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STRUCTURAL ELUCIDATION OF OUTER MEMBRANE LIPOOLIGOSACCHARIDES FROM NEISSERIA GONORRHOEAE

by

CONSTANCE M. JOHN

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

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Structural Elucidation of Outer Membrane Lipooligosaccharides from *Neisseria gonorrhoeae*

by Constance M. John

Abstract

Neisseria gonorrhoeae, the causative agent of gonorrhoea, is a serious pathogen worldwide. A vaccine for gonorrhoea is critically needed given the increased resistance of gonococci to antibiotics and problems in identifying and treating this disease in developing countries. As major cell surface components, the gonococcal lipooligosaccharides (LOS) are potential vaccine targets.

This thesis details efforts to determine the structural basis for LOS variation. Reducing the quantity of sample required to determine the diversity of the oligosaccharide components of gonococcal LOS was a major focus because the LOS are highly heterogeneous, exhibiting both inter- and intra-strain variability. Hydrazino reagents for oligosaccharide derivatization were developed and shown to improve chromatographic and liquid secondary ion mass spectrometric (LSIMS) analyses. High-performance anion exchange chromatography with pulsed amperometric detection was also applied to improve oligosaccharide separation. To analyze the intact LOS, *O*-deacylation of the lipid moiety was performed prior to LSIMS and methylation analysis.

Structural elucidation of the oligosaccharides from the LOS of F62, a prototype gonococcal strain, revealed two main oligosaccharide components that differ by a non-reducing terminal GalNAc. The smaller structure is bound by monoclonal antibody 3F11, which recognizes glycosphingolipids containing lacto-N-neotetraose, such as paragloboside, a precursor to the ABH blood groups.

Elucidation of the structures of LOS from 1291 wild type and five pyocin-resistant mutant strains of 1291 showed the wild type oligosaccharide to be the same as the smaller F62 component. The oligosaccharides of the mutants differ by a series of single saccharide deletions and are presumed to be biosynthetic intermediates. One exception, mutant strain 1291_b, produced an LOS that varied by more than simple saccharide truncation. This LOS was recognized by a monoclonal antibody that binds the P^k antigen, another blood group antigen.

Similarities in LOS from these gonococcal strains and human glycosphingolipids suggest that the LOS is acting as a host mimic. This could aid the bacteria in evading the host immune system, or increase binding of the bacteria to host membranes. Structural elucidation of LOS from additional strains of *N. gonorrhoeae* will be required to correlate pathology and immunochemistry with the heterogeneous LOS.

List of Abbreviations

°	degrees
µg	micrograms
µl	microliters
µm	micrometers
1D	one-dimensional
2D	two-dimensional
A	amperes
Å	Angstroms
ABEE	4-aminobenzoic acid ethyl ester
ACE	alternating chemical and electron impact ionization
A.D.	years after the birth of Christ
B/E	a linked mass spectrometric scan in which both the magnetic and electric fields are scanned at a constant fixed ratio
BPH	4-butylphenylhydrazine
C	centigrade
CD	circular dichroism
CDC	Centers for Disease Control
CI	chemical ionization
CID	collision induced dissociation
CMP-NANA	cytidine monophospho-*N*-acetylneuraminic acid
COSY	2D correlation spectroscopy
cm	centimeters
Da	daltons
DHHS	Department of Health and Human Services
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
EI	electron impact ionization
ELISA	enzyme-linked immunoassay
eV	electron volts
FAB	fast atom bombardment
FD	field desorption
FID	flame ionization detection
g	grams
g	gravity
Gal	galactose
GalNAc	*N*-acetylgalactosamine
GC	gas chromatography
GC-MS	gas chromatography and mass spectrometry
Glc	glucose
GlcNAc	*N*-acetylglucosamine
h	hours
HBEE	4-hydrazinobenzoic acid ethyl ester
Hep	heptose

Hex	**hexose**
HexNAc	***N*-acetylhexosamine**
HIV	**human immunodeficiency virus**
HOHAHA	**homonuclear Hartmann-Hahn 2D spectroscopy**
HPAE-PAD	**high performance anion exchange chromatography**
with	**pulsed amperometric detection**
Hz	**hertz**
i.d.	**internal diameter**
IgA	**immunoglobulin A**
IgG	**immunoglobulin G**
IgM	**immunoglobulin M**
KDO	**3-deoxy-*manno*-2-keto-octulosonic acid**
keV	**kilo electron volts**
LD	**laser desorption**
LSIMS	**liquid secondary ion mass spectrometry**
LOS	**lipooligosaccharide**
LPS	**lipopolysaccharide**
M	**molar**
mA	**milliampere**
MAb	**monoclonal antibody**
mbar	**millibar**
min	**minutes**
ml	**milliliter**
mN	**millinormal**
mM	**millimolar**
m/Δm	**mass to be measured divided by the difference in the masses to be identified with a 10% valley between them**
mΩ	**milliohm**
Mr	**molecular mass**
ms	**milliseconds**
MS	**mass spectrometry**
MS/MS	**tandem mass spectrometry**
m/z	**mass to charge ratio**
N	**normal**
NOE	**nuclear Overhauser effect**
NOESY	**2D nuclear Overhauser effect spectroscopy**
nm	**nanometer**
nmol	**nanomole**
NMR	**nuclear magnetic resonance spectroscopy**
OPH	**4-octylphenylhydrazine**
PAGE	**polyacrylamide gel electrophoresis**
PI	**gonococcal outer membrane protein I**
PII	**gonococcal outer membrane protein II**
PIII	**gonococcal outer membrane protein III**
PD	**plasma desorption**
PID	**pelvic inflammatory disease**
PEA	**phosphoethanolamine**
PFK	**perfluorokerosene**
PMAA	**partially methylated alditol acetate**

PMN	polymorphonuclear leukocyte
pmol	picomole
PPEADP	phosphotidyl ethanolamine dipalmitoate
ppm	part per million
p.s.i.	pounds per square inch
RI	refractive index detection
s	seconds
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SECSY	2D spin echo correlated spectroscopy
TDPH	4-tetradecylphenylhydrazine
TFA	trifluoroacetic acid
THF	tetrahydrofuran
UV	ultraviolet light
V	volts
VIS	visible light
WHO	World Health Organization
w/v	weight per volume
w/w	weight per weight

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CHAPTER I. INTRODUCTION

A. Etiology

Gonorrhoea is an ancient disease which was described and given its present name in 150 A. D. by the Greek physician Galen. The gonococcus was discovered in discharges from infected patients by Neisser in 1879. Then in 1885 Bumm demonstrated that the gonococcus is the causative agent of gonorrhoea by cultivating the organism and producing the disease in inoculated human subjects. Both *Neisseria meningitidis,* which causes meningococcal meningitis, and *Neisseria gonorrhoeae* are pathogenic species of the genus *Neisseria.* They are Gram-negative aerobic, or facultatively aneorbic diplococci. Both species are parasites of human mucous membranes and do not occur outside of their human hosts in nature.

Currently, the standard method for diagnosis of gonorrhoea is culturing the bacteria *in vitro.* This is because the culture technique is very sensitive and can also be used for *in vitro* tests for antibiotic susceptibility (Easmon and Ison 1987). Identification is made by the presence of Gram-negative diplococci that are positive for cytochrome oxidase. Gonococci require cysteine and iron. Of the carbohydrates they can only utilize glucose. Carbohydrate utilization tests are another method used in identification of colonies of gonococci (Easmon and Ison 1987). With infected males, Gram-stains of urethral discharges have been used with good sensitivity (Goodhart et al. 1982, Manis, Harris, and Gieseler 1984). Other diagnostic methods which are available include coagglutination reagents (Young and Reid 1984), biochemical tests for aminopeptidase

activity (Janda et al. 1985), preformed enzymes, enzyme-linked immune assays (ELISA) (Schachter et al. 1984), immunofluorescence (Ison, McLean, and Gedney 1985) and DNA probes (Easmon and Ison 1987, Totten et al. 1983).

Traditional serological methods, such as phage or bacteriocin typing or classification using simple polyclonal reagents have not been useful for epidemiological studies of *N. gonorrhoeae* (Easmon and Ison 1987). The methods most widely used in typing *N. gonorrhoeae* are auxotyping, which is based on the nutritional requirements of the gonococci when grown on chemically defined media (Catlin 1973), protein 1 serotyping using polyclonal or monoclonal antibodies (Buchanan and Hildebrandt 1981, Knapp et al. 1984) and plasmid analysis (Easmon and Ison 1987). Other techniques which have been used in identification of serotypes are determination of the antibiotic resistance pattern of the strain (Easmon and Ison 1987), variation in their lipooligosaccharide (LOS) components (Apicella 1976) and patterns of lectin agglutination (Schalla et al. 1985). Reference to a strain of *N. gonorrhoeae* in this thesis implies origin from a single cloned organism.

B. Pathology

Gonorrhoea is chiefly transmitted by sexual intercourse. From a single sexual exposure the probability of transmission of gonorrhoea is 50-70% from an infected male and 20-30% from an infected female (Weisner and Thompson III 1980). In approximately 90% of males within two to eight days after infection gonorrhoea will produce symptoms. Approximately 5% of the infected males have only very mild symptoms, and

the remaining 5% are completely asymptomatic but infectious (Hethcote and Yorke 1984). Most commonly the disease is localized to the point of inoculation and, for instance, in males can produce acute urethritis, with difficulty and pain in urination and a purulent penile discharge containing numerous polymorphonuclear leukocytes (PMNs). If untreated such an infection can spread to the seminal vesicles and epididymis, and can lead to urethral stricture (Walter 1982).

Gonococcal infections often present more problems to females. In the beginning of an infection in females the disease is also localized to the point of infection, where it can cause, for example, urethritis and cervicitis. Gonococcal infections are most often initially asymptomatic in females (Wilfert and Gutman 1981). These asymptomatic women often remain untreated until the spread of the infection causes acute salpingitis (inflammation of the fallopian tubes) and associated peritonitis, pelvic inflammatory disease (PID) or disseminated gonorrhoea, or they are uncovered by contact tracing or mass screening. From 10% to 20% of women with cervical gonorrhoea develop PID (Westrom 1980). PID occurs when the fallopian tubes become swollen and inflamed from infection and can be caused by a number of organisms, among them *N. gonorrhoeae*. The gonococcal infection spreads from the vagina and cervix to the uterus and during menstruation to the fallopian tubes. Upon healing, scarring of the fallopian tubes can result in blockage, infertility or ectopic pregnancies. Also, chronic pelvic pain can occur due to adhesions on the ovaries, bowel or other organs. At least 25% of females with PID experience one or more of the following: chronic abdominal pain, ectopic pregnancy and infertility (Westrom 1980).

There are two types of infection from *Neisseria gonorrhoeae* not sexually transmitted. The first, acute valvovaginitis, is a gonococcal infection occurring in females prior to puberty and is transmitted by towels or clothing when shared. An effective bactericidal barrier to the infection is provided by the acid pH of the vagina associated with the hormones of sexual maturity. The second type of infection occurs in the eye as conjunctivitis and opthalmitis. Opthalmia neonatorum is a gonococcal infection of the conjunctiva of a newborn baby that is acquired during delivery. The disease can in almost all cases be prevented by a single application of erythromycin or tetracycline opthalmic ointment to the eyes after birth (Brooks 1985a). The eyes of about 30% of the infants exposed to *N. gonorrhoeae* during birth will become infected if prophylaxis is not provided, and if untreated the infection often causes blindness. Previously, instillation of a 1% silver nitrate solution into the eyes after birth was used for prophylaxis (Wilfert and Gutman 1981). Now, tetracycline or erythromycin is preferred as the 1% silver nitrate solution is relatively unstable and can irritate the conjunctiva. Treatment with penicillin G is not recommended because the amount of future penicillin allergy has been found to increase if penicillin G is used in the eye (Brooks 1985a). Opthalmia neonatorum was the cause of blindness in approximately 24% of the individuals admitted to schools for the blind in the U. S. in 1906-1911, and in only 0.5% of those admitted in 1951-1955 after prophylaxis was routinely administered (Wilfert and Gutman 1981). Other complications can also arise from neonatal gonococcal infections (Brooks 1985a). In adult patients gonococcal conjunctivitis has also been observed, generally transmitted to the eyes by their own hands (Walter 1982).

On a molecular level, infection of the mucosa is characterized by the gonococci crossing the epithelial columnar cells and reaching the submucosa and has been most closely studied in cell tissue culture of human fallopian tubes (Cooper and Jeffery 1985, Cooper, McGraw, and Melly 1986, Ward, Watt, and Robertson 1974). The epithelium of the fallopian tube consists of both ciliated and nonciliated cells in varying proportions depending on the location of the cells. The invasion by gonococci was followed by using scanning and transmission electron microscopy; invasion was found to begin approximately 24 hours after infection, with exocytosis of intact gonococci on the subepithelial or basement membrane after 72 hours of infection (Cooper, McGraw, and Melly 1986). The first step is attachment to the microvilli of nonciliated mucus-producing cells in the epithelium. Then in a second, closer attachment step the microvilli retract, and the gonococci are engulfed in a membrane bound vesicle by the normally nonphagocytic epithelial cells (McGee and Horn 1979). The gonococci are transported through the cell in the phagosomes and exocytosed onto the subepithelial tissue (Cooper, McGraw, and Melly 1986, McGee et al. 1983). No other microorganism is known to occupy phagosomes of normal epithelial cells, whose lysosomal enzymes are not as diverse as those of normally phagocytic cells (Gotschlich 1984). By this mechanism the gonococcus overcome a major barrier of the host, the mucosal epithelium (Blake and Gotschlich 1983).

The first attachment step has been found to be mediated by pili, proteinaceous fimbriate outer membrane components. Both piliated and nonpiliated phenotypes of a strain can be found; however, the inoculum of the piliated version required to cause infection is significantly more than

that of the nonpiliated version (Pearce and Buchanan 1978, Schoolnik, Tai, and Gotschlich 1983, Swanson 1973).

It is not clear which gonococcal outer membrane components are responsible for the second, close attachment, although some evidence suggests the major ligand is protein II (Elkins and Rest 1990, Virji and Heckels 1986). Contradictory evidence also exists which indicates protein II is only associated with binding (Draper 1984, Draper et al. 1980). The ability of a small number of gonococci to survive and multiply in human phagocytic cells (polymorphonuclear leucocytes or PMNs) has been the subject of a number of studies (Casey, Shafer, and Spitznagel 1986, Shafer 1988).

During the migration of the gonococci to the submucosa there is extrusion of ciliated cells from the endothelium. Studies of cell tissue cultures of human fallopian tube mucosa have demonstrated that the LOS and peptidoglycan, both shed by growing gonococci, damage fallopian mucosa by causing loss of ciliary activity and sloughing of ciliated epithelial cells. A minimum dose of purified disaccharide peptide monomers of gonococcal peptidoglycan for damage to ciliary activity was found to be 0.75 μg/ml (Melly, McGee, and Rosenthal 1984). As little as 0.015 μg/ml of gonococcal LOS caused maximal damage to fallopian tube mucosa in organ culture; more extensive toxicity occurred during infection with living gonococci (Gregg et al. 1981). Using light microscopy with immunoperoxidase staining direct evidence has been obtained of attachment, entrance and damage to human fallopian tube mucosal cells by gonococcal LOS in tissue culture (Cooper and Jeffery 1985, Cooper, McGraw, and Melly 1986, Gregg et al. 1981). However, gonococci and gonococcal LOS were not damaging to lapine, porcine or bovine oviduct cells

in tissue culture (Gregg et al. 1981, Johnson, Taylor-Robinson, and McGee 1977, Taylor-Robinson et al. 1974). This result suggests that host specificity is a significant factor in the toxicity of the LOS.

Most of the pathogenic effects of the endotoxin, either lipopolysaccharide (LPS) or LOS, from Gram-negative bacteria are due to the lipid A moiety. Lipid A causes a large number of biological responses, some of which are not harmful (Galanos et al. 1977). The structure of the lipid A moiety is largely conserved in bacteria, differing mainly in fatty acid composition and degree of phosphorylation. The biological activity of free lipid A was found to be very nearly equal intact LPS, whereas free oligosaccharide exhibited no toxicity (Galanos et al. 1977). The degree of sensitivity to lipid A is species dependent. For example, horses, rabbits, dogs and human beings are all very sensitive to endotoxin, whereas some strains of rats, mice and primates exhibit much less sensitivity ($1>10^7$) (Galanos et al. 1977). Some of the biological effects of lipid A are as follows: pyrogenicity, septic shock, blood pressure depression, leukopenia and luekocytosis, bone marrow necrosis, platelet aggregation, and B-cell mitogenicity. Lipid A causes induction of the activity of a number of different biological components: macrophages, prostaglandin and interferon synthesis, tumor necrosis factor, plasminogen activator and complement (Galanos et al. 1977).

In approximately 1% of those infected with *N. gonorrhoeae* the infection disseminates, invading the bloodstream, the skin, joints and various organs. The most common symptom of disseminated infection is acute septic arthritis. The strains causing disseminated gonorrhoea are generally not susceptible to the bactericidal action of normal human serum and complement (Rice and Goldenberg 1981, Rice, McCormack, and Kasper

1980, Schoolnik, Buchanan, and Holmes 1976) are of the transparent phenotype, and tend to belong to a certain protein I serotype (Buchanan et al. 1980). Induction of serum resistance by sialylation of specific LOS epitopes within human tissues has been proposed (Mandrell et al. 1990, Parsons et al. 1988, Schneider et al. 1985)

The bacteria that produce disseminated infection often are locally asymptomatic (O'Brien, Goldenberg, and Rice 1983, Rice and Goldenberg 1981), and serum sensitive strains are more likely to produce severe localized infections and acute salpingitis (Rice, McCormack, and Kasper 1980). Binding to the complement proteins C5-9 by both serum resistant strains and serum sensitive strains is equivalent; however, the binding does not adversely affect the serum resistant strains (Harriman et al. 1982). Evidence has been presented that in cases of disseminated disease there is a delay or decrease in the amount of complement-dependent neutrophil chemotaxis (Densen, Gulati, and Rice 1987, Densen, MacKeen, and Clark 1982).

C. Epidemiology

In the United States during the 1960s reported gonorrhoea rates increased at roughly 15% per year. A national control program was begun in 1972, and in 1976 the number of reported cases began to slowly decline and has continued to decline throughout the 1980s. The total number of cases reported to the Centers for Disease Control (CDC) decreased from 999,937 in 1975 to 878,556 in 1985 (Rice et al. 1987). In 1975 the incidence of gonorrhoea was about 450 cases per 100,000; the goal set by the CDC of reduction to 280 cases per 100,000 for 1990 is likely to be achieved (CDC and

DHHS 1990, CDC and DHHS. 1987). Nevertheless, gonorrhoea cases constitute over half of all the reported communicable diseases in the U.S. Polls of practicing physicians and surveillance studies have shown that reporting of the disease is incomplete and indicate that the annual incidence of gonorrhoea is closer to twice that reported (Hethcote and Yorke 1984).

Annual prevalence of gonorrhoea as high as 26% of the population has been reported in developing countries of Asia, Africa, and South America, and Central America, even though reporting is very incomplete (WHO 1978). In these countries health care resources are lacking for treatment of gonorrhoea or contact tracing. Throughout Africa 10-20% of pregnant women are estimated to have gonorrhoea, with reported prevalence in pregnant women ranging from 3% to 22% (Schulz, Cates Jr., and O'Mara 1987). Therefore, numerous cases of blindness due to opthalmia neonatorum occur in much of the developing world (Schulz, Cates Jr., and O'Mara 1987).

In the United States and Europe in the period 1960-1980 acute salpingitis was found to be an etiologic factor in 30-40% of cases of female infertility and in 40-50% of ectopic pregnancies (Westrom 1980). Pelvic inflammatory disease and acute salpingitis have multifactorial microbial etiology. Among the organisms which produce acute salpingitis (and PID) are *N. gonorrhoeae, Chlamydia treachomatis,* and *Mycoplasma hominis,* which together account for more than 75% of all PID in women under 25 years of age in some areas of Sweden (Westrom 1980). Gonorrhoea alone was found to to be the cause of one-half of the PID cases in some areas of the United States (McCormack 1979). Among women who have had one episode of PID, 12.6% of the 15-24 year old group and 25% of the 25-34 year

old group will be infertile or have an ectopic pregnancy (Westrom 1980). To illustrate the significance of these figures for a current age group the following example is used. One of every 28 females born in 1950 in Europe and the United States would eventually fail to have desired children due to PID and acute salpingitis (Westrom 1980).

The amount of antibiotic resistance in gonococci has greatly increased in the last decade throughout the world. Both chromosomally-mediated and plasmid-mediated resistance occur (Easmon and Ison 1987). Chromosomally-mediated resistance results from random selection of mutants with altered chromosomes. These alterations have increased antibiotic resistance, for example, by affecting the target site of a drug (Cannon 1985), or by reducing the permeability of the outer cell membrane (Judson 1989). Until recently chromosomally-mediated resistance could almost always be overcome by larger doses of antibiotics (Judson 1989). For example, between 1945 and 1972 the standard amount of penicillin used to treat gonorrhoea increased from 100,000 units to 4.8 million units of procaine penicillin G (Judson 1989).

Infections due to penicillinase-producing *N. gonorrhoeae* are increasing. These bacteria are resistant to levels of penicillin and ampicillin greater than those achievable in the serum (Cannon 1985) due to their production of the enzyme β-lactamase, which cleaves the β-lactam ring of penicillin or ampicillin. Plasmids confer the genes for production of this enzyme (plasmid-mediated resistance). Plasmids encoding for the production of β-lactamase had been found previously in other bacteria such as *Haemophilus*. Thus, this may be an example of exchange of genetic material between two different species (Cannon 1985, Judson 1989). Penicillinase-producing *N. gonorrhoeae* first appeared in the United States

in 1976 and have been steadily increasing. In 1986 in Florida, 7% of all gonococcal infections were found to be caused by penicillinase-producing bacteria, with rates of 50% in some counties (CDC 1987). Similar levels of plasmid-mediated resistance had been observed in Asia years earlier. Between 1979 and 1987 the reported number of cases in the United States of penicillinase-producing *N. gonorrhoeae* increased from under 1,000 to over 25,000 (Judson 1989). In California the number of penicillinase-producing infections reached 1% of all reported cases by the end of 1986 in more than a dozen health jurisdictions (Kizer et al. 1989). Resistance to other antibiotics, such as tetracycline, has also been observed (Easmon and Ison 1987), with the first report in the United States of plasmid-mediated resistance to tetracycline in 1985 (Judson 1989). The increasing resistance to antibiotics is especially problematic in the developing countries, as the newer antibiotics tend to be much more costly than the older ones such as tetracycline; also, laboratory antibiotic susceptibility tests are more often required.

Antibiotic resistance was evaluated in 6204 isolates of *N. gonorrhoeae* from 21 U.S. clinics between September 1987 and December 1988 for the Gonococcal Isolate Surveillance Project, a national survey to determine levels of and trends in gonococcal antibiotic resistance and make treatment recommendations (Schwarcz et al. 1990). At least one of the criteria for resistance to penicillin, tetracycline, ceftoxin, or spectinomycin was met by 21% of the isolates; 16.8% exhibited chromosomally-mediated resistance without plasmid-mediated resistance; 2.2% were penicillinase-producing; 1.0% had high-level plasmid-mediated tetracycline resistance; three isolates were resistant to spectinomycin. No resistance was found to ceftriaxone (Schwarcz et al. 1990). The number and wide range of

resistance in isolated strains of *N. gonorrhoeae* support recent recommendations by the CDC that tetracycline, penicillin, ampicillin, and amoxicillin not be used in treatment of gonorrhoea. The CDC suggests that uncomplicated infection be treated with a single 250 mg dose of ceftriaxone intramuscularly, in combination with doxycycline 100 mg orally twice daily for 7 daily for days (CDC 1989).

A report on plasmid-mediated tetracycline and penicillin resistance in the U.S. in 1989 was recently published by the CDC (CDC 1990). All clinics reported penicillin plasmid-mediated resistance, which was found in 7.4% of the isolates. Tetracycline plasmid-mediated resistance was found in 4.9% of the isolates. The highest incidences of penicillin plasmid-mediated resistance were reported in Long Beach (32%), Atlanta (18%), and Boston (15%) (CDC 1990).

Control efforts have had an impact on the prevalence of gonorrhoea in the United States (CDC and DHHS 1990, Wilfert and Gutman 1981). Major efforts are needed in some of the developing countries where resources for health care are minimal. The costs of the disease all over the world in terms of both health care dollars and human suffering are staggering, although, the current worldwide epidemic of human immunodeficiency virus (HIV) is much more deadly. A coordinated effort to control all sexually transmitted diseases would have positive results as the same methods are used to prevent the various sexually transmitted diseases, and the same groups and individuals are at risk for infection. There is some evidence that those diseases which cause genital ulcers, such as syphilis and *Haemophilus ducreyi*, may be independent risk factors which cause increased transmission of HIV (Pepin et al. 1989). Such correlations are extremely difficult to establish because of the role of

behavior in the transmission of these diseases and in the differences in susceptibility to infection and transmission between men and women. In Africa the rate of heterosexual transmission of HIV appears to be much greater than in the United States, though high-risk sexual practices such as anal intercourse are not common (Pepin et al. 1989). Although behavioral and physical differences could cause altered susceptibility to HIV, the possibility that genital infections predispose to HIV transmission provides another reason to coordinate and increase control efforts for all sexually transmitted diseases.

Development of a vaccine against gonorrhoea represents a rational and perhaps preferable alternative to the continued use of antibiotics given the increased antibiotic resistance of gonococci and the inherent problems of identifying and treating this disease in the developing countries. In 1984 a National Institutes of Health Vaccine Panel recommended that the goal in development of a gonococcal vaccine should first be to provide protection for women against PID and secondly to provide mucosal immunity in men (Schoolnik and McGee 1985). During the first half of this century trials of a number of different vaccines were conducted without significant success. A more recent test in 62 human volunteers of a vaccine composed of killed whole bacteria from three different strains of *N. gonorrhoea* also failed to show the development of immunity from infection (Greenberg et al. 1974).

Because immunization with killed whole bacteria has not been effective, some preliminary experiments have been performed to evaluate the potential of some of the individual outer membrane components as vaccines, or for inclusion in a multi-component vaccine. The immunogenicity of outer membrane protein I was evaluated in mice with different lipoidal adjuvants in liposomes and complexes (Jiskoot et al. 1986)

and in rats with adjuvants (Jeurissen, Sminia, and Beuvery 1987). In both trials the protein was found to be immunogenic. Administration of a vaccine composed of pilus to human volunteers resulted in the production of specific genital antibodies of all immunoglobulin classes (McChesney et al. 1982). In another study rabbits were immunized with conjugates of oligosaccharides which had been released by acid hydrolysis from the gonococcal LOS and then covalently bound to either gonococcal pilus or bovine serum albumin. Antibodies to both the intact LOS and the pure proteins were produced (Lambden and Heckels 1982). The *O*-deacylated gonococcal LOS was found to retain antigenic determinants for human opsonic antibodies and to be much less toxic than intact LOS using two different biological assays. Antibodies were produced to the *O*-deacylated LOS in mice after vaccination with conjugates composed of protein covalently linked to the *O*-deacylated LOS (Seid and Sadoff 1981).

Considerable research has been focused on the structure, function, variability, immunogenicity, genetic control and interaction of the components of the outer membrane of *N. gonorrhoea*e. The design and development of a successful vaccine may be dependent on the insights and knowledge derived from studies of the outer membrane components. As more is discovered about the diversity and complexity of the gonococcus it becomes apparent that a multicomponent vaccine, containing multiple serotypes of an outer membrane component and/or more than one outer membrane component may be required.

Experimental evidence suggests that the bactericidal activity of normal human serum against gonococci is primarily associated with activation of the classical complement pathway. Activation of the alternate

pathway also occurs, but such activation is delayed and may be antibody dependent (Ingwer, Petersen, and Brooks 1978).

A number of characteristics of the outer membrane components have to be determined in order to understand their role in the pathogenesis and immunochemistry of gonorrhoea. For example, is the expression of one particular type of outer membrane component a stable attribute of a strain of the bacteria? Also, what is the total extent of the heterogeneity in the outer membrane component expressed *in vivo* by a bacterial strain at any one time and over the course of an infection? Another question to be raised in investigation of variable heterogeneous components of bacteria concerns how a bacterial colony can be maintained so that the expression of the outer membrane components is the same as *in vivo* or the same as the point at which the bacteria was isolated. Many of these problems have been addressed by the use of monoclonal antibodies specific for epitopes found in particular serotypes of the outer membrane components. Structural elucidation of the outer membrane components has in some cases also provided or helped to provide some answers.

D. The outer membrane components of *Neisseria gonorrhoeae*

The cell wall of Gram-negative bacteria such as *N. gonorrhoeae* is composed of two membranes, each a lipid bilayer, one on either side of the rigid peptidoglycan layer (see Fig. 1). The outer membrane of Gram-negative bacteria has been found to consist generally of protein, lipopolysaccharides (LPS) and lipooligosaccharides (LOS), and phospholipids. In *Escherichia coli* and *Salmonella typhimurium* the phospholipids were mainly found in the innermost bilayer of the cell

membrane and the LPS mainly in the outer bilayer (Funahara and Nikaido 1980). Assuming that the outer membrane of the gonococcus has a similar organization, then the cell structures mainly exposed are the LOS and proteins.

Although several different groups have reported the demonstration of gonococcal capsules by the use of the wet india ink technique (Hendley et al. 1977, James and Swanson 1977, Richardson and Sadoff 1977), the capsular material has never been isolated. Considerable effort has been put forth trying to isolate the presumed polysaccharide capsule to no avail. It appears unlikely that a capsule does exist on the gonococcus (Gotschlich 1984).

Gonococci, uniquely among the Gram-negative bacteria, enzymatically break down their peptidoglycan into fragments which are released into the surrounding environment (Rosenthal 1979). In rats the gonococcal peptidoglycan fragments have been found to cause arthropathologies, or joint diseases. Therefore, it has been suggested that the peptidoglycan fragments may play a role in the pathogenesis of the arthritis, which is frequently a symptom of disseminated gonococcal infection (Fleming, Wallsmith, and Rosenthal 1986). Recently, the structures of a number of these biologically active fragments were determined using fast atom bombardment mass spectrometry and tandem mass spectrometry (Martin, Rosenthal, and Biemann 1987).

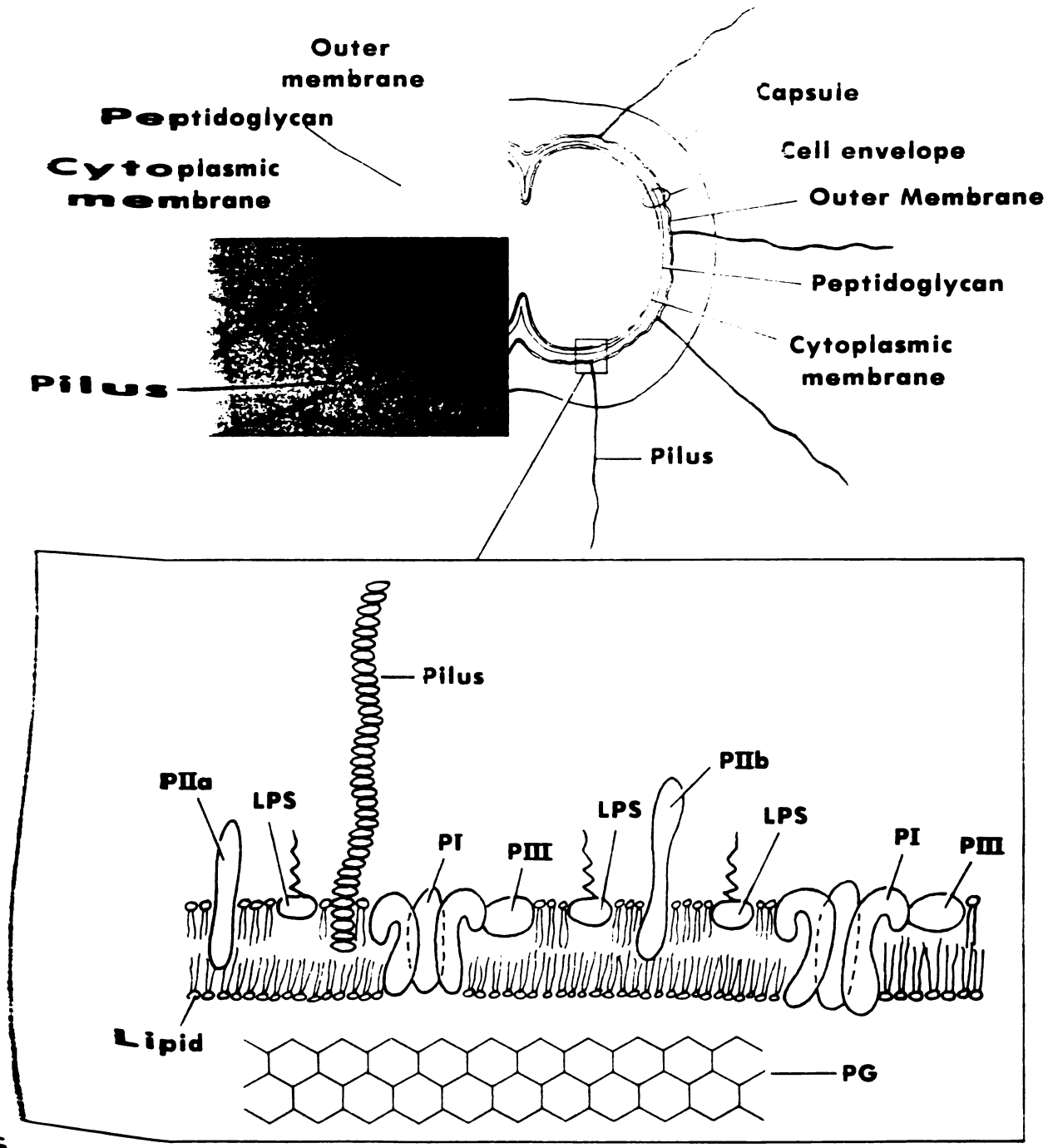


Figure 1. The upper portion illustrates the structure of the the cell wall of *N. gonorrhoeae* as viewed by transmission electron microscopy, or after negative staining with uranyl acetate. In the lower portion is a schematic of the gonococcal outer membrane showing the outer membrane components. This figure is used with permission of Dr. G. F. Brooks. The evidence for the existence of a polysaccharide capsule is lacking.

D.1. Protein I

There are three major types of protein on the outer cell membrane of gonococci, which are simply classified as proteins I, II, and III. Protein I is the most abundant of all the outer membrane proteins; approximately 60% of the mass of the outer cell membrane is composed of protein I (Brooks 1985b). Experimental evidence suggests that protein I is a voltage-dependent porin protein, with some anion selectivity. Protein I can translocate from the gonococcal outer membrane into other membranes, both natural and artificial (Gotschlich et al. 1987). It is similar in size to the porin proteins found in *E. coli* and exists as a trimer (Gotschlich et al. 1987). Nine serotypes of protein I have been identified (Buchanan and Hildebrandt 1981) and now are divided into two major classes on the basis of biochemical and immunochemical differences: A (serotypes 1-3); and B (serotypes 4-9) (Knapp, Sandstrom, and Holmes 1985). Each strain of gonococcus appears to produce one of the nine serotypes of protein I (Buchanan and Hildebrandt 1981). Bactericidal antibodies that recognize protein I have been observed (Gotschlich 1984), and there is evidence that PID is less likely to recur upon reinfection with *N. gonorrhoeae* with the same protein I serotype (Buchanan et al. 1980).

Neither heat nor reducing agents affect the migration of protein I in SDS-PAGE (Blake and Gotschlich 1983). Indications of structural differences between the two major classes of protein I have been observed. Protein IB has an apparent M_r of 34,000-38,000 as determined by migration in SDS-PAGE and can be cleaved by both trypsin and chymotrypsin. Sites susceptible to proteolytic cleavage are found on the surface of protein IB,

and strains expressing protein IB tend to be found in cases of serum sensitive infections (Gotschlich et al. 1987, Schmitt, Layh, and Buchanan 1986). Protein IA is slightly smaller than IB and is resistant to both trypsin and chymotrypsin. Protein IA serotypes tend to occur in serum resistant gonococci which cause disseminated infections (Gotschlich et al. 1987, Martin, Turgeon, and Nathiew 1986, Odum, Buchanan, and Knapp 1987). Other structural differences in the surface-exposed region of the protein I serotypes expressed by serum resistant strains compared to serum sensitive ones have been found (Judd, Tam, and Joiner 1987). Using monoclonal antibodies conserved epitopes have been located on protein IB (Fletcher et al. 1986, Teerlink et al. 1987), and one antibody to a conserved site was found to be bactericidal in the presence of complement (Virji, Zak, and Heckels 1986). Recently the genes encoding for protein IA (Carbonetti and Sparling 1987), and for protein IB (Gotschlich et al. 1987) were cloned and expressed.

A stable association between protein I and the gonococcal LOS was shown after exposure to boiling solutions containing 4% 2-mercaptoethanol and 2% SDS. The LOS and protein I could be dissociated by 2D electrophoresis (Hitchcock 1984). An association between the LOS and an outer membrane component, such as protein I, could alter the immune response to either antigen, possibly inducing tolerance. In another study association was found between the exposed portion of both protein IA and protein IB, the type of LOS expressed by the strain, and the H.8 antigen (Judd and Shafer 1989). The H.8 antigen is a surface macromolecule expressed on the surface of all strains of both *N. meningitidis* and *N. gonorrhoeae* (Cannon et al. 1984). Using monoclonal antibodies, the surface expression of protein I and the neisserial antigen H.8 were found to

be independent and highly variable in degree in gonococci from the same strain (Robinson et al. 1987).

D.2. Protein II

Colonies of gonococcus vary in appearance when observed by the light reflected from the substage mirror of a colony microscope from opaque to intermediate and transparent. This quality varies with a rate of $1:10^{-3}$ cell divisions and is due to expression of some types of a heat-modifiable protein of 24,000-30,000 daltons, termed protein II (Gotschlich 1984, Swanson, Kraus, and Gotschlich 1971). At least six different types of protein II, varying in primary structure and antigenic determinants, can be expressed by a single strain (Barritt et al. 1987, Lambden and Heckels 1979). Protein II appears to cause the gonococci to adhere to each other within a colony (Swanson, Kraus, and Gotschlich 1971). Protein II has also been implicated in adhesion of the bacteria to human cells (James et al. 1980, Lambden and Heckels 1979, Virji and Everson 1981) and could be involved in the second, close adhesion step in the penetration of the mucosa by the gonococcus. However, evidence obtained using radiolabeled protein II containing outer membranes, non-labeled outer membranes lacking protein II, and protein II specific IgG antibodies suggests that protein II may not be the ligand that is involved in adhesion, but rather is associated with increased binding (Draper 1984). A study of the chemical nature of binding to opacity-associated protein II concluded that the binding was mainly to proteins, rather than carbohydrates, and that the forces were more complicated than simple ionic or hydrophobic interactions (Bessen and Gotschlich 1987).

One study found that gonococci expressing protein II tend to be more sensitive to serum bactericidal effects than those gonococci that do not express the protein (James et al. 1982). The role of pili and protein II in interactions with human PMNs was explored using gonococcal strain P9 expressing different pili and types of protein II. Those variants that did not express protein II adhered less to PMNs and were resistant to phagocytosis. The variants expressing protein II were killed more effectively and stimulated less chemiluminescence (Virji and Heckels 1986). Comparison of pilated versus non-pilated gonococci did not show any correlation with neutrophil adherence or phagocytosis. These results suggest that the capacity of the gonococcus to turn on and off the expression of protein II could be immunologically advantageous (Virji and Heckels 1986). Similar results were obtained in a study of 95 monoclonal antibodies (MAbs) reacting with intact nonpiliated gonococci. Only two of the 95 MAbs were able to inhibit the adherence to human neutrophils and induction of neutrophil chemiluminescence. Both of these antibodies bound only to protein II and apparently had the same specificities; however, they were raised using two strains with different types of protein II (Elkins and Rest 1990).

D.3. Protein III

The third type of outer membrane protein, protein III, has an apparent M_r of 31,000 in the presence of mercaptoethanol as determined by electrophoretic motility in SDS-PAGE (Blake and Gotschlich 1983). There appears to be only one type of protein III, but it may not be expressed by all gonococcal strains (Judd 1982, Rice et al. 1986, Swanson, Mayer, and Tam

1982). Protein III is a target of IgG antibodies from normal human serum that block killing by complement (Rice et al. 1986), and it appears to be closely associated with protein I (Blake and Gotschlich 1983). The blocking is due to the competition of IgG antibody with bactericidal antibody for binding to the gonococcus. These IgG blocking antibodies enhance complement activation resulting in complement binding at sites that did not cause serum killing (Joiner et al. 1985).

D.4. Pilus protein

The gonococcal pili are linear molecules from 1 μm to 4 μm in length and are composed of up to 10,000 identical subunits, termed pilins, which have a M_r of 17,000-21,000 depending on the strain (Brinton et al. 1978, Buchanan et al. 1973). The pili are the mediators of the initial attachment to epithelial cells. Upon culture some gonococci no longer produce pili, and such bacteria are much less infective. Generally, antisera from laboratory animals prepared against pili from one gonococcal strain will only cross react with about 10% of the pili from other strains (Brinton et al. 1978, Buchanan et al. 1973). Nevertheless, this data also supports the existence of antigenic determinants which are common to all the serotypes of gonococcal pili (Brinton et al. 1978).

Initial studies on the safety of intact pilus vaccines have demonstrated that there are no serious side effects (Brinton et al. 1981, Tramont et al. 1981). The elicited antibodies were able to inhibit adhesion of the bacteria to buccal epithelial cells, particularly if the bacteria were of the same strain. However, the induced antibodies were cross-reactive and could inhibit the adherence of other strains to a significant degree

(Tramont et al. 1981). After immunization with intact pili the challenge dose of the bacteria had to be increased 30-fold to be infective (Brinton et al. 1978). In another experiment it was found that after two immunizations with intact pili, none of 18 subjects, but seven of 22 controls were infected when challenged with the same gonococcal strain (Brinton et al. 1981). No protection to a heterologous strain was evident (Gotschlich 1984). Similar results have been obtained in studies of adhesion of gonococci using polyclonal anti-pilus antisera (Virji and Heckels 1984). While these results are promising in terms of the possible development of a vaccine, the antigenic diversity of the pili presents a major problem.

Pili have a conserved N-terminal region of 29 amino acids, and the first residue is N-methylphenylalanine (Hermodson, Chen, and Buchanan 1978). Each pilin, or pilus subunit, usually has two methionine residues, one at position 7 and one at about 90. Using cyanogen bromide to cleave the protein, the second fragment, amino acids 8-89, has been found to be a conserved region of the molecule important in binding but normally immunorecessive. The third fragment, residues 90-160, includes the carboxy terminus and was found to be highly variable. In this third fragment are the only two cysteine residues in the protein, and they form a disulfide bridge (Gotschlich 1984, Schoolnik, Tai, and Gotschlich 1983). Also, the third fragment determines the type-specific antigenicity and thus, the serologically diversity of the pili. The second fragment comprising a conserved region of the intact pilus was found to be immunogenic, and the elicited antibodies were able to bind heterologous pili (Schoolnik, Tai, and Gotschlich 1983). The safety and efficacy of this peptide for immunization of of volunteers is being studied by several laboratories (Gotschlich 1984).

The regulation of the variation in pilus expression involves both expression and silent loci within the genome, which each contain multiple variable and conserved regions (Haas and Meyer 1986, Segal et al. 1986). By the process of gene conversion small variable genetic segments are thought to be transferred from silent to expression loci. This rearrangement of the gene then produces the antigenic diversity observed in the pili. Evidence for further complexity in the expression of the pili has been presented: the production of an over-length pilin (L-pilin) and extracellular release of soluble pilin (S-pilin) (Haas, Schwarz, and Meyer 1987).

In another study genomic DNA containing the expression portion of the pilin gene *(pil E)* was cloned and sequenced (Nicolson et al. 1987). Apparent sites of specific epitopes within the pilin protein were determined and predictions made of the secondary structure of various regions of the protein. The recognition site of MAb SM1, which reacts with all pili, was found to occur in a conserved region of the molecule, between amino acids 48 and 53. (Nicolson et al. 1987).

D.5. IgA Protease

N. gonorrhoeae and a number of other organisms, including *N. meningitidis, Haemophilus influenzae* and *Streptococcus pneumoniae* produce a protease that cleaves human IgA1 immunoglobulin in the hinge region (Gotschlich 1984, Plaut et al. 1975). The protease may inactivate IgA1 antibodies found on the epithelia of the human host. The gonococcal protease has been partially purified and characterized (Blake and Swanson 1978), and the gene coding for the protease has been cloned and expressed.

The M_r of the cloned product is approximately 130,000 (Koomey and Falkow 1984).

D.6. Lipooligosaccharide

Lipooligosaccharide (LOS) is a term which we and our colleagues have used to differentiates the glycolipids of Gram-negative bacteria such as *N. gonorrhoeae, N. meningitidis* and *Haemopilus influenzae* from the glycolipids or lipopolysaccharides of the Gram-negative enteric bacteria such as species of the *Escherichia* and *Salmonella* genera (Griffiss et al. 1988). In most living cells glycoproteins are found that interact in the external environment. These glycoproteins are often involved in various binding events and signal transduction. Bacteria do not produce glycoproteins (Sairam 1985), but the glycosylated lipids on the outer membrane are assumed to perform functions similar to those of the glycoproteins of mammalian cells (Griffiss et al. 1988). These glycolipids are also thought to help stabilize the membrane and to act as a barrier (Galanos et al. 1977).

The carbohydrate moieties of both glycoproteins and bacterial glycolipids exhibit a great deal of heterogeneity. Carbohydrates are synthesized, and then assembled by a series of enzymes called glycosyltransferases. This heterogeneity may be partly due to the fact that carbohydrates, unlike proteins, are not direct gene products (Sairam 1985).

Both the LPS and LOS of Gram-negative bacteria are acidic, amphipathic molecules, and as described above are responsible for some of the pathological responses produced during an infection. The lipid A moiety itself has been found to be the causal agent of endotoxicity (Galanos

et al. 1977). The structures of LPS from a number of different species of enteric bacteria have been determined (Galanos et al. 1977). These LPS are composed of three different regions: 1) the membrane-associated lipid A which is a glucosamine disaccharide, both *N*- and *O*-acylated by a number of different fatty acids; 2) inner and outer oligosaccharide core regions that are quite similar in a number of different species, containing 3-deoxy-*manno*-2-keto octulosonic acid (KDO) and L-*glycero*-D-*manno*-heptose along with phosphoester residues, such as phosphoethanolamine and/or ethanolamine pyrophosphate; and 3) the numerous, repeating, identical oligosaccharide units that are usually immunodominant and are called the O-antigens. The O-antigens can contain a wide variety of monosaccharides (Galanos et al. 1977). The O-antigens greatly increase the mass of the LPS; for example, the M_r of the LPS from *E. coli* is $>10^6$. These enteric bacteria inhabit environments rich in bile acid, which acts as an emulsifier of fats during digestion. The long, neutral glycan of the glycolipids would help stabilize the outer lipid membrane in this environment (Griffiss et al. 1988).

In contrast to the LPS of the enteric bacteria, the glycolipids of bacteria that colonize mucosal surfaces of the respiratory and genital tracts, such as *N. gonorrhoeae* and *H. influenzae,* are much smaller and are termed lipooligosaccharides (LOS). These LOS have total M_rs in the range of 3,000 to 7,000 (Schneider et al. 1984) and the entire glycan portion is similar in size to the core region of LPS. However, unlike the core region of the LPS, a great deal of heterogeneity has been found in the composition, size and antigenic determinants of the oligosaccharide portion of the LOS (Griffiss et al. 1987, Mandrell et al. 1986, Schneider et al. 1984, Phillips et al. 1990). The structure of the lipid A portion of *N. gonorrhoeae* shown in Figure 2 was determined using laser desorption mass spectrometry and ^1H

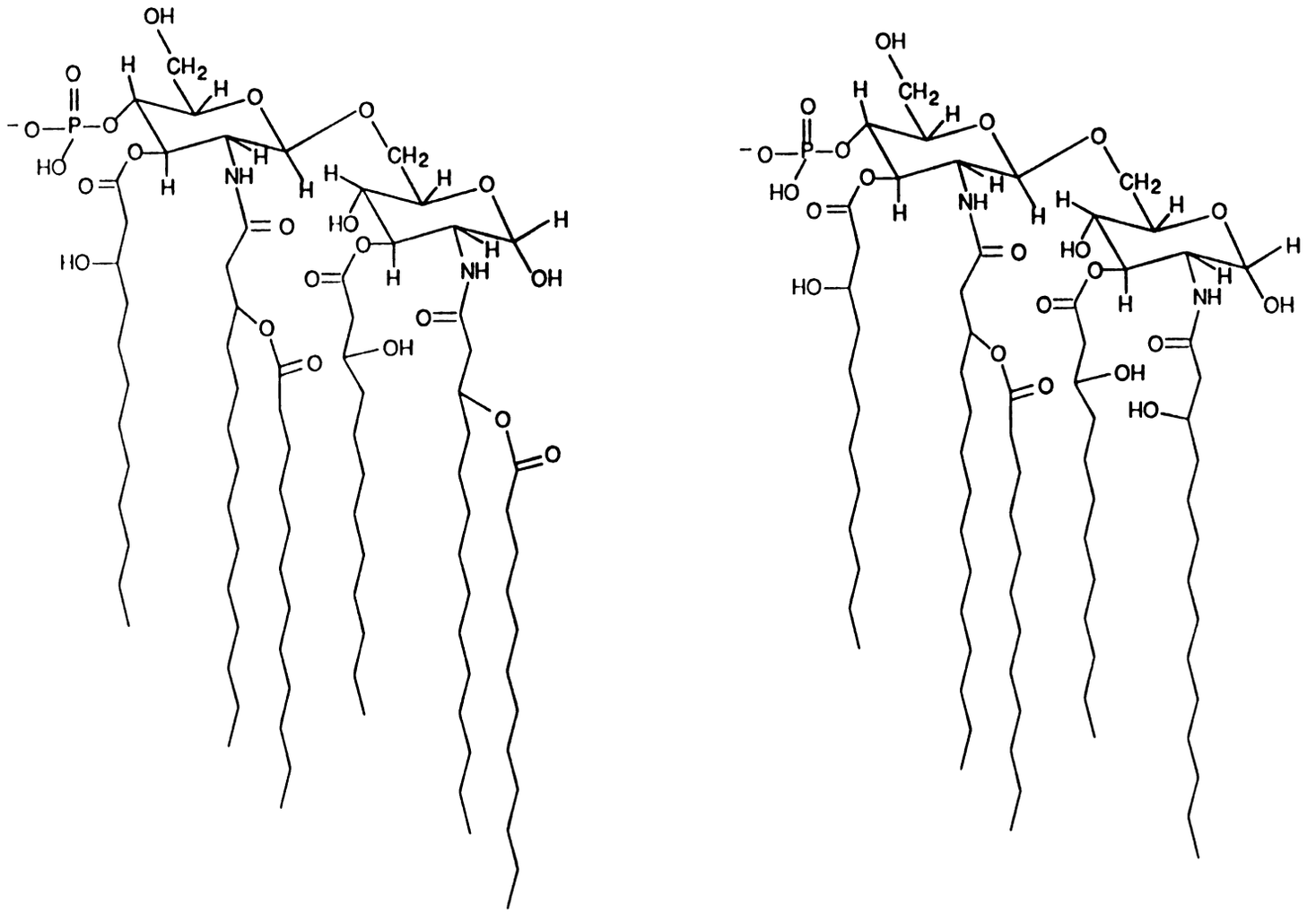


Figure 2. Two of the structures determined for lipid A from *N. gonorrhoeae* strain F62 by Takayama et al. (Takayama et al. 1986).

NMR (Takayama et al. 1986). It is similar to the lipid A moieties in other Gram-negative bacteria, except that the *O*-linked fatty acids are lauric (C_{12}) rather than myristic (C_{10}) (Schneider et al. 1982, Takayama et al. 1986).

The gonococcal outer membrane lipooligosaccharides (LOS) play a major role in the immune response to *N. gonorrhoeae*. Maeland first demonstrated that the gonococcal endotoxins were immunogenic (Maeland 1966, Maeland 1969). In one study, using sixty strains of *N. gonorrhoeae* bactericidal antibody could be absorbed from normal and immune serum by gonococci that had been treated with trypsin or autoclaved, and by human red blood cells coated with gonococcal LOS (Glynn and Ward 1970). Using a bactericidal assay and a gonococcal strain causing disseminated disease, it was later shown that a patient's whole serum or IgG fraction, with complement, could reduce the number of gonococci more than ten-fold. The LOS or outer membrane fractions, but not the outer membrane proteins, could inhibit the bactericidal activity in either the patient's convalescent serum or rabbit antiserum (Rice and Kasper 1977).

A comparison was made of the immunogenicity of highly purified LOS, pili and proteins I and II using rabbit IgG monoclonal antibodies prepared to the individual components and complement. The antigen most effective in induction of the bactericidal response was the LOS component, followed by the the two outer membrane proteins (Ward et al. 1978). Pili were not very effective (Ward et al. 1978). In studies of human sera LOS antibodies of the IgM class were found to be responsible for most of the bactericidal activity (Rice, McCormack, and Kasper 1980, Schoolnik, Ochs, and Buchanan 1979). Gonococcal strain F62 can be killed by complement in the absence of antibodies, and can directly activate the alternate complement pathway (Ward et al. 1978). In animal models vaccination

with gonococcal LOS has been found to elicit protection, such as in mice (Diena et al. 1975, Senior, Jessop, and Demarco de Hormaeche 1988) and chick embryos (Diena et al. 1978).

The general immunological basis of the resistance of some strains of *N. gonorrhoea* to human serum was investigated using 17 gonococcal strains and normal human serum from six donors (Schneider et al. 1982). The gonococcal strains were classified as serum resistant or serum sensitive based on the survival rate of the gonococci in undiluted sera from the donors. By adding homologous immune rabbit sera or the corresponding IgG fraction to the human serum lysis of the serum resistant strains proceeded completely. Thus, together with specific antibody, human complement could kill the serum resistant bacteria. Removal of IgA from the human serum did not increase immune lysis of a serum resistant strain (Schneider et al. 1982). On the other hand, serum IgA antibodies can block IgM and IgG stimulated immune lysis of serum resistant strains of *N. meningitidis* (Griffiss and Bertran 1977). Human IgM, purified from IgA or IgG, was able to initiate immune lysis of two of the serum sensitive strains, but not two serum resistant strains. Incubation of LOS extracted from four serum sensitive strains with the human serum inhibited the immune lysis by the human serum of three of the four serum sensitive strains. Incubation of the LOS extracted from three serum resistant strains with the human serum did not affect the bactericidal activity against the same serum sensitive strains (Schneider et al. 1982). These experiments indicated that serum resistance occurs when antibodies specific for lytic loci are absent from human serum, and they offer added support to the evidence that the LOS is a target of bactericidal antibody (Schneider et al. 1982). Furthermore, it was shown that obvious

size or composition differences in the LOS could not be correlated with serum resistance or sensitivity (Schneider et al. 1982).

These preliminary works established the importance of the gonococcal LOS in the immune response to infection and suggested that at least some of the evasion of the immune system by the gonococcus may be due to the LOS. Numerous studies have since been performed to understand more specifically the nature of the interactions between the LOS, the immune system and the other components of the outer membrane.

The expression of the gonococcal LOS has been found to vary if grown under glucose-limiting conditions (Morse et al. 1983). In addition the antigenic determinants recognized by murine monoclonal antibodies to gonococcal LOS (Mandrell et al. 1985a) vanished and reappeared after treatment of the LOS with NaOH, EDTA and divalent cations (Mandrell et al. 1985b). In other studies of variation in LOS *in vitro* the expression of two epitopes was found to vary and revert with high frequencies at the single cell level. Nevertheless, the expression of the epitopes were found to be a stable attribute of the total population of a colony (Apicella et al. 1987, Schneider et al. 1988).

What is known about amount of variation in the LOS that occurs *in vivo*? Recent work using immunoelectron microscopy analyzed the role of the LOS in the pathogenesis of gonococcal infections *in vivo*. Ultrathin cyrosections of infected epithelial cells were incubated with specific LOS monoclonal antibodies. The results demonstrated stable expression of LOS epitopes during attachment, internalization and intracellular processing of the gonococci over 18 hours (Weel, Hopman, and Van Putten 1989). This is in contrast to observed differences in the expression of LOS epitopes in

gonococci over a period of days. Antigenic and physical differences were observed in LOS isolated from infected volunteers after days when compared to the LOS of the infecting strain [Griffiss, J. McL., unpublished observations]. Physical and antigenic differences were also found in LOS isolated from guinea pigs after immunization with LOS-containing phenol extracts and challenges with gonococci. The extract was immunogenic but not protective, although transient protection (about two days) was observed in most animals. In some of the guinea pigs only gonococci with a different type of LOS than the parent strain were found after the challenge. Also, only 0-3% of the original parent strain survived treatment with antibodies isolated from the inoculated guinea pigs in which the gonococcal variants were found (Senior, Demarco de Hormaeche, and Jessop 1989). The gonococci with variant LOS isolated from the inoculated animals were resistant to killing by the elicited antibodies. Smaller LOS was expressed by three of four gonococcal isolates from the transiently protected guinea pigs. Also, some gonococcal variants producing altered LOS were isolated from guinea pigs that were not inoculated. (Senior, Demarco de Hormaeche, and Jessop 1989). These data suggest that selection for gonococcal variants producing altered LOS can occur *in vivo* and that only a multivalent LOS vaccine or vaccine component is likely to confer protection (Senior, Demarco de Hormaeche, and Jessop 1989).

The expression of a number of epitopes within the LOS has been found to be dependent on the presence of the lipid A moiety. This is probably due to the effect of the lipid A on the conformation of the glycan (Yamasaki et al. 1985, Yamasaki et al. 1988). In other experiments the expression of epitopes in the gonococcal LOS was investigated. Four mouse monoclonal antibodies to the LOS were prepared, two IgM antibodies, 3F11 and 1-1-M,

and two IgG antibodies, 2-1-L8 and 1-1-G (Mandrell et al. 1985a). The binding of the MAbs to 22 gonococcal LOS was assayed and two IgM antibodies, 3F11 and 1-1-M, bound 21 and 12 out of 22 LOS, respectively. The IgG antibodies, 2-1-L8 and 1-1-G, bound to five and seven out of 22 LOS, respectively. Monoclonal antibody 2-1-L8 bound to three of the five LOS from serum resistant strains and to none of the LOS from serum sensitive strains (Mandrell et al. 1985a).

Antibody to LOS from human serum has been isolated using affinity chromatography with LOS from *E. coli*. The antibodies were tested for bactericidal activity against three serum-resistant strains, and two serum sensitive strains of *N. gonorrhoeae*. The IgM fraction was bactericidal for the two serum sensitive strains, while the IgG fraction was bactericidal for two of the three serum resistant strains. The IgA fraction did not kill either serum sensitive or resistant strains, but was able to block IgG-mediated killing for one of the two serum sensitive strains killed (Apicella et al. 1986). Thus, the presence of loci for which bactericidal IgM antibodies exist can be correlated with serum sensitivity (Apicella et al. 1986).

The relationship between serum sensitivity and serum resistance and the expression of a specific LOS epitope has also been studied (Schneider et al. 1985). The IgG MAb 2-1-L8 specific for an epitope within a 3.6 kilodalton component of the LOS of strain L8 of *N. meningitidis* (Mandrell et al. 1985a) was used. The MAb bound to the LOS from six out of seven serum resistant strains, but to none of the LOS of 14 serum sensitive or serum intermediate strains. The expression of the 3.6 kilodalton component by three of the serum resistant strains was determined using SDS-PAGE. Two of the three resistant strains examined expressed the 3.6

kilodalton component; the strain that did not was the one that did not bind 2-1-L8. Therefore, the expression of the 3.6 kilodalton LOS epitope recognized by MAb 2-1-L8 was found to be strongly correlated with serum resistance (Schneider et al. 1985).

Experiments conducted with the murine monoclonal antibodies to gonococcal LOS, 3F11 and 06B4, have revealed crossreacting antigens on gonococci, red blood cells and some meningococci (Apicella et al. 1981, Mandrell, Griffiss, and Macher 1988). These cross-reacting antigens appear to be located on one of the nonreducing termini of some of the LOS from meningococci of three different strains of serotypes L2, L3, L5, L7, and L9. The nonreducing terminal tetrasaccharide of these serotypes (Galβ1→4GlcNAcβ1→3Galβ1→4Glc) is identical to the oligosaccharides which compose the terminal tetrasaccharide of lacto-N-neotetraose of paragloboside (Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→1Cer), a precursor of major blood group glycosphingolipids (Gamian et al. 1986, Hakomori 1981, Jennings, Lugowski, and Ashton 1983, Jennings et al. 1988, Mandrell, Griffiss, and Macher 1988).

To determine the specificity of the 3F11 and 06B4 MAbs, the MAbs were tested with ten different glycosphingolipids of known structure. Both of the antibodies were found to bind to neolacto glycosphingolipids that have the terminal structure Galβ1→4GlcNAc, if the *N*-acetyl lactosamine is unsubstituted. The antibodies did not bind glycosphingolipids with a terminal Galβ1→3GlcNAc structure, or to *N*-acetyl lactosamine glycosphingolipids having a terminal sialic acid or fucose attached to the penultimate glucosamine moiety. Glycosphingolipids from the lacto and globo series and those with less than four monosaccharides were also not recognized (Mandrell, Griffiss, and Macher 1988).

