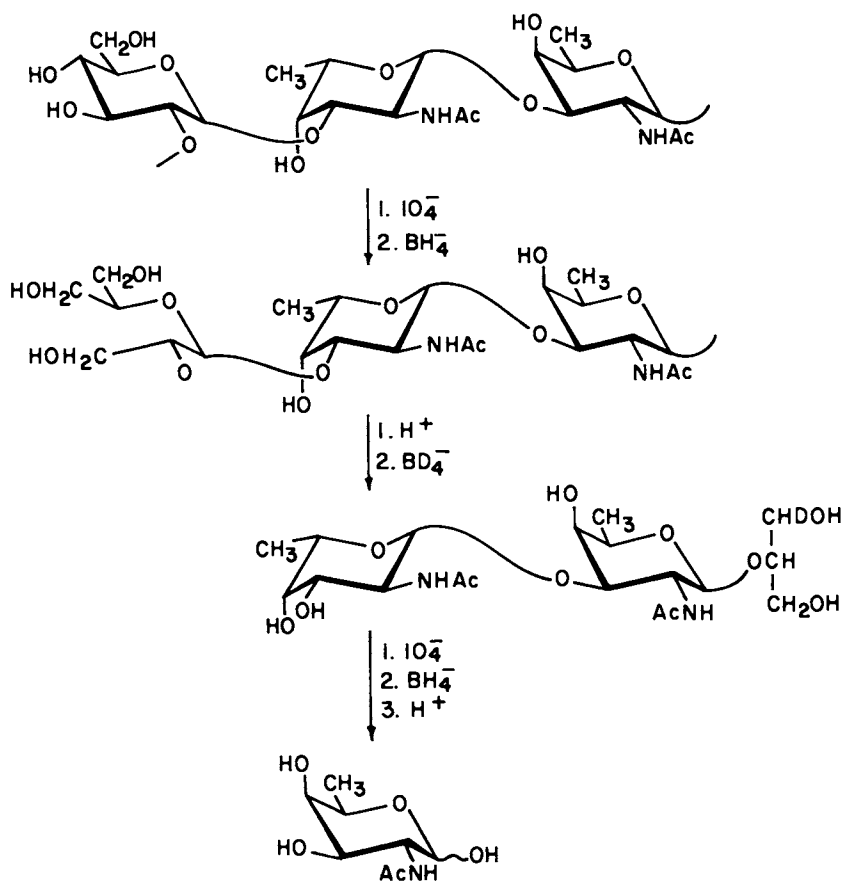


Figure 3. Smith degradation of the type 2 O-chain.

L-fucosamine, and a glycerol residue was isolated and its constitution supported by chemical-ionization mass spectrometry (Figure 4). A second iteration of the Smith degradation cycle led to a loss of the L-fucosamine and retention of the D-fucosamine constituent. From this information it is possible to formulate the repeating sequence as being $\rightarrow 2$ -D-Glcp-(1 \rightarrow 3)-L-FucpNAc-(1 \rightarrow 3)-D-FucpNAc. The formation of a small proportion of 2,3,4,6-tetra-O-methyl-D-glucose in the methylation analysis suggests that glucose is probably the non-reducing terminus of the chain.

The structure thus established (18) for the repeating unit was further consolidated, especially as regards the anomeric linkages, by high resolution proton and carbon-13 n.m.r. spectroscopy (Figures 5 and 6), where inspection of the anomeric-signal regions provided strong indication that the glucose residues are β -linked, the L-fucosamine residues α -linked, and the D-fucosamine residues β -linked. These attributions are evident from the spin couplings of the anomeric proton signals, and the non-equivalence of their acetyl and C-methyl group signals in the proton spectrum, and by similar peak multiplicity in the carbon-13 spectrum.

The same linkage sequence has been recently described for the O-polysaccharide of the lipopolysaccharide of Lanyi strain 0:7a,b of *Pseudomonas aeruginosa*; this strain is possibly identical to Fisher type 2 (20).

Immunotype 5. The type 5 lipopolysaccharide was similarly degraded to separate the lipid A, the core oligosaccharide, and the O-chain components. The O-chain material was hydrolyzed to yield a mixture of two sugars identified by g.l.c. as L-rhamnose and D-quinovosamine (3,18). The ratio of these was 2:1 (g.l.c.) in initial batches of the lipopolysaccharide; later isolates from the organism cultured under somewhat different conditions showed a ratio of 1:1 for these sugars. Paper chromatography of hydrolyzates from O-chain of the later batches indicated a third sugar component, having chromatographic mobility suggestive of an amino uronic acid. Further indication for the amino uronic acid was provided by hydrolysis of the carboxyl-reduced polysaccharide, which again gave rhamnose and quinovosamine, but now in association with 2-amino-2-deoxygalactose, suggesting that a third constituent in the repeating unit is 2-amino-2-deoxygalacturonic acid. The amino groups in the type 5 O-chain are N-acetylated, and certain of the hydroxyl groups are also O-acetylated, as indicated by infrared spectroscopy.

Methylation linkage-analysis of the type 5 O-chain indicated the rhamnose and the quinovosamine residues to be linked through their 3 positions (21), and the galactosaminuronic acid residues through their 4 positions. In the proton n.m.r. spectrum (Figure 7), the H-1 signal of the rhamnose residue may be clearly recognized, but the two other signals in the anomeric region are superposed; however, the spacings and chemical shifts are

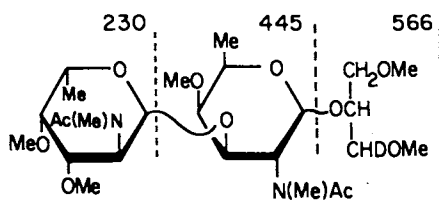


Figure 4. Chemical ionization fragmentation pattern of the Smith fragment of the type 2 O-chain.

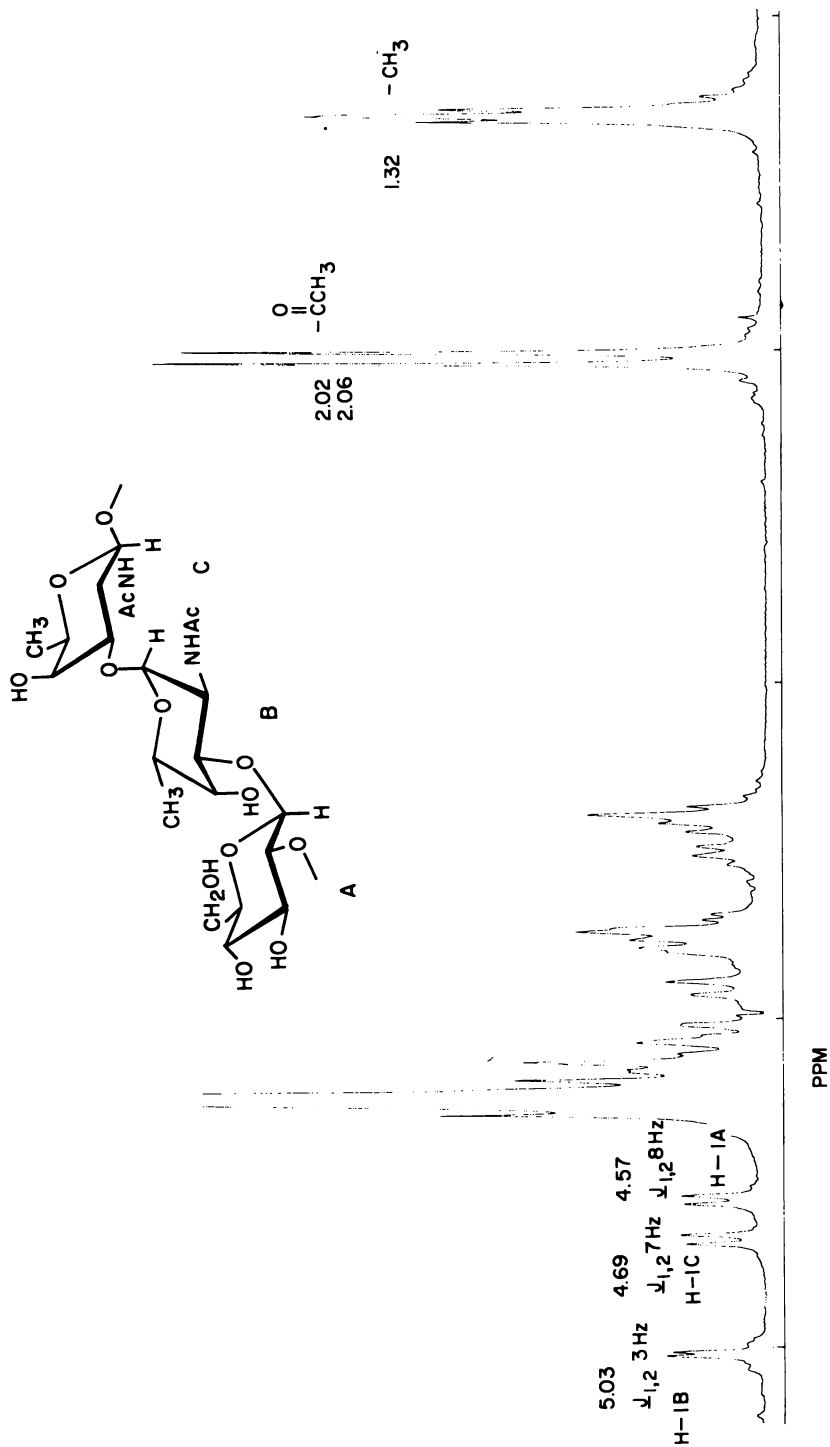


Figure 5. Proton NMR spectrum (300-MHz) of type 2 O-chain.

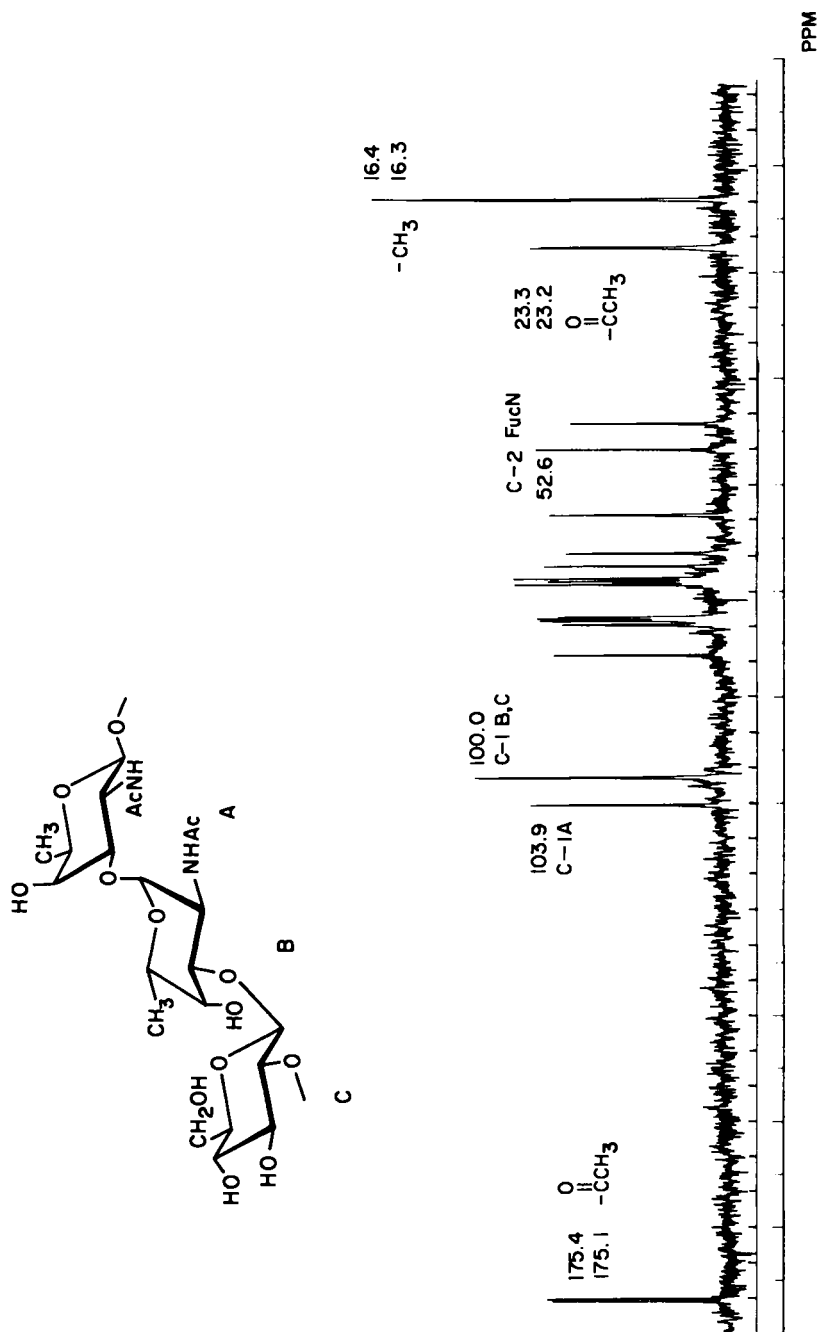


Figure 6. Carbon-13 NMR spectrum (75-MHz) of type 2 O-chain.

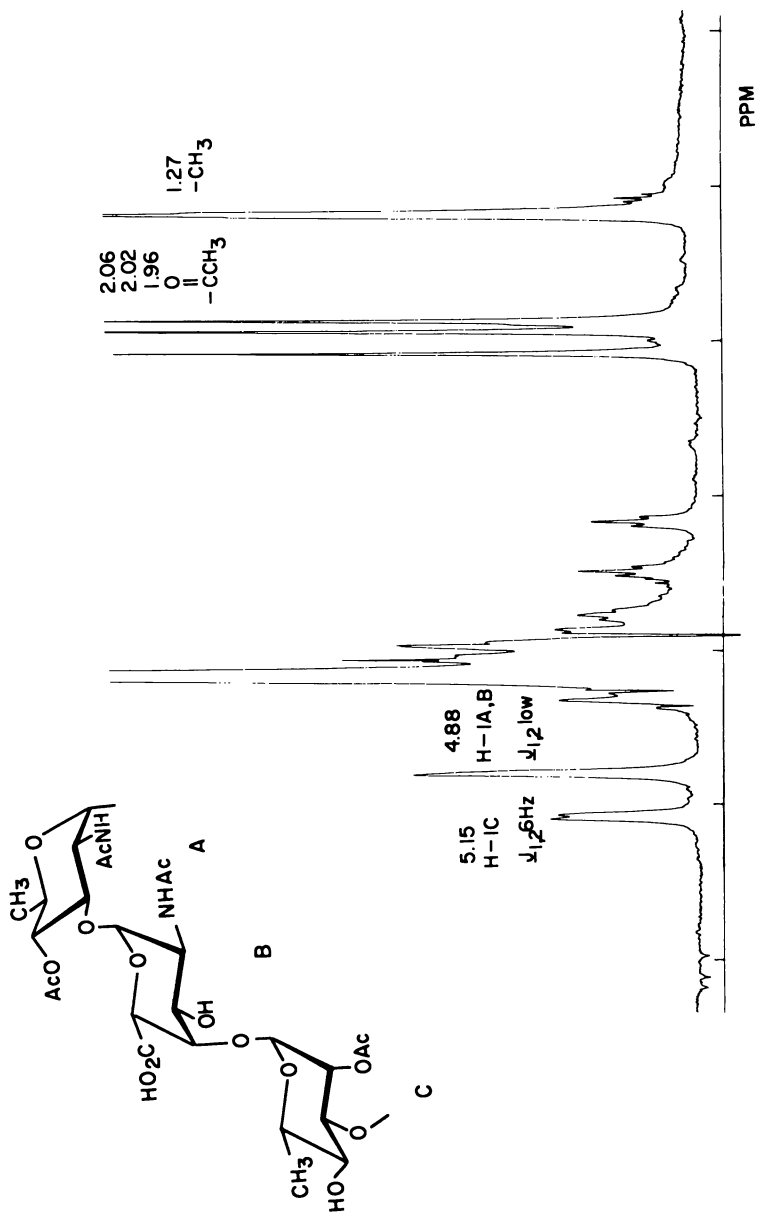


Figure 7. Proton NMR spectrum (300-MHz) of type 5 O-chain.

indicative of the α -D configuration of the quinovosamine residues and the α -L configuration of the rhamnose residues; very probably the amino uronic acid component has the α -L configuration. These data are further supported by the results of carbon-13 n.m.r. spectroscopy (Figure 8).

The O-chain sequence of Lanyi strain 0:2a,b and 0:2b,c *Pseudomonas aeruginosa* corresponds to the sequence of the later batches of Fisher type 5, indicating that these strains may be the same (22).

Immunotype 1. The O-chain from Fisher type 1 lipopolysaccharide was subjected to methylation linkage-analysis and characteristic fragments isolated included the 4-methyl ether of D-quinovosamine, the 2,3,6-trimethyl ether of D-glucose, and the 3,4-dimethyl ether of L-rhamnose, suggesting that the repeating unit contains rhamnose residues linked through their 2 position, glucose units linked through their 4 position, and quinovosamine residues linked through their 3 positions. A probable sequence for the repeating units of the type 1 O-chain is formulated (Figure 9) on the basis of studies of its proton- and ^{13}C -n.m.r. spectra (Figures 10 and 11). It is evident from the spectra that other signals are present in the anomeric region, and it is possible that formyl or other acyl groups are present in this O-polysaccharide. The ^{13}C -n.m.r. spectrum displays three signals in the anomeric region, and the field positions of these signals are in support of the proposed sequence, although final, definitive proof of the sequence has not yet been achieved.

Synthetic Oligosaccharides

Synthetic chemical studies on oligosaccharide sequences related to the determinants have been made with a view to preparing artificial antigens and immunoabsorbents. The established procedure (23) involves production of an oligosaccharide bearing a suitable glycosidic spacer-arm having a functional group at the end of the chain capable of being converted into a reactive electrophile, which serves to attach the molecule to amino groups on the surface of a protein or on a suitable, derivatized solid support (Figure 12). The initial synthetic targets were the methoxycarbonyloctyl glycosides (24) of D-quinovosamine bearing one or two α -L-rhamnosyl residues linked through O-3. These glycosides could be attached to such proteins as human serum albumin or tetanus toxoid to produce potential artificial antigens. Immunoabsorbents could be produced by attachment to a solid support such as poly(acrylamide) or surface-functionalized glass beads. Immunoabsorbents of this type have considerable potential value for such applications as the isolation of hyperimmune sera, for the purification of monoclonal antibodies, as tools in the study of antigen-antibody interactions, for facilitating the immunotyping of different

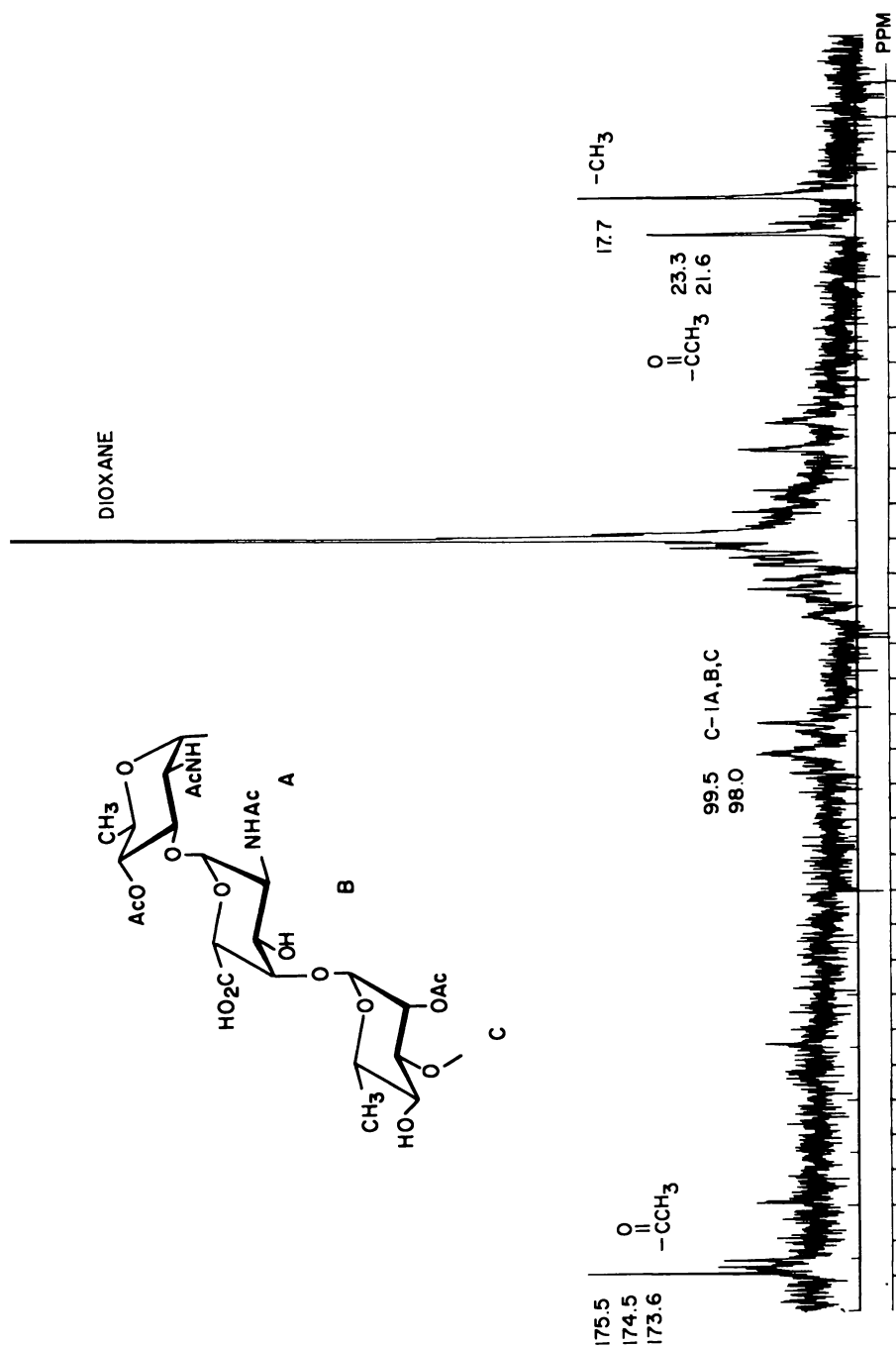


Figure 8. Carbon-13 NMR spectrum (50-MHz) of type 5 O-chain.

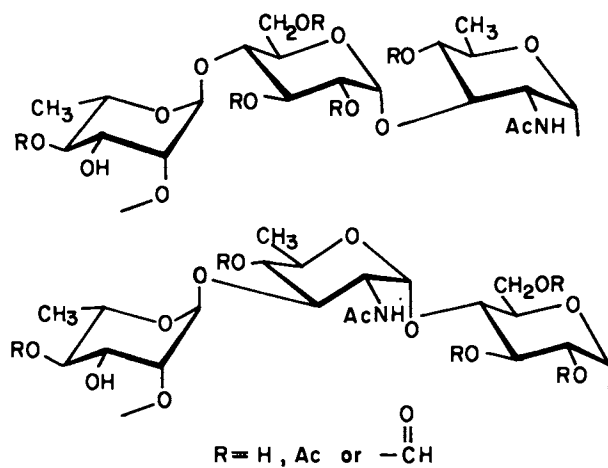


Figure 9. Possible repeating units of type 1 O-chain.

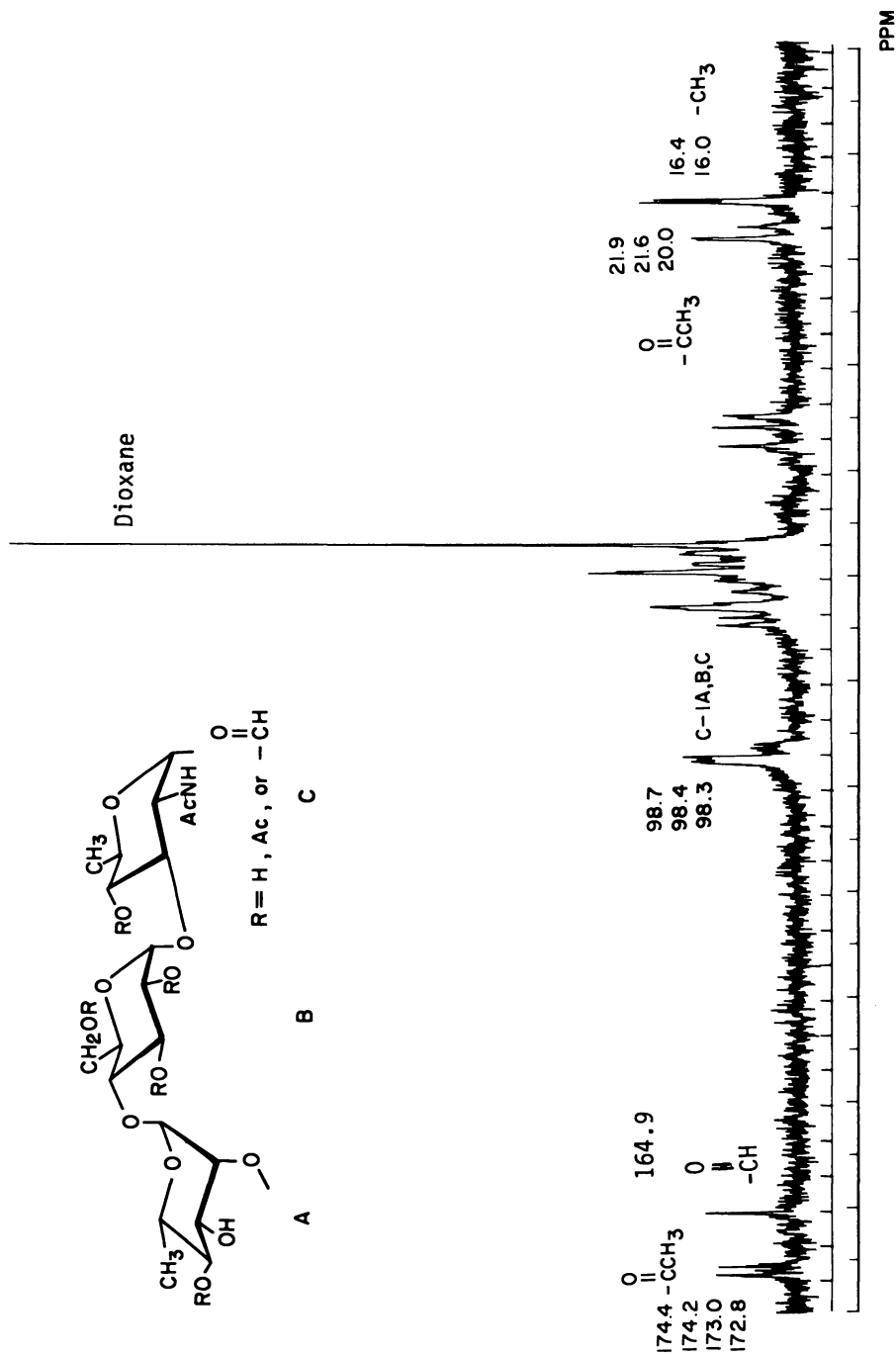


Figure 11. Carbon-13 NMR spectrum (50-MHz) of type 1 O-chain.

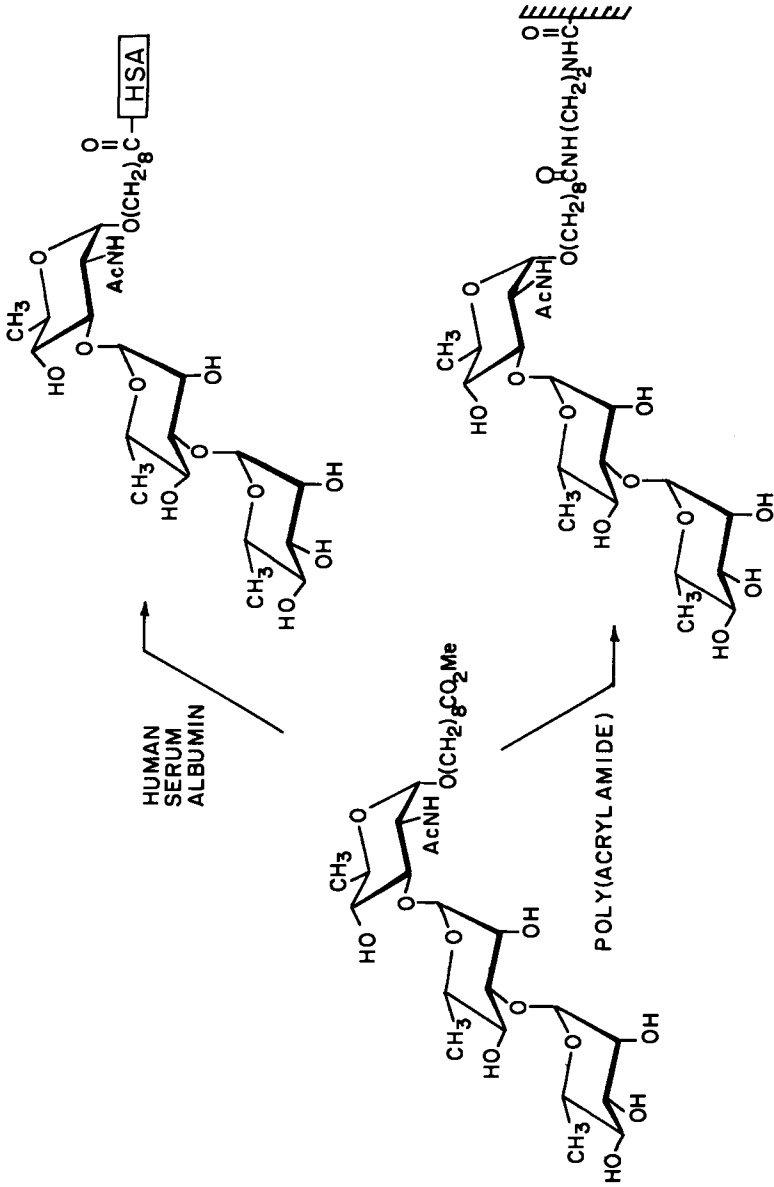


Figure 12. Potential uses of synthetic oligosaccharides.

strains of the organism, and in a practical sense, for developing a method for the preparation of bulk quantities of purified antibody that could be used for the passive immunotherapy of infections caused by the specific strain of the organism.

This synthesis was designed to explore specific synthetic methodology that could be useful, with adaptations, for the determinant sequences of the various antigens in the series, and was targeted on the basis of analytical results on earlier batches of the type 5 antigen that contained approximately 2 rhamnose residues per quinovosamine residue and no detected uronic acid. On this basis, a trisaccharide model was selected having two rhamnose residues linked to quinovosamine uniformly by 1-3 linkages, with a methoxycarbonyloctyl group in glycosidic attachment to the quinovosamine residue.

For procedural reasons in exploration of the synthesis, the β -glycoside was the initial target, as the identical anomeric configuration at that glycosidic center was not considered to be an obligatory feature. The syntheses were designed to be of practical utility, and considerable effort was made to ensure that preparatively useful steps were developed throughout.

The readily accessible 2-acetamido-3,4,6-tri-O-acetyl-2-deoxy- α -D-glucopyranosyl chloride ("acetochloroglucosamine") was condensed with methoxycarbonyloctanol in benzene in the presence of mercuric cyanide to give the β glycoside in good yield (Figure 13). O-Deacetylation of this product, followed by benzylidenation, gave the 4,6-benzylidene acetal. This product has HO-3 "isolated" for subsequent glycosylation, and the O-benzylidene group provides the basis for the ultimate conversion into quinovosamine by deoxygenation at C-6.

Attachment of the two rhamnose residues was first attempted by prior construction of the appropriate (1-3)-linked rhamnose disaccharide. Methyl α -L-rhamnopyranoside was allowed to react with tri-O-acetyl- α -L-rhamnosyl bromide ("acetobromorhamnose") under the Koenigs-Knorr conditions. Higher reactivity was anticipated at O-3; no significant reactivity at the (axial) HO-2 group was expected, and the 4-position is more hindered. Difficulties were encountered, however, in the conversion of this glycoside into the desired disaccharide glycosyl bromide, as the conversion involved an acetolysis step that was relatively low yielding, and so the resultant glycosyl halide of the disaccharide was not available in high yield. Nevertheless (Figure 14), it was allowed to react with the protected amino sugar glycoside to afford the trisaccharide glycoside having the overall framework of the desired target. The low yield of the disaccharide glycosyl halide made this sequence less than ideal for the desired purpose and so recourse was made to stepwise introduction of the rhamnose residues.

The protected amino sugar glycoside was treated with "acetobromorhamnose" under Koenigs-Knorr conditions and an excellent yield of the corresponding 3-O- α -L-rhamnosyl derivative

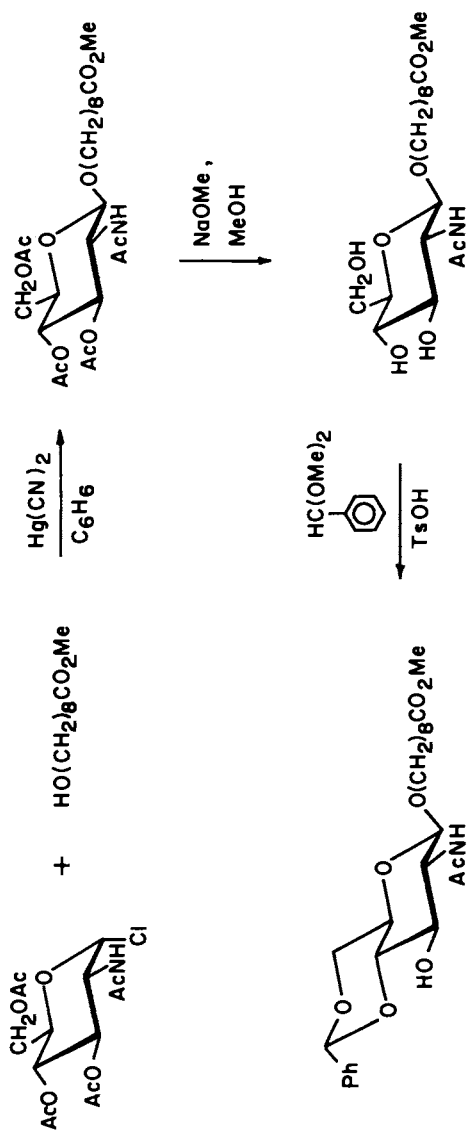


Figure 13. Synthesis of the monosaccharide glycoside.

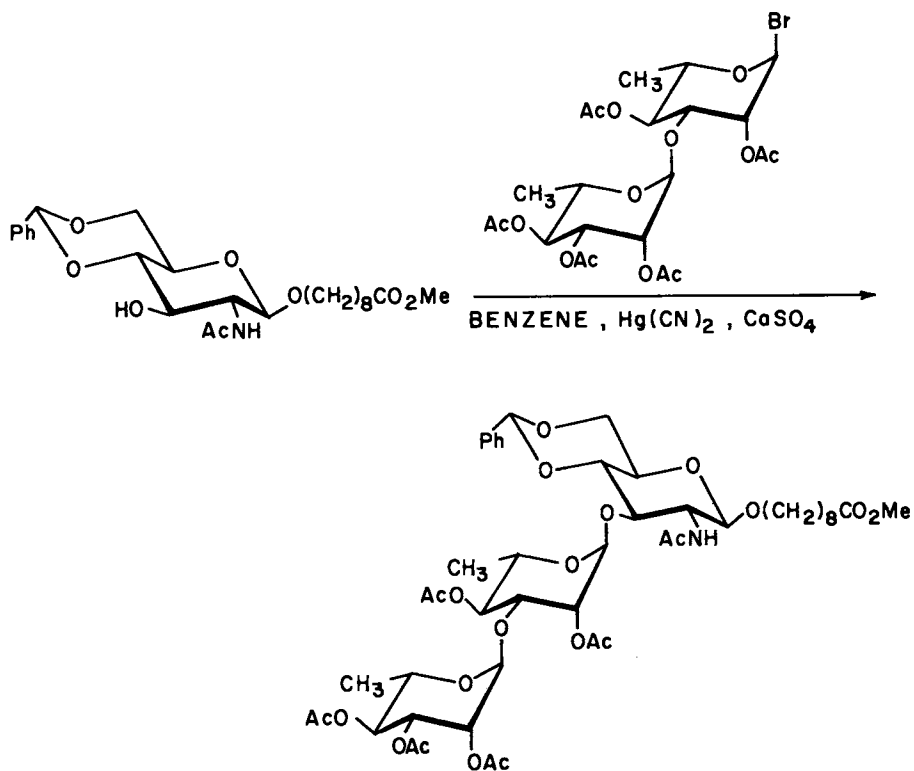


Figure 14. Attachment of disaccharide to monosaccharide glycoside.

was obtained (Figure 15). O-Deacetylation of this product, followed by subsequent treatment with tri-O-acetyl- α -L-rhamnosyl bromide, afforded a major product constituting the trisaccharide derivative linked through O-3 of the middle rhamnose residue, together with a minor proportion of the 4-linked isomer. These were readily differentiated on the basis that the latter reacted with periodate and could be converted into a monoisopropylidene acetal, whereas the major product did not react. The major product was, therefore, the desired 3-linked isomer, identical with that formed by the first approach. Although the yield in this step was only 25%, it was the only relatively low-yielding step in the whole sequence.

Treatment of the trisaccharide derivative with N-bromosuccinimide opened up the benzylidene acetal ring in the anticipated manner to give the product having a primary bromide and a secondary benzoate group (Figure 16). Subsequent reduction of this product with hydrogen and Raney nickel, followed by O-deacylation, led to the target glycoside in which the amino sugar had been converted into the quinovosamine structure and the α -L-rhamnosyl residues had been successively linked through (1 \rightarrow 3) linkages to the methoxycarbonyloctyl glycoside of the amino sugar (Figure 16).

This entire sequence demonstrated the practical feasibility of the trisaccharide synthesis and furnished the basis for a parallel synthesis in which the amino sugar glycoside was initially subjected to anomerization in the presence of an excess of the glycosidation alcohol and a Lewis acid; the anticipated operation of the anomeric effect assured a high level of conversion into the thermodynamically more-stable α anomer, which could be isolated crystalline in good yield (Figure 17). O-Benzylidenation of this product, followed by sequential reaction with "acetobromorhamnose", gave the 3-linked disaccharide, which was in turn O-deacylated and allowed to react with a further mole of tri-O-acetyl- α -L-rhamnosyl bromide to yield the desired trisaccharide structure; again reaction at the 3-position was strongly favored (Figure 17).

In the final stages, the benzylidene acetal was again opened by the action N-bromosuccinimide, and subsequent reduction and deacylation steps were accomplished as before to yield the target α glycoside.

The foregoing synthesis provides a basis for the chemical production of immunoadsorbents and artificial antigens based on this structure and variants thereof incorporating one, two, or more sugar residues in predetermined sequences. Variants of this general theme are expected to be of use in generating partial or entire repeat-sequences of the O-antigens in other immunotypes.

Further work currently in progress will provide ultimate tests as to the validity of this approach toward the proposed objectives.

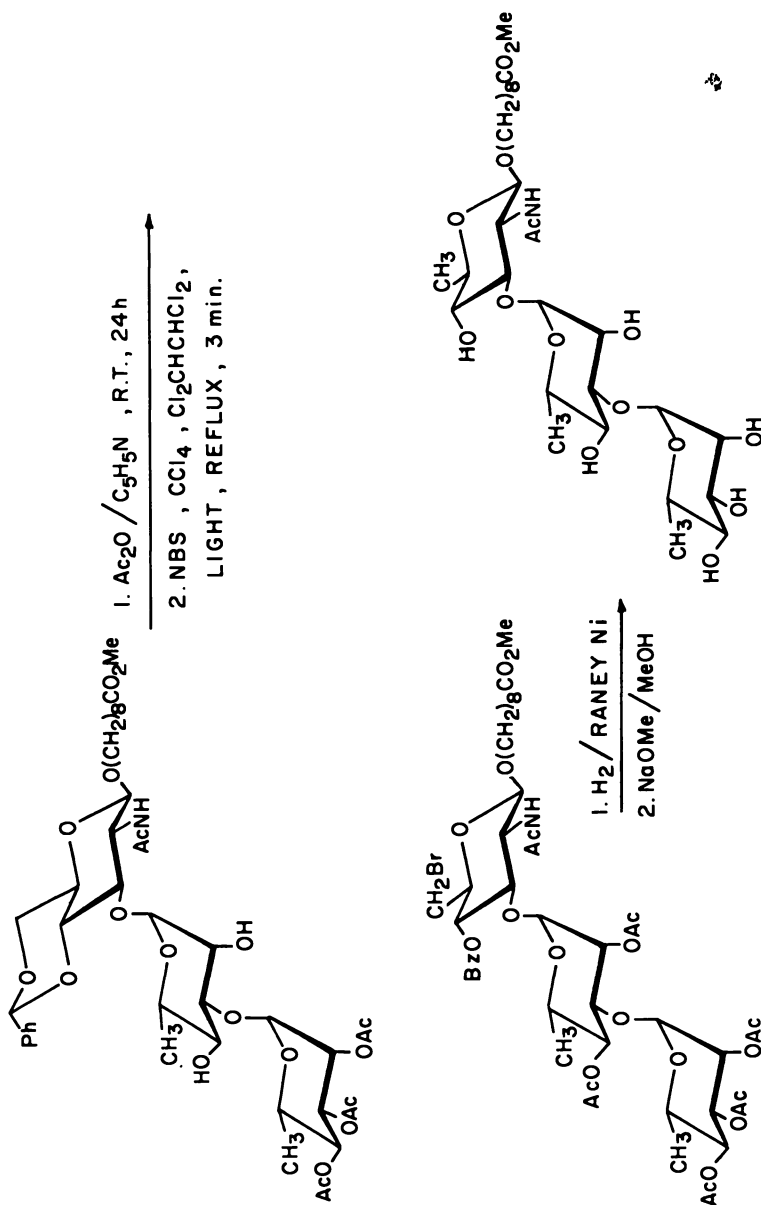


Figure 16. Generation of the quinovosamine structure.

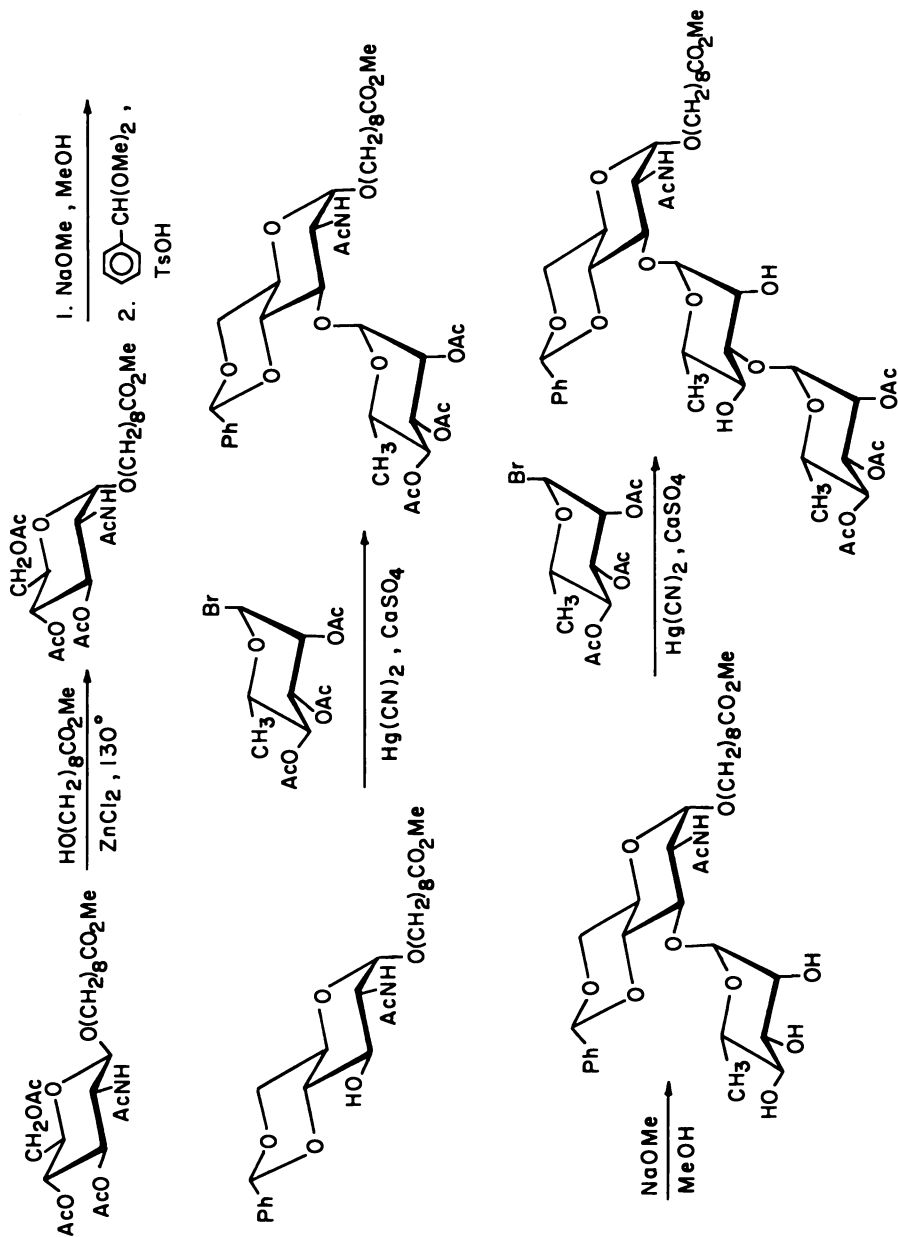


Figure 17. Synthesis of the trisaccharide α -glycoside.

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Synthesis of *Shigella flexneri* O-Antigenic Repeating Units

Conformational Probes and Aids to Monoclonal Antibody Production

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The primary structures of a large number of bacterial O-antigens are now well defined (1,2) and these data may be used to rationalize the serological classification of enterobacteriaceae (2). Since this classification is based upon antibody binding to cell wall polysaccharides, appreciation of the three dimensional structure of the antigen will enhance the understanding of subtle serological interrelationships and cross-reactions. The interpretation of the shapes of O-antigens of known primary structure is just beginning and in this chapter we describe how synthetic oligosaccharides are prepared and how models of O-antigen conformation are inferred from NMR experiments on such compounds. Also outlined is the manner in which the same oligosaccharides may be incorporated into the protocol of the hybrid-myeloma (hybridoma) technique for production of monoclonal antibody toward O-antigens. In this way oligosaccharides for which the three dimensional structure is well established may be used to select specific antibodies. These are of both practical use in serodiagnosis, and are ideally suited to the study of the molecular and stereochemical basis of antibody binding.

Structure and Conformation

The structures of the O-antigens of *Shigella flexneri* were elucidated by Kenne and his co-workers (3-5). A tetrasaccharide $\{2\text{Rha}(\alpha 1-2)\text{Rha}(\alpha 1-3)\text{Rha}(\alpha 1-3)\text{GlcNAc}(\beta 1)\}$ is the repeating unit of the simplest of the *S. flexneri* O-antigens, the variant Y. The serogroups 1-5 possess this basic repeating unit structure but in addition carry α -D-glucopyranosyl and O-acetyl substituents. Since the variant Y structure possessed glycosidic linkages involving only the ring hydroxyl groups and was devoid of either branching residues or charged groups, this O-antigen was considered a good model to investigate antigen-antibody interaction. This reasoning rested upon the assumption

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that the preferred solution conformation of an antigen was the most likely conformer to be bound by an antibody, and that the geometry of a simple, fairly rigid polymer, composed of linear repeating units, would most easily provide this conformational information. At the time this project was started the work of Rees and his co-workers (6,7) had already established that homopolysaccharide and diheteroglycan shape could be satisfactorily explained by consideration of the two glycosidic torsional angles ϕ and ψ . These define the conformation of a glycosidic linkage involving a secondary hydroxyl group. If the linkage involves the primary hydroxyl of a hexopyranoside a third torsional angle ω defines the extra degree of freedom that 1 \rightarrow 6 linkages possess. As S. flexneri O-chains are devoid of 1 \rightarrow 6 linkages the conformation of the Y O-antigen could be modelled with some confidence, provided that due consideration was given to the exo-anomeric effect. Lemieux's work had established, that to a good approximation the glycosidic conformation could be anticipated by setting the O-1 to aglyconic carbon bond antiperiplanar to the C-1 to C-2 bond (8,9). Analysis of X-ray data (10) and theoretical calculations supported this approach (11,12). In this arrangement the non-bonded interactions seemed to be best accommodated when the C-1 to O-1 bond eclipsed the aglyconic carbon to hydrogen bond. Thus it appeared that a reasonable model of the Y O-antigen conformation could be predicted with some confidence, a view later substantiated by semi-empirical calculations and NMR measurements (13).

Oligosaccharides corresponding to the antigenic determinants of the Y polysaccharide were required to either map the antibody combining site, or if suitably derivatised, to elicit antibodies. Such artificial antigens (14) could then provide antibodies that would react with the O-antigen and have a known binding specificity. We therefore synthesized a range of di-, tri- and tetrasaccharides corresponding to the Y O-antigen. At the time these haptens were intended to provide polyclonal but monospecific antibodies, but as a result of subsequent developments the structures also proved to be invaluable aids to the hybrid-myeloma (hybridoma) technique (15).

Synthesis of Oligosaccharides

The details of the synthetic approach we have adopted for the preparation of all possible di- and trisaccharide sequences of the Y polysaccharide repeating unit have been published (16-19), together with the syntheses of two of the four possible tetrasaccharides (19,20). The synthesis of a third tetrasaccharide sequence not previously reported is described below.

Sequential Chain Extension. The general synthetic approach adopted in our published work was to employ sequential chain extension reactions rather than block synthesis. Since two of

the four glycosidic linkages in the repeating unit were to the O-2 position of an α -L-rhamnopyranose unit a general blocking procedure was required to provide this unit in suitably protected form. This was effectively done by adopting the general concepts of Garegg and his co-workers (21) and Lemieux and his co-workers (22), whereby the base stability of 1,2-orthoesters was exploited to provide benzylated 1,2-orthoesters. These derivatives possessing persistent blocking groups at positions O-3, O-4, and O-6 and a latent acetate at O-2 have considerable synthetic value. Accordingly 3,4-di-O-benzyl-1,2-O-(1-methoxyethylidene)- β -L-rhamnopyranose could be quantitatively converted to 2-O-acetyl-3,4-di-O-benzyl- α -L-rhamnopyranosyl chloride (1) (18,23) and this derivative was particularly effective in silver trifluoromethanesulphonate (triflate) promoted Koenigs-Knorr reactions. Thus good yields of rhamnose oligosaccharides containing both 1+2 and 1+3 linkages were obtained by coupling this glycosyl halide with selectively blocked 3,4-di-O-benzyl- α -L-rhamnopyranoside derivatives (4 and 6), and with the 2,4-di-O-benzoyl- α -L-rhamnopyranoside (2) (18-20). The general scheme of this chemistry is illustrated in Figure 1, which describes the synthesis of the tetrasaccharide sequence: GlcNAc(β 1-2)Rha(α 1-2)Rha(α 1-3)Rha(α)-O-R. Other features of the synthetic scheme worthy of note are the selective removal of acetate esters in the presence of benzoate esters (18-20) and the stability of the phthalimido functionality to transesterification conditions (16). Furthermore, the conversion of the phthalimido group to an acetamido group was achieved under selective hydrazinolysis conditions (16), which preserved the integrity of the ω -methoxycarbonyloctyl ester grouping, an essential element in the efficient coupling of the carbohydrate hapten to protein (14). The use of 3,4,6-tri-O-acetyl-2-deoxy-2-phthalimido- β -D-glucopyranosyl bromide (7) (24) had been dictated by the low reactivity at the O-2 position of 3,4-di-O-benzyl- α -L-rhamnopyranosides (e.g. 6). For example, ω -methoxycarbonyloctyl 3,4-di-O-benzyl- α -L-rhamnopyranosides had failed to react with either 2-acetamido-3,4,6-tri-O-acetyl-2-deoxy- α -D-glucopyranosyl chloride or the corresponding 1,2-oxazoline derivative (16); however, use of the bromosugar 7 gave good yields of the desired oligosaccharides.

Block Synthesis. In contrast to this published work we have recently investigated the use of block synthesis to obtain a related frame shifted tetrasaccharide sequence, Rha(α 1-3)Rha(α 1-3)GlcNAc(β 1-2)Rha(α)-O-R, previously unavailable to us. This structure was considered essential since serological screening had shown the trisaccharide Rha(α 1-3)GlcNAc(β 1-2)Rha(α)-O-R to possess higher activity than either of the previously synthesized tetrasaccharides, Rha(α 1-2)Rha(α 1-3)Rha(α 1-3)GlcNAc(β)-O-R or

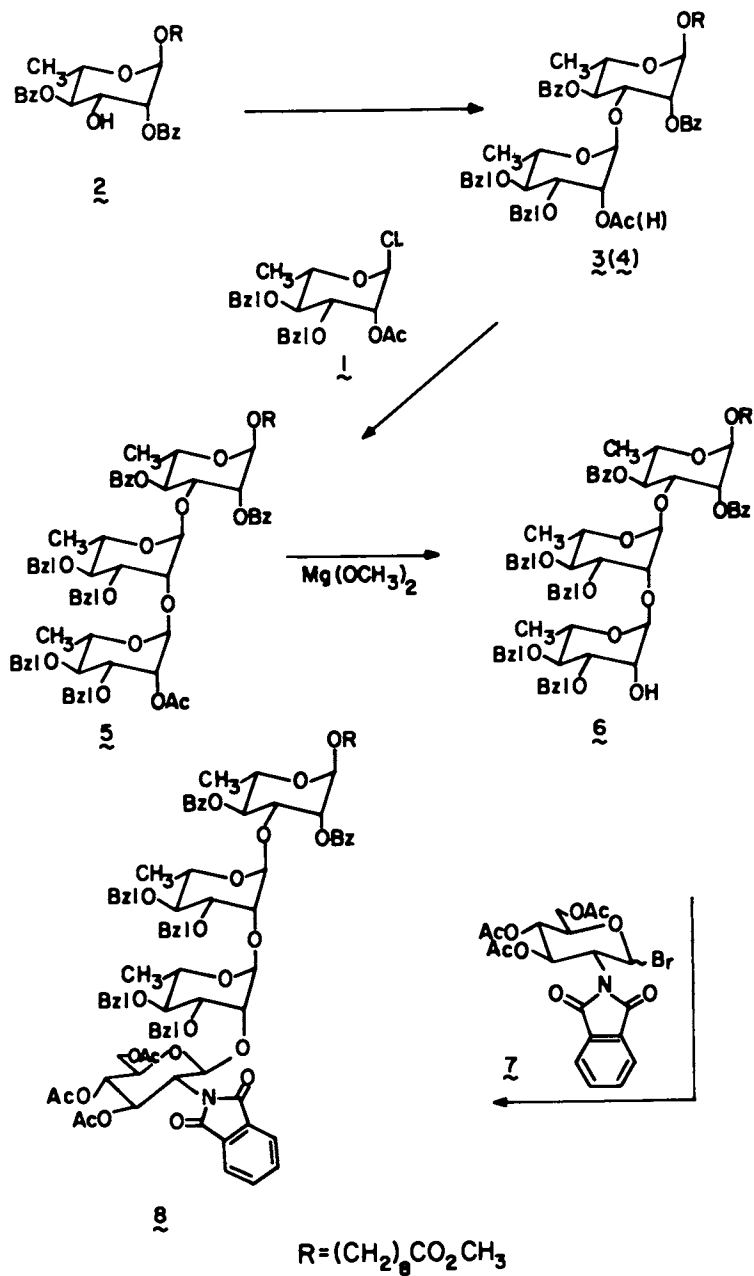


Figure 1. Synthesis of a chemical repeating unit of the *Shigella flexneri* variant Y O-antigen, employing a sequential chain extension strategy.

GlcNAc(β 1-2)Rha(α 1-2)Rha(α 1-3)Rha(α)-O-R. The synthesis of the new tetrasaccharide is summarized in Figure 2.

The synthesis of disaccharide 9 was accomplished by the reaction of ω -methoxycarbonyloctyl 3,4-di-O-benzyl- α -L-rhamnopyranoside (19) with 3,4,6-tri-O-acetyl-2-deoxy-2-phthalimido- β -D-glucopyranosyl bromide (24) (7) as previously described (16,17). The selectively protected disaccharide glycoside 9 constitutes one of the two building units of the target tetrasaccharide. In order to prepare the second disaccharide for the block synthesis, methyl 2,4-di-O-benzoyl- α -L-rhamnopyranoside (10), made according to the general method of Garegg and Hultberg (25), was reacted with either tri-O-acetyl- α -L-rhamnopyranosyl bromide (11) or 2-O-acetyl-3,4-di-O-benzyl- α -L-rhamnopyranosyl chloride (1), in dichloromethane with respectively mercury(II) cyanide or mercury(II) cyanide together with mercury(II) bromide. Disaccharides 12 and 13 were obtained in 93% and 75% yields, respectively, and their conversion to disaccharide glycosyl halides was investigated under a variety of conditions. Initially the reaction of 12 with trimethylbromosilane (23) was studied but even under the most forcing conditions examined, an eight molar excess of trimethylbromosilane in toluene at 80° for 18 hr with or without catalytic amounts of zinc bromide, the starting material 12 was recovered unchanged. The most satisfactory conditions for conversion of 12 to 14 proved to be the reaction with dibromomethyl methyl ether (27) in absolute dichloromethane. In the presence of catalytic amounts of zinc bromide 12 gave 14 in 50% yield, after 15 hr at -15° and 5 hr at 0°, followed by column chromatography on silica gel. Monosaccharide bromides were produced as side products and these, together with the major product, were trapped and isolated as the isopropyl glycosides 15 and 16 in order to confirm their structure. Conversion of the disaccharide glycoside 13 to its corresponding glycosyl halide was not possible using dibromomethyl methyl ether due to the lability of the benzyl ether groups. However, acetolysis of 13 gave the 1-acetate 17 from which the corresponding glycosyl halide could be readily obtained. Use of a derivative of this type would provide a tetrasaccharide intermediate from which a pentasaccharide could readily be prepared in a fashion analogous to the conversion of 5 via 6 to 8 (Figure 1).

When bromide 14, in molar excess, was reacted with the selectively blocked disaccharide 9 in dichloromethane in the presence of mercury(II) cyanide as catalyst and 4Å molecular sieve as the acid acceptor, a 90% yield of the fully blocked tetrasaccharide 18 was obtained after column chromatography. Removal of blocking groups was accomplished by the hydrogenolysis of the benzyl ether and benzylidene acetal groups in acetic acid, followed by transesterification. The deblocked tetrasaccharide 19 had NMR parameters in agreement with its structure. This could be confirmed by comparison with chemical shift data for the

previously synthesized oligosaccharides. These have been exhaustively studied by ^1H and ^{13}C NMR and the results correlated with structure and solution conformation (13). Horton and Samreth have reported the preparation of a derivative similar to 12 but its conversion to a glycosyl halide proceeded via acetolysis and conventional treatment with hydrogen bromide-acetic acid. Their disaccharide bromide gave only poor yields of a trisaccharide on reaction with a 2-acetamido-4,6-O-benzylidene-2-deoxy- β -D-glucopyranoside (28).

Conformation by NMR and Semi-empirical Calculations

Investigation of the preferred solution conformations of the oligosaccharides synthesized by the above mentioned procedures was carried out by semi-empirical calculations and NMR methods (13). The calculations are a type of hard sphere treatment which take into account an energy contribution due to the *exo*-anomeric effect. The calculations referred to as HSEA calculations have been developed and used by Lemieux *et al.* (29,30) particularly for the blood group determinants. The method and its use in conjunction with ^1H and ^{13}C NMR is well reviewed and explained by Bock (31). In outline, experimental NMR measurements that provide conformational information, namely chemical shifts, coupling constants, spin lattice relaxation rates ($1/T_1$) and nuclear Overhauser enhancements (nOe), are used in combination with HSEA calculations to identify consistent oligosaccharide conformations. Heteronuclear three-bond, C-O-C-H coupling constants ($^3J_{13\text{C},1\text{H}}$) can provide information about the glycosidic torsional angles (31,32). Proton chemical shift differences caused by short (2.7Å) proton-oxygen distances can also be related to conformational features (13). The magnitudes of both spin lattice relaxation rates and nOe parameters are intrinsically dependent upon proton-proton internuclear distances (33). These distances, especially those between protons on adjacent pyranose residues, can be related to the glycosidic conformation. The anomeric proton and the aglyconic proton are always in close proximity (8,9,29-31), and if a second proton-proton distance between pyranose residues is available these two distances can then define a unique pair of ϕ, ψ values for the glycosidic linkage involved. An example of the qualitative relationship of T_1 to conformation is shown in Figure 3. Since the contribution by adjacent protons (j) to the relaxation of a particular proton (i) is proportional to r^{-6} , where r is the internuclear distance between proton i and proton j , only those protons in close proximity materially affect the relaxation rate ($1/T_1$) of proton i (33). In the case of the trisaccharide $\text{Rha}(\alpha 1-2)\text{Rha}(\alpha 1-3)\text{Rha}(\alpha 1-0-R$ (a-b-c) H-1 of rhamnose unit b receives a relaxation contribution (in addition to that from H-2 of rhamnose b) from H-3 of the 'reducing' rhamnose c. However the geometry of the terminal $\alpha 1 \rightarrow 2$ linkage places H-5 of rhamnose a close to H-1 of b

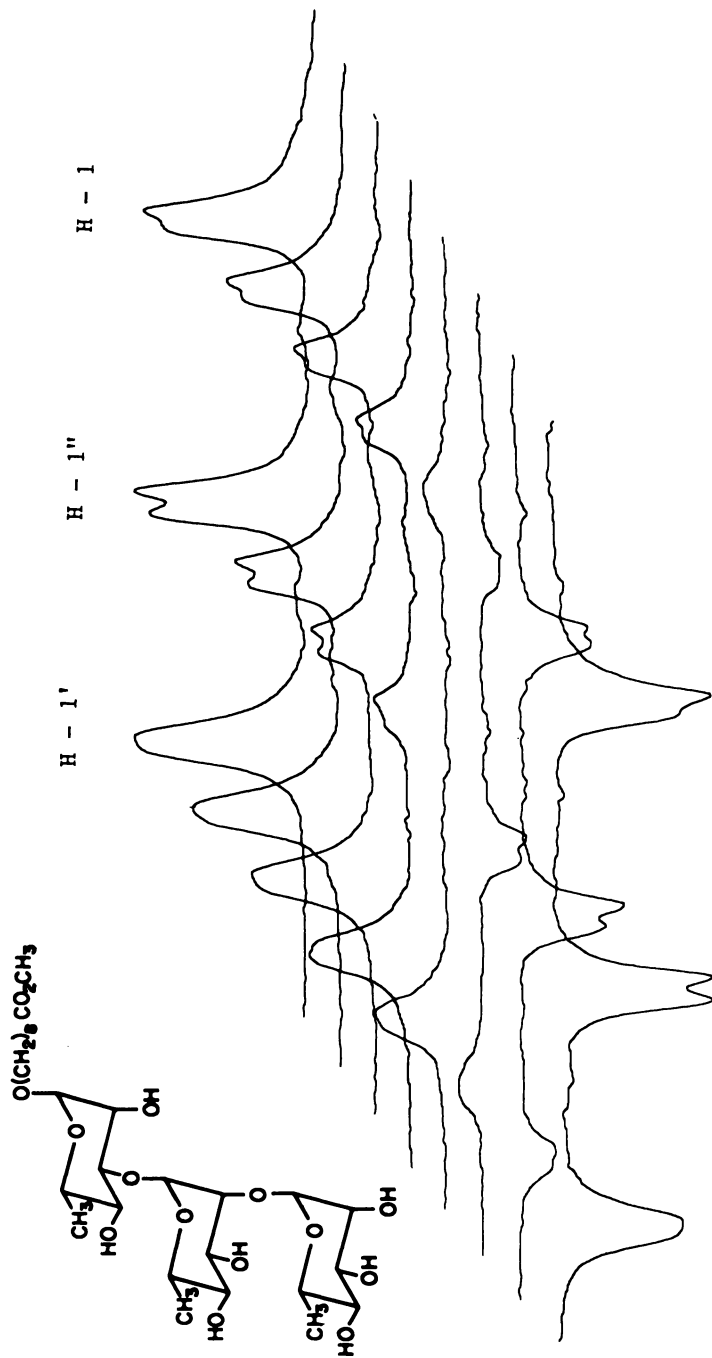


Figure 3. Relaxation of the three anomeric protons of a rhamnose trisaccharide sequence belonging to the Y O-antigen.

(Figure 4). Therefore, in comparison to the other anomeric protons H-1a and H-1c, which receive only one substantial inter-ring relaxation contribution, proton H-1b receives two such contributions; H-5a/H-1b and H-1b/H-3c. Thus H-1b relaxes much faster than either H-1a or H-1c (13,18). The dependence of nOe upon distance also involves an r^{-6} term and these measurements provide a more convenient method for estimating conformational preference, especially when performed in the difference mode (34). This method was used extensively for the *S. flexneri* oligosaccharides and the Y-polysaccharide and the results were in excellent agreement with HSEA calculations of conformational preference (13). The shape of an octasaccharide derived from this model and consisting of two repeating units is presented in Figure 5. It can be noticed that the methyl groups C-6b, C-6a and C-6c bracket the polar acetamido group along one edge of the Y-polysaccharide surface. We have proposed this as a likely site for antibody binding (13) and for this reason we synthesized the tetrasaccharide 19.

Monoclonal Antibodies

At the outset of this work we had intended to raise polyclonal antibodies to our synthetic haptens. However, developments in immunology provided a dramatic advance when Kohler and Milstein (15) showed that myeloma antibody of predefined specificity could be systematically generated via somatic cell fusion techniques (15,35). The technique is summarized in Figure 6. A Balb/C mouse is immunized with the antigen of interest either as a pure antigen, or a component of a cell or a crude extract. After a suitable time interval to allow the development of a good immune response the spleen cells (B-lymphocytes) of the mouse are mixed with myeloma tumour cells, adapted to tissue culture. The two cell populations are induced to fuse by polyethylene glycol (PEG) and subsequently only hybrid cells are permitted to grow because the selective medium in which the fused cells are plated selects against both parental cell types. The culture supernatants from each micro-well are analyzed after two weeks for antibody activity against pure antigen. Active hybrid wells are cloned and re-screened for antibody activity. Since these cloned cell lines possess the properties of tumour cells they may be stored and propagated at will. As ascites these tumour cell lines produce large quantities (up to 100 mg/mouse) of monoclonal antibody. Such homogeneous molecules are ideal for structural studies and investigations of antibody-antigen binding. The production of such hybridoma lines to specific carbohydrate determinants is facilitated by the availability of chemically defined antigens and we have demonstrated this for the human blood group B determinant (36). However, for anti-LPS antibody we have also incorporated the synthetic antigens into a screening protocol

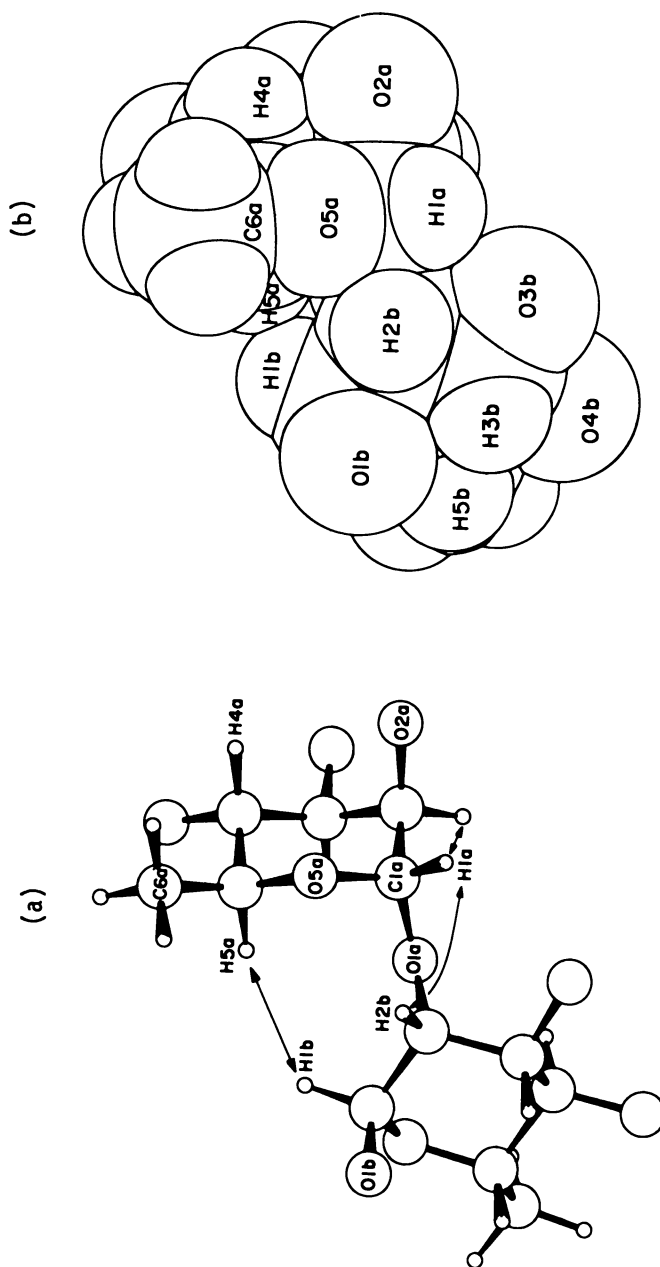


Figure 4. Ball and stick (a) and CPK (b) plots of the disaccharide Rha(α-1-2)Rha showing the proximity of protons H5a to H1b and H1a to H2b and H2a.

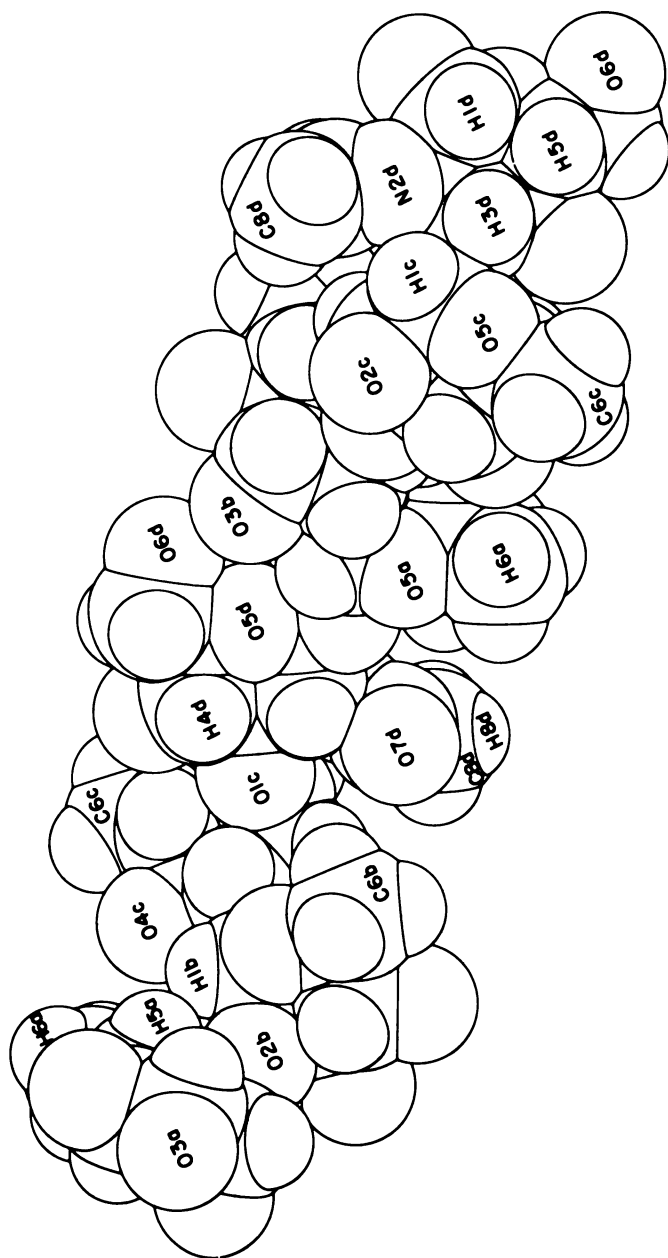


Figure 5. CPK plot of an octasaccharide composed of two repeating units of the Y O-antigen. The ϕ, ψ pairs used to generate the model were derived from published work (13) and correspond to the minimum energy conformers of each glycosidic linkage.

