ADVANCES IN CARBOHYDRATE CHEMISTRY AND BIOCHEMISTRY

Volume 57

Derek Horton

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Advances in Carbohydrate Chemistry and Biochemistry

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PREFACE

The 57th volume of *Advances* reflects sustained trends in the carbohydrate field toward strong emphasis on biological aspects, both fundamental and applied, and a major proportion of the current issue is devoted to this viewpoint. The article by Monteiro (Ottawa, Ontario) on the lipopolysaccharides of *Helicobacter pylori* presents a comprehensive account of the chemical structure, biosynthesis, and potential pathogenic role of these bacterial cell-surface components in a wide range of disorders of the human stomach. It is noteworthy that less than two decades have elapsed since this microbiological basis for gastric ulcers and related diseases became recognized. The structural work presented here reveals in particular a remarkable similarity between the saccharide O-chains of the lipopolysaccharides and the sialy1-Lewis human blood-group antigens, a factor that may aid in the survival of the organism in the host and which presents a major challenge in the development of effective therapeutic vaccines.

Fifteen years ago, Benito Casu (Milan) contributed an authoritative article to this series on the structure and biological activity of heparin. Although this glycosaminoglycan has been used clinically for many decades in treating thromboembolic diseases, precise details of its mode of action have been lacking. Major developments in analytical methods and in our understanding of biosynthetic processes, together with recognition of the complementary role of heparan sulfate in polysaccharide–protein binding interactions involving antithrombin and other proteins, have permitted understanding of these interactions in much greater molecular detail. These developments, and their significance in our understanding of cell-surface interactions and in therapeutic applications, are presented here by Casu and Lindahl (Uppsala).

Unger (Vienna) contributes a major article devoted to physiological aspects of carbohydrates, in a wide-ranging survey of the oligosaccharide ligands of mammalian lectins (selectins) that function as cell-adhesion receptors. The recognition of the essential role of selectin–carbohydrate interaction in the inflammatory response and the key involvement of the sialy1-Lewis^x oligosaccharide and its analogues in the carbohydrate determinant has stimulated an enormous research effort on many fronts in the quest for effective therapeutic agents. This has led to great advances in the three-dimensional understanding of carbohydrate structures and their protein-binding domains, although the weakness of these interactions still presents difficulties in the design of practical anti-inflammatory drugs.

Replacement of the ring oxygen atom in sugars by other atoms, especially by sulfur or nitrogen, has long presented a challenge to synthetic chemists, and the recognition that such analogues have potential as inhibitors of glycoprocessing enzymes has stimulated much recent activity in this area. In this volume, Fernandez-Bolaños and Maya (Seville), together with Al-Masoudi (Konstanz), survey the

chemistry of sugars having sulfur in the ring, leading to products that have shown useful potential as, for instance, oral antithrombotic agents, antidiabetic agents, and agents for treatment of HIV infections.

The lives and scientific work of two legendary figures in the carbohydrate field are commemorated in this issue. George A. Jeffrey was a pioneer crystallographer whose doctoral work in the laboratory of Haworth on the structure of glucosamine hydrobromide, a monumental task at the time, firmly established the chair conformation of the pyranose sugars and set the stage for the stereochemical correlation of the carbohydrates with the amino acids and proteins. The account presented here by French (New Orleans) documents his work during six decades in developing and applying crystallographic techniques for characterization of sugars and their derivatives in the solid state. He was the undoubted authority on all aspects of the molecular geometry of crystalline sugars and for many years contributed extensively to these *Advances.*

The article by Miyake (Kawasaki), Maeda (Tokyo), and this writer details the long career of Sumio Umezawa devoted to the chemistry and medicinal applications of antibiotics, especially the aminocyclitols (aminoglycosides), a field dominated by Sumio and his microbiologist/biochemist brother Hamao from the earliest days of streptomycin through to practical semisynthetic analogues developed by Sumio that have enjoyed wide clinical application. The complementary articles by the two Umezawa brothers in Volume 30 of this series remain a definitive reference work on these antibiotics.

With this volume we welcome David C. Baker to the Board of Advisors.

Washington, DC DEREK HORTON
July, 2001

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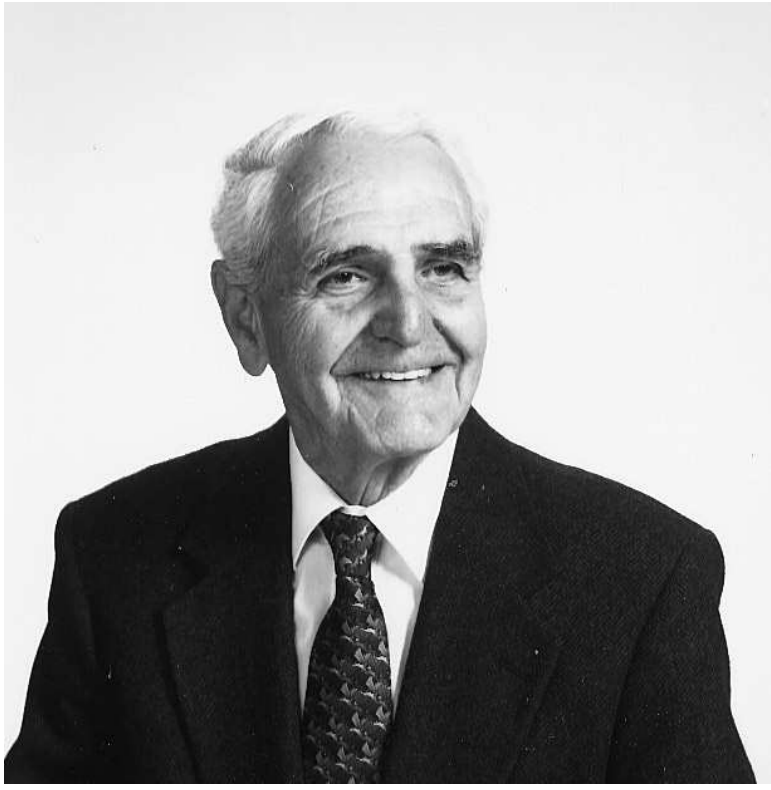

George H. Jaffray

GEORGE ALAN JEFFREY

1915–2000

The 20th century saw the discovery and maturation of the science of the diffraction of X-rays and neutrons by crystals. Through his innovations and exploitation of these technologies and molecular modeling, University Professor George Alan "Jeff" Jeffrey became the preeminent crystallographer and structural carbohydrate chemist of his time. This conservatively sized, energetic Briton made his home in Pittsburgh, Pennsylvania. There, he authored most of his 150 papers on the molecular structures of carbohydrates and the factors, such as hydrogen bonding and the anomeric effect, that determine their preferred geometries. His insights on carbohydrates bear double weight because his career was interspersed with work on other substances, with a total of more than 315 published works. Jeff's results form much of the basis for our understanding of these materials and should lead to the more complete exploitation of carbohydrates in the coming years.

When Jeff started his research in 1936, his crystallographic thesis project would require years of manual calculations and the results would leave substantial room for improved accuracy. At that time, the question was, "Can this structure be solved?" The determination of structures of carbohydrates, which generally lack a center of symmetry, was, in Jeff's words, "like playing chess against Nature." In the mid-1960s, when he was re-entering the world of carbohydrate crystal structures, computers had improved the situation to where the question was, "Would solving this structure be worth the months of effort?" Shortly thereafter, the phase problem for non-centrosymmetric structures was rendered much more tractable with "direct methods," for which Herbert Hauptman and Jerome Karle shared the 1985 Nobel Prize in chemistry. Now the question is, "Do you have a crystal, and is the day after tomorrow soon enough?" Of course, problem structures exist, and the determination of some large molecules is a heroic accomplishment. Still, for reasonably simple molecules and many large ones, crystallography identifies the types of all atoms, and the locations of each atom are determined with an accuracy of three to five significant figures. This allows a complete structural description of the molecule and the thermal motion of each atom. Deviations of the structural details from those of simpler molecules support the various theoretically proposed factors that dictate the molecular shape of carbohydrates.

Jeff's hallmark achievements include his Ph.D. thesis research on the crystal structures of glucosamine hydrobromide and hydrochloride. That work, published in *Nature,* established the chair form of the pyranoid ring some 30 years before a Nobel Prize was shared by Sir Derek Barton and Odd Hassel for their roles in establishing conformational analysis. Jeff later lamented that the configuration at C–2 of his thesis salts, the primary objective of the work, was established slightly earlier that year by others in the same laboratory who used chemical means, but the determination of the pyranoid chair shape remains as a landmark.

Another major achievement was his development of the Crystallography Laboratory at the University of Pittsburgh, well supported by the National Institutes of Health for many years, and designated as a Center of Excellence by the National Science Foundation. During its existence, more than 230 faculty, staff, visiting professors, and students worked in the only such department in the United States. In all, 76 students got Ph.D.s in crystallography; in addition, seven got M.Sc. degrees. He was an outstanding teacher and instilled the significance of carbohydrate chemistry and crystallography in generations of students and associates. He was always the leader with a good plan, and it was easy to obey the sign on his desk: "Be reasonable, do it my way!"

Jeff was a pioneer in the use of computing machines for the calculations needed for structural determinations that would otherwise be repetitious and tedious and take years. For Jeff, calculations in the pre-computer era were additionally frustrating because the mechanical calculating machines available in England worked in pounds, shillings, and pence, back when a pound was worth 20 shillings and a shilling worth 12 pence.

Not content to merely produce the information, Jeff regularly wrote in-depth summaries of the experimental findings. Many of these were written in collaboration with his former student, now Professor, Muttaiya Sundaralingam at The Ohio State University. They were published in these *Advances* and in *Molecular Structure by Diffraction Methods,* and they brought together the available information when the science was just getting started. Such analyses presaged the Cambridge Crystal Structure Database, a marvelous tool for easily gathering such information. In the preparation of those reports, Jeff recalculated all the authors' structural data and pointed out errors. Quite frequently he found that crystallographers had been cavalier in depicting the wrong enantiomer. As a result, biochemists and even some modelers, who regarded X-ray structures as the word of God, had been frequently confused. Jeff did a great service by his unflinching pursuit of accuracy and clear presentation.

Early on, his idea was to plan a program involving the determination of several, perhaps even a dozen, related structures. Comparing the results of these several structures, and including other chemical and physical information, gave overall patterns that constituted much more meaningful contributions to chemistry than

just an isolated structure. He also established and quantified principles through statistical tabulations of the experimental data in other reviews, and these were often published in *Carbohydrate Research.*

Jeff used theory to place the structural results in context. A series of seven papers, "The Application of ***ab initio*** Molecular Orbital Theory to the Structural Moieties of Carbohydrates," began nearly 30 years ago. While he relied on others to develop quantum mechanical methods and software, Jeff did take a role in augmenting molecular mechanics software for reproduction of the bond-length and bond-angle variations around the anomeric center that differed from the more standard values in ordinary alcohols and ethers.

His first book on hydrogen bonding involved manual entry of many crystal-structure coordinates and tedious determination of the hydrogen-bonding systems in more complete detail than in the original individual reports. The interest in hydrogen bonding, in particular, led to more than 30 papers that documented his exploitation of the ability of neutron-diffraction methods to precisely locate hydrogen atoms.

Finally, Jeff realized that the bond-length and bond-angle data that were the basic reported information of the crystallographer were not the most interesting aspects of carbohydrate structure. He was a leading advocate of the discussion of the torsion angles that define molecular shape in detail. He also supported the use of puckering parameters that describe the shape of carbohydrate rings. Those two (for furanose rings) and three (for pyranose rings) parameters allow shorthand descriptions of the important differences in ring shapes. All of this emphasis on shape arose, of course, because of the absolute dependence of biochemical and many chemical and physical properties of carbohydrates on their molecular geometry.

Jeff was born to Beatrice and George Jeffrey on July 29, 1915, in Cardiff, the capital of Wales in the United Kingdom. His father, an accountant, was killed in World War I when Jeff was 2 years old, and he was raised by his mother. Despite the stresses of single parenthood in those times, she lived to be 101 years old, spending her last years in Pittsburgh. When Jeff was 7, they moved to Birmingham. As a schoolboy he was an amateur chemist, and his mother banished his experiments from her kitchen to a wooden shed as far from the house as possible. Surviving those informal investigations into molecules such as nitrogen triiodide, hydrogen cyanide and sulfide, nitrous oxide, and chloroform, he went to King Edward's High School in Birmingham, England. He then took his B.Sc. in chemistry with honors (in 1936) and Ph.D. in chemistry (in 1939) in Sir Norman Haworth's Chemistry Department at the University of Birmingham. He was one of the last students before Haworth's retirement in 1948. Jeff came in the same decade that many of the fundamental chemical aspects of carbohydrates were being learned with certainty in that laboratory. His Ph.D. advisor was Professor Ernest Gordon Cox, later Sir Gordon Cox. During World War II, Jeff worked on rubber structures at the British Rubber Producers Research Association, and in 1942, he married Maureen

Ward of London. She was late to the wedding because of an air raid. Jeff and Maureen's daughter, Susan, was born in 1945 and now resides in Pittsburgh.

In 1945 he returned to work with his Ph.D. advisor, taking a position as a lecturer in physical and inorganic chemistry in Cox's Department of Inorganic and Structural Chemistry at the University of Leeds. Cox and Jeffrey not only solved inorganic structures but also published several papers on the use of punched-card computing machines for crystallographic calculations. In 1951, Jeff came to the University of Pittsburgh as a visiting professor on a Fulbright scholarship. During that visit, the family toured most of the eastern United States as well as national parks in the West in a 12-year-old Dodge: pretty adventurous.

After returning to the United Kingdom, he was awarded the D.Sc. at the University of Birmingham in 1953. He then emigrated to the United States, becoming a statistic in the "brain drain," a mass migration from the poorly funded universities in postwar Great Britain. His son Paul was born just 8 weeks before crossing the Atlantic Ocean. Paul is now the director of product development for Pfizer Pharmaceuticals in New York City.

Jeff came to Pittsburgh as a full professor of physics and chemistry in the Departments of Physics, Chemistry, and Earth and Planetary Sciences and taught quantum mechanics for the chemists and X-ray crystallography for the physicists. In 1964, Maureen, who had trained in England as a medical doctor, started, with Jeff's help, importing diffraction equipment, primarily the German-made Stoe Instruments as well as some English tools. In 1966 he gained the title of "University Professor" and upon his official retirement in 1985, he became Emeritus Professor. Jeff's success depended not only on scientific excellence but also on his and Maureen's ability to make his co-workers feel like members of his crystallographic family. Because of Jeff's advocacy and accomplishments, the Crystallography Laboratory became the Department of Crystallography in 1969, and Jeff served as its chair from then until his retirement in 1985, except for 2 years' leave of absence (1974–1976) to pursue neutron-diffraction studies at Brookhaven National Laboratory. The instrument importing business was given up before he went to Brookhaven. Ultimately, Jeff missed the students from the university setting and happily returned to Pittsburgh.

In his initial years at Pitt, Jeff worked on improving the technology available to crystallographers, and he persuaded the university to acquire an IBM 650 computer in 1956. He undertook various crystallographic projects, including studies of clathrate hydrates and inclusion hydrates. In 1964, Jeff got back into the carbohydrate arena by writing a review on crystal structure analysis in carbohydrate chemistry for *Advances.* After giving a seminar on clathrates at Ohio State University, Jeff met with Professor Derek Horton, who had also been in Haworth's laboratory in Birmingham. Their discussions on extended versus nonextended conformations of acyclic sugar derivatives further stoked Jeff's interest in returning to carbohydrate structures. These doings opened the metaphorical floodgates for the output of what was described to me with some envy by my father, Dexter French, as a "factory" for

the determination of carbohydrate structures. The production line included an automated diffractometer that made it practical to study carbohydrates. Jeff redid his thesis project and then embarked on new structures. Racemic DL-arabinose was the first of the new structures in 1967, followed by the glucurono- and galacturono-1,4-lactones. The lactone crystal structures were important because they lent credence to the proposed half-chair form of the intermediate in several carbohydrate reaction mechanisms. Methyl β-maltoside (the third disacccharide to be determined after sucrose and cellobiose) and mannitol came next. The next carbohydrate paper also was characteristic of a part of Jeff's work—a re-refinement of β-glucose and β-cellobiose. The accuracy and the detailed results were improved. Often Jeff had better technology available, but he was also very demanding in the quality of results, and if he felt that a published hydrogen-bonding system was not logical, that was sufficient reason to reinvestigate.

Work on ribitol and xylitol followed and then 1,6-anhydroglucopyranose. A glucitol–pyridine complex was reported next, and then *epi*-inositol. The first of the neutron-diffraction papers was on glucitol and then came methyl 1-thio- and 1,5-dithio-α-D-ribopyranoside structures. Structures of allitol and iditol and potassium gluconate were reported, followed by 1,6-anhydromannofuranose. The first reported trisaccharide crystal structure was his work on the nonreducing molecule 1-kestose. It is sucrose with an additional fructofuranosyl group attached to the 1′-position. The central fructofuranose ring has the rarely found "Southern" shape, although the terminal furanose ring has the more usual "Northern" shape. Later, Jeff's papers on the tetrasaccharides stachyose and nystose became the primary references on those structures. Nystose has one more fructose residue than 1-kestose, and one question for the nystose determination was whether its 1-kestose moiety would retain the "Southern" ring shape. The answer was no. Thus, the variation in furanoid ring shape was caused by hydrogen bonding and the crystal field, rather than being chemically induced. Even at this writing, only 17 trisaccharide structures have been determined, and only four acyclic tetrasaccharides. The biggest difficulty with such large structures now is in obtaining suitable crystals. Often, only microcrystals are formed. They give only a powder pattern that does not readily allow a complete structural determination. *trans-O-*β-D-Glucopyranosyl methyl acetoacetate was reported and then came the neutron-diffraction study of methyl α-D-altroside. The structure of D-glucaro-1,4-lactone was solved.

These early experimental reports on carbohydrates were matched by some very early applications of computational chemistry to the theory of carbohydrate structure. Collaborating with Professor John Pople, 1998 Nobel Laureate in chemistry, then conveniently located at neighboring Carnegie Mellon University and with Leo Radom, an Australian on a Fulbright Fellowship at Carnegie Mellon University, Jeffrey studied various aspects of the anomeric effect, using the simplifying assumption that the preference for axial glycosidic oxygen atoms could be modeled by dihydroxy- and dimethoxy-methane.

The first of these papers, published in 1972, was truly pioneering work. It provided a complete two-dimensional potential-energy surface for both of the C–O rotations in dihydroxymethane. It also provided optimized bond lengths for various torsion angles so the changes could be compared to the various geometries found in experimentally determined crystal structures, and there was a decomposition of the energies to assess the relative contributions of dipole–dipole interactions, hyperconjugative stabilization, and steric interactions. A recent review of this nearly 30-year-old work cited its tremendous impact on experimental carbohydrate chemists and stated that its remarkably high standards contributed to the generally good reputation that theory enjoys in the carbohydrate chemistry community. Similar computational studies are still being undertaken today, but with improved levels of theory that take advantage of the huge advances in computer speed, as well as the improved theory and software. Besides this series of ***ab initio*** papers, Jeff authored a number of studies based on molecular mechanics. This writer journeyed to his lab in Pittsburgh to make unpublished studies on cellobiose in the late 1970's using MM1-CARB, a version of Professor N. L. Allinger's program that had been modified for use with carbohydrates by Jeffrey and Dr. Robin Taylor. Even though we used the university mainframe, we had to chose which atoms would be allowed to move during energy minimization of the crystal structure. Back then, the computer was just too slow to adjust all 45 atoms in a reasonable time. In a number of later papers, Jeff addressed the problems of trying to model solid-state structures with isolated models, chiefly involving the complications from artifactual intramolecular hydrogen-bonding that occurs because of the lack of neighboring molecules in the isolated model.

Even Jeff sometimes overlooked the theoretical aspect of his career, as indicated by the subtitle of his chapter in a book on modeling carbohydrates, "An Experimentalist Looks at Modeling." In that paper, he cautioned against the pitfalls lurking for the inexperienced who were using "Canned Science." Jeff was very supportive of modeling work and actually discouraged this author from getting involved first hand in experimental work. Also, he was disinclined to share authorship credit with those who had made the crystals that he studied.

By 1980, Jeff had published 80 papers related to carbohydrates and hydrogen bonding, not including the clathrate work. This effort was recognized by the American Chemical Society's Division of Carbohydrate Chemistry. That year, they presented him with the Claude S. Hudson Award. Along with many of the other winners, Jeff lent such prestige to the award that it became an award of the ACS itself, with eligibility extended to carbohydrate chemists worldwide just 3 years later. I was privileged to speak at his awards banquet, filling in for another who had to cancel at the last minute, and perhaps as an emissary from my father, a previous Hudson awardee, who was getting an award from the Division of Agriculture and Food Chemistry the same night. This was despite Jeff's opinion

that only an Englishman could do a proper job of "roasting" a winner at the awards banquet. Perhaps he wanted only a half-baked job! The following year, there was a symposium with 19 papers in his honor at the American Chemical Society's meeting in Atlanta, Georgia. Other awards included the Buerger Award of the American Crystallographic Association, in 1988, and the Haworth Award of the Royal Society of Chemistry, in 1998. He also won the Pittsburgh Award of the ACS in 1978, an Alexander von Humboldt U.S. Senior Scientist Award for 1983–1984, and a Doctor Honoris Causa, Universidade Technica de Lisboa, Portugal, 1991.

In celebration of his 80th birthday, a symposium sponsored by the Pittsburgh Diffraction Society was held in Thaw Hall on the Pitt campus, opened by Professor Sundaralingam. Topics dear to Jeff's heart included the structure and function of proteins, a theoretician's perspective, studies of carbohydrates and lipids, and water and hydrates. Speakers came from around the world, including Professors Chien Ho, Carnegie Mellon University; Donald Abraham, Medical College of Virginia; Richard Bader, McMaster University in Hamilton, Ontario; Wolfram Saenger, Berlin; Martin Caffrey, Ohio State University; Stanley Nyburg, King's College, London; Helen Berman, Rutgers University in Piscataway, New Jersey; Dietrich Mootz, Heinrich Heine Universität, Düsseldorf, Germany; John Finney, University College, London; Terence Sabine, Australian Nuclear Science and Technology Organisation; and two U.S. government researchers, Dr. Daniel Carter, Marshall Space Flight Center, Huntsville, Alabama, and this writer, from the U.S. Department of Agriculture in New Orleans. Also chairing sessions, but not presenting, were Professors Laurens Anderson from the University of Wisconsin, and Bryan Craven, who succeeded Jeff as the chairman of the Crystallography Department.

Jeff was extremely aware of the value of interchange of ideas with other scientists. Not only were there more than 80 formally designated visiting scientists of his laboratory in Pittsburgh over the years, but many others, such as I, came for a week or so. Jeff also traveled extensively to spread the word. With students and collaborators in more than 30 countries, he had a welcome at many of the world's airports. He was a UNESCO advisor to Pakistan in 1967, visiting professor at the University of São Paulo, Brazil, 1970, and a senior Fulbright lecturer in Lisbon, Portugal, in 1973. He so enjoyed Portugal that he kept an apartment on the Algarve coast for many years. He was the Hassel Lecturer of the Norwegian Chemical Society in 1975 (Hassel was the co-winner of the 1969 Nobel Prize). In retirement, he was the director of NATO Advanced Study Institutes in Portugal in 1987 and in Spain in 1990. Domestically, he served as an ACS tour speaker three different years and was the Robert Welch Foundation Lecturer in 1979. One year, he brought Professor Wolfram Saenger from Berlin to New Orleans to meet with Professor Richard Reeves, then at the Louisiana State University Dental School. Reeves, whose cupraammonium complexing work gave early evidence for the

chair conformation of glucose in solution in the late 1940s by chemical methods, was the custodian of the Claude S. Hudson Collection, a couple of cigar boxes of rare carbohydrates. The idea was that Reeves was giving out half of whatever was available for research to each person requesting a sample, and the two crystallographers were seeking grist for their mills. Until his death, Jeff retained memberships in the American Crystallographic Association, the American Chemical Society, and the Royal Society of Chemistry, and he was a Fellow of the British Institute of Physics. He was a corresponding member of the Academia Brasileira de Ciencias and an Honorary Member of the Sociedade Brasilera de Crystalografia.

Jeff spent most of his time on matters related to science. His diversions included enthusiastic attendance at the opera and playing tennis with Maureen several times per week in suitable weather; also, he was the gardener in the family. An earlier interest in mountain climbing was abandoned because of "inadequate mountains" in the eastern United States.

After 1980, Jeff's research interests expanded to include liquid crystals based on carbohydrates. These structures depend in part on hydrogen bonding for stability. Along with many other studies of hydrogen bonding in other carbohydrates, they led to Jeff's extensive and thorough book coauthored with Professor Saenger on hydrogen bonding in biological molecules. That book was followed by a shorter but updated text in 1995.

Jeff served the scientific community well. He was the treasurer (1954–58), vice-president (1962), and president (1963) of the American Crystallographic Association and was also active in the International Union of Crystallography, often serving as a delegate. He was the U.S. co-editor of *Acta Crystallographica* from 1973 to 1984, and he served on the editorial board of *Carbohydrate Research* from 1980 until his death. In the early years of his editorship with *Acta Crystallographica,* he also checked the correspondence between the tables of atomic coordinates and the derived bond lengths and angles. This was a time before computer generation of manuscripts, and those values were retyped from computer printouts by the authors or their secretaries. Jeff found so many transcription errors that he demanded a fee of $5 for each such mistake that he found before a manuscript could be published in *Acta Crystallographica.* Although the policy did not survive very long, Jeff made his point. He was no less demanding of his own work, of course. If a referee was less than enthusiastic about one of Jeff's own papers, he was likely to abandon it.

Jeff's retirement was professionally active, and he also enjoyed the added time for gardening. Sadly, he contracted amyotrophic lateral sclerosis (Lou Gehrig's disease) and it progressed rapidly. His death on February 13, 2000, came in his 85th year, too soon for his family and for the crystallographic and carbohydrate communities. The Pittsburgh Diffraction Society established The Jeffrey Fund to award student scholarships for attendance at Congresses of the International Union of Crystallography. It received many generous contributions.

Acknowledgments

The writer based some of this biography on material published by Professor Bryan Craven, by Dr. Serge Pérez, and by Professor Christopher Cramer. Some anecdotes were provided by Professor Derek Horton. Mrs. Maureen Jeffrey graciously provided information, and Mrs. Joan Klinger, Jeff's secretary for many years, also kindly provided materials used in this effort. Dr. Wendell Binkley retrieved information from the archives of the Division of Carbohydrate Chemistry of the American Chemical Society.

Alfred D. French

梅沢純夫

S. Umezawa

SUMIO UMEZAWA

1909–2000

Even at the age of 90 Sumio Umezawa was active and full of energy. He presided at a March 2000 Council meeting of the Microbial Chemistry Research Foundation (MCRF), but two days later, in the early morning of March 30, he suddenly passed away as the result of a ruptured aneurysm. He had been a fixture on the Japanese scientific scene for so long that his co-workers felt that his presence would continue indefinitely.

Sumio Umezawa was born in Nihonbashi, Tokyo, Japan, on November 22, 1909, the eldest of six remarkably talented sons of Junichi and Takako Umezawa. His father, Junichi, graduated from the Medical Department of the University of Tokyo in 1910 and continued his studies in biochemistry in the same university until 1913, when he was appointed Director of the Obama Hospital, in Fukui. In 1919, he moved back to Tokyo with his family to return to his alma mater and study for the Ph.D. His father's work awakened Sumio's interest in chemistry at a young age.

Following his primary school education, Sumio went to Musashi High School in Tokyo, where he enjoyed the atmosphere and benefitted from the school's policy of developing students' skills in oral communication in the English language. He continued to attend this school even after his family moved in 1923 to Sapporo, Hokkaido, when his father became chief director of the Sapporo Hospital of the National Railways Corporation. Sumio's full life at Musashi influenced his parents' decision to enroll all of their sons in the same private high school, although they lived in Sapporo and later in Moji, Fukuoka, both cities far distant from Tokyo. Their efforts to provide a fine education for their sons were amply rewarded. Sumio's younger brothers also had distinguished scientific careers: Hamao [1914–1986, microbial chemist, director of the Microbial Chemistry Research Foundation (MCRF) and Institute of Microbial Chemistry, MCRF, and professor at the University of Tokyo]; Kuniomi (1916–, geologist, vice-minister of the Science and Technology Agency); Tsutomu (1918–1998, physician, chief director of the Health Center of the Metropolitan Police Board); Minoru (1922–, physicist, professor at the Centre de Recherches Nucléaires, Strasbourg); and Hiroomi (1924–1995, physicist, professor of theoretical physics, University of Alberta).

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In 1930, Sumio entered the Faculty of Science, Hokkaido Imperial University (now Hokkaido University), in Sapporo. He carried out his thesis work in the laboratory of Professor Harusada Suginome (who had just returned from Sir Robert Robinson's laboratory at Oriel College, Oxford), and he graduated in 1933. Following his postgraduate course at the same university (1933–1935) as an honor-roll student, he was appointed research assistant (1935–1940) and then associate professor of organic chemistry (1940–1942) at his alma mater. In 1942, he moved to the Fujihara Institute of Technology at Hiyoshi, Yokohama, as an associate professor of organic chemistry. This institute had just been established by Ginjiro Fujihara in 1939 and was donated in 1943 to Keio University as its Faculty of Engineering (now the Faculty of Science and Technology). He made great contributions to the university in education, administration, and research as one of the earliest faculty members up until his retirement in 1975. In 1944 he was appointed professor of organic chemistry in the Department of Applied Chemistry and served a term (1965–1969) as dean of the Faculty of Engineering. When the Institute of Bioorganic Chemistry, MCRF, was established in 1974, he became its director and served in this capacity until the last day of his life. In 1975 he was granted the title of Professor Emeritus of Keio University.

Sumio Umezawa did not begin his career as a carbohydrate chemist. His first series of papers (1936–1939) described the synthesis and substitution reactions of selenophenes and the first synthesis of selenophthene by the reaction of acetylene with heated selenium. For this work he received the Ph.D. (doctor of science) in 1942 from his alma mater. Sumio occasionally talked to his students on how he had successfully obtained selenophthene in his handmade glass apparatus. One day, after many trials and errors in preparing the compound, he noticed tiny particles inside the reaction tube. When he used the apparatus without cleaning in the next experiment, crystalline selenophthene was formed in good yield. The seed crystals initially formed must have initiated the crystallization. Later a reader of his paper wrote to say that he was unable to reproduce the procedure. Sumio attributed his success to good fortune in using his own apparatus, which contained the requisite seed crystals.

Sumio Umezawa became interested in the chemistry of natural products when he joined Suginome's group in 1941 and learned of their work on isolation and structure of *Aconitum* alkaloids.

At Keio University, Sumio Umezawa initiated his lifelong work on the chemistry of antibiotics. His research began with processes for production of penicillin to comply with a request from the military government. In cooperation with his younger brother, Hamao, he achieved the first isolation of penicillin in Japan in 1944, as its barium and calcium salts. His happy days in the new laboratory ended abruptly when, on the night of April 15 through the early morning of April 16, 1945, about 80% of the facilities of his faculty were burned down as a result of a U.S. air raid. Along with the members of his faculty, he had to find a new place to

continue research and education. It was a critical situation for them as to whether they would be able to maintain their faculty. After times of adversity at temporary locations, they opened a new campus in 1949, at Koganei, Tokyo. However, they were not satisfied with the buildings, which had been used for storage, were old and small, and were far from fulfilling their dream of reestablishing the faculty. They finally moved back to Hiyoshi, Yokohama, between the summer of 1971 and the spring of 1972. As dean of the faculty, Sumio played a major role in preparation for this move.

On May 25, 1945, Shinzo Koizumi, President of Keio University, was brought to Keio Hospital suffering severe burns resulting from the fire of his house, caused during the largest-ever U.S. air raid on the midtown area of Tokyo. At the request of the surgeons at the hospital, Sumio brought his experimental sample of penicillin to prevent Koizumi from developing a dangerous infection. This fortunate treatment turned out to be one of many successful clinical trials of penicillin in Japan at that time. Sumio's early studies on penicillin were accomplished in conjunction with Tetsuo Suami (now professor emeritus of Keio University), Kenji Maeda (vice-director of the Institute of Microbial Chemistry), and Saburo Nakada (instructor at Keio University).

Sumio Umezawa's work in the field of aminoglycoside antibiotics started just after World War II, when he became concerned with the production of streptomycin. Later he turned his attention to structural studies[1] on kanamycin, a compound discovered in 1957 by his brother Hamao. Among his students in this project were Yukio Ito (Tanabe Seiyaku Co.) and Shunzo Fukatsu (Meiji Seika Kaisha). Ito laid the groundwork for the total synthesis of kanamycins, and Fukatsu played an important role in industrial production of dibekacin (described later) in the early 1970s.

The remarkably successful clinical application of aminoglycoside antibiotics attracted the attention of many organic chemists. With his continuous interest in this field for more than 20 years, Sumio Umezawa accomplished the first total synthesis of many aminoglycosides, including paromamine,[2,3] neamine,[4] trehalosamine,[5] kanamycins A,[6,7] B,[8,9] and C,[10–12] butirosin B,[13] tobramycin,[14,15] streptomycin,[16] dihydrostreptomycin,[17,18] and neomycins B[19] and C.[20,21]

Many of Sumio's students who took part in this long-term project received the Ph.D. and went on to senior positions in universities, research institutes, and industry. Shinkiti Koto, who played an important role in the synthesis of paromamine and kanamycins, became an associate professor at Kitasato University. Kuniaki Tatsuta, who studied the synthesis of neamine, trehalosamine, and kanamycins, became a professor at Waseda University. Yoshio Nishimura (kanamycin B, neomycin C), Daishiro Ikeda (butirosin B), and Yoshikazu Takahashi (streptomycin, dihydrostreptomycin) continue their work at the Institute of Microbial Chemistry, while Yasushi Takagi (tobramycin) and Toshiaki Miyake (tobramycin) work at the Institute of Bioorganic Chemistry. Hiroshi Sano (dihydrostreptomycin, Kyowa Hakko Kogyo Co.), Tetsuro Yamasaki (dihydrostreptomycin, Takasago International

Corp.), and Takayuki Usui (streptomycin and neomycin B, Meiji Seika Kaisha) hold positions in industry.

Although the development of resistance to antibiotics remains a serious concern in present-day chemotherapy, Sumio's synthetic work, combined with his brother Hamao's pioneering work on the mechanism of resistance of resistant bacteria, opened a rational approach to the chemical modification of aminoglycoside antibiotics. Sumio and co-workers modified the 3-hydroxyl group in the 6-amino sugar component of kanamycin with the objective of preventing inactivating enzymes from phosphorylating the antibiotic at the 3-position. He then took advantage of his experience in the total synthesis of kanamycins and successfully constructed 3′-*O*-methyl[22] and 3′-deoxy[23,24] kanamycin A. The latter was found to exhibit remarkable antibacterial activity against resistant bacteria as well as against common bacteria, whereas the former was almost devoid of activity.

Once deoxygenation had proved fruitful for effective modification, Sumio Umezawa and co-workers conducted extensive studies on regioselective deoxygenation of various natural aminoglycoside antibiotics. One of these semisynthetic antibiotics is 3′,4′-dideoxykanamycin B,[25,26] which achieved notable clinical use as a valuable drug for resistant infections, under the generic name dibekacin. The task of preparing this semisynthetic aminoglycoside was assigned to Tsutomu Tsuchiya (vice-director of the Institute of Bioorganic Chemistry), who had just rejoined Sumio's laboratory after postdoctoral study in Professor Derek Horton's laboratory at The Ohio State University, Columbus, Ohio. It was fortunate that Horton (now at American University) and Tsuchiya had just accomplished a modification of the Tipson–Cohen procedure, originally reported for introducing unsaturation into acyclic sugars, for cyclic monosaccharides. This modification was successfully applied to regioselective 3′,4′-unsaturation of the pseudotrisaccharide kanamycin B. Other natural aminoglycosides likewise deoxygenated include ribostamycin, kanamycin A, streptomycin, dihydrostreptomycin, butirosins A and B, and lividomycin B. For this deoxygenation project, Isamu Watanabe (5″-deoxylividomycin B,[27] 3′-deoxybutirosins A[28] and B,[28,29] Kowa), Hiroshi Sano (3″-deoxydihydrostreptomycin,[30] Kyowa Hakko Kogyo) and Teruo Kishi (6-deoxydihydrostreptomycin,[31] Kyowa Hakko Kogyo) joined Sumio's group from the pharmaceutical industry and later received the Ph.D.

During this synthetic work, Umezawa developed several techniques for the selective protection of hydroxyl and amino groups in aminoglycosides. These include the carbamate[32] and zinc-chelate protection of kanamycins,[33] developed in collaboration with Tsuchiya and Takagi. The latter procedure became crucial for the industrial production of amikacin from kanamycin A and arbekacin (habekacin) from dibekacin.

In 1966, Umezawa, in conjunction with Tsuchiya and Tatsuta, discovered the "TACu reagent"[34] for determining configuration in aminoglycosides and aminocyclitols. The reagent specifically forms a copper(II) chelate between vicinal,

diequatorial hydroxyl and amino groups, but not between vicinal hydroxyl groups, and it was used in determining the absolute structure of kanamycin.[35] Later, this reagent was successfully applied in structural elucidation of synthetic tobramycin (3′-deoxykanamycin B),[15] when the early 60-MHz NMR spectrometer proved of no use in determining the position of deoxygenation in the pseudotrisaccharide.

Through his extensive studies on aminoglycosides, Sumio Umezawa surely achieved a dream of many chemists, namely making an important contribution to human welfare. His success progressed through logical phases: structure determination of a natural product, its total synthesis, discovery of a compound having better activity by utilizing the techniques for total synthesis, preparation of a promising analogue from the original natural product, and finally its development as a medicinal product. It must be emphasized that he and co-workers carried out most of the work without any of the modern analytical instruments, such as superconducting NMR, FAB-mass spectrometry, and HPLC, which are taken for granted these days.

Moreover, Sumio Umezawa had broad interests in the chemistry of other sugar-containing antibiotics. Bleomycins are useful antitumor agents discovered by Hamao Umezawa and co-workers in 1966 and are glycopeptides composed of a novel hexapeptide, a terminal amine, and a disaccharide. Collaboration with Hamao's group led to elucidation of the structure of the disaccharide portion[36,37] common to the bleomycins. For this work Shoji Omoto (Meiji Seika Kaisha) received his Ph.D., and Umezawa, with Tsuchiya and Miyake, synthesized the disaccharide.[38] Later, he and co-workers successfully achieved the first total synthesis of bleomycin A2[39] in collaboration with the groups of Hamao Umezawa and Masaji Ohno (Professor of the University of Tokyo).

Umezawa and co-workers also conducted chemical modifications of macrolide antibiotics containing more than one sugar residue. This pioneering research afforded a number of semisynthetic macrolides, such as the 4′-deoxy,[40] 23- amino,[41] and 3,4′-dideoxy[42] derivatives of mycaminosyltylonolide, which are active against both gram-positive and gram-negative bacteria. These studies opened up an important new aspect for the macrolide antibiotics. Notably, 3,4′-dideoxymycaminosyltylonolide was subjected to clinical trials, although unfortunately it failed to reach the standard required for marketing. These chemical modifications of macrolides were realized in collaboration with Akihiro Tanaka (4′-deoxy and 23-amino derivatives), Shuichi Sakamoto (23-*C*-substituted derivatives[43]), and Shunji Kageyama (3,4′-dideoxy derivatives), who had joined Sumio's group from Yamanouchi Pharmaceutical Co. for their Ph.D. studies.

Sumio Umezawa also pursued many other topics, including synthetic studies on different groups of antibiotics and related compounds, isolation and structural elucidation of several new microbial metabolites, and new organic reactions. In addition to the co-workers already mentioned, Mitsuhiro Kinoshita (sarkomycin[44,45] and antimycin A3,[46] professor emeritus of Keio University), Shonosuke

Zen (nitroacetic acid derivatives,[47–49] professor emeritus of Kitasato University), Seiichiro Ogawa (2-aminocyclohexyl D-glucosaminides,[50,51] professor at Keio University), and Eisuke Kaji (cyclic α-amino acids,[52] professor at Kitasato University) also studied in Sumio's laboratory and took up senior positions in academia. For a rather recent project on fluorinated antibiotics, Takahiro Torii (3-fluoro-3-demethoxysporaricin A,[53] Kowa) and Eijiro Umemura (5-deoxy-5-fluoronetilmicin,[54] Meiji Seika Kaisha) came from pharmaceutical firms and obtained their doctoral degrees.

Sumio Umezawa's research is described in some 300 scientific papers and reviews,[55–58] including a landmark article in this series.[55] His last paper[59] was concerned with the structural determination of a cyclic lipoundecapeptide, pholipeptin, which was accomplished in cooperation with the team of his nephew, Kazuo Umezawa (professor at Keio University). He was impressed by the capability of the latest two-dimensional NMR techniques that allowed elucidation of the whole structure. As the head of his research group, Sumio always faced the challenges provided by antibiotics, and he had what it took to spur his co-workers to fruitful accomplishments. It is certain, however, that his remarkable academic career would have been quite different without the collaborations with his brother, Hamao, with his lifelong colleague Tsutomu Tsuchiya, and with many other competent co-workers.

Sumio Umezawa served on many committees of academic societies and the Japanese Ministry of Education. He was president of the Society of Synthetic Organic Chemistry, Japan (1964), served as Vice-President (1965–1966) and President (1980–1981) of the Chemical Society of Japan, and was given honorary membership by the Society in 1981. He was a member of the Editorial Board (1953–1990) and emeritus member (1991–2000) of the *Journal of Antibiotics*. He was also a member of the Editorial Advisory Board of *Carbohydrate Research* (1974–1990). He was an expert member of the Science Technology Council to the Cabinet and an expert member of the University Establishment Council at the Ministry of Education. He served as vice-president (1972–1994) and president (1994–2000) of the Microbial Chemistry Research Foundation and as a councilor of many societies, including the Japan Antibiotics Research Association (Tokyo) (1948–1995) and the Japan Chemotherapy Research Association (Tokyo) (1959–2000).

Sumio Umezawa's achievements have been recognized by many awards, including the Gijuku Prize (Keio University) in 1949 for "Chemical Studies on Penicillin" and the Fukuzawa Prize (Keio University) in 1963 for "Studies on Antimicrobial Substances and Related Compounds." He also received the Chemical Society of Japan Award for "Studies on Antimicrobial Substances and Related Compounds" in 1964 and the Japan Academy Prize for "Studies on the Synthesis of Aminoglycoside Antibiotics" in 1980. Sumio was deeply moved when he received the Fujihara Prize in 1982 for "Total Synthesis of Streptomycin," since this award

commemorated the founder of his own faculty, in which he had worked for 33 years. For his notable contribution to synthetic studies and development of antibiotics, he received in 1991 the Special Prize from the Society of Synthetic Organic Chemistry, Japan. In the autumn of 1981, the Emperor of Japan conferred on him the Second Order of the Sacred Treasure (Kun Nito, Zuihosho) for his distinguished service.

In the autumn of 1974, 5 months before his retirement from Keio University, he became director of the newly established Institute of Bioorganic Chemistry, near Keio University. The colleagues and students in his laboratory at Keio moved to the institute with him. Among them were Tsuchiya, Ikeda, Nishimura, Takagi, and Miyake. He accepted senior year and graduate students from Keio University to his institute every year. Starting in 1981, he also accepted students from Kitasato University. Sumio enjoyed having young students in his institute. While drinking at parties with staff members and students, he often talked of his own encounters with well-known persons in Japan or abroad. One night at a party he talked about his hobbies. When he was a student he was enthusiastic about practicing kendo, but later he changed his bamboo sword in favor of a golf club and became a good golfer. At his institute, he took care of a total of 144 students from the two universities in carrying out their thesis work for graduation, master's degrees, or doctoral degrees. These include the present staff members of the institute, Tetsuo Shitara, Yoshiaki Takahashi, Yoshihiko Kobayashi, and Hiromi Sohtome.

It was a difficult period for Sumio when his beloved wife, Yoshiko, developed Parkinson's disease. While she was hospitalized, Sumio left his institute earlier than usual to visit her in hospital. After battling the disability for several years, she passed away on April 25, 1997. This was the saddest incident in Sumio's life. Fortunately, he was not left alone at that time; his first daughter, Miyoko, lived with him in his condominium at Shinjuku, Tokyo, and his second daughter, Asako, lived in the same building with her husband, Miki Wadachi, professor of physics at the University of Tokyo, and their son, Hiroki. He was very attached to his family. On his way back home Sumio often bought doughnuts for his family at a Mister Donut shop in front of Hiyoshi Station, the railroad station nearest to his institute.

On October 15, 1985, he was baptized Francisco John at St. Ignatius Church at Yotsuya, Tokyo, where his wife had been a member for a long time. He attended Bible class at this Catholic church on Saturdays and enjoyed studying the Bible with young people. Church activities became more important in Sumio's life after his wife's death. However, in contrast to his vigorous appearance, he never got over the loss of his wife, and seemed impatient to join her. His death at the end of March, in the last year of the 20th century, came when the cherry trees he loved around his institute came into blossom. The respected "Mr. Antibiotics of Japan" is sadly missed by his colleagues, students, and friends.

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SUGAR DERIVATIVES HAVING SULFUR IN THE RING

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I. INTRODUCTION

Since the synthesis of 5-thio-D-xylopyranose in 1961,[1,2] the first sugar analogue having sulfur replacing the ring oxygen atom, there has been a growing interest in the synthesis of sugar mimetics that have atoms in the ring other than oxygen, such as nitrogen,[3–6] sulfur, selenium,[7–10] phosphorus,[11,12] and carbon.[13,14] These compounds have became important targets because of their use as probes for understanding the recognition processes by sugar-binding proteins and their potential value as glycosidase inhibitors. The chemistry of sugar heteroanalogues having

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sulfur in the ring was described in two pioneering articles of this series by Horton and Hutson[15] in 1964 and by Paulsen and Todt[16] in 1968. Two more articles covered the literature up to 1980.[17,18] The vast literature accumulated since then has described a diversity of useful routes for the synthesis of such compounds[19–21] and has demonstrated specific biological activities[22] and chemical properties that are a consequence of replacing the ring oxygen atom by sulfur.[23]

Replacement of the ring oxygen by sulfur leads to close mimics. However, the sulfur atom is larger and more polarizable than oxygen, the carbon–sulfur bond is longer [C–S 1.82 Å, (0.182 nm) C–O 1.43 Å (0.143 nm)], weaker, and less polar than the carbon oxygen bond (electronegativities on the Sanderson scale: O, 3.654; S, 2.957; C, 2.746); and the endocyclic C–S–C angle (95–100°) is more acute than that for a cyclic oxygen. These differences are responsible for the significant differences in the anomeric effect, conformational behavior, chemical reactivity, molecular recognition by proteins, and metabolic stability, when comparing sulfur-in-the-ring sugar analogues with their oxygen counterparts.

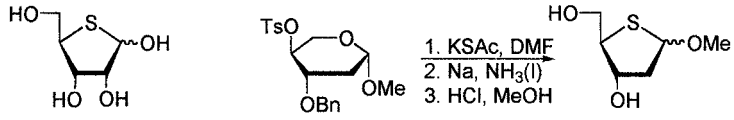
The 4-thio or 5-thio sugar analogues have attracted less attention as pharmaceuticals than did the imino sugar analogues, which are strong glycosidase inhibitors.[5] However, it has recently been shown that 1,5-dithio-β-D-xylopyranosides[24] are orally active antithrombotic agents; Salacinol[25] and Kotalanol,[26] unique structures comprising the "1-deoxy-4-thio-D-arabinofuranosylsulfonium" cation and an internal polyhydroxyalkyl sulfate anion, are potent α-glucosidase inhibitors, isolated from *Salacia reticulata* and traditionally used for diabetes treatment in Indian medicine; and (−)-β-L-2′,3′-dideoxy-3′-thiacytidine (Lamivudine, 3TC), a dideoxynucleoside analogue in which the 3′-CH_2 group has been replaced by a sulfur atom, is an effective and nontoxic drug for the treatment of HIV infections, approved by the United States Food and Drug Administration.[27]

These results have prompted preparation of this chapter, which attempts to present an organized discussion of the studies described, mainly in the past two decades, on the chemistry of sugar derivatives containing sulfur in the ring, including mono- and oligo-saccharides, 4′-thionucleosides, and 3′-deoxy-3′-thianucleoside analogues. Discussions on the biological and therapeutic activities of these families of compounds are also presented in each section. The term thia sugar is restricted to structures where a methylene group, not oxygen, is replaced by the sulfur atom.[28] The term thio sugar is a class name for sugar analogues with sulfur replacing the ring oxygen, and thio should be preceded by the locant of the sulfur atom.[28]

II. Five-Membered Rings

1. Synthesis of 4-Thioaldoses: Methods for Introducing a Sulfur Atom in a Sugar Moiety

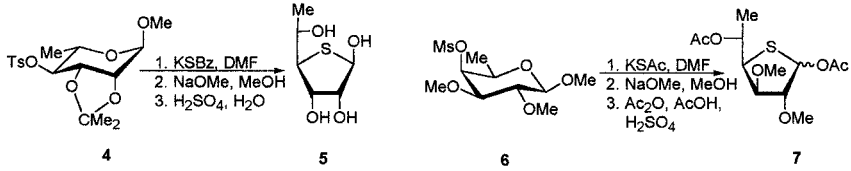
a. Nucleophilic Displacement of a Sulfonyloxy Group.—The syntheses of carbohydrates having sulfur in the furanose ring generally involve displacement of a sulfonyloxy group at C-4 of a suitable protected aldose (or at C-5 of a ketose)



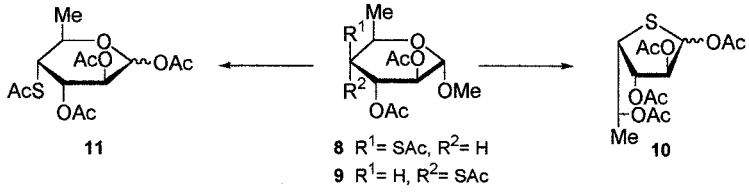
SCHEME 1

using thioacetate, thiobenzoate, or thiocyanate as nucleophilic agents, followed by deprotection to allow spontaneous cyclization.[17] The first 4-thioaldoses to be synthesized by this method were 4-thio-L-[29,30] and D-ribofuranoses (**1**),[30–32] starting from D- and L-lyxose, respectively. Similarly, inversion at C-4 led to the synthesis of peracylated 4-thio-D-xylofuranose[33] starting from 2,3-di-*O*-benzoyl-4-*O*-tosyl-β-L-arabinopyranoside. Fu and Bobek reported[34,35] a 14-step synthesis of a separable mixture of α and β anomers of methyl 2-deoxy-4-thio-D-*erythro*-pentofuranosides (**3**) from L-arabinose, through the tosylate **2** of the L-*threo* configuration.

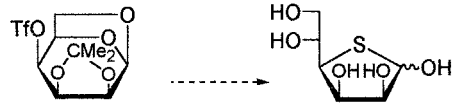
Crystalline 6-deoxy-4-thio-L-talofuranose (**5**), obtained by Owen and Ragg[36] from the L-rhamnopyranoside **4,** was the first described 4-thiohexofuranose. The same authors prepared the 4-thio-D-glucofuranose derivative **7** from the D-galactopyranoside **6.**[36] The *ido* (**8**) and *altro* (**9**) derivatives, prepared[37] from the corresponding 4-mesylates (of the *altro* and *ido* configurations), behaved differently on acetolysis (Ac_2O, H_2SO_4). The idopyranoside **8** gave the furanose form **10,** whereas **9** gave the pyranose form **11.** ^{1}H NMR spectroscopy of the deacetylated compounds showed that 6-deoxy-4-thio-D-idose adopted the furanose form in aqueous acetone, whereas the *altro* compound was an equilibrium mixture of three components. The 4-thio-D-mannofuranose **13** was prepared[38] from triflate **12** by treatment with sodium thiobenzoate, followed by acetolysis and deprotection.



SCHEME 2



SCHEME 3

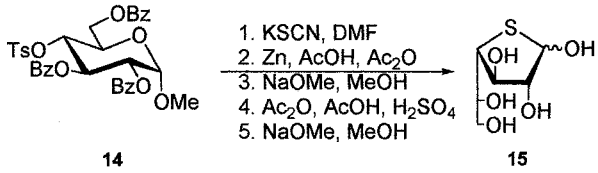


12 13

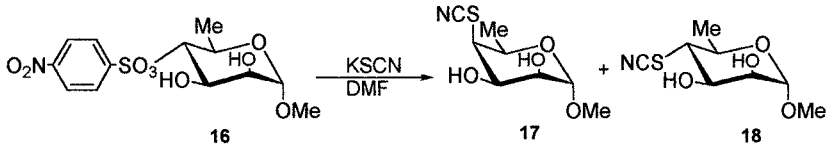
SCHEME 4

The 4-thio-D-galactofuranose **15** has been synthesized[39] from the α-D-glucopyranoside **14** as described in Scheme 5. 6-Deoxy-4-thio-D-galactofuranose[40] and 4-thio-L-rhamnofuranose[41] were prepared in a similar way, the key step being the displacement of the sulfonyloxy group of 6-deoxy-D-gluco- and L-talo-pyranosides, by the thiocyanate ion with inversion at C-4. However, the 4-*O*-(*p*-nitrophenylsulfonyl) derivative **16** led to a 2 : 1 mixture of the 4-thiocyano derivatives **17** and **18** with inversion and retention of the C-4 configuration.[42] Neighboring-group participation of OH-3 could account for the formation of the minor isomer.

Recently, synthesis of the galactofuranose **20,** the first 2-amino-2-deoxy-4-thiohexofuranose, has been reported.[43] Acetolysis or hydrolysis with aqueous HCl of **19** gave a complex mixture. However, treatment of **19** with Amberlite IR-120(H^+) in water or in methanol yielded **20** or its methyl glycosides, respectively.

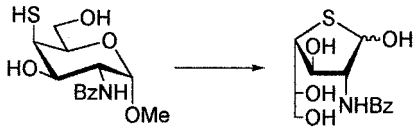


14 15



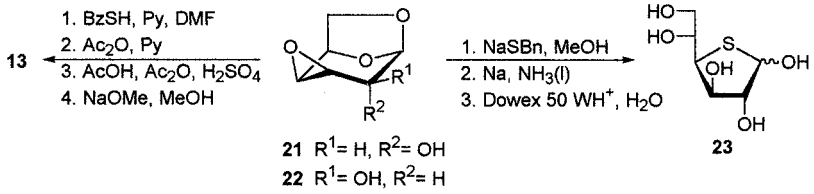
16 17 18

SCHEME 5



19 20

SCHEME 6



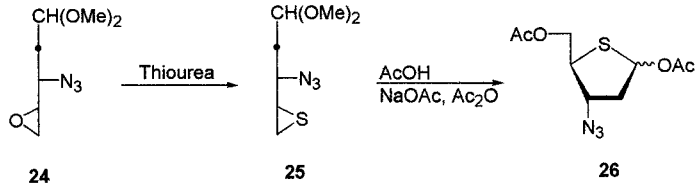
SCHEME 7

Conformational analysis of 4-thiohexofuranose derivatives having α- and β-D-galacto and D-manno and α-D-talo configurations, has been carried out by Cicero and Varela.[44] The anomeric substituent prefer a quasiaxial orientation and the bulky side chain tends to take a quasiequatorial disposition.

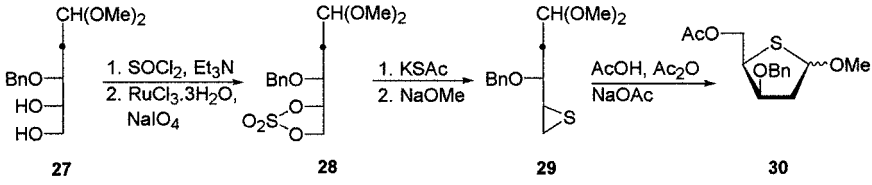
b. Oxirane Ring-Opening.—Oxirane ring-opening at C-4 of 3,4-anhydro sugar derivatives by an RS^- group has also been used in the syntheses of 4-thio-D-glucofuranose (**23**)[45] and 4-thio-D-mannofuranose (**13**),[46] which were prepared from 1,6 : 3,4-dianhydro-β-D-galactopyranose (**21**) and the talopyranose **22,** respectively. Similarly, the synthesis of ethyl 4-thio-α-D-lyxofuranoside was achieved[47] from ethyl 3,4-anhydro-β-L-ribopyranoside.

One of the key steps in the synthesis of 3-azido-2,3-dideoxy-4-thio-D-*erythro*-pentofuranoses (**26**) from D-xylose is the transformation of 4,5-oxirane **24** into the 4,5-epithio derivative **25** by reaction with thiourea.[48] Introduction of a thioacetate group at C-5 of a hexose derivative, followed by chain degradation with periodate to excise C-1, was used in the first synthesis of methyl 2-deoxy-4-thio-D-*erythro*-pentofuranosides, carried out by Nayak and Whistler,[49] starting from D-glucose. The same procedure was used in the synthesis of 4-thio-D-arabinofuranosides.[50]

c. Opening of Cyclic Sulfates of *vic*-Diols.—Santoyo *et al.*[51] have described an expeditious one-pot synthesis of thiiranes from cyclic sulfate of terminal *vic*-diols and have shown its applicability to the synthesis of 4- and 5-thio sugars. 2-Deoxy-4-thio-D-*threo*-pentofuranoside (**30**) was prepared in seven steps starting



SCHEME 8

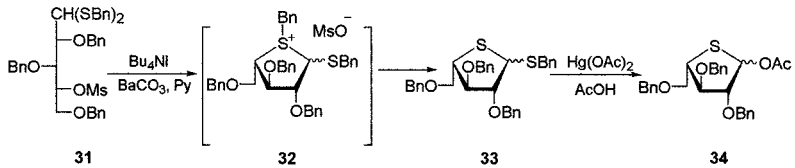


SCHEME 9

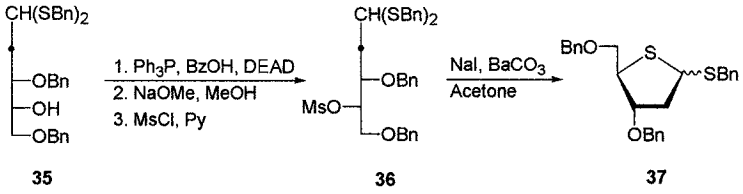
from L-arabinose diethyl dithioacetal in 9% yield. The sequence *vic*-diol **27** → cyclic sulfite → cyclic sulfate **28** → acyclic sulfate potassium salt → thiirane **29** shows the key steps of the method.

d. Cyclization of Dithioacetals.—A convenient route to 4-thio sugars involves intramolecular cyclization of dibenzyl dithioacetals of aldoses having a sulfonyloxy group at C-4.[52–62] This method was first described by Harness and Hughes[63] for the synthesis of benzyl 1,5-dithiopentopyranosides. Numerous sets of reaction conditions for the cyclization have been used, for example, heating in dry acetone containing barium carbonate and sodium iodide.[52–54,60] The essential role that the iodide ion plays is to remove the *S*-benzyl group from the intermediate cyclic sulfonium ion by nucleophilic displacement. Thus, the 4-thio-L-arabinofuranose derivative **34** was obtained[62] from D-xylose in six steps (51%), by preparation of its benzylated methyl glycoside, ring opening ($PhCH_2SH$, conc. HCl), mesylation and cyclization of the dithioacetal **31** to yield **33** (94%) via sulfonium ion **32.** The 1,4-dithiofuranoside **33,** as a 4 : 1 anomeric mixture, was transformed into the acetate **34** (76%) by means of mercuric acetate in acetic acid.

Walker *et al.*[54,55] have described by this method a seven-step synthesis of 4-thio-2-deoxy-D-*erythro*-pentose (**37**) from 2-deoxy-D-*erythro*-pentose via dithioacetals **35** and **36,** involving inversion at C-4 by Mitsunobu reaction and final cyclization of the dithioacetal, accompanied by further inversion at C-4. Secrist *et al.*[59] have synthesized **39** from the ribose derivative **38** using the same method. Similarly, Imbach *et al.* have prepared 1,4-dithio-D-ribofuranosides **39** from L-lyxose[56] and from D-ribose,[58] and 1,4-dithio-L-lyxofuranosides **40** from D-ribose.[57] Mackenzie

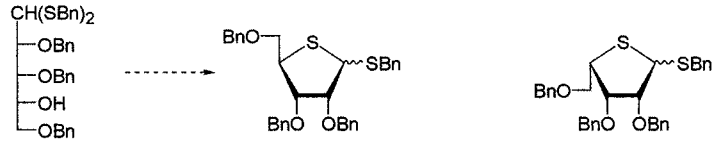


SCHEME 10



35 **36** **37**

SCHEME 11

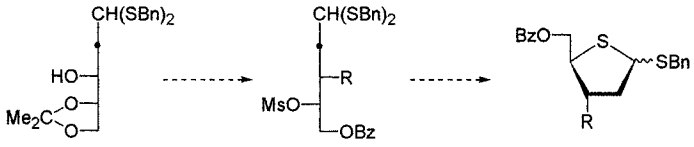


38 **39** **40**

SCHEME 12

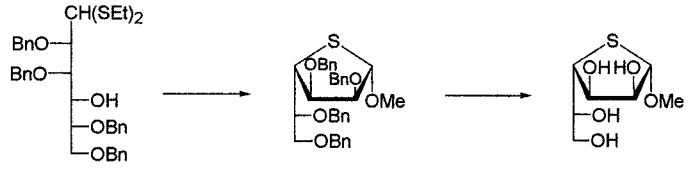
and co-workers[60] have prepared the 3-substituted 2-deoxy-4-thio-D-*erythro*-pentose derivatives **44** and **45** from L-arabinose, via dithioacetals **41**–**43.** The key steps are inversion at C-3 with the Mitsunobu reagents (Ph_3P, diisopropylazodicarboxylate, DIAD) and benzoic acid or the complex $Zn(N_3)_2(C_6H_5N)_2$,[64] respectively, followed by cyclization. Intramolecular cyclization of variously substituted 4-*O*-*p*-tolylsulfonyl-D-glucose dibenzyl dithioacetals yielded the corresponding 1,4-dithio-D-galactofuranosides.[53]

Classon *et al.*[65] have described ring closure of the 4-unprotected D-mannose diethyl dithioacetal **46,** without a leaving group, with Ph_3P and triiodoimidazole, to give the α-D-talofuranoside in 86% yield. Treatment with bromine in methanol, followed by hydrogenolysis, yielded the α-glycoside **48.** The Ph_3P and triiodoimidazole reagent system had been previously used for replacing a hydroxyl group by iodine, and so one of the sulfur atoms in **46** must have intervened before the



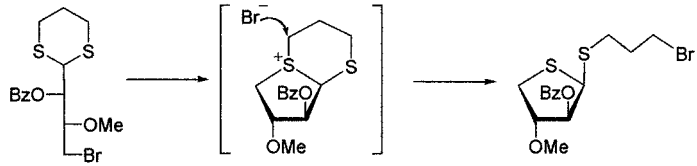
41 **42** R= OBz **43** R= N_3 **44** R= OBz **45** R= N_3

SCHEME 13



46 **47** **48**

SCHEME 14

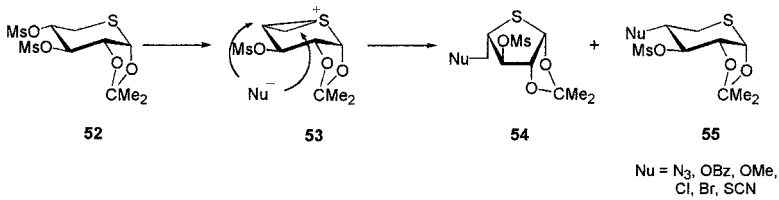


49 **50** **51**

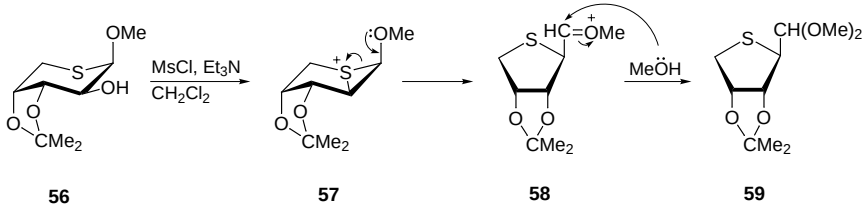
SCHEME 15

nucleophilic attack of iodide anion could occur upon the presumed 4-phosphonium intermediate of **46.** Huang and Hui[66] reported the synthesis of **37** by ring closure of D-*erythro*-pentose dibenzyl dithioacetal (**35**) with PPh_3, I_2, and imidazole. Two subsequent publications[57,67] showed conclusively that they had obtained the isomer of the L-*threo* configuration, by a single inversion at C-4. Using this method, the group of Secrist[68] described the synthesis in 83% yield of benzyl-2,3,5-tri-*O*-benzyl-1,4-dithio-D-arabinofuranoside, the D enantiomer of **33,** from L-xylose by cyclization of the 2,3,5-tri-*O*-benzyl-L-xylose dibenzyl dithioacetal with PPh_3, I_2 and imidazole. Similarly, the same group prepared **37**[69] from 2-deoxy-D-*erythro*-pentose by an alternative procedure to that described in Scheme 11, going directly to the final product from the inverted alcohol of L-*threo* configuration using PPh_3, I_2, and imidazole in the cyclization step. Intramolecular cyclization of the D-threose dithiane **49** led to 1,4-dithio-β-D-threofuranoside **51** in quantitative yield via bicyclic sulfonium intermediate **50.**[70]

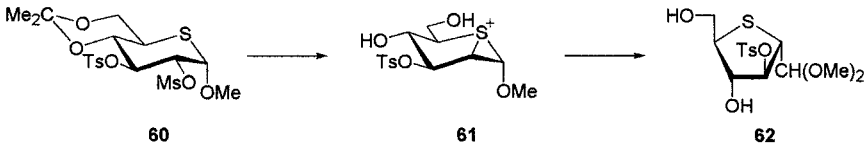
e. Conversion of 5-Thio Sugars.—Transannular participation of the ring sulfur atom of 5-thio sugar derivatives in displacement reactions is facilitated by the larger lone-pair orbital of sulfur (3p) compared to that of oxygen (2p). This allows the synthesis of some 4-thio sugar analogues.[71–73] Thus, the dimesylate **52,** treated with various nucleophiles, gave the 5-substituted 4-thiofuranosides **54** via episulfonium ion **53,** as well as minor amounts of 4-substituted-5-thiopyranoses **55,** with configurational retention when azido or benzoate anions are used as nucleophiles.[71] Analogously,[73] the attempted mesylation of 5-thio-β-D-arabinopyranoside **56** led



SCHEME 16



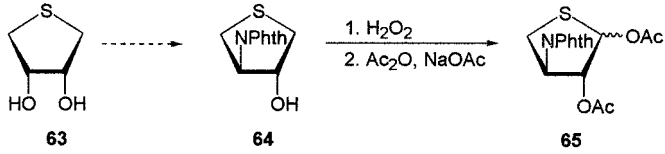
SCHEME 17



SCHEME 18

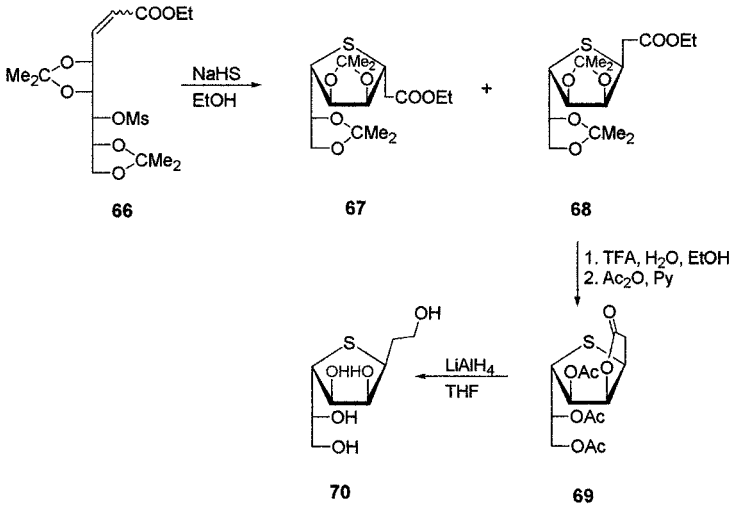
to the 2,5-epithio-D-ribose dimethyl acetal **59** in 82% yield. The reaction proceeded via episulfonium (**57**) and carboxonium (**58**) ions. In contrast, compound **60** was stable because episulfonium ion formation would have led to a strained *trans*-fused bicyclic system. Removal of the acetal with HCl in methanol yielded **62** (96%).[72] Similar participation of the ring sulfur atom was observed for the acid methanolysis and acetolysis of 5-thioglucopyranosides.[74,75]

f. Pummerer Rearrangement.—McCormick and McElhinney prepared racemic 4-thio-tetrofuranoses by Pummerer rearrangement of *cis* or *trans* thiolan-3,4-diol-1-oxides, which were prepared from 1,4-dichlorobutane-2,3-diols and sodium sulfide.[76–80] For example, the 3-deoxy-3-phthalimido-DL-threofuranose **65** was obtained[80] from racemic diol **63** through *S*-oxidation of thiolane **64** with hydrogen peroxide, followed by Pummerer rearrangement with Ac_2O–NaOAc. The isolation of only a 3-deoxy-3-phthalimido-tetrose derivative agrees with the tendency favoring Pummerer rearrangement toward O rather than N in β-substituted sulfoxides.[79]



63 **64** **65**

SCHEME 19



66 **67** **68** **70** **69**

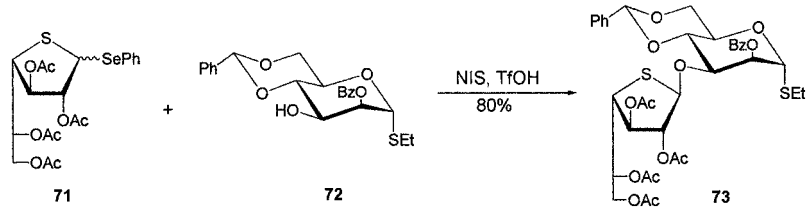
SCHEME 20

2. Synthesis of Thioanhydro-aldonic Acid Derivatives

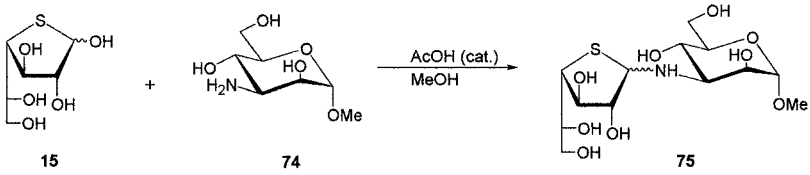
The synthesis of polyhydroxylated thiolanes has recently been described[81] by a procedure that involves introduction of a thiol group in a sugar moiety by the conjugate addition of the hydrogen sulfide anion to the double bond of an α,β-unsaturated ester, followed by internal nucleophilic displacement of the sulfonyloxy group. Treatment of mesylate **66,** itself prepared from D-mannose, with NaSH in ethanol led to a mixture of octonates **67** and **68** (90%, 1 : 1.5 ratio), that could not be separated by chromatography. Acid-catalyzed lactonization of **68** allowed chromatographic separation of the mixture. Reduction of lactone **69** with $LiAlH_4$ furnished 3,6-thioanhydrooctitol **70.** Following a similar sequence of reactions, 3,6-anhydro-2-deoxy-6-thio-L-*arabino*-hexitol was prepared from L-erythrose.

3. Synthesis of 4-Thiofuranosyl-Containing Oligosaccharides

Pinto *et al.*[82] have reported the synthesis of 4-thiogalactofuranosyl analogues of di-, tri-, and tetra-saccharides corresponding to fragments of the



SCHEME 21



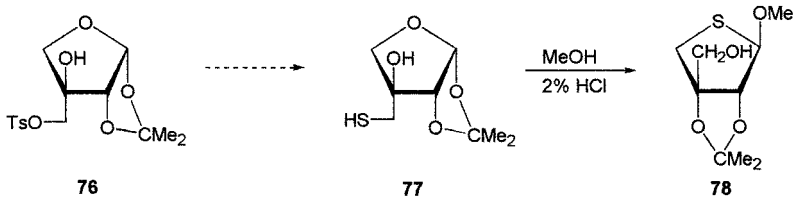
SCHEME 22

glycosylinositol phospholipid (GIPL) from the protozoan, *Trypanosoma cruzi*, that causes Chagas disease. The synthesis of disaccharide **73** was based on the selective activation of 4-thio-1-selenogalactofuranoside **71** as glycosyl donor over an ethyl 1-thioglycoside as glycosyl acceptor with NIS/TfOH. Disaccharide **73** could also be employed as a donor in a trisaccharide synthesis with the same activator, although the considerably slower glycosylation reaction caused anomerization of the 4-thiogalactofuranoside component, because of competition for I^+ between the sulfur atom of the aglycone and the sulfur atom of the galactofuranoside. The selenoglycoside **71** was prepared in 94% yield by reaction of peracetylated 4-thiogalactofuranose with phenylselenol and $BF_3{\cdot}Et_2O$. New heteroanalogues of GIPL fragments from *Trypanosoma cruzi* containing a 4-thiogalactofuranosyl residue at the nonreducing unit and a nitrogen atom in the interglycosidic linkage have been prepared.[83] Acid-catalyzed condensation of 4-thio-D-galactofuranose **15** and methyl 3-amino-3-deoxy-α-D-mannopyranoside (**74**) was performed using acetic acid in refluxing methanol, without the use of protecting groups on the monosaccharide units. This led to a nonseparable $\alpha : \beta$ mixture (1 : 3) of **75** in 52% yield.

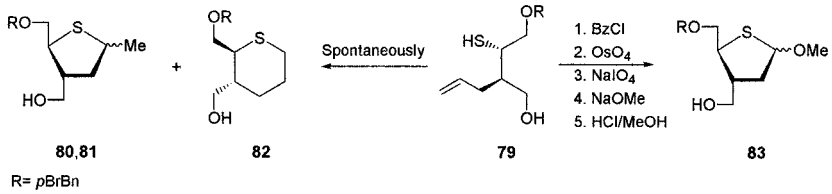
4. Synthesis of Branched 4-Thiofuranoses

In the branched-chain series, the 4-thioapiofuranoside **78** was the first reported[84] example prepared from the apiose derivative **76** via acid-catalyzed methanolysis of thiol **77,** with migration of the acetal group.

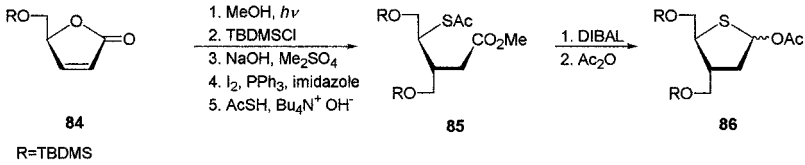
Samuelsson *et al.*[85] have prepared 4-thiofuranosides **83** via regioselective opening of an episulfide with allylmagnesium bromide. This furnished thiol **79**



SCHEME 23



SCHEME 24

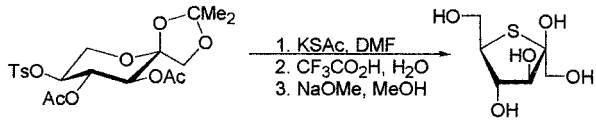


SCHEME 25

that underwent spontaneous transformation into the unseparable mixture of **80**–**82.** Benzoylation of **79** followed by oxidative cleavage of the alkenic bond, ring closure, and treatment with HCl–MeOH afforded **83.** The 4-thio-D-*erythro*-pentofuranose **86** has been prepared starting from butenolide **84,** via thioacetate **85,** which was reduced with diisobutylaluminum hydride (DIBAL), and the resultant hemiacetal was trapped with acetic anhydride.[86]

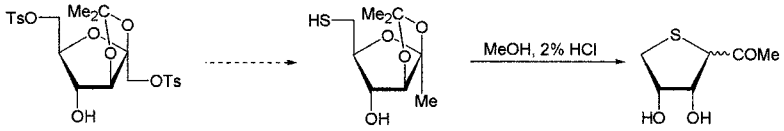
5. Synthesis of 5-Thioketoses

5-Thio-D-fructofuranose (**88**) was prepared from 5-*O*-tosyl-1,3-*O*-isopropylidene-α-L-sorbopyranose (**87**) by the standard sequence of reactions shown in Scheme 26.[87] The ^{1}H and ^{13}C NMR spectra of **88** in aqueous solution show only the signals of the furanose α and β anomers in the ratio of 11 : 89,[88] whereas the NMR spectra of D-fructose in D_2O show the presence of the β-pyranose, β-furanose, α-furanose, and α-pyranose in the ratio of 6 : 3 : 1 : trace, respectively.[89] Partial hydrolysis or methanolysis of **90,** prepared[90] from 1,6-di-*O*-tosyl-D-fructofuranose (**89**), gave 3,6-anhydro-1-deoxy-6-thio-D-*arabino* (or D-*xylo*)-hexulose (**91**).[91] Nucleophilic attack of the ring sulfur atom on C-3



87 88

SCHEME 26



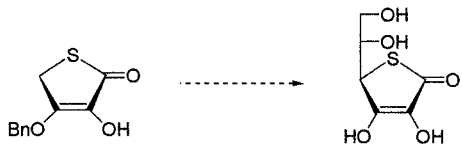
89 90 91

SCHEME 27

under acidic conditions has been argued as the explanation of the formation of the thioanhydrohexulose **91.**

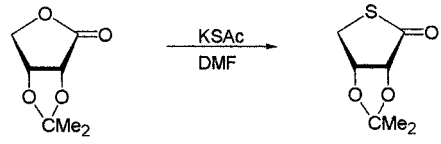
6. Synthesis of 4-Thio Sugar Lactones

Synthesis of the racemic analogue **93** of ascorbic acid was accomplished[92] in 43% yield via the unsaturated thiolactone **92** by aldol-like chain extension with *tert*-butyldimethylsilyloxyacetaldehyde and butyllithium (*threo*/*erythro* 6:1), followed by deprotection. Only a few attempts to prepare sugar thiolactones from aldonic acids have been reported. Thus, 4-thio-D-erythrono-1,4-lactone (**95**) was obtained[93] by nucleophilic attack of potassium thioacetate at C-4 of the 2,3-acetal derivative **94** of D-erythronolactone. 4-Thio-L-lyxono-1,4-lactone (**98**) was prepared[94] from tosylate **96,** which was treated with sodium methoxide and then thiourea to afford the 4,5-thiirane derivative **97.** Regioselective opening of the thiirane ring and simultaneous thiolactonization with KOAc in AcOH–DMF, followed by deacetalation, yielded **98.** A similar approach was employed for the synthesis of 4-thio-D-ribono-1,4-lactone, starting from D-gulono-1,4-lactone.[94] Deoxygenation promoted by SmI_2 of the 5-silyl derivative of 2,3-di-*O*-benzoyl-4-thio-D-lyxono-1,4-lactone led to the 2,3-dideoxy-thiolactone **99.**[95]



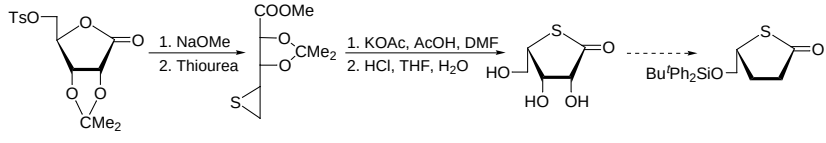
92 93

SCHEME 28



94 **95**

SCHEME 29

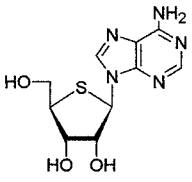


96 **97** **98** **99**

SCHEME 30

7. Nucleosides

a. 4′-Thio-*ribo, arabino,* and *xylo*-Nucleosides.—During the past decade a good deal of effort has been devoted to the synthesis of 4-thio sugar precursors, stimulated by the potent biological activities shown by some nucleoside analogues having sulfur in the pentose ring.[96,97] 4′-Thioribonucleosides are the first reported examples of such nucleosides. 4′-Thioadenosine (**100**) was prepared first (1964) by Reist *et al.*[30] by condensation of peracetylated derivatives of 4-thio-D-ribofuranosyl chloride with the chloromercuri salt of 6-benzamidopurine, followed by deprotection. The L enantiomer[30] of **100** and 4′-thio-β-D-xylofuranosyl and arabinofuranosyladenines[33] were prepared in a similar way. It was stated[98] to cause a 50% reduction in growth of *Streptococcus faecalis* with a concentration of **100** of 4.5×10^{-7} *M*. This compound also showed remarkable inhibition of *S*-adenosylhomocysteine hydrolase,[99] and 4′-thioinosine (**101**) was shown to be resistant to phosphorylase enzymes,[100] providing evidence that the presence of sulfur in the sugar ring of nucleosides stabilized the *N*-glycosyl linkage to the hydrolytic cleavage catalyzed by nucleoside phosphorylase. Further examples were reported by Whistler *et al.,* who synthesized 4′-thiopyrimidine nucleosides,[101–104] including 5-halo-4′-thiouridine[105] and the antibiotic 4′-thiotoyocamicin[106] (**102**). Some of these compounds, such as 4′-thio analogues of 5-fluorouridines[105] and *arabino*-cytosine[103,104] nucleosides, showed potent antitumor activities, especially against leukemia L-1210[104,105,107] and other cell lines, but were found to be too toxic to be used as pharmaceuticals and were inactive against HIV and herpes simplex virus (HSV).[56]



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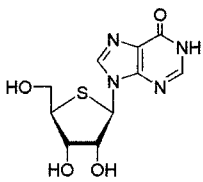

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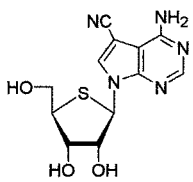
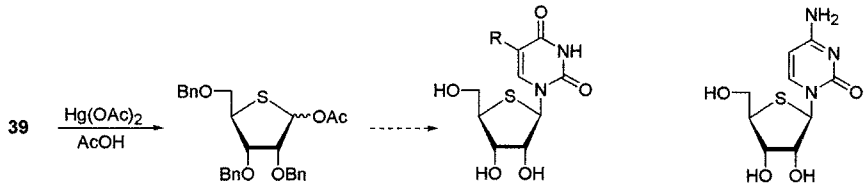


102

Imbach *et al.*[56] have reported syntheses of 4′-thiothymidine (**104**) and 4′-thiouridine (**105**) starting from L-lyxose, following the dithiobenzyl acetal cyclization method and the Hilbert–Johnson silyl condensation reaction of **103** with silylated thymine (77% yield, $\alpha:\beta$ 43 : 57) and uracil (74% yield, $\alpha:\beta$ 47 : 53), followed by deprotection. These nucleosides showed no activity against a number of DNA and RNA viruses at doses up to 1.0 m*M*. The same group has also prepared[58] 4′-thiocytidine (**106**) and 4′-thioadenosine (**100**) by coupling **103** (prepared from D-ribose in an eight-step synthesis) with N^4-benzoylcytosine or adenine. 4′-Thiocytidine **106** was also obtained[108] from the uridine analogue **105.**

Secrist *et al.*[59] synthesized 2-chloro-4′-thioadenosine (**108**) starting from D-ribose, and the latter was also transformed into **39** using the dibenzyl dithioacetal cyclization method. Removal of the benzyl groups, followed by acetylation and treatment with $Hg(OAc)_2$, yielded **107.** Coupling of **107** with 2,6-dichloropurine in the presence of $SnCl_4$ led to a 3 : 1 β/α mixture of protected nucleosides in 70% yield. After separation of the anomers, the β anomer furnished **108** on treatment with ethanolic NH_3. In a search for stable antisense RNA analogues, Imbach *et al.*[109,110] prepared 4′-thio-oligoribonucleotides, which showed more resistance to nuclease-catalyzed hydrolysis than the corresponding 4′-oxygen counterpart.