Binding of the MAbs 3F11 and 06B4 to human erythrocytes, from adults, children and infants, at different temperatures was recently determined. With adult red blood cells, MAb 3F11 agglutinated all of the 47 samples at 4°C, while 06B4 agglutinated 80% of the same samples, even though 06B4 was used at seven-fold higher concentrations than 3F11 (Mandrell, Griffiss, and Macher 1988). Neither antibody agglutinated red blood cells from mice or rabbits. Agglutination by MAb 3F11 was two to three orders of magnitude less at 22°C, and four to six orders of magnitude less at 37°C than at 4°C. The MAb 3F11 agglutinated only 3 of 13 erythrocyte samples from infants under seven months old but reacted similarly with erythrocytes from children aged one to four years. After treatment of the erythrocytes with either neuraminidase or trypsin all of the infant samples could be agglutinated by MAb 3F11. Also, enzymatic treatment of the erythrocytes greatly reduced (>tenfold) the amount of antibody required to agglutinate both the adult and infant cells (Mandrell, Griffiss, and Macher 1988). These data suggest that the epitope recognized by 3F11 was being expressed by infant erythrocytes but was blocked by sialic acid residues and/or by membrane glycoproteins. In contrast to the increased agglutination of erythrocytes by 3F11 at 4°C compared to higher temperatures (22 and 37°C), the reaction of the MAb with LOS was not affected by temperature. The increased agglutination of adult erythrocytes at 4°C is probably caused by conformational shifts in glycoproteins on the erythrocytes that block the 3F11 epitope (Mandrell, Griffiss, and Macher 1988).

The demonstration of human cross-reacting antigens in this study was limited to those on erythrocytes. It has been previously established that the Ii antigens and paragloboside are expressed on human lymphocytes,

monocytes, polymorphonuclear leukocytes and other granulocytes (Fukuda et al. 1985, Macher and Klock 1980, Macher, Lee, and Westrick 1982, Spitalnik et al. 1985), and also in human milk, urine, saliva and semen (Race and Sanger 1975). The presence of cross-reacting antigens in the host cells could provide a number of advantages to the gonococcus. Such antigens may provide a mechanism by which the bacteria can evade the immune system since there is likely to be little or no response to antigens similar to the self. Also, the LOS of the gonococcus could mask other potentially lytic loci within the outer membrane (Mandrell, Griffiss, and Macher 1988). Structures similar to those expressed in the human tissues could increase binding by the host cells to the gonococcus. For example, surface lectins on human cells that recognize a terminal disaccharide of the structure Galβ1→4GlcNAc and/or and internal Galβ1→4Glc have been found (Leffler and Barondes 1986, Sparrow, Leffler, and Barondes 1987). Similar structures might be modified *in vivo* by host enzymes which could further increase the similarity of the bacterial antigen to the host cells.

There is evidence that *in vivo* silylation by host enzymes occurs in the gonococcal LOS (Mandrell et al. 1990). In a series of experiments using human serum and guinea pig and human red and white blood cells, substances were found that induced serum resistance to gonococcal strains. It appears that transfer of a sialic acid moiety from host cytidine monophospho-*N*-acetylneuraminic acid to the LOS may be occurring *in vivo*. The sialylation of the LOS apparently can block the binding of bactericidal antibody to the gonococcus, and thereby induce serum resistance (Mandrell et al. 1990, Nairn et al. 1988, Parsons et al. 1988).

A system of classification of the epitopes expressed by neisserial LOS has been proposed (Griffiss et al. 1988). Four different classes of epitopes

are defined by this system: restricted, common, partially conserved and conserved epitopes. The restricted epitopes are found only in LOS of a particular mass. The best example of this class of epitope is associated with an LOS of 3.6 kilodaltons and serum resistance (Griffiss et al. 1988, Schneider et al. 1985). Common epitopes are expressed within some of the LOS of most strains and are exemplified by the epitopes recognized by 3F11 and 06B4 (Apicella et al. 1981, Mandrell, Griffiss, and Macher 1988). Partially conserved epitopes are common epitopes that are expressed by more than one type of the LOS expressed by a single strain, and conserved epitopes are common epitopes found in all the LOS of a single strain.

Genetic regulation of the biosynthesis of gonococcal LOS has been focus of several studies (Petricoin III and Stein 1989, Stein et al. 1988). DNA was isolated from a serum resistant strain, MUG116, of *N. gonorrhoeae* that is recognized by MAb 2-1-L8 and was used to transform a serum sensitive strain, DOV, to reactivity with 2-1-L8. Analysis of the LOS of the transformed strain by immunoblot after separation by SDS-PAGE indicated that LOS of a different type was being produced, corresponding to the LOS of the donor strain. The transformants all produced multiple LOS components, of which one was recognized by 2-1-L8. Those transformants that produced the same type of LOS components as DOV tended to remain serum sensitive, even though the LOS components of the serum resistant strain were also expressed. Nevertheless, the transformants that reacted strongly with MAb 2-1-L8 were serum-resistant (Stein et al. 1988).

In further work with strain MUG116 a gene bank was created in a cosmid vector (Petricoin III and Stein 1989) in order to express genes involved in LOS biosynthesis. One cosmid was isolated that could transform gonococcal strain FA5100, a pyocin-resistant mutant of strain

FA19 (Guymon, Esser, and Shafer 1982, Shafer et al. 1984). Pyocin is a bacteriocin isolated from *Pseudomonas aeuroginosa* that was found to kill most strains of *N. gonorrhoeae* (Morse et al. 1976, Sidberry and Sadoff 1977). Those strains that are resistant to pyocin produce altered LOS (Blackwell and Law 1981, Connelly et al. 1981, Guymon et al. 1980, Morse and Apicella 1982, Sadoff, Zollinger, and Sidberry 1978, Winstanley et al. 1984). The exact mechanism by which the pyocin causes alterations in the LOS has not been determined. However, pyocin has been found to contain single-stranded DNA [Apicella, M. A., unpublished observations]. The LOS of pyocin resistant strain FA5100, unlike the parent strain FA19, does not react with any of the available MAbs that recognize the gonococcal LOS (Shafer et al. 1984). The FA5100 transformants containing DNA from strain MUG116 produced a number of LOS components in different combinations, including the same LOS as the MUG116, the donor strain. Analysis of DNA from FA5100 by Southern blot indicated that large chromosomal deletions or rearrangements had not occurred. Thus, the authors concluded that the gene or genes they had cloned were integral in the LOS biosynthesis (Petricoin III and Stein 1989).

Additional information related to LOS expression and serum resistance was obtained in experiments with the sac-3 genetic determinant (Stephens and Shafer 1987). The serum resistant strain FA19 had been found to differ from FA899, a serum sensitive transformant of FA19, in the type of outer membrane protein I and the sac-3 genetic locus. New transformants of FA19 were formed using DNA from strain FA899. The sac-3 locus was shown to be involved in LPS expression and in determining serum sensitivity. The changes observed in protein I could not be

correlated with the serum susceptibility or expression of the LOS (Stephens and Shafer 1987).

Independent of its serine protease activity, the lysosomal enzyme cathepsin G from human polymorphonuclear leuckocytes (PMNs) was found to be bactericidal for gonococci *in vitro* independent of its serine protease activity (Shafer 1988). Increased binding was observed of enzymatically inactive, but bactericidal cathepsin G to a pyocin resistant mutant strain of FA102, when compared with binding to the parent strain FA102. The pyocin-resistant mutant strain produced a truncated LOS with less carbohydrate (Shafer, Onunka, and Martin 1986). The outer membrane protein profile of the two strains is similar, but the mutant strain was at least ten times more susceptible to enzymatically active or inactivated cathepsin G. Interaction of cathepsin G with at least three outer membrane proteins was demonstrated. The study concluded that the bactericidal activity of cathepsin G with the outer membrane proteins may be inhibited by the carbohydrate portion of the LOS (Shafer 1988). This may be one explanation for the survival of a few gonococci within human phagocytic cells.

Recently, in our own laboratory the first partial structures of the oligosaccharides from a gonococcal LOS were determined using liquid secondary ion mass spectrometry (LSIMS), tandem mass spectrometry with collision-induced-dissociation (CID), and traditional linkage analysis (Gibson et al. 1989). The generalized structure is shown in Figure 3. This oligosaccharide was from the LOS of pyocin-resistant strain JW31R (Morse and Apicella 1982). The JW31R LOS was found to be unique among the LOS of 22 gonococcal strains in that it was not recognized by MAb 3F11 (Mandrell et al. 1985a). As shown in Figure 3 the JW31R oligosaccharides

do not contain the Gal→GlcNAc→Gal sequence that is the apparent determinant for the binding of 3F11 (Mandrell, Griffiss, and Macher 1988). Four different major species were identified that varied by the addition of a single phosphoethanolamine (PEA) and/or Gal. Also, seven minor components were found which were truncated versions of the larger four species (Gibson et al. 1989). The significance of this structure and its relationship to the parent strain is not yet known, but preliminary evidence suggests that the wild type JW31 LOS does contain a Gal→GlcNAc→Gal structure, unlike this pyocin mutant LOS.

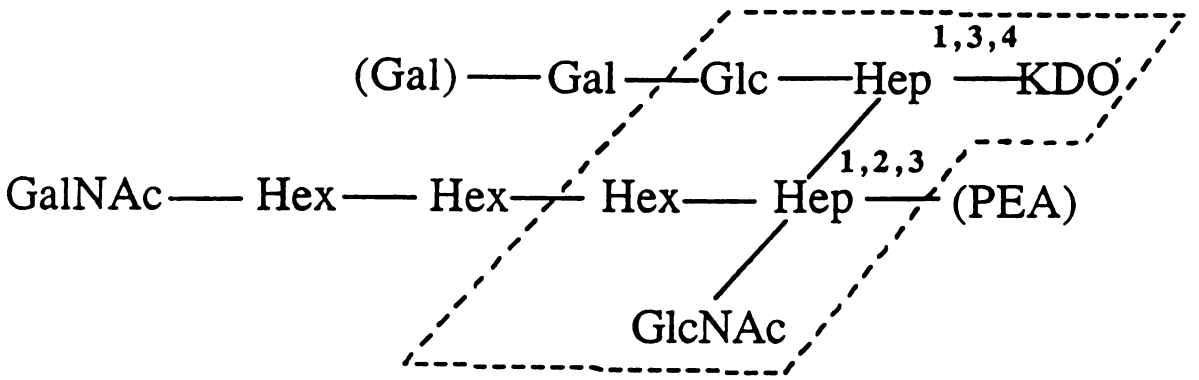


Figure 3. The structure of the oligosaccharides from the lipooligosaccharides from *N. gonorrhoeae* strain JW31R (Gibson et al. 1989).

E. Research goals

Specific structural information about the oligosaccharide components of the LOS from *N. gonorrhoeae* was essentially nonexistent when I began my research work in 1987, particularly in comparison to the amount of immunological and biological data that was available. Except for the work with JW31R described above, the direct structural data was limited to compositional analyses and M_r profiles obtained by comparative electrophoretic motility using SDS-PAGE. A complete or even partial understanding of the structural basis for the role of the LOS in the immunology and pathology of *N. gonorrhoeae* was the long-term goal of the work described in this thesis. Due to the inter- and intra-strain heterogeneity in the gonococcal LOS this goal requires the determination of a large number of oligosaccharide structures and their correlation to binding properties, epitopes or pathogenicity. One of the limiting factors of the analyses of these oligosaccharides is the production of the LOS in sufficient quantity in a timely and efficient manner, and their subsequent separation and purification. Thus, in order to reduce the amount of LOS required the use and development of microscale chemical and chromatographic methods were the first strategy to be employed in my research effort. Simultaneously, structural analysis of the oligosaccharides isolated from the LOS of a select group of gonococcal strains was also underway.

CHAPTER II. METHODS DEVELOPMENT

A. Techniques of structural elucidation of oligosaccharides

The elucidation of the complete structure of an oligosaccharide often requires purification of the sample to a single species, and then determination of the following structural features:

1. monosaccharide composition
2. anomeric configuration of each glycosidic linkage
3. ring form, pyranose or furanose, of the monosaccharides
4. the sequence of the monosaccharides
5. the substitution pattern of the monosaccharides
6. configuration, D or L, of the monosaccharide
7. axial or equatorial conformation of the hydroxyl groups
8. attachment and structures of non-carbohydrate substituents

In addition to these features the secondary and tertiary structures need to be determined to provide a complete picture of the overall molecule (Sweeley and Nunez 1985).

The number of different primary structures possible for a relatively small oligosaccharide is large. For instance, allowing only five hexose sugars in the D-pyranose form there are approximately 983,000 different possible structures. This is due to the different branching and linkage positions possible, such as 1,2,3-Hex vs. 1,3,4-Hex, the different monosaccharides formed by the arrangement of the hydroxyl groups in either the α or β position, (Glc vs. Gal) and the α or β configuration at the anomeric carbon (Glcα1 vs Glcβ1). Elucidation of oligosaccharide

structures has lagged somewhat behind structural elucidation of other biochemical molecules such as peptides, proteins, and DNA at least partly due to the difficulty in determining the primary structures. In Figure 1 a schematic illustrating the steps required to assign the total primary structure of an oligosaccharide is shown, along with techniques that can be used in such assignments.

The strategy that we have developed in our laboratory for the structural elucidation of oligosaccharides is primarily based on mass spectrometric analyses. The general structural data that can be obtained by the various components of this strategy are illustrated in Figure 1. Both branching and sequence of an oligosaccharide can be largely deduced by LSIMS and MS/MS analysis, along with the generic monosaccharide composition in terms of Hex (hexose), HexNAc (*N*-acetylhexosamine), etc. The exact monosaccharide composition, linkages and ring forms can be determined by methylation analysis which involves chemical derivatization and hydrolysis of the oligosaccharide followed by EI (electron impact) and/or CI (chemical ionization) GC-MS. To determine anomeric configuration, the positions of the substituents of disubstituted or trisubstituted monosaccharides, and additional sequence information further analyses are required. Techniques which can be used to obtain this information are nuclear magnetic resonance (NMR), oxidation by chromic trioxide followed by LSIMS analysis, and hydrolysis by enzymes specific for certain anomeric linkages i.e. endo- and exoglycosidases. All of these techniques have different advantages and disadvantages in terms of amounts of sample, time, and instrumentation which are required.

A wide range of different analytical techniques, chemical reactions and derivatizations are employed in determination of oligosaccharide

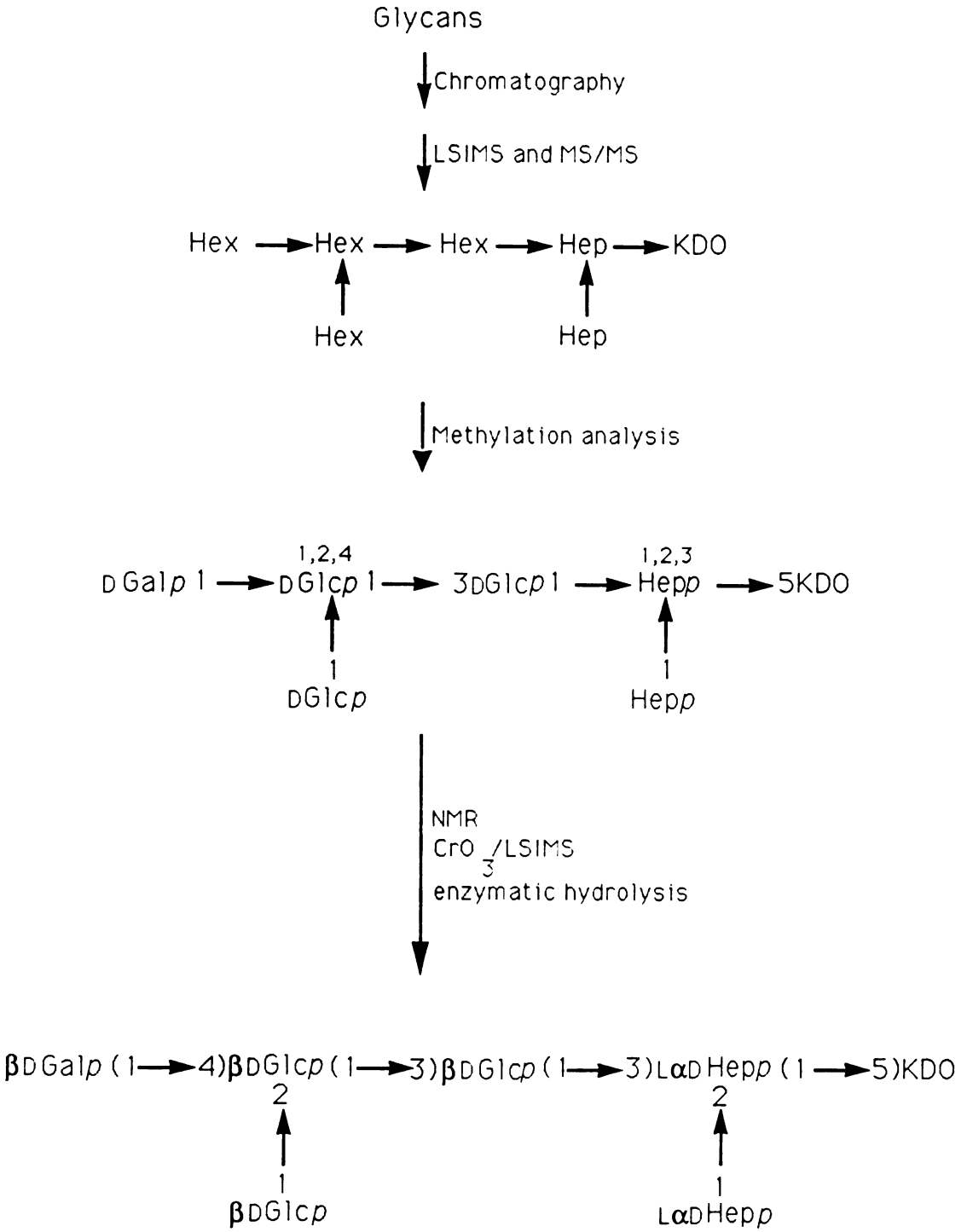


Figure 1. The application of analytical techniques to structural elucidation of carbohydrates is illustrated. Elucidation of all the structural details normally requires the use of a number of different techniques.

structures. The field of oligosaccharide analysis has grown significantly with the advent of soft ionization methods for mass spectrometry and two-dimensional NMR, increasing array of ingenious chemical derivatizations, use of glycohydrolases specific for certain monosaccharides and anomeric configurations, and the use of circular dichroism. There is a considerable amount of literature on structural analysis of carbohydrates and I will not attempt to comprehensively review and describe all of the more traditional and newer methods in structural elucidation of carbohydrates. Nevertheless, it is important to briefly describe some of the more important techniques so as to put into context the methods that I have developed for carbohydrate analyses.

A.1. Chromatographic separations and analyses of oligosaccharides

Most biologically derived oligosaccharides are heterogenous mixtures. Separation of these mixtures into single structurally homogeneous components is a significant challenge. It is also difficult to determine when an oligosaccharide has been purified to a single component. The techniques that are commonly utilized include gel filtration chromatography (Kobata, Yamashita, and Takasaki 1987), paper and thin-layer chromatography (TLC), paper electrophoresis, ion exchange chromatography (Hellerqvist and Sweetman 1990), both normal and reverse phase HPLC (Honda 1984, McGinnis, Laver, and Biermann 1989), gas-chromatography (Biermann 1989, Eisenberg 1972, Laine, Esselman, and Sweeley 1972, Nilsson and Zopf 1982, Sweeley and Walker 1964), and lectin affinity chromatography (Blake and Goldstein 1982). Underivatized

carbohydrates can not generally be detected by ultraviolet or visible absorption, or fluorescence because they lack both a chromophore and a fluorophore. The carboxyl moiety of acetylated oligosaccharides does have absorbance at 205 nm and has been used to easily detect peaks containing approximately 7 μg of acetylated carbohydrate (Wells and Lester 1979). Among the methods of detection often used for carbohydrates is refractive index (RI) detection, which is not very sensitive; more than 100 μg of oligosaccharide is required. Reduction of reducing termini to alditols with sodium borotritide (Beeley 1985), and various postcolumn labeling schemes in which chromophores or fluorophores are added to the carbohydrate have been used with excellent sensitivity. Also, electrochemical techniques have been used for detection with good sensitivity (Honda 1984).

Qualitative and quantitative analyses of oligosaccharides are often performed by complete hydrolysis of the oligosaccharide, derivatization of the resulting monosaccharides, and then GC separation with flame ionization detection (FID) and/or GC-MS analysis. The rate of acid hydrolysis of different glycosidic bonds varies and use of both internal and external standards is recommended. Reduction of the monosaccharides to their alditols followed by peracetylation or permethylation are common derivatization schemes for GC and GC-MS (Radford and Dejongh 1980, Beeley 1985, Fox, Morgan, and Gilbart 1989, Hellerqvist and Sweetman 1990). With both peracetylation and permethylation underderivatization of the carbohydrates is a common problem.

Another often used technique for quantitative and qualitative GC analysis is the preparation of trimethylsilyl derivatives of oligosaccharides (Kakehi and Honda 1989, Laine, Esselman, and Sweeley 1972, Sweeley and Walker 1964). Preparation of the trimethylsilyl derivatives, while not

difficult does require careful work. The products are not very stable, and are best analyzed on the day of preparation. The trimethylsilylated monosaccharide methyl glycosides produce multiple peaks because of the equilibrium between the pyranoside and furanoside ring forms and the α– and β–anomers which complicates the analysis. Trimethylsilyl derivatives can also be used for mass spectrometric analysis; however, the increase in mass produced by the derivatization can be a disadvantage. One advantage of the analysis of alditol acetates of monosaccharides by GC is that single peaks are produced for each monosaccharide because of the reduction to the alditol (Laine, Esselman, and Sweeley 1972).

One recently developed technique has demonstrated great resolving power and sensitivity, high-performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD) (Hardy and Townsend 1988, Johnson and Polta 1986, Townsend et al. 1988). Neutral carbohydrates are weak acids; the hydroxyl groups have pKa's ranging from about 12-14. Thus, they are partially or completely ionized at the high pH of the eluant which is typically 10-200 mM NaOH. As anions, the carbohydrates interact with the solid phase of the ion-exchange column, and are retained and separated. The inherent stereochemistry of the oligosaccharides created by the arrangement of the hydroxyl groups and their associated pKa's is reflected in the separations. With carbohydrates containing acidic groups, such as sialic acid, retention and separation can be achieved at a lower pH due to the negative charge on the acidic moiety (Townsend et al. 1989). Very little difficulty with epimerization, aldose-ketose conversion, β-elimination, or other base-catalyzed reactions of carbohydrates occur in the time frame required for the chromatography, which is only about 30 to 60 min. A very small portion of the carbohydrates are detected by electrochemical oxidation

(<5%) at the surface of a metal electrode using pulsed amperometry. The detection is very sensitive, and nearly all of the carbohydrates can be recovered for further analysis. For direct analysis by LSIMS the carbohydrates must be desalted, as the very high concentration of sodium hydroxide and sodium acetate has been found to hinder the analysis. An on-line anion membrane suppressor can be used to exchange the sodium ions for protons from a counterflow of sulfuric acid.

HPAE-PAD has been used for compositional analyses of oligosaccharides since monosaccharides can also be resolved by this method (Hardy, Townsend, and Lee 1988). Very sensitive (low pmol range) and quantitative detection of monosaccharides can be obtained. One advantage of this technique is that no chemical modification of the oligosaccharide is required. This shortens the time needed for analysis besides eliminating a potential source of error, i.e. underderivatization, in compositional analyses based on chemical modification. Also, the monosaccharides elute as single peaks.

A.2. Mass spectrometry

Analysis of underivatized oligosaccharides or monosaccharides using the more traditional MS ionization modes, such as electron impact (EI) and chemical ionization (CI) with ammonia or isobutane has not been generally possible because the carbohydrate undergoes pyrolysis before volatilization. Two of the most widely utilized derivatization procedures for EI- and CI-MS analysis of carbohydrates are permethylation (Hakomori 1964, Waeghe et al. 1983, Ciucanu and Kerek 1984) and peracetylation (Bourne et al. 1949, Radford and DeJongh 1980, Dell, Oates, and Ballou

1983). In these procedures each of the free hydroxyls becomes a methoxy or acetate group, increasing the volatility of the molecule so that MS analysis can be performed. Both of these procedures are relatively simple to perform, but in permethylation great care must be taken to keep the reagents and molecules as dry as possible. Permethylation requires an extraction step after the derivatization prior to the analysis. Peracetylation is a relatively clean procedure and requires no sample purification step. However, esters such as acetates have quite limited thermal stability and, therefore, limit the column temperatures which can be used. Underderivatization is a problem in both these schemes, and can complicate the analysis. In peracetylation the total mass of the oligosaccharide is substantially increased. Depending on the type of MS instrumentation available, this may be a limiting factor.

A number of derivatization schemes have evolved which are combined with MS analysis using EI or CI ionization to provide composition analysis, and sequence analysis of derivatized intact oligosaccharides. The position of the glycosidic linkages and the ring size of an oligosaccharide can be determined by mass spectrometric analysis of permethylated oligosaccharides. GC-MS analysis is generally possible of intact permethylated carbohydrates containing up to four or five monosaccharides (Rauvala et al. 1981). Permethylation is often performed after reduction which prevents alkaline peeling or β-elimination reactions which can occur in the strongly basic conditions required (Finne, Krusius, and Rauvala 1980, Hellerqvist and Sweetman 1990).

In traditional methylation analysis partially methylated alditol acetate (PMAA) derivatives are formed (Bjorndal et al. 1970, Hakomori 1964, Hellerqvist and Sweetman 1990). After permethylation of the intact

oligosaccharide the sample is hydrolyzed to the component monosaccharides which are then reduced to alditols with sodium borohydride or sodium borodeuteride. The free hydroxyl groups are then acetylated. The resulting PMAAs, therefore, have methyl groups attached to the hydroxyls that were free in the intact oligomer and acetate groups on the substituted positions. In addition to providing the position of the glycosidic linkages and the ring sizes, the relative retention time of the PMAAs is characteristic of both the position and arrangement of the various methoxy and acetate groups, and, thus, provides compositional information.

The traditional method of methylation linkage analysis does not distinguish residues which were 1,4-linked and in the pyranose ring form, from those which were 1,5-linked and in the furanose form. Two different schemes which address this problem have been developed. In one method the permethylated oligosaccharide is reductively cleaved with boron trifluoride etherate (BF_3-Et_2O) and trimethylsilyl trifluoromethanesulfonate as a catalyst in the presence of triethylsilane to produce 1,5-anhydro alditols. Thus, the ring form of the residue remains intact. The free hydroxyls present after the reductive cleavage are then acetylated to establish the location of the glycosidic bonds (Bennek, Rice, and Gray 1986, Rolf and Gray 1982). Another method combines partial hydrolysis of the oligosaccharide with methylation analysis. After permethylation of the partially hydrolyzed oligosaccharide the reducing terminal residue is reduced to an alditol with sodium borodeuteride, and then permethylation is performed. These derivatized fragments are purified by HPLC, and both analyzed intact and subjected to hydrolysis, reduction, and then acetylation as in traditional methylation analysis (McNeil et al. 1982).

In the last decade technological development of soft ionization techniques for mass spectrometry, often referred to as desorption methods, has improved the ion transmission efficiency of high mass polar molecules such as oligosaccharides, without derivatization of all the hydroxyl groups. Field desorption (FD) (Beckey 1977, Linscheid et al. 1981), direct chemical ionization (DCI) (Reinhold 1987), laser desorption (LD) (Conzemius and Capellen 1980), plasma desorption (PD) (Macfarlane 1980, Macfarlane and Torgerson 1976), fast atom bombardment (FAB) (Barber et al. 1981, Surman and Vickerman 1981), and liquid secondary ion mass spectrometry (LSIMS) (Aberth et al. 1982) are among the most significant of these methods. The recent advent of tandem mass spectrometry (MS/MS) as a tool of analytical chemists has greatly enhanced carbohydrate structural methods by providing the means for separating mixtures and obtaining extensive fragmentation.

MS/MS enables a single isotopically pure ^{12}C component to be selected in mixtures in MS-1. In a typical analysis the parent ion is collisionally activated in the field free region between MS-1 and MS-2 using helium or some other gas, and the parent and daughter ions are mass analyzed by MS-2. One of the important advantages of MS/MS analysis is the combination of a soft ionization technique with collisionally induced dissociation (CID), which can produce extensive and characteristic fragmentation of large polar molecules (Carr et al. 1985). The increased fragmentation is produced in the collision cell between MS-1 and MS-2 when the ions possessing a high degree of kinetic energy collide with neutral gas atoms or molecules. A portion of the kinetic energy is translated into internal or vibronic energy which increases the number and types of bond cleavages of the ion (Gillece-Castro and Burlingame 1990a,

Gillece-Castro and Burlingame 1990b, Rose and Johnstone 1982). Another advantage of MS/MS analysis is that low mass fragment ions are not obscured by ions formed from the matrix as is the case in LSIMS and FAB analysis.

Among the soft ionization techniques most utilized for oligosaccharides are FAB which has a primary beam of xenon atoms, and LSIMS in which the primary beam is cesium ions (Carr et al. 1985, Dell 1987, Webb et al. 1988, Hellerqvist and Sweetman 1990,). In both ionization methods the primary beam ionizes and sputters a sample from the surface of a polar liquid matrix in the form of protonated $(M+H)^+$ or deprotonated molecular ions $(M-H)^-$ and fragment ions. The protonation and deprotonation events are assumed to result from interaction with the polar protic liquid matrix. While both molecular ions and fragment ions can be obtained of underivatized oligosaccharides, relatively large amounts of sample are required (1-10 nmol) and the fragmentation is generally too limited to sequence the entire oligosaccharide. Various chemical strategies have been successfully employed with oligosaccharides to increase the sensitivity of detection, degree of fragmentation and, thus, the amount of sequence information that can be obtained in FAB or LSIMS.

In LSIMS or FAB analysis a polar liquid matrix such as glycerol, or combinations such as a mixture of glycerol/thioglycerol are generally used. A very hydrophilic molecule such as an underivatized oligosaccharide does not have much surface activity in such a matrix, and, thus, the sensitivity of detection is decreased (Ligon 1990). For this reason use of a less polar matrix, tetraethyleneglycol, has been recommended for underivatized oligosaccharides (Gillece-Castro and Burlingame 1990a). In general, derivatization of oligosaccharides to produce more hydrophobic moieties

increases the surface concentration of the oligosaccharide in the polar liquid matrix. This in turn increases the sputtering efficiency of the sample and the ion yield.

Two widely utilized chemical derivatization methods for FAB and LSIMS analysis of oligosaccharides are peracetylation and permethylation, first used in EI- and CI-MS as discussed above. Both peracetylation and permethylation increase the surface activity of the oligosaccharide within the polar liquid matrix and, thereby, the sensitivity of detection (Dell 1987, Dell 1990). Underacetylation and both under- and overmethylation of an oligosaccharide can produce multiple products and complicate the analysis (Dell 1987, Gibson, B. W. unpublished observations). The increase in mass, particularly upon acetylation, of large oligosaccharides can be a problem due to lower sputtering and transmission efficiency at higher masses.

Several groups have explored the use of reducing terminal derivatives which also impart hydrophobicity to the oligosaccharide and, thereby, increase the surface activity and sensitivity of detection in LSIMS. In addition, these reducing terminal derivatives contain a sensitive UV chromophore, and allow for HPLC separation and detection of small quantities of sample (<1 μg). The most widely utilized scheme for reducing terminal derivatization for LSIMS analysis has been reductive amination with ethyl 4-amino benzoate (ABEE) (Gillece-Castro et al. 1987, Matsuura and Imaoka 1988, Poulter et al. 1989, Wang et al. 1984, Webb et al. 1988). Abundant molecular ions have been obtained in both the negative- and positive-ion modes with derivatives of ABEE, but there is more extensive fragmentation in the negative-ion mode. Furthermore, derivatization and detection of pmol quantities of oligosaccharides is possible (Webb et al. 1988). Extension of the alkyl chain of the ester on 4-amino benzoate has been found

to further increase the sensitivity of detection by at least an order of magnitude (Poulter et al. 1988, Poulter and Burlingame 1990).

Recent work with reductive amination of oligosaccharides by phosphotidylethanolamine dipalmitoate (PPEADP) and LSIMS analysis has also demonstrated great sensitivity of detection (Lawson et al. 1990). With PPEADP-maltopentaose a limit of detection by LSIMS of 100 fmol was found. Using this derivative, negative-ion spectra were obtained with abundant molecular ions and fragment ions providing sequence information. The sensitivity of detection of oligosaccharides in the negative-ion mode may be attributed to both the increase in surface activity and the presence of a charged phosphate group (Lawson et al. 1990).

Various chemical reactions of carbohydrates are combined with mass spectrometric analysis, especially FAB and LSIMS, to aid in sequence determination and structural elucidation of oligosaccharides. Various types of depolymerization schemes are common (Aspinall 1982, Aspinall 1987), such as partial acid hydrolysis (Beeley 1985), partial methanolysis (Dell 1987), partial acetylosis (Beeley 1985), and base-catalyzed degradations (Aspinall 1987). When such reactions are performed on methylated oligosaccharides, organic solvents can be used and free hydroxyl groups are found only at sites of chemical cleavage or fragmentation upon subsequent mass spectrometric analysis (Aspinall 1987). Partial degradation by enzymatic digestion can be performed on very small quantities of underivatized material and can yield compositional along with sequence information, and in many cases, reveal anomeric configuration as well (Beeley 1985). The products of enzymatic hydrolysis are analyzed by a number of different techniques, included mass spectrometry. However, the specificity of the glycohydrolase enzymes is quite limited. Most linkages

found in bacterial glycans are not susceptible to enzymatic hydrolysis with the currently available enzymes.

Another method of depolymerization is deamination with nitrous acid in which glycosidic bonds involving hexosamine or *N*-sulphated hexosamine can be selectively cleaved (Beeley 1985). Residues of *N*-acetylhexosamine can be de-*N*-acetylated by hydrazine prior to the deamination reaction (Michon et al. 1990).

Periodate oxidation of vicinal diols is a classical technique of carbohydrate structural elucidation which has been applied to analysis of oligosaccharides. When combined with soft ionization mass spectrometry, or by performing methylation linkage analysis before and after periodate treatment, periodate oxidation can yield sequence and linkage information (Beeley 1985). In the Smith degradation technique the oligosaccharide is treated first with periodate. The aldehyde groups of the oxidized oligosaccharide are then reduced to alcohols with sodium borohydride. Mild acid hydrolysis can selectively remove the acetals formed by ring cleavage without hydrolysis of glycosidic bonds, and/or the product can be permethylated (Beeley 1985, Hellerqvist and Sweetman 1990).

Anomeric configuration can be revealed using GC-MS by identification of which monosaccharides in the acetylated oligosaccharide are resistant to chromic acid oxidation. Axially linked (most α-linkages) peracetylated sugars are resistant to chromic acid oxidation, and equatorially linked (most β-linkages) are oxidized to 5-keto-hexulosonates (Beeley 1985, Khoo and Dell 1990, Hellerqvist et al. 1972, Hellerqvist and Sweetman 1990).

A.3 Circular dichroism

Circular dichroism (CD) and UV spectroscopic techniques have been developed as an alternative to methylation analyses by Nakanishi and colleagues (Nakanishi et al. 1984, Park, Takeda, and Nakanishi 1987, Takeda, Zask, and Nakanishi 1987, Wiesler et al. 1990). The chemical steps of this method have evolved over the last six years and now the oligosaccharides are derivatized with the chromophore 4-bromobenzoyloxy. The per(bromobenzoylated) oligosaccharides are then hydrolyzed by either of two variations of the acetolysis/ bromination reaction. After hydrolysis the free hydroxyls are derivatized with the chromophore 4-methoxycinnamoyloxy (Wiesler et al. 1990). The ratio of the two chromophores and the number of linkages of the monosaccharide can be determined by UV analysis. The CD spectra of each derivatized monosaccharide is unique for the sugar residue and for the pattern created by the two chromophores. This pattern reflects the linkages of the original monosaccharide. The monosaccharide derivatives can be analyzed by mass spectrometry to obtain further information as required.

A CD spectral library has been recently published of approximately 150 different glycopyranosides (Wiesler et al. 1990). The CD data for the furanosides must be obtained experimentally as the furanosides are more flexible conformationally than the pyranosides. The CD spectra expected for the pyranosides can be calculated with good accuracy (Wiesler et al. 1990). The method for the glycopyranoside structural analysis does not rely on the use of synthetic standards and requires only nmol quantities of sample. From the spectra the identities of the monosaccharide, linkage pattern, and absolute conformation can be deduced. This is an interesting

and potentially very valuable new technique in structural elucidation of carbohydrates.

A.4. Analysis of oligosaccharides by NMR

Carbohydrates are well-suited for analysis by NMR because of their normal elemental components, ^{1}H and ^{13}C. One aspect of oligosaccharide structure simplifying analysis by NMR is that the majority of sugar residues are in the pyranose form in a rigid chair conformation. Also, due to steric and electronic factors, the glycosidic bonds between the residues exist in no more than a few major conformations (Barker et al. 1982a, Lemieux 1978, Lemieux, Kota, and Voisin 1979, Sweeley and Nunez 1985).

One-dimensional (1D) NMR analysis of complex oligosaccharides has been very useful in the determination of anomeric configuration. Almost all oligosaccharide protons have chemical shifts between 3.6 and 4.3 ppm (Bock and Thogerson 1982). The assignment of chemical shift and coupling constants in the crowded region of 1D spectra of many carbohydrates is therefore difficult. However, the anomeric or H-1 protons of each residue have chemical shifts between 4 and 6 ppm where few other resonances occur. Chemical shifts and coupling constants can be used to distinguish α– and β–anomeric configuration. Except for carbohydrates in the *manno*-configuration, protons in the α-anomeric configuration have smaller J vicinal coupling constants (≈1-3 Hz) and tend to have greater chemical shifts (usually >5 ppm). Protons in the β-anomeric configuration have larger vicinal coupling constants (≈7-8 Hz), and tend to have smaller chemical shifts (usually <5 ppm) (Michon et al. 1990).

Techniques such as homo- (Bock and Thogerson 1982) and heteronuclear decoupling (McFarland and Rycroft 1983) and isotope enrichment have been used for structural elucidation in some cases (Barker et al. 1982b, Christofides and Davies 1983, Sweeley and Nunez 1985). One NMR technique widely used in oligosaccharide sequence analysis is 1D nuclear Overhauser effect (NOE) difference spectroscopy. Saturation of a selected nucleus produces an alteration in the population of an energy level of a nearby nucleus which is revealed by the change in the size of the peak for that nucleus. The protons involved must be no greater than approximately 3 Å apart to show measurable NOEs. This technique is useful in establishing inter-residue connectivities and anomeric conformations as irradiation of the anomeric proton will affect the protons of the aglycone across the glycosidic linkage only if they fall within this range (Sweeley and Nunez 1985).

Relaxation experiments involve the spin-lattice relaxation time (T1) which is the rate at which the NMR signal intensity decreases from the point of sample irradiation to the acquisition of the free induction decay (FID) signal. The T1 of a nucleus is, therefore, a product of the environment of that nucleus (Sweeley and Nunez 1985). Once a resonance has been assigned to a specific sugar residue then NOE and relaxation techniques can be used to assign other resonances and assist in determining sequence and linkage (Bock and Thogerson 1982, Dabrowski 1987).

The development of two-dimensional (2D) NMR in the 1970's was made possible by the coupling of high-field superconducting magnets with powerful computers, and the introduction of pulsing sequences that reveal more structural features (Aue, Batholdi, and Ernst 1976, Macura et al.

1981, Maudsley and Ernst 1977). The data matrices obtained by these sequences, although really containing three-dimensional information, are known as 2D NMR (Koerner, Prestegard, and Yu 1987).

There are two general types of ^1H 2D NMR experiments that have been used for oligosaccharide elucidation, *J*-resolved and correlated. In *J*-resolved experiments one frequency axis contains coupling (*J*) information and the other contains chemical shift information. Resonance intensity is the third dimension and is presented as a contour. In correlated experiments both axes contain chemical shift information with resonance intensity again shown as a contour. In both types of 2D experiments either homonuclear or heteronuclear analyses can be performed.

Proton homonuclear *J*-resolved 2D NMR has not been used a great deal for oligosaccharide analysis due to insensitivity and inability to provide *J*-connectivity (Koerner, Prestegard, and Yu 1987). Scalar correlated and dipole correlated experiments are among the main types of correlated 2D experiments. Scalar correlated experiments reveal through-bond connectivities by scalar couplings through transfer of transverse magnetization, and are also referred to as *J*-correlated and chemical shift correlated spectra. Correlation spectroscopy (COSY) and spin echo correlated spectroscopy (SECSY) are two versions of scalar correlated 2D NMR. Dipole correlated spectra reveal through-space connectivities. One type of dipole correlated experiment used in oligosaccharide analysis is 2D nuclear Overhauser effect spectroscopy (NOESY) (Koerner, Prestegard, and Yu 1987).

The techniques of ^1H 2D NMR have been very useful in analysis of oligosaccharides and glycoconjugates due to the presence of one proton on each of the ring carbons of most carbohydrates, except ketose sugars. This

means that a "vicinal *J*-coupled connectivity" exists such that each proton can lead to the next proton (Koerner, Prestegard, and Yu 1987). Because the anomeric protons fall within a relatively uncrowded region of the spectrum they provide a starting point for identification of isolated monosaccharide spin systems. The close proximity of the anomeric protons and the linkage site protons allows through-space coupling to be revealed by 1D and 2D NOE experiments so that the J-connectivity of the glycosidic bond can also be followed. Thus, the entire oligosaccharide can be viewed as continuously coupled from one end to the other (Koerner, Prestegard, and Yu 1987). The problem of crowding in the 3-5 ppm region of the spectra of oligosaccharides can be overcome through 2D NMR techniques such as homonuclear Hartmann-Hahn (HOHAHA) spectroscopy which shows relayed connectivities (Bax and Davis 1985), and multiple quantum filter COSY, which produces greatly simplified spectra (Piantini, Sorensen, and Ernst 1982). Also, 2D heteronuclear ^{13}C-^{1}H spectroscopy can be used for unambiguous inter-residue connectivity by providing chemical-shift correlations (Lerner and Bax 1986, Lerner and Bax 1987).

The amount of oligosaccharide required for these different types of NMR analyses varies. The following general estimates of the minimal amounts of oligosaccharide required will obviously vary depending on the size, complexity, homogeneity and purity of the sample:

1D. ^{1}H 20-100 μg, ^{13}C 0.5-1 mg

2D. J-correlated 0.5 mg, J-resolved 1 mg, NOE 2 mg,
^{13}C-^{1}H 5-10 mg, ^{13}C-^{13}C 500 mg

(Koerner, Prestegard, and Yu 1987, Sweeley and Nunez 1985). Typically a great deal more than these minimum quantities are needed for NMR experiments. Also in general, much larger amounts of sample are

necessary to perform ^{13}C NMR of oligosaccharides than ^{1}H NMR. The natural abundance of ^{13}C is only 1.1% and ^{13}C is less sensitive (1/64 of ^{1}H) (Sweeley and Nunez 1985).

Analysis of oligosaccharide structure by NMR, particularly 2D NMR, has been used to determine the identity of individual monosaccharides residues, the linkages between the residues, and the sequence of the monosaccharides (Dabrowski 1987). The complete primary structure of oligosaccharides has been determined almost entirely from NMR analyses (Abeygunawardana, Bush, and Cisar 1990, Dabrowski et al. 1988). More commonly, NMR is used with a number of other techniques in the determination of oligosaccharide structure (Michon et al. 1990, Romanowska et al. 1988). However, the only current method to determine the three-dimensional structures of oligosaccharides in solution is NMR. (X-ray crystallography can be used to determine three-dimensional structures of crystallized oligosaccharides.) The assignment of oligosaccharide structure by NMR can in many cases be simplified by reference to the ever-increasing amount of published NMR data (Bock and Thogerson 1982).

B. Development of hydrazino reagents

The rationale for and the results obtained from the development of aromatic hydrazino reagents for chromatographic separation and LSIMS analysis (both MS and MS/MS) of bacterial oligosaccharides are presented in this section of Chapter II. Structural elucidation of the oligosaccharide portion of the LOS from *N. gonorrhoeae* was the overall objective of these

studies. One priority in this research was to minimize the amount of LOS needed such that more structures could be determined in a given time period, and perhaps gel-eluted material could eventually be used. Thus, the task of improving the methods for microscale structural analyses of bacterial oligosaccharides was undertaken, including the synthesis and use of hydrazino reagents.

B.1. Materials and methods

Materials

Maltoheptaose, 3-deoxy-*manno*-2-keto octulosonic acid (KDO), LPS from *Salmonella typhimurium* TV 119 Ra mutant, and 4-aminobenzoic acid ethyl ester (ABEE) were all obtained from Sigma (St. Louis, MO). The compound 4-aminobenzoic acid butyl ester (ABBE) was purchased from Fluka (Buchs, Switzerland). As part of a collaborative project, 4-butylphenylhydrazine, 4-octylphenylhydrazine, and 4-tetradecylphenylhydrazine were a gift from Edward M. Allen and James W. Webb at Illinois State University in Normal, Illinois. Details of the synthesis of these hydrazines will be published elsewhere. Aqueous HF (48%) was purchased from Mallinckrodt (Muskegon, MI). Acetic anhydride was obtained from Supelco (Bellefonte, PA). Acetonitrile and water for HPLC separations were obtained from Burdick and Jackson (Muskegon, MI) as their HPLC grades. All other reagents and solvents used were of reagent grade. Ultramark® 1621, used in the calibration of the Kratos MS50 mass spectrometer, was obtained from PCR Research Chem. Inc. (Gainesville, FL).

Methods

Isolation and purification of the oligosaccharides from JW31R LOS. The LOS from a pyocin-resistant *N. gonorrhoeae* (strain JW31R) was isolated and prepared by our collaborator Dr. M. A. Apicella at the State University of New York at Buffalo. The gonococci were washed three times with acetone and then air-dried and the LOS was extracted by the procedure of Westphal and Jann using hot phenol and water (Westphal and Jann 1965). The water phase was carefully removed and dialyzed repeatedly against distilled water. Nucleic acids were removed by ultracentrifugation and insoluble lipids by extraction with 80% EtOH (Bertram, Griffiss, and Broud 1976, Gibson et al. 1989, Schneider et al. 1982). The oligosaccharide fraction was isolated from the LOS by cleaving between the Lipid A and oligosaccharide moieties with 1% acetic acid at 100° C for 2 hours. The insoluble lipids were then precipitated by centrifugation at 47,800 x *g* for 20 min. The precipitate was washed with 5 ml of H_2O and centrifuged at 47,800 x *g* for 10 min, repeating the washing and centrifugation twice. The combined supernatants were lyophilized, dissolved in 1 ml of H_2O, centrifuged, and again the supernatant was lyophilized. The oligosaccharide fraction was applied to a Bio-Gel P-4 column (2.6 x 90 cm, <400 mesh) in 100 mM ammonium acetate (pH 7) and fractions collected in 4.5 ml intervals in the same buffer. Fraction 52 from this P-4 column was further desalted over a Bio-Gel P-2 column. Refractive index detection was used with both columns. (Gibson et al. 1989). The resulting oligosaccharide mixture was derivatized with 4-aminobenzoic acid ethyl

ester (ABEE) (Wang et al. 1984) and separated by reverse phase HPLC as described later.

Purification of the oligosaccharides from *Salmonella typhimurium* TV 119 Ra mutant. The oligosaccharide was prepared according to the procedure of Johnson and Perry (Johnson and Perry 1976). The LOS was dissolved in 1% acetic acid (2 mg/ml) and hydrolyzed at 90° C for 90 min and then lyophilized. The products were suspended in 10 ml of 50 mM pyridinium acetate buffer (pH 5.2), and centrifuged at 3400 x *g* for 10 min. The supernatant was lyophilized, resuspended in 1-2 ml of the pyridinium acetate buffer and centrifuged. The soluble oligosaccharide was gel-filtered on a Sephadex G-50 (1.5 x 30 cm) column equilibrated with the same buffer. Phenol sulfuric acid tests were performed on each fraction to locate the eluting oligosaccharides (Dubois et al. 1956). Briefly, this test consisted of adding 200 µl of 5% w/v phenol and 1 ml of concentrated sulfuric acid to 100 µl of a 1 ml sample (every other sample was tested) and vortexing. The mixture was allowed to sit for 10 min at room temperature, transferred to cuvettes and the VIS absorbance at 490 nm recorded. Those samples containing oligosaccharide had increased absorbance compared to those not containing oligosaccharide. Fractions testing positive were combined and lyophilized and then desalted over a Bio-Gel P-2 column in 1% acetic acid. Phenol sulfuric assays were again used to locate the fractions containing oligosaccharide, and these fractions were then pooled and lyophilized.

Isolation and purification of the oligosaccharides from JW31 LOS. The LOS was isolated from gonococcal strain JW31, as previously described for

the LOS from strain JW31R, by our collaborators at Walter Reed Army Hospital (Dr. H. Schneider) (Gibson et al. 1989, Schneider et al. 1982). Approximately 14 mg of LOS was dissolved in 7 ml of 1% acetic acid, and heated to 100° C with stirring for 2 hours. The hydrolysates were centrifuged at 5000 x *g* for 20 min, and the supernatant removed. The precipitate was washed with 3 ml of H_2O, centrifuged again and the supernatant removed and combined with the first. The pooled supernatant was lyophilized, then dissolved in 2 ml H_2O and centrifuge filtered using Microfilterfuge tubes (Rainin). The resulting oligosaccharide was divided into three fractions and each fraction was purified by gel filtration HPLC using two 600 x 7.5 mm Bio-Sil TSK-125 columns (BioRad) connected in series with a Waters 6000A pump and refractive index detection. The oligosaccharide samples were eluted in 1% acetic acid at a flow rate of 1 ml/min and fractions collected in 1 ml volumes.

Derivatization of the oligosaccharide from JW31R with ethyl 4-aminobenzoate (ABEE) and butyl 4-aminobenzoate (ABBE). A reagent mixture was first prepared using 0.165 g ABEE, 41 µl of glacial acetic acid, 350 µl methanol and 0.035 g $NaCNBH_3$. The oligosaccharides were placed in 5 ml reacti-vials with 10 µl H_2O and 40 µl of the reagent mixture and then heated at 80° C for 30 min. Each sample was then cooled and 0.5 ml each of H_2O and $CHCl_3$ added to each tube. After thorough mixing, the H_2O layer was removed and the remaining $CHCl_3$ was extracted twice with 0.5 ml of H_2O. All three aqueous layers were then combined and dried under vacuum. The dried oligosaccharide material was then suspended in a small amount of H_2O and separated by reverse phase HPLC as described below. The procedure for the derivatization of these oligosaccharides with

ABBE was similar, except that the reagent mixture was made with 0.193 g of ABBE, 41 µl glacial acetic acid, 0.035 g $NaCNBH_3$, and 700 µl MeOH. To each sample, 10 µl H_2O and 70 µl of the ABBE reagent mixture was added.

Acetylation of primary amines in the oligosaccharide from *S. typhimurium* TV 119 Ra mutant. Several drops of MeOH were added to approximately 100-150 µg of oligosaccharide in a 3 ml reacti-vial. The MeOH was dried under a N_2 stream and several more drops of MeOH were added and again dried. Keeping the dry oligosaccharide under a N_2 stream the following were added in order, with vortexing after each addition: 500 µl MeOH, 50 µl pyridine, and 50 µl acetic anhydride. The sample was capped and the reaction proceeded at room temperature for 30 min. The sample was dried under N_2 and several drops of MeOH were added followed by drying the sample again under N_2.

HF-treatment of oligosaccharides. To remove phosphoester moieties, approximately 2 mg of the oligosaccharide fraction from *S. typhimurium* LOS was placed in a 1.5 ml polypropylene tube with 200 µl of cold 48% aqueous HF solution for 24 to 96 hours at 4° C (Jennings, Hawes, and Adams 1973). Although we used a 96 hour HF-treatment in our earlier work on JW31R OS (Gibson et al. 1989) the HF-treatment for 24 hours was found to greatly reduce glycosidic bond cleavage without a noticeable effect on overall yields. The excess HF was removed under vacuum in a polypropylene desiccator with a solid NaOH in-line trap. As HF is extremely toxic great care must be taken to work in a well-ventilated hood.

Synthesis of ethyl 4-hydrazinobenzoate (HBEE) and butyl 4-hydrazinobenzoate (HBBE). To prepare HBEE approximately 4 g of ABEE were diazotized and reduced (Kochetkov et al. 1959). To 4 g of ABEE (0.024 moles) dissolved in 22 ml H_2O 8.5 ml of concentrated HCl wasw added. The solution was cooled to -5° C. A cooled solution (-5°C) of 1.65 g of $NaNO_2$ (0.024 moles) in 8.5 ml H_2O was then added, and the mixture stirred at -5°C for approximately 1 h. Then this mixture was slowly poured with stirring into the cold solution (-5°C) of 10.2 g of $SnCl_2$ in 10.2 ml concentrated HCl. A solution of 40% NaOH (9 ml) was added to this mixture. Approximately 30 ml of MeOH was added and the solution filtered using a Buchner funnel and filter paper. Then most of the MeOH was removed with a rotary evaporator until product crystals formed. The solution was filtered again and the filtrate air-dried. This product was recrystallized twice more for further purification. Just enough boiling MeOH (≈5 ml) to dissolve the crystals was added and the solution was kept at 4°C until crystals formed. The MeOH was decanted and the crystals were washed with cold MeOH and allowed to air-dry. Next, the crystals were dissolved in 8 ml of MeOH at room temperature and H_2O was added dropwise until cloudiness was observed. The mixture was warmed until the cloudiness disappeared, and then cooled to room temperature. The resulting crystals and solution was filtered and the product stored at -20°C. The product was a white crystalline solid and the overall yield was approximately 10%. The product was found to be a single major peak by reverse phase HPLC with UV detection at 320 nm. The sample was dissolved in 20% acetonitrile in H_2O and injected onto a C-18 column (Vydac, 25 cm x 4.6 mm i.d.) equilibrated with the same acetonitrile solution, followed by a 1%/min gradient of increasing acetonitrile. Under these conditions baseline separation was obtained. The

product HBEE eluted at about 10 min (≈ 27% CH_3CN) and the starting material ABEE eluted at 15 min (≈32% CH_3CN). High resolution mass spectrometric analysis of the recrystallized product confirmed the chemical composition $C_9H_{12}N_2O_2$ (experimental m/z 180.0908, calculated m/z 180.0899). Although originally a yellow color, the final quite pure product was white.

In an analogous manner, 4-hydrazinobenzoic acid butyl ester (HBBE) was also prepared by diazotization and reduction of 1.9 g of butyl 4-aminobenzoate as described above for the synthesis of HBEE. Approximately 15 ml of MeOH at room temperature was added, the solution filtered and then some of the MeOH removed using a rotary evaporator. After crystals formed the mixture was filtered and the crystals air-dried. The product was further purified by extraction with H_2O/MeOH/CH_2Cl_2 (10:70:20). The pure HBBE was found in the yellowish organic phase, and the solvent was evaporated with a rotary evaporator. The overall yield was approximately 10%. The purity of the product was determined using the same HPLC method described for HBEE. High resolution mass spectrometric analysis of this compound confirmed the expected elemental composition of $C_{11}H_{16}N_2O_2$ (experimental m/z 208.1218, calculated m/z 208.1212).

Derivatization of oligosaccharides with HBEE, HBBE, and 4-butylphenylhydrazine (BPH). The oligosaccharide samples (from 10 to 100 nmol) were placed in 1 ml glass reacti-vials and approximately 2 equivalents of a hydrazine reagent solution was added. The reagent solution contained 1-2 μg/μl of hydrazine in MeOH with approximately 1 μl of glacial acetic acid per 100 μg of the hydrazine. The total volume was

brought up to 40 µl with MeOH, followed by the addition of 10-20 µl of H_2O. The resulting reaction mixture was then heated at 80° C for 30 min or, alternatively, 45 min at 60° C as either procedure yielded approximately the same amount of product. The mixture was cooled and dried under N_2, and the oligosaccharide derivatives were dissolved in H_2O for HPLC separation.

Derivatization of oligosaccharide from *S. typhimurium* TV 119 Ra mutant with 4-tetradecylphenylhydrazine (TDPH). HF-treated oligosaccharide from *S. typhimurium* TV 119 Ra mutant (≈100 µg) was placed in a 1 ml glass reacti-vial and three molar equivalents of TDPH (55 µg) were added. To the sample vial were added 45 µl of MeOH, 5 µl of H_2O and 0.5 µl of acetic acid. The reaction mixture was heated with intermittent sonication at 80° C for 30 min. The mixture was cooled and the oligosaccharide derivatives were dissolved in 10% acetonitrile/H_2O for HPLC separation as described in the next section.

HPLC purification of derivatized oligosaccharide. Separations of the oligosaccharide derivatized with HBEE, HBBE and BPH were carried out using reverse phase HPLC. A C_{18} column (Vydac, 25 cm x 4.6 mm i.d.) was used on a Rainin gradient HPLC system equipped with a Kratos 783 variable UV detector. The column was equilibrated with H_2O containing 0.05% trifluoroacetic acid (solvent A) and the oligosaccharides were eluted with a linear gradient of 0 to 40% (or 50%) acetonitrile containing 0.05% trifluoroacetic acid (solvent B) in 40 (or 50) min at a flow rate of 1 ml/min. Purification of the oligosaccharide derivatized with TDPH was similar except that a C_4 column was used (Vydac, 25 cm x 4.6 mm i.d.) due to the greater hydrophobicity of TDPH, and the oligosaccharides were eluted with

a linear gradient of 10 to 100% solvent B in 45 min at a flow rate of 1 ml/min. The HPLC purification of the corresponding amino benzoate ester (ABEE and ABBE) derivatized oligosaccharides was the same except no acid was added to the HPLC solvents. The eluting hydrazino benzoate and alkyl phenyl hydrazino oligosaccharide derivatives were detected at 335 nm with the exception of the first few separations in which 320 nm was used. The amino benzoate oligosaccharide derivatives were detected at 320 nm. The oligosaccharides were dried down on a Savant liquid concentrator prior to analysis by LSIMS. In some cases, an ABI 1000S diode array detector was used to obtain complete UV spectra of the eluting oligosaccharide derivatives in order to determine the λ max of the derivatized oligosaccharides.

Quantitation by GC. Samples of oligosaccharides from the Ra mutant of *S. typhimurium* and gonococcal pyocin-resistant strain 1291_b were HF-treated for 24 hours and then hydrolyzed in 1N methanolic HCl. The resulting methyl glycosides were trimethylsilylated for quantitation analysis by GC (Sweeley and Walker 1964) with mannitol as an internal standard. Prior to hydrolysis and trimethylsilylation, portions of these samples were derivatized with HBEE according to the procedure described above. These HBEE derivatives were purified by HPLC analysis and all the eluting sample peaks and the void volume were collected. The portions of these samples collected from the HPLC were then also hydrolyzed in 1N methanolic HCl, trimethylsilylated, and quantitated using GC with mannitol as an internal standard. A Varian model 3700 GC with a flame ionization detector was used, and was equipped with a DB-1 capillary column (Scientific Products, 30 m x 0.25 mm i.d.). The carrier gas was He,

and H_2 and O_2 gas were used for the flame ionization detector. From 0.5-1.5 µl splitless injections were made at 115° C. After 45 s the splitter was turned on, and after 8 min the temperature of the GC turned up to 150° C. Upon reaching 150° C a program was begun increasing the temperature by 4° C/min to 280° C. All peaks were integrated using a Hewlett Packard 3390A integrator. After normalization to the internal standard, a comparison was made of the relative areas of the peaks of the trimethylsilylated monosaccharides from the (1) starting material, (2) that portion of the starting material collected from the HPLC as the HBEE derivatives, (3) and the starting material recovered unreacted from the void volume of the HPLC analysis. This was done to accurately determine the overall yield of the reaction, and to determine the portion of the starting material which could be recovered unreacted in the void.

LSIMS analysis. Oligosaccharide-containing HPLC fractions were analyzed on a Kratos MS-50S double-focusing mass spectrometer operating at a mass resolution of 1500 to 2500 (m/Δm, 10% valley) depending on the mass range of interest. Approximately 1/2-1 µl of glycerol/thioglycerol (1:1) was applied to the stainless steel probe tip prior to analysis by LSIMS. Then the oligosaccharide sample dissolved in water was added to the liquid matrix. A Cs^+ ion primary beam of 10 keV was used to sputter and ionize the sample and the resulting secondary ions were accelerated at 6 or 8 kV. Scans were typically taken at 300 s per decade and spectra were recorded at a Gould electrostatic recorder as described previously by Falick et al. (Falick, Wang, and Walls 1986). The spectra were manually calibrated with Ultramark® 1621 to an accuracy of better than ±0.2 Da.

Tandem MS/MS analysis. A four-sector 10,000 Da mass spectrometer (Kratos Concept II HH, Manchester, England) equipped with an optically-coupled diode array detector on MS-II was used to acquire all MS/MS spectra (Walls et al. 1990). LSIMS spectra were taken in negative ion mode using a Cs^+ beam energy of 18 keV. The deprotonated molecular ions $(M-H)^-$ were selected in MS-I and passed through to the collision cell filled with helium. The helium cell pressure was adjusted to attenuate the parent molecular ion by approximately 2/3. The resulting fragment or daughter ions were then separated in MS-II and detected in successive 4% mass windows using a magnet jump with a constant B/E ratio. Daughter ions were detected on a one-inch optically-coupled diode array detector.