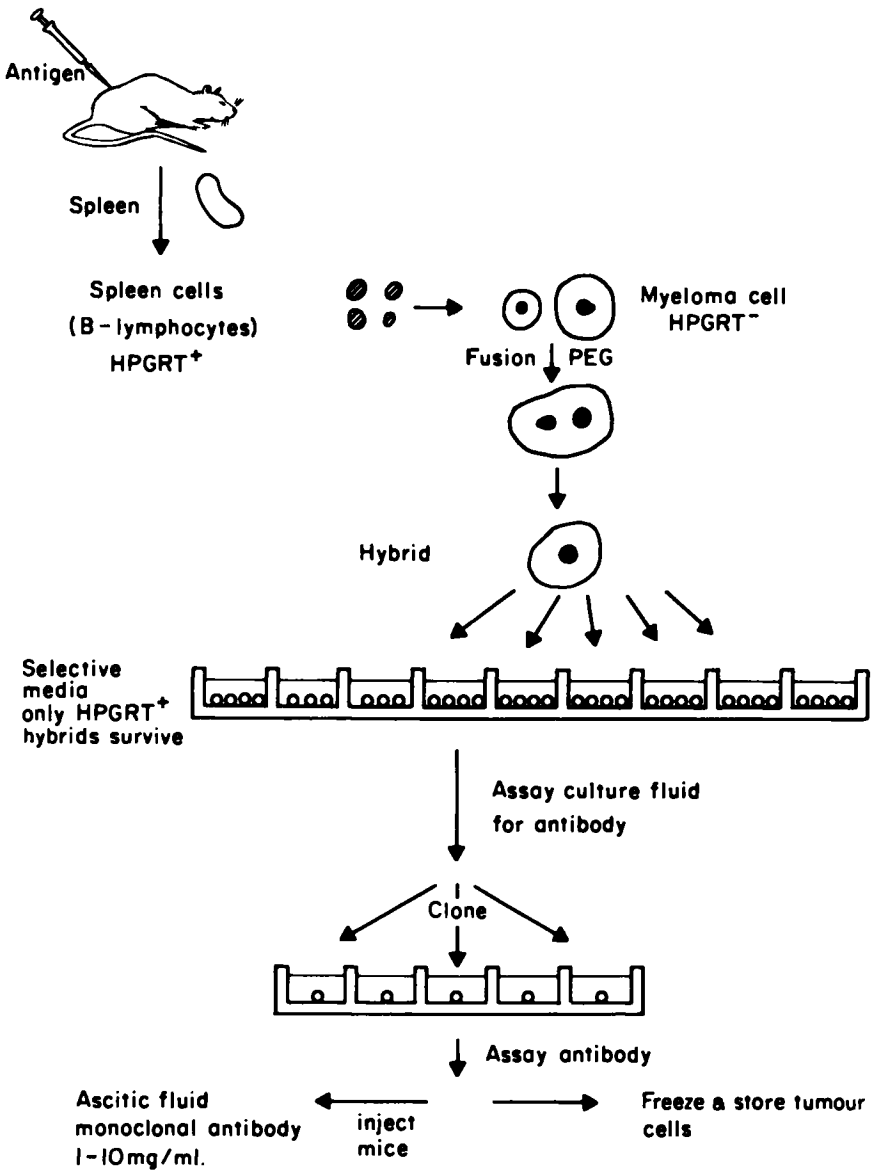


Figure 6. Schematic of the hybrid-myeloma protocol.

which allows the identification of clones with precise binding specificity. Thus consideration of Figure 7 shows how glycoconjugates or artificial antigens may be used at the screening stage to select antibody activity directed toward the oligosaccharide moiety of the conjugate. In the variation shown the mouse was immunized according to II (cells possessing the determinant, *i.e.* bacterial cells bearing the LPS). By this method we have isolated two clones which precipitate the Y O-antigen and these are being subjected to detailed analysis.

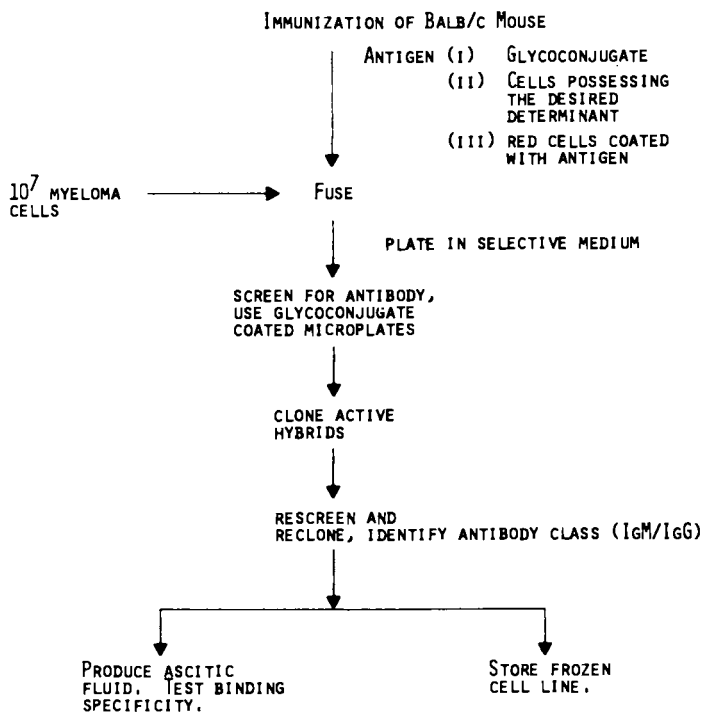


Figure 7. Flow chart of the hybrid-myeloma protocol incorporating the artificial antigens as immunizing antigens and screening aids.

Summary

In conclusion, it has been shown that chemical synthesis of oligosaccharides not only supplies chemically defined antigens but through NMR measurements and HSEA calculations these oligosaccharides permit a model of O-antigen conformation to be inferred. Furthermore, the same defined structures may be used in conjunction with somatic cell fusion techniques to facilitate and streamline the production of monoclonal antibodies with precisely defined binding characteristics. The combination of these various facets holds great promise for deeper appreciation of the molecular process at work in antibody-antigen recognition, and hence the serological classification of bacteria according to their cell wall polysaccharides.

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Synthesis of O-Antigenic Polysaccharides

Pathways for the Polymerization of Oligosaccharide Repeating Units

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In recent years, syntheses of a number of oligosaccharide repeating units of O-antigenic polysaccharides from Salmonella (1-4), as well as some other bacteria (5,6) have been performed. Moreover, the rapid progress of modern oligosaccharide synthesis opens the way for the preparation of a large number of synthetic oligosaccharide repeating units and their close analogues. It, therefore, becomes feasible to try to solve the next problem - a synthesis of the O-antigenic polysaccharides themselves, with the aim of studying their biological properties and the possibilities of their practical use in medicine.

The architectonics of O-antigenic polysaccharides unambiguously indicates that the most appropriate approach to their synthesis is to assemble the biopolymer out of 'prefabricated' repeating units. The practical solution of this problem can be achieved by several pathways (cf. 7); the following three most obvious ones will be discussed.

1. Extension of a polymeric chain through sequential addition of oligosaccharide repeating units (step-wise synthesis).

2. Polymerization of oligosaccharides by chemical methods.

3. Enzymatic polymerization of oligosaccharides.

The most serious, general problem in the realization of any of these approaches is to ensure strict regio- and, especially, stereospecificity of the linkage formation between the repeating units which are by themselves complex, polyfunctional compounds. At the same time, each of these approaches requires the solution of specific, synthetic problems and has its own advantages and limitations. Experience accumulated in our laboratory on the problem will be discussed in this paper.

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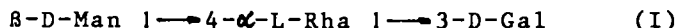
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Stepwise Synthesis

Stepwise addition of repeating units is the most obvious and, at the same time, the most laborious pathway for the production of the biopolymer. A proper functionalization of the oligosaccharide repeating unit is required for implementation of this approach. Two components are generally required for oligosaccharide synthesis: A glycosyl synthon which contains a substituent at C-1 which may serve as a good leaving group for nucleophilic substitution, and an aglycone synthon with a single, free or activated, hydroxyl group, and having all other functional groups temporarily protected.

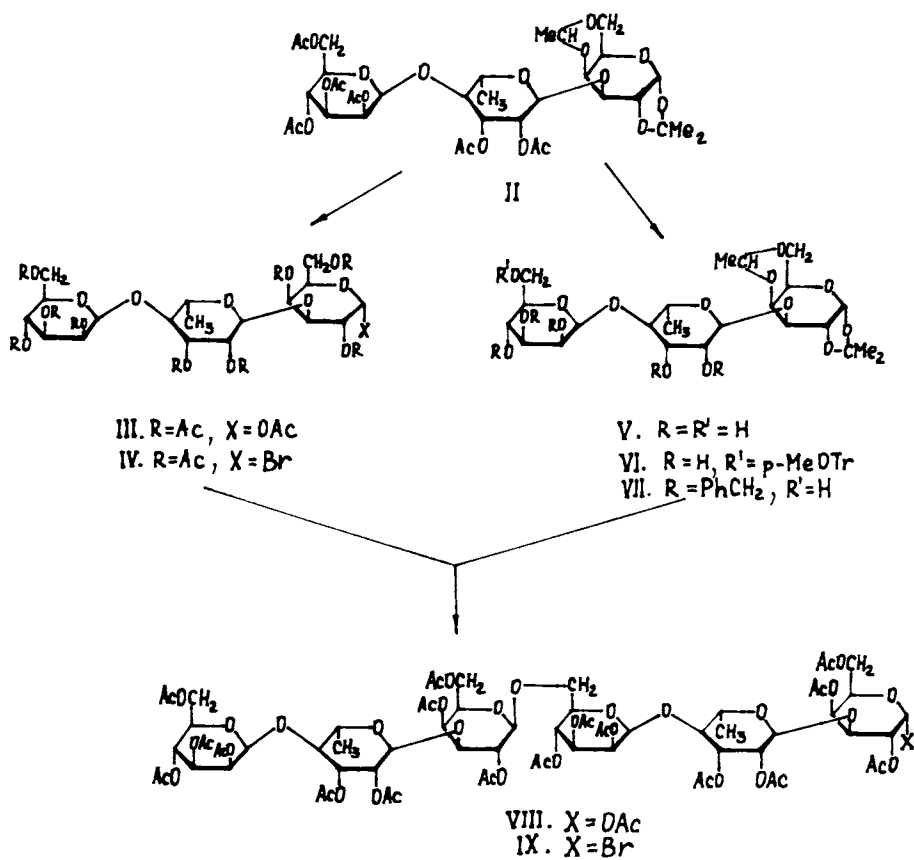
The proper selection of glycosyl and aglycone synthons is essential for the development of a standard procedure for the stepwise synthesis of polysaccharides. Their preparation from the same oligosaccharide precursor seems the most practical.

The synthesis of hexa- and nonasaccharides (8), the 'dimer' and the 'trimer' of the trisaccharide I may be presented as an example for the stepwise synthesis of the *Salmonella newington* polysaccharide which contains the repeating units I joined through the β -galactosyl 1 \rightarrow 6-mannose linkage (9).



The synthesis of the trisaccharide I has been elaborated at this laboratory in several modifications (1, 10, 11). It is most convenient to begin the stepwise synthesis of the polysaccharide from the protected derivative II (12). To prepare the glycosyl synthon, II was converted into the decaacetate III, which was treated with HBr in a $\text{CHCl}_3\text{-CH}_2\text{CO}_2\text{H}$ mixture at 0° to give the glycosyl bromide IV. The preparation of III is certainly possible from the derivatives of the trisaccharide I obtained by different methods (1, 11). The purity of IV is of decisive importance for the success of the subsequent condensation, and, therefore, its preparation, as well as that of the other halogenoses derived from complex oligosaccharides, is to be strictly controlled.

For preparation of the aglycone synthon, II was deacetylated with $\text{Et}_3\text{N-MeOH}$, the only primary hydroxyl group in V was protected by methoxytritylation, the resulting VI was benzylated, and, after de-methoxytritylation with 1% $\text{CF}_3\text{CO}_2\text{H}$ in CH_2Cl_2 , the aglycone component VII was obtained, which may serve as a



standard derivative for the extension of the block-polymer chain.

Condensation of the glycosyl bromide IV and the aglycone VII in the presence of $\text{Hg}(\text{CN})_2$ in CH_3CN , with the use of a high-vacuum technique to completely exclude moisture (cf. 13), gave the hexasaccharide derivative in a yield of about 80% after chromatographic purification which is necessary to remove traces of the α -anomer formed during the condensation. Removal of protective groups gave the free hexasaccharide, a 'dimer' of I, which was converted into the acetate VIII.

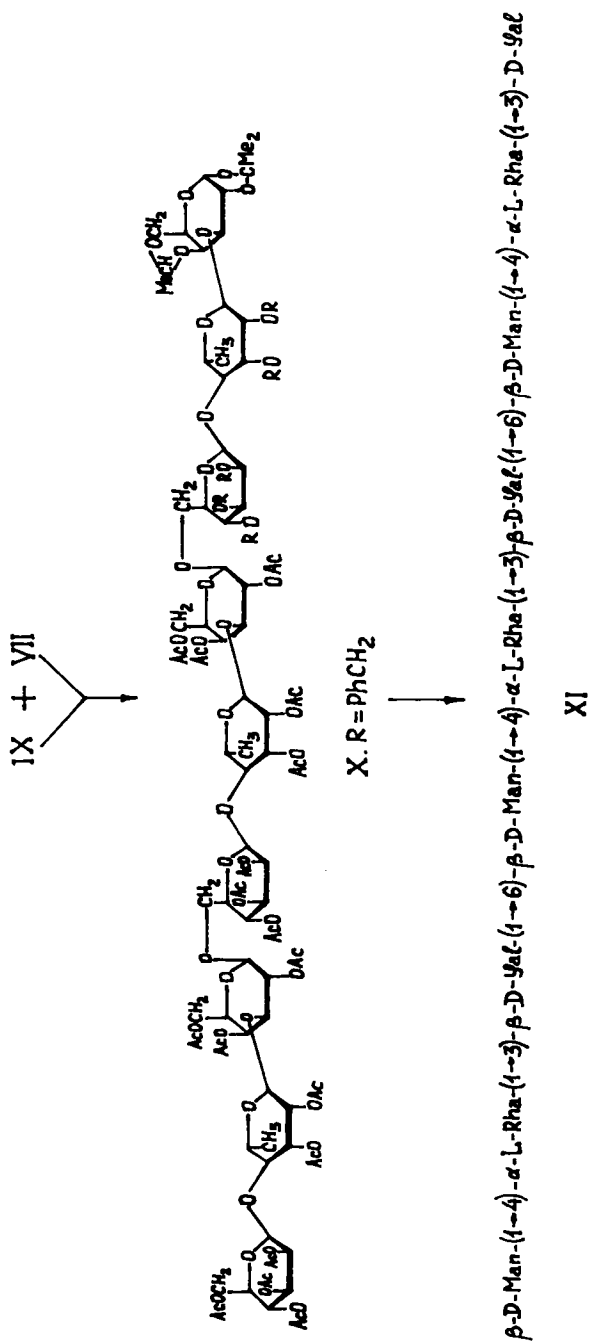
For the next stage of the stepwise synthesis, the preparation of the 'trimer' of I, VIII was converted into the glycosyl bromide IX which was condensed with the aglycone synthon VII in a similar manner to give the protected nonasaccharide X (yield 57%). After removal of the protecting groups, the 'trimer' of the repeating unit I, compound XI was obtained.

It is clear that the stepwise extension of the polymer chain can be continued further following the standard procedure: conversion of an oligomer peracetate into a glycosyl bromide followed by condensation with the standard aglycone VII. It is, however, necessary to bear in mind that an especially stringent control is required for the preparation of higher glycosyl bromides and that the yields of condensation products would probably decrease with increasing chain length of the oligomer.

This route to polysaccharides, or, to be more precise, higher oligosaccharides, can probably be extended sufficiently far, although it is certainly very laborious; the purification of the oligopolymers obtained from anomeric impurities formed at condensation also naturally becomes more complicated with the growth of the molecular weight. At the same time, only this pathway allows to obtain oligomers with a strictly defined degree of polymerization.

Chemical Polymerization of the Repeating Unit

Polymerization of the repeating unit is the most straightforward pathway to reach the objective - to prepare a block-polymer consisting of regularly repeating oligosaccharide units. To obtain a polysaccharide of a strictly regular structure, fully corresponding to the natural one, polymerization must be performed with absolute regio- and stereospecificity. Even a single structural or stereochemical error in the linkage of two neighbouring units may yield a polymer with a distorted three-dimensional structure and, therefore,

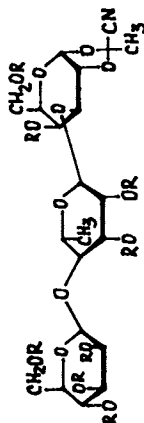
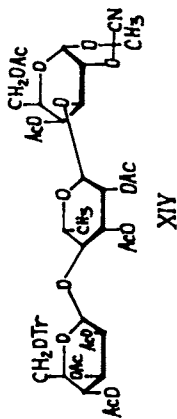
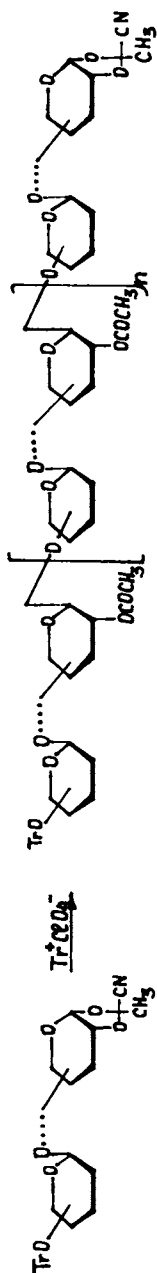


alteration of physico-chemical and biological properties may be expected. The removal of impurities of a wrong linkage type does not seem possible by present purification methods. The solution of this problem is not trivial, and stringent requirements of stereospecificity of glycosidic bond formation are to be met by the glycosylation reaction on which polycondensation has to be based. The solution became possible after a sufficiently general method had been developed for synthesis of polysaccharides of a regular structure, namely, the polycondensation of O-trityl ethers of mono- and oligosaccharidic 1,2-cyanoethylidene derivatives, catalyzed by tritylium perchlorate (for reviews, see 14, 15).

The method allows one for the first time to obtain various types of homo- and heteropolysaccharides in which the mono- or oligosaccharide repeating units are linked by 1,2-trans-glycosidic bonds. Strict regio-specificity is ensured by placing an O-trityl group at the appropriate site of the monomer, and by temporary protection of all other hydroxyl groups. The most essential feature, however, is that, in this reaction, absolute stereospecificity of glycosidic bond formation is achieved, which is not possible by the previously-used glycosylation methods, Koenigs-Knorr reaction and orthoester synthesis. Such stereospecificity of the reaction is rather unusual for substitution at the glycosidic centre of sugars; it is most probably a result of the push-pull mechanism of the reaction (16). The synthesis of a heteropolysaccharide by this approach requires the preparation of a suitable derivative of an oligosaccharide repeating unit which combines features of the glycosyl and aglycone synthons, *i.e.* possesses 1,2-O-cyanoethylidene and O-trityl groups at appropriate sites of the molecule and which therefore may be used as a monomer for polycondensation.

The synthesis of the S. newington O-antigenic polysaccharide by the chemical polymerization pathway will be discussed as an example (16, 17). The first and the most difficult task consists in the preparation of the monomer itself, *i. e.*, the properly functionalized, oligosaccharide repeating unit. The most usual approach is to introduce the required functions into the already-existing oligosaccharide molecule.

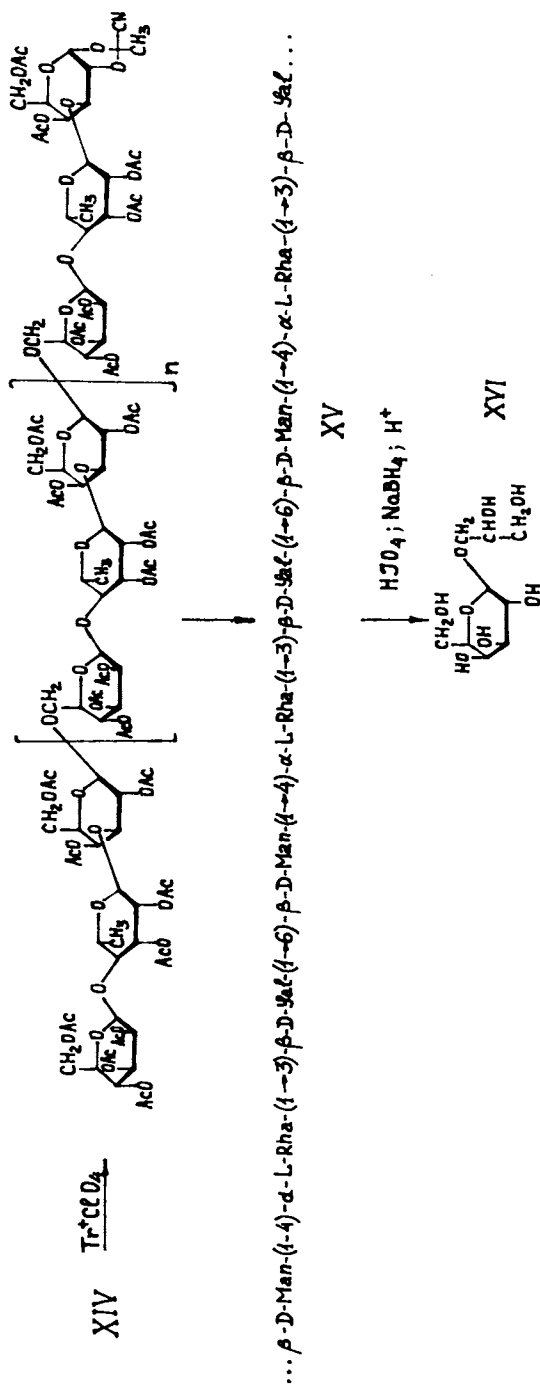
For this purpose, the synthetic trisaccharide derivative II was converted, through the decaacetate III, into the acetohalogenose IV which, by treatment with KCN in CH_3CN was transformed into the 1,2-cyanoethylidene derivative XII (18). Compound XII, by very mild treatment with dilute CH_3ONa , was converted into



XIII having free hydroxyl groups. The next stage was critical, as it was necessary to achieve a regiospecific tritylation at C-6 of the mannose residue in XIII which has two primary hydroxyl groups. A mixture of both isomeric mono-O-trityl ethers and the di-O-trityl-ether was actually formed. Fortunately, after acetylation, it proved possible to isolate the required O-trityl ether XIV, although in moderate yield.

Monomer XIV was introduced into the polycondensation reaction. The reaction was performed in CH_2Cl_2 solution in the presence of 10 mol. % of tritylium perchlorate at room temperature, the removal of traces of moisture being essential. The latter was most conveniently achieved by using a high-vacuum technique although, for larger runs, it is also possible to use standard equipment. The reaction practically came to an end after 50-60 h; it was then finally terminated by the addition of $\text{CF}_3\text{CO}_2\text{H}$ and, after neutralization and solvent removal, the polymer obtained, without purification, was subjected to de-acetylation. The free polysaccharide XV was isolated by precipitation.

Naturally, XV was a mixture of polymer-homologues from which a fraction with mol. mass of ca. 5000 was isolated by gel filtration in 60% yield. This corresponds to a degree of polymerization of 9-10 for the trisaccharide unit, similar to the average degree of polymerization of the natural polysaccharide. The structure of the polymer obtained fully corresponds to that of the natural *S. newington* polysaccharide. Its hydrolysis gave only mannose, rhamnose, and galactose in the ratio of 1:1:1. Methylation with subsequent standard analysis yielded a series of partially-methylated polyols (1,5,6-tri-O-acetyl-2,3,4-tri-O-methylmannitol, 1,4,5-tri-O-acetyl-2,3-di-O-methyl-rhamnitol, and 1,3,5-tri-O-acetyl-2,4,6-tri-O-methyl-galactitol in equimolar quantities), fully confirming the linkage types of monosaccharide residues and indicating the complete regiospecificity of the polycondensation reaction. Especially-thorough checking was performed to ascertain the stereospecificity of the polycondensation. This was done by subjecting XV to Smith degradation which led to the destruction of the mannose and rhamnose residues leaving intact the galactose residue linked to glycerol, formed as a result of oxidation of the mannose residue. The galactosyl linkage in 1-O-galactopyranosylglycerol XVI corresponds to the galactosyl-mannose linkage in the synthetic polysaccharide XV. A thorough analysis of XVI proved it to be the pure β -anomer, *i.e.* confirming the β -configuration of the galactose-mannose linkage in XV.



This finding is indicative of the absolute stereospecificity of the polycondensation reaction. The identity of the structure of XV with that of the natural polysaccharide isolated by a well-known method (19) from S. newington follows also from the comparison of the $^{13}\text{C-NMR}$ -spectra of both samples (see 16).

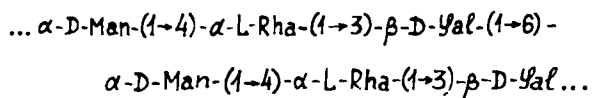
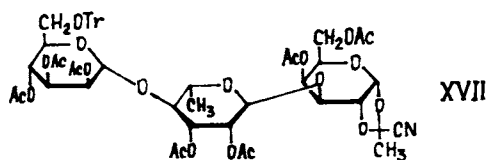
Interesting results were obtained in the immunological studies of the synthetic polysaccharide XV. The synthetic sample was found to be a strong inhibitor of the passive haemagglutination reaction in the Salmonella O-3-anti-3 serum system. At the same time, the closest analogue of XV, the synthetic polysaccharide XVIII with the α -configuration of the mannosyl-rhamnosyl linkage (prepared in a similar way from the derivative of the trisaccharide XVII, ref. 16) showed hardly any inhibitory activity in this test. These data clearly indicate the high biological specificity of the synthetic O-antigen.

Polycondensation of the repeating units of O-specific polysaccharides as the O-trityl ethers of 1,2-cyanoethylidene derivatives is thus a quite promising way for preparation of some natural, biologically active, O-specific polysaccharides and their analogues. At present, a multistage synthesis of another of two similar antigens is in progress in this laboratory, and the necessary modifications are being worked out to extend the method to the preparation of polysaccharides which include aminosugars and uronic acids.

Enzymatic Polymerization of Repeating Units (Chemico-enzymatic Approach)

Present knowledge of the mechanism of biosynthesis of O-antigenic polysaccharides (20) suggests another new approach to their preparation which may be called a chemico-enzymatic approach. In this case, an oligosaccharide, which is the biological repeating unit of the polysaccharide, has to be converted chemically into the polyprenyl pyrophosphate oligosaccharide, an intermediate in the biosynthesis of the polymer. The product should then be treated with an enzyme, O-antigen polymerase, which effects polycondensation of the oligosaccharide units with absolute regio- and stereospecificity of formation of inter-unit linkages, due to the specificity of the enzymatic reaction.

From a preparative point of view, it is essential that the specificity of the polymerase with respect to the structure of the polyprenol residue in the biosynthetic intermediates is not strict. In the Salmonella



XVIII

system, a readily available plant polyprenol, moraprenol from mulberry leaves which was isolated in this laboratory (21) may effectively substitute for the practically unavailable bacterial polyprenol (22). It is interesting that even the synthetic, short hexaprenol derivative was found to participate in the polymerization reaction (23).

As an example of use of the chemico-enzymatic approach, the synthesis of the S. anatum polysaccharide (24) which consists of trisaccharide I units joined by α -galactosyl-1 \rightarrow 6-mannose linkages, will be discussed.

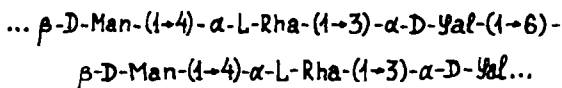
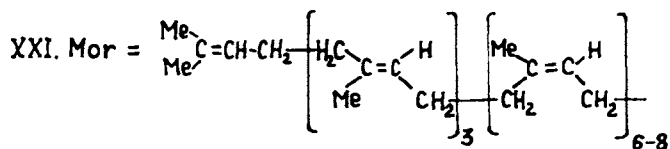
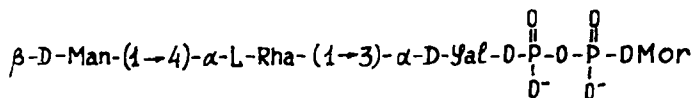
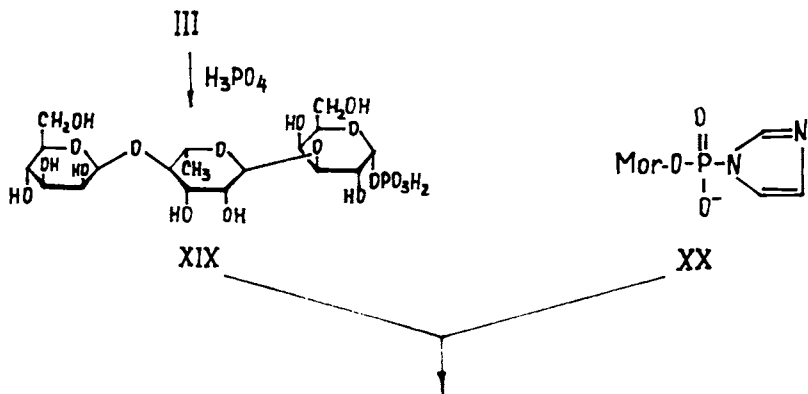
The first stage of the synthesis is the preparation of the substrate for enzymatic polymerization, the polyprenyl pyrophosphate-galactosyl-rhamnosyl-mannose XXI. Since the most convenient way to control the polycondensation reaction is the use of isotopic methods, a procedure for incorporation of tritium into trisaccharide I was developed (25). Labeled trisaccharide was then converted into the glycosyl phosphate XIX through interaction of its peracetate III with anhydrous phosphoric acid (26). Conditions were found under which the reaction is accompanied by minimal destruction and yields the α -phosphate of the trisaccharide.

The synthesis of XXI was accomplished by direct condensation of XIX with moraprenyl phosphoimidazolidate XX in a THF-DMSO-mixture under mild conditions (27). This new method makes it possible to prepare polyprenyl pyrophosphate oligosaccharides directly from unprotected phosphates of oligosaccharides, thus avoiding the need for de-protection procedures which may be accompanied by extensive destruction of polyprenyl pyrophosphate sugars.

The enzymatic step of the synthesis - polymerization of trisaccharide units of XXI - was performed by treatment of XXI with a cell envelope preparation containing the O-antigen polymerase from S. anatum (group E₁).

After splitting of the polyprenyl pyrophosphate residue from the reaction products by mild acid hydrolysis, and gel filtration on Sephadex G-15, a polysaccharide was obtained in about 80% yield. The investigation of its structure by the use of methods similar to those described above has shown it to be a strictly regular polysaccharide XXII consisting of five repeating units of I linked by α -galactoside bonds. The configuration of the linkage formed in the course of enzymatic polymerization was confirmed by Smith degradation of the product, resulting in the formation of α -galactosyl-glycerol.

The synthesis of the S. anatum O-antigenic poly-



XXII

saccharide illustrates the potential of the chemico-enzymatic method. It allows one to prepare also those polysaccharides with the repeating units linked through 1,2-cis-glycosidic bonds. Satisfactory methods for their synthesis through chemical polycondensation are not yet developed.

Probably even more important for wide, preparative use of the method is the fact that the specificity of the Salmonella O-antigen polymerases towards the structure of the trisaccharide unit was found to be not so strict as might be expected (28, 29). A number of analogues of XXI were converted into modified polysaccharides by the polymerases from S. anatum (group E₁), S. senftenberg (group E₄), and S. typhimurium (group B) although yields of the polymers are generally lower than those of the natural polysaccharides. For example, it was possible to demonstrate formation of the modified polysaccharides of S. anatum in which a D-galactose residue is replaced by D-fucose, D-glucose, D-talose, or 4-deoxy-D-xylo-hexose residues. 3-Deoxy-D-arabino-hexose or D-glucose residues may substitute for a D-mannose residue.

Although the preparative aspect of the synthesis of modified polysaccharides has still to be elaborated, there can be no doubt that a wide-spread use of this approach is possible. It seems that, similarly to the case of the chemical polycondensation approach, the synthesis of the corresponding enzymatic polymerization substrates (the polyprenyl pyrophosphate oligosaccharides) remains an essential part of the method. As exemplified by the synthesis of the polysaccharide of S. anatum, the chemical synthesis of these complex compounds has been comprehensively elaborated and can be regarded as sufficiently general. It should be added that partial enzymatic synthesis of polyprenyl pyrophosphate oligosaccharides is also possible using, in some stages, the corresponding nucleotide diphosphate sugars and glycosyl transferases. It is remarkable that, in this case, a way is also open to prepare analogues of natural substrates, since glycosyl transferases, as well as polymerases, proved to possess a not-too-narrow specificity towards the structure of their substrates (30). In fact, it has been possible to obtain several polysaccharides (24, 28, 29, 31) by using a combination of chemical and enzymatic reactions for the assembly of the oligosaccharide residue on the polyprenyl pyrophosphate residue, and subsequent enzymatic polymerization.

Concluding this brief discussion of the pathways for synthesis of O-antigenic polysaccharides by the

polymerization of repeating oligosaccharide units, it should be once again emphasized that the three routes mentioned differ in their possibilities and objectives. The stepwise synthesis yields polysaccharides (or, to be more precise, oligosaccharides containing 3-4 repeating units) with a strictly defined degree of polymerization which cannot be achieved by the other two methods.

The chemical polycondensation method has as yet been elaborated only for polymers with 1,2-trans-glycosidic linkages between the repeating units, but within these limits it seems to be a rather broad chemical method. It is the first purely chemical method for the synthesis of complex polysaccharides, as was demonstrated before for polypeptides and polynucleotides.

Finally, the chemico-enzymatic method has probably the widest possibilities, but further experiments are required to determine whether its enzymatic stage may be scaled-up.

All three approaches can be expected to find application in the preparation of various O-antigenic polysaccharides and their analogues to solve different problems of molecular immunology.

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Immunology and Immunochemistry of Synthetic and Semisynthetic *Salmonella* O-Antigen-Specific Glycoconjugates

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The lipopolysaccharide (LPS) molecule is an interesting component of the envelope of all bacteria belonging to the Enterobacteriaceae. The polysaccharide part constitutes a substantial portion of the cell envelope. The recognition of an antigenic specificity in the polysaccharides, the O-antigens, has led to the common use of antibodies for identification of these bacteria through serotyping. The O-polysaccharide is covalently linked to lipid A where the well-known noxious endotoxic activity of all Enterobacteriaceae resides (1). The toxic activity of lipid A has often been considered to be the greatest biological significance of the LPS, and the importance of the O-polysaccharide has been grossly overlooked. In this communication we will review our research, which has focused on the O-polysaccharide chains with three specific goals in mind:

- (i) The use of small synthetic saccharide haptens identical to regions of various O-polysaccharide chains for the production of specific diagnostic antisera,
- (ii) the use of bacteriophage-associated endoglycosidases for the production of larger saccharides from O-polysaccharide chains, and
- (iii) the production and use of Salmonella O-antigen-specific saccharide glycoconjugates (glycoproteins and glycolipids) for studies of humoral and cell-mediated immune reactivity after salmonellosis, and for studies of their use as immunogens, e.g., vaccines.

Salmonella Oligosaccharide-Protein Conjugates for the Production of O-Antigen-specific Antibodies

The serogroup classification of Salmonella bacteria, according to the Kauffmann-White scheme (2), is based on the antigenic specificities which reside in the polysaccharide chain (the

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O-antigen) of the lipopolysaccharide (LPS) of the bacterial cell envelope. The polysaccharide chain, which as far as we know is composed of polymerized repeating units comprising from three to five monosaccharides (3), contains several antigenic determinants: one specific for the serogroup and one or more in common with other serogroups (2). Conventional procedures for the preparation of antisera against Salmonella O-antigens involve immunization of rabbits with whole heat- or formalin-killed bacteria. However, this procedure results in the production of antibodies of several different specificities, including antibodies with specificity for surface entities other than the O-antigen. To render these antisera O-antigen-specific, absorptions with other bacteria are done in order to remove antibodies with undesired specificities. Absorptions are, however, often incomplete and the titer of the desired antibodies may decrease as well.

We assumed that if saccharides identical to known antigenic determinants of Salmonella O-antigens could be synthesized or obtained by partial hydrolysis of O-polysaccharide chains, and coupled to immunogenic carrier molecules, the resulting glycoconjugates would be suitable as antigens for eliciting in rabbits antibodies with more defined O-antigenic specificity. We have used the O-antigens of Salmonella serogroups A-E as a model system in these studies since the structures of the O-chains are well established (4).

Synthetic Disaccharide Haptens

In serogroups A, B and D the tetrasaccharide repeating units are identical except for the nature of the dideoxyhexose linked to the D-mannose residue (Figure 1). Even before the structure of the O-chain was elucidated it had been convincingly shown that paratose for serogroup A, abequose for serogroup B, and tyvelose for serogroup D were immunodominant sugars in the serogroup specificity (5). Dideoxyhexoses, covalently linked to ovalbumin or bovine serum albumin (BSA) as the carrier protein gave rise to antibodies in rabbits. The antibodies could precipitate the synthetic antigen, but did not agglutinate the bacteria (5). This was in agreement with the results of Goebel (6, 7) and McCarthy (8) who did not find any antibacterial antibodies in rabbits immunized with artificial antigens containing only the terminal sugar of an immunologically active oligosaccharide unit. Therefore the first synthetic work was directed to produce disaccharides with the dideoxyhexose in the non-reducing end position.

The synthesis of the disaccharides entailed the preparation of 3,6-dideoxyhexosyl (paratosyl, abequosyl and tyvelosyl) precursors suitable for joining to an appropriately derivatized D-mannose residue (9, 10). In order to couple the synthesized disaccharides to carrier proteins it was necessary to have an aglycone containing a suitable reactive group such as a *p*-isothiocyanatophenyl group

(11). Therefore the *p*-nitrophenyl α -glycosides of the disaccharides were prepared. For the coupling to protein the *p*-nitrophenyl compounds were catalytically hydrogenated to yield the corresponding *p*-aminophenyl compounds. These were then reacted with thiophosgene resulting in the desired *p*-isothiocyanatophenyl derivatives. In aqueous solution at pH 8-9, the *p*-isothiocyanatophenyl groups of these disaccharide derivatives efficiently reacted with free amino groups (lysine) of the carrier protein (11) to give the artificial antigens Parp(α 1 \rightarrow 3)D-Manp(α 1 \rightarrow)BSA (O-antigen 2), Abep(α 1 \rightarrow 3)D-Manp(α 1 \rightarrow)BSA (O4) and Tyvp(α 1 \rightarrow 3)D-Manp(α 1 \rightarrow)BSA (O9) (12).

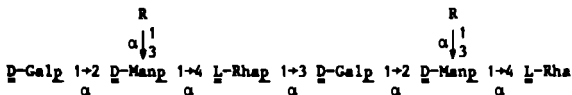
When rabbits were immunized with the glycoconjugates suspended in Freund's complete adjuvant antibodies with specificity for the haptenic disaccharides were produced, as estimated in passive hemagglutination and complement-mediated bactericidal tests, and enzyme-linked immunosorbent assay (ELISA) (12-15).

The specificity of the antibody response for the haptenic groups is illustrated for antibodies elicited against the Parp(α 1 \rightarrow 3)D-Manp(α 1 \rightarrow)BSA conjugate and the *S. paratyphi* A (serogroup A, O-antigen 2) system (Figure 2) (16). In precipitation inhibition experiments a variety of oligosaccharides with O-antigen 2, 4 and 9 determinants, obtained through phage-mediated hydrolysis of O-polysaccharide chains (see later sections), as well as various synthetic di- and monosaccharide derivatives were used. An active inhibitor was Parp(α 1 \rightarrow 3)D-Manp(α 1 \rightarrow)OMe, requiring only 5 nmol for 50% inhibition. An even better inhibitor was the *p*-trifluoroacetamidophenyl α -glycoside of this disaccharide, giving 50% inhibition at 1.6 nmol. This compound in addition to the carbohydrate portion of the hapten also shares the structure of the linkage arm used for attachment of the disaccharide to the BSA carrier molecule of the PM-BSA immunogen (Figure 1). Its superior inhibitory activity as compared to the Parp(α 1 \rightarrow 3)D-Manp(α 1 \rightarrow)OMe derivative indicated that at least the phenyl grouping was recognized by the combining site of PM-BSA antibodies.

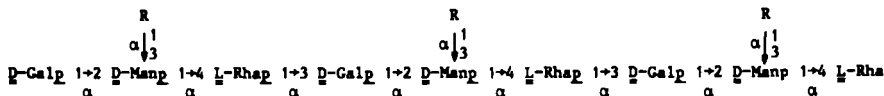
The importance of the complete linkage arm and part of the lysyl residue of the carrier protein in the PM-BSA antibody combining site was demonstrated in an ELISA inhibition assay (Figure 2). The anti PM-BSA serum was depleted of most of its anti-BSA antibodies (>96%) by repeated absorptions with a glutaraldehyde cross-linked BSA immunosorbent. The resulting PM hapten-specific antibodies were then incubated with three different inhibitors before being incubated with PM-BSA antigen-coated tubes. The Parp(α 1 \rightarrow 3)D-Manp(α 1 \rightarrow)OMe glycoside was about 100 fold (50% inhibition at 54,000 nM) less active as an inhibitor than the *p*-trifluoroacetamidophenyl derivative (50% inhibition at 540 nM), which in turn was only one sixth as active as the *p*-isothiocyanatophenyl derivative coupled to ϵ -aminocaproic acid (50% inhibition at 86 nM).

These studies convincingly demonstrated that the anti-glycoconjugate antibodies were complementary to the haptenic disaccha-

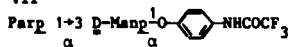
I, III or V



II, IV or VI



VII



VIII

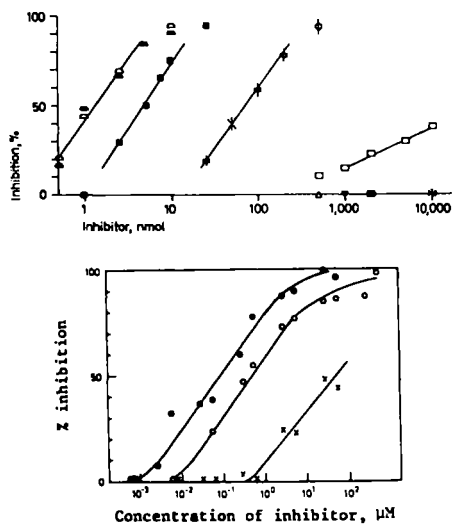
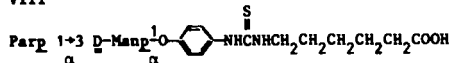


Figure 2. Immunochemical studies of the specificity of rabbit antibodies elicited against $\text{Parp}(\alpha 1 \rightarrow 3)\text{D-Manp-BSA}$ glycoconjugates (02). Top: structures of the Salmonella O-antigen-specific saccharides used. Center: Inhibition of precipitation in mixtures of anti-PM-BSA serum and *S. paratyphi* A PS. Key Center: Ψ , Octasaccharide *S. paratyphi* A. var. Durazzo; Φ , Dodecasaccharide *S. paratyphi* A. var. Durazzo (II); \blacksquare , $\text{Parp} \xrightarrow{1 \rightarrow 3} \text{D-Manp} \xrightarrow{1 \rightarrow \alpha} \text{OME}$; \square , $\text{Parp} \xrightarrow{1 \rightarrow 3} \text{D-Manp} \xrightarrow{1 \rightarrow \alpha} \text{O-}\phi\text{-NHCSNH}(\text{CH}_2)_5\text{COOH}$ (VIII); \blacktriangle , $\text{Parp} \xrightarrow{1 \rightarrow 3} \text{D-Manp} \xrightarrow{1 \rightarrow \alpha} \text{O-}\phi\text{-NHCOCF}_3$ (VII); \square , $\text{Parp} \xrightarrow{1 \rightarrow \alpha} \text{OME}$; Δ , Dodecasaccharide *S. enteritidis* SH 1262 (VI); ∇ , Dodesaccharide *S. typhimurium* SH 4809 (IV); \square , $\text{Abep} \xrightarrow{1 \rightarrow 3} \text{D-Manp} \xrightarrow{1 \rightarrow \alpha} \text{OME}$; \square , $\text{Typ} \xrightarrow{1 \rightarrow 3} \text{D-Manp} \xrightarrow{1 \rightarrow \alpha} \text{OME}$; and $*$, $\text{Abep} \xrightarrow{1 \rightarrow \alpha} \text{OME}$. Bottom: ELISA inhibition of anti-PM antibodies/PM-BSA binding by different disaccharide inhibitors. Key: \times , $\text{PM} \xrightarrow{1 \rightarrow \alpha} \text{OME}$; \circ , VII; and \bullet , VIII. (Reproduced with permission from Ref. 16. Copyright 1980, Int. Arch. All. Appl. Immunol.)

ride, its linkage arm, and part of the carrier molecule. That the part of the combining site recognizing the PM disaccharide discriminated against the AM and TM disaccharides as well as oligosaccharides containing these disaccharides was evident from the precipitation inhibition studies (16). None of these saccharides in concentrations up to 1000 fold higher than the PM disaccharide derivatives showed any inhibitory activity (Figure 2). Thus it could safely be assumed that the PM-ITC-BSA antiserum was specific for the PM disaccharide structure as found in Salmonella serogroup A bacteria.

The assumption was tested in clinical bacteriological studies where Salmonella bacteria belonging to serogroups A, B and D were identified using the PM-, AM- and TM-ITC-BSA rabbit antisera (Figure 1) (13, 14). In an indirect immunofluorescence assay it could be shown that the antisera were specific for all the various Salmonella species within the serogroup, and that no false positive isolates were found. The same excellent specificity was also found when the antibodies of the IgG type were bound through their Fc portions to protein A molecules on Staphylococcus aureus strain Cowan 1 bacteria. Thus, such antibody-coated staphylococci were used as agglutinating reagents, and all three reagents were found to be excellently suited for typing purposes (17).

Salmonella Serogroups C2-C3. The serogroup-specific O-antigen in these two serogroups is O8 (2), and as in O-antigen 4 abequose is the immunodominant sugar (5). Discrimination between the two antigenic determinants results from the fact that in the O8 determinant the dideoxyhexose is $\alpha,1\rightarrow3$ linked to L-rhamnose, whereas the corresponding linkage in the O4 determinant is $\alpha,1\rightarrow3$ to D-mannose (Figure 3).

The disaccharide was synthesized as its p-nitrophenyl derivative and subsequently, as described above, covalently linked to BSA to yield the glycoconjugate AR-BSA (18). Immunized rabbits responded with antibody production with specificity for the saccharide hapten, as demonstrated in ELISA studies (19). When tested in immunofluorescence, the AR-BSA antiserum proved to be of the same excellent specificity for the identification of Salmonella serogroup C2-C3 bacteria as had been shown previously for the other dideoxyhexose-containing disaccharide haptens (Figure 1) (19).

Synthetic Trisaccharide Hapten

Basic information about the immunodeterminant structures for O-antigens 2, 4, 8 and 9 has largely directed synthetic and immunological studies on these antigens. In the O-antigenic polysaccharide chain of bacteria belonging to serogroups E1-E4, O-antigen 3 is serogroup specific (2). The basic structure of the repeating unit of this O-polysaccharide is a trisaccharide (Figure 4), and the accessory O-antigenic determinants found are

a consequence of O-acetyl and D-glucosyl residues linked to this trisaccharide repeating unit. Within the basic structure it was considered, on the basis of complement fixation inhibition studies using oligosaccharides obtained after partial acid hydrolysis of the O-polysaccharide chain, that the D-Manp(β 1 \rightarrow)L-Rhap disaccharide represented the determinant of O-antigen 3 (20). But immunization with a synthetic D-Manp(β 1 \rightarrow 4)L-Rhap(α 1 \rightarrow)BSA glycoconjugate caused only antisera with low hapten-specific titers to be raised. These sera were also found unsuitable for diagnostic purposes.

In order to increase the size of the haptenic determinant the complete basic trisaccharide D-Manp(β 1 \rightarrow 4)L-Rhap(α 1 \rightarrow 3)D-Gal was synthesized, and as before the terminal D-galactopyranose residue was α -linked to a p-nitrophenyl aglycone (21). After conversion of this glycoside to the reactive p-isothiocyanatophenyl derivative it was covalently linked to BSA (β -MRG-ITC-BSA). Immunization of rabbits with the β -MRG-ITC-BSA glycoconjugate resulted in the formation of antibodies with specificity for LPS from Salmonella serogroup E bacteria (Figure 4). Titers seen against the heterologous Salmonella serogroups B and D LPS were significantly lower (100-fold or more).

In indirect immunofluorescence studies we found that the MRG-ITC-BSA serum correctly identified all 30 tested strains belonging to Salmonella serogroup E, whereas no single strain of 90 other non-serogroup E Salmonella isolates were positive. The excellent specificity observed for the D-Manp(β 1 \rightarrow 4)L-Rhap(α 1 \rightarrow 3)D-Galp trisaccharide hapten is of particular interest since in Salmonella belonging to serogroups A, B and D1 the corresponding trisaccharide, having an α instead of a β ,1 \rightarrow 4 linkage between D-Man and L-Rha, is found. However, none of the 50 tested serogroup B and D isolates reacted with the antibodies (Figure 4). Also in the ELISA assay the same type of specificity was observed. This emphasized the importance in terms of immunochemical specificities of the anomeric linkages between the glycosyl residues.

Phage Endoglycosidase-hydrolysed Oligosaccharide Hapten

The structure of the O-antigenic polysaccharide chain in Salmonella serogroup C1 (O antigens 6, 7) has not been established in detail (22). Hence, it has not been possible to identify the structural elements representing the determinant of serogroup O-antigen 07.

In order to obtain structural elements of the O-chain containing the O-antigen 7 determinant we employed a bacteriophage-associated endoglycosidase. By use of the endo- α -mannosidase from the Salmonella serogroup C1-specific bacteriophage 14 the O-polysaccharide from the S. thompson strain IS 40 was hydrolysed into a series of oligosaccharides separable by gel chromatography (23). One of the oligosaccharides, shown by methylation analysis and ^{13}C - and ^1H -n.m.r. to be a decasaccharide with the tentative structure given in Figure 5, was selected for study. ELISA

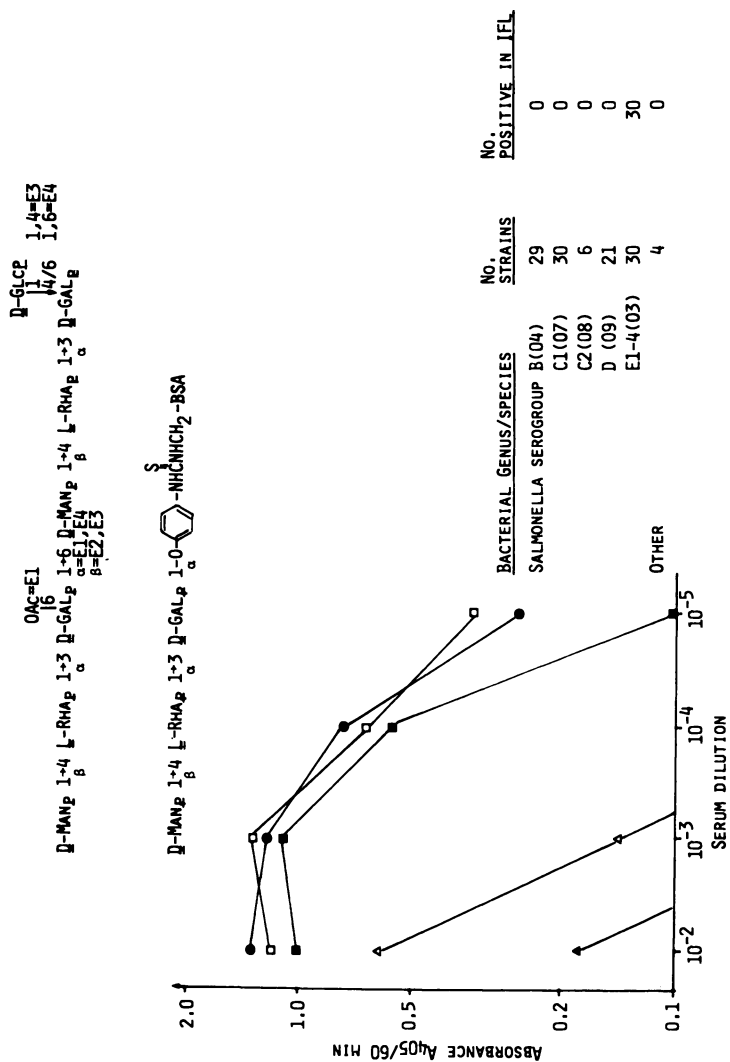


Figure 4. Structures of the O-antigenic polysaccharide chains in Salmonella bacteria of serogroup E and a synthetic trisaccharide glycoconjugate (O-antigen 3-specific). Results of ELISA and immunofluorescence (IFL) studies. Key: ● S. anatum (03, 10); □ S. senftenburg (01, 3, 19); ■ BSA; △ S. typhimurium (04, 5, 12); and ▲ S. enteritidis (09, 12).

LPS COATING ANTIGEN	ELISA TITER ($\times 10^{-3}$)	MICROORGANISMS ASSAYED	NO. POSITIVE IN IFL
MAN 1-2 _{β} MAN 1-3 _{α} GLCNAC 1-2 _{β} MAN 1-2 _{α} MAN 1-2 _{α} MAN 1-2 _{β} MAN 1-3 _{α} GLCNAC 1-2 _{β} MAN 1-2 _{α} MAN	1590	SALMONELLA C1 (07)	50/50
S. CHOLERAE-SUIS (06,7)	7	OTHER SALMONELLA	0/480
S. NEWPORT (06,8)	<1	OTHER ENTEROBACTERIA	3/364
S. TYPHIMURIUM (04,12)	<1	CANDIDA STRAINS	8/9
S. TYPHIMURIUM ROUGH			

Figure 5. Tentative structure of the Salmonella serogroup C1 (O-antigen 7)-specific deca-saccharide. Results of ELISA and immunofluorescence (IFL) studies.

inhibition studies showed that the decasaccharide contained the 07 determinant (24). The reducing terminal D-mannose residue of the decasaccharide was reacted with 2-(4-aminophenyl)-ethylamine and the resulting labile N-alkyl derivative was reduced in situ nascendi in buffered aqueous sodium cyanoborohydride to yield the corresponding secondary amine (25). The process is illustrated in Figure 6. The arylamine was subsequently converted by thiophosgene treatment to the corresponding isothiocyanatophenyl derivative, which then was coupled to give the S. thompson oligosaccharide-BSA glycoconjugate (Os-BSA) (Figure 5).

Rabbits immunized with the Os-BSA conjugate responded with hapten-specific antibodies recognizing the O-antigen 7 determinant in Salmonella C1 bacteria as judged by ELISA using various LPS as antigens (Figure 5). As before, the rabbit Os-BSA antiserum was tested in immunofluorescence against a large number of enteric bacterial strains (Figure 5) (24). All 50 Salmonella C1 isolates scored as positive, whereas none of 480 non-C1 isolates were positive, confirming the 07 specificity of the Os-BSA antiserum. Out of 364 other tested enterobacteria 3 E. coli strains were positive, as were 8 of 9 Candida yeast isolates. These findings are not surprising because reciprocal cross-reactions between Salmonella C1 and some E. coli strains are well known (26) and in yeasts a mannan consisting of an $\alpha,1\rightarrow6$ linked poly-D-mannose backbone with branches of $\alpha,1\rightarrow2$ and $\alpha,1\rightarrow3$ linked D-mannose residues is a principal cell-wall constituent (27).

Within the C1 oligosaccharide hapten it is likely that several different regions elicit complementary antibodies. Hopefully, further structural studies and the synthesis of a few oligosaccharides may help us identify an 07 antigenic determinant with even higher specificity for Salmonella serogroup C1.

In conclusion we have shown that (i) the synthesis of the haptenic disaccharides identical to the O-antigen determinants 02, 04, 08 and 09, (ii) the synthesis of the trisaccharide determinant for O-antigen 3, and (iii) the isolation of a decasaccharide with the O-antigen 7 determinant, and the subsequent covalent linkage of these saccharides to immunogenic carrier proteins has enabled the production of glycoconjugates well suited for eliciting antibodies of desired specificity. The 6 antisera are sufficient for the reliable serogroup-specific identification of more than 95% of Salmonella isolates in most countries (28).

Salmonella O-Antigenic Polysaccharides and Bacteriophage Adsorption

The first step in bacteriophage infection is the adsorption of the phage to a receptor on the bacterium. Almost every structure on the surface of a bacterial cell, or extending from it, can act as (or include) phage receptors (29). The O-antigenic polysaccharide chains of the LPS are no exception in this respect. Since it has been estimated that 10^3 to 10^5 LPS molecules are found on each bacterial cell (30), it is evident that part of the

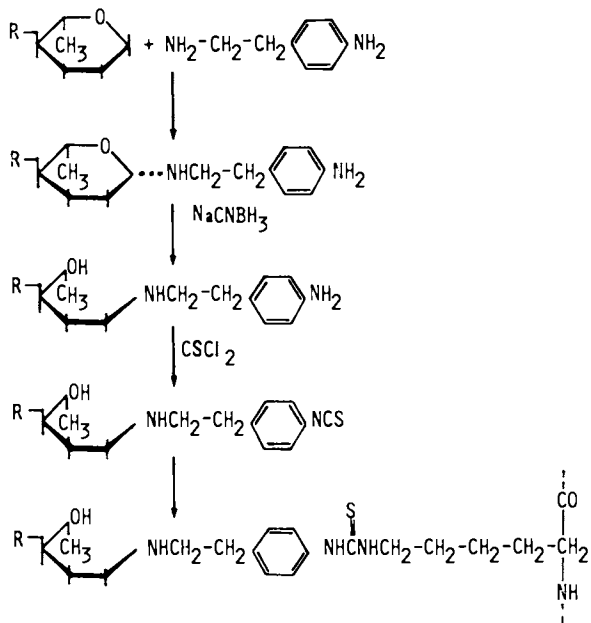


Figure 6. Preparation of saccharide-isothiocyanatophenyl glycoconjugates.

bacterial surface (15-25%) is covered by LPS, and it is the polysaccharide chains that are exposed on the surface.

One of the characteristics of the O-antigen-specific phages is that during infection they hydrolyse glycosidic bonds within the O-polysaccharide, thereby destroying the receptor (31). This endoglycosidase activity is a property of the tail-like phage attachment protein in all cases studied (32). Thus, the adsorption involves formation of an enzyme-substrate complex, the enzyme being an integral part of the phage tail and the substrate being the O-antigenic polysaccharide chain.

We have taken advantage of the hydrolytic activity of these phage proteins for the preparation of series of saccharides of various size. Such large saccharides cannot otherwise be produced by synthetic methods or commonly used partial acid hydrolysis procedures.

Phage P22 lyses bacteria belonging to serogroups A, B and D. Rough mutants, lacking the O-polysaccharide chain, and semi-rough mutants, with one repeating unit only, are resistant (33, 34). This implies that one repeating unit is insufficient as a receptor. The O-polysaccharide chains of serogroups A, B and D differ only in the nature of the dideoxyhexose linked to C-3 of D-mannose (Figure 1). Structural studies of the end products after interaction between the phage and the O-polysaccharide revealed D-galactose residues at the non-reducing ends and L-rhamnose residues at the reducing ends (30, 33, 35). This established that the P22 phage enzyme hydrolyses the L-Rhap(α 1 \rightarrow 3)D-Gal linkage within the repeating unit of the O-polysaccharide chain.

Exploratory studies using 18 different phages active on *Salmonella* serogroups A, B and D revealed that in effect all phages had hydrolytic activity and that all of them shared specificity for the L-Rhap(α 1 \rightarrow 3)D-Galp linkage (33, 36-38). There are, however, minor differences in terms of enzyme specificity. The substrate, *i.e.* the O-antigenic polysaccharide chain, can carry several substituents on the basic tetrasaccharide repeating unit in form of D-glucose residues or O-acetyl groups (Figure 7).

(i) The presence of a D-glucose residue α ,1 \rightarrow 4 linked to the D-galactosyl group of the repeating unit (Figure 7, II), as in the antigenic determinant O12₂, is compatible with hydrolysis by phages P22 and P27 (37). The data even suggested that these phages preferentially hydrolysed the L-Rhap(α 1 \rightarrow 3)D-Gal linkage where the D-galactose residue was glucosylated (37). However, the α ,1 \rightarrow 4 linked D-glucose residue made the substrate resistant to the phage 9NA and KBl endo- α -rhamnosidases.

(ii) The presence of a D-glucose residue α ,1 \rightarrow 6 linked to the D-galactose of the repeating unit (Figure 7, III), a consequence of lysogenic conversion with phage P22, made it impossible for the phage P22 and P27 enzymes to hydrolyse the O-chain (37). However, the α ,1 \rightarrow 6 linked D-glucose, the antigenic determinant O1, permitted hydrolysis of the O-chain by the phage 9NA and KBl enzymes (37). For these enzymes the data suggested that hydroly-

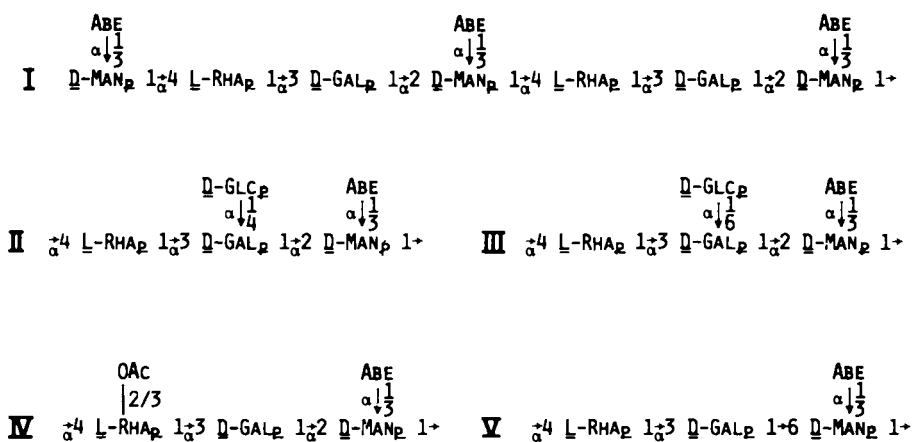


Figure 7. Structures of the O-antigenic polysaccharide chain of *Salmonella* bacteria of serogroup B (I), and various modifications of it (II-V).

sis of the L-Rhap(α 1 \rightarrow 3)D-Gal linkage occurred equally well irrespective of whether the D-galactose residue was glucosylated at position 6.

(iii) Lysogenic conversion of *S. typhimurium* with either of phages A3 or A4 makes the bacteria resistant to adsorption of these two phages, and also reduces the adsorption rate of phage P22 (38). Structural studies revealed that the lysogenic conversion causes an O-acetylation of the L-rhamnose residue at C2/3 (Figure 7 - IV). This O-acetylation is apparently sufficient to prevent hydrolysis of the L-Rhap(α 1 \rightarrow 3)D-Galp linkage by the A3 and A4 phage endoglycosidases. Whether it also prevents phage P22, 9NA, KBl, and P27 enzyme activity, or if it only reduces the substrate affinity, remains to be elucidated.

(iv) A change of the linkage between successive repeating units from α ,1 \rightarrow 2 to α ,1 \rightarrow 6, as occurs upon lysogenization with phage P27 (Figure 7 - V), had a profound effect upon the enzyme-substrate interaction since none of the phage P22, P27, 9NA or KBl enzymes could hydrolyse the L-Rhap(α 1 \rightarrow 3)D-Galp linkage (37). This change in linkage is accompanied by appearance of O-antigen 27 specificity.

(v) The dideoxyhexose in each of the serogroups A, B and D is different, as was discussed above. It is paratose in A, abequose in B, and tyvelose in D (Figure 1). Since LPS and PS from bacteria of all three serogroups were hydrolysed it is apparent that the nature of the dideoxyhexosyl substituent did not influence the substrate specificity of the phage enzymes. Phage P22 apparently requires the presence of the dideoxyhexosyl group, since an abequose-deficient LPS was not cleaved by the phage enzyme (33). On the other hand, two other phages, 28B and 36, with endoglycosidase activity against the L-Rhap(α 1 \rightarrow 3)D-Galp linkage were able to cleave the abequose-deficient LPS (36).

(vi) Periodate oxidation of the L-rhamnose residues followed by sodium borohydride reduction made the *S. typhimurium* PS substrate inert to the phage 28B and 36 enzymes (36).

It is evident from what has been stated above that in spite of the fact that all 18 phages active on smooth *Salmonella* bacteria of serogroups A, B and D hydrolyse the L-Rhap(α 1 \rightarrow 3)D-Galp linkage in the O-polysaccharide chains, small but definitive differences in their substrate specificity exist. The bacteriophage tail has been considered an organ which is clearly in the process of evolution, since it exhibits a more or less complete gradation in morphology and functional complexity (39). Our results support this hypothesis. It would be interesting to investigate whether the observed differences in enzyme specificity are the result of minor (e.g. single amino acid) or major (e.g. oligopeptide) differences in the primary structure of the tail protein.

Production of Different Sized Saccharides

The phage enzyme-bacterial PS substrate interaction resulted in the release of oligosaccharides. When crude preparations,

e.g. saccharides released from the phage-PS mixture through a dialysis bag, were subjected to chromatography on a Bio-Gel P-2 column a series of oligosaccharides was isolated (Figure 8) (36). The product from the cleavage of *S. typhimurium* PS by phage 28B showed peaks corresponding to a dodeca- (E) and an octasaccharide (F), respectively. The product from phage 36 showed essentially the same elution pattern, but in addition to the dodeca-(E') and octasaccharides (F'), a tetrasaccharide (G') was also isolated. The sizes and structures of the oligosaccharides were confirmed by methylation analysis and n.m.r. (36).

With phage 28B we observed that in front of the dodecasaccharide (peak A) there were saccharides of still larger size. By pooling several of the forefractions and subsequently subjecting them to repeated chromatography on Bio-Gel P6 large sized saccharides were isolated (Figure 8). Thus, saccharides with 32 sugar residues (C'' - a hexamer) and 20 residues (D'' - pentamer) were obtained (40). The structures of these saccharides were confirmed by methylation analysis and n.m.r. The yields of these large sized saccharides are much smaller than those of the octa- and dodecasaccharides - usually <1% for each saccharide.

It is evident, however, that the use of phage endoglycosidases can facilitate the production of series of saccharides otherwise unobtainable through either chemical degradation or synthetic procedures. Below we will illustrate with some examples the use of these saccharides as antigens as well as haptens in immunogens.

Salmonella O-Antigen Glycoconjugates

It is generally accepted that two types of immune response are involved in the host defence against salmonellosis - cellular and humoral (41). In attempts to control salmonellosis by active immunization of humans, or of animals in experimental model systems, living vaccines have been superior to killed vaccines; consequently, cellular immunity has been considered to be of greater importance for the host resistance (42, 43).

The specificity of the host defence elicited by killed vaccines has been attributed to the O-antigenic specificity of the vaccine strain (44, 45). Experience with live oral vaccines in mice (46, 47) and man (48) has convincingly demonstrated that also here the presence of the O-antigenic polysaccharide chain is an important requisite for a protective host response.

Neither the mechanisms of the host defence for eradication of challenging *Salmonella* bacteria, nor the relative importance of the host defence against the various individual surface components of the bacterial cell envelope are well understood. Since, as was stated above, the O-antigenic polysaccharide chains apparently play an important role in eliciting an effective host defence of either humoral or cellular nature, we have used the various saccharides described earlier for studies of this problem.

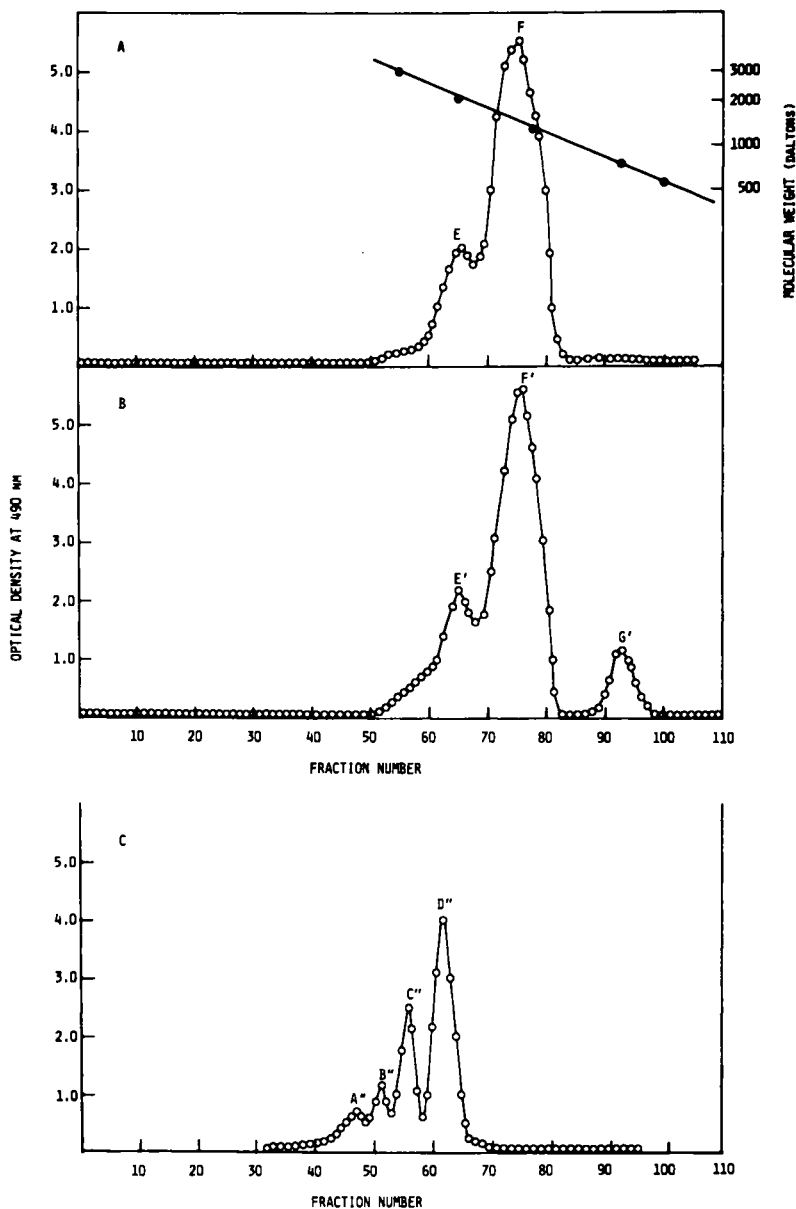


Figure 8. Gel chromatography on Bio-Gel P2 of oligosaccharide preparations obtained after phage 28B (A) and phage 36 (B) hydrolysis of partially delipidated *S. typhimurium* LPS, and on Bio-Gel P6 of pooled fractions 50-60 (C). (A and B reproduced with permission from Ref. 36. Copyright 1979, J. Virol.)

In this section we will report on the use of these saccharides as antigens for studies of the immune response, and as immunogens when conjugated to immunogenic carrier proteins for the elicitation of immune responses.

Antibody Determination in Human Sera

The detection of the appearance of, or increase in, antibody titer against a microorganism is often used to confirm a suspected clinical and/or cultural diagnosis of an infectious disease. In this respect Salmonella infections are no exception. Since antibodies formed against the O-antigen are used for establishing a Salmonella serogroup-specific diagnosis, extracted LPS found an early use for diagnostic purposes when sensitive enzyme immunoassays were developed a decade ago (49). The acceptance of these techniques emphasized the importance of using defined antigens for antibody detection in order to obtain a specificity comparable to the sensitivity of the assay.

Sera collected from patients with verified salmonellosis and from healthy blood donors were titrated against LPS from Salmonella serogroups A, B and D (Figure 9) (51). The difference in end-point titer between sera from patients with organisms homologous to the test LPS antigen and sera from blood donors was always statistically significant. Between patient groups, e.g. B and D, the difference was less pronounced and at times not significant. This is not as surprising as one may be tempted to believe since with the exception of the nature of the dideoxyhexose residue the O-antigenic polysaccharide chains are identical (Figure 1).

The cross-reactivity caused by the common $\rightarrow 2$ D-Manp($\alpha 1 \rightarrow 4$) L-Rhap($\alpha 1 \rightarrow 3$)D-Galp($\alpha 1 \rightarrow$ trisaccharide unit was considerably reduced by periodate oxidation followed by reduction with sodium borohydride, which opens the pyranose ring of the L-rhamnosyl unit between C-2 and C-3 (51). Such LPS preparations with modified L-rhamnose residues, derived from Salmonella serogroups B and D, were subsequently used for the titration of sera collected during an epidemic caused by Salmonella typhimurium (serogroup B). The median relative ELISA titer against the modified B0 antigen was 3200, as opposed to 100 against the modified D0 antigen (51). Extensive use of these antigens over the last few years has revealed, however, that at times the heterologous antigens estimate antibody titers which are considered as positive. We surmised that this is a consequence of the existence of common determinants in the LPS antigens with modified rhamnosyl residues, e.g. in the $\rightarrow 3$ D-Galp($\alpha 1 \rightarrow 2$)D-Manp($\alpha 1 \rightarrow$ region. Titrations using the synthetic D-Galp($\alpha 1 \rightarrow 2$)D-Manp(1)-BSA conjugate as antigen confirmed the hypothesis (52).

In order to further increase the specificity of these assays we have used two synthetic oligosaccharide conjugates as antigens: Abep($\alpha 1 \rightarrow 3$)D-Manp($\alpha 1 \rightarrow 4$)L-Rhap($\alpha 1 \rightarrow 3$)D-Galp($\alpha 1 \rightarrow$)BSA (AMRG-ITC-BSA) and Abep($\alpha 1 \rightarrow 3$)D-Manp(1)-O-(CH₂)₆-BSA [AM-(CH₂)₆-BSA] (53). A

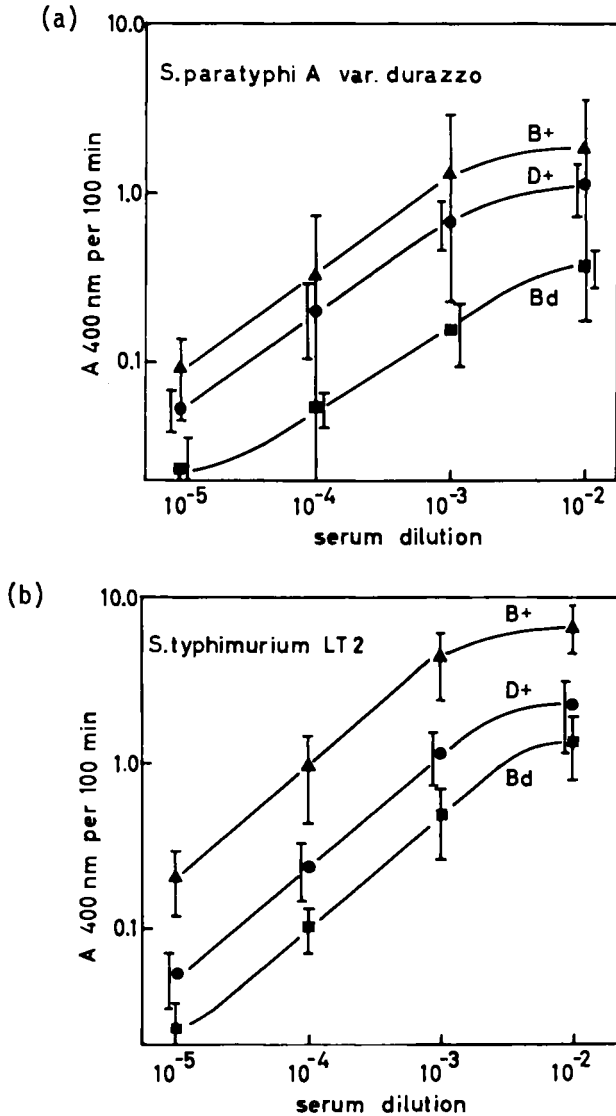


Figure 9. Titration of human sera in ELISA against LPS from (a) *S. paratyphi A* (02, 12), (b) *S. typhimurium* (04, 5, 12), and (c) *S. typhi* (09, 12). Key (a,b,c.): B, 12 patients with Salmonella serogroup B infection; D, 41 patients with Salmonella serogroup D infection; and Bd, 36 healthy blood donors. The horizontal bars indicate the 95% confidence limits. (Reproduced with permission from Ref. 50. Copyright 1973, Int. Arch. All. Appl. Immunol.)