The Secrist group also prepared 4′-thio-β-D-arabinofuranosylpurine nucleosides **111**–**114**[68] and the cytosine analogue (4′-thio-ara-C)[111] **116** from L-xylose, which was transformed, by the dithioacetal cyclization method, into precursors



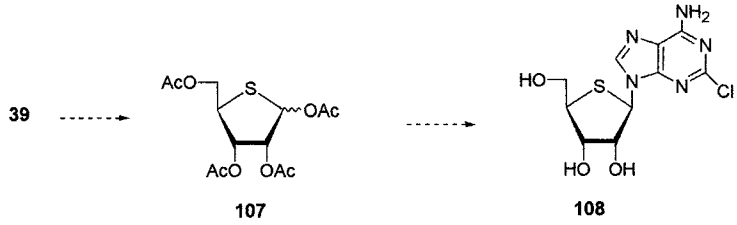
39

103

104 R= Me
105 R= H

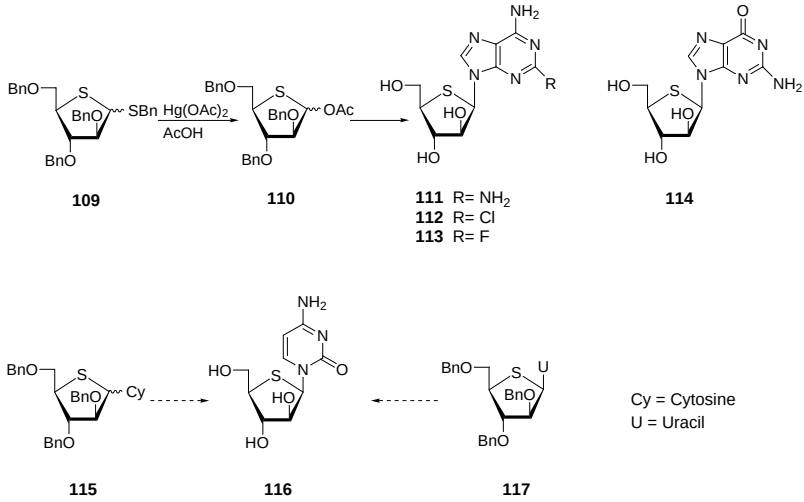
106

SCHEME 31

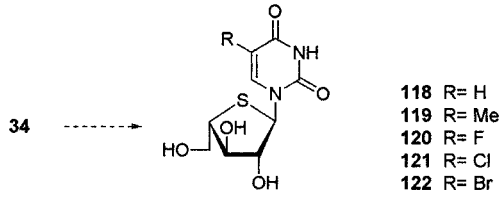


SCHEME 32

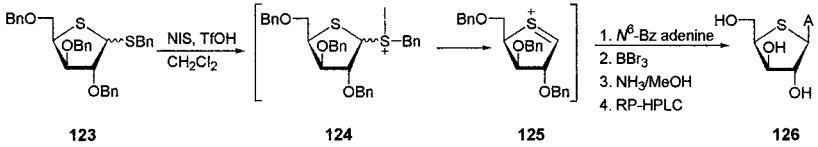
109 and **110.** Coupling *in situ* of **109** with silylated cytosine, using Me_3SiOTf as catalyst, gave a $2:1$ $\alpha:\beta$ mixture of the protected nucleoside **115** (77%), which was deblocked with BCl_3 to afford an anomeric mixture of free nucleosides that was resolved by chromatography of the 5′-dimethoxytrityl derivatives. Alternatively, compound **116** was prepared from the 4′-thiouridine analogue **117** by standard procedures. Evaluation of the purine nucleosides **111**–**114** as potential anticancer agents showed that only **111** and **114** had significant cytotoxicity. 4′-Thio-ara-C (**116**) was cytotoxic to most human tumor cell-lines at unimolar concentration, but was especially curative against HCT-116 colon tumor and effected complete regression of CAKI-1 renal tumors. Otatani and Whistler had previously described the remarkable antitumor activity of **116** against KB cells ($IC_{50} = 0.42\,\mu M$).[104] The activity of **116** is not surprising as 1-β-D-arabinofuranosylcytosine (ara-C, Cytarabine) has been used clinically against acute leukemia and lymphoma,[112,113] although it produced megalobastosin and chromosomal alteration in bone marrow.[112,114]



SCHEME 33



SCHEME 34

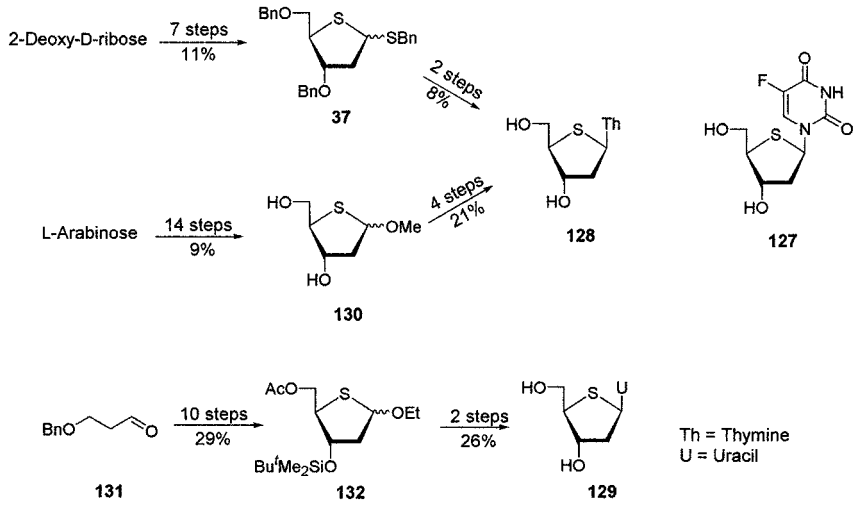


SCHEME 35

Voss *et al.*[62,115] prepared 4′-thio-L-arabinofuranosyl-uracil (**118,** $\alpha:\beta$ 5 : 2) and -thymine (**119,** $\alpha:\beta$ 5 : 1) nucleosides and the 5-halopyrimidine analogues **120**–**122** ($\alpha:\beta$ 13 : 5, 12 : 5, 10 : 6), by coupling the corresponding silylated bases with 4′-thio-L-arabinofuranose **34.** Debenzylation with boron tribromide yielded the free nucleosides **118** and **119** as an α,β mixture. Reverse-phase HPLC and debenzylation led to the pure α and β anomers of the 5-halopyrimidine analogues. No *in vitro* antiviral activity has been found for these compounds.

The synthesis of 4′-thioxyloadenosine **126,** described in the pioneering work by Reist *et al.*,[33] has been improved[116] by direct glycosylation of *N*-benzoyladenine with the 1,4-dithio-D-xylofuranoside[58] **123** in the presence of *N*-iodosuccinimide and catalytic triflic acid, followed by deprotection. Based on the ratio of isomers obtained ($\alpha:\beta$ 20 : 34) an iodium ion-promoted elimination–addition reaction mechanism has been proposed, as outlined in Scheme 35.

b. 2′-Deoxy-4′-thionucleosides.—Special attention has been focused on the synthesis of 2′-deoxy-4′-thionucleosides and 2′,3′-dideoxy-4-thionucleosides in a search for more active and more specific antiviral agents ever since the introduction of 3′-azido 2′,3′-dideoxythymidine (AZT)[117] for inhibiting reverse transcriptase coded by human immunodeficiency virus (HIV),[118] a causative agent of acquired immunodeficiency syndrome (AIDS). The drugs approved so far by the FDA in the United States for the treatment of AIDS include AZT (zidovudine), 2′,3′-dideoxyinosine (ddI, didadosine), 2′,3′-dideoxycytidine (ddC, zalcitabine), and 2′,3′-didehydro-2′,3′-dideoxythymidine (d4T, stavudine), and all of them act as reverse transcriptase inhibitors.[27]

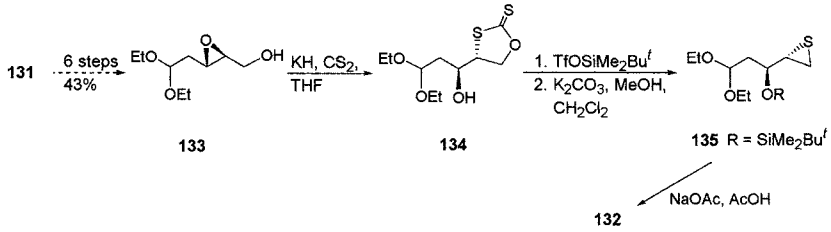


SCHEME 36

Fu and Bobek[119] reported in 1978 the synthesis of 2′-deoxy-5-fluoro-4′-thiouridine (**127**) and its α anomer, the first 2′-deoxy-4′-thioribonucleosides to be prepared. In 1991 the groups of Walker,[54,120] Secrist,[121] and Uenichi[122] simultaneously reported the syntheses of 2′-deoxy-4′-thioribopyrimidine nucleosides starting from 2-deoxy-D-*erythro*-pentose, L-arabinose, and a non-sugar precursor, respectively. All of these preparations involved the intermediacy of a convenient 2′-deoxy-4-thio sugar precursor. Thus, 2′-deoxy-4′-thiothymidine (**128**) was prepared via **37**[55] and via **130,**[34,35,121] and 2′-deoxy-4′-thiouridine (**129**) was prepared via **132**[122] (Scheme 36). Optimization of the conditions allowed the synthesis of **37** from 2-deoxy-D-*erythro*-pentose (seven steps, Scheme 11) on a kilogram scale with a yield of around 50% and involving no chromatography.[96] Another high-yielding synthesis of **37** (six steps, from 2-deoxy-D-*erythro*-pentose) has also been reported.[69]

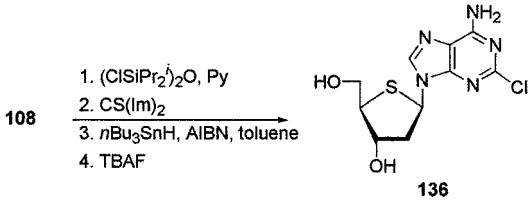
The synthesis of **132,** starting from 3-benzyloxypropanal (**131**), involved the ring opening of an optically active epoxide **133** with a xanthate anion (Scheme 37).[122] The stereoselective synthesis of **133** by Sharpless epoxidation allowed preparation of the 2-deoxy-4-thio-D- and L-*erythro*-pentoses,[123,124] which were transformed into the corresponding pyrimidine nucleosides with silylated uracil and Me_3SiOTf, and then deprotected with Bu_4NF.

A synthesis of a D-2′-deoxy-4′-thiopurine nucleoside proposed by Secrist[59] involved deoxygenation at C-2′ of the 4′-thioribopurine nucleoside. Thus, 2-chloro-2′-deoxy-4′-thioadenosine (**136**) was obtained from the ribo analogue **108** by 3′,5′-*O*-protection, followed by successive thioesterification with $CS(Im)_2$, radical

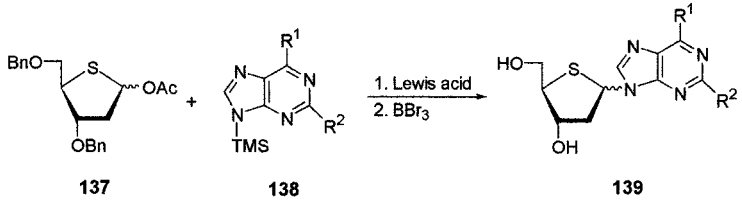


SCHEME 37

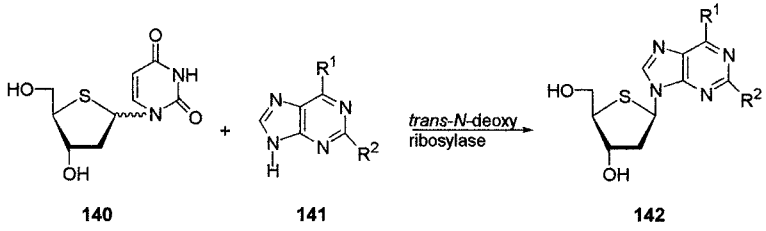
deoxygenation with (*n*-butyl)$_3$SnH, and deprotection (Scheme 38). 2′-Deoxy-4′-thiopurine nucleosides **139** were also prepared[69] by condensation of the 2-deoxy-4-thiosugar acetate **137** with silylated purine bases (**138**) in the presence of a Lewis acid, followed by deprotection with BBr_3, although by this coupling method the α anomer was always formed predominantly over the β anomer ($\alpha : \beta$ 9 : 1). The desired β anomer **142** could be obtained by transglycosylation of an anomeric mixture of 2′-deoxy-4′-thiouridine (**140**) with a purine base (**141**) in the presence of the transfer enzyme trans-*N*-deoxyribosylase.[125]



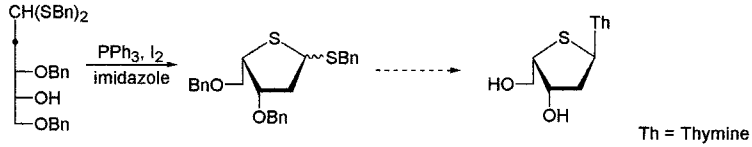
SCHEME 38



SCHEME 39

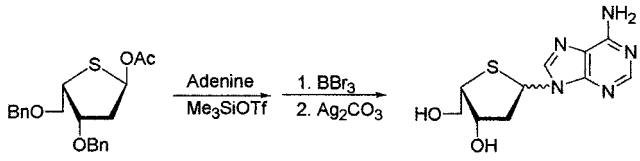


SCHEME 40



35 **143** **144**

SCHEME 41

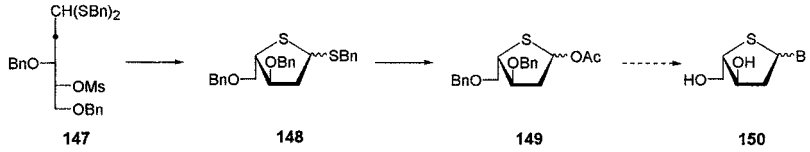


145 **146**

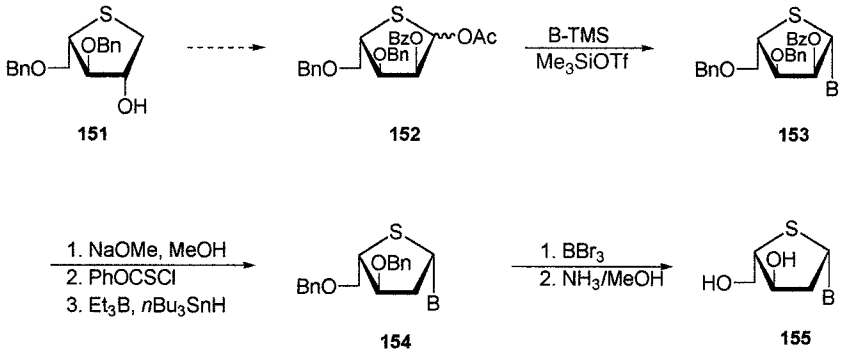
SCHEME 42

Huang and Hui[66] prepared the nucleoside **144,** of L-*threo* configuration, via 1,4-thioglycoside **143,** by a route involving ring closure of 3,5-di-*O*-benzyl-2-deoxy-D-*erythro*-pentose dibenzyl dithioacetal **35** (seven steps, from 2-deoxy-D-*erythro*-pentose) with PPh_3, I_2, and imidazole, although they erroneously proposed structure **128** and its precursor **37.** Voss *el al.*[126] prepared **144,** as well as the uridine, the 5-halouridine, and 5-nitrouridine analogues, in good yields by condensation of **143** with the silylated pyrimidine bases in the presence of *N*-iodosuccinimide, followed by debenzylation with BBr_3. The adenosine analogue **146** was prepared[126] by condensation of thio sugar **145** with adenine in the presence of Me_3SiOTf, and deprotection. The β anomer of **146** could be crystallized as a monohydrate and its configuration was proved by X-ray structural analysis.

L-2′-Deoxy-4′-thio-1′-purine and pyrimidine nucleosides **150** were synthesized[127] via 2-deoxy-4′-thio-L-ribofuranose **149.** This thio sugar was prepared from D-xylose in nine steps and 17% overall yield, by a route involving cyclization of the dibenzyl dithioacetal derivative **147** to afford **148,** the enantiomer of **37.** Similarly, 2′-deoxy-4′-thio-L-adenosine[128] **155** was also obtained from D-xylose via the 4-thio-L-arabinitol derivative **151,**[129] which was transformed into the glycosyl donor **152** by inversion at C-2,[130] under Mitsunobu conditions, followed by oxidation and Pummerer rearrangement. Condensation of **152** with silylated 6-chloropurine led to the β anomer **153** as the predominant product (60%), which could be formed by the neighboring-group effect of the C-2 benzoyl group. Debenzoylation of **153** followed by deoxygenation at C-2, using modified Barton's conditions [PhOCSCl, then $(n\text{-}Bu)_3SnH$], gave the 2′-deoxynucleoside **154,** convertible in two steps (BBr_3, then NH_3 methanol) into **155.**



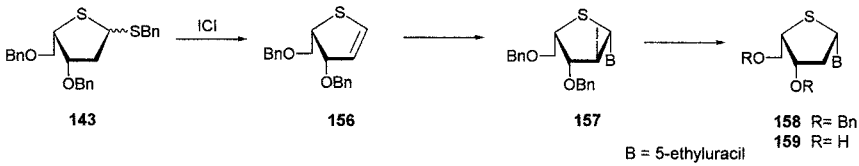
SCHEME 43



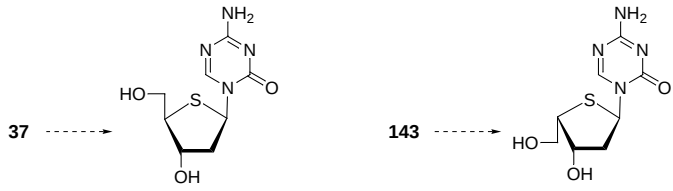
SCHEME 44

Miller *et al.*[131] described the synthesis of 2′-iodonucleoside **157** by a one-pot route from 1,4-dithioglycoside **143.**[67] Treatment of **143** with 2,6-di-*t*-butyl-4-methylpyridine and iodine monochloride, followed by reaction with 2,4-bistrimethylsilyloxy-5-ethylpyrimidine, gave the 2′-iodonucleoside **157** in 70% yield. Reductive deiodination with Bu_3SnH and AIBN, followed by debenzylation with BBr_3, gave the L-*threo* nucleoside **159** in 58% yield.

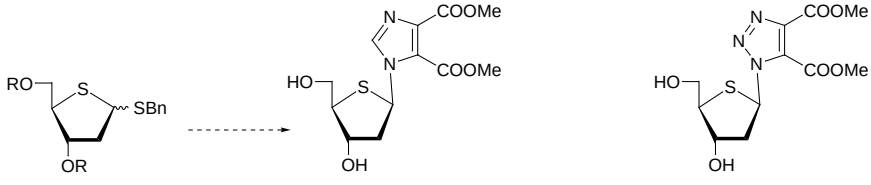
Secrist *et al.* have described[132] the syntheses of a novel class of 2′-deoxy-4′-thionucleosides, carrying 5-azacytosine of D-*erythro* and L-*threo* configurations **160** and **161,** via the 1,4-dithioglycosides **37**[69] and **143,**[67] respectively. Both nucleosides were inactive against hepatitis B virus (HBV) *in vitro.* Simons *et al.*[133] reported the synthesis of 2′-deoxy-4′-thioimidazole nucleoside **163** by coupling the *p*-nitrobenzoyl thio sugar **162** with 4,5-bis(methoxycarbonyl) imidazole in



SCHEME 45



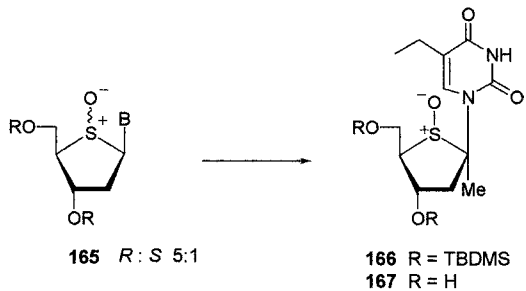
SCHEME 46



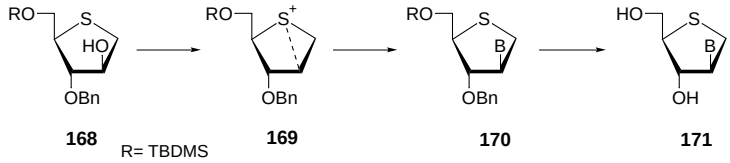
SCHEME 47

the presence of NIS ($\alpha:\beta$ 3 : 1, 60%), followed by separation of the anomers by chromatography and deprotection. These imidazole and triazole nucleoside analogues[134] showed no or poor antiviral activity.[133,134]

Walker *et al.*[135] studied the oxidation of 2′-deoxy-5-ethyl-4′-thiouridine with sodium metaperiodate to afford a separable mixture of *R* and *S* sulfoxides **165** (5 : 1, 62%). The *R* isomer was converted, after protection, by reaction with LDA to the 1′anion, which was treated with a number of electrophiles to furnish novel nucleosides analogues, such as **166,** which was deprotected to **167** with triethylamine–trihydrogen fluoride.



SCHEME 48



SCHEME 49

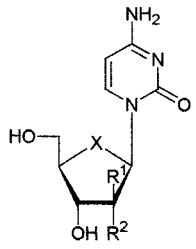
A new class of iso-4′-thionucleosides[136] **171,** with the base moiety at the 2′ position, was synthesized from D-glucose by the coupling of 1,4-anhydro-4-thio-D-arabinitol **168**[137] with purine and pyrimidine bases using the Mitsunobu reaction. The reaction gave predominantly β isomers **170** ($\alpha : \beta$ 1 : 6), when acetonitrile was used as solvent. The reaction proceed via competition between a direct SN2 reaction and an episulfonium intermediate **169.**

2′-Deoxy-4′-thio-1′-D-pyrimidine nucleosides[54,69,96,120,123,138,139] showed potent anti-HSV (herpes simplex virus) and antitumor activities against leukemia L1210, H-Ep-2, and CCRF-CEM cell lines, as well as KB cells in culture. 2′-Deoxy-4′-thio-1′-D-purine nucleosides[96,125] exhibit potent anti-HBV (hepatitis B virus) and anti-HCMV (human cytomegalovirus) activity, but they were found to be highly nephrotoxic when tested *in vivo.*[125] 2′-Deoxy-4′-thiothymidine (**128**) has been shown to be toxic to a range of viruses and host cells, and is also incorporated into L1210 cells, where it is stable and not readily removed by DNA repair enzymes.[140]

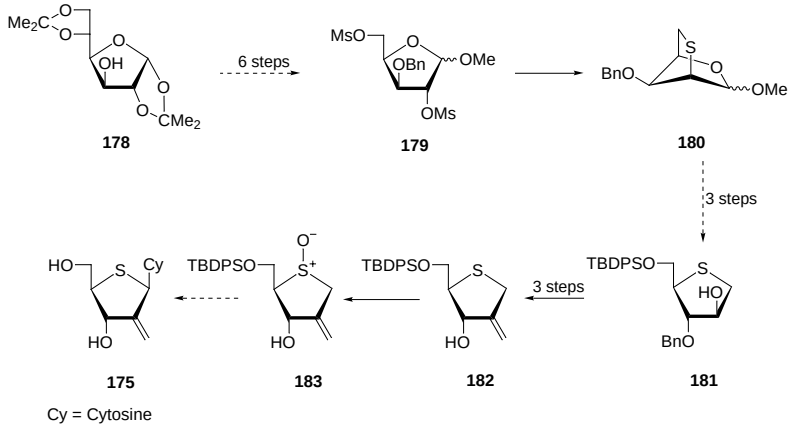
c. 2′-Deoxy-2′ Substituted 4′-Thionucleosides.—The potent antitumor activity against various solid tumors, as well as leukemia, of some cytidine analogues having various 2′-deoxy-2′-substituents, such as 2′-deoxy-2′,2′-difluorocytidine[141,142] (Gemcitabine, **172**), 2′-deoxy-2′-methylenecytidine[143,144] (DMDC, **174**), and 1-(2-azido-2-deoxy-β-D-arabinofuranosyl)cytosine[145] (Cytarzid, **176**), prompted Yoshimura and co-workers[137,146] to prepare the 4′-thionucleoside analogues **173, 175** (4′-thioDMDC), and **177** (4′-thioFAC) via different routes. Thus, 2′-methylene compound **182,** prepared in a 13-step sequence from 1,2 : 5,6-di-*O*-isopropylidene-D-glucose (**178**), was the key intermediate for the synthesis of **175.** In six steps, **178** was converted (Scheme 45) into an anomeric mixture of dimesylate **179,** which was treated with Na_2S to yield bicyclic **180.** Acid hydrolysis, hydride reduction, and protection with the *tert*-butyldiphenylsilyl (TBDPS) group produced 1,4-anhydro-4-thioarabinitol (**181**). Oxidation with Me_2SO–Ac_2O and subsequent Wittig reaction, followed by deprotection of the benzyl group by BCl_3, led to **182.** Oxidation with *m*-chloroperoxybenzoic acid (*m*-CPBA) to the sulfoxide **183** and Pummerer-type glycosylation allowed coupling of the sulfoxide with the silylated *N*-acetylcytosine in the presence of

Me_3SiOTf to produce the protected nucleoside ($\alpha:\beta$ 2.5 : 1), which was deprotected to furnish the anomeric mixture of **175,** separated by HPLC (α 34%; β 13%). Various 5-substituted uracil analogues of **175** were synthesized by similar sila-Pummerer-type glycosylation.[147]

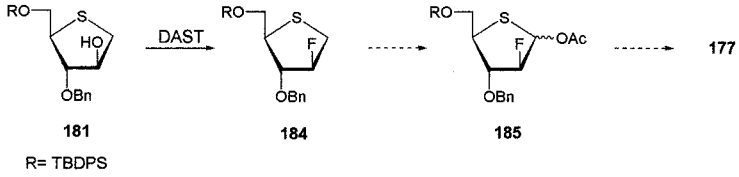
Treatment of **181** with diethylaminosulfur trifluoride (DAST) introduced a fluorine atom, with retention of the 2-stereochemistry, yielding **184,** which was converted to the 1-*O*-acetyl derivative **185** by the usual oxidation and Pummerer rearrangement. Coupling of **185** with silylated N^4-acetylcytosine in the presence of $SnCl_4$ (93%, $\alpha:\beta$ 2.9 : 1) and deprotection gave **177.**[146] The 1,4-anhydroarabinitol derivative **181** was also the key step in the synthesis of **173**[146] and a series of 4′-thio-D-arabinonucleosides.[148] An alternative method[149,150] for the synthesis of **177** employed stereoselective glycosylation ($\alpha:\beta$ 1 : 4) of 1-bromide **188,** which was prepared from D-glucose via the 3-fluoro compound **186** and the 5,6-thiirane **187.**



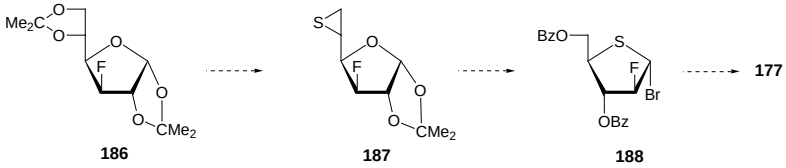
172 $R^1 = R^2 = F$; X = O
173 $R^1 = R^2 = F$; X = S
174 $R^1 = R^2 = CH_2$; X = O
175 $R^1 = R^2 = CH_2$; X = S
176 $R^1 = N_3$; R^2 = H; X = O
177 R^1 = F; R^2 = H; X = S



SCHEME 50



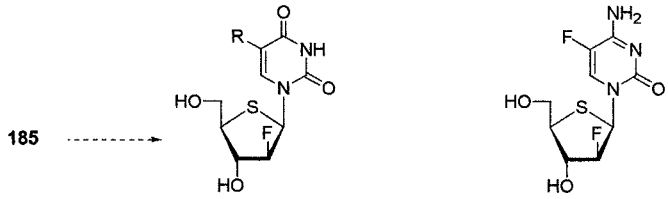
SCHEME 51



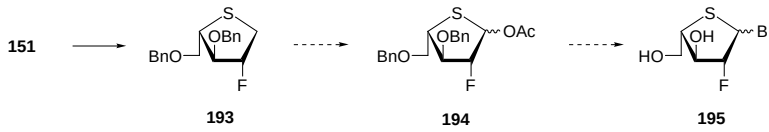
SCHEME 52

Nucleosides 4′-thioDMDC (**175**) and 4′-thioFAC (**177**) were found to have potent antineoplastic activity *in vitro* against human T-cell leukemia CCRF-HSB 2 ($IC_{50} = 0.0091$ and 0.051 μg/mL) and solid tumor KB cells ($IC_{50} = 0.12$ and 0.015 μg/mL),[146] and **177** showed prominent antineoplastic activities *in vivo.*[151,152] However, 4′-thiogemcitabine **173** did not show any activity.[146] 4′-ThioFAC (**177**) was highly effective against human tumors, even by oral administration.[152]

Several 2′-deoxy-2′-fluoro-4′-thio-D-arabinofuranosylpyrimidine nucleosides [β anomers of 5-iodouracil (**189**), 5-ethyluracil (**190**), and 5-chloroethyluracil (**191**) derivatives], also prepared from the 1-*O*-acetyl derivative **185** and silylated pyrimidine in the presence of Me_3SiOTf, showed potent and selective activity against herpes simplex virus (HSV-1 and HSV-2).[153,154] In the purine series, guanine and 2,6-diaminopurines, prepared in a similar way, showed prominent antiviral activities with slight cytotoxicity. The 5-fluorocytosine derivative (5F-4′-thioFAC) **192** showed potent antitumor activity against both leukemia and solid tumors, although it was 10 times less active than 4′-thioFAC **177.**[154]



SCHEME 53

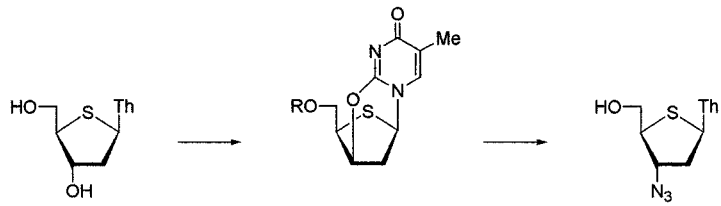


SCHEME 54

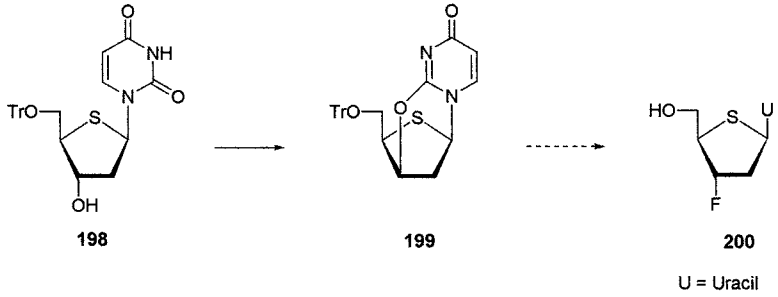
2′-Deoxy-2′-fluoro-4′-thio-L-*arabino*-nucleosides **195** were synthesized[155] starting from D-xylose via the 4-thio-L-arabinitol derivative **151** as a key intermediate (Scheme 54). Introduction of fluorine at C-2 of **151** with DAST yielded **193,** which was transformed into thio sugar **194** by oxidation and Pummerer rearrangement. Coupling of **194** with the base and debenzylation afforded **195.** The thymine analogue exhibited a moderate activity against both HSV-1 and HSV-2.

d. 2′,3′-Dideoxy-2′ or 3′-Substituted 4′-Thionucleosides.—Syntheses of the 4′-thio analogue of AZT, namely, 3′-azido-2′,3′-dideoxy-4′-thiothymidine (**197**), have been reported by different routes. Tritylation of **128** and treatment with DAST gave the 4′-thio-2,3′-anhydrothymidine **196** (R = Tr), which on treatment with sodium azide followed by deprotection afforded **197** (23%, from **128**).[156] Similarly,[54,120] **197** was also obtained via **196** (R = H), prepared from **128** using 2-chloro-1,1,2-trifluorotriethylamine reagent.[157] An alternative strategy for the formation of thioAZT (**197**) and related uridine and 5-halouridine nucleosides has been developed by Villa and co-workers[48] and involves the introduction of the azido group early in the sequence of steps (Scheme 8), thus generating the 3-azido-4-thioribofuranose **26** (13 steps, 2%, from D-xylose), which is then readily converted into several nucleosides by standard procedures. ThioAZT and related uridine nucleosides showed minimal cytotoxicity and none of them were found to possess appreciable activity against HIV-1 or HIV-2.[48,54]

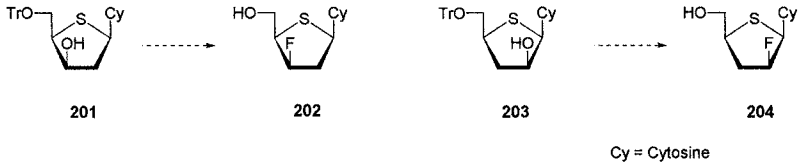
A facile fluorination of 2′- or 3′-deoxy-4′-thiopyrimidine nucleosides was proposed by Marques and co-workers.[158] Thus, fluorination of 2′-deoxy-4′-thiouridine



SCHEME 55



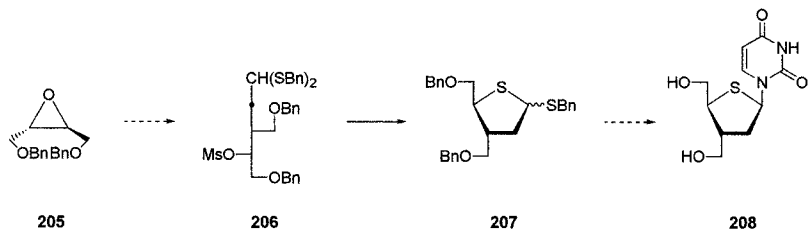
SCHEME 56



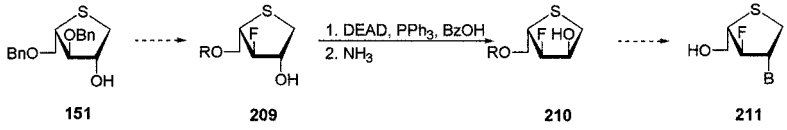
SCHEME 57

198 with DAST produced the anhydronucleoside intermediate **199,** which gave the fluorinated nucleoside **200** on reaction with KF. Similarly, fluorination of 2′-deoxy **201** and 3′-deoxy-4′-thio-β-D-*threo*-cytidine nucleosides **203** with DAST afforded the fluoro analogues **202** and **204** with retention of the configuration at C-3 and C-2, respectively.[159]

The 2′,3′-dideoxy-3′-*C*-(hydroxymethyl)-4′-thionucleoside **208** was prepared[86] from the chiral epoxide **205** via dithioacetal **206,** which was transformed into the benzyl 1,4-dithiopentofuranoside **207** (93%) with sodium iodide and triethylamine. Coupling of **207** with bis(trimethylsilylated) pyrimidines in the presence of NIS, and deprotection (BBr_3) afforded **208.** Compound **208** has also been prepared by other routes.[86,160]



SCHEME 58

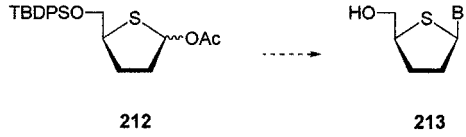


SCHEME 59

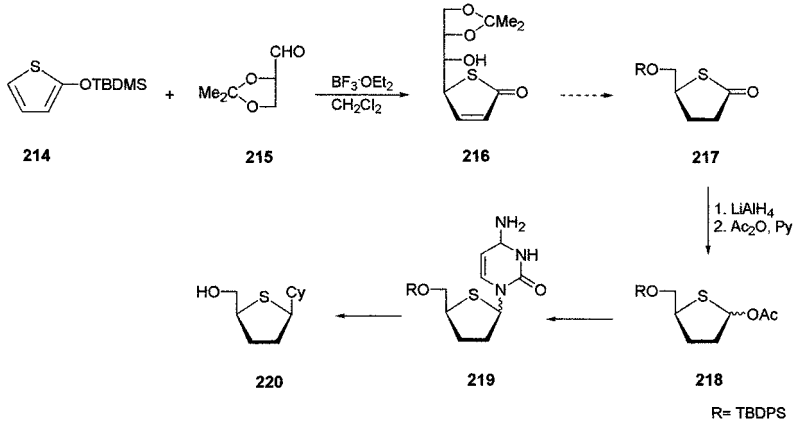
The iso-4′-thiopurine and pyrimidine L-nucleosides **211** were synthesized[130] from 1,4-thio-L-arabinitol (**151**) via 3-fluoro derivative **209,** which was transformed into **210** by a Mitsunobu reaction. Coupling of **210** with purine and pyrimidine bases by the Mitsunobu reaction, followed by deprotection, gave the desired **211.**

e. 2′,3′-Dideoxy-4′-thionucleosides.—A series of 2′,3′-dideoxy-4′-thionucleosides (**213**) of purines and pyrimidines have been synthesized,[156] via the 2,3-dideoxy-4-thio sugar precursor **212,** starting from L-glutamic acid. Only 2′,3′-dideoxy-4′-thiocytidine showed significant anti-HIV activity.

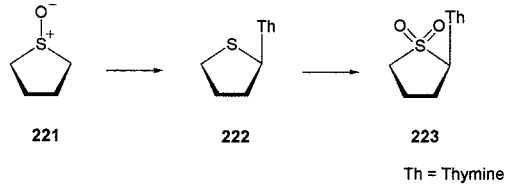
2′,3′-Dideoxy-4′ thiocytidine **220** was prepared[161] by diastereoselective addition of the thiophene derivative **214** to 2,3-*O*-isopropylidene-L-glyceraldehyde (**215**) in the presence of BF_3 etherate, which resulted in preferential formation of the 4-*S*-adduct **216** (73% isolated yield). Transformation of **216** into the thiolactone **217** followed by reduction and acetylation gave the dideoxythio sugar **218,**



SCHEME 60



SCHEME 61



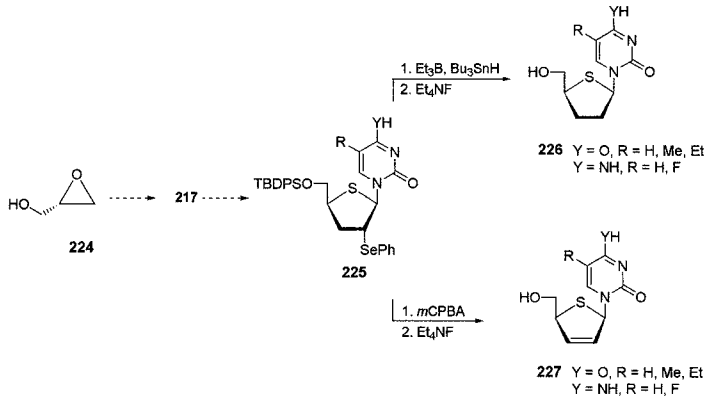
SCHEME 62

which was coupled with cytosine, following a modification[156] of the Vorbrüggen protocol,[162] to yield **219** as a separable 1 : 1 α/β anomeric mixture. The 4′-thiocytidine analogue **220** showed moderate activity *in vitro* against HIV. The L series was prepared in a similar way using D-glyceraldehyde as a chiral source.

O'Neil and Hamilton[163] have described the synthesis of a dideoxy-4′-thionucleoside analogue **222** lacking the 4′-hydroxymethyl group, using a Pummerer-type glycosylation reaction, by coupling the sulfoxide **221** with the silylated thymine in the presence of Me_3SiOTf, Et_3N, and ZnI_2. Oxidation of **222** yielded the sulfone **223.**

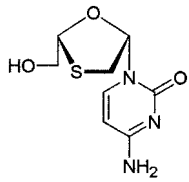
Synthesis of 2′,3′-dideoxy (**226**) and 2′,3′-didehydro-2′,3′-dideoxy-4′-thiopyrimidinenucleosides (**227**) were described[164] starting from *S*-glycidol (**224**). The key intermediates were thiolactone **217** and phenylselenated thio sugar **225.** The L enantiomers were obtained in the same way from *R*-glycidol. The 4-thio analogue of β-L-d4-cytidine, the enantiomer of **227** (R = H, Y = NH), showed marked anti-HBV and anti-HIV activity.

f. 3′-Thianucleosides, 3′-Oxa-4′-thionucleosides, and 3′-Thia-4′-thionucleosides.—The therapeutic effectiveness of 1,3-oxathiolane nucleoside 2′,3′-dideoxy-3′-thia-β-L-cytidine (**228**) (3TC, Lamivudine, Epivir) in the treatment



SCHEME 63

of chronic hepatitis B and HIV infections[27,165–173] has promoted intense research during the last decade on its synthesis[97,174] and pharmacological properties.

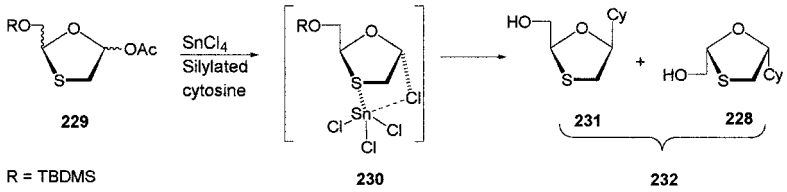


228 (3TC)

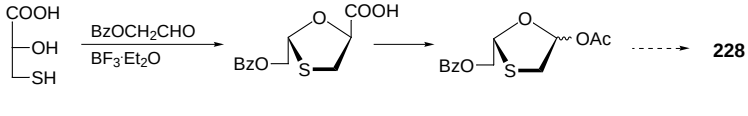
Belleau *et al.*[175,176] reported the first synthesis and biological activity of racemic-3TC [(±)-BCH-189] **232** and Liotta *et al.*[177] described the stereoselective coupling of the racemic thia sugar **229** (prepared from a non-sugar precursor) with silylated cytosine in the presence $SnCl_4$ to give exclusively the racemic β anomers **232** ($\alpha : \beta < 1 : 300$), after deprotection with tetrabutylammonium fluoride (TBAF).

The enantiomerically pure (+)-D-3TC **231** was synthesized by Chu *et al.* from 1,6-thioanhydro-D-mannose[178] or from 1,6-thioanhydro-D-galactose[179] via the D enantiomer of the thia sugar **229,** whereas (−)-L-3TC was obtained from 1,6-thioanhydro-L-gulose via the L enantiomer of **229.**[180] The condensation step of the thio sugars with *N*-acetylcytosine and other pyrimidines and purines in the presence of Me_3SiOTf afforded separable anomeric mixtures ($\alpha : \beta$ 1 : 2).[181] The use of $SnCl_4$ had to be ruled out because it causes racemization of the sugar precursor, presumably via ring opening at C-4.[182]

Another synthesis of L-3TC **228** was carried out from (2*S*)-3-thiolactic acid **233** in four steps.[182] Condensation of **233** with benzoyloxyacetaldehyde to give a separable (2 : 1) mixture of **234** and its C-2 diastereisomer, followed by oxidation of **234** with lead tetraacetate, yielded **235.** The acetate **235** was condensed with persilylated cytosine in the presence of Me_3SiI to obtain an α/β (1 : 1.3) mixture of benzoylated 3TC.



SCHEME 64



233 **234** **235**

SCHEME 65

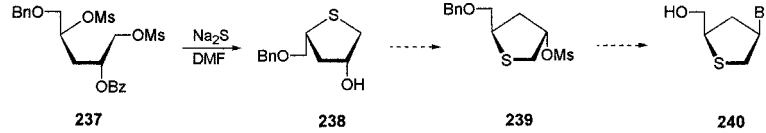
The racemic (±)-3TC **232** and other pyrimidine and purine analogues were resolved by various methods (such as separation on HPLC with a chiral column,[183,184] enzyme-catalyzed hydrolysis of its 5′-*O*-butanoyl ester derivative,[185] or enzymatic resolution of its monophosphate derivative with a nucleotidase derived from *Crotalus atrox* venom[186]). Resolution of racemic thia sugar precursors has also been described.[174,187,188]

The β-L isomer **228** and its 5-fluoro derivative (−)-FTC have been found to be the most potent anti-HIV and HBV (hepatitis B virus) among the four possible isomers and were reported as the first examples of "L-like" nucleosides showing more potent antiviral activities and much less toxicity than their corresponding D counterparts.[181,189–191] The explanation lies in their catabolism and anabolism. The L enantiomers of 3TC and FTC are phosphorylated faster than the D enantiomers. The L forms are not substrates for cytidine deaminase whereas the D forms are.[174] 3TC and FTC, as well as other anti HIV nucleosides, are prodrugs that must be activated to the triphosphate level before they can inhibit reverse transcriptase in the infected cell.

Oxathiolane tetrazole nucleosides analogues of 3TC[192,193] were found to be inactive against HIV-1. Analogues of 3TC substituted at C-4, furnished by nucleophilic displacement of a sulfonamide or imidazole by a variety of nitrogen nucleophiles, did not show activity against HIV-1 in MT-4 cells, but revealed good anti-HBV activity.[194] Substitution at N-3 or at C-4 resulted in appreciable decrease of anti-HIV activity.[194] 1,3-Oxathiolane 5-azapyrimidine, 6-azapyrimidine, and fluoro-substituted 3-deazapyrimidine nucleosides, prepared from 3TC, did not show activity against three tumor cell lines (L1210, $B_{16}F_{10}$, CCRF-CEM), or against HIV and HBV, except the 5-fluoro-3-deazapyrimidine nucleoside, which exhibited moderate anti-HBV activity.[195]

A series of novel 3′-thiacarbocyclic nucleosides **240,** carrying purine and pyrimidine bases, have been prepared from D-glucose.[196,197] The key steps were the treatment of dimesylate **237** with Na_2S, inversion at C-4 by the Mitsunobu reaction (BzOH, Ph_3P, and DEAD), and coupling of mesylate **239** with the nucleoside base in the presence of potassium carbonate, followed by deprotection. None of these nucleoside were active against HIV-1.

Mansour *et al.*[198–201] have reported the synthesis and the anti-HIV activity of a new class of oxathiolane nucleosides, 2′-deoxy-3′-oxa-4′-thiacytidine **244** [(−)-dOTC], its enantiomer [(+)-dOTC], and their 5-fluoro analogues

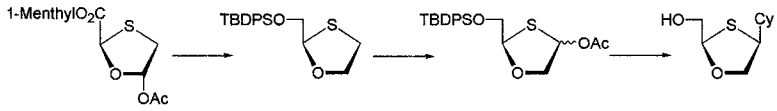


237 **238** **239** **240**

SCHEME 66

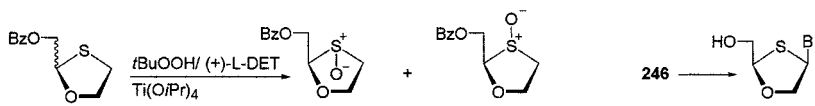
[(−)-dOTFC and (+)-dOTFC]. They are structurally related to 3TC and FTC, but the oxygen and the sulfur in the furanosyl group are transposed. The synthetic strategy for **244** included transposition of the acetoxy group from C-5 in oxathiolane **241** to the C-4 center by removal of the acetoxy group (Et_3SiH, Me_3SiOTf), followed by reduction ($NaBH_4$) of the menthyl ester and protection of the primary alcohol. Subsequent oxidation of **242** and Pummerer rearrangement afforded **243.** Vorbrüggen coupling of **243** with silylated cytosine, separation of the anomers, and deprotection furnished the nucleoside **244.** Its enantiomer and the 5-fluoro analogues were prepared in a similar way. The four nucleosides are potent inhibitors of HIV replication *in vitro* and the racemic nucleoside dOTC is in clinical development for the treatment of HIV-1 infection.[201]

Palumbo *et al.*[202–204] have developed a new asymmetric synthesis of 3′-oxa-4′-thionucleosides in high enantiomeric excess. Treatment of benzoyloxyethanal with mercaptoethanol in the presence of a Lewis acid gave **245,** which by a modified Sharpless oxidation[205] led to the separable chiral sulfoxides (*E*) **246** (60% ee) and (*Z*) **247,** in an 82 to 18 ratio. The (*E*) isomer, treated with thymine or cytosine, under Pummerer-type glycosylation[206] gave **248** after separation of the α anomer and debenzoylation.



241 **242** **243** **244**

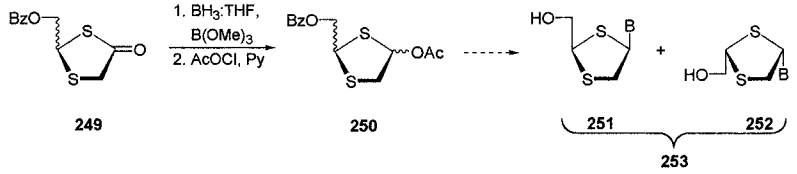
SCHEME 67



245 **246** **247** **248**

E (ee = 60%) : Z = 82:18

SCHEME 68



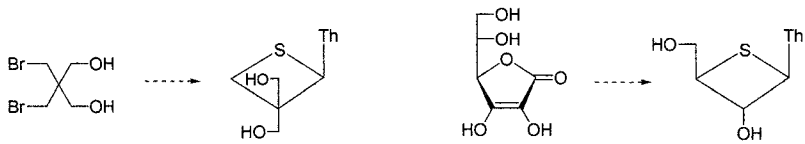
249 250 251 252 253

SCHEME 69

Nguyen-Ba *et al.* have described[207,208] the synthesis of 2′,3′ -dideoxy-3′-thia-4′-thio-β-nucleosides (**251**), a novel class of 1,3-dithiolane nucleosides possessing a second sulfur atom at the 3′-position, that have shown good to excellent antiviral activity. Treatment of mercaptothioacetic acid with benzoyloxyethanal in the presence of ZnI_2 gave racemic thiolactone **249** that by reduction and acetylation afforded **250.** Coupling 3-thia-4-thio sugar derivative **250** with the silylated base in the presence of $SnCl_4$, followed by deprotection and separation of the trans isomer, led to the racemic cis nucleoside **253.** Separation of enantiomers **251** and **252** was undertaken by chiral HPLC. Both enantiomers displayed similar antiviral activity. Treatment of the mixture **253** with cytidine deaminase converted only **251** into its corresponding uracil derivative.

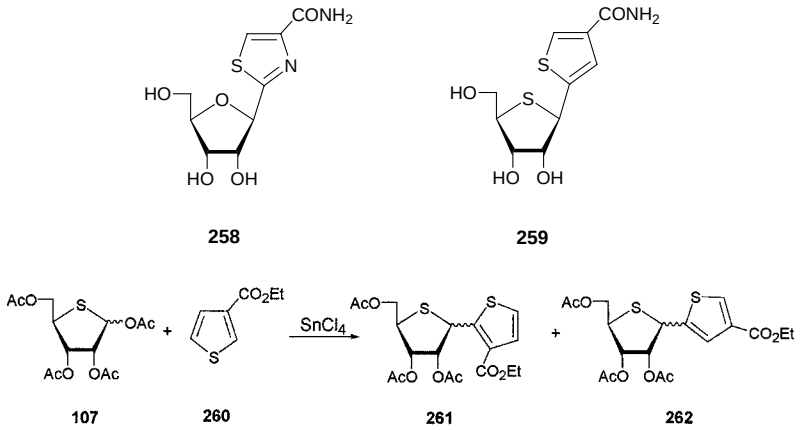
The thietane nucleosides **255** and **257** have been prepared[206] from the commercially available diol **254** and from L-ascorbic acid **256,** via the Pummerer-type glycosylation of the corresponding sulfoxides in the presence of thymine, Me_3SiOTf, Et_3N, and ZnI_2 as a key step. These nucleosides did not show significant antiviral activities.

g. 4′-Thio-*C*-nucleosides.—The synthesis of thiophenthiofurin **259,** a *C*-thioribonucleoside analogue of thiazofurin **258,** has been recently described.[209] Thiazofurin is a synthetic thiazole *C*-nucleoside that has demonstrated clinical efficacy as an antitumor agent.[210] Reaction of 4-thioribofuranose **107** with thiophene **260** in the presence of $SnCl_4$ gave a mixture of 2- and 5-glycosylated regioisomers (**261** α,β and **262** α,β, 56%). Deacetylation with methanolic ammonia, treatment of the ethyl ester with ammonium hydroxide, and chromatography of



254 255 256 257

SCHEME 70

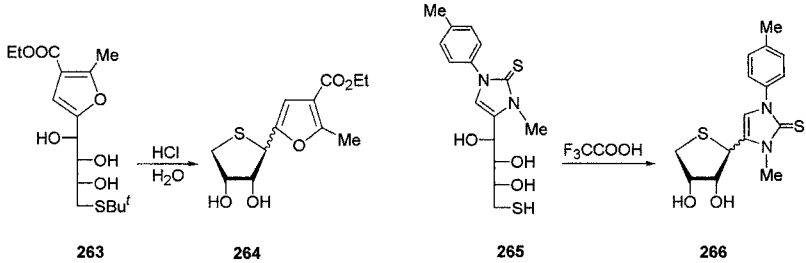


SCHEME 71

the mixture yielded **259.** Compound **107** was synthesized starting from D-gulono-1,4-lactone.[211] Thiophenthiofurin **259** was found to be cytotoxic *in vitro* toward human myelogenous leukemia K562, albeit 39-fold less than thiazofurin **258.**

Syntheses of 4′-thio-α and β-D-erythrofuranosylfuran **264** (α,β ~1 : 6)[212] and imidazoline[213] *C*-nucleosides **266** (α,β ~3 : 2) have been described by acid treatment of 4-(D-arabinitol-1-yl)heterocycles **263** and **265,** respectively. The conformation of the β anomer of **266** in the solid state[214] and the preponderant conformer in solution were the same.

h. Conformational Properties of 4′-Thionucleosides.—^{1}H NMR conformational analyses of $^3J_{HH}$ coupling constants and NOE enhancements of 4′-thionucleosides in D_2O, complemented by *ab initio* calculations, have been recently reported[215] and show the influence of the S–C–N anomeric and other stereoelectronic effects on the $N \rightleftharpoons S$ pseudorotational equilibrium. This equilibrium for the 2′-deoxy-4′-thio-β-ribonucleosides is shifted slightly toward *S*-type conformers,



SCHEME 72

whereas the 4′-thio-β-ribo nucleosides exhibited an approximately 50 : 50 ratio and the α analogues displayed strong preference for *N*-type conformers. The S–C–N anomeric effect is weaker than the O–C–N anomeric effect in their 4′-oxo counterparts. NMR data for about one hundred 2′-deoxy-4′-thionucleosides have been examined as indicators of anomeric configuration.[216]

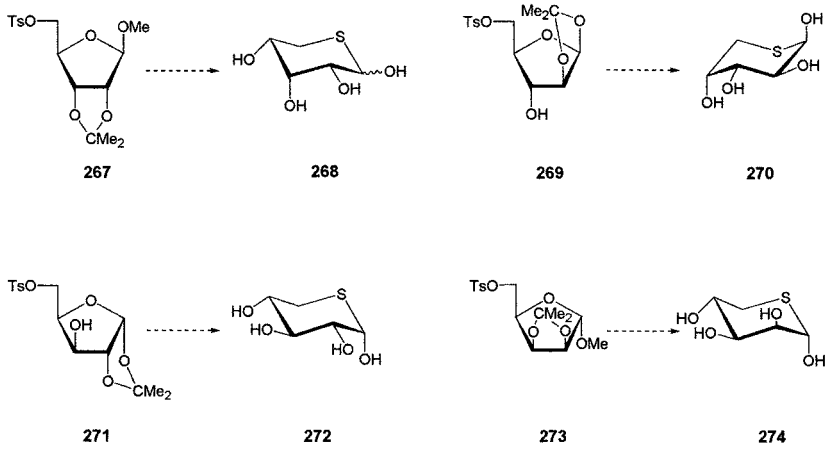
X-Ray crystal data for numerous 4′-thionucleosides have been reported.[96,126,134,214,217–219]

III. Six-Membered Rings

1. 5-Thiopentoses

a. Synthesis of 5-Thiopentoses.—5-Thioaldopentopyranoses are readily prepared by displacement of a sulfonyloxy group at C-5 of a suitable protected pentose by a sulfur nucleophile (such as AcS^-, BzS^-, BnS^-) followed by deprotection of the sulfur and O-1 atoms to allow spontaneous cyclization. Thus, 5-thio-D-ribopyranose **268** was obtained in three steps (formation of the *S*-acetate, deacetylation with sodium methoxide in methanol, and hydrolysis of the glycoside with diluted sulfuric acid) from the β-D-ribofuranoside **267**[220–223] or from 1-*O*-acetyl-2,3-*O*-isopropylidene-5-*O*-mesyl-D-ribofuranose.[224] The presence of sodium borohydride during the base-catalyzed *S*-deacylation is recomended to avoid the formation of disulfides.[223] Similarly, 5-thio-D-arabinopyranose (**270**) was obtained from tosylate **269**[225] and from 1,2,3-tri-*O*-acetyl-5-*O*-tosyl-D-arabinofuranose.[226,227] A mixture of the anomers of methyl 5-thio-L-arabinopyranoside was prepared in low yield from the L enantiomer of **269.**[228] The synthesis of **270** has also been described,[229] starting from the 5-tosylate of 2,3-*O*-isopropylidene-D-arabinose diethyl dithioacetal with conversion into the 5-*S*-benzoate, removal of the ethylthio groups with mercuric oxide, deacylation, and hydrolysis of the isopropylidene group. The same sequence of reactions was repeated with the corresponding L forms. 5-Thio-D-xylopyranose (**272**) and its per-*O*-acetyl derivative were prepared from tosylate **271,** by introducing the 5-thio group with NaSCN,[1] KSAc,[2,230] NaSBn,[231,232] and KSBz,[233] and peracetylated 5-thio-L-xylopyranose was prepared[234] from the L enantiomer of **271,** according to a procedure similar to that reported by Adley and Owen.[230] 5-thio-D-lyxopyranose (**274**) was obtained from tosylate **273.**[225]

All 5-thiopentoses show the expected preference for the thiopyranose ring in solution, due to the greater nucleophilicity of sulfur over oxygen.[221,235,236] All crystallize as pyranoses: α- and β-D-*ribo* **268,**[221] β-D-*arabino* **270,**[225] α-D-*xylo* **272,**[231] and α-D-*lyxo* **274.**[225] All give only pyranosides (α/β ratio: 35 : 65, 8 : 92, 93 : 7, and 91 : 9, for the *ribo, arabino, xylo,* and *lyxo* configurations) on methanolysis,[225] whereas their oxygen counterparts give pyranosides (α/β ratio: 15 : 85, 34 : 66, 69 : 31, and 90 : 10) along with furanosides (22.6, 28.3, 5.1 and

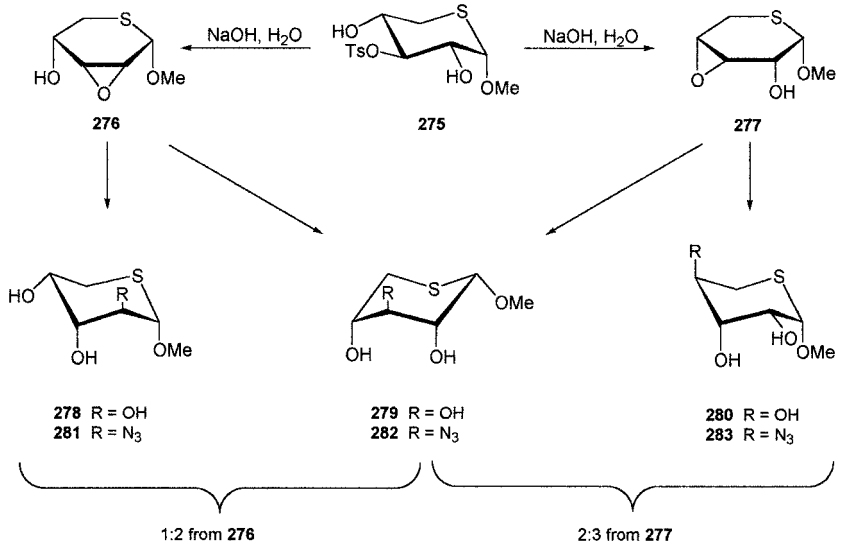


SCHEME 73

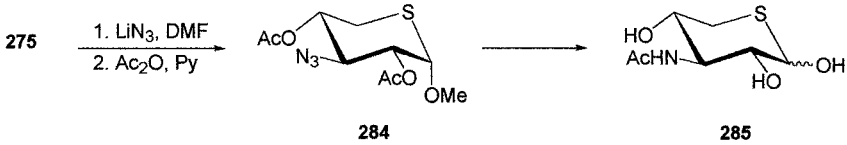
1.4%, respectively).[237] The favored conformation for the anomers of 5-thio-D-pentoses and their methyl glycosides is $^1C_4$ for the β-D-***arabino,*** $^4C_1$ for the α-D-***ribo,*** α- and β-D-***xylo,*** α-D-***lyxo,*** and a mixture of the $^1C_4$ and $^4C_1$ forms for the β-D-***ribo,*** α-D-***arabino,*** and β-D-***lyxo*** compounds.[225,231,238,239] The conformational behavior of 5-thiopentoses was explained[225,239] as depending on the increased puckering of the ring (leading to less strain between adjacent axial and equatorial groups and to an increase in strain between adjacent equatorial groups) and the "hockey-stick effect" (namely, the increased repulsive force between axial lone-pair of electrons on the sulfur and a 1,3-related heteroatom substituent) found in 5-thio sugars in comparison with their oxygen analogues.

Hughes and Munkombwe[240] described the formation of methyl 5-thiopento-pyranosides **278**–**280,** in the D-***arabino,*** D-***xylo,*** and L-***lyxo*** series, by the diaxial ring opening of epoxides **276** and **277** upon heating with aqueous sodium hydroxide. The epoxides were obtained from the tosylate **275.**[241]

b. Synthesis of Azido or Aminodeoxy-5-thiopentoses.—Reaction of tosylate **275** with lithium azide in DMF proceed with retention of configuration to yield, after acetylation, the azido compound **284** (63%).[242] The reaction occurred through the epoxides **276** and **277,** as confirmed by treatment of their acetyl derivatives with LiN_3, which furnished a 1 : 2 and 2 : 3 mixture of the azido compounds **281**–**283,** as shown in Scheme 74. The epoxides **276** and **277** were also opened with methanolic ammonia to give mainly the 3-aminodeoxyxyloside and 4-aminodeoxylyxoside, respectively. Reduction of **284** followed by acetylation, acetolysis, and Zemplén *O*-deacetylation gave crystalline **285.** 4-Acetamido-4-deoxy-5-thio-L-lyxose was similarly prepared from **283.**

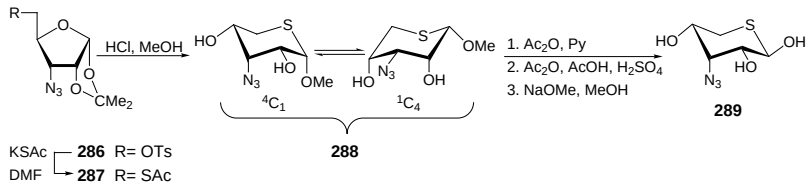


SCHEME 74

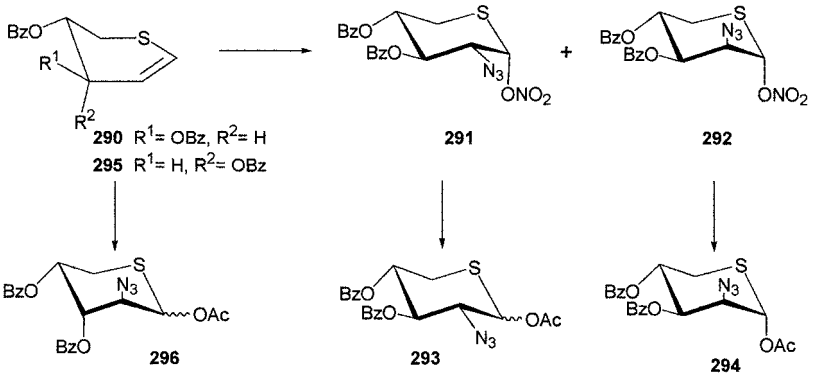


SCHEME 75

The synthesis of 3-azido-3-deoxy-5-thio-β-D-ribopyranose (**288**) was realized[243] from the tosylate **286** via thioacetate **287** in five steps (Scheme 76). Treatment of **287** with acidic methanol gave a septarable 1 : 2 mixture of the methyl α- and β-pyranosides. The α anomer **288,** whose ^{1}H NMR spectrum suggested an equilibrium of the $^1C_4$ and $^4C_1$ conformations, was transformed into **289** by conventional reactions. 1,2,3-Tri-*O*-acetyl-3-azido-3-deoxy-5-thio-D-xylopyranose was prepared via the xylo analogue of **286,** starting form D-glucose.[244]



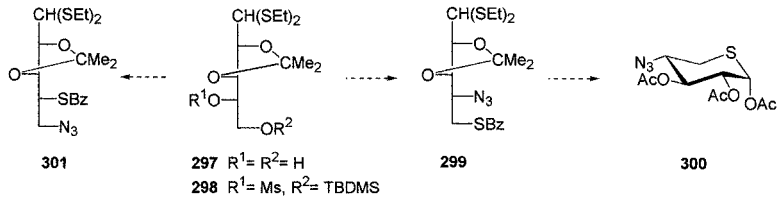
SCHEME 76



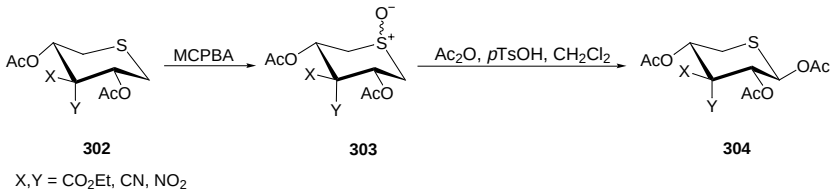
SCHEME 77

Azidonitration of the 5-thio-D-xylal **290** with sodium azide in acetonitrile and, subsequently with ceric ammonium nitrate, afforded a separable 1 : 1 mixture of the D-xylo (**291**) and the D-lyxo derivatives (**292**).[244] They were converted into the corresponding 1-*O*-acetyl derivatives **293** and **294,** on treatment with sodium acetate in acetic acid. In a similar way, azidonitration of 3,4-di-*O*-benzoyl-5-thio-D-ribal (**295**) afforded a mixture of the corresponding 2-azido-1-*O*-nitro derivatives that were converted, without separation, into the corresponding 1-*O*-acetyl derivatives, from which a 3 : 7 mixture of the α and β anomers of **296** could be isolated in 72% yield.[245] The steric arrangement of the axially oriented 3-*O*-benzoyl substituent in the erythro compound **295,** which in compound **290** occupies an equatorial position, explains the differences found in the addition of the azido group at C-2 of both glycals.

L-Arabinose diethyl dithioacetal was converted, via its 2,3-*O*-isopropylidene derivative **298** and its thiobenzoate **299,** into 4-azido-4-deoxy-5-thio-α-D-xylopyranose triacetate (**300**).[246] Ditosylation of **297,** displacement of the 5-tosyloxy group by treatment with potassium thiobenzoate, and exchange of the 4-tosyloxy group with sodium azide in DMF gave the 5-azido-4-*S*-benzoyl-D-xylose derivative **301,** isolated as the main compound (65%) via a 4,5-episulfonium intermediate.



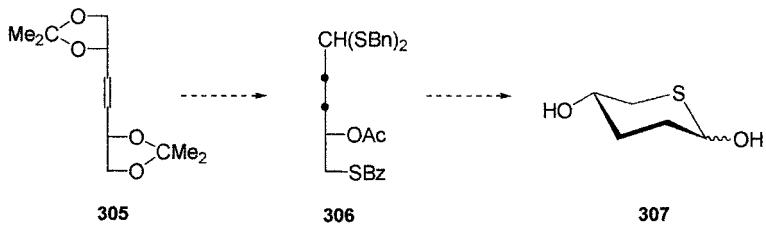
SCHEME 78



SCHEME 79

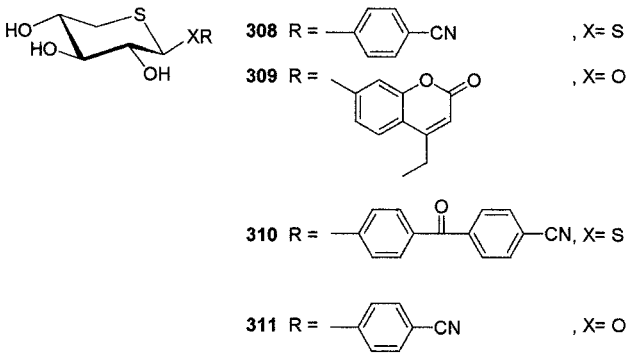
c. Synthesis of Branched 5-Thiopentoses.—The synthesis of branched 3-deoxy-5-thio-β-DL-pentopyranoses **304** from non-carbohydrate precursors has been achieved by oxidation of *meso*-3,5-diacetoxytetrahydrothiopyran derivatives **302,** followed by Pummerer rearrangement of the corresponding sulfoxides **303.**[247] The Pummerer reaction was stereospecific and the β isomers were obtained. L-Menthyl cyanoacetate was used for the synthesis of chiral 3-deoxy-5-thiopentopyranoses **304** (X = CO_2Menthyl-L, Y = CN).[248]

d. Synthesis of Deoxy-5-thiopentoses.—Methyl 2-deoxy-5-thio-β-D-*erythro*-pentopyranoside[223,232] and methyl 3,4-di-*O*-acetyl-2-deoxy-5-thio-α-D-*threo*-pentopyranoside[249] were prepared from 2-deoxy-D-*erythro*- and *threo*-pentose, respectively, via the appropriate ω-*O*-tosyl derivatives. 2-Deoxy-5-thio-D-*erythro*-pentose was obtained by Wong *et al.,* in moderate yield (33%), by 2-deoxyribose-5-phosphate aldolase-catalyzed aldol reaction of acetaldehyde and racemic 3-thioglyceraldehyde.[250] 3-Deoxy-5-thio-β-D-*erythro*-pentopyranose was prepared starting from D-xylose, via the key intermediate 3-deoxy-1,2-*O*-isopropylidene-5-*O*-tosyl-α-D-*erythro*-pentofuranose.[251] 2,3-Dideoxy-5-thio-D-*glycero*-pentopyranose **307** was obtained by Kuzsmann *et al.*[223] from hex-3-enitol **305** via the dibenzyl dithioacetal **306,** which was treated with boron trifluoride etherate in the presence of mercuric oxide, to obtain the aldehyde. Zemplén deacylation gave **307** as a mixture of the α and β isomers in a 2 : 1 ratio. The crystalline acetylated α and β anomers adopted the $^4C_1$ and the $^1C_4$ conformations, respectively.



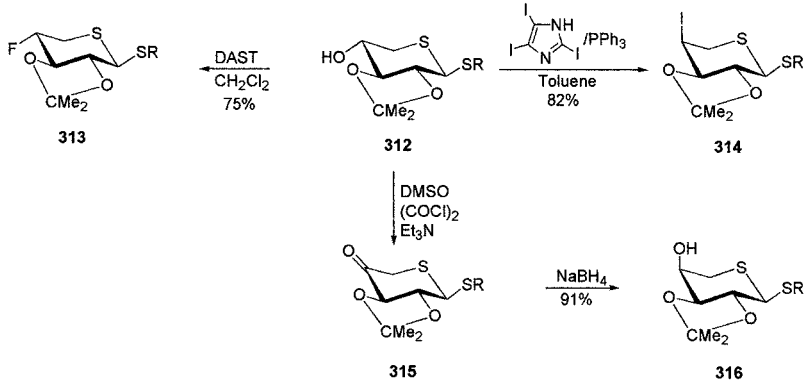
SCHEME 80

e. Aryl 5-Thiopyranosides as Antithrombotic Agents.—5-Thio-β-D-xylopyranosides such as Beciparcil (**308**), Iliparcil (**309**), and Naroparcil (**310**) have shown potent oral antithrombotic activity in mammals. These xylopyranosides have aglycones that make the compounds able to penetrate the plasmic membrane. This activity has triggered extensive research, focused on the structure–activity relationship, by modification of the sugar as well as of the aglycon moiety.[24,252–262] An improved synthesis of Iliparcil (**309**), by glycosylation of 4-ethylumbelliferone with 2,3,4-tri-*O*-acetyl-5-thio-D-xylopyranosyl bromide in the presence of ZnO–$ZnCl_2$,[263] a suitable candidate for clinical development, has been reported. Beciparcil (**308**), one of the most active compounds, failed in the clinical trials because of some unexpected side effects.[24]

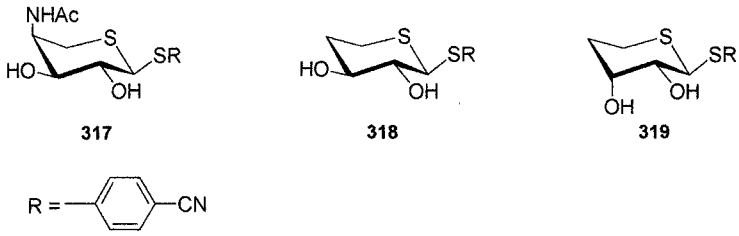


In order to investigate the role of the individual hydroxy groups on the biological activity of Beciparcil, Horton *et al.* have described its conversion into the 2- and 4-methyl ethers[264] via the isopropylidene 3,4-acetal, and 2,3-acetal **312,** respectively. The 3-methyl ether derivative was obtained by protection as a 2,4-cyclic phenylboronate.[265] The 4-deoxy-fluoro and iodo derivatives **313** and **314** were prepared by treatment of **312** with DAST[264] and with triiodoimidazole–PPh_3.[265] Conversion of **308** into the corresponding glycos-4-ulose **315** by Swern-type oxidation[266] (dimethyl sulfoxide–oxalyl chloride–triethylamine), followed by reduction with sodium borohydride, gave **316** predominantly (91%).[267] Oximation–reduction of **315** led to **317.** Conversion of Beciparcil into the 4-deoxygenated products **318** and **319** has also been described.[254,265]

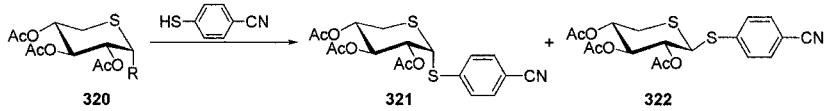
Kuszmann *et al.* have shown that the oral antithrombotic activity of Beciparcil (**308,** ED_{50} 25 mg/kg) determined in rats using the Pescador's model[268] could be significantly increased by changing the chirality of the pentopyranose moiety at C-3 (D-ribopyranoside, ED_{50} 10 mg/kg)[223] or C-2 and C-3 (D-arabinopyranoside, ED_{50} 3.5 mg/kg).[229,269] A similar increase in this biological activity was achieved by substituting each hydroxyl group of **308** by an azido group (ED_{50} 7, 10, and



SCHEME 81



7 mg/kg, for OH-2, -3, -4, respectively).[244,246] The activity of the 2-azido-2-deoxy-D-arabinopyranoside analogue (ED_{50} 3 mg/kg) was practically equal to that of the D-arabinopyranoside, but the 2-azido-2-deoxy-D-lyxopyranoside analogue showed a higher activity (ED_{50} 2 mg/kg).[245] Replacement of the 2-OH group or the 2-OH and 3-OH groups by H also increases biological activity (ED_{50} 7 and 3 mg/kg, respectively).[249] All of these Beciparcil analogues, and Beciparcil itself,[233] were prepared by glycosylation reaction of 4-cyanobenzenethiol with the appropriate 5-thio-D-xylopyranosyl donor (Scheme 82). 4-Cyanophenyl 1,5-dithio-β-D-glucopyranoside[270] as well as thioglycosides with bridged bicyclic structures derived from hexoses[271–274] possess significant antithrombotic activity.



Glycosyl Donor	Promoter	α,β ratio	Yield
R = OAc	Me_3SiOTf	3:1	90%
R = $OC(=NH)CCl_3$	$BF_3{\cdot}Et_2O$	1:1	60%
R = Br	ZnO	1:4	76%

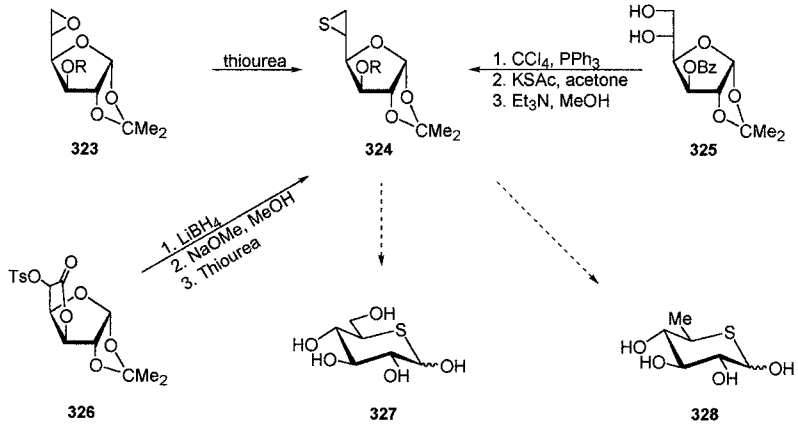
SCHEME 82

In the L-series, 4-cyanophenyl 5-thio-β-L-xylopyranoside, prepared from L-xylose, was found to maintain about 50% of the antithrombotic activity of its D enantiomer **311.**[234] 4-Cyanophenyl 1,5-dithio-β-L-arabinopyranoside and its D enantiomer showed a similar activity.[229]

2. 5-Thiohexoses

a. Synthesis of 5-Thiohexoses.—Most syntheses of 5-thioaldohexoses are based on the introduction of a thiol group on C-5. A classical approach to this funtionalization involves the opening of a 5,6-episulfide at C-6 by a nucleophile, such as an acetate ion. The episulfide is easily produced by reaction of a terminal oxirane ring with a thiourea or thiocyanate ion.[17] This method has been used for the synthesis of 5-thio-D-glucose **327** via thiiranes **324** (R = Bn)[275–278] and **324** (R = H).[75] An alternative synthesis of **327** involved the formation of 5,6-thiirane **324** (R = Bz) via the 5,6-dideoxy-5,6-dichloro sugar of the L-*ido* configuration, which was prepared from 3-benzoyl-1,2-*O*-isopropylidene-α-D-glucose (**325**), by reaction with CCl_4–PPh_3 followed by displacement of the primary chlorine ion with potassium thioacetate and subsequent treatment with triethylamine in methanol.[279] Another synthesis of 5-thio-D-glucose involved the preparation of **324** (R = H) from the 6,3-lactone **326.**[280] Reduction of the thiirane ring of **324** (R = H) with $LiAlH_4$ afforded the corresponding 6-deoxy derivative, along with dimer, trimer, and polymers.[281]

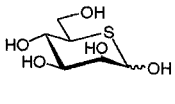
5-Thio-D-mannose **329,**[282] 5-thio-D-galactose **330,**[283] 5-thio-L-altrose **331,**[284] and 5-thio-L-fucose **332**[285] were synthesized using as the key steps the conversion of a terminal oxirane into thiirane with thiourea, and the opening of the thiirane



SCHEME 83

ring with NaOAc–AcOH–Ac_2O[282–284] or $LiAlH_4$.[285] Similarly, syntheses of 5-thio-L-idose **333**[51,230,286] and 6-deoxy-5-thio-L-idose[51,230] involved episulfide opening, prepared from 1,2-*O*-isopropylidene-α-D-glucofuranose by various routes.

A different approach for preparing 5-thioaldohexoses is based on the nucleophilic displacement of a leaving group by an RS^- group. Thus, 5-thio-L-rhamnose **334** was prepared from L-rhamnose by routes that uses the reaction of methyl 5-chloro-5,6-dideoxy-2,3-*O*-isopropylidene-β-D-gulofuranoside, or the corresponding 5-tosylate, with KSAc.[287] Synthesis of 5-thio-L-talose **(335)** involved ring contraction during the reaction of methyl 2,3-*O*-isopropylidene-4-*O*-tosyl-α-L-rhamnopyranoside with KSBz.[17,288] 5-Thio-L-fucopyranose tetraacetate was synthesized from D-arabinose, the key steps being the one-carbon elongation at C-5 and the introduction of a sulfur atom at that carbon, via substitution of a 5-tosylate with KSAc.[227,289]



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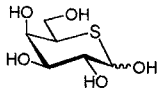

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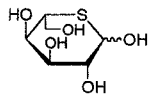

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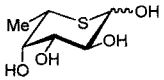

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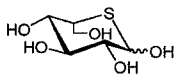

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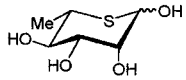

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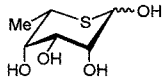
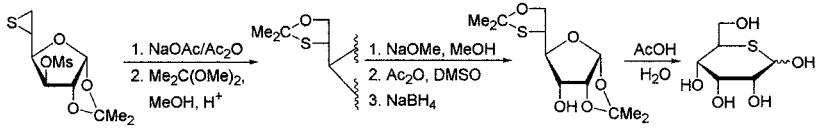


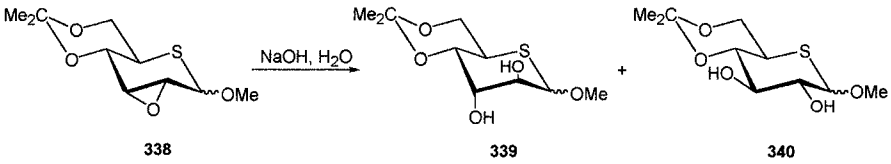
335

5-Thioaldohexoses can also be prepared by transformation of previously formed 5-thio sugars. Thus, 5-thio-D-allose (**337**) was prepared from the D-gluco-5,6-episulfide **336,** as shown in Scheme 84, via epimerization at C-3 by an oxidation–reduction sequence.[290] Epoxide opening of methyl 2,3-anhydro-4,6-*O*-isopropylidene-5-thio-α-D-allopyranoside (**338**) with aqueous sodium hydroxide gave mixtures of the corresponding *altro* and *gluco* derivatives **339** and **340** in an 85 : 15 ratio, whereas the β-anomer afforded the *altro* and *gluco* derivatives in almost equal amounts (51 : 49).[291]



336 **337**

SCHEME 84



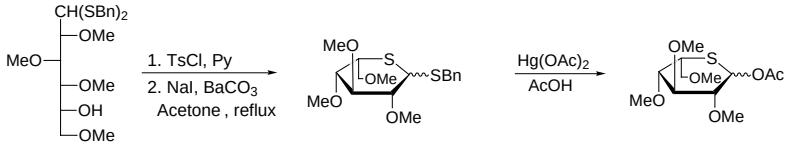
338 **339** **340**

SCHEME 85

Benzyl 1,5-dithio-L-idopyranoside (**342**) was obtained by intramolecular cyclization of the dibenzyl dithioacetal of D-glucose having a sulfonyloxy group at C-5 (Scheme 86).[53] This reaction was first observed with 5-*O*-tosyl-L-arabinose dibenzyl dithioacetal by Harness and Hughes[63] in 1971, and later reexamined.[292] It was shown that protection of the hydroxyl groups, prior to the cyclization with sodium iodide and barium carbonate, increased the yield considerably.[292]

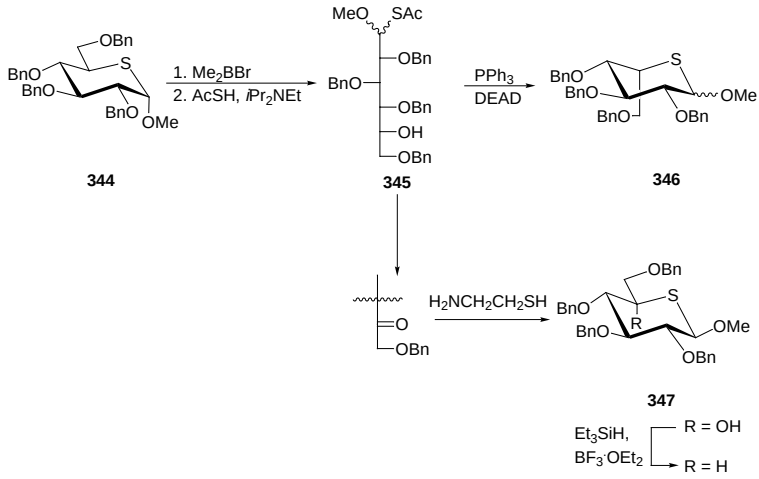
Conversion of aldopyranosides into 5-thioaldopyranosides via acyclic monothioacetals was proposed by Hashimoto *et al.*[293,294] The method involves the ring opening of pyranosides with dimethylboron bromide[295] and thioacetic acid, giving the acyclic *S*-acetyl *O*-methyl monothioacetal, followed by the intramolecular cyclization between C-5 and S-1 of the 5-hydroxymonothioacetal with the Mitsunobu reagents. In this way, perbenzylated methyl 5-thio-L-idopyranoside (**346**) was prepared from methyl tetra-*O*-benzyl-β-D-glucopyranoside (**344**), as shown in Scheme 87. Synthesis of the 5-thio-D-glucopyranoside **347** was achieved by Swern oxidation of **345,** followed by *S*-deacylation with 2-aminoethanothiol, and deoxygenation at C-5 with Et_3SiH in the presence of $BF_3 \cdot OEt_2$. This strategy was applied to the synthesis of 5-thio-L-galactose.