B.2. Results and Discussion

Various alkyl esters of 4-aminobenzoic acid have been previously used in this laboratory as reducing terminal derivatizing reagents for oligosaccharides (Poulter et al. 1988, Webb et al. 1988, Poulter and Burlingame 1990). In this reaction scheme the amino group of the amino-benzoate esters forms a Schiff base with the carbonyl of the reducing terminus of the oligosaccharides, which is then reduced to the secondary amine with $NaCNBH_3$. These esters are excellent UV chromophores and also impart hydrophobicity to the oligosaccharides. The increased hydrophobicity of these oligosaccharide derivatives allows the oligosaccharides to be separated by reverse phase HPLC as well as dramatically increasing the sensitivity of their detection by LSIMS. In addition, these derivatives have been shown to increase molecular ion abundances and fragmentation of *N*-linked oligosaccharides isolated from

various glycoproteins, presumably, by increasing the surface activity of the oligosaccharide, enhancing sputtering efficiency and charge retention on the reducing terminus (Webb et al. 1988). Increasing the hydrophobicity of the benzoate by lengthening the alkyl chain increases sensitivity by a factor of 40 over the methyl 4-aminobenzoate derivative (Poulter et al. 1988, Poulter and Burlingame 1990), presumably by increasing the surface concentration and sputtering efficiency of the oligosaccharides in the LSIMS matrix.

To evaluate this strategy for the structure elucidation of LOS-derived oligosaccharides, we attempted to derivatize various oligosaccharide fractions from JW31R, a pyocin-resistant strain of *Neisseria gonorrhoeae*, whose partial structures had previously been determined in our laboratory (Gibson et al. 1989). In one experiment, a fraction from a Bio-Gel P-4 column that contained at least two related oligosaccharides (M_r 1838 and 1596) with KDO at the reducing terminus was derivatized with ABEE. Reverse phase HPLC separation of the resulting oligosaccharide ABEE derivatives showed the presence of multiple components, several of which were well resolved (Fig. 2).

Negative ion LSIMS analysis of two of the later eluting partially resolved peaks gave two molecular ions at m/z 1700 and 1862[1] (peak 4) and m/z 1942 (peak 5). These masses were consistent with the decarboxylated ABEE derivatives of three major oligosaccharide structures; $Hex_5HexNAc_2Hep_2KDO$ (M_r 1838), $Hex_4HexNAcPEAHep_2KDO$ (M_r 1596)

[1]In this thesis, unless stated otherwise, the nominal mass of the ^{12}C-containing isotope of the molecule has been used. The masses of the atoms composing the molecule rounded to the nearest integer are summed to determine the nominal mass. The exact mass is the sum of the exact masses of the atoms comprising the molecule containing only the most abundant isotopes of each atom, i.e. ^{12}C=12.00, ^{1}H=1.007825. The average mass of the molecule is the average of the mass of a molecule containing all of the different isotopes of the component atoms in their naturally occurring ratios.

and $Hex_5HexNAcPEAHep_2KDO$ (M_r 1758), (see Fig. 2). In addition, these oligosaccharide derivatives produced more abundant molecular ions with more extensive fragmentation than the corresponding underivatized material. In fact, the spectrum of the largest oligosaccharide contained reducing terminal Y-type fragment ions at m/z 1780, 1739, 1618, 1577, 1415, 1253, and a very small ion at m/z 858, that correspond to cleavage at every glycosidic linkage, except between KDO and heptose (Fig. 3).

The nomenclature proposed by Domon and Costello for putative fragment ions of oligosaccharides has been used in many of the spectra presented in this thesis (Domon and Costello 1988). In Figure 4, a diagram illustrating the basic fragment types according to this nomenclature is shown. Briefly, Y- and Z-type ions correspond to fragments produced by cleavage of the glycosidic bond with charge retention on the reducing terminus. B- and C-type ions also are formed by glycosidic bond cleavage but with charge retention on the non-reducing terminus. Both A- and X-type fragment ions are produced by ring cleavage, and are postulated to be formed by a concerted mechanism in which more than one bond is broken (Domon and Costello 1988). According to this nomenclature the branch of an oligosaccharide with the greatest mass is designated as the α branch, the next in mass is called the β branch and the γ branch would be the smallest of three branches.

A spectrum similar to that shown in Figure 3 was also obtained for the smaller decarboxylated oligosaccharide from JW31R (M_r 1596) containing PEA. In general, fragment ions produced from Hep-Hep cleavages in two-sector LSIMS of gonococcal oligosaccharides with reducing terminal derivatives are often either not observed, or of very low abundance. Indeed, this was the case for the Hep-Hep cleavage product at

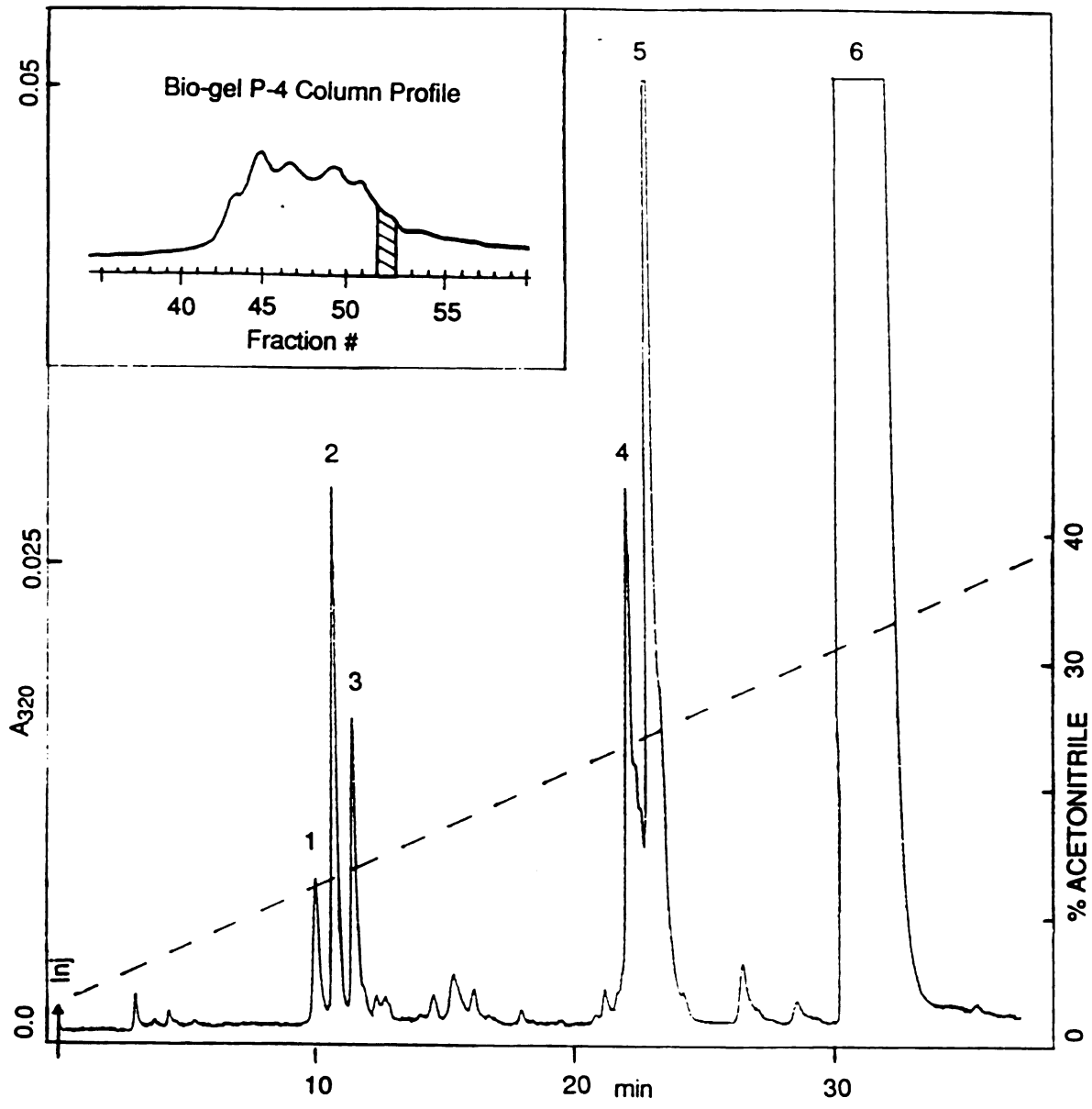


Figure 2. Reverse-phase HPLC chromatogram of JW31R oligosaccharides (fraction 52) derivatized with ABEE. Approximately 50 μg of starting oligosaccharide was used. The inset shown is the P-4 column profile of the JW31R oligosaccharides with fraction 52 marked. Peak 1 was not identified and peak 6 was excess unreacted ABEE.

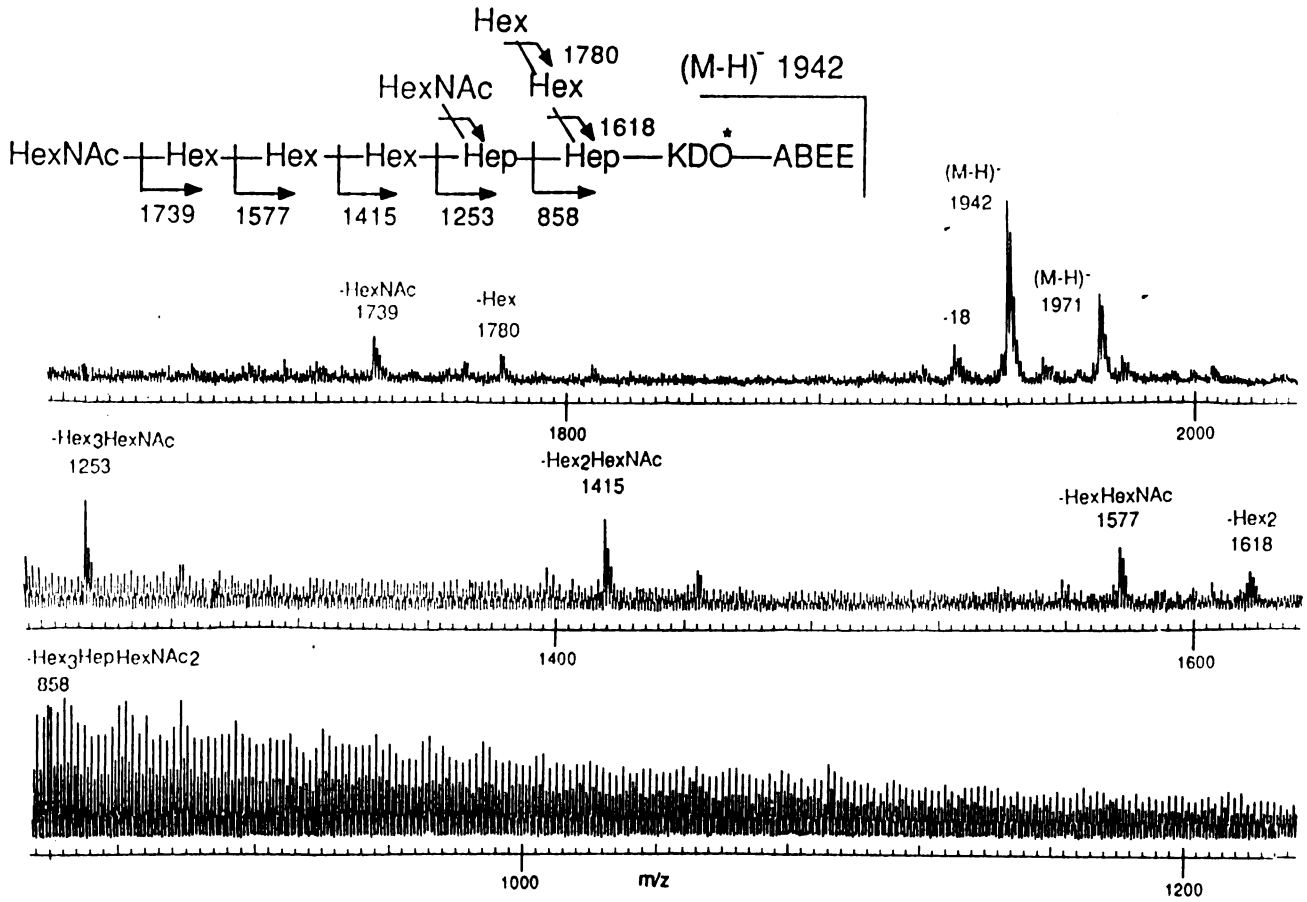


Figure 3. Negative-ion LSIMS spectrum of decarboxylated JW31R oligosaccharide from fraction 52 after derivatization with ABEE. The less abundant molecular ion at m/z 1971 corresponds to a second unresolved component of similar structure. The labeled peaks (1780, 1739, 1618, 1577, etc.) are all Y-type fragment ions. Small amounts of X- and Z-type fragments can be seen +28 and -18 Da from these Y-type fragment ions.

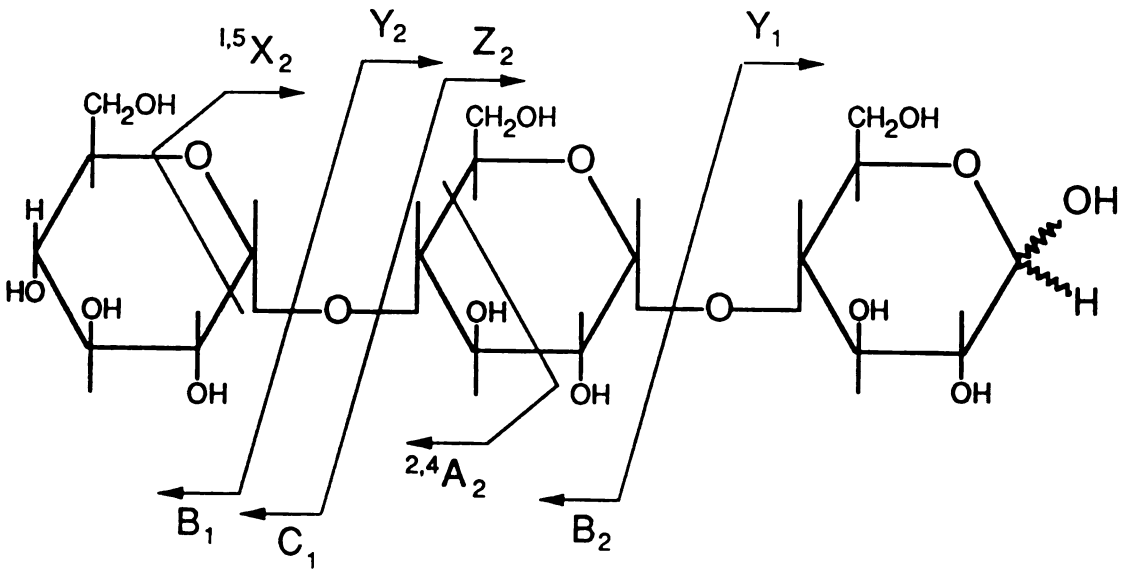
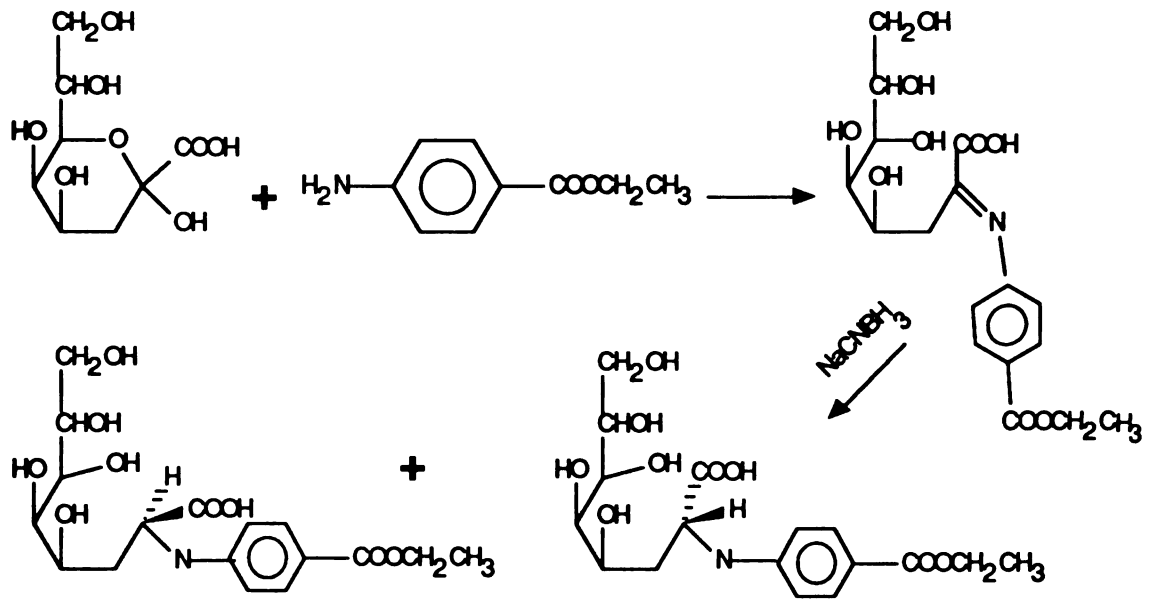


Figure 4. Illustration of the nomenclature proposed by Domon and Costello (Domon and Costello 1988) and given to the oligosaccharide fragment ions in both LSIMS and MS/MS spectra.

m/z 858 in the LSIMS spectrum of the gonococcal JW31R OS (Fig. 3) which was observed at a signal to background ratio of only approximately 2/1. Peaks 2 and 3 (Fig. 2) both were found to produce the same molecular ion at m/z 1986 and corresponds to the oligosaccharide $Hex_5HexNAc_2Hep_2KDO$ without decarboxylation (M_r 1838). After reductive amination with ABEE, the C-2 carbon of the reducing terminal KDO moiety is a chiral carbon as can be seen in Scheme 1, and peaks 2 and 3 may represent diastereomers.

Unfortunately, attempts to apply this derivatization procedure to other neisserial LOS were not as successful, and low yields were generally found (< 5%). In Figures 5A and 5B are shown typical HPLC

Scheme I



chromatograms of the products of the reaction of ABEE with oligosaccharide isolated from the LOS of gonococcal strains 1291b and 1291c. To understand the origin of these low yields, we investigated the derivatization procedure using both KDO and a known oligosaccharide standard prepared from commercially available *Salmonella typhimurium* TV 119 Ra LPS. This latter oligosaccharide was released from the lipid

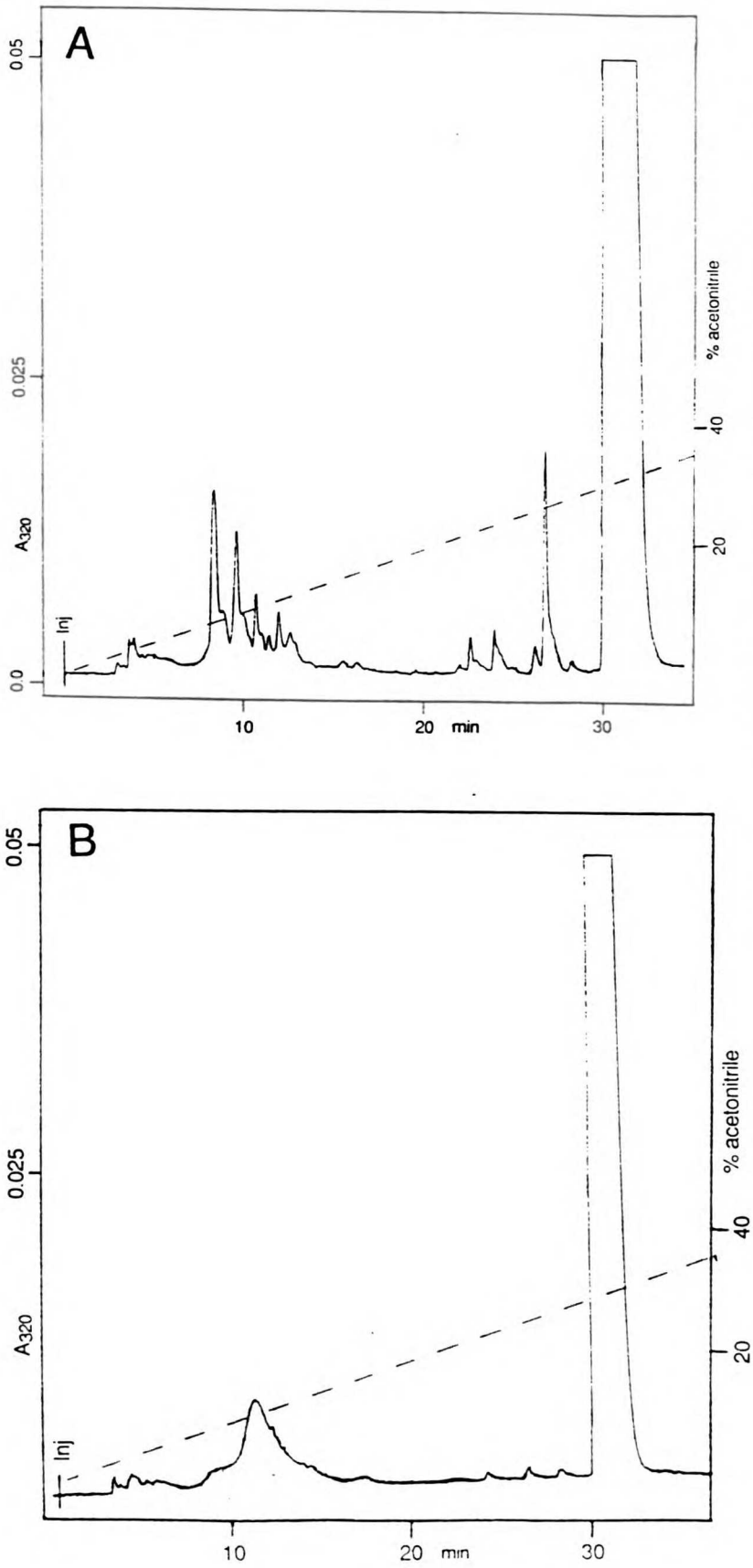


Figure 5. Reverse-phase HPLC chromatograms of (A) 1291_b and (B) 1291_c oligosaccharides after derivatization with ABEE. Approximately 50 μg of each starting oligosaccharide was used. In both chromatograms the large peak that eluted just after 30 min was excess unreacted ABEE. Nothing could be detected of either oligosaccharide in subsequent negative-ion LSIMS analysis.

under mild acid conditions and has KDO at the reducing terminus, but lacks the long repeating O-specific side chain polysaccharide typical of LPS structures. It is, therefore, similar to LOS-derived oligosaccharides in both size and composition.

According to the published LPS structure of this rough *Salmonella* mutant (Luderitz et al. 1974), a single oligosaccharide should result with a composition of $HexNAcHex_4Hep_3PEA(H_2PO_3)_2$ KDO (M_r 1948). LSIMS analysis of this oligosaccharide fraction showed two main structures with M_r 1948 and 2071 (see Fig. 6A). The larger molecular weight component differs by 123 Da from the smaller one, and corresponds to the addition of a second PEA. This difference was confirmed by aqueous HF treatment (96 hours) of this two component mixture that yielded a single common dephosphorylated species with M_r 1665 (see Fig. 6B).

After reaction with ABEE, the untreated oligosaccharides (M_r 1948 and 2071) could be identified as two separated components by reverse phase HPLC, but the yield of the expected ABEE-oligosaccharides was still low (< 5%). To determine if the primary amines on the phosphoethanolamine moieties of the oligosaccharide from *S. typhimurium* were hindering the reaction of the reducing terminal sugar KDO with ABEE, these amino groups were acetylated. However, the yield of the reaction with ABEE still appeared to be very low (<5%) (see Fig. 7A).

In contrast, after the derivatization of the HF-treated oligosaccharide fraction (M_r 1665) with ABEE a number of different oligosaccharide components were produced in comparable yield to the intact species based on UV absorbance (see Fig. 7B). Analysis of these separated components by LSIMS identified the major component as the expected ABEE derivative at m/z 1813 (peak 1 in Figure 7B), as well as the corresponding anhydro (M-

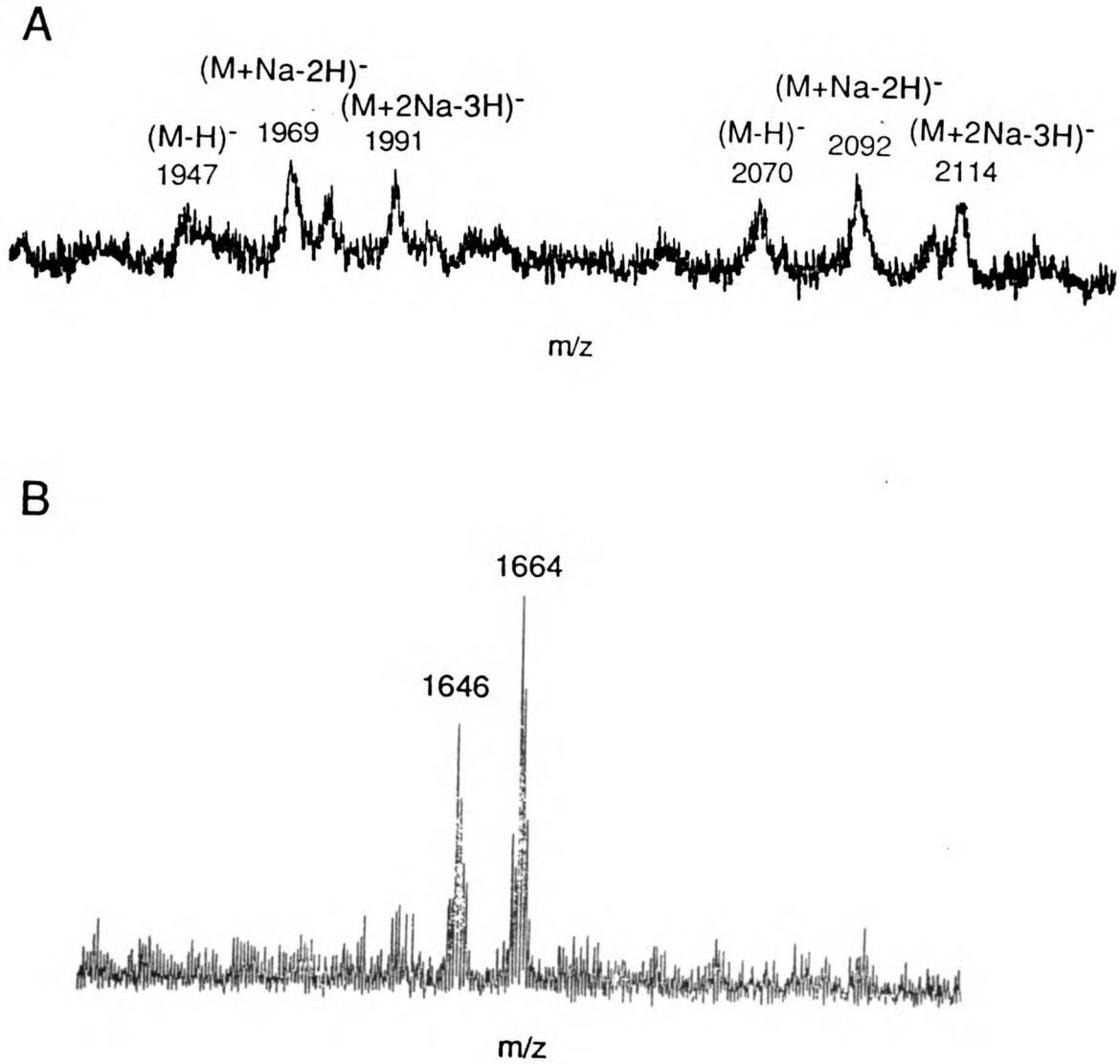


Figure 6. Negative-ion LSIMS spectra (A) of underivatized oligosaccharides from the TV119 Ra mutant of *S. typhimurium* and (B) the HF-treated underivatized *S. typhimurium* oligosaccharides. In panel A the two molecular ions, $(M-H)^-$, at 1947 and 2070 differ by 123, corresponding to one PEA moiety. The ions at 1969 and 2092 are the sodium adducts $(M+Na-2H)^-$ of the two molecular ions and the ions at 1991 and 2114 are the disodium adducts, $(M+2Na-3H)^-$, of the two molecular ions. In panel B the ion at m/z 1646 corresponds to an anhydro species $(M-H_2O-H)^-$.

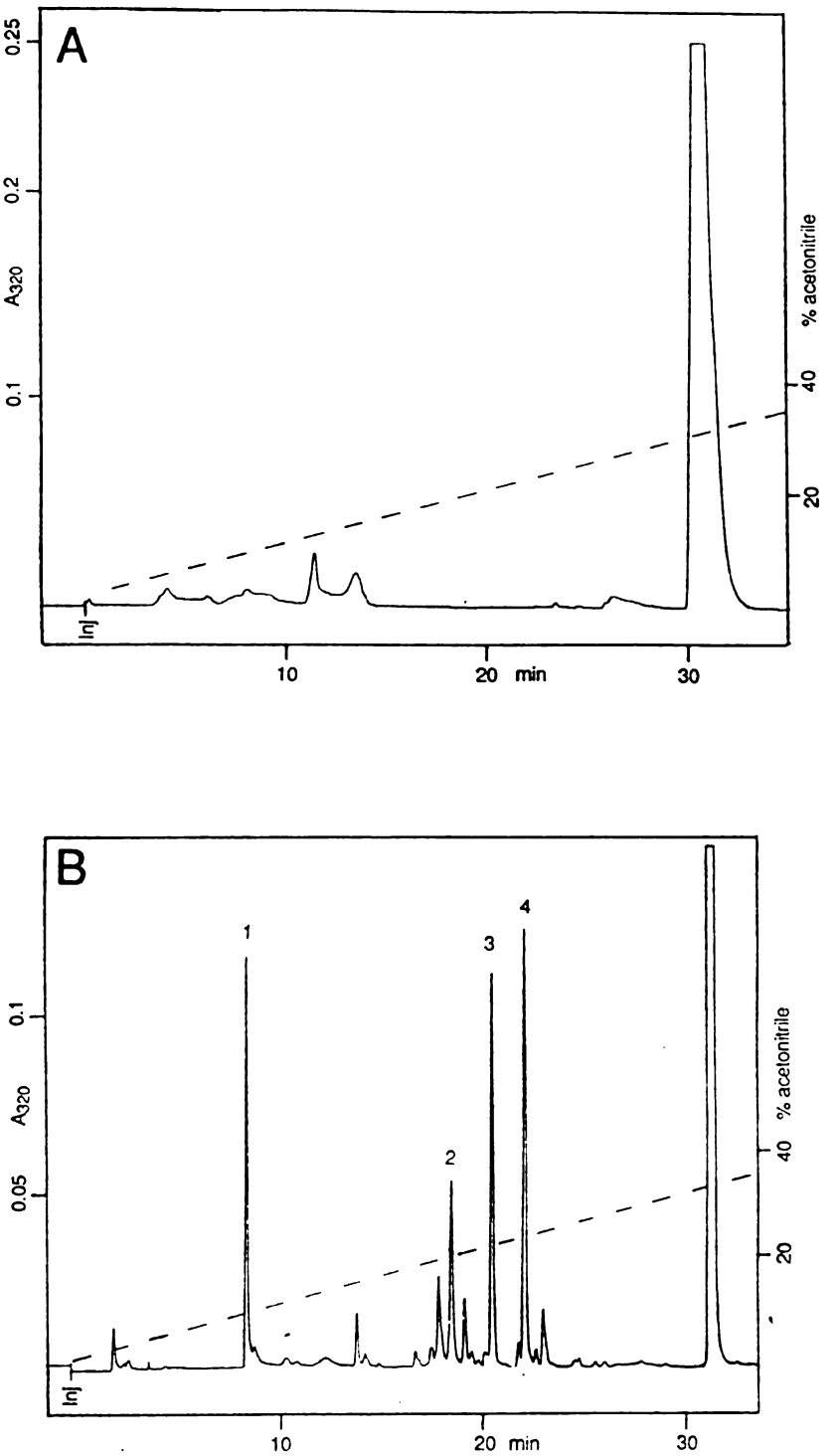


Figure 7. Reverse-phase HPLC chromatogram of (A) about 130 μg of the starting oligosaccharides from the TV119 Ra mutant of *S. typhimurium* after *N*-acetylation of phosphoethanolamine moieties followed by derivatization with ABEE and (B) about 95 μg of the starting *S. typhimurium* oligosaccharides derivatized with ABEE after dephosphorylation. In both chromatograms excess unreacted ABEE eluted just after 30 min.

H_2O-H)$^-$ and decarboxylated (M-CO_2-H)$^-$ analogs. However, several other major late eluting components were also identified as ABEE oligosaccharides that were non-KDO containing subfragments of the larger intact species (peaks 2, 3 and 4 in Figure 7B). The intact species appeared to be present in only about equal molar ratio with all of the smaller fragments which did not contain a reducing terminal KDO after the derivatization with ABEE. These truncated species appear to be arising from non-specific glycosidic bond cleavage during the HF treatment, and their identities were subsequently established by LSIMS analysis. No fragments containing KDO were found, only the intact oligosaccharide. In fact, decreasing the HF reaction time from 96 to 24 hours significantly reduces the number of these fragments.

These discrepancies suggest that the yields of the reaction with ABEE (or ABBE) when KDO was the reducing terminal sugar were considerably lower than when heptose, glucose, galactose or hexosamine residues were at the reducing terminus. This reduction in yield probably stems from the unfavorable initial formation of the Schiff base between the aromatic amino group of ABEE (or ABBE) and the hindered C-2 ketone of the α-keto acid moiety of KDO, compared to the more favorable reaction with aldose sugars.

To explore this reaction in more detail, a set of experiments were carried out with the KDO monosaccharide. Under standard conditions using ABEE, four major species were observed in negative LSIMS after HPLC separation (Fig. 8A); two partially resolved isomers with m/z 386 and two later eluting components that correspond to the more hydrophobic decarboxylated KDO-ABEE (m/z 342) and an anhydro species (m/z 368). As the derivatization itself could form two diastereomers, it was expected they might be partially resolved by HPLC. The formation of anhydro forms of

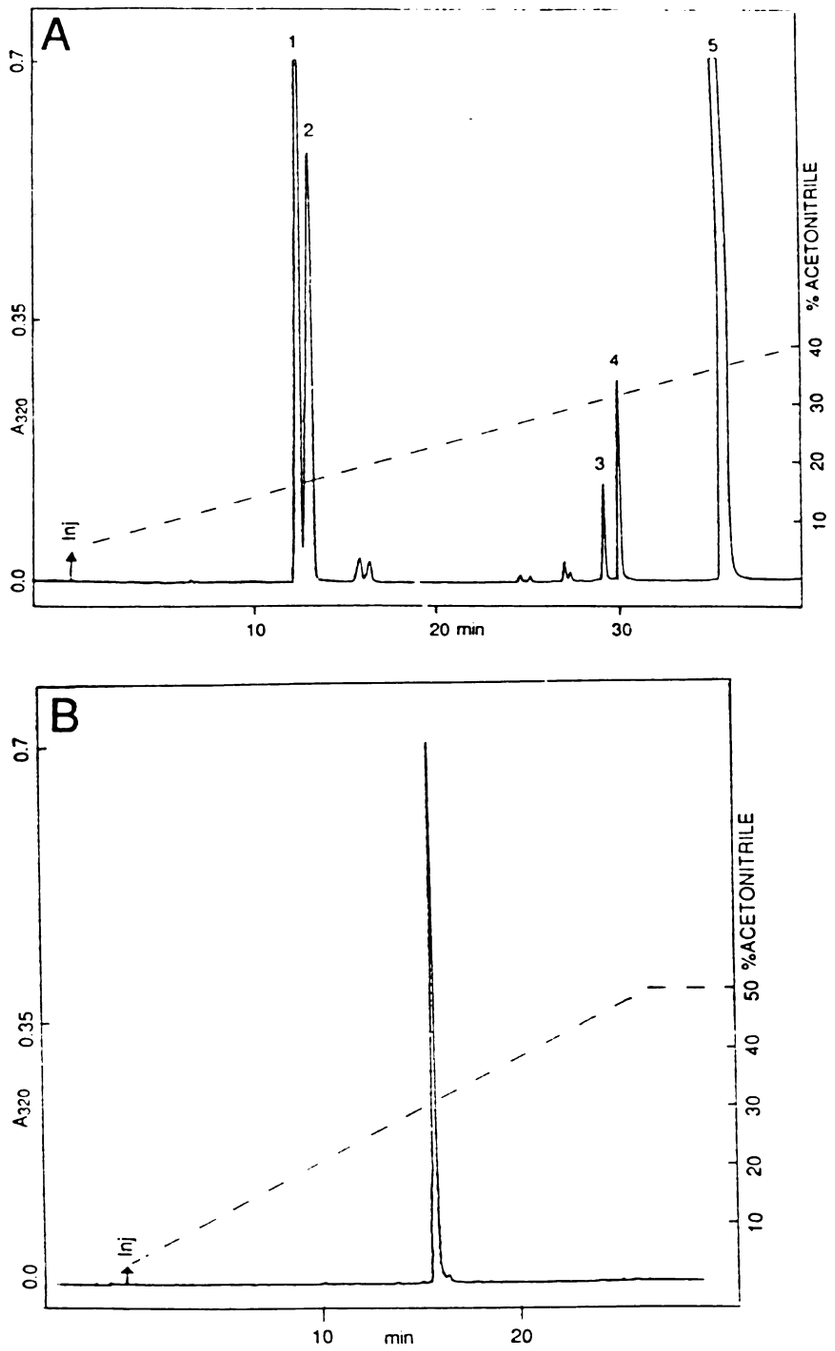
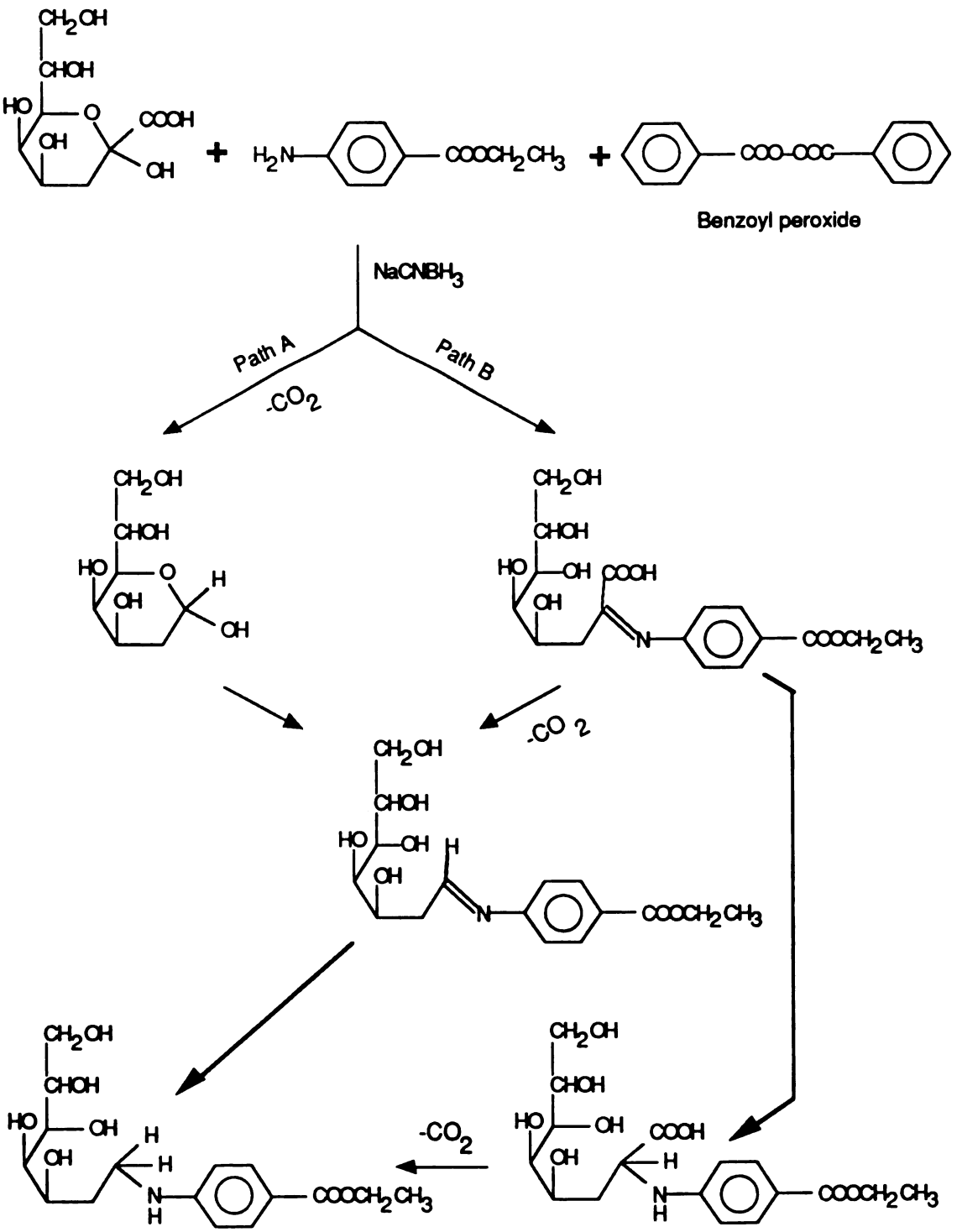


Figure 8. Reverse-phase HPLC chromatogram of (A) KDO derivatized with ABEE and (B) KDO derivatized with HBEE. About 108 μg of KDO starting material was used in panel A, whereas the aliquot injected in panel B represents about 3 μg of KDO starting material. In panel A analysis by negative-ion LSIMS showed that peaks 1 and 2 have the mass of the molecular ion (m/z 386), peak 3 was decarboxylated, peak 4 an anyhydro species, and peak 5 unreacted ABEE.

KDO has been previously reported and was also observed in our previous experiments with the *Salmonella* ABEE oligosaccharides. Formation of 4,7-anhydro and 4,8-anhydro octonic acid from KDO in hot acetic acid was reported by Volk and co-workers (Volk, Salomonsky, and Hunt 1972). Two different products were found upon heating KDO at 100°C for 75 min in 1% formic acid, the bicyclic compound 2,7-anhydro-3-deoxy-α-D-*manno*-2-octulofuranosonic acid and 5-(D-*erythro*-1,2,3-trihydroxypropyl)-2-furoic acid (McNicholas, Batley, and Redmond 1987). The structure(s) of the anhydro product(s) observed in mass spectrometric analysis of both derivatized KDO and the derivatized oligosaccharides from the Ra mutant of *S. typimurium* was not determined.

Since a significant portion of the KDO formed the decarboxylated analog, it seemed possible that this irreversible side reaction could be used to increase the overall yield. If KDO was undergoing decarboxylation prior to the reductive amination, then the primary amine would be reacting with an aldose sugar rather than the ketone group of KDO. For example, if decarboxylation of KDO was occurring through a free radical mechanism, the addition of dibenzoyl peroxide might substantially improve the yield (see Scheme 2). The addition of this free radical initiator to the reaction mixture did in fact increase the extent of the decarboxylated product from 10-30% to about 80% of the total products, but it also slightly lowered the product yield. The same increase in the decarboxylated product could also be obtained by addition of dibenzoyl peroxide after the derivatization and extraction steps were performed, by incubating in methanol at 80° C for 30 min. Thus, it appears the decarboxylation occurs after the formation of the reduced KDO-ABEE derivative when the overall yield could no longer be effected. Attempts to directly decarboxylate KDO under similar conditions with

Scheme 2



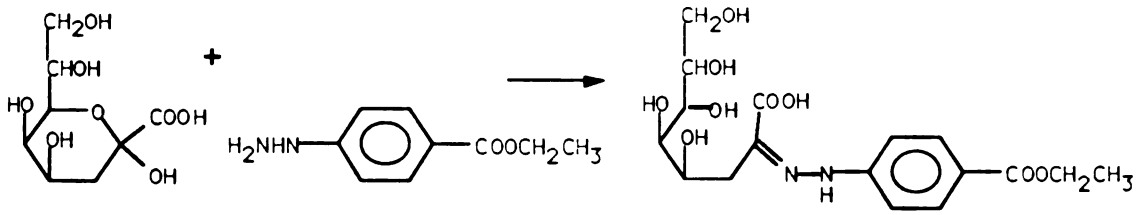
dibenzoyl peroxide were not successful and support our conclusion that decarboxylation is occurring from the final reduced KDO-ABEE product. Increasing the yield of KDO-ABEE by lengthening the reaction time from 45 to 90 min, or increasing the temperature from 80° to 100° C, were also unsuccessful.

While it may be possible to find reaction conditions that will increase the overall yield of this reaction, no such conditions were found. Furthermore, since this procedure appeared to require in some cases a dephosphorylated oligosaccharide for an acceptable yield, an alternative derivatizing agent was sought that would accommodate oligosaccharides that still contained one or more phosphodiester groups. The reason that the yield of the reaction was so poor with some oligosaccharides containing phosphoethanolamine (PEA) is not clear. One possibility is that some of the oligosaccharides containing PEA tend to form aggregates and, thus, sterically hinder the reaction.

It was essential that the alternative reagent retain the chromatographic and mass spectrometric advantages of the amino benzoate ester derivatives. The simplest strategy towards designing such a reagent was to prepare the more strongly nucleophilic hydrazino analogs of ABEE and ABBE, namely the ethyl and butyl 4-hydrazinobenzoates (HBEE and HBBE). The resulting hydrazones formed by reaction of KDO with these two hydrazines would be expected to be more stable to hydrolysis than the imines formed with the amino-reagents and would, therefore, not require a subsequent reduction step to stabilize the products (see Scheme 3). As shown in Figure 8B, the reaction of KDO with HBEE produced one major single peak in the HPLC chromatogram, obtained with 3 μg of KDO (aliquot of starting material injected). This contrasts with the much larger amount

of KDO (aliquot injected represented 108 μg of starting material) that produced the HPLC chromatogram shown in Figure 8A.

Scheme 3



The subsequent application of these same hydrazines to the preparation of hydrazone derivatives of KDO-containing oligosaccharides from HF-treated and non-HF-treated *S. typhimurium* preparations was also successful. These latter hydrazone oligosaccharides could, in fact, be easily isolated by reverse phase HPLC under similar conditions used for the amino benzoate derivatives. The yield of this reaction based on the relative UV absorbance during HPLC separation was estimated to be greater than the amino alkyl benzoates by at least a factor of 5 (See Fig. 9A).

Quantitation of the yield of the HBEE derivatization of HF-treated oligosaccharide from the Ra mutant of *S. typhimurium* and a pyocin mutant strain of *N. gonorrhoeae* 1291 (see Methods) confirmed that the yield was high. To quantitate these yields trimethylsilyl derivatives were made from hydrolyzed aliquots of each sample prior to the derivatization with HBEE, and after the derivatization with HBEE and subsequent HPLC purification, and from that portion of the oligosaccharide sample which was recovered in the void volume. Our results indicated approximately 65% of the oligosaccharide samples were derivatized with HBEE. More recent work has suggested that performing the reaction at room temperature produces similar yields. As shown in Figure 9B the optimum wavelength

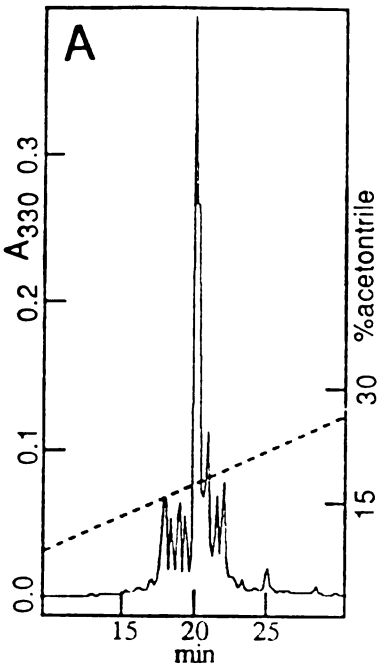
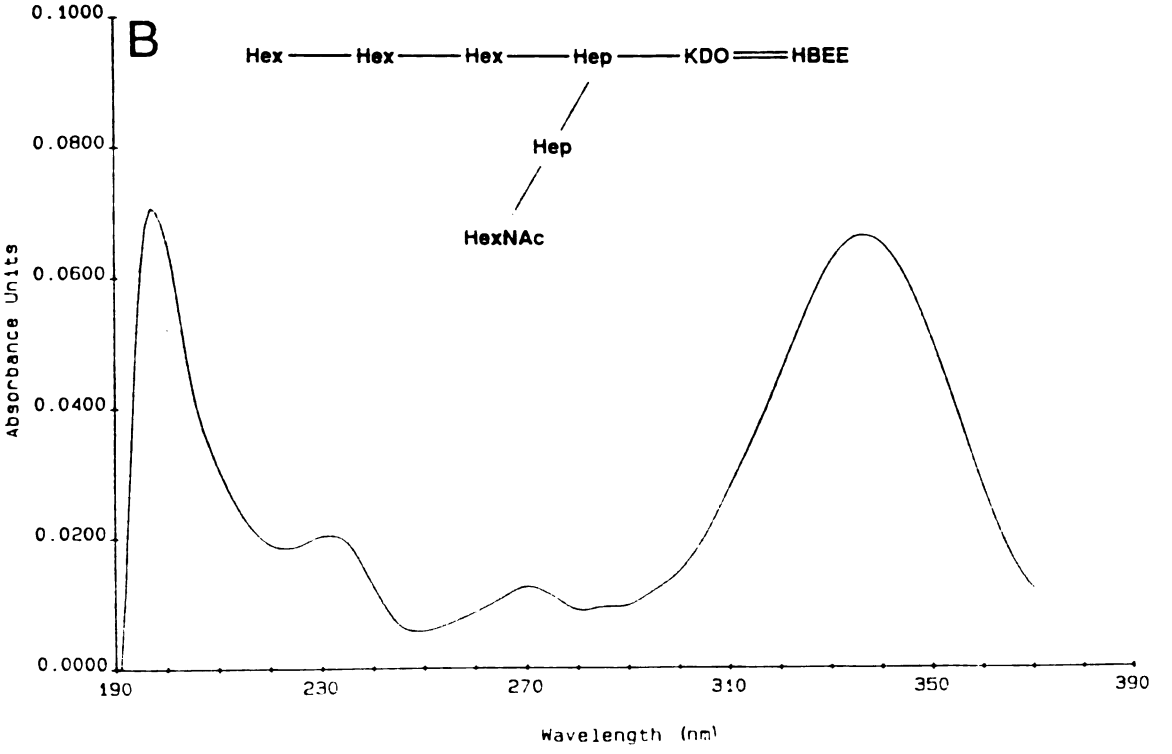


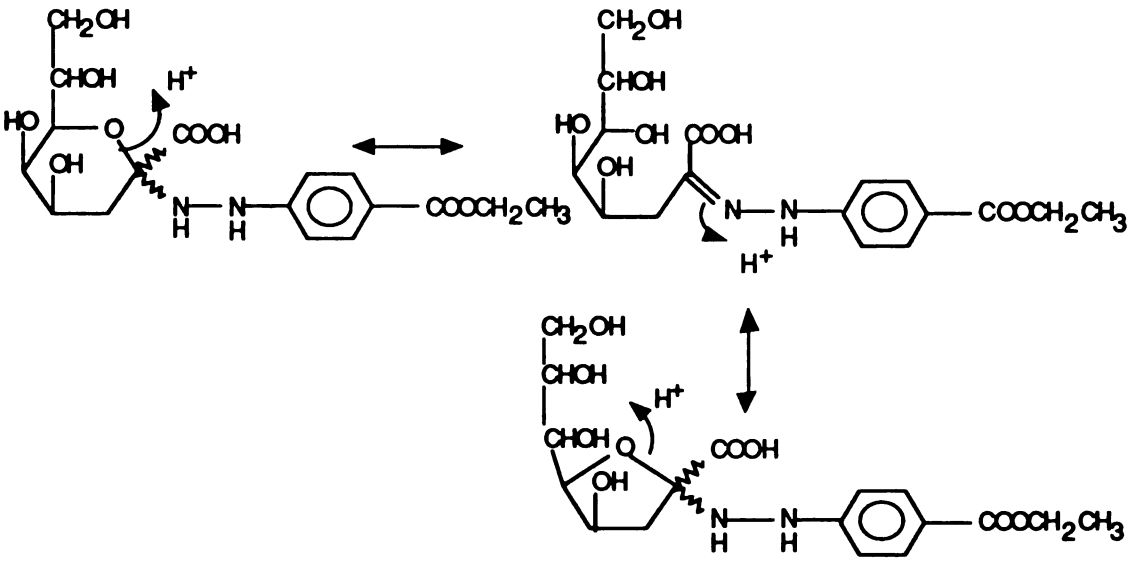
Figure 9. A portion of the reverse-phase HPLC chromatogram of (A) the HBEE derivative of the HF-treated oligosaccharides from the TV119 Ra mutant of *S. typhimurium* and (B) a UV spectrum of oligosaccharide of the gonococcal pyocin-resistant mutant strain 1291_b derivatized with HBEE. The longer wavelength λ max in the UV spectrum is about 335 nm. In panel A approximately 30 μg of starting oligosaccharide was used and the derivatization was performed at room temperature. A very similar yield was obtained from the reaction at 80°C.



for analysis of oligosaccharides derivatized with HBEE containing KDO at the reducing terminus is 335 nm (λ max).

In subsequent mass spectrometric experiments, these derivatized oligosaccharides produced excellent quality LSIMS spectra with abundant sequence ions that could be readily interpreted (Fig. 10). Only a small molar excess of the hydrazines is required to produce a quantitative reaction with ketose sugars using the described reaction conditions. The addition of TFA to the HPLC solvents improved the peak shapes (see Materials and methods) and is now used in all separations. Lowering the pH of the HPLC solvents had been reported to minimize formation of cyclic hydrazino isomers of KDO that are in equilibrium as shown in Scheme 4 (McNicholas, Batley, and Redmond 1984). A multiplet of peaks were not

Scheme 4



observed from the reaction of KDO with HBEE as seen in the prior work with p-nitrophenylhydrazine (McNicholas, Batley, and Redmond 1984), but under neutral conditions the peak was broad. It is possible that under neutral conditions more than one cyclic isomer of the HBEE-oligosaccharide is formed but not separated under the conditions

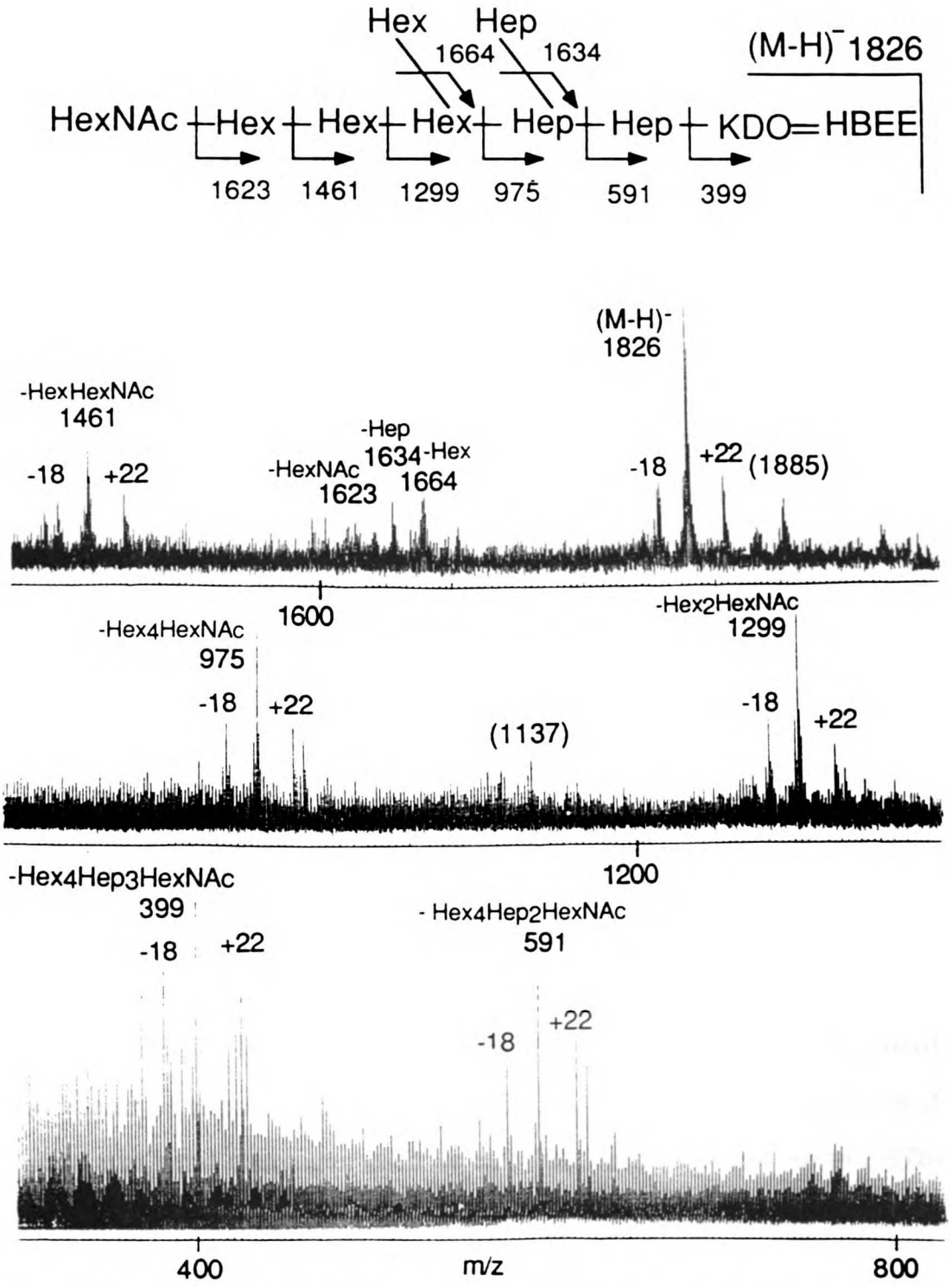


Figure 10. Negative-ion LSIMS spectrum of HF-treated oligosaccharide from the TV119 Ra mutant of *S. typhimurium* derivatized with HBEE. Masses are give for Y-type fragment ions (Domon and Costello 1988). Loss of 18 Da corresponds to either a Z-type ion or an anhydro species formed during isolation or workup. The addition of 22 Da is the sodium adduct, $(M+Na-2H)^-$.

described. Alternatively, at lower pH, protonation of the carboxylic acid may improve the peak shape, or the number of components in equilibrium may be lowered due to reduced tautomerization of the hydrazone double bond in the open chain form.

The mass spectrometric behavior of both the amino and hydrazino benzoate ester derivatives of the KDO-containing oligosaccharides showed several striking characteristics in regards to both molecular ion abundances and the presence of sequence information. In two-sector negative-ion LSIMS spectra of the unbranched ABEE-oligosaccharides (i.e. maltoheptaose) fragment ions with charge retention on the reducing terminus show a roughly linear increase in abundance with decreasing molecular weight (Webb et al. 1988). However, the signal-to-background ratio is better with higher mass fragments due to the increase in matrix-related ions at lower mass. We have found large variations in the fragment ion abundances in negative-ion two-sector LSIMS of branched oligosaccharides derivatized with an ester of 4-amino- or 4-hydrazino benzoate. This can be seen in the negative ion LSIMS spectrum of the HBEE derivative of the oligosaccharide from *S. typhimurium* TV 119 Ra mutant shown in Fig. 10. In this spectrum, the low abundance of fragment ions that would be expected for a linear structure or that may be products of two remote site bond cleavages such as m/z 783 (loss of Hep from m/z 975) and 1137 (loss of Hex from m/z 1299) reveal branch points in this structure.

In the negative ion MS/MS spectra of the same oligosaccharide derivatized with ABBE (Fig. 11) a decrease in ion abundances of reducing terminal fragment ions is generally observed in the lower mass range. As in the spectra obtained by two-sector LSIMS without CID, ions that result from two remote site bond cleavages are of low abundance such that it is

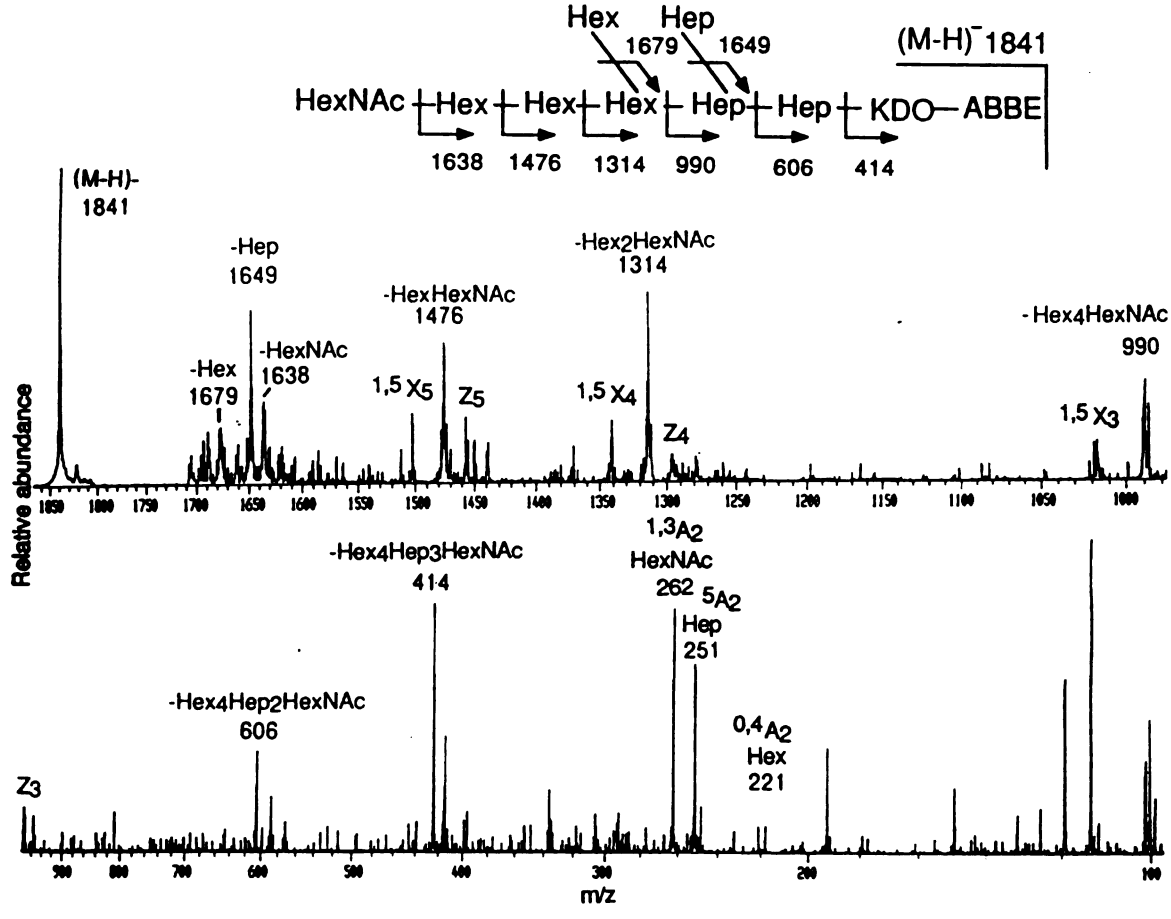


Figure 11. Negative-ion four-sector (MS/MS) CID LSIMS spectrum of the ABBE derivative of *S. typhimurium* TV119 Ra mutant oligosaccharide after HF-treatment. Masses are give for reducing terminal Y-type fragment ions, while reducing terminal X- and Z- and the non-reducing terminal A-type fragment ions are labeled (Domon and Costello 1988).

somewhat difficult to distinguish them from background ions. These data suggest that identification of branching points by careful consideration of fragment ions and their relative abundances is also possible in the negative ion MS/MS spectra of oligosaccharides with reducing terminal derivatives.