(continued)

(c)

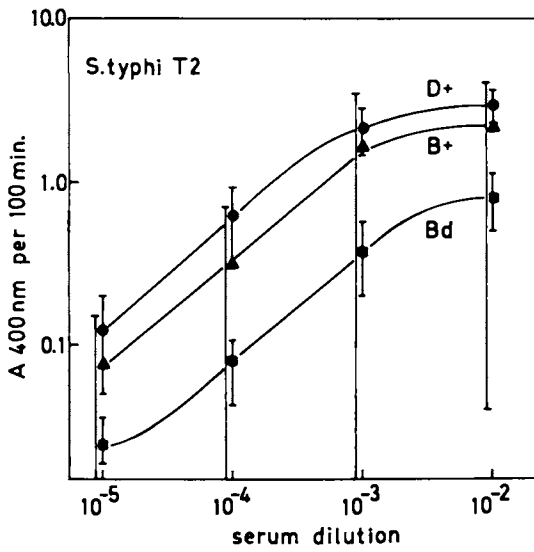


Figure 9c

comparison of the usefulness of these two glycoconjugates and the modified Salmonella BO LPS antigen was done on sera collected from 10 patients with a verified Salmonella BO (04,12) bacterial infection and 10 patients with a verified Salmonella DO (09,12) infection. A typical result is shown in Figure 10. From one patient 5 serum samples were collected over a 12 months period and assayed. Approximately the same antibody titers were found against all three antigens, i.e. in this patient we concluded that most of the antibodies formed were directed against the Abe_p(α 1 \rightarrow 3)D-Man_p disaccharide, which is the determinant of O-antigen 4 (Figure 1). The median IgG titers in convalescent sera (collected 2-6 weeks after onset of Salmonella serogroup B infection) from all 10 patients showed the same tendency: titers were 1380, 1510 and 950 against the Salmonella BO-modified LPS, AMRG-ITC-BSA and AM-(CH₂)₆-BSA antigens, respectively. The corresponding median titers in sera from 10 Salmonella DO-infected patients were 380, 135, and 60, respectively. These results demonstrate the excellent specificity of the AMRG- and AM-glycoconjugates.

Cell-mediated Immune Reactions and Specificity for Salmonella O-Polysaccharides

In Vivo Testing. In demonstrations of cell-mediated immune responses such as delayed hypersensitivity reactions in skin, most investigators have used crude extracts of whole Salmonella bacteria (54, 55). This had made it difficult to evaluate which component(s) had elicited the cell-mediated immunity. In a study of the specificity of delayed hypersensitivity reactions in Salmonella infected cattle to intracutaneous injection of test antigen solutions, it was found that the animals reacted mainly against extracts from O-antigenically homologous bacteria (56). This strongly suggested that the O-polysaccharides were important in this context.

In a subsequent study calves were experimentally infected orally with a sublethal dose of the calf virulent strain S. typhimurium SVA44 and skin tested 3 weeks later (Table I) (57). The LPS from S. typhimurium SH4809 (04,5,12) in a dose of 50 μ g elicited within 48 h in infected calves an increase in double skin fold thickness, with a mean of 4.5 mm. The corresponding figure in the uninfected calves was 1.3 mm, a highly statistically significant difference. Injection of an O-antigenically unrelated LPS (S. thompson), or of poly- or oligosaccharides from the S. typhimurium O-polysaccharide, failed to elicit a skin reaction in both groups of calves. Furthermore the [GM(A)R]₇-ITC-BSA glycoconjugate failed to give a positive reaction (data not shown). However, the covalent linkage of the same octasaccharide structure via the isothiocyanate route to dodecylamine instead of BSA yielded a glycolipid which upon injection in infected calves gave rise to a highly significantly increased skin swelling as compared to the swelling seen when the same semisynthetic glycolipid was

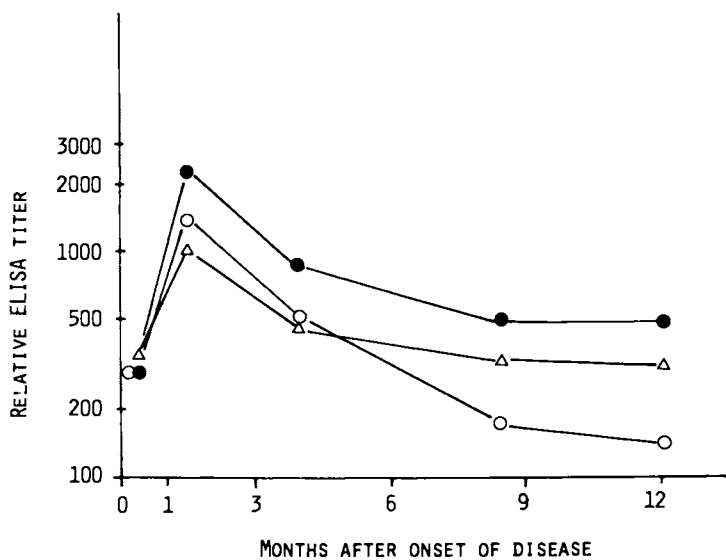


Figure 10. IgG antibody titers against three Salmonella O-antigen 4-containing antigens as estimated by ELISA in serum samples from a patient with *S. typhimurium* infection. Key: Δ , Salmonella BO LPS IO₄ OX; \bullet , AMRG-BSA; and \circ , AM-(CH₂)₆-BSA.

TABLE I. Salmonella typhimurium Infection in Calves: Delayed Skin Reactivity with Specificity for O-Antigenic Polysaccharide Chain

No.	<u>S. typhimurium</u> LPS (04,5,12)	<u>S. thompson</u> LPS (06,7)	<u>S. typhimurium</u> or OLS (04,12)	<u>S. typhimurium</u> PS glycolipid	<u>S. typhimurium</u> Lactobiosyl glycolipid
<u>S. typhimurium</u> infected calves	8 4.5 (0.8)	0.9 (0.8)	0	2.9 (0.6)	0.3 (0.5)
Uninfected controls	10 1.3 (0.7)	0.7 (0.7)	0	0.4 (0.5)	0.1 (0.4)
Significance infected vs. controls	P<0.0005	0.25<P	0.25<P	P<0.0005	0.25<P

injected in uninfected control calves (Table I). That the swelling was caused by a specific reactivity of the immune system of the calves with the saccharide portion and not by reaction with the dodecylamine moiety of the glycolipid was evident from the negative results seen with control lactobiosyl glycolipid. We surmise that the skin swellings seen with the O-antigenic glycolipid but not with the O-antigenic glycoprotein indicate that in these types of immune reactions the O-antigenic determinants must be presented in such a way as to maintain a high local density of antigen epitopes (58), and that the glycolipid can "stick" to membranes of the host cells.

In Vitro Testing. From the same calves used in the studies of skin reactivity peripheral blood lymphocytes (PBL) were collected, and the uptake of ^3H -thymidine, stimulated with among others O-antigenically homologous and heterologous LPS and PS, was followed as a measure of the cell-mediated immunity elicited by the S. typhimurium infection (Figure 11) (59). PBL collected from the infected calves showed a significantly higher uptake of ^3H -thymidine than PBL from the uninfected control calves when incubated with S. typhimurium LPS and PS, as well as with a porin preparation and with a crude extract containing LPS and porins, as well as other cell components. The level of stimulation caused by the O-antigenically unrelated S. thompson LPS (06,7) was significantly lower than that caused by the S. typhimurium LPS (57).

These data show that bovine PBL from Salmonella infected calves can respond to purified homologous O-antigenic PS. The responder cell type was found in a B-cell-depleted and T-cell-enriched lymphocyte population. These results may seem remarkable since they appear to contradict a mass of evidence that polysaccharides are T-lymphocyte-independent antigens. However, most of the studies supporting this T-cell independency have been based on experiments utilizing homo- and heteropolysaccharides which lack the sticky hydrophobic characteristics of the lipid A moiety of the LPS. Our hypothesis is that because of this stickiness the LPS released during a Salmonella infection can adhere to, or insert into, host cell membranes generating O-antigen tagged cell membranes which are recognized by immunocompetent cells able to elicit cell-mediated immune responses.

Salmonella O-Antigen-specific Glycoconjugates as Vaccines

The endotoxic activity associated with Gram-negative bacterial infections resides in the lipophilic lipid A part of the LPS molecule (1). This toxic activity manifests itself in a number of ways, e.g., pyrogenicity, transient leucopenia followed by leucocytosis, hypotension, bone marrow necrosis, abortion, the Shwartzman reaction, etc. As a consequence the commonly used killed whole cell enterobacterial vaccines have to be given in

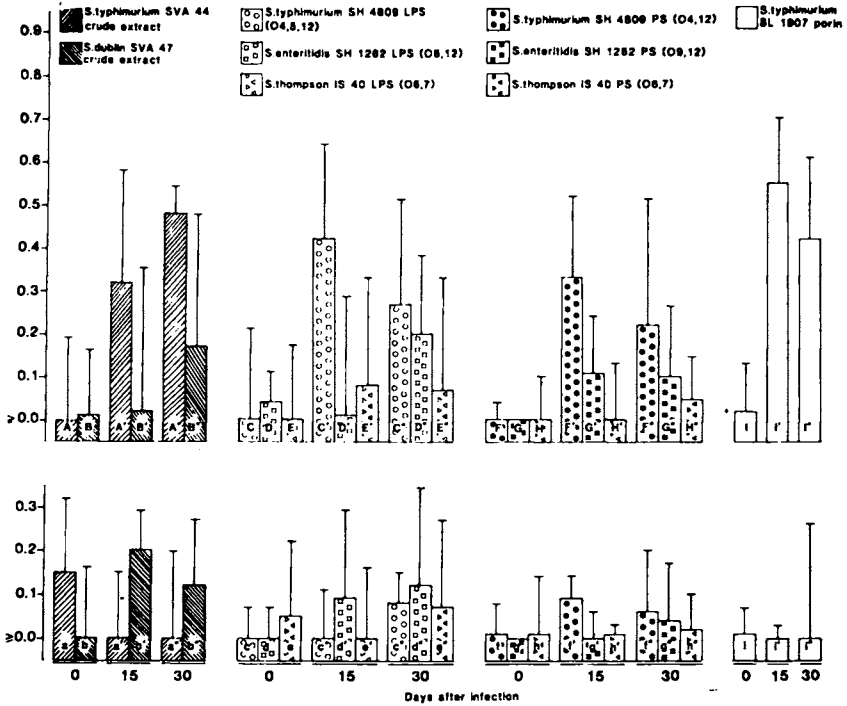


Figure 11. ³H-Thymidine uptake in PBL from *S. typhimurium*-infected and uninfected calves stimulated with various antigens. Each bar shows the mean of log values of SIs, with standard deviations indicated. Statistical analyses were made using Student's t test. (Reproduced with permission from Ref. 59. Copyright 1982, Infect. Immun.)

Levels of statistical significances: A-a, 0.05 < P < 0.1; A'-a', 0.01 < P < 0.025; A'' a'', P < 0.0005; B-b, 0.25 < P; B'-b', 0.1 < P < 0.25; B''-b'', 0.25 < P; C-c, 0.25 < P; C'-c', 0.0025 < P < 0.005; C''-c'', 0.05 < P < 0.1; D-d, D'-d', and D''-d'', 0.25 < P; E--e, E' e', and E'' e'', 0.25 < P; F-f, 0.1 < P < 0.25; F'-f', 0.001 < P < 0.0025; F''-f'', 0.05 < P < 0.1; G-g, 0.1 < P < 0.25; G'-g', 0.05 < P < 0.1; G''-g'', 0.25 < P; H h, H'-h', and H''-h'', 0.25 < P; I i, 0.25 < P; I'-i', 0.01 < P < 0.025; I''-i'', 0.05 < P < 0.1.

quite low doses to minimize the risk of endotoxic side effects. Since a vaccine desirably should contain the O-antigenic polysaccharide chain (46-48) it should therefore also at the same time be devoid of the toxic lipid A moiety. Removal of lipid A by chemical means leaves a non-toxic polysaccharide but this is, however, non-immunogenic (5). The preparation of segments of the O-antigenic polysaccharide chain, either by the synthesis of small di- to tetrasaccharides (10, 11, 18, 21) or through phage enzyme-mediated partial hydrolysis of the O-polysaccharide to yield mono- up to octamers of a tetrasaccharide repeating unit (36, 40), and the subsequent covalent linkage of these saccharides to immunogenic carrier molecules has given us non-toxic immunogenic O-antigen-specific glycoconjugates (60).

The antibody titers seen in rabbits immunized with *S. typhimurium* O-antigen-specific saccharide-BSA conjugates are shown in Table II. The characteristics of the antibody responses in rabbits and mice with respect to anti-hapten antibody response (16, 25, 60-63) can be summarized as follows:

- (i) Each saccharide-protein conjugate elicits a saccharide-specific antibody response.
- (ii) Saccharide-specific antibody titers are often high and comparable to those elicited by heat-killed bacteria when the immunization schemes are identical.
- (iii) The smallest hapten, e.g. the 3-O- α -abequopyranosyl- α -D-mannopyranoside disaccharide (AM-ITC-BSA) elicits the most specific antibody response (O4) but only in rabbits (Table II). Mice failed to respond to saccharides smaller than a tetrasaccharide.
- (iv) Larger saccharide haptens elicit antibody responses with O4 and O12 specificity (Table II). In this respect the antibody responses seen after immunization with either the synthetic tetrasaccharide conjugate (AMRG-ITC-BSA), identical to the biological repeating unit (Figure 1), or with the tetrasaccharide conjugate [GM(A)R-ITC-BSA] where the saccharide was obtained through phage endoglycosidase (Figure 7) are interesting. Evidently antibodies with O4 specificity dominate, but O12 antibodies start to appear. The next larger hapten - the octasaccharide in the [GM(A)R]₂-ITC-BSA conjugate - elicits O4 and O12 antibodies with combining sites very similar, or identical, to those of antibodies elicited by the O-polysaccharide chains of heat-killed bacteria. Indeed, from immunization with a pentadecasaccharide conjugate (AMR[GM(A)R]₃-ITC-BSA) the antibody titers estimated with the homologous LPS and with a hapten conjugate (of a carrier other than BSA) were identical (data not shown).

TABLE II. Antibody Response in Rabbits Immunized with Various *S. typhimurium* Saccharide-BSA Conjugates*.

Immunogen and O-antigen specificity	ELISA end-point titer x 10 ⁻³ against**		
	<i>S. typhimurium</i> SH4809 (04,5,12) LPS	<i>S. enteritidis</i> SH1262 (09,12) LPS	<i>S. thompson</i> (06,7) LPS
AM-ITC-BSA (04)	8250	10	20
AMRG-ITC-BSA (04,12)	585	25	<1
GM(A)R-ITC-BSA (04,12)	900	65	3
[GM(A)R] ₂ -ITC-BSA (04,12)	3320	735	30
[GM(A)R] ₃ -ITC-BSA (04,12)	1430	675	20
[GM(A)R] ₅ -ITC-BSA (04,12)	480	15	<1
<i>S. typhimurium</i> SH4809 (04,5,12) heat-killed bacteria	22,300	8,200	<1

* New Zealand white rabbits (2-4 per immunogen) were injected (3-6 doses) at monthly intervals with the indicated saccharide-protein conjugate (10 µg/dose) or heat-killed *S. typhimurium* SH4809 (1 x 10⁹ bacteria/dose). The glycoconjugates were suspended 1:1 in Freund's complete adjuvant.

** Figures given are mean values. The coating antigen dose was in all instances 1 µg/ml.

- (v) The anti-saccharide antibody response appears to be optimal when the molar ratio of saccharide to BSA is around 20. Theoretically there are 57 sites in the BSA molecule to which the hapten can be linked.
- (vi) Antibodies with specificity for the linkage arm between the hapten and carrier are formed as demonstrated for the disaccharide haptens. The larger the saccharide the less pronounced is this tendency.

The characteristics of the antibody response discussed above did not relate to their biological activity. Therefore a series of experiments was done in order to investigate these aspects.

- (i) The bactericidal activity, e.g. the ability of elicited rabbit antibodies plus guinea pig complement to kill bacteria when mixed and incubated at +37°, was assayed for the anti-AM-ITC-BSA (04) and TM-ITC-BSA (09) antisera (12, 15). These antisera were as effective in this respect as were antisera elicited by whole heat-killed bacteria. Furthermore they were, as expected, absolutely specific, e.g. a bactericidal effect was seen only on bacteria with an O-antigen identical to the antibody-eliciting haptenic structure.
- (ii) In clearance studies, where the ability of the antibodies to aid in the clearance of bacteria from the blood was followed, we found that passively administered rabbit antibodies elicited against the AM-ITC-BSA and [GM(A)R]_{2,3}-ITC-BSA conjugates were as efficient as anti-O-antibodies elicited by whole heat-killed bacteria (64). When mice were actively immunized with the glycoconjugates the larger saccharide haptens elicited an antibody response which was also efficient in promoting clearance. There was a strict correlation between the antibody titer and the clearance rate (64).
- (iii) In in vitro phagocytosis experiments where the ability of individual sera collected from mice before and after immunization with the saccharide-ITC-BSA conjugates were tested for their ability to promote the uptake of bacteria by activated mouse peritoneal exudate cells in the presence of complement we found that also here the immune sera elicited by the [GM(A)R]_n-ITC-BSA conjugates (n = 2 or 3) were efficient (P < 0.001 vs. preimmune sera) for O-antigenically homologous but not heterologous bacteria (64).

- (iv) In passive immunization experiments rabbit antisera elicited by the glycoconjugates were found to protect mice against challenge infection with $100 \times LD_{50}$ doses (5×10^6 bacteria given intraperitoneally) of an O-antigenically homologous *S. typhimurium* strain, but not against challenge with an O-antigenically heterologous *S. enteritidis* strain (the bacteria were isogenic with exception of the O-antigen) (65). From experiments where serial dilutions of the rabbit antisera were given prior to challenge with $25 \times LD_{50}$ doses we could conclude after plotting the anti *S. typhimurium* LPS (04,12) antibody titer against the percentage of surviving mice that all antisera were equally efficient irrespective of whether they had been elicited by a glycoconjugate or heat-killed bacteria (Figure 12).
- (v) Injection of bacterial endotoxins into healthy man or animals results, among other things, in a fever response (66). It has long been recognized that repeated injections of the endotoxin can lead to tolerance to the pyrogenic activity. Virtually all studies have been done with preparations containing the toxic lipid A component, either in its native state or after various more or less efficient chemical detoxification procedures. Since lipid A is required for the immunogenicity of the endotoxin the degradation procedures have left investigators either with only a partially detoxified product, or a completely detoxified product which accordingly has been rendered nonimmunogenic. It has therefore previously been impossible to establish if endotoxin tolerance can be acquired in the absence of the lipid A component.

The problem was addressed using a glycoconjugate consisting of the *S. typhimurium* SH4809 dodecasaccharide covalently linked to human serum albumin ($[GM(A)R]_3$ -ITC-BSA) (67). The conjugate, shown to be free of confaminate protein and endotoxic activity, elicited in rabbits immunized either intravenously or intrapluteally a definite tolerance (Figure 13). The intravenously immunized group 1 showed, when injected with the parent *S. typhimurium* SH4809 LPS, a mean fever index statistically different from the control group ($P < 0.025$). When the immunogen had been administered intrapluteally in Freund's complete adjuvant (group 2) the evoked tolerance was even more striking ($P < 0.01$). That the elicited tolerance was specific for the O-antigen was demonstrated by the lack of tolerance seen when immunized rabbits (group 3) were injected with a *S. thompson* LPS (having identical lipid A and core saccharide regions, but a different O-polysaccharide chain). These findings

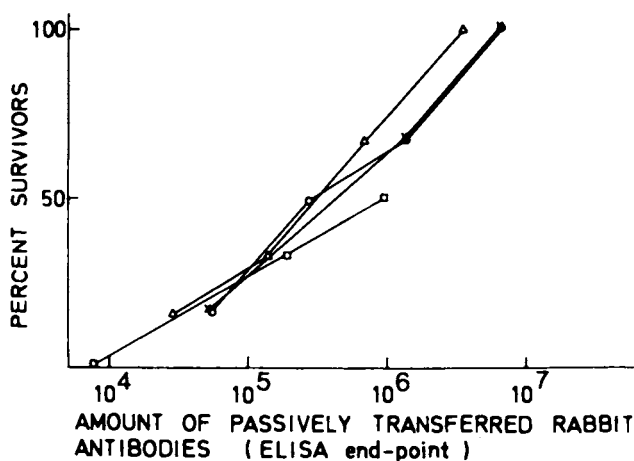


Figure 12. Protective efficiency in mice of passively transferred rabbit antisera against intraperitoneal challenge with *S. typhimurium* bacteria (1.1×10^6 bacteria = $25 \times LD_{50}$ dose). Percentage of survivors plotted against ELISA titers with *S. typhimurium* LPS as antigen. Key: □, anti whole heat-killed bacterial serum (04, 12 specific); Δ, anti [GM(A)R]₂-ITC-BSA serum (04, 12); *, anti [GM(A)R]₂-ITC-BSA serum (04, 12); and ○, anti AM-ITC-BSA serum (04). (Reproduced with permission from Ref. 65. Copyright 1981, Infect. Immun.)

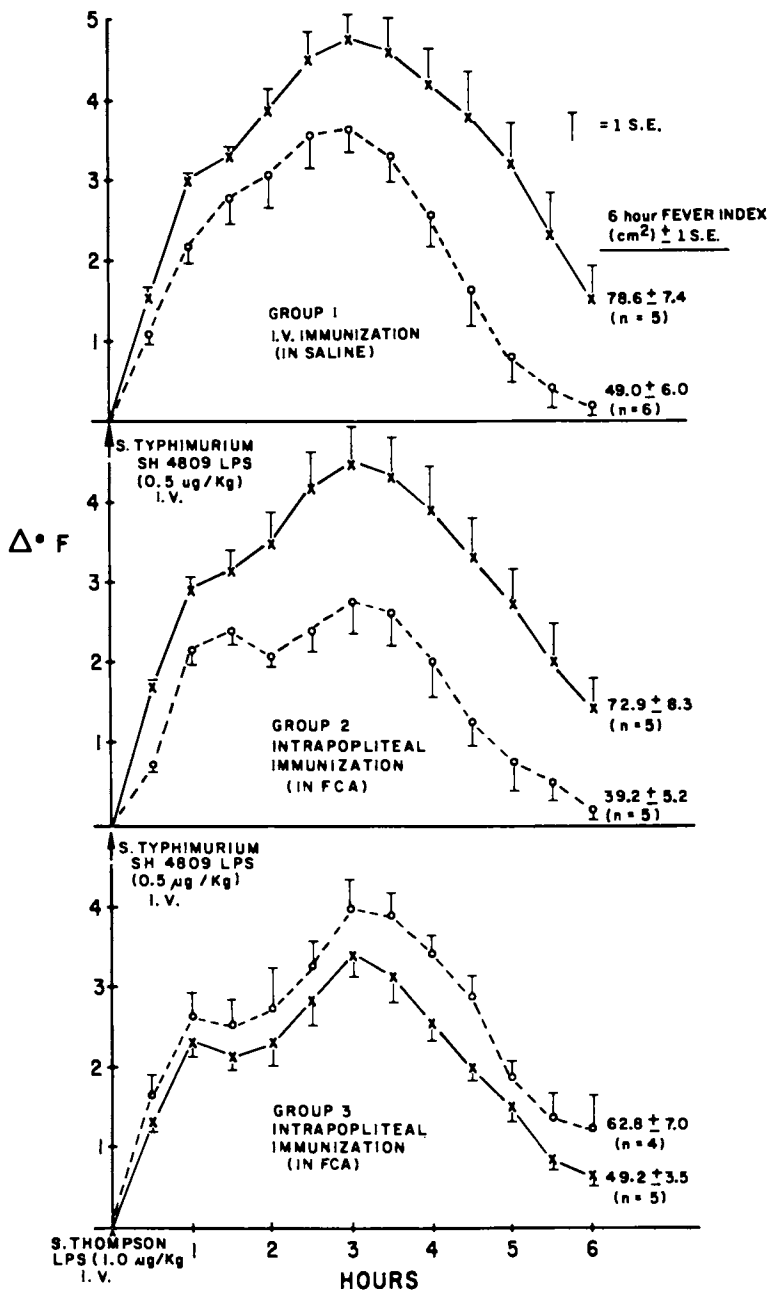


Figure 13. Pyrogenic responses in rabbits immunized to Salmonella LPS. Key: x, control; and o, dodeca 4809-ITC-HSA immunized.

TABLE III. Protective Value of Saccharide-Protein Conjugates as Vaccines in NMRI Mice.

Immunogen*	<u>S. typhimurium</u> SH2201 LD ₅₀ ***
Saline	6 x 10 ⁴
Saline + FCA**	80 x 10 ⁴
AM-ITC-BSA + FCA (04)	64 x 10 ⁴
[GM(A)R] ₂ -ITC-BSA + FCA (04,12)	570 x 10 ⁴
<u>S. essen</u> heat-killed bacteria + FCA (04,12)	1400 x 10 ⁴

*NMRI mice were vaccinated with 5 µg doses on days 0, 19 and 27 and with 10 µg doses on days 42 and 71. Mice were challenged on day 81.

**FCA = Freund's complete adjuvant.

***LD₅₀ values were calculated according to Reed and Muench from the 30-day survival figures of NMRI mice after intraperitoneal challenge with graded doses of S. typhimurium SH2201.

clearly indicate that immunity directed solely against the O-antigen specific portion of the endotoxin molecule is capable of protecting against the pyrogenic activity of the toxophore (i.e. lipid A) component. This directly confirms the previously postulated importance of O-antigenic polysaccharides in the late phase tolerance to bacterial endotoxins (68).

- (vi) The protective active immunity which could be elicited by the glycoconjugates was investigated by immunizing outbred NMRI mice with the immunogens suspended in Freund's complete adjuvant and then challenging the mice with virulent S. typhimurium bacteria (Table III) (69). We found that the [GM(A)R]₂-ITC-BSA conjugate indeed elicited a protective immune response, although not as efficient as that elicited by heat-killed bacteria. The AM-ITC-BSA conjugate failed to elicit a protective immune response, which was in accordance with the observation that the disaccharide haptens repeatedly fail to elicit hapten-specific immune responses in mice. The inferior protective ability of the glycoconjugates as compared to the heat-killed bacteria may have several explanations. Firstly, immunity directed at porins and other outer membrane components unrelated to the O-antigen is of importance (70). Secondly, the saccharide haptens obtained through

phage endoglycosidase activity have an α -D-galactosyl group at their non-reducing ends, whereas the native O-chain terminates with the $\text{Abe}_p(\alpha 1 \rightarrow 3)\text{D-Man}_p$ group (Figure 1). An immune response with specificity for such a terminal α -D-galactosyl group would probably have little, if any, protective effect.

Conclusions

Our investigations have shown that saccharides representing either portions of the repeating unit, the repeating unit, or several repeating units, of the *Salmonella* O-antigenic polysaccharide chain can be produced by synthesis or enzymatic hydrolysis of the O-polysaccharide, and that such saccharides can be covalently linked to carrier molecules. These saccharides and glycoconjugates are useful in studies of the role of the O-polysaccharide in host-parasite interactions. The studies have demonstrated the immunological importance of the O-polysaccharide chain. The following basic findings were made:

- (i) O-Antigen specificity, demonstrable as humoral immunity with exclusive specificity for the O-polysaccharide chain, conferred protection against salmonellosis in an experimental animal model,
- (ii) O-Antigen specificity, demonstrable as cell-mediated immunity with exclusive specificity for the O-polysaccharide chain, was shown in both in vivo and in vitro experiments, and
- (iii) O-Antigen specificity was demonstrable as late phase endotoxin tolerance.

We feel confident in stating that through the use of defined O-antigenic saccharides it is now possible to study on a molecular level selected aspects of host-parasite interactions. These studies will conceivably increase our understanding of certain infectious diseases and definitely hold the prospect for the development of more refined diagnostic procedures and hopefully also vaccines.

Acknowledgment

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Synthesis of Oligosaccharides Containing 3-Deoxy-D-*manno*-2-octulopyranosylono (KDO) Residues

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3-Deoxy-D-manno-2-octulosonic acid (KDO) is a characteristic, ketosidically-linked constituent of the lipopolysaccharide (LPS) in practically all the strains of Gram-negative bacteria examined so far (1, 2). Additionally, in an increasing number of cases, KDO has been found as a constituent of capsular polysaccharides from Escherichia coli (3) or Neisseria strains (4). The significance of these bacterial, cell-surface macromolecules in the pathophysiology and diagnosis of Gram-negative bacterial infections has stimulated investigations into the biosynthesis and immunochemistry of structures containing KDO. In this context, model oligosaccharides involving KDO residues and representing partial structures of LPS or capsular polysaccharides have become of interest as artificial haptens, model substances for spectroscopy, or biochemical probes. In the present chapter, the state of efforts to synthesize KDO-containing oligosaccharides is briefly reviewed.

KDO-containing oligosaccharides as partial structures of the 'inner core' of LPS.

Following the identification of KDO as a constituent of LPS, studies by Osborn and her group (5) have revealed that, in LPS, KDO (or 'the KDO region') is located at the reducing end of the polysaccharide chain, linking the core segment to lipid A. Later, the application of differential color reactions based on thiobarbituric acid (TBA tests) (6, 7) has led to the view that KDO is present, in LPS from Salmonella or E. coli, in the form of a branched trisaccharide (Fig. 1). In this model, a branchpoint KDO residue (KDO I), ketosidically-linked to the second glucosaminyl residue of lipid A, is substituted in position 4 or 5 by a

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ketosidically-linked KDO residue (KDO II) which frequently carries a 7-O-phosphorylethanolamine group; a third KDO residue (KDO III), bearing on its O-5 the remainder of the 'core' oligosaccharide together with the O-antigenic chain, would then be ketosidically linked to O-7 or O-8 of KDO I (Fig. 1).

Due to the limitations of the TBA assays (8), it has not been possible, on this basis, to define more clearly the constitution of the KDO trisaccharide. On the other hand, detailed spectroscopic analyses of the KDO region of LPS have not been reported. In fact, the anomeric configurations of the KDO residues in LPS are still unknown, whereas such configurations have been assigned to the KDO residues present in a number of capsular polysaccharides (9, 13, 31) (cf. the chapter by Jann in this volume).

Only recently, progress has been made toward the elucidation of structure of the KDO region of LPS by physical methods. Thus, n.m.r. measurements seem to indicate that in LPS from several strains, two rather than three KDO residues are present (10). Brade, et al. (11) have detected what is presumably a disaccharide $\text{KDO}_{p2} \rightarrow 4$ or 5 KDO, following hydrolysis (acetate buffer, pH 4.5) of LPS from several strains of Salmonella. The finding that the polysaccharide chain is not released from lipid A under these conditions has led Brade, et al. to speculate that only one KDO residue is located within the main polysaccharide chain, and that the remaining two are part of a disaccharide, side branch (Fig. 2a). According to this interpretation, the KDO-trisaccharide of the heptose-less, R 595 mutant of S. minnesota would be a linear, rather than a branched, trisaccharide. Both the originally-proposed, branched trisaccharide (Figs. 1 and 2b), and the linear structure suggested recently, would have in common a disaccharide of the constitution $\text{KDO}_{p2} \rightarrow 4\text{KDO}$, as found by Prehm, et al. for the LPS of the 'deep-rough', E. coli mutant BB 12 (7). The $\text{KDO}_{p2} \rightarrow 4\text{KDO}$ linkage has also been proposed (12) for a product containing two KDO residues, isolated following the enzyme-catalyzed transfer *in vitro* of radioactive KDO from CMP-KDO onto a lipid-A precursor molecule.

Recent work in the authors' laboratory has been directed at the definitive elucidation of structure of the KDO-trisaccharide by n.m.r.-spectroscopic methods. For this purpose, the LPS from the heptose-less, S. minnesota R 595 mutant is being studied, which has been reported (6) to contain, aside from lipid A, only three KDO residues. The preparation of a homogeneous pseudo-tetrasaccharide, containing besides the three KDO re-

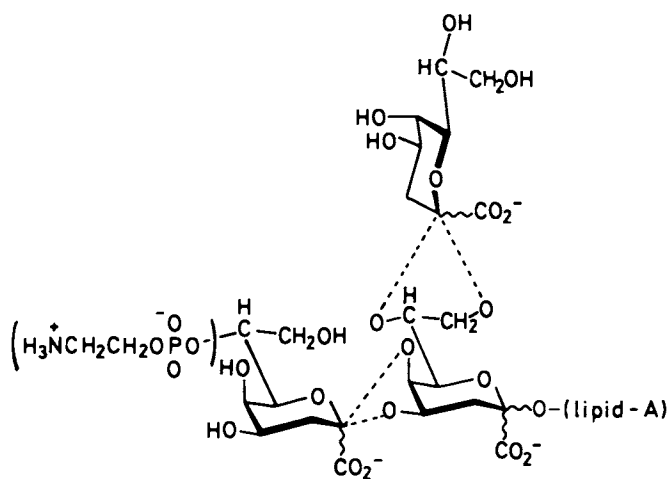


Figure 1. Linkages of the KDO region of LPS from Salmonella strains as proposed in Refs. 5 and 6.

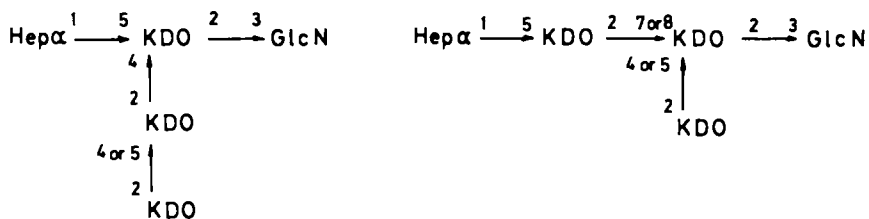


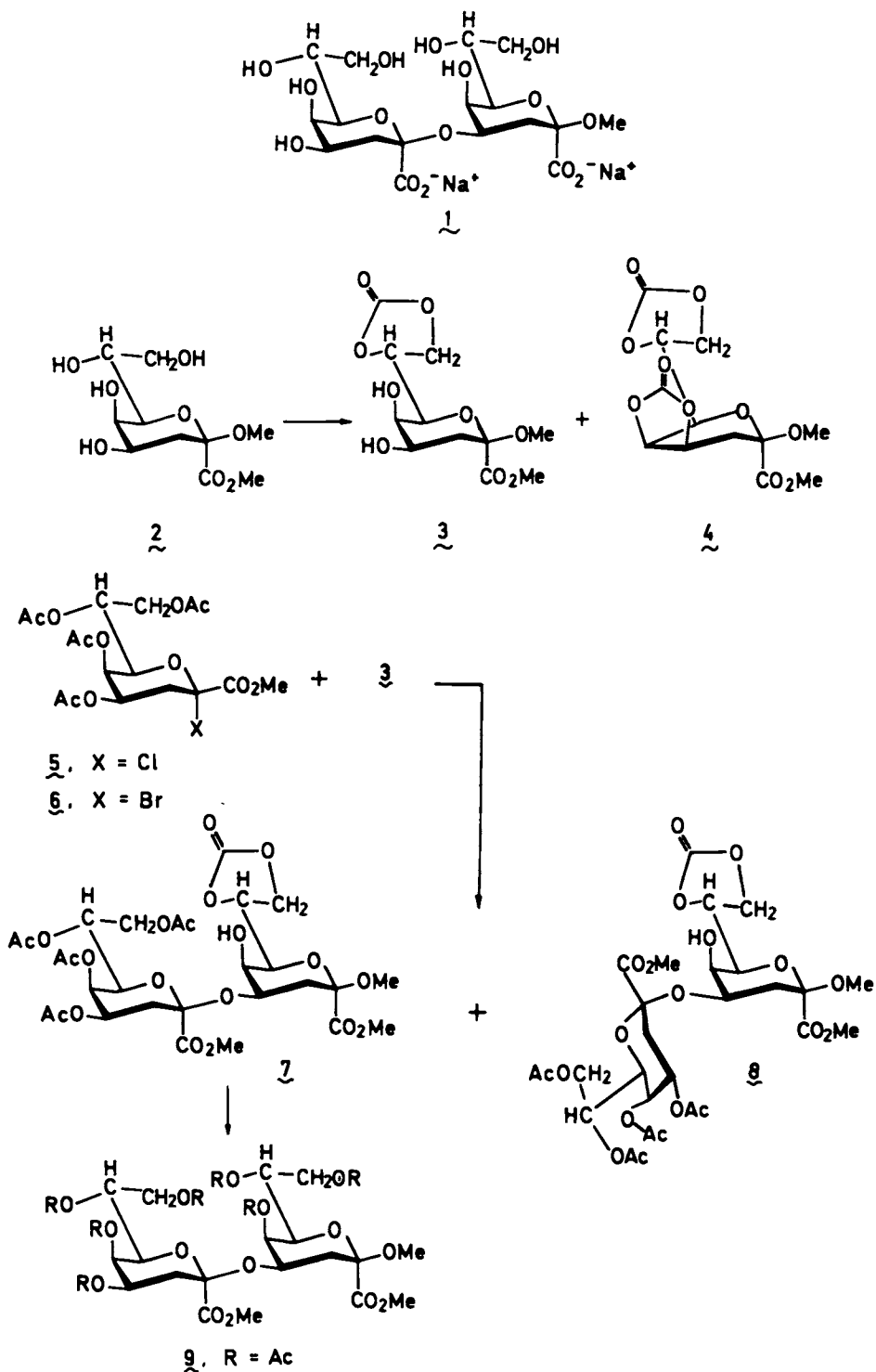
Figure 2. Hypothetical linkages of KDO in Salmonella LPS: (left) according to Brade et al. (11); (right) according to Refs. 5 and 6; cf. Figure 1.

sidues only 2,5-anhydromannitol, is currently being performed as follows. Exhaustive hydrazinolysis of the mutant LPS (previously purified by conventional methods) (6) would free the carbohydrate 'backbone' of O-fatty-acyl, N-fatty-acyl, and O-phosphono groups (cf. the chapter by Rietschel in this volume). Treatment with nitrous acid of the resulting, diamino-dideoxy-pentasaccharide would then afford the desired pseudotetrasaccharide, suitable for spectroscopic studies. A similar degradation has previously been performed by Mireille Rietschel at the Max-Planck-Institut in Freiburg (unpublished results). Preliminary results from the authors' laboratory (observation of the ¹H-n.m.r.-signals attributable to the deoxyprotons of KDO residues, δ 1.80 - 2.40 p.p.m., and TBA assays) indicate that, as expected, the KDO portion of the LPS from S. minnesota R 595 is stable to the conditions of hydrazinolysis and nitrous-acid-deamination. It is hoped that n.m.r.-spectral analysis of the samples thus obtained, in comparison to the model compounds whose syntheses are described below, will lead to a definitive elucidation of structure of the 'KDO region' in the near future.

Selection of target structures for syntheses of model oligosaccharides corresponding to the KDO region of LPS. As a first approach, the synthesis of a 2 \rightarrow 4-linked, disaccharide of KDO residues was attempted. In the absence of conclusive data establishing the anomeric configuration(s) of the KDO residues in LPS, the disaccharide 1, having the KDO residues in the β -anomeric configuration, was chosen considering the established (9, 13) β -anomeric configuration of the KDO residues in the capsular polysaccharides from N. meningitidis 29e (13) and E. coli O6 K13 H1 (9) (cf. the chapter by Jann in this volume).

An aglyconic derivative of KDO, having O-7 and O-8 temporarily blocked, was required for the synthesis of the model disaccharide 1. Koenigs-Knorr reaction of 3 with a suitable halide derivative of KDO would be expected to occur exclusively at the (equatorial) O-4 while OH-5, being axially-disposed and experiencing additional steric shielding by H-7, would be practically unreactive. X-ray crystal structure analysis of KDO derivatives (14, 15) has shown that H-7 is oriented parallel to the axial substituents of the ring carbon atoms.

Synthesis of the disaccharide 1. The reaction of the known (13) methyl ketoside-methyl ester derivative 2



with 4-nitrophenyl chloroformate (16) in pyridine at low temperature afforded preponderantly the crystalline bis-carbonyl derivative 4, but also some of the syrupy 7,8-0-monocarbonyl derivative 3. Compounds 3 and 4 were easily separated by chromatography on silica gel. The unwanted compound 4 may be re-cycled into the starting material 2 by Zemplén saponification. The modified Koenigs-Knorr reaction (17, 18) (silver carbonate, Drierite, dichloromethane, room temperature, over night) of 3 with methyl (3-deoxy-4,5,7,8-tetra-0-acetyl- α -D-manno-2-octulopyranosyl)onate chloride 5 (13) afforded a very low yield of several materials which were too little for further processing and characterization. When, in the Koenigs-Knorr glycosidation of 3, following a suggestion by Paulsen (19), the ketopyranosyl chloride 5 was replaced by the bromide 6 (available in excellent yield from the reaction of methyl 3-deoxy-2,4,5,7,8-penta-0-acetyl- α -D-manno-2-octulopyranosonate (19-21) with titanium tetrabromide) a fair yield of a ca. 10:1 mixture of the β and α -anomers 7 and 8 of methyl [methyl 7,8-0-carbonyl-3-deoxy-4-0-methyl (3-deoxy-4,5,7,8-tetra-0-acetyl-D-manno-2-octulopyranosyl)ono- β -D-manno-2-octulopyranosid]onate was obtained. Separation of the anomers by h.p.l.c. (22) followed by Zemplén saponification of the β , β -derivative 7 afforded a methyl dicarboxylate disaccharide which was per-0-acetylated (acetic anhydride-pyridine-4-dimethylaminopyridine) to give the hepta-0-acetyl derivative, 9. Conversion of 9 into the target compound 1 was performed by sequential Zemplén saponification and treatment with 0.2 N aqueous sodium hydroxide, as previously described for the corresponding monosaccharide derivatives (13, 21).

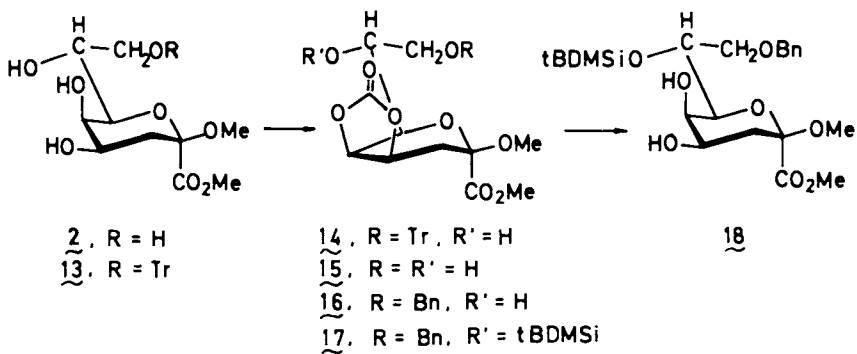
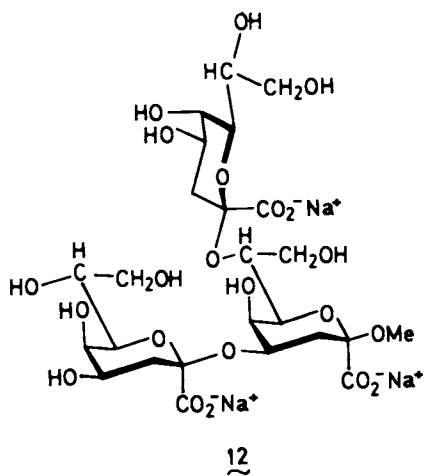
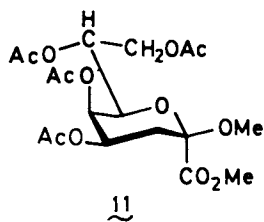
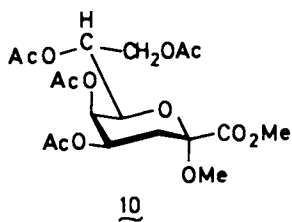
Constitutional and configurational characterization by $^1\text{H-n.m.r.}$ -spectroscopy of the disaccharide derivative 9. The $^1\text{H-n.m.r.}$ -spectrum of 9 (250 MHz, CDCl_3) can be interpreted to indicate both the constitution of the disaccharide and the anomeric configuration of the newly-introduced KDO residue (KDO II, 2 \rightarrow 4-linkage), as follows. Regarding constitution, the presence of seven lines attributable to 0-acetyl groups, of one signal attributable to a ketosidic methoxy group (s, 3 H, δ 3.37 p.p.m.), and of two signals (2s, 6 H, 3.80 and 3.86 p.p.m.) attributable to ester methoxy groups, is in keeping with the over-all structure of 9. There are nine signals due to hydrogen atoms adjacent to acetoxy groups, of which, by first-order analysis, two each are assigned to the geminal hydrogen atoms of C-8 and C-8' (4 H), to H-7 and H-7' (2 H), and to H-5 and

H-5' (2 H). The remaining signal is attributed to H-4' (1 H). Therefore, of the carbohydrate hydroxyl functions, only O-4 is not part of an ester grouping and would be engaged in the interglycosidic linkage, as confirmed by the chemical shift of H-4 (3.95 p.p.m.). The assignment of this signal to H-4 has been verified by INDOR with H-5 (22). The ¹H-n.m.r.-spectrum of 9, in addition, supplies evidence for the β-anomeric configuration of KDO II. Empirical correlations among the shift values of H-4 in the n.m.r.-spectra of ketosidic, O-acetylated KDO derivatives show that, for the α-ketosides, the shift values (δ) of the H-4-signals are significantly larger (by ca. 0.4 p.p.m.) than for the β-ketosides. This difference is clearly apparent from the comparison of the ¹H-n.m.r.-spectra of the two anomeric, O-acetylated ketoside esters 10 and 11. (13, 21) (δ H-4 in the α-ketoside 10: 5.36, in the β-ketoside 11: 4.91 p.p.m.). The shift difference can be explained by de-shielding of the H-4, in α-ketosidic derivatives, through 1,3-*syn*-interaction with the anomeric oxygen atoms. In the spectrum of disaccharide 9, the signal corresponding to H-4' occurs at δ 4.90 p.p.m. and KDO II in 9 would therefore be of the β-anomeric configuration. Both the constitutional and configurational assignments made on disaccharide 9 are independently confirmed by the x-ray crystal structure analysis of the disaccharide derivative 22 (15), the synthesis of which is discussed later in this chapter, and which has been converted into 9 by stereochemically-unambiguous reactions.

Synthesis of the branched, model trisaccharide, 12.

The nature of the protecting groups employed in the preparation of disaccharide 1 discussed above does not permit the extension of the synthesis to the branched type of KDO trisaccharide shown in Fig. 1. To obtain another model compound for the structure elucidation of the natural KDO trisaccharide by spectroscopic and immunochemical methods, a route was devised which would permit the synthesis of the model trisaccharide 12. This compound contains KDO II in a β2→4-linkage, and KDO III in a β2→7-linkage. For a first attempt, preference was given to the 2→7-linked over the 2→8-linked model because precedent exists, in KDO-containing, capsular polysaccharides, for O-7 as a linkage point in the side-chain of KDO residues (cf. the chapter by Jann in this volume).

Preparation of a KDO derivative 18, representing KDO I (the branchpoint) of the branched trisaccharide 12. A

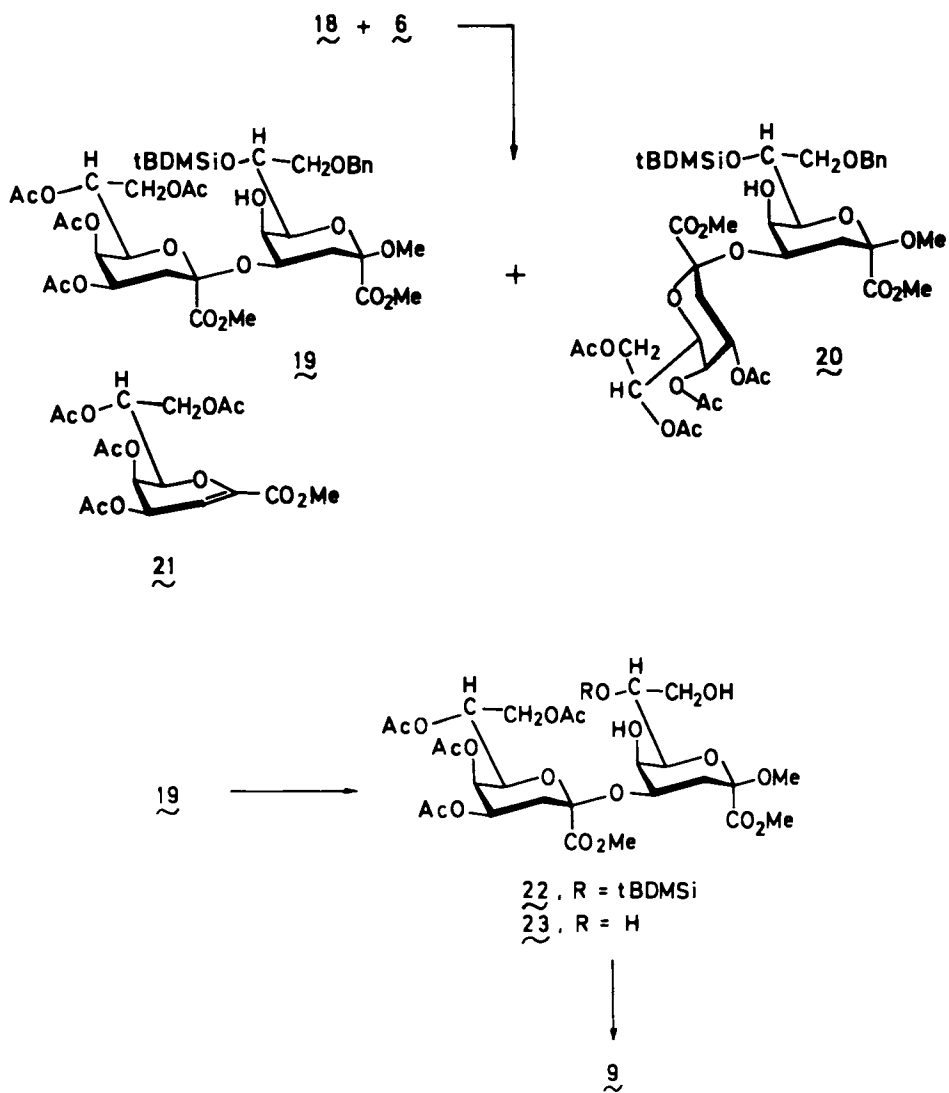


monosaccharide aglycon suitable as the KDO I residue in 12 would have to contain the free 4,5-diol system for glycosidation at the equatorial OH-4, but would have to be protected temporarily at O-7 and persistently at O-8. Ideally, the temporary or persistent functions of the two side-chain protecting groups should be interchangeable with a view toward the eventual synthesis of a model trisaccharide containing KDO III in a 2 \rightarrow 8-linkage. Compound 18, methyl (methyl 8-O-benzyl-7-O-*tert.*-butyldimethylsilyl-3-deoxy- β -D-manno-2-octulopyranosid)onate was designed (22) as fulfilling these demands and was synthesized as follows. The known (13) methyl ester-methyl ketoside 2 was converted into the 8-O-trityl ether 13 (ca. 100%) using trityl pyridinium tetrafluoroborate in acetonitrile (23). 4,5-O-Carbonylation of 13 [4-nitrophenyloxycarbonyl chloride (16) in pyridine] gave a good yield of the trityl ether-carbonate 14 which was de-tritylated by hydrogenolysis (24) to afford the 7,8-diol-4,5-carbonate 15 in practically quantitative yield. Compound 15, by way of its 7,8-O-dibutylstannylidene derivative (25, 26) was regioselectively 8-O-benzylated (dibutyltin oxide, benzene, reflux; then benzyl bromide, dimethyl formamide, 95 $^{\circ}$), in fair yield (16). Finally, the aglycon 18 was obtained in high yield by sequential 7-O-*tert.*-butyldimethylsilylation (27) (compound 17) and Zemplén saponification.

Alternate synthesis of the 2 \rightarrow 4-linked disaccharide

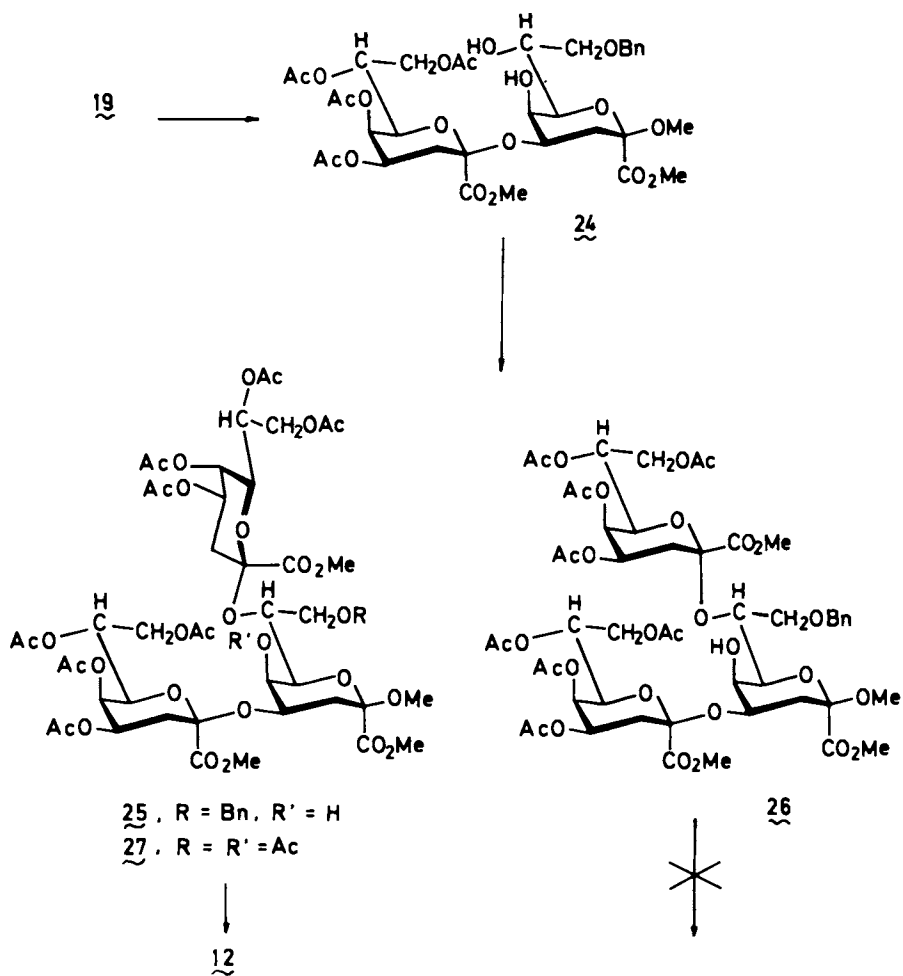
1. Upon glycosidation of 18 with the methyl ester-ketopyranosyl bromide 6 under the modified Koenigs-Knorr conditions given for the synthesis of 7 above, a 7:1 mixture of the β and α -, 2 \rightarrow 4-linked disaccharide derivatives 19 and 20 was obtained in 27% yield. The major part of the halide 6 is converted into the glycal derivative 21 under the reaction conditions. Products arising from glycosidation at O-5 were not detected. The anomers 19 and 20 were separated by preparative h.p.l.c. (22). The configurations of the interglycosidic 2 \rightarrow 4-linkages in 19 and 20 were assigned on the basis of the chemical shift values for H-4' in the respective disaccharide derivatives (see the analogous argument for the configurational assignment of compound 9 above).

Catalytic hydrogenation of 19 over palladium catalyst gave a practically quantitative yield of the crystalline disaccharide derivative 22, the constitution, and configurations at all chiral centers, of which have been verified by x-ray crystal structure analysis (15). De-O-silylation of the 5,8-diol 22



using 2% aqueous hydrogen fluoride in acetonitrile (28) afforded in excellent yield the 5,7,8-triol 23 which, on per-O-acetylation (acetic anhydride-pyridine-4-dimethylaminopyridine) gave the disaccharide dimethyl ester-heptaacetate 9, identical with the material obtained previously by way of glycosidation of the 7,8-O-carbonyl aglycon, 3, as discussed above. The conversion of 9 into 1, having been performed (21) as discussed previously in this chapter, the glycosidation of 18 constitutes an alternate route to the disaccharide 1.

Attachment of the KDO III residue. Setting the stage for trisaccharide synthesis, the β 2 \rightarrow 4-linked derivative 19 was de-O-silylated by treatment with 2% aqueous hydrogen fluoride in acetonitrile (28) to give the 5,7-diol 24 in practically quantitative yield. The structure of the product was ascertained by ¹H-n.m.r.-spectroscopy. The 5,7-diol 24 was subjected to glycosidation with the KDO halide 6 (under the modified Koenigs-Knorr conditions mentioned above for the synthesis of 19), to give a 35% yield of a 6:4, β : α -mixture (anomeric at C-2'') of the 2'' \rightarrow 7- β 2' \rightarrow 4-linked, branched trisaccharides 25 and 26. As with the disaccharide derivatives 19 and 20, preparative h.p.l.c. was required to separate the anomers (22). As crystalline derivatives of the trisaccharides 25 or 26 are still lacking, the constitutional and configurational assignments were made on the basis of the 250 MHz-¹H-n.m.r.-spectra of these compounds and of certain of their derivatives, by the reasoning discussed previously in this chapter. Regarding constitution, the following information was derived from the n.m.r.-spectrum of the deca-O-acetyl-trimethyl ester 27, obtained by sequential, catalytic hydrogenation (palladium oxide in methanol) and per-O-acetylation (acetic anhydride-pyridine-4-dimethylaminopyridine) from 25. i. The spectrum of 27 contains three signals characteristically attributable to H-5 of KDO residues acetylated at O-5 (H-5, δ 5.46; H-5' and H-5'', 5.28 p.p.m.). This excludes O-5 as a site of glycosidation. ii. The only signals attributable to hydrogen atoms adjacent to aglyconic, secondary oxygen functions, are those due to H-4 and H-7. The anomeric configurations of the newly-attached, KDO III-residues have been deduced from the chemical shifts of the H-4''-protons in the spectra of 25, 26, and 27. By the previously-discussed argument, KDO III in 25 and 27 would be of the β -anomeric configuration (H-4'' of 25: δ 4.92; of 27: 4.93 p.p.m.) and, conversely, KDO III in 26 would be of the α -anomeric configuration (H-4'': δ 5.37 p.p.m.).

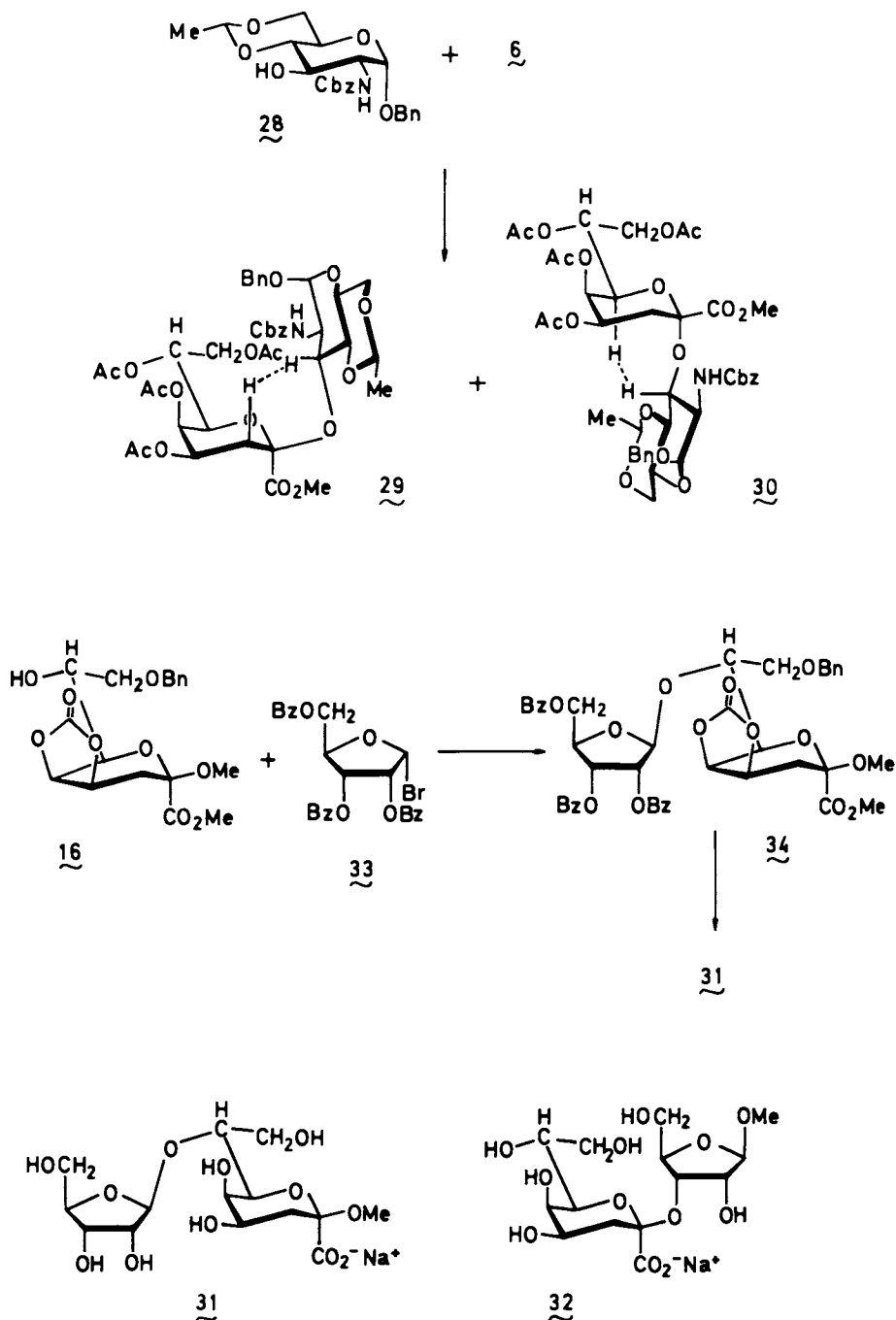


The protecting groups of 27 have been removed by stepwise treatment with sodium methoxide in methanol, then with 0.2 N sodium hydroxide as previously described for the analogous, monosaccharide derivatives (13, 21), to give trisodium methyl [3-deoxy-4,7-di-O-(3-deoxy- β -D-manno-2-octulopyranosyl)onate- β -D-manno-2-octulopyranosid]onate 12 in an over-all yield of 0.8% from 2. Removal of the protecting groups from the C-2"- α -anomeric trisaccharide derivative 26 has been attempted, but not successfully accomplished. De-O-benzoylation under the conditions used with 25 proceeded with extreme sluggishness and, on Zemplén saponification, products containing only two carboxymethyl groups were obtained. This finding may indicate a propensity for lactonization in 26.

Synthesis of disaccharides corresponding to the linkage between lipid A and KDO.

Based on a previous report which had implicated position 3' of lipid A in the linkage to KDO (29), Paulsen and Hayauchi (19) have prepared the anomeric pair of disaccharide derivatives 29 and 30, which they have converted into the disaccharides β KDOp2 \rightarrow 3Glc_pNac and α KDOp2 \rightarrow 3Glc_pNac. Initial experiments had shown that the formation of either 29 or 30 from 28 and the KDO bromide 6 under the 'classical' Koenigs Knorr conditions does not proceed to a preparatively useful extent. However, glycosidation of the aglycon 28 with halide 6 under 'Helferich' conditions (mercuric cyanide, mercuric bromide 3:1, nitromethane, 20^o), affords the α -ketosidic compound 30 in acceptable yield, accompanied by a minor amount of the β -ketoside 29. The β -anomeric configuration was assigned on the basis of the nuclear Overhauser effect (NOE) between the axial H-3 of the KDO moiety and H-3 of the glucosamine moiety in compound 29. Conversely, with the α -anomer 30, an NOE has been observed between H-3 of the glucosamine moiety and H-6 of the KDO moiety (19).

Recent evidence (cf. the chapter by Rietschel in this volume) indicates that, in LPS from Proteus mirabilis, KDO may be linked to O-6' of the lipid A-backbone (the glucosaminyl disaccharide). Conceivably, if the involvement of a primary oxygen function in this ketosidic linkage were substantiated, this would considerably facilitate attempts at the synthesis of oligosaccharides containing the lipid A-KDO linkage.

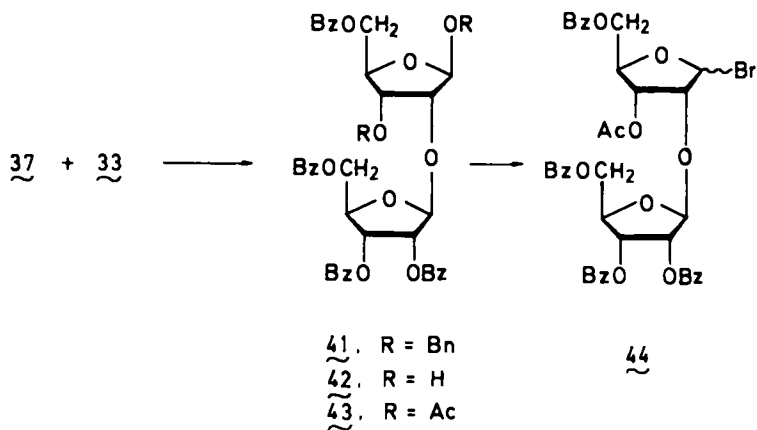
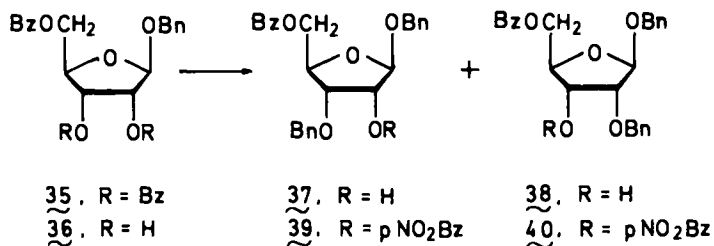


Synthesis of KDO-containing oligosaccharides as 'repeating units' of capsular polysaccharides.

Following the elucidation of the structures of a number of KDO-containing, capsular polysaccharides (see the chapter by Jann in this volume), it became desirable to synthesize oligosaccharides corresponding to 'repeating units' of these polysaccharides. Compounds of this type are of interest for the construction of synthetic haptens, and as steps toward the eventual, total synthesis of capsular polysaccharides, e. g. by way of the block polymerization approach described in the chapter by Kochetkov in this volume. Artificial haptens have potential applications both in fundamental investigations of immunochemistry (e. g., determinations of immunodominant groups) and in the production of antisera (or monoclonal antibodies) for diagnostic and therapeutic purposes (see the chapter by Bundle in this volume).

After the discovery by Taylor (3) of an *Escherichia coli* strain (LP 1092) that produces a capsular antigen containing two ribofuranosyl and one KDO residue per repeating unit (30, 31), interest in our laboratory has centered on those capsular antigens containing KDO and ribose (cf. Fig. 9 and 10, chapter by Jann in this volume). In particular, initial synthetic efforts are concerned with the disaccharides $\beta\text{Ribf1} \rightarrow 7\beta\text{KDOPOME}$ 31 and $\beta\text{KDOP2} \rightarrow 3\text{RibfOME}$ 32 which can be considered model compounds corresponding to repeating units of the K 23 antigen (32) or de-O-acetylated K 13 antigen (33). For a synthesis of 31, the intermediate 16 [methyl (methyl 8-O-benzyl-4,5-O-carbonyl-3-deoxy- β -D-manno-2-octulopyranosid)onate] has been glycosidated with 2,3,5-tri-O-benzoyl-D-ribofuranosyl bromide 33 (34) in the presence of silver triflate (35) to afford the $\beta\text{1} \rightarrow 7$ -linked disaccharide derivative 34 in excellent yield. Following sequential catalytic hydrogenation over palladium catalyst, Zemplén saponification, and saponification by the action of 0.2 N sodium hydroxide, the disaccharide sodium methyl 3-deoxy-7-O-(β -D-ribofuranosyl)- β -D-manno-2-octulopyranosid onate 31 has been obtained (36). By contrast, glycosidation with the KDO halide 6 at O-3 of either of the ribofuranose derivatives 36 or 38, under modified Koenigs-Knorr conditions (17, 18, 22), has not been achieved thus far.

As an approach to the synthesis of trisaccharide 46, which corresponds to a repeating unit of the K antigen from *E. coli* LP 1092, the intermediate 16 has also been glycosidated with the disaccharide halide 44 under the conditions given by Hanessian (35).

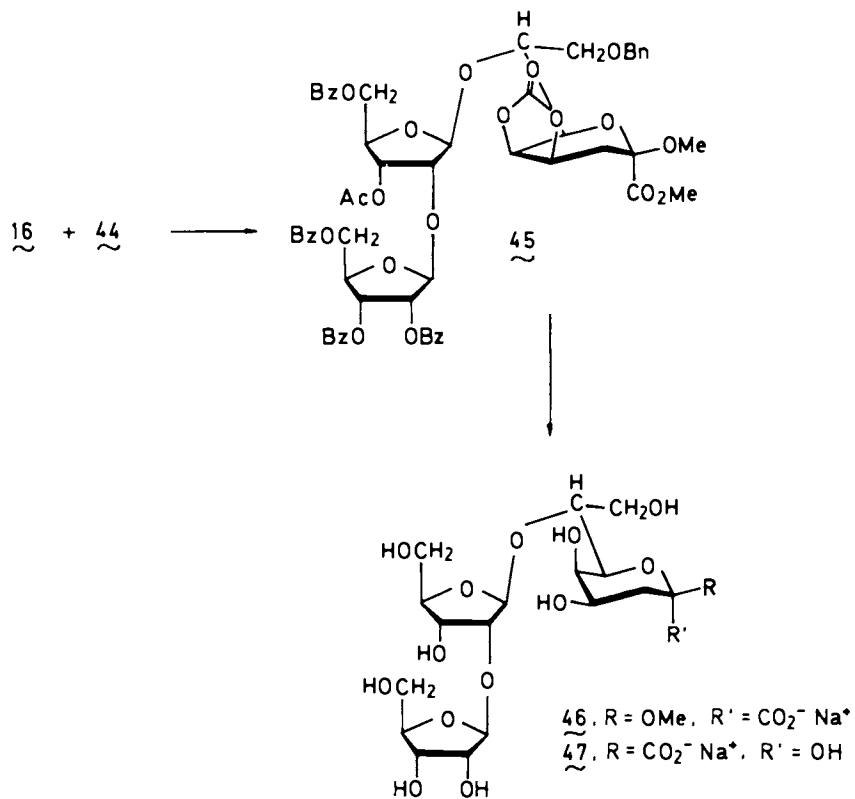


The disaccharide-halide 44 has been prepared as follows. Zemplén saponification, under carefully-controlled conditions, of the known (37) benzyl 2,3,5-tri-0-benzoyl- β -D-ribofuranoside 35 afforded the crystalline benzyl 5-0-benzoyl- β -D-ribofuranoside 36 in good yield. Mono-0-benzylation of 36, by way of its 2,3-0-stannylidene derivative (25, 26), gave a 55:45 mixture of the 3-0- and 2-0-benzyl ethers 37 and 38. These compounds were characterized as their crystalline 2-0- and 3-0-*p*-nitrobenzoates 39 and 40. The crystalline 3-0-benzyl ether 37 was then subjected to glycosidation with 2,3,5-tri-0-benzoyl-D-ribofuranosyl bromide 33, under catalysis by silver triflate (35) to afford the disaccharide derivative 41 in excellent yield. Hydrogenation of 41 over palladium catalyst gave the partially-protected, reducing disaccharide 42 which was 0-acetylated (acetic anhydride-pyridine) to give 5-0-benzoyl-1,3-di-0-acetyl-2-0-(2,3,5-tri-0-benzoyl- β -D-ribofuranosyl)- β -D-ribofuranose 43 (65% from 41). Compound 43 was converted into the bromide 44 by the action of titanium tetrabromide in dichloromethane.

Glycosidation of the aglycon 16 with 44, as catalyzed by silver triflate (35), gave an acceptable yield of the trisaccharide derivative 45, the structure of which was verified by the interpretation of 250 MHz-¹H-n.m.r.-spectra. Following removal of the hydroxyl- and carboxyl-protecting groups, 45 afforded the model trisaccharide 46, suitable for comparison of its ¹³C-n.m.r.-spectrum to that of the intact, capsular polysaccharide from *E. coli* LP 1092 (36). Selective, mild-acid catalyzed hydrolysis of 46 is expected to give the reducing trisaccharide 47, presumably identical to the material obtained after analogous hydrolysis of the LP 1092 polysaccharide by us (30) and by Jennings, et al. (31). Similar studies with the corresponding, disaccharide repeating unit of the K 23 antigen are currently in progress in the authors' laboratory.