New routes to 5-thiohexoses include the synthesis of 5-thio-D-glucose based on the radical-promoted rearrangement of cyclic thionocarbonate **348** to give a 3 : 2 mixture of 5,6-*S*,*O*-thiolcarbonate **349** and its 5,6-*O*,*S*-thiolcarbonate isomer, conducted with tributyltin hydride–azobisisobutyronitrile (AIBN), followed by treatment with NaOMe–MeOH, to give a readily separable mixture of episulfide **324** and 1,2-*O*-isopropylidene-6-thio-α-D-glucofuranose.[296,297] Vogel *et al.*[298] have reported the synthesis of peracetylated 5-thio-DL-allose by modification of the Diels–Alder adduct formed from furan and 1-cyanovinyl acetate, using the "naked



341 **342** **343**

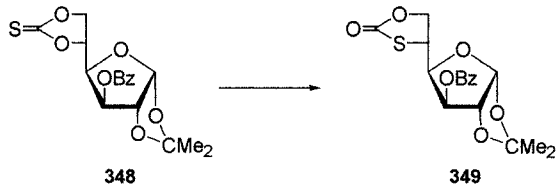
SCHEME 86



SCHEME 87

sugar approach." Similarly, the optically pure adduct of furan and 1-cyanovinyl (1*R*)-camphanate was transformed into 5-thio-L-allose. The conversion of a terminal epoxide into a episulfide was one of the key steps.

b. Biological Activity.—5-Thioglucose (**327**) exhibits a variety of biological activities including antispermatogenic effects in rats,[299–301] cytotoxicity against hypoxic tumor cells,[302] inhibition of D-glucose transport across membranes,[303–305] and inhibition of plant growth[306] and of enzymic degradation of glycogen.[307,308] It causes loss of weight in mice without disrupting normal eating habits, by reducing carbohydrate assimilation.[309] It also shows moderate inhibitory activity toward α-glucosidase from brewer's yeast ($K_i = 0.75$ mM) and weak inhibition against β-glucosidase from sweet almond ($K_i > 10$ mM).[310] 5-Thio-D-glucose 6-phosphate was shown to bind hexokinase from bovine brain ($K_i = 7$ μM) in preference to D-glucose 6-phosphate ($K_i = 0.38$ mM).[311] Similarly, 5-thio-D-glucopyranosyl phosphate binds phosphorylase from potato ($K_i = 0.75$ mM) in preference to D-glucopyranosyl phosphate ($K_i = 5.4$ mM).[312] Methyl



348 349

SCHEME 88

5-thio-α-D-glucopyranoside and the corresponding sulfoxide and sulfone show some inhibition against α-glucosidase from brewer's yeast (K_i >5.0, >5.5, >2.0 m*M*).[310] 5-Thio-D-glucal was found to be a competitive inhibitor of α-D-mannosidase from jack beans, with $K_i = 3.9$ m*M*.[313] Deoxythionojirimycin ("1-deoxy-5-thio-D-glucose") is a weak inhibitor of α- and β-D-glucosidases,[314] and deoxythiomannojirimycin ("1-deoxy-5-thio-D-mannose")[315] is a weak competitive inhibitor of α-D-glucosidase ($K_i = 1.0$ m*M*) and is inactive toward β-D-glucosidase, whereas the parent imino sugar analogues are potent inhibitors of α and β-D-glucosidases.[316] The crystal structure of a D-xylose isomerase–5-thio-D-glucose complex, showing a possible hydrophobic interaction between His-53 and the ring sulfur atom, has been reported.[317] The greater sweetness of 5-thio-α-D-glucopyranose and 6-thio-β-D-fructopyranose, compared to their oxygen counterparts when tested in crystalline form, have been related to differences in intramolecular hydrogen bonding.[318]

Hashimoto *et al.* reported that 5-thio-L-fucose (**332**) is a potent inhibitor of α-L-fucosidase from bovine epididymis ($K_i = 42$ μM),[285] whereas replacement of the 1-OH group with a methoxyl or methylthio group led to a decreased activity (methyl 5-thio-α-L-fucopyranoside, $K_i = 690$ μM and 1,5-dithio-α-L-fucopyranoside, $K_i = 2.3$ m*M*).[319] *p*-Nitrophenyl 1,5-dithio-α-L-fucopyranoside ($K_i = 3.3$ μM), one of the strongest glycosidase inhibitors so far known aside from the imino sugars, showed higher affinity for the enzyme than *p*-nitrophenyl 5-thio-α-L-fucopyranoside ($K_i = 118$ μM), and this was attributed to increased hydrophobicity at the aglycone part by virtue of the glycosidic sulfur atom.[320] The inhibitory activities of 5-thio-D-arabinose (**270**)[227] and 5-thio-L-galactose[294] against α-L-fucosidase from bovine kidney ($K_i = 770$ and 960 μM, respectively) were about 9–11 times larger than that of **332** ($K_i = 84$ μM). These results indicate that the α-L-fucosidase strictly recognizes the methyl residue of 5-thio-L-fucose.

c. Anomeric Effect of 5-Thio Sugars.—Replacement of the ring oxygen atom in D-glucose by sulfur alters the anomer population from $\alpha/\beta = 38:62$[321] to 85 : 15[322] in aqueous solution. Methanolysis of D-glucose gives the pyranosides in 2 : 1 α/β ratio, as compared to 4 : 1 for methanolysis of 5-thio-D-glucose (**327**).[290] These results seem to suggest that the anomeric effect is "considerably" greater for 5-thio sugars,[236] in accordance with the term "anomeric effect" as referred to the *tendency* of an electronegative substituent at C-1 of a pyranoid ring to assume the axial rather than the equatorial orientation in contrast to prediction based only on steric grounds.[323] In contrast, theoretical calculations[324,325] have shown a decreased anomeric effect in the S—C—O arrangement as compared to O—C—O.

The most widely accepted explanation for the anomeric effect is the stabilizing $(n_X \rightarrow \sigma^*_{C-Y})_{endo}$ and $(n_Y \rightarrow \sigma^*_{C-X})_{exo}$ orbital interactions for the axial glycosidic bond, whereas the equatorial glycosidic bond can accomodate only the stabilizing $(n_Y \rightarrow \sigma^*_{C-X})_{exo}$ orbital interaction, where X is the ring heteroatom and Y the

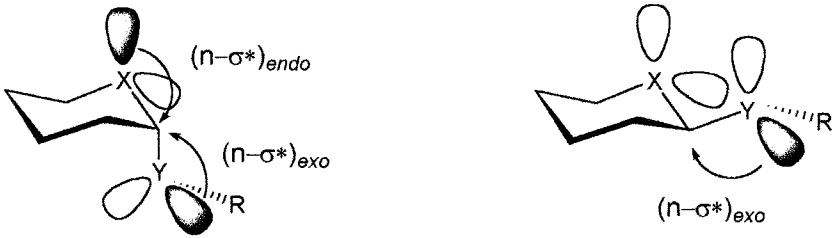
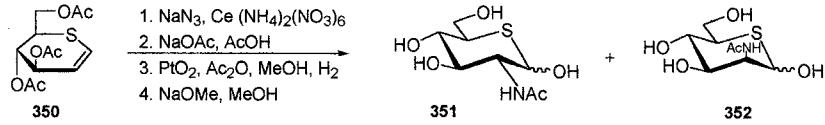


FIG. 1. The n–σ^* hypothesis to explain the anomeric effect in glycopyranosides. (Adapted from ref. 340).

anomeric substituent[326–331] (Fig. 1). The overlap of these bonding and antibonding orbitals, smaller for sulfur than for oxygen because of the longer C–S bond, may explain the decreased anomeric effect.[324] The weak anomeric effect of 5-thio sugars has been invoked to explain why the bromination of peracetylated 5-thio-D-glucopyranose with HBr in acetic acid gives a mixture of anomeric glycosyl bromides, in contrast to the behavior of the oxygen counterpart, which gives only the α glycosyl bromide. Furthermore, the S–C-1 bond is not shortened by the anomeric effect in peracetylated 5-thio-α-D-glucopyranose in the crystal state.[332] The crystal structures of other carbohydrate 5-thio analogues have been reported.[333–338]

5-Thio sugar derivatives may appear to possess a stronger anomeric effect because of difficulties in evaluating this effect. Its magnitude is usually defined as the difference of the free energy difference ($\Delta\Delta G$) for the equilibrium studied (such as the $\alpha \leftrightharpoons \beta$ equilibrium in glucose) and the conformational energy for the same substituent (for instance, OH) in cyclohexane.[327] However, the steric requirements of a group in the anomeric position of the heterocycle are different from those encountered in a cyclohexane derivative. The C–O and the C–S bonds are shorter and longer, respectively, than the C–C bond (1.43 and 1.82 versus 1.54); therefore, steric congestion of an axial substituent at C-1 (1,3-diaxial interactions with H-3 and H-5) is greater and smaller, respectively, than that of the same substituent in cyclohexane. This makes the magnitude of the anomeric effect tend to be underestimated for hexopyranoses and overestimated for 5-thiohexopyranoses.

Hashimoto ***et al.***[339,340] have studied the relative nucleophilicity of the two sulfur atoms in phenyl and ethyl 1,5-dithioglucopyranosides, in relation to n $\rightarrow \sigma^*$ interactions, measuring the rate of electrophilic oxidation. The alkyl or aryl α-glycoside is oxidized mainly at the glycosidic sulfur atom (S-1), whereas the β-glycoside is oxidized mainly at the ring sulfur (S-5) of the aryl glycoside and at S-1 of the ethyl glycoside. Pinto ***et al.***[341] have calculated the anomeric effect of *N*-phenyl peracetylated 5-thioglucopyranosylamine (0.85 kcal mol^{-1}) and *N*-phenyl peracetylated glucopyranosylamine (1.54 kcal mol^{-1}), and they estimated the increase of the *endo*-anomeric effect upon protonation to be approximately 1.0 kcal mol^{-1}. They



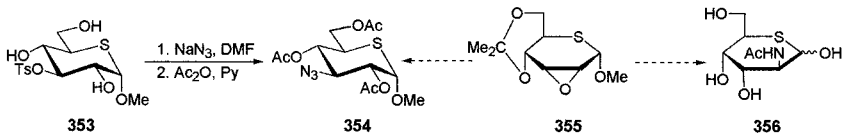
SCHEME 89

found no evidence to support the existence of a generalized "reverse anomeric effect" in neutral or protonated *N*-aryl glucopyranosylamines or in their 5-thio analogues.

d. Synthesis of Azido and Amino Derivatives of 5-Thioaldohexoses.—Several syntheses of 2-acetamido-2-deoxy derivatives of 5-thio-D-glucose **351,**[342–345] 5-thio-D-mannose,[346] and 5-thio-D-galactose,[347] through oxirane → thiirane transformation, from 2-acetamido-2-deoxy-D-glucose, mannose, and galactose, respectively, have been reported. An alternative route to **351** from methyl 4,6-*O*-isopropylidene-3-*O*-tosyl-5-thio-α-D-glucopyranoside by oxidation at C-2, desulfonylation, formation of the oxime, and reduction with $LiAlH_4$ has been described.[348] 2-Acetamido-2-deoxy-5-thio-D-mannose (**352,** 35%) and **351** (15%) were obtained from 2,3,6-tri-*O*-acetyl-5-thio-D-glucal[349] (**350**) by successive azidonitration with cerium (IV) ammonium nitrate and sodium azide, acetolysis, reduction, and deacetylation. 5-Thio-D-glucal was obtained from commercially available 5-thio-D-glucose by the classical synthesis of glycals (i, Ac_2O, pyridine; ii, HBr, HOAc; iii, Zn, HOAc).[350]

A synthesis of 6-amino-6-deoxy-5-thio-D-glucopyranose, in which the key step was the stereospecific addition of B*n*SH to the alkene 3-*O*-acetyl-5,6-dideoxy-1,2-*O*-isopropylidene-6-nitro-α-D-*xylo*-hex-5-enofuranose, was described.[351] Methyl 3-azido-3-deoxy-5-thio-α-D-glucopyranoside triacetate (**354**) was obtained from the *gluco*-tosylate **353** by treatment with sodium azide in DMF under reflux, followed by acetylation.

Treatment of acetylated methyl 5-thio-3-*O*-tosyl-α-D-glucopyranoside gave the corresponding 3-azido-3-deoxy-5-thio-α-D-allopyranoside, with inversion of configuration, isolated in modest yield (10%).[352] Treatment of oxirane **355** with

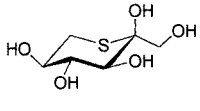


SCHEME 90

sodium azide and ammonium chloride in hot aqueous ethanol gave a 70 : 30 mixture of the corresponding *altro* and *gluco* azides, which were transformed by conventional reactions into 2-acetamido-2-deoxy-5-thio-D-altrose (**356**) and the methyl 3-azido-3-deoxy-5-thio-α-D-glucopyranoside, respectively.[353] A synthesis of 3-amino-3-deoxy-5-thio-D-allose by oximation–reduction of di-1,2-*O* : 5,6-S,*O*-isopropylidene-5-thio-α-D-*ribo*-hexofuranos-3-ulose, followed by acid-catalyzed deprotection, has been reported.[354]

3. Synthesis of 6-Thioketohexoses

6-Thio-β-D-fructopyranose was prepared from 2,3-*O*-isopropylidene-1,6-di-*O*-tosyl-D-fructofuranose (**89**) by a low-yielding route.[90] Displacement of only the 6-tosyloxy group with NaSBn was the key step. A high-yield synthesis of 6-thio-β-D-fructopyranose, by D-glucose isomerase-catalysed interconversion of 6-thio-D-glucose and 6-thio-D-fructose, has been described.[355] 6-Thio-β-L-sorbopyranose (**357**) was prepared in 85% yield by condensation of "dihydroxyacetone" phosphate and (*R*)-3-thioglyceraldehyde catalysed by fructose-1,6-diphosphate aldolase from rabbit muscle, followed by removal of the phosphate group with acid phosphatase. By a similar procedure, 6-thio-L-tagatose (**358**) and 6-thio-L-fructose (**359**) were also prepared.[356] Reduction of acetylated **357** and **359** with triethylsilane in the presence of $BF_3 \cdot Et_2O$ yielded "1-deoxy-5-thio-D-glucose" and "-L-mannose," respectively.



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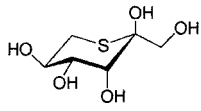

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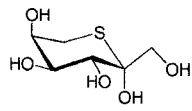
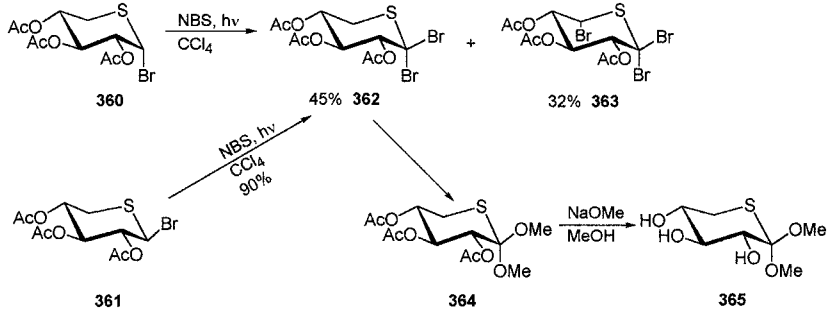


359

4. Synthesis of 5-Thio Sugar Lactones

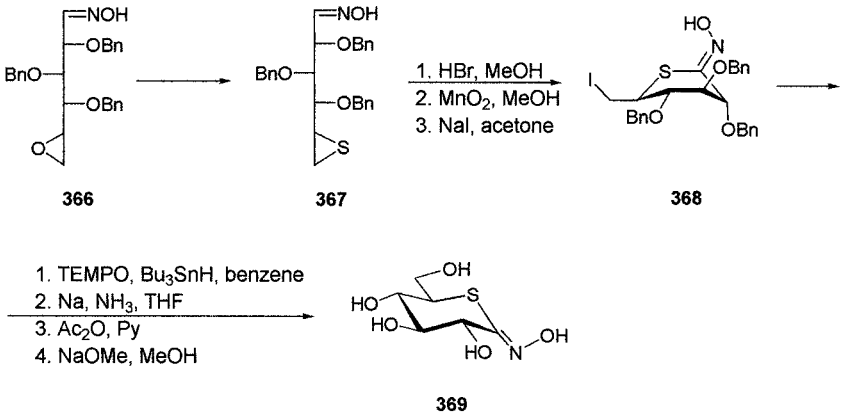
2,3,4,6-Tetra-*O*-alkyl (methyl, benzyl) derivatives of 5-thio-D-glucono-1,5-lactones were synthesized via hydrolysis of the corresponding methyl 5-thioglucopyranosides followed by oxidation with Me_2SO–Ac_2O.[75] Radical bromination of peracetylated 5-thio-α-D-xylopyranosyl bromide **360** with *N*-bromosuccinimide in CCl_4, under irradiation by visible light, gave mixtures of dibromide **362** and tribromide **363** as the main compounds, whereas the β anomer **361** could be cleanly transformed into **362,** as shown in Scheme 91.[357] Bromides **360** and **361** are readily obtained from the corresponding peracetates on treatment with hydrogen bromide in acetic acid. Treatment of **362** with silver triflate in the presence of alcohols



SCHEME 91

yielded the 5-thio sugar ortholactone **364,** deprotected under Zemplén conditions. An anomeric mixture of 2,3,4,6-tetra-*O*-acetyl-5-thio-D-glucopyranosyl bromide yielded the corresponding tribromide, analogous to **363,** after prolonged heating with *N*-bromosuccinimide under irradiation by visible light.

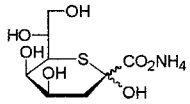
5-Thio-D-gluconohydroxyimino-1,5-lactone (**369**) was synthesized[358] in 10 steps from tri-*O*-benzyl-D-glucose, which was transformed into the L-*ido* epoxide **366.** Reaction of **366** with thiourea yielded the D-*gluco*-thiirane **367.** Successive ring opening of **367** with HBr in MeOH, oxidation by active MnO_2, and displacement of the bromide atom gave the iodide **368,** which on reaction with 2,2,6,6-tetramethylpiperidin-1-oxyl radical (TEMPO), in the presence of Bu_3SnH, followed by Birch reduction, gave **369,** isolated via its pentaacetate. Compound **369** was a poor inhibitor of sweet almond β-glucosidase.[359]



SCHEME 92

5. Synthesis of 6-Thioketoaldonic Acids

3-Deoxy-6-thio-D-*manno*-2-octulosonic acid (6-thio-Kdo) **370** was prepared via 4-thio-D-mannose.[38] Its ammonium salt failed to inhibit CMP-Kdo synthetase.[38] The 6-thio isosteres of *N*-acetylneuraminic acid (6-thio-Neu5Ac) **371** and its 5-epimer **372** were prepared[360] by a metal-catalyzed aldol condensation between 2-acetamido-2-deoxy-5,6-*O*-isopropylidene-3-thio-D-mannofuranose[361] and oxaloacetic acid. Synthesis of 6-thio-4-amino-4-deoxy-(**373**) and 4-deoxy-4-guanidino-Neu5Aen (**374**), from **371,** has been described by von Itzstein *et al.*[362] Compounds **373** and **374,** thio isosteres of potent influenza virus sialidase inhibitors[363,364] (IC_{50} values of 1×10^{-6} *M* and 5×10^{-9} *M*, respectively), were found to be as bioactive as their oxygen counterparts.



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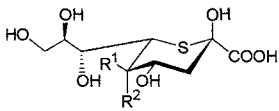
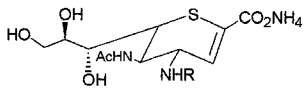


371 R^1 = NHAc, R^2 = H
372 R^1 = H, R^2 = NHAc



373 R = H
374 R = C(=NH)NH_2

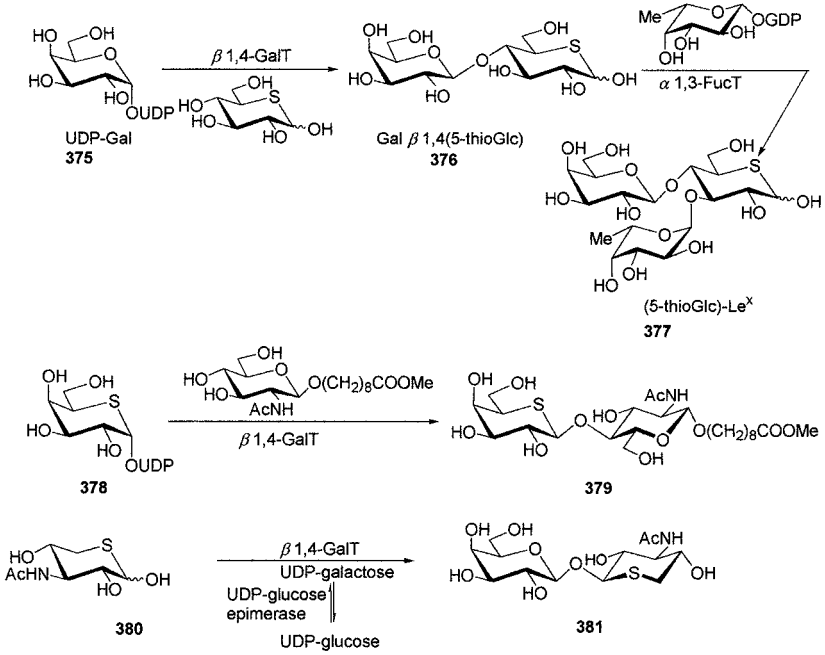
6. Synthesis of 5-Thio Sugar Nucleosides

The number of nucleoside analogues having a 5-thio-pentopyranosyl or hexopyranosyl sugar moiety is quite limited.[101,365,366] Synthesis of some 1-(5-thio-β-D-xylopyranosyl)-pyrimidine and -lumazine nucleosides, by reaction of the silylated bases with tetra-*O*-acetyl-5-thio-D-xylopyranose in the presence of Me_3SiOTf, have been described.[365] In a similar way, 1-(5-thio-β-D-glucopyranosyl)-6-azauracil nucleosides have been prepared by reaction of penta-*O*-acetyl-5-thio-D-glucopyranose with silylated 6-azauracil in the presence of Me_3SiOTf.[366]

7. Synthesis of Oligosaccharides Having a 5-Thio Sugar Moiety

Oligosaccharides containing 5-thiopyranosyl units are potentially resistant to hydrolysis by *exo* or *endo* glycosidases and constitute tools to investigate oligosaccharide–receptor interactions.

a. Chemical–Enzymatic Synthesis.—Wong *et al.* have described the enzymatic synthesis of disaccharides and trisaccharides having sulfur in the ring of the reducing monosacccharide units.[367–371] β-1,4-Galactosyltransferase from bovine milk was used (β-1,4-GalT, EC 2.44.1.22) as the catalyst for galactosylation of 5-thio-D-glucose to form the corresponding disaccharide β-Gal-(1→4)-5*S*Glc (**376**),[367] whereas the galactosidase reaction formed mainly

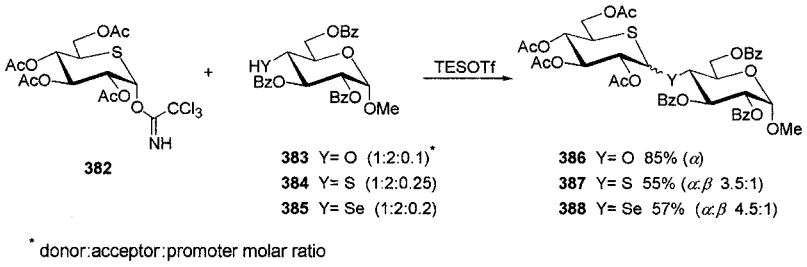


SCHEME 93

β-Gal-(1→6)-5*S*Glc).[368] The galactosyl 5-thioglucose **376** is a good substrate for α-1,3-fucosyltransferase α-1,3-FucT,[372] and fucosylation of this compound with α-1,3-FucT gave the trisaccharide (5*S*Glc)-Le^x analogue **377**[370] (Scheme 93).

Hindsgaul *et al.*[373] have reported the enzymatic synthesis of the 5′-thio-*N*-acetyllactosamine derivative (5′*S*LacNAc-OR) **379,** a disaccharide having sulfur in the ring of the nonreducing sugar moiety. Galactosyltransferase catalyzes transfer of 5-thio-D-galactose from uridine 5′-(5-thiogalactopyranosyl diphosphate) (UDP-5*S*Gal) **378** to an *N*-acetylglucosamine derivative (Scheme 93). Incubation of UDP-5-thioglucose with UDP-Glc 4′-epimerase, in the presence of GalT, also resulted in the conversion of **378** into **379.** Replacing the ring oxygen of LacNAc with sulfur increases its stability almost 200-fold toward digestion by the galactosidase from *Escherichia coli.* UDP-5′-Thio-D-galactose and UDP-5′-thio-D-glucose were chemically synthesized from methyl 5-thio-D-glucopyranoside. UDP-5*S*Glc was prepared via 5-thio-D-glucopyranosyl phosphate.[374,375]

UDP-*N*-acetyl-5-thio-D-galactosamine (UDP-5*S*GalNAc) was active as a donor substrate of lactose synthase, the complex of galactosyltransferase (EC 2.4.1.38) and lactalbumin. By this method the disaccharide β-5*S*GalNAcβ(1→4)GlcNAc was prepared.[376] UDP-5*S*GalNAc was synthesized from an *N*-acetylgalactosamine



SCHEME 94

derivative via ring opening in an *S*-acetyl *O*-methyl monothioacetal–recyclization approach.[294]

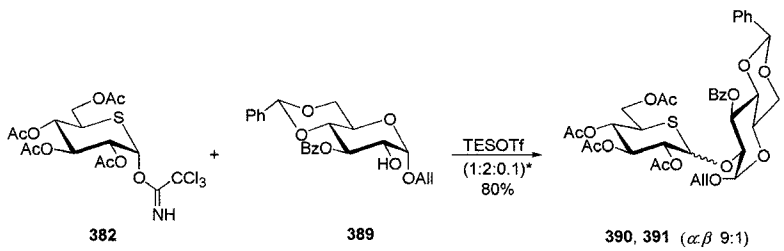
β-1,4-GalT was found to transfer β-galactose to the β-anomeric position of 3-acetamido-3-deoxy-5-thio-D-xylose (*N*-acetyl-5-thiogentosamine) (**380**) to afford the $\beta,\beta(1 \leftrightarrow 1)$-linked disaccharide **381** in 40% yield[377] (Scheme 93).

b. Chemical Synthesis Using a 5-Thioglycopyranosyl Donor.—Pinto reported chemical syntheses of 5′-thio analogues of methyl α-maltoside with oxygen, sulfur, or selenium in the interglycosidic linkage (5′-thiomaltoside, 4,5′-dithiomaltoside, and 4-seleno-5′-thiomaltoside).[21,378,379] The 5-thioglucopyranosyl trichloroacetimidate **382** was used for glycosylation of the 4-OH, 4-SH, and 4-SeH groups of glucopyranoside acceptors **383**–**385,** in the presence of triethylsilyl triflate as catalyst. The notable α-selectivity is attributed to the greater stability of the α anomers. 5′-Thiomaltoside, 4,5′-dithiomaltoside, and 4-seleno-5′-thiomaltoside were shown to be competitive inhibitors of maltose binding by glucoamylase G2, with K_i values of 1.34, 2.04, and 0.80 m*M*, respectively.[379]

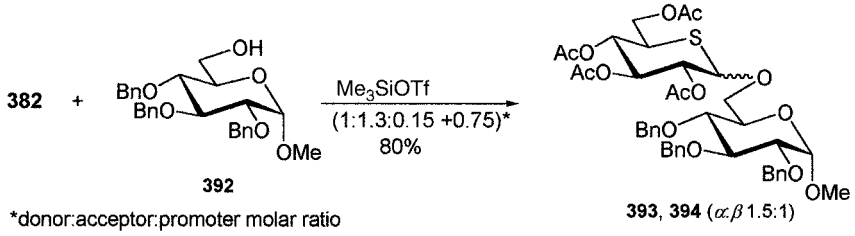
Compound **382** was also employed as a glycosyl donor for glycosylation of the glucopyranosyl acceptors **389** and **392** with 2-OH and 6-OH positions free, respectively, in order to prepare allyl 5′-thiokojibioside (**390**) and methyl 5′-thio-isomaltoside (**393**) (Schemes 95 and 96).[380,381] Nitrosyl tetrafluoroborate-mediated glycosylation of **392** with phenyl 1-seleno-5-thio-α-D-glucopyranoside afforded only the α-disaccharide (35%), and no β-disaccharide was detected.

A mixture of protected *n*-propyl 2,5′-dithio-β-kojibioside (**396**) and 2,5′-dithiosophoroside **397** was obtained by glycosylation of thiol **395** with the trichloroacetimidate **382**[382] (Scheme 97).

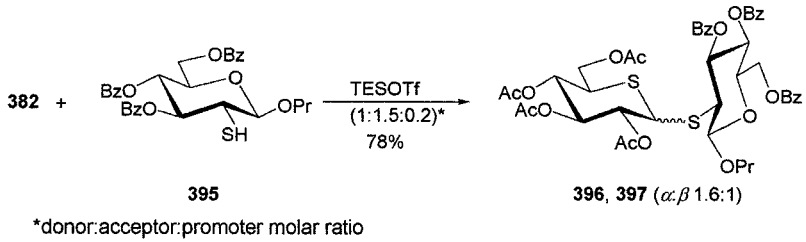
Acid-catalyzed condensation of 5-thioglucose with methyl 4-amino-4-deoxy-α-D-glucopyranoside led to an interconverting anomeric mixture of methyl 5′-thio-4-*N*-α-maltoside (**399**) and methyl 5′-thio-4-*N*-α-cellobioside (**400**).[383] This mixture was shown to competitively inhibit binding of maltose by glucoamylase G2, with a K_i value for **399** of 4 ± 0.3 μM. Compound **399** is also a competitive inhibitor of α-glucosidase from brewer's yeast (K_i 0.5 m*M*).[384] Acetylation



SCHEME 95

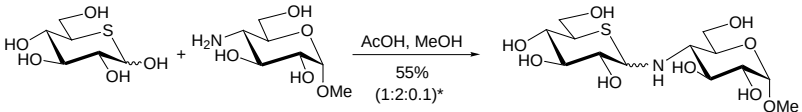


SCHEME 96



SCHEME 97

of the mixture yielded anomers that were separable and did not mutarotate. Likewise, methyl 5′-thio-2-*N*-β-kojibioside, in equilibrium with methyl 5′-thio-2-*N*-β-sophoroside, was prepared by condensation of 5-thioglucose with methyl 2-amino-2-deoxy-β-D-glucopyranoside.[383] The synthesis of methyl 2-amino-2-deoxy-2-*N*-(5-thio-α,β-mannopyranosyl)-α-D-mannopyranoside and methyl 3-amino-3-deoxy-3-*N*-(5-thio-α,β-mannopyranosyl)-α-D-mannopyranoside, by condensation of **329** with the appropriate aminodeoxy sugar, showed better yields using mercuric chloride instead of acetic acid as catalyst.[385] Transferred NOE experiments performed on **399** and methyl 5′-thio-4-*S*-α-maltoside suggested that



327 **398** **399**, **400** (α:β 1:2.5)

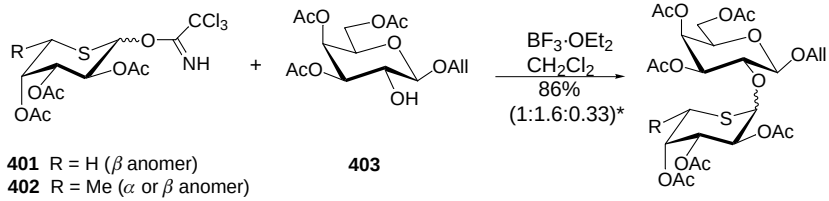
*donor:acceptor:promoter molar ratio

SCHEME 98

both hetero analogues are bound by glucoamylase in conformations in the area of the global energy minimum.[386]

The glycosylation of *N*-acetyl-D-glucosaminides and D-galactosides by peracetylated 5-thio-D-arabinopyranosyl and 5-thio-L-fucopyranosyl trichloroacetimidates (**401** and **402**), using $BF_3 \cdot OEt_2$ as catalyst, gave mainly α-linked disaccharides.[226] Allyl 2-*O*-(5-thio-α-L-fucopyranosyl)-β-D-galactopyranoside, prepared by this procedure, showed activity toward α-L-fucosidase from bovine epididymis ($K_i = 30\ \mu M$) and from *Bacillus* sp. K40T (0.21 m*M*). Other 5-thio-L-fucose-containing disaccharides having α-(1→3), α-(1→4), and α-(1→6)-GlcNAc linkages were synthesized and were shown to be potent competitive inhibitors of bovine α-L-fucosidese ($K_i = 31$–$91\ \mu M$).[387] 5-Thio-L-fucose-containing Lewis X trisaccharide analogues, β-Gal-(1→4)[α-5*S*Fuc-(1→3)]-GlcNAc-OMe and β-Gal-(1→4)[β-5*S*Fuc)-(1→3)]-GlcNAc-OMe, were synthesized via 5-L-thiofucosylation of methyl *O*-(2,6-di-*O*-benzoyl-3,4-*O*-isopropylidene-β-D-galactofuranosyl)-(1→4)-2-azido-6-*O*-benzoyl-β-D-glucopyranoside by the trichloroacetimidate method, using $BF_3 \cdot OEt_2$ or $ZnCl_2 \cdot OEt_2$ as promoters.[388] The latter permitted an α-selective 5-thiofucosylation, whereas with the former both α- and β-anomers were formed in almost equal quantity. Similarly, 5-thio-L-fucose-containing blood group antigen H-type 2 trisaccharide, α-5*S*Fuc-(1→2)-β-Gal-(1→4)-GlcNAc-O$(CH_2)_8CO_2$Me, was synthesized by 5-thio-L-fucosylation of the 2′-OH group of a 1,6-anhydro-2-azido-2-deoxy-lactose with **402** as the glycosyl donor and $BF_3 \cdot OEt_2$ as the promoter.[389] This trisaccharide bound to the anti-H monoclonal antibody more strongly than the natural H-type 2. This result suggests that the antibody binds the upper face of the fucose ring by hydrophobic interaction. The weaker binding to *Ulex europaeus* lectin-1 has been explained by the lack of the aromatic group that recognizes the upper face of the fucose ring in this lectin.

Hashimoto *et al.*[390] have prepared 5-thiomannose-containing oligomannoside mimics, α-5*S*Man-(1→6)-Man, α-5*S*Man-(1→3)-Man, α-5*S*-Man-(1→6)-[α-Man-(1→3)]-Man, α-Man-(1→6)-[α-5*S*Man-(1→3)]-Man, and α-5*S*Man-(1→6)-[α-5*S*Man-(1→3)]-Man, employing per-*O*-benzylated 5-thiomannopyranosyl trichloroacetimidate as the glycosyl donor. In all glycosylation reactions, α-glycosides were obtained stereoselectively as single anomers.



401 R = H (β anomer)
402 R = Me (α or β anomer)

403

404, **405** (α:β 98:2)

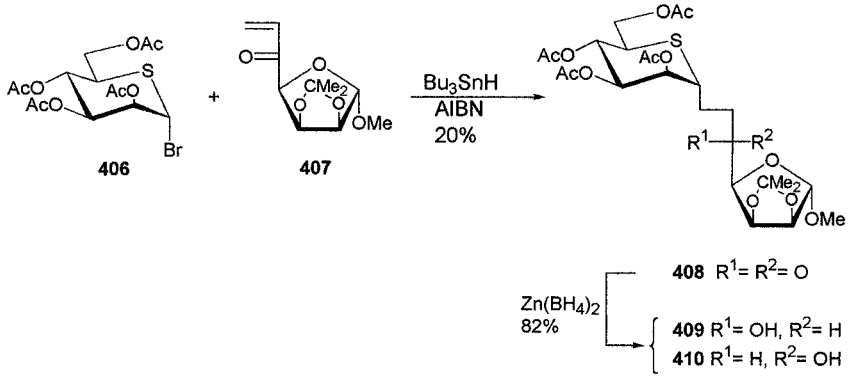
*donor:acceptor:promoter molar ratio

SCHEME 99

The 5-thio-*C*-mannosyl residue-containing trimannose, α-5*S*Man*C*-(1→6)-[α-Man-(1→3)]-Man, was synthesized via a glycosyl radical addition of thiomannosyl bromide to enone **407.** The reduction of **408** with $Zn(BH_4)_2$ mainly afforded the desired *manno* compound **409** (D-*manno:* L-*gulo* = 4 : 1), which was transformed by conventional reactions into the trisaccharide analogue.[391]

Glycosylation of 1,2 : 3,4-di-*O*-isopropylidene-D-galactose (**412**) with tri-*O*-acetyl-5-thio-L-arabinopyranosyl bromide (**411**) in the presence of silver triflate and tetramethylurea gave the 5′-thiodisacharide **413.** Compound **411** was obtained from acetylated 5-*O*-tosyl-L-arabinose dibenzyl dithioacetal by cyclization with sodium iodide–barium carbonate in butanone (82%) followed by bromination with Br_2 in CCl_4 (76%).[292]

Ichikawa *et al.*[392] have described the preparation of disaccharides **421**–**423** by glycosylation reactions of acceptors **415**–**417** with 5-thio-D-glucopyranosyl trichloroacetimidate (**414**), as shown in Scheme 102. Ding and Hindsgaul[393] have chemically synthesized 1-deoxy-3-*O*-(5-thio-α-D-glucopyranosyl)-mannojirimycin.

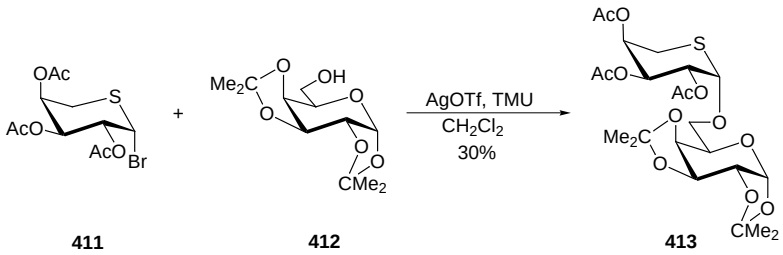


406

407

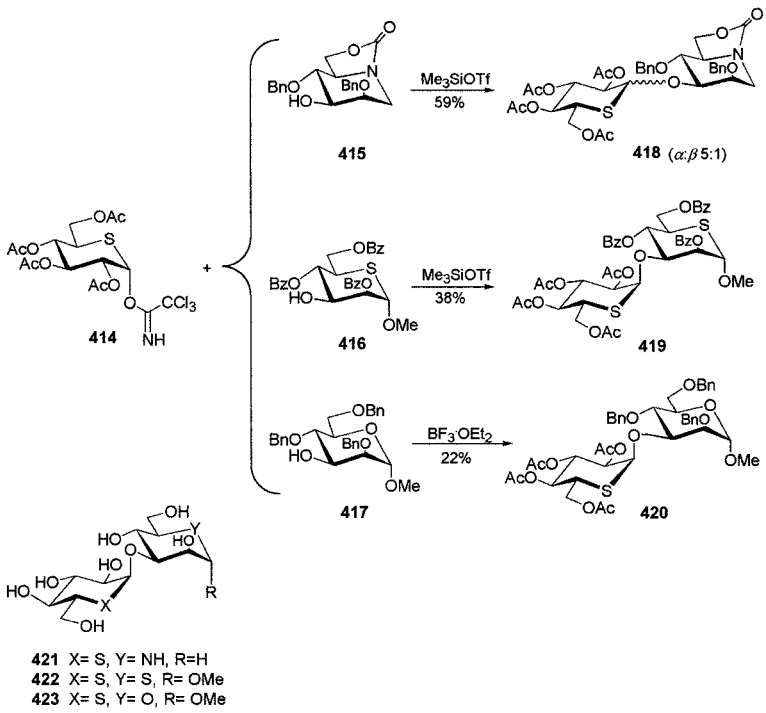
408 R^1= R^2= O
409 R^1= OH, R^2= H
410 R^1= H, R^2= OH

SCHEME 100



411 **412** **413**

SCHEME 101

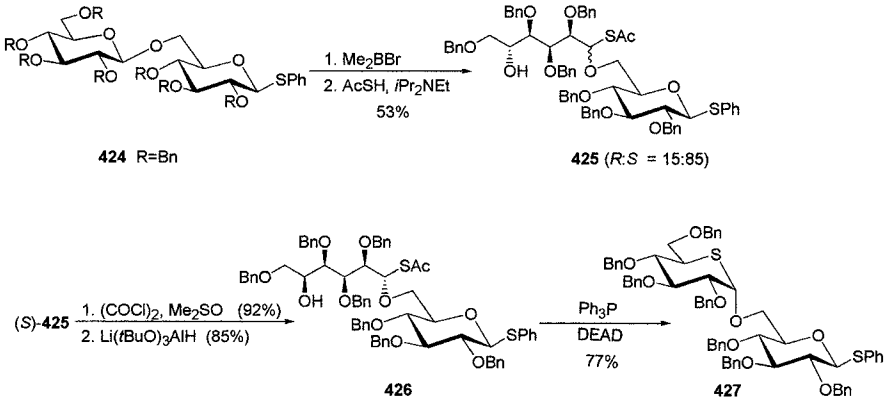


414 **415** **416** **417** **418** (α:β 5:1) **419** **420**

421 X= S, Y= NH, R=H
422 X= S, Y= S, R= OMe
423 X= S, Y= O, R= OMe

SCHEME 102

c. Synthesis through a Nonglycosylating Chemical Strategy.—A nonglycosylating synthesis of methyl 5′-thio-α-isomaltoside was developed by Hashimoto *et al.*[394] The opening of the nonreducing β-pyranoside ring of phenyl 1-thio-β-gentiobioside (**424**) with dimethylboron bromide and thioacetic acid gave the monothioacetal **425** as a 15 : 85 diastereomeric mixture. The selected aglycone was nearly inert under the ring-opening conditions. Inversion of the OH-5′ group by conventional oxidation–reduction to give **426,** followed by intramolecular



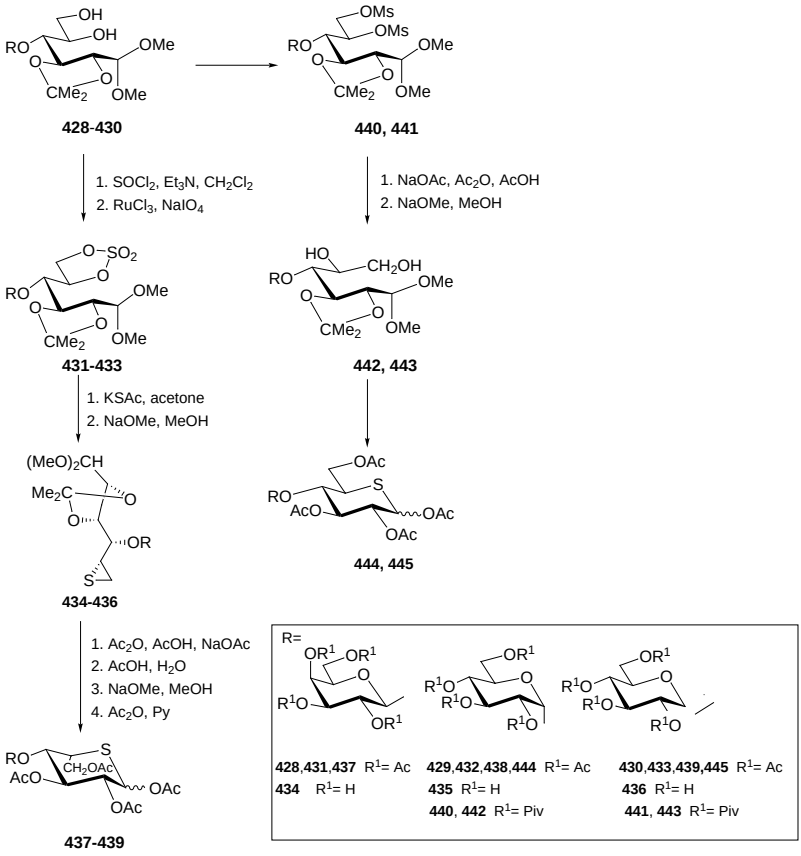
SCHEME 103

cyclization under Mitsunobu conditions, afforded **427,** which was transformed into methyl 5′-thio-α-isomaltoside in four steps.

Santoyo-González *et al.*[395] reported the synthesis of 5-thiodisaccharides from lactose, maltose, and cellobiose. These disaccharides were transformed in several steps into the partially protected 4-*O*-glycosyl-2,3-*O*-isopropylidene-D-glucose dimethyl acetals **428**–**430** through kinetic acetonation and selective hydrolysis of the acetal functions. These disaccharide diols were then transformed[396] into the corresponding thiiranes **434**–**436,** via the 5,6-cyclic sulfates **431**–**433.** Nucleophilic ring-opening of the episulfide ring with sodium acetate, followed by acidic hydrolysis, Zemplén *O*-deacetylation and acetylation gave the acetylated 4-*O*-glycosyl-5-thio-L-idopyranoses **437**–**439**. Acetylated 5-thio-maltose and -cellobiose **444** and **445** were synthesized from the 5,6-di-*O*-mesyl derivatives **440** and **441** by inversion at C-5 with sodium acetate and *O*-de-acetylation to afford the diols **442** and **443,** followed by the sequence diols→cyclic sulfates→thiirane→thio sugars.

IV. SEVEN-MEMBERED RINGS

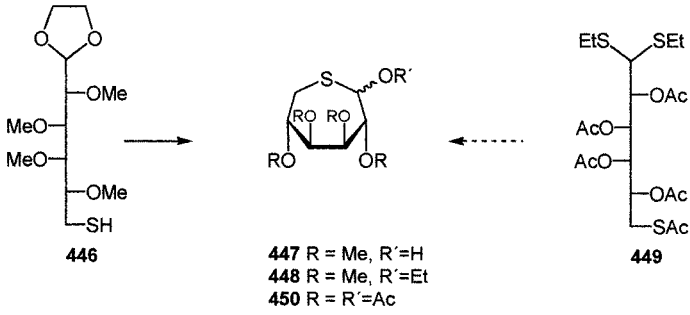
6-Thioaldoses can form hemiacetal seven-membered septanose rings, provided that C-5 and C-4 hydroxyl groups are protected to avoid the formation of the more favorable furanose and pyranose rings.[16] Thus, acid hydrolysis of **446** gave 2,3,4,5-tetra-*O*-methyl-6-thio-D-galactoseptanose (**447**) and ethanolysis afforded an anomeric mixture of the ethyl thioseptanosides **448.**[397] The isomerization to



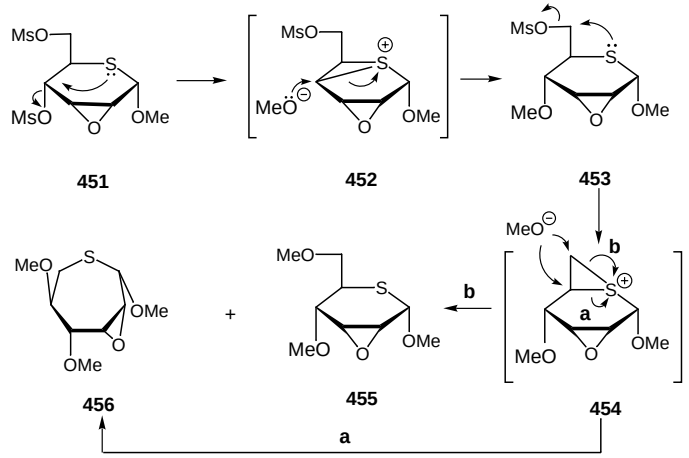
SCHEME 104

the open-chain form occurs much more readily for seven-membered rings having sulfur in the ring than for the five- or six-membered ring analogues, as shown with model compounds.[398] Removal of the ethylthio and the *S*-acetyl groups of **449** with mercuric chloride and mercuric oxide, and then hydrogen sulfide, followed by conventional acetylation, yielded the crystalline α and β anomers of 6-thio-D-galactoseptanose pentaacetate (**450**)[399] (Scheme 105).

The L-taloseptanoside[72] **456** was formed in 74% yield by ring expansion upon heating the dimesyl compound **451** in methanol containing barium carbonate. Participation by the ring sulfur atom and formation of intermediate episulfonium ions **452** and **454,** as shown in Scheme 106, has been suggested. The pyranoside **455** was also isolated as minor product (13%).

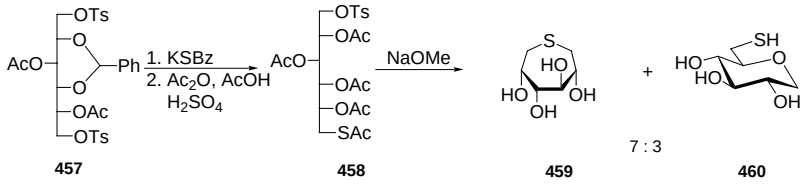


SCHEME 105

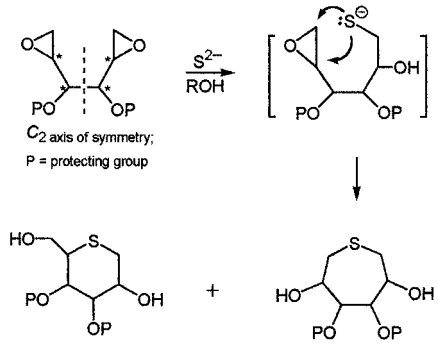


SCHEME 106

1,6-Anhydro-1-thio-D-mannitol and galactitol were synthesized by nucleophilic displacement of the 1,6-disulfonyloxy groups of 1,6-di-*O*-mesyl-D-mannitol and galactitol; 1,2 : 5,6-dianhydrohexitols are likely intermediates.[400] 1,6-Anhydro-1-thio-D-glucitol[401] (**459**) was prepared from the 2,4-*O*-benzylidene-1,6-di-*O*-tosyl-D-glucitol derivative **457,** as shown in Scheme 107.

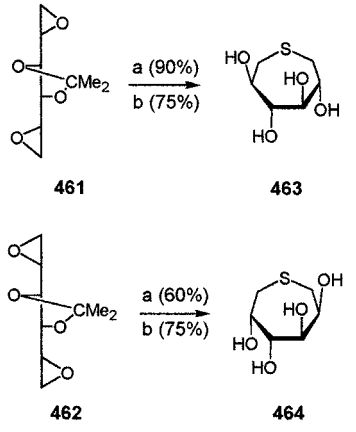


SCHEME 107

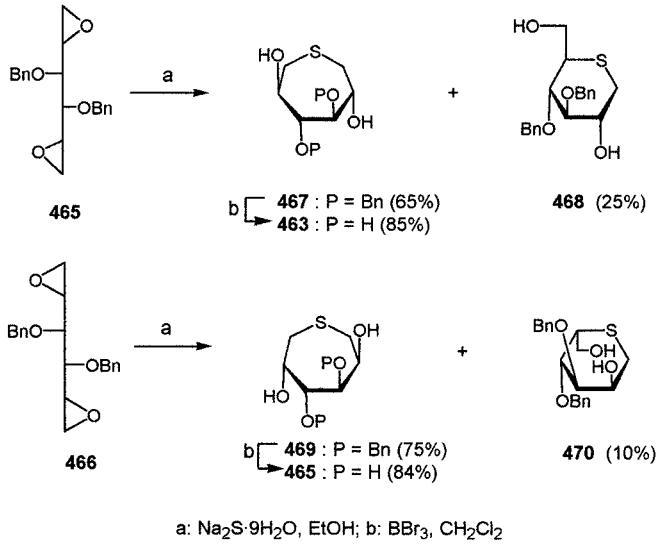


SCHEME 108

Thiocyclization of enantiopure C_2-symmetric bis-epoxides, such as the 3,4-*O*-isopropylidene-L-iditol (**461**) and D-mannitol (**462**) derivatives, to give 1,6-anhydro-1-thio-L-iditol (**463**) and D-mannitol (**464**) on reaction with sodium sulfide, has been described[402,403] (Scheme 109). Improved yields were obtained[314] using alumina-supported sodium sulfide reagent as the nucleophile. Nucleophilic ring-opening of the flexible bis-epoxides **465** and **466,** where the 3,4-diol is protected as a dibenzyl ether, with 2 eq. of sodium sulfide gave mixtures of 1,6-anhydro-1-thioalditols (**467**/**468**) and 1,5-anhydro-1-thioalditols (**469**/**470**), respectively (Scheme 110). The proposed mechanism of the thiocyclization is depicted in Scheme 108. Debenzylations were carried out with BBr_3. The L-*ido*-thiepan **463** showed a weak inhibition of α-D-glucosidase ($K_i = 3.9 \times 10^{-3}$ *M*), and its corresponding sulfone was an even less potent inhibitor of that enzyme.



SCHEME 109

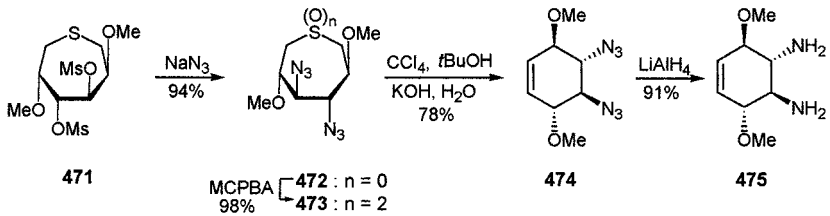


a: $Na_2S{\cdot}9H_2O$, EtOH; b: BBr_3, CH_2Cl_2

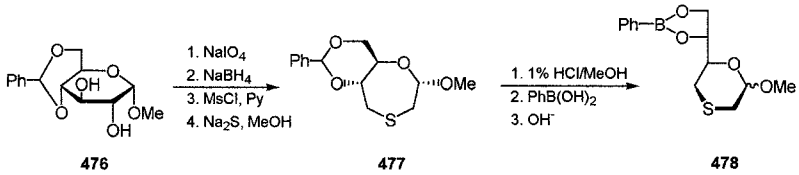
SCHEME 110

Cerè *et al.*[404–406] have described the transformation of dimesylated 1,6-anhydro-1-thio-D-mannitol (**471**), prepared from mannitol,[402] and its transformation into the 2,3-diaminoconduritol derivative **475,** with complete configuration inversion at the C-4 and C-5 chiral carbon atoms. A stereospecific ring contraction to afford polyfunctionalized thiolane derivatives from a thiepane derivative, promoted by NaN_3, was described, starting from the D-glucitol analogue of **471.**[407]

Some sugar derivatives containing two heteroatoms in six- and seven-membered rings have been reported.[408,409] Successive treatment of the methyl glucoside derivative **476** with periodate, borohydride, methanesulfonyl chloride–pyridine, and sodium sulfide–boiling methanol afforded the seven-membered 4-oxathiane derivative **477,** which, after successive transformations, gave the 1,4-oxathiane analogue **478** (Scheme 112).



SCHEME 111

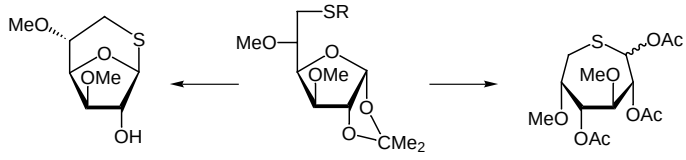


SCHEME 112

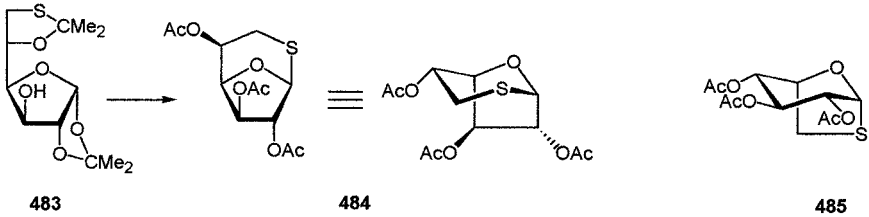
1,6-Anhydro-1-thiohexopyranoses,[178,179,410,411] readily formed by cyclization in a hexopyranose bearing a thiol group at C-1 (or C-6) and a leaving group at C-6 (or C-1), and 1,6-anhydro-1-thiohexofuranoses[397,412] may be considered as sulfur-containing bridged septanoside forms of 6-thiohexoses. It should be taken into account that 1,5-anhydro-6-thiohexoseptanoses and 1,4-anhydro-6-thiohexoseptanoses are not recommended names.[413]

Hydrolysis of **479** (AcOH, H_2O, H_2SO_4) gave the 1,6-anhydro-1-thiohexofuranose **481,** in 52% yield and acetolysis of **480** (AcOH, Ac_2O, H_2SO_4) afforded a small yield, of crystalline 3,5-di-*O*-methyl-6-thio-D-glucoseptanose triacetate (**482**).[397] Similarly, aqueous acid hydrolyis of **483** (1 *M* HCl) followed by acetylation led to the 1,6-anhydro-1-thio-α-L-idofuranose derivative **484** in 55% yield.[414] The expected anhydropyranose **485** was not formed (Scheme 114). In contrast, 1,6-anhydro-5,6-dithio-β-L-idopyranose (**487**) was obtained[415] by the action of acid on 1,2-*O*-isopropylidene-5,6-dithio-β-L-idofuranose (**486**), and acid hydrolysis of 1,2-*O* : 5,6-*S*,*O*-diisopropylidene-5-thio-α-D-glucofuranose gave 1,6-anhydro-5-thio-β-L-idopyranose in addition to 5-thio-L-idose.[286] Related experiments on di-1,2-*O* : 5,6-*O*,*S*-isopropylidene-6-thio-α-D-glucofuranose and 1,2 : 3,4-di-*O*-isopropylidene-6-thio-α-D-galactopyranose gave the corresponding 1,6-epithiohexofuranoses **488** and **489,** respectively.

4-Cyanophenyl (and 4-nitrophenyl) 2,5-anhydro-1,6-dithio-α-D-glucoseptanosides (**494**) and α-L-guloseptanosides (**495**),[272] and the corresponding 4-azido analogues,[273] have been prepared by Kuszmann *et al.* via the 1,6-thioanhydro derivative **491,** which was formed from mesylate **490.** Oxidation and Pummerer

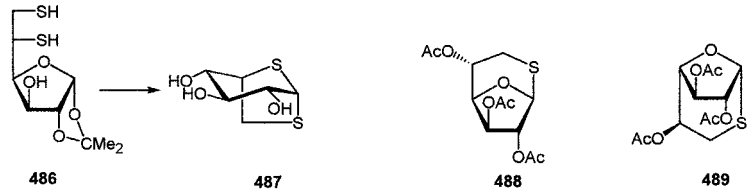


SCHEME 113



483 **484** **485**

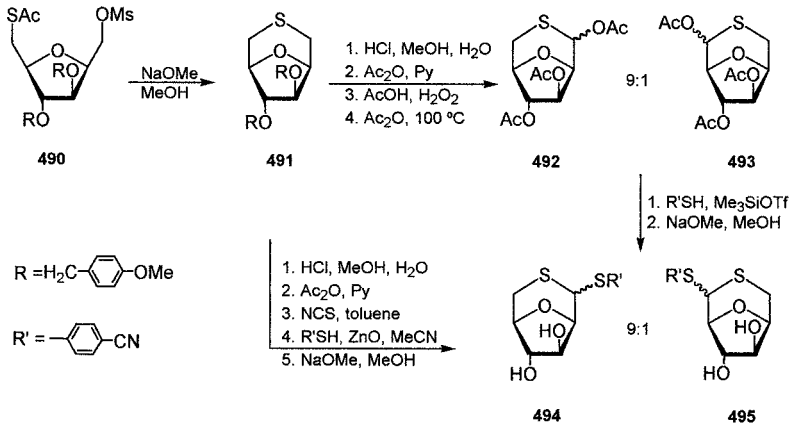
SCHEME 114



486 **487** **488** **489**

SCHEME 115

rearrangement gave the peracetylated 2,5-anhydro-6-thio-α-D-gluco-(**492**) and α-L-guloseptanoses **(493),** which were transformed into the corresponding 1,6-dithio-α-D-gluco- and α-L-guloseptanosides by reaction with 4-cyanobenzenethiol in the presence of trimethylsilyl triflate. Another route to **494** and **495** involved the chlorination with *N*-chlorosuccinimide of the methylene groups that flanked the sulfur atom and the glycosidation of the aglycone in the presence of zinc oxide as promoter. These thioglycosides have displayed a stronger oral antithrombotic effect than Beciparcil in rats.

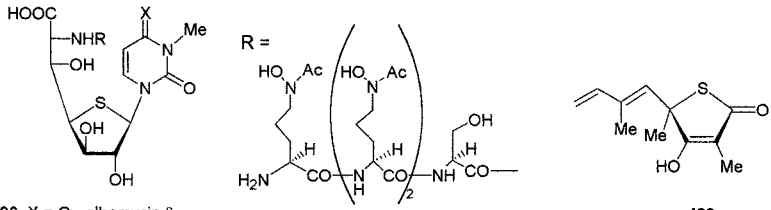


490 **491** **492** **493** **494** **495**

SCHEME 116

V. Naturally Occurring 4-Thio and 5-Thio Sugar Analogues

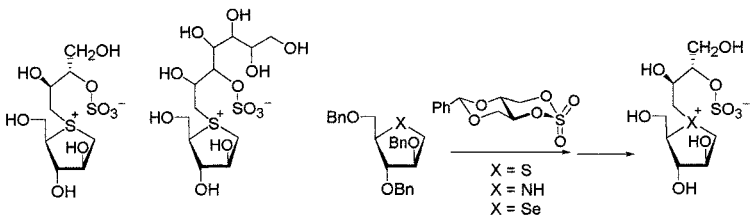
The first thio sugar analogues to be discovered in nature were the antibiotics albomycins δ_1, δ_2, and ε **496**–**498,** isolated from ***Streptomyces*** sp. WS 116 (DSM 1692).[416] Enzymatic hydrolysis of these albomycins afforded 4′-thionucleosides **496** (R = H) and **497** (R = H).[417,418] The isolated[419] antibiotic thiolactomycin showed itself to be a potent inhibitor of bacterial fatty acid biosynthesis.[420–424] The asymmetric synthesis of the (5*S*) enantiomer of the antibiotic thiolactomycin confirmed the (5*R*) configuration of the natural product.[425] 5-Thio-D-mannose **(329),** isolated from the marine sponge ***Chlathria pyramida*** (Lendenfeld), was the first example of a reducing 5-thio sugar occurring naturally.[426]



496 X = O, albomycin δ_1
497 X = $NCONH_2$ albomycin δ_2
498 X = NH albomycin ε

499

Salacinol[25] **500** and kotalanol **501,**[26, 427,428] unique structures comprising a "1-deoxy-4-thio-D-arabinofuranosylsulfonium" cation and an internal polyhydroxyalkyl sulfate anion, have been isolated from roots and stems of ***Salacia reticulata*** Whight, extensively used as a specific remedy for diabetes in Ayurvedic traditional medicine. The structure of salacinol was confirmed by X-ray analysis.[25,26] Salacinol and kotalanol were found to be competitive inhibitors and exhibited very potent inhibitory activities against the intestinal α-glucosidase of rats. The IC_{50} values of **500** were 0.84 μg/mL to sucrase, 3.2 μg/mL to maltase, and 0.59 μg/mL to isomaltase; those of **501** were 0.58, 2.8, and 1.9 μg/mL; whereas those of

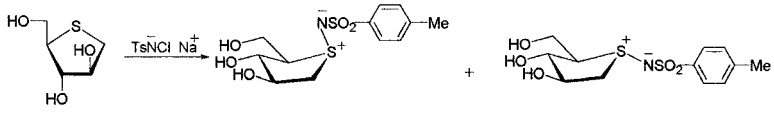


500

501

500 X = S
501 X = NH
502 X = Se

SCHEME 117



503 **504** **505**

SCHEME 118

acarbose, clinically used for the treatment of diabetes mellitus, were 1.1, 1.2, and 100 μg/mL, respectively.[26] Salacinol inhibited the increase of serum glucose levels more strongly in sucrose-loaded rats than did acarbose. Synthetic routes to salacinol and its 1-deoxy-4-thio-D-arabinofuranosyl-ammonium and -selenonium analogues (Scheme 117) have recently been described.[429]

Iminothio sugars **504** and **505,** structurally similar to salacinol, were prepared by treatment of 1,4-anhydro-4-thio-D-arabinitol with chloramine-T.[430] Although they were prepared as potential transition-state-mimic glycosidase inhibitors, compound **504** was found to be only a weak inhibitor of β-glucosidase from brewer's yeast ($K_i = 1.7$ mM).

ACKNOWLEDGMENTS

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Helicobacter pylori: A WOLF IN SHEEP'S CLOTHING: THE GLYCOTYPE FAMILIES OF *Helicobacter pylori* LIPOPOLYSACCHARIDES EXPRESSING HISTO-BLOOD GROUPS: STRUCTURE, BIOSYNTHESIS, AND ROLE IN PATHOGENESIS*

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I. INTRODUCTION

1. *Helicobacter pylori*

In 1983, the generally accepted dogma that gastric diseases in humans were mainly a consequence of stress, alcohol consumption, cigarette smoking, stomach

* Dedicated to the loving memory of my father Sr. Artur Monteiro.

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acidity, and dietary factors[1] was challenged by Warren and Marshall,[2] who alleged that infection by the gastric gram-negative microaerophilic bacterium *Helicobacter pylori* (0.6 × 3.5 microns), initially designated *Campylobacter pyloridis*, may be responsible for the onset of gastritis and gastric ulcers in humans. Approximately one century before, Bizzozero (1893), Salomon (1899), and Balfour (1906) reported the presence of spirochetal bacteria in dog and monkey gastric tissue, and more recently, in the mid-1970s, Steer and coworkers observed the presence of spiral bacteria in the human stomach and indeed a possible association with inflammation was reported.[3] The successful *in vitro* cultivation of *H. pylori* by Warren and Marshall spearheaded an explosion in *H. pylori* research and its role in gastric diseases. *H. pylori* colonizes the human gastric mucosa of more than half of the world's human population[4] and can persist in the stomach of hosts with or without clinical symptoms for a lifetime. It is now well accepted that colonization by *H. pylori* leads to chronic superficial gastritis, chronic active gastritis, and peptic ulcers.[5] In addition, *H. pylori* infection has also been associated with the development of more severe diseases, such as gastric cancer and mucosa-associated lymphoid tissue lymphoma,[6,7] and, consequently, this gastric bacterium has now been classified a category 1 (definite) human carcinogen[8] (Fig. 1). Although it remains largely unproved and contradictory, *H. pylori* infection has been connected with ischeamic and coronary heart diseases.[9] One recent article has also reported the presence of *H. pylori* ureC and cagA genes in the stomachs of children who have succumbed to sudden infant death syndrome (SIDS).[10] Several virulence mechanisms involving urease, the cytotoxin VacA, and the genes that compose the pathogenicity island *cag* have been linked to roles in the pathogenesis of *H. pylori*.[11]

This article focuses on the chemical structure, biosynthesis, and potential pathogenic role of the lipopolysaccharides (LPSs) from *H. pylori*. The structures of LPSs isolated from *Helicobacter* species found in nonhuman primates, which give rise to similar gastric symptoms in their respective hosts, are also described. LPSs are glycolipid structures carried by bacteria on their cell surfaces, which are actively involved in biochemical interactions between the bacterium and its host.[12]

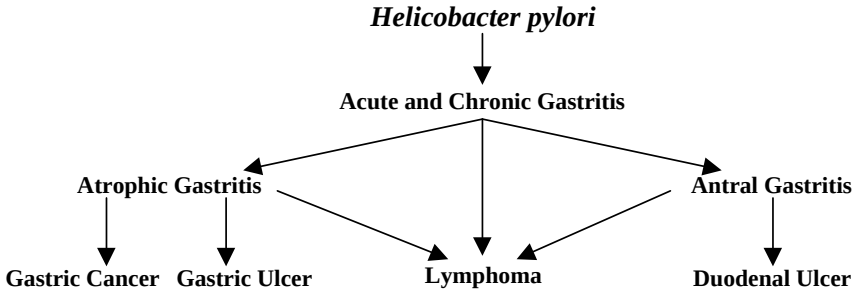


FIG. 1. Genesis of gastric-related clinical outcomes due to *H. pylori* infection.

LPSs from *H. pylori* have been determined to be able to bind to the mucosal mucin receptor, leading to the disruption of the integrity of the gastric mucosa; have been implicated in the stimulation of pepsinogen, leading to penetration of the gastric epithelium by the action of pepsin; have shown an high-affinity binding to laminin; and have also been implicated in the ability of the bacterium to bind to gastric mucosa.[13–15] In most instances, these interactions between *H. pylori* and the host were observed without regard to the LPS chemical structure or to differences between LPSs from different strains. However, recent pathogenic studies, which will be described here, employing well-defined *H. pylori* isogenic mutants carrying modified LPS molecules have shown that these LPS structures are indeed important players in *H. pylori* pathogenesis.

2. Lipopolysaccharides

A common method for classifying bacteria is the gram-stain technique,[16] which distinguishes two groups of bacteria on the basis of differences in their cell walls. Gram-positive bacteria, when compared with gram-negative bacteria, have simpler cell walls, mostly being composed of peptidoglycans.[17] The peptidoglycan of most bacteria is composed of alternating units of *N*-acetylglucosamine and *N*-acetylmuramic acid joined by β-(1 $\rightarrow$ 4) glycosidic bonds. Cell walls of gram-positive eubacteria can also contain teichoic acids, which are extracellular polymers of polyols linked by phosphodiester bonds,[18] and the nonphosphorylated teichuronic acid-type chains which are composed of acidic constituents such as uronic acid. Gram-positive bacteria, especially streptococci, may also produce lipoteichoic acids and various acid-soluble proteins and often carry, as antigenic markers, capsular polysaccharides (CPSs).[19] The addition of an outer membrane in gram-negative enteric bacteria gives it a more complex molecular architecture of which LPSs are the main components.

Gram-negative bacteria have a comparatively low peptidoglycan mass component. In enteric bacteria the peptidoglycan is covalently joined to some lipoproteins forming a connecting bridge between the peptidoglycan and the outer membrane. The external part of the outer membrane is composed of cell wall LPS. LPSs are complex molecules that occur in two forms. The high molecular weight (high M_r) LPSs with M_r values greater than 10,000 amu, commonly referred to as smooth-form LPSs,[20] (S-form LPSs), are derived from the so-called smooth-form organisms, reflecting the surface texture of bacterial colonies. Based mainly on previous pioneering investigations performed on *Escherichia* and *Salmonella*, S-form LPSs may be divided into three covalently linked regions (Fig. 2): the endotoxic component lipid A, the core oligosaccharide (OS) of approximately 8 to 10 sugar residues in a nonrepetitive sequence, and the O-antigen chains (O-chains) consisting of regularly repeating OS blocks. Normally, low molecular weight (low M_r) rough-form LPSs (R-form LPSs) are also present, and this glycan, devoid of the

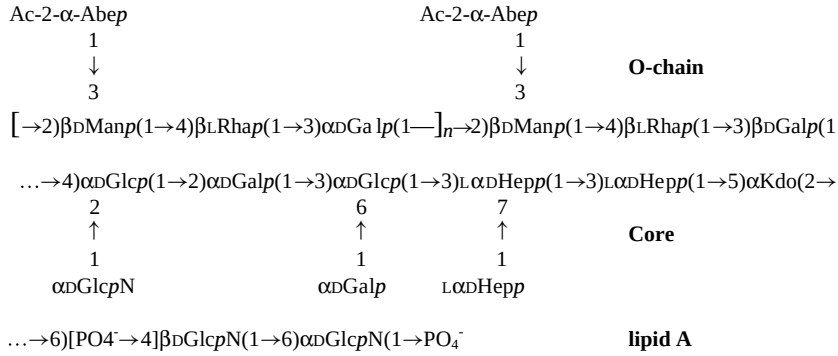


FIG. 2. * The three LPS covalently linked regions: the lipid A, which also contains fatty acids (not shown); the core oligosaccharide; and the O-chain of *Salmonella enterica* serovar Typhimurium.[27]

O-chain, consists of core OS and lipid A only, and thus, in the so-called R-form organisms (incomplete LPS) only the R-form LPS is present. In some bacteria, e.g., *Neisseria,* O-chain regions are entirely absent with strain-to-strain variations being reflected in differences in the nonrepetitive core OS regions, and some researchers have coined the term lipo-oligosaccharides (LOSs)[21] as being more appropriate and literally correct. Frequently, between S- and R-form LPSs, one also finds LPS structures of semi-rough-form (SR-form) that contain a complete core OS and a small number of O-chain repeating blocks.

The terms "smooth" and "rough" originally referred to the texture of the surface of bacterial colonies. Smooth colonies of bacteria seem to be linked to the presence of surface components that promote a compact cellular orientation. This terminology was then extended to label LPS components of gram-negative bacteria, and, as in this article, often reflect the presence (high M_r S-form; O-chain → core → lipid A) or absence (low M_r R-form; core → lipid A) of O-chains. Here, S-, SR-, and R-forms refer to O-chain extension in LPS structures and do not necessarily describe colony morphology. Some gram-negative bacteria possess extracellular polysaccharides (PSs), also referred to as capsular or exopolysaccharides, which are polymers similar to O-chains[22] in that they possess repeating OS blocks.

The location of these glycan molecules on the bacterial outer membrane makes them an important component in the pathogenesis of endotoxemia and septic shock syndromes, and in the survival and overall biological functions of the bacterium.[23] Moreover, LPSs with differences in chemical structures of the O-chains often provide a molecular basis for serological classifications within the same genus.[24]

* Here, and elsewhere in this article, hyphens are omitted from the extended symbolic oligosaccharide sequences, to achieve a more compact presentation than in the standard IUPAC-IUBMB format.

The methods developed by Westphal and colleagues that led to the isolation of LPS by the water–phenol extraction method[25] provided the material on which studies could be carried out, including those of ***Helicobacter*** LPSs that are discussed here.

The lipid moiety, termed lipid A, anchors the LPS to the bacterial cell through hydrophobic and electrostatic forces.[26] Detailed structural investigations using chemical, enzymatic, and spectroscopic methods[28,29] carried out on lipid A from ***S. typhimurium*** and ***E. coli*** have shown that this lipid moiety is the most conserved region of the LPS.[30] The backbone of the lipid A domain consists of a (1 → 6) linked β-D-glucosamine disaccharide [β-D-Glc*p*N-(1 → 6)-α-D-Glc*p*N] to which phosphate groups are attached to the α-glycosidic hydroxyl group at C-1, and ester linked at O-4′, usually with (***R***)-3-hydroxy fatty acids[31,32] as lipophilic substituents, two of them ester linked at O-3, and the other two attached as ***N***-acyl substituents on both GlcN residues.

The core OS region is covalently attached through the reducing end to the lipid A by an acid-sensitive ketosidic linkage from 3-deoxy-D-***manno***-octulosonic acid (Kdo), an immutable component of the core, to O-6 of the outer glucosamine unit of the lipid A. Typically, the nonrepetitive core oligosaccharide is composed of four basal sugars, L-***glycero***-D-***manno***-heptose (LD-Hep), glucose (Glc), galactose (Gal), and Kdo, but may also contain phosphate residues. The first detailed investigations of the chemical structure of core OSs were carried out on those from R-form LPSs from ***E. coli*** and ***S. typhimurium***.[33] Genetic defects, natural or induced, resulted in the generation of mutants displaying enzymatic lesions and resulting in the formation of incomplete R-form LPS of low M_r with shortened glycan chains ranging from the complete core OS in the Ra mutant through to the shortest Re or deep rough mutant.[34] Recently, the complete biosynthesis for the ***E. coli*** (R1) core OS assembly has been described.[35] Kdo has also been observed at the nonreducing end of core OSs of ***Rhizobium etli***[36] and ***Klebsiella*** (Evgeny Vinogradov, personal communication).

Only a single core OS region has been found in LPSs from a range of wild-type strains of ***S. typhimurium,*** but considerable variation is encountered in the structures of the O-chains with specificities conferred by different OS repeating units. Some species, such as ***Neisseria,*** only elaborate R-form LPS of low M_r devoid of repetitive O-chains. In this situation, strain-to-strain variations, in the absence of the biosynthetic machinery for O-chain elongation, are reflected in differences in the nonrepetitive core OS regions of these LOSs. Aspinall and co-workers have also shown that various ***Campylobacter*** species produce LOS molecules that express ganglioside-like antigens, and, simultaneously, these ***Campylobacter*** species also furnished high M_r PSs.[37–39]

The structures of complete high M_r LPSs are dominated by extended O-chains with regular OS repeating units (Fig. 2), which are a source of the bacteria's antigenicity and often serve as receptors for bacteriophage attachment.[40] Antigenic

O-chain PSs have been characterized[41] with a wide range of constituent sugars, which may be accompanied by nonglucan substituents such as phosphate and amino acids. Biosynthetic studies have shown that many O-chains are extended in a blockwise manner from OS blocks that have been assembled on an antigen carrier lipid. This key intermediate permits transport of the polar OS across the outer membrane for insertion at the proximal "reducing" terminus of the growing O-chain.[42] The occurrence of regular repeating units provides a framework within which to interpret data acquired in the course of structural investigations. Recent studies have shown that a biosynthetic incomplete O-chain repeating glycan block can be added to the core OS.[43]

In Fig. 2, it can be observed that the O-chain polymer of *Salmonella* has been determined to be attached to the O-4 position of the outer α-D-Glc*p* unit of the core OS.[27] However, this is one of very few cases where the covalent connection of O-chain to core has been unambiguously sited. The many variable O-chains connected to the core in *Salmonella* are responsible for assignment of serogroups. The O-chain-like CPSs have the same type of structural arrangement in having glycan repeating blocks as the building units of their glycan chains,[44] and in some species, the CPS and O-chain PS can also share a common structure.[45] However, these CPSs are independently held by the outer membrane, for example, through lipid anchors such as di-*O*-acylglycerol units.[46] A few of these antigenic CPSs have been used in a glycoconjugate format (CPS + protein) as protective vaccines against the respective bacterium's infection.[47]

3. Approaches Employed for Elucidation of *Helicobacter* Lipopolysaccharide Structures

The complete structural elucidation of carbohydrates, whether true PSs or glycoconjugates/glycolipids, requires that information must be obtained on: (*i*) monosaccharide composition, including the anomeric and enantiomeric configurations of sugar residues; (*ii*) the sites of linkage between the sugar units; (*iii*) the sequence of glycosyl residues; and (*iv*) siting of any noncarbohydrate substituents. To this end, in the studies to be described, many well-established procedures and analytical methods were used and these are listed with key references. However, as the investigations on *H. pylori* LPSs progressed, new problems were encountered, altered strategies had to be adopted, and departures from standard operating procedures became necessary. This section attempts to consider some of the practical limits of standard procedures rather than repeating readily accessible standard recipes. Consideration is given to the approaches that were sought to deal with new situations and emphasis is placed on the strategies employed to solve structural questions rather than on detailed experimental procedures.

A recurring problem has been the limited quantities of *H. pylori* LPS preparations that have been available because of the fact that *H. pylori* is a very fastidious

grower *in vitro*. The cell material, as received from clinical settings, contained small amounts of impurities; the presence of some of them was revealed in later LPS analyses, e.g., the ubiquitous phthalate plasticizers during mass-spectrometry analysis and the formation of ribitol pentaacetate (from ribose in RNA) during sugar analysis. Because of the limited quantities of material, in most cases, it was impractical to attempt to remove them. A more important consideration was that of carbohydrate microheterogeneity, but again there was little choice but to proceed with the material available. Any attempt to fractionate milligram quantities would have given too little of any subfraction to yield useful information. With careful selections of experiments to give crosschecked results, the major structural features of these unusually complex LPSs were established with the highest degree of confidence. The conclusions reached in these studies will provide the necessary basis for any assessments of microheterogeneity that would require an informed choice of separation procedures, e.g., using histo-blood group antigens as ligands for affinity chromatography.

Contrary to other gram-negative bacteria, most of the *H. pylori* LPS material is easily soluble in aqueous solutions, and thus the water-soluble LPS preparations were examined directly by sugar analysis on the basis of those amenable to the alditol acetate procedure.[48] In most instances, methylation linkage analysis and mass-spectrometry studies were also conducted at this stage. Analysis at this point also assured that acid-sensitive constituents, such as fucose and sialic acid, were not missed. In the case of incomplete solubilization, or of suspected contamination with salts or other impurities, the preparation was separated by gel-permeation chromatography (GPC) on Bio-Gel, preferably on the P-2 grade, and then re-examined. Insoluble gels were delipidated by heating in 1% acetic acid, or sometimes at buffer pH ~6.5, and the soluble liberated saccharides were fractionated on Bio-Gel P-2. Account was taken of the possibility that glycosidic linkages, other than the ketosidic linkage of Kdo to lipid A, might be cleaved, and two such situations from these investigations are discussed.