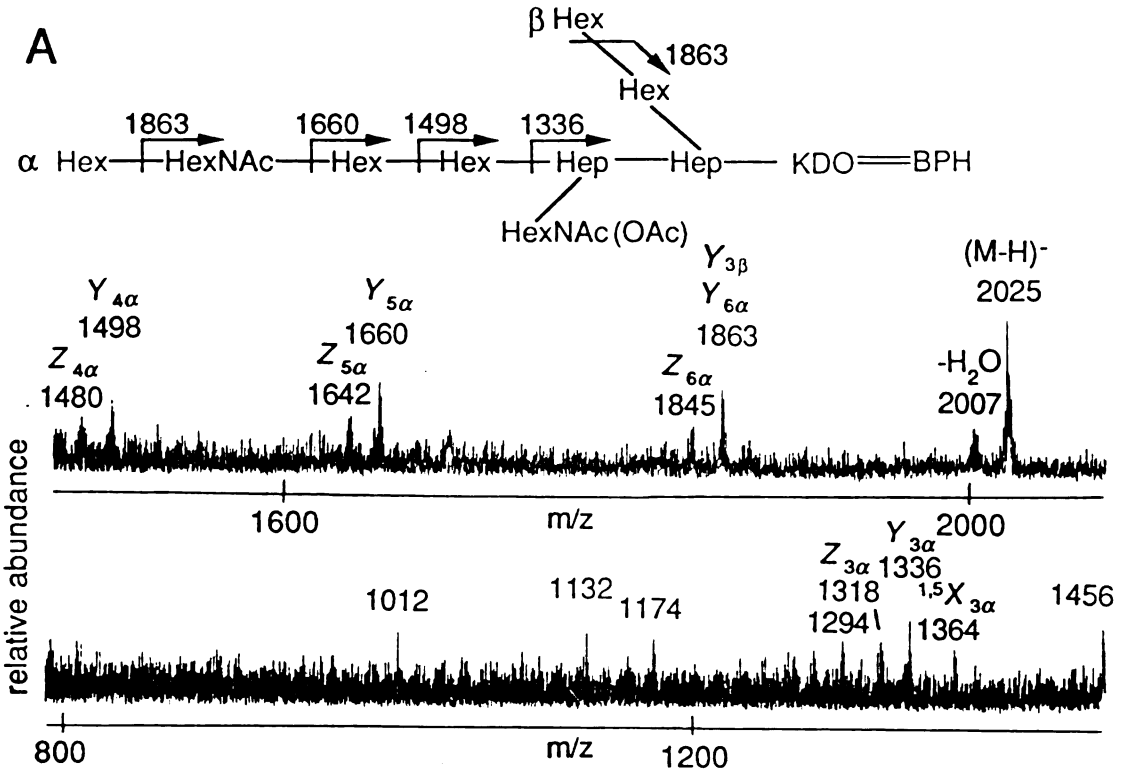
In this four-sector CID LSIMS analysis of the oligosaccharide from *Salmonella* derivatized with ABBE, all glycosidic bonds were cleaved with charge retention on the reducing terminus (Fig. 11). This MS/MS spectrum was obtained to determine using a known compound the fragmentation patterns and quality that could be generally expected in analysis of these type of derivatized oligosaccharides. The sequence of the oligosaccharide (in terms of hexose, *N*-acetylhexoseamine, heptose, and KDO) can easily be readily deduced from the mass differences of the daughter ions, as is also possible in the spectrum obtained by two-sector LSIMS analysis without CID (Fig. 10). This is in contrast with the negative-ion LSIMS spectra of HBEE- or ABXE-derivatized gonococcal oligosaccharides where very little glycosidic bond cleavage of the Hep-Hep bonds occurred, except under CID conditions (see Fig. 3). In LSIMS analysis of permethylated oligosaccharides from the gonococcal strain JW31R cleavage of the Hep-Hep bond was enhanced compared to other glycosidic bonds (Phillips et al. 1990).

To further explore the utility of aromatic hydrazines as reducing terminal derivatizing reagents for reverse phase HPLC purification and mass spectrometric analysis of KDO-containing oligosaccharides isolated from Gram-negative bacteria, and for other complex oligosaccharides, particularly those from glycoproteins, a series of hydrazino reagents was synthesized. Edward M. Allen and Dr. James W. Webb from the Department of Chemistry, Illinois State University, Normal, Illinois,

prepared the following five alkyl phenylhydrazine reagents: 4-butylphenylhydrazine, 4-octylphenylhydrazine, 4-decylphenylhydrazine, 4-dodecylphenylhydrazine, and 4-tetradecylphenylhydrazine. The reagents were synthesized by diazotization of the appropriate primary amines, followed by reduction in a similar procedure to that described for the synthesis of HBEE and HBBE. Details of the synthesis are to be published elsewhere. To use and evaluate for our work with KDO-containing oligosaccharides generous amounts of three of these reagents were given to us, 4-butylphenyhydrazine (BPH), 4-octylphenylhydrazine (OPH), and 4-tetradecylphenylhydrazine (TDPH).

As these reagents are more hydrophobic than HBEE more retention of derivatized oligosaccharides was obtained in reverse-phase HPLC. The generally extensive fragmentation observed in LSIMS analysis of HBEE derivatives of KDO-containing oligosaccharides was also seen in the spectra of oligosaccharides derivatized with these alkyl phenylhydrazine reagents. In addition, there appeared to be a significant increase in the signal-to-background ratio of the molecular and fragment ions in the LSIMS spectra of these more hydrophobic derivatives. compared to spectra of oligosaccharides derivatized with HBEE or HBBE.

In Figure 12, the negative-ion LSIMS spectrum is shown of the 4-butylphenylhydrazine derivative of oligosaccharide isolated from JW31, the parent strain of the pyocin-resistant mutant strain JW31R (Gibson et al. 1989). The molecular ion at m/z 2025 (1880 Da + 146 Da for BPH) is 42 Da larger than one of the major components of JW31R, which had a M_r of 1838 and a proposed composition of $Hex_5HexNAc_2Hep_2KDO$ as stated above. The M_r of the major component in the first peak of the chromatogram shown in Fig. 11 was 1838 the same as that found in JW31R. The mass difference of



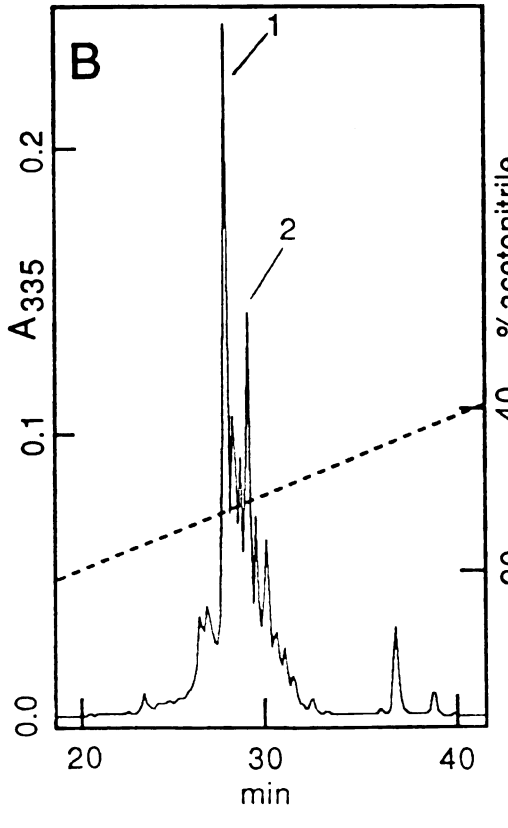


Figure 12. Negative-ion LSIMS spectrum (A) of HF-treated oligosaccharide from gonococcal strain JW31 derivatized with 4-butylphenylhydrazine from peak 2 as shown in (B) a portion of the reverse-phase HPLC chromatogram of the derivatized oligosaccharide. LSIMS analysis of peak 1 in panel B showed a molecular ion at m/z 1983. The difference between these two molecular ions is 42, corresponding to an *O*-acetate moiety not found in oligosaccharides from JW31R. The tentative structure shown is consistent with the presumably analogous oligosaccharide of the pyocin-resistant mutant of JW31, strain JW31R (see Chapter I, Fig. 3) (Gibson et al. 1989).

42 Da probably corresponds to the addition of an *O*-acetate moiety, as has been found on the non-reducing terminal HexNAc of the oligosaccharides from the LOS of some *Neisseria meningitidis* strains (Michon et al. 1990). No evidence was obtained of *O*-acetylation in the analysis of the oligosaccharides from JW31R (Gibson et al. 1989). Thus, the lack of *O*-acetylation may be one of the key differences in the oligosaccharides of JW31R produced by treatment with pyocin. Other LSIMS spectra of both derivatized and underivatized oligosaccharides from JW31 contained evidence of this 42 Da mass shift which corresponds to one *O*-acetate group. The data also indicated the presence of one PEA group in the oligosaccharides of JW31 just as in some of the oligosaccharides from strain JW31R.

There is one quite significant difference between this LSIMS spectrum of oligosaccharides from JW31 and the spectra of the JW31R oligosaccharides (Gibson et al. 1989). The fragment ions at m/z 1863 and 1660 suggest that there is a non-reducing terminal Hex attached to a HexNAc, as these ions respectively correspond to loss of Hex (m/z 1863), and of HexHexNAc (m/z 1660) from the molecular ion. An ion corresponding to a loss of a non-reducing terminal HexNAc, or loss of a HexNAc plus an *O*-acetate can not be observed. However, in LSIMS spectra of other gonococcal oligosaccharides which do contain a non-reducing terminal HexNAc attached to Hep occasionally the fragment ion for the loss of the HexNAc residue was not observed. If the largest branch of the oligosaccharide from JW31 does contain the sequence Hex-HexNAc-Hex-Hex as suggested by this spectrum, then this oligosaccharide could contain the lacto-N-neotetraose epitope recognized by the murine MAb 3F11 (see Introduction D.6.) In a study of LOS from 20 different gonococcal strains the LOS of the pyocin

resistant strain JW31R was the only strain not recognized by the mouse monoclonal antibody 3F11. The LOS from JW31 was weakly recognized with 15-49% of maximum plateau binding (Mandrell et al. 1986). In prior LSIMS analysis of gonococcal oligosaccharides with reducing terminal derivatives (John and Gibson 1990) very limited fragmentation of the Hep-Hep bonds was observed, as is also the case in this spectrum.

The spectrum shown in Figure 12 is dominated by reducing terminal Y-type fragment ions, with some accompanying less abundant reducing terminal Z-type fragment ions. The ion at m/z 1456 is 42 Da less than the Y-type ion at m/z 1498 for loss of $Hex_2HexNAc$. Similarly, the ion at m/z 1294 is 42 Da less than the Y-type ion at m/z 1336 for the loss of $Hex_3HexNAc$. Similarly, the ion at 1132 is 42 Da less than the Y-type ion at 1174 for loss of $Hex_4HexNAc$. Thus, the fragment ions at m/z 1456, 1294, and 1132 appear to the the product of two remote site bond cleavages, one Y-type glycosidic bond cleavage and the other cleavage of the *O*-acetyl moiety.

Two additional ions in the spectrum in Figure 12 at m/z 1174 and 1012 correspond to the loss of $Hex_4HexNAc$ and $Hex_5HexNAc$ from the molecular ion. If these ions are the product of a single-bond cleavage then the overall structure is different than that shown above the spectrum in Figure 12. The structure of the oligosaccharide shown is only one of the possibilities for the sequence of the oligosaccharide with the proposed composition which would be consistent with the spectrum. As suggested in the upper panel of Figure 13B one possibility is that the fragment ions at m/z 1012 and 1174 are the products of remote site two-bond cleavage. Generally, it is my experience that fragment ions from remote site two bond cleavages in negative-ion LSIMS analysis of oligosaccharides with reducing terminal derivatives have been of only very low abundance.

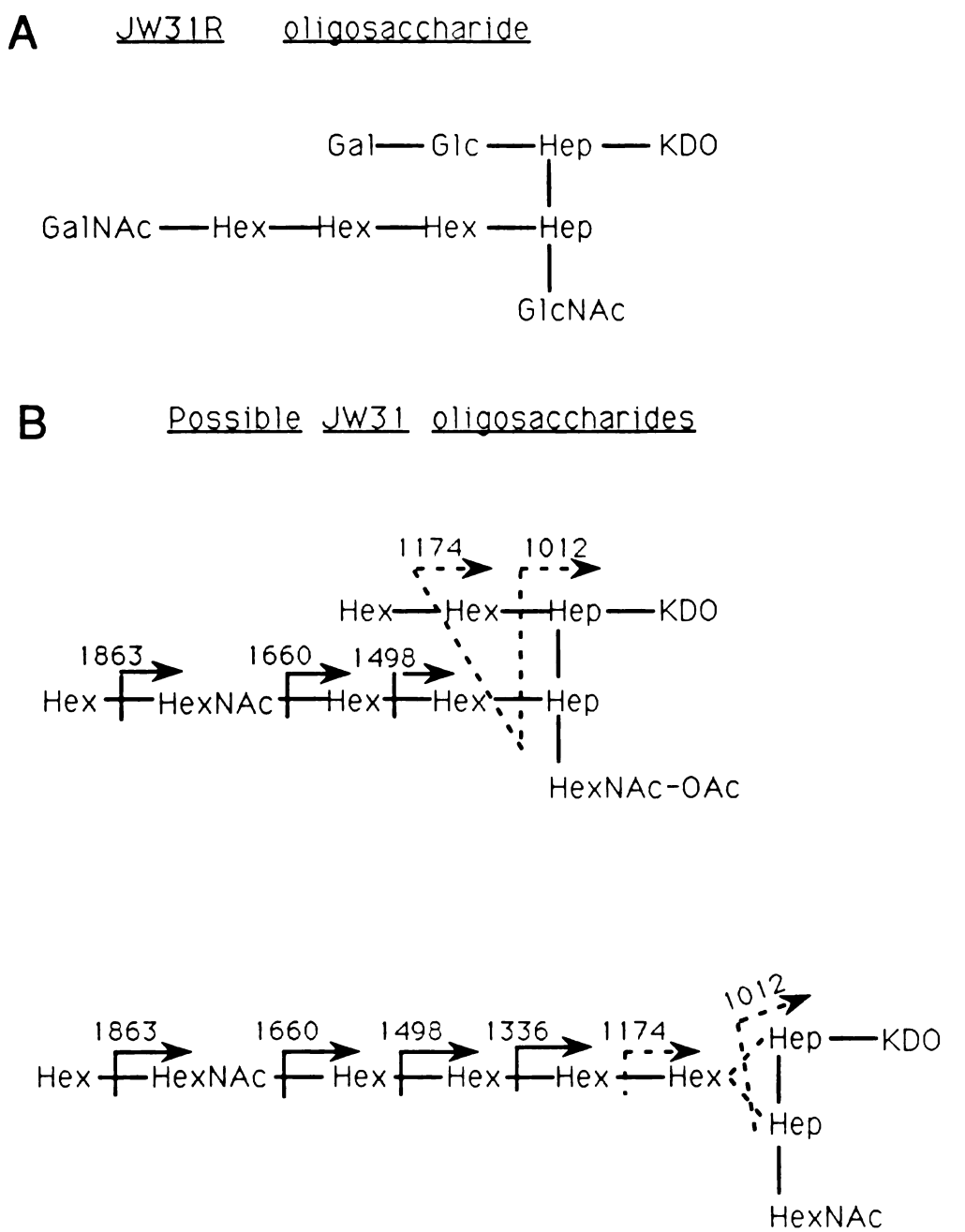


Figure 13. The structure of oligosaccharide from strain JW31R (A) with M_r 1838 and (B) two possible structures of the oligosaccharide from strain JW31 based on the spectrum shown in Figure 12.

Nevertheless, this could be an example of fairly prominent products of remote site two bond cleavage, perhaps initiated by some favorable, but undetermined, spatial arrangement.

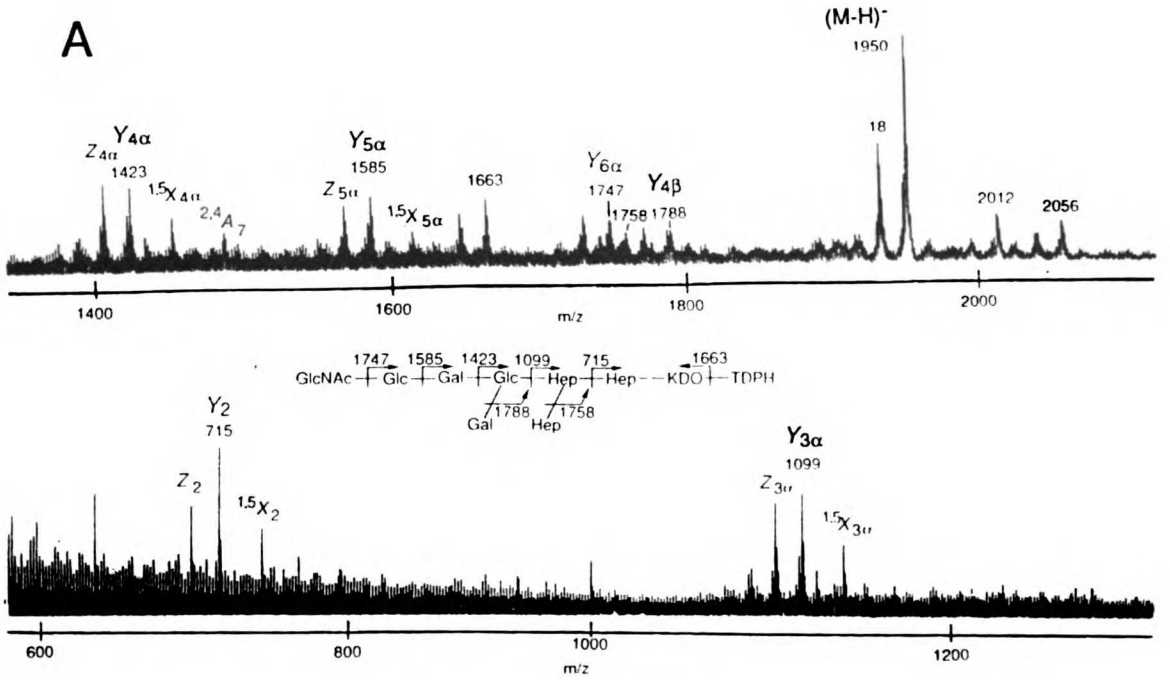
Another possibility is that the oligosaccharide from JW31 has three branches, the major branch being Hex→HexNAc→Hex→Hex→Hex→Hep. The second and third branches would then consist of a single HexNAc and Hex residues. Another possibility is a biantennary structure with one branch of the sequence Hex→HexNAc→Hex→Hex→Hex→Hex→Hep, and the other a single HexNAc moiety. This latter possibility is illustrated in the lower panel of Figure 13B. When some of the fragment ions are presumed to be the products of remote site cleavage of two bonds, it is somewhat difficult to decide which are the products of these remote site two-bond cleavages and which are the products of single bond cleavages or concerted multi-bond ring cleavages of the A- or X-type. The lack of cleavage of the Hep-Hep bond makes the complete assignment of the sequence of this oligosaccharide impossible from this spectrum alone.

Certainly, these remote site double bond cleavages may generally occur less frequently than the single bond cleavages or the concerted two bond cleavages of the A- and X-types. However, there is another simple reason that the ion abundance of remote site two bond cleavage products may often appear to be less than those produced by single bond cleavage, or multiple bond cleavages of the A- or X-type for which a concerted mechanism is postulated. If such remote site two bond cleavages are produced by the same mechanisms as the single glycosidic bond cleavages and the concerted multi-bond ring cleavages, then the possible combinations of just the reducing terminal fragment ions are X-X, X-Y, X-Z, Y-Y, Y-Z, and Z-Z. These double fragmentations, considering loss of two

Hex residues, would produce losses of 268, 296, 314, 324, 342, and 360 Da from the molecular ion. Thus, the abundance of the ions corresponding to loss of two Hex residues by remote site double bond cleavage with charge retention on the reducing terminus could be spread over six different fragment ions, possibly reducing the abundance of any one fragment ion to a large degree. Similar combinations of remote site double bond cleavages involving charge retention on the non-reducing terminus, or fragmentation processes involving charge retention on the reducing terminus and the nonreducing terminus can also be envisioned depending on the sites involved.

The spectrum in Figure 14 of the TDPH derivative of HF-treated oligosaccharide from the LOS of the *S. typhimurium* Ra mutant TV119 was obtained with approximately 1 nmole of derivatized oligosaccharide. The greater abundance of the fragment ions in the LSIMS spectrum of the TDPH derivative of the *S. typhimurium* oligosaccharide (Fig. 14) is notable upon comparison with the spectra of the HBEE derivative of the same oligosaccharide (Fig. 10). In both of these spectra the sequence and branching of the oligosaccharide can be determined from the fragment ions.

The aromatic hydrazino reagents described are significantly better for reducing terminal derivatization of bacterial oligosaccharides containing KDO then similar amino reagents. The yield of the reaction of HBEE with the phosphorylated bacterial oligosaccharides was much better in general, compared to reaction with ABEE. The yield of the reaction of HBEE with dephosphorylated oligosaccharides was also good (≈65%), and was apparently much greater than the yields with ABEE. The quality of the LSIMS data and HPLC separations are essentially equivalent to



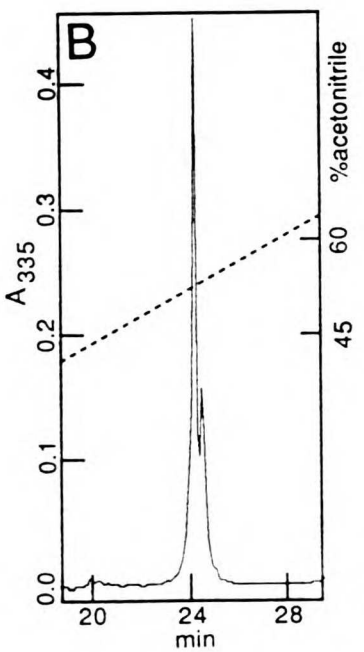


Figure 14. Negative-ion LSIMS spectrum (A) of the HF-treated oligosaccharide from the TV119 Ra mutant of *S. typhimurium* derivatized with 4-tetradecylphenylhydrazine and (B) a portion of the reverse-phase HPLC chromatogram of the derivatized oligosaccharide. Masses are given for reducing terminal Y-type fragment ions, while reducing terminal X- and Z- and the non-reducing terminal A-type fragment ions are labeled. The LSIMS spectrum shown in panel A was obtained with approximately 1 nmol of derivatized oligosaccharide.

oligosaccharides derivatized with similar amino reagents. In addition, much smaller quantities of the hydrazino reagents than the amino compounds are required for derivatization, and no reduction of the hydrazone product is required. The derivatization is easier and faster than the reductive amination reaction requiring no extraction step. Even HPLC purification of the product is optional. The method seems ideally suited for analyses on the microscale which would be required for low μg and sub μg amounts of oligosaccharide.

The alkyl phenylhydrazines seem to provide increases in sensitivity of detection by LSIMS similar to the increases in sensitivity obtained with oligosaccharide derivatives of the longer chain esters of 4-aminobenzoate (Poulter and Burlingame 1990). As discussed previously, increasing the hydrophobicity of the oligosaccharide can also increase the surface activity of the sample within a hydrophilic liquid matrix. An increase in surface activity should be directly related to an increase in sputtering efficiency of the molecule during LSIMS analysis and, therefore, its sensitivity of detection (Ligon 1990). Careful comparisons need to be made of the sensitivity of detection using bacterial oligosaccharides derivatized with the various hydrazino reagents before a conclusion can be made about which is the better derivatizing agent. With the longer alkyl hydrazines problems may be encountered due to low solubility in the solvents used for the derivatization of oligosaccharides, and in aggregation during analysis. The extension of the use of these hydrazino reagents to other types of oligosaccharides is currently the focus of study by our collaborators Dr. Webb and Mr. Allen at Illinois State University, Normal, Illinois. They are attempting to optimize the reaction conditions and characterize the quality

of LSIMS analysis of hydrazino derivatives of oligosaccharides typical of those isolated from glycoproteins found in mammalian systems.

C. Mass spectrometric analysis of *O*-deacylated LOS

Isolation of the oligosaccharide portion of LOS requires initial mild acid hydrolysis to cleave the glycosidic bond of KDO, which is more acid labile than any of the other glycosidic bonds of the LOS. Thus, the oligosaccharide isolated after this procedure then contains a single reducing terminal KDO. However, elucidation of the number, sequence, and linkages of other KDO moieties occurring in the LOS is not possible after the acetic acid hydrolysis. Other acid labile moieties may be completely or partially removed by the mild acid hydrolysis. Biologically significant structural variation in the lipid A portion of the LOS could be overlooked if the lipid portion or the entire LOS is not routinely analyzed, along with the glycan moiety. For these reasons, an attempt was made to develop methods to analyze the LOS intact, or after mild chemical treatment which would preserve the KDO residues and other acid labile moieties.

This section describes the development of methods of analysis of *O*-deacylated LOS by LSIMS, and by GC-MS after the formation of partially methylated alditol acetates. The composition of the LOS from *N. gonorrhoeae* strain FA5100 and of *N. meningitidis* strain 6275 were determined by LSIMS analysis of the *O*-deacylated LOS. By this method a single sialic acid residue, a relatively acid labile monosaccharide, was located on a non-reducing terminus of the meningococcal LOS. This is the

first mass spectrometric evidence for the presence of a sialic acid group in the LOS from *N. meningitidis*.

C.1. Materials and methods

Materials

LOS from the SL1181 Re and the TV119 Ra mutants of *Salmonella typhimurium* and anhydrous hydrazine were obtained from Sigma (St. Louis, MO). Triethylborohydride (Superhydride), sodium hydroxide pellets (99.9%) and sodium borodeuteride (98% D) were purchased from Aldrich (Milwaukee, Wisconsin). Aqueous HF (48%) was obtained from Mallinckrodt (Muskegon, Michigan), iodomethane from Fluka (Buchs, Switzerland), and acetic anhydride from Supelco (Bellefonte, Pennsylvania). The water, methanol, and methylene chloride were obtained from Burdick and Jackson (Muskegon, Michigan). The other reagents and solvents used were of reagent grade.

Methods

O-Deacylation of the LOS. LOS samples were *O*-deacylated according to the procedure of Helander et al. (Helander et al. 1988). The LOS (0.2-2 mg) was placed in 25 mL glass centrifuge tubes with screw caps and 200 µl of anhydrous hydrazine was added to each. The samples were shaken in a water bath at 37° for 20 min with intermittent sonication and then cooled to -20°. Approximately 1 ml of chilled acetone (-20°) was added dropwise. The precipitated LOS was centrifuged at 12,000 x *g* for 20 min and the

supernatant decanted. The addition of chilled acetone and centrifugation were repeated once and the supernatant decanted. After the addition of 0.5 ml of H_2O, the precipitated LOS was lyophilized.

HF-treatment of *O*-deacylated LOS. From 0.2-0.5 mg of *O*-deacylated LOS was placed in a 1.5 ml polypropylene tube and cold aqueous 48% HF solution was added to a concentration of 10 μg/μl. The sample was kept at 4°C for 18-24 h, and then the HF solution was removed as described in Chapter II, B.1.

LSIMS analysis of *O*-deacylated LOS. LSIMS analysis of *O*-deacylated LOS was performed in negative-ion mode as described in Chapter II, B.1. The LOS was dissolved in a mixture of MeOH and H_2O (1:1). For the liquid matrix various combinations were tried: thioglycerol and glycerol; pure triethanolamine; and triethanolamine in combination with thioglycerol and glycerol. None of these matrices produced dramatically better results. Phosphorylated LOS did produce somewhat more abundant molecular ions in pure triethanolamine.

Mild acid degradation of the *O*-deacylated LOS of the pyocin-resistant gonococcal strain FA5100. Approximately 85 μg of *O*-deacylated LOS was placed in an 8 ml glass tube with a screw cap, 100 μl of 20 mM acetate buffer (pH 4.4) was added. The sample solution was heated at 70°C for three hours (Brade, Galanos, and Luderitz 1983b). The sample was evaporated to dryness in a Speed-Vac concentrator (Savant), and 100 μl of H_2O added and evaporated. The addition of H_2O and evaporation were repeated three times.

Methylation analysis of *O*-deacylated LOS. The linkage analysis was performed on *O*-deacylated LOS using the microscale method of Levery and Hakomori (Levery and Hakomori 1987) which we modified for use with powdered NaOH as the base in the permethylation step (Larson et al. 1987). The *O*-deacylated LOS was placed in five ml glass reacti-vials with teflon-lined stopcock caps. The samples were dried *in vacuo* overnight over powdered P_2O_5. To each sample 100 µl of distilled DMSO was added using a syringe through the stopcock. In the permethylation step the samples were flushed with N_2 after the addition of reagent with a syringe through the stopcock. The samples were sonicated for 1 min, vortexed, sonicated again for 1 min, vortexed and then sonicated for 20 min. Using a mortar and pestle, 99.9% sodium hydroxide pellets were crushed under N_2, approximately 10 mg was added to each sample along with a stir bar and then 25 µl CH_3I was added with a syringe. The samples were stirred at room temperature for 30 min. Extraction of the permethylated LOS was performed by adding 400 µl of CH_2Cl_2 and 400 µl of H_2O in that order, after first removing the stir bar. The samples were vortexed, spun, and then the lower organic layer was removed and placed in a clean vial. The extraction of the H_2O with CH_2Cl_2 was repeated three times and the combined organic layer was washed with H_2O three times. The organic layer was evaporated under N_2 and dried *in vacuo* over powdered P_2O_5.

The methyl esters that should have been formed on the KDO moieties were then reduced using one of several different methods. The LOS has very limited solubility in aqueous solvents, such as those used in the procedure for oligosaccharide samples, so other procedures were developed. In the first method, identical to the one we had used for oligosaccharides (York et al. 1985), 125 µl of a 10 mg/ml solution of $NaBD_4$

in H_2O and 250 µl of EtOH was added to each and the reaction proceeded for two hours at room temperature. In a second method 250 µl of a solution of 30 mg $NaBD_4$ in 1 ml tetrohydrofuran/EtOH (50:50) was added to each sample, and the reaction mixture stirred at room temperature for 12 hours. A few drops of acetic acid were added to destroy the excess $NaBD_4$. After evaporation with addition of EtOH as needed, borate was removed by repetitive additions of methanol containing 1% acetic acid followed by evaporation under N_2 at 35° (Levery and Hakomori 1987). As previously the samples were dried overnight *in vacuo* over P_2O_5.

Alternatively, a third method was tried starting with 0.04 µmoles of sample followed by the addition of 25 µl of cold 1 M triethylborohydride (Superhydride) in tetrohydrofuran. Triethylborohydride can be used in organic solvents and is air and moisture sensitive, and must be handled with caution. The reaction mixture was kept on ice for 10 min, and then quenched by dropwise addition of H_2O. The organoboranes were oxidized by adding 8 µl of 3 M NaOH followed by 8 µl of 30% H_2O_2. The resulting borate was removed as the methyl ester by adding methanol containing 1% acetic acid as described above and the samples were dried overnight *in vacuo* over P_2O_5.

Hydrolysis of the permethylated LOS was performed using two different procedures. In the first method, the samples were hydrolyzed in 250 µl of 95% acetic acid, 1.4% sulfuric acid and 3.6% H_2O for 16 h at 80°C, diluted with 250 µl of H_2O, and heated for 5 h at 80°C. In the second more mild procedure, 75 µl of 2M trifluoroacetic acid (TFA) was added to each sample, the vials vortexed and then heated for 1 h at 121°C (Waeghe et al. 1983). The TFA was evaporated under N_2 at room temperature until just dry, and 75 µl of MeOH was added, the vial vortexed and the MeOH

evaporated under N_2. Similarly toluene was also added, the vial vortexed, and the toluene evaporated to further remove excess H_2O. The samples were dried overnight *in vacuo* over P_2O_5.

In the second reduction step 300 µl of a 10 mg/ml solution of $NaBD_4$ in 0.01M NaOH was added to each sample. The vials were left loosely capped at 4°C overnight, and 2 drops of acetic acid were added to quench the excess $NaBD_4$. Borate was removed by evaporation of the methyl ester as described above. Again, the samples were dried overnight *in vacuo* over P_2O_5.

The free hydroxyl groups were acetylated by the addition of 300 µl of acetic anhydride with a syringe followed by heating for 2 h at 100°C with intermittent sonication. The samples were dried under N_2 adding 1-2 ml of toluene. The partially methylated alditol acetates (PMAAs) were extracted with CH_2Cl_2 as described in the permethylation step and dried under N_2.

Gas chromatography with mass spectrometry (GC-MS) of the PMAAs was performed on a VG70SE mass spectrometer equipped with a Hewlett Packard 5890 gas chromatograph and an on-column injector (J&W Scientific). Samples were injected at 60°C onto a 30 m DB-1 column (J&W Scientific) with a 1 µm film thickness. After 4 min the oven temperature was raised to 140°C, the program starting temperature. The temperature program was 140-280°C at 4°/min. The data was automatically calibrated using a VG Opus data system with perfluorokerosene as an external mass calibrant.

C.2. LSIMS analysis of *O*-deacylated meningococcal LOS

In the LPS or LOS of Gram-negative bacteria the lipid A moiety is ketosidically linked to one or more 3-deoxy-D-*manno*-octulosonic acid

residues (KDO) (Ghalambor, Levine, and Heath 1966, Unger 1981) followed by one or more heptose (Hep) residues. The rest of the glycan is typically composed of combinations of glucose, galactose, *N*-acetylglucosamine and *N*-acetylgalactosamine residues. Various non-carbohydrate substituents have been found in neisserial LOS such as phosphate, phosphoethanolamine, and *O*-acetate (Galanos et al. 1977, Gibson et al. 1989, Jennings, Lugowski, and Ashton 1983, Jennings et al. 1988, Jennings et al. 1980, Jennings, Hawes, and Adams 1973, Michon et al. 1990, Phillips et al. 1990). A structural model has been proposed for the neisserial LOS based on the limited structural data available (Griffiss et al. 1988). These data suggest that the LOS of the neisserial species may possess a significant degree of homology.

The first step in the structural analysis of the oligosaccharide portion of the outer membrane glycolipids of Gram-negative bacteria usually is to separate the glycan from the lipid moiety. This is carried out by mild acid hydrolysis of the LOS (or LPS) which cleaves the acid-labile glycosidic bonds of any KDO residues that occur (see Methods). The carboxyl group of KDO may participate in the hydrolysis, as free lipid A is more difficult to isolate from LPS in which the carboxyl group of KDO has been methylated (Galanos et al. 1977). Thus, only a single reducing terminal KDO is normally found attached to the oligosaccharide after this hydrolysis. Any other acid labile moieties that are present may also be partially or completely hydrolyzed in this process, such as *O*-acetate, other *O*-acyl groups, sialic acid, and fucose.

The structures of monophosphoryl lipid A derived from gonococcal strain F62 have been previously determined (Takayama et al. 1986). These structures consist of (apparently 1→6 linked) glucosamine disaccharides

substituted with *N*- and *O*-linked fatty acids on the 2 and 3 positions. Four different major components were identified, along with nine minor ones. The variations occurred in the fatty acid distribution (Takayama et al. 1986). Two of these major compounds are illustrated in Chapter 1, Figure 2. The expression of lipid A could vary to some extent from one strain to another, although most of the heterogeneity in the LOS has been found to be due to the glycan moieties (Griffiss et al. 1987). Inter-strain heterogeneity in the lipid A structure may be overlooked if structural analysis of the lipid A, or the entire LOS, from different strains is not performed.

Intact underivatized (and peracetylated) mycobacterial glycolipids ranging from approximately 1700 to 2750 Da in mass have been analyzed by plasma desorption mass spectrometry (Jardine et al. 1986, Jardine et al. 1989). However, analysis of intact LOS by LSIMS has not been reported. This is probably due to the large molecular weight of the intact LOS (3,200-7,500 Da), inhomogeneity, and possibly low ionization efficiency. The low ionization efficiency may likely be due to the formation of micelles and other LOS aggregates. In an attempt to develop a method to more directly analyze the LOS from *N. gonorrhoeae* in our laboratory it was decided to *O*-deacylate the LOS prior to analysis by LSIMS (see Methods), thereby decreasing the total size and hydrophobicity. The first effort was directed toward the LOS of the SL118 Re mutant of *S. typhimurium*. The LOS of the Re mutant is composed of a diphosphoryl lipid A moiety plus KDO_3PEA (Galanos et al. 1977). While the composition of the *O*-linked fatty acids varies greatly in the lipid A from *Salmonella*, the N-linked fatty acids are all D-3-hydroxylmyristic acid (Galanos et al. 1977). The lipid A has phosphate groups in the 1 and 4' positions which are partially substituted by L-4-aminoarabinose. Some of the lipid A units may be linked through the

phosphate groups to other lipid A units (Galanos et al. 1977). The negative-ion LSIMS spectrum obtained of the LOS after *O*-deacylation was difficult to interpret (data not shown). The apparent molecular ion $(M-H)^-$ at m/z 1584 was 150 Da less than the expected molecular ion (m/z 1734). The signal/background ratio of the molecular ion was only about 3:1, and a number of potassium adducts were apparent $(M+K-2H)^-$, $(M+2K-3H)^-$ and $(M+3K-4H)^-$. The observation of some salt adducts would be predicted due to the presence of phosphate. There was no evidence of fragment ions.

The same *O*-deacylation procedure was carried out on LOS from meningococcal strain 6275, and the LOS from gonococcal pyocin-resistant strain FA5100. In addition, a portion of the *O*-deacylated LOS from both samples was HF-treated. When negative-ion LSIMS analyses of these samples were performed the results were much better in general, both in terms of signal to background ratios of the molecular and fragment ions, and the presence of fewer significant salt adducts.

The negative-ion LSIMS spectrum of the *O*-deacylated HF-treated LOS from strain 6275 revealed the presence of a non-reducing terminal moiety of 291 Da, the expected mass for *N*-acetylneuraminic acid or sialic acid moiety (see Fig. 15). In this spectrum the masses for the molecular and the fragment ion given correspond to the exact mass of the ^{12}C-containing isotope, rather than the nominal mass. The nominal mass of the molecular ion for the *O*-deacylated LOS from meningococcal strain 6275 would be at m/z 2798, whereas the exact mass is slightly greater than 2799, as shown in Figure 15. A number of relatively abundant fragment ions can be observed, and there is very little evidence of salt adducts.

Enzymatic and immunochemical evidence for the presence of sialic acid in the oligosaccharide from the LOS of meningococcal strain 6275 has

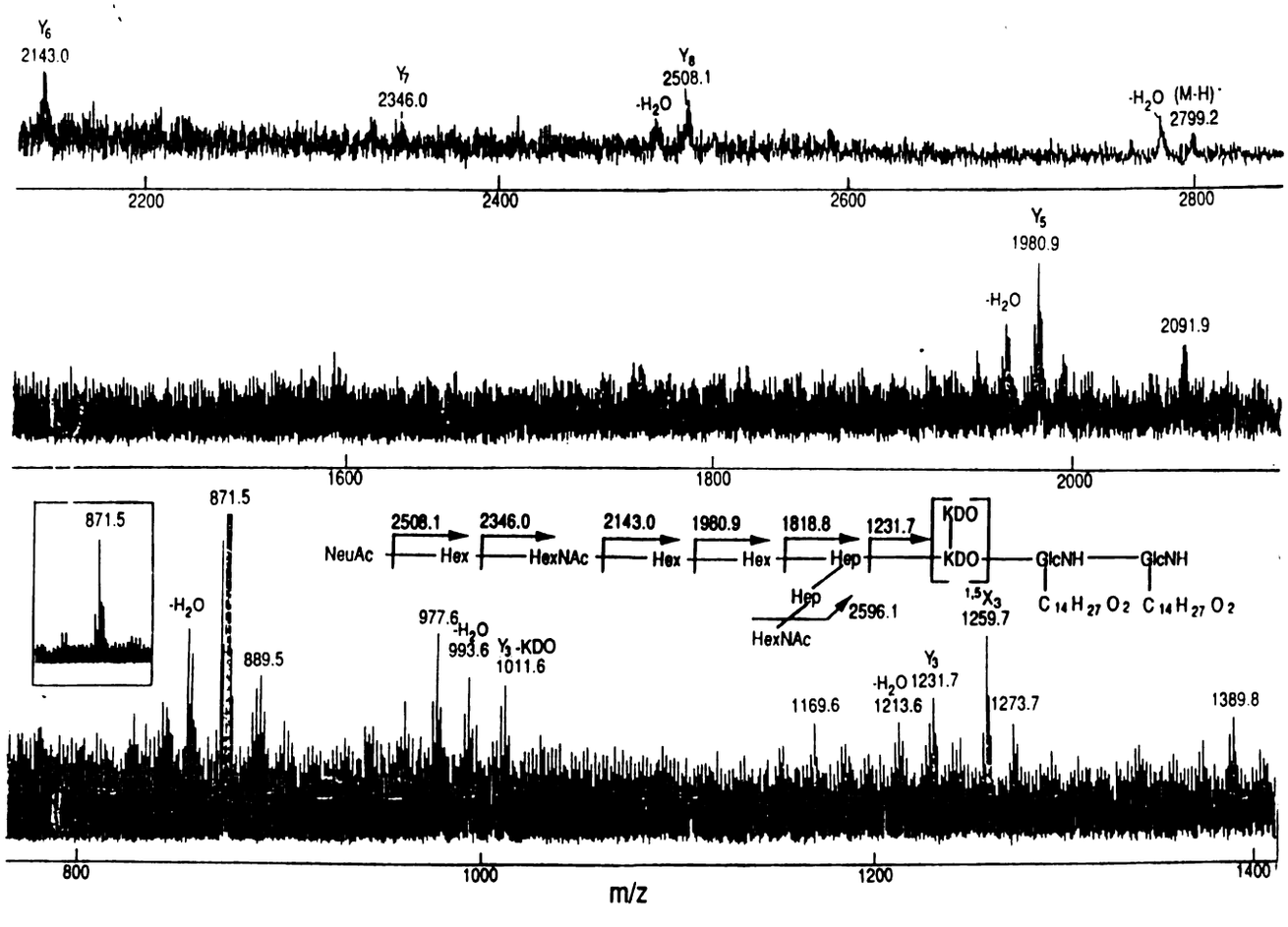


Figure 15. Negative-ion LSIMS spectrum of *O*-deacylated HF-treated LOS from *Neisseria meningitidis* strain 6275. The exact masses are reported in the spectrum; the nominal mass of the molecular ion at m/z 2799.2 is 2788. The tentative structure shown is most consistent with the data and structures reported for meningococcal LOS [Jennings, Lugowski, and Ashton 1983, Michon et al. 1990) and for lipid A from *Neisseria gonorrhoeae* (Takayama et al. 1986).

been also been obtained (Mandrell et al. in press). Although sialic acid had been found in compositional studies of meningococcal LOS (Griffiss et al. 1984, Hammond, Kingsbury, and Weiss 1968, Jennings et al. 1980, Jennings, Hawes, and Adams 1973, Mergenhagen, Martin, and Schiffman 1963) others have suggested that the sialic acid determined from the thiobarbituric acid colorimetric assay was thiobarbituric acid-reactive KDO or a contaminant from the meningococcal capsule (Hackenthal 1969).

To determine if after the acid hydrolysis the 291 Da moiety was still present, the LOS of strain 6275 was hydrolyzed in 1% acetic acid at 80° for 2 hours and a portion of the resulting oligosaccharide was HF-treated. Contrary to the results of the analysis of the *O*-deacylated LOS, LSIMS analysis of derivatized and underivatized HF-treated and non-HF-treated oligosaccharide failed to produce any evidence of sialylation (Mandrell et al. in press). Apparently the acid-sensitive neuraminic acid moiety was completely hydrolyzed even using this more mild acid treatment. This data suggests that other strains of meningococcus may also produce sialylated LOS, but the neuraminic acid moieties are hydrolyzed in the initial treatment with acid used to isolate the oligosaccharide portion and are, therefore, not detected in the subsequent structural analysis (Dell et al. 1990, Jennings, Lugowski, and Ashton 1983, Mandrell et al. in press, Michon et al. 1990). The presence of the sialic acid group on meningococcal strain 6275 was found to modify the expression of the 3F11 and 06B4 LOS epitopes, which recognize Galβ1→4GlcNAc moieties on mammalian glycosphingolipids (Mandrell et al. in press).

The composition of the lipid A moiety of the meningococcal LOS from strain 6275 determined from the measured mass of the LOS after *O*-deacylation and HF-treatment appears to be identical to what would be

expected for the gonococcal LOS based on the published lipid A structure (Takayama et al. 1986). The proposed structure shown in Figure 15 is derived from the spectrum shown as well as other spectra of the oligosaccharide from strain 6275 (Mandrell et al. in press), and is consistent with structures determined for meningococcal OS by Jennings and co-workers (Jennings, Lugowski, and Ashton 1983, Jennings et al. 1980). The molecular ion at m/z 871 corresponds to the monophosphoryl *O*-deacylated lipid A moiety which would be formed during the treatment with hydrazine or HF.

The linkages of the KDO residues in the core region have not been determined for any gonococcal or meningococcal LOS prior to our investigation. The loss of the second KDO residue from the fragment ion at m/z 1231.7 (exact mass) in Figure 16A suggests that the two residues are in series, rather than branched as shown. On the other hand, the fragment ions in the spectrum of *O*-deacylated gonococcal LOS shown in Figure 2A can be more readily explained by the presence of a non-reducing terminal KDO moiety. Although it is possible that the sequence of the core region of the oligosaccharide in the LOS of *N. gonorrhoeae* and *N. meningitidis* varies, the similarity of other structural parameters and the cross-reactivity of the LOS immunologically suggest otherwise. More data is required to determine the sequence of the KDO residues.

The assignment of several significant ions in the spectrum in Figure 15 could not be determined i.e., m/z 1389.8, 1273.7, 1169.6 and 977.6 (exact masses). Presumably, these ions originated from some type of heterogeneity in the sample, perhaps introduced by partial degradation during the HF or hydrazine treatment.

C.3. LSIMS analysis of *O*-deacylated LOS from the pyocin-resistant strain FA5100.

Unlike the parent strain FA19 the LOS of the pyocin-resistant gonococcal strain FA5100 does not react with any of the available MAbs that recognize gonococcal LOS (Shafer et al. 1984), as discussed in Chapter 1, D.6. Petricoin and Stein (Petricoin III and Stein 1989) isolated and cloned a gene from a wild-type strain that could transform strain FA5100 to normal LOS biosynthetic capability. Very little information about the structure of the LOS of strain FA5100 was available. Therefore, we began a collaboration with Dr. Stein and one of our long term collaborators, Dr. J. McL. Griffiss, to determine the structure of this LOS.

The oligosaccharide portion of the LOS was presumed to be very small based on SDS gel data and compositional analysis, and may, therefore, contain only KDO. The *O*-deacylation procedure used previously in the analysis of intact *O*-deacylated LOS appeared to be the best way to obtain molecular weight and sequence information.

Shown in Figure 16A is the negative-ion LSIMS spectrum of the *O*-deacylated LOS from FA5100. Two clusters of ions are apparent in the spectrum, one cluster in region of m/z 960 and the other in the region of m/z 1600. The molecular ion at m/z 951 corresponds to the mass of the lipid A moiety expected after *O*-deacylation (872 Da) (Takayama et al. 1986), but containing two phosphate groups rather than one. The ion at $(M-H)^-$ 933 is the anyhydro version of the diphosphoryl species at m/z 951. The ions at m/z 955 and 973 are the sodium adducts of the ions at m/z 933 and 951.

The cluster of ions with greater mass appear at first to represent slightly more complex structural heterogeneity. However, upon close

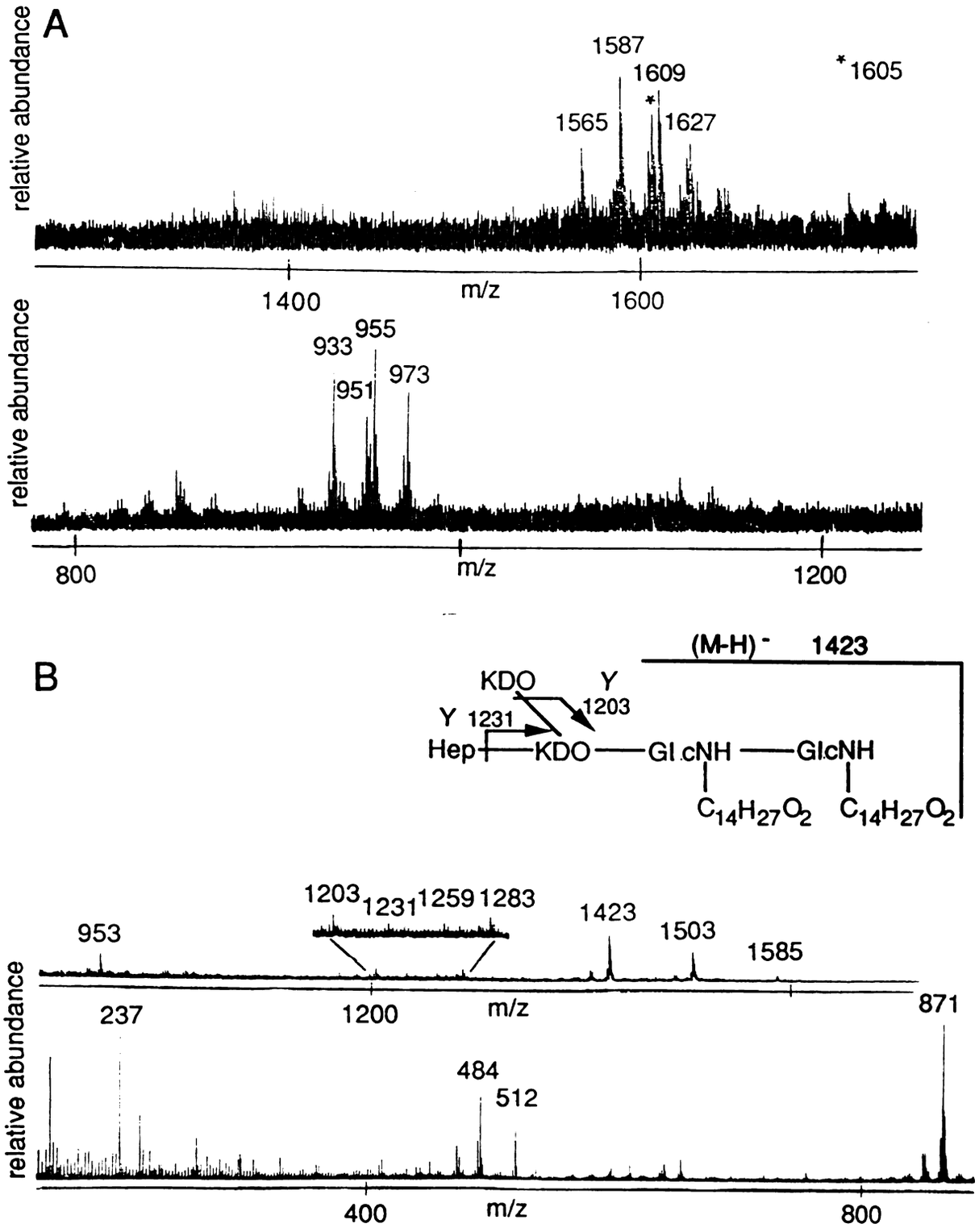


Figure 16. Negative-ion LSIMS spectra (A) of the *O*-deacylated LOS from the pyocin-resistant gonococcal strain FA5100 and (B) of the HF-treated *O*-deacylated LOS. Both spectra are consistent with the structure shown in panel B, with the addition of two phosphoryl moieties to the non-HF treated sample. See the text for a complete discussion of the ions in these spectra.

examination all of these ions can be related quite simply to the lower mass cluster of ion peaks. The difference between the ion at m/z 933 and the ion at m/z 1565 is 632 Da, which corresponds to the mass of 2 KDOs and Hep. The ion at m/z 1587 is almost certainly the sodium adduct of the ion at m/z 1565 and would be expected due to the presence of phosphate. In the lower mass cluster of ions the smallest significant peak is the intact diphosphorylated lipid A at m/z 951. The corresponding ion for the addition of $HepKDO_2$ which is found at m/z 1583 is of too low apparent abundance to be detected in this spectrum. However, the ion at m/z 1605 is the sodium adduct of the diphosphorylated lipid A moiety plus 2 KDOs and Hep (22+951+632), while the ion at 1627 is the disodium adduct. Again, the observation of salt adducts could be predicted due to the presence of phosphate.

The negative-ion spectrum of the HF-treated *O*-deacylated LOS from strain FA5100 shown in Figure 16B supports the structure proposed for the fragment ions in the spectrum of Figure 16A. In Figure 16B the molecular ion at m/z 1423 corresponds to lipid A without phosphate groups plus 2 KDOs and Hep (791 Da + 632 Da). The molecular ion at $(M-H)^-$ 1503 is the monophosphoryl version, although the precise position of the phosphate on lipid A cannot be determined from this ion. An abundant molecular ion is also present at $(M-H)^-$ 871 corresponding to the monophosphoryl lipid A moiety indicating removal of the phosphate by HF treatment did not go to completion. The low mass ion at m/z 237 is quite likely the fragment ion for KDO itself (M_r 238) and might be expected to be abundant due to the negative charge stabilization of the carboxylic acid. Bond cleavage between the two $GlcNH_2$ moieties of monophosphoryl lipid A would be predicted to produce an ion at m/z 484 and is seen in this spectrum. The ion at m/z 512 could be

a reducing terminal ring cleavage fragment ion of the $^{1,5}X$ type, in which case the remaining phosphate would be on the reducing terminal saccharide. Heterogeneity in the *N*-linked fatty acid moieties of the lipid A that increased the chain length by two CH_2 units could also produce an ion 28 Da larger, such as m/z 512.

As seen in the enlarged section of the spectrum in Figure 16B the reducing terminal fragments at m/z 1259 and m/z 1231 are the *X* (-164 Da) and *Y* (-192 Da) fragment ions for the loss of Hep from the molecular ion at m/z 1423. The reducing terminal fragments at m/z 1231 and 1203 are the *X* (-192 Da) and the *Y* (-220 Da) fragment ions for the loss of KDO from the molecular ion at m/z 1423. Finally, the ion at 1283 is the *Y* fragment ion for the loss of KDO from the molecular ion at m/z 1503. These fragment ions are quite clear, even though they are of low abundance. This data suggests that both the Hep and KDO moieties are found on reducing termini of the molecule. Thus, all the major fragment ions observed in these two spectra support the composition of KDO_2Hep plus a diphosphoryl lipid A moiety for the FA5100 LOS as shown above the spectrum in Figure 16B.

These spectra provided valuable structural data, although the samples were heterogenous. Analysis of the intact LOS instead of the oligosaccharide portion allows detection of heterogeneity in the lipid A portion of the molecule as well as increasing the capability to detect acid labile moieties on the LOS. A relatively large amount of sample was required to produce reasonably abundant signals in LSIMS analysis (>10 nmol) suggesting that other ionization methods may prove to be preferable to LSIMS for the analysis of intact LOS. These data, however, represent only a few structures and the ultimate sample requirements remain to be determined. Increasing the purity of the LOS and optimizing the

conditions used in obtaining the spectra, such as better matrices, might greatly improve the quality of the spectra and the sensitivity of detection of *O*-deacylated LOS by LSIMS.

Two procedures for purification of LPS have recently been published, one a gel filtration method (Wu, Tsai, and Frasch 1987) and the other a HPLC procedure (Kol et al. 1987). In LSIMS analysis of the LOS I experimented with various mixtures of thioglycerol/glycerol as a matrix, and with triethanolamine. With phosphorylated LOS, triethanolamine seemed to produce the best results, although the effect was not dramatic. Clearly, a whole range of matrices should be tried in the LSIMS analysis of the LOS. Very sensitive detection has been reported for neoglycolipid derivatives in negative-ion FAB analysis using as a matrix diethanolamine, tetramethylurea, and *m*-nitrobenzyl alcohol (2:2:1) (Lawson et al. 1990). This combination might also be an appropriate matrix for analysis of the *O*-deacylated LOS.

C.4. Methylation analysis of *O*-deacylated LOS

Structural elucidation of the KDO region of the LPS and LOS of Gram-negative bacteria has been slower than elucidation of the rest of the molecule. Isolation of the oligosaccharide from the lipid has been accomplished by hydrolysis of the acid-labile ketosidic linkages of the KDO residues. KDO can undergo side reactions under the conditions of the acidic hydrolysis used to isolate the oligo- or polysaccharide (Unger 1981). Colorimetric methods have been most often used in quantitative analysis of KDO (McNicholas, Batley, and Redmond 1984). However, these

colorimetric techniques are subject to a number of limitations. Various modifications of the periodate-thiobarbituric acid assay for KDO have perhaps most often been used (Unger 1981). Other deoxy sugars also react in this procedure (McNicholas, Batley, and Redmond 1984).

To investigate the structural features of the KDO moieties the LOS must be analyzed more or less intact, or the hydrolysis must be performed in such a manner as to produce at least di- or trisaccharides containing more intact KDO residues than a single reducing terminal one (Brade, Galanos, and Luderitz 1983a, Brade, Galanos, and Luderitz 1983b, Unger 1981). Determining the molecular weight for the intact LOS by mass spectrometry, together with composition data for the rest of the LOS, should unambiguously establish the number of KDO residues. Although the sequence of oligosaccharides can often be deduced from LSIMS data, this depends on both the extent and nature of the fragmentation. The fragment ions most often observed in negative-ion LSIMS analysis of oligosaccharides with reducing terminal derivatives are the products of one bond cleavages and ring cleavage products of the A- or X-type (Domon and Costello 1988). This was discussed in Chapter II B.2. (see Fig. 4, Chapter II B.2.). Fragment ions that are appear to be the products of remote site two bond cleavages are also observed. However, most commonly the ion abundance of the products of remote site two bond cleavages are significantly less than other fragment ions and, thus, can be distinguished from the other fragment ions. During LSIMS analysis the type of fragment ions that would be produced by KDO moieties in positions other than the reducing terminal residue remains to be determined. Thus, elucidation of the sequence of KDO moieties by LSIMS analysis at this point would require the study of LSIMS fragmentation of one or more model compounds containing

more than one reducing terminal KDO to determine if the fragmentation was sequence specific and reproducible.

At least two different strategies have been devised to determine the linkage of reducing terminal KDO in methylation analysis (Jennings et al. 1980, Taylor and Conrad 1972, York et al. 1985). The strategy of York et al. (York et al. 1985) was used to establish the linkage of the reducing terminal KDO in the oligosaccharide isolated from gonococcal LOS using less than 30 nmol of oligosaccharide (see section 4.C.)

Determination of the sequence and linkage of the KDO residues present in LOS or LPS from one bacterial strain has in the past required tens of milligrams of sample produced by mutant strains with truncated glycolipids. Various series of chemical treatments of the LPS or LOS were then required (Unger 1981). In one case, these treatments consisted of permethylation, treatment with periodate, complete Smith degradation followed again by treatment with periodate, acid hydrolysis followed by assaying for reactivity with thiobarbituric acid and, finally, analysis by GC-MS (Prehm et al. 1975). More recently the linkages of the KDO region of the LPS from a rough mutant of *S. minnesota* were determined by Brade and co-workers using Smith degradation of the LPS, permethylation of the intact and Smith-degraded LOS, methylation analysis of the oligosaccharide using GC and GC-MS (Brade, Moll, and Rietschel 1985). Again the analysis required tens of milligrams of LOS (>70 mg). A KDO disaccharide from the LOS of an Re mutant of *S. godesberg* was also investigated by Brade and co-workers (Brade et al. 1984). In this case, the disaccharide was first reduced and then permethylated and analyzed by GC-MS, in both EI and CI modes. The resulting spectra were compared with a known disaccharide sample containing a 5-linked KDO. Both ^{1}H

and ^{13}C nuclear magnetic resonance analyses of the disaccharide were also performed and compared to a synthetic disaccharide. While the structural analysis of this disaccharide was quite elegant, slightly over ten milligrams of purified disaccharide was needed (Brade et al. 1984).

While these investigations are examples of methods which could be used to solve the structural questions pertaining to the KDO-containing region of LPS and LOS, several disadvantages are apparent. The greatest problem with these approaches is the large amount of sample required. The other major disadvantage is the number of chemical steps involved, which increases both the time required for the analysis and the amount of sample which is needed.