Acknowledgments

The authors are grateful to their colleagues and friends, most notably Stephen Hanessian, Otto Lüderitz, András Neszmélyi, Helmut Brade, Barbara and Klaus Jann, Peter Taylor, and Ernst Th. Rietschel for advice and assistance freely given, and for communication of results prior to publication. The work of C. Kratky has been supported by the Österreichischer Fonds zur



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Synthesis and Use of 3-Deoxy-D-manno-2-octulosonate (KDO) in *Escherichia coli*

Potential Sites of Inhibition

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The pathway of KDO biosynthesis and utilization can be described as an essential minor branched pathway in carbohydrate metabolism in Gram-negative bacteria; the pathway is initiated with the key intermediate in the hexose-monophosphate shunt, D-ribulose-5-phosphate. Analogues of D-arabinose-5-phosphate and KDO have been synthesized and tested as possible inhibitors or alternate substrates for D-arabinose-5-phosphate isomerase, KDO-8-phosphate synthase and CMP-KDO synthetase. The substrate analogues synthesized were weak inhibitors of the enzymes involved in KDO biosynthesis. Two intermediates in the biosynthesis of KDO (D-arabinose-5-phosphate and KDO-8-phosphate) have been shown to be weak inhibitors of D-gluconate-6-phosphate dehydrogenase. The specific activities of the enzymes of the KDO pathway (initiating with D-glucose-6-phosphate dehydrogenase and ending with CMP-KDO synthetase) were measured in crude extracts as a function of the specific growth rate and indicate that the level of the individual enzymes are relatively constant. All the specific activities were at least 15 fold greater than the specific activity of CMP-KDO synthetase, suggesting that this may be the rate limiting step in the KDO pathway. Since the specific activities of the individual enzymes involved in the pathway are relatively constant, the overall regulation of carbon flow through the KDO pathway only requires sequential weak end-product inhibition of the enzymes involved to meet the cellular demand for KDO utilized in LPS synthesis.

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Our interest in KDO (3-deoxy-D-manno-2-octulosonate) began with the finding that this unique eight-carbon sugar is a site specific molecule found almost exclusively in the lipopolysaccharide (LPS) portion of the outer membrane of Gram-negative bacteria. George Hitchings, our Research Director at the time, inspired us by his example of thoroughly studying specific biosynthetic pathways of importance to the cell, to study the synthesis and metabolism of KDO with the goal of understanding the regulation of this pathway and finding a new antibacterial agent with this novel and specific site of action.

Gram-negative bacteria possess both an inner cytoplasmic membrane and an outer membrane (1). The inner membrane is the site of the electron transport system, the site of active transport, the location of the enzymes involved with fatty acid biosynthesis and phospholipid biosynthesis; it also functions to retain the intracellular constituents within its boundaries. The outer membrane, as described by Nikaido and co-workers (2-7) is an asymmetric, unique membrane that provides a protective barrier for the fragile, evolutionarily conserved underlying peptidoglycan layer found in Gram-negative bacteria. It is an asymmetric membrane, in that the bulk of the phospholipids are located on the inner surface of this bilayer (8), while the LPS molecules extend outward from the surface. Proteins of the outer membrane have been well studied (9,10) and are distributed within and on both surfaces. The outer membrane of Gram-negative bacteria is unique in that it contains, in addition to proteins and phospholipids, the lipopolysaccharide molecules that are the subject of this symposium.

The structure of the lipopolysaccharide region from *Salmonella typhimurium* provides a good model system and is shown in Figure 1. The LPS is composed of four major components; (1) Lipid A (a phosphorylated diglucosamine molecule containing seven fatty acids) is the fundamental building block of the LPS structure. Attached to Lipid A is the backbone region composed of two or three molecules of KDO and two heptose residues. Connected to the backbone region is a pentasaccharide outer core region which contains glucose, galactose and N-acetylglucosamine. Attached to the outer core portion of the molecule is the O-antigen, whose composition and structure varies from one species to another and thereby determines the major somatic antigen specificities.

The biosynthesis of the LPS is initiated at the Lipid A portion of the molecule and proceeds toward the non-reducing terminus. Mutants defective in either the biosynthesis or the activation and transfer of most of the individual sugars are known (2). These mutants produce incomplete saccharide chains and translocate the resulting incomplete structures to the outer surface. The use of these mutants (described in Figure 1) has

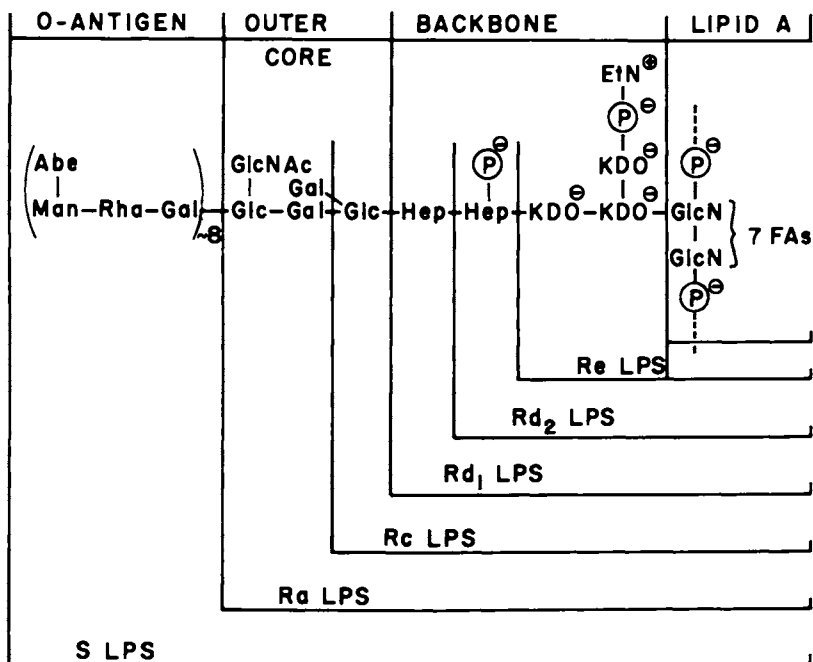


Figure 1. Schematic representation of the LPS of *Salmonella typhimurium*. This drawing was derived from the work of many laboratories (referenced in 2, 3, 45, 46).

Abbreviations: Abe, abequose; Man, mannose; Rha, rhamnose; Gal, galactose; Glc, glucose; GlcNAc, N-acetyl-glucosamine; Hep, L-glycero-D-mannoheptose; KDO, 3-deoxy-D-manno-octulosonic acid; Ein, ethanolamine; FA, fatty acids; P^{\ominus} , phosphate. Mutants that produce incomplete polysaccharide are designated Ra to Re and are indicated by the solid lines; S LPS refers to wild type LPS. Position and number of fatty acids linked to lipid A have been updated by Westphal et al. (46).

allowed the major portion of LPS biosynthetic pathway to be understood.

Elucidation of the pathway for the biosynthesis of Lipid A and the backbone region is still incomplete (11). The first known step in the synthesis of Lipid A was obtained by Rick and Osborn (12,13) who used a temperature sensitive mutant of Salmonella typhimurium that was defective in the synthesis of KDO-8-phosphate. The defect in this mutant lies in the apparent K_m for D-arabinose-5-phosphate of its KDO-8-phosphate synthase. In the absence of exogenous D-arabinose-5-phosphate at 42°C, this mutant ceases the synthesis of LPS immediately and more importantly protein synthesis, RNA synthesis and cellular growth are inhibited after one generation. When KDO synthesis is blocked by an increase in the growth temperature, this mutant accumulates a Lipid A precursor devoid of KDO and containing only four of the normal seven fatty acid esters (13,14). Munson *et al.* (15) have shown that this isolated precursor *in vitro* will accept two KDO residues and that the attachment of KDO is necessary for the maturation of the Lipid A precursor. Rick and Osborn's data (13) suggest that the structural integrity of the KDO-Lipid A region of the LPS is essential for growth. The cessation of macromolecular synthesis may well constitute a regulatory response to the inhibition of LPS synthesis.

From the chemotherapeutic point of view, the LPS biosynthetic pathway is an attractive target, since mutants producing incomplete LPS are more susceptible to antibiotics and less pathogenic. The LPS structure has four unique components: (1) Lipid A itself, (2) β -hydroxymyristic acid (3) L-glycero-D-manno-heptose and (4) KDO. The biosynthesis and metabolism of KDO became our primary target for the following reasons: (1) KDO is a unique eight carbon sugar found only in Gram-negative bacteria, (2) with the exception of the K-antigens, it is a site specific molecule; (3) KDO is the direct link between Lipid A and the growing polysaccharide chain; (4) KDO is required for Lipid A maturation and cellular growth; and (5) several enzymes in the pathway had been identified (16-19).

Our approach to this problem was to isolate and purify the enzymes involved in the synthesis and metabolism of KDO, to investigate the substrate specificity of these enzymes, to synthesize specific substrate analogues with the hope of defining a specific inhibitor of the pathway and lastly, to study the entire pathway in detail to learn the mode of regulation.

Enzymes of KDO Synthesis and Metabolism and Their Inhibition. The KDO pathway can be thought of as a minor branched pathway in carbohydrate metabolism initiating with the key intermediate in the hexose-monophosphate shunt, D-ribulose-5-phosphate. As shown in Figure 2 the biosynthesis and utilization are known to involve at least five sequential reactions:

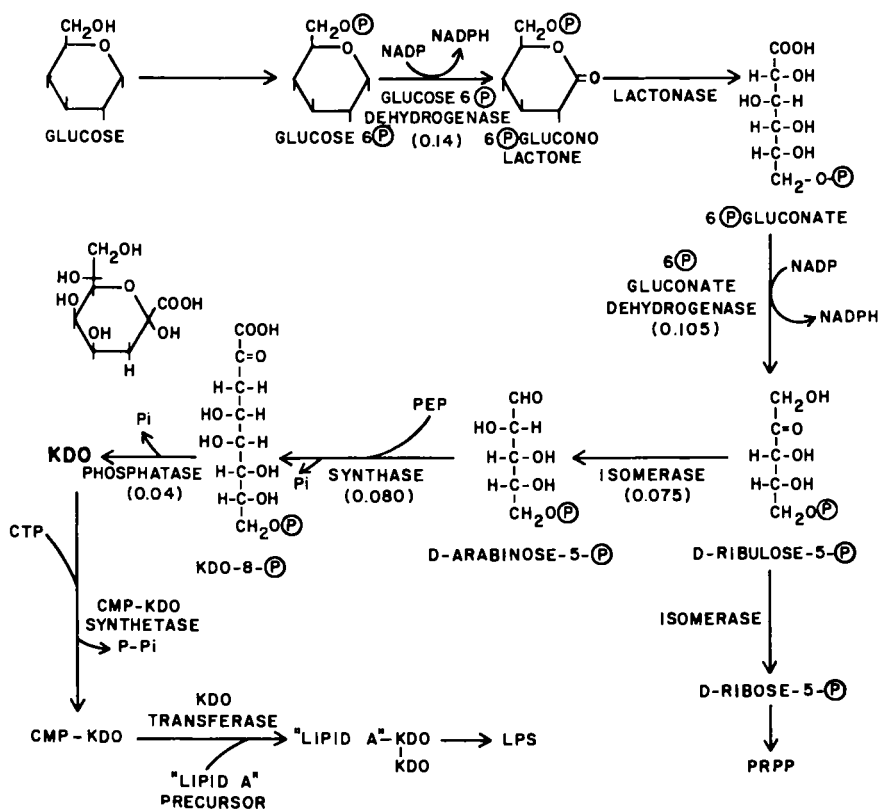


Figure 2. Expanded pathway for the synthesis and use of KDO. The pathway initiates with D-glucose-6-phosphate, shows the branch point whereby D-ribulose-5-phosphate can be used to synthesize either D-arabinose-5-phosphate or D-ribose-5-phosphate and terminates with the transfer of KDO to the lipid A precursor. The numbers in parenthesis below the various enzymes correspond to their specific activities (μ moles per minute per mg protein).

- (1) D-ribulose-5-phosphate \rightleftharpoons D-arabinose-5-phosphate
- (2) D-arabinose-5-phosphate + PEP \longrightarrow KDO-8-phosphate + P_i
- (3) KDO-8-phosphate \longrightarrow KDO + P_i
- (4) KDO + CTP \longrightarrow CMP-KDO + PP_i
- (5) CMP-KDO + Lipid A Precursor \longrightarrow KDO-KDO-Lipid A Precursor + CMP

The reactions, 1-5 respectively, are catalyzed by D-arabinose-5-phosphate isomerase, KDO-8-phosphate synthase, KDO-8-phosphate phosphatase, CMP-KDO synthetase and KDO transferase (s). Using *E. coli B*, we have isolated and purified the second, third and fourth enzymes to homogeneity and studied their properties. The fifth enzyme has been partially purified by Osborn's laboratory (15) and we have not improved on its purification or further studied its properties.

D-Arabinose-5-phosphate isomerase, the first key enzyme in the synthesis of KDO, catalyzes the interconversion of D-ribulose-5-phosphate and D-arabinose-5-phosphate. This enzyme was briefly studied by Volk (18) and later by Lim and Cohen (19). Due to the instability of the enzyme, we have only purified this enzyme 100-fold. The reversible reaction is readily monitored by measuring the formation of the keto-sugar from the aldo-sugar by the method of Dische and Borenfreund (20). The K_m values for D-ribulose-5-phosphate and D-arabinose-5-phosphate are 0.9 to 1.5 and 1 to 3 x 10⁻⁴ M, respectively.

Our approach to the inhibition of this key enzyme was to prepare structural analogues of the substrate, D-arabinose-5-phosphate, in order to probe the binding requirements of the enzyme. Based on previous work by others (21,22,23) a mechanism for the isomerase reaction that involves the *cis* enediol intermediate shown in Figure 3, was used as a model. This is a least motion model utilizing a 1,2-hydrogen shift mechanism. A basic group on the enzyme is postulated to remove the pro-S proton on C-1 of D-ribulose-5-phosphate and reprotonate the substrate at C-2 on the same face of the enediol. If the product cyclizes before departing from the enzyme surface then β -D-arabinose-5-phosphate should be the kinetic product or the kinetic substrate for the reverse reaction. The inhibition data with phosphorylated analogues (Table I) and non-phosphorylated analogues (Table II) of cyclic and non-cyclic sugars suggest the following:

- (1) Phosphorylated analogues are better inhibitors than non-phosphorylated analogues.
- (2) Among the phosphorylated analogues, the aldonic acids are better enzyme inhibitors than the corresponding alcohols, and the alcohols are better inhibitors than the corresponding aldehydes. This may reflect the acid and alcohol molecules ability to mimic the postulated enediol intermediate geometry.
- (3) The best inhibitor is D-erythronic acid-4-phosphate (Compound 25, Table I), which has one less carbon than the normal substrate. This is very similar to the inhibitor-substrate

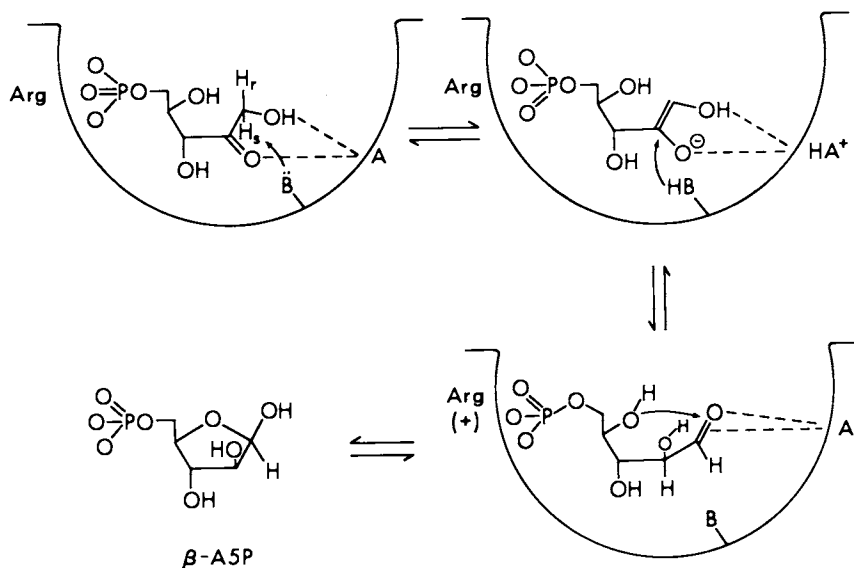
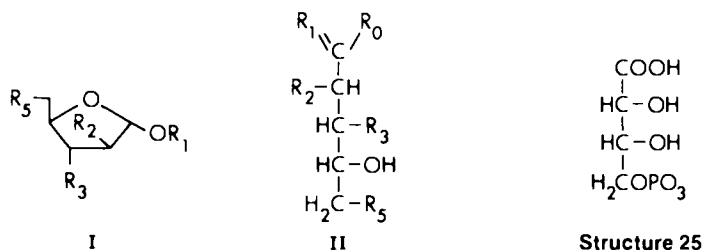


Figure 3. Plausible mechanism for the conversion of D-ribulose-5-phosphate isomerase to D-arabinose-5-phosphate by D-arabinose-5-phosphate isomerase. It is a least-motion model consistent with the mechanisms of similar enzymes (21-23).

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TABLE I. Inhibition of D-Arabinose-5-phosphate isomerase by Substrate Analogues



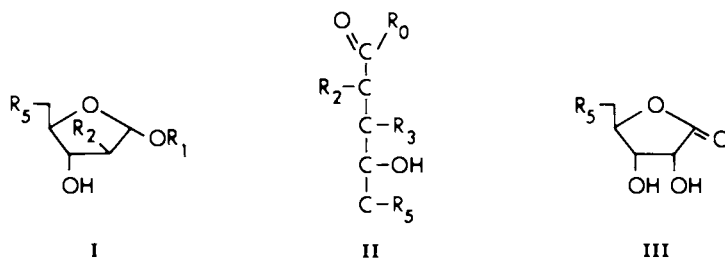
Parent Structures

Compound #	Structure I				I_{50} (mM) Value
	R_1	R_2	R_3	R_5	
1 (A-5-P)	H	OH	OH	-OPO ₃	0.2 (Km)
2 (R-5-P)	H	α OH ^a	OH	-OPO ₃	12.0
3	H	OH	H	-OPO ₃	10.0
4	H	H	OH	-OPO ₃	N.A.
5	H	OH	OMe	-OPO ₃	N.A.
6	H	OH	F	-OPO ₃	9.2
7	H	F	OH	-OPO ₃	N.A.
8	Me	OH	F	-OPO ₃	13.7
9	H	OH	OCH ₂ Ph	-OPO ₃	N.A.
10	H	OH	OH	-PO ₃	N.A.
11	H	OH	OH	-CH ₂ PO ₃	—

Compound #	Structure II					I_{50} (mM) Value
	R_0	R_1	R_2	R_3	R_5	
12	OH	O	OH	OH	-OPO ₃	0.08
13	OH	O	α OH ^a	OH	-OPO ₃	2.6
14	OH	O	F	OH	-OPO ₃	0.7
15	OH	O	OH	F	-OPO ₃	9.3
16	OH ^b	O	H	OH	-OPO ₃	N.A.
17	OH	H,H	OH	OH	-CH ₂ PO ₃	12.8
18	H	NOH	OH	OH	-OPO ₃	14.6
19	OH	H,H	OH	OH	-OPO ₃	0.1
20	OH	H,H	α OH ^a	OH	-OPO ₃	3.0
21	OH	H,H	H	OH	-OPO ₃	N.A.
22	OH	H,H	F	OH	-OPO ₃	0.8
23	OH	H,H	OH	F	-OPO ₃	15.0
24	OH	H,H	OH	OCH ₂ Ph	-OPO ₃	35.0
25	—	—	-O,OH	OH	-OPO ₃	0.03

^aAn α implies a ribo structure^bDenotes a lactone

TABLE II. Inhibition of D-Arabinose-5-phosphate Isomerase by Non-Phosphorylated Analogues



Parent Structures

Compound #	Structure I			I_{50} (mM) Value
	R_1	R_2	R_5	
1 (A-5-P)	H	OH	-OPO ₃	0.2 (Km)
2	H	OH	OH	N.A.
3	Me	OH	OH	N.A.
4	H	α OH ^a	OH	N.A.
5	Me	F	OH	N.A.
6	H	OH	ONO ₂	N.A.
7	H	OH	OSO ₂ NH ₂	32.0
8	1H	OH	OCONHSO ₃	32.0

Compound #	Structure II				I_{50} (mM) Value
	R_0	R_2	R_3	R_5	
9	NH ₂	α OH	OH	OH	N.A.
10	OH	α OH	OH	OH	N.A.
11	NHNH ₂	α OH	OH	OH	22.0
12	NHOH	α OH	OH	OH	5.3
13	OH	OH	OH	OH	N.A.
14	NHOH	H	H	COOH	N.A.
15	OH	=O	H	COOH	3.3

Compound #	Structure III		I_{50} (mM) Value
	R_5		
16	(4-NH ₂)PhSO ₃		15.2
17	MeSO ₃		14.0
18	MeC(O)CH ₂ CO ₂		45.0
19	Cl		N.A.
20	NH ₂ SO ₃		N.A.
21	-OSO ₃ ⁻		60.0
22	OH		N.A.

^aAn α implies a ribo structure
N.A. denotes no activity

relationship for D-glucose-6-phosphate and triose-3-phosphate isomerases (22).

(4) Aldonic acid phosphates substituted at the two position retained inhibitory activity but substitution at the three position destroyed inhibitory activity.

(5) The alditol phosphate analogue of D-arabinose-5-phosphate was a 3-fold less potent inhibitor than D-erythronic acid-4-phosphate. Again substitution at the 2 position by fluorine or hydroxyl functions allowed retention of inhibitory activity but substitution at C-3 by fluorine or O-benzyl inactivated the compound.

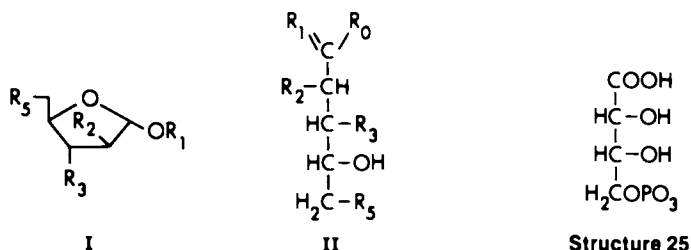
(6) Inhibitors with $K_i \ll K_m$ values were inactive in antimicrobial testing probably because of the inability of the phosphorylated compounds to penetrate the bacterial cell.

The second enzyme in the sequence studied was KDO-8-phosphate synthase. This enzyme was purified to homogeneity (24). This enzyme catalyzes the condensation of D-arabinose-5-phosphate and PEP to yield KDO-8-phosphate and inorganic phosphate. One can assay this irreversible reaction either by measuring the formation of KDO-8-phosphate or the release of P_i (25). The latter is the method of choice, since a number of analogues were found to interfere with the thiobarbituric acid assay. The enzyme has an apparent K_m for PEP of 6×10^{-6} M and an apparent K_m for D-arabinose-5-phosphate of 2×10^{-4} M.

A number of sugars and sugar phosphates were tested both as inhibitors of the reaction and as possible alternate substrates for the enzyme. The naturally occurring sugars and sugar phosphates tested included: D-arabinose, L-arabinose, D-erythrose, D-galactose, D-glucose, D-arabinitol, D-erythritol, D-erythronic acid, D-erythrose-4-phosphate, D-glucose-6-phosphate, D-ribulose-5-phosphate, D-ribose-5-phosphate, glycerol-3-phosphate, 2-phosphoglyceric acid, 3-phosphoglyceric acid, 2-deoxyglucose, maltose-1-phosphate, sedoheptulose-7-phosphate, 6-phosphogluconate, 2-deoxyribose-5-phosphate, 2-deoxygluconate-6-phosphate, D-xylulose-5-phosphate, sorbitol-6-phosphate, N-acetylneuraminic acid, N-acetylneuraminic acid-9-phosphate and D-fructose-1,6-diphosphate. KDO-8-phosphate synthase exhibited an absolute specificity for PEP and D-arabinose-5-phosphate in the synthetic reaction. Only D-ribose-5-phosphate, of the phosphorylated sugars tested, inhibited the formation of KDO-8-phosphate; it was shown to be a competitive inhibitor with an apparent K_i of 5×10^{-4} M (24). The synthetic reaction is also weakly inhibited by both KDO-8-phosphate and KDO (I_{50} values of 10 and 30×10^{-3} M, respectively) but not by inorganic phosphate.

The data in Table III indicate that the enzyme KDO-8-phosphate synthase is more difficult to inhibit with phosphorylated substrate analogues than is D-arabinose-5-phosphate isomerase. Non-phosphorylated substrate analogues (Tables II) were also tested as inhibitors or substrates of KDO-8-phosphate synthase but as with D-arabinose-5-phosphate isomerase, these analogues were

TABLE III. Inhibition of KDO-8-phosphate Synthase by Substrate Analogues



Parent Structures

Compound #	Structure I				I_{50} (mM) Value
	R_1	R_2	R_3	R_5	
1 (A-5-P)	H	OH	OH	-OPO ₃	0.02 (Km)
2 (R-5-P)	H	α OH ^a	OH	-OPO ₃	5.0
3	H	OH	H	-OPO ₃	N.A.
4	H	H	OH	-OPO ₃	N.A.
5	H	OH	OMe	-OPO ₃	4.8
6	H	OH	F	-OPO ₃	9.0
7	H	F	OH	-OPO ₃	9.9
8	Me	OH	F	-OPO ₃	N.A.
9	H	OH	OCH ₂ Ph	-OPO ₃	N.A.
10	H	OH	OH	-PO ₃	17.1
11	H	OH	OH	-CH ₂ PO ₃	Substrate

Compound #	Structure II					I_{50} (mM) Value
	R_0	R_1	R_2	R_3	R_5	
12	OH	O	OH	OH	-OPO ₃	N.A.
13	OH	O	α OH ^a	OH	-OPO ₃	N.A.
14	OH	O	F	OH	-OPO ₃	0.4
15	OH	O	OH	F	-OPO ₃	14.0
16	OH ^b	O	H	OH	-OPO ₃	N.A.
17	OH	H,H	OH	OH	-CH ₂ PO ₃	—
18	H	H,H	α OH ^a	OH	-OPO ₃	—
19	OH	H,H	OH	OH	-OPO ₃	16.5
20	OH	H,H	α OH ^a	OH	-OPO ₃	N.A.
21	OH	H,H	H	OH	-OPO ₃	N.A.
22	OH	H,H	F	OH	-OPO ₃	N.A.
23	OH	H,H	OH	F	-OPO ₃	22.0
24	OH	H,H	OH	OCH ₂ Ph	-OPO ₃	N.A.
25	—	—	= O,OH	OH	-OPO ₃	50.0

^aAn α implies a ribo structure.^bDenotes a lactone.

N.A. denotes no activity.

very weak (I_{50} values $\gg 10$ mM) inhibitors or inactive. While several compounds had weak inhibitory properties, only 2-deoxy-2-fluoro-D-arabinonate-5-phosphate (Compound 14, Table III) had an $I_{50} < 1$ mM. A consistent structure activity relationship within this set of compounds was not found, but some conclusions could be drawn. In general, methylfuranosides and alditol phosphates were the weakest phosphorylated analogues tested. The aldonic acid phosphates (Table III) were inactive except for the 2- and 3-fluoro analogues (Compounds 13 and 14). The aldose phosphates (Compounds 2,5,6 and 7; Table III) were relatively better inhibitors (I_{50} values of 5-10 mM) of KDO-8-phosphate synthase than of D-arabinose-5-phosphate isomerase. These data suggest that the 3-, 4- and -5 carbon substitution and configuration of the substrate D-arabinose-5-phosphate are important for even weak inhibitor binding. (Substitution of the phosphate at the 5 position of D-arabinose-5-phosphate by OH, ONO_2 , Cl, NH_2SO_2 , OSO_3 [see Table II] greatly diminished inhibitory activity.)² A variety of substituents at C-2 and C-3 did not destroy weak inhibitory activity although a bulky lipophilic substituent at C-3 destroyed the activity noted with the 3-O methyl derivative (compare compounds 5 and 9, Table III).

The third enzyme in the pathway, KDO-8-phosphate phosphatase, has been purified to homogeneity (26). Because of its absolute specificity, it should be a focal point for chemotherapeutic studies. The apparent K_m for KDO-8-phosphate was determined to be 5.8×10^{-5} M in the presence of 1.0 mM Co^{++} or Mg^{++} . This specific KDO-8-phosphate phosphatase was separated from enzymes, present in crude extracts, having phosphatase activity on other phosphorylated compounds by column chromatography on DEAE-Sephadex (26). Three distinct peaks of activity were detected. Fractions from each peak were pooled and the rates for the hydrolysis of five compounds were measured. Peak A possessed phosphatase activity for D-glucose-6-phosphate, D-arabinose-5-phosphate, D-ribose-5-phosphate and p-nitrophenylphosphate; Peak B dephosphorylated D-arabinose-5-phosphate, D-ribose-5-phosphate and D-glucose-6-phosphate. Peak C, which was well separated from the other two peaks, could only utilize KDO-8-phosphate as a substrate. KDO-8-phosphate was not hydrolyzed by the phosphatases present in peaks A and B.

The specificity of KDO-8-phosphate phosphatase is shown in Table IV. None of the phosphorylated sugars tested could be dephosphorylated by this enzyme, nor were they inhibitors of the reaction. All of the sugars tested (including KDO-8-phosphate) could be dephosphorylated by alkaline phosphatase. It is important to note, however, that the cells used for the isolation of KDO-8-phosphate phosphatase were grown in the presence of high inorganic phosphate which repressed the synthesis of alkaline phosphatase. It should also be noted that alkaline phosphatase is a well characterized periplasmic enzyme whereas KDO-8-phosphate

TABLE IV. Specificity of KDO-8-phosphate Phosphatase on Phosphorylated Compounds.

Compound	Specific Activity	
	KDO-8-Phosphate Phosphatase	Alkaline Phosphatase
KDO-8-phosphate	1.34	0.60
p-Nitrophenylphosphate	<0.01	0.99
Phosphoenolpyruvate	<0.01	0.62
D-Arabinose-5-phosphate	<0.01	0.56
Glucose-6-phosphate	<0.01	0.60
2-Deoxyglucose-6-phosphate	<0.01	0.64
Fructose-6-phosphate	<0.01	0.52
Fructose-1,6-diphosphate	<0.01	0.62
Galactose-1-phosphate	<0.01	0.57
Galactose-6-phosphate	<0.01	0.53
2-Deoxyribose-5-phosphate	<0.01	0.58
Ribulose-5-phosphate	<0.01	0.60
Ribulose-1,5-diphosphate	<0.01	0.48
Mannose-1-phosphate	<0.01	0.50
Mannose-6-phosphate	<0.01	0.58
Maltose-1-phosphate	<0.01	0.40
Erythrose-4-phosphate	<0.01	0.46
Phosphoglycolic acid	<0.01	0.67
α -Glycerophosphate	<0.01	0.70
Reduced KDO-8-phosphate	<0.01	0.39

Reaction mixtures contained in a volume of 0.5 ml: 50 μ moles HEPES pH 7.0, 0.5 μ moles CoCl_2 , 45 μ g KDO-8-phosphate phosphatase or 5 μ g alkaline phosphatase, and 0.5 mg of the appropriate compound, incubated at 35°C. Data for both enzymes are expressed as specific activities (μ moles per min per mg protein) under the above conditions of incubation.

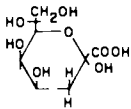
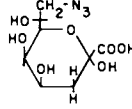
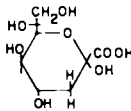
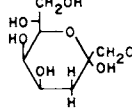
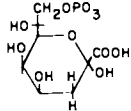
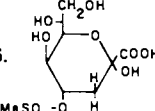
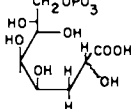
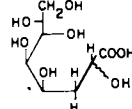
synthesized by KDO-8-phosphate synthetase is a cytoplasmic constituent.

It was shown previously (24) that KDO-8-phosphate was a weak end-product inhibitor of the synthase reaction. Both of the end products of this reaction, KDO and inorganic phosphate are weak mixed-function inhibitors of KDO-8-phosphate phosphatase. The reduced form of KDO-8-phosphate (the C-2 carbonyl was reduced to the corresponding diastereoisomeric alcohol with NaBH_4 , Table V, 3 red.), an open chain analogue, was neither an inhibitor of KDO-8-phosphate synthase nor was it a substrate or inhibitor of the phosphatase reaction. These findings indicate that the mechanism of KDO-8-phosphate synthase does not involve the formation of a linear intermediate and that the KDO-8-phosphate phosphatase requires the phosphorylated substrate in the ring form rather than the linear form.

CMP-KDO synthetase (cytidine-5'-triphosphate:cytidine-5'-monophosphate-3-deoxy-D-manno-octulosonate cytidyltransferase), the next enzyme in the pathway, catalyzes the formation of the nucleotide sugar, CMP-KDO from CTP and KDO. This enzyme was first studied by Ghalambor and Heath (17). We have purified this enzyme to homogeneity (27). The apparent K_m values for CTP and KDO in the presence of 10 mM Mg^{++} were determined to be 2×10^{-4} M and 2.9×10^{-4} M, respectively. The enzymatic reaction was dependent upon the addition of CTP, KDO and Mg^{++} but did not require a reducing agent. The formation of CMP-KDO was not inhibited by the addition of CDP, CMP, KDO-8-phosphate or N-acetylneuraminic acid to the complete reaction mixture. In agreement with Ghalambor and Heath (17), neither KDO-8-phosphate nor N-acetylneuraminic acid could substitute for KDO in the reaction mixture. Pyrophosphate, one of the end products, is a weak inhibitor of the reaction with an apparent I_{50} value of 5.0 mM. The addition of CMP, CDP, or any of the other mono- or di-nucleotides did not inhibit the reaction.

Although other natural sugars were tested as possible alternate substrates the specificity for KDO was absolute. However, the nucleotide CTP could be replaced by deoxy-CTP as a nucleotide acceptor when the reaction was carried out at both pH 8.0 and pH 9.5. Also, UTP was an effective nucleotide acceptor when the reaction was carried out at pH 9.5 but much less so when tested at pH 8.0. No other nucleotide tested acted as a substrate or inhibited the formation of the NaBH_4 -resistant thiobarbituric acid positive reaction. The apparent K_m values, determined at pH 9.5 for CTP, deoxy-CTP and UTP were 2.0×10^{-4} M, 3.4×10^{-4} M, and 8.8×10^{-4} M, respectively. The apparent V_{max} values for CTP, deoxy-CTP and UTP were 3.8×10^{-4} , 6.7×10^{-4} , and 3.3×10^{-4} moles per minute per mg of protein. Repeated attempts to isolate the unstable product of the reaction, CMP-KDO, utilizing a variety of experimental approaches were unsuccessful. The reverse reaction (17) could not be demonstrated by us, probably for the same reasons.

**TABLE V. Analogues of KDO Tested as Inhibitors and Substrates of
CMP-KDO Synthetase**

Compound	K_m	Compound	K_m
1. 	0.3 mM	4. 	0.6 mM
2. 	N.A.	5. 	N.A.
3. 	N.A.	6. 	0.25 mM
 ③ RED	N.A.	 ① RED	N.A.

Dr. F. M. Unger's group at Sandoz Forschungsinstitut independently recognized the potential antibacterial significance of KDO inhibition and have thoroughly explored the chemistry of KDO. His work has been summarized in a recent review (28). Dr. Unger's laboratory has synthesized a number of KDO analogues and in a collaborative effort with us, some of these compounds were tested as alternate substrates or inhibitors in the CMP-KDO synthetase reaction. As shown in Table V, the 4-methanesulfonyl-gluco-KDO (Compound 6) and the KDO-8-azide (Compound 4) were alternate substrates for the reaction with K_m values very similar to that of KDO. Our concurrent experiments demonstrated that the reduced form of KDO (Table V), as expected, was neither a substrate nor an inhibitor of the CMP-KDO synthetase.

The final enzyme in the pathway, CMP-KDO transferase, the enzyme that catalyzes the transfer of KDO from CMP-KDO to the Lipid A acceptor has been studied in Mary Jane Osborn's laboratory. We have not further purified this enzyme nor studied its properties in detail. The in vitro reaction described by Munson et al. (15) utilized a detergent solubilized enzyme preparation to catalyze the transfer of two KDO residues to the Lipid A precursor.

Physiological Studies Concerned With the Regulation of KDO Biosynthesis and Utilization. The KDO pathway can be thought of as a minor pathway in carbohydrate metabolism initiating with the key intermediate in the hexose-monophosphate shunt, D-ribulose-5-phosphate. We have expanded our study of the enzymes leading to the synthesis of KDO to include D-glucose-6-phosphate dehydrogenase and D-gluconate-6-phosphate dehydrogenase, the enzymes responsible for the formation of D-ribulose-5-phosphate as shown in Figure 2. Studies concerned with the regulation of the hexose-monophosphate shunt (29-33) have thus far ignored the potential role that products of KDO metabolism might play as possible metabolic effectors of this pathway. We set out to learn more about the regulation of the KDO pathway and what were the key rate limiting steps in the formation of and the utilization of KDO. This section deals with the effects of some of the metabolites of the KDO pathway on the enzymes of the hexose monophosphate shunt, on the enzymes of the KDO pathway and the effect of the growth medium and growth rate on the concentration of the enzymes of the KDO pathway.

In vivo measurements of lipopolysaccharide synthesis in E. coli B have indicated that two nanomoles of KDO must be synthesized per minute per mg of protein in order to meet the cellular requirement for LPS synthesis under the normal conditions of growth on glucose-minimal medium (27). We have measured the specific activities of the enzymes involved in KDO synthesis in crude extracts of E. coli B including those enzymes responsible for the synthesis of D-ribulose-5-phosphate, the precursor of D-arabinose-5-phosphate. D-Ribulose-5-phosphate is a key intermediate in carbohydrate metabolism as shown in Figure 2, since it is the direct precursor of both D-ribose-5-phosphate and D-arabinose-5-phosphate.

TABLE VI. Kinetic Parameters for the Enzymes of the KDO Pathway

Enzyme	Specific Activity Crude	K_m (mM)	V_{max} $\mu\text{moles min}^{-1}\text{ml}^{-1}$
D-Glucose-6-P dehydrogenase	.14	.19	4.9
D-Gluconate-6-P dehydrogenase	.10	.017	1.8
D-Ribulose-5-P Isomerase	.075		
a) D-Ribulose-5-P		.09	
b) D-Arabinose-5-P		.1-.3	
KDO-8-P synthase	.08		
a) D-Arabinose-5-P		.02	.012
b) PEP		.006	.010
KDO-8-P Phosphatase	.04	.06	.51
KDO-CMP Synthetase	.002		
a) KDO		.29	.62
b) CTP		.20	.38
c) deoxy CTP		.34	.67
d) UTP		.88	.33

D-Glucose-6-phosphate dehydrogenase, D-gluconate-6-phosphate dehydrogenase, KDO-8-phosphate synthase, KDO-8-phosphate phosphatase and CMP-KDO synthetase have been purified to homogeneity and characterized. Munson, Rasmussen and Osborn (15) have partially purified one KDO transferase. D-Arabinose-5-phosphate isomerase is very unstable and has only been purified about 100 fold. D-Ribose-5-phosphate isomerase activity is approximately 20x that

Measurements of the crude specific activity (μ moles of product synthesized per minute per mg of protein in the supernatant after a 50,000 x g centrifugation) of the two isomerases in *E. coli* indicated that the conversion of D-ribulose-5-phosphate to D-ribose-5-phosphate was approximately 20- to 30-fold greater than the conversion of D-ribulose-5-phosphate to D-arabinose-5-phosphate. This rate of reaction strongly pulls the reaction substrate to D-ribose-5-phosphate, since the isomerase reaction at equilibrium strongly favors the formation of the aldo-sugar over the key intermediate D-ribulose-5-phosphate.

The pathway for the synthesis and transfer of KDO to the Lipid A precursor now includes 8 (or 9) enzymatic steps initiating with the substrate D-glucose-6-phosphate. The sequential enzymes of the pathway are listed in Table VI with their specific activities determined from crude extracts, their K_m values determined in crude extracts and their V_{max} values determined for those enzymes that have been purified. The first seven enzymes are completely soluble; we have seen no evidence in our work utilizing various methods of isolation or in the work of others to indicate that any of these enzymes are even loosely membrane bound. The eighth enzyme, as shown by Munson et al. (15) is tightly bound to the inner membrane. The intracellular concentrations of D-arabinose-5-phosphate, KDO-8-phosphate and of KDO were determined to be within 20 percent of their K_m values. The rate limiting step in this pathway appears to be the formation of CMP-KDO since the activity of this enzyme in crude extracts is identical to the measured rate of LPS synthesis in cells grown under the same conditions.

The overall metabolic control of this pathway appears complicated. In the previous section, we have shown that most of the enzymes are weakly inhibited by their end products. As shown in Table VII D-glucose-6-phosphate dehydrogenase is only weakly inhibited by three of the fourteen common intermediates tested. There was no inhibition by D-arabinose-5-phosphate, KDO or KDO-8-phosphate, the novel intermediates of the pathway. The weak inhibitors were PEP, CMP and ATP which are common intermediates involved in many synthetic reactions. D-Gluconate-6-phosphate dehydrogenase was inhibited by several intermediates of the KDO pathway as shown in Table VIII. The inhibitors from the KDO pathway included D-arabinose-5-phosphate but not D-ribose-5-phosphate and KDO-8-phosphate but not KDO or the reduced form of KDO-8-phosphate. D-Ribulose-5-phosphate inhibited the reaction as did D-fructose-1,6-diphosphate in agreement with previous results from Fraenkel's laboratory (30-31) although ATP was found not to inhibit the purified enzyme when present in the reaction mixture at 10 mM. In agreement with the observation by Brown and Wittenberger (29), we found that preincubation of the enzyme with D-fructose-1,6-diphosphate before initiating the reaction with the substrate effected an initial non-linear response (2 min) which then reached a steady state. Velocities for K_i determinations

TABLE VII. Inhibition of D-Glucose-6-P Dehydrogenase

Potential Inhibitors	I_{50} (a)
D-Ribose	> 10 mM
D-Ribose-5-P	> 10
D-Arabinose	> 10
D-Arabinose-5-P	> 10
PEP	8.9
D-Ribulose-5-P	> 10
KDO	> 10
KDO-8-P	> 10
CMP	6.9
CTP	> 10
ATP	7.6
PRPP	> 10
D-Fructose-1,6-P	7.8
CMP-KDO	—

(a) Glucose-6-P concentration in I_{50} determinations was $4 \times K_m$.

TABLE VIII. Inhibition of D-Gluconate-6-Phosphate Dehydrogenase by Intermediates of the KDO Pathway

Potential Inhibitors	$I_{50}(a)$	K _{ii}	K _{is}
D-Ribose	> 10 mM		
D-Ribose-5-P	> 10		
D-Arabinose	> 10		
D-Arabinose-5-P	.80	1.7 mM	.18 mM
PEP	8.0		
D-Ribulose-5-P	.60	.88	.20
KDO	> 10		
KDO-8-P	1.9	2.4	.23
CMP	> 10		
CTP	> 10		
ATP	> 10		
PRPP	4.4		
D-Fructose-1,6-P	.04	.23	.009
CMP-KDO	—	—	—

(a) Gluconate-6-phosphate concentration in I_{50} determinations was $8 \times K_m$.

were measured at this steady state rate while the control rates were still linear. Double reciprocal plots of the data indicated that all the inhibitors tested exhibited mixed type inhibition as defined by Segel (35). Computer analysis of the data gave apparent K_{ii} (K_i derived from the $1/v$ intercept) and K_{is} values (K_i derived from the slope of the reciprocal plot) for the effective inhibitors shown in Table VIII. The best inhibitor of D-gluconate-6-phosphate dehydrogenase tested to date was the physiological regulator, D-fructose-1,6-diphosphate with an I_{50} value of 0.04 mM, when the substrate was present at 8 times the K_m value, as compared to I_{50} values of 0.6 mM, 0.8 mM and 1.9 mM for D-ribulose-5-phosphate, D-arabinose-5-phosphate and KDO-8-phosphate, respectively. This greater inhibition value was reflected in both a lower K_{ii} value and a much lower K_{is} value for D-fructose-1,6-diphosphate than exhibited by the other three compounds.

Since it has been demonstrated in other pathways that more than one metabolite can interact with a key enzyme to more effectively regulate the overall rate of the pathway, we tested the inhibitors of D-gluconate-6-phosphate dehydrogenase in combination to see if the inhibition was independent, antagonistic or synergistic as defined by Harvey (34). In these measurements, the rate of the reaction in the absence of any inhibitor is represented by v_0 ; in the presence of inhibitor 1 (I_1) or inhibitor 2 (I_2) the rate is expressed by v_1 or v_2 , respectively. Synergism would occur if the two inhibitors together produced an inhibition greater than expected from the inhibitors when acting alone. As shown in Table IX, none of the combinations of inhibitors were synergistic with one another which would have indicated that the inhibitors were acting at a different site to more effectively regulate the pathway at the level of D-gluconate-6-phosphate dehydrogenase. As illustrated, most of the inhibitors were antagonistic to one another thus indicating that they were competing for the same binding site.

A second method utilized to investigate the regulation of this pathway was to measure the intracellular concentration of the various enzymes as a function of the specific growth rate. When an individual protein is found to be present at a constant specific activity over a wide range of growth rates and physiological conditions, the rate of synthesis of this protein per genome must be proportional to the growth rate. The synthesis of such constitutive proteins is considered to be under metabolic control (36,37) as compared to those proteins whose synthesis is either repressed or derepressed (38-40). Metabolic control may be a characteristic of crucial enzymes that lack specific active control mechanisms. Different modes of regulation can be accounted for (38,39) if the enzyme concentration can be shown to vary with the specific growth rate. If, however, as shown in Table X that the concentration of the individual enzymes remains relatively constant over a wide range of growth conditions then this suggests that metabolic control or the rate of the normal flow of carbon

TABLE IX. Effect of Combinations of Inhibitors on D-Gluconate-6-phosphate Dehydrogenase

I_1	I_2	$a(I_1)$	$a(I_1/I_2)$	Type Inhibition
Arabinose-5-P	KDO-8-P	0.516	0.736	antagonistic
Ribulose-5-P	Fructose-1,6-P	0.460	0.932	antagonistic
KDO-8-P	Fructose-1,6-P	0.566	0.531	independent
KDO-8-P	Ribulose-5-P	0.629	0.929	antagonistic
Arabinose-5-P	Ribulose-5-P	0.595	0.807	antagonistic
Arabinose-5-P	Fructose-1,6-P	0.632	1.07	antagonistic

a) Fractional activities (a) in the presence of these inhibitors (I) are defined as: $a(I_1) = v_1/v_0$; $a(I_2) = v_2/v_0$, $a(I_1/I_2) = a(I_1, I_2)/a(I_2) = V_{1,2}/V_2$. The type of inhibition is independent if $a(I_1/I_2) = a(I_1)$; synergistic if $a(I_1/I_2) < a(I_1)$ and antagonistic if $a(I_1/I_2) > a(I_1)$.

TABLE X. Effect of Medium and Growth Rate on the Concentration of the Enzymes in the KDO Pathway

Enzyme	Specific Activity (μ moles/min/mg protein)				
	Medium	I	II	III	IV
Glucose-6-(P) Dehydrogenase		0.2000	0.1950	0.2250	0.2824
Gluconate-6-(P) Dehydrogenase		0.1340	0.1600	0.1845	0.2289
KDO-8-(P) Synthase		0.0811	0.0846	0.0825	0.0822
KDO-8-(P) Phosphatase		0.0782	0.0268	0.0781	0.0600
CMP-KDO Synthetase		0.006	0.0037	0.006	0.007
Medium	Growth Rate (k)				
I. Glycerol Minimal	0.60				
II. Glucose Minimal	0.90				
III. Medium A	1.40 (Protease Peptone + Beef Extract)				
IV. Medium A + Glucose	1.50				

E. coli B cells were grown at 37°C. The cells were harvested (OD = 0.76) disrupted by a French Pressure cell and the supernatant was assayed after centrifugation at 216,000 xg for 2 hours. In all media tested, the specific activities did not vary significantly as a function of growth phase ($A_{600} = 0.2, 0.76, 1.2$). Enzyme specific activities determined from cells grown in a Glucose-limited chemostat, $k = 0.028-0.4$, were in agreement with those of batch cultures presented above except that the specific activities for glucose-6-phosphate dehydrogenase and gluconate-6-phosphate dehydrogenase were reduced 20% at the slower growth rates.

determined the *in vivo* rate of this pathway, with the enzymes acting on their substrates at a rate determined by the concentration of that substrate. As can be noted in Table X, the specific activities of D-glucose-6-phosphate dehydrogenase and D-gluconate-6-phosphate dehydrogenase increased 40 percent and 70 percent respectively over a 2.5-fold increase in the specific growth rate, while the level of KDO-8-phosphate synthase remained constant under all conditions tested, even in a very slow growing chemostat culture. In batch cultures using glucose-minimal medium both the specific activities for KDO-8-phosphate phosphatase and CMP-KDO synthetase levels were lower but in the glucose limited chemostat and in the other batch cultures this level was relatively constant at about 0.07 and 0.006 μ moles per minute per mg of protein. D-Arabinose-5-phosphate isomerase, which is not listed in the table also remained constant at a specific activity of 0.095 μ moles per minute per mg of protein. Again, as shown in Table X, it appears that the overall rate limiting step in the synthesis and utilization of KDO is at the level of CMP-KDO synthetase, the last cytoplasmic enzyme in the pathway. The extreme lability of CMP-KDO may indicate that the transferase acts on this substrate in a zero-order fashion or that, once made, this product is bound to the enzyme until the time for the transfer of the KDO residue.

Discussion

The elucidation of the lipopolysaccharide structure and the studies concerned with determining the pathway for LPS biosynthesis were made possible by the use of the various mutants defective in the various stages of LPS synthesis (Figure 1). The innermost region of the LPS structure, the Lipid A-(KDO)₂ region, is however necessary for bacterial growth and viability since mutants defective in its biosynthesis have only been isolated as temperature sensitive mutants. The importance of KDO synthesis to Lipid A maturation and LPS synthesis, as well as cellular growth and viability has been demonstrated by Rick and Osborn (11, 12 see also 10 and 13). From a chemotherapeutic point of view, the inhibition of any enzyme catalyzing a step in the biosynthesis or transfer of KDO would necessarily result in the inhibition of LPS synthesis and thus bacterial growth. Considering that the same biosynthetic conversions do not take place in mammalian metabolism, inhibitors of these enzymes would probably act with a high degree of selectivity and possess a novel site of action.

The isolation and purification of the enzymes involved in KDO biosynthesis was necessary for the study of potential inhibitors of the individual reactions. A number of phosphorylated substrate analogues were synthesized and tested as inhibitors of D-arabinose-5-phosphate isomerase since this enzyme is the first direct reaction involved in KDO biosynthesis. (We have found no evidence that D-arabinose-5-phosphate is required in any other biosynthetic reaction in *E. coli* and *Salmonella typhimurium*; it

can be transported and utilized because of the reversible nature of the isomerase reaction.) As shown in Table I, analogues of the parent structure with the aldose oxidation state are at best very weak isomerase inhibitors. Substitution at any of the functional hydroxyl groups greatly diminished the binding of the inhibitor to the enzyme when compared to the K_m value of the substrate, D-arabinose-5-phosphate. Analogues in the alcohol series (whereby the aldehyde group at C-1 was reduced to the alcohol) indicated that D-arabinitol-5-phosphate (Compound 19, Table I) was an effective inhibitor of the isomerase reaction (K_m/I_{50} value = 2), substitution at C-2 by fluorine diminished the activity by 8-fold whereas substitution at C-3 by fluorine reduced the inhibitor activity by 150-fold. The best inhibitors of the isomerase reaction were the carboxylic acid analogues (the aldehyde group at C-1 was oxidized to a COOH function); 4-phosphoerythronate (Compound 25, Table I, K_m/I_{50} value = 6.6) and 5-phosphoarabinonate (Compound 12, Table I, K_m/I_{50} = 4) were the best inhibitors of D-arabinose-5-phosphate isomerase found. The inhibitor data suggests that the enediol model proposed in Figure 3 for the isomerase reaction may be correct and that the greater inhibition exhibited by the acid and alcohol analogues reflects their ability to mimic the enediol geometry. It should be mentioned that Woodruff and Wolfenden (41) found that 4-phosphoerythronic acid was a strong competitive inhibitor of spinach D-ribose-5-phosphate isomerase, the enzyme that catalyzes the interconversion of D-ribulose-5-phosphate to D-ribose-5-phosphate. The K_i values for 4-phosphoerythronic acid determined with the spinach D-ribose-5-phosphate isomerase and the *E. coli* D-arabinose-5-phosphate isomerase were between 4-6 μ M. This latter finding suggests that D-arabinose-5-phosphate isomerase may not be a good target enzyme for inhibitors that are substrate analogues.

The requirement of the remaining enzymes, KDO-8-phosphate synthase, KDO-8-phosphate phosphatase and CMP-KDO synthetase, for their natural substrates, D-arabinose-5-phosphate + PEP, KDO-8-phosphate and KDO + CTP, respectively, was specific and the inhibition studies with substrate analogues were disappointing. Of the compounds tested as potential substrates of KDO-8-phosphate synthase, only the isosteric phosphonate analogue (Compound 11, Table III) of D-arabinose-5-phosphate was an alternate substrate (see Ref. 28). There were a number of weak competitive inhibitors of the synthase reaction (Compounds 2, 5, 6, 7, 15, and 19, Table III) the best inhibitor of the synthase reaction was 2-deoxy-2-fluoro-D-arabinonate-5-phosphate (compound 14, Table III).

The requirement of the phosphatase that dephosphorylates KDO-8-phosphate is very specific (Table IV). None of the phosphorylated sugars tested was either an inhibitor or an alternate substrate for this phosphatase. The specificity of this enzyme, the fact that none of the other intracellular phosphatases catalyze the hydrolysis of KDO-8-phosphate and the fact that KDO-8-phosphate needs to be dephosphorylated for subsequent metabolism

suggest that this enzyme is a prime target for inhibition studies related to chemotherapy.

The stringent substrate specificity exhibited by the isomerase, the synthase and the phosphatase was not exhibited by CMP-KDO synthetase. Dr. Unger's group, at Sandoz Forschungsinstitut, has synthesized a number of KDO analogues (reviewed in Reference 28), as potential inhibitors of CMP-KDO synthetase or subsequent reactions involving the elongation of the polysaccharide chain. None of the seven KDO analogues tested was an effective inhibitor of CMP-KDO synthetase, including the reduced form of KDO (Compound 1 reduced, Table V). Two of the compounds tested, the KDO-8-azide and the 4-methanesulfonyl-gluco-KDO (Compounds 4 and 6, Table V) were effective substrates but neither of the compounds inhibited the specific growth rate of *E. coli* in minimal medium.

Studies concerned with the individual enzymes in the pathway have allowed us to optimize the enzyme reactions (25,42,43), to purify the individual enzymes and to utilize these purified enzymes to synthesize radiolabelled substrates with high specific activity. Potential inhibitors of this specific pathway can now be screened with relative ease for the following reasons: (1) all of the enzymes can be monitored independently; (2) initiating with KDO-8-phosphate synthase the pathway can be measured totally by initiating the reaction with ^{14}C -PEP and assaying the radioactivity in ^{14}C -KDO-Lipid A Precursor, even using crude membranes that contain both the Lipid A Precursor and CMP-KDO transferase; (3) initial compound screening should show low MIC values against Gram-negative bacteria and high MIC values against Gram-positive organisms and (4) if LPS synthesis is inhibited by inhibiting any of the enzymes in KDO biosynthesis, the growth of *E. coli* or *Salmonella typhimurium* test strain should be very susceptible to inhibition on plates containing 2 $\mu\text{g}/\text{ml}$ of Crystal Violet. Mutants of the Rc phenotype are not inhibited by this concentration of Crystal Violet, however, Rd mutants are severely inhibited.

The data concerned with the overall regulation of the KDO pathway (Tables VI-X) indicate that there is no strong regulation either in the catalytic sense or in the rate of synthesis of the enzymes involved. The data in Table X suggest that the enzymes in KDO biosynthesis are synthesized constitutively at a constant fraction of the total protein. This means that the enzymes required for KDO synthesis are present in sufficient quantities to meet the LPS synthetic demand. If the enzymes of the KDO pathway normally act at about the rate of their K_m then positive regulation or increased enzyme activity can only^m be achieved by increasing the concentration of D-arabinose-5-phosphate which will, in turn, increase the concentration of the other substrates. Since the isomerase reactions (both D-arabinose-5-phosphate and D-ribose-5-phosphate) are in equilibrium then this suggests that increasing the rate of carbon flow through D-gluconate-6-phosphate, as noted in Table X, would increase the rate of KDO biosynthesis. Since the data in Table VI indicate that the overall

rate limiting step in the KDO pathway (Fig. 2) is at the level of CMP-KDO synthetase (this rate is very similar to the rate of overall LPS synthesis) then the restriction on carbon flow through the pathway requires very little regulation. Both KDO-8-phosphate phosphatase and KDO-8-phosphate synthase are weakly inhibited by their end products KDO and KDO-8-phosphate respectively. If the concentration of KDO increased, this would inhibit the phosphatase reaction and cause an increase in KDO-8-phosphate concentration. If the concentration of KDO-8-phosphate increased enough to inhibit the synthase reaction then the concentration of D-arabinose-5-phosphate would increase. Since this isomerase reaction is an equilibrium reaction, excess D-arabinose-5-phosphate would be converted to D-ribulose-5-phosphate and then to D-ribose-5-phosphate. We have shown that D-ribose-5-phosphate is a competitive inhibitor of KDO-8-phosphate synthase (Table III) and that D-ribulose-5-phosphate and D-arabinose-5-phosphate are inhibitors of D-gluconate-6-phosphate dehydrogenase (I_{50} values 0.6 mM and 0.8 mM, respectively) whereas D-ribose-5-phosphate is not an inhibitor of the dehydrogenase. If the concentration of D-ribose-5-phosphate increased due to the slowing of RNA synthesis then this would have two effects: 1) the increase in D-ribose-5-phosphate would inhibit the formation of KDO-8-phosphate and cause an increase in the concentration of D-arabinose-5-phosphate and 2) since D-ribose-5-phosphate isomerase also catalyzes an equilibrium reaction, then the concentration of D-ribulose-5-phosphate would increase. Both of these compounds would then inhibit D-gluconate-6-phosphate dehydrogenase and slow the carbon flow through D-ribulose-5-phosphate. Thus, the overall regulation of carbon flow through the KDO pathway can be controlled by a weak system of end product inhibition since the specific activities of the individual enzymes involved in the pathway remain relatively constant. Moreover, the specific activity of CMP-KDO synthetase in crude extracts of *E. coli* B grown on glucose minimal medium is in the range required for LPS synthesis *in vivo* (2 nmoles per min per mg protein), and would suggest that the formation of CMP-KDO may be the rate limiting step in LPS synthesis.

The isolation of mutants in *S. typhimurium* by Rick and Osborn (11,12) and mutants in *E. coli* by Nishijima and Raetz (44) that accumulate the Lipid A precursor indicate that KDO synthesis and Lipid A synthesis are not coordinately controlled. The initial steps in the synthesis of the Lipid A precursor are totally unknown. The temperature sensitive mutants of *E. coli* isolated by Nishijima and Raetz (44) that are defective in phosphatidylglycerol phosphate synthesis at 42°C and accumulate the Lipid A precursors indicate that there is some relationship between the synthesis of phosphatidylglycerol and LPS. The reasons for the accumulation of the Lipid A precursors in this *E. coli* mutant are not obvious. We have shown that CDP-diglyceride, one of the substrates for phosphatidylglycerol phosphate synthesis, is an inhibitor of D-arabinose-5-phosphate isomerase with an I_{50} value

of 0.10 mM. We do not know if the concentration of CDP-diglyceride increases in this temperature sensitive mutant of *E. coli* at 42°C or the intracellular location of CDP-diglyceride; we assume it is membrane bound and we have shown that D-arabinose-5-phosphate isomerase is a cytoplasmic protein.

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Capsular Polysaccharides of Uropathogenic *Escherichia coli*

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In this lecture I will characterize capsular polysaccharides of coli bacteria which cause extraintestinal diseases, such as pyelonephritis. To put these studies into perspective, the main pathogenic mechanisms of Escherichia coli, and the role of capsules will first be discussed briefly.

As indicated in Figure 1, pathogenic coli bacteria may either be invasive or enteropathogenic. Both types of pathogens initially adhere to epithelial cells. Enteropathogenic coli bacteria dock in on their receptors on the outer face of the epithelial plasma membrane and remain outside of the epithelium. Their pathogenic effect is exerted by virtue of exotoxins, also called enterotoxins, which perturb metabolic processes of the host cell without apparent tissue or cell damage. Invasive coli bacteria penetrate the plasma membrane of epithelial cells - often deep into tissue of organs or into the blood stream. The specific pathogenic effects of invasive coli bacteria are not well understood. A number of bacterial products, such as endotoxin, mediate these effects which often lead to tissue damage, e.g. renal scarring in pyelonephritis (1-3). It is striking that invasive E.coli have a high negative surface charge and enterotoxigenic coli do not. The most prominent negatively charged surface components of Escherichia coli are the acidic capsular polysaccharides (4-5), the subject of this lecture.

Once penetrated into the tissue or circulation of the host, invasive coli bacteria are exposed to the unspecific host defense which consists mainly of the bactericidal action of serum complement and of phagocytes. This is a decisive stage in an infection, especially since antibodies which greatly assist in the defense are produced only later. Pathogenic bacteria have developed counteracting strategies, as shown in Figure 2. These are put into effect predominantly through capsules. Overcoming of the unspecific host defense may be brought about by the shielding effect of the extracellular layer of the capsule. In this effect, which appears basically to be a physical one, charge seems to play a role. The host defense does not become effective until specific anti-capsular antibodies are formed which then neutralize the protective effect of the capsule in a process called opsonization (4). The capsular K12 antigen is an example of a shielding capsule. Another means of

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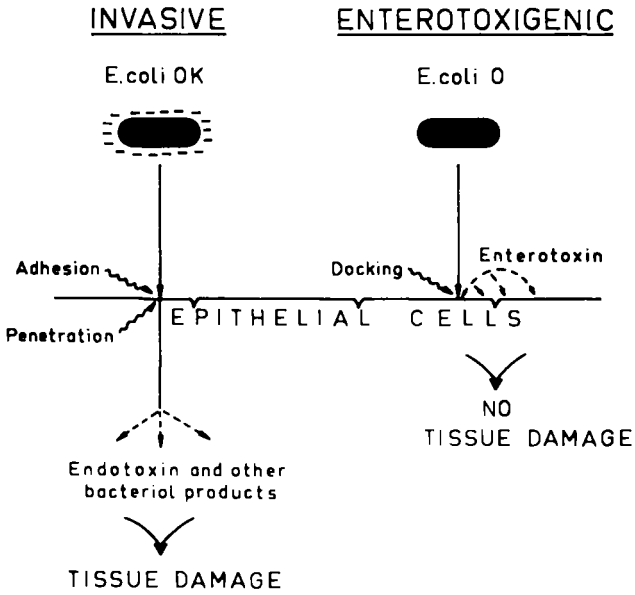


Figure 1. Mechanisms of pathogenicity of Escherichia coli.

INVASIVE BACTERIA ENCOUNTER HOST DEFENSE
(PHAGOCYTES, COMPLEMENT, ANTIBODIES)

BACTERIAL STRATEGIES OF COUNTER ACTION
ARE PREDOMINANTLY PUT INTO EFFECT BY

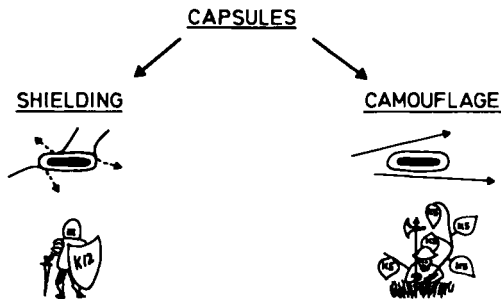


Figure 2. Counteracting strategies of invasive bacteria against unspecific host defense.

bacterial self-defense, which is based on structural relations, can best be described as camouflage. Possibly, due to structural similarities with host material, the bacterial cell cannot be recognized as foreign and antibodies against it can not or only inefficiently be formed by the host. In such cases the unspecific preimmune phase of the host defense is prolonged. This camouflage effect, in addition to shielding, is typically exerted by the *E.coli* K1 and K5 polysaccharides. It should be pointed out that although these protective mechanisms are demonstrated in bacteraemia, they are less obvious in urinary tract infections.

E.coli capsules can surround the bacterial cell as thick copious layers which are rather easy to demonstrate. As shown in Figure 3, an electronmicroscopic picture of an *E.coli* thin section, the capsules extend far into the surrounding of the cell. Another capsular form, exhibited by many coli bacteria, is shown in Figure 4. Such capsules have a quite thin and patchy appearance. Both forms of capsules are genetically fixed and there is no interchange between thick and thin capsules. Although such a differentiation may seem somewhat superficial, there is chemical and physical evidence supporting this distinction. Furthermore, for the isolation of the different capsular antigens the respective coli bacteria have to be cultivated under different conditions (5). Therefore, the distinction of capsular antigens from thick and thin capsule will be used in the following.

As indicated in Figure 5, both capsules are composed of acidic polysaccharides. The polysaccharides forming thick capsules have much higher molecular weights (up to several hundred thousands) than those forming thin and patchy capsules. The latter usually have molecular weights far below 50 000 daltons and may be as small as 5000 daltons. There are also characteristic differences in the chemical composition of the two types of capsular polysaccharides. The acidic constituents of the polysaccharides forming thick capsules are hexuronic acids (in most cases glucuronic acid). In some of these polysaccharides pyruvate substitution was also found. In the acidic polysaccharides of thin capsules, hexuronic acids are rarely found. Instead, we encounter N-acetylneuraminic acid and frequently 2-keto-3-deoxymannosooctonic acid (KDO). It is striking that capsular polysaccharides from thick capsules never contain aminic sugars, a characteristic which they share with the capsular polysaccharides of *Klebsiella* (4,5). The latter bacteria also form copious capsules. The polysaccharides forming thin capsules are typical of invasive coli bacteria which cause urinary tract infections, bacteraemia, or neonatal meningitis. Interestingly, bacteraemia and neonatal meningitis can also be caused by *Neisseria*, containing N-acetylneuraminic acid or KDO (4).

With respect to polysaccharides, N-acetylneuraminic acid and KDO are unusual components. Rather, N-acetylneuraminic acid is a constituent of glycoproteins and certain glycolipids - and KDO is characteristically found in enterobacterial lipopolysaccharides, where it forms the linkage region between the lipid and carbohydrate moieties.

The biochemical relationship between these two carbohydrates is shown in Figure 6. In their biosynthesis, phosphonenolpyruvate may

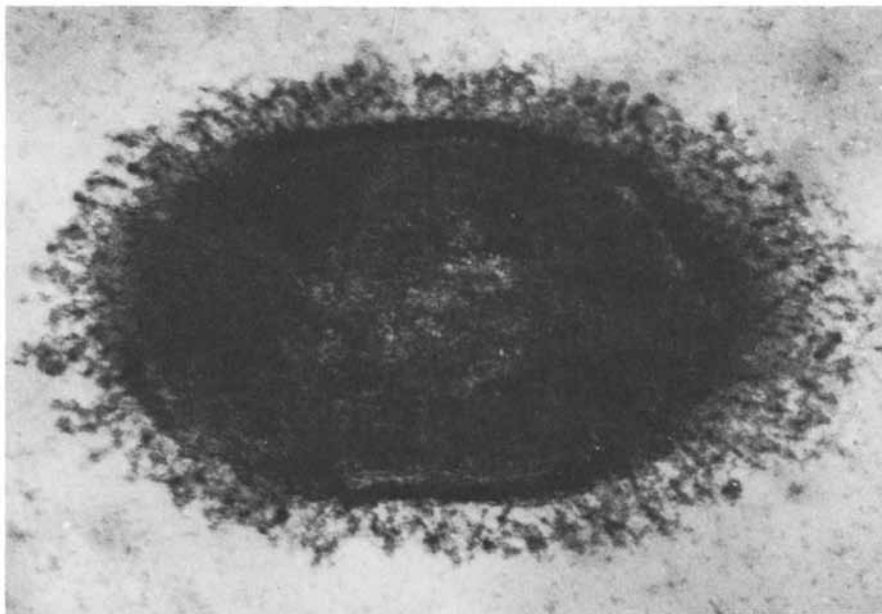


Figure 3. Electron micrograph image of an E. coli thin section showing a thick capsule.

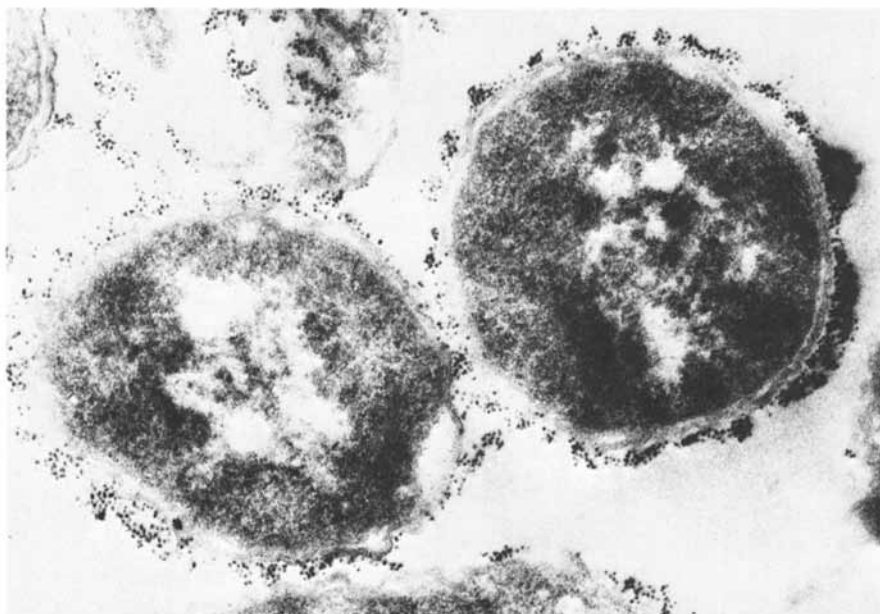


Figure 4. Electron micrograph image of an E. coli thin section showing a thin capsule.

COLI BACTERIA MAY HAVE :

THICK COPIOUS CAPSULES

K(A) ANTIGENS ARE ACIDIC POLYSACCHARIDES

MOLECULAR WEIGHTS ABOVE 100 000

ACID COMPONENTS :

HEXURONIC ACIDS

CAPSULES ARE COMPARABLE TO :

KLEBSIELLA CAPSULES

THIN PATCHY CAPSULES

K ANTIGENS ARE ACIDIC POLYSACCHARIDES

MOLECULAR WEIGHTS BELOW 50 000

ACID COMPONENTS :

HEXURONIC ACIDS

KDO

NANA

CAPSULES ARE COMPARABLE TO :

NEISSERIA CAPSULES

Figure 5. Description of two capsule types found on E. coli.

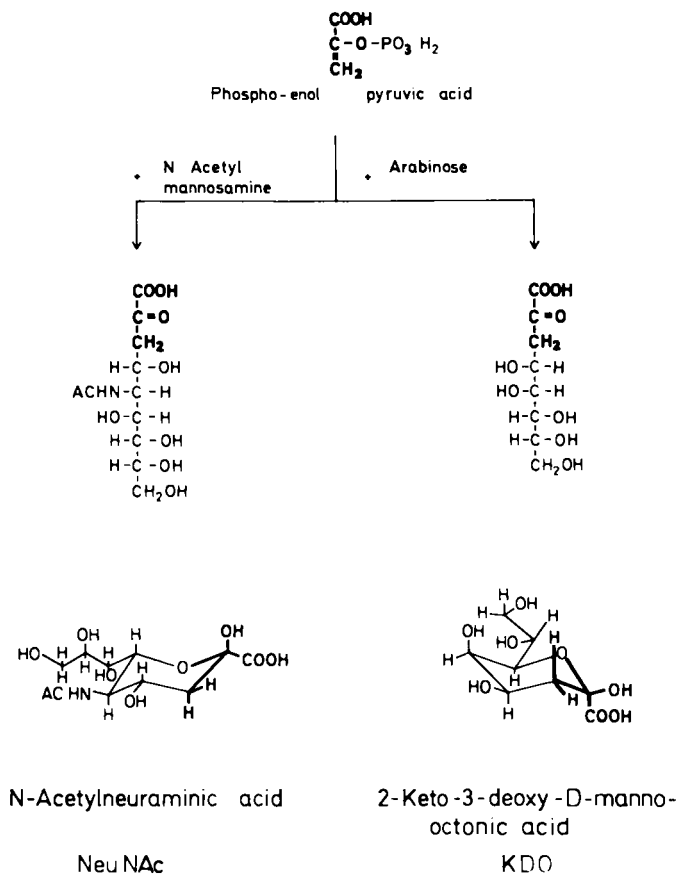


Figure 6. The biosynthesis of N-acetylneuraminic acid and KDO.

condense in very similar reactions either with N-acetylmannosamine to yield N-acetylneuraminic acid, or with arabinose to yield KDO.