Detailed investigations on *H. pylori* LPSs were commenced in the early 1990s as an extension of ongoing studies dealing with the closely related genus *Campylobacter*. Preliminary sugar composition analyses had been performed by Anthony Moran[49] and Armando MacDonald.[50] In the previous *Campylobacter* studies,[51,52] satisfactory separations were achieved of high-M_r glycans, comparable to CPSs,[53] from the aqueous layer of the phenol–water extraction, and the core OSs liberated from LOS of low M_r in the water-insoluble gel. Indeed, in the *Campylobacter* studies, by the criteria of molecular size from GPC and composition analysis, no significant overlap was observed and no evidence was obtained for materials of intermediate size, e.g., of carbohydrate chains from semi-rough strains that might contain core OS and a single O-chain repeating unit. At the outset, similar separation techniques were used with *H. pylori* LPSs, but as the knowledge on LPS structural features and behaviors was accumulated different strategies were

adopted, and eventually the purifications and fractionations were carried out using the intact LPSs in order to study the O-chain regions.

During the preparation of delipidated OSs and PSs from *H. pylori* by delipidation on heating in 1% acetic acid, limited hydrolysis of fucopyranosyl linkages occurred. Here, the desirability of obtaining carbohydrate devoid of associated lipid A for nuclear magnetic resonance (NMR) studies had to be balanced against the slight loss of fucose components. A more important factor was that separations of LPS fractions, on the basis of solubility or size, provided access to a range of molecules of different sizes spanning the gap between the inner core OS region and the regularly extending O-chains. The situation was found to vary from strain to strain since not all LPS preparations yielded fractions with a full complement of variations in size and structure. Separations of water-soluble LPS preparations and of derived OS and PS on the basis of molecular size were effected by GPC using polyacrylamide gels covering the anticipated size range. In practice it was found that GPC on Bio-Gel P-6, supposedly spanning the molecular mass range 5000–6000 Da, failed to remove LPS with shorter chains from those with relatively long O-chains. LPS with extended chains were separated satisfactorily on GPC using Bio-Gel P-2 (mass range 100–1800 Da) with retardation of shorter chain material. The extent of fractionation and the quantities of differently sized OSs liberated on delipidation of water-insoluble LPS gels varied from one preparation to another. In favorable cases, fractions were obtained ranging in size from the smallest inner core OS through to those of core OS with attached O-chains only slightly less extended than those from initially soluble high M_r LPS. In the examination of *H. pylori* LPSs, advantage was taken of OS fractions showing a progression of chain extension to derive partial structures from which a composite structure for the complete PS chain could be proposed.

Typically, the *H. pylori* isolates were frozen immediately after isolation ($-70\,^{\circ}C$). Cultures were subsequently thawed and plated onto brain heart infusion agar (1.2%, w/v) plates supplemented with 0.5% (w/v) yeast extract and 0.5% (v/v) fetal bovine sera. The *H. pylori* strains were allowed to grow for 3 days under microaerobic conditions at $37\,^{\circ}C$, subcultured into brain heart broth, and allowed to grow for an additional 3 days under the same conditions with agitation.

Sugar analyses were conducted routinely by hydrolysis of LPSs with aqueous trifluoroacetic acid followed by reduction with $NaBD_4$ and acetylation to give alditol acetates for separation, detection, and characterization by combined gas–liquid chromatography and mass spectrometry (GLC-MS).[54] Retention times of derivatives were compared with those of available configurationally defined reference compounds. Reference compounds were also required for determinations of absolute configurations by the chiral glycoside method using 2-(*R*)- and 2-(*S*)-butanol in the formation of acetylated glycosides for GLC analysis.[55] Aside from the decomposition of ketose sugars on treatment with strong acid, the normal conditions of hydrolysis were accompanied by the formation of nonreducing compounds

such as 1,6- and/or 1,7-anhydro sugars as evidenced by lack of incorporation of ^2H on treatment with $NaBD_4$. L-*glycero*-D-*manno*-Heptose was one such sugar regularly encountered in these studies, so that both the heptitol heptaacetate and the anhydro-heptose tetraacetate were included in quantitative analyses.[56] Chemical sugar linkage analysis was performed by the methylation method.[57] Although usage of capillary GLC columns of different lengths and polarities discriminated most of the partially methylated alditol acetate derivatives (PMAAs), it was common, because of the complexity of *H. pylori* LPSs, for these sugar derivatives to elute without baseline separation and in some instances simultaneous elution occurred. Thus, these analyses had to be carefully studied and crosschecked with other available data.

The technique for elucidation of sequence and total glycose composition used up to now has been fast-atom bombardment–mass spectrometry (FAB-MS).[58] The methylated LPS and OS derivatives were used. Mainly, two types of key information were obtained by FAB-MS. First, when observed, molecular weights were obtained through the formation of pseudomolecular ions by the addition of a proton $[M + H]^+$ and/or another cation in the positive-ion mode. Second, the sequence of the individual sugar units in a chain was elucidated by the detection of three types of ions in the mass spectra (Fig. 3). The most important involved glycosidic cleavage forming glycosyloxonium ions that gave exact incremental masses of the glycose components. This, however, yielded no information on linkage sites or stereochemistry. Another type of cleavage pathway involved the observation of a secondary ion formed by β-elimination of the substituent at the O-3 position of the glycosyloxonium sugar unit (Fig. 3). The mass difference between the parent glycosyloxonium ion and the secondary ion yielded information about the nature of the substituent at O-3. The other useful type of ion arose from a double cleavage process that involved a hydrogen transfer where two glycose units were cleaved, resulting in ions which contained neither the original nonreducing nor the reducing end. FAB-MS has proved to be the most fruitful and industrious technique in the *H. pylori* LPS studies described here. The observed preferential cleavage at the GlcNAc unit (reducing end)[59,60] yielding A type primary glycosyl oxonium ions and the subsequent secondary ion from β-elimination have aided greatly in these structural assignments. Electrospray mass spectrometry (ES-MS),[61] and, when necessary, its combination with high-performance liquid chromatography (HPLC-ES-MS), provided information on the carbohydrate molecule's molecular weight. In some cases, unusual mass increments in the FAB-MS spectrum pointed to the presence of non-sugar substituents such as phosphate moieties.

One-dimensional (1D) NMR investigations usually revealed genuine characteristics present in the glycan molecule, for example, methyl groups and carbonyl carbons from *N*-acetylglucosamine (GlcNAc) and fucose (Fuc) units. The detection of the downfield placed (typically between δ_H 4.3 and 5.7; δ_C 95 and 105) anomeric resonances and the measurement of their respective vicinal coupling

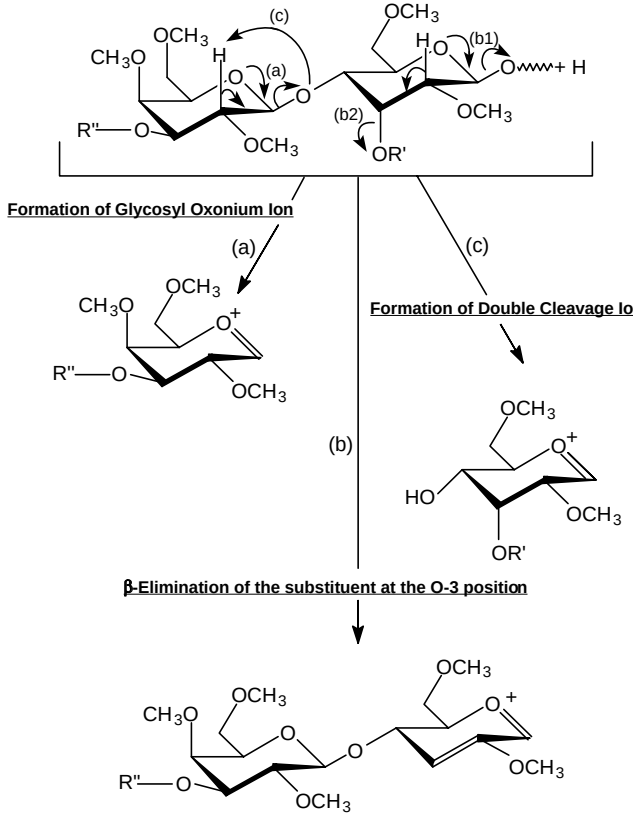


FIG. 3. Useful fragmentation pathways of permethylated glycans in FAB-MS.

constants ($^3J_{C,H}$) and one–bond proton–carbon coupling constants ($^1J_{C,H}$) gave information regarding the anomeric configuration of each component in the molecule. However, the ^{1}H NMR spectra of *H. pylori* carbohydrate molecules tended to have overlapping anomeric resonances that made it difficult to unambiguously detect all the anomeric signals. Two-dimensional (2D) NMR experiments had to be employed in order to disentangle overlapping resonances,[62] and in some cases the anomeric coupling constants were measured by obtaining the 1D subspectrum by "slicing" the $H^{1,2}$ cross peaks in the 2D ^{1}H–^{1}H COSY spectrum. When anomeric configurations could not be deciphered, serological experiments using commercially available monoclonal antibodies (mAbs), specific for histo-blood groups, helped in sugar configuration assignments.

After gathering all the information obtainable by 1D NMR techniques, and with previous knowledge of the type of sugars present from composition analysis, 2D NMR spectrometry was frequently employed. The experiments listed here are

presented in chronological order. Homonuclear correlation spectroscopy (COSY)[63] and total correlation spectroscopy (both 1D and 2D TOCSY)[64] experiments allowed the assignment of the protons in the sugar ring by observing the scalar coupling through cross peaks between vicinal protons, which is made possible by the transfer of magnetization from one proton nucleus to the other through their respective covalent bonds. In theory, the assignment of all the sugar ring protons is possible, but, in these investigations, this fact was rarely the case since overlapping of ring protons, especially H-3 and H-4, made it impossible to make unambiguous assignments. The assignment of the independent pathways required that at least one resonance be unambiguously located, that typically being the anomeric resonance. The measurement of the vicinal proton coupling constants around the ring most of the time affords information about the configuration of the sugar residue.

Experiments that produce ^{1}H NMR resonances engineered by through-space energy transfer based experiments, which take advantage of nuclear Overhauser enhancement effects (NOE),[65] were also used. 2D NOESY and 2D rotating frame NOE (ROESY)[66] were two useful experiments used to detect close spatial proximity between protons. After assignment of the proton resonances from COSY and TOCSY experiments, these spatial interactions were used to establish ring conformation and configuration as well as aiding in linkage site determination. *Inter*-residue spatial connectivities were helpful in confirming sites of glycosidic linkages. The linkage assignments deduced from NOE connectivities were cross-checked with data from chemical analysis. An important limitation of these NOE-based experiments was the possible presence of resonances originating via through-bond interactions that might have given rise to unwittingly incorrect assignments. This was overcome by comparing NOESY and ROESY spectra where the latter is known for avoiding through-bond connectivities. However, ROESY experiments were only successful on some OSs of low M_r (up to 10 sugar residues); various attempts to obtain ROESY spectra on PSs were not successful.

^{13}C NMR was a reliable source of detection and identification of carbon nuclei in the saccharide molecule. However, greater quantities were required for 1D ^{13}C experiments as compared with ^{1}H NMR spectroscopy and often extended periods for acquisition (sometimes 3 days) were required. The ^{1}H–^{13}C heteronuclear multiple quantum correlation (HMQC)[67] was one experiment that allowed the assignment of carbon resonances, by correlation with their proton nuclei, and where smaller quantities were used. A conscious 1D ^{31}P NMR investigation quickly revealed the presence of phosphate or a 2-aminoethyl phosphate unit in the core OS. 2D ^{1}H–^{31}P HMBC NMR spectroscopy was a reliable method for efficient detection and sometimes placement of the phosphate substituents.

In order to gain information into the fine structure of carbohydrate molecules, selective chemical and enzymatic degradations were performed. A useful chemical degradation used in these studies was the Smith degradation,[68] where

periodate oxidation results in cleavage of the bonds between free vicinal diols. Characterization of the comparatively simpler products yielded information about the structure of the parent molecule. Some structural problems were also solved by the action of a specific enzyme, endo-β-galactosidase, selective for the hydrolysis of 3-substituted β-Gal*p* units that are linked to a linear 4-substituted β-Glc*p*NAc residues in an *N*-acetyl-polylactosamine (polyLacNAc) structure.

After the initial detection of histo-blood group epitopes in *H. pylori* LPSs by chemical analyses, serological analysis such as enzyme-linked immunosorbent assay (ELISA) and immunoblots employing commercially available mAbs specific for histoblood group antigens were repeatedly performed to quickly detect the presence of blood-group determinants in *H. pylori*. These serological results were used hand-in-hand with chemical data in determining the complex structures of *H. pylori* LPSs.

The objective of this article is to discuss the chemical structure, biosynthesis, and pathogenic role of the LPSs from strains of *H. pylori* in order to understand the broader biological implications of this organism. The molecular structure of these LPS molecules will provide the basis for serological differentiation between strains, and may be the basis of potential glycan-based therapeutics to combat *H. pylori* infections.

II. The Chemical Structure of *Helicobacter pylori* Type Strain Lipopolysaccharide: The Lewis X O-Chain

Differences between *H. pylori* isolates had been observed by an ability to distinguish strains based on electrophoretic patterns in sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) in combination with serological analyses with strain-specific antisera raised in rabbits.[69] In considering such structural variations, account must be taken of possible modifications of LPS expression during *in vitro* cultivation. The modifications may include a decrease of O-chain length on repeated passages on solid medium and its reversal when grown in a fluid medium.[70]

This section describes the first LPS structure to be investigated, that of *H. pylori* type strain NCTC 11637 (ATCC 43504).[71,72] An account of the structural elucidation of the complete PS component, from O-chain through to the inner core OS regions, of LPS from the type strain is discussed here. Penner and co-workers, by using homologous antisera raised in rabbits, assigned *H. pylori* NCTC 11637 to the O : 1 serogroup.[69] The LPS preparations obtained possessed a high degree of molecular microheterogeneity, but what appeared to be a complication proved to be an advantage in furnishing OS molecules whose structures were to shed light on the nature of the chemical linkages connecting O-chains to the inner region of the LPS.

Initially, the overall approach to the structural investigations was based on that used in previous studies on LOSs and CPSs from *Campylobacter* species in which it had been observed that insoluble gels from phenol–water extractions of bacterial cells yielded mainly low-M_r LOSs with core OSs linked to lipid A. The aqueous phases from such extractions gave high-M_r glycans with extended polymers, where carbohydrate polymers with no attachment to lipid A, such as the teichoic acid-like polymer from *C. coli* serotype O : 30 or the poly(tetraglycosylphosphates) from *C. lari*,[52] were found, and thus this possibility had to be kept in mind when deciphering the molecular makeup of *H. pylori* LPSs. The general strategy adopted for the analysis of *H. pylori* type strains set the stage for future *H. pylori* LPS investigations.

This section describes studies of the water-soluble S-LPS together with the isolation of an OS remaining after chemical and enzymatic degradation of the parent S-LPS. Mutually supportive evidence came from studies on OSs (OS-1, OS-2, and OS-3) from the SR-form LPS, of presumed low M_r, but in this preparation showing progressive chain extensions from the inner core OS region leading to O-chain initiation. From these observations a structural model for *H. pylori* LPS was developed, and a structure is proposed for the complete PS chain from the nonreducing terminus of the O-chain through an intervening region to the Kdo-terminated core OS.

S-form LPS of *H. pylori* type strain was almost completely water-soluble. It was observed that treatment of the water-soluble LPS from the S-form organism under standard conditions with dilute acetic acid, to cleave the ketosidic linkage of the Kdo terminus to lipid A, resulted in some liberation of fucose. In order to avoid inadvertent loss of fucose residues the key experiments for the characterization of the O-chain were, therefore, repeated on untreated water-soluble LPS. Glycose analyses showed that the main constituents were L-Fuc, D-Gal, and D-GlcNAc from the O-chain region. D-Glc, DD-Hep, and LD-Hep were also detected, in minor amounts, which composed the core OS part of the LPS. The anomeric configurations of the dominant sugar residues were defined by ^{1}H and ^{13}C NMR as those of α-L-Fuc, β-D-Gal, and β-D-GlcNAc.[72] The ^{31}P NMR spectrum for lipid-free PS showed a single resonance at δ 3.79 at pD = 6 assignable to a phosphoric monoester. Sugar linkage analysis performed on the LPS showed the presence of terminal Fuc, 3-linked Gal, and 4-linked and 3,4-linked GlcNAc residues in the approximate molar ratio of 5 : 9 : 4 : 4, together with the sugar residues (approximately 1 molar equivalent each) of variously substituted Glc, DD-Hep, and LD-Hep units arising from the inner region of the LPS (core region).[72] The major constituents appeared to be consistent with the presence of polyLacNAc chains of 3-substituted Gal and 4-substituted GlcNAc residues, with approximately half of the latter carrying at O-3 terminal Fuc residues. An additional feature was the formation of a small proportion (<1 molar proportion) of nonterminal 3-substituted Fuc residues. This constituent was postulated to arise from fucobiose

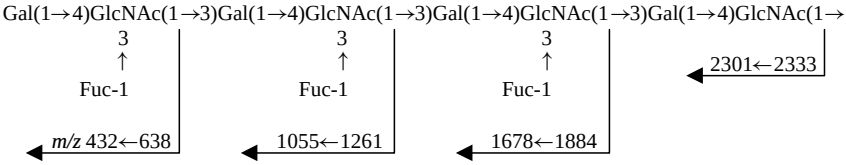


FIG. 4. The composite structure, along with *m/z* values from FAB-MS spectrum, of the polymeric Le^x O-chain of *H. pylori* type strain.

units and this possibility seemed to be confirmed with the detection of periodate-resistant residues in the product from Smith degradation (*vide infra*). Indeed, in the following sections that deal with the structure of other *H. pylori* LPSs, a difucosylated GlcNAc antigen, α-L-Fuc-(1 → 3)-α-L-Fuc-(1→ 4)-β-D-GlcNAc, is described.

The FAB-MS spectrum of the methylated LPS indicated the presence, at the nonreducing end, of a trisaccharide similar to the human blood-group antigen Lewis X (Le^x) [*m/z* 638 → 432(638–206)], β-D-Gal-(1 → 4)-[α-L-Fuc-(1 → 3)]-β-D-GlcNAc-(1 → (Fig. 4). Higher *m/z* ions (Fig. 4) showed a polymeric O-chain composed of internal Le^x repeats (fucosylated polyLacNAc) with some randomly Fuc-free *N*-acetyl-lactosamine [LacNAc; β-D-Gal-(1 → 4)-β-D-GlcNAc-(1 →] blocks.

More detailed information for the distribution of linkages in the branched polyLacNAc O-chain was obtained from two series of degradations. The Smith degradation sequence yielded an essentially linear fucose-free polyLacNAc, β-D-GlcNAc-[→ 3)-β-D-Gal-(1 → 4)-β-D-GlcNAc-(1-]$_n$→, arising from the O-chain region of the LPS. A minor component of low M_r was obtained when the material from the Smith degradation was fractionated by GPC on Bio-Gel. Hydrolysis of this component gave Glc and threitol in equimolar amounts, and the compound was characterized as 2-*O*-β-D-glucopyranosyl-D-threitol. The significance of this degradation product within the LPS structure did not become apparent until after the inner regions of the lowM_r SR-form LPS had been examined.

The second degradative approach was by the action of *Bacteroides fragilis* endo-β-D-galactosidase. The specificity of this enzyme in the depolymerization of incompletely fucosylated polyLacNAc glycans, whereby cleavage takes place at other than terminal galactosyl linkages to non-fucosylated GlcNAc residues,[73] led to the formation of two categories of oligosaccharides (Fig. 5): (*a*) those containing a nonreducing terminal α-D-Gal from a single glycosyl cleavage and (*b*) those containing a nonreducing β-D-GlcNAc end-group resulting from internal regions of the chain by cleavage at linkages to two unbranched β-D-GlcNAc residues.

The results of these experiments are summarized in Fig. 5 and the structures of oligosaccharides **1**–**6,** even when in admixture, were unambiguously assigned. Disaccharide 1 and tetrasaccharide 3 arose from unbranched internal GlcNAc-Gal

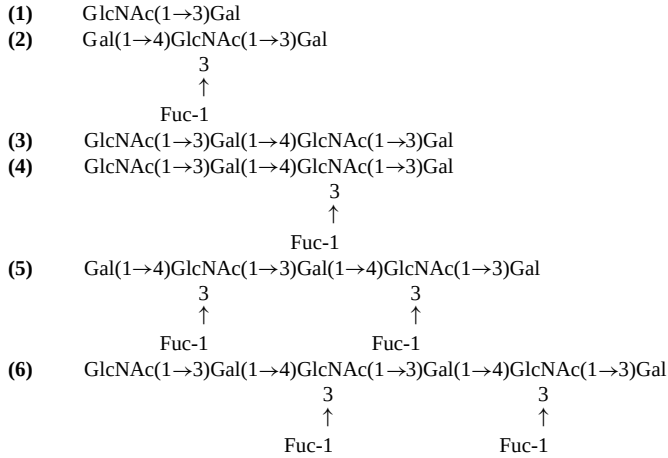


FIG. 5. Glycans from degradation of *H. pylori* type strain high M_r LPS with endo-β-D-galactosidase.

segments without Fuc side chains, and Fuc-containing pentasaccharide 4 and octasaccharide 6 derive from branched internal regions. Tetrasaccharide 2 and heptasaccharide 5 were assigned to nonreducing terminal sequences that incorporate monomeric and dimeric Le^x determinants.

The most striking conclusion from the results of this part of the investigation was the recognition of bacterial components closely resembling in structure the extended oligomeric type-2 Le^x chains found in fucosylated polyLacNAc glycan conjugates[74] and glycolipids,[75] and in many adenocarcinomas[76] with fucosylated type-2 chains. Polymeric type-2 chains $[\rightarrow 3)\beta$-D-Gal$(1 \rightarrow 4)$ β-D-GlcNAc$(1 \rightarrow]_n$ are mainly found in adenocarcinoma cells, whereas type-1 $[\beta$-D-Gal$(1 \rightarrow 3)\beta$-D-GlcNAc$(1 \rightarrow]$ and 2 $[\beta$-D-Gal$(1 \rightarrow 4)\beta$-D-GlcNAc$(1 \rightarrow]$ *N*-acetyl-lactosaminoglycan monomeric units are mostly encountered in normal human erythrocytes.[77] ELISA and immunoblot experiments, using intact *H. pylori* type strain whole cells and intact LPS, with several Le^x mAbs showed strong positive reactions, thus underlining the results obtained by chemical analyses. Evidence for the connection of the repetitive structure in the O-chains to the LPS core emerged from studies on SR-form LPS.

Since SR-LPS of *H. pylori* NCTC 11637 isolated from phenol–water extraction was partially water-insoluble, and could be isolated as a pellet by high-speed centrifugation, detailed studies of core OS structure were carried out on material obtained after treatment with aqueous acetic acid under standard conditions. Compositional analysis of the liberated OS crude preparation showed the presence of significant amounts of sugar constituents from O-chains in addition to Hep and Glc core units. Separation of the OS preparation by GPC on Bio-Gel afforded in

[P→7]LαDHep(1→5)Kdo

LαDHep(1→3)[P→7]LαDHep(1→5)Kdo

DαDHep(1→2)LαDHep(1→3)[P→7]LαDHep(1→5)Kdo

αDGlc(1→4)βDGal(1→7)DαDHep(1→2)LαDHep(1→3)[P→7]LαDHep(1→5)Kdo

FIG. 6. Core OS molecules present in fraction OS-3 showing the progressive addition of core units.

succession three fractions of which that of lowest M_r, OS-3, had a composition showing an absence of O-chain constituents. The first two fractions OS-1 and OS-2 were less completely resolved, but were sufficiently different, in extent of O-chain sugar constituents, to justify separate examination. Parallel studies of the fractions involved ^{1}H and ^{31}P NMR of the parent OS, FAB-MS of methylated derivatives, and accompanying linkage analyses.

The lowest M_r fraction, OS-3, was in fact a mixture of core-related linear molecules (Fig. 6) containing a minimal disaccharide composed of the innermost LD-Hep (phosphorylated) and its neighboring Kdo residue, and three more extended glycans that allowed for the determination of the core OS backbone. The backbone of the core OS of *H. pylori* type strain was composed of a phosphorylated (monoester phosphate) hexasaccharide in which the typical core sugars, LD-Hep, Glc, and Gal, were present, but, in addition, this core OS also possessed the less common DDHep as a constituent.

In addition to those in OS-3, compositional analysis of OS-2 showed the presence of Fuc, GlcNAc, a second DD-Hep and, on average, less than one extra Gal, and ~2 further Glc residues per chain; linkage analysis showed that these hexose residues were distributed between four locations. Lack of uniformity in the placing of the additional Gal and Glc residues was indicated by the less than stoichiometric proportions of methylated sugar derivatives in linkage analysis, which showed the introduction of the new structural units and the creation of new branch points in OS-2. The locations of the chain extensions were assessed from variations in the fragment ions shown by FAB-MS, and Fig. 7 shows the proposed structure for OS-2 with an interpretation of the origins of the newly observed fragment ions in the FAB-MS of the methylated derivative. With the known structural units of OS-2 the ion at *m/z* 682 was of unambiguous structure, Fuc-(1 → 3)-GlcNAc-(1 → 7)-DD-Hep, and the other glycosyloxonium ions could only have originated from cleavage at Hep residues. It is suggested that the ready formation and presumed relative stability of these ions may be a consequence of the anomeric effect of sugar residues having the α-D-*manno* configuration. In agreement with the detection of fragment ions at *m/z* 682 and 886, linkage analysis showed the addition of single Gal end groups to some of the GlcNAc residues with the formation of a Le^x terminus. Linkage analysis of OS-2 showed that both 3- and 6-linked Glc residues were present,

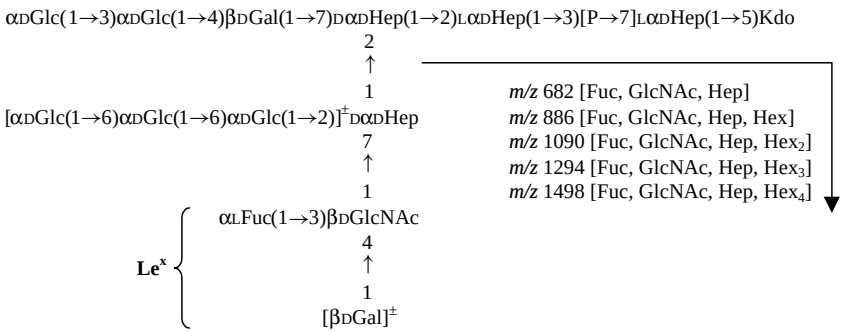


FIG. 7. Structure of molecule OS-2 with *m/z* ions from FAB-MS of methylated derivative. In some OS-2 molecules, the structure GlcNAc-(1 → 3)-Fuc (1 → 7)-DD-Hep-(1 → core may also be present.

and indeed a 2D COSY and 2D TOCSY experiments indicated the presence of five anomeric signals with the α-D-Glc configuration, but of unequal intensity.

In the light of the earlier observation that 2-*O*-α-D-glucopyranosyl-D-threitol was isolated from Smith degradation of S-form LPS, it is proposed that these differently linked α-D-Glc residues may be assigned to specific locations. Only one type of Glc residue would be resistant to periodate oxidation and the formation of threitol could only arise from a 4-linked Gal residue in the core OS after chain extension with an extra α-D-Glc residue attached by a (1 → 3) linkage [α-Glc-(1 → 3)-α-Glc-(1 → 4)-β-Gal-(1 → inner core]. It is, therefore, implied that the O-2 of approximately 50% of the outer DD-Hep residues is the site of attachment of an α-D-Glc residue and on some chains up to two further α-D-Glc residues in (1 → 6) linkages. ES-MS of permethylated OS-2 showed a series of pseudomolecular ions corresponding to the above-mentioned OSs together with those of compositions of Fuc, Hex_5, GlcNAc, Hep_4, Kdo, and Fuc, Hex_6, GlcNAc, Hep_4, Kdo, in a further demonstration of the size heterogeneity of OS-2.

Linkage analysis data for OS-1 indicated the presence a more extended Le^x O-chain than that present in OS-2, and hence resembling intact S-form LPS. FAB-MS of the permethylated OS-1 showed fragment ions derived from terminal sequences of dimeric Le^x blocks. A noteworthy feature was the absence of 6-linked α-D-Glc residues; this dextran side chain was also absent in the high-M_r S-form LPS. Interestingly, in LPS knockout mutants, this glucan reappears and becomes one of the prominent moieties of *H. pylori* isogenic mutants with truncated LPSs lacking Lewis O-chains.

The preceding experiments performed on the OSs liberated from SR-form LPS provided essential evidence for the connection of the polymeric Le^x O-chain to the inner core segment through an O-chain—β-D-GlcNAc-(1 → 7)-DD-Hep—core connection. Confirmation of this aspect of structure was sought by degradation of the high-M_r S-LPS. A more extended controlled treatment of LPS with aqueous

αDGlc(1→3)αDGlc(1→4)βDGal(1→7)DαDHep(1→2)LαDHep(1→3)[P→7]LαDHep(1→5)Kdo
2
↑
1
DαDHep
7
↑
1
β-DGlcNAc

FIG. 8. Structure of the core-related oligosaccharide (OS-4) that originated from chemical and enzymatic degradation of S-form LPS from *H. pylori* type strain. OS-4 is composed of the core OS and one GlcNAc unit from the Le^x O-chain region.

acid resulted in complete defucosylation, in addition to cleavage of the keto-sidic linkage of Kdo to lipid A. Complete depolymerization of these unbranched polyLacNAc chains was achieved on treatment with endo-β-galactosidase to give the previously characterized disaccharide 1 (Fig. 5) and a core-related OS, OS-4 (Fig. 8). Analysis of OS-4 showed the presence of a single GlcNAc residue as a nonreducing terminus of the O-chain, together with most of the residues from the inner region of the S-form LPS that were present in OS-2. This terminal GlcNAc unit was connected to the 7-substituted DDHep residue [*m/z* 260 ($GlcNAc^+$) and 508 ($GlcNAc\text{-}Hep^+$) in FAB-MS]. However, a notable difference was the absence of the 6-linked α-D-Glc residues attached to the outer of the two DD-Hep units as observed in high-M_r molecules from S-form LPS and OS-1.

The LPS of *H. pylori* type strain was shown to contain a partially fucosylated type-2 polyLacNAc O-chain (polymeric Le^x/LacNAc) of 8 to 10 disaccharide repeating units, where approximately half the GlcNAc units carried a Fuc residue. This O-chain was terminated by a Le^x epitope (Fig. 9). The sugar constituents LD-Hep, Glc, Gal, Kdo, and the less common DD-Hep made up the core OS region which was shown to be covalently attached to the polymeric Le^x/LacNAc O-chain domain by a GlcNAc → DD-Hep glycosidic linkage. Figure 9 shows the complete LPS molecule of the first examined *H. pylori* strain from the O-chain termini to the Kdo of the inner core oligosaccharide.

These pioneering investigations on *H. pylori* LPS were undertaken within the framework of the classical model for LPS structure. This structural model is based on a regular O-chain, synthesized by polymerization of OS repeating blocks, assembled on a lipid carrier and transferred to a nonrepetitive core OS linked via glyculosonic acid residues, most commonly Kdo, to the endotoxic component, lipid A. The results presented in this section on the structure of the LPS from the first *H. pylori* strain examined, comprising the O-chain and the intervening and core OS regions, may be considered in terms of the structural proposal shown in Fig. 9. The O-chains of the LPS were shown to consist of partially fucosylated type-2 polyLacNAc chains with 4-substituted GlcNAc residues in the backbone

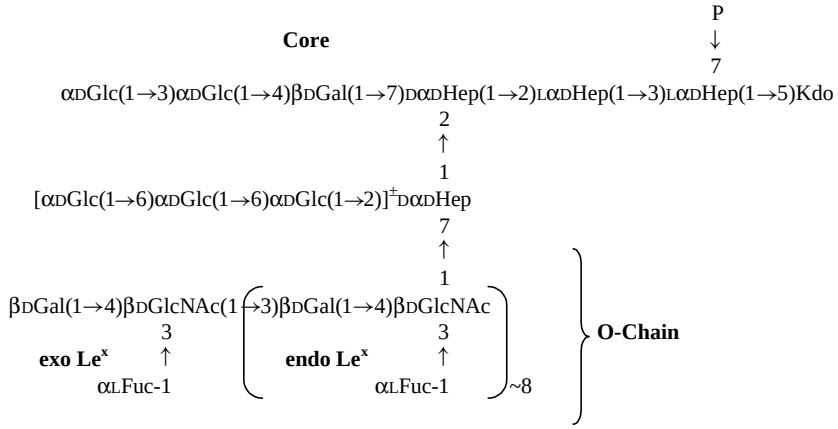


FIG. 9. The structure of the complete core and O-chain regions of the *H. pylori* type strain LPS showing the polymeric Le^x O-chain.

and to approximately half of which α-L-Fuc residues were attached at O-3 forming structures analogous to the histo-blood group determinant, Le^x. From the FAB-MS experiments on the products arising from the action of endo-β-galactosidase on LPS, there was evidence for monomeric and dimeric Le^x units at the chain terminus, and, from the FAB-MS of methylated S-form LPS, fragment ions were observed for trimeric Le^x units.

The inner regions of the LPS structure were delineated by the characterization of OS fractions liberated from the presumed incomplete SR-LPS. A core OS fraction representing the region to which the O-chain might be directly linked was isolated. Chromatographic separation of this core OS mixture gave three fractions. Each fraction OS displayed natural microheterogeneity, but they differed sufficiently from each other in overall structure to provide evidence for the progression of glycosylations leading to the assembly of the mature LPS. Thus, the phosphorylated hexasaccharide, OS-3 (Fig. 6), defined the inner core OS region. The next higher M_r fraction, OS-2 (Fig. 7), with a micro-heterogeneous collection of molecules, pointed to three types of chain extension: (*i*) an extension of the backbone core OS by attachment of a second α-D-Glc residue in a $(1 \rightarrow 3)$ linkage, (*ii*) the introduction of branching from the DD-Hep residue in the inner core through a second such residue to which the first Le^x unit was to be developed, and (*iii*) in some chains yet further branching in a sequence of up to three α-D-Glc residues in $(1 \rightarrow 6)$ linkages; and the OS-1 fraction of highest M_r showed the development of the O-chain structure with up to two Le^x/LacNAc repeating units. However, in OS-1, as in the complete S-form LPS, the 6-linked α-D-Glc residues were no longer present, and their peculiar role in the LPS assembly is not yet known.

Complementary evidence from controlled degradation of the S-form LPS to give the core-associated glycan OS-4 (Fig. 8) confirmed the proposed connection of the single remaining GlcNAc residue in O-chain via an intervening DD-Hep residue to the inner core. In the complete LPS structure (Fig. 9), present estimates suggest that there are at least nine Le^x/LacNAc repeating units in this O-chain. The detailed distribution of Fuc residues is not known, but indications are that fucosylation of GlcNAc in *H. pylori* seems to be an unsystematic process.

The most significant conclusion from these initial investigations concerns the elaboration by *H. pylori* of LPS with oligomeric Le^x epitopes in mimicry of structures expressed mainly on glycolipids in certain human cell surface glycoconjugates. Although these tumor-associated polymeric carbohydrate antigens are found on normal cells, e.g., on granulocytes, their abundant expression is limited to malignant cells. Nevertheless, Le^x epitopes are expressed in normal gastric tissue, which thus poses the question as to whether mimicry of this blood-group antigen by *H. pylori* in the gastric mucosa may camouflage the bacterium from the host and thus aid survival of *H. pylori* in that environment. Conversely, the presence of antibodies against *H. pylori* in patients has been found to strongly correlate with the presence of autoantibodies against human antral gastric mucosa.[78] Since antibodies cross-reacting with the gastric mucosa have been demonstrated in mice immunized with *H. pylori*, the expression of Le^x-like epitopes on the surface of the bacterium may play a role in the development of an autoimmune reaction contributing to disease. The further implications of these surface structures in interaction between the bacterium and the human host will require biological experiments with chemically defined LPSs.

III. The Lewis Y and Sialyl Lewis X in *Helicobacter pylori* Lipopolysaccharides

As structural studies on the LPS of the *H. pylori* type strain were proceeding, and the first evidence for O-chains expressing structures analogous to the type-2 Le^x blood-group was developing, Boren *et al*.[79] reported differences in the binding of *H. pylori* strains to human gastric mucosal cells carrying the type-1 Le^b antigen. The binding of one such strain, *H. pylori* P466, from a patient with dyspeptic syndrome, to gastric mucosa was inhibited by a neoglycoconjugate carrying the Le^b antigen, whereas another *H. pylori* strain, MO19, from an asymptomatic patient, did not bind to gastric mucosa. Although LPS was not implicated in the interaction of this *H. pylori* strain with gastric mucosa, and indeed a bacterial cell-surface adhesin, *babA*,[80] has been implicated in this adhesion, these observations prompted a comparative examination of LPS from the P466 *H. pylori* strain. This section describes the expression of type-2 Le^y and Sialyl Le^x blood-groups by the LPS of *H. pylori* P466.[81,82]

The structure of the LPS from *H. pylori* type strain (preceding section) served as a reference point, and the strategies employed in its elucidation were to provide the guidelines in the examination of LPS from other *H. pylori* strains. With no formal distinction between R- and S-form LPS of strain P466, bacterial cell extracts were divided into soluble high-M_r LPS, with extended outer O-chains, and partially soluble low-M_r LPS. OS fractions comprising inner core and developing O-chains were liberated and separated by GPC for detailed study. With considerable microheterogeneity in each of these fractions, compositional and linkage analysis gave only average values for constituent residues. Assuming that smaller molecules comprise the first steps in the progressive development of the mature LPS, as with type strain LPS, the evidence for these defined components could be turned to advantage in the elucidation of the structure of the complete molecule.

Water-soluble high-M_r LPS was examined directly without cleavage from lipid A in order to avoid cleavage of any relatively acid-sensitive fucopyranosyl linkages. The main constituents were L-Fuc, D-Gal, and D-GlcNAc, with smaller amounts (~2 molar proportions) of Glc, DD-Hep, and LD-Hep. The identities and anomeric configurations of the principal sugar residues were defined as α-Fuc, β-Gal, and β-GlcNAc. Linkage analysis performed on the P466 intact LPS showed the presence of all the sugar linkage units previously observed in *H. pylori* type strain, but, in addition, 2-substituted Gal was also detected. The significance of the 2-substituted Gal residue arising from a Fuc-(1 → 2)-Gal unit was seen in the FAB-MS spectrum of the permethylated LPS that showed a fragment ion at *m/z* 812 of composition Fuc_2, Gal, GlcNAc which could have arisen from either a type-1 Le^b or a type-2 Le^y determinant, but the detection of a secondary fragment ion at *m/z* 606, from β-elimination of a terminal Fuc residue (206 amu) from O-3 of GlcNAc, and not at *m/z* 402, pointed to a terminal Le^y blood-group determinant (Fig. 10). Fragment ions of higher *m/z* were observed with increments of Fuc, Gal, GlcNAc, with secondary ions observed from loss of a terminal Fuc residue indicating a regular chain extension of Le^x units in a type-2 fucosylated polyLacNAc chain. The presence of both Le^y and Le^x was also confirmed serologically (ELISA and immunoblot) employing mAbs specific for these blood-group antigens.

For confirmation that the polyLacNAc backbone consisted of regular Gal-(1 → 4)-GlcNAc repeating units, the soluble high-M_r LPS was heated with aqueous 5% acetic acid in which cleavage from lipid A was accompanied by complete defucosylation. 3-Substituted Gal and 4-substituted GlcNAc residues were the main units of the final product with no detectable 3-linked GlcNAc residues. The proportion of Gal end groups indicated an average chain length of 4–5 disaccharide units. FAB-MS supported a regularly repeating structure for the polyLacNAc backbone chain of the O-chain region of the P466 LPS with a series of glycosyloxonium ions at *m/z* 464 [Gal-GlcNAc], 913 $[Gal\text{-}GlcNAc]_2$, 1362 $[Gal\text{-}GlcNAc]_3$, 1811 $[Gal\text{-}GlcNAc]_4$, and 2260 $[Gal\text{-}GlcNAc]_5$from preferential cleavage at GlcNAc residues.

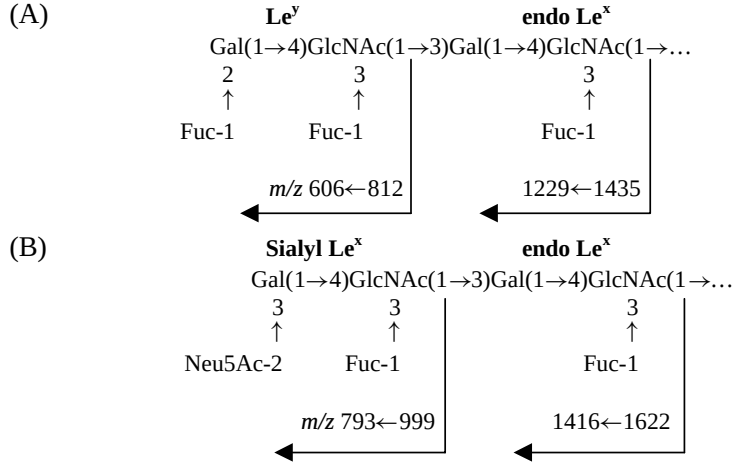


FIG. 10. The O-chain of *H. pylori* strain P466 carrying a type-2 Le^y (A), or a type-2 sialyl Le^x (B) epitope at the nonreducing terminus of a Le^x O-chain.

Treatment of the water-insoluble P466 LPS preparation in acetate buffer at pH 6.5, with cleavage of the ketosidic linkage to lipid A, gave a mixture of OSs which was separated by GPC to give a series of fractions (OS-1, OS-2, OS-3) or subfractions. Molecule OS-3 gave qualitatively similar analyses for composition and linkage types to the linear OS-3 glycan from *H. pylori* type strain (Fig. 5). Sugar constituents of the "Lewis O-chain" region, notably Fuc and GlcNAc, were absent. OS-3 contained D-Glc, D-Gal, DD-Hep, and LD-Hep and chemical and spectroscopic data revealed that P466 OS-3 was analogous to the phosphorylated core backbone OS-3 from the type strain (Fig. 5): α-D-Glc-(1 → 4)-β-D-Gal-(1 → 7)-D-α-D-Hep-(1 → 2)-L-α-D-Hep-(1 → 3)-[P → 7]-L-α-D-Hep-(1 → 5)-Kdo. Compositional analysis and ^{1}H NMR for OS-2 pointed to an average incremental addition to the core OS-3 region of 2 Fuc, 1 Glc, 2 Gal, 2 GlcNAc, and 1 DD-Hep. Using the structure of the OS-2 fraction from the type strain as a guide (Fig. 6), linkage analysis showed that the increment could correspond to a core extension by a single 3-substituted Glc residue (in the core OS backbone), and the addition, as a side chain to the DD-Hep in the core, of a second DD-Hep residue (7-substituted) from which O-chain development would ensue with the attachment of up to 3 Le^x and 1 Le^y units. Glycan P466 OS-2 had a structure similar to that of OS-2 from the type strain (Fig. 7), with the exception of the absence of the side-antenna dextran. In fraction P466 OS-1, extension of the growing Lewis O-chain approached that in the complete water-soluble high-M_r LPS.

The LPS just discussed of *H. pylori* P466 possessed type-2 polymeric Le^x O-chains of 4 to 5 repeat units, but instead of being completed at the nonreducing

end by the Le^x epitope, as in the *H. pylori* type strain LPS, the O-chain of P466 strain is terminated by the Le^y antigen (Fig. 10). The O-chain of the P466 strain was also shown to be covalently connected to the core OS region, which had the same structure as the core OS region from the *H. pylori* type strain (Fig. 6), through a similar GlcNAc-(1 → 7)-DD-Hep bridge.

Additional serological studies also showed a positive reaction between P466 LPS (from a separate growth) and a mAb (sLeX) specific for the sialyl Le^x epitope. This P466 LPS was then chemically analyzed to determine if sialyl Le^x was indeed present. Sugar composition analysis performed by the typical trifluoroacetic acid hydrolysis on this intact P466 LPS revealed the presence of the sugars found in the previous analyzed P466 LPS, and in addition, a sugar analysis specific for detection of neuraminic acid (methanolysis) indeed showed the presence of sialic acid. The intact P466 LPS was consequently methylated and its FAB-MS spectrum showed the characteristic ion of terminal neuraminic acid at *m/z* 376 and its correspondent secondary ion at *m/z* 344 [376 − 32(CH_3OH)]. Moreover, ions *m/z* 999 and 793 [999 − 206] represented the whole type-2 sialyl Le^x epitope {Neu5Ac-(2→3)-Gal-(1 → 4)[Fuc-(1 → 3)]-GlcNAc}. In addition to sialyl Le^x, terminal Le^x epitope [*m/z* 638→432], and traces of Le^y [*m/z* 812 → 606] were also observed. Thus, *H. pylori* P466 was also shown to have the ability to elaborate the cancer-associated sialyl Le^x antigen in its LPS. The sialyl Le^x was expressed simultaneously with Le^x and, in lesser amounts, the Le^y antigen. These Lewis blood-group termini were connected to the a core OS similar to that of the type strain.

The lower degree of chain elongation (4–5 repeats), the absence of 6-substituted Glc side chains, and the replacement at the nonreducing end of type-2 Le^y and sialyl Le^x epitope for Le^x are the marked differences in molecular structure from that of *H. pylori* type strain (Fig. 9). The production of Le^y and sialyl Le^x by *H. pylori* P466 shows a further ability, in addition to Le^x in type strain, of *H. pylori* to produce structures homologous to human blood-group antigens. Here, competition, environmental, and genetic factors for glycosylation of Gal in a type 2 LacNAc structure, fucosylation at O-2 for Le^y, and sialylation at O-3 for sialyl Le^x will dictate which LPS structure will dominate. Sialyl Le^x is frequently expressed on human cancer cells serving as ligands for E-selectin, and thus plays a role in tumor formation and in hematogenous metastasis of cancer.

IV. The Heptoglycan Domain in *Helicobacter pylori* Lipopolysaccharides

This section describes studies on the LPSs from *H. pylori* strains MO19,[79] and serogroups O : 6[69] and O : 3[69] that possess oligomers of DD-Hep (heptoglycan) between the core OS and the Lewis O-chain. In contrast to the P466 strain discussed in the preceding section, the MO19 strain did not bind to gastric mucosa expressing the Le^b antigen.[79] As studies on these LPSs proceeded, evidence was obtained for

the presence, as a major structural feature, of chains of 3-substituted DD-Hep, a rather rare sugar, which created a distinct LPS structural domain.

Analysis of the water-soluble LPSs of *H. pylori* strain MO19 and serostrain O : 6 showed the presence of 2 L-Fuc, 3 Gal, 1 GlcNAc, 4 Glc, 10 DD-Hep, and 2 LD-Hep. The ^{1}H NMR spectra of MO19 and O : 6[83] LPSs showed one dominant α-anomeric resonance at δ5.08 (unresolved doublet) which could be attributed to the overriding DD-Hep component detected in the chemical analysis. In addition to anomeric resonances from residues in the inner core oligosaccharides, equal intensity signals were observed for two αFuc, one βGlcNAc, and two αGal residues. Methylation linkage analysis for the MO19 and O : 6 LPSs showed derivatives from three regions of structure: (1) two terminal Fuc, and one each of 2-linked Gal, and 3,4-linked GlcNAc; (2) most prominently seven 3-substituted DD-Hep and three 6-substituted Glc residues, the locations of which will be considered later; and (3) those residues probably arising from a similarly linked core OS region to those in the type strain NCTC 11637 (Section II) and P466 (Section III) LPSs. In addition to the prominent 3-substituted DD-Hep units, two other structural units, not seen in the type strain or P466 LPSs, were a 2- and 6-substituted DD-Hep. The FAB-MS of methylated LPSs of MO19 and O : 6 showed that these LPSs carried a Le^y (*m/z* 812 $\rightarrow$ 606). In accordance with compositional and linkage analysis data, no other abundant ions were observed arising from an extended Lewis O-chain; the next ion in the chain extension at *m/z* 1016 showed an increment for a hexose residue, which was consequently identified as being a 3-substituted Gal. The most striking observation from the linkage analysis was the presence of multiple 3-substituted DD-Hep residues. Based on proton assignments from various NMR studies, especially a NOESY experiment that showed *inter*-residue connectivities from the anomeric H-1 to H-3 of the adjacent 3-linked DD-Hep, extended chains of DD-Hep residues were observed, -[$\rightarrow$3)-D-α-DHep-(1-]$_n\rightarrow$. 6-Substituted Glc residues were also prominent, but these data gave no direct information on their location in the overall structure. The water-soluble MO19 and O : 6 LPSs, without prior removal of the lipid A component, were submitted to a Smith degradation in which the reduction was performed with $NaBD_4$ and each afforded one PS fraction. These PSs had a composition of 1 mol of [$^2H_1$]erythritol, Gal, and GlcNAc and 7 mol of [$^2H_1$]Man. The NMR spectrum showed anomeric signals for β-Gal and β-GlcNAc, and a major anomeric signal for a sugar with the α-*manno* configuration as an unresolved doublet. Linkage analysis showed the presence of one terminal GlcNAc, one 3-substituted Gal, and about seven 3-substituted Man residues. The FAB-MS gave fragment ions for the terminal trisaccharide segment at *m/z* 260 [GlcNAc], *m/z* 464 [GlcNAc-Gal], and 669 [GlcNAc-Gal-Man-6-^{2}H], thereby defining the sequential connection of the GlcNAc residue of the Le^y epitope via the 3-linked Gal residue to the 3-linked DD-Hep oligomer. The erythritol moiety, from the proximal terminus of the Smith degradation product, could only arise from the oxidative–reductive degradation of a 6-substituted or a 2,7-disubstituted

DD-Hep residue. The following structure may therefore be proposed for the Smith degradation products of MO19 and O : 6 LPSs:

β-D-GlcNAc-(1→3)-β-D-Gal-(1-[→3)-α-D-Man-6-[^{2}H]-[(1-]$_{\sim 7}$→D-Erythritol,

and thus for the outer region of MO19 and O : 6 LPSs:

Le^y **Heptoglycan**

β-D-Gal-(1 → 4)-β-D-GlcNAc-(1 → 3)-β-D-Gal-(1-[→ 3)-D-α-D-Hep]$_{\sim 7}$(1 → 6/7)DD-Hep → ⋯

2 3
↑ ↑
α-L-Fuc-1 α-L-Fuc-1

The LPSs from *H. pylori* MO19 and O : 6 contained a single Le^y epitope at the nonreducing end of the LPS molecule which was attached through a Gal unit to a heptoglycan of 3-linked DD-Hep units. These LPSs were also shown to have an inner core OS attached to Kdo representing the reducing terminus of the PS molecule from the LPS (Fig. 11). The backbone of the LPS core OS from these

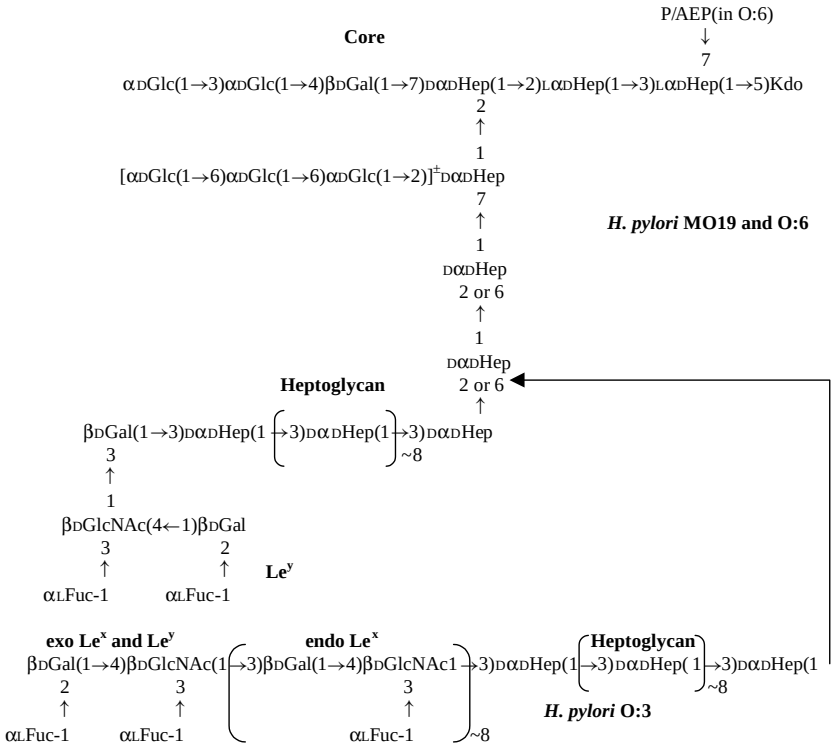


FIG. 11. The chemical structures of core OS, heptoglycan and O-chain regions of *H. pylori* strain MO19, and serostrains O : 6 and O : 3.

H. pylori were proven to be similar to those previously described for *H. pylori* type strain and P466 (Figs. 5 and 6).

Studies on another *H. pylori* strain, serostrain O : 3,[83] that also raised strain-specific antisera,[69] yielded the polymeric Le^x O-chain units terminated by a Le^x or Le^y, as previously found in the elongated O-chains from *H. pylori* type strain and P466, respectively. The LPS of *H. pylori* O : 3 was also shown to carry a heptoglycan domain similar to that observed in strains MO19 and O : 6. Hence, *H. pylori* O : 3 shared all the structural features present in strains previously discussed, namely, a polymeric fucosylated type-2 polyLacNAc O-chain and a heptoglycan region.

To deduce the nature of the connection between the O-chain and the heptoglycan oligomer in *H. pylori* O : 3, two degradations were performed. First, the O-chain was defucosylated with acetic acid to furnish a linear polyLacNAc chain. With the intention of reaching the first GlcNAc unit (reducing end) of the O-chain, the polyLacNAc was treated with endo-β-galactosidase. From this series of reactions one high-M_r product was obtained, and structural analyses of this molecule showed one terminal βGlcNAc unit, five 3-linked D-α-D-Hep residues, and other sugar derivatives from the core OS regions. The FAB-MS spectrum revealed ions at *m/z* 260 for GlcNAc, 508 for GlcNAc-Hep, 756 for GlcNAc-Hep-Hep, and 1004 for GlcNAc-Hep-Hep-Hep. These composite ions in combination with chemical analysis showed that the connection between the Lewis O-chain and the heptoglycan domain, in *H. pylori* O : 3, was through a GlcNAc residue rather than a Gal unit as in strains MO19 and O : 6. Thus, the degraded *H. pylori* O : 3 molecule had the following structure:

$$\beta\text{D-GlcNAc(1-[}\rightarrow 3)\text{D-}\alpha\text{D-Hep(1-]}_{\sim 5} \rightarrow \text{Core OS},$$

and hence for the outer part of the LPS the following structure is proposed:

exo Le^x and Le^y **endo Le^x** **Heptoglycan**

$$\begin{array}{lll} \beta\text{-D-Gal}(1\rightarrow 4)\beta\text{-D-GlcNAc}(1\text{-}[\rightarrow 3)\beta\text{-D-Gal}(1\rightarrow 4)\beta\text{-D-GlcNAc}(1\text{-}]_{\sim 7}\rightarrow[\rightarrow 3)\text{-D-}\alpha\text{-D-Hep}]_{\sim 5}(1\rightarrow \text{Core OS} \\ \begin{array}{c}2\\|\\1\\ \alpha\text{-L-Fuc}^{\pm}\end{array} & \begin{array}{c}3\\|\\1\\ \alpha\text{-L-Fuc}\end{array} & \begin{array}{c}3\\|\\1\\ \alpha\text{-L-Fuc}^{\pm}\end{array} \end{array}$$

H. pylori serogroup O : 3 contains an LPS structure that possesses both the polymeric Lewis O-chains, as encountered in *H. pylori* type strain and strain P466, and an intervening heptoglycan domain, a region shown to be present in *H. pylori* strains MO19 and O : 6.

The limited quantities available of LPS from MO19, O : 3 and O : 6 strains were insufficient to establish the formal connection of this outer heptoglycan to the core OS region. However, on the basis of linkage analysis, the following sugar residues, additional to those already placed in the outer O chain→heptoglycan and the inner core OS regions, remained to be accommodated. Two types of units were those of

α-D-Glc residues, a single 3-substituted Glc residue, which probably arose from extension of the outer region of core OS as in the P466 and type strain LPSs (Fig. 5), and 6-substituted Glc residues, probably mutually linked as in similar short chains in OS-2 from the type strain (Fig. 6). Three types of DD-Hep residues, additional to that in the inner core phosphorylated hexasaccharide and those in the 3-linked heptoglycan oligosaccharide, were units of 2-, 6-, and 2,7-substituted residues. The absence of 7-substituted DD-Hep residues in the MO19 and O : 6 LPSs, and the presence in less than 1 molar quantity in O : 3 LPS, implies that this residue in the inner core OSs occurs as one of two branched residues in the LPS (see bolded sugars below):

Glc → Glc → Gal → 7)-**DD-Hep-(1** → LD-Hep → LD-Hep[P] → Kdo

2

↑

1

$[Glc]_n$ → 2)-**DD-Hep**

7

↑

Heptoglycan

↑

Lewis O-chain

The other 2,7-linked unit is presumably that to which the 6-linked Glc residues are attached in the LPS (see foregoing illustration). Either this second branched unit or the 6-substituted DD-Hep residue could be the residue to which the whole outer chain is linked and gives rise to an erythritol terminus in the Smith degradation product of the MO19 and O : 6 LPSs. The known structural features of the MO19 and O : 6 LPSs are summarized in Fig. 11, which shows: (*a*) the single Le^y terminal epitope linked via a β-D-Gal residue to the heptoglycan domain; (*b*) a region showing three DD-Hep residues and three Glc residues; and (*c*) the inner core OS region extended by an additional Glc residue. The molecular structure of *H. pylori* O : 3 LPS can also be seen in Fig. 11 where the (*a*) outer region of the LPS is composed of an $Le^{x/y}$ terminated long Le^x O-chain connected directly by a GlcNAc to the heptoglycan, (*b*) a region showing three DD-Hep residues and approximately three Glc residues, and (*c*) the inner core OS region. Although the heptoglycan component, D-*glycero*-D-*manno*-heptose, is the biosynthetic presursor of L-*glycero*-D-*manno*-heptose in the biosynthesis of LPS molecules,[84] it is rarely encountered as a component of LPS molecular structures. *H. pylori* MO19, O : 6, and O : 3 LPSs contain in the heptoglycan domain an LPS structural region of a type not previously encountered in LPS structures.

The investigations carried out on *H. pylori* strains MO19 and serogroups O : 3 and O : 6 have revealed marked differences in molecular structures; these structures are shown in their entirety in Fig. 11. Strains MO19 and O : 6 lack the extended fucosylated polyLacNAc O chains but contain a heptoglycan oligomer of 3-linked DD-Hep units inserted between a terminal Le^y antigen and the core OS region. The single Le^y epitope in strains MO19 and O : 6 is attached to the heptoglycan domain via a Gal residue. These heptoglycans probably act as a biological arm in order to present the sole Lewis antigen to host molecules. Serostrain O : 3 showed a combination of the structural characteristics found in the other strains by carrying both the extended Lewis O-chain and the heptoglycan oligomer. However, in serogroup O : 3 the connection of the Lewis O chain to the heptoglycan was effected through a GlcNAc unit. Strains MO19, O : 6, and O : 3 were shown to have the same inner core OS region. The presence of the heptoglycan domain in the LPSs of these *H. pylori* strains represents an additional LPS region (O-chain → **heptoglycan** → core → lipid A) to those common in other bacteria (O-chain → core → lipid A).

V. Semi Rough-Form and Smooth-Form Lipopolysaccharides in *Helicobacter pylori:* The Ubiquitous Phase Variation

This section describes the expression of SR- and S-form LPSs by two *H. pylori* strains of significant importance in *H. pylori* research: strain 26695, whose complete genome sequence has been published[85] and is thus being explored for genes involved in LPS biosynthesis, and the Sydney mouse model strain,[86] which is now widely employed in animal models to study *H. pylori* pathogenesis.

Separately grown batches of *H. pylori* 26695 and Sydney strain cells were studied to detect if there were any major differences between LPS molecules derived from separate growths. A conscious effort was made to grow all bacterial cell batches under the same conditions (pH 7.3–7.5). Altogether, experimental evidence[82] from serological and chemical analyses showed that the LPSs of *H. pylori* 26695 and Sydney strain possessed a core OS similar to those found in other *H. pylori* strains (Fig. 6). However, the core OS of *H. pylori* Sydney strain was phosphorylated by a diester phosphate moiety, 2-aminoethyl phosphate, in place of the more common monoester phosphate. The core OS from these strains could carry either a short O-chain region (SR-form LPS) expressing a variety of single type-1 and type-2 Lewis-related epitopes (Table I), namely, a type-2 Le^y, a type-1 [α-D-Gal*p*-(1 → 3)-β-D-Gal*p*-(1 → 3)-β-D-Glc*p*NAc] and 2[α-D-Gal*p*-(1 → 3)-β-D-Gal*p*-(1 → 4)-β-D-Glc*p*NAc] linear B blood-group, a type-1 Lewis disaccharide [α-L-Fuc*p*-(1 → 4)-β-D-Glc*p*NAc], a type-1 fucosylated Lewis disaccharide [α-L-Fuc*p*-(1 → 3)-α-L-Fuc*p*-(1 → 4)-β-D-Glc*p*NAc] and in lesser amounts, LacNAc, Le^x, and GlcNAc. Alternatively, from separate growths, these LPSs were found to be able to produce an elongated type-2 Le^x O-chain (S-form LPS)

TABLE I

O-Chain Epitopes Present in SR-Form LPSs of *H. pylori* 26695 and Sydney Strain; Interpretation of *m/z* Ions in the FAB-Mass Spectrum of the Methylated Intact SR-Form LPSs

Primary *m/z* Ion	Secondary *m/z* Ion from β-Elimination or from β-Cleavage		Proposed Structure
260	228 (260 − 32)		$GlcNAc^+$
434	402 (434 − 32)	Lewis disaccharide	Fuc-(1 → 4)-$GlcNAc^+$
464	432 (464 − 32)	LacNAc	Gal-(1 → 4)-$GlcNAc^+$
508			GlcNAc-(1 → 3)-Hep^+
608	576 (608 − 32)	fucosylated Lewis disaccharide	Fuc-(1 → 3)-Fuc-(1 → 4)-$GlcNAc^+$
638	432 (638 − 206)	Lewis X	Gal-(1 → 4)-$GlcNAc^+$
	450 (638 − 288)		3 ↑ Fuc-1
668	636 (668 − 32)	Type-2 B blood-group	Gal-(1 → 3)-Gal-(1 → 4)-$GlcNAc^+$
668	228 (668 − 440)	Type-1 B blood-group	Gal-(1 → 3)-Gal-(1 → 3)-$GlcNAc^+$
682			Fuc-(1 → 4)-GlcNAc-(1 → 3)-Hep^+
			GlcNAc-(1 → 3)-Fuc-(1 → 3 or 7)-Hep^+
812	606 (812 − 206)	Lewis Y	Gal-(1 → 4)-$GlcNAc^+$ 2 ↑ Fuc-1, 3 ↑ Fuc-1
856			Fuc-(1 → 3)-Fuc-(1 → 4)-GlcNAc-(1 → 3)-Hep^+

terminated mainly by a type-2 Le^x epitope. The coexpression of the blood-groups described above and the production of SR- and S-form LPSs reflects a high degree of phase variation within *H. pylori* LPS molecules.

The communally accessible complete genome sequence of *H. pylori* 26695 is shaping investigations dealing with the characterization and role of genes in *H. pylori* pathogenesis, including those responsible for LPS biosynthesis. Knowledge about the structure of 26695 LPS will facilitate the identification of "active" genes involved in sugar syntheses and in sugar glycosylations. Although some genes involved in sugar assembly mechanisms may be present in *H. pylori* genomes, it does not necessarily imply that they are expressed constantly *in vitro* by their LPSs. For example, in the *H. pylori* 26695 and J99[87] genomes, a gene postulated to be linked with the biosynthesis of the sugar nucleotide of sialic acid was characterized; however, the results obtained showed that sialic acid was not a constituent of the 26695[82] or J99[82] LPS molecules. The absence of sialic acid may be due to *in vitro* growth conditions and, conceivably, it may be that *in vivo* sialic acid is an LPS component; however, the presence of Le^y in *H. pylori* 26695

LPSs would impede the fabrication of any terminal sialyl Le^x epitope. Sialyl Le^x was observed to be a member of *H. pylori* P466 LPS[82] (Section III); thus the full mechanism for the production of this antigen in *H. pylori* must, in some strains, be in place. These results indicated that at any time during LPS biosynthesis, a mixture of Lewis glycoforms might be present as O-chain members (Table I). The presence of incomplete Le^x and Le^y antigens imply that the building of Lewis determinants in *H. pylori* takes place through a sugar-by-sugar addition and not by a block-by-block synthesis as found in other enteric bacteria. In *H. pylori* there must be genes involved in the biosynthesis of Le^x and Le^y, those being fucosyltransferases (FucTs), that place a terminal α-L-Fuc*p* on O-2 of a terminal β-D-Gal*p* and on O-3 of β-D-Glc*p*NAc, and the galactosyltransferase (GalT) that adds β-D-Gal*p* to O-4 of β-D-Glc*p*NAc. The presence of the Le^x determinant in *H. pylori* 26695 LPS suggests that the $(1 \rightarrow 3)$-α-fucosyltransferase may act prior to fucosylation at O-2 of β-D-Gal in the fabrication of Le^y in *H. pylori* LPS biosynthesis. The presence of Le^y impedes the creation of sialyl Le^x since both epitopes use the terminal βGal residue as the acceptor for fucosylation and sialylation, respectively. The difucosyl antigen, α-Fuc-(1-3)-α-Fuc, found in *H. pylori* is uncommon, but it has been found in certain oligosaccharides-alditols.[88] The presence of this difucosylated antigen may vary from strain to strain depending on the degree of activities of the glycosyltransferases involved in its biosynthesis. This same argument is also true for other Lewis-related structures expressed by *H. pylori* LPSs, in that, even within the same *H. pylori* strain, the degree of fucosylation [Fuc-$(1 \rightarrow 2)$-Gal, Fuc-$(1 \rightarrow 3)$-GlcNAc, and Fuc-$(1 \rightarrow 4)$-GlcNAc] varies between separate bacterial cell growths in which similar conditions were employed, which implies that fucosylation of any specific LacNAc region is not an imperative event.

The switch between SR-form LPS (Lewis $\rightarrow$ core $\rightarrow$ lipid A) and S-from LPS [$(\text{Lewis})_n \rightarrow$ core $\rightarrow$ lipid A] in *H. pylori* is most likely dependent on the activity of two glycosyltransferases: first, the LacNAc GalT that places a β-D-Gal at O-4 of β-D-GlcNAc; premature fucosylation of GlcNAc at either O-4 [Fuc-$(1 \rightarrow 4)$-GlcNAc] or O-3 [Fuc-$(1 \rightarrow 3)$-GlcNAc] may hamper galactosylation of GlcNAc, thus stopping O-chain (polyLacNAc) progression; and secondly, in the same manner, fucosylation at O-2 of β-D-Gal to create a Le^y epitope will halt O-chain extension since the β-D-Gal unit may no longer be able to be glycosylated at O-3 by β-D-GlcNAc. This would explain the structural differences observed, in that the short O-chains of SR-form LPSs, of both *H. pylori* 26695 and SS1, carried predominantly the Le^y epitope and the fucosylated GlcNAc antigens, in contrast to the longer O-chains of S-form LPSs that expressed a low concentration of Le^y and carried mostly LacNAc and Le^x at the nonreducing terminus. *H. pylori* P466, which carried Le^y and sialyl Le^x at the nonreducing terminus, also had a lower degree of O-chain extension than O-chains terminated by Le^x (type strain). Therefore, it would seem that there is a direct relationship between the degree

of (1 → 2)-α-fucosyltransferase activity and Le^y production and consequently the length of Lewis O-chains. These results suggest that low-M_r SR form LPSs are more likely to express Le^y than their high-M_r S-form LPS counterparts and that, presumably, fucosylation takes place on a completed linear polyLacNAc to afford a Le^x O-chain. Intrastrain variation in the expression of long O-chains of two strains of *H. pylori* has also been observed by silver staining in SDS-PAGE and by immunobloting with homologous antisera.[89] The factors controlling the length of Le^x O-chain polysaccharide may be various, from environmental conditions to gene regulation, where all these factors may be interdependent. It is therefore very clear that at any time in *H. pylori* biosynthesis many LPS forms might be present in different concentrations. This intra-LPS variation, within the same *H. pylori* strain, carries certain implications with regard to the study of LPS isogenic mutants of *H. pylori,* in that the evaluation of LPS knockout mutants by SDS-PAGE may not be sufficient to determine the outcome of the mutation, because, at any time, the LPS of the parent strain may change from a S-form to a SR-form.

As an assurance, sugar composition analyses were performed on intact whole cells of *H. pylori* 26695 and Sydney strain, and no sugar components, other than those belonging to the LPS molecules, were detected that would have originated from a non-LPS glycan, thus ruling out the presence of any non-LPS polysaccharide from *in vitro* grown cells (such as a CPS) in these strains. It is also worth noting that at this early stage of *Helicobacter* research, and because of the massive general circulation, one must ensure the correct identity of any strain, especially important strains such as the type strain and mouse model strains. For example, two unrelated versions of the reference *H. pylori* type strain NCTC 11637 have been shown to be in common usage and we have also detected two different types of LPSs, each from two sources (see Table III).

H. pylori Sydney strain is often used in studies of *H. pylori* pathogenesis in mouse models. The side-branch (1 → 6)-α-glucan typically present in the core OS of some *H. pylori* strains (Fig. 6) was absent in the LPS of Sydney strain. The core of *H. pylori* Sydney strain carried a 2-aminoethyl phosphate in the innermost LD-Hep residue, instead of the more common monoester phosphate. These results do not clarify the role of LPS in the ability of *H. pylori* Sydney strain to colonize the gastric mucosa of mice. Besides, other *H. pylori* strains that were able to successfully colonize mice or primates have been reported in the literature.[90–92] However, one may raise the following questions: Which type of LPS (SR-form LPS with a sole Lewis antigen or an S-form LPS with a polymeric Lewis O-chain) is most prominent in *in vivo* colonization and in adherence mechanisms? And which LPS-forms and antigens take part in *H. pylori* pathogenesis? These same arguments also need to be addressed when discussing the behavior of *H. pylori* in humans. A plausible prospect may be that the LPS-form and fine structure may change during and/or after colonization of the host's mucosa because of particular host factors and/or the stage of pathogenesis.

VI. Type-1 Histo-Blood Groups in *Helicobacter pylori* Lipopolysaccharides and Their Prevalence in Asian Strains: The Lewis A, B, D, and Blood-Group A Determinants

After the characterization of structures analogous to type-2 Le^x and Le^y blood-group antigens in *H. pylori* LPSs, a series of serological investigations, using commercially available histo-blood group mAbs, looked for the presence of blood-group determinants in a wide range of *H. pylori* strains.[93,94] In addition to confirming the presence of Le^x and Le^y in a large number of Western (North America and Europe) *H. pylori* strains (>80%), these serological studies also hinted at the presence of type-1 blood-group antigens Le^a and Le^b in a small number of strains. Detailed chemical analyses were then performed on some strains suspected of carrying type-1 Le^a and Le^b. This section describes structural studies revealing that *H. pylori* strains may possess LPSs with structural homology to type-1 Le^a $\{\beta$-D-Gal*p*-$(1 \rightarrow 3)$-$[\alpha$-L-Fuc*p*-$(1 \rightarrow 4)]$-β-D-Glc*p*NAc$\}$ and Le^b $\{\alpha$-L-Fuc*p*-$(1 \rightarrow 2)$-β-D-Gal*p*-$(1 \rightarrow 3)$-$[\alpha$-L-Fuc*p*-$(1 \rightarrow 4)]$-β-D-Glc*p*NAc$\}$ human blood-group antigens, and that they may carry them simultaneously with type-2 Le^x and Le^y blood-group determinants. The expression of the blood-group A epitope $\{\alpha$-D-Gal*p*NAc-$(1 \rightarrow 3)$-$[\alpha$-L-Fuc*p*-$(1 \rightarrow 2)]$-β-D-Gal*p*$\}$ by *H. pylori* LPS and the propensity of *H. pylori* LPSs isolated from Asian hosts to produce type-1 histo-blood-group antigens are also noted.

Several *H. pylori* strains[95] were determined to simultaneously express type-2 Le^x and type-1 Le^a structures in their LPS O-chain regions (Table II). With complementary data from chemical and spectroscopic analyses, the FAB-MS spectra of the same methylated LPS preparation yielded two β-elimination secondary ions, those being *m/z* 432 [638 − 206(FucOH)] as we have previously seen for type-2 Le^x, and *m/z* 402 [638 − 236(GalOH)] which characterized the type-1 Le^a determinant (Table II). Another Lewis blood-group trisaccharide comprising Fuc, Gal, and GlcNAc, the type-1 Le^d $\{\alpha$-L-Fuc*p*-$(1 \rightarrow 2)$-β-D-Gal*p*-$(1 \rightarrow 3)$-β-D-Glc*p*NAc$\}$, also known as H-type-1, was also shown to be an LPS component of *H. pylori* $\{$*m/z* $638 \rightarrow 228$ [638 − 410(Fuc-GalOH)] (Table II). The structural tetrasaccharide isomers, type-2 Le^y and type-1 Le^b $\{$*m/z* $812 \rightarrow 402$ [812 − 410(Fuc-GalOH)] in FAB-MS$\}$ were also coexpressed in some *H. pylori* strains (Table II).

Just as the majority of *H. pylori* strains possessed LPSs with only type-2 Lewis antigens [Le^x, Le^y, and LacNAc], a class of strains was also observed to carry strictly type-1 Lewis determinants. These *H. pylori* strains did not express any type-2 Lewis antigens, but furnished solely type-1 Le^a, Le^d, and Le^b (Table II). Of particular interest, some *H. pylori* strains, which followed the paradigm of other strains in producing Lewis blood-group antigens, also elaborated LPSs structures analogous to the blood-group A family $\{\alpha$-D-Gal*p*NAc-$(1 \rightarrow 3)$-$[\alpha$-L-Fuc*p*-$(1 \rightarrow 2)]$-β-D-Gal*p*$\}$. The FAB-MS spectra of methylated LPSs from these blood-group A-containing *H. pylori* strains (Table II) showed *m/z* 260 for terminal GalNAc, *m/z* $883 \rightarrow 228$ for the complete monofucosyl

TABLE II

Interpretation of the Ions from the FAB-Mass Spectrum of Methylated Intact *H. pylori* LPSs. The Secondary Ions Shown Originate from β-Elimination of the Residue at O-3 of the GlcNAc at the Reducing End. The / Symbol Indicates that Either Structure is Possible

Primary Ions (*m/z*)	Secondary Ions (*m/z*)		Proposed Structure
H. pylori UA948			
638	402 (638 − 236)	Type-1 Lewis A	Fuc-(1 → 4)-GlcNAc$^+$ 3 ↑ Gal-1
638	432 (638 − 206)	Type-2 Lewis X	Gal-(1 → 4)-GlcNAc$^+$ 3 ↑ Fuc-1
886			Lewis A/X → 7-DDHep$^+$
1087	1055 (1087 − 32)		Lewis A/X → LacNAc
1261	1055 (1261 − 206)		Lewis A/X → Lewis X
H. pylori UA955			
434	402 (434 − 32)	Lewis Disaccharide	Fuc-(1 → 4)-GlcNAc$^+$
638	432 (638 − 206)	Type-2 Lewis X	Gal-(1 → 4)-GlcNAc$^+$ 3 ↑ Fuc-1
812	606 (812 − 206)	Type-2 Lewis Y	Gal-(1 → 4)-GlcNAc$^+$ 2 3 ↑ ↑ Fuc-1 Fuc-1
1057	851 (1057 − 206)	Le Dis → Lewis X, type-1 and 2 regions	Fuc-(1 → 4)-GlcNAc-(1 → 3)-Gal-(1 → 4)-GlcNAc$^+$ 3 ↑ Fuc-1
1087	1055 (1087 − 32)		Lewis X → LacNAc
1261	1055 (1261 − 206)		Lewis X → Lewis X
1261	1229 (1261 − 32)		Lewis Y → LacNAc
1435	1229 (1435 − 206)		Lewis Y → Lewis X
2058			Lewis Y → Lewis X → Lewis X
H. pylori J233			
464	432(464 − 32)	Type-2 LacNAc	Gal-(1 → 4)-GlcNAc$^+$
464	228 (464 − 236)	Type-1 Lewis C	Gal-(1 → 3)-GlcNAc$^+$
638	228 (638 − 410)	Type-1 Lewis D(H Type-1)	Fuc-(1 → 2)-Gal-(1 → 3)-GlcNAc$^+$
913		Type-2 i-antigen	LacNAc → LacNAc
1087		(Type-1 and -2 regions)	Lewis D → i-antigen
H. pylori F-58C			
464 (weak)	432 (464 − 32)	Type-2 LacNAc	Gal-(1 → 4)-GlcNAc$^+$
638	402 (638 − 236)	Type-1 Lewis A	Fuc-(1 → 4)-GlcNAc$^+$ 3 ↑ Gal-1
668	228(668 − 440)	Type-1 linear B blood-group	Gal-(1 → 3)-Gal-(1 → 3)-GlcNAc$^+$

(*continued*)

TABLE II—*Continued*

Primary Ions (*m/z*)	Secondary Ions (*m/z*)		Proposed Structure
916			linear B blood-group → 7-DDHep$^+$
886			Lewis A → 7-DDHep$^+$
H. pylori R-58A			
638	402 (638 − 236)	Type-1 Lewis A	Fuc-(1 → 4)-GlcNac$^+$ 3 ↑ Gal-1
886			Lewis A → 7-DDHep$^+$
H. pylori F-15A			
608	576 (608 − 32)	Type-1 fucosylated Lewis disaccharide	Fuc-(1 → 3)-Fuc-(1 → 4)-GlcNAc$^+$
638	402 (638 − 236)	Type-1 Lewis A	Fuc-(1 → 4)-GlcNAc$^+$ 3 ↑ Gal-1
668	228 (668 − 440)	Type-1 and 2 linear	Gal-(1 → 3)-Gal-(1 → 3)-GlcNAc$^+$
	636 (668 − 32)	B blood-group	Gal-(1 → 3)-Gal-(1 → 4)-GlcNAc$^+$
812 (weak)	606 (812 − 206)	Type-2 Lewis Y	Gal-(1 → 4)-GlcNAc$^+$ 2 3 ↑ ↑ Fuc-1 Fuc-1
886			Lewis A → 7-DDHep$^+$
H. pylori R-7A			
260	228 (260 − 32)		GalNAc$^+$
464 (weak)	432 (464 − 32)	Type-2 LacNAc	Gal-(1 → 4)-GlcNAc$^+$
608 (weak)	576 (608 − 32)	Type-1 fucosylated Lewis disaccharide	Fuc-(1 → 3)-Fuc-(1 → 4)-GlcNAc$^+$
638	432 (638 − 206)	Type-2 Lewis X	Gal-(1 → 4)-GlcNAc$^+$ 3 ↑ Fuc-1
709 (weak)	228		GalNAc-(1 → 3)-Gal-(1 → 3)-GlcNAc$^+$
883	228	Type-1 monofucosyl A blood-group	GalNAc-(1 → 3)-Gal-(1 → 3)-GlcNAc$^+$ 2 ↑ Fuc-1
886			Lewis X → 7-DD-Hep$^+$(from core)
1057	402	Type-1 difucosyl A blood-group	GalNAc-(1 → 3)-Gal-(1 → 3)-GlcNAc$^+$ 2 4 ↑ ↑ Fuc-1 Fuc-1
1087	881	LacNAc → Lewis X	Gal-(1 → 4)-GlcNAc-(1 → 3)-Gal-(1 → 4)-GlcNAc$^+$ 3 ↑ Fuc-1
1087	1055	Lewis X → LacNAc	Gal-(1 → 4)-GlcNAc-(1 → 3)-Gal-(1 → 4)-GlcNAc$^+$ 3 ↑ Fuc-1

TABLE II—*Continued*

Primary Ions (*m/z*)	Secondary Ions (*m/z*)		Proposed Structure
1261		Lewis X → Lewis X	Gal-(1 → 4)-GlcNAc-(1 → 3)-Gal-(1 → 4)-GlcNAc$^+$ 3 3 ↑ ↑ Fuc-1 Fuc-1
1305			difucosyl A blood-group → 7-DD-Hep$^+$
1332			monofucosyl A blood-group → LacNAc$^+$
1335			Lewis X → LacNAc → 7-DD-Hep$^+$ LacNAc → Lewis X → 7-DD-Hep$^+$
H. pylori strains CA2, H428 and H507			
812	402 (812 − 410)	Type-1 Lewis B	Gal-(1 → 3)-GlcNAc$^+$ 2 4 ↑ ↑ Fuc-1 Fuc-1
812	606 (812 − 206)	Type-2 Lewis Y	Gal-(1 → 4)-GlcNAc$^+$ 2 3 ↑ ↑ Fuc-1 Fuc-1
1261	1229 (1261 − 32)		Lewis B/Y → 3-Gal-1 → 4-GlcNAc$^+$
1435	1229 (1435 − 206)		Lewis B/Y → Lewis X$^+$
1884			Lewis B/Y → Lewis X → LacNAc$^+$ Lewis B/Y → LacNAc → LacNAc$^+$
H. pylori strains CA4, CA5, CA6, and GU2			
638	402 (638 − 236)	Type-1 Lewis A	Fuc-(1 → 4)-GlcNAc$^+$ 3 ↑ Gal-1
638	432 (638 − 206)	Type-2 Lewis X	Gal-(1 → 4)-GlcNAc$^+$ 3 ↑ Fuc-1
638 (CA4, CA5)	228 (638 − 410)	Type-1 Lewis D	Fuc-(1 → 2)-Gal-(1 → 3)-GlcNAc$^+$
1087 (CA4, GU2)	1055 (1087 − 32)		Lewis A/X/D → LacNAc$^+$
1261	1055 (1261 − 206)		Lewis A/X/D → Lewis X$^+$
1710	1688 (1710 − 32)		Lewis A/X/D → Lewis X → LacNAc$^+$
1884 (CA4)	1688		Lewis A/X/D → Lewis X → Lewis X$^+$

type-1 blood-group A moiety {α-D-Gal*p*NAc-(1 → 3)-[α-L-Fuc*p*-(1 → 2)]-β-D-Gal*p*(1 → 3)-β-D-Glc*p*NAc}, and *m/z* 1057 → 402 indicating also the presence of the difucosyl type-1 blood-group A {α-D-Gal*p*NAc-(1 → 3)-[α-L-Fuc*p*-(1 → 2)]-β-D-Gal*p*(1 → 3)[α-L-Fuc*p*-(1 → 4)]-β-D-Glc*p*NAc}. The *H. pylori* strains found to have LPS with blood-group A structures also expressed the Le^x and LacNAc antigens (Table II), and, indeed, LPS O-chains consisting of blood-group A and LacNAc sections covalently attached were also characterized [*m/z* 1332 (monofucosyl blood-group A → LacNAc)].