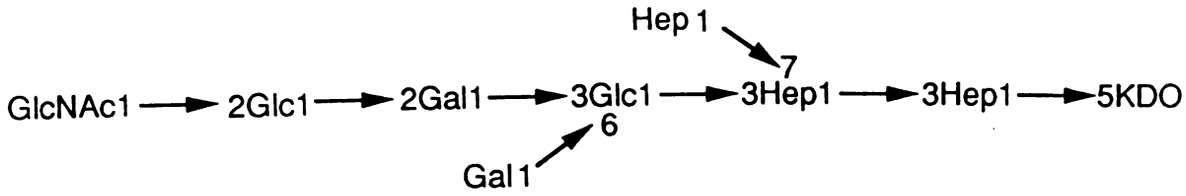
One of these reports discussed permethylation of intact LOS of a mutant which produced a truncated oligosaccharide (Prehm et al. 1975). It seemed possible that a microscale strategy could be devised and incorporated into methylation analysis that would establish in one experiment the position of the linkages of all the sugar residues of the oligosaccharide from the LOS, including KDO. I attempted such a scheme by performing a complete methylation analysis of the gonococcal LOS. (A description of the general procedure used in methylation analysis of the gonococcal oligosaccharides is contained in this thesis in Chapter 4.C.)

The first step in methylation analysis is solubilization of a dry sample in distilled dimethyl sulfoxide (DMSO). The LOS of the Ra mutant of *S. typhimurium* and of the wild type gonococcal strain 1291 was not soluble in DMSO, even after extensive sonication and vortexing. However, the *O*-deacylated LOS from both was readily soluble in DMSO.

One part of the method to determine the linkage of reducing terminal KDO in oligosaccharide samples by methylation analysis is an extra

reduction step after permethylation. This step reduces the methyl ester on the C-1 of KDO to an alcohol. This reduction is performed with $NaBH_4$ in $EtOH/H_2O$ (2:1) as described in the Methods section of 4.C. A more logical solvent combination for the permethylated LOS is a mixture of tetrahydrofuran (THF) and EtOH (1:1), and this was used first with $NaBH_4$. In a second experiment a 1 M solution of triethylborohydride (Superhydride) in THF was used to reduce the methyl ester. Triethylborohydride is a more powerful reducing agent than $NaBH_4$ and would be expected to reduce the ester more quantitatively (see Methods). Triethylborohydride is soluble in organic solvents, such as THF, which should better solvate the permethylated LOS also. Otherwise, the reaction conditions for the methylation analysis were the same as those used for oligosaccharide samples.

As shown in the total ion chromatogram in Figure 17, the methylation analysis of the HF-treated LOS of the Ra mutant of *S. typhimurium* was successful using this technique. The LOS from a wild type gonococcal strain (1291) was also subjected to this procedure with good results (data not shown). But in both cases there was no evidence of a peak corresponding to the expected derivatives of KDO. For the oligosaccharide from the Ra mutant of *S. typhimurium* peaks from 1,2,4,6,7-*O*-acetyl-5,8-*O*-methyl-3-deoxyloctitol (from 2,4,7-KDO), or a 1,2,5,6-*O*-acetyl-4,7,8-*O*-methyl-3-deoxyloctitol (from 2,5-KDO), or 1,2,6-*O*-acetyl-4,5,7,8-*O*-methyl-3-deoxyloctitol (from non-reducing terminal KDO) should have been observed (Aspinall 1987). While there may be an advantage in performing methylation analysis without first isolating the oligosaccharide portion of the LOS, the goal of these experiments was to develop a relative simple method which could be used on a microscale to determine the linkages of



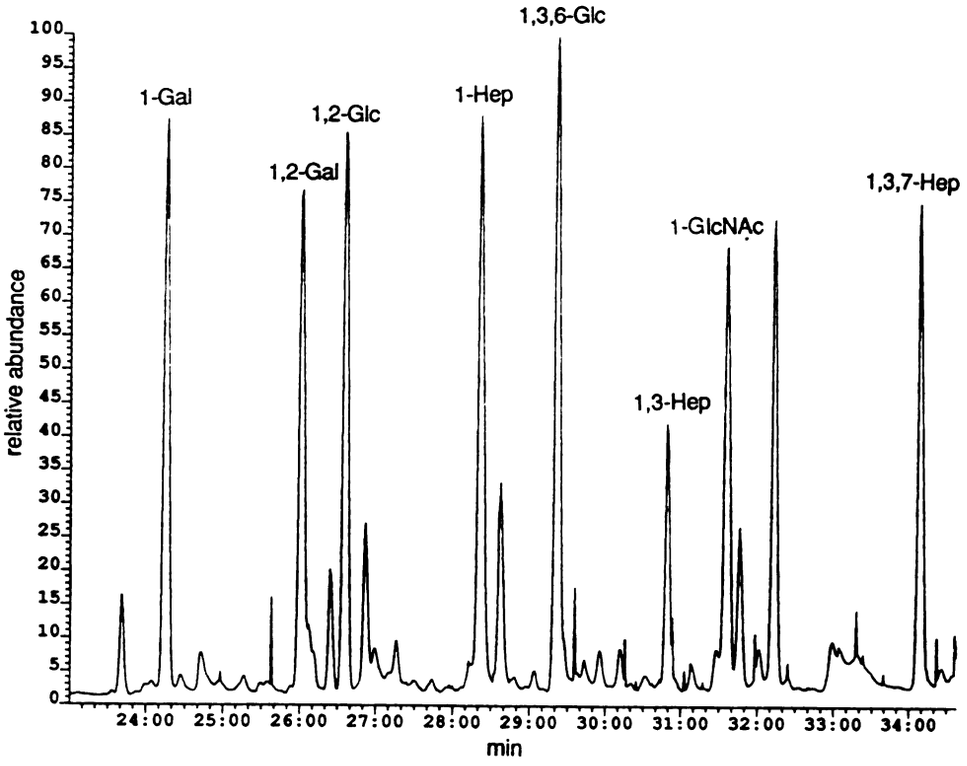


Figure 17. Total ion chromatogram of the PMAAs from methylation analysis of the *O*-deacylated HF-treated LOS from the TV119 Ra mutant of *S. typhimurium*. The peaks are labeled with the monosaccharide from which the PMAA was derived. Unlabeled peaks represent various types of non-carbohydrate impurities.

the entire oligosaccharide including KDO. Although these initial experiments were not successful in providing information on the linkage of KDO, it is still possible that a modification of this procedure will produce a stable PMAA derivative of KDO.

Experiments to develop this technique were continued using LOS from the gonococcal pyocin-resistant strain FA5100. LSIMS data previously obtained of the *O*-deacylated LOS from FA5100 suggested a composition of two KDOs and one Hep plus a diphosphoryl lipid A moiety (see Chapter II, C.3.). It was postulated that the conditions of the acid hydrolysis step in the methylation analysis had been too harsh for the KDO moieties, which may have been degraded. Several different mild acid hydrolyses had been used by Brade and co-workers in the isolation of oligosaccharides containing two or three KDO residues (Brade, Galanos, and Luderitz 1983b, Brade, Moll, and Rietschel 1985). Work in our laboratory had established conditions for acid hydrolysis of oligosaccharides containing reducing terminal KDO to produce monosaccharides for compositional analysis with limited degradation of the KDO (see Methods) (Phillips et al. 1990).

To investigate this further my colleague, Lorri Reinders, and I carried out a permethylation of HF-treated and non-HF treated *O*-deacylated LOS from FA5100, and HF-treated oligosaccharide released from the LOS by mild acid hydrolysis (see Methods). For each sample approximately 85 μg of *O*-deacylated LOS starting material was used. Methylation analysis of all three samples was performed using mild hydrolysis conditions for glycosidic bond cleavage (see Methods). The results of the methylation analysis when analyzed by GC-MS in both the CI and EI mode were disappointing (data not shown). The methylation analysis had produced the expected alditol acetate derivative for a non-

reducing terminal Hep (1,5-di-*O*-acetyl-2,3,4,6,7-penta-*O*-methylheptitol). In CI analysis both the $(M+H)^+$ and the $(M+NH_4)^+$ ions were found in one scan at m/z 368 and m/z 385. However, there was no evidence of ions that could be associated with PMAA derivatives of KDO.

One reason that the reaction sequence may not have produced the desired products is that the esterification and reduction of the KDO residues may be very inefficient, even though a number of different strategies were tried. The reduction of the methyl ester on KDO with $NaBH_4$ (York et al. 1985) was successful with reducing terminal KDO (see Chapter IV, C.1.) but the final yield was much less than the yield of the PMAAs of the other sugar residues. The procedure employing a water soluble carbodiimide has been used successfully to reduce the carboxylic acid of reducing terminal KDO moieties (Jennings et al. 1980, Taylor and Conrad 1972). Perhaps the reduction of the carboxylic acid of the KDO residues on the *O*-deacylated LOS could be performed similarly with better results.

Such large amounts of sample may have been consumed in the past in elucidation of the KDO-containing region of LPS and LOS due to the instability of the KDO moiety, resulting in degradation of the molecule during sample preparation steps. This may also be the case in this procedure used to isolate a stable KDO octitol. I have attempted to solve these structural questions by reducing the amount of sample by a factor of >500 to 100-200 μg, but still using larger quantities than is normally used for methylation analysis in our laboratory (≈10-30 μg). Perhaps this is an overly ambitious first goal, and the amount of sample used should be increased somewhat until the conditions have been optimized. Relatively larger amounts of the LOS from the smaller of the pyocin-resistant mutants of gonococcal strain 1291 are available in the laboratory than of the LOS

from FA5100. Also, the commercially available LOS from the Re mutant of *S. typhimurium* could be used as a standard since the structure has previously been determined, and has some features in the KDO region that are similar to the gonococcal LOS. Although the experimentation to date with methylation analysis of the intact LOS did not provide the desired data it is possible that this strategy may yet prove to be a valuable approach.

D. Separation and compositional analysis of oligosaccharides isolated from LOS by HPAE-PAD.

The LOS from *N. gonorrhoeae* has been found to exhibit a high degree of heterogeneity, particularly in the oligosaccharide portion (Gibson et al. 1989, Griffiss et al. 1987, Mandrell et al. 1986, Schneider et al. 1984). Considerable structural variation has also been found in the LOS expressed *in vivo* as compared to the LOS *in vitro* using immunochemical techniques and comparative electrophoretic motilities. This was discussed in some detail in Chapter I, D.6. Although evidence was obtained of structural variation *in vivo*, neither the structures of the initial or the variant LOS were determined [Senior, 1989 #38; Griffiss, J. McL. manuscript in preparation].

Purification of the oligosaccharide portion of the LOS and/or the LOS to homogeneity became increasingly important during our structural analysis work. Purification came to be viewed as an even more essential component of the strategy to be employed in future analyses. One of the advantages of MS/MS analysis of heterogenous samples is the ability to select a single, isotopically pure parent ion in MS-1, and analyze exclusively

the daughter ions of this parent in MS-2. Thus, MS/MS analysis provided the answers to structural questions, in some instances, which were otherwise ambiguous due to heterogeneity issues. However, particularly when trying to determine the entire repertoire of LOS produced *in vitro* by one strain, including the minor components, good chromatographic separation of these components could be an invaluable aid. The technique of high pH anion exchange chromatography with pulsed amperometric detection (HPAE-PAD) had not previously been applied to oligosaccharides isolated from bacterial LOS containing KDO on the reducing terminus. Initial exploration of the use of this technique for purification of the gonococcal oligosaccharides is described in this section.

D.1. Materials and methods

Materials

The 18MΩ water for the HPAE chromatography was acquired by purification of deionized water using a Millipore milli-Q system. The 50% (w/w) NaOH solution was obtained from Mallinckrodt (Muskegon, MI). The LOS from the TV119 Ra mutant of *S. typhimurium*, and the monosaccharides KDO, galactose, glucose, and glucosamine were purchased from Sigma (St. Louis, MO). The monosaccharide D-*glycero*-D-*gulo*-heptose was obtained from Aldrich (Milwaukee, Wisconsin).

Methods

HPAE-PAD. A Dionex BioLC (Dionex, Sunnyvale, CA) system operated by a Dell 210 computer with AI-450 software was used for the HPAE-PAD chromatography. This system contained a Carbo-Pak PA1 column (4 x 250 mm), a pulsed amperometric detector, and a gradient pump. The pulse potentials and durations were as follows: E_1 = 0.05V (t_1 = 480 ms); E_2 = 0.60V (t_2 = 120 ms); E_3 = -0.60V (t_3 = 60 ms). The response time of the PAD was set at 3 s. Four eluants were used, H_2O, 1 M NaOH, 200 mM NaOH and 1 M NaOAc. The NaOH solutions were prepared as directed by Dionex by dilution of a 50% (w/w) NaOH solution purchased from Mallinckrodt. To desalt the samples in preparation for analysis by LSIMS the effluent was passed though a Dionex anion micromembrane suppressor. To provide the protons for the suppressor a flow of 100 mN H_2SO_4 at 10 ml/min was obtained by pressurization with He (25 p.s.i.).

The following procedure was used for HPAE-PAD chromatography of oligosaccharide from the LOS of the TV119 Ra mutant of *S. typhimurium*. From 15-60 nmol of the oligosaccharide was dissolved in H_2O for the analysis. For elution a linear gradient of 0-500 mM NaOAc in 60 min with an isocratic concentration of 50 mM NaOH was employed with a flow rate of 0.5 ml/min. After collection of the samples they were neutralized, and derivatized with HBEE for LSIMS analysis as described in Chapter II, B.1.

The compositional analysis of the monosaccharides was performed as follows (Phillips et al. 1990): Approximately 10 nmol of the HF-treated oligosaccharide from the LOS of the TV119 Ra mutant of *S. typhimurium* was dissolved in 200 µl of H_2O, 200 µl of 4 M TFA was added, and the solution heated for 3 h at 100° C. The sample was dried in a Speed-Vac concentrator, dissolved in a small amount of H_2O, and dried again. The sample was then dissolved in 100 µl of H_2O and a 25 µl aliquot was injected

onto the Carbo Pak PA-1 column for analysis. A flow rate of 1 ml/min of the following gradient was used for elution: 16 mM NaOH isocratic for 20 min; then a linear gradient for 5 min to 100 mM NaOH and 100 mM NaOAc; a linear gradient for 15 min to 160 mM NaOAc with NaOH isocratic at 100 mM. Standards of KDO, Glc, Gal, $GalNH_2$, and $GlcNH_2$ were used to determine response factors for the monosaccharides. The heptose moieties in the oligosaccharide from *S. typhimurium* are L-*glycero*-D-*manno*-heptose residues. However, this monosaccharide is not available commercially so D-*glycero*-D-*gulo*-heptose was used as a standard for the initial phase of this work.

D.2. Results and discussion.

One recent development in the chromatographic separations of carbohydrates is the method of HPAE-PAD, which was previously described in Chapter II, A.1. Excellent resolution of mixtures of both oligosaccharides and monosaccharides has been obtained with this technique (Hardy and Townsend 1988, Townsend et al. 1989, Townsend et al. 1988). In our laboratory gel filtration chromatography followed by reducing terminal derivatization and reverse-phase HPLC have been routinely used to purify mixtures of gonococcal oligosaccharides. However, in many cases single components are still not obtained.

In application of HPAE-PAD to these oligosaccharides containing reducing terminal KDO one of the first questions was the stability of the KDO moiety. To determine if the KDO residue would withstand the high pH, the HF-treated oligosaccharide from the LOS of the TV119 Ra mutant of *S. typhimurium* was purified by HPAE-PAD. During the analysis several

peaks were collected, which were then derivatized with HBEE and separated by HPLC (see Fig. 18). The hydrazone-oligosaccharides were analyzed by negative-ion LSIMS which confirmed the oligosaccharide had survived intact. Peak one (Fig. 18) produced the expected molecular ion as well as a lesser amount of the anhydro species that had been previously observed (John and Gibson 1990), as discussed in Chapter II, B.2. The mass spectrometric analysis indicated peak two contained only anhydro species, even though the shape of the peak indicates there are apparently two different components (see Fig. 18). The HPAE-PAD separation of the intact oligosaccharide from the anhydro was very similar to the separation of the derivatized oligosaccharide from the derivatized anhydro oligosaccharide by reverse-phase HPLC. Apparently KDO survived the high pH of the HPAE-PAD separation intact. Some formation of the anhydro had been observed previously after acid hydrolysis to isolate the oligosaccharide from the LOS, and after derivatization with both HBEE and ABEE. This technique was next applied to oligosaccharide isolated from the LOS of gonococcal strain F62 (see Chapter III, A. and Fig. 19).

HPAD-PAD chromatography can provide quantitative compositional analysis of oligosaccharides (Hardy, Townsend, and Lee 1988). However, monosaccharide standards are required for the components of the oligosaccharide to determine the response factor for each monosaccharide. Also, depending on the components present in the oligosaccharides the conditions required for acid hydrolysis can vary. For example, most neutral monosaccharides can be quantitatively hydrolyzed in 2 M TFA by heating at 100° C for 2-6 h. Due to the presence of the protonated amino groups, the glycosidic bonds of amino sugars are more resistant to acid hydrolysis (Hellerqvist and Sweetman 1990). Therefore, quantitative

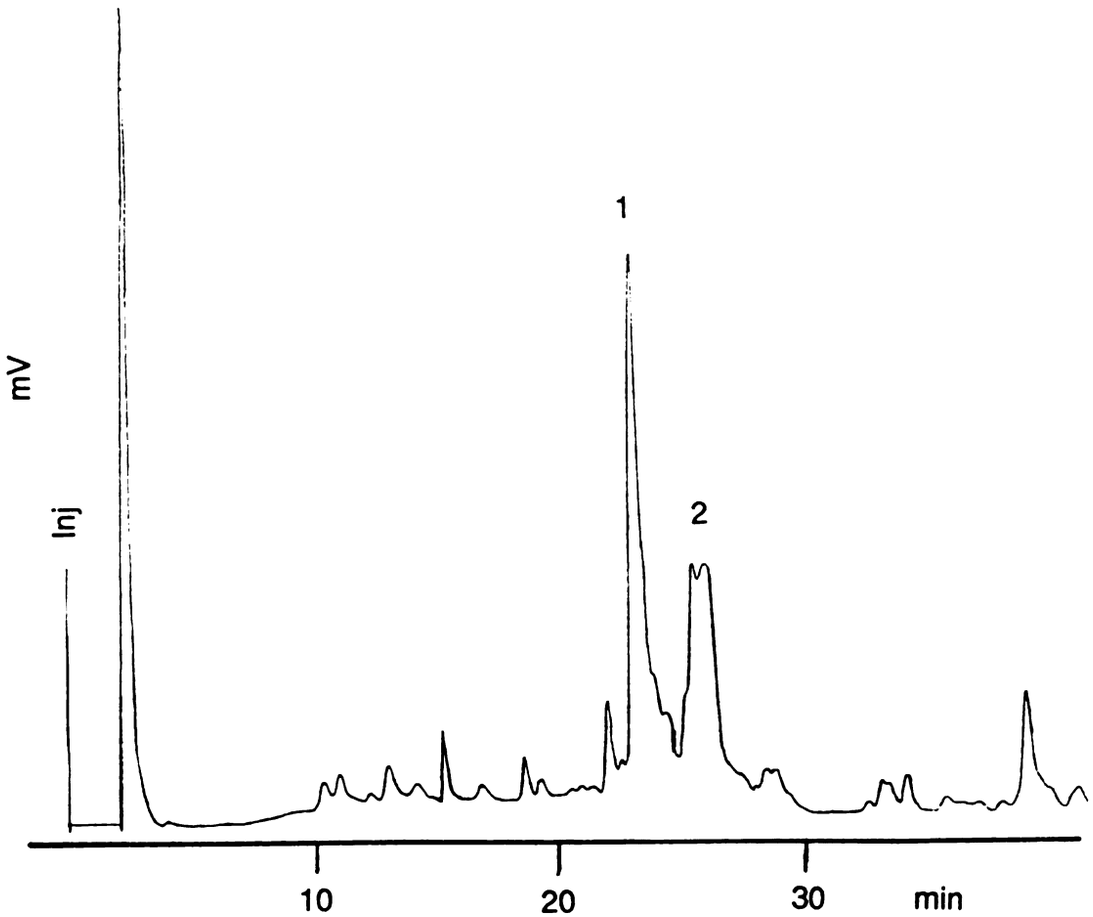


Figure 18. Separation of dephosphorylated oligosaccharide from the TV119 Ra mutant of *S. typhimurium*. Subsequent LSIMS analysis of the HBEE derivative of peak 1 showed the expected molecular ion, whereas peak 2 was an anhydro species. The large peak shortly after the injection is due to the start of the NaOAc gradient. The conditions for the separation are given in Methods.

hydrolysis of amino sugars requires more vigorous conditions, such as heating for 6 h at 100° C in 4 M HCl (Hardy, Townsend, and Lee 1988). It is preferable to use an oligosaccharide standard also for analysis by HPAE-PAD. Nonreducing terminal residues hydrolyze two to five times faster than analogous internal residues. Both free sialic acid and 2-deoxy residues are subject to acid catalyzed degradation (Hellerqvist and Sweetman 1990).

The oligosaccharide from the Ra mutant of *S. typhimurium* was hydrolyzed to yield monosaccharides and analyzed by HPAE-PAD to serve as a standard for compositional analysis (see Methods). Response factors and retention times for the compositional analysis were determined by injection of monosaccharide standards. No standard was available for L-*glycero*-D-*manno*-heptose, which is the heptose component *S. typhimurium*. Instead D-*glycero*-D-*gulo*-heptose which is available commercially was used to obtain a rough approximation of the response factor of the heptose moieties in the oligosaccharide. In this initial effort, different concentrations of acid were tried and the length of hydrolysis varied from 1-3 h. Particularly for the KDO and Hep residues the ideal hydrolytic conditions will have to be determined experimentally. The results reported below have been normalized to Glc, and were obtained from a 3 hour hydrolysis in 4 M TFA. The number in parenthesis is the number of residues of each monosaccharide present in the oligosaccharide of the Ra mutant of *S. typhimurium*.

Hep	0.92	(3)
KDO	0.51	(1)
Glc	2.00	(2)
Gal	2.41	(2)
$GlcNH_2$	1.03	(1)

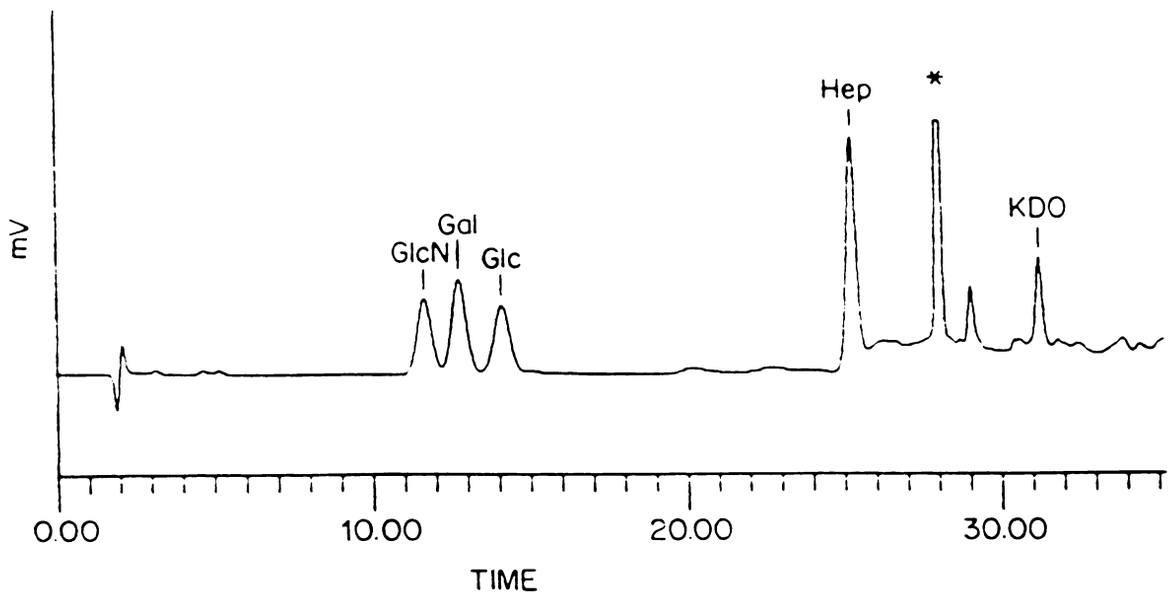


Figure 19. Compositional analysis by HPAE-PAD of the dephosphorylated oligosaccharide from the TV119 Ra mutant of *S. typhimurium* after hydrolysis in 2M trifluoroacetic acid. The peak with an asterisk is due to the beginning of the NaOAc gradient. The conditions for the separation are given in Methods.

The quantitation of the Hep and the KDO moieties was the least accurate. The KDO may possibly be degrading while the hydrolysis is occurring. The response factor may be greater for D-*glycero*-D-*gulo*-heptose compared to L-*glycero*-D-*manno*-heptose. Another possibility is that the Hep residues of the oligosaccharide may not be completely hydrolyzed under these conditions. As stated above, more experimentation is required to determine the best conditions for the hydrolysis. If the accuracy of this procedure can be improved for the KDO and the Hep residues, the speed, ease, and sensitivity offer great advantages for quantitative compositional analysis of bacterial oligosaccharides.

CHAPTER III. OLIGOSACCHARIDES OF STRAIN F62

Considerable effort has been expended to determine the relationship between the outer membrane LOS of *N. gonorrhoeae* and the response of the human immune system to infection by this bacteria (see Chapter I, D.6.). In particular, the LOS from strain F62 has been among the subjects of a number of studies (Mandrell et al. 1986, Mandrell et al. 1985a, Schneider et al. 1982, Schneider et al. 1984). Previous to our work some analysis of the physical components of the LOS from F62 and their immunochemical properties had been carried out. Strain F62 appeared to be typical in terms of the immunochemistry of a serum-sensitive strain (Schneider et al. 1982, Mandrell et al. 1986). The structure of the lipid A moiety of gonococcal strain F62 was previously determined (see Fig. 2, Chapter I) (Takayama et al. 1986). The LOS from strain F62 was selected for further structural elucidation because it is prototypical of the gonococcal LOS.

The molecular size of the LOS from nine of the strains of *N. gonorrhoeae* including F62 was approximated by ascending recycling chromatography, and the carbohydrate composition of the LOS assayed by GLC of the trimethylsilyl derivatives (Schneider et al. 1982). Using colorimetric methods the oligosaccharide composition of the F62 LOS was found to contain ≈25% hexose and ≈6% KDO (w/w). The molar ratios of the sugars occurring in the oligosaccharides was as follows: Gal 1.00; Glc 0.68-0.69; $GalNH_2$ 0.47-0.40; $GlcNH_2$ 1.18-1.00; and KDO 1.05-0.88. No obvious differences were apparent in the composition or molecular size of LOS from serum-resistant versus serum-sensitive strains (Schneider et al. 1982). In further experiments by Schneider and co-workers the molecular size of LOS from various strains of *N. gonorrhoeae* was estimated by electrophoretic

migration in SDS-PAGE using silver-staining to visualize periodate-oxidized LOS components (Tsai and Frasch 1982) and LOS from rough mutants of *S. minnesota* as molecular weight standards (Schneider et al. 1984). The gonococcal LOS in general was found to be heterogeneous, consisting of two or more components with molecular masses ranging from 3,200 to 7,000 Da. Two major components were visualized in the LOS from F62 with molecular mass estimations of 5,400, 4,500 Da and a very minor component of approximately 4,000 Da in mass.

A comprehensive study (described in Chapter I, D.6.) of antigenic and physical characteristics of the LOS of *N. gonorrhoeae* strains employing murine MAbs also included the LOS of strain F62 (Mandrell et al. 1986). Using solid-phase radioimmunoassay and SDS-PAGE it was found that strains with multiple LOS components usually bound more than one MAb. Some components bound more than one MAb while other MAbs bound more than one LOS component. The LOS from F62 bound three of four IgM MAbs and none of the four IgG MAbs. Both IgM MAbs 3F11 and 06B4 were found to be specific for highly conserved epitopes within the gonococcal LOS; the MAbs bound to 19 of 20 strains tested, with the exception of the LOS of the pyocin-resistant mutant strain JW31R. Both of these MAbs, 3F11 and 1-1-M, bound strongly to separate components within the F62 LOS. MAb 3F11 bound to the smaller LOS component of F62 (M_r≈4,500), and 1-1-M bound to the larger component (M_r≈5,400) (Mandrell et al. 1986). A number of the slow-migrating, large F62 LOS aggregates were recognized by the MAb 1-1-M as shown by the radioimmunoassay that were not silver-stained (Mandrell et al. 1986).

In subsequent studies that were previously discussed in Chapter I, B.6., the mouse MAb 3F11 agglutinated erythrocytes from all adult humans

tested. The mouse MAb 06B4 agglutinated 80% of the samples (Mandrell, Griffiss, and Macher 1988). These MAbs were found to recognize glycosphingolipids with a terminal *N*-acetyllactosamine (Galβ1→4GlcNAc) structure. The terminal tetrasaccharide (lacto-N-neotetraose) of paragloboside, Gal1β→4GlcNAcβ1→3Galβ1→4Glcβ1→1Cer, a glycosphingolipid precursor of the major blood group antigens, terminates in this disaccharide (Mandrell, Griffiss, and Macher 1988). A comparison was made of the binding affinity of 3F11 and 06B4 for branched and linear *N*-acetylactosamine glycosphingolipids. The MAb 3F11 had a higher affinity of branched-chain glycosphingolipids, while 06B4 had a higher affinity for linear ones. However, the exact binding specificity for the various sugars in these structures was not fully established in this work.

The specificity of the MAbs 3F11 and 06B4 was determined in general in the work by Mandrell et al. (Mandrell, Griffiss, and Macher 1988). On the other hand, the range of LOS components that are recognized by these MAbs, and the role of specific sugar residues in binding can only be determined by structural analysis of the complete repertoire of the LOS and correlation of the structures with MAb binding. Thus, structural elucidation of the oligosaccharides from the F62 LOS was begun with these goals in mind.

A. Purification and separation

The LOS from strain F62 was extracted from acetone-powdered organisms by a modification of the hot-water phenol procedure of Westphal and Jann (Westpahl and Jann 1965). The oligosaccharide fraction was prepared from the LOS by two different procedures (A and B). In procedure

A the oligosaccharides were purified in a manner similar to that used by Yamasaki et al. (Yamasaki et al. 1988). Briefly, this consisted of hydrolysis of 20 mg of LOS at 100°C for 2 h in 10 ml of 1% acetic acid. The mixture of oligosaccharide and lipid was then centrifuged at 47,800 x *g* for 20 min to precipitate the insoluble lipid A. The precipitate was washed with H_2O (3 x 5 ml, 47,800 x *g* for 10 min) and the supernatants combined and lyophilized, dissolved in H_2O, centrifuged and the supernatant lyophilized once again. Then the oligosaccharide was dissolved in 100 mM ammonium acetate (pH 7) and applied to a Bio-Gel P-4 column (2.6 x 90 cm, <400 mesh) equilibrated with the same buffer. Ascending chromatography was accomplished with an LKB 2130 peristaltic pump with a flow rate of ≈20 ml/h. Fractions (4.5 ml) were collected and the oligosaccharides located by refractive index detection.

In procedure B the oligosaccharides were purified by hydrolysis of the LOS at 90°C for 90 min in 1% acetic acid (2 mg/ml) as described for the oligosaccharides from the Ra mutant of *S. typhimurium* in Chapter II, B.1. using the procedure of Johnson and Perry (Johnson and Perry 1976). The solution was lyophilized, the products suspended in 50 mM pyridinium acetate buffer (pH 4.3) and centrifuged at 3400 x *g* for 10 min and the supernatant lyophilized. The oligosaccharides were resuspended in the pyridinium acetate buffer, centrifuged and the supernatant gel-filtered on a Sephadex G-50 column (1.5 x 30 cm) equilibrated with the same buffer. Gravity flow was used to collect fractions in 2 ml intervals. The oligosaccharides were located by spotting each fraction on a silica TLC plate and then applying an anisaldehyde spray reagent. The spray reagent was composed of 25 ml glacial acetic acid, 0.5 ml sulfuric acid, and 0.25 ml anisaldehyde. After application of the spray reagent the TLC plate was

heated by placing on a hot plate. Upon heating, the spots from the various fractions that contained oligosaccharides developed a distinctive dark outline. Fractions containing oligosaccharide were pooled and lyophilized. The oligosaccharides were then dissolved in 100 mM ammonium acetate buffer and purified on a Bio-Gel P-4 column as described in procedure A above. Fractions were collected in 3.3 ml intervals . Figure 1A shows the P-4 profile of oligosaccharides from F62 purified by procedure A, and Figure 1B shows a P-4 profile of oligosaccharides from F62 purified by procedure B.

Oligosaccharides from pooled P-4 fractions (see Fig. 1B) were HF-treated as described in Chapter II, B.1. HPAE-PAD was performed on the HF-treated oligosaccharides as described in Chapter II, D.1. and on some occasions the peaks were collected, dried and derivatized with HBEE as described earlier (Chapter II, B.1.). The best separations (see Fig. 2) were obtained with constant 50mM NaOAc with the following NaOH gradient: (i) isocratic at 10 mM for 5 min; (ii) linear to 100 mM in 10 min; (iii) linear to 300 mM in 50 min; (iv) isocratic at 300 mM for 5 min.

In the earliest eluting fractions from the P-4 column three main peaks were observed in HPAE-PAD. Both peaks 1 and 2 as shown in Figure 2 were seen, plus a smaller peak which eluted just prior to peak 1. In the middle fractions the two peaks shown in Figure 2 were observed of about equal size. But in the later eluting fractions from the P-4 column just one main peak corresponding to peak 2 (see Fig. 2) was observed. Although there is a shoulder on peak 2 only a single component was observed in LSIMS analysis. The subsequent negative-ion LSIMS analysis of the HBEE derivatives of the collected peaks revealed that the M_r of the oligosaccharides corresponding to peak 1 and 2 was 1717 and 1514, respectively. The mass difference of 203 Da corresponds to one HexNAc

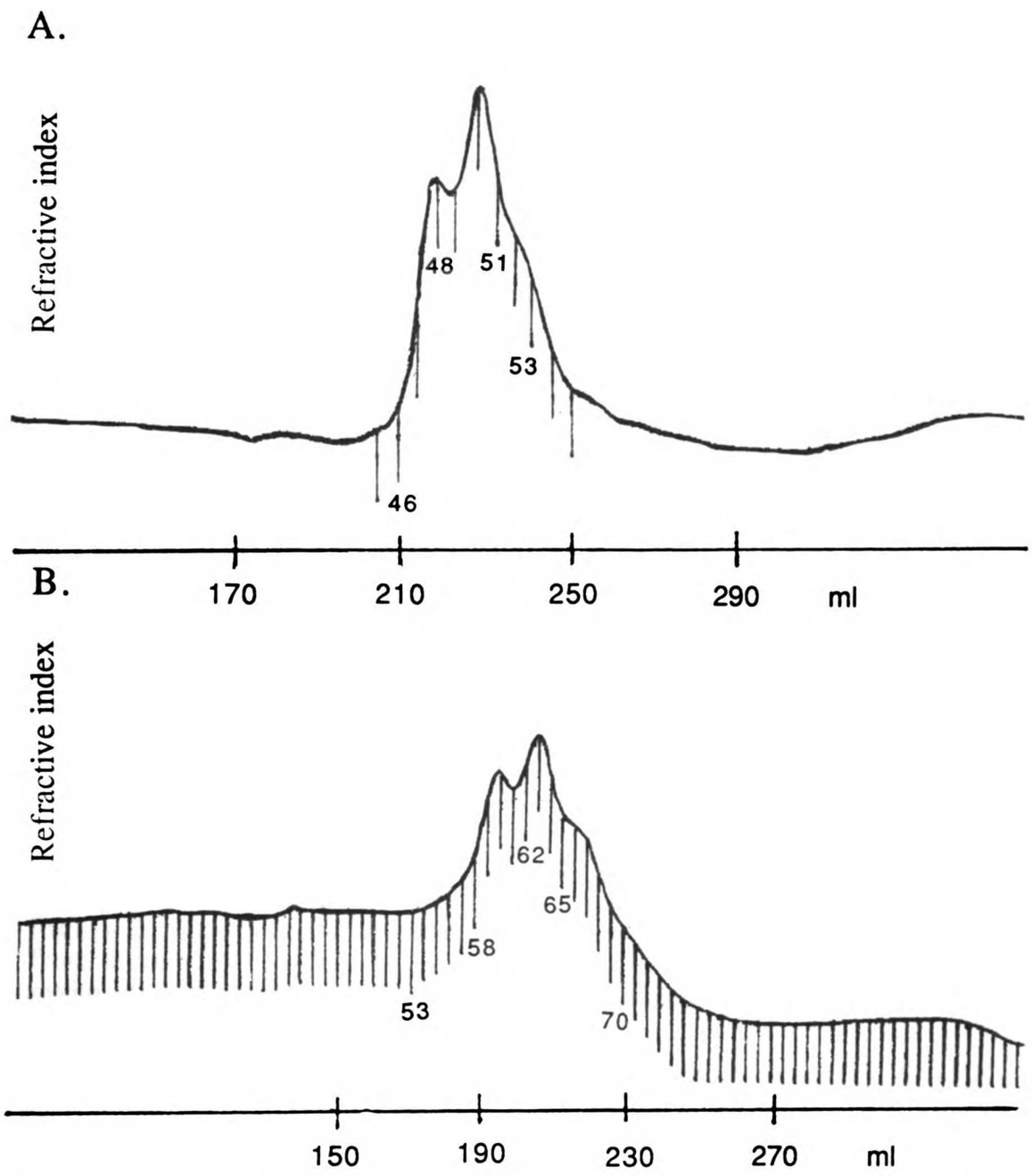


Figure 1. P-4 profile of F62 oligosaccharides (A) prepared according to procedure A as described in the text, with fractions 46, 48, 51, and 53 labeled. P-4 profile of F62 oligosaccharides (B) prepared according to procedure B as described in the text, with the fractions pooled for methylation analysis labeled. The void volume of the column (V_0) was approximately 145 ml.

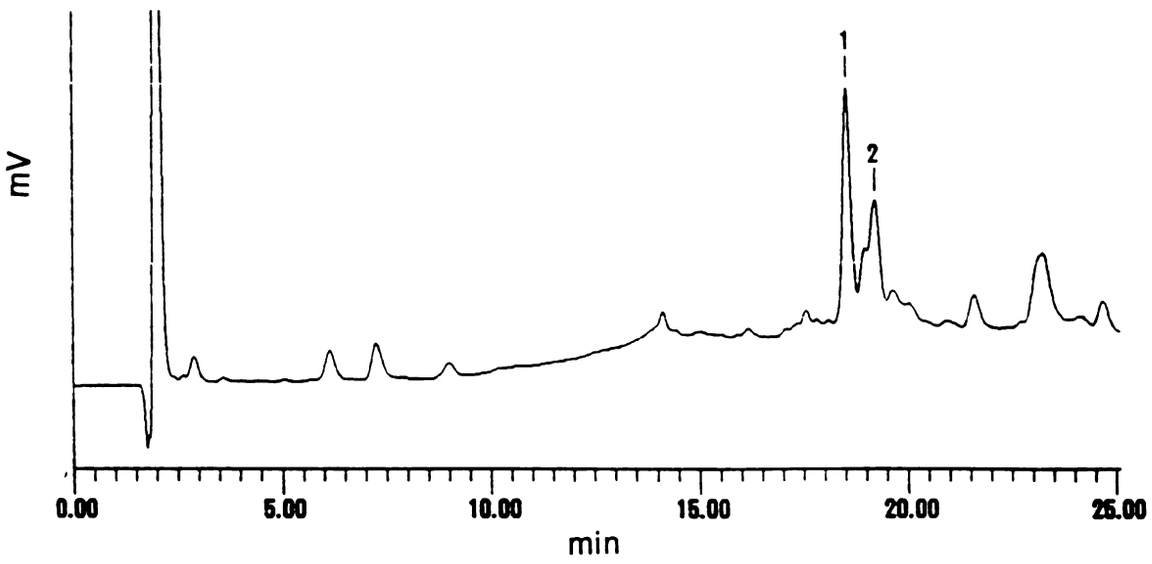


Figure 2. Separation of approximately 2 nmol of HF-treated oligosaccharide from strain F62 by HPAE-PAD. Peaks labeled 1 and 2 represent the two major oligosaccharide species with M_r 1717 and 1514, respectively.

moiety. Nothing could be observed in negative-ion LSIMS analysis of the third peak found in the earlier eluting P-4 fractions.

B. LSIMS analysis

A number of different components were found by LSIMS analysis of both HF-treated and non-HF-treated underivatized oligosaccharides released by acetic acid hydrolysis from the LOS of strain F62. In Figure 3 a portion of the negative-ion LSIMS spectra are shown of non-HF treated underivatized oligosaccharides from fractions 46, 48, 51, and 53 from Bio-Gel P-4 size-exclusion chromatography (see Fig. 1B). The LSIMS spectra were acquired as described in Chapter II, B.1. Abundant deprotonated molecular ions were observed, $(M-H)^-$, along with their sodium adducts, $(M+Na-2H)^-$ and some anhydro species. In these negative-ion spectra of underivatized oligosaccharides, little fragmentation occurred even though a relatively large amount of sample was used for each spectrum (≈5-10 nmol). This is a common problem with underivatized oligosaccharides and is presumably a consequence of being highly polar and, therefore, of low surface activity in the polar matrix. Another factor may be that there is no obvious site for negative-charge stabilization other than the carboxyl group of KDO and phosphate group in those oligosaccharides not treated with HF.

The nominal mass of HexNAc (203 Da) is the same as one PEA (123 Da) plus one phosphate moiety (80 Da). HF-treatment (described in Chapter II, B.1.) of oligosaccharides removes phosphate and phosphoester moieties, as well as some other components such as *O*-acetyl moieties. Therefore, a comparison of the spectra shown in Figure 3 with the LSIMS spectra of the same fractions after HF-treatment (data not shown) established the number

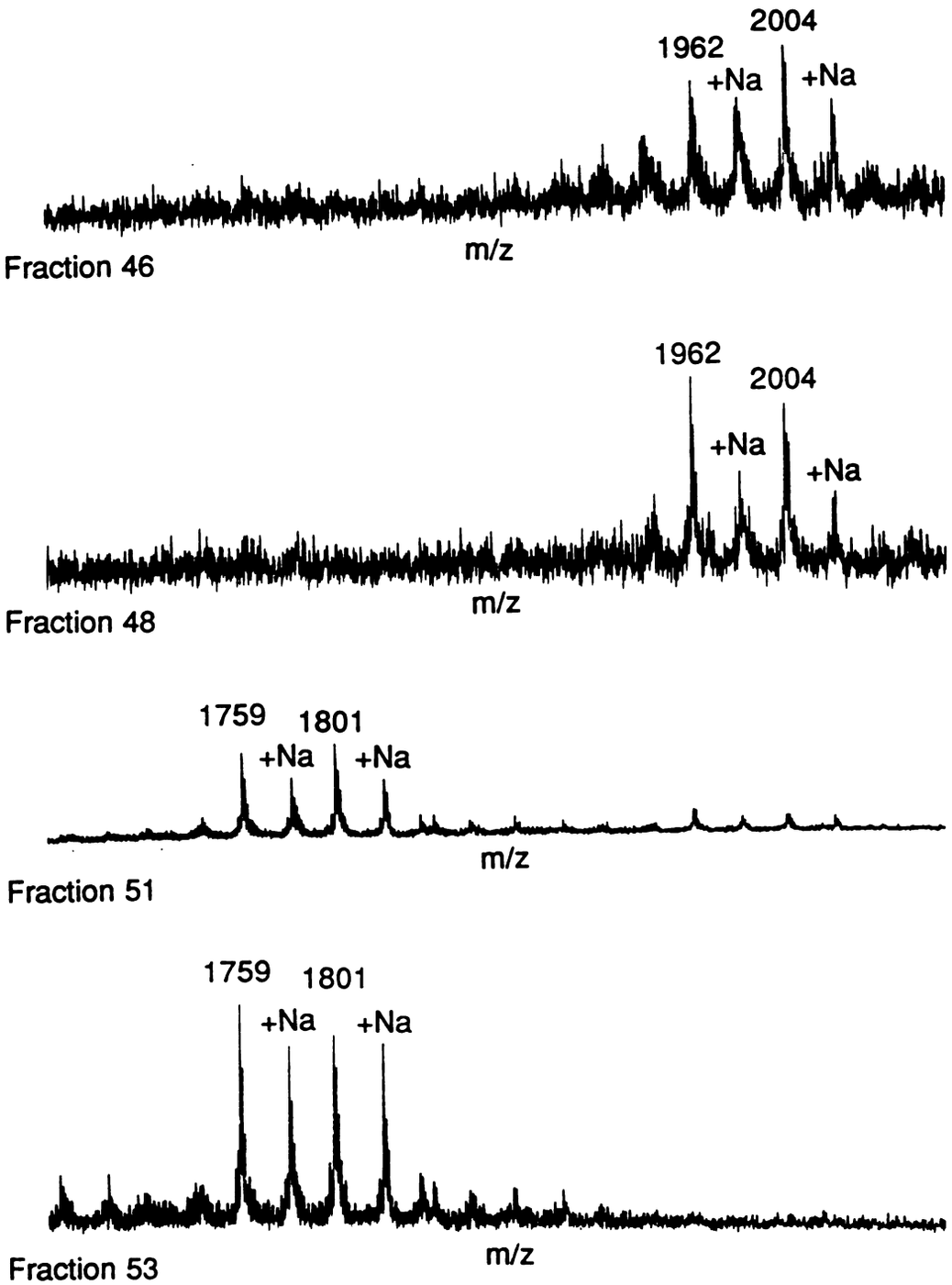


Figure 3. Negative-ion LSIMS spectra of underivatized oligosaccharides from fractions 46, 48, 51, and 53 (see Fig. 1A). Peaks 22 Da larger than the molecular ions $(M-H)^-$ are presumed to be sodium adducts $(M+Na-2H)^-$.

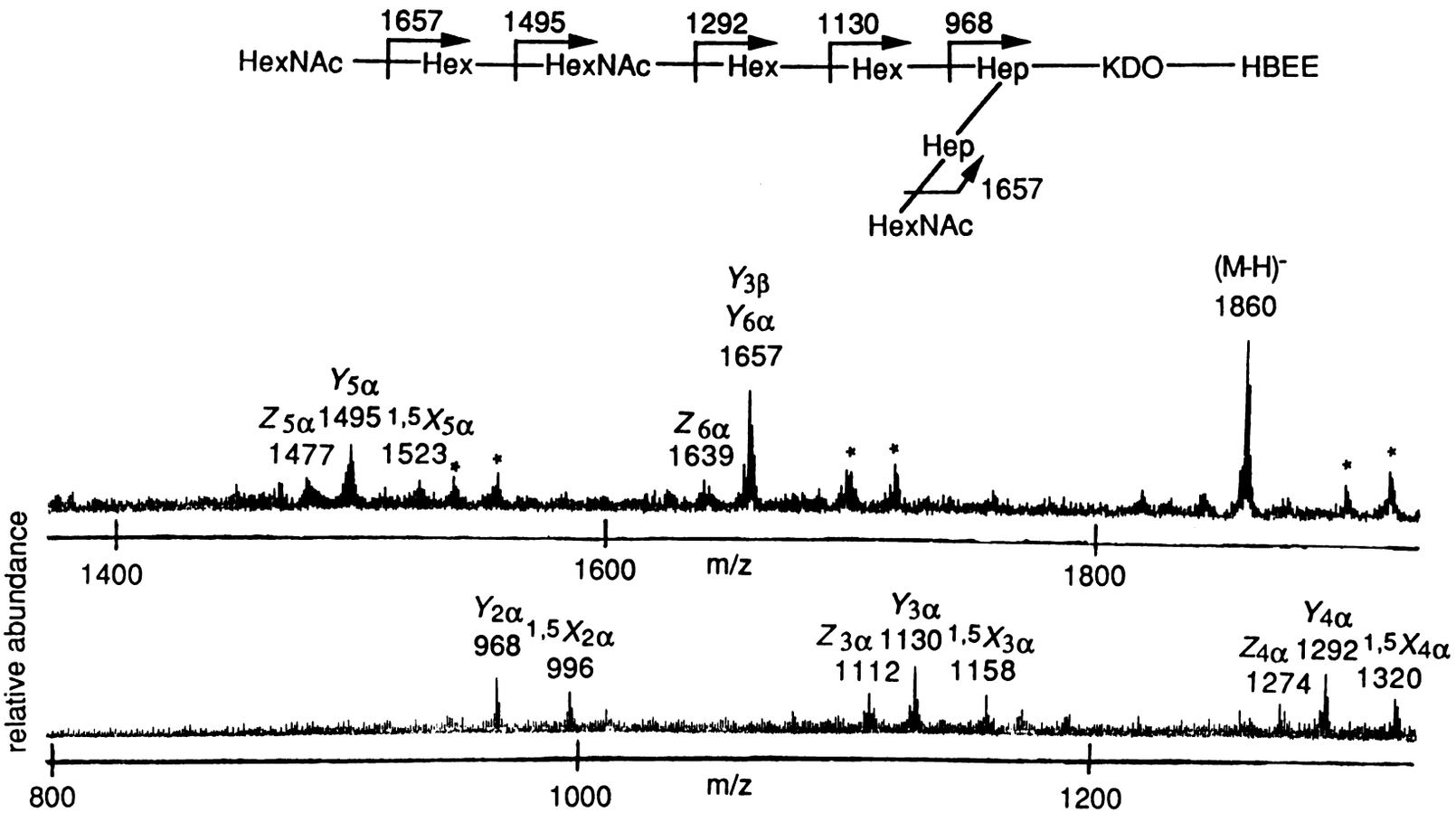


Figure 4. Negative-ion LSIMS spectrum of anhydro HF-treated oligosaccharide from F62 derivatized with HBEE. The molecular and fragment ions marked with an asterisk were produced by the *O*-acetylated oligosaccharide (60 Da larger) and the *O*-acetylated anhydro oligosaccharide (42 Da larger). The fragment ions are labeled according to the nomenclature devised by Domon and Costello (Domon and Costello 1988).

of PEA, phosphate, and HexNAc moieties found in the corresponding oligosaccharides.

In Table 1, the ions, the corresponding molecular weights and the proposed compositions for each significant ion observed in the spectra are listed (see Figure 3). In fractions 46 and 48, two major components with M_r of 2005 and 1963 were identified that differ by 42 Da, the mass of one acetyl moiety. Many additional components were also identified in fractions 51 and 53 as listed in Table 1. Many or all of the smaller molecular mass components listed in Table 1 (not all of which can be viewed in the portions of the spectra shown in Figure 3) may be fragment ions of the major components of fractions 51 and 53 (listed in bold in Table 1) rather than molecular ions. Indeed, because the major fragment ions are of the Y-type it is often impossible to distinguish chemical from gas phase fragments. Among the components and fragment ions observed the number of PEA groups varied from zero to two, the *O*-acetate groups varied from 0-1, the HexNAcs from 1-3, and the Hex moieties from 2-3. However, the main differences observed both within and between fractions correspond to a single acetate group (42 Da mass shift) and a single HexNAc moiety (203 Da mass shift).

Both HF-treated and non-HF-treated oligosaccharides from F62 were derivatized with HBEE, purified by reverse-phase HPLC and analyzed by negative-ion LSIMS (see Chapter II, B.1.). An example of the spectra obtained is shown in Figure 4. This spectrum is from an early eluting oligosaccharide from the P-4 column (the equivalent of fraction 46 in Figure 1A) that was subsequently HF-treated and approximately 100 nmol derivatized with HBEE. The spectrum shown (see Fig. 4) was obtained with approximately 3 nmol of derivatized oligosaccharide. The molecular and

TABLE 1*

	$(M-H)^-$	M_r	Proposed Composition
46	**2004**	**2005**	**KDO, Hep_2, $HexNAc_3$ Hex_3 PEA_2 OAc**
	1962	**1963**	**KDO, Hep_2, $HexNAc_3$, Hex_3, PEA_2**
48	**2004**	**2005**	**KDO, Hep_2, $HexNAc_3$, Hex_3, PEA_2, OAc**
	1962	**1963**	**KDO, Hep_2, $HexNAc_3$, Hex_3, PEA_2**
51	2004	2005	KDO, Hep_2, $HexNAc_3$, Hex_3, PEA_2, OAc
	1962	1963	KDO, Hep_2, $HexNAc_3$, Hex_3, PEA
	1881	1882	KDO, Hep_2, $HexNAc_3$, Hex_3, PEA, OAc
	1839	1840	KDO, Hep_2, $HexNAc_3$, Hex_3, PEA
	1801	**1802**	**KDO, Hep_2, $HexNAc_2$, Hex_3, PEA_2, OAc**
	1759	**1760**	**KDO, Hep_2, $HexNAc_2$, Hex_3, PEA_2**
	1716	1717	KDO, Hep_2, $HexNAc_3$, Hex_3
	1639	1640	KDO, Hep_2, $HexNAc_2$, Hex_2, PEA_2, OAc
	1597	1598	KDO, Hep_2, $HexNAc_2$, Hex_2, PEA_2
53	1881	1882	KDO, Hep_2, $HexNAc_3$, Hex_3, PEA, OAc
	1839	1840	KDO, Hep_2, $HexNAc_3$, Hex_3, PEA
	1801	**1802**	**KDO, Hep_2, $HexNAc_2$, Hex_3, PEA_2, OAc**
	1759	**1760**	**KDO, Hep_2, $HexNAc_2$, Hex_3, PEA_2**
	1716	1717	KDO, Hep_2, $HexNAc_3$, Hex_3
	1678	1679	KDO, Hep_2, $HexNAc_2$, Hex_3, PEA, OAc
	1639	1640	KDO, Hep_2, $HexNAc_2$, Hex_2, PEA_2, OAc
	1636	1637	KDO, Hep_2, $HexNAc_2$, Hex_3, PEA
	1598	1599	KDO, Hep_2, HexNAc, Hex_3, PEA_2, OAc
	1597	1598	KDO, Hep_2, $HexNAc_2$, Hex_2, PEA_2
	1555	1556	KDO, Hep_2, $HexNAc_2$, Hex_3, OAc

* All of the fragment ions are listed which were observed at a signal/background ratio greater than two in the spectra from the Bio-Gel P-4 fraction. The major portion of the spectra are shown in Figure 3. The major ions, and corresponding masses and compositions are in bold. It is likely that the lower mass ions are fragments formed during the mass spectrometric analysis.

fragment ions observed in this spectrum support the compositions proposed in Table 1 for the oligosaccharides found in fractions 46 and 48. The molecular ion at m/z 1860 would correspond to an anhydro species with the composition of $KDOHep_2HexNAc_3Hex_3$. The molecular ion at m/z 1902, marked with an asterisk, is the anhydro species containing the *O*-acetate group. The molecular ion marked with an asterisk at m/z 1920 also corresponds to the oligosaccharide containing an *O*-acetate group. The length of HF-treatment was reduced to 18-24 h to minimize the amount of glycosidic bond cleavage; however, the removal of *O*-acetate and phosphoesters is not as complete as with the longer reaction times (48-96 h) (see Chapter II, B.2.). Reducing terminal Y-type fragment ions (marked by asterisks) from these two less abundant molecular ions can also be observed. The peaks for the reducing terminal Y-type fragment ions (Domon and Costello 1988) of the most abundant molecular ion at m/z 1860 are the most prominent, but reducing terminal $^{1,5}X$- and Z-type ions can be seen as well (see for example m/z 1639, 1523, 1477, 1320, and 1274). Fragment ions for cleavage between the two Hep moieties are not observed in this spectrum. For instance, the expected peak for a Y-type loss of HexNAcHep from $(M-H)^-$ 1860 is not apparent at m/z 1465.

Other negative-ion LSIMS spectra were also obtained (data not shown) of F62 oligosaccharides derivatized with ABBE that had a composition of $KDOHep_2HexNAc_2Hex_3$ based on the observed molecular ion at m/z 1690. In these spectra, a major ion was found for the reducing terminal Y-type fragment produced by loss of Hex from the molecular ion at m/z 1528 $((M-H)-Hex)^-$. Other fragment ions were also observed consistent with a structure of Hex→HexNAc→Hex→Hex→ for the largest branch. For example, Y-type fragment ions were observed in the spectrum at m/z 1325

(for loss of HexHexNAc) and 1163 for the loss of ($Hex_2HexNAc$) that would correspond to this branch. Thus, the HexNAc moiety found to vary between the early and late eluting oligosaccharides in both HPAE-PAD and size exclusion chromatography apparently is the non-reducing terminal HexNAc attached to the largest branch (see Fig. 4). In negative-ion LSIMS spectra of F62 oligosaccharides, as in general with gonococcal oligosaccharides derivatized with the amino or hydrazino benzoates (John and Gibson 1990), the fragment ions for cleavage of Hep-Hep bonds were either not observed or fairly weak. The lack of cleavage between the Hep-Hep residues makes it difficult to assign the specific oligosaccharide branches to the first or second Hep.

C. Methylation linkage analysis.

C.1. Materials and methods.

Methylation analysis. Methylation analysis was performed using the microscale method of Levery and Hakomori (Levery and Hakomori 1987). In this procedure the methylsulfinyl carbanion solution was prepared from sodium hydride (Aldrich, Milwaukee, WI) and distilled dimethyl sulfoxide as described and aliquots were stored at -80° C for future use. The basicity of the solution was determined using triphenylmethane (Sigma, St. Louis, MO) as an indicator (Rauvala 1979). The sources of the other materials used in the methylation analysis are listed in Chapter II, C.1.

GC/MS. GC/MS of the partially methylated alditol acetates was performed by electron ionization on a Kratos MS-25 mass spectrometer equipped with a

Varian 3700 GC with He as the carrier gas and an on-column injector (J & W Scientific). A 30 m DB-1 column with a 1 μm film thickness (J & W Scientific) was used for the separation with a temperature program from 120-280° C at 4° C/min. The spectra were recorded at 1 s/decade and were automatically calibrated with perfluorokerosene as an external mass calibrant.

C.2. Results and discussion.

In Table 2 the relative ion abundance of characteristic fragment ions are shown for the PMAAs of the component monosaccharides of the HF-treated F62 oligosaccharides from the P-4 fractions listed (see Fig. 1B). A plus sign indicates that a particular saccharide was identified but without standards to attempt quantitation. In Figure 5A and B the total ion chromatograms (TICs) are shown from the GC/MS analysis of the PMAAs from fractions 53-57 and 58-61.

According to the compositions proposed in Table 1 for the molecular ions observed in the P-4 fractions of underivatized F62 oligosaccharides and from LSIMS analysis of the HPAE-PAD purified oligosaccharides (see Chapter III, A and Fig. 2) the major difference between the larger and the smaller oligosaccharides is a HexNAc moiety (203 Da). More evidence supporting this conclusion is present in the results of the methylation analysis shown in Table 2. While PMAAs from both terminal GlcNAc and 1,4-GlcNAc residues were present in the oligosaccharide from all fractions, PMAAs derived from terminal GalNAc were present in all but the latest eluting fractions 71-78. Thus, the variable HexNAc moiety must have been a terminal GalNAc.

Table 2. Relative ion abundance of characteristic fragment ions of the corresponding PMAA derivatives from Bio-Gel P-4 fractions.

	1-Glc	1-Gal	1,4-Gal	1,4-Glc	1,3-Gal	1,2,4-Hex	1,2-Hep	1,3,4-Hep	1-GlcNAc	1-GalNAc	1,4-GlcNAc
53-57	—	0.4	0.4	1.1	1.1	—	+	+	1.0	0.7	2.2
58-61	0.2	0.9	0.1	1.2	1.0	—	+	—	0.9	1.1	1.6
62-64	—	1.2	0.4	0.7	1.6	+	+	+	0.3	0.4	2.8
65-70	—	1.0	—	1.1	1.4	—	—	+	0.7	0.5	0.8
71-78	0.4	0.6	0.1	2.4	0.3	+	+	—	0.8	—	0.8

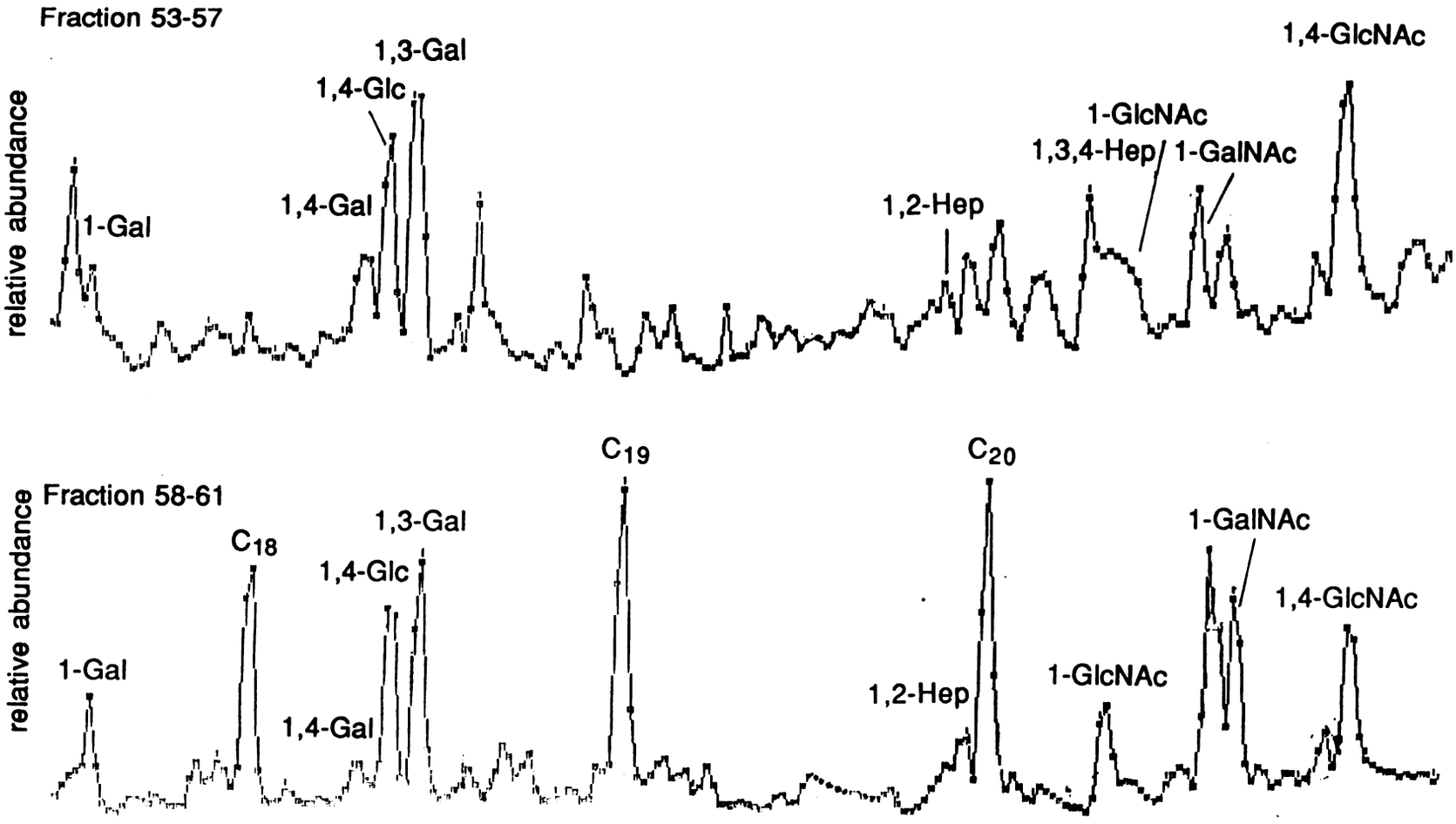


Figure 5. The total ion chromatogram (TIC) for the methylation analysis of HF-treated oligosaccharides from fractions 53-57 (A) as shown in Figure 1B. The TIC for the methylation analysis of the HF-treated oligosaccharides from fractions 58-61 (B) as shown in figure 1B.