Both N-acetylneuraminic and KDO can be detected and determined in similar reactions which make use of the carboxyl-ketodeoxy region of the molecule. Both compounds also exhibit characteristic signals in proton- and carbon-13 NMR. In proton NMR the protons on C3 exhibit characteristic signal patterns (6,7). This is shown with KDO in Figure 7. The apparent triplet around 1.8 ppm arises from the axial proton and the quartet is due to the equatorial proton. In the α - and β -configurations of KDO the carboxylgroup (C1) has different positions relative to the sugar ring, and as a result the influence of this group on the equatorial proton is also different. In α -KDO the equatorial proton on C3 is found at higher field (around 2 ppm) than in β -KDO (around 2.4 ppm). Figure 7 also shows the 0 - 3.5 ppm region of proton magnetic resonance spectra exhibited by the K12, K13 and K14 polysaccharides of uropathogenic *E.coli*. The typical pattern of KDO-C3 protons are apparent and the chemical shifts show that all three polysaccharides contain β -KDO. The K12 polysaccharide exhibits characteristic signals of methyl groups, indicative of the presence of 6-deoxy sugars. The K14 polysaccharide shows a signal which can be assigned to the methyl residue of an acetamido group and which indicates the presence of an N-acetylaminosugar in the K14 polysaccharide. Figure 8 shows the ^{13}C -NMR spectra of the same three capsular polysaccharides. The most prominent signals of the KDO residue are the C3 signal around 36 ppm, the C2 signal around 105 ppm and the C1 signal around 175 ppm. Other characteristic non-KDO signals are those of methyl and carbonyl residues of N-acetylaminogroups in the K14 spectrum and those of methyl carbon atoms in the K12 spectrum. The region of glycosidic carbon signals shows two signal each in the K13 and K14 spectra and three signals in the K12 spectrum, which permits the conclusion that the repeating unit of the K12 polysaccharide is a trisaccharide and those of the K13 and K14 polysaccharides are disaccharides (8; Jann, B. *et al.*, in preparation; Schmidt, M.A. and Jann, K., in preparation).

In addition to NMR spectroscopy analysis of native and de-O-acetylated capsular polysaccharides we have used more conventional methods such as methylation combined with gas chromatography and mass spectrometry, as well as chemical reactions. These studies lead to the structures of some KDO containing capsular polysaccharides which are shown in Figure 9 (8,9; Schmidt, M.A. and Jann, K., in preparation; Vann W.F., personal communication). The structural formulae of the K12, K13 and K14 polysaccharides are in agreement with the NMR spectra shown in the previous two figures. Thus, the different chemical shifts of the two rhamnose-carbonyl C atoms in ^{13}C -NMR are obviously due to the different substitutions of these sugar units in the K12 polysaccharide. It can be seen that the KDO containing capsular antigens are a group of very similar polysaccharides and that only small differences in the nature and linkage of the sugar constituents are found. It is also apparent that these capsular polysaccharides have a very high charge density with mostly two sugar units per charge. As

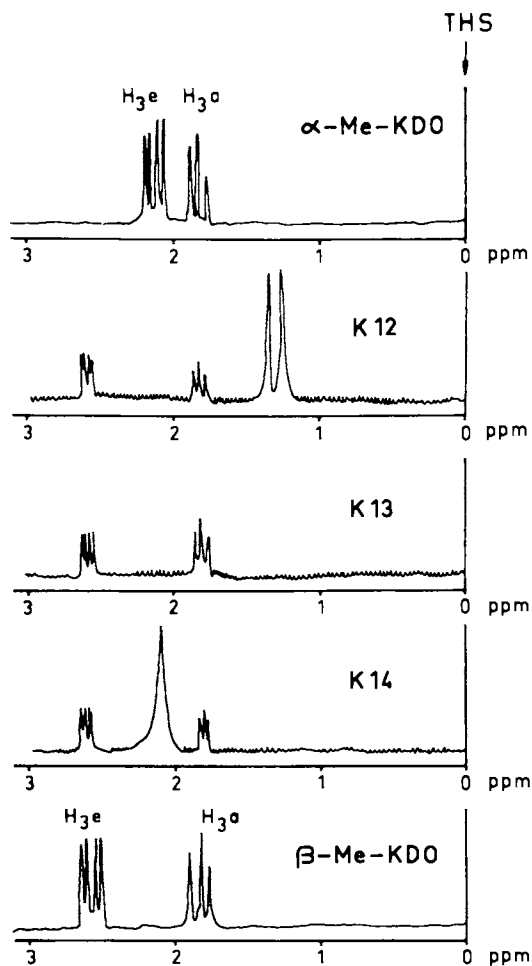


Figure 7. Characteristic proton signals of KDO and KDO-containing K antigens.

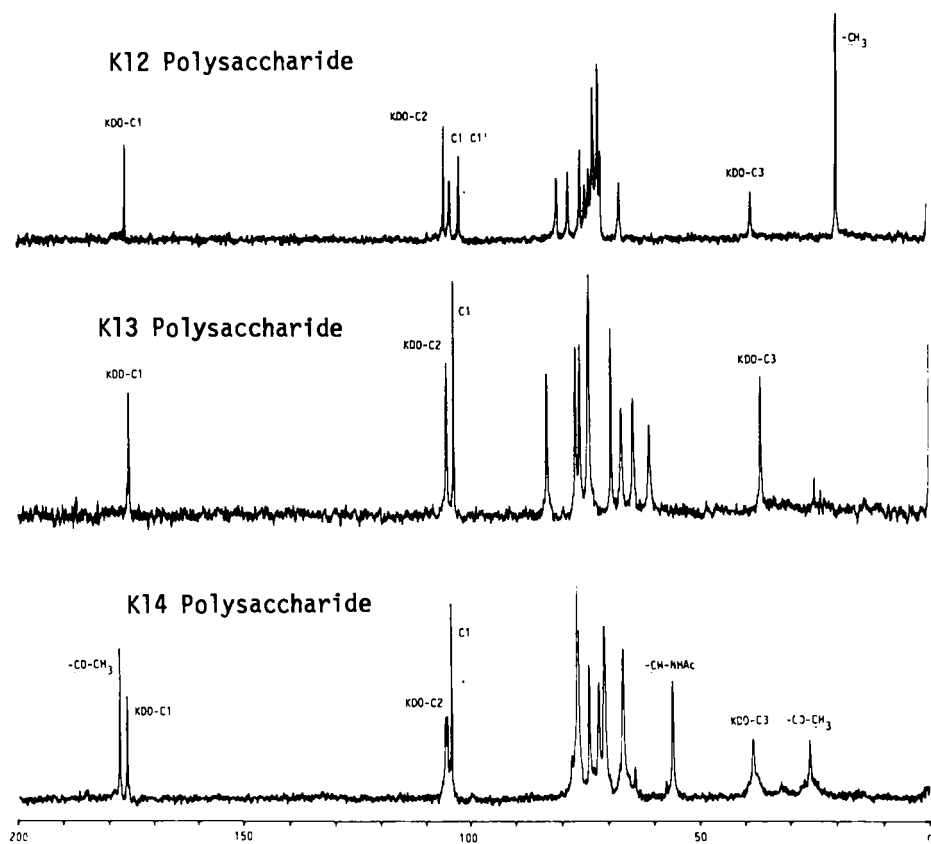


Figure 8. Carbon-13 NMR spectra of three capsular polysaccharides.

shown in Figure 10, the K6, K13, K20 and K23 polysaccharides are particularly closely related. They all have the same backbone structure of alternating 3-linked β -ribose and 7-linked β -KDO. The K13, K20 and K23 polysaccharides differ only in acetylation and the position of the O-acetyl group, respectively. The K6 polysaccharide is not acetylated and appears as a β -ribosyl derivative of the K23 polysaccharide (7, 9 but see also 10)..

All the KDO containing capsular polysaccharides shown in Figure 10 cross react in conventional antisera, mainly due to the fact that O-acetylation is never complete. In comparing native and modified polysaccharides we found that the immunodominant group in all these polysaccharides is KDO, acetylated or not. Closer serological study can be performed with monoclonal antibodies (Söderström, T, personal communication). It was found that a monoclonal K13 antibody reacts neither with the K20 nor with the K23 polysaccharides. Evidence was found that this antibody is specific for the β -(4-O-acetyl-KDO)-determinant of the K13 polysaccharide.

Working with monoclonal K12 antibodies we have found a cross reaction of the capsular K12 polysaccharide with R-lipopolysaccharides of rough *E.coli* K-12. This was supported by chemical analysis: We have not only detected rhamnose in the K-12 R-lipopolysaccharides but also isolated the disaccharide rhamnosyl-KDO from one of them. A possible structural basis for this cross reactivity is shown in Figure 11. We assume that the monoclonal antibody recognizes the rhamnosyl-KDO determinant in both polymers, the K12-specific capsular polysaccharide and the cell wall lipopolysaccharide of the rough strain *E.coli* K-12. The comparison of a terminal with a 2-linked sugar seems to be permissible in view that both are similarly exposed. In corroboration of these findings, the recently isolated coliphage K12 which has as host *E.coli* bacteria encapsulated with the capsular K12 polysaccharide, can also be propagated on the serologically cross reacting *E.coli* K-12 rough strains. This provides an example of serological and bacteriophage cross recognition of a capsular and a cell wall antigen.

Not all capsular polysaccharides of uropathogenic and invasive coli bacteria contain KDO. Some of the non-KDO capsular polysaccharides are shown in Figure 12 (11-16). These are the K1, K2, K5 and K51 polysaccharides. The K1 polysaccharide is a poly- α -2,8-N-acetylneuraminic acid and has been known as colominic acid since many years (11). Its structure which is identical with that of the capsular antigen of *Neisseria meningitidis* B has first been reported by McGuire and Binkley (11) and was later confirmed by two research groups (12, 13). The K2 antigen is reminiscent of certain teichoic acids of Staphylococci, polymers which have not been found in Gram-negative bacteria before (12). It consists of galactose, glycerol and phosphate in linear sequence and contains galactofuranose and galactopyranose in the same chain. The glycerol phosphate moiety of the K2 antigen has the sn-3 configuration (17). This indicates that in the biosynthesis of the K12 polymer, glycerolphosphate is transferred from CDPglycerol like in the biosynthesis of the teichoic acids, and not from diphosphatidyl glycerol like in the biosynthesis of the lipoteichoic acids. The K5 polysaccharide

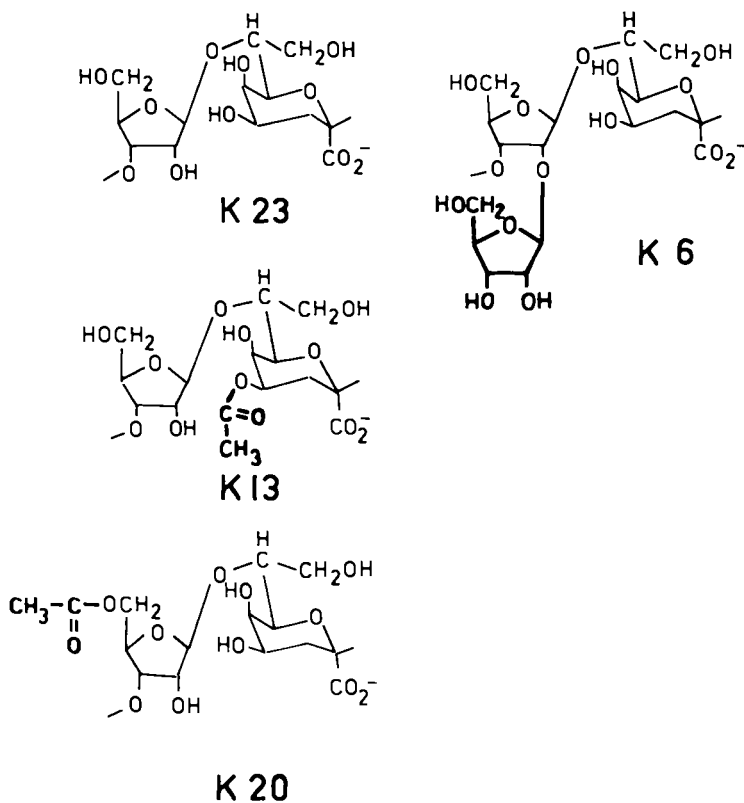
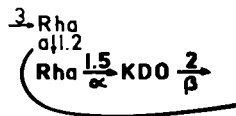


Figure 10. Structures of KDO-containing polysaccharides.

**K 12 CAPSULAR
POLYSACCHARIDE**



**LINKAGE REGION
OF K-12 (R) LPS**

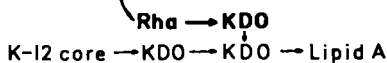


Figure 11. Proposed specificity of monoclonal anticapsular K12 antibody.

has a very simple structure with a repeating unit of β -glucuronyl-1,4-N-acetylglucosamine. The K51 polysaccharide is a per-O-acetylated poly N-acetylglucosamine-1-phosphate (Jann, B. and Jann, K., unpublished). The linkage between the monomers is not known.

Like with the KDO containing K-polysaccharides, one can observe also in this group of polysaccharides antigen modification by O-acetylation: the K1 antigen and the K2 antigen occur in non acetylated and in acetylated forms (14, 18). Bacteria with the non acetylated polymers (K1ac⁻ and K2a) seem to be more pathogenic.

The KDO containing and the non-KDO polysaccharides of invasive *E.coli* have several features in common. One of them is their low molecular weight in combination with a high charge density (5, 19). Considering these properties, it appears difficult to envisage their retention by the bacterial cell with capsule formation. On closer examination, we found that the low molecular capsular polysaccharide antigens contain a hydrophobic moiety, as shown in Figure 13 with the K12 antigen as an example. This lipid part is a phosphatidic acid, linked to the reducing end of the polysaccharides through a phosphodiester bridge (20). The same results were reported on some Sialic acid polysaccharide antigens (21), although the phosphatidic acid was not isolated in this study. By radioreduction the reducing sugar was found to be KDO (20). We assume that these polysaccharides are kept in association with by the bacterial cells through hydrophobic interactions of the lipid moiety with the cell wall which results in capsule formation. Since the linkage between polysaccharide and phosphatidic acid is very labile, these capsules are released from the bacteria relatively easily.

As mentioned earlier, encapsulated coli bacteria override the unspecific host defense with the help of their capsular polysaccharides. Some capsular polysaccharides may even escape immune recognition, as is the case with the K1 antigen (3, 22, see also 23) and the K5 antigen (I. and F. Ørskov, personal communication). The immune response against these polysaccharides is at best extremely low, and this is probably one of the reasons why coli bacteria with these antigens are particularly virulent. An explanation of this fact can be attempted by structural comparisons, as shown in Figure 14 with the K1 antigen. The recognition of this capsular polysaccharide by the immune system may be hampered by its structural relation to tri- and also certain disialo-gangliosides. These gangliosides also contain α -N-acetylneuraminy-2,8-N-acetylneuraminic acid. The immune response to the K1 polysaccharide probably depends on the copy number and/or expression of these gangliosides on host cell surfaces.

A similar situation exists with the K5 antigen. Figure 15 shows that this capsular polysaccharide is practically identical with a precursor of heparin. It was in fact shown recently that the K5 polysaccharide is a substrate for the de-N-acetylase which initiates the processing of this precursor to heparin. Structural relatedness may not be the only cause for the very low immunogenicity of a coli capsular antigen, but it appears quite suggestive.

K	REPEATING UNIT	IMMUNOGENICITY
92	$\overset{9}{\alpha}\text{-NeuNAc} \overset{2,8}{\alpha}\text{-NeuNAc} \overset{2}{\alpha}$	yes (-9)
IAC+	$\overset{8}{\alpha}\text{-NeuNAc} \overset{2,8}{\alpha}\text{-NeuNAc} \overset{2}{\alpha}$ $\begin{array}{c} 7/9 \\ \text{OAc} \end{array}$ $\begin{array}{c} 7/9 \\ \text{OAc} \end{array}$	yes (-OAc)
IAC-	$\overset{8}{\alpha}\text{-NeuNAc} \overset{2,8}{\alpha}\text{-NeuNAc} \overset{2}{\alpha}$	no

TRISIALO GANGLIOSIDES:

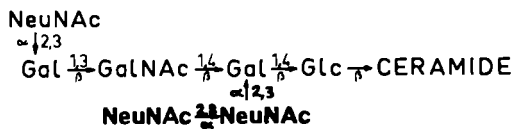
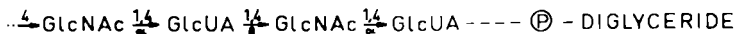


Figure 14. Possible reason for the low immunogenicity of the K1 polysaccharide.

K5 POLYSACCHARIDE :



INTERMEDIATE OF HEPARIN BIOSYNTHESIS:

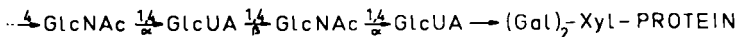


Figure 15. Possible reason for the low immunogenicity of the K5 polysaccharide.

The lack of immunogenicity is not only detrimental for an infected host, but is also an impasse in serological diagnosis of the K antigen in question. One can circumvent this calamity with the aid of specific bacteriophages. K1-Specific bacteriophages are known (24, 25), and we have recently isolated a K5 specific bacteriophage (26), which is now widely used for the detection of the K5 polysaccharide on pathogenic *E.coli*. As shown in Figure 16, coliphage K5 hydrolyzes its receptor polysaccharide with the formation of mainly a decasaccharide. This has N-acetylglucosamine at the reducing end, which shows that coliphage K5 acts as an endo- α -N-acetylglucosaminidase (D.S. Gupta *et al.* (1982), FEMS Microbiol. Lett., in press).

Since anticapsular antibodies neutralize the pathogenic properties of capsules, it is desirable to have a vaccine on the basis of the capsular polysaccharides, which induces such antibodies. It is, however, known that the isolated and purified polysaccharides of invasive coli bacteria are practically not immunogenic *per se*. To obtain a polysaccharide vaccine, the capsular polysaccharides have therefore to be converted into good immunogens. This can be done by coupling them to immunogenic carrier proteins. We are pursuing this work in our laboratory and some of the reactions we are using are shown in Figure 17. The polysaccharides are coupled to proteins which have been substituted with a spacer ending in a primary amino group. The coupling is achieved by reductive amination of the reducing end of the polysaccharide with the amino group of spacer. Alternatively, carboxyl groups are reacted with the spacer amino group; or the polysaccharide is first reacted with cyanogen bromide and subsequently condensed with the spacer amino group. In these reactions it is of great help to know the structure of the respective polysaccharide. Using such reactions we have prepared protein conjugates of the K12, K13 and K14 polysaccharides, and those of the K1 and K5 polysaccharides are just being made (unpublished work). The polysaccharide-protein conjugates were immunogenic and in first experiments the K12 and K13 conjugates protected rats and mice against *E.coli* pyelonephritis ((B. Kaijser, B. Jann, and K. Jann, in preparation). This shows that the shielding effect of the *E.coli* capsules could be overcome.

These examples may serve to illustrate that the combination of chemical and immunological approaches can be of great help in the attempts to fight bacterial infections. As summarized in Figure 18, chemical characterization and modification of purified capsular polysaccharides will not only advance our knowledge of their structures, but also enable us to produce specific antibodies with untoxic polysaccharide immunogens. These in turn, we can use for the immunochemical characterization of the polysaccharide antigens. The most rewarding result, however, is the protection of man against infection with bacteria from which the polysaccharides were isolated. As shown on the bottom of Figure 18, specific antibodies induced with derivatives of the capsular polysaccharides will overcome the self defense of the bacteria by opsonization and thus shorten the unspecific preimmune phase of a bacterial infection in which we are most vulnerable.

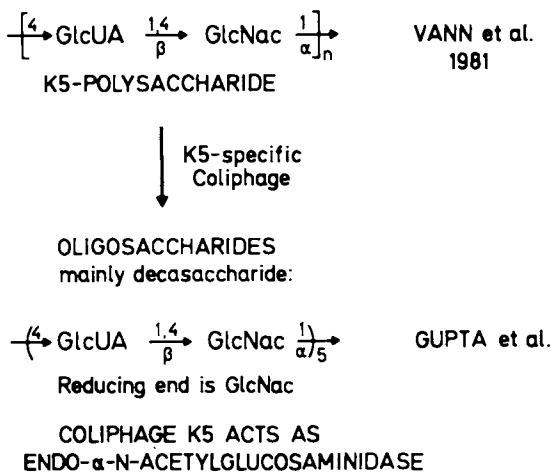


Figure 16. Structure of the K5 polysaccharide and action of a K5-specific coliphage.

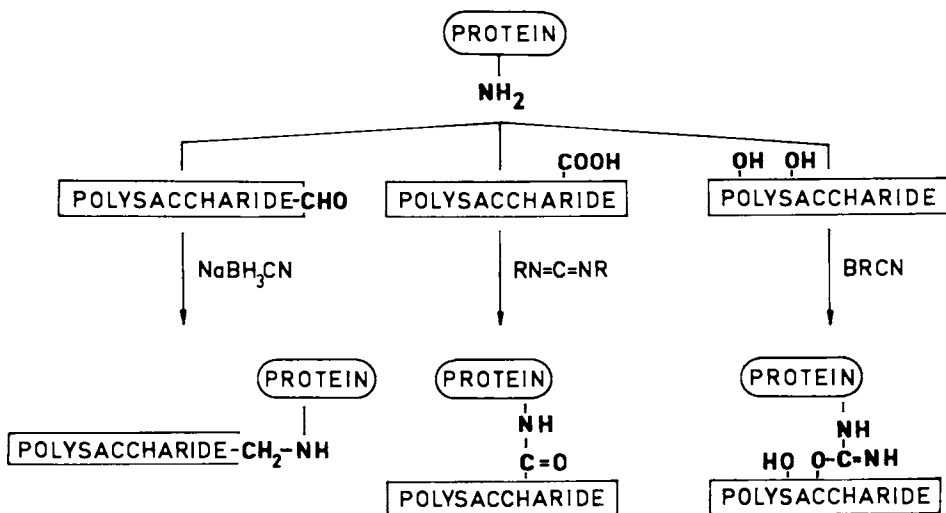


Figure 17. Coupling of capsular polysaccharides to protein.

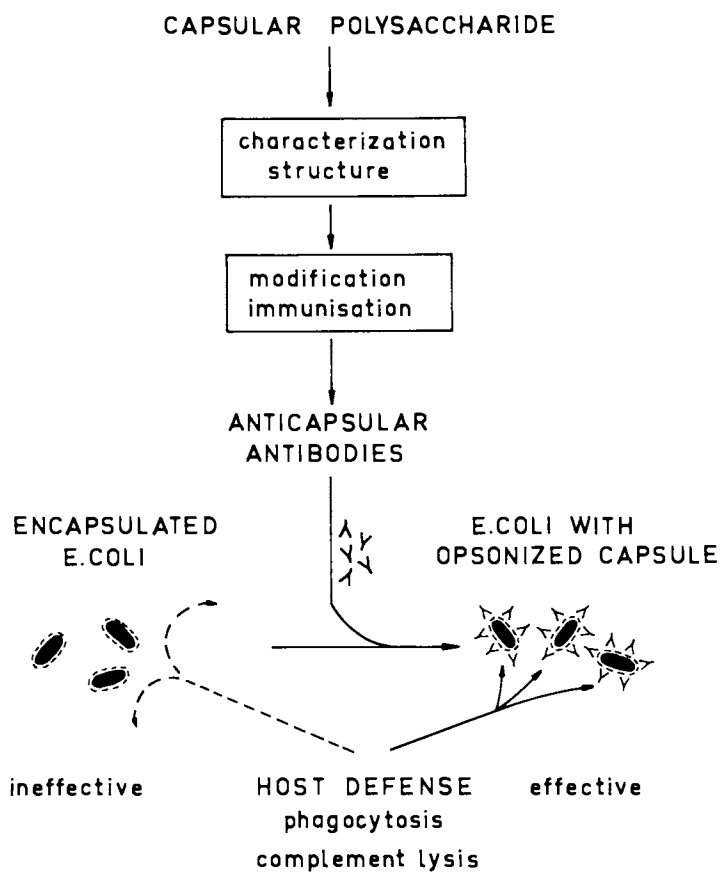


Figure 18. Specific antibodies induced with derivatives of the capsular polysaccharide overcome bacterial self-defense by opsonization.

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Analysis of the Primary Structure of Lipid A

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Gram-negative bacteria such as Salmonella, Escherichia coli, Pseudomonas and Vibrio contain in their cell walls various types of macromolecules which share the property of consisting of a hydrophilic and a hydrophobic region. These amphiphiles include capsular antigens (K-antigens) (1,2), the enterobacterial common antigen (ECA, present in Enterobacteriaceae) (3,4), the lipoprotein (LP, first detected in E. coli) (5,6) and lipopolysaccharides (LPS, also designated O-antigens or endotoxins) (7,8). In isolated form, these amphipathic molecules express a number of common biological properties: they all bind to mammalian red blood cells, they are (in general) immunogenic, and they all are likely to be involved in bacterial virulence. Thus, the similar amphiphilic make-up of these bacterial products finds its expression in similar biological activities. However, in a number of other biological effects, these amphipathic molecules differ markedly. Thus, lipopolysaccharides are potent pyrogens in man and rabbits. Furthermore, they are mitogens for B-lymphocytes (mouse) and they induce lethal toxicity in various experimental animals (7). The lipoprotein (E. coli) is also a B-cell mitogen; it is, however, devoid of pyrogenicity and lethal toxicity (6). K-antigens and the enterobacterial common antigen lack all of these latter activities.

Investigations performed in various laboratories during the last decades have shown that most of the biological effects mentioned (which are shared or expressed individually by these amphiphiles) are mediated by, or dependent on, their lipid regions (9). In view of the similar and differing biological activities of the lipid portions, chemical studies were performed in order to characterize structural components which determine those common and distinct activities. Such studies showed that the lipophilic portion of K-antigens and ECA consist of a glycerol phosphate backbone carrying fatty acids which are also found in free phospholipids of Gram-negative bacteria (mainly 16:0, 16:1, 18:0, 18:1) (1,2,4). The lipid component of lipoprotein was found to be made up of a diglyceride, bound by a thioether linkage to the mercapto

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group of a cysteine residue, the α -amino group of which is acylated. The fatty acids present comprise 16:0, 16:1, 17:cyclo and 18:1 (5).

Of the lipid portions of bacterial macromolecular amphiphiles, that of lipopolysaccharides is the structurally most complex. For its designation the term lipid A has been coined. More specifically, it was suggested that the lipid, as it is present in intact lipopolysaccharide, should be called lipid A, while the lipid in a separated form should be termed free lipid A (10,11). This nomenclature will be used throughout this paper. In the following, ways and methods will be described which have been used to elucidate the chemical structure of lipid A. The present discussion will deal in more detail with the elucidation of the structure of Salmonella lipid A. Relative to this structure, chemical features of other lipid A's will then be discussed.

LIPID A STRUCTURE

Treatment of Salmonella lipopolysaccharides with 0.1 N acetic acid (2.5 h, 100°C) yields a precipitate which represents free lipid A. In acid hydrolysates (4 N HCl, 12 h, 100°C) of free lipid A, three main constituents are detected, i.e. 2-amino-2-deoxy-D-glucose (D-glucosamine, ~17%, w/w), inorganic phosphate (~12%) and long-chain, nonhydroxylated fatty acids (12:0, 14:0, 16:0) as well as larger amounts of (R)-3-hydroxytetradecanoic acid (total fatty acids ~60%). (For other lipid A constituents, see below). In first, systematic studies, partial structures of free Salmonella lipid A were obtained and identified as D-glucosaminyl-4-phosphate and N-3-hydroxytetradecanoyl-D-glucosamine (12, 13). In addition, it was found that mild alkali treatment of free lipid A released part of the fatty acids. These findings taken together indicated that the lipid was made up of phosphorylated D-glucosamine residues carrying ester- and amide-bound fatty acids. Molecular weight determinations of free lipid A gave values of approximately 2000 (13). This suggested that more than one substituted D-glucosamine residue is present in a lipid A unit. Subsequent studies proposed that glucosamine residues in lipid A are interlinked by 1,4-phosphodiester bonds (14); however, in other investigations, evidence for glycosidically interlinked glucosamine residues was obtained (15).

A number of investigators have since then been concerned with the structural make-up of Salmonella lipid A and have shown that lipid A itself is an amphiphile consisting of a hydrophilic and a hydrophobic region. Here, approaches of our laboratory to the elucidation of these regions will be described.

Hydrophilic Region

The Lipid A Backbone An important step for the isolation and structural analysis of the hydrophilic region of lipid A was the

development of procedures for the selective degradation of lipopolysaccharides (16,40). The individual steps involved in one of these pathways are schematically shown in Fig. 1. *Salmonella* lipopolysaccharide (isolated from a Re mutant of *Salmonella minnesota*) is first treated with alkali (0.2 N NaOH, 1 h, 100°C). By this procedure, ester-bound fatty acids are removed, while under these conditions amide-linked acyl groups are not liberated. The preparation obtained (alkali-treated lipopolysaccharide, LPS-OH, yield 97% based on glucosamine) contains, besides 3-deoxy-D-manno-octulosonic acid (KDO) and N-acyl groups, organic phosphate and D-glucosamine in a molar ratio similar to that found in native lipopolysaccharide (2.2:2.0). Treatment of preparation LPS-OH with acid (0.1 N HCl, 10-30 min, 100°C) yields a precipitate (lipid A-OH; yield relative to LPS-OH = 88%, based on glucosamine) which is free of KDO but which still contains about 1 mole of organic phosphate relative to 2 moles of D-glucosamine. Of the latter, 1 mole is detectable in the direct Morgan-Elson reaction without hydrolysis and N-acetylation. Since LPS-OH gave a negative direct Morgan-Elson test, and since, in the acidic hydrolysate, about half of the phosphate bound to LPS-OH is detected as inorganic phosphate, it is concluded that this was originally linked to position 1 of a glucosamine residue. Reduction of lipid A-OH with NaBH₄ (10 mg/0.1 ml, pH 10, 12 h, 50°C) yielded a material (lipid A-OH_{red}, yield 67%, based on glucosamine and relative to lipid A-OH) which was negative in the direct Morgan-Elson reaction and which contained 1 mole each of organic phosphate, glucosamine and glucosaminitol (the latter detected by the use of an amino acid analyser). Thus, 50% of the glucosamine present in lipid A-OH had been reduced, indicating the presence of a (phosphorylated) glucosamine-disaccharide in lipid A. Lipid A-OH_{red} was then freed from amide-bound fatty acids and organic phosphate by treatment with hydrazine (anhydrous N₂H₄, 40 h, 100°C). The material obtained was applied to high-voltage paper electrophoresis and, by means of ninhydrin, a spot was identified which comigrated with N-deacetylated chitobiositol (M_{GlcN} = 1.15). The eluted material contained glucosaminitol and glucosamine in a molar ratio of 0.8 to 1, and was free of phosphate. From these data it was concluded that lipid A contained a D-glucosamine-disaccharide. Treatment of the N-acetylated disaccharide preparation (yield 40%, based on glucosamine and relative to lipid A-OH_{red}) with β-N-acetyl-glucosaminidase quantitatively released D-glucosamine within less than 30 minutes. This showed that the D-glucosamine residue of the reduced pseudodisaccharide was present in the pyranose form and that it was β-linked.

In order to further study the anomeric configuration of glucosamine and the linkage site of glucosamine to glucosaminitol, the N-acetylated and reduced disaccharide was permethylated according to Hakomori (17) and subjected to gas-liquid chromatography.

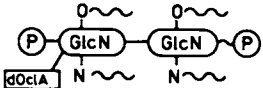
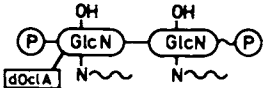
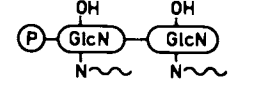
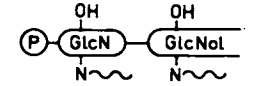
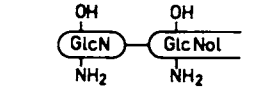
Fraction	Schematic	Formula	P		Reducing	
			mol/mol	GlcN	GlcN	GlcNol
LPS			1.1 (2.2)	1.0 (2.0)	0	0
	↓ OH ⁻					
LPS-OH			1.1 (2.2)	1.0 (2.0)	0.01	0
	↓ H ⁺					
Lipid A-OH			0.5 (1.0)	1.0 (2.0)	0.48 (1.0)	0
	↓ H ⁻					
Lipid A-OH _{red}			0.92	1.0	0	0.8
	↓ N ₂ H ₄					
Lipid A Backbone (reduced)			0	1.0	0	0.8

Figure 1. Schematic representation of the preparation of the lipid A backbone of *Salmonella minnesota Re* lipopolysaccharide. (Reproduced with permission from Ref. 16. Copyright 1976, Eur. J. Biochem.).

The peak obtained eluted like a synthetic, $\beta(1\rightarrow6)$ -linked, reduced and derivatized D-glucosamine disaccharide and could be clearly distinguished from the $\alpha(1\rightarrow6)$ as well as from the $\beta(1\rightarrow3)$ and $\beta(1\rightarrow4)$ -linked isomers (18). Mass spectrometric analysis of the material demonstrated the pattern characteristic for a reduced, permethylated $(1\rightarrow6)$ -linked N,N'-diacetylglucosamine disaccharide including the characteristic fragment at $m/z = 218$ (18). The other major fragments present have been assigned to the A-series fragmentation ($m/z = 154, 228$ and 260 , glucosamine), D-series fragmentation ($m/z = 142, 276, 346$ and 378 , glucosaminitol) and C-2/C-3 cleavage in the alditol moiety of the disaccharide ($m/z = 88$ and 130). Fragments characteristic for $(1\rightarrow3)$ or $(1\rightarrow4)$ -linked N-diacetylglucosamine disaccharides such as $m/z = 133, 390, 419$ and 422 were absent (18).

These data unequivocally prove that the D-glucosamine disaccharide isolated from *Salmonella* lipid A contains a $\beta(1\rightarrow6)$ linkage. In earlier studies, Gmeiner et al., using a different approach, had also come to the conclusion that, in *Salmonella* lipid A, a $\beta(1\rightarrow6)$ -linked D-glucosamine disaccharide was present (19). Thus, our results confirm these earlier findings.

As discussed above, one of the two phosphoryl groups present in lipid A is linked to the glycosidic hydroxyl group of the reducing glucosamine residue in the disaccharide. The location of the second phosphoryl residue was identified in the following way (20). *Salmonella minnesota* Re lipopolysaccharide was treated with hydrazine (10 h, 100°C), mild acid, and NaBH_4 . By this procedure, and after purification, a reduced glucosaminyl disaccharide was obtained which contained one phosphate residue. When this material had been exhaustively oxidized with periodate, glucosamine (-phosphate) was no longer detectable. If, however, the sample was first N-acylated and then oxidized, glucosamine (-phosphate) was detectable in an amount identical to that present in the sample prior to oxidation. These findings indicate that the ester-bound phosphate group is linked at position 4' of the nonreducing glucosamine residue of the disaccharide.

In a similar way it was demonstrated that in *Salmonella minnesota* Re LPS, KDO is bound to the nonreducing glucosamine residue in the lipid A backbone, presumably to position 3' (20). Recent experiments of our laboratory involving methylation analysis indicate that, in *Proteus mirabilis*, the polysaccharide component is linked to position 6' of the lipid A backbone (21).

In summary, these data show that *Salmonella* lipid A contains a 2-amino-6-O-(2-amino-2-deoxy- β -D-glucopyranosyl)-2-deoxy-D-glucose unit which carries phosphoryl residues in position 1 and 4', the glycosyl phosphate being α -linked (37). This structure is called the lipid A backbone.

A very similar or identical backbone has later been identified in a number of other enterobacterial lipid A's including that of *Escherichia coli*, *Proteus mirabilis*, *Shigella sonnei*, *Shigella flexneri*, *Yersinia enterocolitica* and *Serratia marces-*

cens. In addition, a phosphorylated $\beta(1\rightarrow6)$ -linked D-glucosamine disaccharide has been identified in lipid A of Pseudomonas aeruginosa, Pseudomonas alcaligenes, Xanthomonas sinensis, Chromobacterium violaceum, Rhodopseudomonas gelatinosa, Rhodospirillum tenue, Vibrio cholerae, Rhizobium trifolii, Aeromonas salmonicidae, Fusobacterium nucleatum, Selenomonas ruminantium and Myxococcus fulvus (for literature compare (8)).

Substituents Linked to Phosphate Groups of the Lipid A Backbone

Substituents of the ester-linked phosphate group Lipopolysaccharide of a Re-mutant of Proteus mirabilis (R45) is similar to the lipopolysaccharide of the Salmonella minnesota Re-mutant (R595) in that it contains a phosphate to glucosamine ratio of approximately 2.4:2.0 ((21), Fig. 2). When the degradation procedure described for Salmonella, however, was applied to P. mirabilis lipopolysaccharide, an unexpected result was obtained (Fig. 2). On treatment of Proteus lipopolysaccharide with alkali (0.2 N NaOH, 1 h, 100°C) a material was obtained (LPS-OH, yield 61%) which contained only 1.2 moles of phosphoryl groups per 2 moles of glucosamine. This was in contrast to Salmonella LPS-OH where about 2 moles of phosphate per glucosamine disaccharide were identified. The phosphoryl group still present in LPS-OH of Proteus could be quantitatively liberated by treatment with acid (0.2 N HCl, 30 min, 100°C), indicating that it had been glycosidically bound to the backbone disaccharide. This finding suggested that the phosphate residue removed by alkali had been bound as an ester, and it was assumed to be substituted by an unknown group, facilitating (by cyclisation ?) its alkali-catalyzed liberation.

In order to identify the unknown residue, the dialysate, after alkaline hydrolysis of P. mirabilis lipopolysaccharide (0.2 N NaOH, 1 h, 100°C), was subjected to paper electrophoresis, the molybdate-positive region (neutral) treated with phosphatase and again analysed by paper electrophoresis. Both ninhydrin and AgNO₃-staining revealed a spot which co-migrated with authentic 4-amino-4-deoxy-L-arabinose. Analyses of the material with the amino acid analyser showed a peak with an absorption ratio (440 nm : 590 nm) of about 5.9 and co-eluting with authentic 4-amino-4-deoxy-L-arabinose (38). These results indicated that the material released from lipopolysaccharide by alkali was identical with 4-amino-4-deoxy-L-arabinosyl-1-phosphate. The nature of this aminopentose was further studied and its amount determined by combined gas-liquid chromatography/mass spectrometry. Proteus lipopolysaccharide was N-acetylated, hydrolysed (0.02 N HCl, 10 min, 100°C), and the released material reduced and peracetylated. On gas-liquid chromatography, a single peak (0.38 µg/mg LPS = 1.0 mol per 2 moles glucosamine) was obtained, the mass spectrum of which showed inter alia fragments at $m/z = 288$ (M), 217, 145 and 144, expected for peracetylated 4-amino-4-deoxy-arabinitol.

4-Amino-4-deoxy-L-arabinose had been previously found

Fraction	Schematic	Formula	(P)	GlcN	Reducing	GlcNol
			mol/mol	GlcN	GlcN	GlcNol
LPS			1.2 (2.4)	1.0 (2.0)	0	0
	↓ OH ⁻					
LPS-OH			0.56 (1.1)	1.0 (2.0)	0	0
	↓ H ⁺					
Lipid A-OH			0.15	1.0 (2.0)	0.57 (1.1)	0
	↓ H ⁻					
	↓ N ₂ H ₄					
Lipid A Backbone (reduced)				1.0		0.86

Figure 2. Schematic representation of the preparation of the lipid A backbone of *Proteus mirabilis* Re lipopolysaccharide (21).

attached to the ester-linked phosphate group in Chromobacterium violaceum lipid A (22) and in subsequent studies it was also identified as a non-obligatory constituent of various Salmonella species (11,23). After deamination of Salmonella and Proteus lipopolysaccharide, 4-amino-4-deoxy-L-arabinose is no longer detectable. It, thus, is present in the pyranose form. In Salmonella, 4-amino-4-deoxy-L-arabinose appears to be β -linked (37). In the other lipopolysaccharides mentioned, the anomeric configuration of the aminopentose has not been established.

It is emphasized that examples are known where the ester-bound phosphate group is not substituted (8,35,36).

Substituents of the glycosidically-linked phosphate group In ^{31}P -nuclear magnetic resonance studies on Salmonella minnesota Re lipopolysaccharide which had been subjected to mild hydrazine treatment (30 min, 60°C), a signal at +11 ppm (reference: H_3PO_4) was seen which was shown to be due to a pyrophosphodiester group (23). After treatment of the material with mild acid (0.05 N HCl, 20 min, 100°C) this signal disappeared and, in the hydrolysate (besides inorganic phosphate) phosphorylethanolamine could be detected. Quantitative determinations showed that the amount of phosphorylethanolamine (and inorganic phosphate) released, and the amount of pyrophosphodiester groups as determined by ^{31}P nmr, were comparable and it was concluded that the Salmonella lipid A disaccharide, at its reducing end, has a pyrophosphorylethanolamine substituent. The substitution of the glycosidic phosphate group of the Salmonella lipid A backbone by pyrophosphorylethanolamine, however, is not quantitative. By chemical and spectroscopic analyses, the degree of substitution was determined to be dependent on the lipopolysaccharide preparation (bacterial batch) and to be in the order of 20-60%.

Other studies showed that the glycosidic phosphate group of lipid A's is also substituted in Chromobacterium violaceum (by D-glucosamine, (22)), in Rhodospirillum tenue (by furanosidic D-arabinose, (30)) and, partially, in Escherichia coli (by phosphate, (36)). In a number of bacterial groups, however, the glycosidic phosphate appears to be unsubstituted (for literature see (8)).

Hydrophilic Region - Summary

The findings described may be summarized in the following way:

The structural principle identified in many lipid A's as the hydrophilic backbone, a phosphorylated $\beta(1\rightarrow6)$ -linked D-glucosaminyl disaccharide, is present in closely related and taxonomically remote groups of bacteria. Therefore it represents an ubiquitous and common component of lipid A's from various Gram-negative bacteria. A D-glucosaminyl disaccharide being $\beta(1\rightarrow6)$ -linked has, to our knowledge, not been found in other natural substances. Hence, this structure is unique and characteristic for Lipid A.

The lipid A backbone, depending on its bacterial source, may be substituted at its phosphate groups by, in general, nitrogen-containing residues which are not acylated. So far only one group, namely 4-amino-4-deoxy-L-arabinose has been identified as a substituent of the phosphate residue ester-bound to the nonreducing glucosamine group of the backbone. Different types of groups, however, may be linked to the glycosidic phosphoryl residue attached to the reducing glucosamine in lipid A of distinct bacteria.

In contrast to the backbone structure which represents a constant portion of lipid A the phosphate groups with their various substituents comprise a variable region of lipid A. This natural, obviously restricted interbacterial diversity of phosphate substituents demonstrates that lipid A's of different origin are distinct in their fine structure. In some cases (like *Salmonella* and *Escherichia*) the substitution of the backbone phosphate group is not quantitative. This implies a certain heterogeneity of a given lipopolysaccharide preparation expressed on the level of its lipid A component.

Hydrophobic Region

Nonhydroxylated and hydroxylated fatty acids (C10-C18) comprise the hydrophobic region of lipid A's. Among these, cyclopropane and, in general, unsaturated fatty acids are lacking. In the following, procedures are described which were applied for the determination of the nature, quantity and binding site of fatty acids present in *Salmonella* lipid A (39).

Total fatty acids were liberated by subjecting *Salmonella minnesota* Re lipopolysaccharide (or free lipid A) to acidic (4 N HCl, 5 h, 100°C) followed by alkaline (1 N NaOH, 1 h, 100°C) hydrolysis. After extraction (chloroform), the free fatty acids were converted into their methyl esters (diazomethane) and analysed by combined gas-liquid chromatography/mass spectrometry. Alternatively, the fatty acids of lipid A are transesterified by treatment of lipopolysaccharide with methanolic HCl (2 N HCl in water-free CH₃OH, 18 h, 85°C). By these procedures the following fatty acids were identified (in approximate amounts relative to 2 moles glucosamine): dodecanoic (12:0, 1.1 mole), tetradecanoic (14:0, 0.8 mole), hexadecanoic (16:0, 0.9 mole), 2-hydroxytetradecanoic (2-OH-14:0, 0.1 mole), and 3-hydroxytetradecanoic acid (3-OH-14:0, 4 moles). In total, therefore, approximately 7 moles of fatty acids are present per mole of lipid A backbone. The stereochemistry of the hydroxylated fatty acids was determined by gas-liquid chromatography of their diastereomeric methoxyacyl-L-phenylethylamide derivatives (24). It was found that 2-hydroxytetradecanoic acid possesses the (S), and the predominating 3-hydroxytetradecanoic acid the (R) configuration.

Ester-bound fatty acids The lipid A backbone provides hydroxyl and amino groups as possible linkage sites for fatty acids. In order to determine the nature of ester-bound fatty acids (linked to hydroxyl groups) Salmonella lipopolysaccharide was treated with alkali (0.2 N NaOH, 1 h, 100°C), a procedure expected to selectively cause O-deacylation. In the alkaline hydrolysate, approximately 1 mole of each 12:0, 14:0 and 16:0 and 2 moles of 3-OH-14:0 were found (the latter figure includes artificially formed Δ^2 -14:1). This shows that all nonhydroxylated fatty acids, as well as half of the amount of 3-OH-14:0, present in Salmonella lipid A are ester-bound. When O-deacylation was performed with sodium methylate (0.25 N NaOCH₃ in CH₃OH, 56°C, 1 h) the gas-liquid chromatogram showed peaks corresponding to 12:0 and 16:0 (as methyl esters) in the expected amounts. It was, however, surprising to find that the methyl ester of 14:0 was lacking from the chromatogram, that only 1 mole of 3-OH-14:0 was present in the methanolysate, and that about 1 mole of 3-methoxytetradecanoic acid had been formed.

These unexpected results were then tentatively explained as being the result of an alkali-catalyzed β -elimination reaction involving an ester-bound 3-hydroxytetradecanoic acid residue which is acylated at its 3-hydroxyl group by tetradecanoic acid (Fig. 3). Such a structure, on treatment with methylate, should be converted into Δ^2 -tetradecenoic acid ester and subsequently, by a nucleophilic addition of methylate, to racemic 3-methoxytetradecanoic acid methyl ester. Thus, 1 mole of (R)-3-O(tetradecanoyl)-tetradecanoic acid ester is transformed to 1 mole of (R,S)-3-methoxytetradecanoic acid ester. The β -eliminated (not transesterified) product (14:0) should be present as the free fatty acid and, therefore, not be migrating like a methyl ester on gas-liquid chromatography. This free fatty acid should, however, elute as the methyl ester if the methanolysate is additionally carboxymethylated (Fig. 3). A corresponding experiment showed that indeed tetradecanoic acid methyl ester was present on chromatograms after carboxymethylation of the methanolysate. These findings support the reaction sequence delineated above (25).

Therefore, of the 2 moles of ester-bound 3-OH-14:0 one is 3-O-acylated by tetradecanoic acid; the other should carry a free 3-hydroxyl group. This latter assumption was proven to be correct by the following experiment. Lipid A was treated with t-butyldimethylsilylchloride, ester-bound fatty acids were transesterified by NaOCH₃ and analysed by gas-liquid chromatography. Besides the other expected fatty acid methyl esters (12:0, 16:0, 3-OCH₃-14:0) approximately 1 mole (per 2 moles glucosamine) of the t-butyldimethylsilylether of 3-hydroxytetradecanoic acid methylester could be identified (26).

Amide-linked fatty acids The fact that O-deacylation of lipopolysaccharide with NaOCH₃ (56°C) released (in addition to 12:0, 14:0 and 16:0) only 2 moles of the 4 moles of 3-OH-14:0 present in

lipopolysaccharide, suggested that the remaining portion of 3-OH-14:0 (2 moles) was amide-linked. This assumption was supported by the finding that, by strong alkaline hydrolysis (4 N KOH, 5 h, 100°C) of O-deacylated lipopolysaccharide (LPS-OH), 3-OH-14:0 was the only fatty acid liberated (1.4 mol/2 moles glucosamine). On treatment of LPS-OH with acid (4 N HCl, 4 h, 100°C) and subsequent alkali (1 N NaOH, 1 h, 100°C), or by acidic methanolysis (2 N HCl in CH₃OH, 18 h, 85°C) also 3-OH-14:0 was released (1.6 mol/2 moles glucosamine). In addition, however, larger amounts of trans- Δ^2 -tetradecenoic acid (0.3 mol/2 moles glucosamine) were present in the latter hydrolysate. Since in acid hydrolysates of untreated lipopolysaccharide this unsaturated acid was present in significantly smaller amounts (0.1 mol/2 moles glucosamine) it was concluded that Δ^2 -14:1 represented an artifact generated by an alkali-induced β -elimination reaction of substituted 3-OH-14:0 residues. Subsequently, it was shown that on oxidation (IO₄/MnO₄⁻) of LPS-OH (but not of untreated lipopolysaccharide), dodecanoic acid (12:0, 0.5 mol/2 moles glucosamine) was liberated. In addition, treatment of LPS-OH (but not of lipopolysaccharide) with deuterium gas(D₂) yielded a material from which deuterated 14:0 could be liberated (27). These findings confirmed that, in lipopolysaccharide, amide-bound 3-OH-14:0 is, at the 3-hydroxyl group, substituted by a so far unknown residue.

In order to characterize the unknown substituent, free lipid A was subjected to procedures supposed to selectively release N-acyl residues. It was expected that these procedures would liberate 3-OH-14:0 still carrying the substituent in question. Among the procedures available for selective cleavage of amide-bound acyl residues, the method described by Kraska *et al.* (28) was applied first. This method involves methylation of acylamido group-containing compounds with methyl iodide in the presence of silver salts (Fig. 4). Silver salts are supposed to complex the free electron pair of the amide nitrogen, thus preventing N-methylation but rather enforcing methylation of the carbonyl oxygen yielding a methyl acylimidate. With mild acid at room temperature (10% H₃PO₄, 15 min) the acylimidate is cleaved whereby the originally amide-linked acyl residue is liberated in the form of its methyl ester.

When this method, in a slightly modified form, was applied to Salmonella free lipid A, mainly two compounds were liberated which were detected by gas-liquid chromatography and characterized by mass spectrometry. The spectrum of the first compound was identical with that of authentic 3-hydroxytetradecanoic acid, 3-O-acylated by dodecanoic acid (3-O-dodecanoyl-tetradecanoic acid methyl ester, 3-O(12:0)-14:0) (27). The mass spectrum of the second peak showed, inter alia, characteristic fragments at 496 (M⁺), 465 (M-31), 239, 240 and 241, thus corresponding to 3-O-hexadecanoyl-tetradecanoic acid methyl ester (3-O(16:0)-14:0). Therefore, the two components present in Salmonella lipid A in amide linkage and released by the above described procedure

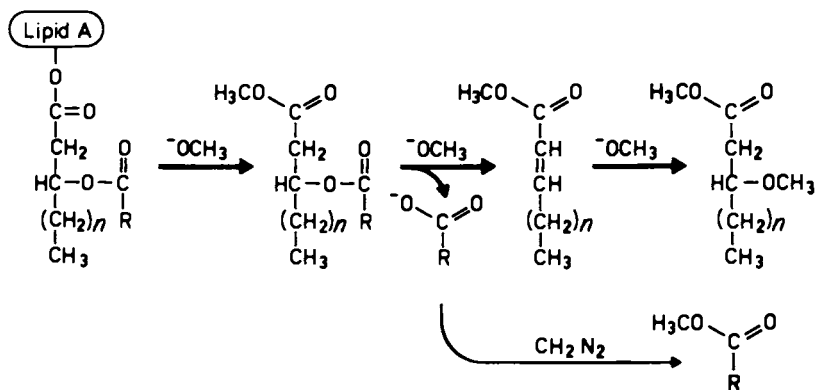


Figure 3. β -Elimination reaction involving a 3-acyloxyacyl group present in lipid A in ester linkage.

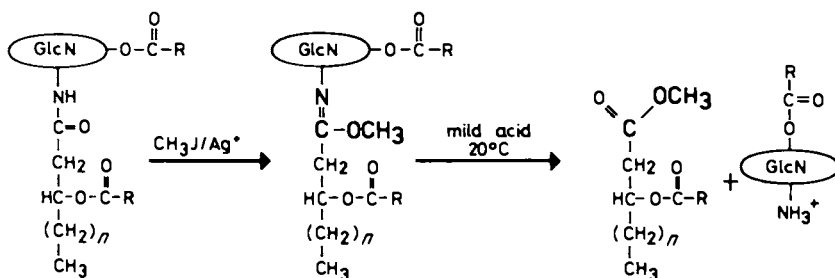


Figure 4. Steps involved in the selective liberation of amide-bound acyl groups from free lipid A. (Reproduced with permission from Ref. 27. Copyright 1982, Federation of European Biochemical Societies.)

represent 3-OH-14:0 carrying at their 3-hydroxyl groups 12:0 and 16:0, respectively.

Quantitative analyses revealed that approximately 1 mole 3-O(12:0)-14:0 is present per mole of lipid A backbone (27). The yield of 3-O(16:0)-14:0 was lower (0.3 mol/2 moles glucosamine) than expected with regard to the amount of 16:0 present in free *Salmonella* lipid A (0.8 mol/2 moles glucosamine). This low yield may find its explanation in the fact that acylimidate formation is greatly influenced by the anomeric configuration of the substituted hexosamine. Thus, treatment of benzyl-2-acetamido-3,4,6-tri-O-acetyl-2-deoxy- β -D-glucopyranoside with $\text{CH}_3\text{I}/\text{Ag}^+$ largely yields the O-methylated product (yield 83%), while no detectable amount of methylacetimidate is formed with the α -anomer (28). In *Salmonella* lipid A, the nonreducing D-glucosaminyl residue is present in the β -pyranose form. Hence, the formation of the methyl acylimidate is expected to occur to a large extent. The reducing glucosamine residue of the lipid A backbone which carries at its glycosidic hydroxyl group a phosphoryl group is likely to possess the α -configuration (37). Thus, methyl acylimidate formation should take place to only a minor extent. The fact that 3-O(12:0)-14:0 is detected in the expected amounts (~1 mole equivalent) could therefore mean that this group is linked to the nonreducing glucosamine residue. Furthermore, the small yield of 3-O-(16:0)-14:0 could indicate that this residue is bound to the reducing glucosamine residue of the backbone. This latter hypothesis is in accordance with the finding that, from free lipid A (*Salmonella*), treated with $\text{BF}_4^-\text{O}(\text{C}_2\text{H}_5)_3^+$ (29), 3-O(12:0)-14:0 and 3-O(16:0)-14:0 are liberated in similar amounts (although in low yield, ~10%). Interestingly, this reaction proceeds equally well with both α and β anomeric substrates.

A similar distribution of fatty acids has also been detected in lipid A of other bacteria (Fig. 5). Thus, in *Fusobacterium nucleatum*, 2 moles of (R)-3-OH-14:0 are ester-bound, one of which is 3-O-acylated by 14:0. In amide linkage, (R)-3-O(14:0)-16:0 is present. In *Vibrio cholerae*, a dimer of (R)-3-OH-12:0 is bound as an ester while (R)-3-O-(14:0)-14:0 and (R)-3-O-(16:0)-14:0 are amide-linked. The lipid A component of *Chromobacterium violaceum* possesses 2 moles of (R)-3-OH-10:0 in ester linkage. The amide-bound acyl groups are represented by (R)-3-OH-12:0 residues which are 3-O-acylated by 12:0 and (S)-2-OH-12:0. In *P. mirabilis*, 3-OH-14:0 is, like in *Salmonella*, ester- and amide-bound. In this case, however, exclusively 14:0 substitutes the 3-hydroxyl groups of both O- and N-linked 3-OH-14:0.

Hydrophobic Region - Summary

From the conclusion that 12:0 and 16:0 are quantitatively bound to hydroxyl groups of amide-linked 3-OH-14:0, it follows that, of the fatty acids present in *Salmonella* lipid A, only (R)-3-hydroxytetradecanoyl residues (4 mol/2 moles glucosamine) are

Bacteria	O-Acyl	N-Acyl
Salmonella minnesota	(R)-3-OH—14:0 (R)-3-O(14:0)-14:0	(R)-3-O(12:0)-14:0 (R)-3-O(16:0)-14:0
Fusobacterium nucleatum	(R)-3-OH—14:0 (R)-3-O(14:0)-14:0	(R)-3-O(14:0)-16:0
Vibrio cholerae	(R)-3-O((R)-3-OH-12:0)-12:0	(R)-3-O(14:0)-14:0 (R)-3-O(16:0)-14:0
Chromobacterium violaceum	(R)-3-OH—10:0	(R)-3-O(12:0)-12:0 (R)-3-O((S)-2-OH-12:0)-12:0

Figure 5. Ester- and amide-bound acyl groups present in lipid As of different bacterial species.

bound directly to the backbone disaccharide, 2 moles to hydroxyl and 2 moles to amino groups. In contrast to previous assumptions, the nonhydroxylated fatty acids (and 2-OH-14:0) are not linked to the lipid A backbone, but are attached in a specific distribution as esters to hydroxyl groups provided by ester- and amide-bound 3-OH-14:0 residues.

Therefore, all biologically active lipid A preparations studied so far contain as the dominating lipophilic constituent ester- and amide-bound (R)-3-hydroxy fatty acids which carry, at their 3-hydroxyl group, other fatty acids. (R)-3-acyloxyacyl groups are found in evolutionary distinct groups of Gram-negative bacteria. This structural principle, therefore, represents common and ubiquitous feature of lipid A's of various origin. Since (R)-3-acyloxyacyl groups are, in general, missing in other lipids of Gram-negative bacteria, they are characteristic and unique for lipid A.

Salmonella Lipid A

The findings discussed in this paper provide a basis for the construction of a structural model of Salmonella lipid A. Figure 6 shows our present view of this structure. Salmonella lipid A consists of a $\beta(1\rightarrow6)$ -linked D-glucosamine disaccharide which carries phosphoryl groups in position 4' of the nonreducing, and in position 1 of the reducing glucosamine residue. The pyranosidic α -D-glucosaminyl structure (reducing glucosamine) shown in Fig. 6 has recently been proven (37). The phosphate group in position 4' is partially (approximately 20-60%) substituted by non-acylated 4-amino-4-deoxy-L-arabinose, which has been shown to be β -linked (37). The phosphate residue occupying the glycosidic hydroxyl group in position 1 is partially (approximately 30-60%) substituted by phosphorylethanolamine residues.

The amino group of the nonreducing glucosamine residue of the disaccharide is acylated by a (R)-3-O-dodecanoyl-tetradecanoyl group. The amino group of the reducing glucosamine residue carries (R)-3-hydroxytetradecanoic acid which is substituted, at the 3-hydroxyl group, by hexadecanoic acid. It is likely that this latter substitution is quantitative. One backbone hydroxyl group represents the attachment site of the polysaccharide portion in intact lipopolysaccharide. It is presently assumed that in Salmonella this is the hydroxyl group in position 3' of the nonreducing glucosamine residue. Hence, this hydroxyl groups is unsubstituted in free lipid A. The hydroxyl groups in positions 3 and 4 as well as 6' could carry the ester-bound fatty acid residues. These comprise 1 mole of each (R)-3-hydroxytetradecanoic and (R)-3-O-(tetradecanoyl)-tetradecanoic acid. In this latter structure, approximately 10% of tetradecanoic acid are present as the α -oxidation product, i.e. (S)-2-hydroxytetradecanoic acid. However, the sum of 3-O(14:0)-14:0 and 3-O(2-OH-14:0)-14:0 equals approximately 1 mole equivalent.

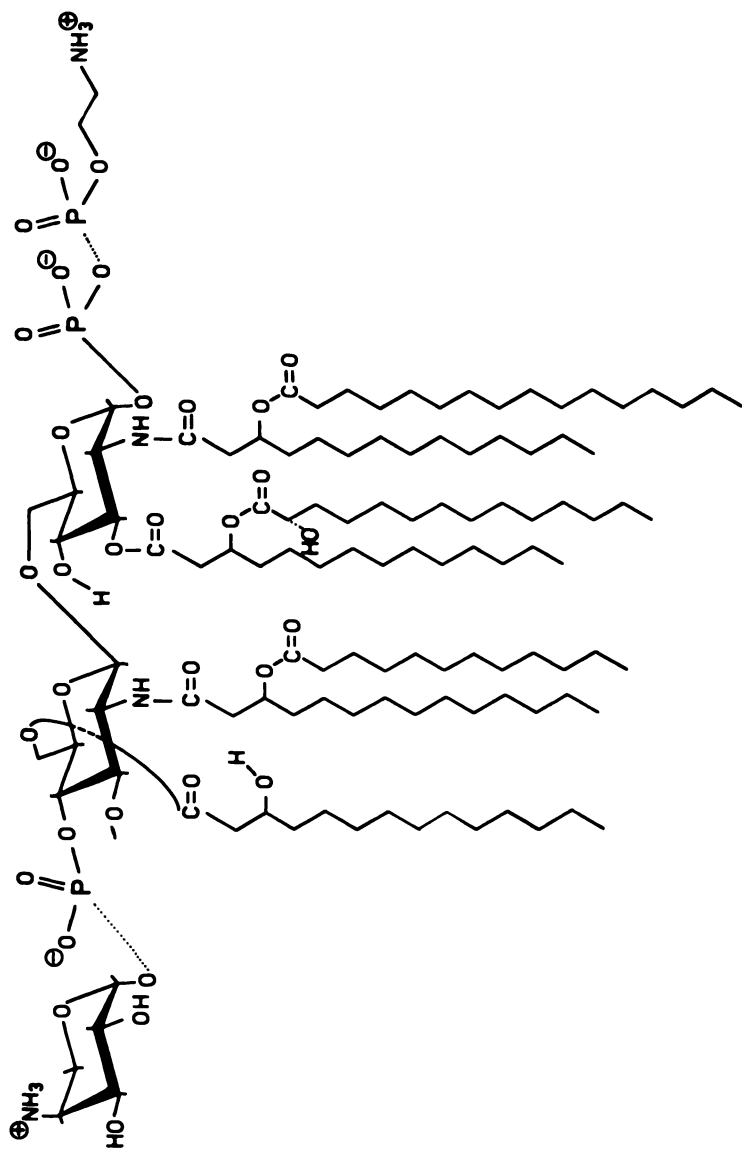


Figure 6. Proposed chemical structure of the lipid A component of *Salmonella* lipopolysaccharides (8, 32).

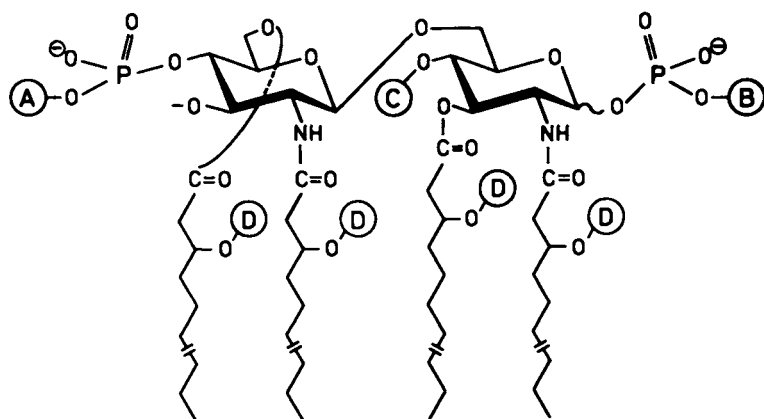
It is obvious that, for the binding of the two moles of O-acyl groups present, three hydroxyl groups are available on the lipid A backbone. There could be a random distribution of the two (R)-3-hydroxytetradecanoyl residues over the three available hydroxyl groups, and also of tetradecanoic and 2-hydroxytetradecanoic acid over the two 3-hydroxyl groups of these residues. Such a statistical binding pattern would create a great number of Salmonella lipid A structures, and consequently, an enormous heterogeneity. Alternatively, the 2 moles of O-acyl residues are linked to two defined hydroxyl groups. From such a distribution, it would follow that one hydroxyl group of the backbone is not acylated. In Rhodospirillum tenue, the hydroxyl group in position 4 of the lipid A backbone carries a glucosaminyl residue (30) and, thus, cannot be acylated. It, therefore, is tempting to speculate that also in Salmonella it is the hydroxyl group in position 4 which does not carry a fatty acid and that one of the two O-acyl groups is bound to the hydroxyl group in position 3 and the other one to that in position 6' as shown in Fig. 6. It is emphasized, however, that experimental data supporting this distribution are presently not available. Figure 6 does not show the trace amounts of (R)-3-O(decanoyl)-tetradecanoic and (R)-3-O(dodecanoyl)-dodecanoic acid recently identified in Salmonella minnesota lipid A (27).

General Structure of Lipid A's

The general principles governing the architecture of Salmonella lipid A also apply to the lipid A component of other Gram-negative bacteria. Figure 7 represents a proposal for a general structure of the biologically active lipid A's studied so far. Characteristic for, and present in, most lipid A's is a diphosphorylated, $\beta(1\rightarrow6)$ -linked, D-glucosaminyl disaccharide which carries in amide linkage (R)-3-acyloxyacyl residues. Also common to most lipid A's are ester-bound (R)-3-hydroxy fatty acids which may be 3-O-acylated. Individual lipid A's, however, differ from each other (see Fig.7) by 1. The presence and nature of residues attached to the backbone phosphate groups (positions A and B), 2. Other substituents of the glucosaminyl-disaccharide, such as D-glucosamine (in Rhodospirillum tenue, position C), 3. The type (normal, branched) and chain length of fatty acids, and 4. The facultative 3-O-acylation of ester-linked 3-hydroxy fatty acids (position D). Variation of these parameters creates a larger number of related, but not identical, chemical structures, *i.e.* lipid A structures. (For a more detailed discussion of these structures compare (8,31)).

CONCLUDING REMARKS

Subsequent to the first structural proposals, our views on the chemical structure of lipid A have changed considerably. Today, a quarter of a century later, certain principles of the lipid A



*Figure 7. Proposed general structure of lipid As of different bacterial origin (34). Circled letters indicate points of structural variation of different lipid As. The figure does not take into account the recent finding (21) that, in *Proteus mirabilis* Re lipopolysaccharide, the polysaccharide portion is linked to position 6' of the lipid A backbone and not to position 3' as indicated.*

structure (backbone, fatty acid composition) are well recognized and established. Other structural details, however, remain to be studied and elucidated (e.g. the location of ester-bound acyl and 3-acyloxyacyl groups). Furthermore, present proposals for structural models of lipid A are fragmentary in that they account only insufficiently for the heterogeneity of lipid A. As discussed, lipopolysaccharides and free lipid A are amphiphiles which form aggregates and micelles, a property which has so far largely prevented the successful, direct application, to bound lipid A, of modern analytical, physicochemical techniques (such as nuclear magnetic resonance). Such techniques have, however, been applied to substructures of lipopolysaccharides and lipid A which were prepared by controlled degradation. The strategy of investigating isolated substructures of lipid A harbours, however, the innate disadvantage that during preparation and purification of one fragment, another may escape detection. The sum of the presently known components of *Salmonella* Re lipopolysaccharide (consisting of KDO and lipid A) amounts to almost 100% (8). Thus, it is unlikely that a major lipid A component has been overlooked, but this possibility cannot be ruled out at present. In summary, proposals for lipid A structures remain provisional. Such proposals should be regarded as structural models which indicate the general architecture of lipid A and which summarize its known elements. They certainly cover the major part of lipid A but they may be incomplete.

Despite these uncertainties several laboratories have now started to chemically synthesize lipid A substructures and analogues (32). These preparations are presently being compared with (native) free lipid A by physicochemical methods of analysis, and are being tested in a variety of biological systems (33). It is obvious that these synthetic samples will be of great significance in the verification of the structural proposals made for lipid A.

The synthetic lipid A preparations will furthermore help to answer the question as to the nature of groups which are essential for the mediation of the biological activities of lipid A. Among these are activities which are shared by all known amphiphiles from Gram-negative bacteria (e.g., binding to mammalian cells), activities which are common to some molecules (such as B-lymphocyte mitogenicity which is expressed by lipid A and the lipid component of the lipoprotein), and finally, activities which are specific to lipid A (pyrogenicity, lethality). It is known that, for affinity to cell membranes, fatty acids in amphiphiles are essential. For this activity to be expressed, no structural restriction with regard to the type of long chain fatty acid and the nature of the polyol backbone to which fatty acids are attached is known. Evidently, more stringent structural requirements have to be met to endow a bacterial amphiphile with immunological or endotoxic activities. Thus, the mitogenically active minimal structure of the lipoprotein has been shown to be N-hexadecanoyl-L-cysteine (methyl ester) (6). It is hoped that also the now avail-

lable lipid A analogues, the chemical synthesis of which is described and discussed in this symposium will, in the near future, help to define structures and conformations which determine the endotoxic activities characteristic for lipopolysaccharides and lipid A.

ADDENDUM (May 1983)

Since the completion of the present manuscript (December 1982) further chemical and physicochemical studies have shed new light on the structure of lipid A. The results of these recent investigations are briefly summarized below.

As described in this paper KDO is, in Proteus mirabilis, linked to position 6' of the lipid A backbone (21). This was also found to be true for Salmonella minnesota (41) and Escherichia coli (42,43). Therefore, the linkage point of KDO (i.e. the saccharide portion) in enterobacterial lipopolysaccharides is the primary hydroxyl group position 6' of the lipid A glucosaminidiacaride and not, as was previously assumed, the hydroxyl group in position 3'. In addition in free E. coli lipid A, the attachment points of the two ester-bound (R)-3-hydroxytetradecanoyl residues were, by 2D-NMR analysis, identified as positions 3 and 3' of the lipid A backbone (44). This finding is in accord with the demonstration that the ester-bound (R)-3-hydroxytetradecanoyl group present in a monomeric lipid A precursor molecule (E. coli) is linked to the hydroxyl group in position 3 (45). Further, in free lipid A of E. coli the hydroxyl group on C-4 was shown to be free (44). This result is supported by our recent finding (46) that in free lipid A of E. coli and S. minnesota the hydroxyl group in position 4 can be methylated by diazomethane (in the presence of SiO₂). Finally, by subjecting free lipid A fractions of S. typhimurium to positive fast atom bombardment mass spectrometry it was demonstrated that (R)-3-O-(14:0)-14:0 is bound to the nonreducing and (R)-3-OH-14:0 to the reducing glucosamine unit of the backbone, respectively (47).

In light of this new evidence the Salmonella lipid A structure (Fig. 6) requires certain modifications. A proposal for the chemical structure of Salmonella lipid A which takes in account the above discussed new findings and which is in accord with the present knowledge is presented in Fig. 8.

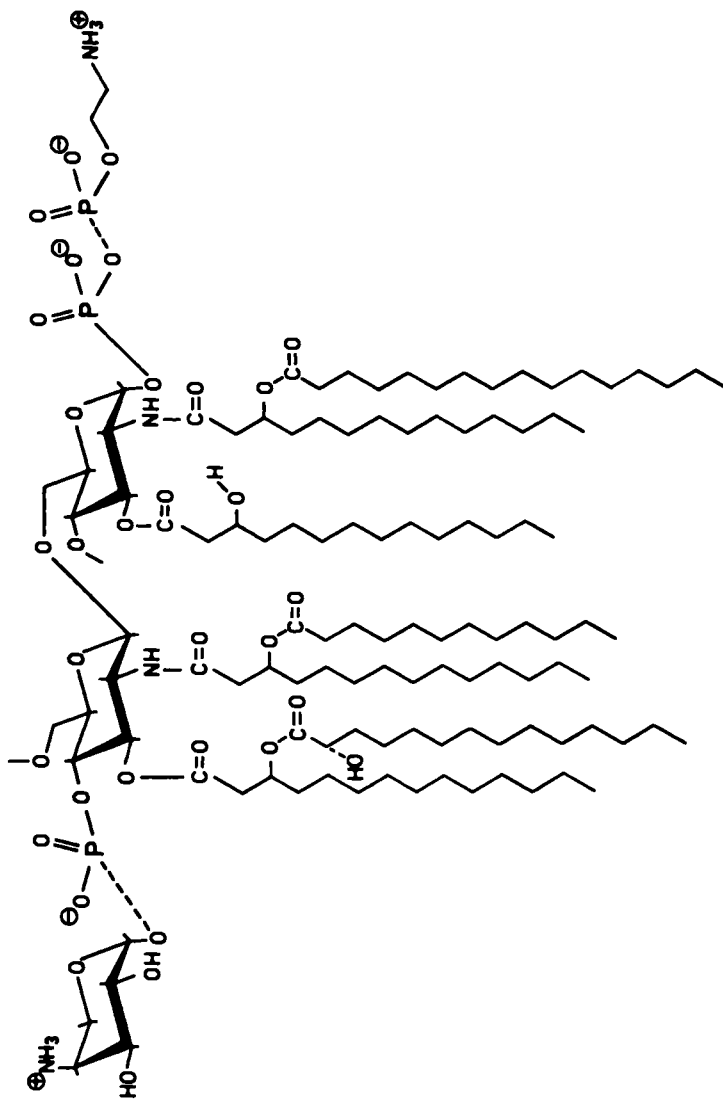


Figure 8. Chemical structure of *Salmonella* lipid A according to present knowledge (May 1983).

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Use of Endotoxin in Cancer Immunotherapy and Characterization of Its Nontoxic but Active Lipid A Components

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When crude endotoxin from the heptose-less mutant of Salmonella typhimurium is combined with trehalose dimycolate from mycobacteria in oil droplets and injected directly into established tumors (line 10 hepatocellular carcinoma) in syngeneic guinea pigs, rapid regression of the tumors occurs and over 90% of the animals are cured. The three required components for activity in this tumor model are: (a) the endotoxin; (b) the mycobacterial adjuvant, trehalose dimycolate; and (c) a compound satisfying the minimal structural requirement (muramyl dipeptide) for adjuvant activity by bacterial cell wall materials. The mycobacterial cell wall skeleton is able to replace the latter two components.

Since endotoxin is very toxic to humans, a method was sought to render it nontoxic and yet retain its tumor regression activity. We detoxified endotoxic extracts and diphosphoryl lipid A obtained from S. typhimurium G30/C21 by controlled acid hydrolysis to yield a product that retained the ability to synergistically enhance the antitumor properties of mycobacterial adjuvants. The chemical structure of the nontoxic product was established by first purifying it by preparative thin layer chromatography, analyzing the purified lipid A by reverse-phase high performance liquid chromatography, and finally by determining its exact molecular mass by fast atom bombardment mass spectrometry. The nontoxic but active product was shown to be a 4'-monophosphoryl lipid A containing ester-linked lauroyl-, 3-hydroxymyristoyl-, and 3-myristoxymyristoyl groups. This lipid may represent a potential candidate for use in the immunotherapy of human cancer.