Some *H. pylori* strains that by serological means, with Le^b mAbs, were thought to carry the type-1 Le^b antigen were determined to elaborate only substructures

of Le^b. In one case, *H. pylori* strain UA955,[95] the Le^b mAb recognized a Lewis disaccharide structure [α-L-Fuc*p*-(1 → 4)-β-D-Glc*p*NAc] and in another, *H. pylori* J233,[95] recognized another Le^b substructure in the Le^d antigen (Table II). That mAbs putatively specific for the Le^b determinant can detect glycan substructures [Lewis disaccharide and Le^d (H-type-1)] of Le^b indicates their nonspecificity. Both *H. pylori* UA955 and J233 also carried type-2 antigens in their LPS O-chain regions. O-chains of polymeric type-2 Le^x along with O-chains composed of terminal type-1 Le^d and internal type-2 Le^x (Le^d → Le^x → core) were produced by *H. pylori* UA955 LPS (Table II). *H. pylori* J233 also furnished a fucose-free type-2 polyLacNAc, known as the i-antigen [LacNAc → LacNAc → core] O-chain (Table II). The serological studies that showed recognition of the Le^b epitope in *H. pylori* LPSs with the Le^b specific mAb in strains UA955 and J223 were recognizing only substructures of the Le^b antigen. In *H. pylori* UA955 the Le^b mAb presumably detected the α-L-Fuc-(1 → 4)-β-D-GlcNAc Lewis disaccharide, which is a region of the Le^b determinant {α-L-Fuc-(1 → 2)-β-D-Gal-(1 → 3)[**α-L-Fuc-(1 → 4)**]-**β-D-GlcNAc**}, and in *H. pylori* J223, it either recognized the H-1 antigen (Le^d) {α-L-Fuc-(1 → 2)-β-D-Gal-(1 → 3)-β-D-GlcNAc} or Le^c {β-D-Gal-(1 → 3)-β-D-GlcNAc} which are moieties of Le^b {**α-L-Fuc-(1 → 2)-β-D-Gal-(1 → 3)**[α-L-Fuc-(1 → 4)]-**β-D-GlcNAc**}. The Fuc-(1 → 4)-GlcNAc terminal unit in *H. pylori* UA955 either may be a biosynthetic precursor of Le^a or Le^b in *H. pylori* LPS biosynthesis, or may represent a dead-end product due to premature fucosylation of GlcNAc at O-4, inhibiting further galactosylation of GlcNAc at O-3. The cross-reactivity observed between Le^b mAb BG-6 and *H. pylori* J223 LPS seems to be a mAb-specific phenomenon since Le^b mAb 225-Le did not react with J223 LPS. Imberty *et al.*[96] have reported that blood-group determinants have certain conformational dependent "micro-epitopes" toward which different mAbs have dissimilar activities. It is also worth noting that the same Lewis mAb might have different sensitivity toward the same LPS epitopes when tested under ELISA or immunoblot conditions, as indicated by the reaction of Le^b mAb with UA955 in ELISA, but not in immunoblot after removal of the proteins by proteinase K. Isolated *H. pylori* UA955 proteins did not react with the Le^b mAb, suggesting that the observed activity is not to the protein. Also, when whole cells of UA955 were tested in immunoblot the Le^b mAb showed no reaction, suggesting that recognition of UA955 LPS is lost under immunoblot conditions. The same type of phenomenon can also be observed with strain UA1182, whose internal Le^{x}epitopes were well recognized by the Le^x mAb in ELISA, but showed only a weak interaction in immunoblot. These two differences in activities may be caused by conformational changes in the LPS molecules. These studies illustrate the necessity for performing structural rather than serological studies to unambiguously define *H. pylori* LPS antigens.

The expression of type-1 Lewis blood-group antigens suggests that for the biosynthesis of some *H. pylori* LPS molecules there must be glycosyltransferases

of the type-1 family, such as a FucT that places the α-L-Fuc unit at O-4 of β-D-GlcNAc and a GalT that adds β-D-Gal to O-3 of β-D-GlcNAc. The concurrent expression of type-1 and 2 chains, and of various chains with different single-step glycosylation patterns, within a single *H. pylori* strain, represents a complex biosynthetic mechanism used by this organism, which differs from O-chain repeating OS unit addition mechanism of *Escherichia* and *Salmonella* LPS biosynthesis. Various factors, such as differential enzyme kinetics, regulation, and mutation or clonal variation, may control the assembly of *H. pylori* LPS molecules.

The genomes of *H. pylori* strain 26695 and J99 contain two copies of an α-fucosyltransferase and multiple copies may play a role in the expression of the Lewis antigens. Among Western *H. pylori* strains, type-2 Le^x and Le^y antigens are widespread and few seem to express type-1 Le^a and Le^b epitopes. This phenomenon might be due to regulation whereby all *H. pylori* strains contain the genes for the assembly of these alternative structures, but in certain instances some transferases are not expressed; alternatively, only certain strains may contain the appropriate genes required to produce the type-1 antigens. Diversity in fucosylation and thus in Lewis antigen expression among single colonies derived from the same gastric biopsy suggests that this phenomenon might occur *in vivo* as well.[97]

The Le^b epitope is widely expressed in human gastric mucosa[98,99] and thus *H. pylori* with Le^b are capable of mimicking the entire domain of the human gastric mucosa. A protein adhesin produced by *H. pylori* has been ascertained to be involved in adhesion of *H. pylori* to Le^b present in human gastric mucosa[80]; this behavior could extend to the possibility of any *H. pylori* strain that produces Le^b in its LPS being able to append to itself by the same mechanism, giving rise to auto-agglutination and thus formation of colonies. Other *H. pylori* cell-surface proteins have also been shown to be involved in adhesion to histo-blood group antigens.[100]

Parallel structural investigations unveiled the composition of LPS molecules from *H. pylori* strains obtained from Chinese, Japanese, and Singaporean symptomatic hosts.[101] The new findings were: (i) production of type-1 blood-group determinants (Table II) was prevalent in LPSs of Asian *H. pylori* strains, in contrast to Western *H. pylori* LPSs, and, in particular, the O-chain regions of strains F-58C and R-58A carried type-1 Le^a without the accompanying type-2 Le^x; (ii) strain R-7A and H608 were shown to have the capability of producing the type-1 blood-group A antigen; and (iii) strains CA2, H507, and H428 expressed simultaneously the difucosyl isomeric antigens, type-1 Le^b and type-2 Le^y. In contrast to LPSs of *H. pylori* strains isolated from hosts residing in Western countries (Sections II–V), which, overwhelmingly, were found to express type-2 Le^x and Le^y blood-group determinants, these Asian *H. pylori* LPSs also showed a tendency to produce type-1 histo-blood group determinants. The most complex O-chain molecules were found to be present in the LPS of *H. pylori* strain R-7A where an array of histo-blood group glycoforms were observed (Table II). Both blood-group

A type-1 forms, monofucosyl and difucosyl, were detected in *H. pylori* R-7A LPS, and it was shown that the type-1 A blood-group was connected to a type-2 LacNAc backbone, which is unique in histo-blood-group chemistry. Serologically, mAb 3-3a also detected blood-group A in this LPS by ELISA. Moreover, the R-7A strain also furnished a string of type-2 Le^x and LacNAc epitopes as terminal entities and as members of elongated linear chains (Table II).

In some of the foregoing *H. pylori* LPSs, a covalent connection between the O-chains, composed of histo-blood groups, and the core was shown to be formed by a ligation between the reducing end GlcNAc unit of the O-chain and an outer core DD-Hep residue (Tables I and II). Ion *m/z* 886 showed the linkage of the type-1 Le^a or type-2 Le^x epitope to the O-7 position of the outer core DD-Hep. Several higher mass ions in the FAB-MS spectrum of the methylated R-7A LPS, of defined composition and that included the linear heptose (7-substituted DD-Hep) of the outer core region, yielded important evidence for the connection of the type-1 difucosyl blood-group A (*m/z* 1305), and of the elongated type-2 $Le^x \rightarrow$ LacNAc and LacNAc $\rightarrow Le^x$ to the core (*m/z* 1335). These structural interpretations, which showed the covalent linkage between the O-chain region and the core, offered unequivocal proof that these *H. pylori* cell surface glycan molecules were indeed LPSs and were not capsular or exopolysaccharides. However, it is possible that some high-molecular weight Le^x O-chains found in some *H. pylori* strains may be present as non-LPS molecules, since it is not always possible to prove the covalent connection of O-chain to core.

H. pylori was found to carry both difucosyl type-1 Le^b and type-2 Le^y isomeric structures. The coexistent expression of Le^b and Le^y indicated that fucosylation and galactosylation at O-3 or O-4 of GlcNAc were available at any instance in *H. pylori* LPS biosynthesis and may be dictated by an array of internal and/or external factors. The end products (Le^b and Le^y in this case) may also have different functions in *H. pylori* pathogenesis, that is, they may perform particular physical roles in endogenous (bacterium–bacterium) or exogenous (bacterium–host) recognition/adhesion, or may be intrinsic players in the host's immune response pathways. A similar role may also apply to the other *H. pylori* LPS blood-group antigens. In *H. pylori* strains CA4 and CA5 (Table II), fucosylation at O-2 of a terminal Gal of a Gal-(1 → 3)-GlcNAc structure yielded the type-1 Le^d, Fuc-(1 → 2)-Gal-(1 → 3)-GlcNAc. The presence of three different monofucosylated Lewis determinants, Le^a, Le^x, and Le^d, in *H. pylori* strains CA5 and CA4 reflect three [Fuc-(1 → 2), Fuc-(1 → 3), and Fuc-(1 → 4)], possibly self-regulating, fucosylation pathways in the biosynthesis of these LPSs. Parallel serological studies using several commercially available mAbs, specific for histo-blood group epitopes, were also successfully in detecting the blood-group antigens in these *H. pylori* LPS.[82,95,101]

The core OS derivatives (non-histo-blood-group units), obtained from chemical linkage analysis of these *H. pylori* LPSs, were of the same type as found in the core structures described in the preceding sections. All core residues detected fell in line

with the typical core structural regions of *H. pylori:* Glc-(1 → 3)-Glc-(1 → 4)-Gal-(1 → 7)-[DD-Hep-(1 → 2)]-DD-Hep-(1 → 2)-LD-Hep-(1 → 3)-[P or AEP → 7]-LD-Hep. The ^{31}P NMR spectra and the linkage analysis data suggested the presence of a monoester phosphate (PO_4^-) at the O-7 position of the inmost LD-Hep of the strains described here, except in strain R-7A, where a 2-aminoethyl phosphate was shown to replace the monoester phosphate, as in *H. pylori* Sydney strain (Section IV).

The complete genomes of two Western *H. pylori* strains, 26695 and J99, have been determined to be similar. Chemical analyses have shown that the O-chain region of *H. pylori* 26695 and J99 LPSs were composed of type-2 Le^x and/or Le^y antigens, and no type-1 Le^a, Le^b, or blood-group A epitopes were observed.[82] The LPS structural differences noted here between the Asian and Western *H. pylori* strains, based on a more prominent expression of type-1 histo-blood group antigens in the O-chain sections of Asian strains, suggest that some significant differences may be encountered between the genomes of Asian and Western *H. pylori* strains.

The apparent strong inclination for the production of type-1 histo-blood group antigens in Asian *H. pylori* LPSs, when compared with Western strains, may be an adaptive evolutionary effect in that differences in the gastric cell surfaces of the respective hosts might be significantly dissimilar to select for the formation of different LPS structures on the resident *H. pylori* strain. Interestingly, in Asian countries ABO non-secretors are relatively more common than in Caucasian populations. A large-scale serological experiment using blood-group mAbs is needed to come to a conclusion on the extent to which Asian *H. pylori* strains express type-1 histo-blood group antigens.

In this section, the molecular mimicry between *H. pylori* LPSs and host molecules was extended to include the type-1 determinants Le^a, Le^b, Le^d, Le^c, and blood-group A. This ability of *H. pylori* to produce various Lewis isoforms permits mimicking all regions of the gastric epithelium, those being the gastric superficial and glandular epithelium, which display mainly type-2 molecules, and the superficial epithelium, which expresses predominantly type-1 chains.[98] Consequently, each *H. pylori* strain, depending on the antigens expressed by its LPS, may have a different ecological niche within the gastric mucosa, and ultimately the role of LPS in pathogenesis and adaptation may differ between *H. pylori* strains.

VII. Glucosylated and Galactosylated Poly-*N*-acetyllactosamine O-Chains in *Helicobacter pylori* Lipopolysaccharides

This section describes two classes of *H. pylori* LPSs that produce type-2 elongated polyLacNAc backbones similar to those described in preceding sections, but, in these cases, decorated by branches composed of end-group units of α-D-Glc*p* or α-D-Gal*p* in addition to the common α-L-Fuc.

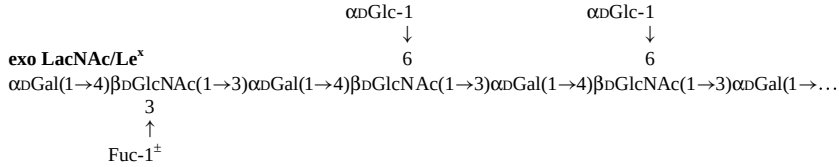
The cloning and characterization[102] of a glycosyltransferase from a clinical strain isolated from endoscopic biopsy specimen from a patient, *H. pylori*

UA861, capable of adding, in an enzyme assay, a α-D-Gal*p* to the O-6 position of a β-D-Glc*p*NAc [α-D-Gal*p*-(1 $\rightarrow$ 6)-β-D-Glc*p*NAc] prompted a structural investigation into the LPS from this strain.[103] Overall, the structural data unanimously pointed to an LPS composed of an O-chain region that comprised a type-2 polyLacNAc linear backbone with α-D-Glc*p* branches appended to the O-6 position of some backbone GlcNAc residues $\{\rightarrow 3)$-β-D-Gal*p*-(1 $\rightarrow$ 4)[α-D-Glc*p*-(1 $\rightarrow$ 6)]-β-D-Glc*p*NAc-(1 $\rightarrow\}$. Unexpectedly, no α-D-Gal*p*-(1 $\rightarrow$ 6)-β-D-Glc*p*NAc structure was witnessed in this analyses of *H. pylori* UA861 LPS. The ^{1}H NMR spectrum of the water-soluble intact LPS showed three dominant anomeric resonances for α-Glc (δ 4.99), β-GlcNAc (δ 4.69), and β-Gal (δ 4.47). ^{1}H–^{1}H *inter* NOE connectivities revealed the α-Glc-(1 $\rightarrow$ 6)-β-GlcNAc and β-Gal-(1 $\rightarrow$ 4)-β-GlcNAc connections in line with the observed 4,6-disubstituted GlcNAc derivative in the chemical linkage analysis. A LacNAc antigen [*m/z* 464 $\rightarrow$ 432 (464 $-$ 32) in FAB-MS] affected termination of the nonreducing end in this glucosylated O-chain, and no fucosylated Lewis determinant was detected in this outermost region of LPS. Only sole randomly placed internal Le^x moieties were observed. The LPS of *H. pylori* serogroup O : 4, from Penner's serotyping system,[69] also produced glucosylated polyLacNAc chains similar to those observed in the just-described *H. pylori* strain UA861 (Aspinall; Monteiro, unpublished results).

Aspinall[104] also investigated the LPS structure of *H. pylori* strain 471, which, unlike some *H. pylori* strains, lacked the ability to stimulate pepsinogen secretion. The proposed structure of this LPS comprised a typical *H. pylori* type-2 polyLacNAc O-chain backbone that was adorned with branches of α-D-Gal*p* units. Similar to the branching Glc residues in *H. pylori* UA861, these end-group Gal units were attached at O-6 of the backbone GlcNAc $\{\rightarrow 3)$-β-Gal*p*-(1 $\rightarrow$ 4)-[α-D-Gal*p*-(1 $\rightarrow$ 6)]-β-D-Glc*p*NAc-(1 $\rightarrow]$. The nonreducing terminus of this galactosylated LPS O-chain was composed of Le^x and Le^y, and also of nonfucosylated LacNAc and β-Gal-(1 $\rightarrow$ 4)-[α-D-Gal*p*-(1 $\rightarrow$ 6)]-β-D-Glc*p*NAc. A limited number of internal Le^x units were also detected in this galactosylated polyLacNAc O-chain. The resonance of H-1 α-D-Gal*p* side-units was observed at δ 4.98 and noticeable *inter* NOE connectivities were detected between this anomeric signal and H-6 (δ 3.90) and H-6′ (δ 4.15) of β-D-Glc*p*NAc confirming the Gal-(1 $\rightarrow$ 6)-GlcNAc linkage. Figure 12 displays the structures of the glucosylated and galactosylated polyLacNAc O-chains just described. The core OSs of the glucosylated and galactosylated LPSs discussed earlier possessed the same structural arrangement as parallel regions of previously investigated *H. pylori* strains (Fig. 6).

The most striking structural feature of the LPSs from *H. pylori* strains UA861 and 471 and serostrain O : 4 is the replacement of Fuc by Glc and Gal and therefore the absence of heavily fucosylated type-2 polyLacNAc O-chains, and consequently the limited number of internal Le^x antigens, which are a common characteristic component of *H. pylori*. The low degree of Le^x expression, and, occasionally, the absence of terminal Le$^{x/y}$, carries important implications with regard to pathogenic differences among *H. pylori* strains, such as possible bacterial–epithelial cell

***H. pylori* strain UA861 and serostrain O:4**



***H. pylori* strain 471**

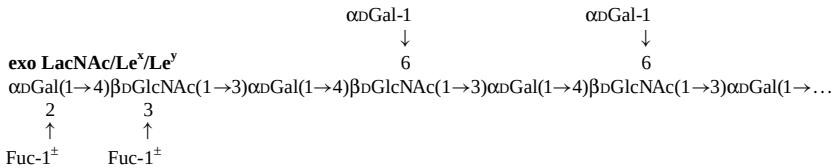


FIG. 12. The chemical structures of the glucosylated *H. pylori* UA861 and O : 4, and of the galactosylated *H. pylori* 471. A limited number of endo Le^x units are also present.

interactions, and in their role in a postulated autoimmune disease component linked with *H. pylori* strains that carry Lewis blood-group antigens. Although one would not expect *H. pylori* strains devoid of complete terminal Lewis epitopes, such as *H. pylori* UA861, to be recognized by mAbs raised against terminal Lewis antigens, in fact this strain did react with mAbs for Le^x, so that the internal α-1,3-Fuc unit must be accessible to react both in ELISA and immunoblot.[102] The characterization by chemical means of the glucosylated polyLacNAc amended the earlier observation that *H. pylori* UA861 produced an unusual α-(1 → 6)-galactosyltransferase. Since this *H. pylori* strain carries a terminal α-D-Glc residue at the O-6 of GlcNAc, this particular glycosyltransferase is likely to be a glucosyltransferase rather than a galactosyltransferase or a glycosyltransferase with dual specificity. This irregularity might have arisen from the use of UDP-Gal as the acceptor substrate in the earlier transferase assays.[102] The structural studies must take precedence over transferase assays with chemical substrates, but direct evidence awaits the cloning and characterization of the postulated α-(1 → 6)-glucosyltransferase. However, the galactosylated polyLacNAc of *H. pylori* 471 should indeed express an α-(1 → 6)-galactosyltransferase. The diversity in polyLacNAc glycosylation observed here is most likely controlled at the genetic level. The genes encoding the FucTs that control Lewis antigen fabrication and the GlcT and GalT responsible for GlcNAc glycosylation may be present in some strains and absent from others. Alternatively, all *H. pylori* strains may contain a full complement of transferase genes and other factors may control either their expression or assembly of these complex carbohydrates. Diversity at both the levels of individual genes (microdiversity) as well as in gene order (macrodiversity) seems to be present in *H. pylori*.

The ornamentation of *H. pylori* LPS polyLacNAc chains with Glc and Gal residues does not appear to be a very common characteristic of *H. pylori* LPSs. The exact purpose(s) of these hexose units is not known, but they could be involved in stabilizing, or creating, LPS conformers that are required in *H. pylori* pathogenesis, especially when, as in these cases, fucosylation of these LPSs is limited. Indeed, additional reexaminations of these *H. pylori* strains revealed that fucosylation, and conversely glucosylation and galactosylation, of these LPSs varied vastly from batch to batch preparations. These variable degrees of polyLacNAc glycosylations reflect an unpredictable mechanism that is controlled by a flexible set of biosynthetic events.

VIII. *Helicobacter pylori* FROM ASYMPTOMATIC HOSTS EXPRESSING HEPTOGLYCANS, BUT LACKING LEWIS O-CHAINS

This section describes the chemical structures of *H. pylori* LPSs from hosts with no explicit signs of severe disease. Serological analyses with histo-blood-group monoclonal antibodies did not detect the presence of any blood-group antigen in these *H. pylori*, and thus were non-typeable by this procedure. Data from chemical- and spectroscopic-based experiments unanimously showed that these *H. pylori* manufactured LPS with heptoglycans of 2- and 3-linked D-*glycero*-α-D-*manno*-heptopyranose units (as seen in Section IV), but are completely devoid of Lewis O-chains.[105] An *H. pylori* isolate with a similar LPS structure was shown to be capable of colonizing mice, which suggests that the presence of Lewis O-chains is not absolutely required for colonization. The absence of O-chains with histo-blood groups may cause *H. pylori* to become inept in adhering to human receptor cells, and/or also the presence of elongated heptoglycans may impede exposure of disease-causing bacterial cell-surface molecules. These two factors may render such *H. pylori* incapable of creating exogenous contacts essential for pathogenesis of severe gastroduodenal diseases.

H. pylori strains Hp1C2, Hp12C2, Hp65C, Hp7A, Hp75A, and Hp77C from asymptomatic hosts were used for serological analysis and chemical manipulations. The SDS-PAGE profile of these non-typeable LPSs showed the presence of high-M_r LPS material. As mentioned, ELISAs and immunoblots with blood-group mAbs fail to detect any histo-blood-group antigen in these LPS or whole-cell preparations. Sugar analysis performed on the seven intact LPS preparations revealed the presence of L-Fuc, D-Glc, D-Gal, D-GlcNAc, DD-Hep, and LD-Hep in the approximate ratios of 1 : 5 : 1 : 1 : 17 : 1.5, respectively. DD-Hep was the dominant component of these LPSs. Sugar linkage analysis showed that each LPS was composed of terminal Fuc, Glc, and DD-Hep, traces of 3-substituted Fuc, 4-monosubstituted Gal, 3- and 6-monosubstituted Glc, 4-monosubstituted GlcNAc, 2-monosubstituted LD-Hep, 3,7-disubstituted LD-Hep, 2,7-disubstituted DD-Hep, and, most predominantly, 2- and 3-monosubstituted DD-Hep units

indicative of extended linear heptan chains. FAB-MS provided the first solid evidence for such heptoglycans, in that the FAB-MS spectrum of each methylated LPS yielded sequential glycosyl oxonium ions from cleavage at successive Hep residues. The FAB-MS spectra of these methylated *H. pylori* LPSs afforded A-type primary ions, and corresponding secondary ions from loss of methanol, at *m/z* 263 → 231 [Hep$^+$], *m/z* 511 → 479 [Hep → Hep$^+$], *m/z* 759 → 727 [Hep → Hep → Hep$^+$], *m/z* 1007 → 975 [Hep → Hep → Hep → Hep$^+$], and *m/z* 1255 [Hep → Hep → Hep → Hep → Hep$^+$] that pointed to the presence of DD-Hep oligomers. In addition, ions at *m/z* 189 [Fuc$^+$], *m/z* 682 [Fuc,GlcNAc,Hep]$^+$, *m/z* 930 → 898 [(Fuc,GlcNAc,Hep) → Hep$^+$], and *m/z* 1179 → 1146 [(Fuc,GlcNAc,Hep) → Hep → Hep$^+$] suggested that some heptoglycans were terminated at the nonreducing end by Fuc and GlcNAc units, possibly in a Fuc-(1 → 4)-GlcNAc linkage. No ions characteristic of blood-group trisaccharides Le^a or Le^x (*m/z* 638), tetrasaccharides Le^b or Le^y (*m/z* 812), or any other histo-blood group, typical components of *H. pylori* LPSs, were detected in the FAB-MS spectra of the strains examined here. The first evidence showing that some DD-Hep oligomers were constructed by consecutive 3-linked DD-Hep {-[→ 3)DD-Hep(1-]$_n$ →}, was obtained by the isolation of an OS from a Smith degradation composed mainly of 3-substituted Man-C^6-D units along with some nonsubstituted Man-C^6-D representative of a 3-linked mannan. The outer region of these LPSs was thus shown to exhibit a linear 3-linked DD-Hep chain DD-Hep-(1-[→ 3)-DD-Hep-(1-]$_n$ → 3)-DD-Hep-(1 → *inner LPS region.* The ^{1}H and ^{13}C NMR spectra of each delipidated *H. pylori* PS furnished two strong α-anomeric resonances, as unresolved doublets in the ^{1}H NMR spectrum, at δ_H 5.40/δ_C 101.0 and δ_H 5.09/δ_C 103.0 emanating from two major sugars, in this case, with the *manno* configuration, the 2- and 3-linked DD-Hep LPS constituents. Unequivocal evidence supporting the presence of linear heptoglycans in these strains was also obtained from a 2D NOESY experiment that yielded *inter*-space NOE connectivities between H-1 at δ 5.09 and H-3 at δ 4.02 for the 3-linked DD-Hep oligomer and H-1 at δ 5.40 and H-2 at δ 4.05 for the 2-linked DD-Hep oligomer. Other NOE connectivities involving H-1 at δ 5.40 were also observed, but they could not be unambiguously assigned and may suggest that this DD-Hep-(1 → 2)-DD-Hep linkage is highly flexible. One NOE signal involving H-1 at δ 5.40 and H-3 at δ 4.02 indicated that amidst the 3-linked heptoglycan oligomers some 2-substituted DD-Hep units may be inserted between the 3-linked DD-Hep residues [. . . → 3)-DD-Hep-(1 → 2)-DD-Hep-(1 → 3)-DD-Hep-(1 → . . .].

In the lower molecular weight LPS fractions, 2-, 3-, and 6-substituted DD-Hep residues were present in equimolar proportions, which indicated that these units are always co-expressed in these heptoglycan-rich strains. Also, in some low-M_r LPS subfractions, 3-substituted Fuc was an equimolar component suggesting the presence of a rare Fuc-(1 → 3)-Fuc moiety that is found in some *H. pylori* structures and/or a GlcNAc-(1 → 3)-Fuc structure.

The *H. pylori* Sydney strain[86] (Section V), widely and successfully used in mice models that study *H. pylori* pathogenesis, followed the *H. pylori* LPS pattern in that it carried Le^x and Le^y antigens in the O-chain region.[82] The usage of *H. pylori* Sydney strain and its genetically induced mutants carrying truncated LPS has revealed that these LPS structures may play a role in colonization; a mutated *H. pylori* Sydney strain carrying a rough-form LPS devoid of "Lewis O-chain," created by insertional mutagenesis of HP0826 (β4-*galT* gene), has been shown to still be able of colonizing the murine model, although with less efficiency (Section X). It was also shown that an *H. pylori* isolate, HpPJ1, producing a heptoglycan-rich LPS devoid of Lewis O-chains and structurally similar to those described earlier, was able to colonize CD1 mice, but in lesser numbers when compared with the mouse model standard Sydney strain. The fact that *H. pylori* strain HpPJ1 was able to persistently colonize the stomachs of CD1 mice for a period of at least 12 weeks implied that complete histo-blood group O-chains are not an absolute prerequisite for colonization.

H. pylori strains isolated from asymptomatic hosts, which were untypeable using mAbs specific for Lewis antigens (Le^a, Le^b, Le^x, or Le^y), were found to produce LPS molecules devoid of "blood-group O-chain" regions. Instead, they contained elongated heptoglycans, oligomers of D-α-D-Hep, DD-Hep-(1-[$\rightarrow$3)-DD-Hep-(1-]$_n \sim$ [$\rightarrow$2)-D-Hep-(1-]$_n \rightarrow$ core OS, in which some were capped by an incomplete Lewis antigen (Fig. 13). This family of *H. pylori* strains also contained 6-substituted DD-Hep as a common component in the inner regions of LPS; this unit was also found to be present in *H. pylori* strains that contain extended heptoglycans (Section IV). These three heptosyl linkages, 2-, 3- and 6-substituted DD-Hep units, seem to be always coexpressed. A common feature of the *H. pylori* LPSs described here, and those of heptoglycan-rich strains, was the production of an accompanying side-antenna dextran [(1$\rightarrow$6)-α-D-glucan] (Fig. 13). The core regions, within the limits of detection, were composed of the same sugar units as other *H. pylori* isolates.

The individuals from which these strains were isolated all had positive signs of *H. pylori* infection, positive urea breath test, as well as positive histology or culture for *H. pylori*, but no signs of overt disease were detected. The increased proportion of *H. pylori* untypable by Lewis specific mAbs identified among the *H. pylori* from asymptomatic subjects may be an evolutionary trend toward a decreased immune response. If the Lewis, or any other, blood-groups are the inflammatory agent responsible for immune activation, a decrease in the presentation of these antigens may lead to a decrease in immune-related symptoms and symptomatic colonization. Previous studies of inflammatory responses in relation to *H. pylori* Lewis antigens did not include any untypeable *H. pylori* isolates; thus, it will be necessary to investigate the immune response, colonization, and inflammation capabilities of these isolates in both *in vitro* and *in vivo* model systems to see if any differences are observed. Previously, the LPS of another asymptomatic

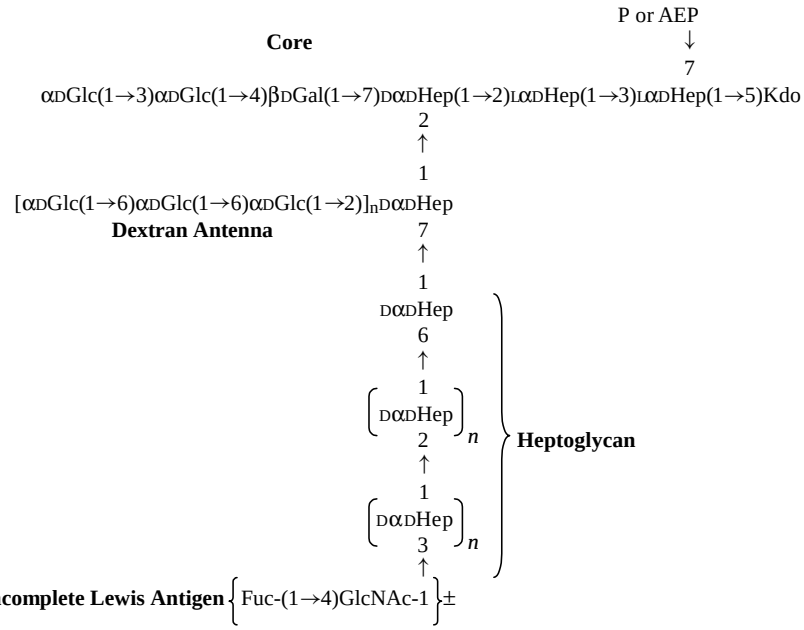


FIG. 13. Chemical structure of the heptoglycan-rich LPSs of *H. pylori* strains from asymptomatic hosts that lack histo-blood-group O-chain regions. A small number of LPSs may also contain a Hep/GlcNAc(1 → 3)Fuc antigen as a nonreducing terminus.

isolate, MO19 (Section IV), was chemically characterized and shown to contain an elongated heptoglycan similar to those found here, with some being capped by a sole Le^y. This intervening region was also found in the LPS of two other isolates, those being serogroups O : 3 and O : 6 of the Penner serotyping system.[69] The presence of this heptoglycan region may provide increased length and flexibility to the LPS such that it may cover the bacterial surface and consequently interfere with the involvement of bacterial virulence factors with the epithelium of host cells. Therefore, in addition to the absence of long Lewis blood-group O-chains, this intervening region may increase LPS flexibility that may allow masking of other *H. pylori* products on the bacterial surface, preventing a symptomatic *H. pylori* infection. Indeed, the heptoglycan-rich asymptomatic *H. pylori* MO19, found to be unable to bind to the gastric mucosa, may be impeded in adhering because the heptoglycan "covers" certain adhesin(s). Also, protective antibodies directed at the dominant heptoglycan domain in these *H. pylori* might confer some protection against infection.

The production of these types of LPSs may be controlled at the genetic level. The complete order of synthesis of the Lewis antigens by *H. pylori* has not been elucidated, but the untimely fucosylation of GlcNAc here (Fuc → GlcNAc) could

impede galactosylation of this residue and thus obstruct the building of a complete Lewis determinant. This addition of Fuc would effectively terminate O-chain elongation. Additionally, these *H. pylori* strains may contain inactive β-1,4-GalT and β-1,3-GalT glycosyltransferases, both responsible for Lewis O-chain formation, Le^x and Le^a, respectively, and thus resulting in the absence of Lewis blood-groups. LPSs composed of heptoglycans without Fuc-GlcNAc capping were common in these strains, which may also implicate the GlcNAcT responsible for adding GlcNAc to the heptoglycan, the GlcNAcT responsible for O-chain initiation. These *H. pylori* isolates may also lack enzyme activity of a second GlcNAcT that places GlcNAc at O-3 of Gal, the GlcNAcT responsible for O-chain elongation. Phase variation in *H. pylori* LPS may be controlled by an "on" and "off" switching mechanism of several glycosyltransferases. These heptoglycan-rich isolates must contain in their armor highly "active" heptosyltransferases. These glycosyltransferase activities may be controlled by environmental factors, which dictate the presence or absence of Lewis O-chains, thus yielding the desired LPS structure for the present stage of pathology.

These results indicate that the presence of Lewis O-chains in *H. pylori* seems to be important for development of pathology, but not colonization. These isolates all colonized human individuals, but it is not clear if they expressed any Lewis antigens at the initiation of infection. This study demonstrates that complete Lewis antigens in *H. pylori* do not appear to be absolutely required for initial colonization in the murine model. Therefore, Lewis antigens are unlikely to be an absolute prerequisite for colonization of the human stomach either, although the data obtained in this study strongly suggest that blood-group O-chains are a requirement for *H. pylori* pathology. The *H. pylori* that do not express complete Lewis antigens appear to augment the cell surface by elongating the heptoglycan region and adding a dextran, possibly in lieu of the Lewis antigens.

IX. The Lipopolysaccharides of *Helicobacter* Species from Nonhuman Primates

Many nonhuman primates have been observed to carry their own *Helicobacter*-like organism, which, like *H. pylori* in humans, gives rise to gastric diseases in their respective hosts. *H. mustelae* in ferrets, *H. felis* in cats, *H. acinonyx* in cheetahs, *H. bilis* in mice, and *H. suis* in pigs are some examples of *Helicobacter* species distributed among the animal kingdom that colonize the host's stomach. *H. hepaticus* colonizes the liver of mice, and infrequently *H. heilmannii* is also found in the human stomach. *In vitro* growth of these *Helicobacter* species is extremely difficult and in some cases yet unachievable.

Only the LPS of *H. mustelae* (ATCC 43772) from ferrets, a commonly used *Helicobacter* animal model, has been investigated in detail.[106] The LPS of *H. mustelae* followed the example of *H. pylori* LPSs in that it also expressed a

histo-blood group antigen, that being the monofucosyl type-1 A blood-group {α-D-GalNAc($1 \rightarrow 3$)[α-L-Fuc-($1 \rightarrow 2$)]-β-D-Gal-($1 \rightarrow 3$)-β-D-GlcNAc} in mimicry of the host's gastric cell-surface molecules.[106,107] The blood-group A in ***H. mustelae*** LPS completed a core OS of which the inner portion showed similarities to parallel regions {β-D-Gal-($1 \rightarrow 3$)-LD-Hep-($1 \rightarrow 3$)-LD-Hep-($1 \rightarrow 5$)-Kdo} of ***Campylobacter*** LOSs. No elongated O-chains were found in ***H. mustelae*** and, thus, its LPS was more akin to low-M_r LOSs found in ***Neisseria, Campylobacter,*** and ***Haemophilus*** containing structures homologous to mammalian cell-surface glycomolecules. No DD-Hep was detected in ***H. mustelae*** LPS. The lipid A structure of ***H. mustelae*** type strain ATCC 43772 presented two different lipid A moieties.[108] The ***H. mustelae*** lipid A structures observed differed from that of the related human gastric pathogen, ***H. pylori. H. pylori*** lipid A molecules are sparsely acylated, with most strains containing a sole C_{18} component, and are only monophosphorylated by a 2-aminoethyl phosphate at the anomeric position,[109,110] β-D-Glc*p*N-($1 \rightarrow 6$)-α-D-Glc*p*N-($1 \rightarrow$ PEA. One ***H. mustelae*** lipid A structure contained a bisphosphorylated β-($1 \rightarrow 6$)-linked D-glucosamine disaccharide with hydroxytetradecanoic acid in amide linkages. The ***H. mustelae*** lipid A was found to be heterogeneous with two major molecular species, a pentaacyl and a hexaacyl species. Unlike the structure described for the lipid A of ***H. pylori,***[109,110] this lipid presented phosphate groups at both the C-1 and C-4′ positions in the backbone, and contained no octadecanoyl fatty acid.[108] The C-4 and C-6′ hydroxyl groups of the backbone disaccharide were unsubstituted, the latter being the proposed attachment site of the core OS. This lipid A was similar to that of ***Haemophilus influenzae*** with tetradecanoyl-oxytetradecanoic acids at the C-2′ and C-3′ positions of the nonreducing GlcN. The other lipid A structure had a different fatty acid composition with C_{16}-OH replacing most of the amide-linked C_{14}-OH. Structural heterogeneity in this case was also due to the presence of a minor molecular species having hydroxytetradecanoyloxy-tetradecanoic acid at the C-3′ position.

H. felis (ATCC 49179), ***H. hepaticus,*** and ***H. acinonyx*** LPSs were observed to be composed of similar sugar constituents as those found in ***H. pylori,*** namely, Fuc, Glc, Gal, DD-Hep, LD-Hep, GalNAc, and GlcNAc, and thus have the potential to produce blood group-related antigens. Of particular interest, the Fuc component in ***H. felis*** was present as a 3,4-disubstituted unit[111] and not as an end group, as seen in ***H. pylori*** and ***H. mustelae,*** and therefore cannot carry the known blood-group epitopes with terminal Fuc, but may produce glycan structures similar to those present in its host's gastric niche not yet identified. The other linkage types of ***H. felis*** LPS represent a branched molecule that includes terminal Glc and Gal, 2- and 2,7-substituted DD-Hep, 4-substituted GlcNAc, 3-substituted Gal and Glc, 4-substituted Gal, 2-substituted Man, and 2-substituted LD-Hep.[111] The molecular mimicry shown by ***H. mustelae,*** in producing blood-group A in its LPS, furthers the belief that ***Helicobacter*** LPS may have a significant role in disease pathogenesis.

X. Classification of *Helicobacter pylori* Lipopolysaccharides into Glycotype Families

The LPSs of *H. pylori* strains share the common characteristic of expressing epitopes in their O-chains similar to human histo-blood group antigens. However, fine points in overall and fine structural details differentiate these LPS molecules. With this fact in mind, and in order to create a systematic grouping of different *H. pylori* LPSs, the LPSs from the strains of which the chemical structures are known were classified into specific glycotype families (Table III).

The first, and apparently the most common in North American and European *H. pylori* strains, glycotype A, represents *H. pylori* LPSs composed of polymeric type-2 Le^x O-chains (more than one Lewis repeat), terminated at the nonreducing end by Le^x, Le^y, or sialyl Le^x, attached to the core. The type-2 Lewis polyLacNAc backbone is composed of a β-Gal-(1 $\rightarrow$ 4)-β-GlcNAc linkage in which the addition of β-Gal to O-4 of β-GlcNAc is affected by the *H. pylori* β-1,4-galactosyltransferase,[112] HP0826 in *H. pylori* 26995 genome[85] and JHP765 in *H. pylori* J99 genome,[87] which was annotated as a lex2B/lob1 homologue. This β-1,4-galactosyltransferase gene involved in *H. pylori* O-chain synthesis diverges from the predicted pattern of LPS gene homologies with respect to O-chain and core specificity. When the *H. pylori* β-1,4-galactosyltransferase gene is inactivated the resulting LPS is truncated and thus becomes devoid of any Lewis O-chain, which in turn negatively affects the bacterium's colonization capability[112] in the murine model. The formation of the type-2 polyLacNAc backbone of glycotype A LPS is also dependent on the activity of the *H. pylori galE* gene, HP036/JHP1020, encoding a galactose epimerase activity.[113] Disruption of *H. pylori*'s *galE* gene leads to an LPS without a Lewis O-chain and an outer core region [αGlc-(1 $\rightarrow$ 3)-αGlc-(1 $\rightarrow$ 4)-βGal], and the absence of these LPS sections was shown to disrupt adhesion between the bacterium and the host gastric cells.[113] The complementary *H. pylori* β1,3-GlcNAcT involved in type-2 polyLacNAc production [βGlcNAc-(1 $\rightarrow$ 3)-βGal] has not yet been identified. Fucosylation of β-GlcNAc [α-L-Fuc-(1 $\rightarrow$ 3)-β-D-GlcNAc] and β-Gal [α-L-Fuc-(1 $\rightarrow$ 2)-β-D-Gal] is the essential final step for the fabrication of type-2 Le^x and Le^y. The *rfbM* (*manA/C*) *H. pylori* gene, HP0043/JHP37, encodes a GDP mannose pyrophosphorylase that is one of the precursors for the synthesis of GDP-Fuc. Upon interruption of *H. pylori rfbM* gene,[113] fucosylation of the LacNAc block did not occur and the resulting LPS molecule did not express Le^x or Le^y, but rather a linear polyLacNAc O-chain. Also, the *H. pylori* gene involved in the conversion of GDP-mannose to GDP-fucose, *wbcJ* (HP0045/JHP38), was found to be induced at the transcription level by exposure to acid.[114] As seen only by SDS-PAGE, disruption of *wbcJ* gene led to an LPS without O-chain,[114] and this isogenic mutant was comparatively more sensitive to acid stress, which hinted at the fact that *H. pylori* may change its LPS makeup in response to pH levels *in vivo*. However, because *H. pylori*, after

rapidly migrating through the mucus layer toward higher pH, takes up residence the a neutral milieu (pH 7) of the epithelial surface, and because a wide array of structures, with and without Fuc, are often observed in a single LPS preparation, a predetermined role of an *in vivo* acidic environment on the outcome of LPS structures is not obvious. The synthesis of Le^x in *H. pylori* takes place in a manner similar to that observed in mammalian cells, that is, a type-2 LacNAc moiety is fucosylated by a α-1,3-fucosyltransferase (FucT) using GDP-Fuc as the donor molecule.[115,116] The published genome sequences[85,87] contain two α-1,3-FucT genes, *futA* (HP0379/JHP1002) and *futB* (HP0651/JHP596). In *H. pylori,* a final addition of Fuc, by an α1,2 FucT, *futC* (HP0093|0094/JHP86), onto O-2 of Gal in a Le^x structure furnishes the difucosylated Le^y antigen.[117] The Le^y production via Le^x in *H. pylori* differs from the fabrication of Le^y in mammalian cells, which takes place by fucosylation [Fuc-(1 → 3)-GlcNAc] of an H type-2 moiety [Fuc-(1 → 2)-Gal-(1 → 4)-GlcNAc] rather than Le^x. Induced mutations of the mentioned FucT genes in glycotype A *H. pylori* strains resulted in LPS preparations devoid of Le^x and/or Le^y antigens. Glycotype A LPSs may also possess a type-2 sialylated antigen, sialyl Le^x, as found in strain P466 (Section III). Two potential *H. pylori* genes involved in sialylation are ***neuA*** HP0326/JHP309, an acylneuraminate cytidyltransferase, and ***neuB*** HP0178/JHP166, a sialic acid synthase. Fucosylation of the polyLacNAc O-chains present in glycotype A LPSs has been observed to be a highly random process, and thus these O-chains contain many fucose-free internal LacNAc repeats amid the Le^x moieties, resulting in a high degree of phase variation. Appelmelk *et al.*[118] suggested that in *H. pylori* the *futB* gene product readily fucosylates a terminal nonreducing LacNAc to yield an exo Le^x, whereas the *futA* gene product tends to fucosylate internal LacNAc repeats to afford endo Le^x units. Alterations in the polyC or polyA length, and in other regions, that lead to an *on* or *off* status of *H. pylori futA* and *futB* genes are suspected to be related to active or inactive fucosylation and thus to Le^x expression.[119] At least in part, phase variation in *H. pylori* LPS seems to be mediated by frameshift mutations in the polyC tract of the FucT encoding genes. As described in this article, the expression of Le^x and Le^y antigens in *H. pylori* LPSs varies between strains; therefore, each *H. pylori* strain must possess different levels of FucTs activities, so that the relative levels of *fucA, fucB,* and *fucC* genes in a particular strain dictate the expression pattern of Le^x and Le^y within that strain. Le^x-carrying *H. pylori* strains that do not produce Le^y probably do not make α-1,2-FucT or carry one with very low activity. Such *H. pylori* strains may have a tendency to produce S-form LPSs with elongated Le^x O-chains, whereas strains with a highly active α-1,2-FucT tend to elaborate SR-form LPS with a sole Le^y antigen in the O-chain (Section V) since fucosylation of the nonreducing Gal halts O-chain extension by preventing addition of GlcNAc to O-3 of Gal. In glycotype A LPSs, the ligation between the Lewis O-chain and the core OS is affected by a O-chain–GlcNAc-(1 → 7)-DD-Hep–core or a O-chain–GlcNAc-(1 → 3)-DD-Hep–core linkage.

TABLE III

Classification of *H. pylori* Lipopolysaccharides into Glycotype Families

Glycotype (Lewis Type)	Strain	O-Chain[a]	Heptoglycan	Core[b]	(LPS-form)
					Smooth-form LPS
A (Type-2)	NCTC11637	Le^x-$[Le^x]_n$		Core	
A	P466,	$Le^{x/y}$-$[Le^x]_n$		Core	
		Sialyl Le^x-$[Le^x]_n$		Core	
A	26695	Le^x-$[Le^x]_n$		Core	
A	J99	$Le^{x/y}$-Le^x		Core	
A	SS1	Le^x-$[Le^x]_n$		Core	
A[126]	AF1, 007	$Le^{x/y}$-$[Le^x]_n$		Core	
A	UA1182	$Le^{x/y}$-$[Le^x]_n$		Core	
					Smooth-form LPS
B[c] (Type-2)	O : 3	$Le^{x/y}$-$[Le^x]_n$	Heptoglycan	Core	
					Smooth-form LPS
C	MO19	$Le^{y\pm}$	Heptoglycan	Core	
C	1C2, 12C2,	(Fuc,GlcNAc)$^{\pm}$	Heptoglycan	Core	Asymptomatic hosts
	62C, 7A, 75A,		Heptoglycan	Core	
	77C, PJ1		Heptoglycan	Core	
C	O : 6	$Le^{y\pm}$	Heptoglycan	Core	
					Smooth-form LPS
D[d] (Type-2)	UA861, O : 4	$[LacNAc]_n$		Core	
		|			
		α-Glc			
					Smooth-form LPS
E[d] (Type-2)	471	$[LacNAc]_n$		Core	
		|			
		α-Gal			
					Smooth-form LPS
F (Type-1 and 2)	UA948	$Le^{a/x}$-$[Le^x]_n$		Core	
F	UA955	$Le^{dis/x/y}$-$[Le^x]_n$		Core	

F	J223	$Le^{c/d}\sim[LacNAc]_n$	Core	
F	NCTC11637	Le $^{d/x/y}$-Le^x	Core	
F	R-7A/H608	Type-1 A blood-group-LacNAc	Core	
F		Le^x-Le^x	Core	
F	CA2/H428/H507	$Le^{b/y}$-$[Le^x]_n$	Core	
F	CA5, CA4	$Le^{a/d/x}$-$[Le^x]_n$	Core	
F	GU2	$Le^{a/x}$-$[Le^x]_n$	Core	
				Semi rough-form LPS
G (Type-1 and 2)	26695	$Le^{dis/x/y}$	Core	
		Fuc-1 → 3-Fuc-1 → 4-GlcNAc	Core	
		GlcNAc-1→3-Fuc		
		Linear B blood-group	Core	
G	SS1	Le^y	Core	
		Fuc-1 → 3-Fuc-1 → 4-GlcNAc	Core	
		Linear B blood-group	Core	
G	F-15A	$Le^{a/y}$	Core	
		Fuc-1 → 3-Fuc-1 → 4-GlcNAc	Core	
		Linear B blood-group	Core	
G	CA6	$Le^{a/x}$	Core	
				Semi rough-form LPS
H (Type-1)	UA915	$Le^{b/d}$	Core	
H	UA1111	Le^b	Core	
H	F-58C	Le^a	Core	
		Linear B blood-group	Core	
H	R-58A	Le^a	Core	
I		*H. pylori* strains not expressing histo-blood-group LPS molecules		

[a] The Le^x elongated O chains also contain randomly placed LacNAc units.

[b] All cores share a similar structure. However, the inner most LD-Hep unit may either carry a monoester phosphate or a 2-aminoethyl phosphate at position O-7.

[c] The solidus (/) indicates that either structure is possible.

[d] The α-D-Glc and α-D-Gal side chains are attached to the O-6 position of the GlcNAc. The UA861 O-antigen chain also expresses one internal Le^x. The 471 LPS also contains O-3-fucosylated GlcNAc units.

Glycotype B comprises LPSs with glycotype A-like Lewis O-chains, but which are attached to the heptoglycan domain [DD-Hep oligomer] which in turn is adjoined to the core OS. LPSs with short O-chains carrying a sole Lewis epitope attached to the heptoglycan (similar to that of glycotype B), or just carrying the heptoglycan attached to core, are grouped into the glycotype C family. The heptoglycan domain found in glycotype B and C LPSs must employ an α-1,3-heptosyltransferase (DD-HepT) with the ability of forming homopolymeric heptan linear chains. These heptoglycans may be capped at the nonreducing end by a long Lewis O-chain (glycotype B) connected by a GlcNAc-(1 $\rightarrow$ 3)-DD-Hep linkage, or by a single Le^y (glycotype C) that is attached by a Gal-(1 $\rightarrow$ 3)-DD-Hep linkage. Glycotype C also contains *H. pylori* strains from asymptomatic hosts that were shown to contain mainly uncapped heptoglycan regions in which some were terminated by an incomplete Lewis antigen. Two important, yet unidentifiable, glycosyltransferases involved in heptoglycan-rich glycotypes are a β-1,3-GlcNAcT and a β-1,3-GalT that form the covalent attachment between the Lewis O-chain and heptoglycan domain. A similar β-1,7-GlcNAcT must also be involved in connecting the Lewis O-chain to the core DD-Hep in glycotype A. Characterization of genes involved in these connections will be crucial for creating LPS mutants that are completely devoid of O-chain constituents and are composed only of core/heptoglycan regions in order to study the role of Lewis O-chains in *H. pylori* pathogenesis.

The polyLacNAc O-chains of *H. pylori* LPSs can also be glucosylated or galactosylated, and these LPSs were assigned to glycotypes D and E, respectively. These glycotypes also contained, in small amounts, Le^x and Le^y antigens. *H. pylori* strains containing glycotypes D and E must contain gene products for encoding α-1,6-GlcT and α-1,6-GalT activities. Branching of polyLacNAc O-chains by Glc and Gal units may be a requirement for certain *H. pylori* strains to adopt necessary LPS conformations required for successful pathogenic duties.

Glycotype F represents S-form LPSs that have the ability of producing type-1 and type-2 Lewis blood-groups simultaneously. Glycotype G contains SR-form LPSs also coexpressing type-1 and type-2 histo-blood groups, and glycotype H LPSs express solely type-1 blood-groups in the O-chain region. The expression of type-1 LPS antigens in these *H. pylori* glycotypes depend on the activity of α-1,4-FucT, for fucosylation of GlcNAc at O-4, and of β-1,3-GalT, for galactosylation at O-3 of GlcNAc for Le^a and Le^b production. In *H. pylori* strain UA948, one of the FucT genes, ***futA***, has been shown to encode an enzyme with both α-1,3 (for type-2 Le^x) and α-1,4-FucT (for type-1 Le^a) activities, while the other gene, ***futB***, encodes a truncated protein without any FucT activity.[120] This reflects a high level of interstrain gene diversity. Fucosylation at O-4 of GlcNAc to give Le^a occurs when a Le^c acceptor [Gal-(1 $\rightarrow$ 3)-GlcNAc] is available, a pattern similar to Le^a production in mammalian cells. Galactosylation of GlcNAc at O-3, in *H. pylori* Le^a

fabrication, appears to involve the β-1,3-GalT,[121] HP0619. This β-1,3-GalT gene is responsible for galactosylating GlcNAc to create the type-1 backbone, β-Gal-(1 → 3)-β-GlcNAc (Le^c), of Le^a, H type 1, Le^b, and maybe type-1 blood-group A. For the synthesis of type-1 Le^b in *H. pylori,*[122] the α-1,2-FucT fucosylates Le^a, a pathway not usually found in production of Le^b in mammalian cells. This *H. pylori* α-1,2-FucT can also fucosylate a fucose-free type-1 Le^c antigen to afford the H type-1 antigen (Le^d), which is found in some *H. pylori* strains. Practically, no production of Le^b through fucosylation, by α-1,4-FucT, of H type-1 takes place in *H. pylori*. Also, inactivation of the *futC* (α-1,2-FucT) gene[122] in Le^b-producing strains leads to LPSs solely expressing Le^a, underlining the fact that Le^b production in *H. pylori* is mainly channeled through Le^a. Because of the apparent tendency of Asian *H. pylori* strains to produce type-1 Le^a and Le^b antigens, these strains must have, in comparison to Western strains, gene products with higher α-1,4-FucT and β-1,3-GalT activities. *H. pylori* LPSs containing type-1 antigens seem to be only of the SR-form, since no elongated type-1 chains of Gal-(1 → 3)-GlcNAc repeats have yet been detected in *H. pylori,* which suggests that a possible β-1,3-GlcNAcT that adds GlcNAc onto a type-1 Gal-(1 → 3)-GlcNAc acceptor does not exist in *H. pylori*. Type-1 blood-group A has also been detected among Lewis antigens in *H. pylori* LPS preparations. In *H. pylori,* the presence of GalNAc-(1 → 3)-Gal-(1 → 3)-GlcNAc implies that fucosylation at O-2 of Gal in blood-group A synthesis can take place after the addition of GalNAc to O-3 of the same Gal unit. Some *H. pylori* strains may also make use of *futA, futB,* and *futC* genes to create the Fuc-(1 → 3)-Fuc linkage in the difucosylated antigen, Fuc-(1 → 3)-Fuc-(1 → 4)-GlcNAc. The bridging of the type-1 Lewis O-chains to the core OS in glycotypes F, G, and H is similar to that in glycotype A, where the reducing-end GlcNAc of the O-chain is ligated to O-7 of DD-Hep from core. An atypical *H. pylori* polysaccharide has been found in some strains from a single source in Denmark,[123] which contained no blood-group structures and no similarities to core or heptoglycan regions, and, therefore, glycotype I is reserved here for this *H. pylori* LPS and for any other that in future will be characterized and which will not express any histo-blood-group molecules. There are now six non-*pylori Helicobacter* species that have been isolated from diarrheic humans: *H. pullorum, H. canis, H. rappini, H. fennelliae, H. cinaedi,* and *H. canadensis.*[124] Comparison of ribosomal DNA sequences does not always give conclusive evidence for species-level identification and may be deceptive; hence, one must be careful not to misidentify helicobacters.[125]

Fine structural and genetic data strongly suggest that *H. pylori* LPS Lewis O-chains are assembled by a sugar-by-sugar addition rather than by the most common block-by-block mechanism found in gram-negative bacteria. Perhaps all *H. pylori* strains, given the agreeable conditions, have the ability to generate any of these LPS glycotypes.

XI. Concluding Remarks and Future Directions

The detection of LPSs in *H. pylori* expressing structures homologous to human histo-blood-group antigens stands as one of the most important and crucial discoveries in both *H. pylori* and bacterial-LPS research. The production of Lewis determinants, blood-group A, and linear blood-group B by *H. pylori,* occasionally by a single strain, is a perfect example of molecular mimicry, and of the capability that bacteria possess to elaborate glycan structures similar to those present in their biological niche. In this case, *H. pylori* carry LPSs with structures similar to cell-surface glycolipids and glycoconjugates expressed by the human gastric mucosa. No exact reason for this observed molecular mimicry has yet been categorically established; however, several reasons why *H. pylori* produce histo-blood-group antigens can be put forth. The most obvious is based on the molecular mimicry premise, in that *H. pylori* produces host-like gastric-glycan structures in order to mimic its immediate surrounding and thus avoid being detected by the host's immune system, leading to an uninterrupted long life. A parallel situation is also found in ferrets, in that *H. mustelae* mimics the host's gastric molecules by producing an LPS with a blood-group A structure. Two facts point to this possibility: first, *H. pylori* have a very low endotoxic activity, and, second, the organism, if left undisturbed, can survive in the gastric mucosa for the host's lifetime. Interestingly, *H. pylori* seem to stop colonization in the normal gastric mucosa after this region becomes occupied by cancerous malignancies. This molecular mimicry reasoning supports the fact that *H. pylori* may wish to remain anonymous while living in the host's normal gastric flora. Ultimately, in some cases, *H. pylori* may perform some beneficial role for the host. Another motive may be that *H. pylori* makes use of histo-blood group LPS antigens for initial colonization via adhesion of these LPS epitopes to the host's gastric epithelial cells. The detection of pedestal formations formed by Le^x–Le^x homotypic interactions may be important in the interaction of the bacterium with gastric eukaryotic cells.[99] In fact, isogenic LPS mutants devoid of Lewis O-chains have been shown to lose their capability of adhering to, and colonizing, the human gastric epithelial tissue when compared with the wild-type strain.[112,113] These adhesion properties of *H. pylori* LPS can also be used endogenously so that the bacteria can attach to each other, thus forming colonies that may aid in survival and in colonization. Alternatively, during infection, antibodies directed at the *H. pylori* Lewis O-chain may bind to gastric epithelial molecules that express similar antigens, giving rise to an autoimmune component,[127] especially in chronic type B gastritis and gastric and duodenal ulcers. Yokota and co-workers, based on serological and immunological studies with Japanese *H. pylori* strains, have also suggested that a region of the LPS, but not the Lewis antigens, may also be antigenic and highly immunogenic in humans.[128] From the structural results described here, these prospective antigenic epitopes may be any region of the core OS $\{\alpha$-Glc-$(1 \rightarrow 3)$-α-Glc-$(1 \rightarrow 4)$-

β-Gal-(1 → 7)-[α-Glc-(1 → 6)-α-Glc-(1 → 2)-D-α-D-Hep-(1 → 2)]-D-α-D-Hep-(1 → 2)-L-α-D-Hep-(1 → 3)-[AEP/P → 7]-L-α-D-Hep-(1 → 5)-Kdo}, lipid A, or any of the O-chain structures not detectable by Lewis blood-group mAbs, those being α-Fuc-(1 → 3)-β-GlcNAc, α-Fuc-(1 → 4)-β-GlcNAc, α-Fuc-(1 → 3)-α-Fuc-(1 → 4)-β-GlcNAc, β-Gal-(1 → 3)-β-GlcNAc, β-Gal-(1 → 4)-β-GlcNAc, α-Gal-(1 → 3)-β-Gal-(1 → 3)-β-GlcNAc, or α-Gal-(1 → 3)-β-Gal-(1 → 3)-β-GlcNAc. Ho[129] and Heneghan[130] and co-workers have reported that peptic ulcer disease is associated with increased expression of Lewis antigens in *H. pylori.* The linear B blood-group moiety [α-Gal-(1 → 3)-β-Gal-(1 → 3,4)-β-GlcNAc] present in some SR-form *H. pylori* LPSs may be one of the factors in why humans contain high level of IgG antibodies (known as xeno-antibodies) directed at this epitope, which is involved in organ rejection in xeno-transplantation.[131] Possibly, persons infected with *H. pylori* may have a higher concentration of xeno-antibodies.

The heptoglycan domain present in some *H. pylori* strains probably serves as a biological arm to present the sole Lewis antigen to the host's receptors. Interestingly, many *H. pylori* strains from asymptomatic hosts are devoid of a complete Lewis O-chain and contain this elongated heptoglycan. This heptoglycan region may cover disease-causing outer membrane proteins, which, coupled with the lack of Lewis O-chains, probably impedes these strains in activating pathological responses. Perhaps, after successful colonization by *H. pylori* cells with high-M_r S-form LPSs cells covered by SR-form LPSs, containing a short O-chain with a single blood-group antigen, become prevalent and expose disease-causing cell-surface molecules; in fact, *H. pylori* containing low-M_r SR-LPS LPSs with type-1 antigens (Le^a and Le^b) are very common in Asian countries (Section VI), and in these regions *H. pylori* infections are more symptomatic and severe[132] than in the Western world, where *H. pylori* with high-M_r LPS of type-2 (Le^x and Le^y) antigens are more widespread. Various studies dealing with the virulence of *H. pylori* LPS have been undertaken showing multiple LPS-associated immunogenic responses and behaviors.[133] The performance of any study delving into the immunogenic or pathogenic role of *H. pylori* LPSs needs to take into consideration the chemical structure of the LPS employed, since the variability in histo-blood-group expression differs between strains and thus each LPS may yield different results.

H. pylori has already been the source of several functional glycosyltransferases, FucTs and GalTs, with potential commercial uses, especially in enzymatic synthesis and modifications of histo-blood-group molecules for therapeutic uses. However, in the near future, other histo-blood-group-related glycosyltransferases will surely be identified, with those of special interest being the β-1,3-GlcNAcT (polyLacNAc synthesis), α-1,2-GalNAcT (blood-group A synthesis), α-1,3-Gal (linear blood-group B synthesis), and α-2,3-Neu5AcT (sialyl Le^x synthesis). Other *Helicobacter* species, for example *H. mustelae* and *H. felis,* may also be good sources of histo-blood-group glycosyltransferases. The creation of well-characterized LPS isogenic mutants, by disrupting glycosyltransferases,

for utilization in animal studies will yield answers regarding the role of LPS in *H. pylori* pathogenesis and in which host-immunity pathways this disease operates. One of the leading queries regarding *H. pylori* LPS is whether these molecules, or mimics thereof, can be used as therapeutic agents. The synthesis of glycoconjugate vaccines containing *H. pylori* LPS does not seem to be a viable solution due to the fact that this organism is extremely hard to grow *in vitro*. Unless new methodologies in *H. pylori* growth are devised, the realistic quantities needed for mass production of LPS-based vaccines would most likely not be met. Moreover, employment of vaccines containing Lewis or any other blood-group epitopes almost certainly to be expressed by the host, will probably not confer protection. Other regions of the LPS, synthetic homologues, or peptide mimics, however, may prove highly useful in conferring an immune protection when used in a glycoconjugate vaccine, and these regions may also be useful in developing a serologically based *H. pylori* diagnostic.

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ADVANCES IN CARBOHYDRATE CHEMISTRY AND BIOCHEMISTRY, VOL. 57

STRUCTURE AND BIOLOGICAL INTERACTIONS OF HEPARIN AND HEPARAN SULFATE

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I. INTRODUCTION

Heparin has been in clinical use for decades, to prevent and cure thromboembolic disease. It is only relatively recently that the molecular mechanisms behind the anticoagulant/antithrombotic effects were elucidated. A previous article on the subject in this series[1] highlighted some major advances in our understanding of these mechanisms, in particular regarding the role of antithrombin (AT) and its interaction with the polysaccharide. These relations can now be explained in molecular detail. A most important development of recent years has been the growing awareness of the ubiquitous distribution, structural diversity, and biological importance of heparan sulfate (HS). Formerly by and large an unwanted by-product

0065-2318/01 $35.00

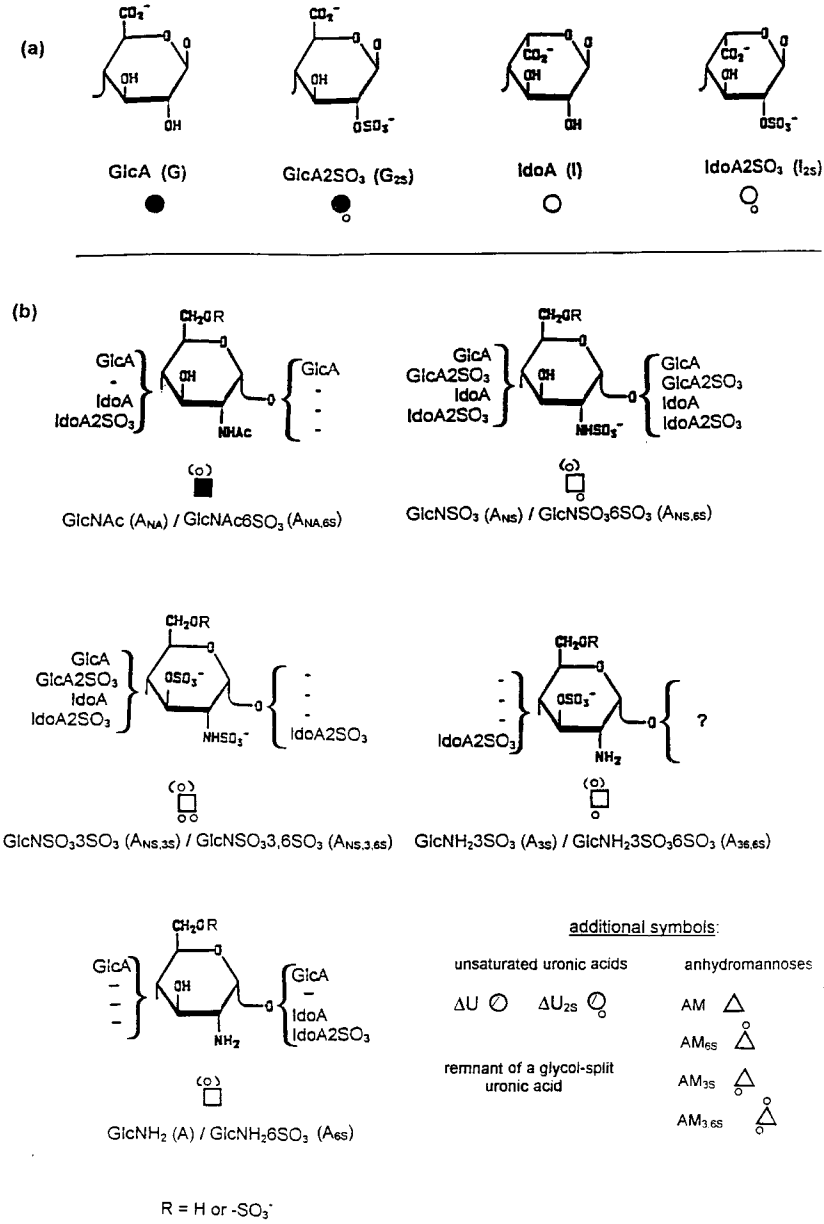


FIG. 1. Monosaccharide components of heparin/HS, with abbreviations and symbols. (*a*) Uronic acid residues. (*b*) Glucosamine residues, with adjacent uronic acid units. The display of variously substituted GlcN residues and adjacent units are based on reported HexA-GlcN and GlcN-HexA

of heparin manufacture, HS is now recognized as a family of multiple, closely related yet distinct polysaccharide species. In fact, heparin may well be considered merely another member of this family. Owing to the structural diversity of the heparin/HS family, the polysaccharides are capable of binding a large number of different proteins, and thus may be conceived as important regulators of a variety of biological processes. Intense research is currently aimed at clarifying the regulatory functions of HS in growth factor and cytokine action. Conversely, several biological effects exerted by exogenous heparin are now being considered in terms of either reinforcing or competing with, HS functions.

Along with the deepened understanding of HS structure and function, there has been growing insight into the mechanisms that control the formation of specific saccharide sequences during the biosynthesis of heparin/HS. In particular, this progress has entailed the molecular cloning of the enzymes involved in the biosynthetic process and on their characterization at the gene level. Various aspects of this development have been discussed in reviews,[2–6] symposium proceedings,[7] and a book.[8] Biological activities of heparin/HS other than anticoagulant/antithrombotic have also been extensively studied and reviewed.[8–14]

The present article will deal mainly with structural aspects and structure–activity relationships of heparin/HS, but also with pertinent findings regarding polysaccharide formation, through biosynthetic processes or chemical synthesis/modification.

II. Biosynthesis

Although heparin and HS are often described in terms of their prevalent disaccharide sequences, the complex biosynthesis of these glycosaminoglycans (GAGs) leads to several combinations of sequences involving nonsulfated and variously sulfated D-glucuronic acid (GlcA), L-iduronic acid (IdoA), *N*-acetyl-D-glucosamine (GlcNAc), *N*-sulfo-D-glucosamine ($GlcNSO_3$), and D-glucosamine (GlcN) units (Fig. 1). All of these residues are α-(1→4)-linked except GlcA, which is β-(1→4)-linked.

Heparin is synthesized in connective-tissue type mast cells, as part of the serglycin proteoglycan.[16] HS is produced by most mammalian (and many other) cells and is bound to a variety of core proteins, in particular syndecans, glypicans, perlecan, and agrin.[17–20] Following translation in the rough endoplasmic

disaccharide sequences, and do not represent demonstrated trisaccharide structures. Gaps indicated by (–) denote combinations that have not, so far, been found; some of these combinations, e.g., GlcNAc-IdoA, apparently do not exist. The question mark at C-1 of the $GlcNH_23SO_3$ unit indicates lack of information regarding any HexA residue in this position. References to reports that demonstrate the sequences are given in Refs. 10 and 16. Novel information regarding structures around 3-*O*-sulfated GlcN units is provided in Refs. 13 and 15. The sequence of two GlcA units adjacent to an N-unsubstituted GlcN residue has been reported in Ref. 14.

reticulum (RER), the core proteins are transported to the Golgi apparatus, where the enzymes responsible for the HS/heparin biosynthesis are located. Selected serine units are O-substituted with the GlcA-Gal-Gal-Xyl- "linkage region" that connects the polysaccharide chain proper with the core protein.[21] The carbohydrate structure of this region is the same for glucosaminoglycan- (heparin/HS) and galactosaminoglycan (chondroitin sulfate, dermatan sulfate) containing proteoglycans (although the substituent pattern may differ),[22,23] and the same glycosyltransferases appear to be involved in its biosynthesis, regardless of the nature of the glycosaminoglycan (GAG) chain.[24] Most of these enzymes have now been cloned (see Ref. 25). The next reaction, transfer of a GlcNAc residue from UDP-GlcNAc to form an α-linkage to C4 of the GlcA unit in the linkage region, appears to be the committing step toward formation of HS/heparin rather than chondroitin/dermatan sulfate. The corresponding enzyme is apparently able to distinguish between core proteins designed for HS and chondroitin sulfate attachment, presumably through recognition of the peptide structural features previously associated with HS addition (a cluster of acidic residues, hydrophobic amino acids, and a close spacing of glycosylation sites).[26] A GlcNAc transferase with the corresponding properties has been partially purified[27] and was more recently cloned.[28] Remarkably, this enzyme is capable of transferring an α-GalNAc unit as well to the same acceptor, thus distinct from the β-GalNAc transfer that initiates chondroitin sulfate formation.[29,30] The α-GalNAc residue may conceivably serve as a stop signal that prevents further GAG formation. Formation of the actual $[\beta\text{-GlcA-}(1\rightarrow4)\text{-}\alpha\text{-GlcNAc-}(1\rightarrow4)]_n$ heparin/HS precursor structure is catalyzed by a GlcA/GlcNAc copolymerase.[31] Cloning of this enzyme remarkably revealed the existence of two isoforms that were identical with the alleged tumor suppression factors EXT1 and EXT2.[32,33] Intriguing recent data suggest that EXT1 and EXT2 function as a hetero-oligomeric complex in the Golgi.[34,35]

Conversion of the $(\text{GlcA-GlcNAc})_n$ precursor structure into the products recognized as heparin/HS occurs through a series of polymer-modification reactions, schematically illustrated in Fig. 2*a*,[4] which also shows the structures of the major disaccharide sequence **1** of the extensively modified NS-domain, and **2** of the unmodified NA-domain. This polymer modification appears to be initiated while the chain is still under elongation.[36] The individual reactions and their concerted action have been described in recent reviews.[3,4] The first modification step, N-deacetylation and N-sulfation of GlcNAc residues, is catalyzed by yet another bifunctional enzyme. The resultant *N*-sulfate groups are prerequisite to all subsequent modifications, which include C-5-epimerization of GlcA to L-iduronic acid (IdoA) units, 2-O-sulfation of GlcA and IdoA units, and 6-O- and 3-O-sulfation of GlcN residues. The process occurs in at least partly stepwise fashion, such that the products of a given reaction will provide the substrate for subsequent reactions. However, most of the reactions do not go to completion, as typically a fraction of the potential substrate residues in each step will escape modification. Such partial

polymer modification is a fundamental feature of the biosynthetic process, and the direct cause of the diversified domain structure of HS chains. In this context heparin may be regarded as a product of less restricted polymer modification, essentially an extended NS-domain (Fig. 2a). A typical product of strictly regulated polymer modification is the hexasaccharide **3** (Fig. 2*b*), which incorporates the minimal (pentasaccharide) AT-binding sequence **4.**

Although the basic mechanisms of biosynthetic polymer modification have been elucidated, the overall organization and regulation of the process remain poorly understood. Most of the enzymes involved are transmembrane proteins, and it has been hypothesized that they form one or more complexes, attached to the Golgi membranes, that attack the polysaccharide chain in processive fashion (see model in Ref. 2). In apparent agreement with this notion, polymer modification is a rapid process, a heparin chain being completed in less than 30 s.[37] Although this model seems appealing it is not established, and we cannot exclude other modes of GAG assembly. Whatever the final solution to this problem, it will have to account for the remarkably stringent control of HS expression, as revealed through compositional analysis of HS from different organs,[38,39] and, in particular, through immunohistochemical application of monoclonal antibodies against different HS epitopes.[40,41]

Recent results of cloning of these enzymes add further complexity. All enzymes (with some exceptions) are transmembrane proteins with type II topology. However, several species occur as genetically distinct isoforms, the functional characteristics of which have only begun to be unraveled, but clearly involve variations in expression pattern as well as in substrate specificity and catalytic properties. The mode of expression and the substrate specificities of such isoforms presumably dictate the structural properties of the final polysaccharide product. Although a detailed account of the various enzyme species falls outside the scope of this chapter, some conspicuous features will be highlighted. The GlcNAc transferase that adds the first GlcNAc residue onto the linkage region was found to belong to the same protein family as the EXT1/EXT2 copolymerase enzymes.[28] The GlcNAc *N*-deacetylase/*N*-sulfotransferase (NDST in Fig. 2) that catalyzes the initial modification of the $(GlcA\text{-}GlcNAc)_n$ polymer has been shown to occur in at least four different isoforms.[42–45] Two of these species, NDST-1 and NDST-2, both show ubiquitous expression in a variety of cells and tissues. Overexpression experiments indicated that the latter form is more prone to generating extended *N*-sulfated sequences, of the type found in heparin.[46] Remarkably, targeted disruption of the NDST-2 gene in mice selectively abolished the biosynthesis of heparin in connective-tissue type mast cells, but had no noticeable effect on HS biosynthesis, or on the apparent health state of the animals.[47] In contrast, NDST-1 knockout mice synthesized undersulfated HS and died before or during the neonatal period, with severe multiple organ defects.[48,49] Also, the GlcN 6-*O*- (6-OST in Fig. 2)[50] and 3-*O*- (3-OST)[13] sulfotransferases show genetic polymorphism, each enzyme

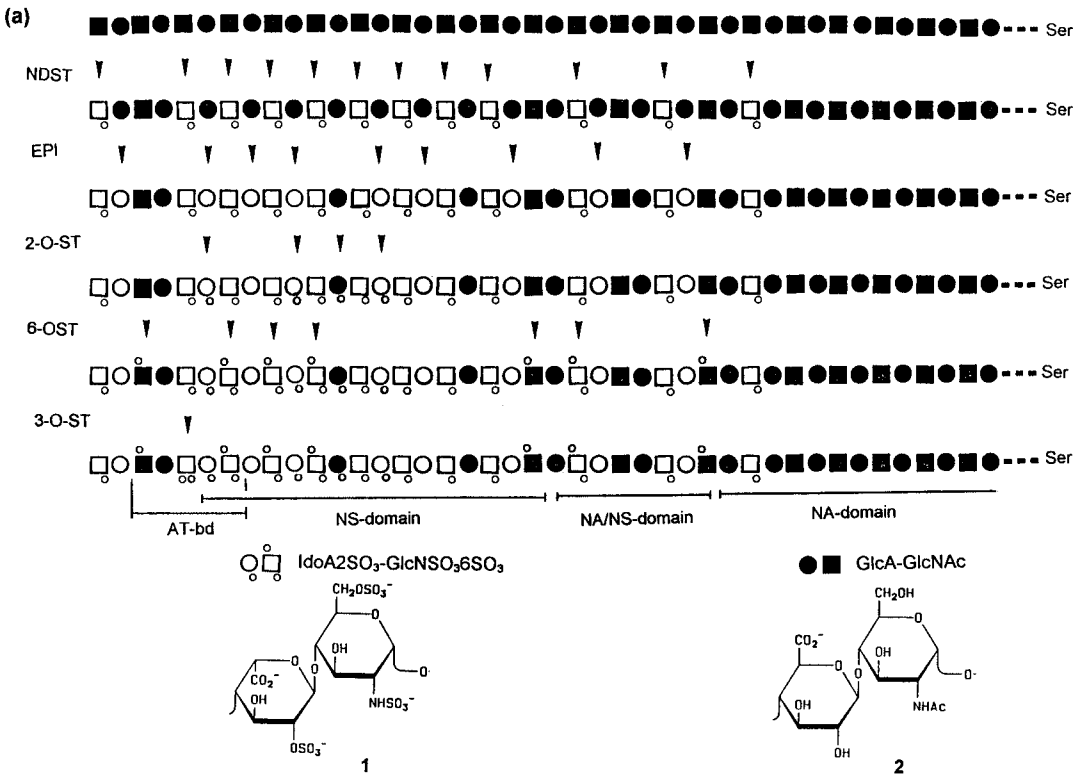


FIG. 2. (*a*) Generation of the typical domain structure during biosynthesis of HS. For definition of symbols see Fig. 1, and the two model disaccharide units shown below the scheme. The target residues for each enzymatic reaction are indicated by arrowheads. NS-domains are composed of contiguous N-sulfated disaccharide units, NA/NS-domains by alternating N-sulfated and N-acetylated units, and NA-domains by contiguous N-acetylated units. AT-bd: AT-binding pentasaccharide sequence. (Modified from Ref. 4.) (*b*) Detailed structure of the AT-binding sequence (units 1–5 between the dotted vertical lines). The circled sulfate groups are all essential for the interaction with AT. (Reprinted with permission from Ref. 4).

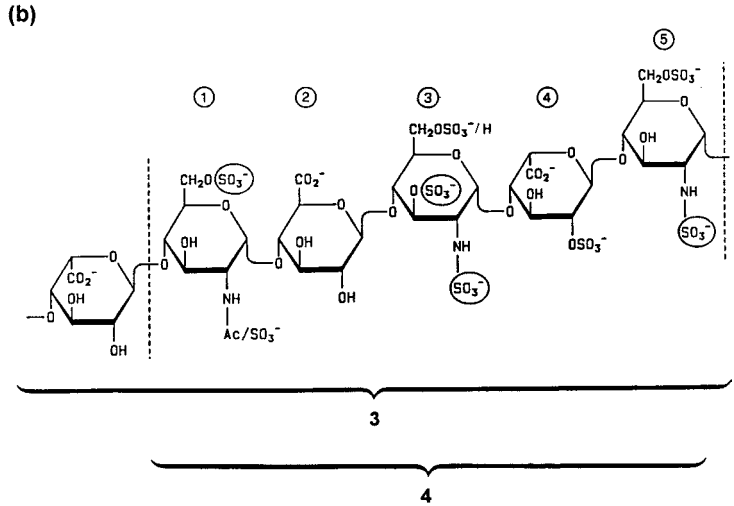


FIG. 2. (*continued*)

occurring in at least three different forms. Interestingly, only single forms of the GlcA C-5-epimerase (EPI)[51] and the IdoA 2-*O*-sulfotransferase (2-OST)[52,53] have been found so far. IdoA units in different structural contexts, such as the "alternating" NA/NS domains and the contiguous NS structures of HS chains, or of heparin, thus may all be generated by the same enzyme. Moreover, the 2-OST sulfates not only IdoA but also GlcA units.[53–55] In fact, overexpression of recombinant 2-OST in human embryonic kidney cells led to selective 2-*O*-sulfation of GlcA units in endogenous HS,[53] suggesting that the relative abundance of such residues in HS from certain tissues[38] may depend on the disposition of 2-OST in the Golgi system of the appropriate cells.