The methylation analysis of the F62 oligosaccharide revealed the presence of a total of four different non-reducing terminal monosaccharides, two HexNAc and two Hex moieties as shown in Table 2. The PMAAs for one terminal GalNAc and one terminal GlcNAc moiety were also present. A spectrum of the PMAA for a single non-reducing terminal Gal residue was found in all fractions, and a spectrum corresponding to an additional non-reducing terminal Glc moiety was found in fractions 58-61 and 71-78.

Fragment ions were observed from the PMAAs that identified the presence of one internal HexNAc and three internal Hex residues. PMAAs derived from a single 1,4-GlcNAc moiety (2-acetamido-2-deoxy-1,4,5-tri-*O*-acetyl-3,6-di-*O*-methyl-glucitol) were present in all the fractions. All of the LSIMS and MS/MS data supported the presence of only one 1,4-GlcNAc per oligosaccharide in all of the oligosaccharides from strain F62. However, assuming the stoichiometry of the 1,4-GlcNAc residue to be one per molecule, the ion abundance of the PMAA derivative for this monosaccharide was consistently higher than the PMAA derivatives for most of the other monosaccharides (see Table 2). The PMAA derivatives of 1,4-Gal (1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl-galactitol), 1,4-Glc (1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl-glucitol), and 1,3-Gal (1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-galactitol) were also found. In the PMAA derivatives from fractions 71-78, the greater abundance of the derivative for 1,4-Glc was assumed to be due to contamination, possibly from the Sephadex column. The oligosaccharides which lack the additional non-reducing terminal GalNAc moiety, if otherwise identical, would contain only two internal Hex moieties plus one terminal Hex. The methylation analysis supported such a structure, as the fragment ions for the PMAAs from 1,4-Gal were

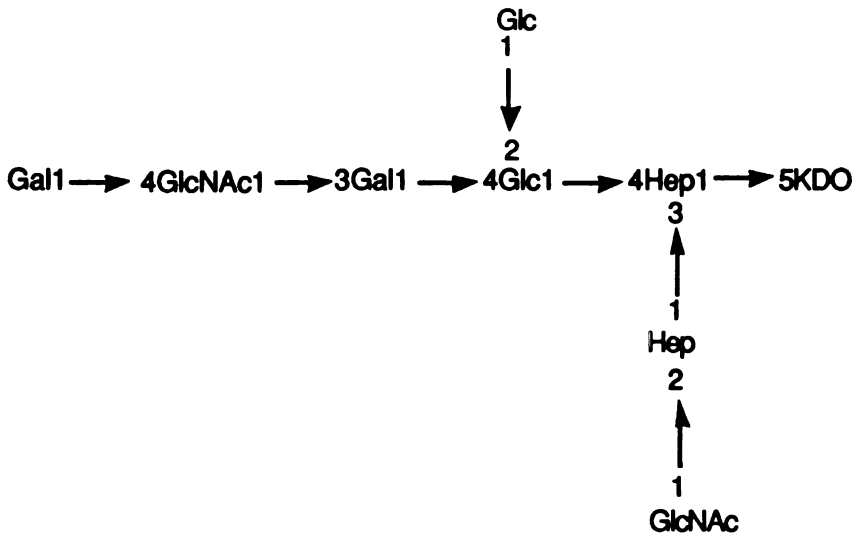
consistently less (and absent in fractions 65-70) than those from 1,4-Glc and 1,3-Gal. However, the spectra of the PMAA derivatives from fractions 62-62 and 71-78 also indicated the presence of a disubstituted Hex residue, a 1,2,4-Hex moiety (1,2,4,5-tetra-*O*-acetyl-3,6-di-*O*-methyl-hexitol) in the F62 oligosaccharides (see Table 2).

The PMAAs that would be expected from the Hep moieties of these glycans were sometimes absent, and when present were generally smaller than the peaks for the PMAAs derived from other monosaccharides. We have previously postulated this to be the result of low recoveries due to steric crowding and acid resistance of this linkage. In amino acid analysis of peptides and proteins, analogous low recoveries are found for Val-Val and other hydrophobic linkages. No standard was available for the Hep moieties. In all the other work in our laboratory with the oligosaccharides from the outer membrane LOS of *N. gonorrhoeae*, two Hep moieties have always been found (Gibson et al. 1989, John et al. in press). In the methylation analyses of the oligosaccharides from these other gonococcal strains, the fragment ions from the PMAAs of the Hep residues were also often of lower abundance than other residues and occasionally absent altogether. Whether these differences are due to incomplete hydrolysis or degradation of the Hep residues during methylation analysis is not clear. However, when the methylation data was correlated with the LSIMS data which had already identified one internal and one di-linked heptose, it was apparent that both a 1,2-Hep (1,2,5-tri-*O*-acetyl-3,4,6,7-tetra-*O*-methyl-heptitol) and a 1,3,4-Hep moiety (1,3,4,5-tetra-*O*-acetyl-2,6,7-tri-*O*-methyl-heptitol) must be present in all the HF-treated oligosaccharides from F62.

Thus, although the methylation analysis confirmed the compositions and sequence predicted from the LSIMS analysis of derivatized and

underivatized oligosaccharides (see Figs. 2, 3, 4 and Table 1) some inconsistencies were still apparent. The presence of two different non-reducing terminal residues and a di-linked Hex moiety in some fractions could not be explained by the proposed structures. One explanation for the inconsistency in the methylation analysis and the LSIMS data is that a less abundant isobaric isomer of the other F62 oligosaccharides with a triantennary structure was also present. Another possible explanation is that an oligosaccharide with a triantennary structure of different mass existed that was less abundant and possibly suppressed in the LSIMS and MS/MS analysis by the more abundant, and possibly more surface active biantennary oligosaccharide. A structure which could produce the data obtained in methylation analysis containing both the non-reducing terminal Glc and the 1,2,4-Hex moiety is shown in Scheme 1. In either case

Scheme 1



it is apparent from the methylation data that the oligosaccharide containing the 1,2,4-Hex moiety and terminal Glc was of much less abundance.

D. MS/MS with CID analysis

Analysis of the oligosaccharides from gonococcal strain F62 by MS/MS with CID was performed as described in Chapter II, B.1. In Figure 6B, the negative-ion MS/MS spectrum of underivatized HF-treated oligosaccharide from fraction 46 is shown (see Fig. 1A). The molecular ion at m/z 1716 corresponds to a composition of $Hex_3HexNAc_3Hep_2KDO$. The origin of the fragment ions in this spectrum is illustrated in Figure 6A. The placement of the linkages in the structure shown is drawn from the methylation analysis data, except for the positions of the two branches on the disubstituted Hep. It is likely that the inner core KDO-Hep region of the gonococcal LOS is conserved and, therefore, the branching of this region of the F62 oligosaccharide would be the same as indicated for the structures of the series of oligosaccharides isolated from pyocin-resistant mutants of gonococcal strain 1291 (see Chapter 4). The inner core region of the LOS from *N. meningitidis* is conserved in the LOS of all the strains examined to date, and identical to the inner core region we have proposed for the LOS of *N. gonorrhoeae* (Jennings, Lugowski, and Ashton 1983, Michon et al. 1990) Structural analysis of the oligosaccharides from these mutant strains suggested the linkage of the largest branch (see Fig. 6A) was to the C-4 of the first Hep moiety, and that the two Hep residues were 1,3-linked. Also, the ordering of the two directly linked Hex moieties has not been established for the F62 oligosaccharide, but rather has been drawn from analogy to the same 1291 series of oligosaccharides and to the structure of lacto-N-neotetraose, the terminal tetrasaccharide of paragloboside, (Galß1→4GlcNAcß1→3Galß1→4Glcß1→1Cer) (see Chapter 1, D.6.). MAbs with specificity for the LOS of F62 also recognize glycosphingolipids

containing the lacto-N-neotetraose moiety (Mandrell et al. 1986, Mandrell, Griffiss, and Macher 1988).

As shown in Figure 6A the extensive fragmentation of the molecule under CID conditions provided unambiguous sequence information indicative of a biantennary structure. All three types of reducing terminal fragments, X, Y, and Z (Domon and Costello 1988) were formed from each of the glycosidic bonds, with the exception of the one between KDO and Hep. In addition, several non-reducing terminal fragment ions helped define the non-reducing termini. The non-reducing terminal fragment ion at m/z 262 was produced by ring cleavage and contains the intact non-reducing terminal *N*-acetylhexosamine residues. At m/z 424 the second A-type non-reducing terminal ion in the series is found, and contains a non-reducing terminal *N*-acetylhexosamine plus a hexose residue. Another non-reducing terminal ^{2,4}A-type fragment ion, produced by ring cleavage of KDO, can be observed in this spectrum at m/z 1538. The abundance of this ion is reminiscent of the abundance of the analogous ion found in the MS/MS spectrum of derivatized oligosaccharide from *Haemophilus influenzae* (Phillips et al. 1990). In this latter case a ^{2,4}A-type non-reducing terminal fragment ion for ring cleavage of KDO at m/z 959 was also one of larger fragment ions. The *Haemophilus* oligosaccharides, however, were derivatized with HBEE and ions from non-reducing terminal A-type ring cleavages of many of the other monosaccharides were also abundant.

Although the ion at m/z 221 could indicate the presence of the same A-type fragment containing a non-reducing terminal Hex, no evidence exists for such a terminus in the rest of the spectra. The spectra shows Y-type fragment ions consistent with the losses of HexNAc (m/z 1513), HexNAcHex (m/z 1351), $HexNAc_2Hex$ (m/z 1148), $HexNAc_2Hex_2$ (m/z 986),

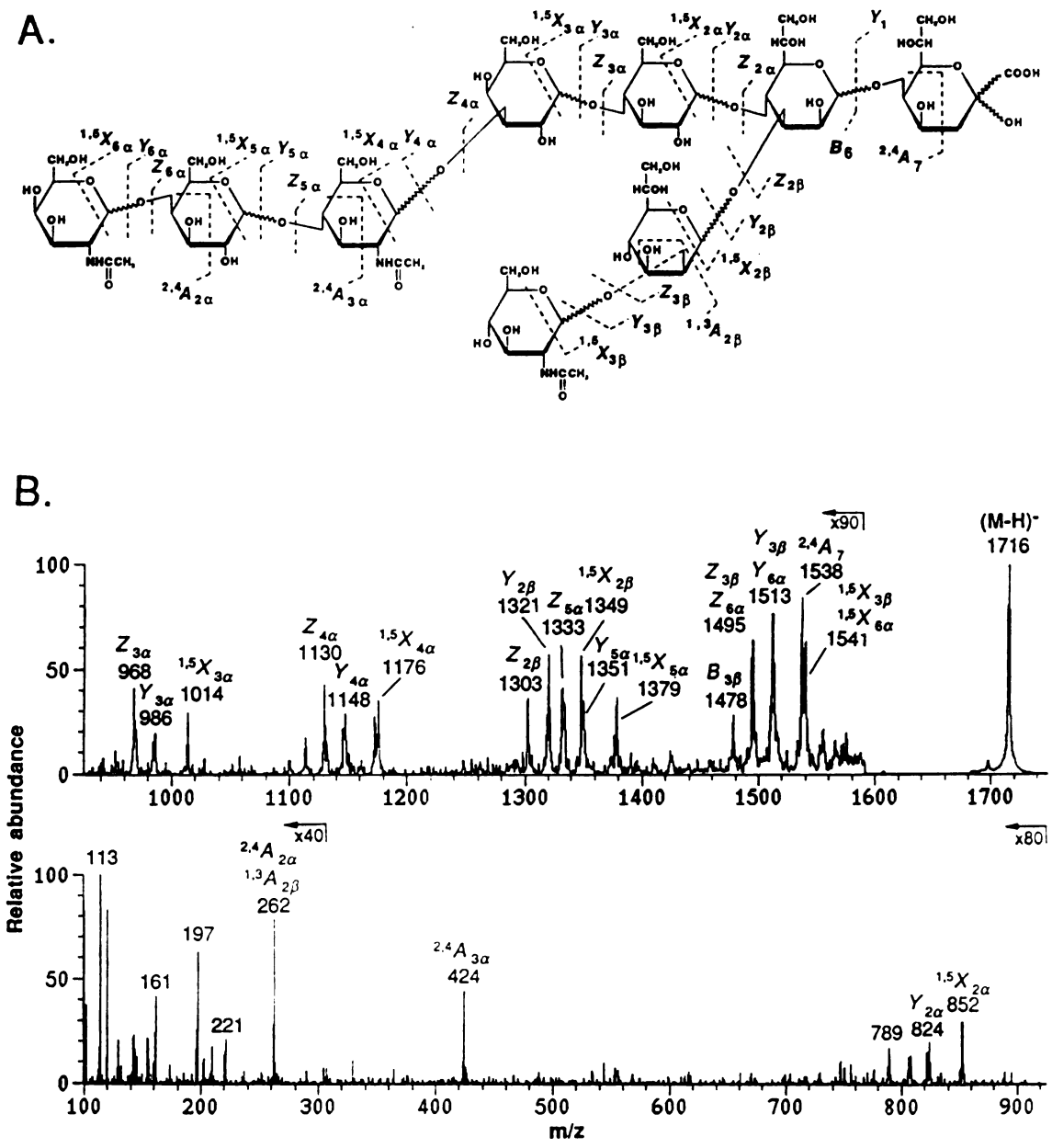


Figure 6. Illustration of the fragment ions and the proposed structure (A) of the oligosaccharide which produced the spectrum shown in panel B. Negative-ion MS/MS with CID spectrum (B) of HF-treated underivatized F62 oligosaccharide.

and $HexNAc_2Hex_3$ (m/z 824) from the molecular ion with gradually decreasing reducing terminal fragment ion abundance with decreasing molecular mass indicative of a linear arrangement of the monosaccharides (John and Gibson 1990). Examination of the negative-ion MS/MS spectra of standard oligosaccharides with reducing terminal derivatives from the Ra mutant of *S. typhimurium* (see Chapter II, Fig. X) and maltoheptaose (Gillece-Castro and Burlingame 1990) have demonstrated a generally decreasing ion abundance of reducing terminal fragment ions of decreasing mass from a linear branch. There is generally a significant decrease in abundance of those fragment ions which would only be produced by remote site two bond cleavages. Thus, for a linear tri-hexose branch there would be a gradually decreasing abundance for the fragment ions from loss of one, two and three Hex residues from the molecular ion. In the case of a branched tri-hexose branch there would be relatively abundant ions for the loss of one and three Hex residues. However, the fragment ions for loss of two Hex residues, the product of two remote site bond cleavages, would be significantly less abundant. This type of pattern appears in the fragment ions of negative-ion MS/MS spectrum of the underivatized spectrum of the F62 oligosaccharides shown in Figure 6B. No evidence of any other branch besides HexNAc-Hep is apparent in the spectrum. One possible explanation for the ion at m/z 221 is the presence of another isobaric molecule containing a non-reducing terminal Hex. The existence of a less abundant isobaric isomer of the F62 oligosaccharides was one of the possible explanations for some of the data obtained by methylation analysis which suggested the presence of a terminal Glc and a disubstituted Hex (see Chapter III, C.2.). This same reason could explain

the presence of a non-reducing terminal fragment ion at m/z 221 for a terminal Hex in this spectrum.

The negative-ion LSIMS spectrum of the underivatized HF-treated F62 oligosaccharide from fraction 51 (see Fig. 1A) is shown in Figure 7B. This oligosaccharide has a molecular ion at m/z 1513 and a composition of $Hex_3HexNAc_2Hep_2KDO$. The origins of the fragment ions present in the spectrum are illustrated in the structure above this spectrum (Fig. 7A). Again, the placement of the linkages of the monosaccharides in the LSIMS derived structure are based on the results of methylation analysis. The positions of the branches on the disubstituted Hep (1,3,4-Hep), and the order of the two directly linked Hex moieties (1,4-Glc and 1,3-Gal) were placed by analogy to the series of oligosaccharides isolated from pyocin-resistant mutants of gonococcal strain 1291 (see Chapter 4). The ordering of these two Hex residues is also in accordance with MAb binding data. As explained above in description of the structure of the F62 oligosaccharide in Figure 6A the inner core region of the neisserial LOS has to date been found to be highly conserved.

The fragment ions found in this spectrum (Fig. 7B) are very similar to those of the MS/MS spectrum in Figure 6B. However, several more non-reducing terminal fragments of the B and C-types (Domon and Costello 1988) are seen in this spectrum than in the MS/MS spectrum in Figure 6B. Also, in this spectrum the extensive fragmentation of the oligosaccharide produced by CID provides unambiguous sequence information. The Y-type reducing terminal fragment for loss of HexNAc-Hep from the molecular ion (m/z 1118) indicates the presence of a branch of that composition, and the losses of Hex, HexHexNAc, $Hex_2HexNAc$, and $Hex_3HexNAc$ reveals the sequence of the other non-reducing terminal branch. The abundance of the

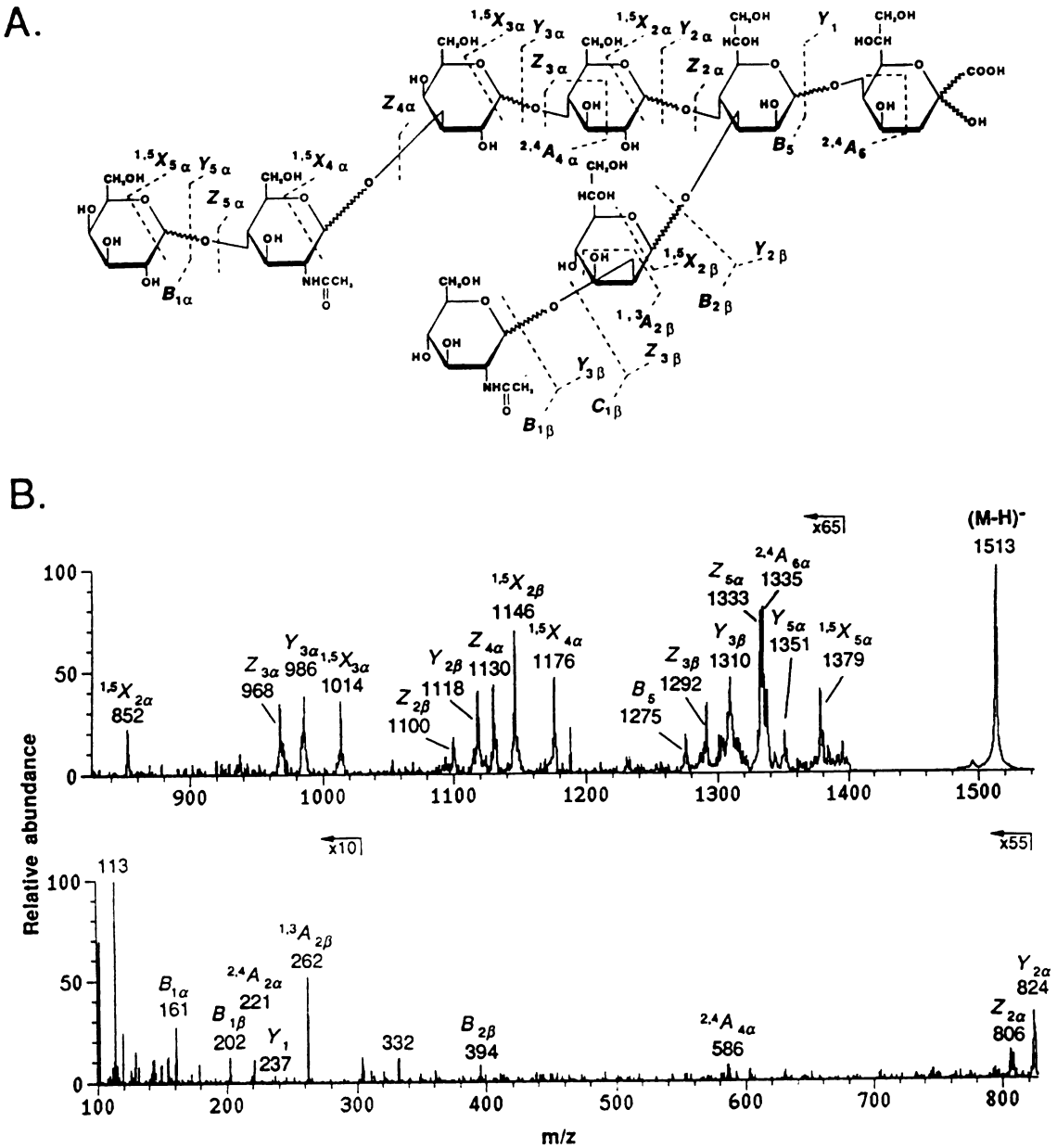


Figure 7. Illustration of the fragment ions and the proposed structure (A) of the oligosaccharide which produced the spectrum shown in panel B. Negative-ion MS/MS with CID spectrum (B) of HF-treated underivatized F62 oligosaccharide.

fragment ions is again indicative of a linear arrangement as there is a gradual decrease in abundance of the reducing terminal fragment ions with decreasing molecular mass, as described above for the spectrum shown in Figure 6B. No evidence suggesting other branching patterns is apparent. As in the other MS/MS spectrum of F62 oligosaccharides shown in Figure 6B, an abundant non-reducing terminal fragment ion produced by ring cleavage of the KDO moiety is present in the spectrum at m/z 1335. In both these negative-ion MS/MS spectra, additional sequence information could obtained due to the extensive fragmentation of the oligosaccharide under CID conditions compared to the same glycan under two-sector LSIMS conditions. One of the advantages of MS/MS with CID for oligosaccharide analysis is the more extensive fragmentation obtained when the ions collide with the neutral gas molecules in the collision chamber between MS1 and MS2.

E. Partial structures.

From the structural analyses described in the preceding section, structures could be proposed for the oligosaccharide components of F62. These structures are shown in Figure 8A. The position of the branches on the disubstituted Hep moiety were not determined for the oligosaccharide from F62 but are drawn by analogy to the oligosaccharides from the pyocin-resistant mutant strains of 1291 and to the meningococcal oligosaccharides (Jennings, Lugowski, and Ashton 1983, Michon et al. 1990). The order of the two directly linked Hex moieties, i.e. Gal→Glc vs. Glc→Gal also has not been established chemically, but is shown in accordance with MAb binding data which suggests the structure of lacto-N-neotetraose, the terminal

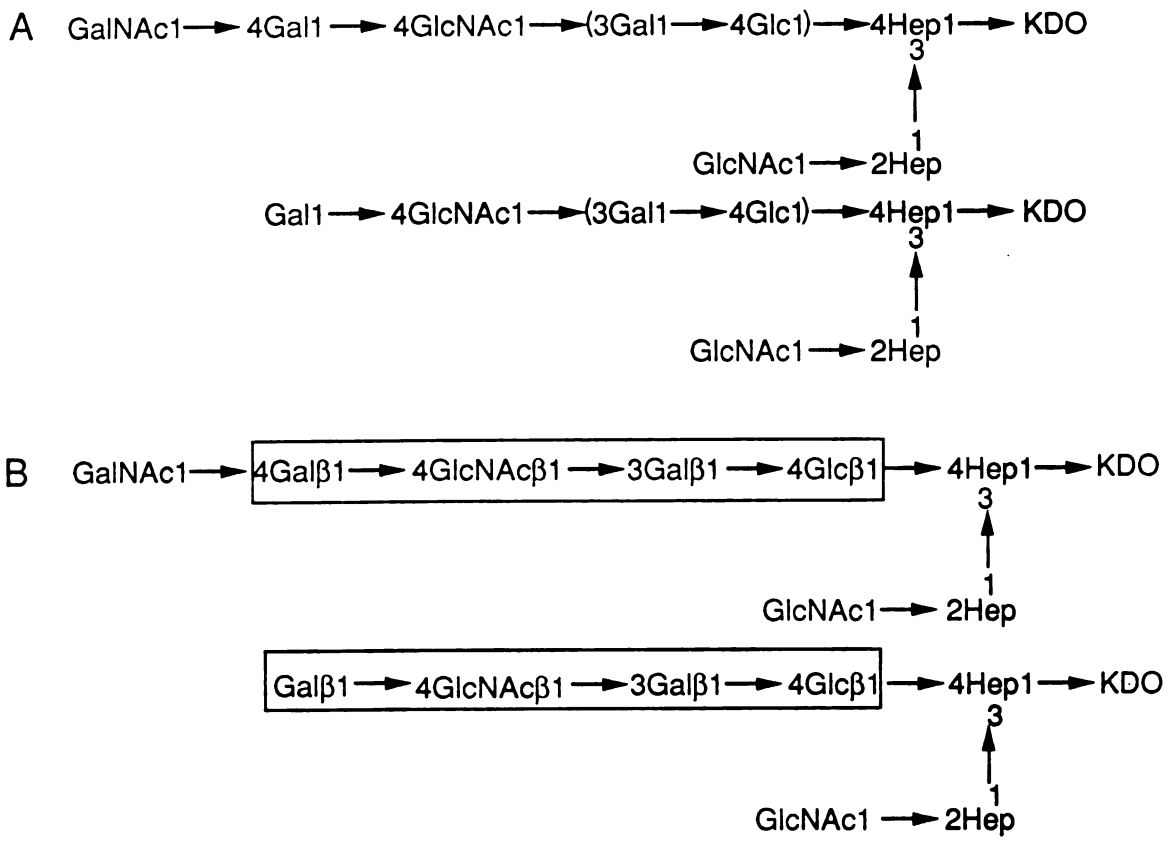


Figure 8. The proposed structures (A) of the major components of the F62 oligosaccharide after HF-treatment and (B) with partial anomeric assignments based on the binding of MAB 3F11 and 06B4.

tetrasaccharide of paragloboside is present in this oligosaccharide. Both the F62 LOS and paragloboside contain the epitope for the MAb 3F11 (Mandrell, Griffiss, and Macher 1988). Recent work with gonococcal oligosaccharides from the LOS of strains 1291 and pyocin-resistant mutants of 1291 has demonstrated the presence of a single *O*-acetate moiety on a non-reducing terminal GlcNAc analogous to the non-reducing terminal GlcNAc present in the F62 oligosaccharide (John et al. in press) (see Chapter IV, D.). For the same group of 1291 oligosaccharides mass spectrometric evidence was also obtained placing a PEA moiety on the C-3 of the second Hep from KDO (John et al. in press) (also see Chapter IV, D).

The smaller of the two structures shown in Figure 8A contains the (non-anomeric) structure of terminal *N*-acetylactosamine (Galβ1→4GlcNAc), the terminus of the terminal tetrasaccharide of paragloboside, lacto-N-neotetraose. In fact, the entire terminal tetrasaccharide (Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1) may very likely be present as shown in Figure 8B. This terminal tetrasaccharide has been found in meningococcal LOS of three strains of different serotypes: L2, L3, L7, L9, and L5 strains (Jennings, Lugowski, and Ashton 1983, Michon et al. 1990). However, the LOS from only 3 out of 8 meningococcal strains that contain this non-reducing terminal tetrasaccharide were found to bind the MAb 3F11 (Mandrell, Griffiss, and Macher 1988). A larger number (5 out of 8) of the meningococcal strains containing the tetrasaccharide were found to bind the MAb O6B4.

The binding of the MAb 3F11 to a smaller LOS component of the F62 LOS oligosaccharide is easily explained by these two structures. MAb 3F11 has been shown to bind oligosaccharides expressing terminal *N*-acetyllactosamine, such as the terminal tetrasaccharide of paragloboside,

lacto-N-neotetraose, but not to those in which the *N*-acetyllactosamine group is internal. For example, if the lacto-N-neotetraose has a terminal sialic acid or fucose moiety the MAb would not be expected to bind (Mandrell, Griffiss, and Macher 1988, Mandrell et al. 1990). In fact, the entire smaller structure found in the F62 oligosaccharide is up to this point identical with a structure found in the LOS of *N. meningitidis* that contains as a non-reducing terminal tetrasaccharide lacto-N-neotetraose (Jennings, Lugowski, and Ashton 1983).

Previous work by Yamasaki and colleagues with the LOS from F62 demonstrated that binding by MAb 3F11 was not reduced by treatment of the LOS with HF for 4 days at 4°C (Yamasaki et al. 1988). Structural elucidation of oligosaccharides after HF treatment in our laboratory has indicated that PEA and *O*-acetate moieties are nearly quantitatively removed from oligosaccharides by such treatment. However, some glycosidic bond cleavages have been found to occur from 96 h treatment with HF. Glycosidic bond cleavage from 96 h HF-treatment was found by LSIMS analysis of oligosaccharides from the Ra mutant of *S. typhimurium* derivatized with ABEE. (These results were described in Chapter II, B.2.). Similar results were obtained with F62 oligosaccharides. Oligosaccharides from Bio-Gel P-4 fractions 46, 48, and 51 (see Fig. 1) were HF-treated for 96 h, derivatized with ABEE, separated by reverse phase HPLC and analyzed by negative-ion LSIMS. Molecular ions were observed in analysis of isolated peaks collected from the HPLC that could only represent truncated oligosaccharides produced by glycosidic bond cleavage. For example, in analysis of oligosaccharides from fraction 51 molecular ions at m/z 924, 721, and 559 were observed corresponding to ABBE (Δ 177 Da) plus $HexNAc_2Hex_2$, $HexNAcHex_2$, and HexNAcHex, respectively. Similar

species were observed in analysis of the HF-treated oligosaccharides from the other fractions, 46 and 48. When shorter HF-treatment (18-24 h) was performed on F62 oligosaccharides, molecular ions were no longer observed corresponding to truncated oligosaccharides lacking Hep and KDO, such as those described from fraction 51. However, incomplete removal of PEA and *O*-acetate moieties has been found with the shorter HF-treatments.

Thus, the results of Yamasaki et al. (Yamasaki et al. 1988) support the conclusion that when the non-reducing terminal GalNAc is present in the larger F62 structure the binding of the MAb 3F11 is apparently blocked. The nearly quantitative removal of the PEA and *O*-acetyl moieties from the glycan did not effect the expression of the epitope. The presence of the basically intact lipid A moiety was found to be important for expression of the epitope suggesting a conformational role for lipid A. For example, after acid hydrolysis of the LOS, by which the lipid is separated from the glycan, there was essentially no binding of the MAb 3F11. Deacylation of the *O*-linked fatty acid moieties of the LOS by hydrazine or NaOH also greatly reduced the binding of the MAb (Yamasaki et al. 1988).

The MAb 3F11 apparently binds only the F62 LOS containing the smaller structure (see Fig. 8) lacking the non-reducing terminal GalNAc (Mandrell et al. 1986, Mandrell, Griffiss, and Macher 1988). The presence of the non-reducing terminal GalNAc must occlude the epitope recognized by 3F11 and inhibit binding. The 3F11 epitope as stated above has been found to be present on all adult erythrocytes tested (Mandrell, Griffiss, and Macher 1988). The presence of bactericidal antibodies to an epitope expressed by human tissue would not be expected and, therefore, the presence of such epitope in LOS should contribute to the serum resistance of a strain.

In support of this conclusion, a HexNAc moiety has been found to be a determinant of the epitope within serum sensitive strains that binds the IgM in normal human serum that initiates immune lysis (Griffiss, Schneider, and O'Brien 1985). Also, an increase was noted in the number of HexNAc moieties found in the LOS of a serum sensitive strain versus a serum resistant strain in compositional studies (Griffiss et al. 1987). This data clearly supports the conclusion that expression of the larger LOS structure shown in Figure 8 could contribute to the serum sensitivity of gonococcal strain F62 by inhibiting immunological recognition of the 3F11 epitope expressed in the smaller structure. In Fig. 8B the structures of the oligosaccharides from F62 most consistent with the MAb binding data are shown.

In regard to the larger of the two major structures, the IgM MAb 1-1-M was found to bind to a larger component of the F62 LOS and to high molecular weight LOS aggregates (Mandrell et al. 1986). The larger component could correspond to those containing the non-reducing terminal GalNAc moiety. The immunological significance of the undetermined structure containing the additional non-reducing terminal Glc and di-linked Hex moieties is not known. Even though this structure is apparently much less abundant *in vitro*, it could be expressed to a much larger extent *in vivo*. The correlation of immunological affinity data with LOS structure is very difficult given the inter- and intra-strain (and *in vivo* vs. *in vitro*) heterogeneity of the gonococcal LOS.

In order to unambiguously determine the immunological affinity of particular LOS structures, a method needs to be developed to purify LOS to homogeneity in sufficient quantity for antibody affinity studies. In addition, it would be important to determine the molecular mass of the purified LOS.

A molecular mass of the intact LOS could be very informative if prior structural work had been performed on the LOS using the variety of techniques we now have available. Alternately, a method of reconstructing LOS epitopes using purified and well characterized oligosaccharides would be desirable. For example, if oligosaccharide that had been previously isolated from the LOS were then coupled with other lipid moieties to fashion neoglycolipids, we might then be able to correlate specific structures with binding by specific antibodies. The longer chain alkylphenylhydrazine compounds, such as 4-tetradecylphenylhydrazine, might serve as excellent reagents for preparing neoglycolipids.

CHAPTER IV. OLIGOSACCHARIDES OF 1291 SERIES

In the study of lipopolysaccharide (LPS) from the *Enterobacteriaceae* family, such as the *Salmonella* and *Escherchia* species, identification of mutant strains producing LPS lacking the long, repeating units called the O-antigens aided the development of the structural and immunochemical models of LPS. These mutants were found to produce truncated LPS with successive saccharide deletions in the relatively invariant core region (Aspinall 1982, Galanos et al. 1977, Luderitz and Westphal 1966). The structures of the rough mutants of *Salmonella* Ra through Re are shown in Figure 1 (Aspinall 1982, Galanos et al. 1977).

Pyocin, isolated from *Pseudomonas aeroginosa*, has been shown to alter the expression of LOS by *Neisseria gonorrhoeae* in surviving, resistant strains. These strains no longer bind pyocin, as described in Chapter I, D.6. Results of previous work has indicated that resistance occurs after exposure to pyocin in approximately one in every 10^6 organisms (Guymon et al. 1980, Guymon, Esser, and Shafer 1982). Pyocin has been classified as a bacteriocin, a class of proteins including the colicins of *E. coli* and the megacins of *Bacillus megaterium* that are lethal to other strains of bacteria. Bacteriocins bind to receptors on the outer cell membrane of bacteria (Tortora, Funke, and Case 1982). The exact mechanism by which pyocin effects LOS expression is not clear, although recent evidence suggests pyocin, rather than a protein, is a tailless bacteriophage. A bacteriophage is a bacterial virus, composed of either RNA or DNA. The structure of bacteriophages varies from simple fibrous forms to more complex ones with contractile tails (Tortora, Funke, and Case 1982). Pyocin has been found to contain single-stranded DNA which may directly insert

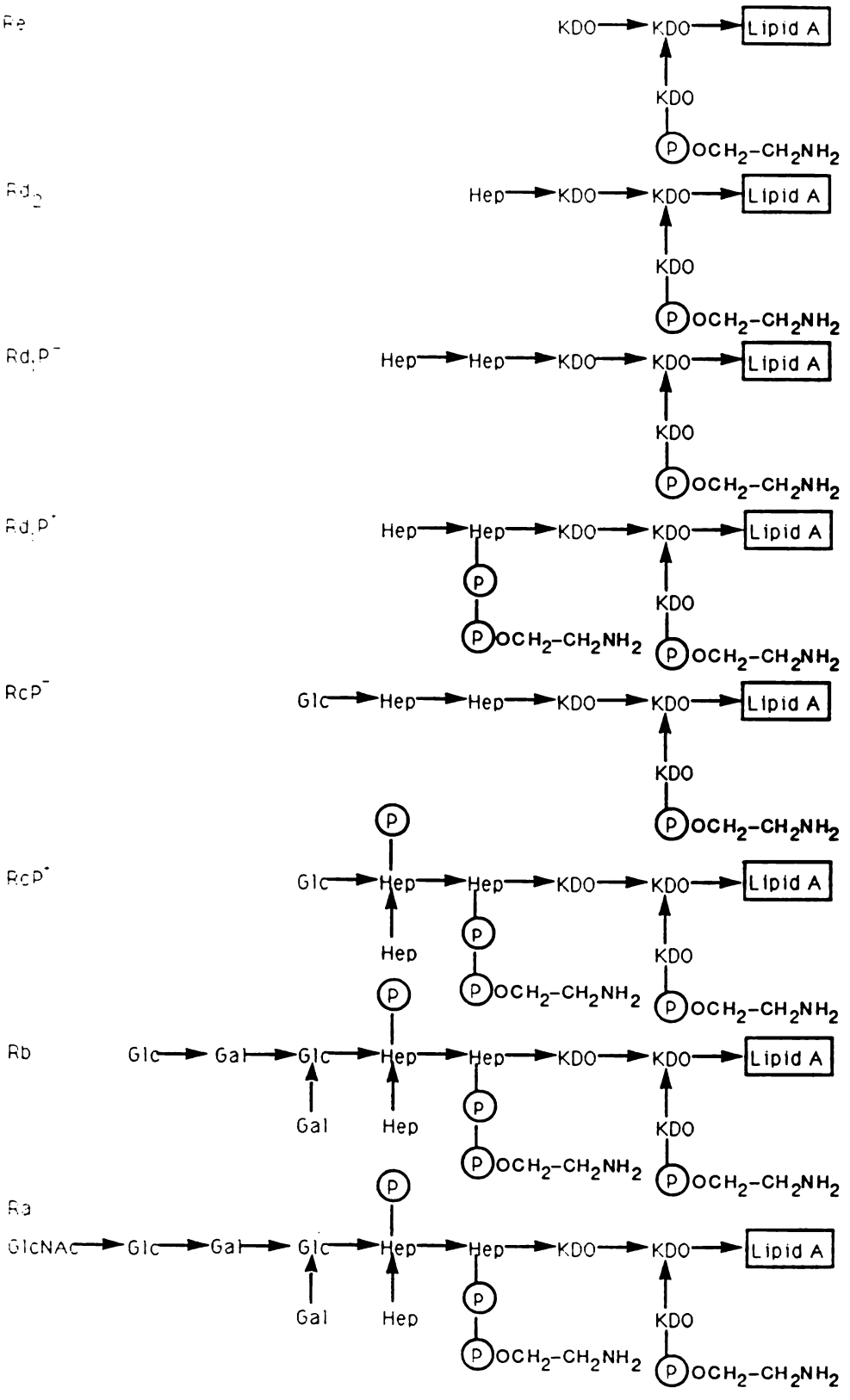


Figure 1. Structures of the Ra to Re LPS mutants of *Salmonella*.

into the gonococcal genome (Apicella, M.A., unpublished observations). The LOS appears to be the receptor for pyocin on the outer cell membrane (Blackwell and Law 1981, Sadoff, Zollinger, and Sidberry 1978). It may be that pyocin-resistant mutants express altered LOS after having one or more of the genes responsible for LOS biosynthesis interrupted by the insertion of the DNA from the bacteriophage. An illustration of the current theory of the mechanism of the generation of pyocin-resistant mutant strains is shown in Figure 2.

Gonococcal strain 1291 was chosen as a parent strain by Dudas and Apicella to produce a series of pyocin-resistant mutant strains because analysis using SDS-PAGE and silver-staining indicated a single major LOS band (Dudas and Apicella 1988). However, some heterogeneity in the LOS is indicated by the presence of small amounts of lighter bands and the breadth of the major band. The lysis of six gonococcal strains by nine different pyocin preparations, A-E, was tested to select a suitable pyocin for the production of mutants. Pyocin C was the only type which lysed all the prototype gonococcal strains, including JW31R, a previously isolated pyocin-resistant strain (Morse and Apicella 1982). After treatment of parent strain 1291 with pyocin C, 25 colonies of mutant strains were isolated and the LOS of the strain analyzed by SDS-PAGE and silver straining after proteinase K lysis (Hitchcock and Brown 1983). The apparent M_r of the LOS of the majority of the resistant strains was less than the apparent M_r (≈4715 Da) of the parent 1291 LOS. Four of these strains were selected after the initial interaction with pyocin C. Strain 1291_b was isolated after retreatment of strain 1291_c with pyocin C, the only one of the five mutant strains still sensitive to pyocin.

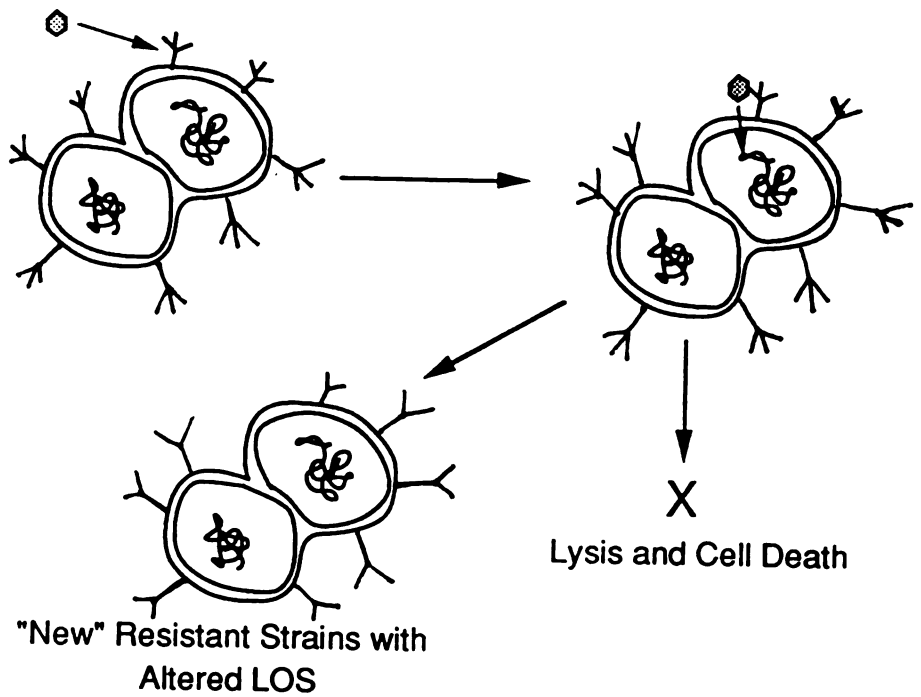


Figure 2. A schematic of the generation of pyocin-resistant mutants of *Neisseria gonorrhoeae*. The pyocin (shown as a small hexagon) binds to the LOS on the outer membrane. The pyocin is lethal to most cells but can give rise to a bacterium with altered LOS (lower left) that is now resistant to pyocin binding.

In Figure 3, a silver-stained SDS-polyacrylamide gel is shown of the PK lysates of the 1291 parent and mutant strains (on the left) and the *S. minnesota* Ra to Re LPS mutants (Dudas and Apicella 1988). The electrophoretic mobility of the LPS of the *S. minnesota* mutants, which differ by sequential saccharide deletions, increases from Ra to Re as the size of the oligosaccharide decreases, as does the LOS of the 1291 mutants. The LOS of the parent strain 1291 and the mutant strains 1291_{a-e} were selected for further structural analysis based on the assumption that the LOS of the mutant 1291 strains may differ by sequential saccharide deletion, and may represent a series of biosynthetic precursors.

A. Purification and separation

LOS from *N. gonorrhoeae* wild-type strain 1291 (1291wt) and the five pyocin-resistant mutants (1291_{a-e}) was generously provided by M. A. Apicella at the State University of New York, Buffalo, New York. The LOS was isolated and purified from acetone dried organisms as previously described (Dudas and Apicella 1988). To isolate the oligosaccharide portion, 10-25 mg of crude LOS from the various 1291 strains was hydrolyzed in 1% acetic acid (2 mg/ml) for 2 h at 100°C. The resulting mixture containing insoluble lipid A and soluble oligosaccharide was then centrifuged for 20 min at 5000 x *g* and the water-soluble oligosaccharide removed. The precipitate was washed with 5 ml of H_2O, centrifuged, and combined with the first soluble fraction and lyophilized. For the LOS from strain 1291_e, 0.1% SDS (w/w) was added to the acetic acid to increase the solubility of this LOS, and was removed after the hydrolysis by a C_{18} Sep-Pak. The low

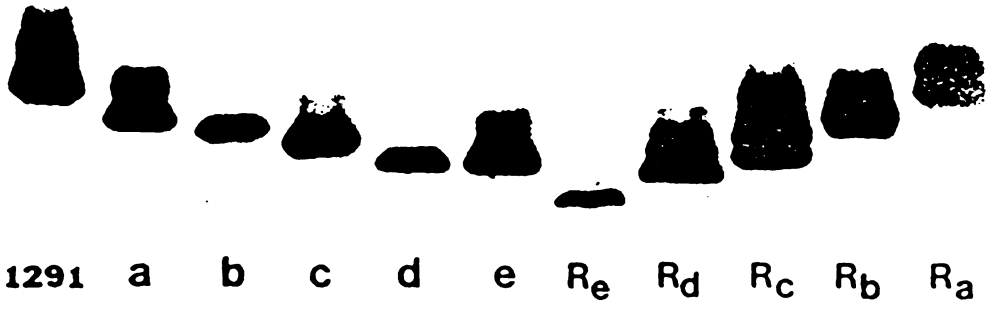


Figure 3. The differences in the electrophoretic motility of the LOS of the wild type and the mutant 1291 strains are demonstrated. This is a silver-stained SDS-polyacrylamide gel. The LPS of the *S. minnesota* Ra through Re mutants are also included on the gel. This figure is reproduced with permission of Dr. M. A. Apicella.

solubility of the 1291_e LOS is likely a consequence of containing only a very small glycan moiety on the otherwise hydrophobic lipid A.

From 1-10 mg of crude lyophilized oligosaccharide was then dissolved in 250-500 µl of 1% acetic acid and centrifuged-filtered through a 0.45 µm nylon Microfilterfuge (Rainin). Oligosaccharide samples less than 1-2 mg were injected onto two TSK-125 Bio-Sil gel filtration HPLC columns (600 x 7.5 mm) linked in series. The oligosaccharides were eluted isocratically with 1% acetic acid at 1 ml/min and monitored by refractive index detection (Knauer RI detector). Fractions of 1 ml each were collected and those containing oligosaccharide were pooled and dried on a Savant Speed-Vac concentrator. Alternately, the oligosaccharides were dissolved in 0.047 M pyridinium acetate buffer (pH 4.3), centrifuge-filtered, and applied to a Bio-Gel P-4 column (1.5 x 84 cm, <400 mesh; BioRad). The column was equipped with a water jacket and both the column and refractive index detector were maintained at 30°C. Upward elution at flow rates of 10-15 ml/h was achieved with a P-1 peristaltic pump (Pharmacia). Again, refractive index detection was used for peak detection and fractions were collected at 7.5 min intervals and evaporated to dryness in a Speed-Vac concentrator. Chromatograms from both the TSK and the Bio-Gel P-4 columns were recorded and stored with a Shimadzu C-R3A Chromatopac integrator.

Examples of chromatographic separations of the oligosaccharides produced by acid hydrolysis of the LOS from 1291wt and each of the 1291 mutant strains are shown in Figure 4. As indicated in this figure, some of the chromatograms shown are from separations on the TSK columns and others from the Bio-Gel P-4 column. In general, better resolution of the individual oligosaccharide components was obtained from the Bio-Gel P-4

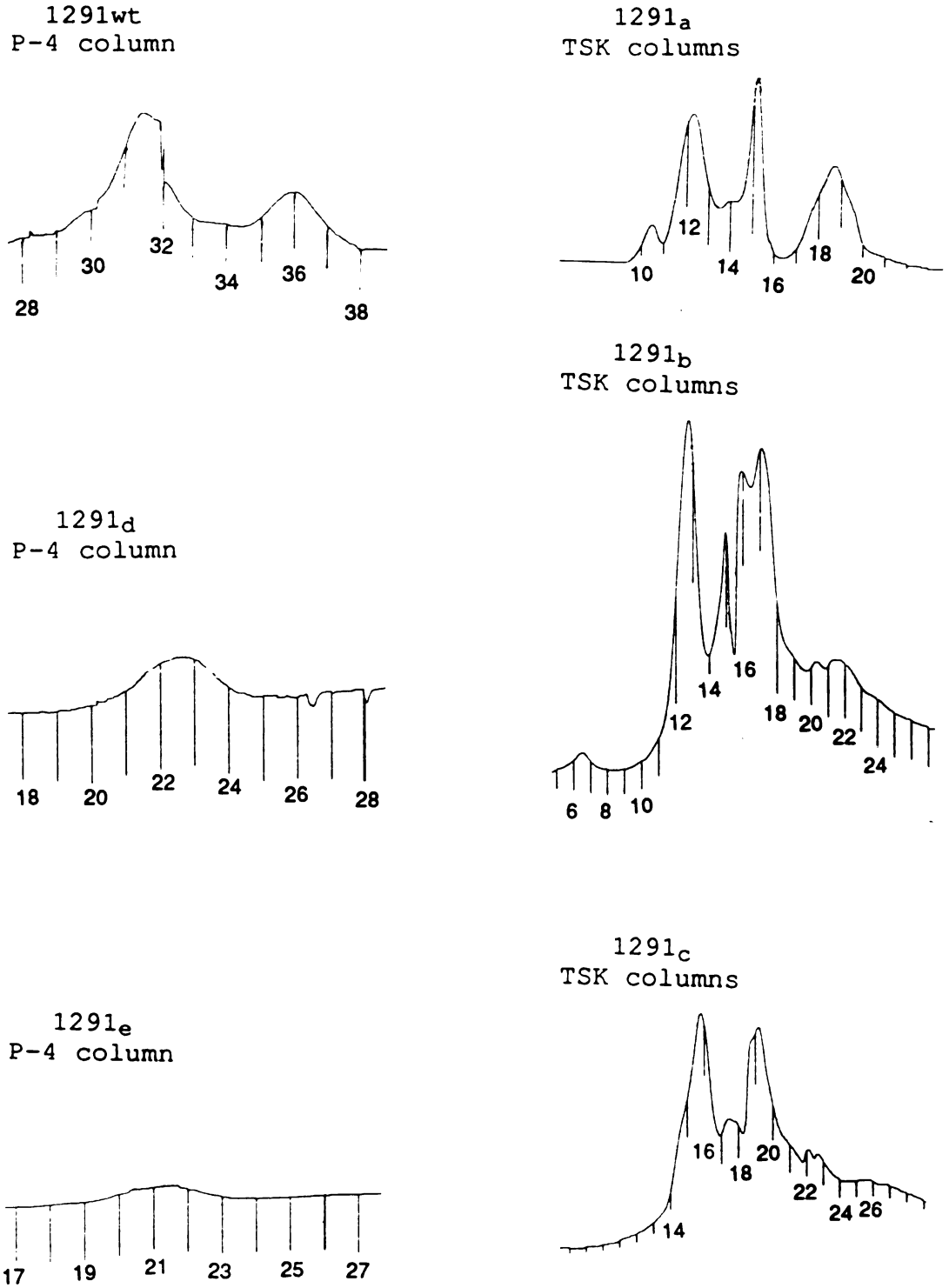


Figure 4. Chromatograms from size-exclusion gel filtration (P-4 on the right and TSK columns on the left) of 1291 oligosaccharides are shown Fractions containing oligosaccharides are described in the text. The void volume of the P-4 column was ≈65 ml and maltoheptaose eluted at ≈102 ml. The flow rate of the TSK columns was 1 ml/min and 1 ml fractions were collected after 25 min. Maltoheptaose eluted in ≈42 and glucose in ≈46 ml.

column. However, more sample was required for separations on the P-4 column with an overall lower recovery, particularly when using smaller amounts of oligosaccharide (1 mg). Another advantage of the HPLC system with the TSK columns was the speed of the analysis. Whereas a typical separation with the HPLC system took 60 min to complete, the P-4 column required ≈12-14 hours, excluding the initial time required for column equilibration and the time for washing after the run (1-2 days). Therefore, when less material was available and/or separation efficiency was not a critical factor, the more rapid and less sample intensive HPLC method was used. Negative-ion LSIMS analysis was performed on fractions from these separations to confirm which peaks contained oligosaccharides.

In the P-4 separation of 1291wt oligosaccharides shown in the chromatogram on the top left side of Figure 4 the oligosaccharide containing fractions 30-33 and 34-38 were pooled. The volumes of these pooled fractions were 75-81 ml and 82-90 ml, respectively. Fractions 20-24 were pooled from the chromatogram of the oligosaccharides of strain 1291_d shown in the middle left side of Figure 4. The volume of elution of the oligosaccharides was 88-98 ml. From the chromatogram of the P-4 separation of 1291_e oligosaccharides shown in the bottom left side of Figure 4, the fractions 20-22 were pooled and found to contain oligosaccharides. The volume of elution of the 1291_e oligosaccharides was 84-89 ml.

From the TSK separation of the oligosaccharides from 1291_a as shown in the top right side of Figure 4 the following fractions were pooled and found to contain oligosaccharides: 9 and 10; 11-13; 14 and 15. The volume of the elution of the oligosaccharides was 33-40 ml. From the chromatogram of the 1291_b oligosaccharides shown in the middle right side of Figure 4, fractions 11-13 and 14-15 were pooled and found to contain

oligosaccharides. The volume of elution of the oligosaccharides was 36-40 ml. As shown in the chromatogram on the bottom right of Figure 4 of the oligosaccharides from 1291_c fractions 13-16 and 17-18 were pooled and found to contain oligosaccharides. The 1291_c oligosaccharides in these fractions eluted from 38-43 ml.

B. LSIMS analysis

Negative-ion LSIMS analysis of derivatized and underivatized oligosaccharides was performed as described in Chapter II, B.1. The resulting spectra of the underivatized oligosaccharides from 1291wt and $1291_{a,b,c,e}$ are shown in Figure 5. The molecular ions, corresponding molecular mass, and the proposed compositions for the molecular ions are listed in Table 1. The molecular ions observed range from m/z 1678 for the 1291wt oligosaccharide to m/z 947 for the oligosaccharide from $1291_{d\ \&\ e}$. It was apparent from the LSIMS analysis of the underivatized oligosaccharides that the mutant strains produced a series of oligosaccharide moieties smaller than the wild type strain. Furthermore, on the basis of the proposed compositions they differed by combinations of Hex and HexNAc. These oligosaccharides could, therefore, represent biosynthetic precursors of the wild type oligosaccharide.

Although not observed in the spectrum of the oligosaccharide from 1291wt in Figure 5, a significant molecular ion was observed in a spectrum of underivatized 1291wt oligosaccharide from a different acid hydrolysis at m/z 1720. This shift of 42 Da from the molecular ion at 1678 corresponds to an additional OAc and a composition of $KDOHep_2HexNAc_2Hex_3PEAOAc_2$. Usually only one *O*-acetate moiety was observed in analysis of the 1291

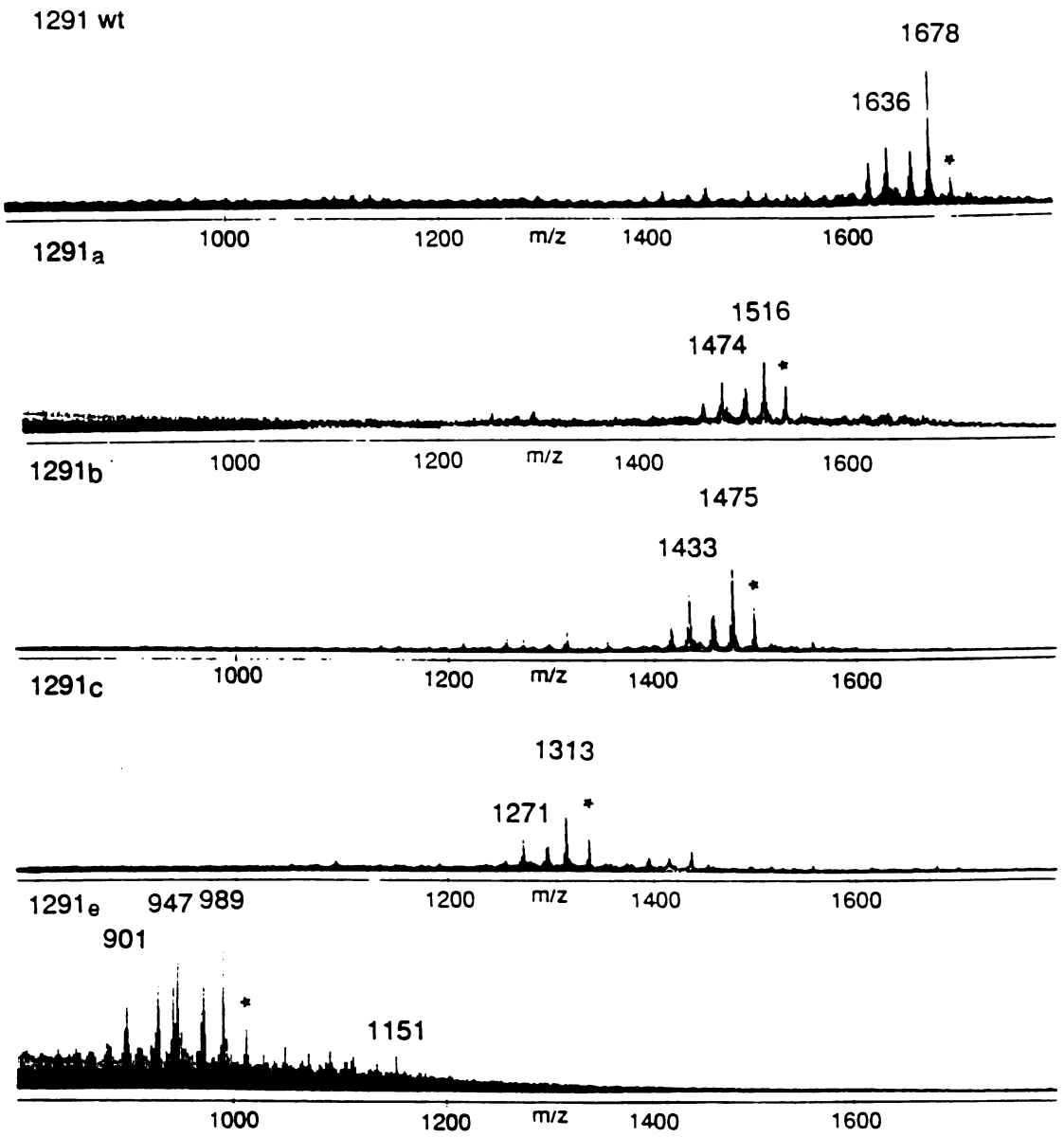


Figure 5. Negative-ion LSIMS spectra of underivatized oligosaccharides from strain 1291wt and mutant strains $1291_{a,b,c,e}$. Only the major ions are labeled in each spectrum; the peaks marked with an asterisk are the sodium adducts, $(M+Na-2H)^-$, of the major species.

Table 1. Compositions of Gonococcal 1291 Oligosaccharides

Strain	$(M-H)^-$	M_r	Proposed Composition
1291wt			
	1678	1679	KDO, Hep_2, $HexNAc_2$, Hex_3, PEA, OAc
	1636	1637	KDO, Hep_2, $HexNAc_2$, Hex_3, PEA
	1555	1556	KDO, Hep_2, $HexNAc_2$, Hex_3, OAc
	1513	1514	KDO, Hep_2, $HexNAc_2$, Hex_3
1291_a			
	1516	1517	KDO, Hep_2, $HexNAc_2$, Hex_2, PEA, OAc
	1474	1475	KDO, Hep_2, $HexNAc_2$, Hex_2, PEA
1291_b			
	1475	1476	KDO, Hep_2, HexNAc, Hex_3, PEA, OAc
	1433	1434	KDO, Hep_2, HexNAc, Hex_3, PEA
1291_c			
	1313	1314	KDO, Hep_2, HexNAc, Hex_2, PEA, OAc
	1271	1272	KDO, Hep_2, HexNAc, Hex_2, PEA
1291_d			
	1151	1152	KDO, Hep_2, HexNAc, Hex, PEA, OAc
	989	990	KDO, Hep_2, HexNAc, PEA, OAc
	947	948	KDO, Hep_2, HexNAc, PEA
	943	944	?
	901	902	?
1291_e			
	1151	1152	KDO, Hep_2, HexNAc, Hex, PEA, OAc
	989	990	KDO, Hep_2, HexNAc, PEA, OAc
	947	948	KDO, Hep_2, HexNAc, PEA
	901	902	?