Early History of Tumor Immunology

The suggestion that the control of cancer may be effected by immunologic methods was made about 100 years ago. At that time, William Coley observed that tumors either partially or totally

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regressed in patients following an acute bacterial infection (1). Today we know that an aroused immune system, which plays a protective role against infectious disease, also plays a major role in the host's defense against cancer. The effective ingredient of Coley's bacterial vaccine appeared to be endotoxin, which caused hemorrhagic necrosis of the tumor. Although the antitumor activity of endotoxin has been extensively studied for at least 50 years, endotoxin had come to be regarded as of questionable value for the treatment of tumors. It rarely led to tumor elimination with the concomitant production of systemic tumor immunity but rather to partial regression followed by resumption of growth.

It was not until the late 1960's that encouraging experimental and clinical results were obtained from the treatment of tumors with bacterial preparations other than endotoxins, primarily with an attenuated antituberculosis vaccine consisting of living bacteria of Mycobacterium bovis strain BCG alone or admixed with tumor cells. However, the use of viable cells of BCG caused complications.

Because of the need for more potent, nonviable immunotherapeutic agents that can be administered without harmful side effects, a major objective of our research effort was to identify, isolate, and evaluate chemically defined microbial components that are effective antitumor agents.

Development of Well-Defined Cell-Free Components

Preparations of BCG cell wall in oil droplets were as effective as viable BCG in inducing regression of established skin tumors (line 10) of inbred guinea pigs and in eliminating lymph node metastases upon intralesional injection (2). By fractionating the BCG cell wall we have obtained components that retain antitumor activity, and have the potential advantages of increased potency and reduced allergenicity (3,4). To isolate and define the cell wall components required for tumor regression, we first digested the cell walls with proteolytic enzymes to remove proteins and then exhaustively extracted them with organic solvents to liberate free lipids. The resulting cell wall skeleton (CWS), an insoluble, polymeric mycolic acid-arabinogalactan-mucopeptide, had reduced antitumor activity compared with cell walls. But full activity was restored when CWS was combined with trehalose dimycolate (P3), obtained from the free lipids of the cell wall (5).

Efficacy of Endotoxin in Tumor Immunology

We found that P3 combined with cell walls of endotoxin containing gram-negative bacteria, such as Escherichia coli and Salmonella, induced complete regression of line 10 tumors (6). This led to a reinvestigation of the value of endotoxins, which were known to be equally powerful microbial adjuvants (poten-

tiators of immune responses) as the above described mycobacterial cell wall fractions, namely CWS and P3.

In agreement with the early findings, when the endotoxin was injected alone into line 10 tumors, we observed a necrotic reaction leading to partial regression and the tumor shortly resumed its growth. However, when certain preparations of endotoxin, in combination with P3 in oil droplets, were injected into established tumors, a high rate of cures concomitant with the development of specific tumor immunity was attained. The animals rejected a subsequent challenge with a lethal dose of line 10 tumor cells (6). The data are summarized in Table I. All of the tumors disappeared when 150 μ g of endotoxin obtained from the rough mutant of Salmonella typhimurium was combined with 50 μ g of

Table I. Synergistic Effect Between Cell Wall Skeleton (CWS) and Endotoxins (ET) in Regressing Line 10 Tumors

Material associated with oil droplets injected into tumors ^a	Dose (μ g)	Cured/Total	Percent cured
ET	150	0/8	0
P3	150	0/217	0
ET + P3	150 + 50	8/8	100
Purified ET + P3	150 + 50	0/8	0
CWS	300	14/16	88
CWS	50	1/16	6
CWS + purified ET	50 + 50	8/8	100
Purified ET + P3 + ACP ^b	150 + 50 + 150	54/67	81
ACP + P3 ^c	150 + 50	0/9	0

^aRibi et al. (7)

^bRibi et al. (8)

^cTakayama et al. (9)

P3. When the endotoxin preparation was freed of peptides, phospholipids, and divalent cations, it lacked the tumor regressive potency. We noted muramic acid, alanine, and glutamic acid among the principal nitrogenous components present in the endotoxic extract, and observed that the proportion of each of these components was lowered significantly during the preparation of purified endotoxin (10). These are the components that make up the minimal structural entity, N-acetylmuramyl-L-alanyl-D-isoglutamine (muramyl dipeptide), of the mucopeptide moiety of the bacterial CWS that is responsible for adjuvant activity (11). We considered the possibility that the product of autolysis of the Salmonella CWS may have been co-extracted with the endotoxic glycolipids. Since the CWS of mycobacteria but not that of Salmonella contains mycolic acid esters (to replace P3 in addition to the peptidoglycan moiety, it was not surprising to find that the tumor regressive potency of the peptide-free endotoxin could be restored by the addition of mycobacterial CWS (10). As little as 50 μ g of CWS and 50 μ g of purified endotoxin sufficed to give 100% regression. A ten-fold lower quantity of purified endotoxin

still led to a cure rate of 78%. The tumor regressive potency of purified endotoxin also could be restored by the addition, to the purified endotoxin plus P3, of a preparation of acetone-chloroform precipitate (ACP), a nontoxic peptide-containing side fraction obtained during the isolation of endotoxin (10). The tumors treated with this endotoxin mixture regressed rapidly (in about one week), whereas tumors treated with a 300 µg dose of CWS plus P3 regressed slowly (in about 2-3 weeks).

The Problem of Toxicity of Endotoxin

Human, horse, dog, and rabbit are known to be highly susceptible to the toxic effects of endotoxin whereas small animals such as guinea pig and mouse are relatively resistant (Table II). As an example, a single injection of 150 µg of endotoxin can be lethal to a horse. Because of this high susceptibility of humans to endotoxin, its potentials for clinical application have not been realized. In the past, endotoxin preparations were usually described by their degree of toxicity and pyrogenicity. We and other investigators had previously explored the possibility of using chemical modification techniques to selectively reduce the toxicity and pyrogenicity of endotoxins while retaining adjuvanticity (15-19). The aim was to provide chemically defined, nontoxic adjuvants which were capable of enhancing nonspecific resistance to bacterial infections and of synergistically enhancing the tumor regressive potency of mycobacterial CWS (10). Because the experiments were done with heterogenous mixtures of endotoxin whose structures are unknown, the observed biological activity could not be related directly to any specific component(s).

An Approach to Purifying Endotoxin and Its Derivatives

It was our working hypothesis that complete solubilization and

Table II. Lethal Dose of Endotoxin and Detox

Test subject	Endotoxin (µg)	Detox ^a (µg)	Detox is less toxic by factor of
Mouse ^b	100-500	>10,000	>20-→100
Guinea pig ^c	>300	ND	--
Horse ^d	150	>20,000	>130
Dog ^e	100	>4,000	>40
Human ^f	1-100	ND	--
Rabbit ^b	1-10	>15,000	>1,500-→15,000
Chick embryo ^g	<0.0008	>10	>10,000

^aCantrell and Ribí, unpublished results

^bMilner *et al.* (12)

^cRibí *et al.* (10) ^dBottoms *et al.* (13)

^eHinshaw *et al.* (14)

^fSusceptibility is estimated to be similar to that of dog and rabbit. ^gTakayama *et al.* (9)

disaggregation are required before one is able to fractionate amphipathic compounds such as mixtures of endotoxic glycolipids. The purified endotoxin prepared by microparticulate gel chromatography appeared to meet these requirements, and work is in progress to fractionate this complex mixture (8). Purified endotoxin was suspended in 0.02 M sodium acetate, pH 4.5, and heated at 100 °C for 30 min according to Rosner *et al.* (20) to cause the liberation of the 2-keto-3-deoxyoctonate (KDO) moiety. The application of two cycles of this treatment to purified endotoxin removed 99% of the KDO without the loss of toxicity (Table III). Since the starting material was prepared from the heptose-less Re mutant of *S. typhimurium*, the purified endotoxin fraction is regarded as a KDO-lipid A complex and the KDO-free residue resulting from the above treatment is then defined as toxic lipid A. The presence of KDO was not essential for toxicity.

Table III. Lethality of KDO-depleted Endotoxic Lipid A

Material	Recovery %	KDO μ mol/mg	Lethality for chick embryos CELD ₅₀ , μ g ^c
Purified endotoxin	--	1.13	0.012
Toxic lipid A ^a	41	0.014 ^b	0.031

^a Purified lipid A was treated with two cycles of sodium acetate pH 4.5, 100 °C, 30 min.

^b 99% of KDO was removed.

^c Ribí *et al.* (8)

Preparation of Nontoxic Lipid A

When purified endotoxin was treated with 0.1 N HCl for 30 min at 100 °C, the resulting nondialyzable residue was also free of KDO but was nontoxic (CELD₅₀, >10 μ g) and nonpyrogenic (FI₄₀, 20 μ g) (Table IV). We have designated this product nontoxic lipid A (Detox) and demonstrated its low toxicity in four animal species (Table II). The nontoxic lipid A in combination with P3 and ACP (or CWS) retained a degree of tumor regressive potency (80% cures) similar to that observed with the toxic lipid A (88% cures) and the purified endotoxin (81% cures).

Results of chemical analysis showed that the glucosamine and total fatty acid contents of the KDO depleted, toxic lipid A and the nontoxic lipid A were essentially the same but that the nontoxic lipid A was significantly lower in the phosphorus content (Table V). The molar ratio of glucosamine:phosphorus:fatty acids was 2:2:4 for the toxic lipid A and 2:1:4 for the nontoxic lipid A. The relative molar distribution of normal fatty acids (lauric, myristic, and palmitic acids) and the 3-hydroxymyristic acid did not indicate a correlation between the content of these components and toxicity. The nontoxic lipid A possessed as high a tumor regression activity when combined with CWS as did the purified

endotoxin from which it was prepared (Table VI)(7, Cantrell and Ribi, unpublished results). Whereas a 150 μg dose of endotoxin was lethal to a horse (Table II), the administration of 20,000 μg of the nontoxic lipid A caused no detectable lethargic reactions or rise in the body temperature.

Table IV. Antitumor Effect of Toxic and Nontoxic Glycolipids

Material tested	CEL _{D50} , μg	Pyrogenicity for rabbits (FI ₄₀ , μg)	Line 10 tumor regression			
			+P3		+ACP +P3	
			cured total	%	cured total	%
Purified ET ^a	0.026	0.049	1/26	4	54/67	81
Toxic lipid A ^a	0.031	ND	ND		7/8	88
Nontoxic lipid A (Detox) ^a	>10	20	ND		27/32	84
ACP ^b	>10	>100	0/26	0	--	

^aRibi *et al.* (8) ^bTakayama *et al.* (9)

Table V. Chemical Composition of Endotoxin (ET), Purified ET, and Lipid A

Material tested	Glucosamine	Phosphorus	KDO	Total fatty acid
ET ^a	638 (2)	941 (2.95)	911 (2.86)	1715 (5.37)
Purified ET ^b	932 (2)	1055 (2.26)	1134 (2.43)	1821 (3.91)
Toxic lipid A ^b	1182 (2)	1122 (1.90)	14.1 (0.02)	2658 (4.49)
Nontoxic lipid A (Detox) ^b	1157 (2)	713 (1.23)	9.8 (0.02)	2510 (4.30)

^aAmano *et al.* (21) ^bRibi *et al.* (8)

Table VI. Synergy Between Endotoxin (ET), Nontoxic Lipid A (Detox), and Mycobacterial Cell Wall Skeleton (CWS) in Regressing Line 10 Tumors^a

Material associated with oil droplets injected into tumors	Dose (μg)	Cured/Total	Percent cured
CWS	50	10/48	20
ET	150	0/8	0
CWS + ET	50 + 50	8/8	100
	50 + 5	6/8	75
Detox	50	0/18	0
CWS + Detox	50 + 50	50/52	96
	25 + 25	9/10	90
	50 + 8	4/8	50

^aCantrell and Ribi, unpublished data

We have shown that the treatment of disaggregated, purified endotoxin with HCl reproducibly led to complete detoxification (CELD₅₀, >10 μg), whereas similar treatment of conventional endotoxin extracts (with CELD₅₀ of 0.004 μg) led to only partial detoxification (CELD₅₀, 0.25 μg). The failure to detoxify this latter extract might be due to the aggregation of the endotoxin and its protective effect on acid-labile groups, resulting in incomplete hydrolysis. This may explain the earlier reported results where a mere 10-fold decrease in toxicity was observed following acid treatment of endotoxic phenol-water extracts (22).

Purification of Lipid A

We showed the existence of a toxic and a nontoxic lipid A fraction in the acid hydrolyzed endotoxin preparation (23). These two fractions were separated and the composition was determined on the purified components so that we could relate specific structural features of lipid A to both toxicity and tumor regression activity (23).

Figure 1 shows the scheme for the preparation of purified lipid A from endotoxin. *S. typhimurium* G30/C21 was extracted by the method of Galanos *et al.* (24) and submitted to one of two different conditions of hydrolysis: (a) 0.1 N HCl [in methanol-water (1:1, v/v)], 100 °C, 45 min, to yield the crude monophosphoryl lipid A (nontoxic), and (b) 0.02 M sodium acetate, pH 4.5, 100 °C for 30 min (two cycles) to yield the crude diphosphoryl lipid A (toxic). The 0.1 N HCl hydrolysis product was fractionated on a Sephadex LH-20 column (23). Each of these fractions was then separated by preparative thin layer chromatography (TLC) on silica gel H (500 μm), with the solvent system chloroform-methanol-water-concentrated ammonium hydroxide (50:25:4:2, v/v) as previously described (23) to yield TLC fractions 1-7 and 1-9 respectively.

Chemical Analysis of Purified Lipid A

The results of the chemical analysis of these fractions are given in Table VII. For the monophosphoryl lipid A (TLC-1, -3, and -5), the glucosamine:phosphate ratio was 1.98 to 2.15, whereas

Table VII. Chemical Analyses of Mono- and Diphosphoryl Lipid A

Content ($\mu\text{mol}/\text{mg}$)	Lipid A			
	Monophosphoryl ^a			Diphosphoryl ^b
	TLC-1	TLC-3	TLC-5	
Phosphate	0.54	0.52	0.58	0.81
Glucosamine	1.07	1.12	1.24	0.76
KDO	0.0	0.0	0.0	0.01
Glucosamine/Phosphate (molar ratio)	1.98	2.15	2.14	0.95

^a Qureshi *et al.* (23)

^b This sample contained 20-25% silica gel.

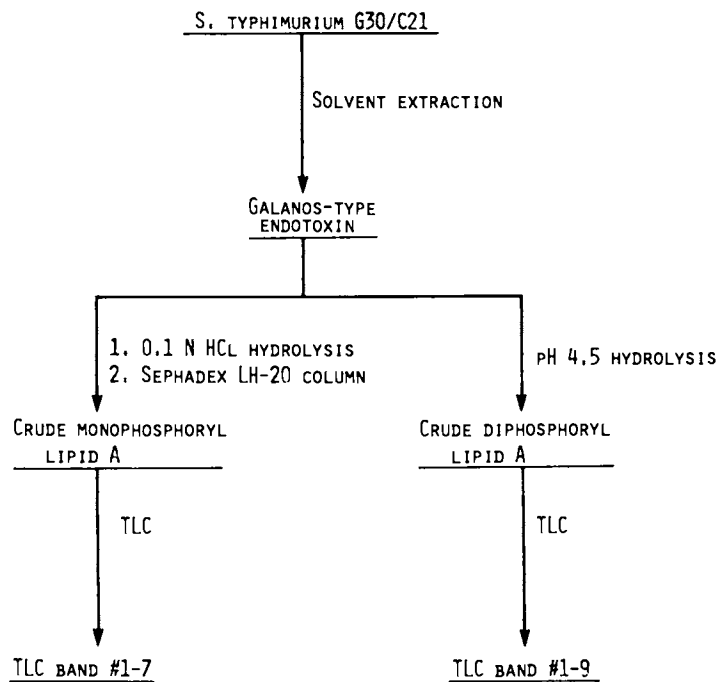


Figure 1. Scheme for preparing and purifying mono- and diphosphoryl lipid A from endotoxin obtained from *S. typhimurium* G30/G21.

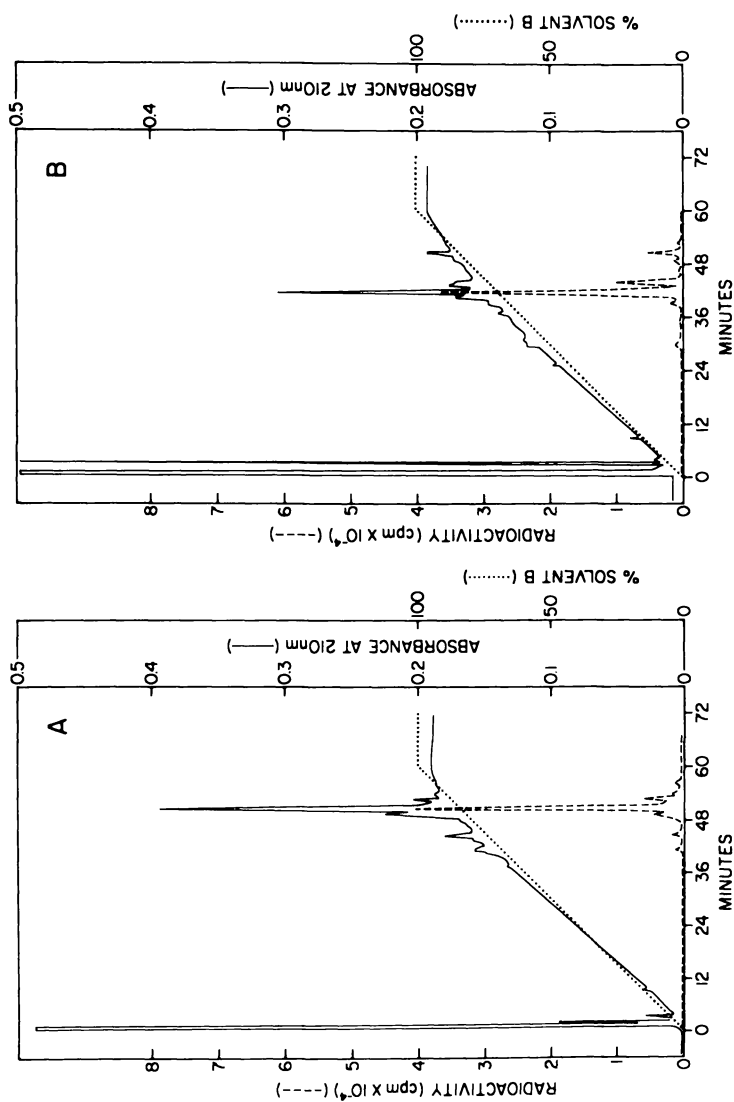


Figure 2. Reverse-phase HPLC of purified [¹⁴C]monophosphoryl lipid A. A, TLC-3; B, TLC-5. The sample sizes were 1.4 and 2 mg, respectively (60,000 cpm each). The labeled lipid As were obtained from *S. typhimurium* G30/G21 grown in the presence of [¹⁴C]acetate as previously described (23).

Conditions: C₁₈-Bonded cartridge, 8 mm x 10 cm (Radial pak A, Waters Associates, Inc.) was used with a mobile phase of a linear gradient of 0-100% isopropyl alcohol-water (9:1, v/v) (solvent B) in acetonitrile-water (9:1, v/v) (solvent A) over a period of 60 min at a flow rate of 3 mL/min. Both solvents contained 5 mM tetrabutylammonium phosphate. For the determination of the radioactivity profile, fractions were collected at 30-s intervals and assayed for radioactivity.

in the unfractionated but TLC-purified diphosphoryl lipid A, the ratio was 0.95. All fractions were virtually devoid of KDO.

High Performance Liquid Chromatography (HPLC) of Monophosphoryl Lipid A

The analysis of the purified monophosphoryl lipid A by reverse-phase HPLC revealed the degree of purity of the fractions TLC-1, -3, -5, and -7 (23). TLC-1 and -3 each gave one major peak (85%), and these peaks had identical elution times. TLC-5 showed one major (72%) and one minor (18%) peak. TLC-7 resolved into one major (48%) and two minor (21 and 16%) peaks. One of the minor peaks in TLC-7 (16%) was the overlapping contaminant of the minor components in TLC-5. Representative results of the HPLC analysis of ¹⁴C-labeled TLC-3 and -5 are shown in Figure 2.

Fast Atom Bombardment (FAB) Mass Spectral Analysis

Purified monophosphoryl lipid A (TLC-1 through -9) were analyzed by FAB mass spectrometry in the negative mode, gave the results summarized in Table VIII. TLC-1 gave a major molecular ion (M-H)⁻ at m/z 1730. Its ester-linked fatty acid composition would be one mole each of 3-hydroxymyristic, lauric, and 3-myristoxymyristic acids per mole of lipid A. TLC-1 also contained a methyl group (possibly as a methyl glycoside or a methyl phosphate) that was probably added to the lipid during acid hydrolysis of the sugar 1-phosphate in the presence of methanol. TLC-3 gave a molecular ion at m/z 1716. Its ester-linked fatty acid composition was identical to that of TLC-1. The major component of TLC-5 gave a molecular ion at m/z 1506. Its ester-linked fatty acid composition would be two moles of 3-hydroxymyristic and one mole of lauric acids per mole of lipid A. Other minor ions were observed in TLC-1 through -7. Finally, TLC-9 gave a molecular ion at m/z 1098. A molecule of this lipid would contain a single ester-linked 3-hydroxymyristic acid residue. The ester-linked fatty acid distribution, molecular formulas of the free acids, and their M_r were determined as shown in Table IX.

Table VIII. FAB Mass Spectral Analysis of Purified Monophosphoryl Lipid A^a

TLC Fraction	m/z (M-H) ⁻	
	Molecular ion	Other ions
TLC-1	1730	1716, 1520, 1294
TLC-3	1716	1520, 1506, 1294, 1280
TLC-5	1506	1534, 1520, 1280
TLC-7	1280	1324, 1308, 1294
TLC-9	1098	None

^a Reprinted from Ref. 23.

Table IX. Ester-linked Fatty Acid Content, Molecular Formulas and Molecular Weights of Fractions of Purified Monophosphoryl Lipid A^a

Monophosphoryl lipid A fraction (major component)	Ester-linked fatty acid ^b			Molecular formula ^c	M _r ^c
	OH-C ₁₄	C ₁₂	MM		
TLC 1	1	1	1	C ₉₅ H ₁₇₉ O ₂₂ N ₂ P	1731.2
TLC-3	1	1	1	C ₉₄ H ₁₇₇ O ₂₂ N ₂ P	1717.2
TLC-5	2	1	0	C ₈₀ H ₁₅₁ O ₂₂ N ₂ P	1507.0
TLC-7	1	1	0	C ₆₆ H ₁₂₅ O ₁₉ N ₂ P	1280.8
TLC-9	1	0	0	C ₅₄ H ₁₀₃ O ₁₈ N ₂ P	1098.7

^a Qureshi *et al.* (23)^b OH-C₁₄, 3-hydroxymyristic acid; MM, 3-myristoxymyristic acid^c Calculated as the free acidRelating the Mono- and Diphosphoryl Lipid A's

The TLC purified diphosphoryl lipid A was hydrolyzed in 0.1 N HCl at 100 °C for 30 min to yield the corresponding monophosphoryl lipid A derivatives as previously described (23). These products were then compared with previously characterized monophosphoryl lipid A fractions by TLC using silica gel H (250 μm) and the previously mentioned solvent system. The TLC fractions -3, -5, -7 of the acid hydrolyzed diphosphoryl lipid A series corresponded with a similarly numbered series of monophosphoryl lipid A fractions (TLC-3, -5, and -7). There appeared to be some breakdown of the monophosphoryl lipid A products to the lower homologues presumably by the acid catalyzed hydrolysis of some fatty ester linkages. TLC-1 and -9 were not analyzed due to small sample sizes.

The TLC purified diphosphoryl lipid A fractions (TLC -3, -5, and -7) were analyzed by FAB mass spectrometry in the negative mode as previously described (23) and the results are shown in Table X. TLC-3 gave a molecular ion (M-H)⁻ at m/z 1796; TLC-5, m/z 1586; TLC-7, m/z 1360. As expected, these values were 80 amu or (PO₃H₂-H) larger than those for the corresponding monophosphoryl lipid A's of the series as shown in Table VIII. These results established the structural relationship between the mono- and diphosphoryl lipid A's.

Toxicity and Tumor Regression Activity of Purified Lipid A

The results of the biological tests carried out on the purified monophosphoryl lipid A fractions are shown in Table XI. The chick embryo lethality test showed that both TLC-1 and -3 were nontoxic, whereas TLC-5 exhibited some toxicity. These results indicate that there might be two levels of toxicity based on structure. The presence or absence of the sugar 1-phosphate group (and possibly some other unknown group) would control the upper

Table X. FAB Mass Spectral Analysis and Molecular Weight Determination of the Series of Purified Diphosphoryl Lipid A Fractions

TLC fraction	Molecular ion m/z (M-H) ⁻	M _r ^a
TLC-3	1796	1797.2
TLC-5	1586	1587.0
TLC-7	1360	1360.8

^a Calculated as the free acid

Table XI. Toxicity and Tumor Regression Activity of Purified Lipid A Obtained from *S. typhimurium* G30/C21

Material tested	CELD ₅₀ , µg	Line 10 tumor regression in guinea pigs	
		Cured/total	Percent cured
Diphosphoryl lipid A ^a	0.033	33/36	92
Monophosphoryl lipid A ^b			
TLC-1	>10	7/8	88
TLC-3	>10	6/8	75
TLC-5	0.199	8/8	100

^a Calculated sum and average of the diphosphoryl lipid A fractions V-VII from the DEAE-cellulose column as reported by Takayama *et al.* (9)

^b Data from Qureshi *et al.* (23)

level of toxicity (macrototoxicity) and the degree or kind of O-acylation could control the lower levels of toxicity (microtoxicity). All three of the purified monophosphoryl lipid A fractions were active in the tumor regression assay. It is to be noted that the diphosphoryl lipid A mixture is both toxic and has tumor regression activity. We have thus associated the lack of toxicity and tumor regression activity to precise chemical structures of lipid A.

Structure of Nontoxic Monophosphoryl Lipid A

The suggested structure for the nontoxic monophosphoryl lipid A is given in Figure 3. The phosphate group would occupy the 4'-position of the 2-deoxy-2-amino-β-D-glucopyranosyl-(1+6)-2-deoxy-2-amino-D-glucopyranose disaccharide. A lauroyl, 3-myristoxy-myristoyl group or a hydrogen (R₂) would occupy the 3- and 4-positions and a 3-hydroxymyristoyl residue (R₁) would occupy the 6'-position of the disaccharide. If there is a free hydroxyl group at position 3 or 4, the lauroyl group would be associated with one of the two nitrogen-linked hydroxy fatty acids (R₁).

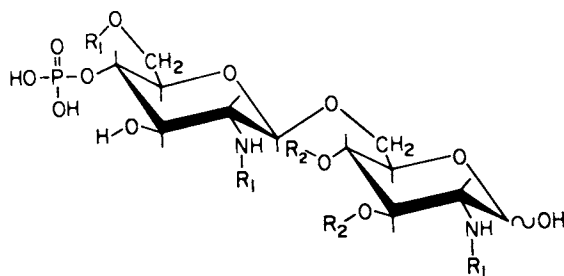


Figure 3. Structure of nontoxic monophosphoryl lipid A. R_1 = 3-hydroxymyristoyl; R_2 = lauroyl, 3-myristoxymyristoyl, or H.

This is the proposed structure of the highly O-acylated and non-toxic TLC fraction 3.

Significance of Nontoxic Lipid A

This nontoxic lipid may represent a potential candidate for use in the immunotherapy of human cancer. In addition, it will be interesting to determine to what extent this nontoxic lipid A can replace the toxic components in eliciting the numerous other biological activities of lipid A.

Acknowledgment

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Chemical Synthesis of Lipid A for the Elucidation of Structure-Activity Relationships

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A synthetic route was established for the preparation of an O,N-polyacyl glucosamine $\beta(1-6)$ disaccharide 1,4'-diphosphate which corresponds to the proposed structure of Salmonella-type lipid A. Many structural analogs were prepared in order to elucidate the true structural requirements for the biological activities of lipid A.

"Lipid A", which is the lipophilic component of bacterial lipopolysaccharide, was shown to possess many of the biological activities of lipopolysaccharide, e.g., lethal toxicity, pyrogenicity, adjuvant activity, antitumor activity, and others. The chemical structure of lipid A from Salmonella species was proposed by the group of Westphal to be an O,N-polyacyl derivative of $\beta(1-6)$ -glucosamine disaccharide 1,4'-diphosphate as shown in Figure 1 (1). Thereafter, this was confirmed by an independent study of Khorana *et al.* on lipid A of E. coli (2). Accordingly, the basic structure of lipid A was shown to be a monomer of the acyl disaccharide diphosphate but not its polymeric form as previously assumed. However, lipid A preparations from natural cell walls always contain many components, probably due to heterogeneity in type and number of acyl groups, lack of phosphate moieties or presence of polar head groups. In other words, natural lipid A had never been obtained as a chemically pure substance. It had not been fully confirmed yet whether the multiple biological activities described for lipid A could have been attributed to a single chemical entity of a definite structure. In this situation, synthetic approach seemed to be very useful to solve the remaining problems, since the proposed structure of lipid A seemed to be no more an inaccessible target for synthetic chemists. We thus decided to start a synthetic study on lipid A for the following purposes:

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- 1) Confirmation of the proposed structure of lipid A.
- 2) Precise investigation on the relationship between chemical structures and biological activities, and elucidation of minimum structural requirements for the activities.
- 3) Preparation of a standardized sample of lipid A for experimental and clinical uses.

Such an approach would only be possible by the use of pure, synthetic materials of various definite structures. Owing to the complex features of the target molecule, this synthetic study is also a worthy challenge from the standpoint of chemists. As the target of our synthesis, we chose Salmonella-type lipid A, which had been studied most extensively, and planned to construct the structure in the following order: 1) Preparation of a suitably protected $\beta(1-6)$ glucosamine disaccharide 2) Stepwise acylation of the desired positions of the disaccharide. 3) Phosphorylation of the 1- and/or 4'-hydroxyl groups. According to this strategy, many structural analogs with various acyl groups can be prepared, using one disaccharide as a common synthetic intermediate.

Synthesis of an O,N-tetradecanoyl disaccharide

First of all, the 2,2'-bis-N-3,4,6'-tris-O-tetradecanoyl $\beta(1-6)$ -glucosamine disaccharide (1) was prepared, which corresponds to a bisdephospho derivative of Salmonella-type lipid A in which all acyl groups are replaced by simple tetradecanoyl residues (3). The monosaccharide components, 2 and 3, were prepared by conventional methods as shown in Figure 2. They were coupled in the presence of $\text{Hg}(\text{CN})_2$ to give a disaccharide 4, whose $\beta(1-6)$ structure was confirmed unequivocally (3). This disaccharide was used as the common synthetic precursor of all derivatives prepared in this study. Because the 3'-oxygen was assumed to be linked to the polysaccharide part in natural bacterial lipopolysaccharide and should be a free hydroxyl group in the final product, it was protected with persistent benzyl group. The glycosidic position was protected with an allyl group, which could be removed at any later stage of synthesis independently of the other protecting groups and as would be necessary for the preparation of glycosyl phosphates. The benzoyl group in 3 was employed in order to increase the solubility of the molecule.

The synthesis of 1 was performed as outlined in Figure 3. After selective removal of both N-acetyl groups with Meerwein's reagent, the free amino functions formed were acylated with tetradecanoyl chloride. All ester-type protecting groups were then removed with alkali, the 4'- and 6'-hydroxyl groups protected by isopropylidene ketal formation, and the remaining two hydroxyl groups on C-3 and -4 were acylated again with tetradecanoyl chloride - pyridine. The glycosidic allyl group was next isomerized into the 1-propenyl group which was cleaved off with mercury salt. Hydrolysis of the 4',6'-O-isopropylidene group and selective 6'-O-monoacylation at low temperature afforded a disaccharide 8

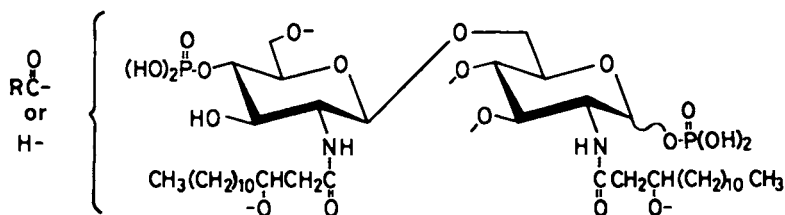


Figure 1. The proposed structure of *Salmonella*-type lipid A.

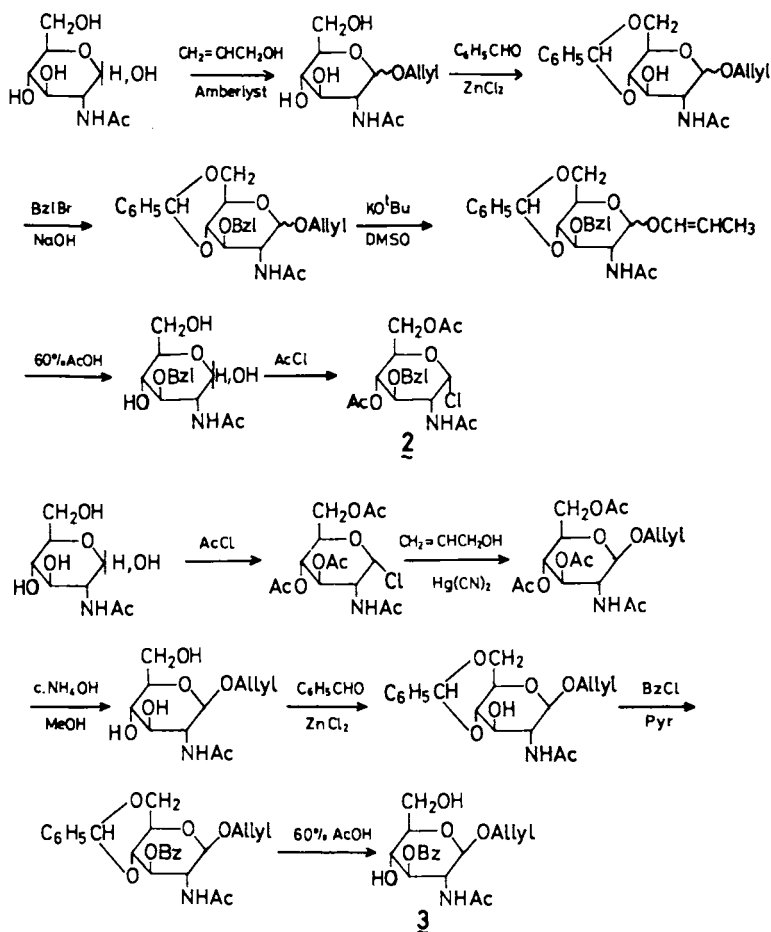


Figure 2. Preparation of the monosaccharide components **2** and **3**.

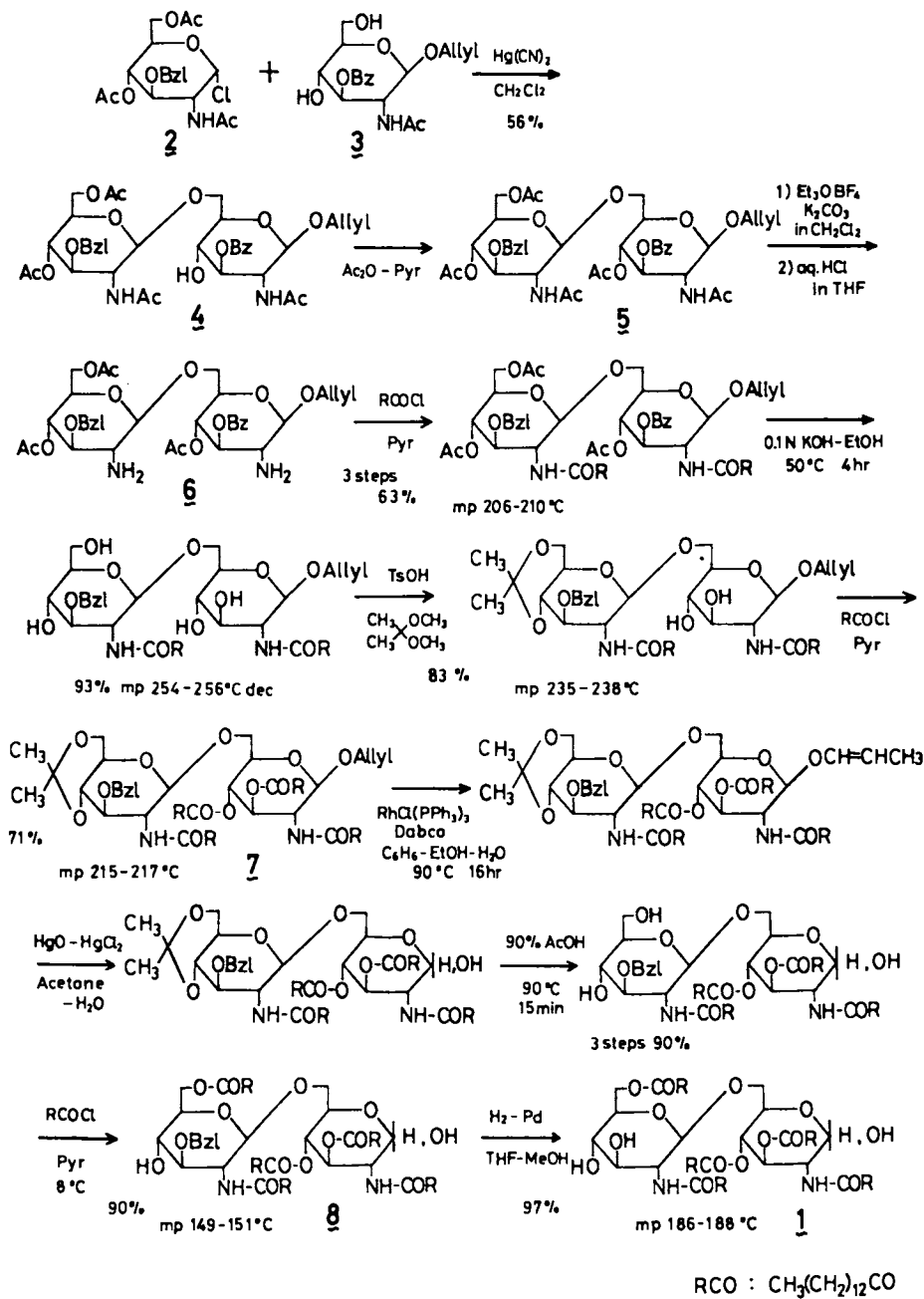


Figure 3. Preparation of the O, N-tetradecanoyl disaccharide 1.

which had all tetradecanoyl residues in the designed positions. This was then hydrogenolyzed to give the desired compound λ .

Now, a synthetic route could thus be constructed to prepare acyl disaccharides corresponding to λ , with any variation of acyl groups on desired positions. Even 3- and 4-hydroxyl groups could be differentiated if necessary in this synthesis, since the latter hydroxyl group has considerably lower reactivity.

Synthesis of phosphates of the O,N-tetradecanoyl disaccharide

The next step of our synthetic study was the preparation of 1- and/or 4'-phosphates of the acyl disaccharide λ , because it seemed to be important to have both the 1- and 4'-monophosphates and the 1,4'-diphosphate in order to know the role of both phosphate moieties for activities. Though we first attempted the direct and simultaneous phosphorylation of the 1- and 4'-hydroxyl groups in λ , this could not be achieved under various conditions. The two phosphate moieties were therefore introduced separately, using different procedures (4).

Preparation of 1-phosphate was performed by use of the known oxazoline procedure (5). We first examined the reaction carefully using a simple model oxazoline ρ and found that both the α - and β -glycosyl phosphates were initially formed in this reaction but that eventually only the α -anomer remained. On reaction of ρ with dibenzyl phosphate, the presence of two new phosphates was observed on TLC. They were identified to be the α - and β -glycosyl phosphates ($\lambda\lambda$ and μ) after deprotection (Figure 4). However, when the reaction mixture was left standing at room temperature, the amount of the β -phosphate μ decreased slowly, and the α -anomer $\lambda\lambda$ was the sole product after 24h. This observation could be explained by assuming that the dibenzyl β -phosphate μ was formed kinetically and anomerized into the thermodynamically more stable α -anomer $\lambda\lambda$. The early authors probably overlooked the presence of the β -anomer in their reaction mixtures since they worked up after 48h.

Although the anomeric configuration of the glycosyl phosphate in natural lipid A had not been elucidated at that time, it was assumed to be α and we employed a sufficient long reaction time for this phosphorylation to secure formation of α -phosphates in the following synthesis. Meanwhile the natural glycosyl configuration was established to be α (6) and our choice is fortunately correct.

Compound λ obtained in the above synthesis (Figure 3) was employed as the starting material for the preparation of the 1-phosphate. Hydrolysis of the isopropylidene group followed by 6-O-acylation and isomerization into the 1-propenyl glycoside afforded $\lambda\lambda$ (Figure 4). On reaction with $\text{HgCl}_2\text{-HgO}$ in dry $\text{CH}_3\text{CN-CHCl}_3$, this was converted into the sufficiently pure oxazoline $\lambda\lambda$, which was treated directly with dibenzyl phosphate. Since the dibenzyl ester of the glycosyl phosphate was not stable and decom-

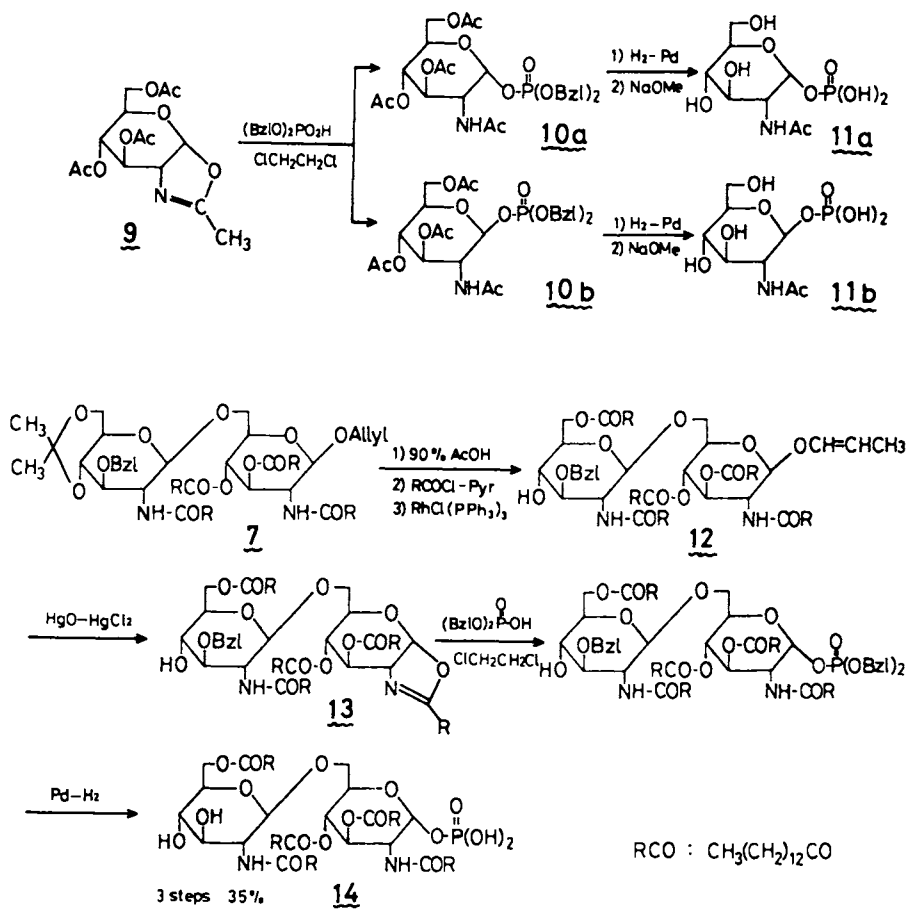


Figure 4. Preparation of glycosyl phosphates by the oxazoline procedure.

posed on standing, the reaction was interrupted after 24h by hydrogenolysis and the product was then purified. Silica gel column chromatography yielded λ_4 which corresponds to the 1- α -phosphate of the above acyl disaccharide λ_1 .

The 4'-monophosphate was also prepared from the same compound λ_2 as above (Figure 5). Although the 4'-hydroxyl group in λ_2 has rather low reactivity, its phosphorylation could be performed with phenyl phosphate and dicyclohexylcarbodiimide (DCC) in pyridine. The reaction product was converted into the benzyl phenyl ester (λ_5) to facilitate purification. In contrast to the above dibenzyl ester of the glycosyl phosphate, the benzyl phenyl ester of the 4'-phosphate was stable and could be purified by silica gel column chromatography after removal of the propenyl group without decomposition. Hydrogenolytic deprotection of λ_6 (first with Pd-black then with PtO₂) afforded the 4'-monophosphate λ_7 .

By combining the above phosphorylation methods, the preparation of the 1- α ,4'-diphosphate became possible. Of the two phosphate residues, the more stable one on the 4'-position was introduced first. Thus, λ_5 was converted, via the corresponding oxazoline, into the fully protected diphosphate λ_8 , which on hydrogenolysis afforded λ_9 .

Synthesis of acyl disaccharides containing (R)-3-hydroxytetradecanoyl moieties

With the limitation that all acyl groups were simple tetradecanoyl, the structure of polyacyl glucosamine disaccharide phosphates corresponding to the basic structure of *Salmonella*-type lipid A could be constructed as described above. However, the synthetic tetradecanoyl-type lipid A analogs showed very poor solubility in water in comparison with natural lipid A preparations. Although some of the synthetic phosphates showed several biological activities, e.g., in the antigenicity test, as already described by Dr. Lüderitz at this symposium, the majority of the typical activities of natural lipid A could not be observed. Such differences in physical and biological properties between synthetic and natural materials might be due to the absence of 3-hydroxy fatty acids in the former. These hydroxy acids are typical components of lipid A and are probably important for its activities.

We then focused on the synthesis of lipid A analogs which contain 3-hydroxy fatty acids. For this purpose, sufficient amount of (R)-3-hydroxytetradecanoic acid (λ_0), which is the commonest hydroxy acid in *Salmonella* lipid A, was first prepared by means of an asymmetric reduction of the corresponding keto ester, i.e., methyl 3-oxotetradecanoate (λ_1) (7). Catalytic hydrogenation of λ_1 in the presence of Raney Ni modified with (R,R)-tartaric acid \sim NaBr (8) afforded the crude (R)-ester in 85% enantiomeric excess. After saponification, the resultant acid was purified through its dicyclohexylammonium salt to give the optically and chemically pure (R)-acid λ_0 in a yield of 61% from λ_1 .

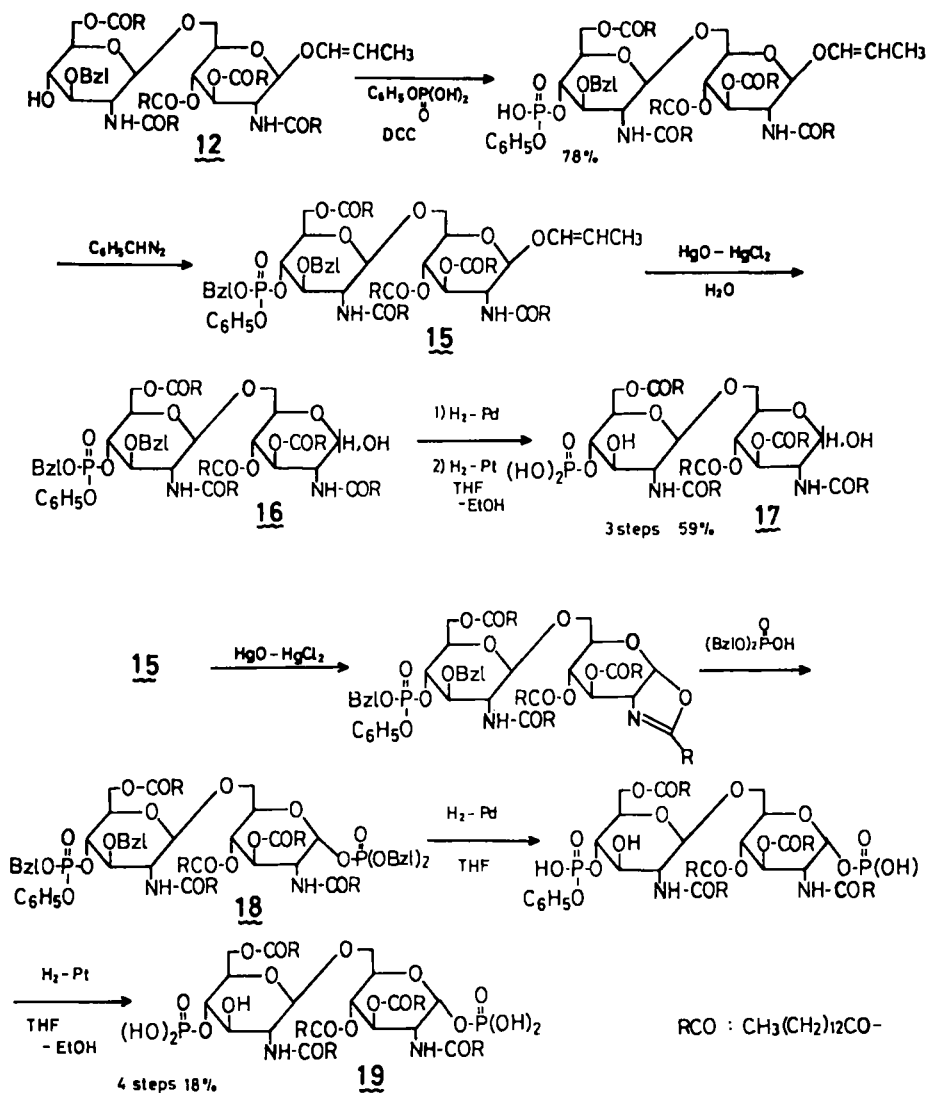


Figure 5. Preparation of 4'-monophosphate (17) and 1- α ,4'-diphosphate (19) of the O,N-tetradecanoyl disaccharide.

The noteworthy advantages of this method are that the pure acid can be prepared, on a hundred gram scale, and that even the unnatural enantiomer, (S)-acid, can be obtained simply by use of (S,S)-tartaric acid for the modification of the Raney-Ni catalyst (7).

Before this acid could be used in lipid A synthesis, its 3-hydroxyl group had to be protected most preferably by benzylation. This was performed as shown in Figure 6. Of the two benzylating procedures, that with trichloroacetimidate described recently by Iversen and Bundle (9) was preferable because of the shorter reaction time needed. Since this hydroxyl group is on the β -carbon atom of the carboxyl function, strong alkali such as NaOH could not be used as it caused β -elimination of benzyl alcohol from the product (22).

With the protected hydroxy acid (22) in hand, it became possible to synthesize acyl disaccharides (24, 25, and 26, see Table I) which contain (R)-3-hydroxytetradecanoyl groups on the amino functions (and even on hydroxyl functions in the case of 26) by means of a strategy similar to that described for the tetradecanoyl derivatives above. The only difference was that coupling of the benzyloxy acid (22) with amino and hydroxyl groups was effected not via acid chloride but with DCC and DCC - 4-dimethylaminopyridine, respectively. The outline of the synthesis of 24 is exemplified in Figure 7. Compound 25 was prepared by removal of all O-protecting groups after N-acylation.

A detailed study of the structure of natural lipid A then revealed the presence of 3-acyloxyacyl groups on the amino functions of the disaccharide (10). (More recently, acyloxyacyl groups on oxygen functions of the disaccharide were also proposed. Cf. the presentation of Dr. Rietschel in this symposium). Since the presence of branched alkyl or acyl chains often influences the physical properties of lipid molecules, we next synthesized the N-(R)-3-tetradecanoyloxytetradecanoyl derivative (27) in order to test the importance of such double acyl structures for the activity. In this synthesis, the above strategy had to be modified slightly. Ester-type protecting groups could not be removed in the presence of the N-double acyl groups which already contain an ester structure in themselves. This problem could be overcome by using the trichloroethoxycarbonyl protecting group which is resistant against Meerwein's reagent and cleavable without disturbing other ester functions in the molecule. The synthesis was performed as shown in Figure 8, the N-acetyl groups of the starting material being kept until all necessary O-acyl groups had been introduced, and the 4'-hydroxyl group protected by trichloroethoxycarbonylation. After that, both N-acetyl groups were cleaved with Meerwein's reagent as above, and the tetradecanoyloxytetradecanoyl groups were introduced. Removal of the protecting groups afforded the desired N-double acyl-type disaccharide (27). This synthetic route would be also very useful in case when one has to prepare various structural analogs which differ from each other only in the amino-bound acyl groups.

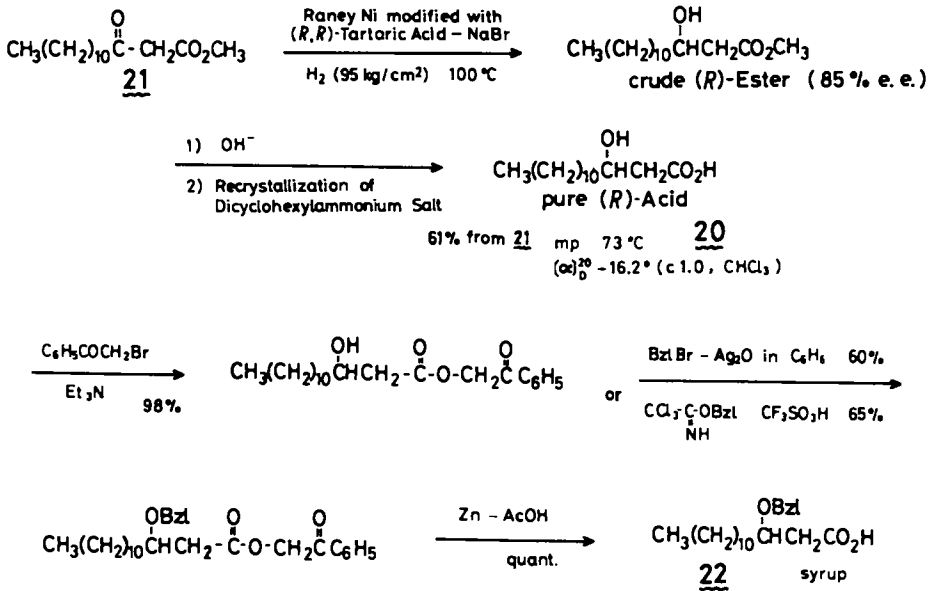


Figure 6. Preparation of (R)-3-hydroxytetradecanoic acid (20) and its 3-O-benzyl derivative (22).

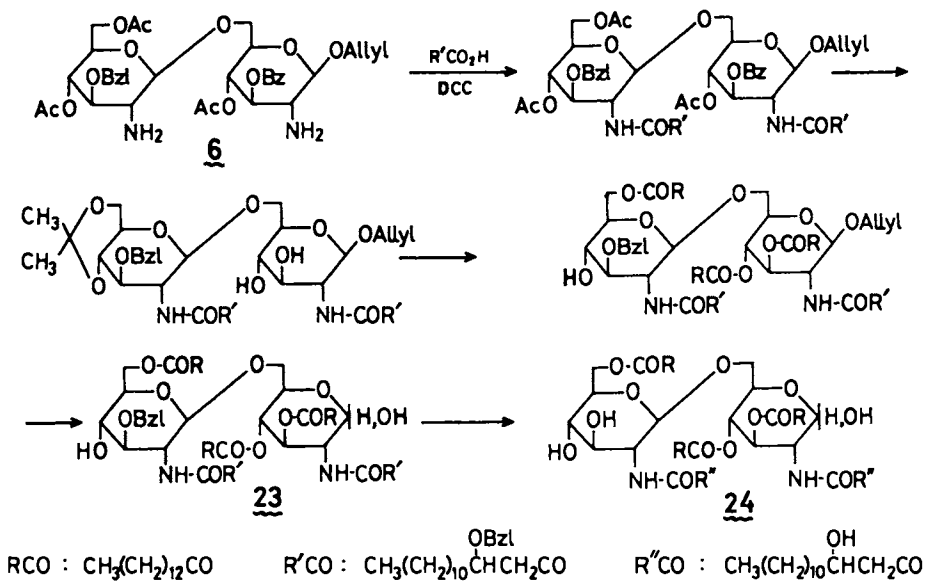
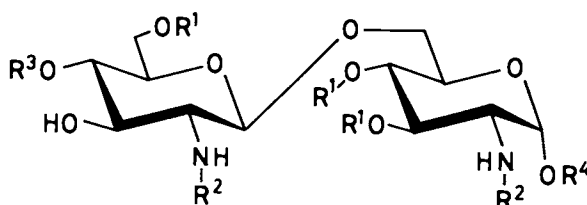


Figure 7. Preparation of the O-tetradecanoyl-N-[(R)-3-hydroxytetradecanoyl] disaccharide 24.

Table I. Structures and physical properties of synthetic lipid A analogs



Compound	R ¹	R ²	R ³	R ⁴	mp (°C)	[α] _D
1	C ₁₄	C ₁₄	H	H	186-188	+11.4° (28°C) ^{4,5)}
24	C ₁₄	C ₁₄ -OH	H	H	192-195 ³⁾	+ 0.41° (28°C) ⁵⁾
25	H	C ₁₄ -OH	H	H	182-183	
26	C ₁₄ -OH	C ₁₄ -OH	H	H	203-204 ³⁾	- 4.7° (20°C) ⁵⁾
27	C ₁₄ -OH	C ₁₄ -O-(C ₁₄)	H	H	176-179	+ 6.7° (20°C) ⁵⁾
17 ¹⁾	C ₁₄	C ₁₄	P	H	187-191	+10.6° (14°C) ⁶⁾
28 ²⁾	C ₁₄ -OH	C ₁₄ -OH	P	H	187-189 ³⁾	- 7.2° (20°C) ⁷⁾
29 ¹⁾	C ₁₄ -OH	C ₁₄ -O-(C ₁₄)	P	H	183-190 ³⁾	+ 7.5° (20°C) ⁷⁾
14 ¹⁾	C ₁₄	C ₁₄	H	P	145-150	+12.9° (18°C) ⁵⁾
40 ²⁾	C ₁₄	C ₁₄ -OH	H	P	177-181	+13.5° (20°C) ⁷⁾
19 ²⁾	C ₁₄	C ₁₄	P	P	208-211 ³⁾	+14.7° (13°C) ⁸⁾
41 ²⁾	C ₁₄	C ₁₄ -OH	P	P	218-222 ³⁾	+12.5° (15°C) ⁹⁾

C₁₄ : tetradecanoyl
 C₁₄-OH : (R)-3-hydroxytetradecanoyl
 C₁₄-O-(C₁₄) : (R)-3-tetradecanoyloxytetradecanoyl
 P : PO(OH)₂

1) Triethylamine salt. 2) Na salt. 3) With decomposition.

4) At 365 nm. 5) c 0.3-1.0 in CHCl₃-MeOH (5:1). 6) c 0.5 in CHCl₃-MeOH-H₂O (15:5:1). 7) c 0.4-1.0 in CHCl₃-MeOH (3:1).

8) c 0.5 in CHCl₃-MeOH-H₂O (50:10:1) 9) c 0.5 in CHCl₃-MeOH-H₂O (30:10:1).

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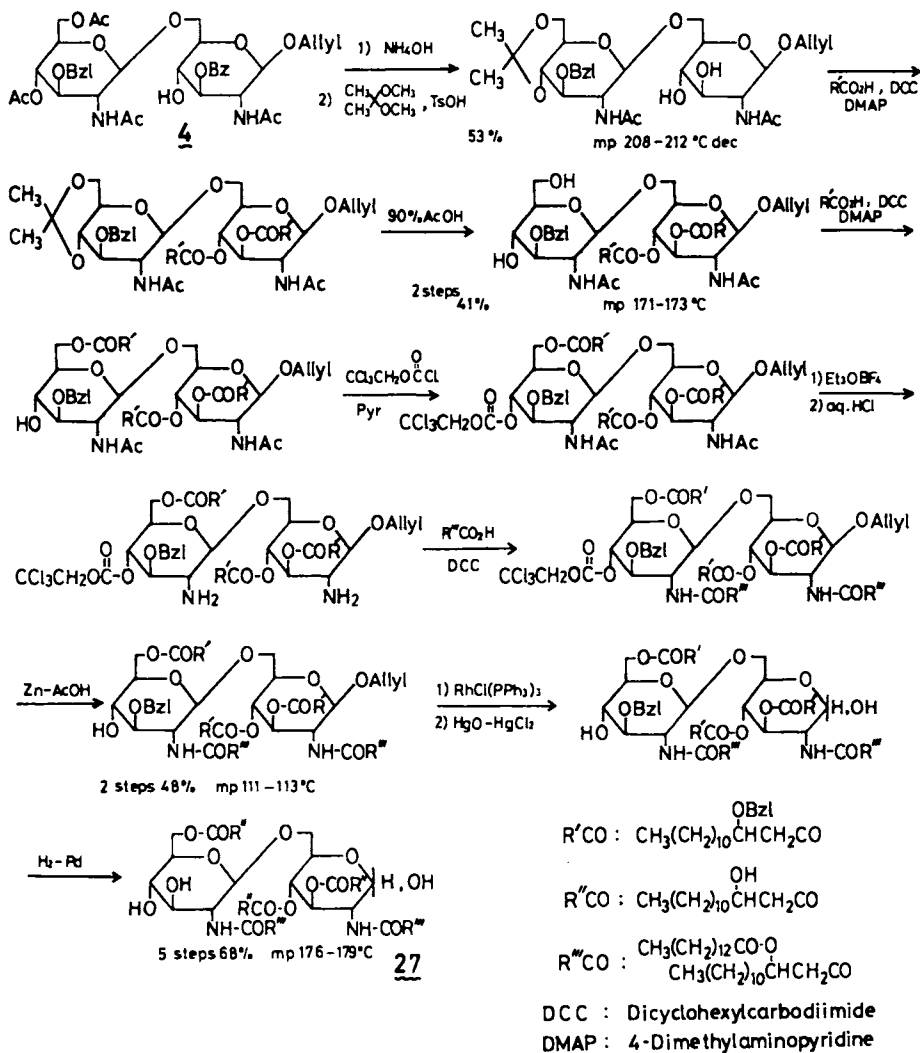


Figure 8. Preparation of the O-hydroxyacyl-N-acyloxyacyl disaccharide 27.

Synthesis of phosphates of N-(3-hydroxyacyl)-type disaccharides

The remaining problem in our synthetic study is the preparation of phosphates of N-hydroxyacyl- and N-double acyl-type disaccharides.

4'-Monophosphates. Concerning the preparation of 4'-phosphates, no difficulties were encountered. Application of the DCC - phenyl phosphate method to the corresponding derivatives with free 4'-hydroxyl groups afforded the 4'-phosphates of N-hydroxyacyl- and N-double acyl-type derivatives (28 and 29) (Figure 9 and Table 1).

New preparative method for α -glycosyl phosphates. A new procedure had to be explored for preparation of glycosyl phosphates of N-(3-hydroxyacyl)glucosamine derivatives, because oxazoline derived from such glucosamine (30) did not react with dibenzyl phosphate in a normal way but underwent elimination to yield unsaturated compound (31) (Figure 10), giving no desired glycosyl phosphate at all. Another known preparative method for glycosyl phosphate via 1-bromide (11) did not give satisfactory results either, though examined under various reaction conditions.

In this situation, we examined the activation of glycosyl oxygen for glycosyl phosphate synthesis (12). Quite recently, a successful example of 1-O-activation was described by Granata and Perlin in the case of glucose (13). They prepared the 1- α and β -phosphates of 2,3,4,6-tetra-O-benzylglucopyranose by reaction of its 1-O-thallium salt with diphenyl phosphorochloridate. This method was applied to a model N-(3-hydroxyacyl)glucosamine derivative (Figure 11). When N-[(R)-3-acetoxytetradecanoyl]-3,4,6-tri-O-acetylglucosamine (32) was converted into its 1-O-thallium salt, and then treated with dibenzyl phosphorochloridate, the product isolated after deprotection (hydrogenolysis followed by removal of O-acetyl groups with NaOMe) was exclusively the β -glycosyl phosphate of N-[(R)-3-hydroxytetradecanoyl]glucosamine (33) ($^1\text{H-NMR } \delta$ 5.05ppm, 1H, t, $J_{1,2}=J_{1,p}=8\text{Hz}$, H-1). No trace of the desired α -anomer was detected.

Since the glycosidic carbon of the starting compound had exclusively the α -configuration as determined by the $^{13}\text{C-NMR}$ spectrum, almost complete anomerization must have occurred during thallium salt formation and/or the phosphorylation step in the above experiment. It could be expected therefore that the desired α -phosphate could be obtained if metalation of the α -glycosyl oxygen and its prompt phosphorylation at low temperature could be effected avoiding anomerization. In fact, the α -glycosyl phosphate of N-[(R)-3-hydroxytetradecanoyl]glucosamine (34) was obtained in a total yield of 83% from 32 by use of butyllithium for metalation. An outline of the procedure is as follows. One equivalent of butyllithium in hexane was added to a stirred solu-

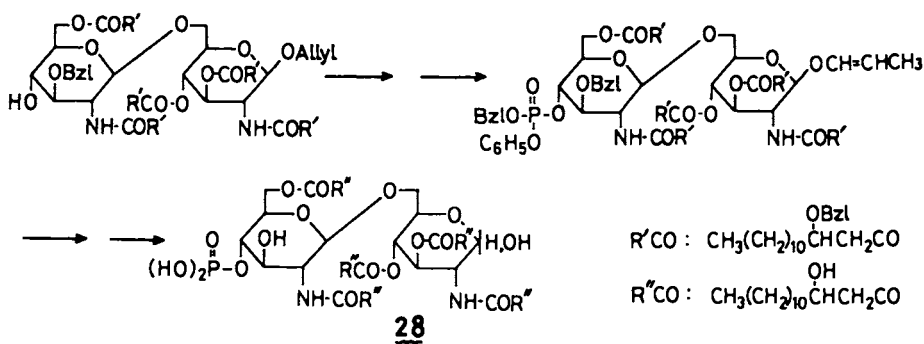


Figure 9. Preparation of the 4'-monophosphate **28**.

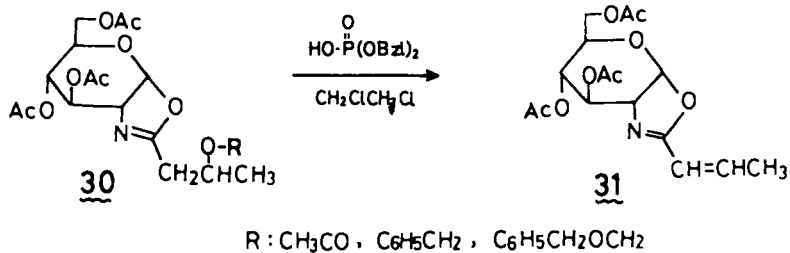


Figure 10. Reaction of the oxazoline **30** with dibenzyl phosphite.

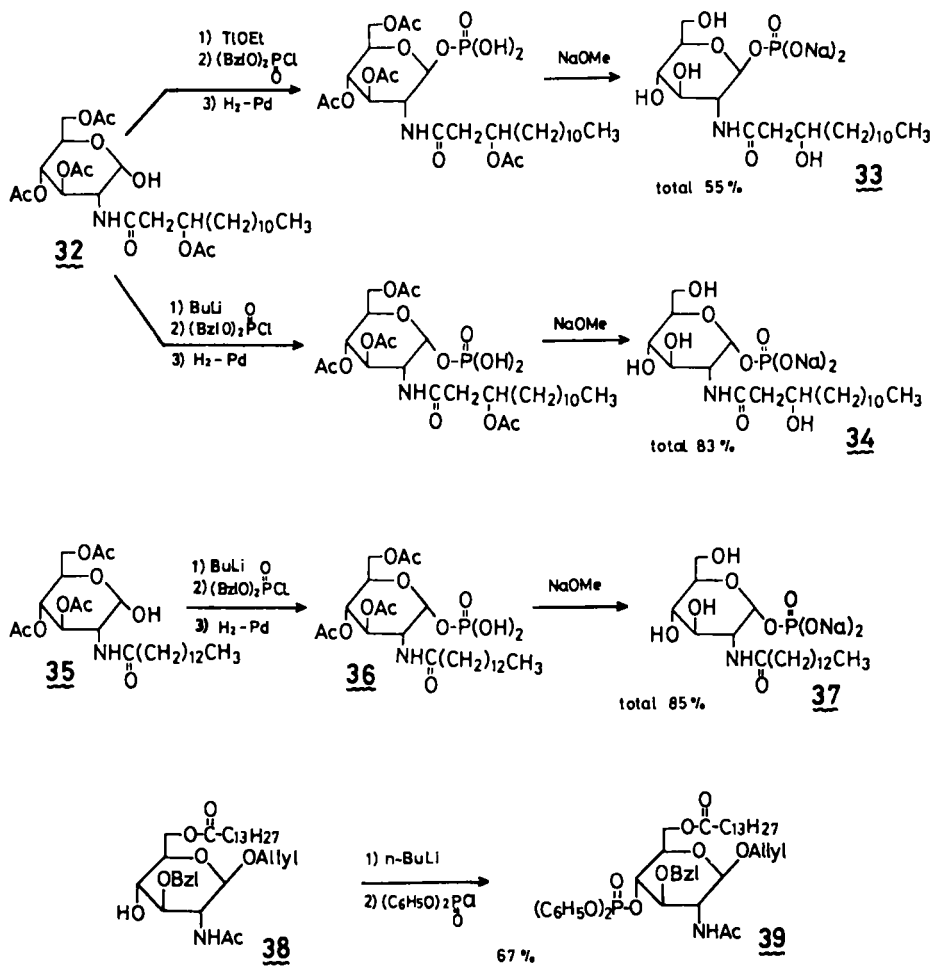


Figure 11. A new phosphorylation method with butyllithium and a phosphorochloridate.

tion of $\underline{32}$ in dry THF at -70°C . After 2min, dibenzyl phosphorochloridate was added and the mixture was stirred at -60°C for 10min. Immediate hydrogenolysis followed by removal of all O-acetyl groups with NaOMe afforded the disodium salt of $\underline{34}$, whose α -configuration was ascertained with $^1\text{H-NMR}$ (δ 5.49ppm, 1H, dd, $J_{1,2}=3\text{Hz}$, $J_{1,p}=7\text{Hz}$, H-1). This method could also be applied to glucosamine derivatives having simple N-acyl groups. For example, 3,4,6-tri-O-acetyl-N-tetradecanoylglucosamine ($\underline{35}$) was similarly converted in a high yield into the corresponding α -phosphate ($\underline{36}$) which was identical with an authentic sample obtained by the known oxazoline procedure. The desired α -glycosyl phosphates were formed selectively, irrespective of the type of N-acyl groups.

Furthermore, this phosphorylation reaction could be applied not only to the glycosyl position but also to other hydroxyl groups. When compound $\underline{38}$ (Figure 11) was treated with butyllithium and diphenyl phosphorochloridate as above, a syrupy 4-phosphate $\underline{39}$ was obtained whose structure was unequivocally confirmed by 360MHz $^1\text{H-NMR}$. A quartet signal of H-4 (δ 4.70ppm, $J_{3,4}=J_{4,5}=J_{4,p}=9\text{Hz}$) indicated the presence of the phosphate moiety on C-4.

This phosphorylation method is thus a very convenient and versatile one, especially adequate for our synthetic study of lipid A as utilized below. It could be confirmed from the above experiments that no cleavage and transposition of acyl groups occurred by the use of butyllithium under the reaction conditions employed.

Synthesis of 1- α -monophosphate and 1- α ,4'-diphosphate. Compound $\underline{23}$, which was obtained above and had two free hydroxyl groups on C-1 and C-4', was used as the starting material for the preparation of the 1- α -monophosphate and 1- α ,4'-diphosphate (Figure 12).

After $\underline{23}$ had been treated with one equivalent of butyllithium and then with dibenzyl phosphorochloridate as above, the mixture was hydrogenolyzed immediately. Of the two free hydroxyl groups in $\underline{23}$, the more acidic one on C-1 was phosphorylated selectively. The desired 1- α -monophosphate $\underline{40}$ was isolated after purification with a silica gel column (CHCl_3 -MeOH- H_2O 40:10:1) as its sodium salt (65% from $\underline{23}$).

By contrast, when the disaccharide $\underline{23}$ was treated with two equivalents each of butyllithium and dibenzyl phosphorochloridate under otherwise similar conditions, the corresponding 1- α ,4'-diphosphate $\underline{41}$ was isolated as the sodium salt (23% from $\underline{23}$) after purification with a silica gel column (CHCl_3 -MeOH- H_2O 30:10:1). In this manner, we now succeeded to phosphorylate both free hydroxyl groups on C-1 and C-4' directly and simultaneously even in the presence of 3-hydroxyacyl moieties on the 2-amino groups of the disaccharide. This was, as already described, not possible by combination of the known procedures.

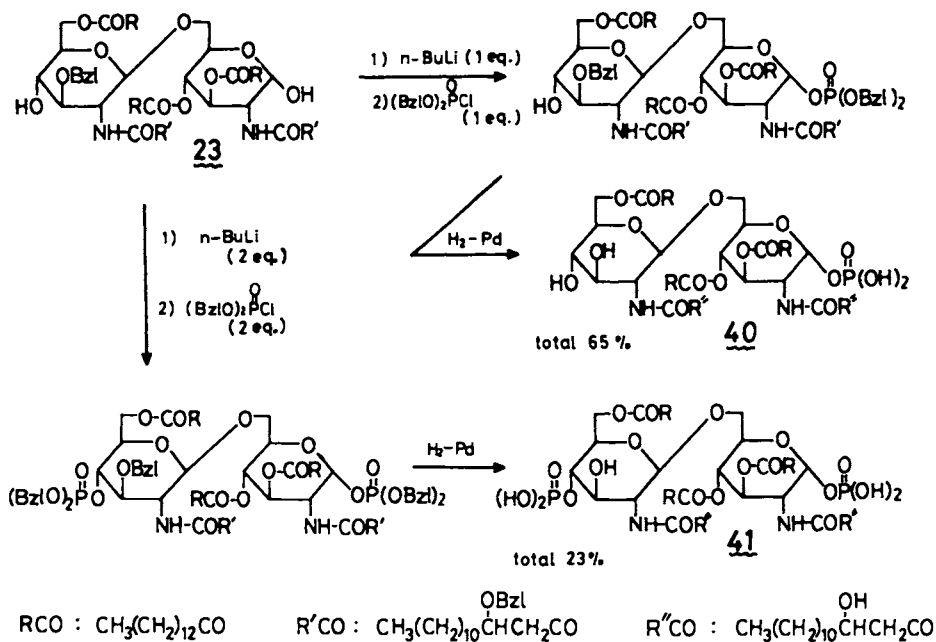


Figure 12. Preparation of the acyl disaccharide phosphates **40** and **41**.