In the future, functional aspects of HS will be increasingly correlated with analyses of epitope structure and the substrate specificities of biosynthetic enzymes. As an example to highlight this development, N-unsubstituted GlcN residues were shown, by an immunohistochemical approach, to be part of native HS structures.[40] Although the mechanism of formation of N-unsubstituted GlcN units is unknown, one of the recently discovered NDST isoforms, NDST-3, is potentially implicated owing to its relatively high *N*-deacetylase activity and low *N*-sulfotransferase activity compared to other NDST isoenzymes.[56] The occurrence of N-unsubstituted GlcN residues was found to correlate with the ability of HS to interact with L-selectin.[57] Moreover, one of the 3-OST isoforms preferentially attacks N-unsubstituted GlcN residues,[15] and the resultant GlcN(3-OSO_3) structure is specifically recognized by the capsular gD glycoprotein of *Herpes simplex* 1 virus, in an essential step of viral penetration of the host cell.[13] The

proposed role of 3-*O*-sulfation also in formation of HS-FGF-FGFR complexes[58] is not supported by more recent experiments.[59] Identification of an increasing number of novel HS sequences underlines the view that HS is not always differentiated from heparin by mere compositional analysis. Although HSs are generally richer in *N*-acetylated disaccharide units and contain fewer *O*-sulfates (especially $IdoA2SO_3$) than heparins,[60,61] a clearcut borderline between the two species is not easily drawn. A more distinctive feature is the typical domain organization of HS chains, as dictated by the patterns of N-substituents and complemented by differential distribution of the various *O*-sulfate groups.[61–64]

III. Structural Analysis

1. General Strategies

Elucidation of heparin and HS structures has been continuously expanded by optimizing classical methods as well as by developing more efficient separation and spectroscopic approaches. Although in favorable cases "fingerprints" of heparin preparations, provided by nuclear magnetic resonance (NMR) spectra, may permit compositional analysis and characterization of sulfation patterns,[65,66] detailed analysis of heparin (and especially of the more complex HS) structures largely requires the characterization of GAG fragments generated by heparin lyases or nitrous acid, or both.[8] Anion-exchange HPLC,[67,68] gradient polyacrylamide gel electrophoresis (PAGE),[68,69] agarose gel electrophoresis,[70] and capillary electrophoresis (CE)[71–73] are examples of methodologies that have contributed to isolate heparin fractions and heparin/HS oligosaccharides amenable to structural analysis by a combination of chemical, enzymatic, and spectroscopic methods.[66]

Mass spectrometry (MS) has definitely become established as an important tool for structural analysis of sulfated oligosaccharides, previously hardly accessible to this analytical approach.[74] These advances have become possible through development of strategies for exploitation of the fast atom bombardment (FAB),[75–77] FAB-tandem (MS-MS-FAB),[78,79] electrospray ionization (ESI),[80–82] and liquid secondary desorption (LSI)[83] MS techniques. More recently, matrix-assisted laser desorption ionization (MALDI) has added a new dimension to MS, because of its almost unlimited, though still largely unexploited, potential to analyze subnanogram amounts of sulfated glycosaminoglycan fragments over a wide range of molecular weights.[84,85] This technique is now used in combination with chemical–enzymatic degradation methods and use of endolytic enzymes (see Section III.3.c),[86,87] and also for sequencing of heparin/HS oligosaccharides.[88] Whenever samples in the milligram range are available, two-dimensional NMR methods may provide assessment of sequence and molecular conformation.[82,89]

2. Compositional Analysis

Heparin/HS chains are not amenable to the classical approach for compositional analysis of polysaccharides, involving cleavage with acids of all glycosidic bonds and profiling the monosaccharide composition of hydrolyzates. In fact, both the negative charge of the uronic acid residues and the positive charge on free amino groups exposed by *N*-desulfation of sulfamino groups stabilize most of the glycosidic bonds of these GAGs. Attempts to circumvent the problem by acid-labilizing the uronic acid to glucosamine bonds by carboxyl reduction have been also unsuccessful because of easy conversion of resulting idose residues into anhydro derivatives.[1,8] Methylation analysis (another common approach to compositional analysis of neutral polysaccharides) has likewise had limited success in the analysis of highly sulfated GAGs, mainly because of difficulties in achieving complete etherification of free hydroxyl groups and subsequent cleavage to methylated monosaccharides without loss of sulfate groups. Efforts toward improvement of nondestructive hydrolysis and quantification of methylated monosaccharides by gas chromatographic (GC) and GC/MS methods permit partial profiling of heparin monosaccharide components.[90]

Also, determination of total uronic acids and total hexosamine, classically performed by colorimetric methods, has lost popularity mainly because different responses are obtained (especially for the uronic acids) depending on the nature of the uronic acid and sulfation patterns of the uronic acid as well as the hexosamine. Chromatographic/colorimetric methods have been specifically developed for determining molar ratios between GlcA and IdoA in heparin/HS hydrolyzates.[91] Again, these methods also have been largely superseded by the successful development of methods for disaccharide analysis, which, in addition to quantification of different uronic acid and glucosamine residues, provide further structural information (see later). Whenever the amount of sample is not a limiting factor, individual residues (especially IdoA, IdoA2SO_3, GlcA, GlcNS, GlcNAc), and the extent of 6-O-sulfation on the glucosamines can be reliably determined by NMR spectroscopy directly on unmodified heparins[65,92] and HSs.[93] Though with less sensitivity, the extent of 3-O-sulfation of glucosamines can also be estimated by direct NMR spectroscopy.[65,92,94,95]

3. Methods of Depolymerization and Sequence Analysis

a. Enzymic Methods.—*Flavobacterium heparinum* and other bacteria induced to grow on GAGs contain a number of heparin/HS degrading enzymes, the most widely exploited ones belonging to the class of lyases, which cleave glycosidic bonds through a β-elimination reaction. Since the early article in this series,[1] extensive studies have been done with purified lyases (reviewed in Refs. 8, 96–99). Recombinant heparin/HS lyases have also become available.[100,101]

The manufacture of heparin/HS-degrading lyases by independent groups has generated some confusion regarding the nomenclature for these enzymes. Some research groups and manufacturing companies distinguish lyases that preferentially act on the most abundant sequence of heparin as "heparinase" and those that more efficiently cleave HS as "heparitinases," this latter nomenclature being based on the earlier designation of HS as heparitin sulfate. Also, the term "heparanase" proposed in the early review in this series as a substitute for "heparitinases"[1] is misleading, since it does not distinguish HS-lyases from the now better known HS-cleaving hydrolytic endoglycosidase (see later). Nowadays, the three major heparin/HS-depolymerizing enzymes are most commonly designated lyase I, II, and III.[8] To avoid confusion with glycosidases, in the present article they will be named heparin lyases I, II, and III.

Heparin lyases cleave glucosaminidic bonds between $GlcNSO_3$ (or GlcNAc) and uronic acid residues (IdoA or GlcA) with different specificities depending on the type and sulfation of the uronic acid and the sulfation state of the preceeding glucosamino residue and the following uronic acid residue. The substrate specificity of heparin lyases as determined using heparin/HS and well-characterized fragments of the polysaccharides is summarized in Fig. 3.[98]

The enzyme most extensively used for cleavage of heparin is heparin lyase I (E.C. 4.2.2.7), which cleaves bonds between $GlcNSO_3(6SO_3)$ and $IdoA2SO_3$ and, thus, preferentially acts on the highly sulfated regions of heparin and HS. Heparin lyase II (no E.C. number) has a much broader specificity than the other two heparinases, since it cleaves linkages between glucosamine and uronic acid units irrespective of the type of uronic acid residue or the sulfation of either residue.[102] In contrast, heparin lyase III (E.C. 4.2.2.8) does not work when the uronic acid is 2-O-sulfated. Although heparin lyase III is generally more active when the uronic acid is GlcA and the glucosamine is *N*-acetylated, cleavage of $GlcNSO_3$–IdoA bonds has also been observed with this enzyme.[102] Requirement of *N*-acetylation for cleavage by lyase III seems more strict when GlcA is the only uronic acid. In fact, whereas both the $(GlcNAc\text{-}GlcA)_n$ sequences of the *Escherichia coli* K5 polysaccharide and $(GlcNSO_3\text{-}GlcA)_n$ sequences of its *N*-sulfated analogue are good substrates for heparin lyase II, heparin lyase III cleaves only the former.[103]

Treatment of heparin/HS with individual lyases generates a variety of oligosaccharides. Heparin preparations from pig instestinal mucosa incubated with heparin lyase I currently generate five major di- and oligo-saccharides, the most abundant one arising from sequences of the trisulfated disaccharide $IdoA2SO_3\text{-}GlcNSO_36SO_3$ (**1**).[104] Probably because the enzyme requires at least three consecutive units of **1** for effective action (see Ref. 1 and references therein), about one-third of these sequences usually remain as tetrasaccharides. A possible reason for reported discrepancies as regards specifities of heparin lyases is the molecular-weight dependence of their activity, the longer chains being usually

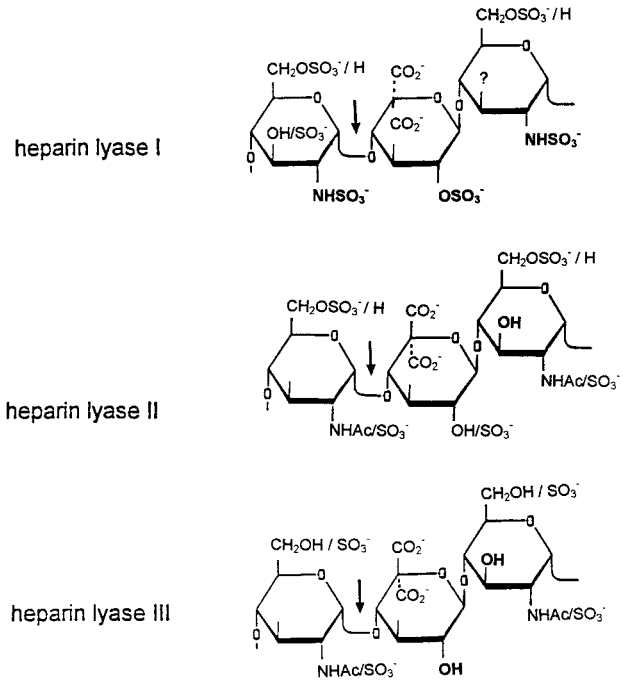


FIG. 3. Substrate specificity of heparin lyases. The arrows indicate the glucosaminidic linkages cleaved by the enzymes. Both configurations of the carboxylate group are shown when both IdoA and GlcA are compatible with lyase action. An unsubstituted hydroxyl group at C-3 of the GlcN residue toward the reducing end is a prerequisite for susceptibility to lyases II and III. Other groups essential for cleavage with the indicated enzyme are shown in bold type. (Adapted from Ref. 98.)

cleaved at higher rates.[104,105] Also, apparent discrepancies as regards the mechanism of action of heparin lyase I can be reconciled by the evidence that, although some internal linkages are cleaved by the enzyme at a detectable rate, this lyase acts predominantly with an exolytic mechanism.[106] By contrast, heparin lyase II is an endolytic enzyme.[107]

As expected, the major fragments generated from HS by heparin lyase III[108] are different from those generated by heparin lyase I from heparin.[104] The most represented sequences in the low-sulfated, "irregular" regions of pig mucosal heparin (accounting for approximatively 20% of the starting polysaccharide) have been identified upon extensive digestion with heparin lyase I and structural characterization of the corresponding oligosaccharides. Eight out of the 10 isolated oligosaccharides shared the trisaccharide sequence -I-A_{NA}-G-.[109] Two hexasaccharides, two octasaccharides, and a decasaccharide isolated from the irregular region of heparin contain 3-*O*-sulfated GlcN residues. One of the tetrasaccharides contains this residue at the reducing end, indicating that 3-*O*-sulfation of GlcN does

not prevent cleavage by heparin lyase I.[110,111] Similarly, 2-*O*-sulfation of GlcA residues is not incompatible with action of heparin lyase I.[112] D-Mannosamine-containing tetrasaccharides and disaccharides identified in lyase digests of HS[113] and heparin,[114] respectively, are artifacts due to alkali-induced C-2-epimerization of the corresponding D-glucosamine disaccharides.[113,114]

Heparins and HSs from different animals, organs, and tissues significantly differ in their relative contents of lyase-generated oligosaccharides.[64,115] On the other hand, the oligosaccharide profiles from human heparin and from pig mucosal heparin are similar.[116] Widely different oligosaccharide profiles as compared with pig mucosal preparations have been reported especially for mollusk heparins[117] and HS,[118,119] the former being unusually rich in 3-*O*-sulfated glucosamine residues. Different oligosaccharide compositions have also been reported for HS from different cell lines (examples in Refs. 119–121). The structures of major disaccharide fragments (**5**–**12**) generated by depolymerization of heparin/HS with lyases are shown in Table IA. Tetra- and higher oligo-saccharides identified so far in lyase digests of heparin/HS (**13**–**90**) are listed in Table IIA.

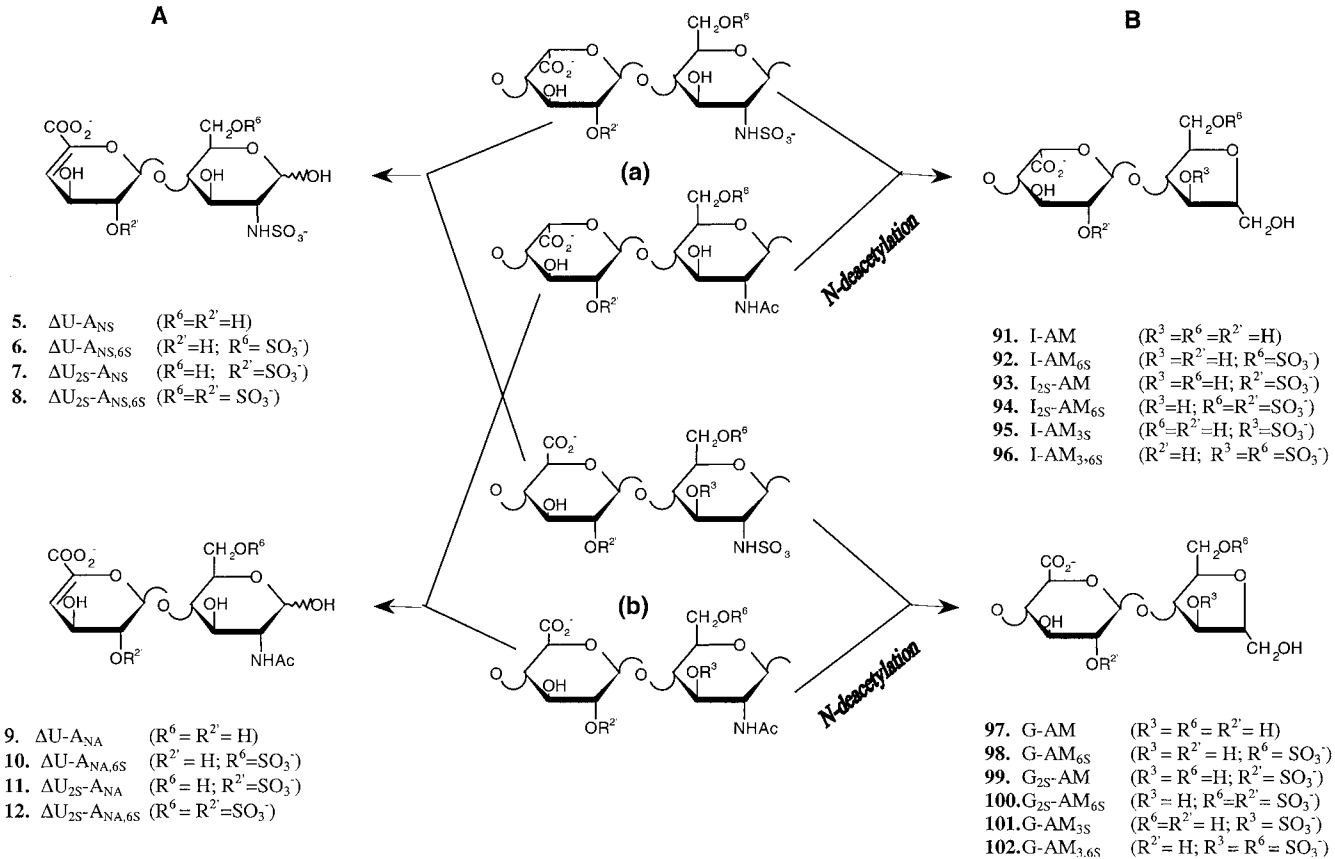
Combined incubation with the three heparin lyases eventually converts heparin and HS to disaccharides, the only sequence invariably resistant to cleavage being the "linkage region."[21,98] The HPLC profiles of the disaccharide pools thus obtained are conveniently used for compositional characterization of heparins and HS of different origin; notably, however, information regarding GlcA/IdoA ratio is lost.

Enzymes other than heparin lyases of potential use in the elucidation of heparin/HS structures are the heparanases. These endoglycosidases, of animal origin (murine metastatic melanoma cells,[145,146] human hepatoma and placenta,[147] and platelets[147–149]) cleave glucosiduronic bonds between GlcA and GlcN residues, including the one within the pentasaccharide sequence of the active site for AT (linkage between residues 2 and 3 in **4**).[150,151] Experiments using various derivatives of the *E. coli* K5 polysaccharide as substrates suggested that the minimal HS-type sequence recognized by heparanase includes 2-*O*-sulfation of the uronic acid residue adjacent to the cleaved glucuronosidic bond (corresponding to residue 4 in **4,** but without requirement for L-*ido* configuration).[151,152] Notably, there is still uncertainty as to the number of existing heparanase enzymes[149,151,152]; only one species has been cloned so far. The availability of recombinant heparanases[147,153,154] is likely to provide further insight into the enzyme specificity as well as an additional tool for structural studies.

b. Chemical Methods.—The most successful chemical method used for cleavage of heparin/HS chains is depolymerization with nitrous acid (reviewed in Refs. 1 and 8). At pH 1.5, the HNO_2 reagent cleaves bonds between N-sulfated glucosamine and uronic acid residues, with formation of 2,5-anhydromannose residues at the sites of cleavage. The NS domains of heparin/HS are thus converted

TABLE I

Disaccharides Generated by Exhaustive Digestion of Heparin/HS with Heparin Lyase (A, Refs. 122–125) and with Nitrous Acid (B, Refs. 150, 155–157). (a) from IdoA-containing Sequences; (b) from GlcA-containing Sequences.



5. ΔU-A_{NS} (R^6=$R^{2'}$=H)
6. ΔU-$A_{NS,6S}$ ($R^{2'}$=H; R^6= SO_3^-)
7. ΔU_{2S}-A_{NS} (R^6=H; $R^{2'}$=SO_3^-)
8. ΔU_{2S}-$A_{NS,6S}$ (R^6=$R^{2'}$= SO_3^-)

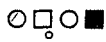
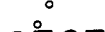
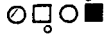
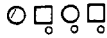
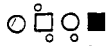
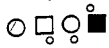
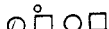
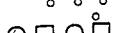
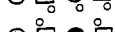
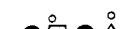
9. ΔU-A_{NA} (R^6 = $R^{2'}$= H)
10. ΔU-$A_{NA,6S}$ ($R^{2'}$ = H; R^6=SO_3^-)
11. ΔU_{2S}-A_{NA} (R^6 = H; $R^{2'}$=SO_3^-)
12. ΔU_{2S}-$A_{NA,6S}$ (R^6 = $R^{2'}$=SO_3^-)

91. I-AM (R^3 =R^6 =$R^{2'}$ =H)
92. I-AM_{6S} (R^3 =$R^{2'}$=H; R^6=SO_3^-)
93. I_{2S}-AM (R^3 =R^6=H; $R^{2'}$=SO_3^-)
94. I_{2S}-AM_{6S} (R^3=H; R^6=$R^{2'}$=SO_3^-)
95. I-AM_{3S} (R^6=$R^{2'}$=H; R^3=SO_3^-)
96. I-$AM_{3,6S}$ ($R^{2'}$=H; R^3 =R^6 =SO_3^-)

97. G-AM (R^3 = R^6 = $R^{2'}$= H)
98. G-AM_{6S} (R^3 = $R^{2'}$ = H; R^6 = SO_3^-)
99. G_{2S}-AM (R^3 = R^6 =H; $R^{2'}$= SO_3^-)
100. G_{2S}-AM_{6S} (R^3 = H; R^6=$R^{2'}$= SO_3^-)
101. G-AM_{3S} (R^6=$R^{2'}$= H; R^3 = SO_3^-)
102. G-$AM_{3,6S}$ ($R^{2'}$ = H; R^3 = R^6 = SO_3^-)

TABLE II

Identified Heparin/HS Fragments Generated by Depolymerization of Heparin/HS with Heparin Lyases (A) and with Nitrous Acid (B)*

A			B				
		Refs.		Refs.			Refs.
Tetrasaccharides							
13		126, 127					
14		126, 127					
15		126, 127	**103** 	155, 157	**104** 		155, 157
16		126, 127					
17		14					
18		126					
19		126, 127					
20		126	**105**	155, 157	**106**		155, 157
21		128	**107** 	150, 155			
22		128	**108** 	150, 155			
23		128					
24		105	**109**	158			
25		129					
26		126, 129					
27		126, 129					
28		127, 128					
29		129					
30		112	**110**	157	**111**		155, 157, 159
31		128					
32		130	**112** 	155			
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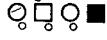
34		128					
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36		131					

TABLE II (*continued*)

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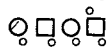
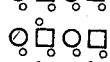
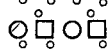
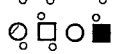
39		112			
40		131, 133			
41		131			
42		112			
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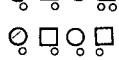
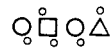
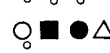
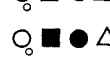
45		15			
46		15			
47		112			
48		130, 133	**114**		160, 161
			115		155
			116		158
Hexasaccharides					
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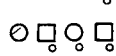
50		135			
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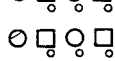
52		87			
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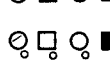
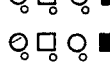
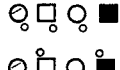
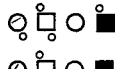
54		127			
55		109			
56		109			
57		109			
58		109			

TABLE II (*continued*)

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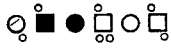
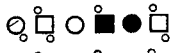
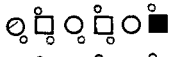
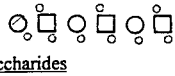
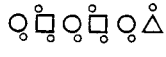
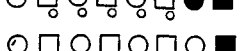
60		111			
61		131			
62		136			
63		136			
64		109, 137			
65		137			
66		137			
67		138, 139			
68		139, 140	**117**		162, 163
Octasaccharides					
69		135			
70		135			
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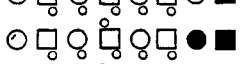
72		135			
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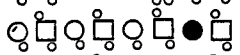
75		140			
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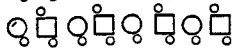
77		142			
78		111			
			118		165
			119		164
			120		150

TABLE II (*continued*)

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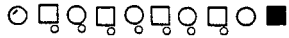
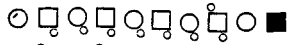
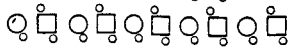
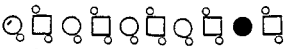
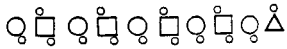
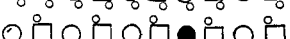
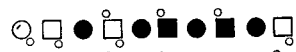
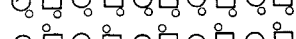
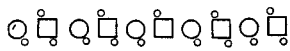
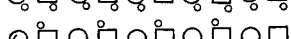
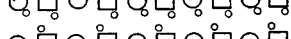
79 	135		
80 	135		
81 	126		
82 	126	**121** 	166
83 	143		
84 	111		
Dodecasaccharides			
85 	121		
86 	142		
87 	142		
Tetradecasaccharides			
88 	142		
89 	142		
90 	144		

* Adapted from Ref. 98. The table does not include odd-number oligosaccharides. [a] Isolated also in nonreduced form[155]; [b] isolated also in nonreduced form[158]; [c] isolated only in nonreduced form[158]; [d] for a revised structure, see Refs. 88, 189.

to disaccharides **91**–**96** (Table IB), while the NA domains are unaffected by the reaction and recovered as oligosaccharides. Sequences of alternating *N*-sulfated and *N*-acetylated disaccharide units yield tetrasaccharide products. *N*-Deacetylation (usually performed with hydrazine) makes the NA domains also amenable to HNO_2 depolymerization, although at pH 3.9, with eventual formation of one or more of the disaccharides **91**–**102.** *N*-Deacetylation followed by exhaustive nitrous acid depolymerization (reactions at pH 1.5 and 3.9; alternatively at pH 3, which will lead to cleavage of *N*-sulfated as well as at *N*-unsubstituted glucosamine residues) thus leads to complete conversion of heparin/HS preparations to disaccharides, the analysis of which (usually performed by HPLC after reduction with $B^3H_4^-$)[155,156] permits profiling in terms of disaccharide composition. Like the depolymerization

with lyases, the reaction with nitrous acid preserves the sulfation patterns of disaccharide units in the original polysaccharides. It has the additional advantage of also preserving the configuration at C-5 of the original uronic acids, thus permitting the distinction of IdoA- from GlcA-containing disaccharides, including 2-O-sulfated IdoA and GlcA, which cannot be differentiated in heparin lyase digests. Possible side-reactions, such as anomalous "ring contraction" (without cleavage) of glucosamine residues and C-5 isomerization of hexuronic acid residues during hydrazinolysis, are minimized or taken into account under controlled reaction conditions.[8,156] Most of the identified HNO_2-generated tetra- and higher oligo-saccharides (**103**–**121,** listed in column B of Table II) have been obtained by reaction at pH 1.5 and contain GlcNAc residues. Others were obtained from NS domains by partial depolymerization with nitrous acid. Although major oligosaccharides are clearly observable in complex anion-exchange HPLC profiles (examples in Refs. 73, 87), their structural analysis requires purification to sufficient homogeneity, most commonly by chromatographic methods,[73,167,168] or by PAGE.[66,167,169]

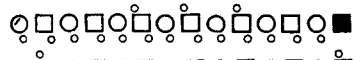
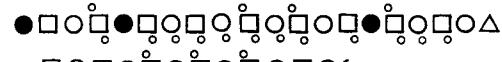
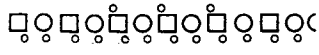
Another classical method for depolymerization of glycosaminoglycan chains is based on cleavage of glycol split residues. The C-2–C-3 bonds of nonsulfated GlcA and IdoA residues are split by oxidation with periodate, generating open-ring residues bearing dialdehyde groups. Under controlled reaction conditions, the reaction does not cause depolymerization or loss of sulfate groups. Cleavage of the heparin/HS chains at the level of glycol-split residues is easily achieved with base, or (upon reduction of the dialdehydes to dialcohols) under mild acid conditions (Smith degradation) (reviewed in Refs. 1 and 8.)

The sizes of fragments obtained by cleavage of exhaustively glycol-split heparin/HS reflect the distribution of unsulfated uronic acid residues along the polysaccharide chains. The corresponding oligosaccharide profiles thus permit determination of the length and relative abundance of sequences bearing 2-*O*-sulfated uronic acid (mainly $IdoA2SO_3$) residues, which influence heparin/HS polyelectrolyte and binding properties (see later). Early reports (summarized in Ref. 1) that GlcA in heparin/HS is oxidized by periodate faster than IdoA, thus permitting an estimate of the two different uronic acid residues, were not confirmed by further studies. In fact, while confirming a sequence-dependence of the kinetics of the periodate reaction,[91] these studies indicate that IdoA is oxidized much more rapidly than most of the GlcA.[170]

c. Domain Mapping; Sequencing.—The potential use of each of the aforementioned methods in generating defined saccharide fragments is illustrated in Table III, which compiles conjectured, extended sequences of the types likely to be generated when these procedures are applied to HS. Thus, NA-domains can be excised in two distinct modes, involving either degradation of the polysaccharide with HNO_2 at pH 1.5, or digestion with heparin lyase I. Notably, the products

TABLE III

Examples of Extended Saccharide Domains Recovered Following Enzymatic or Chemical Depolymerization of HS

Treatment	Product	Reference
Heparin lyase I		121, 171
Heparin lyase III		121, 135, 172
HNO_2 pH 1.5		173, 174
N-Deacetylation; HNO_2 pH 3.9		175–177
Periodate/alkali or Smith degradation		178

obtained may be expected to differ in that HNO_2/pH 1.5 will not leave any intact $GlcNSO_3$ residue, whereas the oligosaccharide released by the lyase may contain such units, provided that the adjacent hexuronic acid residue is nonsulfated (see Fig. 3). NS-Domains may be produced in three different ways, by digestion with heparin lyase III, by treatment with HNO_2 at pH 3.9 after *N*-deacetylation of the polysaccharide, or by periodate oxidation. The products generated by heparin lyase III and by periodate cleavage would be similar, in that the constituent disaccharide units contain exclusively 2-*O*-sulfated hexuronic acid residues, whereas the fragment produced through *N*-deacetylation and HNO_2 treatment would also encompass nonsulfated uronic acid residues. No standard procedure is so far available for the isolation of mixed sequences, containing both *N*-acetylated and *N*-sulfated disaccharide units (NA/NS-domain in Fig. 2). However, comparison of degradation products obtained with HNO_2 at pH 1.5 and, after *N*-deacetylation, at pH 1.5 and 3.9 (complete degradation to disaccharides) provides some information on the composition of these sequences as well. Such studies showed, unexpectedly, that IdoA 2-*O*-sulfate groups are almost exclusively confined to NS-domains, whereas GlcN 6-*O*-sulfate groups are about equally distributed between NS- and NA/NS-domains.[174]

Size-profiling of oligosaccharides obtained by single approaches or by a combination of two or more of these approaches has given indication on the distribution of different domains and, in favorable cases, on location of special sequences along the heparin/HS chains. Selected examples are the characterization of heparin sequences in endothelial HS,[121] information of distribution of $IdoA2SO_3$ residues in HS,[62] end-referenced sequence analysis of HSs[179] and heparin[180] regions near the linkage region, characterization of 3-O-sulfated glucosamine-containing sequences and the localization of trisulfated disaccharides to peripheral domains of glomerular basement membrane HS,[181] localization of $GlcA2SO_3$ in highly sulfated blocks of heparin,[182] identification of variable and constant oligosaccharide

domains in HS of different origins,[64] detection of unsubstituted amino groups in HSs,[14,40] mapping multiple HS domains interacting with multimeric proteins (platelet factor 4,[183] interferon-γ,[184] interleukin 8[185]), and defining the minimal size of single HS domains binding to various proteins (lipoprotein lipase,[166] *Herpes simplex* gC glycoprotein,[175] platelet-derived growth factor A,[176] basic fibroblast growth factor[168,186]). Strategies for defining sequences committed to interactions with proteins involve recovery of protein-binding oligosaccharides by affinity fractionation on immobilized protein, following partial enzymatic or chemical cleavage (see Sections III.3.a and III.3.b) of the polysaccharide ligand. The products obtained are likely to be polydisperse with regard to size and charge density, and therefore need to be further separated to obtain the smallest, least sulfated oligosaccharide species that retain affinity for the protein. The few sequence analyses that have actually been performed so far have generally involved further partial degradation of the isolated oligosaccharide, followed by identification of the resultant, partially overlapping smaller species (see, e.g., the identification of the antithrombin-binding region in heparin).[150,187] The procedure is time-consuming, nonroutine in character, and requires relatively large amounts of material. Novel developments aimed at more general applicability utilize putative protein-binding oligosaccharides that are tagged at the reducing end by a fluorophore[86,188] or a ^{3}H label.[87] The labeled species are subjected to (ideally) random cleavage at *N*-sulfated GlcN units, by limited reaction with nitrous acid, and the fragments generated are further modified by digestion with exolytic glycosidase(s) and *O*-sulfatases in different combinations. The effects of these treatments, as detected by anion-exchange HPLC (^{3}H-labeled samples) or by gel electrophoresis (fluorophore-labeled samples), can be directly translated into sequence information. Alternatively, similar fragmentation patterns (relating to unlabeled oligosaccharides) may be analyzed by processing MALDI mass spectrometry data,[88,189,190] a recent development featuring MALDI analysis of oligosaccharides concomitant with their sequestration by binding to a protein ligand.[191] An alternative approach is based on metabolically radiolabeled (^{3}H and ^{35}S) oligosaccharides, which are degraded in similar fashion and finally analyzed by anion-exchange HPLC.[135]

Sequence information of a different kind may be obtained by co-crystallography of protein–saccharide complexes, as has been described for the proteinase inhibitor AT[192] and for the acidic (FGF-1)[193] and basic (FGF-2)[194] fibroblast growth factors (see further details below). The bound carbohydrate ligands in these cases have been heparin-derived (or synthetic, heparin-related) oligosaccharides. Analysis of crystal structures has allowed precise identification of, in particular, sulfate and carboxylate groups that contribute to protein binding through interactions with defined, predominantly basic, amino acid residues. Such information could be "modeled" into idealized HS sequences, each capabable of selective, and exclusive, interaction with their cognate proteins. The approach suffers, however, from several limitations, one being the amounts required of both protein and saccharide. It

remains to be seen whether this method will become generally applicable to the analysis of HS-derived oligosaccharides.

4. Molecular Conformation

The availability of synthetic and natural oligosaccharides and of advanced NMR methods has contributed a deepened insight into the conformation of heparin/HS sequences in solution. While confirming the $^4C_1$(D) chair conformation for GlcA and GlcN residues, most of the studies have focused on the unique conformational properties of IdoA, as primarily deduced from widely different interproton coupling constants of this residue in different sequences. Combined NMR and molecular mechanics studies (reviewed in Refs. 195 and 196) have provided evidence that L-IdoA may be present in one of the three equienergetic conformations—$^1C_4$, $^2S_0$, or $^4C_1$— or in all three of these forms in rapid dynamic equilibrium. This equilibrium is highly sensitive to intramolecular factors (such as 2-*O*-sulfation of IdoA and the sulfation pattern of neighboring GlcN residues)[197] as well as to intermolecular interactions, such as cation binding (Ca^{2+} ions drive toward the $^1C_4$ form the conformational equilibrium of IdoA2SO$_3$ residues in heparin[198] and heparin oligosaccharides).[198–200] When IdoA or IdoA2SO$_3$ are present inside heparin/HS sequences, only $^1C_4$ (L) and $^2S_0$ (L) contribute to the equilibrium (illustrated in Fig. 4*a*). This equilibrium is displaced toward the $^2S_0$ form when IdoA2SO$_3$ is preceded by a 3-*O*-sulfated GlcNSO$_3$ residue as in the active site for AT, and toward the $^1C_4$ when it is located at the nonreducing terminal. For terminal nonsulfated IdoA, the $^4C_1$ form also contributes to the equilibrium. Three IdoA2SO$_3$ residues in different positions along a hexasaccharide may thus be represented by different proportions of the three conformers. As illustrated in Fig. 4*a*, the small distance in space between protons H-5 and H-2 in the $^2S_0$ conformation permits detection of this form also through observation of inter-proton nuclear Overhauser effects (NOEs). The conformer population of 2-*O*-sulfated IdoA in the sequence GlcNSO$_3$6SO$_3$-IdoA2SO$_3$-GlcNSO$_3$6SO$_3$ (about 60 $^1C_4$:40 $^2S_0$) is reversed (to 40 : 60) in GlcNSO$_3$3,6SO$_3$-IdoA2SO$_3$-GlcNSO$_3$6SO$_3$. The conformer population has been calculated from the NMR spectra of numerous di-, tri-, and larger heparin/HS oligo-saccharides.[197–199,201] The overall conformation (including estimation of interresidue torsion angles) has been defined by molecular modeling and measurement of inter-ring NOEs for the α-methyl glycoside of the synthetic pentasaccharide **4** (GlcNSO$_3$6SO$_3$-GlcA-GlcNSO$_3$3,6SO$_3$-IdoA2SO$_3$-GlcNSO$_3$3,6SO$_3\alpha$OMe),[202,203] for the lyase-derived heparin hexasaccharide **67** (ΔUA2SO$_3$-GlcNSO$_3$6SO$_3$-IdoA2SO$_3$-GlcNSO$_3$6SO$_3$-IdoA2SO$_3$-GlcNSO$_3$6SO$_3$),[82] and for the synthetic heparin trisaccharide mimic Glc2,3,6SO$_3$4OMe-ldoA2SO$_3$-Glc2,6SO$_3\alpha$OMe.[204]

Conformational analysis of longer "regular" heparin sequences[205] gives evidence for regular arrays of three sulfate groups alternating on both sides of a helix

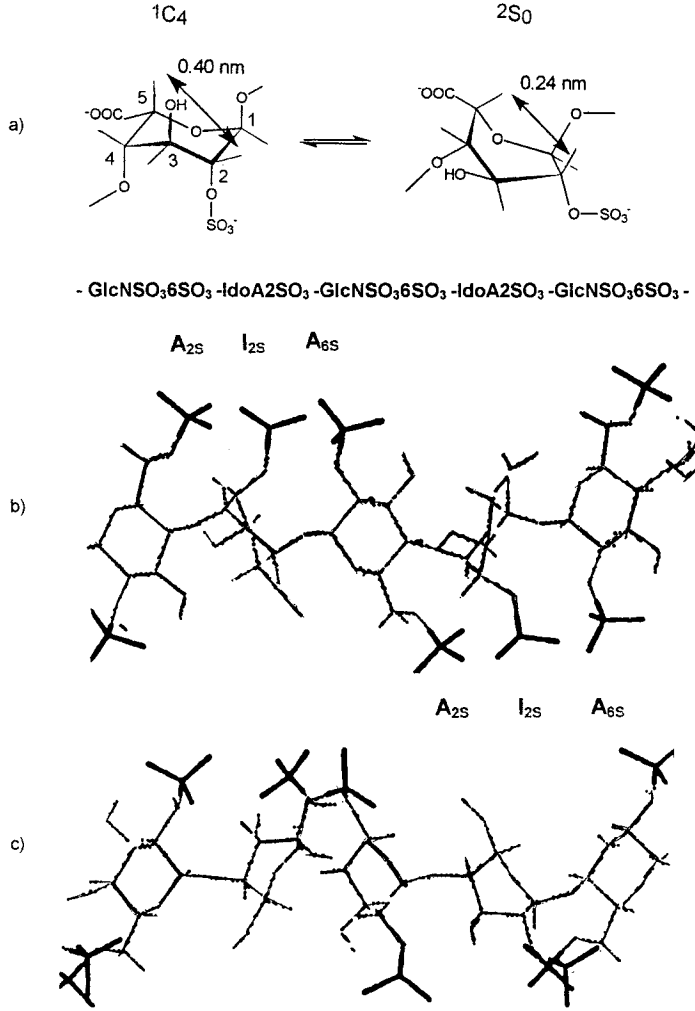


FIG. 4. Conformational equilibrium of IdoA2SO$_3$ residues (*a*).[213] Minimum energy conformation of major sequences of heparin (-IdoA2SO$_3$-GlcNSO$_3$6SO$_3$)$_n$, with L-IdoA residues in the $^1C_4$ (*b*) and $^2S_0$ conformation (*c*). (Reproduced with permission from Ref. 205; © the Biochemical Society).

on models built with the IdoA2SO$_3$ residues in both the $^1C_4$ and in the $^2S_0$ form (Figs. 4*b* and 4*c*). However, as previously noted for pentasaccharide **4,**[202] the distance between the 2-*O*-SO_3^- group of the iduronic residue and the 6-*O*-SO_3^- group on the following amino sugar residue is much shorter when IdoA2SO$_3$ is in the $^2S_0$ than in the $^1C_4$ conformation.[205] Conformational and molecular dynamics studies have also been reported for heparin derivatives in solution such as *N*-deacetylated

heparin,[206] 6-*O*-desulfated heparin,[206] and heparin epoxide,[207] as well as for the *E. coli* capsular K5 polysaccharide.[208] *N*-Acetylation and 6-*O*-desulfation do not significantly affect the minimum energy conformations of heparin chains as depicted in Figs. 4*b* and 4*c*.[206]

The conformational flexibility of iduronic acid residues (also demonstrated by analysis of complete conformational maps)[209,210] has been suggested to play an important role in modulating the protein-binding and associated biological properties of heparin/HS and dermatan sulfate, by providing an easy (energy-inexpensive) mechanism to attain the most effective approach of the anionic groups of the GAG to appropriate basic groups of the protein.[211] A convincing experimental proof of the aforementioned concept was provided by the X-ray diffraction evidence that one of the two IdoA2SO_3 residues in a lyase-derived heparin hexasaccharide co-crystallized with FGF-2 was in the $^1C_4$, and the other in the $^2S_0$ conformation.[194] The 4,5-unsaturated uronic residue of this hexasaccharide is another example of conformational flexibility. As demonstrated by NMR studies for lyase-derived di- and tetra-saccharides[212] and for a hexasaccharide,[82] both the almost equienergetic $^2H_1$ and $^1H_2$ forms of this residue contribute to a conformational equilibrium in solution. High-resolution X-ray diffraction-derived data obtained for the complex of AT with a synthetic heparin pentasaccharide variant of the natural AT-binding sequence,[192] for FGF-2 with lyase-generated tetra- and hexa-saccharides,[194] and for FGF-1 with a heparin decasaccharide fraction[193] have revealed the conformation of heparin oligosaccharide segments outside the actual protein-binding sites as well as of those "locked" into these sites. Although not explicitly stated in the published crystallographic study, L-IdoA2SO_3 bound to AT is in the $^2S_0$ conformation,[192] as also found in aqueous solution for pentasaccharide **4** (methyl glycoside) in the presence of AT[213] and for conformationally locked synthetic oligosaccharides.[214] On the other hand, the conformational equilibrium of IdoA in the weakly AT-binding tetrasaccharide Glc$NSO_3$6SO_3-GlcA-Glc$NSO_3$6SO_3-IdoAαOMe in aqueous solution is definitely shifted toward $^1C_4$ in the presence of the protein,[215] once again showing the ability of IdoA residues to adapt their shape for optimal contacts with the protein. In FGF-2/heparin hexasaccharide co-crystals, the IdoA2SO_3 residue in close proximity of the protein is in the $^1C_4$ form, and the next residue (not directly involved in binding) is in the $^2S_0$ form.[194]

The crystal coordinates of the complex between FGF-1 and a heparin decasaccharide[193] indicate a slightly distorted $^1C_4$ conformation for at least one of the bound IdoA2SO_3 residues.[216] In aqueous solution, both IdoA2SO_3 residues of synthetic heparin tetrasaccharides bound to FGF-1 are in the $^1C_4$ conformation.[216] A unifying theory for the role played by IdoA residues in facilitating protein binding has been offered by the observation—based on analysis of available crystal data—that they favor departure from the linear propagation of heparin/HS helical chains (which would be unfavorable for multi-site contacts) by formation of "kinks" of the chains at the sites of binding.[217]

Extra flexibility along a GAG chain can be artificially induced by splitting (with periodate) the C-2–C-3 bonds of nonsulfated uronic acid residues, glycol-split residues acting as flexible joints between extensively sulfated sequences.[218] On the other hand, glycol-splitting of nonsulfated uronic acids of heparin (including some IdoA residues generated by controlled O-desulfation of IdoA2SO_3 residues) causes a drastic deviation from the chain orientation of unmodified sequences, making the new chain conformation unsuitable for oligomerization of protein molecules along the GAG chain.[219]

IV. Interaction with Proteins

1. General Considerations

The list of "heparin-binding" proteins is extensive and continuously growing. The seemingly nonspecific interactions of multiple proteins with a "single" polysaccharide species, heparin, have tended to obscure the notion of selective binding of these same proteins to specific HS species. Yet it is recognized that the physiological polysaccharide ligands are not heparin, but HS species on cell surfaces or in the extracellular matrix. It is proposed that the extensively sulfated heparin polymer encompasses the variety of combinations of sulfate groups that provide binding sites for selected proteins in different HSs; the redundant sulfate groups in the heparin chain do not contribute to nor do they interfere with the interactions (Fig. 5). Only a few protein-binding sequences in HS chains have been characterized to date (see later). Conversely, the heparin/HS-binding sites in proteins are generally poorly defined, apart from the presence of basic amino acid residues. Linear "consensus sequences" for heparin binding have been proposed,[220] but are not a consistent finding. Alternatively, binding ability has been rationalized in terms of the sterical disposition of basic amino acid residues, that

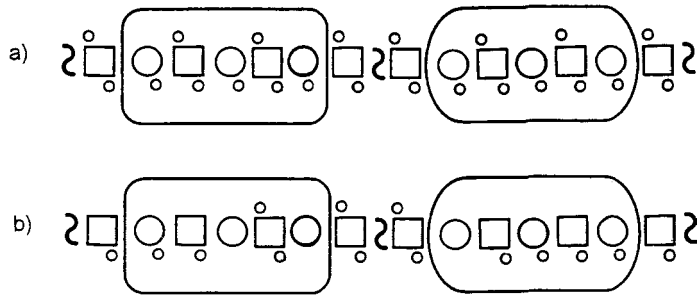


Fig. 5. "Hidden" protein-binding sequences in HS. (*a*) Two different proteins bind to the same, fully sulfated ("regular") heparin sequence. (*b*) The same proteins interact specifically each with one distinct HS sequence. The symbols are the same as in Fig. 1. For further information see the text.

should be located no more than 20 Å (2 nm) from each other in order to contribute to interaction.[221] Indeed, mutational analysis and crystal structures point to the occurrence of composite binding sites for heparin/HS that involve different loops from domains widely separated in the primary sequence (see, for instance, the example with AT later).

It is not always simple to distinguish between specific interactions and nonspecific interactions associated with polyelectrolyte effects. In fact, arrays of closely spaced anionic groups along a polymer chain—not necessarily a GAG or even a polysaccharide—may "saturate" the binding sites of a protein for heparin/HS and induce biological effects similar to those of heparin. On the other hand, polyelectrolyte effects usually strengthen specific GAG—protein interactions both through involvement of secondary binding sites of proteins and through the energetically favored release of cations (especially Na^+) from the polyanion.[222–226]

The charge and molecular weight heterogeneity of heparin has prevented conclusive rationalization of its solution properties. Not unexpectedly, the strongest electrostatic interactions (both with proteins and counterions) have been generally observed for heparins richest in trisulfated disaccharide sequences **1** (such as beef lung heparins),[1] and for heparin fractions with the highest charge density and average molecular weights.[227] These latter have been often found to have also high anticoagulant activities[227] paralleled by high affinity for AT.[228] Inspection of NMR spectra of heparin fractions with the highest charge density[227,229] clearly indicates that these fractions also have the highest content of AT-binding sequences. Whenever cooperative effects are involved, heparin chains containing long sequences of disaccharide **1** are expected to have higher protein-binding properties than chains of approximately the same length and charge density in which these sequences are shorter and separated from each other by undersulfated residues.[230]

Several polyanion–protein interactions are mediated by cations, especially Ca^{2+} ions (examples in Refs. 1 and 231). NMR spectroscopic and circular dichroism studies on heparin and heparin derivatives have shown that binding of calcium ions involve primarily the carboxylate groups of $IdoA2SO_3$ residues, but also the sulfamino groups of the glucosamine residues.[232] Although exchange of Na^+ ions with Ca^{2+} ions causes dramatic changes in the NMR spectra of heparin,[233,234] these changes could be explained in terms of nonspecific counterion condensation and do not imply substantial conformational changes in the polysaccharide.[233] By contrast, binding to heparin of Cu^{2+} ions (which has been shown to enhance angiogenesis)[235] is regarded to be a specific chelation.[236] Site-specific binding to heparin was indicated by NMR studies also for small basic molecules of biological relevance such as histamine.[237]

The following model examples of protein interactions have been selected because they have been analyzed in some depth and may serve to illustrate common concepts and problems in the area.

2. Antithrombin

Heparin has been used clinically for decades to prevent and treat thromboembolic disease and is isolated on an industrial scale from animal tissues, in particular pig intestinal mucosa. The corresponding physiological blood anticoagulant is presumably not heparin, but an HS species that is located on the surface of vascular endothelial cells.[3] Both heparin and HS contain a specific pentasaccharide sequence[3,150,238] that binds and activates the plasma proteinase inhibitor AT. This pentasaccharide sequence (**4**) is present only in a subfraction of heparin and HS preparations. It displays a characteristic structural feature, namely a 3-O-sulfated GlcN residue, that is only rarely seen in other portions of heparin/HS chains.

The effects of heparin/HS on the inhibition of coagulation enzymes by AT are essentially twofold. It was early noted that the minimal saccharide chain required to promote the inactivation of thrombin, a key player in the coagulation mechanism, corresponded to 16–18 saccharide units, thus far longer than the AT-binding pentamer (reviewed in Ref. 239). The explanation to this finding is that the polysaccharide binds to both AT and thrombin, thus bringing the two proteins into close apposition. Whereas AT binds specificially and with high affinity (K_d 1–2 $\times$ 10^{-8} M) to the pentasaccharide domain, thrombin binds nonspecifically at various sites on the same polysaccharide chain, and then diffuses along the polymer to encounter the inhibitor.[240] Applications of various synthetic oligosaccharides/-neoglycoconjugates have shown that thrombin approaches AT from the nonreducing end of the polysaccharide chain.[241–243]

In addition, there is a direct "activation" of AT, due to a conformational change induced by the binding to the pentasaccharide.[192,244] This effect accounts for most of the increase in inhibitory activity toward Factor X_a induced by heparin, but is of marginal importance in thrombin inhibition, which largely depends on the "template" effect (promoting apposition of thrombin and AT) of the polysaccharide. The conformational changes induced in the AT molecule upon pentasaccharide binding have been elucidated primarily through crystallographic studies of the protein, in the presence[192] (Fig. 6; see color insert) and absence[245,246] of pentasaccharide. Notably, intact AT occurs in two forms, the active inhibitory form and a nonphysiological, "latent" form that has adopted an overall conformation similar to that of the cleaved inhibitor generated by reaction with a cognate proteinase. During this process the reactive-site loop, with or without covalently bound proteinase, becomes inserted as a sixth strand in the central β-sheet (A-sheet) of the molecule, along with a weakening of the interaction between the AT and heparin molecules. Comparison of co-crystals of active and latent AT, each form bound to pentasaccharide, with those of active AT in the absence of pentasaccharide thus has defined the conformational changes induced by heparin binding as well as those leading to release of the polysaccharide after completed reaction.[192] Amino acid residues from three distinct domains of the primary structure (Lys11-Arg13; Asn45-Arg47; Glu113-Arg129) have been implicated in interactions with sulfate

and carboxylate groups of the pentasaccharide sequence. The presence of pentasaccharide induces conformational changes at the actual heparin-binding site of AT, but also major rearrangement of the helical domains of the molecule, closure of the A-sheet, and expulsion of the partially inserted reactive site loop, thus facilitating its interaction with the target proteinase.[247]

Binding of high-affinity heparin, via the specific pentasaccharide sequence, to AT is a two-step process, involving formation of an initial complex with a K_d of $\sim 4 \times 10^{-5}$ *M*, followed by conformational changes in both the AT and saccharide moieties.[240,248] Initial assignments of functionally important groups in the pentasaccharide (*N*-sulfate groups of residues 3 and 5, 6-*O*-sulfate and 3-*O*-sulfate groups on units 1 and 3, respectively; formula **4**)[150] were confirmed and extended through systematic studies of binding properties and biological activities of a large number of synthetic oligosaccharides[249] and, more recently, through the crystallization of AT–pentasaccharide complexes.[192] Interestingly, complex formation with AT was found to shift the conformational equilibrium of the IdoA unit 4 toward the twisted-boat $^2S_0$ (L) form, thus presumably promoting the interaction.[213] This finding may help to explain why a 2-*O*-sulfate group at this unit, which also favors the $^2S_0$ (L) conformer, strengthens the binding between saccharide and AT, and increases the anticoagulant activity, without directly interacting with the protein.[213]

3. Fibroblast Growth Factors and Their Receptors

The mammalian fibroblast growth factor (FGF) family involves at least 20 structurally related polypeptides that are expressed, in specific temporal and spatial fashion, during embryonic development, but also in adult vertebrates. The FGFs are of crucial importance to a wide variety of biological processes related to cell proliferation, differentiation, and migration. They interact with high-affinity cell-surface receptors that are composed of an extracellular ligand-binding domain, a transmembrane sequence, and a cytoplasmic domain with protein tyrosine kinase activity. The extracellular domain is composed of three immunoglobuline (Ig)-like domains (D1–D3). Four distinct high-affinity receptor genes (FGFR1–FGFR4) have been described, with further variants due to alternative splicing (for reviews see Refs. 250–252). FGF receptors are activated by oligomerization,[253] and this activation as well as FGF-induced biological responses require the participation of heparin-related polysaccharides.[254,255] This requirement appears to be satisfied by HS proteoglycans, so-called low-affinity FGF receptors, at the cell surface. Most of the available information regarding the role of HS in FGF signaling relates to the acidic (FGF1) and basic (FGF2) FGFs.

The interactions of HS with FGFs[168,256–258] involve primarily the NS-domains (see Fig. 2) of the polysaccharide. Biochemical analyses of FGF2 interactions with heparin and HS oligosaccharides implicated a I-A_{NS}-I-A_{NS}-I_{2S} pentasaccharide sequence (for symbols, see Fig. 1; reducing terminus to the right), notably lacking any 6-*O*-sulfate groups, as the smallest and least sulfated structure capable

of FGF2 binding.[168] A related pentasaccharide sequence, A_{NS}-I_{2S}-A_{NS}-I-A_{NS}, thus also devoid of 6-*O*-sulfation but with the 2-*O*-sulfated IdoA unit in a different position, was inferred from co-crystallography of an FGF2-heparin-6-mer complex[194] (Fig. 7; see color insert). A similar minimal protein-binding size was reported for heparin interacting with FGF1.[193,257] However, studies with partially desulfated heparin preparations[259] and the structure of FGF1-heparin-10-mer cocrystals[193] implicated 6-*O*-sulfate groups in the heparin–FGF1 interaction, confirmed by analysis of FGF1-binding HS NS-domains.[260] More recent sequence analysis of such domains implicated a tri-O-sulfated -I_{2S}-$A_{NS,6S}$-I_{2S}- motif that appeared to be a prerequisite to high-affinity binding.[261] Members of the FGF family thus may recognize HS domains with different structures (see also interactions with small and nonsulfated oligosaccharides).[262] Remarkably, specific HS structures may be modulated during embryonic development (over a few days; murine brain)[263,264] as well as in adult individuals (over many years; human aorta)[260,265] to induce selective, controlled changes in the relative binding of FGF1 and FGF2.

The minimal 4–5-mer domains sufficient for FGF binding (for a review see Ref. 266) fail to promote the mitogenic acitivity of the growth factors. For FGF2, the mitogenic response requires a 12-mer or longer saccharide sequence containing both $IdoA2SO_3$ and $GlcNSO_36SO_3$ residues.[259,267] Somewhat shorter (6/10-mer) fragments appear sufficient to promote FGF1-induced FGFR phosphorylation[268] and cell proliferation.[259,269,270] Essentially two hypotheses have been advanced to rationalize the difference in saccharide size required for mere growth factor binding vs receptor activation. Binding of two cis-oriented FGF molecules along the same HS chain may be needed for interaction with two FGFRs, which are thus dimerized.[271–273] Alternatively, the saccharide extension, in addition to that required to bind an FGF monomer, may serve to interact with the FGFR, thereby bridging the growth factor and its receptor.[231,267,274] Notably, FGF2-induced receptor activation was found to depend on at least one 6-*O*-sulfate group, which thus was a constituent not required for FGF2 binding, being present in the 12-mer saccharide,[267,275] in agreement with the notion of a direct interaction between the saccharide chain and the FGFR. In further support of this concept, an FGF2 monomer covalently bound to a heparin 12-mer was able to induce FGFR activation (stimulate mitogenesis).[276] Indeed, direct binding of heparin to FGFRs has been demonstrated.[231]

Studies of crystal structures involving FGF1 and a heparin oligosaccharide[193] or FGF2 complexed to (part of) FGFR1[277] provide novel and intriguing insight into the possible ways in which heparin/HS may promote FGF action. Analysis of FGF1 dimers in complex with a heparin 10-mer revealed a "sandwich" structure, in which two FGF1 monomers were found to simultaneously interact with opposite faces of the oligosaccharide (Fig. 8; see color insert). Each of these interactions involved 4–5 monosaccharide units. However, considerable variability was noted, in that the saccharide ligand could occupy different positions, shifted by about one disaccharide unit, or even with reversed polarity. A model was proposed by

combining the crystallographic data with the results of mutagenesis experiments, such that the FGF residues found to be important for FGFR binding were mapped onto the molecular surface of the heparin-linked FGF1 dimer.[193] The saccharide thus would promote receptor dimerization by linking two FGF1 monomers, each bound to domains D2 and D3 of different receptor molecules. Notably, this model is distinguished by lack of FGF–FGF as well as of saccharide–receptor interfaces. Strikingly different interaction patterns were inferred from crystallographic data for a complex of FGF2 and a FGFR1 variant composed of domains D2 and D3.[277] Tightly knit dimers of FGF2–FGFR1 complexes were observed, each growth factor interacting with the D2 and D3 domains of one receptor molecule as well as with D2 of the adjoining complex, the dimer being further stabilized by a direct interaction between the D2 domains of each receptor molecule. A positively charged "canyon" between the two D2 domains, extending over the bound FGF2 molecules, was proposed to accommodate a saccharide chain with an optimal size of 12 sugar units. This model thus agrees with previous findings of a minimal 12-mer saccharide required to promote receptor activation by FGF2, as well as with the prediction of direct interaction between this chain and the receptor.[267]

The validity of these models with regard to the role of HSPGs remains unclear; several questions need to be resolved. Do both modes of FGF–saccharide–FGFR interaction indeed occur at the cell surface? If so, do one or both of the models apply to more than one type of FGF and/or FGFR? Interestingly, crystallization of a complex between FGF1 and a two-domain extracellular fragment of FGFR2 revealed FGF1–FGFR2 complexes in dimeric assembly[278] (Fig. 9; see color insert) similar to that described for the FGF2–FGFR1 complex[277] including the predicted composite saccharide binding site. More recent analysis of an FGF2–FGFR1 heparin oligosaccharide complex revealed a somewhat similar assemblage, although the "heparin binding canyon" was occupied by two oligomers in opposite polarity, nonreducing termini facing each other.[279] Significantly different interactions were observed in an FGF1–FGR2–heparin oligosaccharide complex, with a central saccharide molecule linking two FGF1 ligands into a dimer bridge between two receptor molecules.[280] Do HS chains affect growth factor action/receptor activation in yet further ways? To what extent is the mechanism of saccharide involvement determined by the fine structure of the HS chain? It is recalled that the crystallization experiments reported so far have largely been based on highly sulfated heparin oligosaccharides, with structures different from those of the cognate HS sequences expected to apply *in vivo*. Conceivably, some of these problems may be more readily assessed when crystal structures of ternary complexes involving growth factor, receptor ectodomains, and the appropriate HS oligosaccharides become available.

4. Other Proteins

A detailed discussion of the diverse interactions of proteins with heparin/HS would fall outside the scope of this chapter; the reader is referred, in particular,

to a monograph on the subject.[8] Although at least some of the "heparin-binding" proteins are believed to interact with HS *in vivo,* there is still by and large scant evidence for the functional roles of such binding. Nevertheless, an increasing number of proteins are being implicated, at the cell or tissue level, with phenomena that appear to depend on HS binding. Selected examples from a steadily growing list of such target proteins and functions, in addition to those described in more detail above, include the laminin G domain in supramolecular assemblies of the extracellular matrix,[281] lipoprotein lipase in enzyme transport,[282] endostatin in inhibition of angiogenesis,[283] viral envelope glycoproteins in viral infection,[175,284,285] heparin-binding growth-associated molecule (HB-GAM) in neurite outgrowth[286] and neural plasticity,[287] and growth factors in embryonic development.[288] Few of these interactions have been characterized in molecular detail. A particularly intriguing feature is the domain organization of HS chains, the functional significance of which is poorly understood. Notably, oligomeric proteins may bind to HS chains in domain-designed mode, such that separate protein monomers interact with distinct NS domains that are separated by N-acetylated sequences.[183–185]

V. Modified and Novel Structures

1. Low Molecular Weight Heparins

Binding with significant affinity to proteins usually require heparin/HS chains longer than 4–5 monosaccharide residues. Such a requirement is due to the need of involving several (at least two) basic residues on the protein. Moreover, several of the biological activities associated with protein binding involve formation of ternary complexes, that require relatively long chains. (See Ref. 6 and Section IV for discussion of selected examples.) As extensively investigated in connection with the antithrombotic activity, the effects of heparin on the inactivation by AT of different enzymes of the coagulation cascade have different molecular-weight dependences.[1,239] Observations that AT-mediated inhibition of thrombin requires heparin chains containing 16–18 saccharide residues, whereas a similar inhibition of Factor Xa is achievable with much shorter chains (see Section IV.2), induced the concept of low molecular weight heparins (LMWHs) as antithrombotic agents with relatively low thrombin-inhibiting activity (and, conceivably, reduced hemorrhagic risks). Although the assumption that the hemorrhagic potential is associated only with anti-thrombin activity is not correct,[289] LMWHs are gradually replacing conventional "unfractionated" heparin in the prevention of venous thrombosis mainly because of their better bioavailability in subcutaneous administration and more predictable dose-response properties.[290]

Commercial LMWHs have average molecular weight (M_r) of 2500–8000. They are prepared either by size fractionation, or (now prevalently) by chemical or enzymic depolymerization of heparin. Most common manufacturing processes of

LMWHs (reviewed in Ref. 291) involve controlled treatment of heparin with nitrous acid; with heparin lyase I; with benzyl chloride followed by β-elimination with alkali; by radical-catalyzed peroxidative cleavage; or with γ-ray irradiation. Acid cleavage of heparin under sulfating conditions affords a different type of fragments ("supersulfated" LMWH).[292] Characterization of end groups of heparin fragments generated by different depolymerization methods permits the distinction of different classes of LMWHs. Typically, the reducing residues of nitrous-acid generated LMWHs are 2,5-anhydromannose (or anhydromannitol, after reduction), and the terminal nonreducing residues of fragments obtained both by heparin lyase and β-elimination are 4,5-unsaturated uronic acids (prevalently arising from IdoA2SO_3 residues).[291,293] These end residues, as well as terminal residues at the reducing and/or nonreducing ends that are not modified by the depolymerization reaction, can be characterized and quantified by a variety of analytical methods (reviewed in Refs. 230, 291, 294). LMWHs from several commercial sources significantly differ from each other also in their "internal" structures as reflected by disaccharide compositional analysis,[291,294] oligosaccharide mapping,[295] and NMR spectra,[230] as well as by other physicochemical parameters, including Raman spectra and dynamic light scattering.[296] It is still debated whether these differences are attributable to the different depolymerization/purification procedures adopted by the manufacturers, or (at least in part) to structural differences between the parent heparins.

Assuming that LMWHs largely retain the internal structure of parent heparins (including the original AT-binding sequence), the most important parameter for defining a LMWH is its average molecular weight (M_r). M_r values for LMWHs can be evaluated by polyacrylamide gel electrophoresis (PAGE)[297] and by high-performance gel permeation chromatography (HP-GPC).[298,299] HP-GPC will assess both the number-average M_r (M_N), weight-average M_r (M_W), and polydispersity (P) of heparin and MWHs.[297,299] M_w values can also be determined by ^{13}C NMR spectroscopy.[300] These parameters are significantly different for commercial LMWHs from different manufacturers. Not unexpectedly in view of the distinct molecular weight dependence for different mechanisms of thrombin generation and inhibition,[301,302] different types of commercial LMWHs show different anticoagulant profiles both *in vitro* and *in vivo*.[303,304] The latter properties reflect not only the inherent biological activity of the polysaccharide, but also the size dependence of pharmacokinetics in animal models[305] and in humans.[306]

2. Modulation of Sulfation Patterns; Heparin/HS Mimics

Classical methods for removing N-SO_3 groups and for *N*-acetylation of exposed amino groups, for solvolytic *N*,*O*-desulfation (with or without re-*N*-sulfation), as well as for preferential 6-*O*-desulfation (reviewed in Refs. 1, 8) have been widely used for assessing the relative importance of sulfamino/acetamido substituents and 6-*O*-sulfation of glucosamine residues in the expression of biological activities of

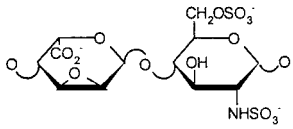
heparin (and, less frequently, of HS). A recently developed procedure affords selective and complete 6-*O*-desulfation virtually without any 2-*O*-desulfation of IdoA units.[307] Selective 2-*O*-desulfation is conveniently achieved by controlled treatment of heparins with alkali. By lyophilization of heparin at pH 11–13, sequences **1** can be quantitatively converted into the "epoxidated" sequences **122.** Lyophilization of **122** at pH >13 affords the 2-*O*-desulfated sequence **123.** However, reflux of **1** in Na_2CO_3, or its treatment with 0.1 *M* NaOH at temperatures $>80^{\circ}C$, affords **124,** in which the original IdoA2SO_3 residues are converted into L-GalA.[308,309] Variants of these reaction conditions may also be applied to the generation of selectively 2-*O*-desulfated heparins.[310–312] The *O*-desulfation reaction with base was reported to involve, in addition, removal of the 3-*O*-sulfate group of GlcN$SO_3$3(6)SO_3 residues typical of the AT-binding site.[311] Under the alkaline conditions used for 2-*O*-desulfation, 3-*O*-sulfated glucosamine residues in both oversulfated heparins[313] and in heparin fractions with high affinity for AT[314] are indeed converted into *N*-sulfoaziridine derivatives (**125**), suggesting that removal of 2-OSO_3 from iduronic acid and of 3-OSO_3 from glucosamine takes place by two different mechanisms.

Selective desulfation has been used to pinpoint structure/function relations for a variety of biological activities *in vitro* and *in vivo*. *N*- and *O*-desulfation of heparin involves loss of overall anticoagulant properties of the original polysaccharides, primarily due to removal of sulfate groups essential for binding to AT (see Ref. 150), but also because desulfation of the regular trisulfated disaccharide sequence **1** abolishes thrombin inhibition by heparin cofactor II,[315] as well as the interaction with thrombin required for its inhibition by AT.[239] Most of these modifications have in fact been deliberately performed with the aim of decreasing the anticoagulant activity of heparin (thought to be associated with bleeding and other side effects) while not affecting other desirable biochemical and biological properties of this versatile GAG. For example, 6-*O*-desulfated heparin still binds to FGF-2,[168,316] and thus inhibits FGF-2-induced angiogenesis.[317] On the other hand, completely 6-*O*-desulfated heparin does not inhibit FGF-2-promoted proliferation of selected cell types.[318] Conversely, 2-*O*-desulfation does not significantly impair the antimetastatic activity of heparin.[319]

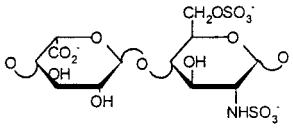
Compared to the desulfation procedures, regioselective chemical sulfation of heparin-related saccharides poses more of a challenge. Re-*O*-sulfation of *O*-desulfated heparin (usually performed on pyrididium salts, with pyridine $\cdot$ SO_3 or $SO_3 \cdot$ DMF in aprotic solvents) fails to restore the sulfation pattern of the original polysaccharide, since it leads to preferential 3-*O*-sulfation (rather than 2-*O*-sulfation) of IdoA residues.[320,321] Under more drastic reaction conditions, IdoA residues are sulfated at both the 2 and 3 positions.[321] On the other hand, 3-*O*-sulfates on IdoA can be removed more rapidly than 2-*O*-sulfates under solvolytic conditions, such that solvolytic desulfation of totally sulfated heparin may yield intermediate species with substantial contents of IdoA2SO_3 as well as GlcN3SO_3 residues.[321,322] Some enrichment in GlcN3SO_3 residues has been

observed also on simple vacuum drying of heparin, through transfer of SO_3^- groups from sulfamino groups.[323] Direct *O*-sulfation of HS preferentially leads to 2-*O*-sulfation of GlcA residues.[324] Contrary to the findings for 2,3-*O*-disulfated IdoA in oversulfated heparin, 2-*O*-sulfate groups are removed preferentially on solvolytic desulfation from 2,3-*O*-disulfated GlcA residues of oversulfated HS.[321]

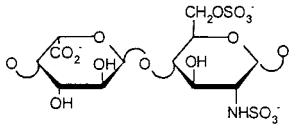
Variously *O*-desulfated or oversulfated heparins and corresponding oligosaccharides have been used for establishing relationships between sulfation patterns and NMR parameters.[325–327] Such derivatives have also been widely used for structure–activity studies. Selected examples of biological activities other than those already described investigated using chemically modified heparins include antiproliferative activity (inhibition of smooth muscle cell growth),[328,329] activities modulated by various growth factors and their receptors,[330,333] and effects on bleeding.[330,334] (See also reviews on heparin in relation to atherosclerosis,[335] inflammation,[336] and thrombocytopenia,[337] which essentially implicate interactions with lipoproteins, selectins, and PF4, respectively.)



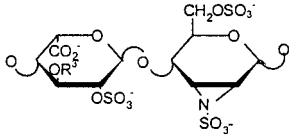
122



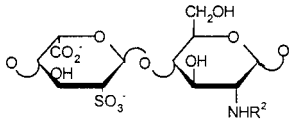
123



124

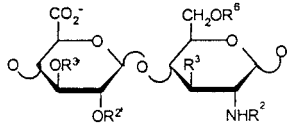


125



126

a) R^2 = Ac
b) R^2=SO_3^-



127

a) R^2 = Ac; R^3=R^6=$R^{2'}$=$R^{3'}$=H
b) R^2= SO_3^-; R^3=R^6=$R^{2'}$=$R^{3'}$=H
c) R^2= R^6=SO_3^-; $R^{2'}$=$R^{3'}$=H
d) R^2=R^6=$R^{3'}$=SO_3^-; $R^{2'}$=H

Sulfation methods have been applied to a variety of polysaccharides, also of nonmammalian origin such as chitosan,[338] to yield heparin-related products.

N-deacetylation/*N*-sulfation reactions were used to convert acharan sulfate, a GAG from the giant African snail *Achatina fulica* predominantly composed of units **126a**[339,340] into *N*-sulfoacharan sulfate (**126b**).[341] Similarly, the microbial *E. coli* K5 polysaccharide **127a** has been converted to sulfaminoheparosan (**127b**).[342] Regioselective *O*-sulfation of **127b** afforded the 6-*O*-sulfated sulfaminoheparosan (**127c**) and its 6,3′-di-*O*-sulfated analogue **127d.** Further sulfation yielded hybrid structures[342] containing minor sequences that differ from the AT-binding pentasaccharide sequence **4** of heparin/HS in having $GlcA2SO_3$ instead of an $IdoA2SO_3$ residue.[343] **127b** is a good substrate for enzymes responsible for the polymer-modification in heparin/HS biosynthesis.[344] Conversion to IdoA of a significant proportion of the GlcA residues of **127b** has been achieved using a C-5-epimerase,[345,346] and the epimerized products have been further *O*-sulfated either enzymically[345] or chemically,[345,347] to afford HS-like species. Indeed, recent experiments have demonstrated that carefully controlled chemical and enzymatic modification of the K5 polysaccharide may yield products with biological activities highly similar to those of heparin.[347]

The antithrombotic properties[348] of sulfated K5 derivatives are presumably due to the presence of minor sequences mimicking the AT-binding site.[342,343] It is less clear why these products are also antimetastatic[349]; however, it is noted that they are also good substrates for heparanase.[151] The structures of oversulfated heparins and HSs have seldom been characterized in detail (for a review, see Ref. 350). Oversulfation invariably involves some increase in the anticoagulant activity of heparin and other polysaccharides, mostly due to non-AT-mediated mechanisms[351] or release of endogeneous anticoagulant species such as Tissue Factor Pathway Inhibitor (TFPI).[352,353] Induction of high anticoagulant activity consistently requires short sequences that bind AT with high affinity and/or longer sequences that bind HC2.[239] Conversely, the nonanticoagulant/nonantithrombotic activities of heparin/HS do not require such sequences.[8,12,354]

Semisynthetic mimics of heparin have been also prepared by linking together (through a bridge formed by reaction of reduced cystamine with iodoacetic acid) the reducing AM residues of HNO_2-generated fragments and nonreducing $GlcNSO_36SO_3$ residues of lyase-generated fragments exposed by removal (with mercuric acetate) of the terminal unsaturated uronic acid residue.[243] The terminal unsaturated uronic acid residues are themselves exploitable for conjugation of di- and oligo-saccharide GAG fragments.[355] These “neo-GAGs” are especially useful for studying the domain organization of complexes involving two protein molecules, such as AT and thrombin (see Section IV.2). Heparin/HS and their fragments have also been conjugated with appropriate probes for detection of binding proteins. Novel probes, such as ^{125}I-DTAF (dichlorotriazinylaminofluorescein)-heparin, heparin biotinylated on occasional free amino groups along the polysaccharide chain, and a heparin biotinylated on amino-group-bearing substituents, have been proposed as substitutes of the less sensitive ^{125}I- and ^{125}I-fluoresceinamine conjugates for detection of histone

and FGF-2.[356] Biotinylation of heparin has also been obtained by reaction of the aldehydo groups of a periodate-oxidized heparin[357] and of terminal AM residues generated by limited nitrous acid depolymerization with biotinyl hydrazide.[358] These latter residues have also been used to conjugate radiolabeled and fluoresceinated tyramine to LMW heparins.[359]

3. Total Chemical Synthesis

Although coverage of total chemical synthesis of heparin/HS sequences is outside the scope of this chapter, important achievements in this field will be mentioned, inasmuch as they have contributed to our understanding of the molecular mechanisms underlying the biological activities of these GAGs. Soon after the structure of the AT-binding sequence was elucidated, a major effort was made toward the chemical synthesis of heparin oligosaccharides partially or totally containing the active sequence for AT. These studies (reviewed in Ref. 249) led to synthesis of pentasaccharide **4** (as methyl α-glycoside, *N*-sulfated at residue 1 and 6-*O*-sulfated at residue 3) as well as of of a number of its variants, including a more active pentasaccharide that carries an extra *O*-sulfate group at position 3 of glucosamine residue 5. Oligosaccharides bearing *O*-methyl substituents on OH groups not involved in binding to AT, as well as "nonglycosaminoglycan" oligosaccharides where the $GlcNSO_3$ residues were replaced by 2-*O*-sulfated glucose residues, have proved to be as active as the natural pentasaccharide.[249] The problem of obtaining sufficiently long saccharide chains for binding both AT and thrombin has been approached first by assessing through chemical synthesis the size of the heparin sequence involved in thrombin inhibition,[360] and identification of a hexasaccharide/pentasaccharide sequence required to promote the inhibition of thrombin.[361] Linking a partially methylated pentasaccharide containing the essential residues and groups of the natural AT-binding sequence, through a neutral hexasaccharide tether, with the aforementioned hexasaccharide or pentasaccharide afforded products with anticoagulant and antithrombotic activities comparable with those of heparin. These experiments unequivocally established the topology of the AT-binding and thrombin-binding regions along the polysaccharide chain, with the former domain linked to the reducing end of the latter sequence (see also Ref. 243). In addition, the product is devoid of major side effects of heparin, such as activation of platelets, which may cause thrombocytopenia, and bleeding.[242,362]

Chemical synthesis has also provided useful models for studying the interaction of heparin/HS oligosaccharides with FGFs and has revealed structural variants compatible with binding to FGF-2 and induction of mitogenic activity.[363,364] Further, synthetic oligosaccharides have helped to assess the binding–facilitating role of nonsulfated IdoA as compared with GlcA residues[364] and to identify the minimal heparin sequence capable of inhibiting receptor binding of FGF-2[365] and FGF-1[366] and inducing binding to (and dimerization of) FGF-1.[85,358,367]

VI. Future Perspectives

HS proteoglycans attract increasing interest from researchers in widely different disciplines, and rapid progress can be foreseen in several areas. Refined methods for microscale structural analysis of HS, with regard to composition, sequence, and conformation, will be prerequisite to such advances. In particular, methods for efficient sequencing of small amounts of HS oligosaccharides are required. To this end, a variety of techniques/reagents will be exploited, including specific exo-enzymes, sequence-specific phage-display antibodies, mass spectrometry, and NMR spectroscopy. These methods will be applied primarily to the analysis of isolated protein-binding domains, but also, in a more extended perspective, to comprehensive "mapping" of HS species on cell surfaces and in different tissues.

The accumulation of sequence information will undoubtedly generate a complex picture of HS as a protein ligand. Certain types of functionally important interactions, such as "monodimensional diffusion" of proteins along HS chains,[368] are likely to depend on nonspecific polyelectrolyte effects rather than sequence-specific binding. Nevertheless, the occurrence of distinct saccharide epitopes in tissues, as visualized by specific monoclonal antibodies (see Section II), clearly points to the need for specificity in a multitude of interactions. So far, most studies in this regard have aimed at identifying the cognate HS sequences that correspond to specified proteins. The reverse question must be considered: Which protein(s) serve(s) as the natural ligand(s) for a given HS sequence?

Other questions that are likely to attract much attention in the near future bear on the actual functional roles of HS–protein interactions *in vivo*. Binding of a protein to the HS chain of a proteoglycan, at the cell surface or in the extracellular matrix, may serve a variety of purposes (storage/protection, protein presentation for interaction with other ligands, activation of protein effector functions).[369] Insights into the functional potential *in vivo* of HS have been provided by developmental biologists, who have uncovered an array of genes, in flies, worms, and mammals, with essential roles in embryonic development. Notably, whereas the structures of these genes have been well established, the functional roles of the corresponding proteins are only recently beginning to be understood. Information from quite diverse fields of research has revealed that a surprisingly large proportion of developmentally important genes are encode either core proteins of HS-proteoglycans, enzymes involved in the biosynthesis of HS chains, growth factors/cytokines depending on HS for their proper presentation/function, or the receptors of such factors.[370] This fruitful interaction between the fields of glycobiology and developmental biology is likely to expand.

Given the functional diversity of HS chains, and the specificity of their interactions with many proteins, the mechanisms behind the biosynthesis of the polysaccharide and its regulation will continue to attract interest. Valuable information is obtained through a variety of approaches, including GAG formation catalyzed by

enzymes in cell-free membrane preparations and cloning, expression, and functional characterization of biosynthetic enzymes, as well as mutational analysis of the biosynthetic process.[371] Models have been conjectured to account for the experimental data (see, e.g., Ref. 372). However, disturbingly little is still known about several key features of the process, such as the "on-off" mode of polymer modification that is a hallmark of HS formation. We need to know more about the interaction between the biosynthetic proteins and their organization in the Golgi membrane; notably, we have no information regarding any auxiliary proteins that lack catalytic activity, yet may be essential for the biosynthetic process. An ultimate goal would be the construction of functional biosynthetic machineries composed of recombinant proteins assembled in artificial membrane systems, which can be instructed to produce an HS chain of predetermined structure.

This last challenge leads to a final aspect of the future perspective, GAG biotechnology. The multitude of physiologically important processes that are mediated through specific HS–protein interactions would presumable trigger the development of saccharide (or glycomimetic) based drugs for clinical use, other than blood anticoagulation. Such a development would again depend on strong input from a variety of separate research areas, including carbohydrate–protein interactions (NMR spectroscopy; crystallography), organic and enzymatic synthesis, cell biology, and physiology.

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THE CHEMISTRY OF OLIGOSACCHARIDE LIGANDS OF SELECTINS: SIGNIFICANCE FOR THE DEVELOPMENT OF NEW IMMUNOMODULATORY MEDICINES*

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*Abbreviations.—The relatively wide scope of the present article requires some use of terms uncommon in carbohydrate chemistry and biochemistry; some of these are explained together with the list of abbreviations. AIBN, azobis(isobutyronitrile). All, allyl. Amino acids, one-letter code: A, Ala, alanine; C, Cys, cysteine; D, Asp, aspartic acid; E, Glu, glutamic acid; F, Phe, phenylalanine; G, Gly, glycine; H, His, histidine; I, Ile, isoleucine; K, Lys, lysine; L, Leu, leucine; M, Met, methionine; N, AsN, asparagine; P, Pro, proline; Q, GlN, glutamine; R, Arg, arginine; S, Ser, serine; T, Thr, threonine; V, Val, valine; W, Trp, tryptophan; Y, Tyr, tyrosine. Angioplasty, the repair of a blood vessel, as by inserting a balloon-tipped catheter to unclog it or by replacing part of the vessel with either a piece of the patient's own tissue or a prosthetic device. Apomucin, apoprotein, the carbohydrate-free

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protein portion of a mucin or other glycoprotein; apposing, placed side by side, juxtaposed. Atherectomy, surgical removal of an atheroma. Atheroma, a mass of yellowish fatty and cellular material that may form in and beneath the inner lining of arterial walls, when it is also referred to as atherosclerotic plaque. Atopy, a property of individuals who are prone to develop strong immediate hypersensitivity responses or allergies that may take the forms of hay fever, asthma, chronic eczema (skin irritation), or food allergies. Bn, benzyl. Bz, benzoyl. cDNA, a DNA molecule that is a copy of a messenger RNA molecule and therefore lacks the introns present in genomic DNA. CD, cluster of differentiation; CD numbers are used to designate leukocyte cell surface antigens. CLA, cutaneous lymphocyte antigen. ClAc, monochloroacetyl. COD, 1,5-cyclooctadiene. COS cells (CV1 origin SV40), a cell line derived from the monkey kidney cell line CV1 and carrying an SV40 (simian virus 40) provirus with a deficient origin of replication. CRD, carbohydrate-recognition domain. CSA, camphorsulfonic acid. Cutaneous, pertaining to skin. DAST, diaminoethylsulfur trifluoride. DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene. DDQ, 2,3-dichloro-5,6-dicyano-1,

4-benzoquinone. DMTST, dimethyl(methylthio)sulfonium trifluoromethanesulfonate. EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide. EGF, epidermal growth factor. ELAM-1, endothelial–leukocyte adhesion molecule 1. E-selectin. ELFT, ELAM-1 (namely E-selectin) ligand fucosyltransferase. Eosinophil, a leukocyte that usually has a bilobate nucleus and cytoplasmic granules that have an affinity for eosin and other acidic dyes. Episome, independent, extrachromosomal DNA molecule. Et, ethyl. G-Protein, any of numerous heterotrimeric GTP-binding proteins that function in intracellular signaling pathways. G-protein coupled receptor, member of an important class of cell-surface receptors that have seven transmembrane α-helices and are directly coupled to a trimeric G-protein. GAG, glycosaminoglycan. GlyCAM-1, glycosylation-dependent cell adhesion molecule-1. GMP-140, granule membrane protein of molecular mass 140 kilodalton, P-selectin. Granulocyte, a circulating white blood cell having prominent granules in the cytoplasm and a nucleus of two or more lobes. Hemolymph, a fluid in the body cavities an tissues of invertebrates, in arthropods functioning as blood and in some other invertebrates functioning as lymph. HEV, high endothelial venule. $HfCp_2Cl_2$, bis-(cyclopentadienyl)hafnium dichloride. HPAEC, high-pH anion-exchange chromatography. HUVEC, human umbilical vein endothelial cells. ICAM-1 (CD 54), ICAM-2 intercellular adhesion molecules-1 or -2, receptors of integrins, belong to the immunoglobulin superfamily. IDCP, iodonium dicollidine perchlorate. Inflammation, redness, swelling, pain, tenderness, heat, and disturbed function of an area of the body, especially as a reaction of tissues to injurious agents. Integrins, a large family of heterodimeric transmembrane proteins that promote adhesion of cells to the extracellular matrix or to the surface of other cells. Intravital, occurring during life. Ischemia, local deficiency of blood supply produced by vasoconstriction or local obstacles to the arterial flow. LAM, leukocyte adhesion molecule. LiTMP, lithium salt of 2,2,6,6-tetramethylpiperidine. LNFIII, lactoneofucopentaose III, CD 15. Lymphocyte, a type of white blood cell having a large spherical nucleus surrounded by a thin layer of nongranular cytoplasm; originates in the thymus or bone marrow. MAdCAM-1, mucosal (vascular) addressin cell adhesion molecule-1. MBn, 4-methoxybenzyl. mCPBA, *m*-chloroperoxybenzoic acid. Me, methyl. Mesentery, the membrane, consisting of a double layer of peritoneum, that invests the intestines, attaching them to the posterior wall of the abdomen, maintaining them in position in the abdominal cavity, and supplying them with blood vessels, nerves, and lymphatics, especially the part of this membrane investing the jejunum and ileum. Me_3Si, trimethylsilyl. MPh, 4-methoxyphenyl. Myocardium, the muscular substance of the heart. Neutrophil, a phagocytic white blood cell having cytoplasmic granules with affinity for neutral dyes, and a lobulate nucleus. NIS, *N*-iodosuccinimide. NMM, *N*-methylmorpholine. PAD, pulsed amperometric detection. PADGEM-protein, platelet activation-dependent granule to external membrane protein, P-selectin. PAGE, polyacrylamide gel electrophoresis. Parenchyma, the specific tissue of an animal organ as distinguished from its connective or supporting tissue. Percutaneous coronary intervention, a procedure to restore coronary blood flow during which skin is penetrated (angioplasty or atherectomy). Perivascular, in the space surrounding a blood vessel. Phth, *o*-phthaloyl-. Piv, pivaloyl, 2,2-Dimethylpropanoyl. PNA, peanut agglutinin. PPME, the phosphomannose-rich core polysaccharide from the yeast ***Hansenula holstii***. PSGL-1, P-selectin glycoprotein ligand-1. QAE-Sephadex, diethyl(2-hydroxypropyl)aminoethyl Sephadex, an anion-exchange resin. Revascularization, the restoration of the blood circulation of an organ or area, achieved by unblocking obstructed or disrupted blood vessels or by surgically implanting vessel replacements. SDS-PAGE, polyacrylamide gel elecrophoresis in the presence of sodium dodecyl sulfate. SSEA, stage-specific embryonic antigen. Spheroplast, a Gram-negative bacterial cell with a cell wall that has been altered or is partially missing, resulting in a spherical shape. TBDPS, *tert*-butyldiphenylsilyl. TCA, trichloroacetic acid. TEA, triethylamine. TFA, trifluoroacetic acid. Tf, trifluoromethylsulfonyl. TfOH, trifluoromethanesulfonic acid. Bu^tMe_2Si, *tert*-butyldimethylsilyl. Vascular endothelium, a thin, smooth layer of cells that line the inner lumen of a blood vessel. VCAM-1, vascular cell adhesion molecule-1, also referred to as INCAM-110 (inducible cell adhesion molecule). Virions, virus particles. VLA, very late activation antigen, a member of the integrin family. WGA, wheat germ agglutinin.