* Other species observed but not reported in this list are sodiated $(M+Na-2H)^-$, anhydro $(M-H_2O)^-$ and an artifact series $(M-H+C_4H_4O_3)^-$ that have been reported earlier.

oligosaccharides. However, some of the *O*-acetate moieties may have been hydrolyzed during the acid hydrolysis of the LOS. Other methods, such as analysis of intact LOS by laser or plasma desorption time-of-flight mass spectrometry, might be able to accurately determine the amount of *O*-acetylation in the LOS (see Chapter II, C.2 and C.3).

Reasonable compositions for all of the more abundant ions could be proposed except for the ions from $1291_{d\ \&\ e}$ observed at m/z 943 and 901. In all other molecular ions, a common core region consisting of $KDOHep_2$ could be proposed for the corresponding oligosaccharides. However, in the LSIMS analysis of the oligosaccharide fractions of 1291_a LOS some molecular ions were observed for which no composition could be found. In one negative-ion LSIMS spectrum of underivatized HF-treated oligosaccharides from 1291_a a molecular ion was observed at m/z 1435, consistent with a composition of $KDOHep_2HexNAc_2Hex_2OAc_2$. Ions were also observed at m/z 1393 and 1351, which are each 42 Da (the mass of an acetate group) less than m/z 1435. In the fraction of the 1291_a oligosaccharide analyzed in the spectrum shown in Figure 3, there only two major species differing by a single *O*-acetate group. These two species appeared to be major components of the 1291_a oligosaccharide. Heterogeneity in the *O*-acetate content was clearly one distinctive feature of these oligosaccharides.

To increase the extent of fragmentation and to better separate these oligosaccharides, HF-treated and untreated oligosaccharides were derivatized with either HBEE or one of the alkylphenylhydrazines for LSIMS analysis as described in Chapter II, B.1. Separation by reverse-phase HPLC was by basically the same method as previously described (see Chapter II, B.1.). However, with the more hydrophobic

alkylphenylhydrazine oligosaccharide derivatives, the samples were dissolved in a solution of 10% acetonitrile/H_2O with 0.05% TFA, and eluted at a higher percentage of acetonitrile than the more hydrophilic HBEE-oligosaccharide derivatives.

In Figure 6, the negative-ion LSIMS spectrum of HF-treated oligosaccharide from 1291wt derivatized with 4-octylphenylhydrazine (OPH) is shown. In this spectrum, an abundant $(M-H)^-$ is present at m/z 1697 which corresponds to an anhydro species $(M-H_2O-H)^-$. The partial structure of this oligosaccharide is shown in the lower left of the figure, and a much less abundant molecular ion is present at m/z 1739 that contains an additional *O*-acetate moiety. Also, two higher mass ions are present at m/z 1803 and 1811 which are apparently due to impurities in the derivatizing agent, as the same mass shift was observed in spectra of other oligosaccharides including standards derivatized with OPH. All of the labeled ions are of the reducing terminal Y-type which dominated the spectrum. Some smaller (unlabeled) reducing terminal ions of the $^{1,5}X$-(Y+28) and Z-type (Y-18) can also be observed, along with Y-type fragment ions of the molecular ion at m/z 1739. Fragment ions from cleavage of all the glycosidic bonds can be observed, with the exception of the Hep-KDO bond. Overall, the extent of fragmentation in this spectrum is much greater than that observed in the negative-ion LSIMS spectra of the underivatized oligosaccharides shown in Figure 3 in which only the molecular ions were easily observed. At most, 2-3 nmoles of derivatized oligosaccharide was required to produce the spectrum shown in Figure 6.

The sequence of the two branches, Hex→HexNAc→Hex→Hex and HexNAc→Hep, can clearly be determined from the fragmentation data. The fragment ion for cleavage of the Hep-Hep bond at m/z 1302 can readily

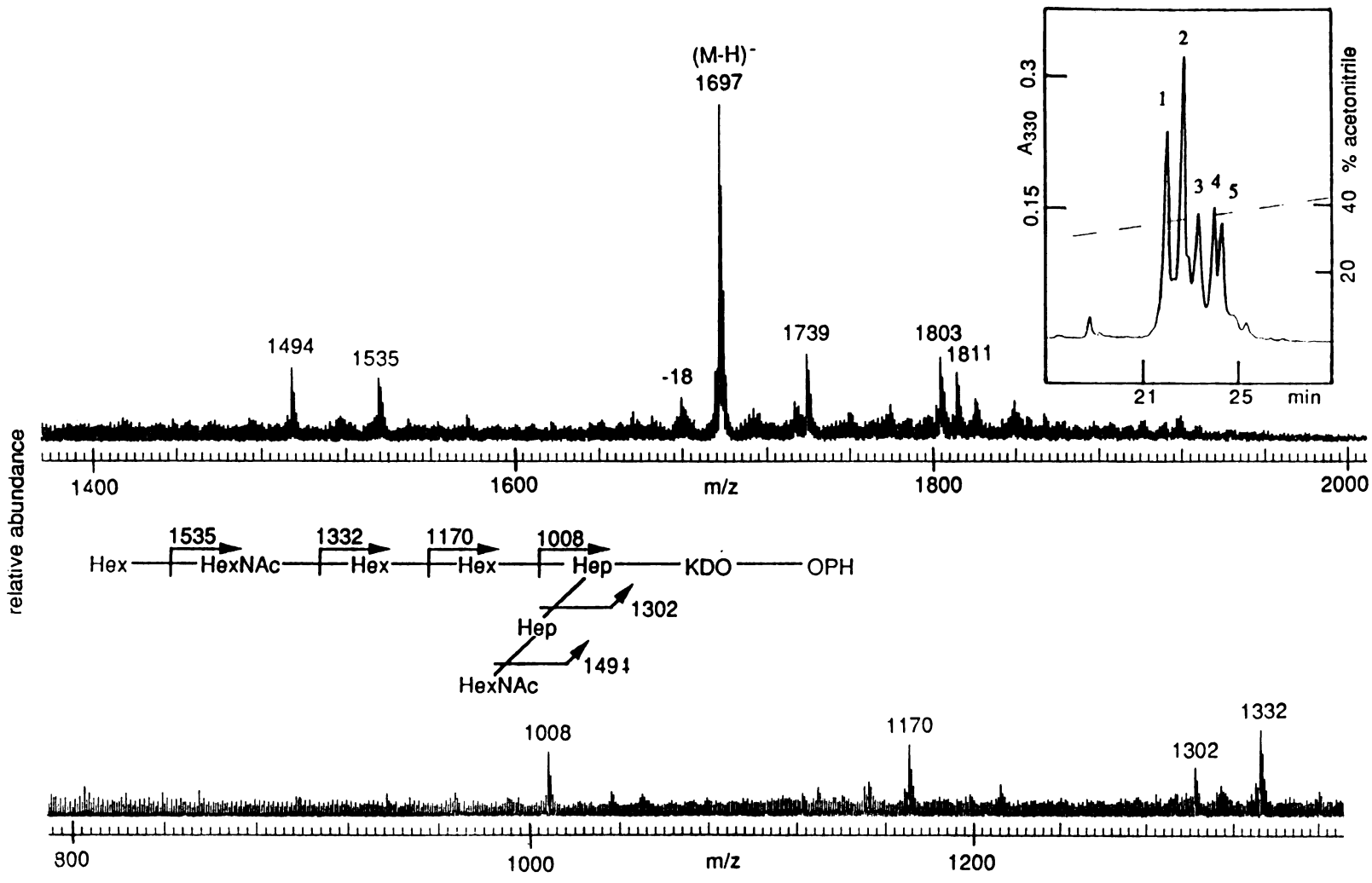


Figure 6. Negative-ion LSIMS spectrum of HF-treated oligosaccharide from 1291wt derivatized with OPH. All the labeled fragment ions are the non-reducing terminal Y-type. The reverse-phase HPLC separation of the oligosaccharide after derivatization with OPH is shown in the inset.

be observed. This is a particularly important fragment since it establishes the linkage of the two branches to the two Hep residues. In many LSIMS spectra of oligosaccharides derivatized with the amino- or hydrazinobenzoates, fragment ions for Hep-Hep bond cleavage was very weak or could not be detected at all.

In the inset of Figure 6 is the HPLC chromatogram of approximately 140 μg of HF-treated 1291wt oligosaccharide starting material derivatized with OPH. The spectrum shown was produced from the oligosaccharide from peak 1, and the same molecular ion for the anhydro oligosaccharide at m/z 1697 was observed in analysis of peak 2. LSIMS analysis of peak 3 revealed a molecular ion at m/z 1739, corresponding to the anhydro *O*-acetylated oligosaccharide. Analysis of peak 4 produced the intact oligosaccharide with $(M-H)^-$ 1715. Finally, peak 5 corresponded to the intact *O*-acetylated oligosaccharide with a molecular ion at m/z 1757. Baseline resolution of these different components was not achieved. Thus, more than one oligosaccharide component was present in the LSIMS analysis shown in Figure 6, although these components are clearly related in structure.

A negative-ion LSIMS spectrum of HF-treated oligosaccharide from 1291_a derivatized with HBEE is shown in Figure 7A. The molecular ion in the spectrum at m/z 1513 corresponds to a composition of $KDOHep_2HexNAc_2Hex_2$. Again, reducing terminal Y-type ions are the dominant fragments in the spectrum. The Y-type fragment ion for loss of HexNAcHep can barely be observed over background at m/z 1118. However, the sequence of the longest branch of the oligosaccharide, HexNAc→Hex→Hex, can easily be determined from this spectrum.

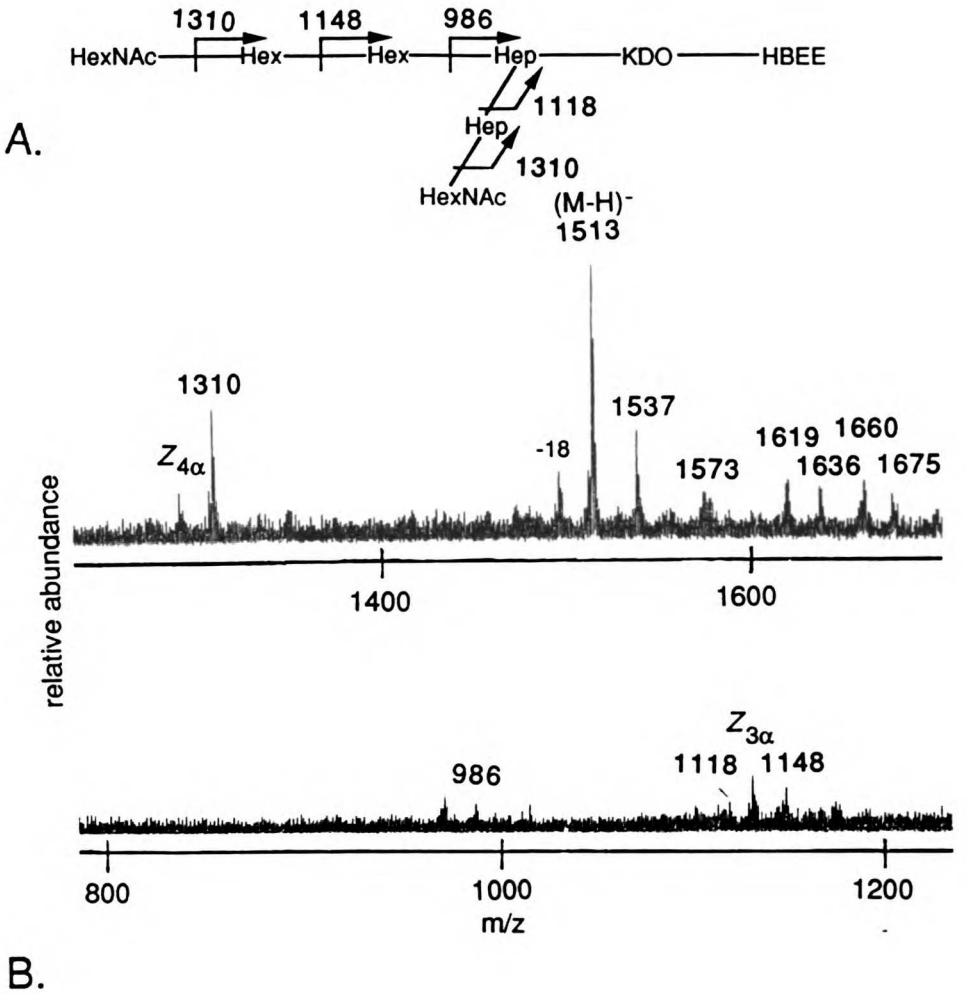
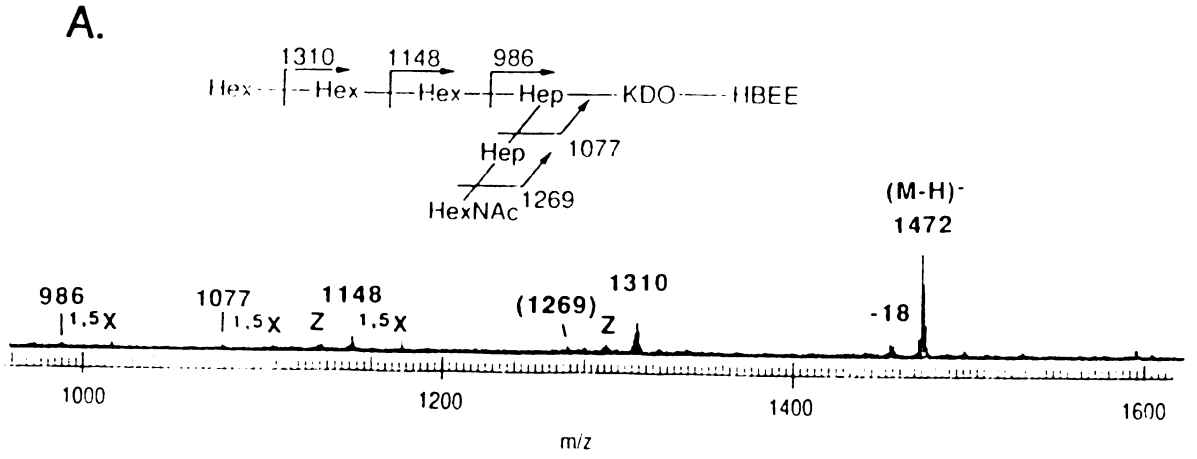


Figure 7. Negative-ion LSIMS spectrum (A) of HF-treated oligosaccharide from strain 1291_a derivatized with HBEE and (B) reverse-phase HPLC separation of the 1291_a oligosaccharide after derivatization with HBEE. In the spectrum the molecular ion at m/z 1537 is the analogous *O*-acetylated anhydro oligosaccharide, the ion at m/z 1636 has an additional PEA moiety, and the ion at m/z 1675 has an additional Hex residue. The composition of the ions at m/z 1573, 1619, and 1660 is not clear.

The reverse-phase HPLC chromatogram shown in Figure 7B was produced with approximately 190 μg of starting 1291_a oligosaccharide after derivatization with HBEE. The spectrum shown was obtained with the oligosaccharide from approximately one-fourth of peak 2. The corresponding anhydro species $(M-H_2O-H)^-$ at m/z 1495 was the main component of peak 1. In peak 3 the major component was the anhydro *O*-acetylated oligosaccharide at m/z 1537.

A negative-ion LSIMS spectrum of HF-treated oligosaccharide from mutant strain 1291_b derivatized with HBEE is shown in Figure 8. A typical reverse-phase HPLC of the HF-treated 1291b oligosaccharide after derivatization with HBEE can be seen in the inset of Figure 8. Approximately 40 μg of oligosaccharide starting material was used for the derivatization. In the LSIMS spectrum the reducing terminal Y-type ions (labeled with masses) are the most prominent, with some smaller $^{1,5}X$- and Z-type fragment ions on either side. Again, fragments ion for cleavage of all the glycosidic bonds, except the Hep-KDO bond can be observed, although some of the ions are not very abundant such as m/z 1269 for the loss of HexNAc, m/z 1077 for the loss of HexNAcHep, and m/z 986 for loss of Hex_3. From this spectrum the sequence of the longest branch appears to be Hex→Hex→Hex, unlike either of the branches of the 1291wt oligosaccharide. However, the other branch would have the sequence HexNAc→Hep as in the oligosaccharide from 1291wt.

Figure 9 shows the negative-ion LSIMS spectrum of HF-treated oligosaccharide from mutant strain 1291_c that had been derivatized with HBEE and separated by HPLC. The reverse-phase HPLC chromatogram in the inset of Figure 9 was obtained from injecting approximately 140 μg of the HF-treated 1291_c oligosaccharide after derivatization with HBEE.



B.

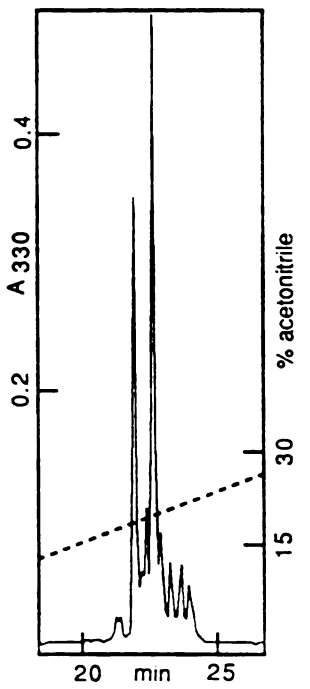


Figure 8. Negative-ion LSIMS spectrum (A) of HF-treated oligosaccharide from strain 1291_b derivatized with HBEE and (B) the reverse-phase HPLC chromatogram of 1291_b oligosaccharide after derivatization with HBEE. The fragment ions in the spectrum labeled with m/z values are of the non-reducing terminal Y-type. Several non-reducing terminal X- and Z-type fragment ions can also be observed in the spectrum.

LSIMS analysis of the labeled peaks (#1-#5) in the HPLC identified as the major component in peak 1 an anhydro species of the structure shown (see Fig. 9). Also identified in the analysis of peak 1 was the anhydro species plus a $C_4H_4O_3$ moiety, a previously observed adduct in gonococcal oligosaccharides. This adduct was observed and partially separated in oligosaccharides from JW31R. This moiety perhaps originates due to degradation of another KDO residue present in the LOS. The elemental composition of the adduct was determined by high resolution mass spectrometric analysis (Gibson et al. 1989). In several analyses of oligosaccharides from the 1291 strains, this adduct could be removed by adding a small amount (0.25 µl) of 1% HCl. This suggests that the adduct is acid-labile.

The spectrum shown in Figure 9 was obtained from peak 2 which had a proposed composition of $KDOHep_2HexNAcHex_2$. The sequence of this oligosaccharide is also shown in Figure 9. There is an abundant molecular ion at m/z 1310 in the spectrum of the oligosaccharide from 1291_c. In addition, reducing terminal Y-type fragment ions for the loss of Hex and Hex_2 can be observed accompanied by much smaller $^{1,5}X$- and Z-type fragment ions. However, fragment ions for the loss of HexNAc at m/z 1107, or HexNAcHep at m/z 915 from the reducing terminus were not observed. A much less abundant molecular ion is also present at m/z 1433 that differs from the molecular ion at m/z 1310 by 123 Da, the mass corresponding to a single PEA moiety.

A molecular ion for the *O*-acetylated species was found in analysis of peak 3 at m/z 1352, and a mixture of molecular ions were observed in analysis of peak 4 corresponding to a combination of anhydro (m/z 1292) and *O*-acetylated (m/z 1352) versions of the oligosaccharide. In the analysis of

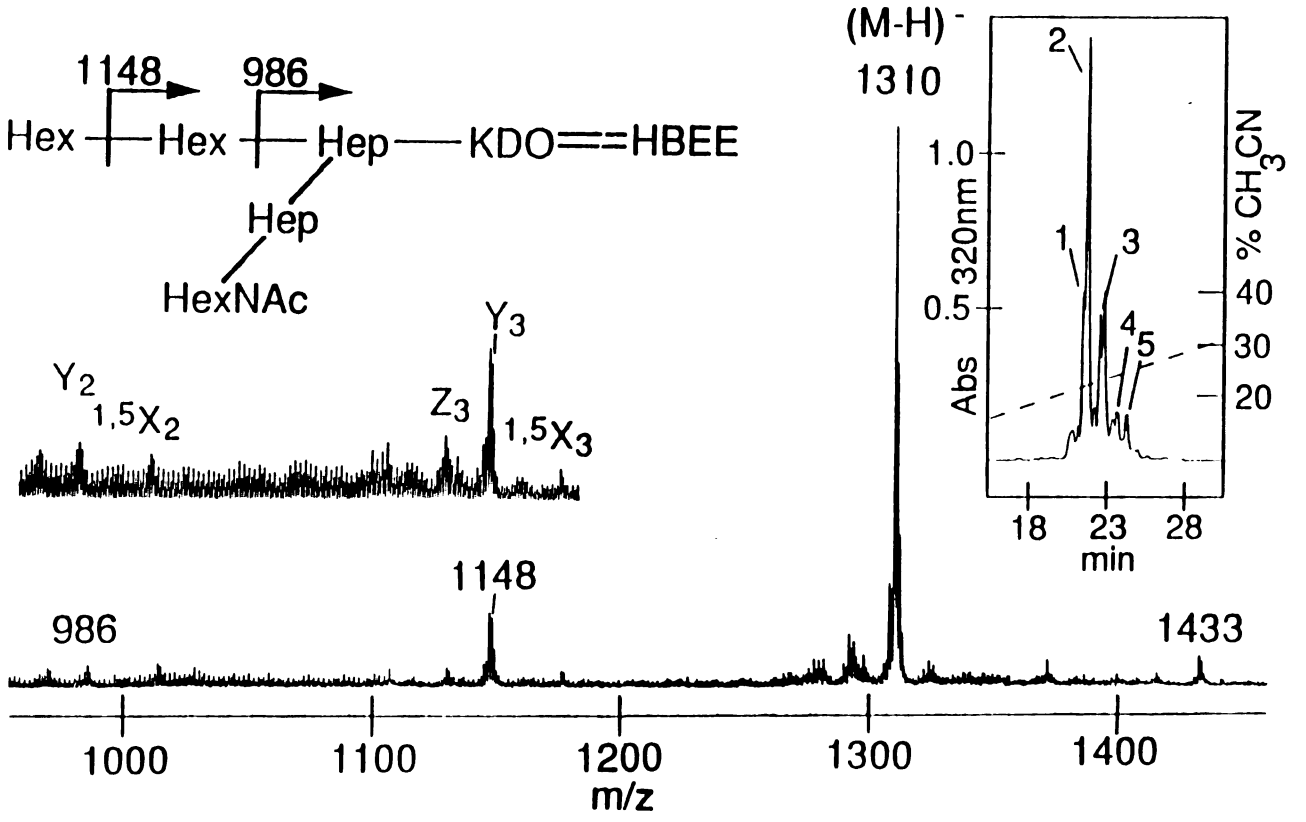


Figure 9. Negative-ion LSIMS spectrum of the HF-treated oligosaccharide from 1291_c derivatized with HBEE. The molecular ion at m/z 1433 contains an additional PEA moiety. The inset shows the reverse-phase HPLC chromatogram of the 1291_c oligosaccharide after derivatization with HBEE.

peak 5, a molecular ion was found at m/z 1717 which would correspond to a composition of $KDOHep_2HexNAc_2Hex_3$. These are the same components as the 1291wt type oligosaccharide and, therefore, appear to be due to some minor amount of reversion of the 1291_c mutant back to wild type or wild-type contamination. The fact that 1291_c was the only mutant not resistant to further treatment with pyocin (Dudas and Apicella 1988) could be due to the expression of this wild type oligosaccharide in the LOS.

C. Methylation linkage analysis

C.1. Materials and methods

Materials

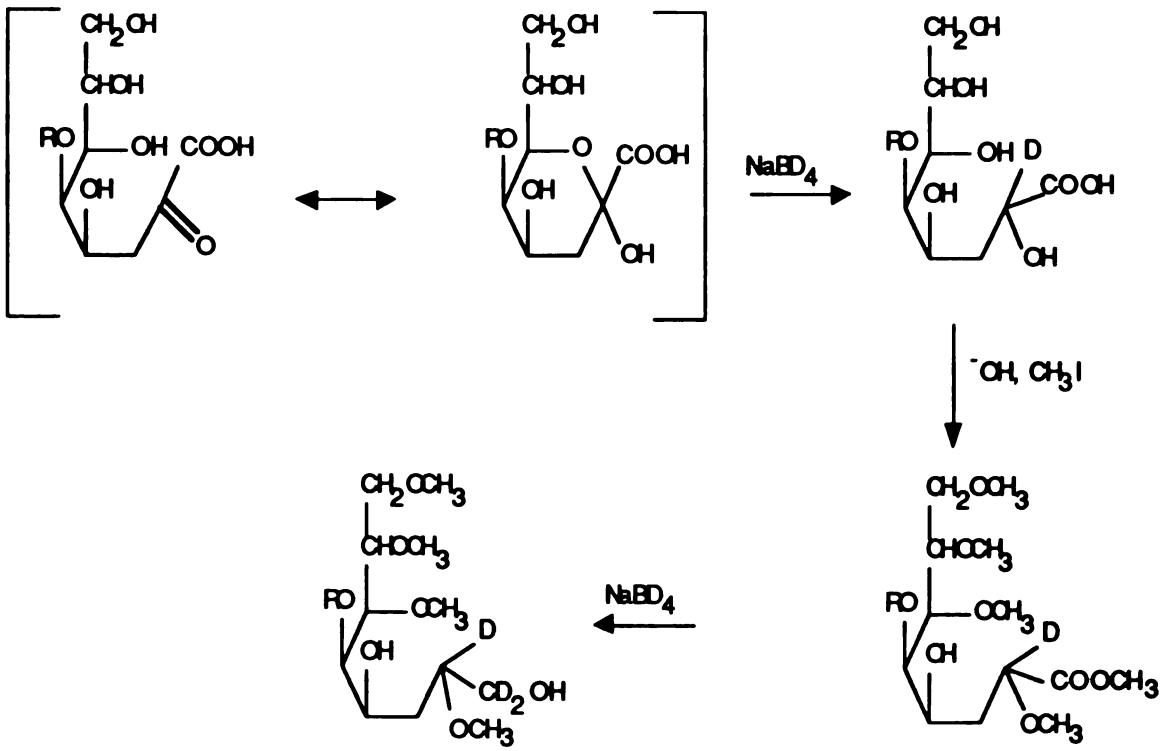
Sodium hydroxide pellets (99.9%) and sodium borodeuteride (98% D) were obtained from Aldrich (Milwaukee, Wisconsin). The iodomethane was obtained from Fluka (Buchs, Switzerland) and acetic anhydride from Supelco (Bellefonte, Pennsylvania). Water, methanol, and methylene chloride were purchased from Burdick and Jackson (Muskegon, Michigan) and the other reagents and solvents were of the reagent grade.

Methods

Methylation linkage analysis was performed using a modified version of the microscale procedure of Levery and Hakomori (Levery and Hakomori 1987) in which the dimethylsulfinyl carbanion was formed by sodium hydroxide rather than sodium hydride. In our experience, the

gonococcal Hep-Hep bonds appear to be more resistant to acid hydrolysis than the other glycosidic bonds. Therefore, longer hydrolysis times (21 h) and slightly more acidic conditions were used to increase the yields of Hep moieties (Stellner, Saito, and Hakomori 1973). To determine the linkage positions of the labile KDO moiety two additional reductions by $NaBD_4$ were performed on several samples. First, as shown in Scheme 1, the ketone group of KDO was reduced to a secondary alcohol and, after permethylation, the C-1 methyl ester of KDO was reduced to a primary alcohol (York et al. 1985).

Scheme 1



For reduction of the ketone group of KDO, 5-50 μg of oligosaccharide was placed in 1 ml Reacti-vials with Mininert valves (Pierce, Rockford, Illinois). The samples were dried overnight *in vacuo* over powdered P_2O_5. To each sample 150 μl of a 10 mg/ml solution of $NaBD_4$ in 1 M NH_4OH was

added and the reaction proceeded at room temperature for 1 h. The remaining $NaBD_4$ was then destroyed by the addition of 50 µl of acetic acid and the solution was evaporated by a stream of N_2 while heating to 35°C. Boric acid was removed by adding and then evaporating 500 µl of methanol with 1-2% acetic acid, repeating seven to eight times.

Prior to permethylation, oligosaccharides or prereduced oligosaccharides (in 1 ml Reacti-vials with Mininert valves) were dried overnight *in vacuo* over P_2O_5. Each sample was dissolved in 100 µl of distilled DMSO which was added with a syringe through the stopcock valve, flushed with N_2 and sonicated for 1 min. Using a mortar and pestle 99.9% sodium hydroxide pellets were crushed under N_2. While flushing with N_2, approximately 10 mg of powdered NaOH, 25 µl of methyl iodide and a stir bar were added to each sample. The samples were stirred at room temperature for 30 min and then extracted with CH_2Cl_2 and H_2O. First, 250 µl of CH_2Cl_2 was added to each sample, followed by the addition of 250 µl of H_2O. The vial was then vortexed and briefly centrifuged in a Speed-Vac concentrator (Savant). The lower organic layer was removed and placed in a second vial and washed twice with 250 µl of H_2O as previously described. All aqueous extracts were combined and then washed twice with 250 µl of CH_2Cl_2. The CH_2Cl_2 layers were then combined with the first. The combined CH_2Cl_2 layers were washed once again with 150 µl of H_2O, dried under a stream of N2, and finally dried overnight *in vacuo* over P_2O_5.

The methyl ester of the KDO moiety was reduced to a primary alcohol by adding 250 µl of ethanol and 125 µl of a 10 mg/ml solution of $NaBD_4/H_2O$. The reduction was carried out at room temperature for 2 h. Excess $NaBD_4$ was then removed by the addition of 50 µl of acetic acid, the samples dried under N_2, and boric acid removed by the addition of acidic methanol as

described above. Again, the samples were dried overnight *in vacuo* over P_2O_5.

The permethylated (or permethylated and reduced) oligosaccharides were hydrolyzed to monosaccharides by heating for 16 h at 80°C in 250 µl of a mixture of 95% acetic acid/1.4% sulfuric acid/3.6% H_2O. The samples were cooled and 250 µl of H_2O was added, followed by heating for an additional 5 h at 80°C. The samples were neutralized after cooling by adding 325 µl of 0.5 M NaOH. The samples were once again dried at 35°C under a stream of N_2. Methanol was added several times to speed the evaporation process and the residue dried overnight *in vacuo* over NaOH.

To reduce the saccharide aldehydes, 300 µl of a 10 mg/ml solution of $NaBD_4$ in 0.01 N NaOH was added to each sample. The sample vials were loosely capped and kept at 4°C overnight. As described above, the remaining $NaBD_4$ was quenched by the addition of acetic acid, the samples dried under N_2, and the residual borate evaporated as the methyl ester by repeated additions of acidic methanol. The partially methylated alditols were dried overnight *in vacuo* over P_2O_5.

To prepare the final partially methylated alditol acetates (PMAAs), 300 µl of acetic anhydride was added to acetylate the free hydroxyl groups and the vials flushed with N_2. The sample vials were heated for 2 h at 100°C with intermittent sonication. After cooling, the excess acetic anhydride was evaporated at 35°C in a gentle stream of N_2 with repeated additions of 500 µl aliquots of toluene. The resulting PMAAs were purified by CH_2Cl_2/H_2O extraction as described previously for the permethylation step. The combined CH_2Cl_2 layers were then evaporated under a stream of N_2 and the PMAAs dissolved in an appropriate amount of CH_2Cl_2 just prior to analysis by GC/MS.

GC/MS analysis of the PMAAs was performed on a VG70SE mass spectrometer equipped with a Hewlett Packard 5890 gas chromatograph and an on-column injector (J & W Scientific). The samples were separated on a 30 m DB-1 column with a 1 μm film thickness (J & W Scientific) using a temperature program of 120 to 280°C at 4°C/min. Electron impact ionization (70 eV) was performed with a trap current of 200 μA, a source temperature of 250°C and an emission current of 1 mA. The scans were acquired at 0.8 s/decade with a 0.15 s interscan delay. Some of the PMAA samples were analyzed by alternating chemical and electron impact ionization (ACE) on the VG70SE mass spectrometer. Conditions for ACE were as follows: 70eV; trap current of 500 μA; emission current of 2 mA; source temperature of 180°C; and source pressure of NH_3 of 1-2 x 10^{-4} mbar (Geyer et al. 1983). With ACE the scan conditions were 0.9 s/decade with a 0.1 s interscan delay. Masses were assigned automatically using a VG Opus data system calibrated with an external PFK reference.

C.2. Results and discussion.

Table 2 shows the relative intensity of characteristic fragment ions are listed for the PMAAs of the monosaccharides identified in each sample. A plus sign indicates the presence of residues for which no standard was available for comparison and quantitation. As will be described more fully, the data obtained from methylation analysis supports the compositions and the structures proposed from the LSIMS analysis of underivatized and derivatized 1291 oligosaccharides. For the oligosaccharides from 1291wt, a terminal GlcNAc and an internal 1,4-GlcNAc residues were found, one non-reducing terminal Hex residue (1-Gal), two internal Hex moieties

Table 2. Relative ion abundance of characteristic fragment ions of corresponding PMAA derivatives.

Strain	1-Glc	1-Gal	1,4-Gal	1,4-Glc	1,3-Gal	1,2-Hep	1,3-Hep	1,3,4-Hep	1-GlcNAc	1,4-GlcNAc
1291wt	–	0.7	–	1.3	0.7	+	–	+	1.0	1.0
1291_a	0.1	0.2	–	0.9	0.7	+	–	+	2.0	0.3
1291_b	–	1.1	0.6	1.5	0.2	+	–	+	1.2	–
1291_c	–	1.2	–	0.9	–	+	–	+	0.4	–
1291_e	0.6	–	–	–	–	+	+	+	1.1	–

(1,4-Glc and 1,3-Gal), and two Hep moieties (1,2-Hep and 1,3,4-Hep). From LSIMS analyses of the HBEE derivatives of the oligosaccharide from 1291wt (see Fig. 6) two non-reducing terminal branches were proposed for the structure, Hex→HexNAc→Hex→Hex and HexNAc→Hep. Correlating this data with the data from methylation analysis the following two structures can be assigned to these branches: Gal1→4GlcNAc1→(3Gal1→4Glc1) and GlcNAc1→Hep.

In the methylation analysis of the 1291 oligosaccharides, PMAAs for both 1,2-Hep (1,2,5-tri-*O*-acetyl-3,4,6,7-tetra-*O*-methylheptitol) and 1,3,4-Hep (1,3,4,5-tetra-*O*-acetyl-2,6,7-tri-*O*-methylheptitol) moiety were were found as shown in Figure 10. In addition, the PMAAs from the oligosaccharides of 1291_e contained a 1,3-Hep residue (1,3,5-tri-*O*-acetyl-2,4,6,7-tetra-*O*-methylheptitol). LSIMS analysis of underivatized oligosaccharides from 1291_e (see Fig. 5 and Table 1) indicated the presence of two major components, one with the composition $HexNAcHep_2KDO$ and the other with an additional non-reducing terminal Glc moiety. From this data a composition of $GlcHexNAcHep_2KDO$ could be proposed. Thus, the smaller component must have the structure HexNAc1→2Hep1→3Hep1→KDO. In the larger structure the non-reducing terminal Glc moiety must be attached to C-4 of the previously 1,3-Hep residue that is directly linked to KDO. This heptose would now become a 1,3,4-Hep.

LSIMS analysis of the oligosaccharides from 1291_c revealed a structure with two Hex groups (see Figs. 5, 9 and Table 1), an additional Hex moiety compared to the largest of the $1291_{d,e}$ oligosaccharides. In the methylation analysis of 1291_c oligosaccharides, a new internal 1,4-Glc moiety was found along with a non-reducing terminal Gal residue. Thus,

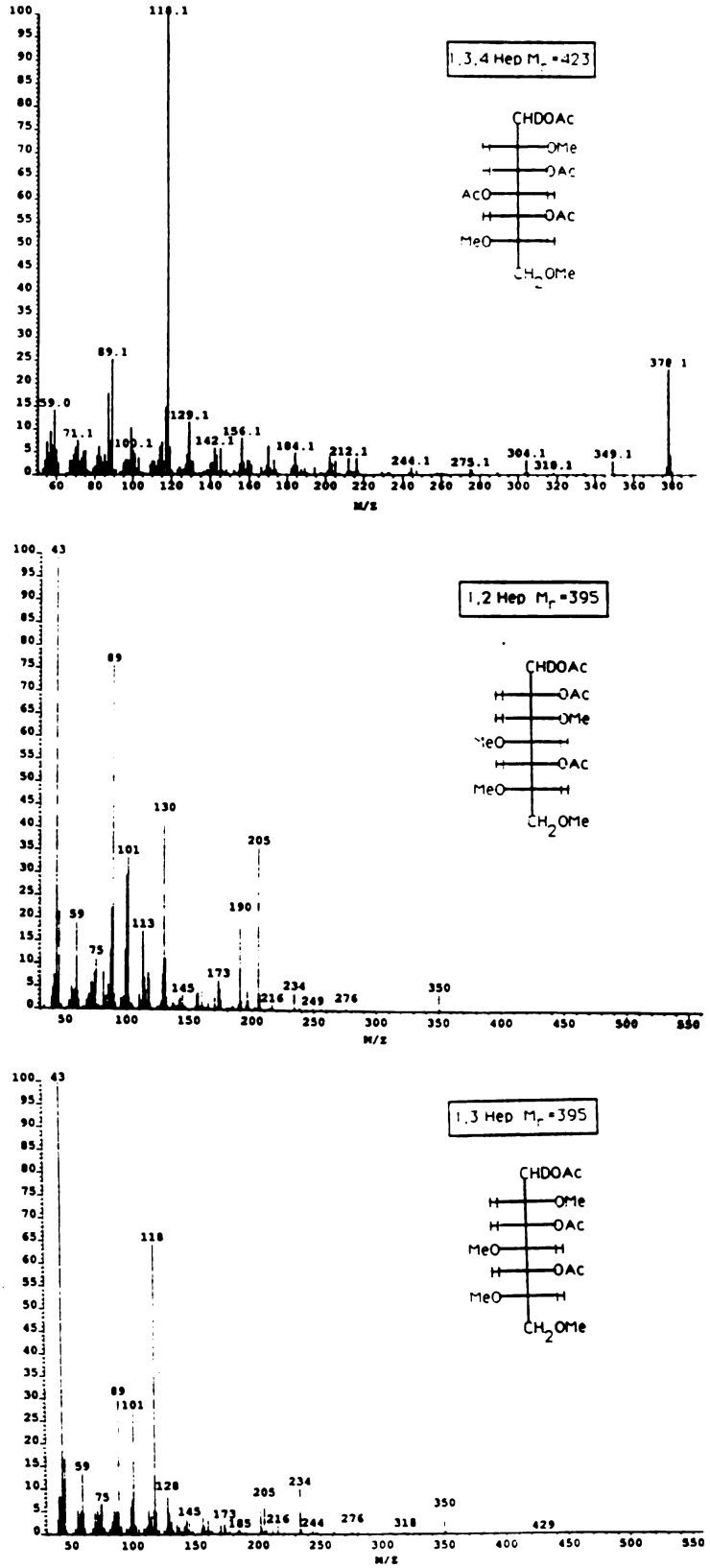


Figure 10. The EI spectra of the PMAAs of the heptose residues from the oligosaccharides of the wild type and mutant 1291 strains.

the saccharide chain was apparently built-up with the addition of a terminal Gal to the C-4 position of the former non-reducing terminal Glc residue.

Consistent with this pattern and with the LSIMS analyses (see Figs. 5, 7 and Table 1), methylation analysis of the oligosaccharides from strain 1291_a indicated a major structure with two internal Hex moieties, 1,3-Gal and 1,4-Glc, and two non-reducing terminal GlcNAc moieties. Again, in this strain the saccharide chain of the major component was elongated compared to the oligosaccharide from 1291_c. The oligosaccharide from 1291_a contained a new non-reducing terminal GlcNAc linked to the C-3 of the former non-reducing terminal Gal residue. The major component of the oligosaccharide from 1291_a differed from the wild type by deletion of the non-reducing terminal Gal residue on the C-4 of the this non-reducing terminal HexNAc residue.

In methylation analysis of the 1291_a oligosaccharide fraction, the scheme of York et al. (York et al. 1985) was employed to determine the linkage of the 1,3,4-Hep to KDO (see Scheme 1). Prior to permethylation the keto moiety of the KDO was reduced with $NaBH_4$ to a secondary alcohol and, after permethylation, the methyl ester on the KDO moiety was reduced to a primary alcohol. Thus, the C-1 position was later acetylated and the C-2 position was methylated as shown in the structure in Figure 11. The resulting spectra (see Fig. 11) of 1,5-*O*-acetyl-2,4,6,7,8-*O*-methyl-3-deoxyoctitol for reducing terminal KDO confirmed the C-5 linkage to the Hep moiety. The same PMAA derivative for a 5-linked reducing terminal KDO has since been identified in methylation analysis of oligosaccharide from 1291_c. It has been assumed at this point that this linkage is conserved among all the 1291 oligosaccharides, as has been the case for the analogous

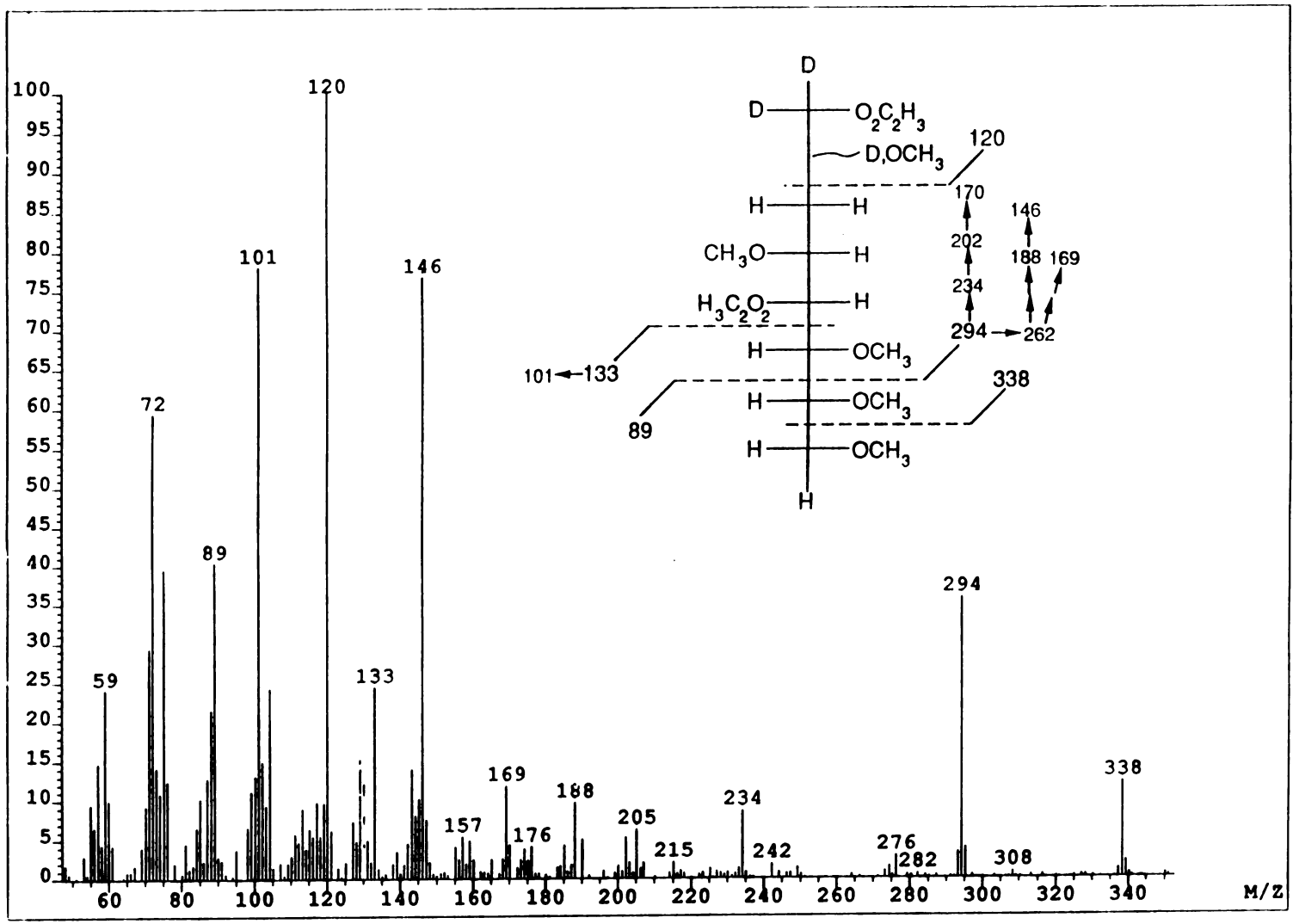


Figure 11. The EI spectrum of 1,5-*O*-acetyl-2,4,5,7,8-*O*-methyl-3-deoxyoctitol, the methylation product of reducing terminal 5-linked KDO using the procedure of York and colleagues (York et al 1985).

KDO C-5 linkage found in the oligosaccharides from *N. meningitidis* (Jennings, Lugowski, and Ashton 1983, Jennings et al. 1980, Michon et al. 1990).

Both LSIMS and methylation analyses of the oligosaccharide from strain 1291_a indicated the presence of some minor components of different structure. As shown in Table 2, the PMAAs for a number of minor monosaccharide components were also identified This included a terminal Gal (1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylgalactitol), a terminal Glc (1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol), and a 1,4-GlcNAc (2-acetamido-2-deoxy-1,4,5-tri-*O*-acetyl-3,6-di-*O*-methylglucitol). Also several different heptoses were identified not found in any of the other 1291 oligosaccharides, such as PMAAs apparently originating from 1,2,7-Hep (1,2,5,7-tetra-*O*-acetyl-3,4,6-tri-*O*-methylheptitol), 1,2,3-Hep (1,2,3,5-tetra-*O*-acetyl-4,6,7-tri-*O*-methylheptitol), and 1,3,6-Hep (1,3,5,6-tetra-*O*-acetyl-2,4,7-tri-*O*-methylheptitol) residues. Although the major component in the oligosaccharide from 1291_a was consistent with the structure from the wild type strain missing the non-reducing terminal Gal residue, the structure of some of these minor components obviously may not be consistent. This data suggests that there may be minor forms of the oligosaccharides from 1291_a differing by more than a single saccharide deletion from the oligosaccharides of the parent strain 1291wt. It should be pointed out, however, that these variant oligosaccharides appeared to be only minor components of the LOS.

In the methylation analysis of the oligosaccharide from the mutant strain 1291_b, a PMAA arising from a 1,4-Gal moiety (1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methylgalactitol) was found which is not present in the other 1291 oligosaccharides. LSIMS analyses of derivatized and underivatized

oligosaccharides from 1291_b supported a non-reducing terminal Hex→Hex→Hex→ branch (see Figs. 2, 6 and Table 1). This structure is also consistent with the methylation data in which approximately equal molar ratios were found for PMAAs for terminal Gal, 1,4-Gal and 1,4-Glc. Apparently, in the oligosaccharide from 1291_b a non-reducing terminal Gal is linked to the C-4 of the adjacent Gal, whereas in the oligosaccharides from 1291wt and 1291_a, a GlcNAc moiety is added to the C-3 position of the analogous Gal moiety. A structure consistent with this data would then be a non-reducing terminal branch: Gal1→4Gal1→4Glc. This trisaccharide had not been found in any of the other oligosaccharide fractions from the other strains of LOS. It may be worth noting that strain 1291_b had been formed after pyocin treatment of strain 1291_c, which had been isolated after treatment of strain 1291wt with pyocin (Dudas and Apicella 1988). Thus, 1291_b is a second generation mutant strain and, therefore, it might be expected to vary from the parent strain to a greater degree.

The non-reducing terminal trisaccharide in the structure of the oligosaccharide from 1291_b that we have proposed, Gal1→4Gal1→4Glc, is very similar to the P^k glycosphingolipid structure, Galα1→4Galβ1→4Glcβ1→1Ceramide. In collaboration with R. E. Mandrell we have investigated the binding of all the 1291 LOS by the commercially available anti-P^k MAb (Monocarb) using a solid phase radioimmunoassay (John et al. in press). The investigation showed the binding of the anti-P^k MAb to outer membrane complexes of the 1291_b LOS was >50 times greater than binding to 1291wt (see Table 3). Also, binding of the outer membrane complexes of 1291wt by the anti-P^k MAb was approximately 4 times greater than the binding of the MAb to strains $1291_{a,c,d,e}$ which had negligible background binding affinities (John et al. in press). The variation in

Table 3. Binding of P^k antigen MAb to outer membrane complexes of *N. gonorrhoeae* 1291wt and pyocin-resistant mutants 1291_{a-e}.

Strain	Counts
1291wt	283
1291_a	73
1291_b	15,443
1291_c	97
1291_d	67
1291_e	67

immunoaffinity supports the differences we have postulated for the structure of the 1291_b oligosaccharide and suggests that this structure may be present as a minor component, or as a biosynthetic precursor of in the 1291wt LOS. Differences have been observed in the expression of gonococcal LOS *in vitro* as compared that isolated from infected hosts. Thus, minor components of the LOS found *in vitro* may be major components of the LOS *in vivo*. Variations in LOS expression may also occur at a particular phase or in a particular site of infection.

As previously postulated (Dudas and Apicella 1988), the oligosaccharides from $1291_{a,c,d,e}$ represent a series of oligosaccharides that differ by sequential saccharide deletion and, as such, could represent biosynthetic precursors of the major 1291wt oligosaccharide. Following the sequential saccharide deletions of the oligosaccharides of the mutant strains, the structure of the larger of the two non-reducing terminal branches of the 1291wt oligosaccharide must be Gal1→4GlcNAc1→3Gal1→4Glc. The same (non-anomeric) tetrasaccharide structure has been identified previously in the oligosaccharides from *N. meningitidis* as Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1 (Jennings, Lugowski, and Ashton 1983, Michon et al. 1990). This tetrasaccharide is also identical to the terminal tetrasaccharide of paragloboside (Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→Cer) recognized by the MAbs 3F11 and 06B4. The epitope recognized by 3F11 and 06B4 was shown to be present in the 1291wt LOS, but not to occur in the LOS from any of the 1291 mutant strains (Dudas and Apicella 1988), which also supports our proposed composite structures.

D. MS/MS with CID analysis

MS/MS analysis with CID of the oligosaccharides from the 1291 parent and mutant strains was performed as described in Chapter II, B.1. In Figure 12, the negative-ion MS/MS spectrum is shown of HF-treated oligosaccharide from strain 1291_b. This glycan had been previously derivatized with HBEE and separated by HPLC and produced a parent ion at $(M-H)^-$ 1454. The oligosaccharide appears to be an anhydro species $(M-H_2O-H)^-$ on the basis of the mass of the molecular ion. This anhydro species could be formed during the isolation or work-up of the oligosaccharide. Anhydro oligosaccharides have been observed previously in gonococcal oligosaccharides and other oligosaccharides containing KDO on the reducing terminus (see Chapter II, B.2.) (John and Gibson 1990).

Reducing terminal fragment ions of the Y-type (Domon and Costello 1988) are the dominant fragments and are observed for all glycosidic bonds in the molecule i.e., m/z 1292, 1251, 1130, 1059, 968 and 381. In addition, reducing terminal X- and Z-type fragments are also present along with most of these Y-type fragment ions. Two non-reducing terminal ions produced by ring cleavage can be observed at m/z 221 and 262, which contain the non-reducing terminal Hex and HexNAc moieties respectively. Abundant reducing terminal fragment ions are present at m/z 1059 (Y_2) and 1041 (Z_2) for cleavage between the two Hep groups, and at m/z 381 (Y_1) for cleavage of the Hep-KDO bond. In general, LSIMS analysis of gonococcal oligosaccharides that have been derivatized with the hydrazino- or aminobenzoate reducing terminal reagents show very weak fragment ions from cleavage between the heptoses. In the higher energy CID

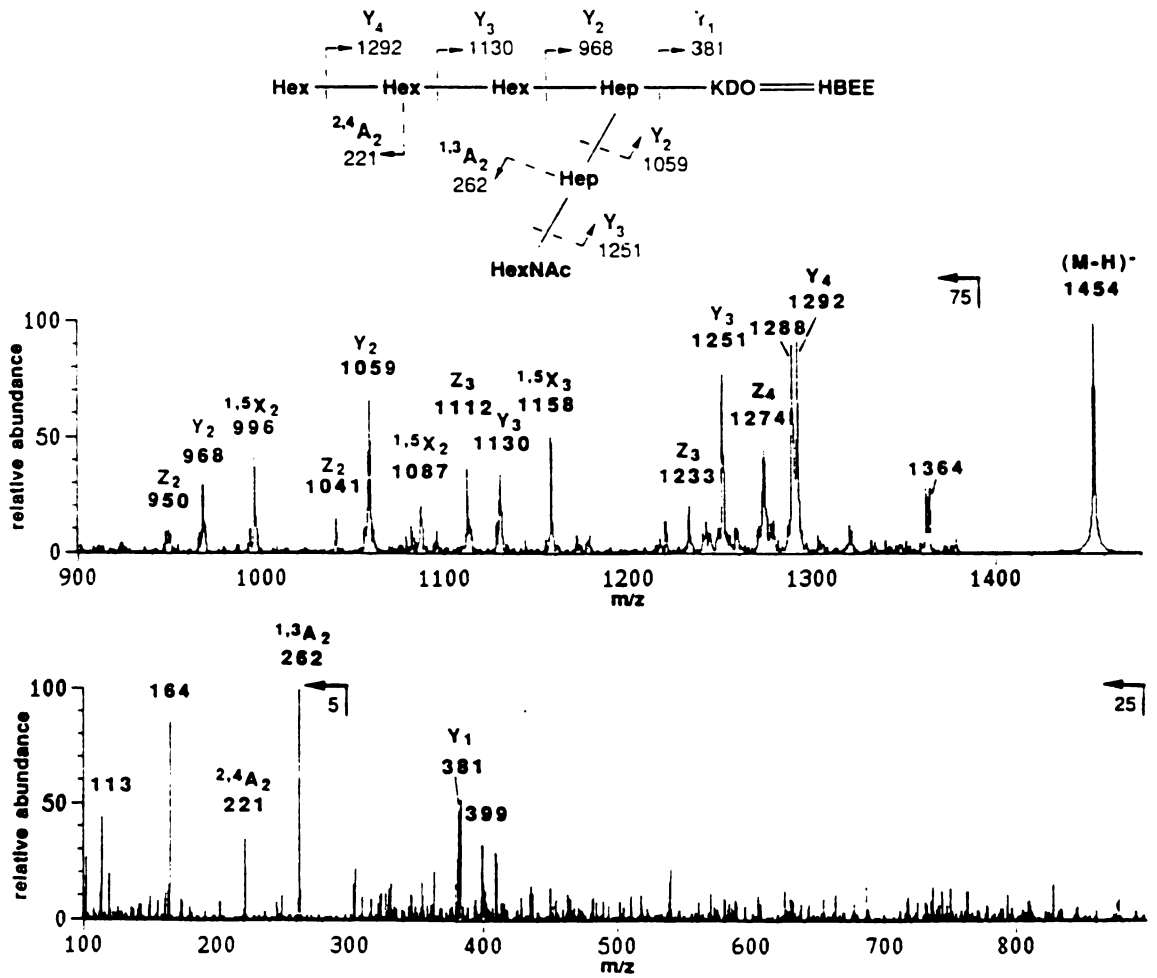


Figure 12. Negative-ion MS/MS with CID spectrum of HF-treated anhydro oligosaccharide from strain 1291_b derivatized with HBEE. The structure at the top illustrates the origin of the Y-type ions labeled in the spectrum.

conditions more fragmentation occurs of the Hep-Hep bonds in these derivatized oligosaccharides.

In the MS/MS spectrum shown in Figure 12, the extent and specificity of the fragmentation throughout the molecule produced by CID allows one to define the structure as Hex→Hex→Hex→(HexNAc→Hep→)Hep→KDO. The composition of the branches is unambiguously determined by the fragment ions, and the lack of those that would be produced by the cleavage of two remote site bonds. For instance, there is not a significant ion at m/z 765 for the Y-type loss of $Hex_3HexNAc$ from the reducing terminus, nor is there any significant ion at m/z 897 for the Y-type loss of HexNAcHepHex from the reducing terminus.

The MS/MS spectrum of HF-treated oligosaccharide from strain 1291_c that had been derivatized with HBEE (m/z 1310) is shown in Figure 13 (lower panel B). The figure in the upper panel illustrates the origin of the fragment ions observed in this spectrum. As was observed previously, Y-type reducing terminal fragment ions for cleavage of all the glycosidic bonds can be seen, along with some reducing terminal X- and Z-type ions. The presence of the Y-type ion at m/z 915 that corresponds to cleavage between the two heptoses was again particularly helpful in assigning the sequence of the inner core region of this molecule, as no fragmentation of the Hep-Hep bond could be observed in the corresponding LSIMS data (see Fig. 9). Two non-reducing terminal A-type ions are also present, one from each of the non-reducing terminal ions at m/z 262 and 221. The ion at m/z 164 ($C_9O_2NH_{10}$) is presumed to be due to the HBEE moiety itself, produced by cleavage between the two nitrogens (see Scheme 2).

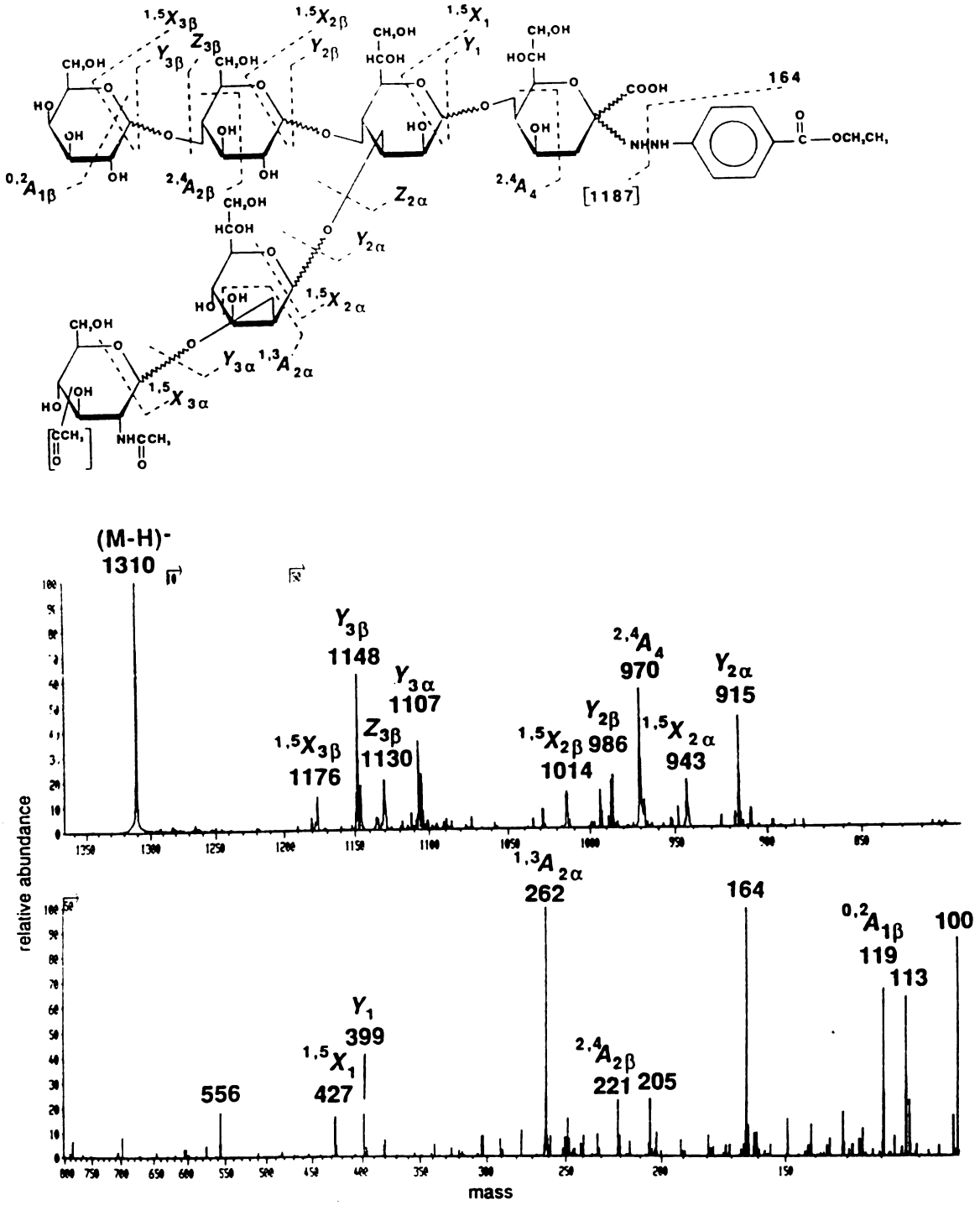
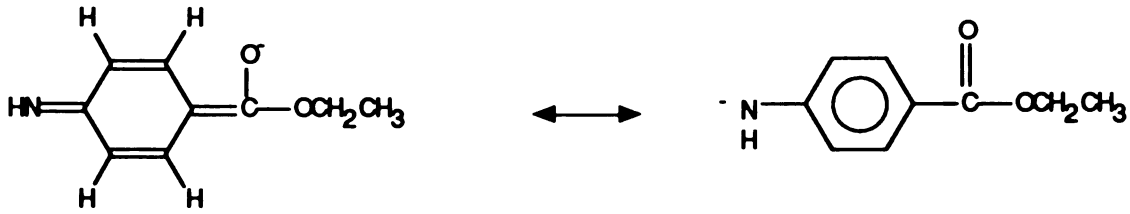


Figure 13. Negative-ion MS/MS with CID spectrum of HF-treated oligosaccharide from strain 1291_c derivatized with HBEE. The origin of the fragment ions for the spectra shown in both Figures 13 and 14 is shown in the structure at the top.