Final remarks

As the result of the present study, a synthetic method is now available for the preparation of any desired derivative of Salmonella-type lipid A with or without phosphate groups. The compounds so far prepared in this study are summarized in Table 1. Any acyl groups including optically active 3-hydroxyacyl as well as 3-acyloxyacyl groups can also be introduced into the desired positions.

Results of biological tests of the synthetic compounds are now being accumulated in many collaborative groups. Some of them were already discussed by Dr. Lüderitz at this symposium. Many of the important activities of natural lipid A were already observed in some synthetic specimens, and the knowledge so far obtained indicates that minimum structures required for individual activities could be different, e.g., one requiring 1-phosphate while the other 4'-phosphate. In general, however, the importance of N-bound double acyl, i.e., N-3-acyloxyacyl, and phosphate groups are quite evidently demonstrated. No doubt, the preparation of many more synthetic derivatives will be required to reach our final goal, which can, however, certainly be approached step by step. The knowledge of the structure of natural lipid A has also much improved in the meantime, as already described by Dr. Rietschel at this symposium. This will certainly facilitate our approach.

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The Convergent Approach to the Synthesis of Lipid A and Its Analogs

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The chemistry and biological activities of lipid A are reviewed in earlier chapters of this volume. As noted there, the components of the lipid A complexes from *Salmonella* species and *E. coli* are derivatives of a disaccharide comprised of two D-glucosamine units linked $\beta,1\rightarrow6$. The disaccharide is substituted at positions 1 and 4' by phosphate functions and on the amino nitrogens by β -hydroxymyristoyl groups. A variable number (up to 5) of ester-linked fatty acyl residues is also present. In the simplest components the phosphates are present as monoester groups.

Once these structural features were established, the synthesis of compounds of the lipid A group appeared feasible, and was undertaken in a number of laboratories. Successful efforts, based on distinctive strategies, are described by Kusumoto *et al.*, Kiso and Hasegawa, and Szabó *et al.* elsewhere in this volume, and in the present chapter. In common with the Osaka and Gifu groups, we were guided by the hypothesis that positions 3,4, and 6' of the lipid A disaccharide are O-acylated, as in structure 1, with any additional acyl groups being carried by the sugar-linked β -hydroxymyristate units. (The assignment of the "double" fatty acyl residue to position 6' in formula 1 is arbitrary.) Also in common with other investigators, we considered it prudent to work with simple, saturated fatty acyl groups while developing our synthesis. The choice of palmitoyl as an appropriate fatty acyl moiety then led us to regard 2 as our eventual target compound.

New data, mentioned elsewhere in this volume, make it probable that the pattern of O-acylation in lipid A differs from that shown in formula 1. However, it appears that the syntheses so far described, including our own, can with slight modification be made to yield products having their O-acyl groups in the same

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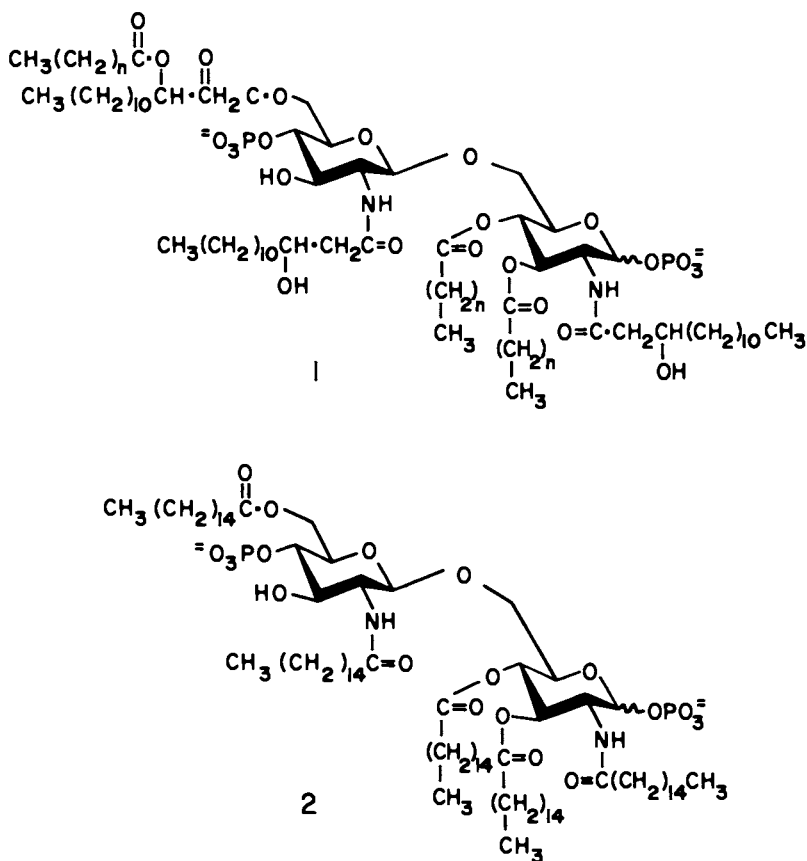


Figure 1. Structures of the simplest component of lipid A (a recent representation) and its pentapalmitoyl analog. Key: Ac, acetyl; All, allyl; Bn, benzyl; Bz, benzoyl; ClAc, chloracetyl; Me, methyl; Pal, palmitoyl; Ph, phenyl; Pre, 1-propenyl; Py, pyridine; Tce, β,β,β -trichloroethyl; Tf, triflyl (trifluoromethylsulfonyl); THF, tetrahydrofuran (oxolane); Tr, trityl; and Ts, p-tosyl.

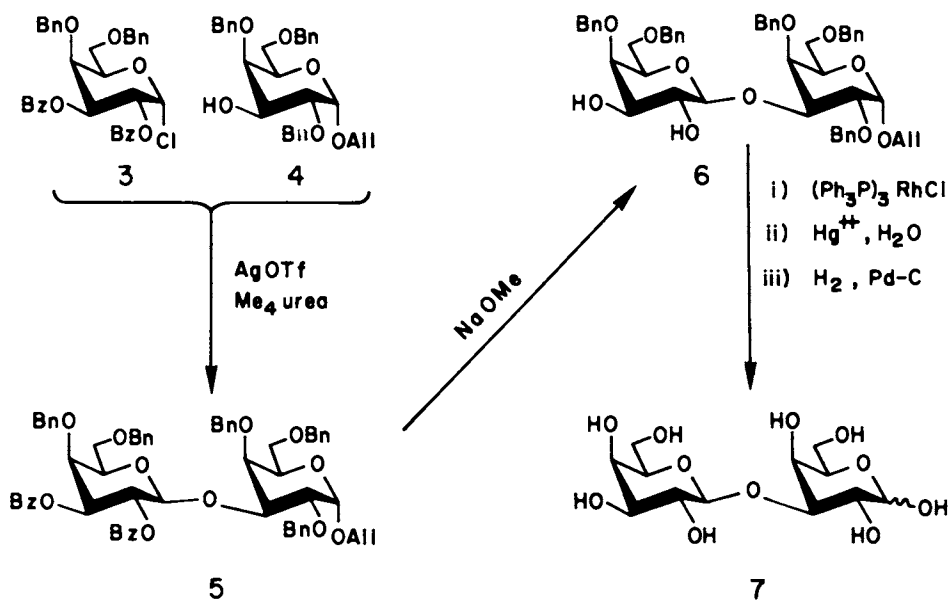


Figure 2. Synthesis of a disaccharide (Gal β -3Gal) by a standard approach. Key to formulas: same as for Figure 1.

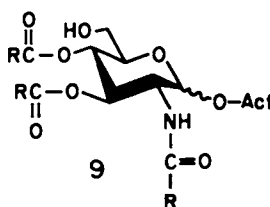
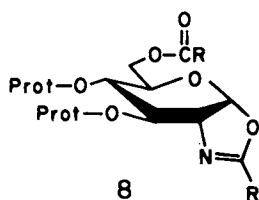
positions as in the natural materials, once these are firmly characterized.

To put our approach to the lipid A structure in perspective, it seems helpful to briefly consider the synthesis of an ordinary, unsubstituted oligosaccharide by the standard method used for such compounds. An example from our recent work (unpublished) is shown in Figure 2, where the end product is 3-O- β -D-galactopyranosyl-D-galactose (Gal β →3Gal, λ). To obtain this disaccharide we first prepared "building block" derivatives of the reducing and non-reducing units (1). The reducing-end building block (4) is substituted with protecting groups at all positions except 3, which is to be the site of the intersugar linkage, and the non-reducing building block (3) is fully substituted. The 1-substituent (chloro) in 3 is an activating group, and the 2-substituent (benzoyl) is the participating neighboring group that directs the formation of a β -glycosidic bond. When intermediates 3 and 4 were ready they were coupled to give the fully protected disaccharide 5. Then, the protecting groups were removed in a series of reactions, one or more for each different kind of protecting group present. The final deprotection reaction gave the free disaccharide λ .

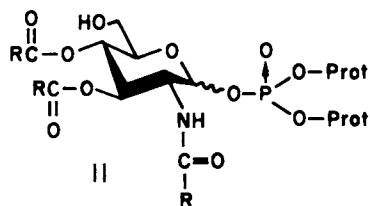
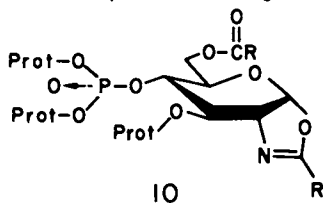
The difference between λ and a lipid A component or analog is that in the lipid A case the final product (for example μ) is highly substituted. It most closely resembles the intermediate 6 in the foregoing disaccharide synthesis, i.e. the coupling product with only a portion of its complement of protecting groups removed. Because the preparation of selectively substituted building block derivatives such as 3 and 4 consumes much of the effort that goes into an oligosaccharide synthesis, we argued that in work toward lipid A the appropriate protecting groups were those that would remain as substituents in the final product. By minimizing the use of temporary protecting groups, which would have to be removed and replaced, we would economize on synthetic steps.

Figure 3 shows the building block derivatives of D-glucosamine that would be needed for the synthesis of lipid A compounds by two variants of this approach. In the first variant, one would couple intermediates such as 8 and 9 to form a fatty acylated disaccharide carrying temporary substituents at positions 1, 3', and 4'. Removal of all temporary substituents would give a dephospho analog of lipid A. Or, one could in stepwise fashion deprotect and phosphorylate position 4', activate and phosphorylate position 1, and remove any remaining protecting groups. An oxazoline, 8, is proposed as the precursor of the non-reducing unit of the disaccharide, in view of our past reliance on these compounds as donors of glucosamine residues in coupling reactions (2). The oxazoline would contain the N-palmitoyl group in latent form. It would also have an O-palmitoyl group at position 6 and protecting groups at positions 3 and 4. The group at position 4 would have to be selectively removable. The reducing-end or

For Dephospho Analogs (Phosphorylation after coupling)



For Complete Analogs



Act = activating group

Prot = protecting group

Figure 3. Building blocks suitable (in principle) for the synthesis of compounds of the lipid A group. Key to formulas: same as for Figure 1.

acceptor unit would be unsubstituted at position 6, where the glycosidic linkage between the two sugars is to be formed. It would carry palmitoyl groups on the nitrogen and on O-3 and O-4. And finally, it would carry an activating substituent at the anomeric position or, more accurately, a substituent that would allow an oxazoline ring to be generated at that position, as a prelude to phosphorylation.

To carry out the convergent synthesis in its purest form, one would use building blocks (10 and 11) having protected phosphate groups already in place. After coupling the only further operations required would be removal of the protecting groups from position 3' and from the phosphate functions.

Direct Derivatization of N-Anisylidene-glucosamine

In our first attempts in the lipid A field we undertook the preparation of building blocks such as those just described, in as direct a fashion as possible (Figure 4). Thus we started with Bergmann's (3) well-known anisylidene Schiff base of D-glucosamine (12), and found that we could successfully tritylate it in the 6-position, then palmitoylate the remaining positions (4). The palmitate function at the anomeric position in the product (14) turned out to be in the β -configuration, which is essential for eventual activation by conversion into an oxazoline. The problem of selectively removing the anisylidene group in the presence of the trityl group looked a little difficult, but we found this could be accomplished cleanly with aniline hydrochloride as the reagent. The deblocking of the nitrogen permitted us to add the N-palmitoyl group, and on detritylation we had a compound (17) meeting the specifications listed above for a reducing-end building block.

To make the non-reducing unit we again started with the Bergmann Schiff base, as shown in Figure 5, and found we were able to selectively palmitoylate it in the 1 and 6 positions (4). The 1-palmitate group in the product (18) again had the β configuration. After removal of the anisylidene group, the nitrogen was palmitoylated to give the 3,4-diol (20). Then, to make a "model" glycosyl donor we acetylated the 3 and 4 positions, and obtained (21). This compound could not of course serve in the actual synthesis, because the acetyl groups are not selectively removable. However, it was readily converted into an oxazoline (22), and the normal behavior of the oxazoline was demonstrated by reacting it with allyl alcohol to give the allyl glycoside (not shown).

Our efforts to carry this scheme further ran into two difficulties. First, attempts to couple a simple oxazoline to the reducing-end building block (17) failed, apparently because of the migration of the 4-palmitoyl group to the 6 position under the influence of the acid coupling catalyst (5). Second, our attempts to make a suitable non-reducing building block from the 3,4-diol

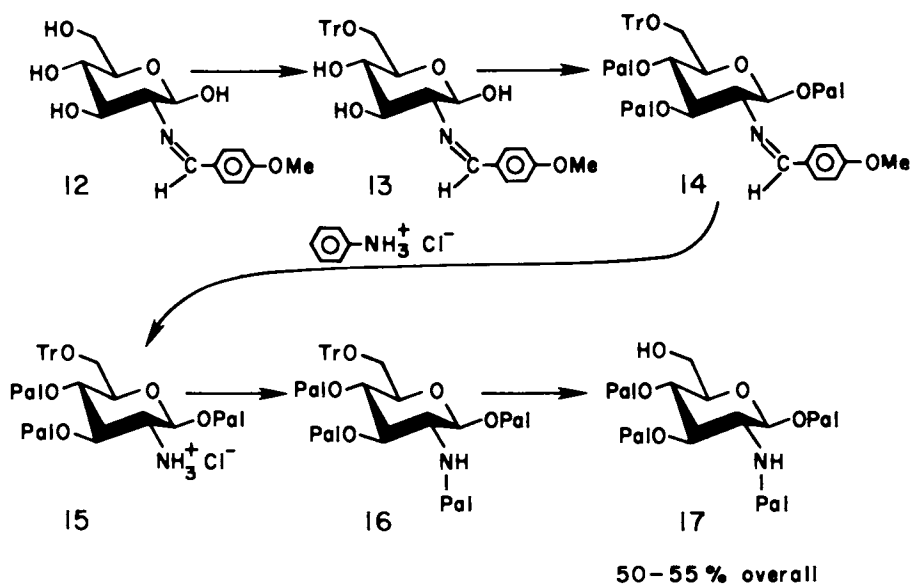


Figure 4. Preparation of a possible reducing-end building block. Key to formulas: same as for Figure 1.

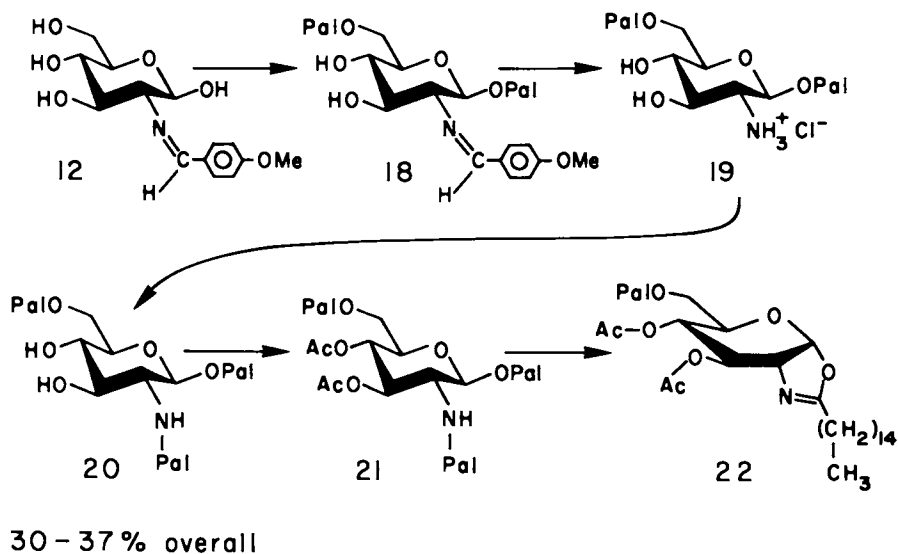


Figure 5. Preparation of a model nonreducing-end building block. Key to formulas: same as for Figure 1.

20 by selectively introducing a benzylsulfonyl protecting group at position 3 were also unsuccessful (5). These results led us to turn for a time to the approach used by the Tokyo and Gifu groups, but we eventually came back to our idea of having most or all of the final substituents in place at the monosaccharide stage. However, we conceded the necessity of making some use of temporary protecting groups in constructing the two building blocks.

Synthesis of a Fatty Acylated, Selectively Deprotected Disaccharide

The initial phase of our eventually successful synthesis (6) is shown in Figure 6. Our starting material was another of Bergmann's compounds (3), the tetra-O-acetate hydrochloride 23, alluded to elsewhere in this volume. This substance is readily N-palmitoylated, and the product (24) can be directly converted, by the procedure of Kiso and Anderson (7), to either the allyl or the benzyl β -glycoside (25) (8). The reason for having both glycosides will be discussed later on. De-O-acetylation of these glycosides was followed by protection of positions 4 and 6 as the benzylidene acetals. The 4,6-benzylidene derivatives (27) were key intermediates.

To make the reducing-end unit (Figure 7) the allyl or the benzyl 4,6-O-benzylidene glycoside was simply palmitoylated at position 3 (27 \rightarrow 28), and the benzylidene group was removed in the normal fashion by treatment with mild acid. No effort was made to protect the 4 position in the products (29) because we assumed that on coupling, glycosylation would occur preferentially at position 6.

In the preparation of the non-reducing unit the 4,6-O-benzylidene allyl glycoside (27-All) was benzylated at position 3, and the benzylidene group was removed, giving the 4,6-diol 31. The further conversion of this diol into the target oxazoline 35 required four further operations, namely isomerization of the aglyconic allyl group to 1-propenyl, the cyclization of the propenyl glycoside, the protection of position 4, and the fatty acylation of position 6. Within limits, the sequence in which these further operations were done was not critical. In the synthesis of the dephospho analog we proceeded as shown in Figure 7. Compound 31 was first selectively palmitoylated at position 6, with one equivalent of palmitoyl chloride in pyridine at low temperature. We found that isomerization of the allyl group to 1-propenyl, using tris(triphenylphosphine)rhodium(I) chloride (Wilkinson's catalyst) (9), was best accomplished before going further. For the protection of the 4 position, we chose the chloroacetyl group, and this was next emplaced by treating the propenyl glycoside 33 with chloroacetyl chloride. Finally, the fully substituted propenyl glycoside 34 was cyclized to the oxazoline 35 by the mercuric chloride-mercuric oxide procedure developed in our laboratory (10).

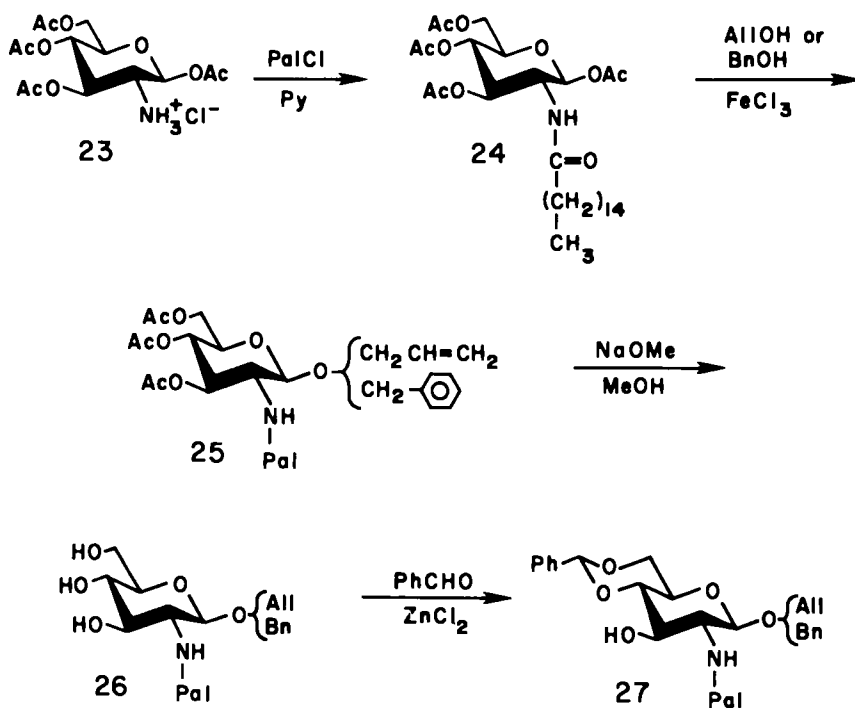


Figure 6. Initial phase of synthesis of a fatty acylated, selectively deprotected disaccharide. Key to formulas: same as for Figure 1.

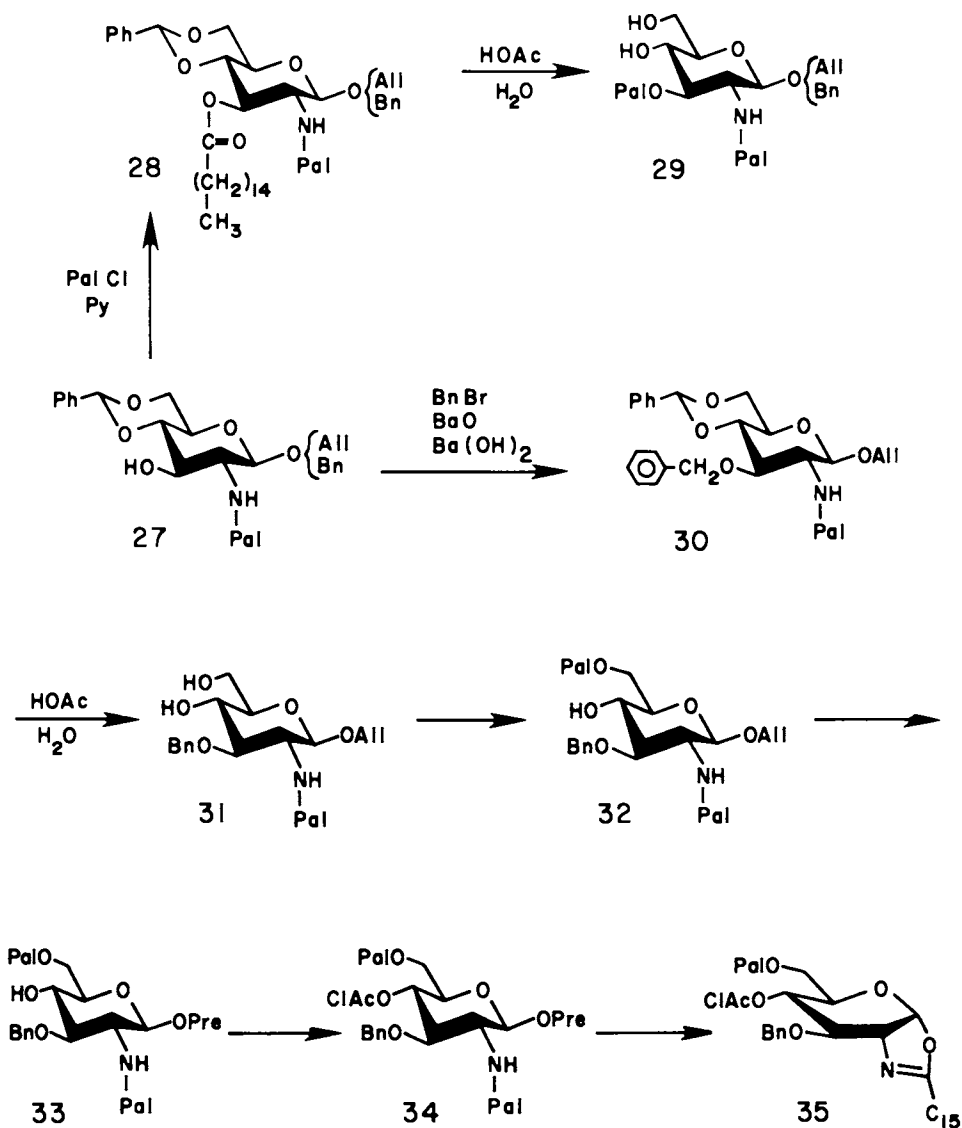


Figure 7. Conversion of intermediates 27 into reducing-end (29) and nonreducing-end (35) building blocks. Key to formulas: same as for Figure 1.

With the building blocks just described, the construction of the fully substituted disaccharide was straightforwardly accomplished as shown in Figure 8. The protected oxazoline 35 readily coupled, under catalysis by *p*-toluenesulfonic acid (2), to the allyl and benzyl glycoside acceptors (29). The idea was that if one wanted to make a complete lipid A analog, with phosphate groups at both ends of the molecule, one would use the allyl glycoside, since this would permit the disaccharide to be converted at a later stage to an oxazoline, the desired intermediate for phosphorylation at the 1-position (11). If one intended to have phosphate only at the 4' position, *i.e.* to make the 1-dephospho analog, which is of considerable interest for biological testing, then one would use the benzyl glycoside. Final deprotection of the product would thus be simplified, as the aglyconic O-benzyl and the 3'-O-benzyl groups could be removed at the same time.

The products of these condensations were the disaccharide glycosides 36, having their 4-hydroxyls free. This is the position to be occupied by the last of the fatty acyl groups, and accordingly it was next palmitoylated, to give the fully substituted disaccharide glycosides 37. Deprotection of the 4' position, as a prelude to phosphorylation, was readily accomplished by treating 37 with thiourea according to the literature procedure (12).

Evidence that the products of O-dechloroacetylation had the structure 38 was provided by ¹H-n.m.r. spectroscopy. In the mid-field portion of the 270 MHz n.m.r. spectrum (Figure 9) one can see two peaks for amide protons, which could be identified by their exchangeability with deuterated solvents. Most importantly, there are two triplet signals, having 9 Hz spacings, at 5.0 and 5.2 ppm, respectively. These must represent ring-proton signals downshifted by acylation, and they therefore verify the presence of palmitoyl groups on O-3 and O-4. The region just above δ 5.0 is largely occupied by peaks due to benzyl methylene groups, but two anomeric signals can also be distinguished in this region. As already demonstrated by the work of Kusumoto *et al.* (this volume), the disaccharide products 38-A11 and 38-B11 could have been converted into a pentapalmitoyl analog of complete lipid A, and the corresponding analog of 1-dephospho lipid A, respectively. Rather than undertake the phosphorylation of these compounds, however, we chose to explore a modification of our synthesis that would permit at least one of the phosphate groups to be incorporated at the monosaccharide stage.

Introduction of the 4'-Phosphate at the Monosaccharide stage

If we return to the structures of the "ideal building blocks" shown in Figure 3, we see that these include a precursor of the non-reducing unit having a protected phosphate group at the 4

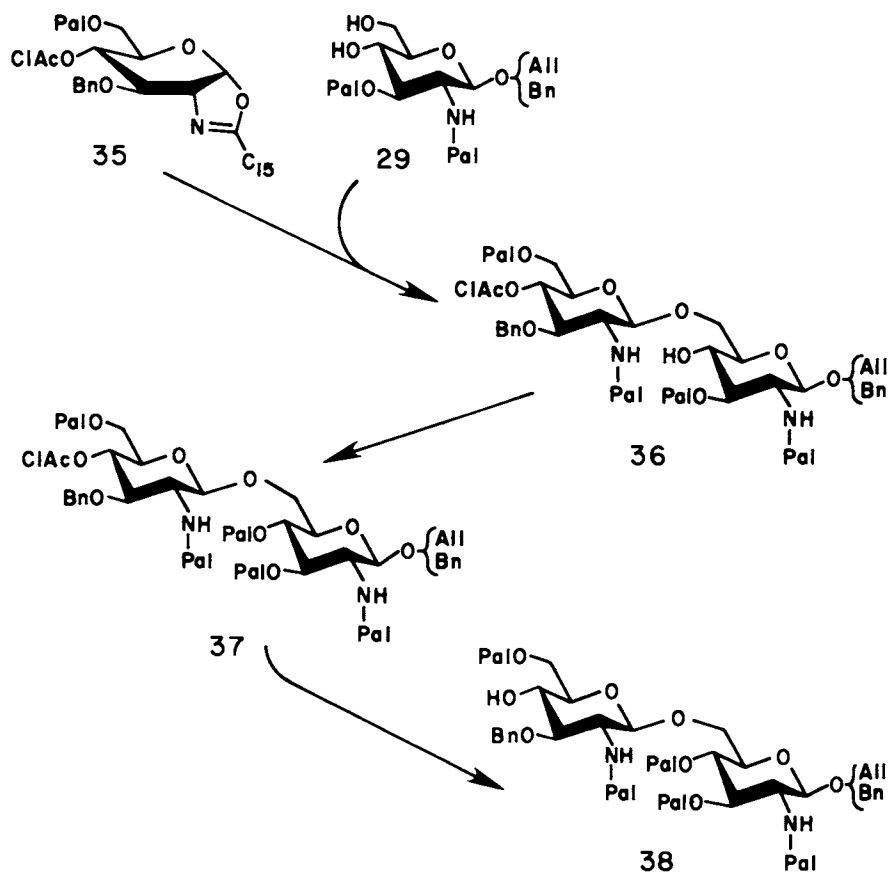


Figure 8. Construction of the selectively deprotected disaccharide from the building blocks 35 and 29. Key to formulas: same as for Figure 1.

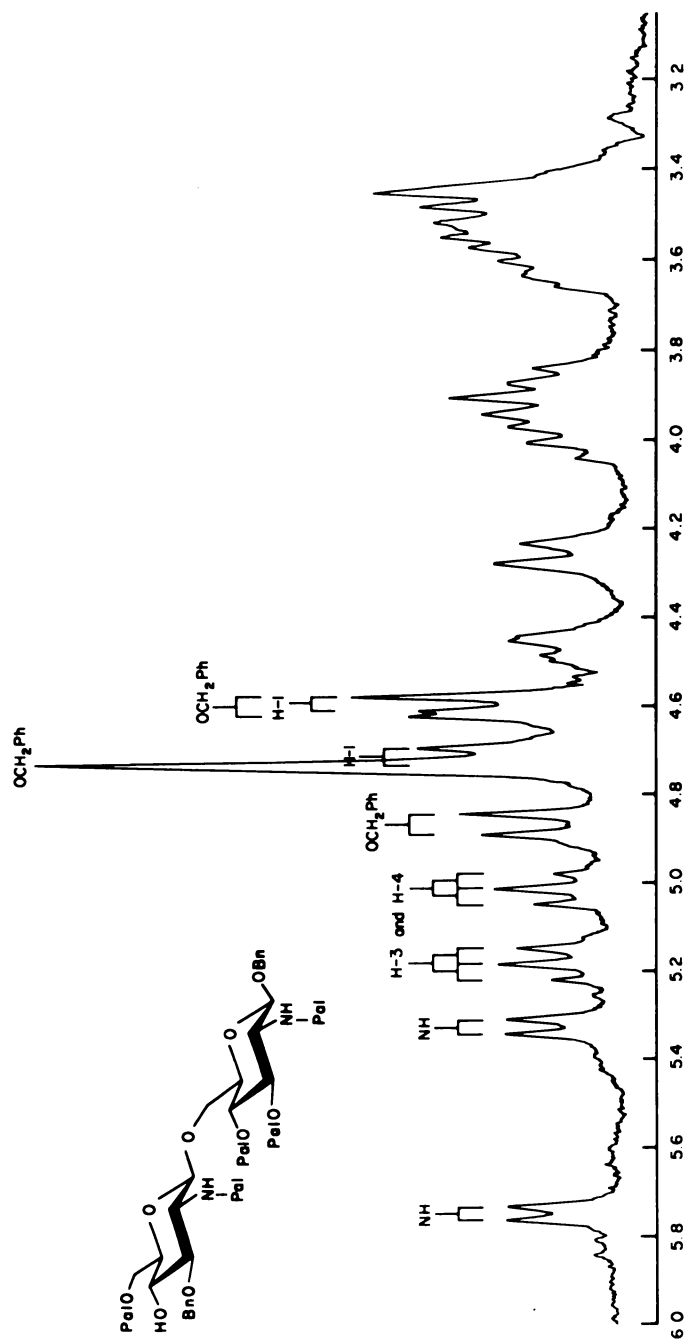


Figure 9. Proton-NMR spectrum (270-MHz) of compound 38-Bn. Key to formula: same as for Figure 1.

position (10), and a precursor of the reducing unit with its 1-phosphate in place, in protected form (11). Structure 11 contains an anomeric phosphotriester function, and because of the lability of these functions its employment as a lipid A synthon may not be feasible. On the other hand a phosphorylated intermediate such as 10 is attractive because using it would eliminate two steps from the synthesis outlined in Figures 7 and 8. These are the emplacement of the temporary protecting group (chloroacetyl) at position 4 of 34, and its later removal (37 → 38).

In our first attempt (Figure 10) to make an oxazoline having structure 10, we phosphorylated the allyl glycoside 32, which has all substituents in place except that at position 4. As indicated in earlier chapters, the hydroxyl in this position can be phosphorylated in various ways. However, it is necessary to choose a method suitable for hydroxyls of low reactivity. In our hands, several common phosphorylating agents gave disappointing results. Diphenyl phosphorochloridate, for instance, converted the glycoside into a product containing no phosphorus--perhaps a product of cyclization between the carbonyl oxygen of the N-acyl group and OH-4. Eventually we turned to the phosphorochloridite method devised by Letsinger and his colleagues (13) for oligonucleotide synthesis, and proceeded as shown in the figure. Compound 32 was treated with β,β,β -trichloroethyl phosphorodichloridite in the presence of 2,6-dimethylpyridine at low temperature to give the phosphite diester chloride 39. Next, the addition of trichloroethanol to the reaction mixture converted 39 into the bis(trichloroethyl) phosphite triester 40. Finally, the addition of iodine, and a little water to provide the oxygen, effected the oxidation of 40 to the desired phosphorylated intermediate 41. An important virtue of the phosphorochloridite procedure is that all of these operations can be done in a single flask, without isolation of intermediates, in a period of less than two hours. However, the product 41 was not suitable for the next projected step in the sequence, namely the isomerization of the allyl group to 1-propenyl. The treatment of 41 with Wilkinson's catalyst caused extensive decomposition. This result led us to change our tactics so that the phosphorylation would be the final step in the production of the building block, and the bis(trichloroethyl)phospho group would not be exposed to any heavy metal reagents.

The allyl to propenyl isomerization in this series had been troublesome for us, and we had some indication that it proceeded better on compounds having at least one hydroxy group. We therefore performed it as the first step in the transformation of the 4,6-unsubstituted allyl glycoside 31 (Figure 10). This indeed gave more reliable conversions, and the resulting propenyl glycoside 43 was next selectively palmitoylated at position 6. Following this the product was converted to the oxazoline 44, either by the mercuric chloride-mercuric oxide method, or more easily by treatment with iodine in oxolane for a few minutes at

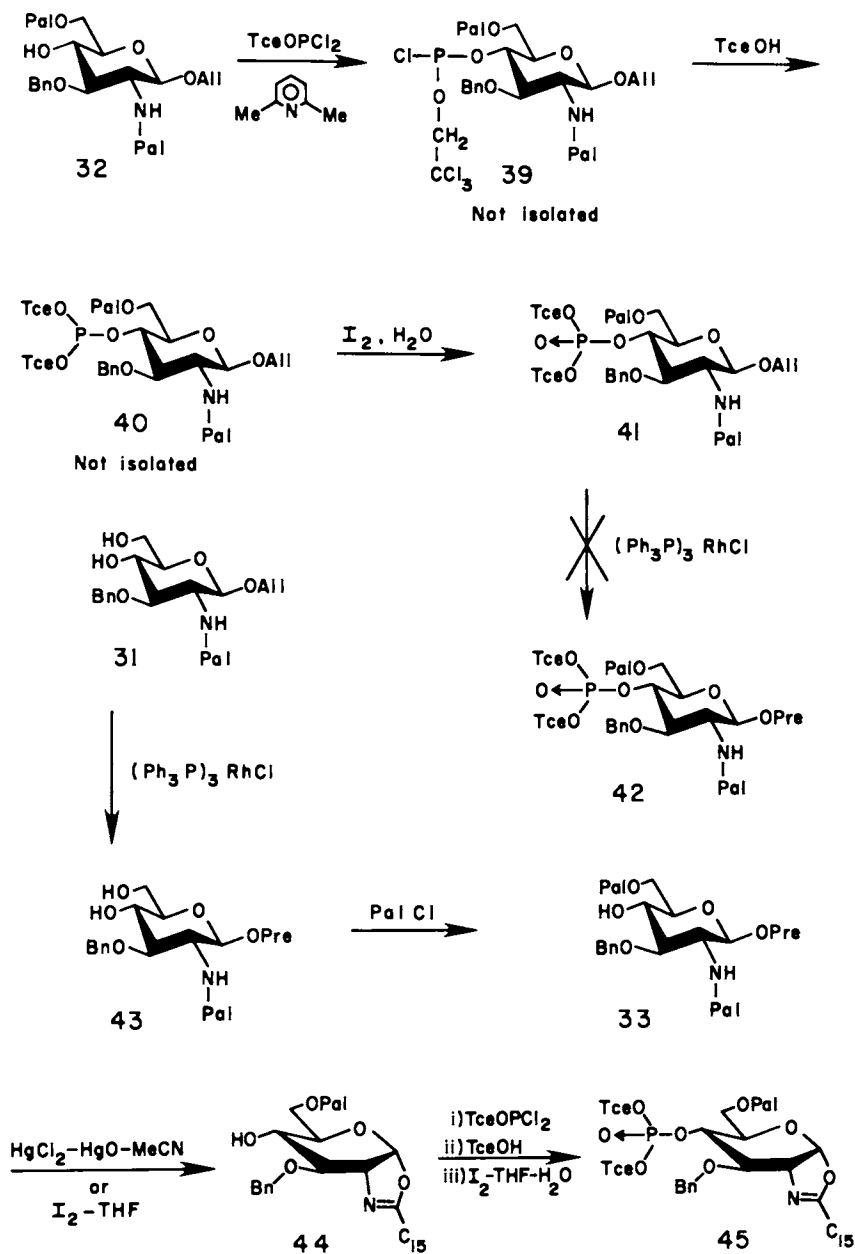


Figure 10. Preparation of the protected, phosphorylated oxaline derivative 45. Key to formulas: same as for Figure 1.

room temperature or below. The fact that iodine would accomplish this conversion was discovered accidentally in the course of an attempt to oxidize the phosphite triester of a propenyl glycoside. In that case the oxidation resulted in the loss of the propenyl group, since water was present. However, when the reaction is run in the absence of water, clean cyclization to the oxazoline takes place (14). The method is much more convenient than the procedure using mercuric salts, because the work-up of the reaction mixture is so simple.

As we had hoped, the phosphorochloridite procedure, which goes at low temperature under very mild conditions, was indeed usable with the oxazoline 44. The treatment of 44 with trichloroethyl phosphorodichloridite, trichloroethanol, and iodine-oxolane-water in succession gave the protected, phosphorylated product 45 in excellent yield, and this product could be purified by rapid passage through a column of silica gel. The ¹H-n.m.r. spectra of the precursor oxazoline 44 and the phosphorylated oxazoline 45 are shown in Figure 12. Particularly evident are signals for the anomeric protons with their characteristically low chemical shift of ~6.0 ppm, and typical spacing of about 6 Hz, intermediate between the spacings normally found for α-glycosides and β-glycosides, respectively. The formation of the phosphorylated product is confirmed by the appearance of numerous new lines in the region 4.5 to 4.8 ppm, due to the methylene protons of the two trichloroethyl groups. The large number of lines results from the magnetic non-equivalence of these protons, and their splitting by the phosphorus atom.

As a reducing-end building block for coupling with the phosphorylated oxazoline the previously used 3-O-palmitoyl benzyl glycoside 29-Bn appeared suitable if the end product was to be a 4'-monophosphate (1-dephospho analog) in the lipid A series. However, if the eventual product was to be the 1,4'-bisphosphate (complete analog), the allyl glycoside 29-All would not be suitable. According to our experience, just described, the conversion of the allyl group to 1-propenyl would not be possible in the coupling product, which would now possess a 4'-bis(trichloroethylphospho) group. Consequently, we performed the isomerization on the allyl glycoside before coupling (Figure 11). This entailed the risk of losing the anomeric protecting group under the acidic conditions of the coupling reaction, but in the event it proved satisfactory.

The acid catalyzed coupling of oxazoline derivatives of glucosamine to sugar acceptors typically involves some loss of the oxazoline by decomposition (2). In view of the possibly sensitive nature of the new, phosphorylated oxazoline 45, we sought to minimize this decomposition by using a very mild catalyst, namely 2,6-dimethylpyridinium p-toluenesulfonate, recently advocated by Gigg and Conant (15). With this catalyst the coupling reactions proceeded rather slowly but in good yield with both the benzyl (29-Bn) and propenyl (29-Pre) glycosides as

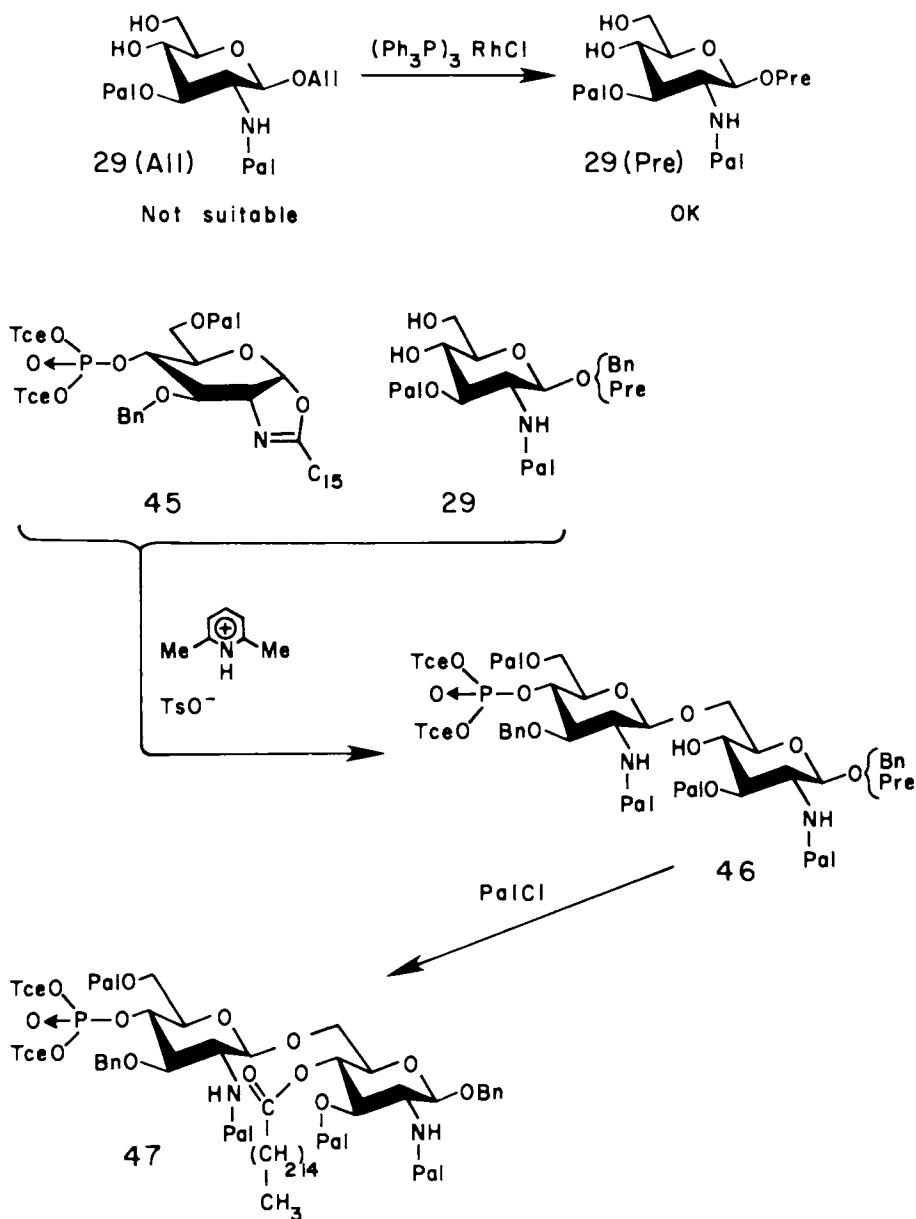


Figure 11. Synthesis of fully protected disaccharide 4'-phosphates. Key to formulas: same as for Figure 1.

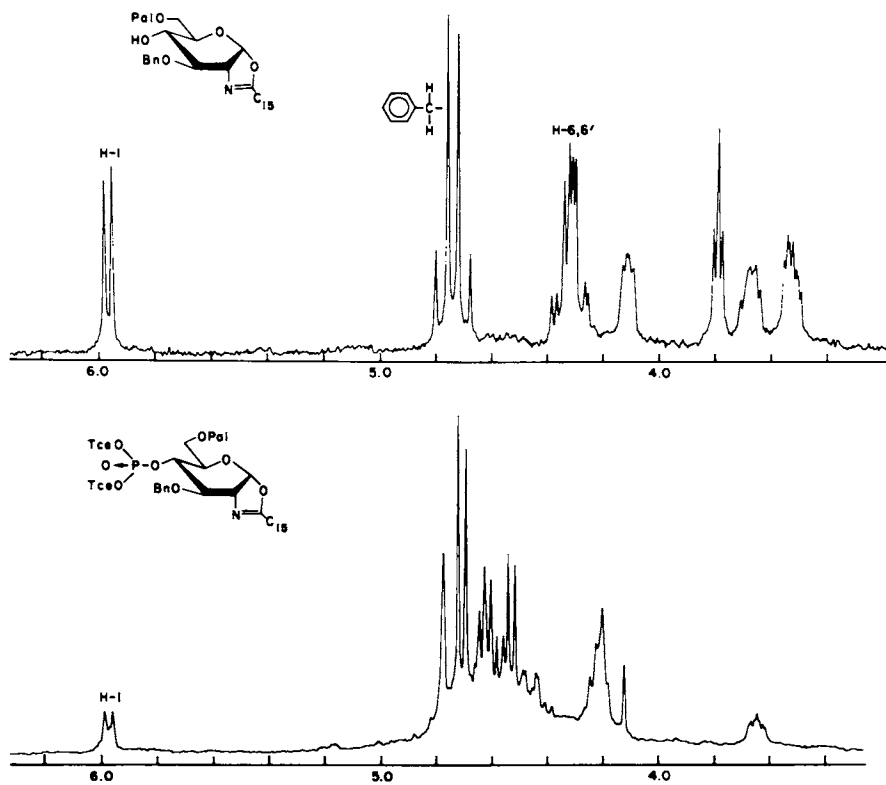


Figure 12. Proton-NMR spectra of the oxazoline intermediates **44** and **45**. Key to formulas: same as for Figure 1.

acceptors, to give the disaccharide products (46) substituted in all positions except 4. As in the unphosphorylated series (Figure 8), this position could next be acylated with palmitoyl chloride, and the acylation was carried out with the coupling product (46-Br) from the benzyl glycoside. Verification of the structure of the fully substituted product 47 is provided by the $^1\text{H-n.m.r.}$ spectrum shown in Figure 13. As in the spectrum (Figure 9) of the corresponding disaccharide without the phosphate group, one can discern two signals for amide hydrogens, and signals for the protons (H-3 and H-4) at the acylated ring positions. Two doublets for anomeric protons can again be found among the peaks for the benzyl methylene hydrogens. The region just to higher field is much more complex than in the spectrum of the 4'-unsubstituted disaccharide because of the many lines of the trichloroethyl groups of the phosphotriester function.

For conversion into the 2,3,4,2',6'-pentapalmitoyl analog of 1-dephospho lipid A, compound 47 requires only the debenzylation of O-1 and O-3', and the cleavage of the trichloroethyl groups from the 4'-phosphate moiety. Work on the deprotection of this compound is in progress.

Compound 46-Pre closely resembles the intermediates used by Kusumoto *et al.* (this volume) and by van Boeckel *et al.* (16) for the preparation of disaccharide oxazolines, which were then elaborated into fully phosphorylated analogs (1,4'-diphosphates). In a formal sense, therefore, the synthesis of 46-Pre effectively constitutes a synthesis, by our revised route, of the 1,4'-bisphosphate having palmitoyl groups at positions 2, 3, 2', and 6'. An additional palmitoyl group could be attached at position 4 if desired.

Acknowledgments

This work was supported by the College of Agricultural and Life Sciences, University of Wisconsin-Madison, and by Grant No. AM 10588 from the National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases, N.I.H.

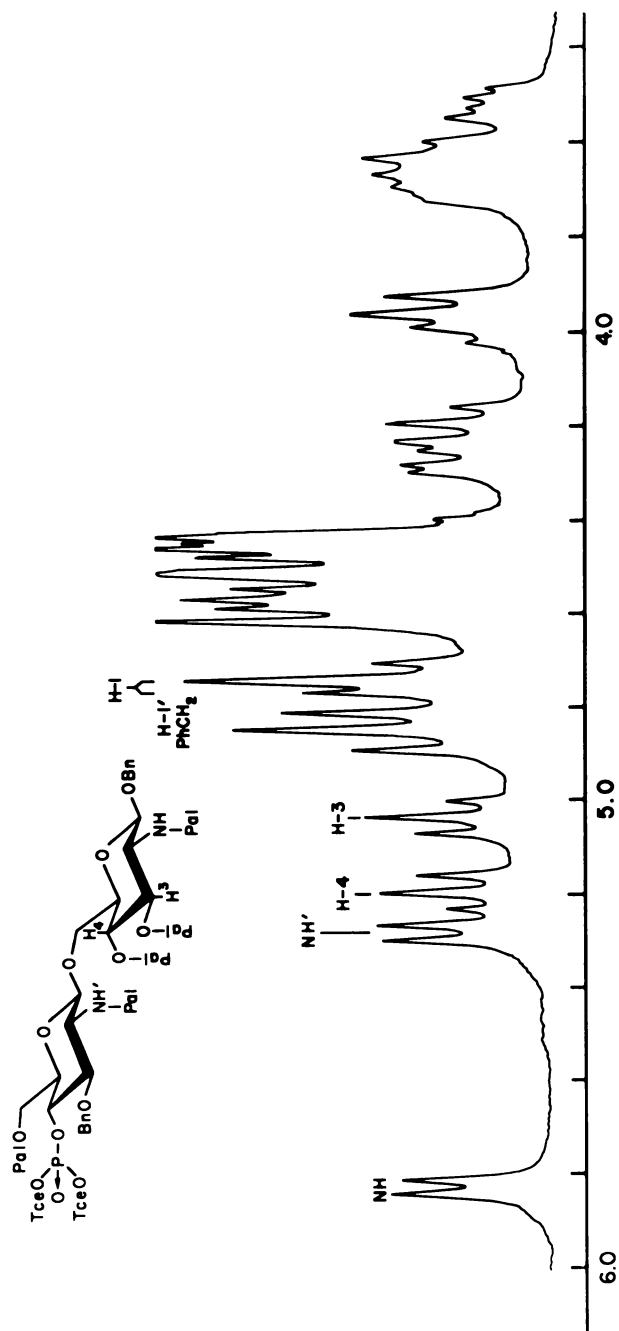


Figure 13. Proton-NMR spectrum of the fully substituted disaccharide 47. Key to formulas: same as for Figure 1.

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Synthetic Studies on the Lipid A Component of Bacterial Lipopolysaccharide

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Endotoxic lipopolysaccharide (LPS) is a prominent, macromolecular component of the outer membrane of Gram-negative bacteria, and most of its biological activities have been shown to be dependent upon structures in the lipid A component (1,2). The fundamental structure of the *Salmonella*-type lipid A (Figure 1) has been established to be a monomer of a $\beta(1\rightarrow6)$ -linked disaccharide of 2-deoxy-2-(*R*-3-hydroxytetradecanoylamino)-D-glucopyranose esterified with fatty acyl and phosphoryl groups (1,3,4). The esterified as well as the amide-linked 3-hydroxy fatty acids have been suggested to be common and prominent constituents that constitute a characteristic marker of lipid A. However, because of the difficulties of isolation and purification of lipid A, the moiety of the structure that is required for manifestation of its biological activities has been obscure. In this situation, the chemical synthesis of lipid A and related compounds is important in elucidating the relationship between chemical structure and activity, as well as in providing a new source of biologically active substances.

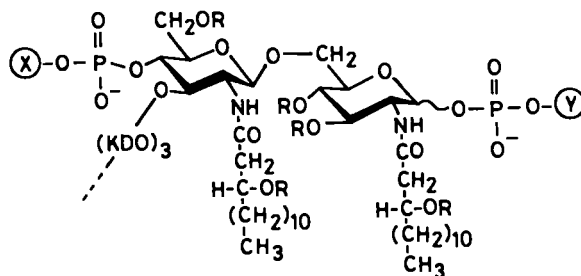


Figure 1. The proposed structure of *Salmonella*-type lipid A. Key: R, fatty acyl or H; X, H or 4-amino-L-arabinose; and Y, H, PO_3^{2-} , or phosphorylethanolamine.

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3-oxo-(4) and 3-hydroxy-(5) tetradecanoyl derivatives. The 3-oxo-tetradecanoyl derivative 4, $[\alpha]_D +11.3^\circ$ (chloroform), was synthesized by treatment of 1 with \underline{N} -(3-oxotetradecanoyloxy)succinimide, which is readily prepared by stirring 3-oxotetradecaic acid (10, 11) with dicyclohexylcarbodiimide (DCC) and \underline{N} -hydroxysuccinimide (HOSu) in dry tetrahydrofuran (oxolane, THF) or 1,4-dioxane. Similarly, the 3-hydroxytetradecanoyl derivative 5, $[\alpha]_D +6.7^\circ$ (chloroform), was obtained by treatment of 1 with 3-hydroxytetradecanoic acid (12,13) activated by DCC and HOSu (14). Compound 5 was prepared also by treatment of 4 with sodium borohydride in methanol solution, and then acetylated to give 6, $[\alpha]_D +3^\circ$ (chloroform).

The ferric chloride-catalyzed glycosylation was conducted by treating compounds 2, 4, 5, and 6 (1 mol equiv.) with benzyl or allyl alcohol (5 mol equiv.) in the presence of anhydrous ferric chloride (1.5 mol equiv.) in dichloromethane (6), to yield the corresponding β -glycosides 3, 7, 8, and 9 in 75-90% yield. Compound 4 was completely converted into 7, $[\alpha]_D -23.6^\circ$ (chloroform), within 3 hr, whereas the conversion 2 \rightarrow 3, 5 \rightarrow 8 (14), and 6 \rightarrow 9 required longer times (at most overnight). The formation of the oxazoline intermediates (12, 15, 16) in the course of the reaction was observed for all of the β -acetates except 4.

As shown in Figure 3, the treatment of compound 8(RS) with (2-methoxyethoxy)methyl chloride (MEMCl) (15) in dichloromethane containing $\underline{N,N}$ -diisopropylethylamine gave 10(RS), and selective tritylation of the primary hydroxyl group afforded 11(R), amorph., $[\alpha]_D -52^\circ$ (chloroform), and 11(S), m.p. 107° , $[\alpha]_D -35.2^\circ$ (chloroform), in the ratio of about 1:1. These diastereoisomers were separated by chromatography on a column of silica gel (16). The optically pure samples of 11(R) and 11(S) thus obtained were, by mild hydrolysis with 70% acetic acid and then acetylation, separately converted into 10(R), m.p. $103-104^\circ$, $[\alpha]_D -23.8^\circ$ (chloroform) and 10(S), m.p. $81-81.5^\circ$, $[\alpha]_D -14.6^\circ$ (chloroform). Removal of the MEM group was achieved by stirring 10(R) or 10(S) (1 mol equiv.) with anhydrous ferric chloride (1 mol equiv.) in dichloromethane (3 mL was used for 80-90 mg of the MEM derivative) for 1 hr at room temperature to give 8(R) (80%) or 8(S) (70%). Generally, the cleavage of MEM ethers has been achieved by treatment with zinc bromide or titanium tetrachloride (15). However, we found that the use of these Lewis acids for 10 caused rapid anomerization of the glycoside. In the course of this study, it was also found that 2-butenyl (crotyl) ethers can be cleaved by the same procedure as just described (the reaction was almost complete within 2 hr using a equimolar amount of anhydrous ferric chloride in dichloromethane at room temperature). For characterization, 8(R) and 8(S) were reacetylated and the structure were analyzed by means of $^1\text{H-n.m.r.}$ at 90 MHz (16).

O-Deacetylation of 8(R) and 8(S), and hydrogenolytic removal of the benzyl groups in the presence of 10% palladium-carbon catalyst gave the desired optically pure 2-deoxy-2-(R- and S-3-hydro-

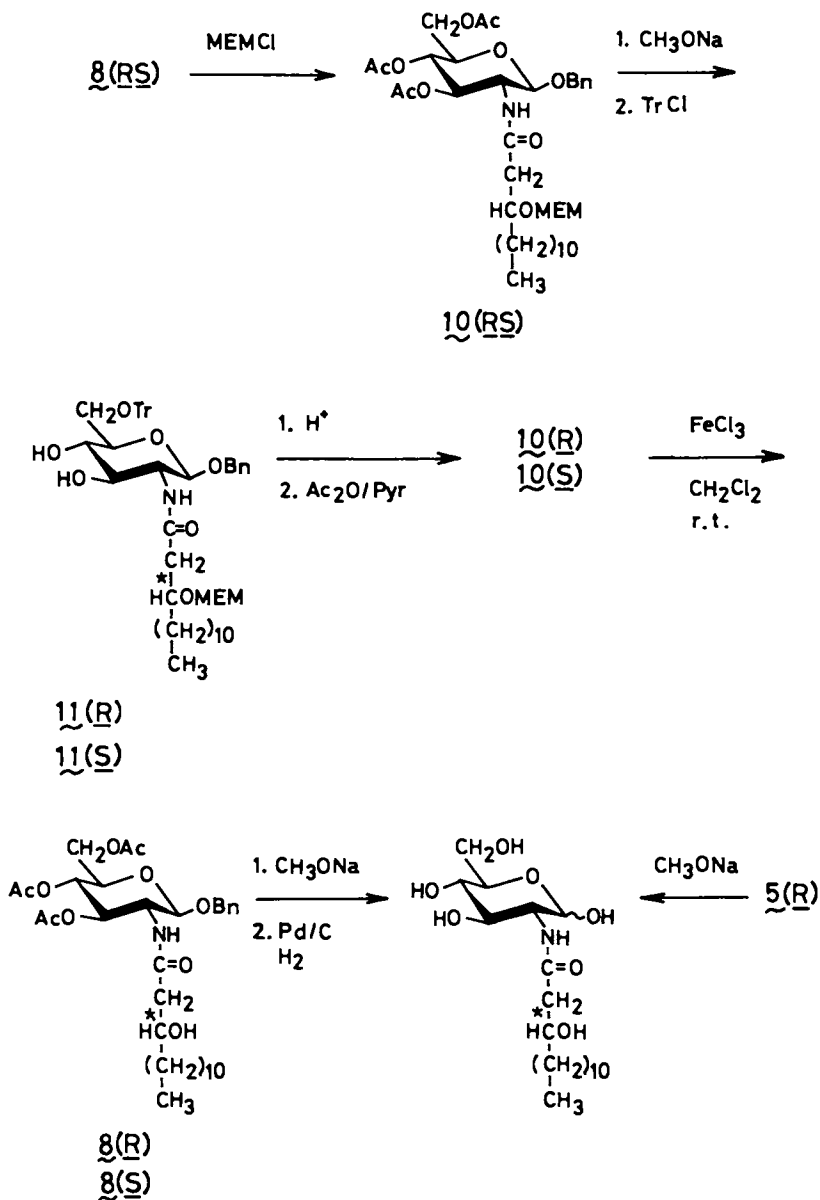


Figure 3. Treatment of compound $\underline{8(RS)}$ with (2-methoxyethoxy)methyl chloride and subsequent regeneration of compounds $\underline{8(R)}$ and $\underline{8(S)}$.

xytetradecanoylamino)-D-glucose: (R), $[\alpha]_D +23.7^\circ$ (2:1 oxolane-water, equil.), $+25.8^\circ$ (3:1 methanol-chloroform, equil.); and (S), $[\alpha]_D +35.0^\circ$ (2:1 oxolane-water, equil.), as amorphous materials. Demary (17) synthesized 2-deoxy-2-(R-3-hydroxytetradecanoylamino)-D-glucose, $[\alpha]_D +26.9^\circ$ (2:1 oxolane-water, equil.), by the direct condensation of activated (R)-3-hydroxytetradecanoic acid, which had been resolved by means of dehydroabietylamine, with 2-amino-2-deoxy-D-glucose. We also prepared the same compound by the treatment of ζ (R) with sodium methoxide in methanol solution.

Synthesis of 2-Acylamino-2-deoxy-D-glucopyranosyl Phosphates

From Oxazolines. The conversion of the 1- β -acetates λ into the corresponding oxazolines μ (Figure 4) was accomplished by the method of Matta and Bahl (18). The resulting oxazolins were all crystallized from ether-petroleum ether in refrigerator (19). Phosphorylation was carried out by treating the oxazolines (1 mol equiv.) with dibenzyl phosphate (2 mol equiv.) in 1,2-dichloroethane or 1,2-dichloroethane-toluene at room temperature according to the procedure reported by Khorlin et al. (20), to give the desired 3,4,6-tri-O-acetyl-2-deoxy-2-(tetra-, hexa- and octadecanoyl, and eicosanoylamino)- α -D-glucopyranosyl dibenzyl phosphates (13) in 38-42% yields. The structures of these phosphates were carefully confirmed by silica gel t.l.c. using the spray reagent described by Dittmer and Lester (21), elemental analysis, FD-MS, and both ^1H - and ^{13}C -n.m.r. data. In the ^1H -n.m.r. spectra in chloroform-d the anomeric protons, without exception, appeared as doublets of doublets due to couplings with ^{31}P ($^3\text{J}_{\text{P},1}$, 6.0 Hz) and H-2 ($\text{J}_{1,2}$ 3.1 Hz) at δ 5.65 ppm as required for α -glucopyranosyl phosphates. Furthermore, in the ^{13}C -n.m.r. spectra, two- and three-bond couplings (22,23) between ^{13}C and ^{31}P , namely $^2\text{J}_{\text{C}-1,\text{P}}$ (5.88-7.35 Hz) at δ 96-97 ppm and $^3\text{J}_{\text{C}-2,\text{P}}$ 7.35-8.82 Hz) at δ 51-52 ppm, were clearly observed. Considering that the three-bond coupling is generally larger than the two-bond coupling, the result well support the assigned structure. Compounds μ were then hydrogenolyzed in the presence of 10% palladium-carbon catalyst to give the corresponding free phosphates, which were subsequently treated with a cation-exchange resin (Na^+) until the pH of the solution became neutral. The resulting ν were lyophilized to afford colorless, amorphous solids (19).

However, the oxazolines ξ (9), η (14) and θ which were also prepared by treating ζ , δ or the corresponding levulinoyl derivative with anhydrous ferric chloride in dichloromethane, did not yield phosphate derivatives by Khorlin's method, just described. The common, major product was 2-(E-1-tridecenyl)-(3,4,6-tri-O-acetyl-1,2-dideoxy- α -D-glucopyranosyl)-[2,1-d]-2-oxazoline (18) [^1H -n.m.r. at 90 MHz in chloroform-d: δ 3.58 (m, 1 H, $\text{J}_{4,5}$ 9, $\text{J}_{5,6}$ 4.3 Hz, H-5), 4.1-4.3 (m, 3 H, H-2,6), 4.93 (m, 1 H, $\text{J}_{3,4}$ 2.3, $\text{J}_{2,4}$ 1.2 Hz, H-4), 5.28 (bd, 1 H, $\text{J}_{2,3}$ \sim 0 Hz, H-3), 5.95 (bd, 1 H, $\text{J}_{1',2'}$ 16 Hz, H-1'), 5.98 (d, 1 H, $\text{J}_{1,2}$ 7.2 Hz, H-1), and 6.67 (dt, 1 H, $\text{J}_{2',3'}$ 6.8 Hz, H-2')].

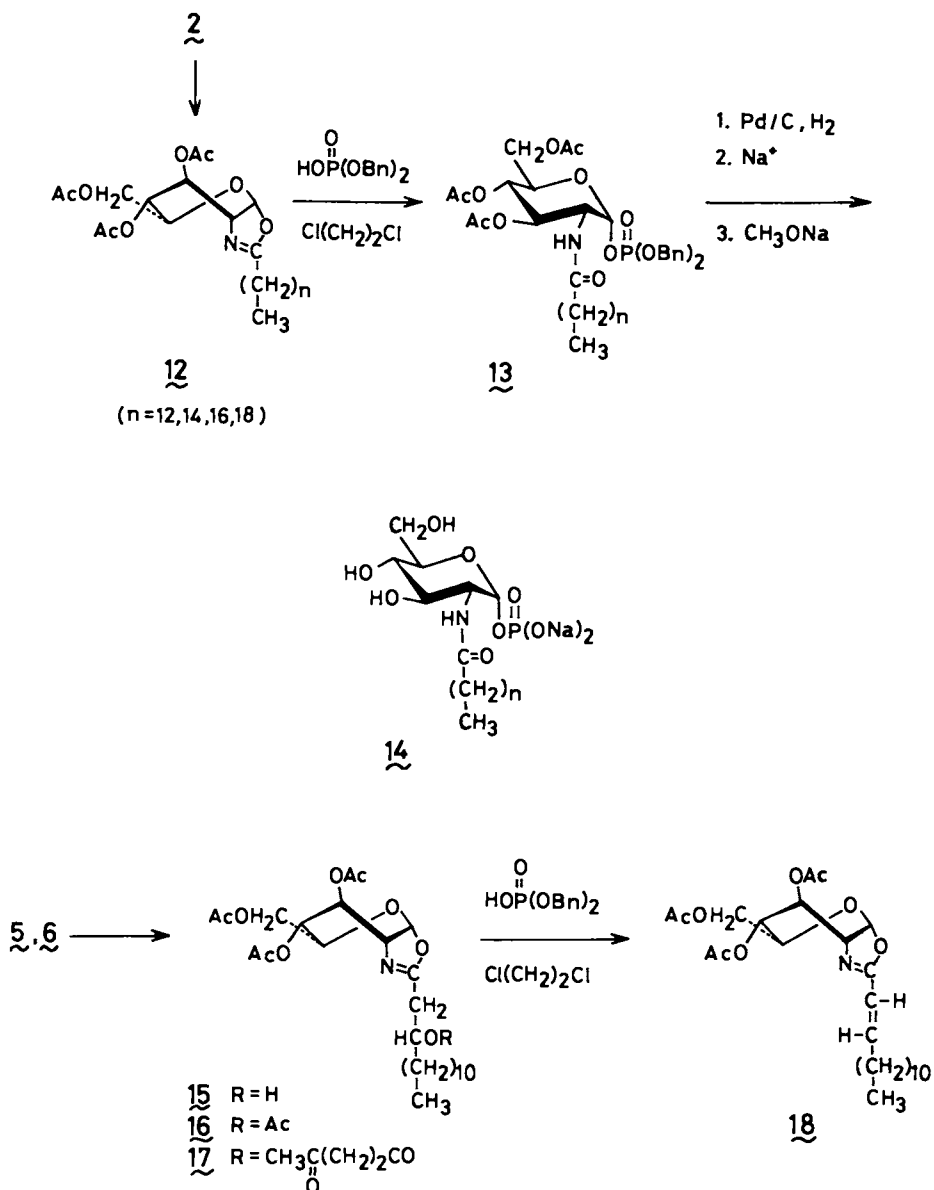
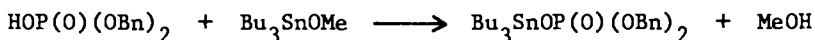
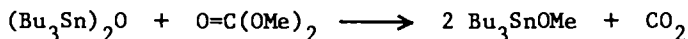


Figure 4. Reactions of 2-(fatty alkyl)oxazolines with dibenzyl phosphate.

From Halides. Since the synthesis of the 1-phosphates of the 3-hydroxytetradecanoyl derivatives by the oxazoline method was unsuccessful as just described, we examined a new procedure using 1-halides as the starting materials (24) (Figure 5).

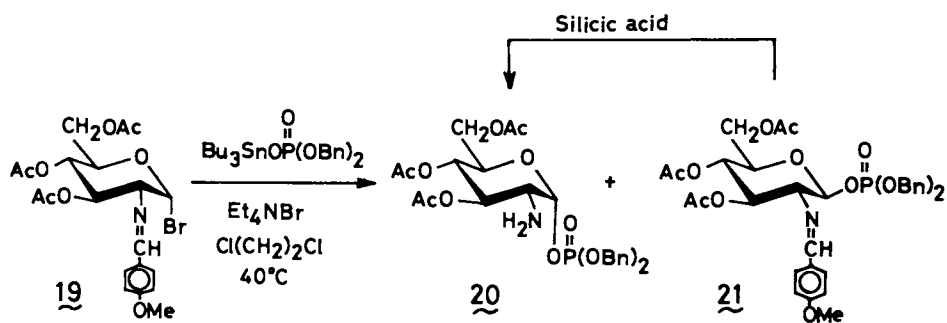
3,4,6-Tri-O-acetyl-2-deoxy-2-(p-methoxybenzylideneamino)- α -D-glucopyranosyl bromide (19) (25) (1 mol equiv.) was treated with dibenzyl tributylstannyl phosphate (1.1 mol equiv.) in dichloroethane containing tetraethylammonium bromide (0.5 mol equiv.) for 3 hr at 40° to give two major glycosyl phosphates, 20 and 21. The dibenzyl tributylstannyl phosphate was prepared by the treatment of dibenzyl phosphate with tributyltin methoxide (26) according to the procedure of Yamaguchi *et al.* (27), and used without purification. A similar procedure was employed for the synthesis of glycosyl esters by Ogawa *et al.* (28).



Although t.l.c. revealed the favored formation of 21, m.p. 128-130°; $[\alpha]_D +4.7^\circ$ (chloroform); i.r. (KBr): 1640, 1610 (C=N), and 1300-1250 cm^{-1} (P=O); $^1\text{H-n.m.r.}$ in chloroform-d: δ 5.6 (t, 1 H, $J_{1,2}$ and $J_{1,p}$ 8 Hz, H-1) and 6.8-7.7 (m, 14 H, Ph), its isolated yield, after chromatography on a column of silica gel (Wako gel C-300) with 100:1 chloroform-methanol, was 33%. This was less than the yield of 20, m.p. 87-89°; $[\alpha]_D +80.9^\circ$ (chloroform; i.r. (KBr): 3680-3240 and 1600 cm^{-1} (NH₂); $^1\text{H-n.m.r.}$ in chloroform-d: δ 5.66 (dd, 1 H, $J_{1,2}$ 3.5, $J_{1,p}$ 6 Hz, H-1) and 7.3 (s, 10 H, Ph). In fact, we found that when the reaction mixture was stirred, before chromatography, with silica gel in 100:1 chloroform-methanol for 20 hr at room temperature, most of the compound 21 was converted into 20.

Treatment of 20 in 1:1 dichloromethane-2,6-lutidine containing a trace of N,N-diisopropylethylamine with freshly prepared (R)-3-acetoxytetradecanoyl chloride [prepared by a slight modification of the method employed for the synthesis of acetylmandelyl chloride (29)] during 3 hr at 0° gave, after chromatography, a syrup of 22 (40%), $[\alpha]_D +48.4^\circ$ (chloroform); i.r. (film): 3240, 1670, and 1540 cm^{-1} (amide); $^1\text{H-n.m.r.}$ in chloroform-d: δ 0.75-2.3 (25 H, CH₃ and CH₂), 1.98-2.02 (12 H, CH₂CO), 5.65 (dd, 1 H, $J_{1,2}$ 3.2, $J_{1,p}$ 6 Hz, H-1), and 7.3 and 7.32 (2 s, 10 H, Ph). Similar treatment of 20 with tetra- and hexadecanoyl chlorides, respectively, gave 13 ($n = 12$ and 14). Their physical properties and spectral data were identical with those of the same compounds prepared by the oxazoline method (Figure 4). Finally, compound 22 was converted, by de-O-acetylation and hydrogenolysis, into 23, $[\alpha]_D +35.3^\circ$ (water).

The new phosphorylation method was further applied to halides having N-benzoyloxycarbonyl (Z) (24 and 25), N-acetyl (26) and N-



Et ₄ NBr Bromide	<u>20</u> (%) ^a	<u>21</u> (%) ^a	Total (%) ^a
0.5	36 (59) ^b	33 (10) ^b	69
1.0	38	27	65
2.0	32	9	41

^aIsolated. ^bReaction mixture was stirred with silica gel (Wako gel C-300) in CHCl_3 -MeOH(100:1) for 20 h at r.t.