I. Introduction

On a huge hill,
Cragged, and steep, Truth stands,
And hee that will reach her,
About must, and about must goe;
And what the hills suddennes resists,
Winne So. . . .

John Donne

Oligosaccharide ligands of selectins have been the focus of intense interest on the part of investigators from a range of disciplines over the past decade. Such ligands, in the form of glycoconjugate glycans, or as model oligosaccharide derivatives, bind weakly but specifically to the carbohydrate-recognition domains of selectins. The selectins are designated E- (for endothelial), P- (for platelet), and L- (for leukocyte) selectin and constitute a three-member family of mammalian lectins that function as cell-adhesion receptors. Interaction of selectins with their oligosaccharide ligands on apposing cells mediates tethering and rolling, characteristic cellular manifestations that precede firm adhesion of cells in certain disease processes. Following recognition of the essential role of selectin–carbohydrate interaction in the extravasation of neutrophil leukocytes during inflammatory responses, a general pharmacological hypothesis was implied in the late 1980s according to which oligosaccharide ligands of selectins or their appropriately modified derivatives could become useful as specifically acting therapeutic agents.

In the present article, an attempt is made to provide a synoptic review of some of the lines of investigation that have converged and synergistically interacted in the past decade in a monumental interdisciplinary effort to develop clinically useful antiinflammatory therapeutics from inhibitors of selectin–ligand interactions. The discussion begins in Section II with a summary of early cell-physiological studies with monoclonal antibodies that led to the independent discoveries of E-, P-, and L-selectins in the fields of vascular physiology, platelet activation, and lymphocyte trafficking. As a result of these investigations, important insights were provided on the cellular distribution and mode of expression of the selectins, and their roles in acute and chronic inflammation. Application of the methods of molecular genetics then led to the molecular characterization and expression cloning of the selectins. Thereby revealed was the typical domain structure of the selectins, most notably the N-terminal lectin domain with its high degree of homology, not only among the selectins but also with previously known, C-(Ca^{2+})-type mammalian lectins such as liver lectin or mannose-binding proteins. Next discussed is the quest for the structures of the carbohydrate determinants that would physiologically bind to the lectin domains of the selectins, leading to the identification of the common sialyl-Lewis^x (sLe^x) tetrasaccharide motif.[1] This structure, shown in Fig. 1 together

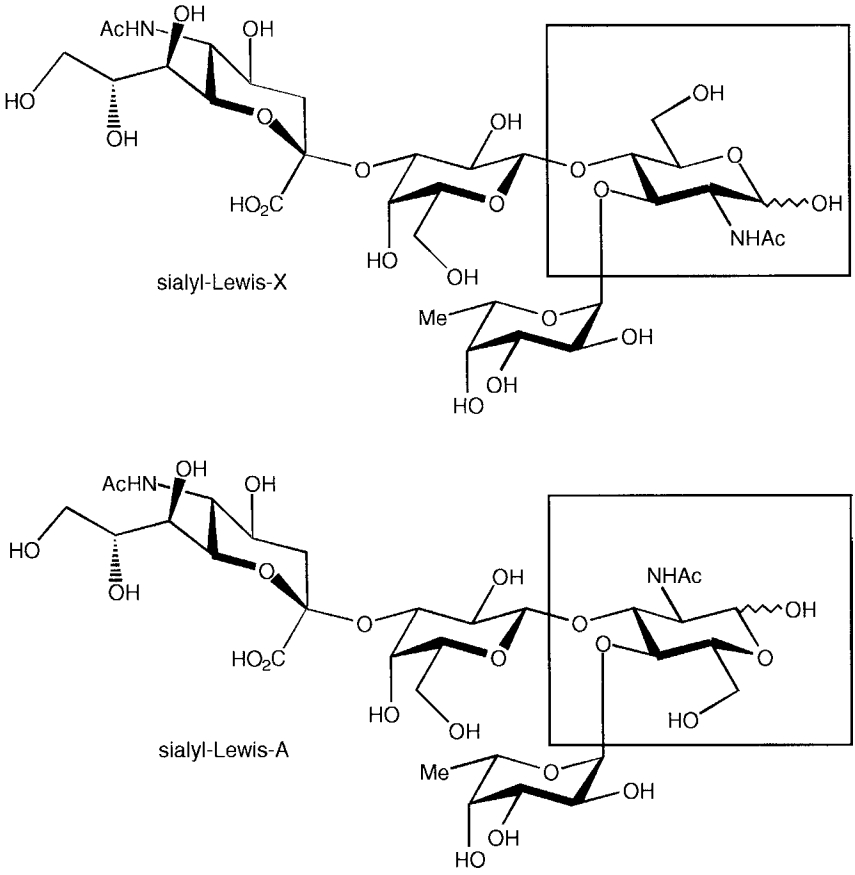


FIG. 1.

with the closely related sialyl-Lewis^a determinant,[2] is pivotal to the present review and constitutes a branched tetrasaccharide sequence composed of a nonreducing *N*-acetylneuraminyl (sialyl) unit linked α-(2 $\rightarrow$ 3) to a central galactosyl unit which is in turn linked β-(1 $\rightarrow$ 4) to a reducing *N*-acetylglucosaminyl unit; attached to the reducing unit is an L-fucosyl branch linked α-(1 $\rightarrow$ 3). In sLe^a, the Gal-GlcNAc linkage is β-(1 $\rightarrow$ 3) and the fucose residue is linked α-(1 $\rightarrow$ 4). Subsequently, for each of the selectins, additional structural features have been discovered that contribute to the specific interactions of the physiological glycoprotein ligands with each selectin. At the end of Section II, a description follows of some typical selectin–ligand binding assays that have been used to measure the binding strength of oligosaccharide analogues and candidate inhibitors.

In Section III, selected references to the roles of E- and P-selectins in disease processes are mentioned to indicate the areas of prospective clinical application of

inhibitors of selectin–carbohydrate interaction. Essentially three main areas have been investigated. One concerns the prevention of tissue damage during acute inflammation, as in reperfusion after occlusion of blood vessels. Second, immune suppression has been considered in conjunction with the prevention of transplant rejection and with potential therapies of autoimmune diseases. Finally, selectin–ligand interactions are thought to mediate certain steps in the process of cancer metastasis. In this context, inhibitors of selectin–carbohydrate interactions have been considered as potential antimetastatic agents.

Based on the highly developed art of oligosaccharide synthesis, organic-chemical, enzyme-catalyzed, and combined organic–enzyme-catalyzed syntheses of the sialyl-Lewis^x structure and related oligosaccharides have been devised. Selected examples of these syntheses are discussed in Section IV, some of them including recently developed procedures of glycoside formation.

Approaches to pharmaceutically applicable inhibitors of selectin–ligand interactions began with target structures modeled on the entire sialyl-Lewis^x tetrasaccharide. However, the scope of these studies was soon focused when structure–activity relationships emerged from consideration of the structures of naturally occurring ligands of the selectins (Section V).

These perceptions were corroborated through binding studies with synthetic oligosaccharide derivatives that contain modified sugar residues in place of the natural *N*-acetylglucosamine, galactose, *N*-acetylneuraminic acid, or fucose residues. Conversely, the carbohydrate-binding domains of the selectins were identified with the aid of fragment peptides, by mutational studies, and through X-ray crystal analyses of E-selectin and, especially, of mannose-binding proteins and their selectin-like mutants. Together, the data indicated that, on the side of the oligosaccharide ligands, complex formation between the selectins and the sialyl-Lewis^x determinant requires the fucose residue and an appropriately spaced negative charge, with some contribution from hydroxyl groups of the galactose residue. According to this model, the functionalities of the *N*-acetylglucosamine residue and those associated with the major portion of the *N*-acetylneuraminic acid residue would not be required for selectin–ligand binding. Accordingly, a typical carbohydrate-binding domain of a selectin would comprise two important structural elements. One is a cluster of three lysine residues opposite the negative charge of the *N*-acetylneuraminic acid unit; the other is a coordination shell around a calcium ion of which the ligands are functional groups of the polypeptide and two hydroxyl groups of the fucose residue.

As outlined in Section VI, this greatly simplified picture has been confirmed by an extensive series of binding studies with sialyl-Lewis^x derivatives that had been synthesized as candidate pharmaceutical agents. In accordance with the incremental refinement of structure–activity relationships over time, the design of inhibitory structures followed a series of models decreasing in complexity. These have been designated the tetrasaccharide, trisaccharide, disaccharide, and monosaccharide

models. As an example of the last category, a candidate pharmaceutical, conveniently accessible by a combined organic and enzyme-catalyzed synthesis, comprises a mannose residue representing the fucose residue of sialyl-Lewis^x, and a phosphate group appropriately spaced at the end of a hydroxylated linker chain to mimic the carboxylic acid function of the *N*-acetylneuraminic acid residue.

In keeping with the physiological roles of selectin–ligand interactions, it follows that candidate inhibitors optimally modeled according to the interactive pattern of the sialyl-Lewis^x oligosaccharide are relatively weak inhibitors in pharmaceutical terms. Two strategies have been successfully employed to produce more potent inhibitors. One involves the attachment to the molecules of aromatic or long-chain alkyl groups that would interact with hydrophobic areas within or adjacent to the carbohydrate-binding domain; alternatively, more potent inhibitors have been produced in the form of divalent or clustered ligands. In certain instances, binding of clustered ligands to target cells elicits biological effects that are not observed with monomeric ligands.

To conclude Section VI, some alternative concepts are briefly-mentioned that relate to inhibition of selectin–ligand interactions. These include soluble forms of selectins, monoclonal antibodies directed against selectins or their ligands, and inhibitors of the biosynthetic pathways of selectin ligand oligosaccharides.

In the final Section VII, arguments are presented concerning the prospects of inhibitors of selectin–ligand interaction becoming clinically successful pharmaceuticals. Such inhibitors might possess important advantages such as exquisite specificity of action and low toxicity. In a number of preclinical studies, the efficacy has been demonstrated of sialyl-Lewis^x derivatives in preventing P-selectin-mediated lung damage in rats following administration of cobra venom toxin or in the reduction of reperfusion injury following experimental ischemia in several animal models. However, clinical studies of selectin antagonists have been successful in limited areas only. In a major prospective indication—prevention of reperfusion injury following coronary infarction—administration of sialyl-Lewis^x derivatives has not provided significant patient benefit. Presumably, the weakly binding inhibitor is only partially effective in preventing cell adhesion, so that a sufficient number of granulocytes can extravasate to mediate the deleterious inflammatory effects.

As a competing concept in anti-adhesion therapy, inhibitors of integrin binding have been successfully developed by pharmaceutical companies over the past decade. These agents are directed at integrin-mediated cell adhesion events that mediate platelet aggregation, and at the interaction between the integrin VLA-4 and VCAM-1, the respective integrin counterreceptor. With their susceptibility to broad chemical variation, strength of receptor binding, and bioavailability following oral administration, anti-integrin receptor therapeutics possess a number of advantageous pharmacological properties that are difficult to achieve with polar small molecules modeled on carbohydrates.

To develop clinically useful therapeutics from inhibitors of selectin–ligand interactions, an approach is suggested that generates lead structures by targeting the hydrophobic segments within or adjacent to the carbohydrate-binding domains of selectins; conceivably, an appropriate combination of rational design, combinatorial chemistry and natural product screening will generate a sufficient number of suitable candidate compounds. Development of such leads may provide more potent therapeutics with the advantageous pharmaceutical properties frequently lacking in carbohydrates and other polar derivatives.

Many of the individual topics addressed in the present chapter have been discussed in the context of excellent specialist reviews. Some of these are cited in the respective sections. Wong and his associates have prepared a comprehensive review[3] entitled "Selectin–Carbohydrate Interactions: from Natural Ligands to Designed Mimics" which provides a complete survey of the literature dealing especially with the medicinal chemistry aspects of selectin inhibitor design; treatment of this aspect has therefore been restricted to a few representative examples in the present article. Vestweber[4] has edited a comprehensive monograph entitled *The Selectins: Initiators of Leukocyte Endothelial Adhesion*.

II. Selectins: Carbohydrate-Binding, Cell-Adhesion Molecules

The topic of selectins and their role as vascular adhesion molecules has been the subject of expert reviews.[5–7]

1. Discovery of E-, P-, and L-Selectins

a. E-Selectin, an Inducible Adhesion Molecule of Vascular Endothelial Cells.—From 1985 onward, several research groups reported that adhesion of leukocytes to vascular endothelial cells is increased subsequent to exposure of the endothelial cells to stimulants such as interleukin-1 (IL-1), lymphotoxin, tumor necrosis factor (TNF), or lipopolysaccharide (LPS).[8] Bevilacqua and his colleagues at Brigham and Women's Hospital, Boston, reported[9] that the inducible adhesion of cells of the promyelocytic cell line HL-60 to vascular endothelium is mediated by an inducible cell-surface protein they termed "endothelial leukocyte adhesion molecule-1 (ELAM-1)". Later, the term ELAM-1 was abandoned in favor of E-selectin.[10] Bevilacqua *et al.*[9] raised two mouse monoclonal antibodies (MAbs), termed H 18/7 and H 4/18, that bind to human E-selectin. From lysates of activated endothelial cells, these MAbs precipitated two polypeptides, a major component of molecular mass 115,000 and a minor component of molecular mass 97,000. The MAbs inhibited cell–cell adhesion in an assay[11] of ^{111}In-labeled polymorphonuclear neutrophils or HL-60 cells adhering to monolayers of human umbilical vein endothelial cells (HUVEC). With the use of the MAbs, surface expression of E-selectin was shown to be inhibited by cycloheximide (an inhibitor of protein biosynthesis[12]) or actinomycin D (an inhibitor of transcription[13]).

b. P-Selectin.—In 1984, McEver and Martin and the group of Barbara and Bruce Furie concurrently reported on the discovery of P-selectin with the aid of mouse MAbs. McEver and Martin[14] had developed MAb S12, specific for a membrane glycoprotein antigen that is exposed on the surface of human blood platelets only after activation of these cells, for example, by the action of thrombin or histamine. Thrombin is a proteolytic enzyme that converts fibrinogen into fibrin as part of the blood clotting process.[15] Histamine is a potent vasodilator and is derived from histidine by decarboxylation.[16] The activation-dependent antigen, later to be characterized as P-selectin, was isolated by immunoadsorption on an S12 antibody–agarose column. On polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE), the native protein had an apparent molecular mass of 138,000 (148,000 under reducing conditions). Staining with both the periodic acid–Schiff reagent[17] and Coomassie Blue[18] indicated P-selectin to be a glycoprotein. The Furie group[19] elaborated a mouse MAb similar to S12, designated KC4. This antibody interacts with P-selectin with a binding constant $K_D = 7.2 \pm 0.4$ nM, roughly comparable to the $K_D = 1.5$ nM found for S12 by McEver and Martin.[14] Isolated by immunoadsorption on a KC4-antibody–agarose column, P-selectin had an apparent molecular mass of 140,000 under both native and reducing conditions. Since P-selectin is present in total extracts of resting platelets, both McEver and Martin and the Furies suggested that it is normally a constituent of internal granule membranes that becomes translocated to the external membrane in the course of the redistribution of glycoproteins ("rearrangement") that accompanies platelet activation. This was confirmed by Stenberg *et al.*[20] and the Furie group[21] through electron microscopy of frozen thin sections with IgG-colloidal gold[20] or indirect immunolabeling with rabbit anti-P-selectin antibody, biotinyl goat anti-rabbit IgG as second antibody, and an avidin–colloidal gold conjugate for visualization.[21] The McEver group, in their early reports, designated[20] P-selectin as GMP-140 (granule membrane protein of molecular mass 140 kDa); the Furie group[21] gave P-selectin the name PADGEM protein (platelet activation dependent granule-external-membrane protein) and reported that GMP-140 and the PADGEM protein are identical; both terms have been abandoned[10] since 1991 in favor of P-selectin (CD62P). In 1987, McEver *et al.* reported that, as in α-granules of platelets, P-selectin is constitutively expressed in Weibel–Palade bodies of vascular endothelial cells.[22] Later, P-selectin from human platelets was characterized[23] by Johnston *et al.* using PAGE, as a glycoprotein of apparent molecular mass 140,000 under reducing and 120,000 under nonreducing conditions. Together with the high content of cysteine (as half-cystine), the difference in electrophoretic mobility suggested the presence of a relatively large number of intrachain disulfide bridges. P-selectin was estimated to contain 12 N-linked glycan chains of the complex type.[24] Although at that stage no reports on the carbohydrate binding of P-selectin had yet appeared, its behavior in the systems studied had been interpreted to indicate a role in the physiological response to vascular damage. McEver *et al.*[25] discovered that P-selectin is present not only in blood

platelets and their precursors, megakaryocytes, but also in HUVEC where it is localized in the membranes of Weibel–Palade bodies, the storage granules for von Willebrand factor.[26] Upon stimulation of the endothelial cells, P-selectin was rapidly redistributed to the plasma membrane. Geng *et al.*[27] confirmed the redistribution of P-selectin to the plasma membrane of vascular endothelial cells during cellular activation. They furthermore found that human neutrophils and HL60-cells bind specifically to COS cells transfected with P-selectin cDNA, and to microtiter wells coated with purified P-selectin. The authors interpreted these findings to indicate that P-selectin expressed on activated endothelial cells mediates rapid targeting of neutrophils to sites of acute inflammation.

c. L-Selectin.—In 1983, Gallatin, Weissman, and Butcher[28] reported on a rat monoclonal antibody, termed MEL-14, that is specific for a surface antigen of the mouse B cell lymphoma 38C-13. Cells of this lymphoma line adhere to the postcapillary, high-endothelial venules (HEVs) of mouse peripheral lymph nodes but not to those of gut-associated lymphoid tissue (GALT or Peyer's patches). The functional significance of the MEL-14 antigen was demonstrated by means of the Stamper–Woodruff assay,[29–31] a technique for quantitative measurements of the adhesion of lymphocytes to HEV in frozen sections of target organs such as peripheral lymph nodes or Peyer's patches. Thus, preincubation of cells of the 38C-13 mouse B-cell lymphoma clone with MEL-14 but not with other antibodies was shown to inhibit their ability to adhere to high endothelial venules. In addition, the MAb MEL-14 was found to inhibit the adhesion of normal lymphocytes to high endothelial venules of peripheral lymph nodes but not of Peyer's patches. Pretreatment of lymphocytes with MAb MEL-14 also inhibited their "homing" into lymph nodes *in vivo*. The MEL-14 antigen, later designated L-selectin, had an estimated molecular mass of 80,000 Da when isolated from normal mesenteric node lymphocytes and of 92,000 in lysates of 38C-13 cells (by SDS-PAGE). Following the observation[32] that L-selectin is shed from the surface of mouse neutrophils upon activation of the cells with phorbol 12-myristate 13-acetate (PMA),[33] Kishimoto *et al.*[34] subjected human peripheral blood leukocytes to PMA activation and raised monoclonal antibodies against the proteins that were shed into the supernatant. In this manner, five MAbs designated DREG-55, -56, -110, -152, and 200 (for being directed against a rapidly down-regulated antigen) were obtained that define an 80–85 kDa protein involved in human lymphocyte binding to peripheral lymph node HEVs. The MAb DREG-56 inhibits, to an extent >90%, the binding of human lymphocytes to HEV within frozen sections of peripheral, but not mucosal, lymphoid tissue.

2. Molecular Characterization and Expression Cloning of E-, P-, and L-Selectins

a. Distinction of C-Type and S-Type Mammalian Lectins.—In his review[35] of 1988, when the polypeptide sequences of the selectins had not yet been

announced, Drickamer discussed two distinct classes of animal lectins termed C-type (for calcium-dependent) and S-type lectins (for requiring free SH-groups). More recently, Barondes *et al.*[36] have distinguished among four animal lectin families including, in addition to C-type and S-type lectins, the P-type lectins and pentraxins. In the course of their molecular characterization, all three selectins have been identified as C-type lectins. The classical example of a C-type lectin is the rat hepatic lectin, a protein that functions as a receptor of asialoglycoproteins in mammalian liver and binds mainly to nonreducing β-D-Gal residues of glycoprotein glycans. The rat hepatic lectin, subject of pioneering investigations by Ashwell and his associates,[37] mediates the endocytosis of the bound glycoproteins and their routing to lysosomes for eventual degradation. The related chicken hepatic lectin fulfils a similar function in birds but is specific for *N*-acetylglucosaminyl residues. Other known C-type lectins included mannose-binding proteins (cf. Section V.5), a sea urchin lectin, and core polypeptides of proteoglycans. Common to these C-type lectins is the size of the carbohydrate recognition domain (CRD) of ~130 amino acids, the requirement of Ca^{2+} ions for binding of carbohydrate ligands, the disulfide oxidation state of the cysteine residues, and the presence of 18 invariant amino acid residues in a conserved pattern within the CRD. An alignment of the amino acid sequences of 39 C-type animal lectins including the E-, P-, and L-selectins and the rat and human mannose-binding proteins is found in a modeling study by Mills[38] of the CRD of human E-selectin.

By contrast, binding of carbohydrate ligands to S-type lectins (later referred to as galectins[36]) is independent of divalent cations. The thiol functions of the cysteinyl residues in S-type lectins need to be in the form of free SH-groups for binding of ligands. Many of the S-type lectins are specific for β-Gal units. Some show selectivity of binding to more complex, Gal-containing glycans or for GalNAc units.[39,40]

b. Molecular Characterization and Expression Cloning of E-Selectin.—

(i) ***Work at Harvard Medical School.—***Molecular cloning[41,42] of E-selectin from human vascular endothelial cells was reported by Bevilacqua and his colleagues[43] in 1989. These authors derived a suitable complementary DNA (cDNA) library[41] by the action of reverse transcriptase on RNA extracted from cultured endothelial cells following stimulation with interleukin-1 (IL-1). This stimulus induces protein synthesis of E-selectin and therefore, E-selectin mRNA would be present in the RNA extracted. The cDNA was then modified with adaptor sequences and ligated into the πH3M vector using the cloning site created by excision with the BstXI restriction endonuclease. Vector πH3M is a single-stranded vector that contains the origin of replication of bacteriophage M13 and is suitable for expression cloning in mammalian cells.[44,45] Following insertion of the cDNA library, the vector was transfected into *Escherichia coli* MC 1061/p3. From the transfected bacterial cells, spheroplasts were prepared by careful removal of the bacterial cell wall and fused with COS cells for transfection. Transfected COS

cells were isolated by a "panning" technique[44,45] as follows. COS cells expressing E-selectin at the cell surface attach strongly to culture dishes coated with the anti-E-selectin monoclonal antibodies H 18/7 or H4/18, whereas cells not expressing the surface antigen would be removed by washing. The episomal DNA from transfected COS cells was isolated, expanded in *E. coli* strain MC 1061/p3, and subjected to three more cycles of transfection by spheroplast fusion and selection by antibody panning. In this manner, a cDNA clone designated pELAM-1 was finally obtained. The E-selectin cDNA of this clone is 3.85 kb long and contains a 5′-untranslated region of 116 bases. The length of the continuous open reading frame is 1830 bases, corresponding to 610 amino acid residues. The cDNA also contains a relatively long 3′-untranslated region of 1898 bases ending in a poly-(A) sequence. This region contains several repeats of a DNA sequence that is transcribed into the messenger sequence AUUUA. This confers instability on the respective mRNA, a feature found with other transiently expressed proteins. The start codon ATG occurs at a consensus initiation site of mammalian protein biosynthesis.[46] Following a signal sequence, the first amino acid residue of mature E-selectin is tryptophan. The mature protein consists of 589 amino acid residues and has a molecular mass of 64 kDa. The N-terminal protein domain is ∼120 amino acid residues long and is related to the C-type lectins. The domain comprising residues 121–155 shows homology to epidermal growth factor (EGF). This is followed by six tandem repeats of ∼60 amino acid residues each, showing homology to complement regulatory proteins. Amino acid residues 536–557 constitute the transmembrane region, which is followed by a cytoplasmic sequence of 32 amino acid residues.

(ii) ***Cloning of E-Selectin at Biogen, Inc.*****—**An alternative approach to molecular cloning of E-selectin was reported by the group of Lobb.[47] Using RNA from HUVEC treated with human IL-1β, these authors created a cDNA library in the animal cell expression vector[45,48] CDM8. To enrich for sequences induced by IL-1β, the library was screened against a subtracted cDNA probe that had been generated from the initial library by two rounds of hybridization with mRNA from uninduced HUVEC.[49] The preparation of subtracted libraries is discussed in a recent book chapter.[50] The resulting sub-library had only 864 colonies, representing ∼0.09% of the original library, and was used to transfect COS cells by spheroplast fusion.[44] The cells were screened after 48 h for their ability to bind HL-60 cells. Transformed COS cells that expressed E-selectin were identified by their formation of rosettes with HL-60 cells labeled with carboxyfluorescein diacetate.[51] In two rounds of panning, rosettes were picked from the plates, and plasmids were rescued into *E. coli* MC1061/p3. In the third round, individual clones were transfected. From the subsequent screen, a full-length cDNA was obtained, designated ELAM/CDM8 and encoding functional E-selectin. Both HL-60 cells and human polymorphonuclear leukocytes adhered to COS cells transfected with this

cDNA. In comparison to the earlier-reported sequence[43] of Bevilacqua *et al.*, the nucleotide sequence determined by Hession *et al.*[47] includes 24 additional bases of 5′-untranslated region and differs at 5 nucleotides. However, only one C → T difference results in an amino acid exchange His-477 → Tyr-477, with the others located in the 3′-untranslated region. Hession *et al.* knew of the lectin nature of E-selectin and L-selectin[52] and examined a number of carbohydrate derivatives for their ability to inhibit the adhesion, to HL-60 cells, of COS cells transformed with E-selectin. Mannose 6-phosphate or fucoidan,[102] which had been known[52] to inhibit the interaction of L-selectin with HEV, was not inhibitory, nor were 10 m*M* concentrations of galactose, galactose 1-phosphate, galactose 6-phosphate, fucose, mannose, or *N*-acetylglucosamine. Fucose, mannose, or galactose coupled to human serum albumin were not inhibitory either. From these findings, Hession *et al.* rightly concluded[47] that the adhesive function of E-selectin may be restricted to a complex carbohydrate structure that cannot be mimicked by simple sugars.

c. Molecular Characterization and Expression Cloning of P-Selectin.— In early 1989, the molecular cloning of P-selectin was announced by McEver and his associates[53] at the University of Oklahoma. These authors prepared a 35-mer (pentatriakontamer) oligonucleotide probe on the basis of the amino acid sequence IVRCDNLGQWTA. This oligopeptide had been identified by Edman degradation[54] as the N-terminal portion of a peptide obtained by protease-catalyzed hydrolysis of reduced and S-alkylated P-selectin. With the aid of this probe, a human umbilical vein endothelial cell DNA library of 1.4 million recombinant λgt11 bacteriophages was screened and three clones identified. None of the three contained the desired full-length cDNA. A fourth P-selectin cDNA, this one of full length, was identified by rescreening of an amplified endothelial cell DNA library, using as a probe a 1 kb restriction fragment of one of the incomplete cDNAs obtained in the initial screening. All four overlapping clones were used to derive the nucleotide sequence of P-selectin cDNA.

The predicted sequence contained an open reading frame of 2490 bases, corresponding to 830 amino acid residues. Six domains of the translated protein were distinguished. Considering a signal peptide sequence of 41 amino acid residues, the mature protein would consist of 789 residues. Its theoretical amino acid composition and molecular mass of 86,244 Da were found to agree with previous experimental data.[23] The N-terminal domain of 118 amino acid residues contains the invariant residues of the C-type lectin motif as discussed by Drickamer[35] (compare Fig. 2). Residues 119–148 constitute the "EGF domain" named for its homology with the epidermal growth factor precursor. The following domain (residues 159–730) consists essentially of nine tandem repeats of 62 amino acid residues each; these show homology to complement regulatory proteins. The subsequent membrane-spanning domain contains mostly hydrophobic amino acid residues.

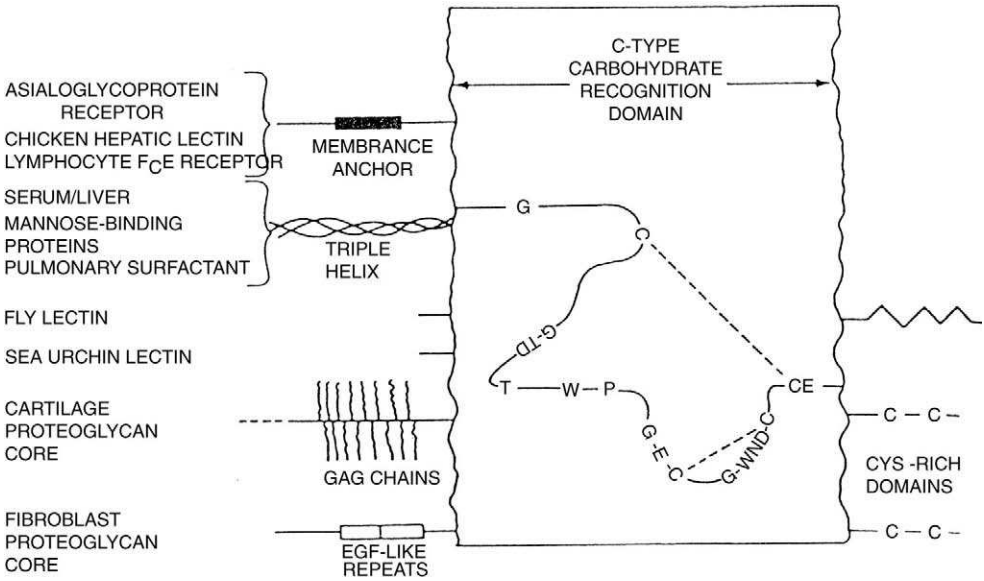


FIG. 2. Summary of structural features of C-type animal lectins. The (nearly) invariant residues found in the common carbohydrate-recognition domain of the C-type lectins are shown, flanked by schematic diagrams of the special effector domains (if any) found in individual members of the family. GAG, glycosaminoglycan; EGF, epidermal growth factor. Reproduced from K. Drickamer, Two Distinct Classes of Carbohydrate-recognition Domains in Animal Lectins, *J. Biol. Chem.*, 263 (1988) 9557–9560 (Ref. 35); © 1988. The American Society for Biochemistry & Molecular Biology; with permission by Professor Kurt Drickamer and The American Society for Biochemistry & Molecular Biology.

Finally, the C-terminal, cytoplasmic domain of P-selectin consists of 35 amino acid residues, several of which are highly charged as expected.

d. Molecular Characterization and Expression Cloning of (Mouse) L-Selectin.—

(i) ***Work Performed at Stanford University.—***Weissman and his associates[55] reported on L-selectin produced in the cell line EL4-MEL14hi. L-Selectin was purified by antibody affinity chromatography on columns of MEL14 antibody,[28] followed by size-exclusion chromatography. The N-terminal pentapeptide sequence was identified as Trp-Thr-Tyr-His-Tyr (WTYHY) by Edman degradation,[54] and a corresponding pentadecamer (15-mer) oligonucleotide probe with 32-fold codon degeneracy was synthesized by the phosphoramidite method.[56] These oligonucleotides were used to screen 7.5×10^5 λ ZAP placques of a cDNA library prepared from EL4-MEL14hi DNA, resulting in the identification of 58 independent isolates. Sequencing and analysis of the isolates led to a single cDNA clone of

∼1550 base pairs that was found to encode the N-terminal pentapeptide sequence WTYHY and a number of predicted downstream amino acids. The nucleotide sequence of the cDNA was determined by the dideoxy chain termination method[57] and comprises a 5′-untranslated region of 54 base pairs followed by a single ATG initiator codon with a flanking sequence typical of eukaryotic translation initiation sites. The open reading frame is 1116 base pairs in length and is followed by a 3′-untranslated region of 327 base pairs. The amino-terminal 118 amino acid residues are homologous to the hepatic lectins of human and rat, and the 45 amino acid residues 74 to 118 correspond to the 50 carboxyl-terminal residues of the carbohydrate binding domain in animal lectins. Characteristic amino acid residues include three cysteine residues at positions 90, 109, and 116, AsN and Trp at positions 75 and 76, Glu-Pro-AsN (80–82), a cluster of three Lys residues at positions 84, 85, and 87, Glu (88), Cys, and Val at positions 90 and 91, and the sequence Gly-Lys-Trp-AsN-Asp at positions 102–106. Amino acid residues 118–155 comprise the EGF-like domain. This is followed by two identical repeats of 62 amino acid residues (156–217 and 218–279), preserving a motif seen in complement regulatory and other proteins. Finally, a hydrophobic transmembrane region (amino acids 295–317) is followed by a cluster of charged amino acid residues, and a hydrophilic cytoplasmic tail of 18 amino acid residues.

(ii) ***Cloning of Mouse L-Selectin at Genentech and the University of California, San Francisco.—***Concurrent work on the molecular characterization of L-selectin was reported[58] by Lasky and Rosen and their associates. A cDNA clone of mouse L-selectin was isolated as follows. L-Selectin was first isolated as a protein of molecular mass ∼90,000 by immunoaffinity chromatography on a MEL-14 antibody column of a detergent lysate of mouse spleen. The N-terminal sequence of the protein (48 amino acid residues) was determined by Edman degradation.[54] Out of this sequence, a sequence of nine amino acid residues EKPMNWENA, comprising residues 7–15, were chosen to produce a hexeicosamer (26-mer) oligonucleotide probe with 32-fold codon redundancy. Using this probe, a mouse spleen DNA library (500,000 independent λgt10 bacteriophages)[41] was screened, and a single hybridizing cDNA clone was isolated. The corresponding phage contains a 2.2 kb EcoRI insert; the longest open reading frame of this insert begins with a methionine codon at position 106–108, a region homologous with other translation initiation sites of eukaryotic mRNAs.[46] Within the open reading frame, a protein sequence is encoded of 372 amino acid residues (corresponding to a molecular mass of 42,200 Da). The sequence contains 10 potential glycosylation sites, all within the extracellular domain of L-selectin. According to sequence similarities and homologies, the total sequence may be subdivided into six domains. Amino acid residues 39–76 correspond to the N-terminal sequence as determined from the isolated MEL-14 antigen. Tryptophan 39 is the probable N-terminal amino acid of the mature L-selectin. The domain corresponding to residues 39–155 was

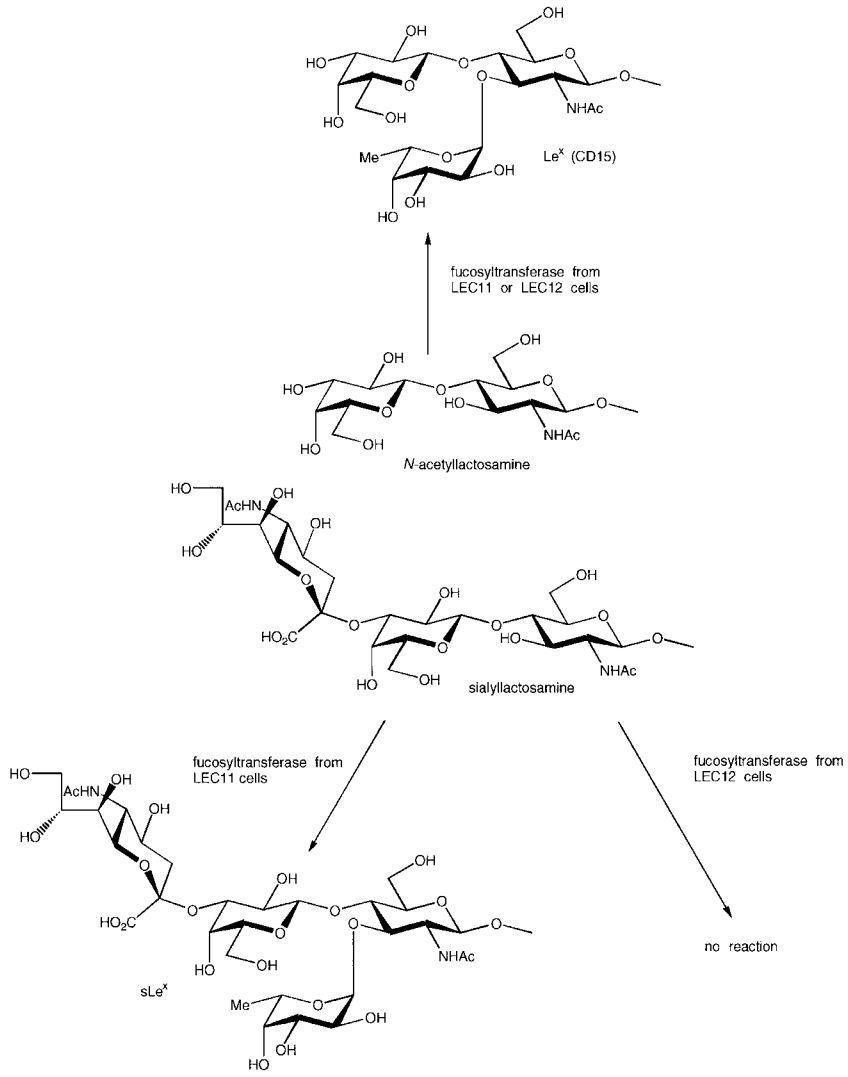
found to be homologous to a high degree with the C-type animal lectins described by Drickamer.[35] The motif comprising amino acid residues 160–193 shows a high degree of homology with the epidermal growth factor family. The last of the motifs of the extracellular portion of L-selectin (residues 197–328) consists of two repeats homologous to proteins that have been shown to bind to the complement components C3b or C4b. The two repeats are precisely identical on both the polynucleotide and protein levels. Finally, L-selectin was shown to contain transmembrane and intracellular domains of 22 and 17 amino acid residues, respectively.[58]

3. Investigations of the Natural Carbohydrate Determinants That Bind to Selectins

The intense interest in the perceived pharmacological potential of E-selectin ligands stimulated investigations, by several research groups, into the nature of the oligosaccharide ligands that mediate the interaction of cells with selectins. The early studies toward this goal have been reviewed[59,60] and more recent developments have been discussed in a chapter by Lowe.[61] Lowe and his associates[62] demonstrated that transfection of the Lewis α-1,3-fucosyltransferase cDNA into COS-1 or CHO cells results in COS-1 derived transfectants that express the sLe^x, Le^x, Le^a, and sLe^a determinants, and in CHO transfectants expressing the sLe^x and Le^x structures. While wild-type COS-1 or CHO cells do not bind to E-selectin, the fucosyltransferase transfectants were shown to bind to TNF-stimulated human umbilical vein endothelial cells (HUVEC).

a. E-selectin.—The identification of sLe^x as a ligand glycan of E-selectin was performed independently by the groups of Paulson[63] at Cytel Corporation and Bevilacqua,[64] then at the Harvard Medical School, and was reported back-to-back in 1990.

To identify the glycan chains that constitute the physiological ligands of E-selectin, the Cytel investigators[63] used two mutant cell lines, termed LEC11 and LEC12, that had been derived from chinese hamster ovary (CHO) cells by Stanley and her associates.[65] Following chemical mutagenesis of CHO cells, Stanley had selected[66] mutants LEC11 and LEC12 on the basis of their resistance to toxic concentrations of the lectins, wheat germ agglutinin (WGA), Lens culinaris lectin (LCA), and Phaseolus vulgaris leukoagglutinin (L-PHA). In contrast to wild-type CHO cells, both LEC11 and LEC12 cells were found[67] to bind an anti-SSEA-1 MAb (a monoclonal antibody directed against Le^x, an antigen that had been designated[68,69] ***stage specific embryonic antigen 1*** on the basis of earlier embryological studies). The appearance of the Le^x determinants on the mutant CHO cell lines was explained[67] by the expression of GDP-fucose:*N*-acetylglucosaminide 3-α-L-fucosyltransferase [α(1,3)fucosyltransferase] activity in LEC11 and LEC12 cells. Differences in substrate specificity and susceptibility to inhibition indicated[70]



SCHEME 1

that the fucosyltransferase activity in each cell line is due to one of two structurally distinct enzymes that is not expressed in the other cell line. Both the enzymes from LEC11 and LEC 12 cells were found[70] to catalyze the fucosylation of lactosamine-type acceptors that do not contain sialyl units (β-D-Gal-(1 $\rightarrow$ 4)-β-D-GlcNAc; Scheme 1) whereas the enzyme from LEC 11 cells catalyzes, in addition, the transfer of fucose units to sialosylated lactosamine portions of glycoprotein glycans

[α-D-Neu5Ac-(2 $\rightarrow$ 3)-β-D-Gal-(1 $\rightarrow$ 4)-β-D-GlcNAc]. This difference in acceptor specificities was more closely defined by Stanley and her associates[71] using a variety of glycolipid acceptors. Thus, the fucosyltransferase from LEC12 cells was shown to catalyze 3′′-fucosylation of lacto-*N*-tetraosylceramide (nLc$_4$OseCer) but not of either the 3′′′- or 6′′′-sialylated derivatives of that glycolipid. In contrast, the enzyme from LEC11 cells catalyzes the transfer of a fucosyl unit to O-3 of GlcNAc in both nLc$_4$OseCer or its 3′′′-sialylated homologue IV^{3}Neu5AcnLc$_4$Ose Cer. Neither fucosyltransferase would catalyze fucosylation of the 6′′′-sialylated glycolipid IV^{6}Neu5AcnLc$_4$Ose Cer. Stanley and Atkinson[72] reported on ^{1}H nuclear magnetic resonance (NMR) evidence for the biosynthesis, in LEC11 and LEC12 cells, of glycoprotein glycans containing the Le^x and sLe^x determinants. The authors grew vesicular stomatitis virus (VSV) in wild-type CHO cells and in the mutant cell lines, LEC11 and LEC12. Virus grown in wild-type CHO cells contains a glycoprotein, termed G protein, that comprises N-linked, biantennary glycans of the complex type.[73] However, when VSV is grown in LEC12 cells, the GlcNAc residues of those antennae that do not contain nonreducing Neu5Ac units become 3-fucosylated. Following harvest of the virus, the G protein was isolated and degraded by pronase digestion. The milligram quantities of glycopeptides containing the biantennary complex type glycans were isolated by affinity adsorption on concanavalin A (conA)-Sepharose. Analysis of the 500 MHz ^{1}H NMR spectra of the glycopeptides in D_2O indicated the presence of fucose residues linked α-(1 $\rightarrow$ 3) to GlcNAc, a type of linkage not formed when VSV is grown in wild-type CHO cells. Resonances diagnostic[74] of the α-(1 $\rightarrow$ 3)-linked fucose residues are those at δ 1.176 and 1.165 (CH_3), 4.83 (H-5), and 5.1 ppm (H-1). Fucosylation of the GlcNAc residues in sialylated complex-type *N*-glycans of G protein occurred when VSV was grown in LEC11 cells, as demonstrated by an immunochemical experiment. Virions grown in LEC12 or LEC11 cells, in their native state or following treatment with neuraminidase, were incubated with an iodine-labeled anti-SSEA-1 (anti-Le^x) MAb and subjected to equilibrium density gradient centrifugation. To virions grown in LEC12 cells, the anti-Le^x MAb bound equally well before or after treatment with neuraminidase. In the case of virions grown in LEC11 cells, the extent of MAb binding was approximately one-third higher following neuraminidase treatment. These findings confirm that Le^x determinants are formed during (viral) glycoprotein biosynthesis in both the LEC12 and LEC11 CHO mutant cell lines, while sLe^x determinants are additionally formed in LEC11 cells.

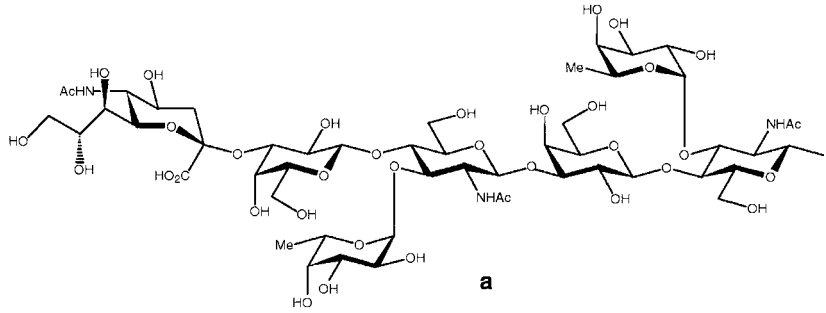
When cell adhesion was examined[63] of LEC11, LEC12, or wild-type CHO cells to interleukin-1-stimulated, human umbilical vein endothelial cells, it was found that LEC11 cells adhered whereas LEC12 or wild-type CHO cells did not. This result strongly suggested that cell adhesion in this case is mediated by sLe^x on LEC11 cells binding to E-selectin on the endothelial cells. To substantiate this interpretation, Paulson and his associates performed several control experiments,

as follows. The participation of E-selectin in the adhesion process was confirmed by the finding that adhesion was inhibited when the endothelial cells had been incubated with a monoclonal anti-E-selectin antibody prior to the assay. Furthermore, none of the cell lines examined would adhere to resting, unstimulated endothelial cells (cf. p. 214). Similarly, participation of sLe^x on the surface of LEC11 cells in the adhesion process was confirmed by the 90% inhibition of adhesion observed following incubation of the LEC11 cells with the monoclonal anti-sLe^x antibody CSLEX1.[75] Preincubation of LEC11 cells with an anti-Le^x MAb resulted in but slight inhibition of cell adhesion. Analogous preincubation of the endothelial cells with the anti-sLe^x MAb did not result in abrogation of binding. Additional confirmation of sLe^x binding to E-selectin was provided through inhibition experiments using liposomes that contain glycolipids comprising sLe^x, Le^x, or unrelated determinants in their glycans. Preincubation of endothelial cells with liposomes containing the glycolipid $III^3V^3Fuc_2VI^3Neu5AcnLac_6Cer$ (s-di-Le^x-ceramide) abrogated cell adhesion between LEC11 and endothelial cells. Liposomes prepared from glycolipids that contained nonreducing Le^x (di-Le^x) determinants effected a lesser degree of inhibition (40%).

Bevilacqua and his associates[64] identified sLe^x as a ligand of E-selectin, through the use of a soluble, chimeric protein molecule that had been genetically constructed from cDNA fragments coding for the extracellular domains of E-selectin and for the hinge, CH2 and CH3 domains of human immunoglobulin G_1 (IgG_1); these regions are constant regions of the heavy chain of the IgG molecule.[76] As a matrix to which cells expressing E-selectin ligands would adhere, the E-selectin/IgG chimera was used bound to a layer of goat antibody directed against human IgG, coated onto plastic wells. Granulocytes and the myeloid cell lines HL-60 and THP-1 were found to adhere to the matrix, as were other myeloid cells and some carcinoma cells. Initially, the ability of cells to adhere to the E-selectin matrix appeared to correlate with the presence of Le^x (CD15) surface determinants on the cell lines examined. However, treatment of cells with neuraminidase both abolished adhesion to the E-selectin matrix and increased the binding to cells of anti-Le^x monoclonal antibodies. This finding suggested that the sialylated form of Le^x (sLe^x) would be the determinant that mediates granulocyte adhesion to the E-selectin matrix. The hypothesis was corroborated by the results of binding experiments with the anti-sLe^x monoclonal antibody,[75] CSLEX1. Indeed, the higher the cell surface density of sLe^x determinants as measured by CSLEX1 binding, the more cells were found to adhere to the E-selectin matrix. Adhesion was completely inhibited by CSLEX1 but not by antibodies to related structures such as CD15 (Le^x) or CD65 {α-D-Neu5Ac-(2 → 3)-β-D-Gal-(1 → 4)-β-D-GlcNAc-(1 → 3)-β-D-Gal-(1 → 4)-[α-L-Fuc-(1 → 3)]-β-D-GlcNAc} (compare **242,** Section V.1). Additional experiments have been performed to demonstrate that the tetrasaccharide determinant α-D-Neu5Ac-(2 → 3)-β-D-Gal-(1 → 4)-[α-L-Fuc-(1 → 3)]-β-D-GlcNAc is indeed the minimum E-selectin ligand. The mucins of amniotic fluid

contain sLe^x determinants.[77] Whereas, on granulocyte surface glycoproteins, the sLe^x tetrasaccharides are linked to β-D-Gal residues of N-glycans,[78] in amniotic mucins they are linked β-(1 → 6) to a 3-substituted α-D-GalNAc residue O-linked to serine or threonine residues of the apomucin. Inhibition by amniotic fluid, or by purified amniotic mucin, of myeloid cell adhesion to the E-selectin matrix indicated that the sLe^x tetrasaccharide functions as the E-selectin ligand, independent of additional constituents of the respective glycoconjugate glycans. Finally, Bevilacqua and his associates[64] converted the N-linked glycan chains of α_1-acid glycoprotein, containing partially sialylated *N*-acetyllactosamine determinants β-D-Gal-(1 → 4)-β-D-GlcNAc, into E-selectin ligands by enzyme-catalyzed transfer of fucose residues from GDP-fucose to the 3-positions of the GlcNAc residues.[79] The α-1,3-fucosyltransferase used had been isolated from human amniotic fluid by affinity chromatography.[80] To determine the extent of E-selectin binding to the glycoprotein containing the modified glycan chains, the following assay system was employed. Fucosylated α_1-acid glycoprotein (or a non-fucosylated control) was adsorbed to plastic wells, and soluble E-selectin-IgG chimeric protein was added. Binding of the chimera was assessed with the aid of a sandwich radioimmune assay, using ^{131}I-labeled goat antibody to human IgG (see Section II.5 for assay systems). As expected, significantly more E-selectin–IgG chimera was bound to the wells that contained fucosylated glycoprotein than to those containing the unfucosylated controls, substantiating once more the role of the sLe^x determinant as the ligand of E-selectin. In α_1-acid glycoprotein, the sLe^x tetrasaccharides are bound to mannosyl residues; the similar extent of E-selectin binding of the sLe^x ligand, irrespective of the type of glycan to which it is attached, was taken to indicate a more general role of the sLe^x tetrasaccharide as the physiological ligand of E-selectin.[64]

(i) ***E-Selectin Ligand Glycans with High Affinity.—***In an elegant and comprehensive investigation, Patel and his associates[81] at Oxford Glycosystems and Biogen Inc. have identified a set of glycans that bind to E-selectin with high affinity. Analytical samples were isolated from carbohydrate libraries that had been prepared from total pools of plasma membrane glycoproteins of cells that display strong, E-selectin-mediated adhesion. Both N- and O-linked glycans were released from the glycoproteins by previously established hydrazinolysis protocols.[82] The oligosaccharides were then radioactively labeled and fractionated to homogeneity by QAE anion exchange chromatography, gel filtration, and high-pH anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD). Since the quantities of glycans were insufficient for NMR spectroscopy, analysis of the oligosaccharides was performed by combinations[83] of glycosidase digestion, gel filtration, controlled acetolysis and matrix-assisted laser desorption (MALD) mass spectrometry. Three glycans **1**–**3** were identified as high-affinity ligand glycans of E-selectin (Fig. 3); all three contain the sialyl



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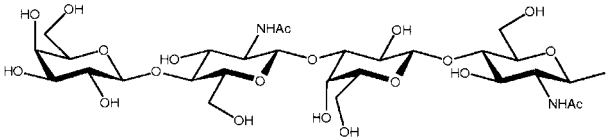

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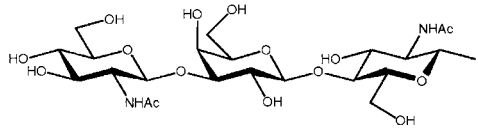

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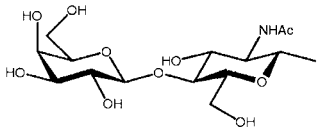

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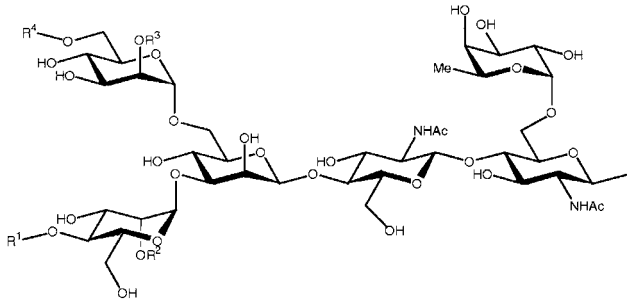


1, R^1 = a, R^2 = c, R^3 = b or d, R^4 = d or b
2, R^1 = a, R^2 = d, R^3 = d or b, R^4 = b or d
3, R^1 = a, R^2 = R^3 = R^4 = d

FIG. 3.

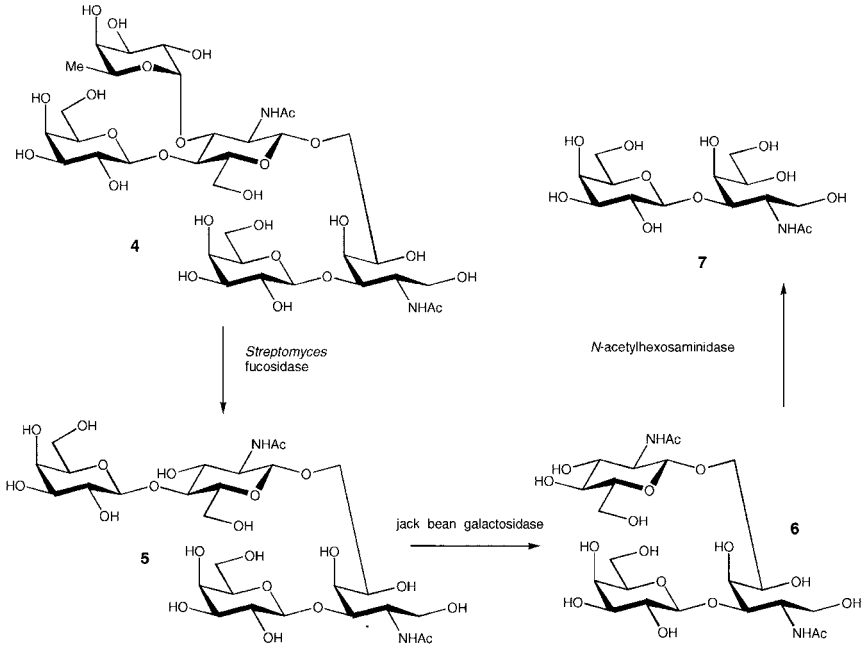
di-Le^x structure α-D-Neu5Ac-(2 → 3)-β-D-Gal-(1 → 4)-[α-L-Fuc-(1 → 3)]-β-D-GlcNAc-(1 → 3)-β-D-Gal-(1 → 4)-[α-L-Fuc-(1 → 3)-]-β-D-GlcNAc on the β-D-GlcNAc-(1 → 4)-α-D-Man-(1 → 3)-branch of tetraantennary N-glycans. All three ligand glycans contain the trimannosyl chitobiosyl core typical of glycoprotein N-glycans. The specificity of the glycans as ligands of E-selectin was confirmed by their binding to an affinity column of recombinant, soluble E-selectin[84] (rsE-selectin) on agarose (estimated dissociation constant $K_d < 1\ \mu M$). On this column, the reducing sLe^x and sLe^a tetrasaccharides were not retained under the chromatographic conditions used for adsorption of the tetraantennary ligands. Finally, the high-affinity ligands were found to bind to antibody C_2E_5, an IgM MAb[85] that inhibits the binding of HL60 and polymorphonuclear cells to both rsE-selectin and activated human endothelial cells. The α-(1 → 3)-linked L-fucose residue that forms part of the epitope recognized by MAb C_2E_5 was previously found[86] to be present on PMNs principally in the form of sialyl-di-Le^x. The three related glycan structures identified by Patel *et al.* are present only on leukocytes or leukocytic cell lines that bind E-selectin. Of the total of surface-associated glycans, structures **1**–**3** represent only a very small percentage. Very likely, the three tetraantennary N-glycans identified by Patel *et al.* represent physiologically relevant ligand glycans for E-selectin.

b. P-Selectin.—During early work directed at the identification of the physiological oligosaccharide ligand of P-selectin, Larsen *et al.*[87] reported that P-selectin binds to the Le^x determinant expressed on leukocytes (lactoneofucopentaose III, β-D-Gal-(1 → 4)-[α-L-Fuc-(1 → 3)]-β-D-GlcNAc-(1 → 3)-β-D-Gal-(1 → 4)-D-Glc; CD-15 antigen). However, Corral *et al.*[88] and Moore *et al.*[89] showed that interactions mediated by P-selectin are greatly decreased by prior treatment of the ligands with neuraminidase. These findings strongly suggested a role of a Neu5Ac unit in the P-selectin–ligand interaction. Indeed, Polley *et al.*[90] demonstrated that the Le^x determinant is only a low-affinity ligand of P-selectin and that E- and P-selectin recognize the same carbohydrate determinant, sLe^x. In an assay measuring the P-selectin-mediated adhesion of activated platelets to neutrophils, antibodies to sLe^x gave complete inhibition while MAbs specific for Le^x partially inhibited the binding. By analogy, wild-type CHO cells that express neither Le^x nor sLe^x did not bind activated platelets. However, the CHO mutant LEC-11 cells,[67,71] which express sLe^x, bound activated platelets almost as effectively as did the HL-60 cells used as a positive control. Binding of platelets to LEC-11 cells was abrogated by prior treatment of the LEC-11 cells with neuraminidase. Similarly, liposomes containing sLe^x glycolipids[63] inhibited the P-selectin-mediated interaction, as did several soluble oligosaccharide derivatives comprising the sLe^x determinant.[91] By contrast, Le^x-containing liposomes or oligosaccharides were not inhibitory.

Larsen *et al.* studied the adhesion to HL-60 cells of CHO cells transformed to express P-selectin or E-selectin at the cell surface.[92] Purified P-selectin inhibited

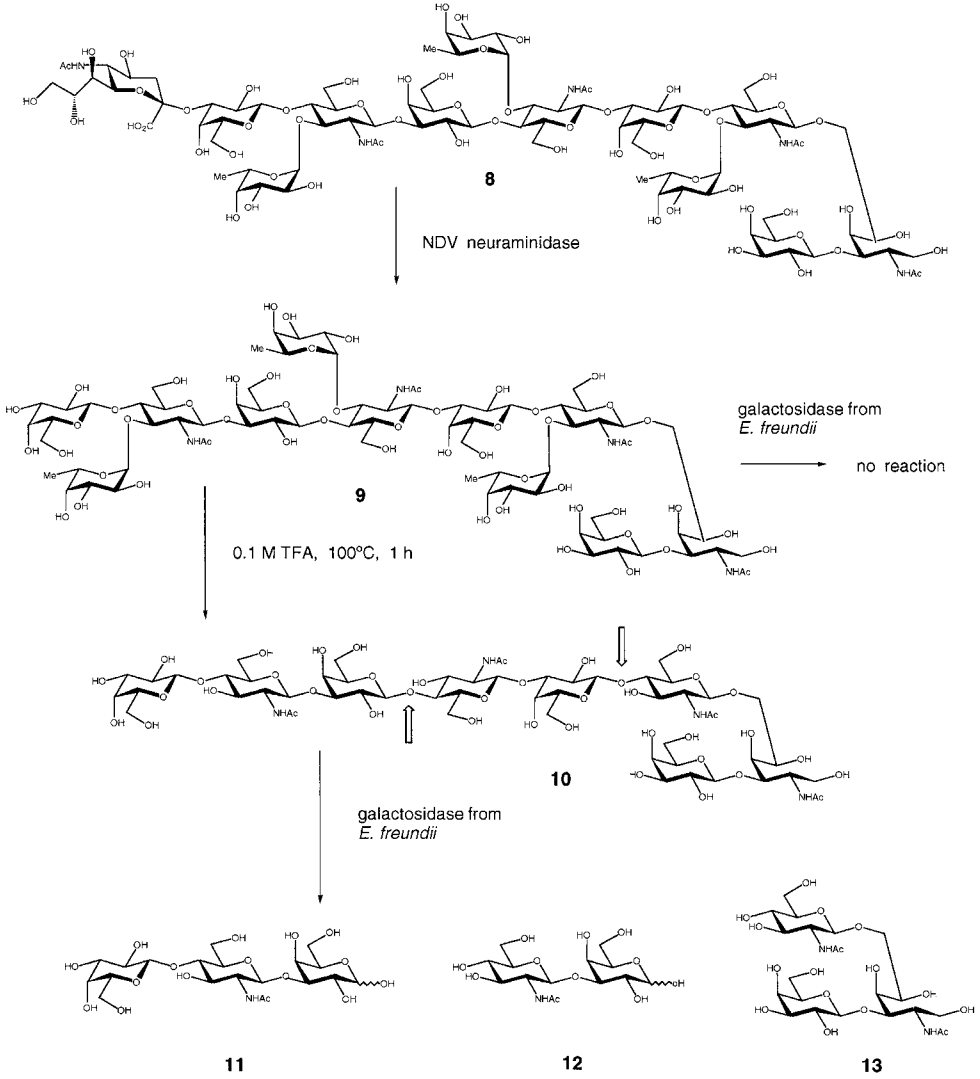
the binding of P-selectin-expressing CHO cells to HL-60 cells, but only partially inhibited the binding of the E-selectin-expressing transformants. By contrast, purified soluble E-selectin inhibited binding to HL-60 cells of both transformants equally and completely. COS cells (which are normally incapable of binding either E- or P-selectin), upon transfection with an α-1,3-fucosyltransferase gene, acquired the ability to bind the E-selectin-expressing transformant CHO cells, but not the P-selectin-expressing CHO cells. Similarly, LEC-11 cells[65] (that express sLe^x) bound to E-selectin- but not P-selectin-expressing CHO cells. On the basis of these data, Larsen *et al.* concluded that the physiological ligands of E- and P-selectins are related but structurally distinct. According to their interpretation, the sLe^x determinant, while sufficient to mediate E-selectin binding, is only a component of the counterreceptor for P-selectin. Complete structural characterization of that molecule would be required to define the nature of the P-selectin ligand.

In the wake of the identification and expression cloning of the P-selectin glycoprotein ligand[93] (PSGL-1), detailed structure studies were performed of the O-glycans that interact with the CRD of P-selectin. Wilkins *et al.*[94] metabolically labeled HL-60 cells with ^{3}H-glucosamine (^{3}H-GlcN) and isolated the glycoproteins PSGL-1 and CD43. The latter glycoprotein does not bind to P-selectin and was included as a reference sialomucin. PSGL-1 was purified by affinity chromatography on a column of P-selectin. Cells were also labeled with ^{3}H-mannose as a precursor of the fucose residues. Initial assessments of monosaccharide composition indicated that both the ratio of GlcNAc to GalNAc and the content of fucose are higher in the glycans of PSGL-1 than in those of CD43. The O-glycans of PSGL-1 and CD43 were released from the glycoproteins by β-elimination (treatment with mild base in the presence of sodium borohydride). Under these conditions, the reducing GalNAc residues of the glycans are converted into *N*-acetylgalactosaminitol (GalNAcol) residues. Separation on columns of Bio-Gel P4 provided essentially three fractions designated P-4-I, P-4-II, and P-4-III. Fraction P-4-I was separated further on a column of Bio-Gel P10, providing fractions P-10-1 and P-10-2. The latter fraction from PSGL-1 was separated further by preparative descending paper chromatography to give a slow-moving fraction containing larger-sized glycans (P-10-2a) and a faster-moving fraction (P-10-2b). From a column of the anion exchange resin, QAE Sephadex, the glycan of fraction P-10- 2a was eluted at $\sim$20 m*M* sodium chloride, indicating it to contain one Neu5Ac residue. The glycan of fraction P-10-2b was eluted at $\sim$70 m*M* NaCl, corresponding to a content of two Neu5Ac residues. The smaller glycans of fraction P-10-2b were next treated with neuraminidase from Newcastle disease virus (NDV), a neuraminidase that catalyzes the hydrolysis of α-(2 $\rightarrow$ 3)- but not of α-(2 $\rightarrow$ 6)-linked Neu5Ac ketosides.[95–97] This treatment released all the Neu5Ac, indicating that only α-(2 $\rightarrow$ 3)-linked Neu5Ac residues are present in the native glycans. The released Neu5Ac was adsorbed on QAE Sephadex, and the neutral glycan derivatives were analyzed by descending paper chromatography. A minor



SCHEME 2

fraction (10%, P-10-2b$_1$) comigrated with the standard pentasaccharide derivative β-D-Gal-(1 → 4)-[α-L-Fuc-(1 → 3)]-β-D-GlcNAc-(1 → 6)-[β-D-Gal-(1 → 3)]-D-GalNAc-ol (**4,** Scheme 2) whereas the major portion (∼90%) comigrated with the standard core-2 tetrasaccharide derivative β-D-Gal-(1 → 4)-β-D-GlcNAc-(1 → 6)-[β-D-Gal-(1 → 3)]-D-GalNAc-ol (**5,** P-10-2b$_2$). Treatment of the P-10-2b mixture with jack bean β-galactosidase did not affect the larger glycan derivative **4** but resulted in the formation from **5** of a material that comigrated with the standard trisaccharide derivative β-D-GlcNAc-(1 → 6)-[β-D-Gal-(1 → 3)]-D-GalNAc-ol (**6**). The β-(1 → 3)-linked Gal residue is resistant to jack bean β-galactosidase. Treatment of the P-10-2b mixture with α-1,3/4 fucosidase from *Streptomyces* did not affect the lighter component **5** but converted the heavier component **4** into the lighter one. When the P-10-2b glycan derivatives were subjected to a combined digestion with *Streptomyces* α-1,3/4 fucosidase, jack bean β-galactosidase, and β-*N*-acetylhexosaminidase, both components were degraded into free ^{3}H-GlcNAc and the ^{3}H-disaccharide derivative β-D-Gal-(1 → 3)-D-GalNAc-ol **7.** From these results, Wilkins *et al.* identified one of the fucosylated mucin glycans of PSGL-1 as α-D-Neu5Ac-(2 → 3)-β-D-Gal-(1 → 4)-[α-L-Fuc-(1 → 3)]-β-D-GlcNAc-(1 → 6)-[α-D-Neu5Ac-(2 → 3)-β-D-Gal-(1 → 3)]-α-D-GalNAc. In view of the larger size of the glycan from fraction P-10-2a, the authors considered



SCHEME 3

the presence of a polylactosamine structure **8** (Scheme 3). Following treatment with neuraminidase from Newcastle disease virus,[95,96] Neu5Ac was removed by adsorption on QAE Sephadex, and the neutral glycan derivative **9** was incubated with endo-β-galactosidase from *Escherichia freundii*, an enzyme expected to catalyze the hydrolysis of the β-D-Gal-(1 $\rightarrow$ 4)-β-D-GlcNAc linkages of a type 2 polylactosamine structure.[98] However, compound **9** was resistant to

this treatment or to a combined treatment with endo-β-galactosidase and β-*N*-acetylhexosaminidase. The authors then considered that the presence of fucose branches on the GlcNAc residues might protect the chain against degradation by the endo-β-galactosidase.[99] Precedent for such behavior had been found in previous work from the group of Cummings[100] on polyfucosylated polylactosamine glycans from *Schistosoma mansoni*. In their native state, these chains are resistant to endo-β-galactosidase but can be degraded by the enzyme after defucosylation by the action of 0.1 *M* trifluoroacetic acid (100 °C, 1 h). When this treatment was applied to the glycan derivative **9** of fraction P-10-2a, a product **10** was formed that was hydrolyzed under catalysis by endo-β-galactosidase from *E. freundii*[98] to afford approximately equimolar amounts of the trisaccharide β-D-Gal-(1 → 4)-β-D-GlcNAc-(1 → 3)-D-Gal (**11**), the disaccharide β-D-GlcNAc-(1 → 3)-D-Gal (**12**), and the trisaccharide derivative β-D-GlcNAc-(1 → 6)-[β-D-Gal-(1 → 3)]-D-GalNAc-ol (**13,** Scheme 3). This result was interpreted by Wilkins *et al.* to indicate structure **8** for the polyfucosylated polylactosamine glycan of fraction P-10-2a. No evidence was found by the authors for O-glycans similar to structures **8** and sialylated **4** in the standard sialomucin, CD43.

c. L-Selectin.—Stoolman and Rosen[101] reported in 1983 that L-fucose (albeit at a concentration of 18 m*M*) and fucoidin (at ~1–5 × 10^{-8} *M*) inhibit the adhesive interaction between lymphocytes and postcapillary venules; these authors also demonstrated that other sulfated polysaccharides such as heparin, dermatan sulfate, or dextran sulfate are not inhibitory and that the inhibition by fucoidin is due to its binding to the lymphocytes. Fucoidin is a sulfated polysaccharide from the seaweed *Fucus vesiculosus;* a revised structure of fucoidin[102] has been proposed by Patankar *et al.* (Fig. 4). These authors have tentatively attributed the inhibitory activity of fucoidin toward selectin–ligand interactions to the presence, on the fucoidin polysaccharide chain, of α-(L)-linked fucose branches and sulfate groups in arrangements that resemble those found in L-selectin ligand glycans (cf. later in this section). Stoolman *et al.*[103] subsequently found that the adhesive interaction between lymphocytes and the endothelium of postcapillary venules in frozen sections of peripheral lymph nodes[29–31] is also inhibited by mannose

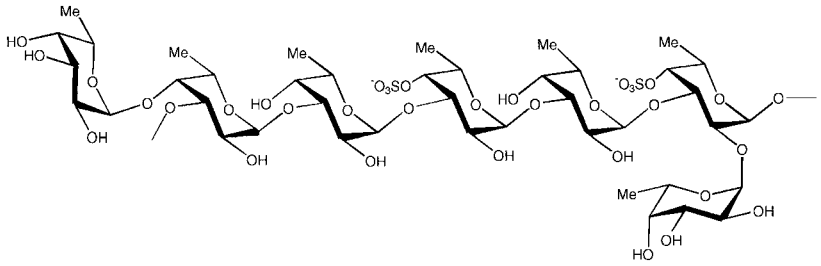


FIG. 4.

6-phosphate, fructose 1-phosphate, and PPME,[104] the phosphomannosyl-rich, core polysaccharide of mannan derived from the yeast *Hansenula holstii*. While mannose 1-phosphate and fructose 6-phosphate are slightly inhibitory, glucose 1-phosphate, glucose 6-phosphate, galactose 1-phosphate, and galactose 6-phosphate are inactive. Yednock *et al.*[105] coupled PPME to intensely fluorescent, amino-derivatized polystyrene beads of 0.6 μm diameter. Such beads bind to peripheral blood lymphocytes six- to eightfold better than underivatized beads. The interaction of lymphocytes with PPME beads is calcium dependent, is sensitive to trypsin, and is selectively inhibited by mannose 6-phosphate, PPME, and fucoidin. Therefore, binding of the lymphocytes to PPME beads was initially considered a valid model for the interaction of lymphocytes with HEV of peripheral lymph nodes. Rosen *et al.* demonstrated that treatment with neuraminidase of tissue sections from mouse peripheral lymph nodes abrogated the binding of lymphocytes to the HEV as determined by a Stamper–Woodruff assay.[106] Similarly, neuraminidase injected intravenously was found to inactivate the sites of attachment of lymphocytes to peripheral node but not to Peyer's patch HEV.[107] True *et al.*[108] demonstrated the importance of Neu5Ac residues as parts of the L-selectin counterreceptors. Among a variety of lectins examined, only *Limax flavus* agglutinin, a lectin specific for Neu5Ac, inhibited the attachment of lymphocytes to HEV in Stamper–Woodruff assays[29–31] of both peripheral and Peyer's patch lymph node sections. The *Limax* agglutinin[109] is a Neu5Ac-specific lectin of broad specificity that occurs in the hemolymph of an invertebrate, the slug *Limax flavus*. Inhibition by the *Limax* agglutinin was abrogated in the presence of 50 m*M* Neu5Ac but not of glucuronic acid. Pretreatment of tissue sections with *Limax flavus* agglutinin also prevented the specific staining of peripheral lymph node HEV by the L-selectin–IgG chimera described by Watson *et al.*[110]

In a major series of investigations, Rosen and his associates studied the structures of L-selectin ligand glycans on samples obtained from the L-selectin ligand glycoprotein GlyCAM-1 following radioactive labeling in lymph node slices *ex vivo*. GlyCAM-1 (initially designated Sgp50 or sulfated glycoprotein of 50 kDa) and CD34 (originally designated Sgp90 or sulfated glycoprotein of 90 kDa) are mucin-type glycoproteins that function as counterreceptors of L-selectin on the surface of the endothelial cells of high endothelial venules. Imai *et al.* have isolated GlyCAM-1 and CD34 by adsorption on Affi-Gel beads coated with an L-selectin-IgG chimera.[111] When GlyCAM-1 or CD34 had been treated with neuraminidase from *Arthrobacter ureafaciens*, they no longer bound to the L-selectin beads, indicating that α-Neu5Ac residues of the glycoprotein glycans are involved in the binding to L-selectin. In addition to $^{35}SO_4^{2-}$, ^{3}H-labeled fucose was found to be incorporated into GlyCAM-1. Treatment of the radiolabeled mucins with *N*-glycanase did not result in the release of significant amounts of radioactivity. By contrast, radioactive materials were released upon treatment with alkaline borohydride, indicating that the glycan chains are O-linked. From their data,

Imai *et al.*[111] tentatively concluded that the L-selectin ligand glycans would be sulfated, fucosylated, and sialosylated O-linked oligosaccharides. Presumably, the inhibitors mannose 6-phosphate or fucoidan would function as mimetics of such mucin glycans. As a next step toward the elucidation of the structures of L-selectin ligand glycans, Imai and Rosen[112] used the β-elimination reaction to release the ^{35}S-labeled sulfated *O*-glycans from the mucins GlyCAM-1 and CD34. The affinity-purified glycoproteins were treated with a solution of sodium borohydride in sodium hydroxide and the released glycan alditols fractionated by gel permeation chromatography on Sephadex G-50. The oligosaccharide alditols were eluted in the vicinity of the elution volume corresponding to a standard, ^{3}H-labeled bis-sialylated galactosylated biantennary alditol from human fibrinogen. The ^{35}S-labeled oligosaccharide alditols were adsorbed on an affinity column of ***Limax flavus*** agglutinin,[109] whence they were eluted with a 50 m*M* solution of Neu5Ac. The oligosaccharide alditols were also subjected to anion-exchange chromatography on QAE-Sephadex A-25 and were separated by elution with a sodium chloride gradient. The major portion of the fucose-labeled chains were eluted at 140–200 m*M* salt whereas the sulfate-labeled chains were eluted preponderantly in the range 400–1000 m*M* salt. Neuraminidase-treated preparations gave similar elution profiles, indicating that the carboxylate groups of the Neu5Ac units contribute but a minority of the negative charges and that the oligosaccharide alditols are retained on the anion-exchange resin mainly by virtue of substitution with sulfate.

Hemmerich *et al.*[113] identified galactose 6-sulfate (Gal6*S*) and *N*-acetylglucosamine 6-sulfate (GlcNAc6*S*) as O-sulfated monosaccharide constituents of the L-selectin ligand glycans of GlyCAM-1. The glycans of GlyCAM-1 were metabolically labeled with [^{35}S]sulfate, [^{3}H]galactose, [^{3}H]glucosamine, [^{3}H]fucose, or [^{3}H]mannose in organ culture of mouse lymph node slices. The labeled GlyCAM-1 was isolated by immunoprecipitation with an antibody directed against the peptide CKEPSIFREELISKD. By SDS-PAGE analysis and fluorography, nearly all of the incorporated radioactivity was present in the vicinity of a molecular mass of 45 kDa. To estimate the extent of sulfation of the glycan chains, anion-exchange chromatography was performed of β-eliminated glycans following metabolic labeling in the presence of chlorate. Previously, Imai, Lasky, and Rosen[114] had shown that chlorate is an inhibitor of the sulfation of GlyCAM-1 but does not affect protein synthesis of the apoprotein or the incorporation of Neu5Ac or fucose into the glycan chains. Significantly, the ability of GlyCAM-1 to bind to L-selectin was dramatically diminished when the mucin had been isolated from an organ culture performed with chlorate present in the medium. This finding was taken to indicate an important role for *O*-sulfate groups in L-selectin–ligand interaction.

As expected, the presence of chlorate in the organ culture medium resulted in a decreased content of those glycan alditols eluted at higher salt concentrations from an anion-exchange column. From the data, the authors estimated[114] that at least 48% of the O-linked glycan chains would be sulfated. To identify the sulfated

monosaccharides among the constituents of the ligand glycans of GlyCAM-1, the mucin was subjected to mild acid-catalyzed hydrolysis (0.2 *M* sulfuric acid, 100 °C, 30 min), and the hydrolyzate separated, by anion-exchange chromatography on DEAE-Sepharose, into unbound materials (neutral sugars), singly charged, and multiply charged materials. Only the uncharged and singly charged components, eluted at concentrations between 50 and 150 m*M* pyridinium acetate, were analyzed further. The glycan preparations derived from organ cultures with [^{3}H]Man or [^{3}H]Fuc as the labeled precursors, upon hydrolysis, afforded all radioactivity in the form of free fucose in the fraction not bound to the anion-exchange resin. Therefore, mannose is not a constituent of the GlyCAM-1 glycans. The fractions of singly charged carbohydrate materials originating from organ cultures labeled with [^{35}S]sulfate, [^{3}H]Gal, and [^{3}H]GlcN were each separated into four well-resolved fractions, designated I through IV, by gel-permeation chromatography over Bio-Gel P4. Each fraction was distinct with respect to the origin of the radioactivity eluted: the radioactivity in fraction I originated from [^{35}S]sulfate and [^{3}H]Gal, in fraction II, from [^{35}S]sulfate and [^{3}H]GlcN, in fraction III, from all three labeled precursors, and in fraction IV, only from [^{3}H]GlcN. Considering that the GlcN precursor may be metabolically converted[115] into GlcNAc, GalNAc, or Neu5Ac, fraction IV was identified as Neu5Ac by comparison with the product released by treatment with neuraminidase of [^{3}H]GlcN-labeled GlyCAM-1.

The constituent monosaccharides of fractions I, II, and III were identified following strong acid-catalyzed hydrolysis of the respective materials (6 *M* hydrochloric acid, 100 °C, 4 h). Under these conditions, all glycosidic linkages or sulfate ester or *N*-acetyl groups are expected to be cleaved.[116] Using Gal and GlcN standards for Dionex high-pH-anion exchange chromatography (HPAEC), fraction I was shown to contain Gal, fraction II, GlcN, and fraction III, both Gal and GlcN. These results were taken to indicate that fraction I corresponds to a galactose sulfate, fraction II to a sulfate of GlcN or GlcNAc, and fraction III to one or more monosulfated forms of Gal-GlcN(Ac) or GlcN(Ac)-Gal. Fractions II and III were characterized further, following solvolysis to remove the sulfate groups (50 m*M* hydrogen chloride in methanol, 5% water, 37 °C, 24 h). In the case of fraction II, solvolytic removal of the sulfate ester group afforded GlcNAc as identified by HPAEC. Fraction II, therefore, contains a sulfate of GlcNAc, not of GlcN. Similar removal of the sulfate ester groups from the materials of fraction III afforded a neutral product that was eluted from a HPAEC column at the position corresponding to *N*-acetyllactosamine **14** [β-D-Gal-(1 $\rightarrow$ 4)-D-GlcNAc; Fig. 5] as distinct from β-D-Gal-(1 $\rightarrow$ 3)-D-GlcNAc or β-D-Gal-(1 $\rightarrow$ 6)-D-GlcNAc. Treatment of this material with exo-β-galactosidase from jack bean gave a quantitative yield of [^{3}H]Gal as expected. Thus, the materials of fraction III were identified as one or more monosulfated forms of *N*-acetyllactosamine. By comparative HPAEC analysis of fractions I and II relative to authentic standards, their identities were established as Gal6*S* **15** and GlcNAc6*S* **16,** respectively. Fraction III, derived from

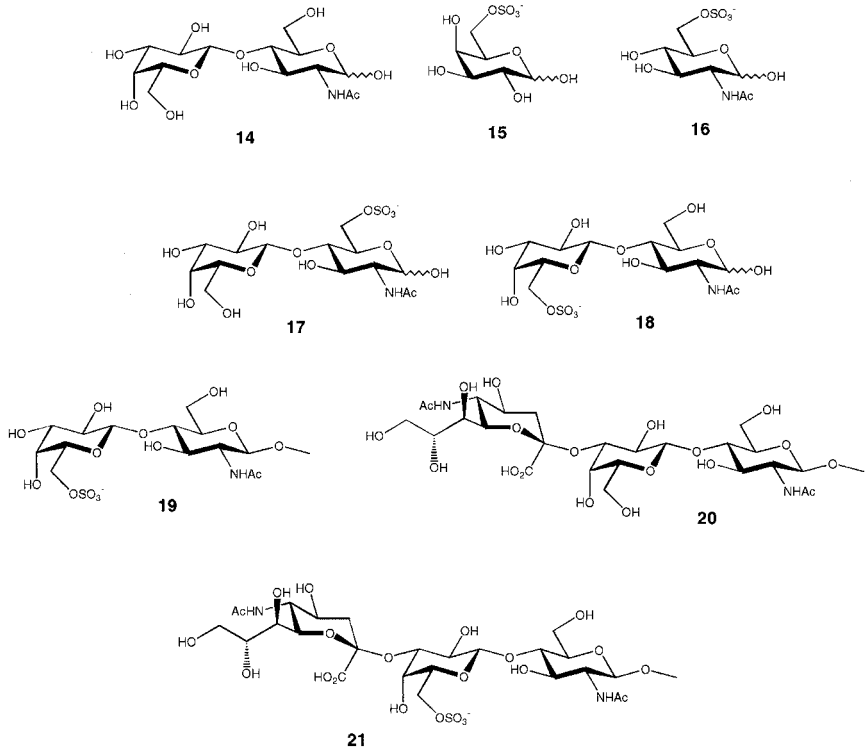


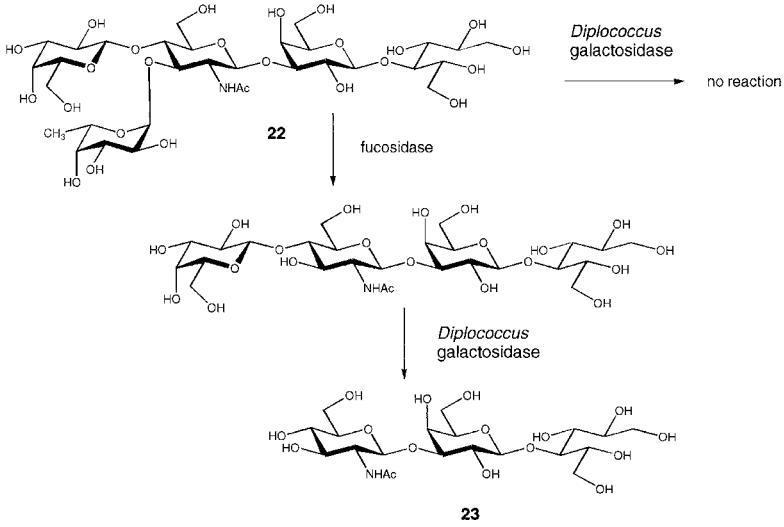
FIG. 5.