In Figure 14 the negative-ion MS/MS spectrum is shown of HF-treated oligosaccharide from 1291_c that had been previously derivatized with HBEE. The $(M-H)^-$ 1352 shows a difference of 42 Da compared to the $(M-H)^-$

Scheme 2



1310 shown in Figure 13. This 42 Da difference can be attributed to the presence of an *O*-acetate moiety, although the exact position was not determined yet. When the HF-treatment of these oligosaccharides was reduced from 4 days to 18-24 hours to decrease the amount of glycosidic bond cleavage observed, other moieties previously removed such as PEA and *O*-acetate were found to be still present as minor components (<10-20%). In this case the oligosaccharide containing a single *O*-acetate moiety but no PEA could be isolated during HPLC purification with HBEE (see inset Fig. 9). The spectrum of the *O*-acetylated oligosaccharides shown in Figure 14 is very similar to that shown in Figure 13. All of the fragment ions containing *O*-acetate have been shifted by 42 Da, such as the reducing terminal Y-type fragment ions at m/z 1190 (Y_3) and m/z 1028 (Y_2). A significant Y-type fragment ion for loss of HexNAc from the reducing terminus is not observed at m/z 1149; however, a significant ion can be seen at m/z 1107 for the loss of HexNAc-OAc. The non-reducing terminal A-type fragment ion containing HexNAc (m/z 262) is seen, but only weakly in the spectrum in Figure 14. However, an abundant fragment ion can be observed at m/z 304 corresponding to the A-type fragment ion for HexNAc-OAc. Thus, when compared with the spectrum in Figure 10 this spectrum

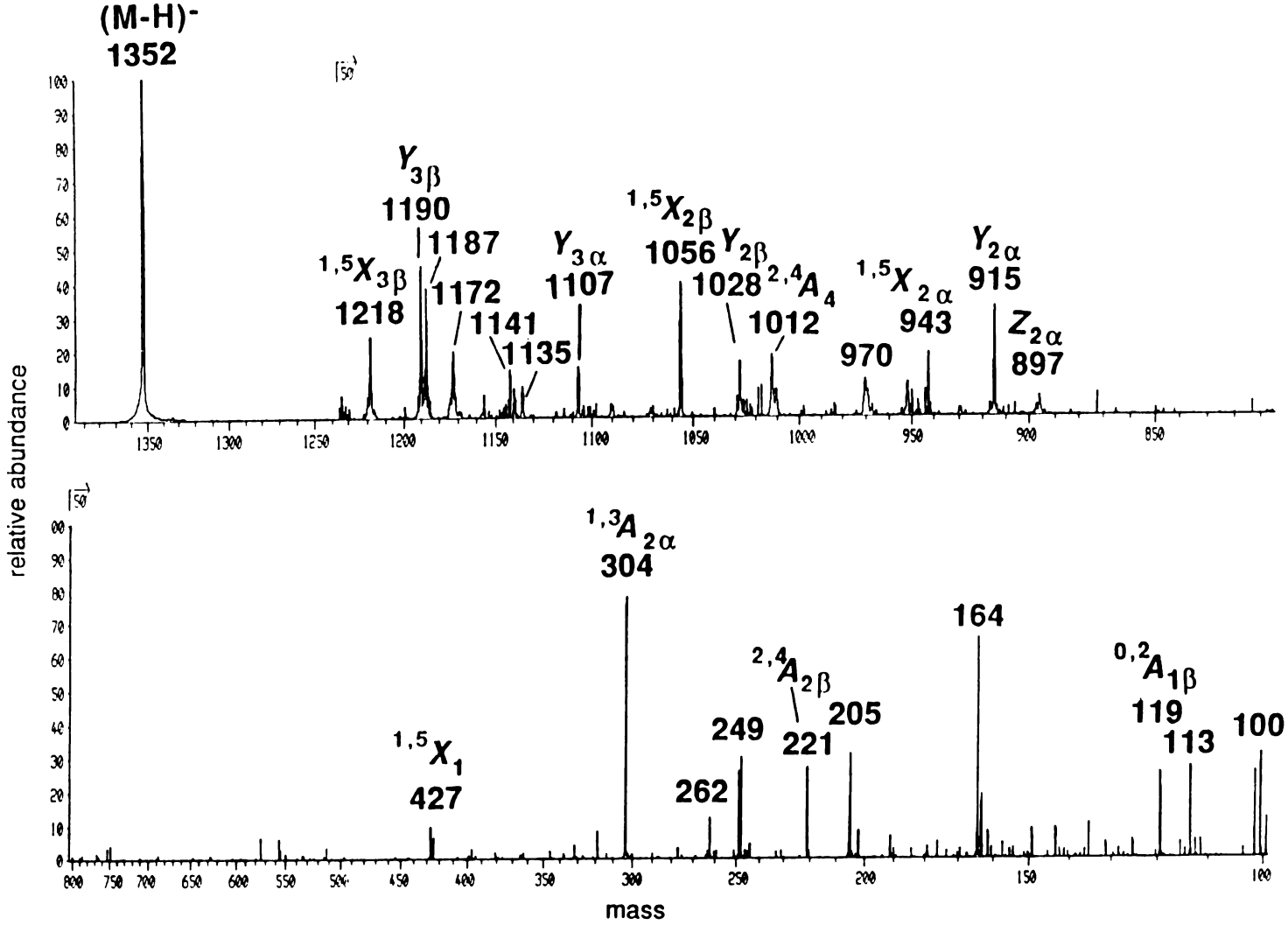


Figure 14. Negative-ion MS/MS with CID spectrum of HF-treated oligosaccharide from strain 1291_c containing an *O*-acetate derivatized with HBEE. The origin of the fragment ions is shown in the structure at the top of Figure 13.

establishes the position of the *O*-acetate group on the non-reducing terminal HexNAc moiety. Assignment of an *O*-acetate moiety observed in meningococcal LOS to the analogous non-reducing terminal HexNAc moiety has also recently been made using mass spectrometric methods (Michon et al. 1990).

Again in the spectrum shown in Figure 14 an abundant ion is present at m/z 164, presumably due to the HBEE moiety (see Scheme 2). A fragment ion is present in the spectrum in Figure 14 at m/z 1187 which may also be produced by cleavage of the HBEE-KDO linkage. This would presumably occur between the two nitrogens of HBEE with some accompanying proton transfer and charge retention on the oligosaccharide. In addition to the labeled ions, the ions at m/z 1135 and 1172 would correspond to the $^{1,5}X_{3\alpha}$ ion and the $Z_{3\beta}$ ion respectively. The origin of the fragment ion at m/z 1141 has not been determined.

In Figures 15 and 16, the MS/MS spectra are shown of oligosaccharides from strain 1291_d that have been previously derivatized with OPH. These oligosaccharide were not treated with HF and would, therefore, still contain PEA. In Figure 15, the parent ion at m/z 1149 gives a mass consistent with a composition of KDOHep$_2$HexNAcPEA plus OPH. Two of three possible reducing terminal Y-type ions at m/z 946 (Y_3) and 439 (Y_1), and two of three possible non-reducing terminal C-type ions at m/z 535 (C_2) and 727 (C_3) can be observed in this spectrum. At m/z 140, an abundant ion for phosphoethanolamine is observed. However, two other ions at m/z 298 and 330 are more difficult to explain (see Scheme 3). The ion at m/z 330 is postulated to be a HepPEA moiety formed by two remote site bond cleavages with a loss of H_2 (M_r of HepPEA plus H_2O is 333, therefore

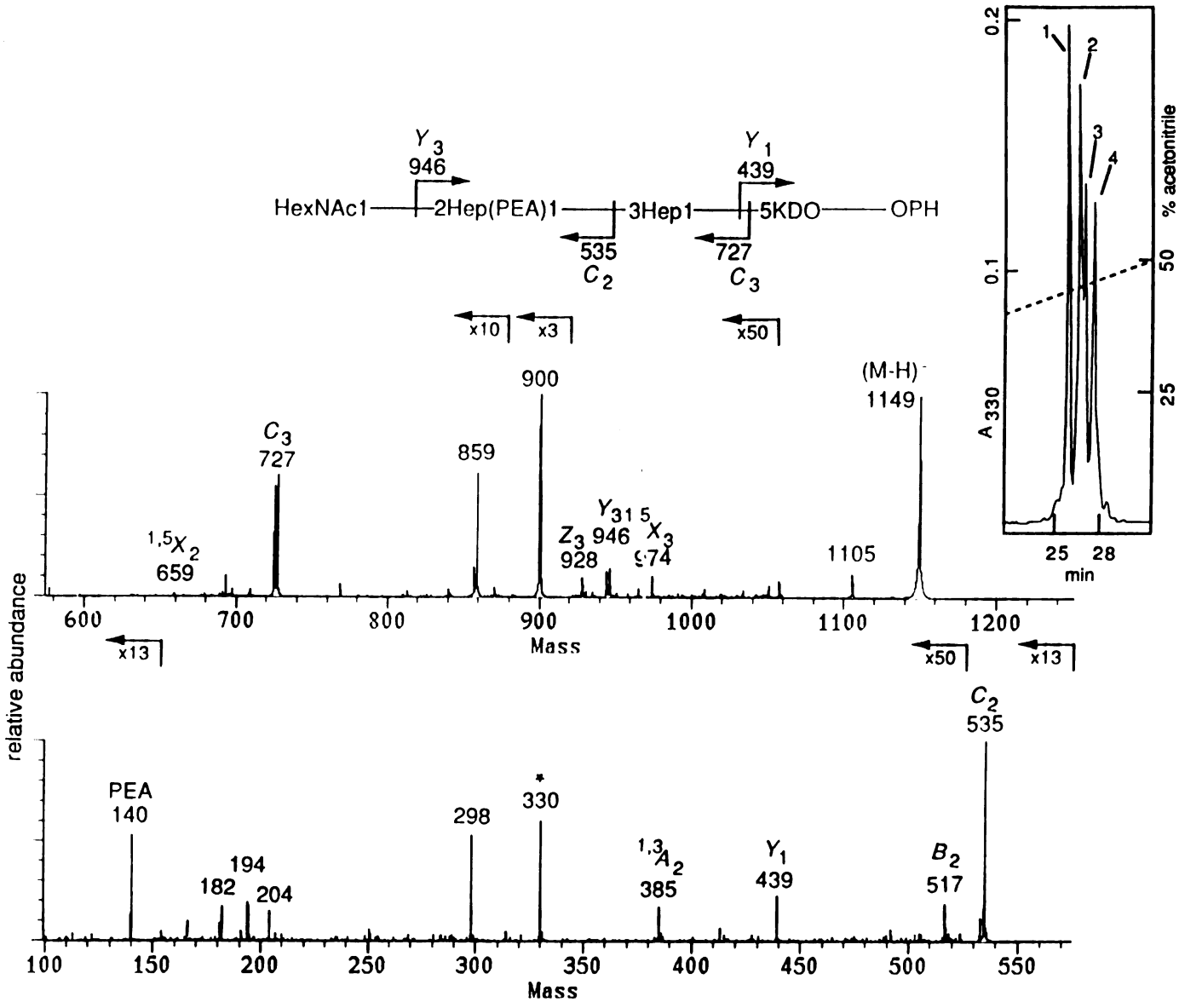


Figure 15. Negative-ion MS/MS with CID spectrum of oligosaccharide from strain 1291_d containing a PEA moiety derivatized with OPH. The inset contains the reverse-phase HPLC chromatogram of the 1291_d oligosaccharide after derivatization with OPH. The origin of some of the fragment ions in the spectrum is shown in the structure at the top.

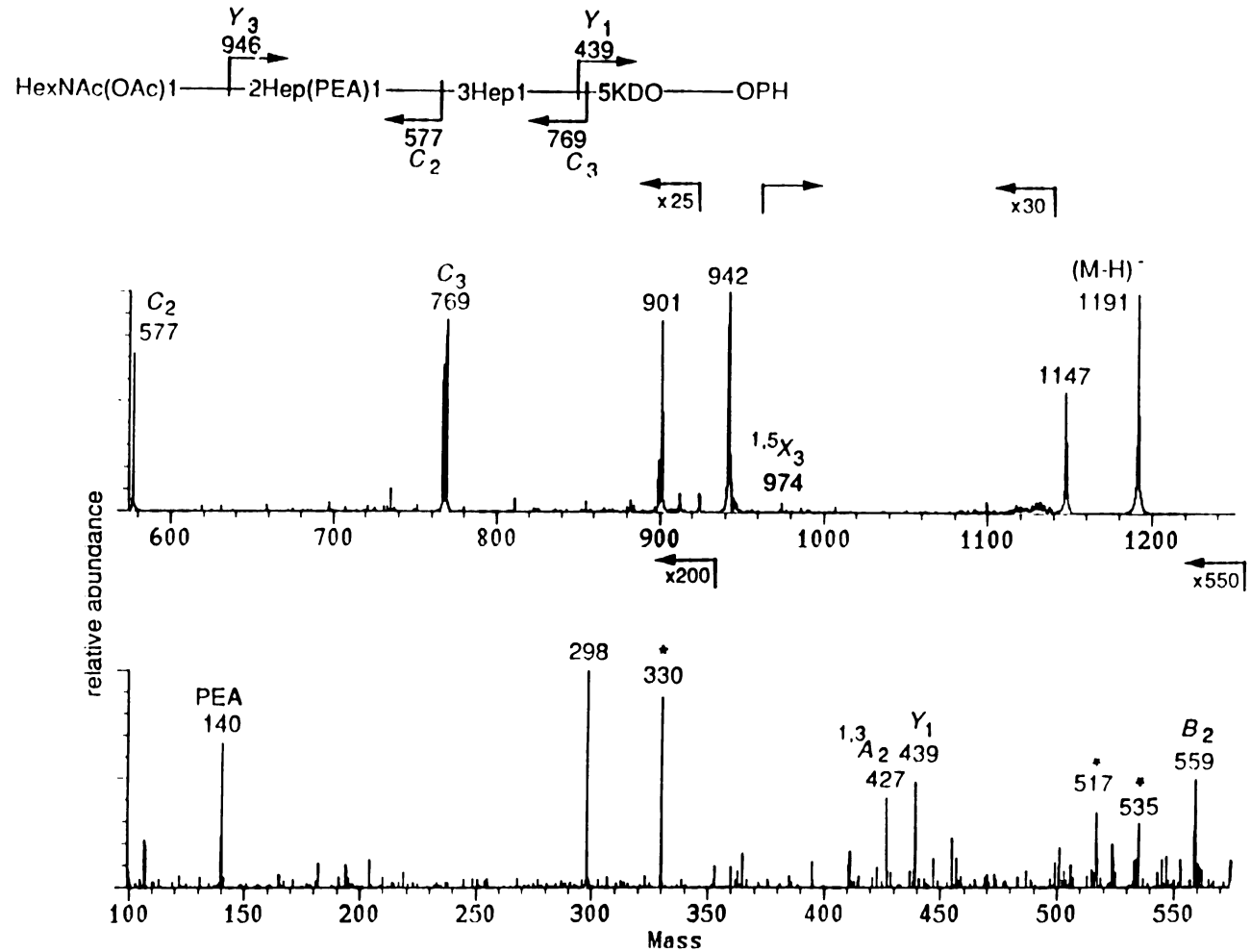
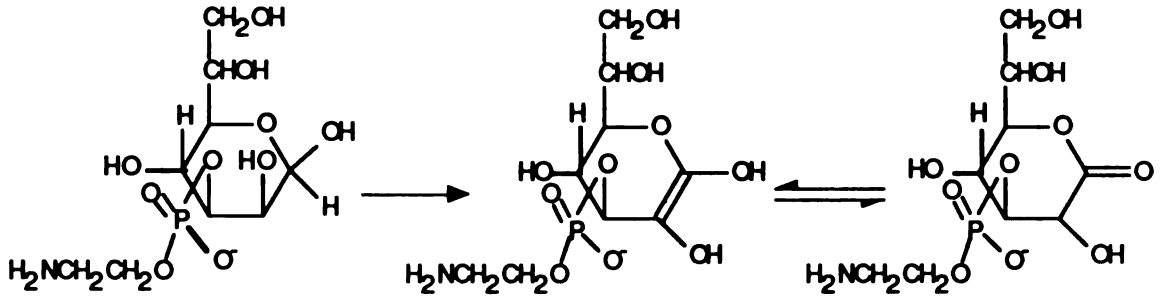


Figure 16. Negative-ion MS/MS with CID spectrum of the oligosaccharide from 1291_d derivatized with OPH, containing both PEA and *O*-acetate moieties. The ions marked with an asterisk are postulated products of remote site two bond cleavages. The structure at the top of the figure illustrates the origin of some of the fragment ions.

(M-2H-H)$^-$ would be 330). The ion at 298 is likely related in some way to the HepPEA moiety at m/z 330.

Scheme 3



In this spectrum shown in Figure 15 there is not a significant ion at m/z 262 for the $^{1,3}A_2$ ion corresponding to the non-reducing terminal HexNAc. However, there is an ion at m/z 385 which is equal to 262 plus 123, the mass shift of one PEA moiety. Is the PEA moiety attached to the HexNAc? All three of the types of reducing terminal fragment ions are present at m/z 974, 946, and 928 for loss of HexNAc itself (without modification) from the molecular ion. Thus, the PEA group is not directly attached to the HexNAc residue. If the postulate for the ion at m/z 330 is correct then the PEA moiety should be linked to one of the Hep residues. In the reducing terminal A-type fragments ring cleavage of the adjacent sugar occurs with charge retention on the non-reducing terminus (see Chapter II, Fig. 4). Two of the carbons of the Hep moiety, C-2 which is glycosidically linked to C-1 of the HexNAc and C-3, are part of the $^{1,3}A_2$ fragment ion for loss of HexNAc. Thus, there is only one possibility for the linkage to the PEA moiety, i.e., the C-3 of the second Hep residue from KDO.

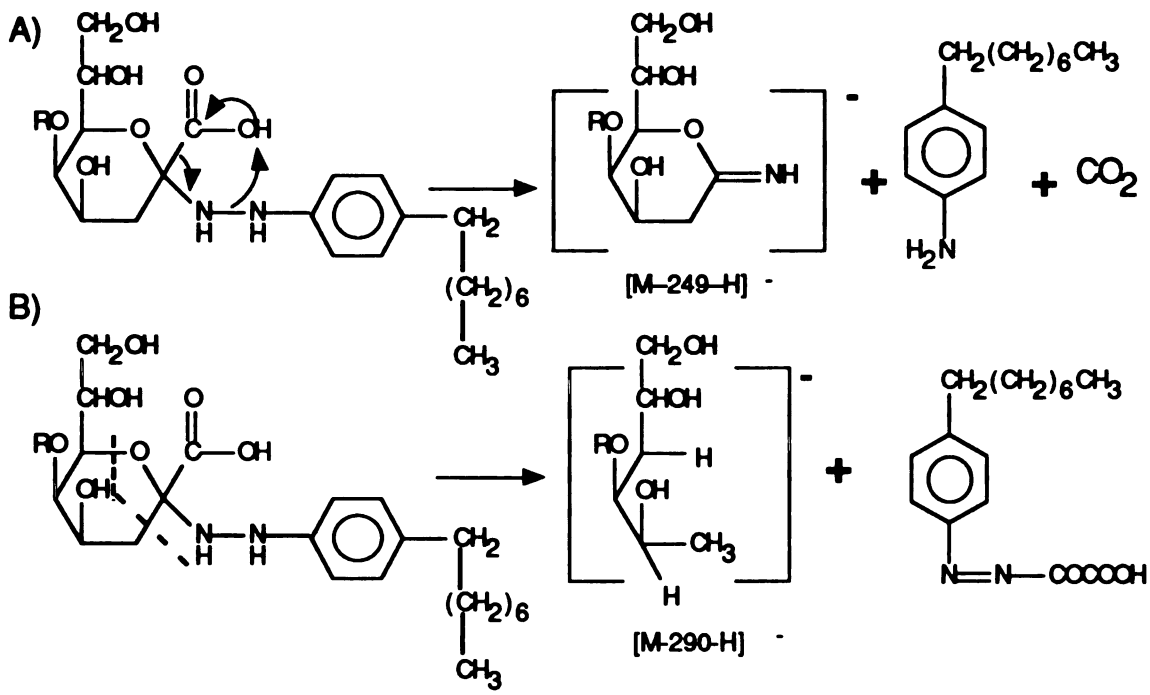
Two abundant ions at m/z 900 and 858 differ from the molecular ion by 249 and 290 Da, respectively. The same losses from the molecular ion can be observed in the spectrum shown in Figure 16 at m/z 942 and 901. The parent ion in the spectrum shown in Figure 16 at (M-H)$^-$ 1191

corresponds to an oligosaccharide of composition KDOHep$_2$HexNAcPEA plus OPH and an *O*-acetate moiety. The previously observed non-reducing terminal $^{1,3}A_2$ fragment ion for loss of HexNAc at m/z 385 (Fig. 15) can be observed at m/z 427 (Fig. 16) shifted by 42 (the mass of *O*-acetate). Again in Figure 16, the ion at mass 140 would correspond to PEA and the ions at m/z 298 and 330 are also present (see Scheme 3). These same low mass fragments ions at m/z 140, 298, and 330 that have been postulated to contain phosphate have also been observed in the MS/MS spectra of other gonococcal oligosaccharides containing PEA. Two of three possible non-reducing terminal C-type fragment ions can be observed in this spectrum also, along with two of three possible reducing terminal Y-type fragment ions. (The Y-type fragment ion at m/z 946 is significant in this spectrum, but obscured by the very abundant ion at m/z 942.) The ions marked with an asterisk at m/z 517 and 535 are presumably products of remote site two-bond cleavages. Cleavage of the *O*-acetate from the B_2 fragment ion at m/z 559 would yield the ion at m/z 517. Similarly, cleavage of the *O*-acetate from the C_2 fragment ion at 577 would yield the ion at m/z 535. The ion at m/z 330 has been discussed previously. Overall, the spectrum shown in Figure 16 supports the assignment of the PEA moiety to C-3 of the second Hep residue from KDO.

The non-reducing terminus of the oligosaccharides whose spectra are shown in Figures 15 and 16 is different by one *O*-acetate moiety. However, fragment ions corresponding to the loss of 249 Da and 290 Da from the molecular ion are present in both spectra (m/z 900 and 859 in Figure 15, and m/z 942 and 901 in Figure 16). Thus, the loss of 249 Da and 290 Da must be from the reducing terminus which is the same in both oligosaccharides. Cleavage between the two nitrogens of the OPH group would produce a loss

of a molecule of 205 Da. If this occurred simultaneously with decarboxylation, the total loss from the parent ion would be 249 Da (see Scheme 4). The loss of mass 290 from the molecular ion could be due to a ring cleavage analogous to the commonly observed $^{1,5}X$-type cleavages, with a 2 proton migration and charge retention on the oligosaccharide (see Scheme 4).

Scheme 4



The HPLC shown as an inset in Figure 15 was obtained by injection of approximately 100 μg of 1291_d oligosaccharide that had been derivatized with OPH, but not treated with HF. The spectrum shown in Figure 15 of the oligosaccharide without an *O*-acetate moiety was obtained from peak 1 of the HPLC. The spectrum shown in Figure 13 of the oligosaccharide containing an *O*-acetate group was obtained from peak 2 of the HPLC chromatogram. The oligosaccharide collected from peaks 3 and 4 produced the same molecular ion in LSIMS analysis at m/z 1191. Thus, these

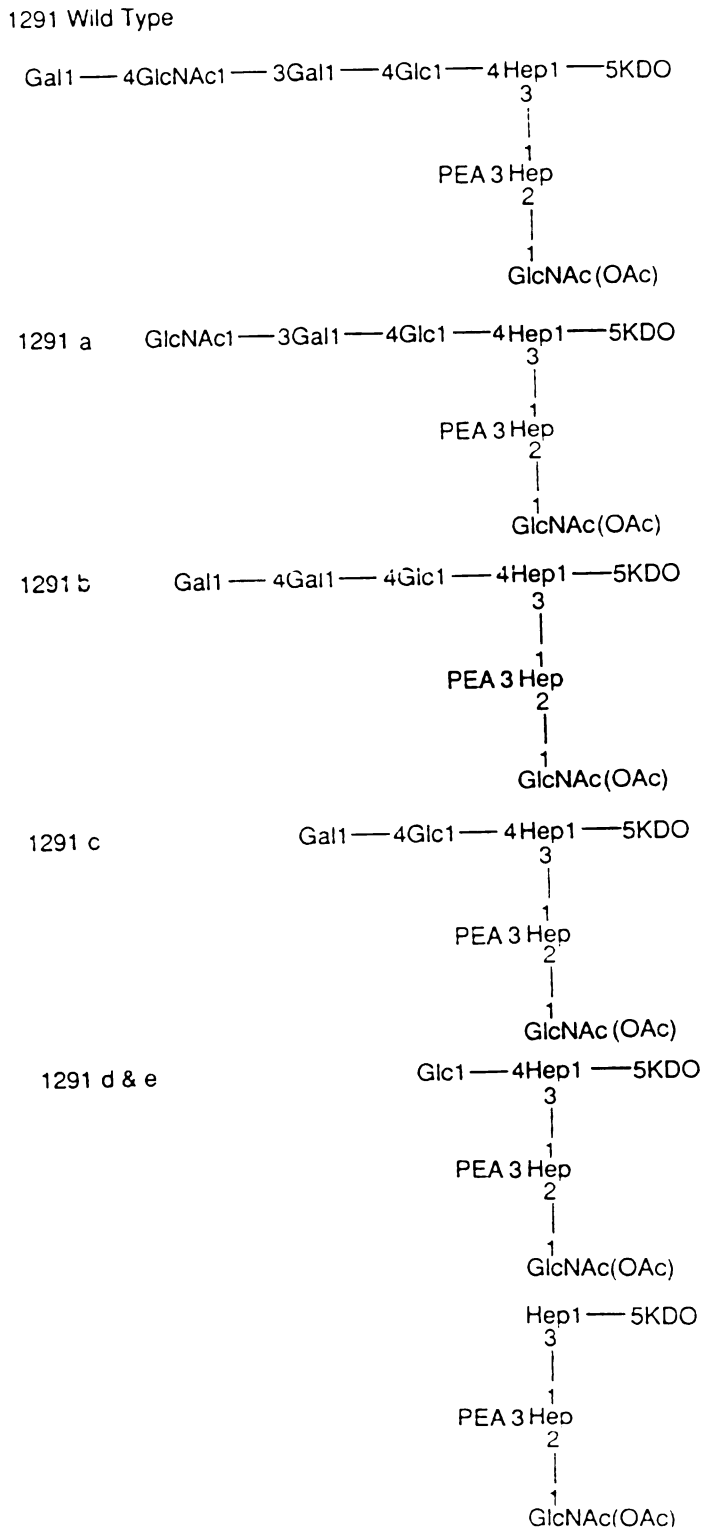
molecular ions were identical to the parent ion in the spectrum shown in Figure 16, collected from peak 2 of the HPLC shown in Figure 15. The spectra of the oligosaccharides from 1291_d shown in Figure 15 and 16 provide further support for the proposed sequence of the inner core region of the glycan, as well as the assignment of the position of the PEA moiety.

Other MS/MS spectra have also been obtained of larger, derivatized gonococcal oligosaccharides containing a PEA moiety. Much of the fragmentation observed in these spectra could not be readily interpreted. In the spectra shown in Figures 15 and 16 of the OPH-derivatized gonococcal tetrasaccharides, a number of unusual fragment ions were observed. The presence of phosphate appears to direct much of the fragmentation. The most abundant ions in these spectra are only observed in oligosaccharides that contain PEA. For example, the fragment ions for loss of 249 Da and 290 Da from the reducing terminus and the fragment ions at m/z 298 and 330 are not usually found in spectra of non-phosphorylated oligosaccharides.

E. Partial structures

The proposed partial structures for the oligosaccharides isolated from the LOS of strains 1291wt and the pyocin-resistant mutant strains 1291_{a-e} are shown in Figure 17. The structures shown are derived from the LSIMS, MS/MS and methylation analysis data. In the LSIMS analysis of underivatized oligosaccharides (see Fig. 15 and Table 1), it appeared that most of the oligosaccharide species contained the PEA moiety but perhaps only 50% were *O*-acetylated. The presence of even a single acetate group

Figure 17. Proposed Partial Structures of Oligosaccharides from Gonococcal 1291Wild Type and Mutant Strains



should increase the surface activity and, therefore, the sensitivity of detection of an oligosaccharide. Although in LSIMS analysis the relative abundance of the molecular ions of the *O*-acetylated oligosaccharides was significantly greater for strains 1291wt and 1291_{a-c} than for the oligosaccharides lacking the *O*-acetate (see Fig. 5), it is likely that the actual ratios are closer to being equal. On the other hand, during the initial acid hydrolysis of the LOS some *O*-acetate groups could have been hydrolyzed and, therefore, the degree of *O*-acetylation present in the oligosaccharide products may not be reflective of the LOS itself. Indeed, it is possible all of the oligosaccharides were *O*-acetylated, but that some of the *O*-acetate groups were hydrolyzed during the initial work-up in acetic acid.

The major component of strain 1291_a is shown in Figure 17. A number of minor species with apparently various monosaccharide components and slightly different structures were also present. The amount of structural variation expressed by 1291_a might very well be significant in terms of the understanding the total repertoire of oligosaccharide structures available to the gonococcus, as well as in comprehending the genetic regulation of the expression of the oligosaccharides.

The structures of lactosylceramide, the precursor of the glycosphingolipids, paragloboside, a precursor of the glycosphingolipid ABH blood group antigens, and the P^k antigen, a precursor of the P blood groups are shown in Figure 18. The oligosaccharide structures which possess human ABH and Lewis blood group activity occur as oligosaccharides in milk and urine. They also occur as part of glycoproteins in bodily secretions and fluids, such as saliva, gastric juices, milk, sweat and tears. These glycoproteins are approximately 80-90%

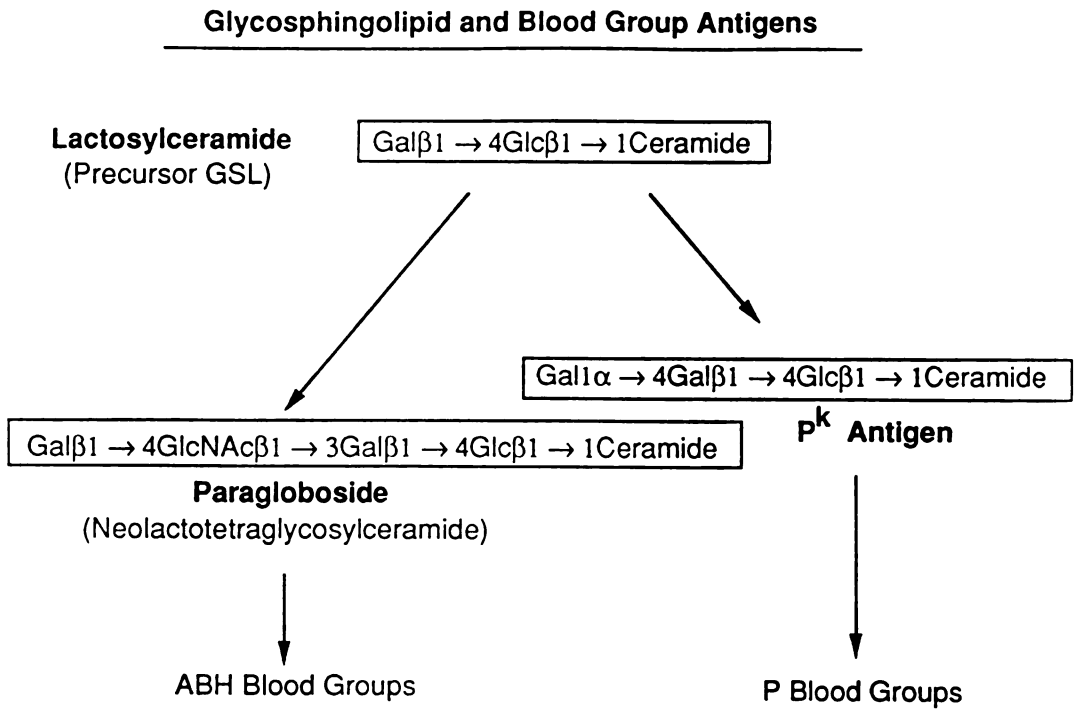


Figure 18. The structures of glycosphingolipid precursors to human blood group antigens are shown. The disaccharide of lactosylceramide is the same as the (non-anomeric) non-reducing terminal disaccharide of the oligosaccharide from 1291_c. The trisaccharide of the P^k antigen is the same as the (non-anomeric) structure of the non-reducing terminal trisaccharide of the oligosaccharide from strain 1291_b. The terminal tetrasaccharide of paragloboside is repeated in non-reducing termini of both the 1291wt oligosaccharide and the smaller of the two main components of the F62 oligosaccharide. It is very likely that the anomeric configurations of these portions of the gonococcal oligosaccharides are identical to those of the glycosphingolipids.

carbohydrate and only 10-20% protein. Glycosphingolipids are mainly found on the surfaces of erythrocytes and tissues (Lemieux 1978). The P^k globotriaosyl ceramide antigen has been found on human red blood cells, fibroblasts, blood vessels, smooth muscle cells of the digestive tract and the urogenital system, and to lesser extent on the glomeruli and tubules in the kidney (Brodin et al. 1988, Kasai et al. 1985).

The similarity of the structure of paragloboside to the oligosaccharides from strain 1291wt, and the similarity of the P^k antigen to the oligosaccharides from mutant strain 1291_b almost certainly has biological significance. The ability of the gonococcus to produce glycolipids containing these structures, which are expressed in human cells, tissues and fluids, could protect the bacteria from recognition by the human immune system. One of the roles of the carbohydrates of glycolipids and glycoproteins is thought to be in cell-cell adhesion. The expression of oligosaccharides similar to glycosphingolipids might increase binding of the bacteria to human tissues and cellular components and, thereby, increase the ability of the bacteria to establish itself.

The expression by the 1291wt strain of the oligosaccharide structure produced by the mutant strain 1291_b appeared to be only a very minor component of the wild type oligosaccharides. However, the biosynthetic capacity of the gonococcus was revealed by the structure of oligosaccharide of the 1291_b mutant strain. The increased binding of the anti-P^k antigen MAb to the wild type oligosaccharides compared to those from $1291_{a,c,d,e}$ suggests that this structure is produced by 1291wt *in vitro*. Since the expression of the LOS has been shown to be different *in vitro* compared to *in vivo* , what may be a minor LOS component *in vitro* may very well be a more major component *in vivo*. Thus, understanding the real significance of

these oligosaccharide structures upon the interactions of the gonococcus with its human hosts will require structural elucidation of the oligosaccharides from the LOS of gonococci from infected patients. However, it can be postulated that the expression of these oligosaccharide structures represents a form of host mimicry by the gonococcus.

The treatment with pyocin did effect the oligosaccharide structures of the wild type LOS. If monosaccharides are sequentially added to the growing oligosaccharide chain by specific glycosyltransferase enzymes, then the absence of one glycosyltransferase would likely block the synthesis of the rest of the oligosaccharide chain. Thus, if pyocin were acting to suppress different genes coding for specific glycosyltransferases in the mutant strains, the production of a series of oligosaccharides by the mutant strains representing biosynthetic precursors to the wild type oligosaccharide would be expected. The recent observation that pyocin DNA is directly inserting into the gonococcal genome (Apicella, M. A., unpublished observations) raises some interesting questions concerning the exact mechanism by which suppression of expression of the glycosyltransferases could be occurring. Does pyocin randomly insert into the genome and, thereby, interrupt the expression of individual genes coding for the specific glycosyltransferases in the resistant surviving strains? Or does pyocin effect some master control site for expression of glycosyltransferases and, thus, oligosaccharide structure?

Certainly, understanding LOS biosynthesis in *N. gonorrhoeae* is of general interest, but more importantly could be relevant for future therapeutic intervention in infection. The success of a carbohydrate vaccine could depend on inclusion of sufficient and specific LOS types. A carbohydrate-based vaccine could prove to be impractical because

recognition by the human immune system of the LOS components of serum-resistant strains may be impossible due to the presence of nearly identical glycans in the host. Another possible problem is that due to the similarity between the LOS and human glycosphingolipids a carbohydrate-based vaccine may cause some auto immune reactions. If biosynthesis of the oligosaccharide portion of the LOS occurs on the outer cell membrane, then biosynthetic precursors would be expected to be exposed. Vaccination with a biosynthetic precursor that could elicit antibody recognition of the precursor could be one strategy to overcome evasion of the bacteria by this form of host mimicry.

Specific glycosyltransferases could potentially prove to be excellent chemotherapeutic targets. Alternatively, perhaps a monosaccharide analog, such as an analog of KDO, would stop the biosynthesis of the LOS prematurely. This could prevent further infection by bacteria by inhibition of the binding of the bacteria to the host tissues, or unmask the bacteria so that it is recognized by the immune system. Investigation of the biosynthesis and structural elucidation of the gonococcal LOS should continue to be active fields of endeavor.

CHAPTER V. CONCLUSIONS

A. Methodological improvements

The development of microscale methods for the analysis of the oligosaccharides of gonococcal LOS was one of the major goals of this work. Using hydrazino reducing terminal derivatives for oligosaccharide separation and analysis, *O*-deacylated LOS for LSIMS, and HPAE-PAD for purification of gonococcal LOS have greatly improved the overall strategy for the structural determination of LOS. The development of these techniques has also reduced the amount of sample required for such analyses. Figure 1 shows a schematic of the incorporation of these procedures into an overall method of sample preparation.

One of the advantages of the reducing terminal hydrazino reagents for oligosaccharides is the simplicity and speed of the derivatization procedure. Another advantage is the quality of the mass spectra, which is apparent in the increased molecular and fragment ion abundances relative to underivatized oligosaccharides (see Chapter IV, Fig. 5 and 6). The quality is similar to that obtained of reductively aminated oligosaccharides (Gillece-Castro et al. 1987, Webb, et al. 1988). Also, a great deal less sample was required for LSIMS analysis of the derivatized oligosaccharides compared to underivatized oligosaccharides. We estimate approximately 15-20 times more underivatized sample would have to be used to obtain the same signal/background ratios of the molecular and fragment ions in LSIMS analysis compared to oligosaccharides derivatized with HBEE. Use of the more hydrophobic phenylhydrazine reagents decreases the amount of

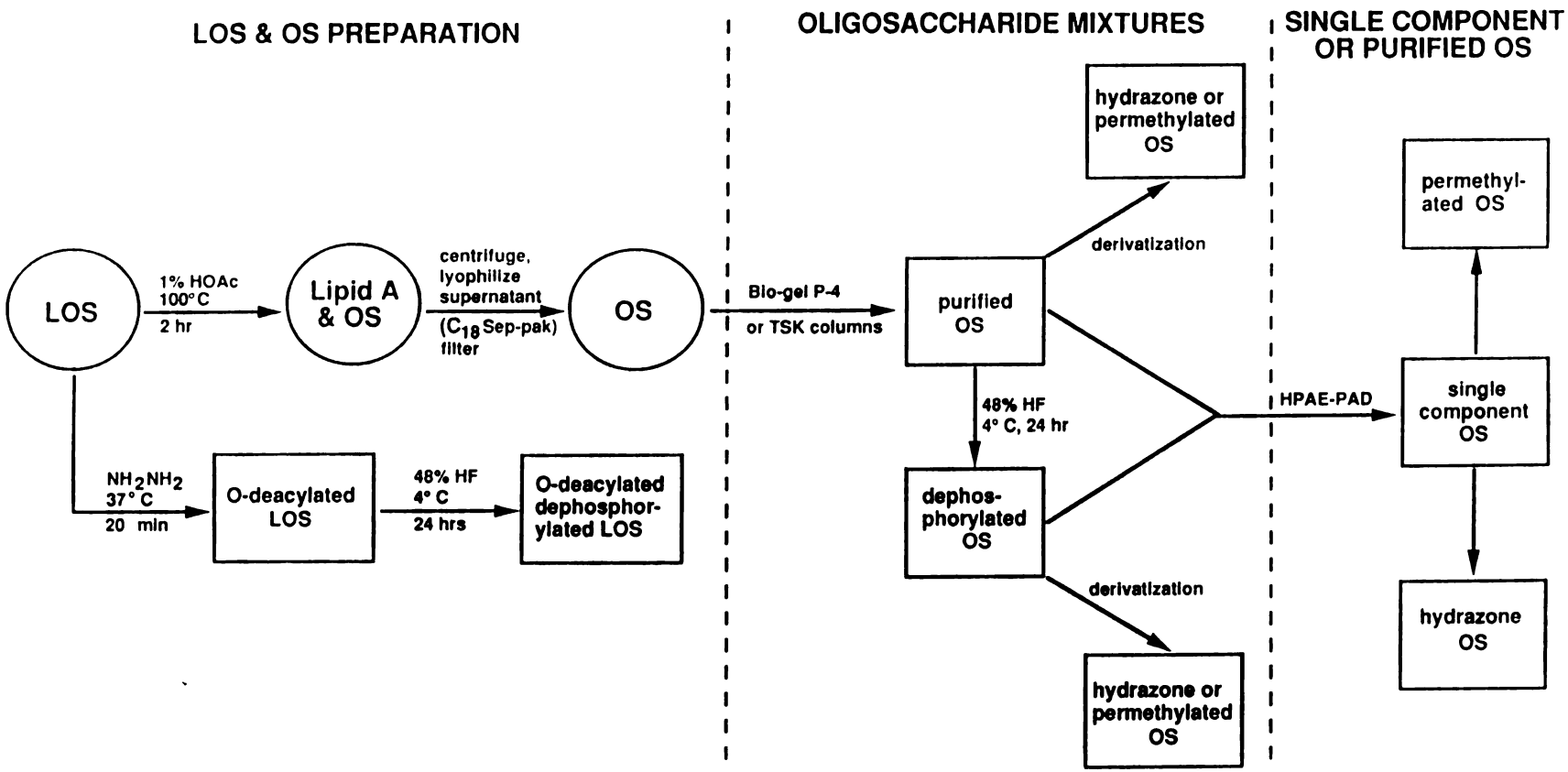


Figure 1. Overall scheme for the preparation of LOS and oligosaccharide isolated from LOS for structural analysis by mass spectrometry.

sample required for analysis compared to oligosaccharides derivatized with HBEE. Furthermore, under- and overderivatization of these bacterial oligosaccharides, which can greatly complicate mass spectral analysis, has not been a problem and would not be expected.

In general, the fragmentation patterns of the derivatized oligosaccharides observed in LSIMS and MS/MS spectra have been very predictable and sequence specific. Much of this fragmentation can be interpreted in light of the nomenclature proposed by Domon and Costello (Domon and Costello, 1988). However, remote-site double bond cleavage is apparently occurring, although less frequently than those produced by simple glycosidic bond cleavage (B, C, Y and Z ions) or those produced by concerted ring cleavages (A and X ions). Thus, the branching pattern of much of the oligosaccharide can usually be determined from LSIMS analysis of the hydrazone-oligosaccharides.

The use of MS/MS with CID was found to increase the amount of fragmentation observed, particularly of the Hep-Hep glycosidic bonds, compared to that obtained with two-sector LSIMS analysis. This enabled us to determine the complete sequence of gonococcal oligosaccharides. The fragmentation of underivatized oligosaccharides in MS/MS with CID analysis was also very extensive and sequence specific (see Chapter III, Figs. 6B and 7B, and Chapter IV, Fig. 13). Spectra obtained from both derivatized and underivatized oligosaccharides made it possible to observe low mass non-reducing terminal fragment ions which would be obscured by matrix ions in two-sector LSIMS analysis. These non-reducing terminal fragment ions can be very informative in many cases. However, though the fragmentation of both underivatized and derivatized oligosaccharides was

quite similar in MS/MS with CID analysis, much less sample was required for derivatized oligosaccharides.

Which of the hydrazino reagents is best for derivatization of bacterial oligosaccharides for LSIMS and MS/MS analysis still needs further investigation. For example, we do not know if the yields of the longer chain alkyl phenylhydrazine reagents with the oligosaccharides from the gonococcal LOS are significantly different than the yields with HBEE. As the hydrophobicity of the derivatizing reagent increases the yield of the reaction would be expected to decrease because the reaction is performed in a fairly polar solvent required to solvate the oligosaccharide. Two other considerations in terms of LSIMS analysis is the overall signal/background ratios obtained of the molecular and fragment ions and the lifetime of the molecular ions. For example, if the molecular ions are so surface active that the fragment ions are largely gone before the entire mass range has been analyzed then some of them will be missed. Considering the current average scan time for the Concept MS/MS instrument is approximately five minutes this is clearly an important consideration in the success or failure of this approach. Thus, the signal/background ratios of the molecular and fragment ions over a number of minutes must be determined to adequately compare these various reducing terminal derivatives.

LSIMS analysis of O-deacylated HF-treated meningococcal LOS after incubation with cytidine monophosphoryl *N*-acetyl neuraminic acid revealed the presence of a non-reducing terminal sialic acid moiety. This moiety would have been completely hydrolyzed in the acetic acid hydrolysis step of the traditional procedure. As sialic acid is a common non-reducing terminal sugar in oligosaccharides from human glycoproteins and glycolipids, sialylation of the bacterial LOS may aid the bacteria in evasion

of the human immune system. Whenever the LOS is subjected to chemical treatments of any type during structural elucidation potentially biologically significant groups may be lost due to hydrolysis of labile linkages or degradation of molecular components. The *O*-deacylation procedure we have utilized with anhydrous hydrazine is quite mild and apparently quite specific for *O*-acyl groups. One disadvantage of this procedure is that the extent of the *O*-acetylation of the oligosaccharide is of interest and the *O*-acetate groups are also removed by the hydrazine treatment. This problem could be circumvented if an enzymatic method were used to specifically *O*-deacylate the fatty acid moieties of the lipid A. Also, potentially other groups lost or degraded during the treatment with hydrazine could be observed in LSIMS analysis after enzymatic hydrolysis of the *O*-acyl moieties.

The relatively poor sensitivity of detection of the *O*-deacylated LOS needs to be investigated. As discussed in Chapter II C., further purification of the *O*-deacylated LOS might improve the sensitivity of detection and a number of different matrices should be tried in LSIMS analysis of the *O*-deacylated LOS. Other methods of ionization could be evaluated for mass spectrometric analysis of intact or *O*-deacylated gonococcal LOS, such as plasma (Jardine et al. 1989) or laser desorption which might improve the desorption of the LOS, and thus the sensitivity of detection. Alternatively, electrospray ionization mass spectrometry could be used, and in at least one case has produced good ion abundance of an *O*-deacylated LOS from *H. influenzae* (N. J. Phillips and B. W. Gibson, unpublished results).

The apparent technical problems in determination of the linkages of the KDO moieties of the LOS was described in some detail in Chapter II,

C.4. Performing methylation analysis on the *O*-deacylated LOS did not reveal the KDO linkages, although it was successful with all the other sugar residues. However, such a strategy may still prove to be workable. Perhaps other methods of reducing the KDO carboxylates may be more efficient than the one used, such as the method of Taylor and Conrad using a water-soluble carbodiimide (Taylor and Conrad 1972). Further experimentation is required to determine the optimal hydrolysis conditions for the permethylated KDO moieties. This might be best approached by hydrolysis of the LOS for different lengths of time under a variety of conditions, followed by isolation of the monosaccharides from the lipid using a C_{18} Sep-Pak and analysis of the resultant monosaccharides by HPAE-PAD. Some differences may exist in the conditions required for hydrolysis of the permethylated LOS compared to the underivatized LOS, particularly in terms of the solvent to be used for the hydrolysis. However, because of the quantitative nature and the ease of the HPAE-PAD analysis this would be a good starting point. Then, based on the results of the hydrolysis of underivatized LOS a set of conditions could be applied for hydrolysis of the permethylated LOS. The products of this hydrolysis could be analyzed by perhaps either LSIMS or probe CI-MS to determine the integrity of the permethylated KDO residues. Another possible improvement in the strategy for determining the linkages of all KDO residues in the LOS is simply to begin with more sample. Quantitative recovery of PMAA derivatives of the KDO residues, while certainly advantageous, should not be necessary to solve the structure. This is particularly true if other methods can be used, such as mass spectrometric analysis of intact or *O*-deacylated LOS, to determine the number of KDO residues per LOS molecule.

The use of high-pH anion exchange chromatography with pulsed amperometric detection (HPAE-PAD) has improved the resolution of oligosaccharides in general as discussed in Chapter II, A.1. and D.2. Particularly with very heterogeneous oligosaccharides, such as those derived from gonococcal LOS, such resolution may prove essential. For example, the results of the methylation analysis of the oligosaccharides from strain F62 were difficult to interpret. The presence of two major and one minor component was postulated to explain these results. The data would have been much clearer if the analysis had been performed on oligosaccharides purified by HPAE-PAD into single components.

A number of different gradients were applied to the separation of gonococcal oligosaccharides by HPAD-PAD with good results. However, it is likely that better resolution can yet be achieved using this system. For instance, although separation of phosphorylated oligosaccharides was performed, most of the analyses were of HF-treated oligosaccharides. Due to the presence of the negatively-charged acidic moieties, separations of oligosaccharides containing acidic groups such as phosphate can be obtained at lower pH than is normally used with neutral oligosaccharides. Separation of these phosphorylated oligosaccharides at lower pH could possibly improve their resolution. Or HPAE-PAD analysis of the phosphorylated oligosaccharides using conditions more similar to those for neutral oligosaccharides might lead to better separations.

As a method of compositional analysis, HPAE-PAD offers an excellent alternative to GC analysis of derivatized oligosaccharides. No derivatization is required, and the method is fast, very sensitive and accurate. In addition, KDO is detected as a single peak. Multiple injections of appropriate standards of known compositions (such as the

monosaccharides derived from the Ra LPS mutant of *S. typhimurium*) and analysis of a number of gonococcal oligosaccharide samples of known composition is necessary before the method is truly established for gonococcal oligosaccharides. Further exploration of oligosaccharide hydrolysis conditions that will maximize the release of intact KDO without promoting its degradation would also improve the application of this method to these and other bacterial oligosaccharides.

B. Biological implications of gonococcal LOS structures

The overall goal of this research described in this thesis was to obtain a better understanding of the structural basis of the role of the LOS in the immunochemistry and pathology of *N. gonorrhoeae*. In Figure 2 a generalized structural model of the gonococcal LOS is shown. To obtain the LOS structures, improvements were made in the methods for structural elucidation, while the analysis of the LOS from gonococcal strains F62 and 1291 was underway. Undoubtedly, structural elucidation of the LOS from additional gonococcal strains is required to conclusively correlate structural features with biological functions.

The structural analysis of the LOS from strain F62 revealed the presence of two major components that differ by a non-reducing terminal GalNAc moiety (see Chapter III). The smaller component contains a terminal *N*-acetyllactosamine which is apparently recognized by the MAb 3F11. This same MAb also bound to all adult erythrocytes tested. The MAb 3F11 recognizes human glycosphingolipids, such as paragloboside, which terminate in Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→1Cer, lacto-N-neotetraose. The components of this entire tetrasaccharide moiety are

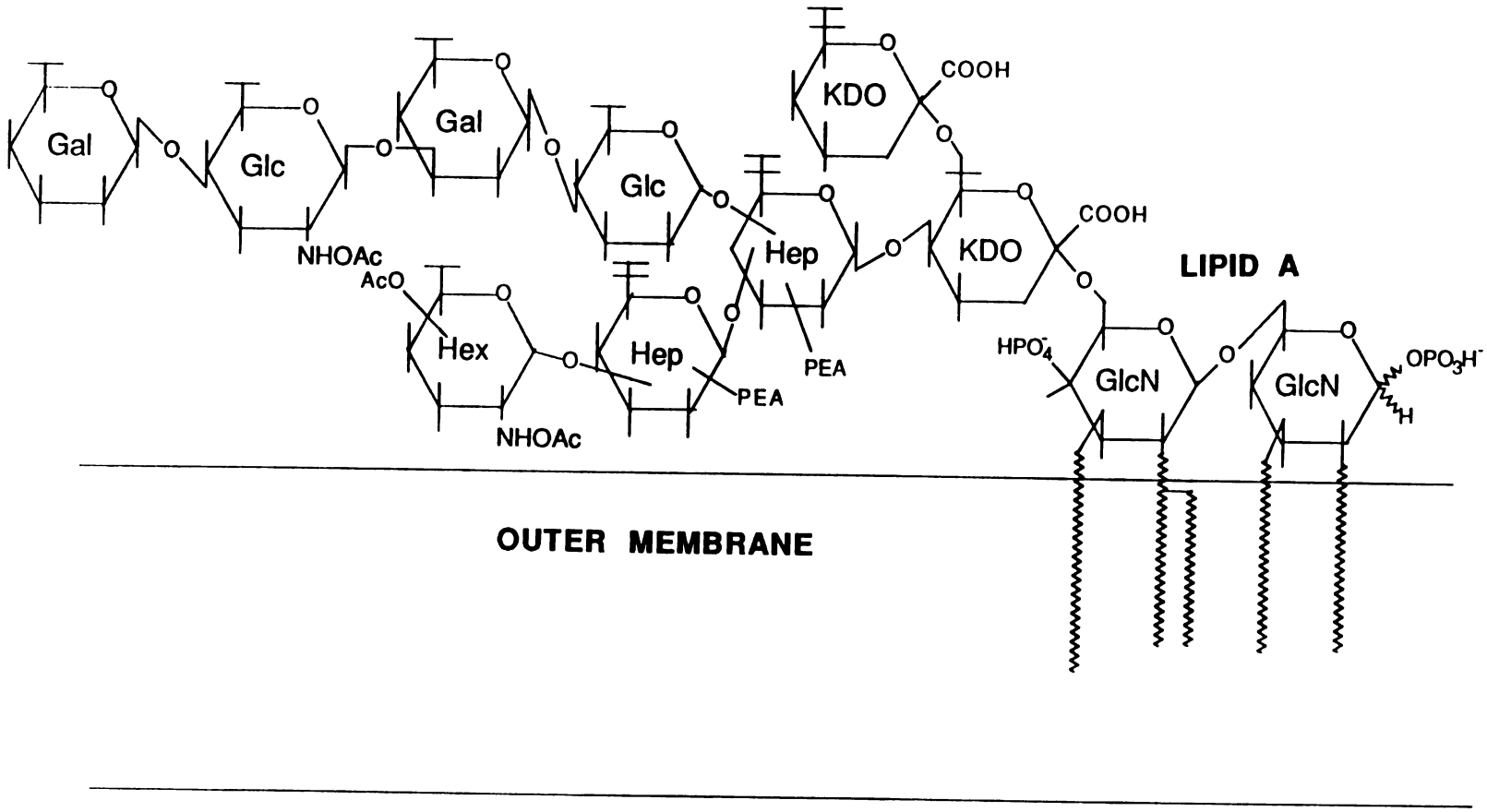


Figure 2. Structural model of the lipooligosaccharide of *Neisseria gonorrhoeae*.

present in the longest branch of the F62 oligosaccharides, and very probably the tetrasaccharide is reproduced in the gonococcal oligosaccharide. However, the anomeric configuration of the monosaccharides of this branch were not independently confirmed. The larger component of the F62 LOS was not recognized by 3F11, and would contain the non-reducing terminal GalNAc which must block recognition of the 3F11 epitope.

The presence of the 3F11 and 06B4 epitopes in the gonococcal LOS (which are also found on the on red blood cells and some other human tissues) could aid the gonococci by several different mechanisms (Mandrell, Griffiss and Macher 1988). Specific adaptations by the gonococcus to its host could produce evolutionary advantages and would be expected. Expression of structures identical to those of human blood group antigens could aid the bacteria in evasion of the human immune system which does not recognize as foreign those molecules that are identical to itself. Human cells have been shown to possess lectins that bind terminal Galβ1→4GlcNAc and internal Galβ1→4Glc (Leffler and Barondes 1986, Sparrow, Leffler and Barondes 1987). Thus, the expression of the F62 oligosaccharides may increase binding of the bacteria to human tissues and cellular components and, thereby, increase the ability of the bacteria to establish itself. As discussed in Chapter I B., in the second step of the gonococcal invasion of the epithelium, bacteria are engulfed in membrane bound vesicles by the normally nonphagocytic epithelial cells, transported through the cell in these phagosomes, and exocytosed onto the subepithelial tissue. No other microorganism is known which occupies phagosomes of normal epithelial cells. Perhaps this crucial step in infection by the gonococcus is made possible by the binding of human lectins to the oligosaccharides of the gonococcal LOS. Another possible benefit to the

bacteria in expression of oligosaccharides similar to mammalian glycolipids and glycoproteins may be glycosylation of the LOS by human glycosyltransferases. This might increase the similarity between the gonococcal oligosaccharides and human oligosaccharides. Conversely, bacterial glycosyltransferases might alter human oligosaccharides creating neoantigens. This could cause the host immune system to attack tissues of the host (Mandrell, Griffiss, and Macher 1988).

These postulates are supported by evidence suggesting that the occurrence of cytidine monophospho-*N*-acetylneuraminic acid (CMP-NANA) in human serum and red blood cells induces gonococcal strains to become serum resistant and alters their LOS (Mandrell et al. 1990). It has been shown that after incubation with CMP-NANA the electrophoretic migration of the smaller LOS component of the F62 is altered and that the binding of this component by 3F11 is greatly reduced. Also, radiolabelled CMP-NANA was found to be exclusively incorporated into the smaller F62 LOS component and analogous components of other gonococcal LOS which were also recognized by MAb 3F11 (Mandrell et al. 1990). Thus, apparently the smaller component of F62 that contains a terminal *N*-acetyllactosamine is sialylated in the presence of CMP-NANA which is found in human tissues. (The analysis of these LOS by LSIMS after *O*-deacylation could provide more direct evidence of the sialylation.)

The heterogeneity of the LOS of strain F62 illustrates the necessity to analyze purified oligosaccharide components of the LOS to avoid the determination of "average" structures. Analysis of unpurified heterogeneous oligosaccharides may not reveal the correct structures but rather some composite structure. In fact, the differences in the oligosaccharide moieties of the LOS may be highly relevant to the

immunochemistry, as is apparently the case with the F62 oligosaccharides. Data suggesting that the LOS expression can vary *in vitro* compared to in an infected host also points out the need to analyze small amounts of heterogeneous LOS samples. Variation of the gonococcal LOS *in vivo* does support a postulated role for the LOS in its interactions with the host. Until the LOS structures are determined which are actually expressed *in vivo* by gonococcal strains of varying pathology, only inferences can be made about the relationship between LOS structure and pathology and immunochemistry. Thus, understanding the immunochemical and pathological role of the LOS appears to require knowledge of specific structural features and the differences between molecules, and the ability to analyze small amounts of heterogeneous material.

Elucidation of the structures of the oligosaccharides of the LOS from the 1291 parent and mutant strains showed a series of oligosaccharides that probably represent biosynthetic precursors varying (except for the 1291_b) by sequential saccharide deletion. As illustrated in Chapter V. Figure 18 at least three similarities between precursors of human glycosphingolipids and the 1291 oligosaccharides can be seen. The wild type oligosaccharide is apparently identical to the smaller F62 structure and is also recognized by MAb 3F11. The oligosaccharide structure expressed by pyocin-resistant strain 1291_b is immunologically and structurally similar to the P^k antigen, another of the human blood group antigens. Also, the oligosaccharides of mutant strain 1291_c were found to express the same terminal disaccharide, Gal1→4Glc, as another human glycosphingolipid precursor, lactosylceramide. Thus, the biosynthesis of the LOS may parallel glycosphingolipid biosynthesis. Correlations of the structures of the LOS from the 1291 parent and mutant strains with MAb recognition of these and

other strains (along with further structural elucidation as required) should reveal the structures of biologically significant epitopes occurring in the LOS and could aid in determining the function of the LOS.

The structures of the LOS from the five pyocin mutants suggest that the expression of a specific glycosyltransferase may have been inhibited in each of the mutant strains. Thus, these strains could possibly be used to identify and isolate specific glycosyltransferase enzymes required in the biosynthesis of the oligosaccharide portion of the LOS. These glycosyltransferases could prove to be excellent chemotherapeutic targets. On the other hand, additional study of the interaction of pyocin with the gonococcal genome and the effect on structure may further understanding of the genetics of the expression of the gonococcal LOS. The expression of a probably very minor component of the wild type LOS *in vitro* by pyocin-resistant mutant strain (1291_b) could be significant in terms of developing a general strategy to be used in determination of the complete repertoire of LOS structures available to one specific strain.

The analysis of the gonococcal oligosaccharides has provided support and the structural basis for existing immunochemical data as well as suggesting new analogies and experiments. The results indicate that the LOS may be a critical component in the interaction of the gonococcus with its human host. As such, the LOS may very likely be a candidate for a carbohydrate-based vaccine or one component of a multi-component vaccine. A number of improvements have been made in the structural elucidation of the LOS, and these could lead to further improvements in the structural analysis. The determination of a much greater number of oligosaccharide structures from the LOS of various gonococcal strains could then be accomplished in the same time frame as required for the

research that produced this dissertation. The knowledge which would be obtained from additional research should enable us to better understand the workings of this pathogen which is exclusively adapted to human beings. Also, such knowledge may provide opportunities for new therapeutic interventions in the infection of human beings by the gonococcus. Thus, it must be concluded that the research described in this thesis should provide stimulus and groundwork for the next phase of the investigation of the structures of the outer membrane lipooligosaccharides from *Neisseria gonorrhoeae*.

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