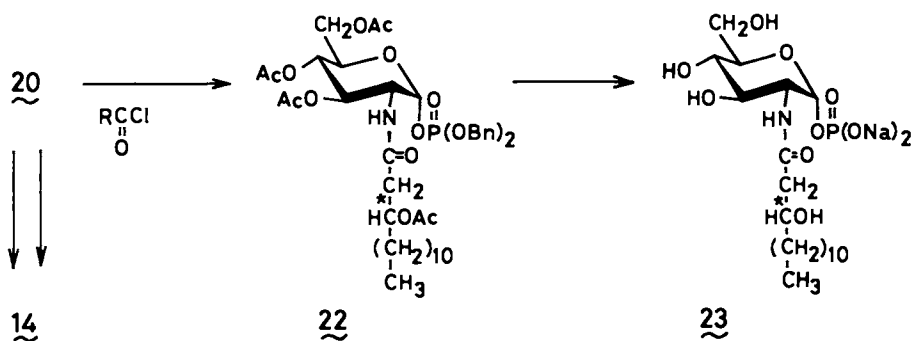


Figure 5. Synthesis of 2-acylamino-2-deoxy-D-glucopyranosyl phosphates using l-halides as starting materials.

3-acetoxytetradecanoyl (27) substituents, as shown in Figure 6. The phosphorylation reaction was conducted for 8 hr at 40–45° and the products were chromatographed on columns of silica gel. From compounds 24 and 25 the α -phosphate ($28\text{-}\alpha$) was obtained in 40–42% yield as needles, m.p. 101–103°; $[\alpha]_D +75.1^\circ$ (chloroform); ^1H -n.m.r. in chloroform- d : δ 5.7 (dd, 1 H, $J_{1,2}$ 3.2 Hz, H-1). Most of the β -phosphate ($28\text{-}\beta$), however, decomposed during the chromatography. On the other hand, compound 26 yielded the corresponding α -phosphate ($29\text{-}\alpha$), needles, m.p. 109°; $[\alpha]_D +63.9^\circ$ (chloroform); ^1H -n.m.r. in chloroform- d : δ 5.62 (dd, 1 H, $J_{1,2}$ 3.1 Hz, H-1), in only 10% yield. For compound 27 , no phosphate derivative was isolated, and instead, the oxazoline 18 (see Figure 4) was obtained as a major product. This result suggested that the neighboring N -acyl group in 27 inhibited the formation of the stable α -phosphate, and even if the β -phosphate was produced in the initial stage of the reaction, the phosphoryl group became a good leaving group to afford the oxazoline 18 .

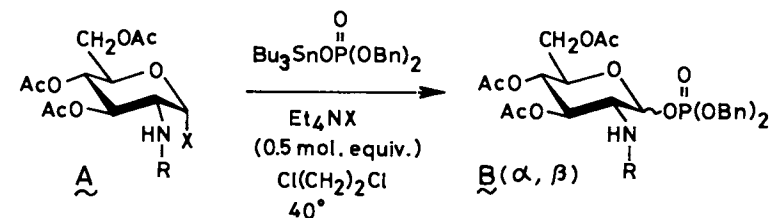
Since tetraethylammonium halides are known to catalyze the anomerization of α - and β -glycosyl halides (30), we next examined the effect of the chloride on the phosphorylation of 25 (Figure 7). Interestingly, the yield of $28\text{-}\alpha$ as well as the total yield increased with an increasing ratio of the catalyst to the glycosyl chloride 25 . Although the reactivity of diphenyl tributylstannyl phosphate was slightly lower than that of the dibenzyl derivative, the diphenyl compound gave a 65% yield of the α -phosphate 30 , amorph., $[\alpha]_D +54.3^\circ$ (chloroform); ^1H -n.m.r. in chloroform- d : δ 5.97 (dd, 1 H, $J_{1,2}$ 3 Hz, H-1).

The presence in bacterial lipopolysaccharides of phosphorylated ethanolamine residues (1) prompted us to experiment with the incorporation of this group. When compound 19 was treated with an activated phosphorylethanolamine derivative, prepared by the procedure shown in Figure 8, the α -phosphate 31 , $[\alpha]_D +66.7^\circ$ (chloroform), was obtained in 46% yield. The reaction is similar to that with dibenzyl tributylstannyl phosphate. Thus, the new phosphorylation method may have wide application in the synthesis of C-1 phosphorylated lipid A analogs.

Synthesis of Disaccharide Analogs of Lipid A

For the reconstitution of a β -linked disaccharide of 2-acylamino-2-deoxy-D-glycopyranose, the coupling procedure employing oxazoline derivatives (31) appeared most suitable. The oxazolines 12 ($n = 12$), m.p. 49–50°, $[\alpha]_D +9.4^\circ$ (chloroform), and 16 , syrup, $[\alpha]_D +21.6^\circ$ (chloroform), were accordingly used as the glycosyl-donors in the disaccharide synthesis (Figure 9). As acceptors, we employed benzyl 3,4-di-O-acetyl-2-deoxy-2-tetradecanoylamino- β -D-glycopyranoside (33) for the coupling with 12 , and the 3-acetoxy-tetradecanoylamino derivative 34 for the coupling with 16 (32).

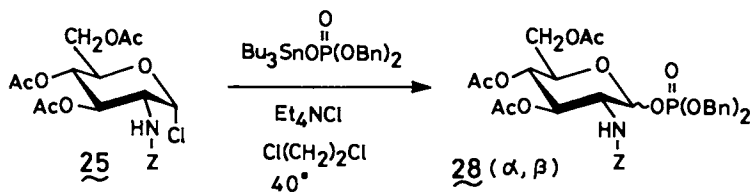
The glycosylation reaction was conducted by adding a solution of 12 or 16 (3 mol equiv.) in 1,2-dichloroethane to a mixture of



<u>A</u>	R	X	α (%)	β (%)	Total (%)	<u>B</u>
<u>24</u>	Z	Br	42	30	72	<u>28</u>
<u>25</u>	Z	Cl	40	30	70	<u>28</u>
<u>26</u>	Ac	Cl	10	-	10	<u>29</u>
<u>27</u>	3-OAc-Myr	Cl	Oxazoline (18)			

Z : PhCH₂OCO. 3-OAc-Myr : CH₃(CH₂)₁₀CHCH₂CO.
OAc

Figure 6. Phosphorylation method described in Figure 5 applied to halides having N-benzyloxycarbonyl (Z) (24 and 25), N-acetyl (26), and N-3-acetoxytetradecanoyl (27) substituents.



Et ₄ NCl Chloride	28(α) (%) ^a	28(β) (%) ^b	Total (%)
0.5	40	30	70
1.0	60	15	75
2.0	64	15	79

^a Isolated. ^b T.L.C.

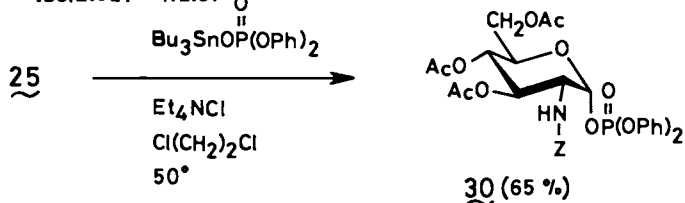


Figure 7. Effect of tetraethylammonium chloride on the phosphorylation of 25.

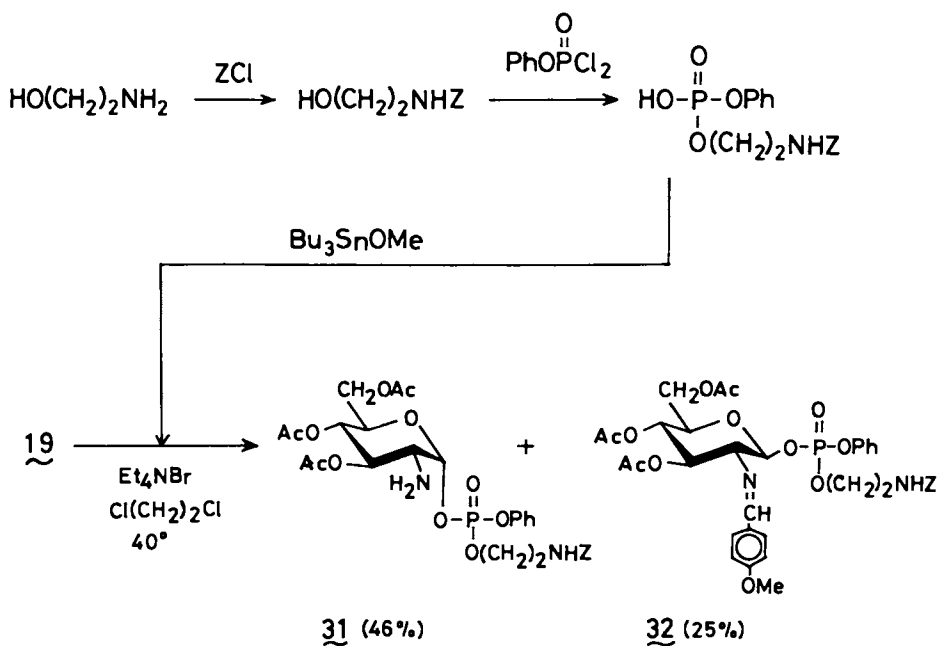


Figure 8. Treatment of compound **19** with an activated phosphorylethanolamine derivative.

acceptor β or β (1 mol equiv.) and *p*-toluenesulfonic acid (0.33 mol equiv.) in dichloroethane. The final concentration of the acid was adjusted to about 20 mM by adding dichloroethane, and the mixture was stirred overnight, at 60-70° when β was the acceptor, or at 50° with β as the acceptor. When the oxazoline had almost completely disappeared, the mixture was extracted with chloroform as described previously (32-34). The resulting residues were chromatographed on columns of silica gel with 100:1 chloroform-methanol to provide 65-70% yields of the desired β -D(1 \rightarrow 6)-linked disaccharides β , $[\alpha]_D -23.0^\circ$ (chloroform), and β , $[\alpha]_D -20.6^\circ$ (chloroform). Finally, the disaccharides β and β were treated with methanolic sodium methoxide and the products hydrogenolyzed in the presence of 10% palladium-carbon catalyst, to give β , m.p. 164-166°, and β , m.p. 191-192°, respectively.

Although the oxazoline method is a general procedure for the synthesis of β -glycosides, the acylamido groups at C-2 of the starting materials are unchangeable to others. We next examined a more flexible method by which a variety of fatty acyl residues can be introduced after the formation of disaccharides (Figures 10 and 11).

Compound β was treated with phthalic anhydride in pyridine at 90° for 0.5 hr, then acetic anhydride was added, and the mixture was stirred for another hour at 90° and worked up. The product β (34) was isolated as needles in 80% yield, and was then converted into 3,4,6-tri-*O*-acetyl-2-deoxy-2-phthalimido- β -D-glucopyranosyl bromide (40) ("glycosyl donor") according to the method reported by Lemieux et al. (36). On the other hand, the treatment of β (1 mol equiv.) with benzyl alcohol (2 mol equiv.) in the presence of anhydrous ferric chloride (1.5 mol equiv.) in dichloroethane (6) gave β (36) in excellent yield. *O*-Deacetylation of β and 4,6-*O*-isopropylidene (37) in dry 1,4-dioxane in the presence of a catalytic amount of *p*-toluenesulfonic acid, gave β , m.p. 214-216°; $[\alpha]_D -78.7^\circ$ (chloroform). The remaining hydroxyl group at C-3 of β was then benzylated with sodium hydride and benzyl bromide in dry 1,4-dioxane to yield benzyl 3-*O*-benzyl-2-deoxy-4,6-*O*-isopropylidene-2-phthalimido- β -D-glucopyranoside, m.p. 121-122°; $[\alpha]_D -6.9^\circ$ (chloroform). This was converted, by mild hydrolysis with 80% acetic acid, into the "glycosyl acceptor" β (37).

The glycosylation reaction was conducted by adding a solution of 40 (1.2 mol equiv.) in dichloroethane to a mixture of acceptor β (1 mol equiv.), silver trifluoromethanesulfonate (1.27 mol equiv.) and tetramethylurea (2.7 mol equiv.) at -30° (39). The mixture was then stirred overnight at 0° and worked up. The product was chromatographed on a column of silica gel to give β (70% yield), $[\alpha]_D +2.2^\circ$ (chloroform); $^1\text{H-n.m.r.}$ in chloroform-*d*: two doublet peaks (J 8.4 Hz) for the anomeric protons at δ 4.95 and 5.50 ppm. *O*-Deacetylation of β and 4',6'-*O*-isopropylidene of the product in 1,4-dioxane gave amorphous β (85% yield), $[\alpha]_D -35.0^\circ$ (chloroform), which was treated with hydrazine hydrate in ethanol under reflux to afford β , $[\alpha]_D -33.6^\circ$ (methanol),

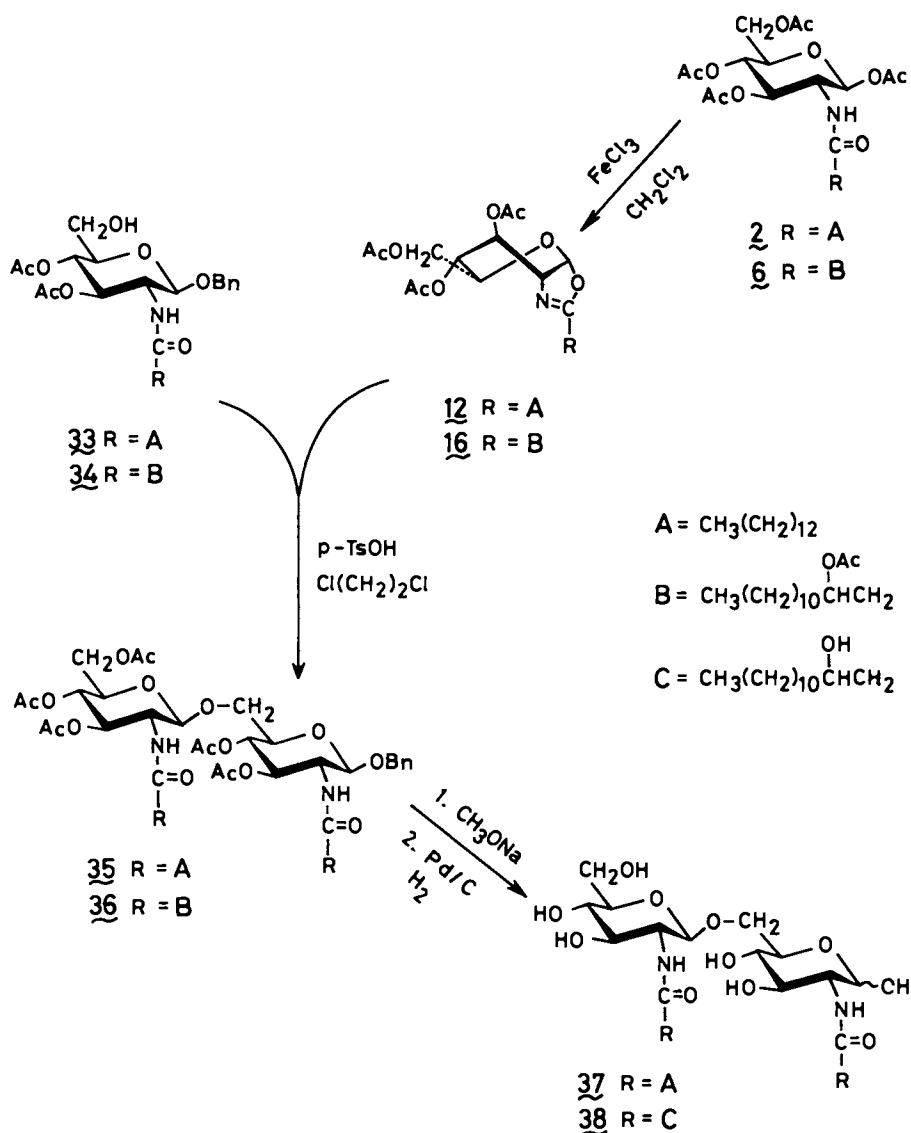


Figure 9. Synthesis of di-N-acyl disaccharide derivatives via the oxazoline route.

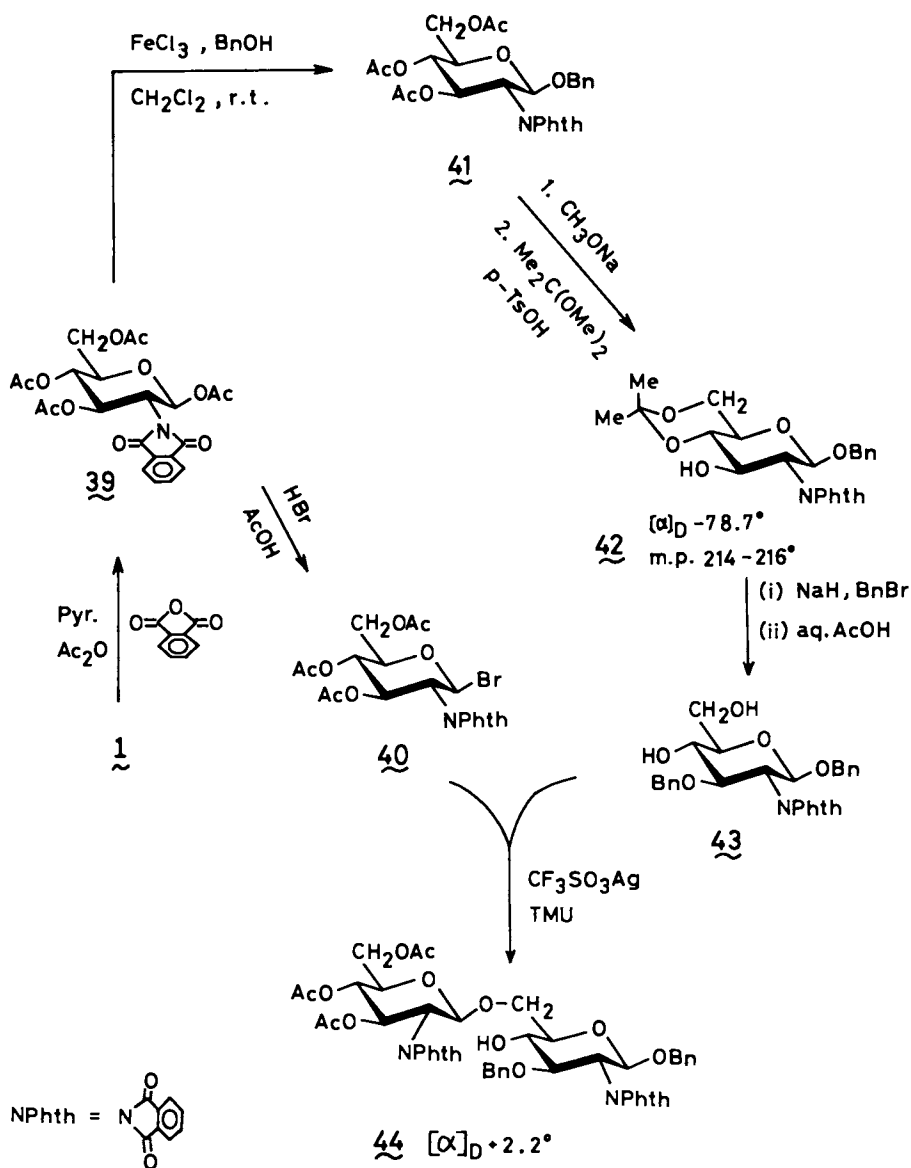


Figure 10. Method for the introduction of a variety of fatty acyl residues after disaccharide formation.

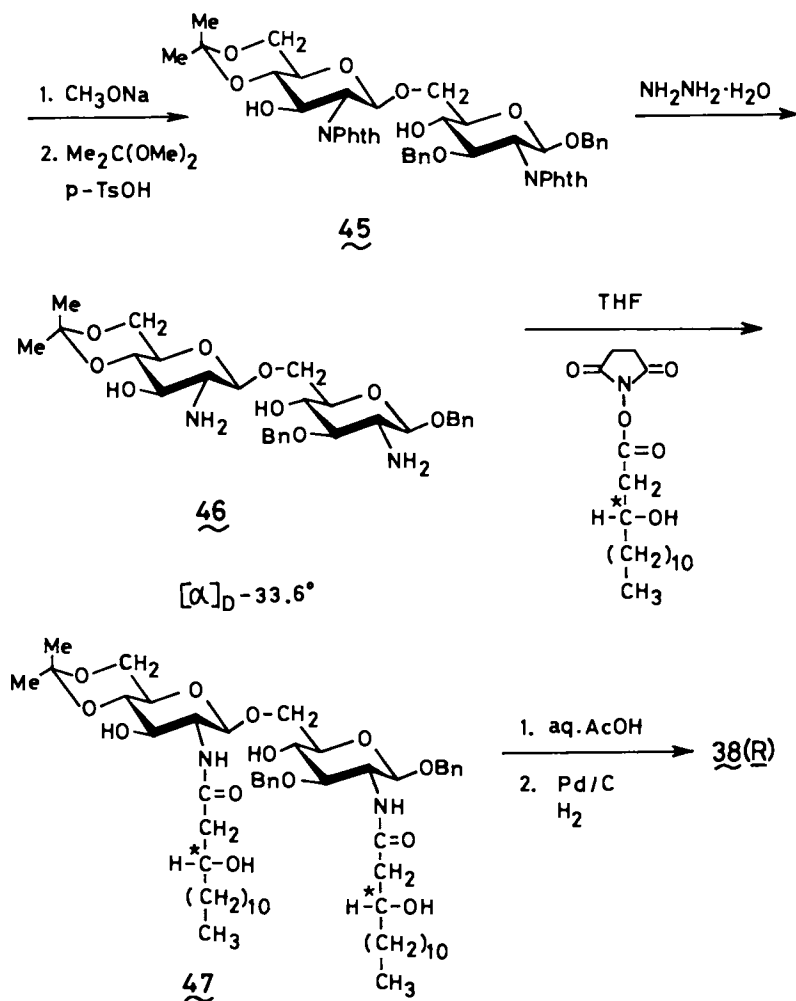


Figure 11. Continuation of the scheme of Figure 10 to yield **38(R)**.

quantitatively. This compound was useful for the preparation of the dephospho disaccharide skeleton of lipid A containing a variety of amide-linked fatty acyl residues. In fact, the treatment of 46 with N-(R-3-hydroxytetradecanoyloxy)succinimide gave 47, which was converted, by O-deisopropylidenation and then hydrogenation, into 38(R).

Synthetic Strategy for Lipid A

In the course of our preceding synthetic studies on the fundamental carbohydrate skeletons of lipid A and their phosphate derivatives, it has been recognized that the β (1 \rightarrow 6)-linked disaccharide 59 (Figure 14) might be a key intermediate for the synthesis of lipid A, since a variety of functional groups can be introduced on the nitrogen and oxygen atoms after formation of the β (1 \rightarrow 6) linkage. For the preparation of the initial precursor 57, allyl 3-O-benzoyl-2-deoxy-2-phthalimido- β -D-glucopyranoside (51) and 4,6-di-O-acetyl-3-O-benzyl-2-deoxy-2-phthalimido- β -D-glucopyranosyl chloride (56) were chosen as the glycosyl acceptor and donor, respectively (Figures 12 and 13).

As shown in Figure 12, the treatment of 39 (1 mol equiv.) with allyl alcohol (2 mol equiv.) in the presence of anhydrous ferric chloride (1.5 mol equiv.) in dichloromethane (Kiso and Anderson, 6) gave 48, which was converted into 49, m.p. 109°; $[\alpha]_D -29.6^\circ$ (chloroform) (87% yield from 39) as described for the synthesis of 42. Compound 49 is a useful, common intermediate for the preparation of the glycosyl acceptor 51 and the donor 56. Benzoylation of 49, to give 50, m.p. 141.5°, $[\alpha]_D +79.1^\circ$ (chloroform), and mild hydrolysis of the isopropylidene group afforded amorphous 51 in almost quantitative yield, $[\alpha]_D +101.2^\circ$ (chloroform). Benzoylation of 49 (Figure 13), on the other hand, gave 52, m.p. 104.5°, $[\alpha]_D +55^\circ$ (chloroform), which was converted in high yield, by O-deisopropylidenation and acetylation, into 53, m.p. 94°, $[\alpha]_D +56.8^\circ$ (chloroform). The allyl group was then cleaved by selenium dioxide (2 mol equiv.) in 1,4-dioxane containing acetic acid (2.5 mol equiv.) (40) to give 54 in 80% yield, m.p. 151-151.5°; $[\alpha]_D +83.5^\circ$ (chloroform); $^1\text{H-n.m.r.}$ in chloroform-d: δ 5.35 (d, 1 H, J 8 Hz, H-1). Next, 54 was acetylated to afford 55, m.p. 147°; $[\alpha]_D +75.6^\circ$ (chloroform); $^1\text{H-n.m.r.}$ in chloroform-d: δ 6.29 (d, 1 H, J 8 Hz, H-1). The glycosyl donor 56, $[\alpha]_D +80.2^\circ$ (chloroform) was easily prepared by treating 55 with anhydrous hydrochloric acid in dichloromethane.

The coupling of 56 (2 mol equiv.) to 51 (1 mol equiv.) was achieved (Figure 14) by using silver carbonate and silver perchlorate as the catalysts in dichloroethane to give 57, $[\alpha]_D +70^\circ$ (chloroform), in 75% yield (this reaction was complete within 2 hr). The use of $\text{CF}_3\text{SO}_3\text{Ag-TMU}$ as the catalysts reduced the isolated yield of 57. O-Deacetylation of 57 and 4',6'-O-isopropylidenation with 2,2-dimethoxypropane in 1,4-dioxane afforded 58, $[\alpha]_D +4.1^\circ$ (chloroform), in 83% yield. The cleavage of phthalimido

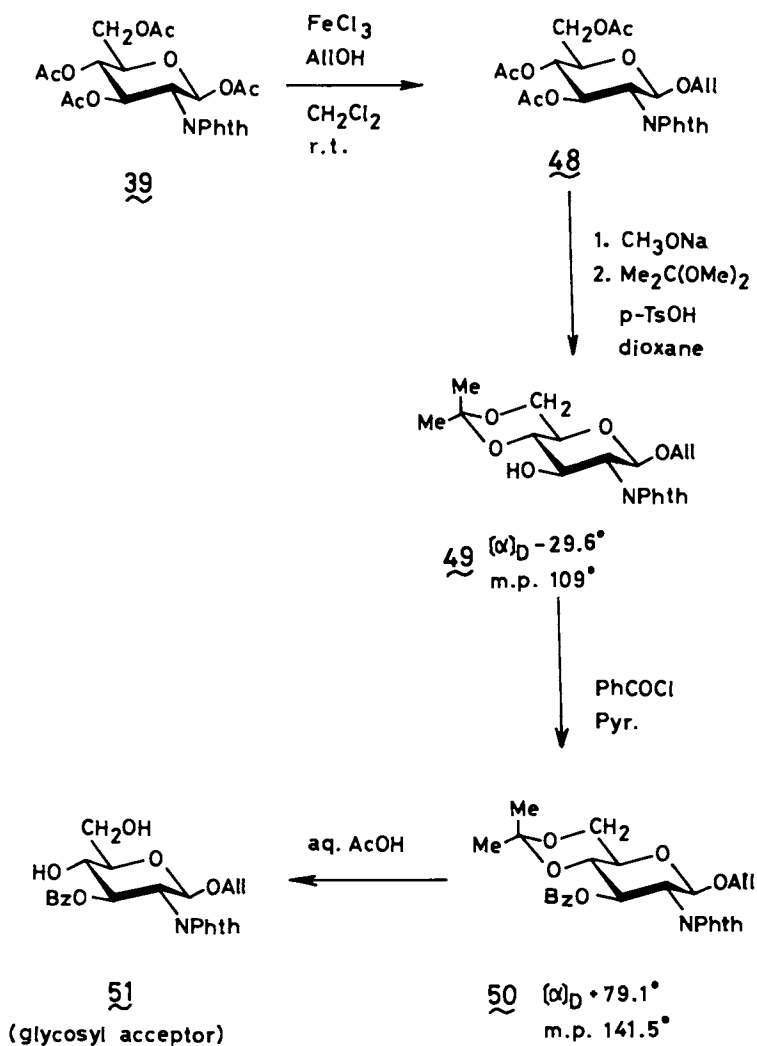


Figure 12. Preparation of a glycosyl acceptor, allyl 3-O-benzoyl-2-deoxy-2-phthalimido- β -D-glucopyranoside (**51**).

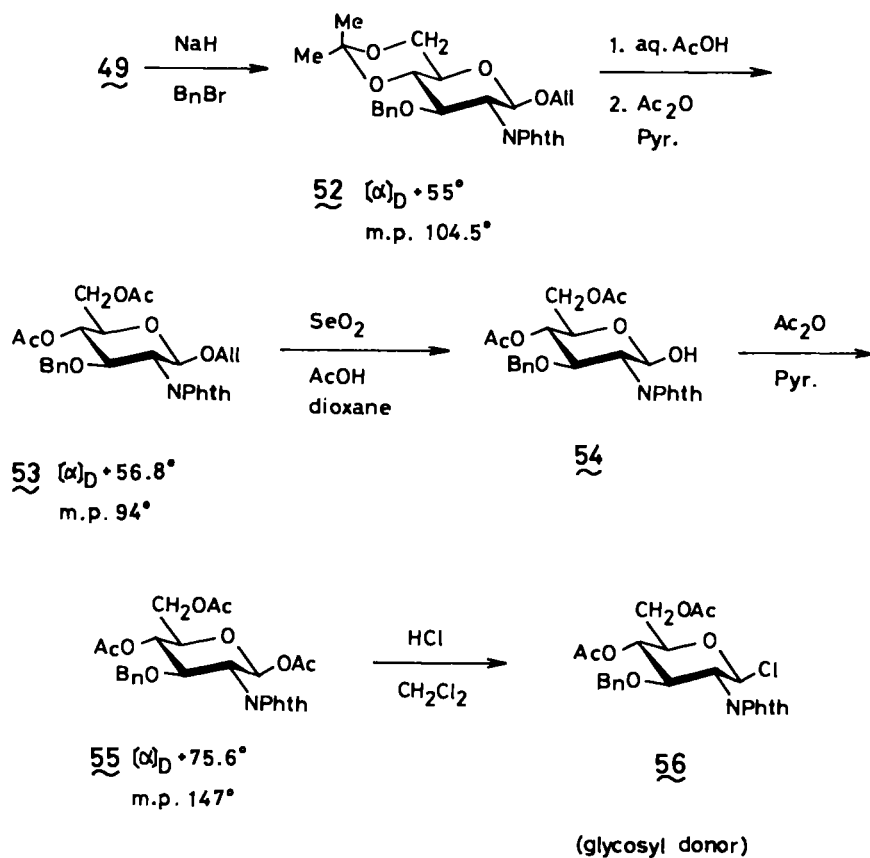


Figure 13. Preparation of a glycosyl donor, 4,6-di-O-acetyl-3-O-benzyl-2-deoxy-2-phthalimido- β -D-glucopyranosyl chloride (**56**).

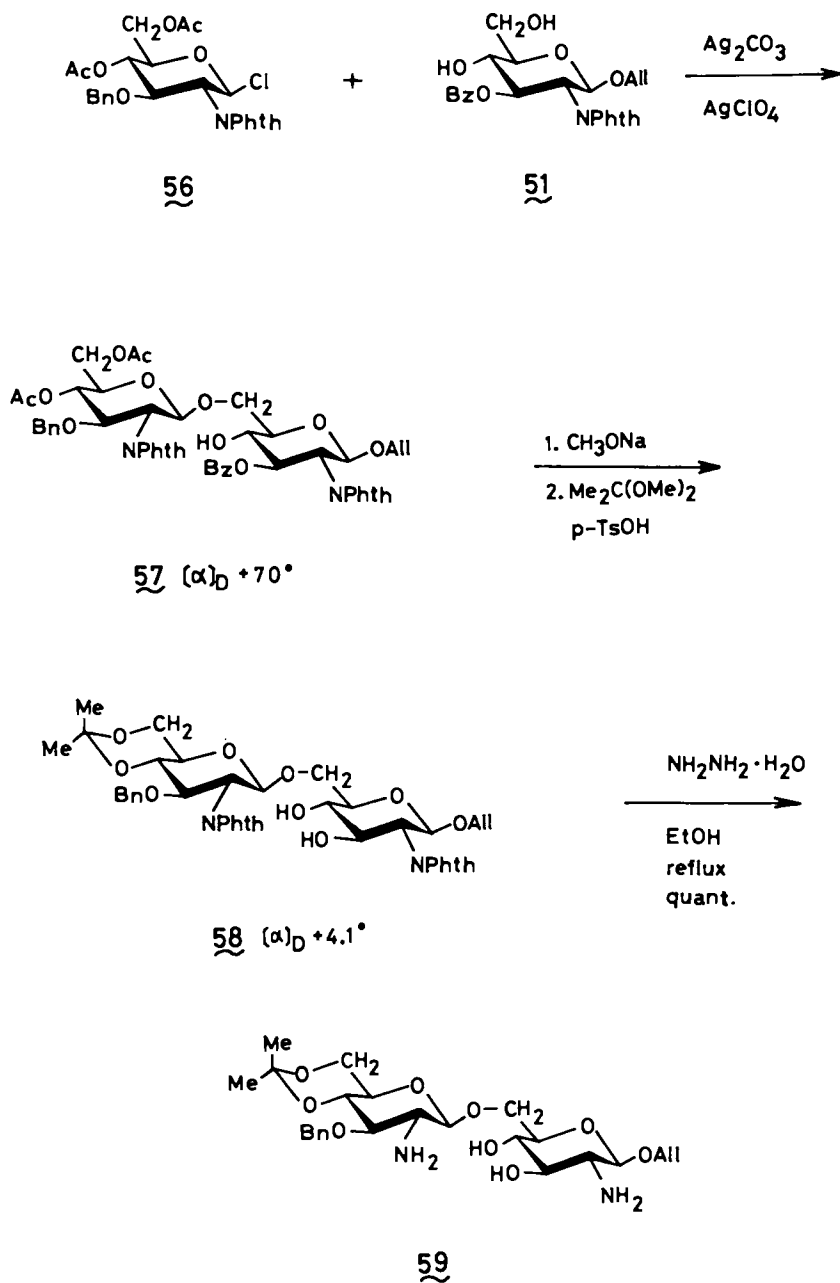


Figure 14. Coupling of **56** (Figure 13) and **51** (Figure 12), and elaboration of the β ,1-6-linked disaccharide **59** as a key intermediate for the synthesis of lipid A.

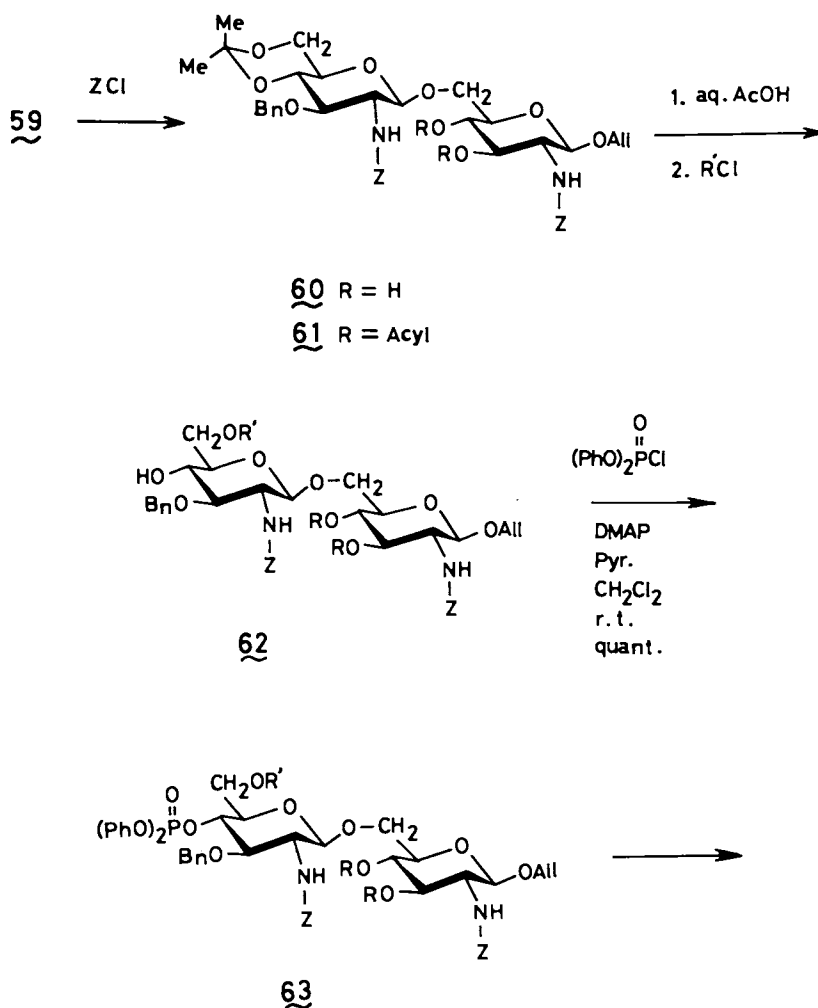
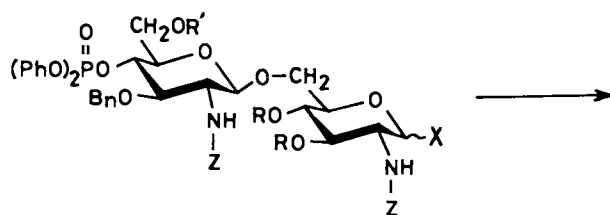
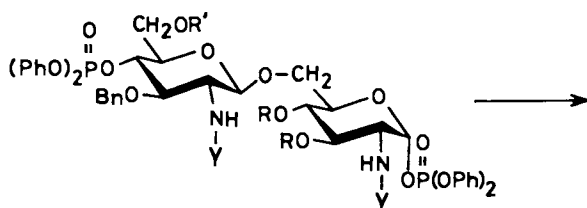


Figure 15. Proposed total synthesis of lipid A using the precursor 59 prepared as in Figure 14.



64 X = OH

65 X = Cl, Br



66 Y = Z

67 Y = H

68 Y = COCH₂CH(CH₂)_nCH₃
 |
 OR''

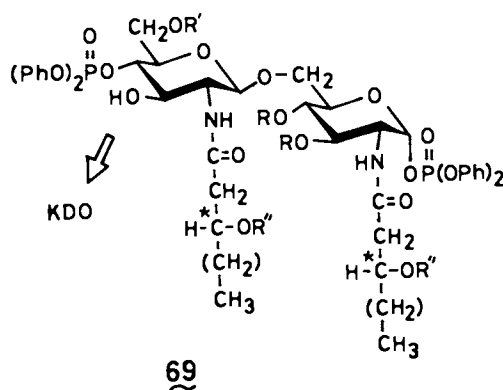


Figure 15. Continued.

groups was carried out by treatment with hydrazine hydrate in ethanol, to give 59 , which was promptly benzyloxycarbonylated to 60 , $[\alpha]_D -23.7^\circ$ (chloroform) [lit. -24.3° (41)].

Our synthetic plan, including the coupling of 3-deoxy-D-mannooctulosonate (KDO) to the oxygen atom at either C-3' or C-6', is shown in Figure 15. In our strategy for the total synthesis of the lipid A, the selective protection of the amino, hydroxy, and phosphate functions by benzyloxycarbonyl (Z), benzyl (Bn), and phenyl (Ph) groups, which can be selectively cleaved by hydrogenolysis using palladium-carbon or platinum oxide as the catalyst, is a most important feature. Since the conversion of 60 into 62 (R = tetradecanoyl) was already achieved by Inage *et al.* (41), a remaining problem is the introduction of the phosphate group at C-4'. When allyl 2-benzyloxycarbonylamino-2-deoxy-3,6-di-O-tetradecanoyl- β -D-glucopyranoside (1 mol equiv.) was treated overnight with diphenyl phosphorochloridate (3 mol equiv.) in dichloromethane containing 4-dimethylaminopyridine (2 mol equiv.) and a small amount of dry pyridine (the amount is not critical for the reaction) at room temperature, the corresponding 4-phosphate derivative was readily obtained in almost 100% yield. The same procedure was then employed for the conversion of 62 (R = Ac, R' = Bz), $[\alpha]_D -8.2^\circ$ (chloroform), into 63 , $[\alpha]_D +22^\circ$ (chloroform). The yield was quantitative and the single product 63 was easily isolated just by conventional extractive processing. Therefore, if we can protect the hydroxyl group at C-6' with another protecting group, such as trityl ether, it will be possible to examine the coupling with KDO derivatives on both C-3' and 6' positions.

Acknowledgments

We are grateful to Mr. H. Nishiguchi, Mr. K. Nishihori, Mr. S. Murase, Mr. H. Ishida, and Mr. T. Kito for their effort which had led to the results described in this article. We would like to thank professor Laurens Anderson, of the University of Wisconsin, Madison, for valuable discussion and advice.

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Synthetic Studies on Structural Elements of the Hydrophobic Region Present in Bacterial Endotoxins

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The disaccharide α -D-(2-deoxy-2-[(3R)-3-hydroxytetradecanamido]- β -D-glucopyranosyl 4-phosphate)-(1 \rightarrow 6)-2-deoxy-2-[(3R)-3-hydroxytetradecanamido]-D-glucose was synthesized by two independent routes. In the first 2-[(3R)-3-acetoxytetradecanamido]-3,6-di-O-acetyl-2-deoxy-4-diphenylphospho- α -D-glucopyranosyl bromide was condensed with 2-[(3R)-3-acetoxytetradecanamido]-1,3-di-O-acetyl-2-deoxy- β -D-glucopyranose, followed by the removal of the protecting groups. In the second the amino groups of a 2-amino-6-O-(2'-amino-2'-deoxy- β -D-glucopyranosyl)-2-deoxy- β -D-glucopyranoside were converted to amido groups by treatment with (3R)-3-acetoxytetradecanoic anhydride; the resulting diamide was then condensed with benzaldehyde and acetylated to give the fully protected 4',6'-acetal. Removal of the benzylidene group, selective protection of position 6', followed by introduction of the diphenylphosphoryl group in position 4', gave the benzyl glycoside of the phosphorylated disaccharide from which, after removal of the protecting groups the free disaccharide was obtained. α and β 1-phosphates of 2-deoxy-2-[(3R)-3-hydroxytetradecanamido]-D-glucopyranose and P¹-2-deoxy-2-[(3R)-3-hydroxytetradecanamido]- α -D-glucopyranosyl-P²-(2-aminoethyl)-pyrophosphate were also prepared.

The aim of the work outlined below is to provide reference compounds of well-defined structures to be used in connection with the analysis of the

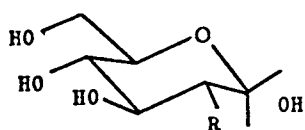
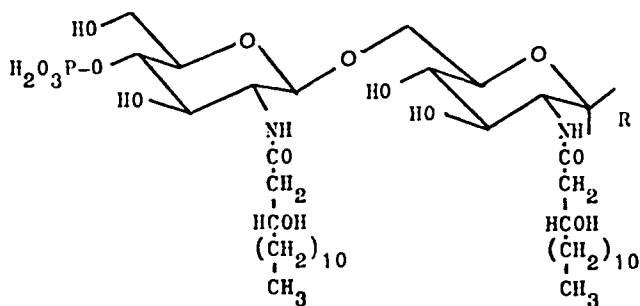
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Bordetella pertussis endotoxin, and with the ultimate hope of correlating structure and biological, in particular immunological, properties of lipopolysaccharides that compose this endotoxin.

The Bordetella pertussis endotoxin is made up of two, separable, protein-free lipopolysaccharides, present in the ratio of 3:2, which account for more than 90% of the endotoxin's mass (1), and a minor, third component, that can be detected by both electrophoresis in polyacrylamide gel in the presence of sodium dodecylsulfate (2) and immunoelectrophoresis. Although they differ in their composition and chemical reactions, the two major, separated lipopolysaccharides have, as far as investigated, identical biological activities. The disaccharide β -D-GlcpN(1 \rightarrow 6)D-GlcN, shown to be present in a great number of endotoxins (3), has also been identified in the 'Lipid A' fragments of both of the major lipopolysaccharides of the B. pertussis endotoxin (4). As chemical degradation established that at least the first of the glucosamine units carried a 3-hydroxytetradecanoic acid residue in amide linkage, and that the periodate resistant, phosphorylated glucosamine unit was also present in the hydrophobic fragment, the working hypothesis was adopted, that the phosphorylated disaccharide 1, identified in the Lipid A fragment of the endotoxin isolated from a deep rough mutant of E. coli by Rosner *et al.* (5), was also present in the pertussis endotoxin. Accordingly its synthesis was attempted.

The necessity of establishing the presence of a structural element present in many endotoxins may be questioned, but it should be remembered that B. pertussis is a rather singular microorganism in every respect (6), whose classification itself is uncertain, and it has been observed by MacLennan, who first isolated this endotoxin (7), that the lethal toxicity of this material was considerably lower than that of enterobacterial endotoxins, an observation confirmed by both Kasai (8) and Nakase (9). It was, therefore, considered imperative not to extrapolate results obtained with endotoxins of other bacteria, but to establish each structural feature of the pertussis endotoxin independently.

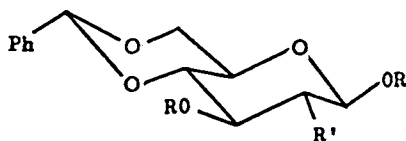
The synthesis of a disaccharide carrying a number of different substituents may be approached in two ways. One is to prepare the disaccharide first, and then to introduce the substituents, the other is to synthesize monosaccharides carrying the required substituents and then join them. The disaccharide 2, isolated by Rosner *et al.* (5) following mild acid hydrolysis of disaccharide 1, has been synthesized by both methods.



1 \sim R = OPO_3H_2 2 \sim R = OH

3 \sim R = NH_3Cl

4 \sim R = N=CHPh

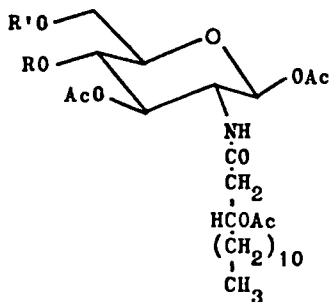


5 \sim R = H, R' = N=CHPh

6 \sim R = Ac, R' = N=CHPh

7 \sim R = Ac, R' = NH_3Cl

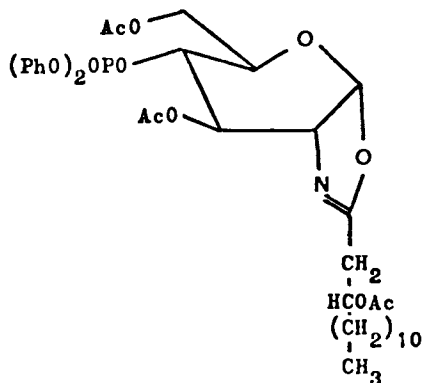
8 \sim R = Ac, R' = $\text{NH-CO-CH}_2\text{-HCOAc-(CH}_2\text{)}_{10}\text{CH}_3$



9 \sim R = R' = H

10 \sim R = H, R' = Ac

11 \sim R = PO(OPh)_2 , R' = Ac



12

In the first synthesis 2-amino-2-deoxy-D-glucose hydrochloride **3** was transformed into the benzylidene-amino derivative **4** (10), which, upon further treatment with benzaldehyde and zinc chloride gave the corresponding 4,6-benzylidene acetal **5**. Treatment of this with acetic anhydride in pyridine at 0-20°C for 16 h gave the β -diacetate **6** in accordance with the well known (11) directing effect of the N-benzylidene group. The latter was then removed by addition of one molar equivalent of hydrochloric acid to the diacetate **6** dissolved in acetone to yield the hydrochloride **7**. The complete sequence can be done by starting with one or two g moles of the sugar **3**, the crude products being used throughout. The overall yield for five steps was 50 to 60%.

The 3-hydroxytetradecanoic acid required in the next step was obtained as the racemic mixture from n-dodecanal and ethyl bromoacetate in a Reformatsky condensation (12), and saponification of the ester formed with potassium hydroxide. The enantiomers were then separated as their dehydroabietylamine salts according to Demary et al. (13). All reactions described below were carried out using first the racemic mixture of the acid, and, when reaction conditions were established, with pure (3R)-3-hydroxytetradecanoic acid.

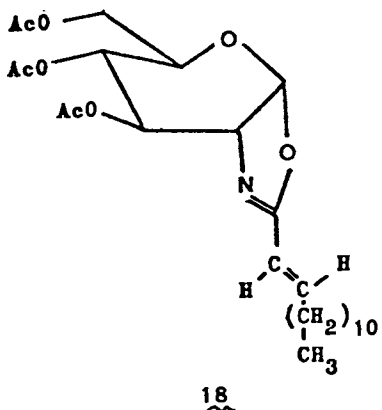
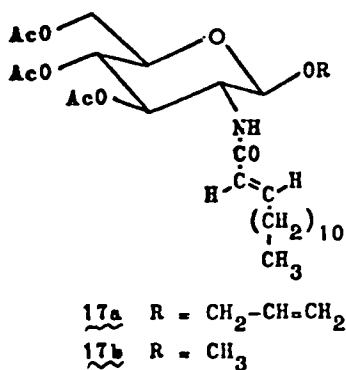
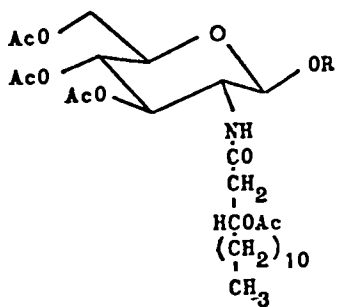
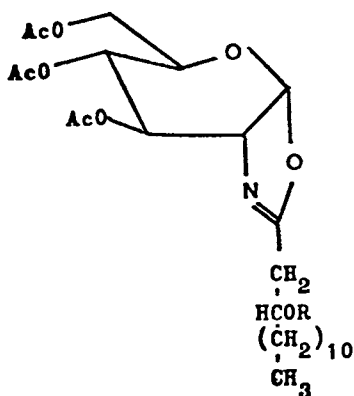
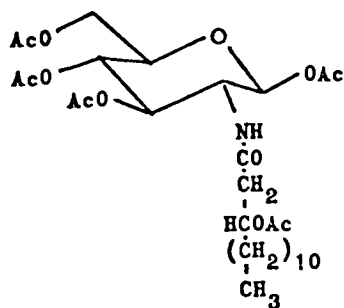
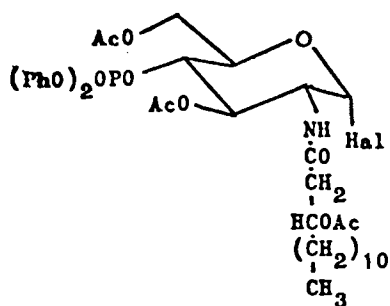
Condensation of 3-hydroxytetradecanoic acid with the hydrochloride **7** was attempted by a number of methods; the best results were obtained when the reaction was carried out in pyridine with equimolar quantities of the reagents, dicyclohexylcarbodiimide, and triethylamine, for 16 hours. The isolated, crude product was then acetylated with acetic anhydride-sodium acetate at 100°C for 1 hour, and the fully acetylated amide **8** was isolated by chromatography. The yield for the two steps was 70%. Removal of the benzylidene group by acid from **8** failed, the β -acetate group being lost simultaneously; it could, however, be accomplished by dissolving the acetal in hot ethanol and submitting it to hydrogenolysis at 50°C in the presence of palladium-on-charcoal. The diol **9** was isolated in 60% yield after chromatography of the crude reaction mixture on silica gel. Selective acetylation of the primary hydroxyl group of the diol **9** occurred when a solution of one molar equivalent of acetyl chloride in dichloromethane was added to the diol in dichloromethane containing one molar equivalent of pyridine; addition was carried out dropwise at -50°C and the reaction mixture was allowed to reach room temperature overnight. The tetraacetate **10** was isolated in 60% yield after column chromatography on silica gel.

Phosphorylation of the free hydroxyl group on C-4 of the tetraacetate **10** was not straightforward: it could,

however, be accomplished in benzene solution with two molar equivalents of diphenylphosphoryl chloride, if 1.2 molar equivalents of pyridine and 4-dimethylamino-pyridine were present. In these conditions phosphorylation was fast (16 h at 20°C) and no, or negligible color development was observed. The diphenylphosphate 11 was isolated in 80% yield. Inage *et al.* (14) have noticed previously that phosphorylation of the 4 position of an analogous disaccharide derivative was not easily accomplished.

From this point on two synthetic routes could be followed: compound 11 could be treated either to yield the oxazoline 12, or the α -halogeno sugar 13, both being expected to lead to the β 1,6-linked disaccharide upon condensation with the diol 9, because of the very low reactivity of the secondary alcohol of this diol.

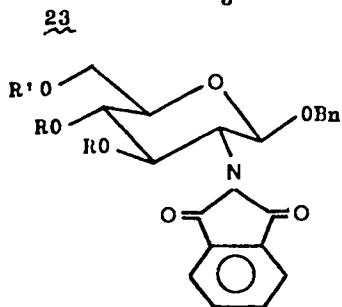
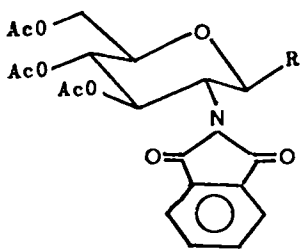
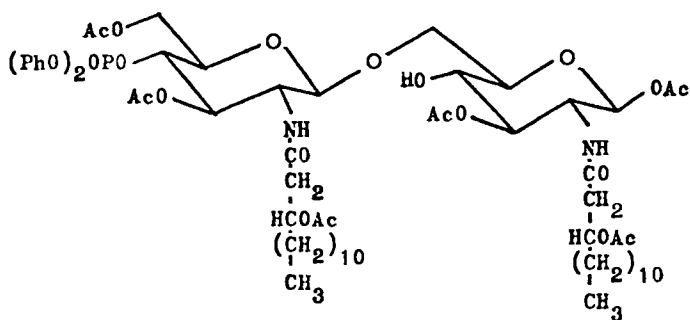
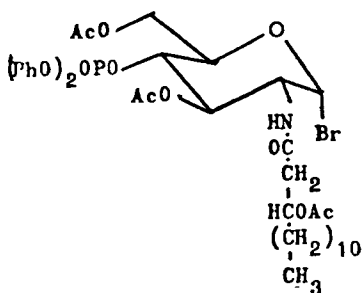
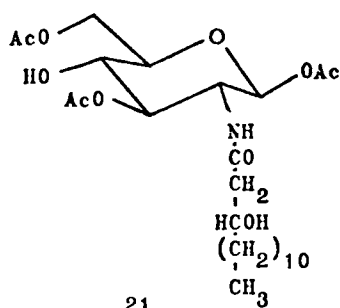
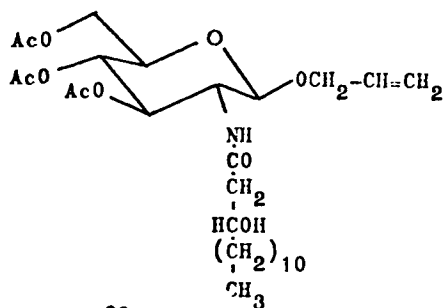
The phosphorylated β -acetate 11 was readily transformed into the oxazoline 12 upon treatment with ferric chloride and was stable enough to be purified by column chromatography on silica gel. However, upon attempts to condense it with the diol 9 in the presence of *p*-toluenesulfonic acid, more than one product was formed. Examination of the ^1H n.m.r. spectra of the major products isolated by t.l.c. led to the conclusion that at least in some of these products fatty acids with olefinic double bonds were present. The reaction was then further examined using, instead of compound 11, 2-(3-acetoxy-tetradecanamido)-1,3,4,6-tetra-O-acetyl-2-deoxy- β -D-glucopyranose 14. The oxazoline 15, formed upon its treatment with ferric chloride, when allowed to react with allyl alcohol (or methanol) in the presence of catalytic amounts of *p*-toluenesulfonic acid, gave essentially two products which were separated by column chromatography and identified. One was the expected β -glycoside 16a,b, in which the fatty acid still carried the acetoxy group attached to the fatty acid; in the other, however, which was also a β -glycoside, the acetoxy group originally present on the fatty acid was lost and the 3-acetoxytetradecanoic acid residue was transformed into a *trans*-tetradecenoic acid residue 17a,b. To find out whether the elimination reaction occurred before or during the condensation, the oxazoline 15 was treated with *p*-toluenesulfonic acid in the absence of any alcohol: elimination occurred rapidly, and the oxazoline carrying the olefinic fatty acid 18 was formed simultaneously; when this was isolated and allowed to react with allyl alcohol or methanol, the corresponding β -glycoside 17a,b was obtained in high yield. It was, therefore, concluded that during the condensation of oxazolines 12 and 15, two reactions occurred simultan-



eously: an acid catalysed elimination of the acetate group attached to the fatty acid residue followed by condensation of the oxazoline with the alcohol to yield β -glycosides with olefinic fatty acids, and direct condensation of the oxazolines with the alcohols to β -glycosides carrying the acetoxy-fatty acids. In a given set of conditions the rates of these two reactions will determine the proportion of the olefinic and acetoxyated products formed.

It is well known that the rate of an elimination reaction depends, among other things, on the nature of the leaving group. The acetoxy group being a better leaving group than the hydroxyl group, it was therefore considered that replacement of the acetoxy group by a non-protected hydroxy group could sufficiently reduce the rate of the elimination reaction to make the direct condensation the major, for practical purposes perhaps the only reaction. This was, indeed, found to be the case: the oxazoline 19 - prepared by condensation of 1,3,4,6-tetra-O-acetyl-2-amino-2-deoxy- β -D-glucopyranose and 3-hydroxytetradecanoic acid with dicyclohexylcarbodiimide and treatment of the product with ferric chloride - when allowed to react with allyl alcohol in the presence of *p*-toluenesulfonic acid gave the β -glycoside carrying the 3-hydroxytetradecanoic acid residue 20 in excellent yield. This result confirmed the previous demonstration by Kiso *et al.* (15) that 1,3,4,6-tetra-O-acetyl-2-deoxy-2-(3-hydroxytetradecanamido)- β -D-glucopyranose gave, upon treatment with benzyl alcohol and ferric chloride, the corresponding β -glycoside in quantitative yield. It is noteworthy that the oxazoline 19 did not undergo self-condensation despite the presence of a free, secondary alcohol within the molecule. As, however, selective phosphorylation of the 4-hydroxy group of compound 21 could not be achieved, we turned to the alternative approach, namely the transformation of the phosphorylated β -acetate 11 into an α -halogeno derivative.

Treatment of the β -acetate with an excess of hydrogen bromide in acetic acid diluted with chloroform, for 2 h at room temperature, gave, in apparently quantitative yield, the bromide 22 which appeared homogeneous upon t.l.c. and according to its n.m.r. spectrum. Because of its instability it was immediately condensed with the diol 9 in a 1:1 mixture of nitromethane-toluene and in the presence of mercury dicyanide and molecular sieve 4 Å; it gave the phosphorylated disaccharide 23. When the synthons 22 and 9 carried D,L-3-acetoxytetradecanamido groups the isolated yield was 20%, but it was reduced to only 7% when derivatives of the D-acids were made to react. It is thought that the lesser solubility



24 R = Cl
25 R = Br

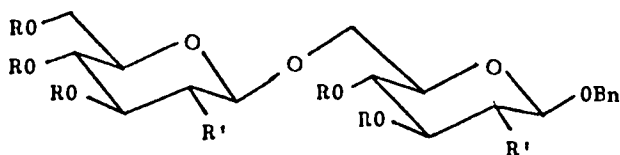
26 R = R' = H
27 R = Ac, R' = C(Ph)₃

of the synthons containing the D-acid in toluene-nitromethane is the main reason for the lower yield obtained with these derivatives.

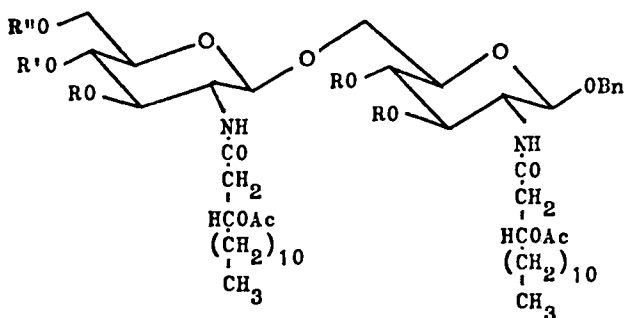
Deprotection of the disaccharide 23 was accomplished by cleaving off the phenyl groups by hydrogenolysis over platinum and the acetyl groups by treatment of the methanolic solution with ammonia. After removal of excess ammonia, the monoammonium salt of the unprotected disaccharide 2 was isolated by precipitation with acetone. The yield was 55%; $[\alpha]_D^{+21}$ (c, 0.255, 1:1 pyridine-methanol). Upon t.l.c. the material appeared to be homogeneous. The structure of this phosphorylated disaccharide is the same as that assigned to "Compound II" by Rosner *et al.* (5): in the chromatographic conditions used by these Authors, the synthetic compound had an R_f value similar to that found for "Compound II" isolated from an *E.coli* endotoxin.

In the second synthesis, in which the β 1,6-linked disaccharide was prepared first and the substituents introduced afterwards, 2-amino-2-deoxy-glucose hydrochloride was transformed into the well known (16) 3,4,6-tri-O-acetyl-2-deoxy-2-phthalimido- β -D-glucopyranosyl chloride (24), from which the corresponding benzyl glycoside was obtained. Deacetylation by Zemplén's method at 0°C gave the triol 26 which, when treated successively with triphenylmethyl chloride and then with acetic anhydride, both in pyridine solution, led to the triphenylmethyl ether 27. Condensation of this with 3,4,6-tri-O-acetyl-2-deoxy-2-phthalimido- β -D-glucopyranosyl bromide 25 (17) in the presence of silver perchlorate according to the method of Bredereck *et al.* (18) gave the fully protected disaccharide 28 in 65-70% yield. It is important not to remove the triphenylmethyl group before condensation, because during purification of the de-tritylated benzyl glycoside on silica gel acetyl-group migration invariably occurs and a mixture of disaccharides is formed in the subsequent condensation reaction. The protected disaccharide 28 gave, after removal of the acetate groups by Zemplén's method and the phthalimido groups by hydrazinolysis (19), the benzyl glycoside of the otherwise unsubstituted disaccharide 29, the yield for the two steps being 85-90%. In the 400 MHz ¹H n.m.r. spectrum the coupling constants J_{1,2} at δ 4.24 and J_{1',2'} at δ 4.30 were both 8 Hz as expected for β -glycosides.

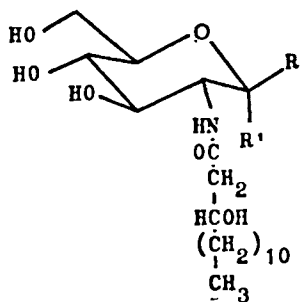
In the next step the 3-acetoxytetradecanoic acid residues were introduced by use of the corresponding anhydride which was prepared from the benzyl ester of the 3-hydroxy acid: this was acetylated with acetic anhydride-sodium acetate at 100°C for 2 h, the acetate



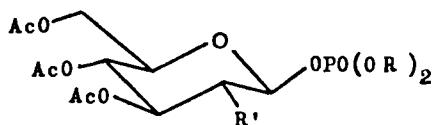
- 28 R = Ac, R' = phthalimido
29 R = H, R' = NH₂
30 R = H, R' = NH-CO-CH₂-HCOAc-(CH₂)₁₀-CH₃



- 31 R = H, R' = R'' = CHPh
32 R = Ac, R' = R'' = CHPh
33 R = Ac, R' = R'' = H
34 R = Ac, R' = H, R'' = CH₂OBn
35 R = Ac, R' = PO(OPh)₂, R'' = CH₂OBn



- 36 R = H, R' = OPO₃H₂
37 R = OPO₃H₂, R' = H



- 38 R = Bn, R' = phthalimido
39 R = H, R' = phthalimido

was isolated and the benzyl group was removed by hydrolysis; treatment of the acetoxytetradecanoic acid thus formed with dicyclohexylcarbodiimide then gave the required anhydride. The overall yield was about 80%.

Treatment, in methanol, of the diamino disaccharide 29 with this anhydride gave an 80% yield of the diamido-disaccharide 30. Although it could be crystallized, this compound was not easily handled, presumably because of its amphipathic nature. The corresponding hepta-acetate, which crystallised easily, was used to characterize the compound completely: indeed, its 400 MHz ^1H n.m.r. spectrum was of first order and all peaks could be assigned unambiguously. The 4' and 6' hydroxy groups of the diamido disaccharide 30 were then protected: upon treatment with α,α -dimethoxytoluene in N,N -dimethylformamide at 60°C it was transformed into the 4',6'-O-benzylidene derivative 31. The crude product, isolated in 95% yield, when acetylated with acetic anhydride in pyridine gave, after purification by column chromatography on silica gel, a 70% yield of the pentaacetyl derivative 32, from which the benzylidene group was removed by treatment with 80% acetic acid at 100°C for 90 minutes; a 90% yield of the crude diol 33 was recovered by lyophilisation. This without further purification, was dissolved in dichloromethane and treated with (benzyloxy)methyl bromide (20) and N,N,N',N' -tetramethylurea: the 6'-(benzyloxy)methylether 34 was obtained. Phosphorylation of the free hydroxyl group of the otherwise protected disaccharide 34 was accomplished in pyridine solution in the presence of 4-dimethylaminopyridine: the reaction was complete within 2 hours at room temperature. The diphenylphosphate 35 thus obtained was purified by column chromatography and recovered by lyophilisation from benzene. The overall yield from the pentaacetyl derivative 32 and by use of the crude products, was 47%.

The benzyl and phenyl groups were removed by hydrolysis over palladium and platinum catalysts, respectively, and the acetyl groups by treatment with ammonia in methanol. In this case too, the monoammonium salt of the phosphorylated disaccharide 2 was isolated; physical and chemical parameters of the compounds obtained by both methods were identical.

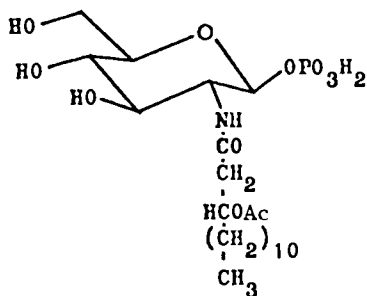
While these compounds may be useful as reference compounds for structural analysis of endotoxins and helpful for orienting investigations aiming at the establishment of the chemical structure/biological activity relationship of these substances, they are still far from representing any 'Lipid A' fragment and even less the hydrophobic region of an endotoxin. From

studies of a number of laboratories it would appear that besides the 4'-phosphate group, at least one other phosphoryl radical is usually attached to position 1 of the disaccharide β -D-GlcpN(1 \rightarrow 6)D-GlcN, known to be present in hydrophobic regions of endotoxins. Accordingly, attempts were made in the first place to elaborate appropriate syntheses for 2-deoxy-2-[(3R)-3-hydroxytetradecanamido]- α -, and - β -D-glucopyranosyl phosphates 36 and 37, hopefully applicable to the synthesis of phosphorylated disaccharides.

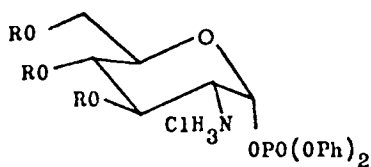
Both anomers of 2-acetamido-2-deoxy-D-glucopyranosyl phosphate have been prepared previously; however, the methods used are not easily adapted to obtain glucosamine derivatives carrying 3-hydroxytetradecanoic acid attached to the amino group.

The starting material for the β anomer 37 was 3,4,6-tri-O-acetyl-2-deoxy-2-phthalimido- β -D-glucopyranosyl chloride 24: it was treated, according to Baluja *et al.* (21), with silver dibenzyl phosphate to yield the β -dibenzyl phosphate 38. After removal of both benzyl groups by hydrogenolysis, the acetyl and phthalimido groups could be cleaved off by treatment of the glycosyl phosphate 39 with the calculated amount of hydrazine at 80°C for 2 h. The phosphorylated glucosamine salt formed was insoluble in methanol and was recovered; it was transformed into the triethylammonium salt. Upon addition of 3-acetoxytetradecanoic anhydride to an alcoholic solution of this salt in the presence of a slight excess of triethylamine, 2-deoxy-2-[(3R)-3-acetoxytetradecanamido]- β -D-glucopyranosyl 1-phosphate 40 was formed. The crude product was deacetylated with methanolic ammonia and the unprotected β -phosphate isolated as the monoammonium salt by column chromatography on silica gel.

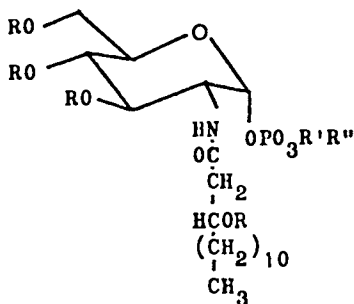
For the synthesis of the α -phosphate, 3,4,6-tri-O-acetyl-2-amino-2-deoxy- α -D-glucopyranosyl bromide hydrobromide (22) was condensed, according to Maley *et al.* (23), with the triethylammonium salt of diphenylphosphoric acid, and from the resulting phosphotriester 41, isolated as its hydrochloride, the phenyl groups were removed by hydrogenolysis. To the resulting methanolic solution of 3,4,6-tri-O-acetyl-2-amino-2-deoxy- α -D-glucopyranosyl phosphate 6 milliequivalents of triethylamine were added followed by 3 mmoles of (3R)-3-acetoxytetradecanoic anhydride: 3,4,6-tri-O-acetyl-2-[(3R)-3-acetoxytetradecanamido]-2-deoxy- α -D-glucopyranosyl phosphate 42 was formed. It was deacetylated *in situ* by saturating the solution with ammonia. The monoammonium salt of 2-[(3R)-3-hydroxytetradecanamido]-2-deoxy- α -D-glucopyranosyl phosphate 43 was isolated by column chromatography on silica gel. The yield, from the phosphotriester 41 was 60%.



40

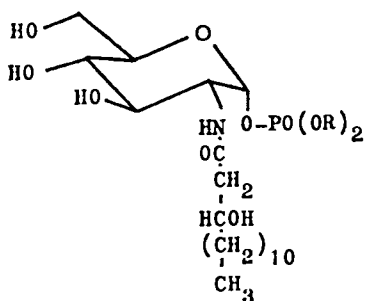


41 R = Ac

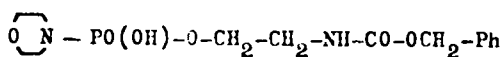


42 R = Ac, R' = R'' = H

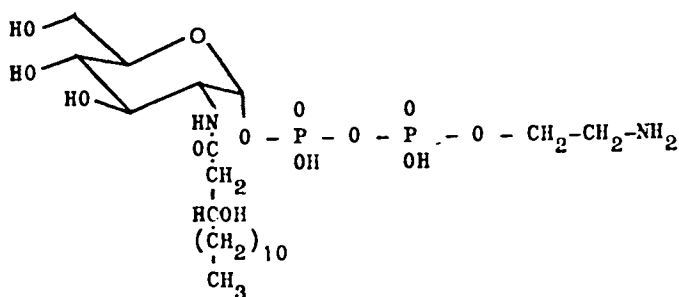
43 R = R' = H, R'' = NH₄



43a R = (n-C₄H₉)₃NH



44



45

It has been observed that Salmonella typhimurium endotoxin released ethanolamine phosphate upon treatment with dilute acetic acid (24). Later ethanolamine pyrophosphate and ethanolamine triphosphate were detected (25) when Pseudomonas aeruginosa endotoxin was similarly treated. Release of ethanolamine pyrophosphate also occurred from the Bordetella pertussis endotoxin in similar conditions (26). P^1, P^2 -pyrophosphoric acid diesters containing ethanolamine have been detected in both the core region (27) and the hydrophobic region (28) of endotoxins. As in the former case the other group esterified appears to be a secondary alcohol of a heptose unit, and as it is known that hydrolysis of the pyrophosphate bond is faster than that of the phosphate ester bond, it is not likely that the pyrophosphoryl ethanolamine found in hydrolysates of endotoxins should originate from such structures. On the other hand it has been reported that in the hydrophobic region of Salmonella sp. (29) and Vibrio cholerae (30) ethanolamine pyrophosphate was attached to the glycosidic position of a glucosamine unit. In order to investigate whether pyrophosphoryl ethanolamine can be set free from this type of structure, a glucosamine derivative carrying (3R)-3-hydroxytetradecanoic acid in amide linkage, and pyrophosphoryl ethanolamine in the α -glycosidic position, was synthesized.

The starting material was the tri-*n*-butylammonium salt of 2-deoxy-[(3R)-3-hydroxytetradecanamido]- α -D-glucopyranosyl phosphate 43a, which was condensed with 2-N-benzyloxycarbonylamino-ethyl-phosphomorpholidate 44 in pyridine solution. The pyrophosphate formed was isolated and the benzyloxycarbonyl group was removed by hydrogenolysis.

It has been shown by Rosner *et al.* (5) that the glycosidic bond of the 3-deoxy-D-manno-2-octulosonic acid (KDO) units present in the endotoxin of the deep rough mutant of Escherichia coli they investigated, was quantitatively cleaved in a 20 mM sodium acetate-acetic acid buffer at 100°C in 1 hour. These being the mildest conditions used so far in the analysis of endotoxins, the behaviour of the pyrophosphate 45 in these conditions was investigated. It was found that no cleavage of either the glycosidic or the pyrophosphate bond occurred. However, upon treatment of the same compound with 0.1M hydrochloric acid for only 5 minutes complete hydrolysis of the glycosidic bond took place, while a substantial proportion of the pyrophosphate linkage was preserved. It follows, that pyrophosphoryl ethanolamine released from certain endotoxins during acid hydrolysis may, indeed, originate from structures such as 45, but does

not prove it. Correlation of the kinetics of ethanol - amine pyrophosphate release with those of the appearance of a reducing glucosamine unit and their molar ratio during the process, would considerably strengthen the hypothesis.

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