[^{35}S]sulfate labeled GlyCAM-1, was subjected to mild acid-catalyzed hydrolysis to afford both Gal6*S* and GlcNAc6*S* with some free sulfate. This finding indicated that fraction III contains a mixture of β-D-Gal-(1 $\rightarrow$ 4)-D-GlcNAc6*S* (**17**) and β-D-Gal6*S*-(1 $\rightarrow$ 4)-D-GlcNAc (**18**). By HPAEC, most of fraction III was separated into two further fractions, designated IIIa and IIIb. Fraction IIIa, upon treatment with jack bean exo-β-galactosidase, afforded GlcNAc 6-sulfate and was thus identified as β-D-Gal-(1 $\rightarrow$ 4)-D-GlcNAc6*S* (**17**). Fraction IIIb was found resistant to hydrolysis catalyzed by jack bean exo-β-galactosidase and was assigned the structure β-D-Gal6*S*-(1 $\rightarrow$ 4)-D-GlcNAc (**18**).[117,118]

To identify the linkage positions of the Neu5Ac and Fuc residues on the sulfated *N*-acetyllactosamine disaccharides, a comparative analysis was performed[119] of GlyCAM-1 glycans as follows. Metabolic labeling was performed with [^{35}S]sulfate, [^{3}H]Gal, and [^{3}H]Man in the presence or absence of sodium chlorate as an inhibitor of O-sulfation.[114] In this manner, radioactively labeled preparations of undersulfated and normally sulfated GlyCAM-1 were obtained. The mucins were subjected to the actions of a panel of exo-glycosidases, and the

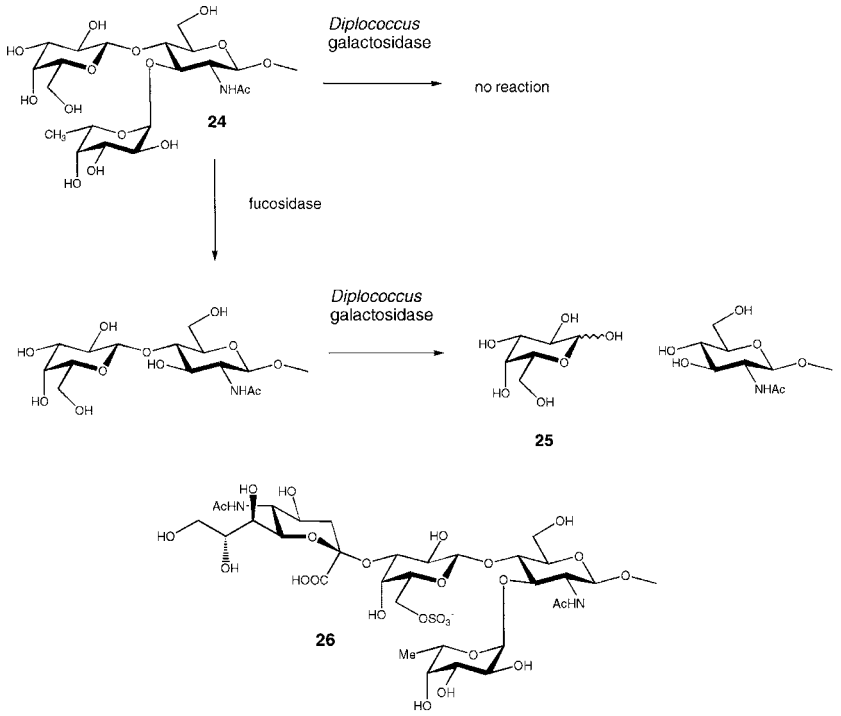
truncated or intact glycoprotein glycans examined regarding their abilities to bind to lectins of different specificities. Hydrolytic removal of the Neu5Ac residue from normally sulfated GlyCAM-1 by the action of the sialidase from *Arthrobacter ureafaciens* afforded a glycoprotein that bound to *Trichosanthes japonica* and *Sambucus nigra* agglutinins.[120–122] These lectins are specific for the structure β-D-Gal6S-(1 $\rightarrow$ 4)-β-D-GlcNAc (**19,** Fig. 5). Significantly, neither the sialylated nor the asialo forms of the undersulfated glycoprotein were bound by these lectins. Undersulfated GlyCAM-1 in the sialylated but not asialo form was found to be bound by the *Maackia amurensis* agglutinin,[123,124] a lectin specific for the determinant α-D-Neu5Ac-(2 $\rightarrow$ 3)-β-D-Gal-(1 $\rightarrow$ 4)-β-D-GlcNAc (**20**). When the hydrolytic cleavage of the glycosidic linkage of the Neu5Ac residue was performed under catalysis by the α-(2 $\rightarrow$ 3)-specific neuraminidase from Newcastle disease virus,[95,96] binding of GlyCAM-1 by the *Limax flavus* agglutinin was decreased by more than 90%. This indicates that the majority of the Neu5Ac residues in GlyCAM-1 are α-(2 $\rightarrow$ 3)-linked. Together, the results of the examination of lectin binding of GlyCAM-1 before or after neuraminidase treatments indicated the presence of the trisaccharide structure α-D-Neu5Ac-(2 $\rightarrow$ 3)-β-D-Gal6S-(1 $\rightarrow$ 4)-β-D-GlcNAc (**21,** Fig. 5).

Previous studies had indicated that GlyCAM-1 contains fucose residues[111] that are essential for binding to L-selectin and are not sulfated.[113] In addition to the ^{3}H-labeled material, a batch of GlyCAM-1 was labeled at the fucose residues using [^{3}H]Man as the precursor. When asialo-GlyCAM-1 (from a fully sulfated preparation) was treated with α-1,3/4-fucosidase from *Streptomyces* sp. 142, 70% of the fucose was released. The intact sialoglycoprotein was unaffected by the action of this enzyme. From the asialo form of the undersulfated glycoprotein, 85% of the fucose was released under analogous conditions. No fucose was released from asialo-GlyCAM-1, in either the fully sulfated or undersulfated forms, by the action of the α-1,2-specific fucosidase from *Corynebacterium.* Isolated glycan alditols, obtained following β-elimination from GlyCAM-1, displayed analogous behavior when subjected to the action of the two linkage-specific fucosidases. These findings were taken to indicate the presence of an α-(1 $\rightarrow$ 3 or $\rightarrow$ 4)-linked fucose residue in the mucin glycans. The release of fucose from asialo-GlyCAM-1 but not from the sialoglycoprotein is in agreement with previous observations on SLe^x-related structures.[125,126] The linkage positions of the fucose units were investigated further. [^{3}H]Gal-labeled GlyCAM-1 was desialylated and the asialoglycoprotein treated with *Streptomyces* α-1,3/4 fucosidase. While the asialoglycoprotein was bound to *Aleuria aurantia* agglutinin, a lectin that binds glycans containing fucose in α-(1 $\rightarrow$ 2)-, α-(1 $\rightarrow$ 3)-, or α-(1 $\rightarrow$ 6)-linkages,[127] the material obtained by the action of the fucosidase was not bound by this agglutinin. Conversely, while the asialoglycoprotein was but weakly bound by the lectin from *Lycopersicon esculentum,* binding of the fucosidase-treated material was tenfold stronger. The *L. esculentum* lectin is specific for β-(1 $\rightarrow$ 4)-linked GlcNAc residues.[128,129] These

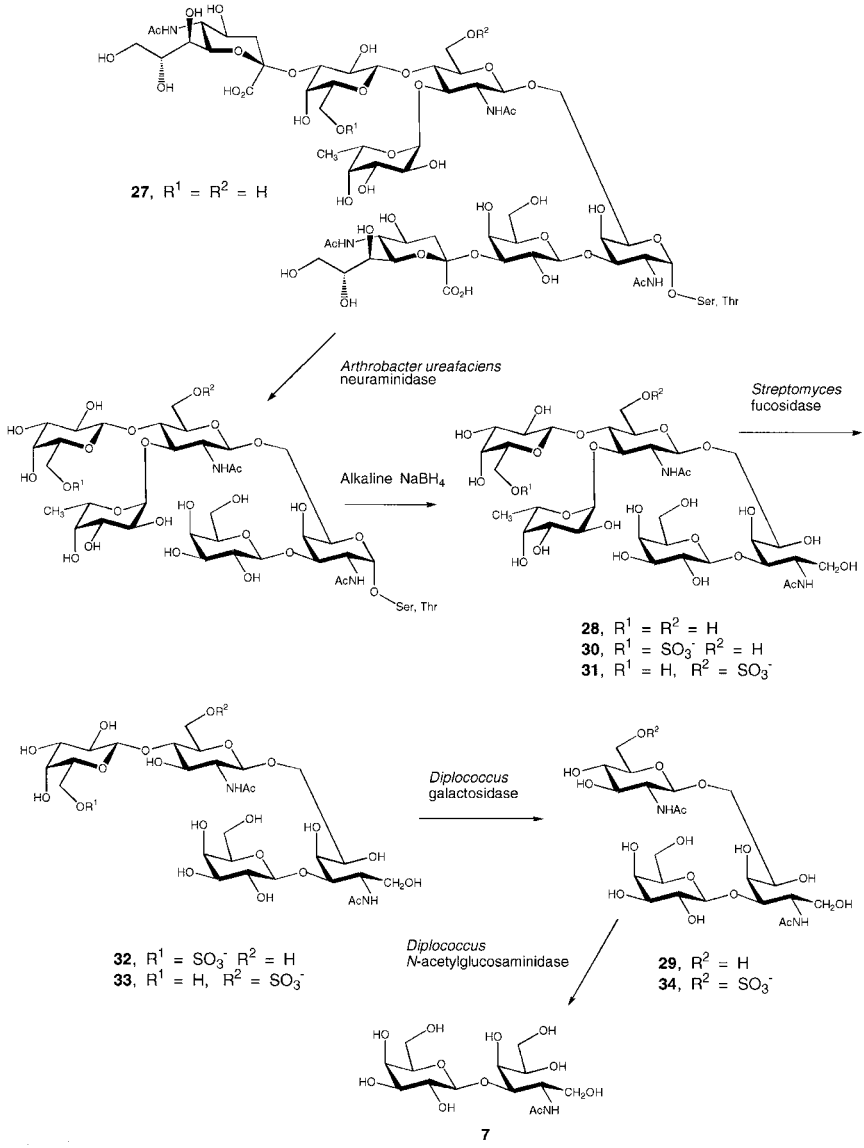


SCHEME 4

findings are in agreement with the Fuc residue being α-(1 → 3)-linked to a β-(1 → 4)-linked GlcNAc residue. Finally, to ascertain the location of the fucose residue relative to the penultimate Gal residue, a further set of experiments was performed. These are based on previous findings by Maemura and Fukuda[130] who had reported that the (1 → 4)-linked β-Gal residue in the Le^x determinant is resistant to the action of β-1,4-galactosidase from ***Diplococcus pneumoniae***.[131] To confirm the previous results, Hemmerich and Rosen subjected the ^{3}H-labeled alditol of lacto-neofucopentaose III (LNF III) β-D-Gal-(1 → 4)-[α-L-Fuc-(1 → 3)]-β-D-GlcNAc-(1 → 3)-β-D-Gal-(1 → 4)-[1-^{3}H]Glc-ol (**22**) to the action of the ***D. pneumoniae*** β-1,4-galactosidase, both in the presence or absence of the α-1,3/4-fucosidase from ***Streptomyces*** (Scheme 4). As expected, Gal was released from the LNF III alditol only subsequent to hydrolytic removal of the fucose residue, affording β-D-GlcNAc-(1 → 3)-β-D-Gal-(1 → 4)-Glc-ol (**23**). No Gal was released by the action of the galactosidase alone on LNF III alditol **22.** When [^{3}H]Gal labeled GlyCAM-1 (containing the glycan part structure **24**) was examined by the same procedure, 39% of the total galactose **25** was released, indicating that one Gal residue is in a penultimate position relative to a Neu5Ac residue and is β-(1 → 4)-linked, presumably to GlcNAc. Preliminary results indicated that another Gal residue was present in β-(1 → 3)-linkage to GalNAc in the mucin glycans. In their study of 1994, Hemmerich and Rosen concluded[119] that the 6′-sulfated sLe^x structure α-D-Neu5Ac-(2 → 3)-β-D-Gal6S-(1 → 4)-[α-L-Fuc-(1 → 3)]-β-D-GlcNAc (**26**) is present as a nonreducing oligosaccharide segment (termed a "capping group") of O-linked glycans of GlyCAM-1.



In a subsequent study from the laboratory of Rosen, the structures of two of the less-complex mucin glycans of GlyCAM-1 have been elucidated.[132] To this end, the authors have applied a combination of techniques suitable for the analysis of tracer amounts of glycoconjugate glycans: metabolic radiolabeling, binding to plant lectins, digestion with specific glycosidases, gel-permeation chromatography, and high-pH anion-exchange chromatography. To probe for the presence of the T-antigenic determinant[133,134] β-D-Gal-(1 $\rightarrow$ 3)-α-D-GalNAc, the binding to PNA-agarose was examined of ^{3}H-labeled GlyCAM-1 before and after removal of the sialyl residues by the action of neuraminidase from ***Arthrobacter ureafaciens***. The native sialoglycoprotein was bound strongly by WGA and not appreciably by PNA. By contrast, the asialoglycoprotein was bound to WGA with low avidity, whereas binding to PNA was enhanced ~25-fold over that of sialo-GlyCAM-1. These findings were interpreted to suggest the presence, in intact GlyCAM-1, of the T-antigen β-D-Gal-(1 $\rightarrow$ 3)-α-D-GalNAc sialylated at position 3 of the Gal residue (compare the conversion of structure **27** into **28** in Scheme 5). From the literature,[135] substitution with a Neu5Ac residue at position 6 of the GalNAc unit had been known not to impair binding of the T-antigenic determinant to PNA.



SCHEME 5

In a subsequent experiment, [^{3}H]Gal-labeled O-glycans were released by β-elimination from undersulfated GlyCAM-1. The oligosaccharide alditols were digested with a mixture of α-1,3-fucosidase from *Streptomyces* and β-1,4-galactosidase and β-*N*-acetylglucosaminidase from *Diplococcus pneumoniae,* in the presence or absence of the *A. ureafaciens* neuraminidase. The enzyme

digests were separated by gel-permeation chromatography over Bio-Gel P4. As a set of calibration standards, alditol derivatives of chitooligosaccharides corresponding to between 1 and 6 glycosyl residues were used. A sample treated with an enzyme mixture not containing the neuraminidase gave rise to an elution profile similar to that of an untreated sample. Treatment of samples with enzyme mixtures that included neuraminidase presumably gave rise to the degradation outlined in Scheme 5 and resulted in the formation of free galactose (55% of the ^{3}H-counts), the T-disaccharide alditol **7** [β-D-Gal-(1 $\rightarrow$ 3)-D-GalNAc-ol] (20%), and higher oligosaccharide derivatives (25%). The disaccharide alditol **7** (Scheme 5) was distinguished from the corresponding *N*-acetylglucosaminitol-containing isomers linked (1 $\rightarrow$ 3), (1 $\rightarrow$ 4), and (1 $\rightarrow$ 6) by comparative high-pH anion exchange chromatography (HPAEC).

The next series of experiments were designed to identify the β-(1 $\rightarrow$ 6)-linkage of the GlcNAc residue in **27** (Scheme 5) to the reducing GalNAc unit of the mucin glycans. Undersulfated, desialylated GlyCAM-1 labeled with [^{3}H]Gal, [^{3}H]GlcNAc, or [^{3}H]Man was subjected to β-elimination.[112] The oligosaccharide alditols were passed over DEAE-Sepharose and the initial, uncharged fractions separated further by gel-permeation chromatography over Bio-Gel P4. Three peaks of radioactivity were observed, designated I, II, and III, and corresponding roughly to molecular sizes of 2.5–4, 4–6, and 6 and more glycosyl units. The material of peak I (compound **28** in Scheme 5) was subjected to the action of a mixture of the linkage-specific glycosidases, α-1,3/4-fucosidase and β-1,4-galactosidase with or without addition of *N*-acetylglucosaminidase. In the absence of the *N*-acetylglucosaminidase, 50% of the radioactivity was released as free Gal, and **28** was converted into a material of molecular size corresponding to a trisaccharide; comparison of chromatographic retention times indicated this material to be identical with the core-2 trisaccharide alditol β-D-GlcNAc-(1 $\rightarrow$ 6)-[β-D-Gal(1 $\rightarrow$ 3)]-D-GalNAc-ol (**29,** Scheme 5). When the materials of peak I had been derived from glycoprotein labeled with [^{3}H]GlcN, no radioactivity was released by the action of the fucosidase–galactosidase mixture, and all of the counts remained associated with the fractions corresponding to oligosaccharides. By contrast, treatment with enzyme mixtures that included the *N*-acetylglucosaminidase produced the T-disaccharide alditol **7** plus 47% of the [^{3}H]GlcN as free GlcNAc. The T-disaccharide alditol **7** was also produced by treatment of the core-2 trisaccharide derivative **29** with the *N*-acetylglucosaminidase. These findings were corroborated by comparisons of the radioactively labeled compounds **7** and **29** to authentic standards by HPAEC. Similar analyses of the larger oligosaccharide alditols from peaks II and III of the BioGel P4 eluate indicated the presence of additional GlcNAc and Fuc residues in these materials. However, detailed structure assignments were not made on the basis of these additional data.

In addition to the sugar analyses of glycans from undersulfated GlyCAM-1, monosulfated chains were also investigated.[132] Separation of desialylated glycan alditols by anion-exchange chromatography afforded fractions corresponding to

mono-, di-, and tri-sulfated glycans. By a specific application of enzyme degradation analysis, the monosulfated fraction was shown to be a 1 : 1 mixture of oligosaccharide alditols sulfated at O-6 of the Gal or GlcNAc units of the Le^x portion (structures **30** and **31,** Scheme 5). Previous results[119] had indicated that the β-galactosidase from jack bean will catalyze the hydrolysis of the β-D-Gal glycosidic linkage in β-D-Gal-(1 $\rightarrow$ 4)-GlcNAc6S (**17**) but not in β-D-Gal6S-(1 $\rightarrow$ 4)-D-GlcNAc **18** (Fig. 5). The β-galactosidase from *Diplococcus pneumoniae,* on the other hand, would not catalyze the hydrolysis of either type of galactoside. When the monosulfated fraction was treated with a mixture of *D. pneumoniae* β-1,4-galactosidase and α-1,3/4 fucosidase, only the fucose residue was released. When the [^{3}H]Gal-labeled glycan alditols **32** and **33** were digested with jack bean galactosidase, approximately half of the radioactivity was left unchanged (**32**) while the other half was equally divided between free galactose and material corresponding in size to the trisaccharide alditol **29** (presumably **34**). Following desulfation by the action of 50 m*M* hydrogen chloride in anhydrous methanol, the assignments of structures **32** and **34** were corroborated by chromatographic comparisons with the corresponding radiolabeled materials from undersulfated glycan chains. In general, detailed investigations of the physiological selectin ligand glycans have led to the identification of structures considerably more complex than the sLe^x or sLe^a determinants initially discovered. Examples of such complex glycans are shown in Fig. 6.

4. Investigations of the Physiological Glycoprotein Ligands of Selectins

a. E-Selectin.—Vestweber and his associates[136] at the Max-Planck-Institut für Immunbiologie in Freiburg, Germany, isolated a 150 kDa glycoprotein from mouse neutrophils and the neutrophil progenitor cell line 32 D cl 3. This protein, later termed ESL-1 (E-selectin ligand-1) is the only protein from the cell extracts that is adsorbed in significant amounts to an affinity column of soluble E-selectin–IgG chimera. Removal of Neu5Ac from ESL-1 by the action of neuraminidase impairs the interaction of the glycoprotein with E-selectin–IgG chimera. Treatment of ESL-1 with endoglycosidase F decreased the apparent molecular mass of the glycoprotein to 135 kDa and abrogated its binding to the E-selectin–IgG chimera. Endoglycosidase F (mannosyl-glycoprotein-β-*N*-acetylglucosaminidase, EC 3.2.1.96) is an enzyme from *Flavobacterium meningosepticum* that catalyzes the hydrolytic cleavage of the β-D-GlcNAc-(1 $\rightarrow$ 4)-β-D-GlcNAc (chitobiose) linkage in N-linked glycoprotein glycans.[137,138] Steegmaier *et al.* reported[139] on the isolation of a mouse cDNA encoding ESL-1. From this cDNA, a sequence of ESL-1 is predicted that is 94% identical with chicken cysteine-rich fibroblast growth factor receptor. Furthermore, a fucosylated chimera of the complete extracellular portion of ESL-1 with the F_c part of human IgG was expressed in CHO cells cotransfected with the cDNA for fucosyltransferase III. This ESL-1 chimera, linked to cyanogen bromide-activated Sepharose, was used for affinity purification of

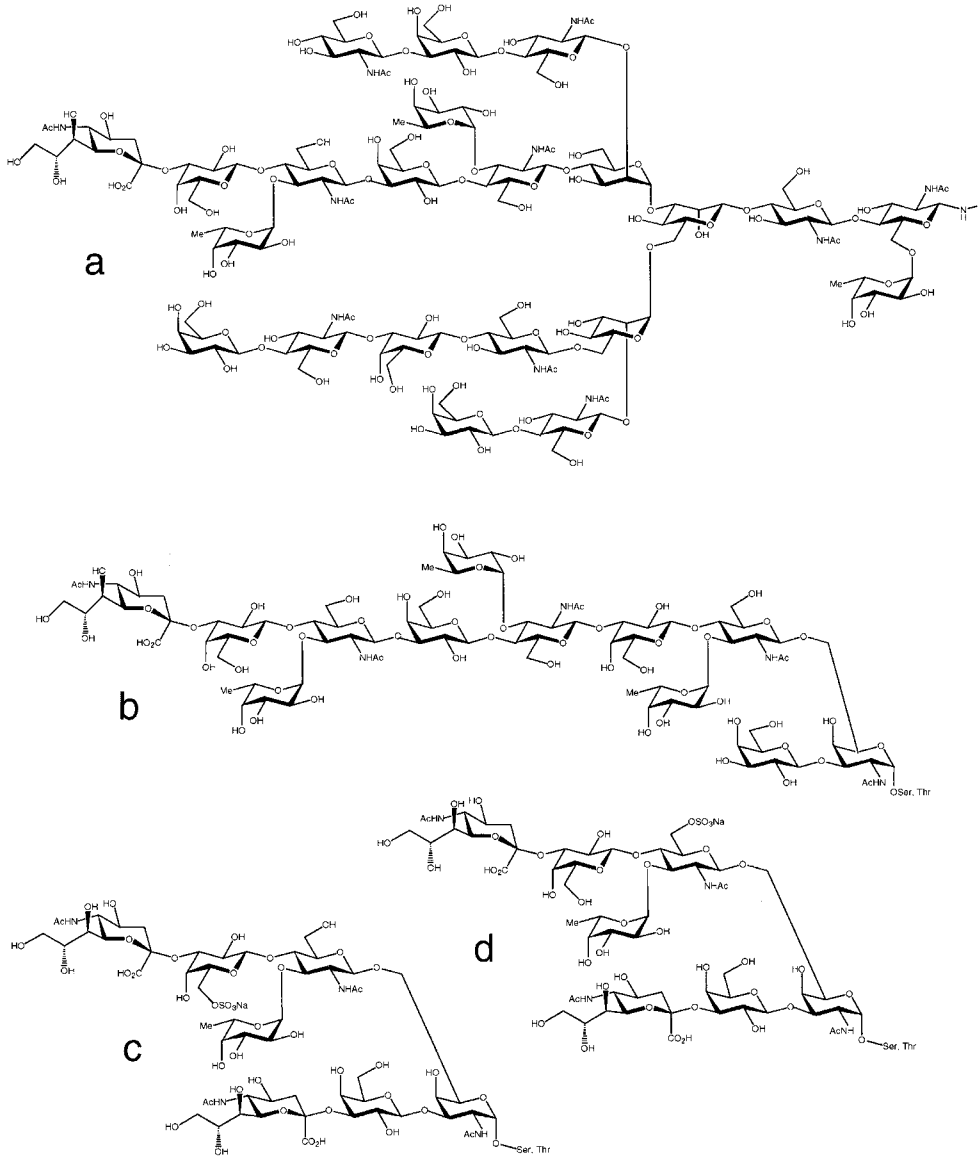


FIG. 6.

anti-ESL-1 antibodies from rabbit antisera. Zöllner and Vestweber,[140] with the aid of an E-selectin–IgG chimera, investigated the expression of E-selectin ligand activity in a variety of CHO cells that each overexpress one of seven different fucosyltransferases. From LEC11 cells[67,70–72] and fucosyltransferase III transfectants, a large number of glycoproteins were found adsorbed to the E-selectin–IgG

chimera. By contrast, in LEC12 cells and CHO cells transfected with the fucosyltransferases IV and VII, ESL-1 was almost exclusively isolated as the preponderant E-selectin ligand. In CHO cells transfected with fucosyltransferases V and VI, selectivity of fucosylation of ESL-1 was apparent but less pronounced. The selective fucosylation of ESL-1 was found to correlate with the production of the cognate antigen of MAb HECA452 (cf. Section VI.9), an antibody specific for carbohydrate epitopes that are bound by E-selectin and are related to, but not identical with, sLe^x. It is tempting to speculate that such subtle differences in the recognition of the sLe^x epitope could arise from its presentation in the context of the sialyl di-Le^x antenna within the tetraantennary *N*-glycans such as **1**–**3** (Fig. 3) described by Patel *et al.*[82] From their data, Zöllner and Vestweber conclude that, for certain fucosyltransferases, ESL-1 is a strongly preferred substrate for the generation of E-selectin-binding carbohydrate structures.[140] Picker *et al.*[141] demonstrated that L-selectin from polymorphonuclear leukocytes, but not from lymphocytes, is glycosylated with sLe^x determinants that enable the PMNs to functionally bind E- and P-selectins. Purified L-selectin from PMNs is bound by the anti-sLe^x antibodies HECA-452 and CSLEX-1. When PMNs are briefly incubated with very low concentrations of chymotrypsin, L-selectin is selectively removed while the cells continue to be viable and other cell-surface constituents remain intact, including most of the sLe^x determinants. However, PMNs treated in this manner showed a markedly diminished ability to bind to E-selectin-transfected COS cells under conditions of shear. These data were taken to support the hypothesis that L-selectin of PMNs plays an important role in presenting sLe^x or related carbohydrate determinants to E-selectin under conditions of flow.

b. P-Selectin Glycoprotein Ligand-1 (PSGL-1).—

(i) ***Identification of PSGL-1.*****—**With the aid of radioiodinated (^{125}I) P-selectin, Moore *et al.* demonstrated[89] that neutrophils and monocytes constitutively express a glycoprotein receptor for P-selectin. The physiological ligand of P-selectin, now designated P-selectin glycoprotein ligand-1 (PSGL-1), is a dimeric sialoglycoprotein with a subunit mass of 120 kDa. PSGL-1 is not present on red blood cells, lymphocytes, or platelets, whether thrombin-activated or quiescent. Binding of [^{125}I]P-selectin to the neutrophils is not inhibited by fructose 1-phosphate, mannose 6-phosphate, or the Man 6-phosphate–containing polysaccharide, PPME. However, binding was inhibited in the presence of 10 m*M* Man 1-phosphate, a substance that does not block the binding of L-selectin to its ligands on high endothelial venules.[105] These findings indicated that the binding interaction is distinct from the L-selectin-dependent adhesion of lymphocytes to HEV. Treatment with proteases (trypsin or elastase) of neutrophils that had been stabilized with diisopropyl fluorophosphate and fixed essentially abolished the ability of neutrophils to bind P-selectin. By incubation of neutrophils with neuraminidases from *Vibrio cholerae*

or Newcastle disease virus,[95,96] the binding capacity of the cells for P-selectin was decreased, by ~30 or 50% respectively, relative to untreated controls. This partial loss of binding capacity was taken to indicate that not all of the α-(2 → 3)-linked Neu5Ac residues of the P-selectin ligand are accessible to the enzymes, or that some of the Neu5Ac residues are substituted, possibly with *O*-acetyl groups.[142] Norgard *et al.* characterized PSGL-1 as a sialomucin.[143] Mild periodate treatment of the sialomucin gave a modified glycoprotein containing Neu5Ac units with partially oxidized side chains (compare Section V.6); binding to P-selectin was not impaired by this modification. Reduction of the partially oxidized material by the action of sodium [^{3}H]borohydride afforded a tritium-labeled glycoprotein ligand. Alternatively, ^{14}C-labeled sialomucin was produced from cultured HL-60 cells following metabolic labeling with [^{4}C]glucosamine. PSGL-1 was found to account for only a small fraction of the total glycoprotein-bound Neu5Ac of neutrophil cells, and for only a small fraction of the sLe^x determinants contained in the membranes of these cells. When PSGL-1 was subjected to β-elimination, most of the radioactivity that had been metabolically introduced in the form of [^{3}H]glucosamine (present as Neu5Ac and GlcNAc) was found in a fraction of low molecular mass; this indicated that the sialylated oligosaccharides mediating the binding of PSGL-1 to P-selectin are O-linked to the polypeptide chain. Finally, PSGL-1 on HL-60 cells was subjected to treatment with *O*-sialoglycoprotease[144] from *Pasteurella haemolytica* A1, an enzyme that can be applied to whole cells and that specifically cleaves polypeptide chains of sialomucins containing a dense array of O-linked sialoglycan chains. Previously, Steininger *et al.*, had reported[145] that HL60 cells and neutrophils following treatment with the *O*-sialoglycoprotease lose their ability to bind P-selectin but still bind E-selectin. Analysis by two-dimensional electrophoresis of [^{35}S]cysteine labeled proteins from HL60 cells before and after treatment with *O*-sialoglycoprotease had indicated that two major proteins of molecular masses 100 and 150 kDa are affected by the treatment. Norgard *et al.* confirmed these results by demonstrating that HL-60 cells exposed to the action of the *O*-sialoglycoprotease had lost their ability to bind to P-selectin, whereas binding to the selectin of sham-treated control cells was unaffected.[143] This finding was taken to indicate that PSGL-1 contains sLe^x as part of closely-spaced or clustered *O*-glycans; the arrangement of the sLe^x-containing glycans in so-called "saccharide patches" on PSGL-1 was speculatively considered as a signal for specific recognition of the ligand by P-selectin. In 1995, three groups of investigators reported that O-sulfation of one of the tyrosine residues (residues 5, 7, and 10 of the mature protein) in the Tyr sulfation consensus site of PSGL-1 is required for physiological binding of PSGL-1 to P-selectin. Sako *et al.*[146] at Genetics Institute have constructed a set of fusion proteins, composed of truncated versions of PSGL-1 (the N-terminal 19, 47, 148, or 253 amino acid residues) and an F_c segment[76] of IgG_1. The fusion protein 19.F_c contains the sulfation site and was capable of binding to P-selectin but not to E-selectin. Using the 148.F_c

fusion protein, Sako *et al.* demonstrated that N-glycosylation sites can be abolished by mutation without affecting binding of the mutants to P-selectin or E-selectin. Next, both myeloid cells (containing natural PSGL-1) and COS cells containing recombinant PSGL-1 were grown in the presence of sodium chlorate, an inhibitor of ATP sulfurylase.[147] This treatment strongly impaired the ability of the cells to bind to a matrix of P-selectin but not their ability to bind to a matrix of E-selectin. Furthermore, mutation to phenylalanine of the Tyr residues at positions 5, 7, and 10 produced PSGL-1 mutants that bound to P-selectin only weakly, similar to those grown in the presence of sodium chlorate. Binding of the mutants to E-selectin was not affected. From their data, Sako *et al.* concluded[146] that two structural determinants are required for binding of PSGL-1 to P-selectin. One is a peptide sequence that comprises the amino acid residues 4–11 (EYEYLDYD) including at least one sulfated tyrosine residue; the other is a closely adjacent, O-linked oligosaccharide modified with sLe^x. For binding to E-selectin, only the sLe^x-containing oligosaccharide component seems to be required. In a back-to-back communication, Pouyani and Seed[148] reported essentially identical results of their concurrent work on PSGL-1 recognition of P-selectin. These authors, through construction of truncated mutants of PSGL-1, similarly localized the tyrosine sulfation consensus and O-linked sLe^x glycan and confirmed that HL60 cells grown in the presence of chlorate have significantly diminished capacity to attach to, and roll on, P-selectin-coated surfaces under conditions of flow. Interestingly, Pouyani and Seed also speculate that the sLe^x and tyrosine sulfate components of PSGL-1 interact with P-selectin not synchronously, but in a stereotyped temporal sequence. A similar, nonconcerted mode of interaction provides one of several possible explanations for the unusually large distance between the Neu5Ac carboxylate group and the apposing ε-amino groups of Lys-111, -112, and -113 in the X-ray crystal structure of an MBP mutant containing bound sLe^x (section V). Wilkins *et al.*[149] metabolically introduced [^{35}S]sulfate into tyrosine residues of PSGL-1. The labeled PSGL-1 was isolated by affinity chromatography over a column of recombinant, soluble P-selectin coupled to the carrier resin Emphaze. Incubation of the labeled PSGL-1 with arylsulfatase[150] (EC 3.1.6.1) from *Aerobacter aerogenes* released $\sim$50% of the sulfate; concomitantly, the ability of PSGL-1 to bind to P-selectin was diminished by $\sim$50%. From their results, the authors suggest a model in which PSGL-1 presents both carbohydrate and tyrosine sulfate as constituents of a recognition site for P-selectin. Picker *et al.* have demonstrated that sLe^x determinants on neutrophil L-selectin will mediate the attachment of neutrophils to P-selectin transfected COS cells.[141]

(ii) ***Cloning and Molecular Characterization of PSGL-1.—***For expression cloning and sequence analysis of PSGL-1, Sako *et al.*[151] ligated cDNA from (the human) HL60 cells into the EcoRI cloning site of vector pMT21. Plasmids from the cDNA library were coexpressed in COS cells with the vector pEA.3/4FT

that contains the cDNA encoding α1,3/4-fucosyltransferase. The cotransfected COS cells were then panned over plates coated with LECγ_1, a chimeric soluble form of P-selectin prepared by fusing the extracellular portion of P-selectin and the F_c portion[76] of human IgG_1. To capture LECγ_1, the plates were first coated with anti-human IgG_1-F_c polyclonal antibody. Plasmids from adherent cells were rescued, amplified, and subjected to additional rounds of expression and panning. Eventually, one pool showed significantly increased binding to the LECγ_1-coated plates. This pool was subdivided and screened further in an adhesion assay of P-selectin-expressing CHO cells labeled with 6-carboxyfluorescein diacetate.[152] Finally, plasmid pPL85 was identified as a positive clone. The cDNA of this clone comprises 1649 base pairs. Following a putative translation initiation site at nucleotide 60, the open reading frame encodes 402 amino acid residues, including a signal sequence of 18 amino acid residues. The extracellular domain contains three N-glycosylation sites and exhibits regions with a high frequency of proline, serine, and threonine residues. There are 15 consecutive repeats of the 10 amino acid sequence A-T/M-E-A-Q-T-T-X-P/L-A/T, with X being P, A, Q, E, or R. Three sites of potential tyrosine sulfation are observed at positions 46, 48, and 51 (5, 7, and 10 of the mature protein). Residues 309–333 constitute the putative transmembrane domain, which is followed by a cytoplasmic tail (residues 334–402). With one exception, no significant homology was detected between PSGL-1 and other known proteins.

c. Glycoprotein Ligands of L-Selectin.—

(i) *GlyCAM-1 and CD34.*—Streeter *et al.*[153,154] raised rat monoclonal antibodies against endothelial cell suspensions isolated from mouse mesenteric and peripheral lymph nodes. MAbs MECA-89 and MECA-367 intensely stained all high-endothelial venules in Peyer's patches, but failed to react with most HEVs in peripheral lymph nodes.[153] The MECA-367 antigen was affinity isolated and characterized as a protein of molecular mass ~58–66 kDa. Conversely, MAb MECA-79 was found to stain all high-endothelial venules in peripheral lymph nodes,[154] but was absent or only weakly detectable on the lumenal surface of most HEV in Peyer's patches. In keeping with the expression of both mucosal and peripheral lymph node endothelial cell specificities in mesenteric lymph nodes, MECA-79 inhibited lymphocyte binding to the HEV of this tissue to an extent of 54%. Furthermore, as determined in Stamper–Woodruff assays,[29–31] MECA-79 inhibits lymphocyte binding to peripheral lymph node, but not Peyer's patch HEV by 95%. Pretreatment of mice with MECA-79 results in substantially (64%) diminished extravasation of lymphocytes into peripheral lymph nodes. Prior to the molecular characterization of mouse L-selectin as a lymphocyte homing receptor and as the cognate antigen of the MEL-14 MAb, substantial evidence had been provided for a C-type lectin interaction mediating the attachment of lymphocytes to peripheral lymph node HEV. In the rat, mouse, and human, lymphocyte homing

to peripheral nodes had been shown to be calcium dependent.[29–31,155] As an important tool for further investigations of L-selectin–ligand interactions, Watson *et al.*[110] constructed a chimeric protein composed of the extracellular region of L-selectin and a human IgG heavy chain.[76] The binding behavior of this soluble chimera resembles that of L-selectin in that it binds to peripheral lymph node high-endothelial venules and inhibits the attachment of lymphocytes to HEV as determined in Stamper–Woodruff assays[29–31] (see Section II.1.c.). Similar to L-selectin, the L-selectin IgG chimera binds to PPME and may be used as a histochemical reagent for the detection or affinity purification of L-selectin ligands. Imai *et al.* identified two mucin-type glycoproteins as counterreceptors of L-selectin in endothelial cells of lymph node HEV.[111] Lymph node slices were metabolically labeled in organ culture in the presence of sodium [^{35}S]sulfate. Following lysis of the tissue slices in the presence of the detergent, Triton X-100, the extracts were cleared by treatment with protein A beads to remove antibody. Subsequently, L-selectin ligand glycoproteins were precipitated with an L-selectin–human-IgG_1 chimera.[110] By treatment of the precipitated complexes with buffer containing Triton X-100, two glycoprotein fractions were released that migrated as diffuse bands on SDS-PAGE. The major band, originally designated Sgp50, is now referred to as GlyCAM-1; a minor band corresponds to another mucin ligand that was initially termed Sgp90; Baumhueter *et al.*, on the basis of Edman degradation[54] studies and anti-CD34-antibody binding to capillaries and HEV of mouse peripheral lymph nodes, have identified[156] Sgp90 as CD34, a sialomucin that had been previously characterized[157] and that is constitutively expressed on endothelial cells in a diversity of blood vessels in mice and humans. A series of complexed beads were prepared from Affi-Gel–protein A beads and were used to isolate and characterize the HEV-derived glycoprotein ligands of L-selectin. Based on the interaction of protein A with the F_c portion of immunoglobulins (Ig), adsorption beads were assembled by incubation with L-selectin–IgG_1 chimera, with CD4–IgG_1 chimera, and with human IgG_1. Beads containing the bound L-selectin–IgG_1 chimera were found to adsorb GlyCAM-1 and CD34, whereas these glycoproteins were not bound to control beads containing human IgG_1 or CD4. Following analogous sulfate labeling experiments with tissue slices from other organs, GlyCAM-1 and CD34 were identified in peripheral and mesenteric lymph nodes whereas Peyer's patches, spleen, thymus, and nonlymphoid organs such as kidney, liver, and brain did not contain materials giving rise to the 50 or 90 kDa bands on SDS-PAGE. In agreement with the nature of L-selectin as a calcium-dependent lectin, the binding of GlyCAM-1 to L-selectin–IgG_1–Affi-Gel–protein A beads required the presence of Ca^{2+} ions. Preincubation of the beads with the monoclonal antibody MEL-14 prevented the binding of GlyCAM-1 while preincubation with a control MAb did not. Binding was completely blocked in the presence of fucoidan but not of other sulfated polysaccharides such as chondroitin sulfates A or B or keratan sulfate. Binding

of the minor component CD34 was similarly subject to inhibition by MAb MEL-14 or fucoidan.[111] The groups of Lasky and Rosen at Genentech and the University of California, San Francisco, reported[158] on cDNA cloning and molecular characterization of GlyCAM-1 (Sgp50). The N-terminal polypeptide sequence of the mucin was determined by Edman degradation and was used to construct a 26-mer (hexeikosamer) oligonucleotide probe of 36-fold codon redundancy. This probe was ^{32}P-labeled and was used to screen a library of ~1 million λgt10 phages that had been constructed from mRNA of mouse peripheral lymph nodes. Thereby, a single positive clone was identified. The cDNA of this clone is 600 base pairs long and contains a translation initiation site within a consensus sequence.[46] The single open reading frame is 453 base pairs long. There is a signal sequence of 19 amino acid residues, and the mature protein (132 amino acid residues) has a serine/threonine content of ~29%. The protein lacks cysteine and has only one potential N-glycosylation site. On the basis of the amino acid sequence, the molecular mass of the apoprotein is 14,154 Da. This figure indicates that the glycan chains account for ~70% of the molecular mass of the glycoprotein (~50,000).

(ii) ***MAdCAM-1.*****—**Berg *et al.* reported[159] that the antibody MECA-79, a MAb that stains L-selectin-binding carbohydrates on HEV of mouse peripheral lymph nodes, also binds MAdCAM-1 (mucosal addressin cell adhesion molecule), a protein with both mucin-like and immunoglobulin-like domains.[160] MAdCAM-1 is also a counterreceptor,[161] in Peyer's patches, of the lymphocyte integrin $\alpha_4\beta_7$. MAdCAM-1 from mouse mesenteric lymph nodes supports rolling of lymphocytes mediated by L-selectin.[159] This rolling interaction was demonstrated using glass tubes coated with MAdCAM-1 (see Section II.5.) Rolling was inhibited by the anti-L-selectin antibody[162] DREG-56 or was abrogated by pretreatment of the MAdCAM-1 layer with neuraminidase.

5. Experimental Systems for Studies of Selectin–Carbohydrate Interactions

Bevilacqua and his co-workers[163] at the Howard Hughes Medical Institute, La Jolla, have reviewed the early work on assay systems suitable for evaluating the potency of low molecular mass candidate inhibitors of E-selectin–carbohydrate interactions. Since then, a large number of static *in vitro* assays have been developed for all three selectins, most of them based on the principle of solid-phase binding assays employing either the selectin or the oligosaccharide ligand, in the context of various matrices, as the solid-phase reactant. Accordingly, the oligosaccharide ligands or selectin equivalents are then added in the fluid phase and their binding to the solid matrix in the presence or absence of inhibitors is assessed with the aid of suitable analytical techniques. In the following, some of the more frequently used, static assay formats are listed. Keeping aware of the limited significance of

static binding results for physiological binding under shear stress, flow chamber assays are also mentioned. Some representative animal models used to study the inhibition of selectin–ligand interactions *in vivo* are listed in Section VII.

a. Static Assays Employing Selectins or Their Functional Equivalents as the Solid-Phase Reactants.—The assay system reported by Goldstein[11] and Bevilacqua[9] is based on a solid phase of HUVEC that display E-selectin; oligosaccharide ligands are present on ^{111}In-labeled PMN, the binding of which to the HUVEC is measured by counting of radioactivity. Following the reports that activated HUVEC express both P-selectin and E-selectin, the need arose for selectin-specific *in vitro* assays. Furthermore, the contingencies associated with radioactivity have generally stimulated developments of automated, enzyme-linked, colorimetric detection methods. In these regards, important progress was made through the construction of selectin–immunoglobulin fusion proteins (selectin–Ig chimeras) containing an N-terminal segment of the selectin molecule including the carbohydrate-binding domain, and part of the constant region of an antibody.[64,110,164,180] The covalent attachment of the antibody fragment, previously applied for the design of immunoadhesins for AIDS therapy,[165] enables either the "capture" of the selectin on a solid phase, or its analytical detection when the oligosaccharide ligand constitutes the solid phase and the selectin–Ig chimera is applied in the fluid phase as a soluble selectin equivalent. Watson *et al.* also demonstrated that administration to mice of an L-selectin–Ig chimera results in significant inhibition of L-selectin–ligand interaction *in vivo*[110] (compare Section VI.9).

(i) *Cell-Free Assays.*—Weitz-Schmidt at Sandoz-Pharma (now Novartis), Basel, Switzerland, and her colleagues at the Zelinsky and Shemyakin Institutes of the Russian Academy of Sciences in Moscow developed a convenient enzyme-linked selectin binding assay.[166] For example, microtiter wells were coated with a rat anti-mouse-C_K monoclonal antibody that was used to capture an E-selectin mouse C_K fusion protein.[76] The ligand oligosaccharides are represented by biotinylated polyacrylamide copolymers[167] with pendant sLe^x or sLe^a oligosaccharide determinants. Binding to the solid phase of the copolymer matrix in the presence or absence of candidate inhibitors is detected with the aid of a streptavidin–peroxidase conjugate.[168] The quantity of bound conjugate is estimated from the rate at which the chromogenic substrate, 2,2′-azino-bis-[3-ethylbenzothiazoline-6-sulfonic acid] (ABTS), is oxidized to colored products of λ_{max} 414 nm under catalysis by the horseradish peroxidase. Scudder *et al.*[169] estimated the binding of purified, $^{35}SO_4$-labeled peripheral node addressins (GlyCAM-1 and CD34) to human L-selectin–F_c chimera bound to protein A–Trisacryl resin. Candidate inhibitors were added to the incubation mixture in final concentrations ranging from 0.2 to 3.2 m*M*. After incubation for 16 h at 4 °C, the labeled addressins bound to

the selectin were removed by centrifugation, and the unbound labeled addressins remaining in the supernatant were measured by scintillation counting. Drickamer and his associates studied the binding of ^{125}I-labeled neoglycoproteins (mannose–BSA or sLe^x–BSA) to wild-type or mutant carbohydrate-recognition domains of mannose-binding proteins immobilized on microtiter plates.[170] Alternatively, binding was measured of ^{3}H-labeled HL60 cells to the same matrix. In a study of mutant CRD of MBP, binding was assessed of the soluble mutant domains to nitrocellulose coated with invertase, a glycoprotein comprising oligomannoside glycan chains. Bound CRD was quantified with the aid of anti-CRD antibody, measured by autoradiography of ^{125}I-protein A bound to the F_c region of the antibody.[171]

(ii) ***Assays Employing Cell-Bound Selectin Ligands as the Fluid-Phase Reactant.—***Bevilacqua's group[163] also described an assay format in which leukocytes are allowed to bind to a solid-phase matrix of selectin fusion protein. For example, microtiter wells were coated with polyclonal goat anti-human IgG antibody or protein A.[172] Staphylococcal protein A is a protein found in the cell wall of the pathogenic bacterium *Staphylococcus aureus* and binds to the F_c portion of most immunoglobulin G (IgG) molecules.[173,174] Both these proteins capture the fusion protein in a manner that results in the selectin domains being exposed for ligand binding on the surfaces of the wells. Next, the wells were incubated with candidate soluble ligand glycans, allowing them to bind to the selectin portions of the fusion protein molecules. Subsequently, polymorphonuclear leukocytes (PMN) were added to the wells and permitted to adhere to those selectin portions not blocked by soluble ligands. Following appropriate washing procedures, adhering cells were fixed by crosslinking with glutaraldehyde and counted under a microscope. The number of cells bound is inversely proportional to the potency of a candidate inhibitor. Norgard *et al.*[143] measured the binding of HL60 cells to a matrix of P-selectin coated onto microtiter wells. The numbers of adherent cells were quantified on the basis of their myeloperoxidase activity.[27] DeFrees *et al.*[175] used a similar assay format. Microtiter wells were coated with recombinant soluble E-selectin (rs-E-selectin) to generate a matrix to which HL60 cells were allowed to bind in the presence or absence of candidate inhibitors. The number of bound cells was estimated from the myeloperoxidase activity released upon lysis of the bound HL60 cells. Myeloperoxidase catalyzes the oxidation of *o*-phenylenediamine by hydrogen peroxide to colored products having an absorption maximum at 492 nm.[176] Revelle *et al.*[177] have reported on binding assays based on selectin- or selectin mutant-mouse IgG fusion proteins captured by goat anti-mouse-IgG coated on magnetic beads (Dynabeads). Such beads have been used to bind HL60 cells labeled with the fluorescent dye calcein AMC-3099. The numbers of bound cells, in the presence or absence of candidate inhibitors, are estimated from the fluorescence intensity of the dye liberated by lysis of the bound cells.

(iii) ***Assays Based on Cell–Cell Adhesion*** **in Vitro.—**In a further leukocyte-binding assay reported by Bevilacqua,[163] cell-bound selectin was used as the solid-phase reactant. Microtiter wells were coated with a confluent monolayer of human umbilical vein endothelial cells (HUVECS). Upon stimulation by tumor necrosis factor, such cells express a large number of copies of E-selectin at the cell surface.[9] Following exposure of the cell monolayer to candidate soluble ligand glycans, polymorphonuclear leukocytes were allowed to adhere as in the assay for adhesion to the fusion protein just discussed. Adhering cells were fixed and counted under a microscope. Larsen *et al.*[92] reported on a quantitative assay of HL60-cell binding to Chinese hamster ovary (CHO) cells expressing P- or E-selectin. CHO cells were grown to confluence on microtiter wells, and ^{3}H-labeled HL60 cells were allowed to bind to the CHO cells. Following appropriate washing procedures, cells were released from the wells and the numbers of bound cells were determined by scintillation counting. Skinner *et al.*[178] employed a rosetting assay previously reported by the Furie group[179] to estimate the extent of P-selectin–ligand interaction in the presence or absence of inhibitors. Counting under a light microscope the formation of rosettes between thrombin-activated platelets and neutrophils or HL60 cells, the percentage of cells with two or more adherent platelets was taken to indicate the extent of P-selectin–ligand interaction.

b. Static Assays Employing Oligosaccharide Ligands as the Solid-Phase Reactants.—

(i) ***Cell-Free Assays.—***Bevilacqua[163] reported on a cell-free, solid phase enzyme-binding assay that allows the determination of binding of selectin–(human)immunoglobulin fusion proteins to a matrix of neoglycoproteins prepared by linking sLe^x or sLe^a glycans to bovine serum albumin (BSA). The neoglycoproteins are coated onto microtiter wells. The selectin portion of the fusion protein will bind to the cognate ligands on the coated wells while the immunoglobulin portion serves as the analyte, binding rabbit anti-human immunoglobulin labelled with horseradish peroxidase. Foxall *et al.*[180] and Ohmoto *et al.*[181] described an assay whereby the binding of a selectin–immunoglobulin fusion protein was measured to a solid phase of synthetic sLe^x pentasaccharide ceramide. For colorimetric detection of the bound fusion protein, biotinylated anti-human IgG (F_c) was employed in conjunction with streptavidin–alkaline phosphatase. The assay used by Ohmoto *et al.* is schematically represented in Fig. 7.

(ii) ***Selectin-Expressing Cells Binding to a Solid Phase of Ligand Glycolipids.—***In the assay described by Berg *et al.*, a panel of neoglycoproteins were coated onto glass wells of eight-chamber slides and were incubated with cells of the murine pre-B cell line L1-2 transfected with E-selectin cDNA. Bound cells were fixed with glutaraldehyde and counted under a microscope.[182] Feizi and

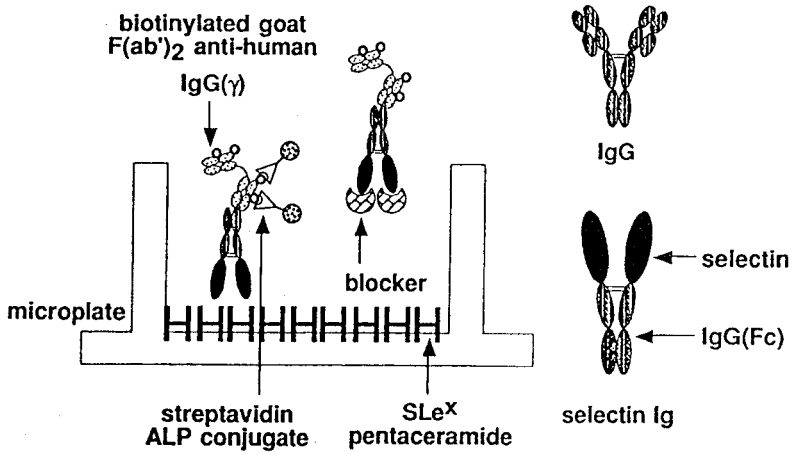


FIG. 7. Schematic diagram of ELISA inhibition assay using selectin–Ig chimera. Reprinted in part with permission from H. Ohmoto, K. Nakamura, T. Inoue, N. Kondo, Y. Inoue, K. Yoshino, and H. Kondo, *J. Med. Chem., 39* (1996) 1339–1343. (Ref. 181); © (1996). American Chemical Society.

her associates[183–185] examined a series of structurally defined neoglycolipids as candidate selectin ligands (compare Section V.1). Neoglycolipids were prepared from naturally occurring glycoprotein glycans by reductive amination[186,187] with L-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, and were characterized by thin-layer chromatography and liquid secondary-ion mass spectrometry.[188] The neoglycolipids served as solid-phase reactants in two types of selectin-binding assays. In a chromatogram binding assay modeled on previous work by Swank-Hill, Needham, and Schnaar,[189] TLC plates were coated with a thin film of Plexigum P28. Subsequently, suspensions were applied of ^{3}H-labeled CHO cells expressing different levels of the transfected full-length E-selectin. Following appropriate washing, the bound cells were fixed with glutaraldehyde and their numbers estimated by fluorography. Alternatively, following the previous example of Blackburn, Swank-Hill, and Schnaar,[190] glycolipids were coated on microtiter wells, and suspensions of ^{3}H-labeled cells were added as the selectin equivalent. Following appropriate washing, the bound cells were released by treatment with trypsin and the calcium-specific chelating agent, ethylene glycol-bis(2-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), and their numbers estimated by scintillation counting. Similar chromatogram and microtiter assays were used by Tiemeyer *et al.*[191] in their search for the physiological ligand(s) of E-selectin. These authors used, as the solid-phase reactant, natural glycolipids extracted by the method of Svennerholm[192] from leukocytes collected during therapeutic leukophoresis of patients with chronic myelogeneous leukemia; the selectin equivalent, in these experiments, was COS cells transfected with the E-selectin-encoding plasmid pl-ELAM, metabolically labeled with $^{32}PO_4$. The cDNA for E-selectin had been obtained

by subjecting to the reverse-transcriptase–polymerase chain reaction (RT-PCR) the total RNA extracted from human umbilical vein endothelium stimulated with interleukin-1 (IL-1).

(iii) ***Cells Expressing Selectin Ligands as the Solid Phase.—***Magnetic beads with adsorbed selectin–mouse IgG fusion proteins have also been used as fluid phase reactants,[177] for example when their binding to a lawn of HL60-cells was estimated from the rate of color development ($\lambda_{max} = 415$ nm) catalyzed by alkaline phosphatase conjugated to a rabbit antigoat antibody. This latter antibody binds the goat anti-mouse IgG antibody used to capture the selectin–mouse IgG fusion proteins to the magnetic beads. Similar arrangements were used when the binding was estimated of beads containing E- or P-selectin mutant chimeras to oligomannoside glycans coated onto nitrocellulose filters or microtiter wells.[177] Skinner *et al.*[178] reported that the binding of ^{125}I-labeled P-selectin to neutrophils or HL60-cells is strongly inhibited by heparin, fucoidan, and dextran sulfate of molecular mass 500 kDa, moderately inhibited by dextran sulfate of 5 kDa and λ- and κ-carrageenan, and not inhibited by chondroitin 4- or 6-sulfate. For measurements of binding, cells were incubated with the iodinated selectin and were centrifuged through 17% w/v sucrose in RPMI medium. The extent of binding was indicated by the radioactivity associated with the centrifuged cell pellets.

c. Dynamic Assays.—

(i) ***Flow-Chamber Models.—***McIntire *et al.*[193] reported on a flow-chamber apparatus suitable for the observation and quantitation of cells rolling on a confluent culture of HUVEC. In a parallel plate arrangement, one side of the chamber was a glass slide on which the HUVEC were cultured. The other side was machined from transparent polycarbonate and was mounted 250 μm apart with the aid of a gasket. Thermostated at 37 °C, the chamber was perfused with solutions or cell suspensions at velocities corresponding to the shear stresses expected to prevail in venules or veins (0.98 to 3.92 dyn cm^{-2}). The interactions of the HUVEC with polymorphonuclear leukocytes (PMN) were visualized by phase-contrast videomicroscopy and digital image processing. In a typical experiment, washed PMN were perfused at 0.98 dyn cm^{-2} over a lawn of HUVECs stimulated with 1×10^{-7} mol L^{-1} of formylmethionyl-leucyl-phenylalanine, a chemoattractant that activates neutrophil integrins; under these conditions, 283 ± 37.3 PMN adhered while 195 ± 20.3 PMN adhered to unstimulated control HUVEC. To HUVEC treated with interleukin-1 (IL-1) and perfused with a PMN suspension at 1.96 dyn cm^{-2}, 371 ± 25.8 PMN adhered whereas 28 ± 2.9 adhered to unstimulated controls. Using a similar flow chamber apparatus, Lawrence and Springer[194] demonstrated that rolling of neutrophils on P-selectin precedes and is a prerequisite for firm adhesion mediated by integrins and ICAM-1. Later, Puri and Springer,[195] with the aid of a similar flow chamber, investigated the formation of covalent linkages between L-selectin and oxidized L-selectin ligand glycans (V.6). Sanders *et al.*[196]

employed a flow chamber assay to study the inhibition of rolling of L-selectin-transfected cells on substrates coated with the purified ligand GlyCAM-1. The results obtained by these latter authors indicate that the specificity of L-selectin for one ligand over another as found in static assays is not necessarily observed in the dynamic conditions of flow; also, inhibitors of L-selectin ligand interaction in static assays may not block the physiologically more relevant phenomenon of selectin-mediated rolling (Section VI.6).

(ii) ***Assay of Cell Rolling in Glass Capillary Tubes.*****—**For their study of mouse pre-B-cell lines transfected with human L-selectin and rolling on MAdCAM-1, Berg *et al.*[159] allowed the L-selectin transformants to flow through glass capillary tubes coated with MAdCAM-1. Cells rolling on the tube wall were readily distinguished from noninteracting cells and were counted under a microscope.

III. Significance of Selectin–Ligand Interactions In Pathophysiology

1. Biological Context of Selectin–Ligand Interactions

a. General Functions of Selectins and Their Ligands.—Selectins and their ligands mediate specific cell-adhesion processes[197,198] whereby blood cells are captured or "tethered" from the blood stream to vascular endothelial cells of specialized blood vessels and then proceed to "rolling"[199] either along the vessel wall or on blood cells that already adhere[200] to the vascular endothelium. During rolling, the blood cells are subject to shear forces and become exposed to additional stimuli such as chemoattractant cytokines[201] (chemokines). Chemokines act on G-protein-coupled receptors of the blood cells, causing among other processes the activation of integrins[202–204] on the blood cell membranes into a form that can bind to endothelial integrin receptors of the immunoglobulin superfamily.[198] The protein–protein interaction of integrins with their receptors results in a more stable form of endothelial adhesion during which the rolling cells will flatten and may eventually extravasate into the surrounding tissues.[205]

b. Occurrence of Selectins and Their Ligands on Blood Cells and Vascular Endothelia.—Selectins and their most frequently studied ligands[206–208] are expressed in several distinct combinations on endothelial cells, leukocytes, and platelets. Endothelial cells may express E- and P-selectins, and the L-selectin ligands, GlyCAM-1, CD34, and MAdCAM-1. Leukocytes express L-selectin, the P-selectin ligand PSGL-1, and the E-selectin ligand,[209] ESL-1. Platelets are known to express only P-selectin. L-Selectin expressed on human neutrophils is glycosylated with E-selectin ligand glycans and binds to an E-selectin–Ig chimera[210]; by contrast, mouse L-selectin is not a ligand of E-selectin.[210] A schematic representation of the occurrence of selectins and their ligands on blood cells and endothelia is given in Fig. 8.

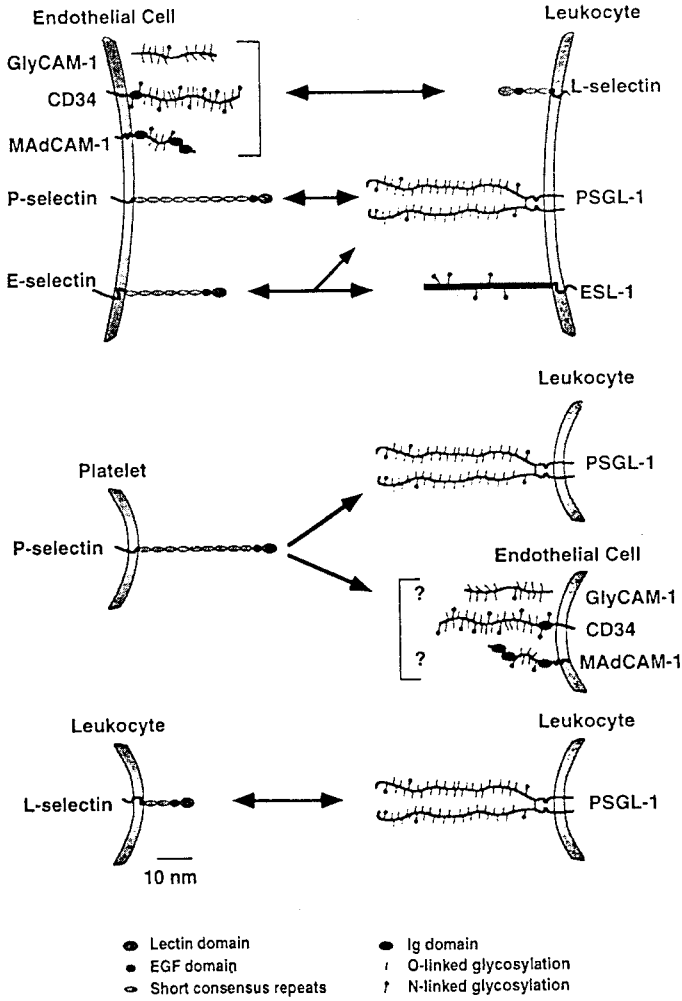


FIG. 8. Distribution of selectins and their glycoprotein ligands. Reprinted with permission from Rodger P. McEver, Selectin-carbohydrate interactions during inflammation and metastasis, *Glycoconjugate J.*, 14 (1997) 585–591 (Ref. 208); © (1997). Kluwer Academic Publishers.

c. Aspects of Selectin–Ligand Interactions under Flow.—McIntire *et al.*[193] have used a flow chamber to study the effect of flow on the adhesion of polymorphonuclear leukocytes (PMN) to cultured human vascular endothelial cells (HUVEC) in the presence or absence of stimulants such as the chemotactic peptide formylmethionylleucylphenylalanine (fMet-Leu-Phe) or the cytokine interleukin-1 (IL-1). Adhesion of PMN to HUVEC at constant shear stress was higher than controls when the HUVEC had been stimulated with fMet-Leu-Phe or

IL-1. Furthermore, McIntire *et al.* demonstrated that relatively small changes in shear stress can have large effects on PMN adhesion under the conditions studied. With the use of a similar flow chamber, Lawrence and Springer[211] have demonstrated that, under flow, tethering and rolling of leukocytes mediated by selectin–ligand interactions is a necessary, preliminary step prior to the firm adhesion of these cells mediated by the interaction of β_2-integrin with ICAM-1. In the flow chamber model, an artificial lipid bilayer represents the vessel wall and contains intercalated P-selectin, ICAM-1, or a mixture of these adhesion molecules. The phenomenon of rolling was demonstrated under conditions of physiologically relevant shear stress *in vitro* using lipid bilayers that contained P-selectin only. When lipid bilayers containing only ICAM-1 were used, rolling was not observed, although, under static conditions, leukocytes were found to attach to membranes containing fourfold lower densities of ICAM-1. Rolling was seen to occur at physiologically relevant site densities of as few as 50 P-selectin molecules per μm^2, at physiological shear stresses. Rolling velocities were found comparable to those previously determined *in vivo*. Lawrence and Springer have also demonstrated how the rolling interaction—based on a relatively weak molecular interaction of oligosaccharide determinants with the carbohydrate-binding domains of selectins—may develop further into the firm adhesion of leukocytes to vascular endothelial cells that precedes extravasation. Neutrophils rolling on a bilayer that contains both P-selectin and ICAM-1 were slowed and brought to a halt by the action of fMet-Leu-Phe, an activator of neutrophil integrins. This firm attachment results in spreading of the neutrophils and has been shown to be inhibited by a monoclonal antibody to CD18 *in vivo*. In the flow chamber model under physiological shear stress, rolling on a selectin was a prerequisite for the firm attachment of leukocytes mediated by the interaction of leukocyte integrins with ICAM-1. The authors have therefore defined a physiological role of P- (and probably E-)selectins as part of a tethering mechanism that functions to slow down leukocytes as they tumble through the microvasculature near sites of inflammation, in preparation for firm attachment, spreading and diapedesis through the vessel wall into the adjacent tissues.

As elucidated during extensive investigations since the early 1980s, the selectins and their ligands are important elements of an elaborate system of traffic signals[212–215] or "area codes" that has evolved to control and coordinate the recruitment, differentiation, activation, and tissue targeting of specialized blood cells during inflammation, repair of vascular injury, and different forms of immune response. Indeed, early perceptions of the importance of selectin–ligand interactions in the pathogenesis of acute and chronic inflammatory conditions, autoimmune diseases, and cancer have motivated the prodigious efforts toward the design and synthesis of therapeutically applicable oligosaccharide derivatives and mimetics outlined in Sections V and VI of this article. In the following paragraphs of this Section III, selected examples are provided of selectin–ligand pairs that participate or are assumed to participate in pathologically significant cell-adhesion processes of granulocytes, lymphocytes, and tumor cells. However, it is

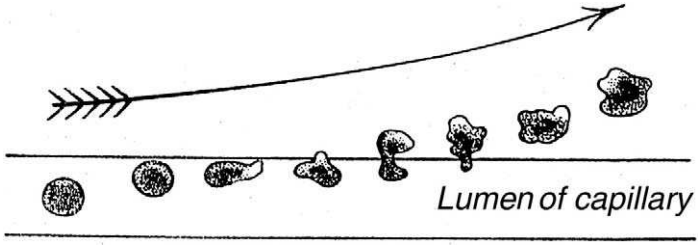


FIG. 9. Extravasation of leukocytes: classical observations. Reprinted with permission from A. Boivin and A. Delauney, *Phagocytose et Infections* (Ref. 219); © Hermann, Paris, 1947.

combinations of selectin and integrin binding, rather than selectin–ligand interactions alone, that mediate functional cell adhesion. Once tethered, a cell needs to be exposed to signaling molecules for activation of integrins and firm adhesion.[216] Moreover, the integrin–receptor interactions that follow tethering and rolling, at least in some cases, appear to constitute specific, pharmacologically inhibitable steps of adhesion processes; this has led to the successful pharmaceutical development of integrin antagonists, whereas clinically interesting selectin inhibitors appear to have emerged only recently (compare Section VII).

2. Selectin–Ligand Interactions in Granulocyte Function

a. Historical Reports.—Experimental observations by intravital microscopy of the tethering, rolling, and extravasation of leukocytes during acute inflammation have been reported since the 19th century.[217,218] In their 1947 monograph *Phagocytose et Infections,* Boivin and Delauney[219] provide a vivid description of leukocyte extravasation (cf. Fig. 9) under the heading " La diapédèse des leucocytes" (author's translation*):

> *In capillary blood vessels with normal blood circulation, the leukocytes maintain a spherical shape, and the rapid blood flow carries them along, together with the erythrocytes. Exceptionally, a leukocyte will adhere to the vessel wall and migrate into the surrounding connective tissues. This state of things will change, however, as soon as inflammation sets in: the blood vessels become extremely dilated and the blood flow slows down considerably. One observes, at this time, the remarkable phenomenon of leukocyte extravasation discovered by* Cohnheim.* *The various leukocytes, polymorphonuclear cells, . . . and large mononuclear cells will migrate into the perivascular connective tissue where they eventually will have to exert their phagocytotic activities . . . . Extravasation may be conveniently studied on the exposed frog mesentery . . . and the sequence of its stages may be followed under the microscope.*

* With permission. © Hermann, Paris, 1947.

* Julius Cohnheim (1839–1884), German pathologist, professor at the universities of Kiel, Breslau, and Leipzig. Worked mainly on inflammation ("theory of alteration"), the fine structure of striated muscle ("Cohnheim striation"), infarction, and the etiology of tumors.

While the erythrocytes keep circulating, albeit much more slowly, in the dilated capillaries, the leukocytes now have a pronounced tendency to adhere to the vessel wall, to attach themselves to the wall as if held back in contact with a surface that has become adhesive. This phenomenon has been called the margination of leukocytes. In this manner, large numbers of leukocytes may accumulate on the inner walls of capillaries within inflamed tissues. Let us follow the fate of one of these leukocytes, a polymorphonuclear cell for example. By virtue of its microvilli, the white blood cell remains capable of a few crawling motions along the vessel wall. However, one of the microvilli quickly inserts itself between two of the vascular endothelial cells that constitute the lining of the blood vessel. The manner in which this passage is opened is still a matter of debate . . . [it is proposed by different authors] *. . . that the opening results from a gradual softening of the adhesive that normally keeps the endothelial cells together . . .* [or] *. . . that the opening by which the leukocytes extravasate is caused by a simple contraction of the endothelial cells under the influence of various stimuli . . . . Be that as it may, the polymorphonuclear cell will force itself through the small, temporary orifice, and thus migrate from the vascular lumen to the perivascular connective tissue. The great extension the polymorphonuclear cell needs to undergo in the process is certainly made easier by the shape of its nucleus that may resemble a sausage or a rosary. As soon as extravasation is complete, the opening in the vessel wall will close again. The whole process may require from a few minutes to a half hour . . . .*

b. Granulocyte Adhesion in Inflammation.—Since the early 1980s, the phenomena described by Boivin and Delauney have become known in consirable molecular detail. The role of selectins in acute inflammation has been discussed in excellent recent reviews.[220,221] Briefly, inflammatory stimuli inside tissues will be produced by invading pathogens or responding monocytes in the form of inflammatory mediators and chemokines that induce vascular endothelial cells (VECs) of adjacent postcapillary venules to express P-selectin and E-selectin. P-Selectin, constitutively stored in granula known as Weibel–Palade bodies, is rapidly displayed on the surface of VECs following fusion of the granule membrane with the plasma membrane. The redistribution of P-selectin from α-granules to the plasma membrane of platelets occurs in response to platelet activators such as thrombin, histamine, complement components, and oxygen-derived radicals. By contrast, E-selectin is elicited by lipopolysaccharide (endotoxin) or the cytokines TNFα and IL-1, and is produced by *de novo* protein synthesis, reaching peak levels at the cell surface 4 h after the initial stimulus.[8] Leukocytes are tethered to and roll on endothelium by means of constitutively expressed selectin ligands such as PSGL-1, ESL-1, and, on human neutrophils, L-selectin glycosylated with sLe^x determinants.[141] These ligands are prominently displayed on the tips of microvilli to facilitate the interaction with P- and E-selectin on VECs. Leukocytes constitutively express L-selectin, which binds to selectin ligands such as PSGL-1 on adherent leukocytes. Tethering of leukocytes to adherent leukocytes may function as a mechanism to expand the pool of leukocytes attracted to an inflamed site. However, during the activation stage that follows tethering, L-selectin

is shed from the leukocyte surface.[222] The P-selectin displayed on platelet surfaces may mediate the attachment of platelets to tethered leukocytes. In this manner, the formation of platelet–leukocyte aggregates immobilized on vessel walls may be initiated by binding of leukocyte PSGL-1 to P-selectin expressed on adherent platelets. Together with other factors such as vascular injury and blood cholesterol, such aggregates may contribute to the formation of atherosclerotic plaques.[223]

c. Ischemia–Reperfusion Injury.—Ischemia–reperfusion injury is an important example of an inflammatory condition in which selectin–ligand interactions play a role and for which administration of sLe^x-related oligosaccharide derivatives has been shown to be beneficial in animal experiments (compare Section VII). The function of selectins in ischemia–reperfusion injury has been discussed in a recent review.[221] Typical of the condition is a rapid burst of oxygen-derived radicals such as superoxide that arises shortly after reperfusion of the ischemic tissue. By the action of such oxygen radicals, P-selectin will be transferred from the Weibel–Palade bodies of endothelial cells to the cell surface, resulting in strongly enhanced adhesion of neutrophils. The resulting accumulation of neutrophils in the damaged tissue will induce vascular dysfunction and cause further injury to heart muscle cells by a number of mechanisms.[221] The prevention of reperfusion injury became a highly desirable therapeutic goal in the wake of the widespread application of thrombolytic therapy in cases of cardiac infarction, which is necessarily followed by reperfusion of the ischemic heart tissue. Examples of investigations demonstrating efficacy of sLe^x-related oligosaccharide derivatives in animal models of ischemia–reperfusion injury are cited in Ref. 221 and in Section VII. In another type of animal model, Mulligan *et al.*[224] have demonstrated the protective effects of sLe^x-related oligosaccharide derivatives against lung injury induced by administration of cobra venom factor in rats (compare Section VII). This model resembles the models based on ischemia–reperfusion in that the acute lung injury is mediated by oxygen radicals and involves the interaction of neutrophil PSGL-1 with P-selectin.

d. E-Selectin in Allergy, Asthma, and Cutaneous Allergic Reactions.—Bochner *et al.*[225] have demonstrated that E-selectin, together with the integrin receptors, ICAM-1 and VCAM-1, mediates the activation of eosinophil and basophil granulocytes and their recruitment to extravascular sites. Eosinophil granulocytes play a prominent role in allergic inflammation and asthma.[226] Bochner *et al.* have also shown that interleukin-1, an elicitor of E-selectin,[8] is released at sites of human cutaneous allergic reactions.[227] When adhesion was examined of human basophils, eosinophils and neutrophils to HUVEC activated by IL-1, monoclonal antibodies specific for E-selectin or ICAM-1 inhibited adhesion of all three types of cells. However, anti-VCAM-1 MAb inhibited the adhesion of eosinophils and basophils but not neutrophils. Accordingly, the VCAM-1 counterreceptor, a member of the

integrin family designated very late activation antigen 4α (VLA-4α), was found expressed on eosinophils and basophils but not on neutrophils. Using immunohistochemical techniques, Groves *et al.*[228] found marked expression of E-selectin on vascular endothelium in cutaneous inflammatory disorders such as allergic contact dermatitis, atopic dermatitis, and psoriasis, and in skin infiltrates associated with benign, premalignant, and malignant proliferation of keratinocytes. Groves *et al.* interpreted their findings to indicate that, in cutaneous inflammatory disease states, mononuclear and other resident cells in the vicinity of blood vessels would functionally interact with the vessel wall, enabling the vascular endothelium to recruit neutrophils and perhaps monocytes to the inflammatory sites.

e. E-Selectin Expression in Septic Shock.—Redl *et al.*[229] studied the expression of E-selectin in baboons, comparing septic vs traumatic/hypovolemic shock by immunohistochemical techniques. Septic shock was induced with live *Escherichia coli* bacteria and was accompanied by widespread expression of E-selectin in capillaries, venules, small veins, arterioles, and arteries of most tissues examined; expression was most pronounced on vessels of the lung, liver, and kidneys (compare Section VI.6). By contrast, animals with traumatic/hypovolemic shock showed minimal evidence of E-selectin expression in all organs studied. Parallel with the antibody staining, granulocyte elastase levels were measured in the plasma as a measure of neutrophil activation, and were found much higher in animals with septic as compared to traumatic/hypovolemic shock. The authors interpreted their findings as being consistent with the higher levels, during sepsis, of circulating lipopolysaccharide (LPS), TNF, and other cytokines, all of which are known inducers of E-selectin expression.[8]

f. Type 2 Leukocyte Adhesion Deficiency.—Etzioni *et al.* have discovered a rare genetic disorder[230] which they termed type 2 leukocyte adhesion deficiency (LAD-2). The clinical features of this condition include mental retardation, short stature, and recurrent bacterial infections accompanied by high leukocyte counts. Examination of the patients' blood group phenotype revealed the presence of the Bombay (hh) blood group antigen.[231] This rare blood group results from failure to attach fucose in α-(1 $\rightarrow$ 2)-linkage to form the H blood group determinant α-L-Fuc-(1 $\rightarrow$ 2)-β-D-Gal. The LAD-2 patients were also negative for the secretor, Le^x and Le^a blood group antigens α-L-Fuc-(1 $\rightarrow$ 2)-β-D-Gal, β-D-Gal-(1 $\rightarrow$ 4)-[α-L-Fuc-(1 $\rightarrow$ 3)]-β-D-GlcNAc and β-D-Gal-(1 $\rightarrow$ 3)-[α-L-Fuc-(1 $\rightarrow$ 4)]-β-D-GlcNac. Whereas the previously known leukocyte adhesion deficiency disorder[232,233] (LAD-1) is based on mutations in the gene coding for the CD18 subunit of integrins LFA-1, Mac-1, and p150,95, in the case of LAD-2, the defect would reside in the biosynthetic pathways of cell-surface carbohydrates. From their observations, Etzioni *et al.* derived the hypothesis that the fucose residues of sLe^x determinants might also be lacking in neutrophils from patients

with LAD-2. When fluorescence-activated cell sorting (FACS) was performed with anti-sLe^x (CSLEX-1),[75] anti-CD18 (MHM23), and appropriate fluorescence-labeled secondary antibodies, control neutrophils from healthy donors were seen to bind both CSLEX-1 and MHM23 while neutrophils from patients with LAD-2 bound MHM23 but no CSLEX-1. Furthermore, neutrophils from normal donors were found to bind to HUVEC activated with interleukin-1β, the binding being largely inhibited by anti-E-selectin antibody. Neutrophils from LAD-2 patients were found not to bind to HUVEC under analogous conditions. From these results, the authors concluded that neutrophils from LAD-2 patients have an adhesion deficiency due to the absence of sLe^x determinants from the cell surface. Since the defects of neutrophils from LAD-2 patients occur in several surface glycans, the biosynthesis of which involves different fucosyltransferases,[234] Etzioni ***et al.*** concluded that some general defect in fucose metabolism underlies LAD-2.

3. Selectin–Ligand Interactions in Lymphocyte Function

Lymphocytes constitute a population of leukocytes centrally important to the specificity of immune responses; they are the only cells in a mammalian organism capable of distinguishing different antigenic determinants. To fulfill its function, the immune system continuously establishes and maintains diverse populations of mature lymphocytes and provides them with the capability to respond to foreign antigen at a variety of effector sites.[76,235]

To accomplish these diverse tasks in an integrated fashion, the immune system employs specialized immune microenvironments in the context of primary, secondary, and tertiary lymphoid organs and other tissues. These microenvironments are linked together and to effector sites of the body through a system of lymphocyte recirculation and homing. Selectin–ligand interactions constitute crucial steps of several lymphocyte homing pathways. Butcher and Picker have expertly summarized[236] the involvement of selectin and integrin interactions in lymphocyte trafficking. For example, homing of lymphocytes from the blood stream into the parenchyma of lymph nodes or GALT proceeds through initial adhesion of the lymphocytes to the endothelial cells of the post-capillary high endothelial venules[29] (HEV; Fig. 10). Different populations of lymphocytes show distinct organ preferences for homing. Thus, T-cells will primarily migrate to peripheral lymph nodes while B cells exhibit a preference for GALT.[237] Homing preferences are also exhibited by certain murine lymphoma cell lines.[238] As discussed in Section II, L-selectin as a lymphocyte homing receptor binds to sulfated glycans of mucin ligands located on HEV of peripheral lymph nodes. The circulating skin-associated T-lymphocyte subset expresses L-selectin and the cutaneous lymphocyte antigen (CLA), a glycoprotein that binds the HECA-452 antibody and presents E-selectin ligand glycans.[239,240] Picker ***et al.*** have shown that these cells bind to COS cells that have been transfected to express E-selectin, and that only memory cells are bound. Shimizu ***et al.*** similarly demonstrated that a population of

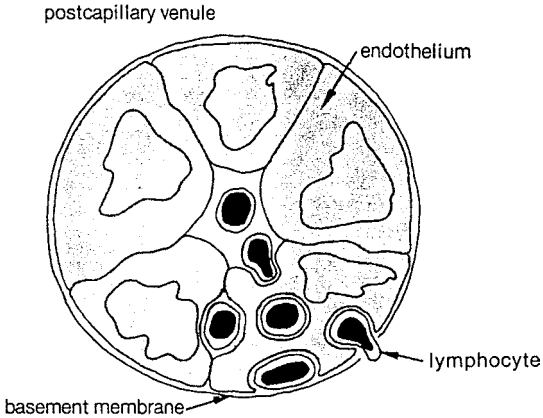


FIG. 10. Diagrammatic cross section of a lymph-node postcapillary venule. Small dark cells are lymphocytes adhering to and migrating across the specialized endothelial walls of these vessels. [Redrawn from J. L. Gowans, *Hosp. Pract.* 3, 3 (1968) 34 from *Immunology.* 2nd Ed., by L. E. Hood, I. L. Weissman, W. B. Wood, and J. H. Wilson (Ref. 235); © 1984. Reprinted by permission of Pearson Education Inc.]. Illustration: Irving Geis. Rights owned by Howard Hughes Medical Institute. Not to be reproduced without permission.

memory T-cells binds to activated endothelium, and that this binding is independent of acute activation events that regulate integrin-mediated adhesion. Both groups of authors conclude that E-selectin may function in chronic inflammation as a tissue-selective adhesion molecule for skin-homing memory T-lymphocytes. Turunen *et al.*[241] demonstrated by means of Stamper–Woodruff assays[29] that during rejection of heart allografts in rats, lymphocytes adhere to the endothelium of the cardiac transplants being rejected. Lymphocyte adhesion was significantly decreased by treating the lymphocytes with an anti-L-selectin MAb, HRL-1, or by treating the tissue sections with neuraminidase or anti-sLe^x or anti-sLe^a MAbs. Normal rat cardial endothelium does not express sLe^x or sLe^a and will not support lymphocyte adhesion. In MRL-*lpr/lpr* mice, a strain that develops massive lymphadenopathy and autoimmunity, the anti-L-selectin MAb Mel-14 blocks lymphocyte homing to peripheral lymph nodes and prevents lymphadenopathy.[242] Yang *et al.*[243] reported that blocking by monoclonal antibodies of L-selectin and the integrin, VLA-4, inhibits insulitis and prevents diabetes in nonobese diabetic mice. The authors suggest that inhibitors acting on these two adhesion mechanisms may provide a new approach to the treatment of autoimmune diseases such as insulin-dependent diabetes mellitus.

4. Potential Roles of Selectin–Ligand Interactions in Cancer

Some of the carbohydrate determinants functioning as selectin ligand glycans have been identified as tumor-associated antigens.[244,245] In the early 1970s, evidence had been well established that profound changes in surface carbohydrate

structures[246] occur upon malignant transformation of cells. As discussed in recent reviews,[247,248] investigators have focused primarily on three types of glycan structure that are increasingly expressed during progression of cancers. These are the Le^x and Le^a structures and their sialylated homologues sLe^x and sLe^a (compare Refs. 75, 208, 249–252), the truncated mucin glycans[253] derived from α-D-GalNAc, β-D-Gal-(1 → 3)-α-D-GalNAc, and α-D-Neu5Ac-(2 → 6)-α-D-GalNAc (the T_n, T, and sialyl-T_n antigens; for a comprehensive treatment, see Ref. 134), and the structures arising from β-(1 → 6)-branching of N-linked glycoprotein glycan cores.[254] Possible pathophysiological functions of these surface glycan changes have been considered[255] and their significance discussed[256] in conjunction with tumor progression and metastasis. Kannagi[247] discusses several studies in which a highly significant correlation was demonstrated between sLe^a expression and a poor prognosis in a total of more than 500 patients with colon cancer in Japan. Tumor cells expressing, in the context of surface glycoproteins, selectin ligand glycans such as sLe^x or sLe^a might derive an advantage in the formation of metastases.

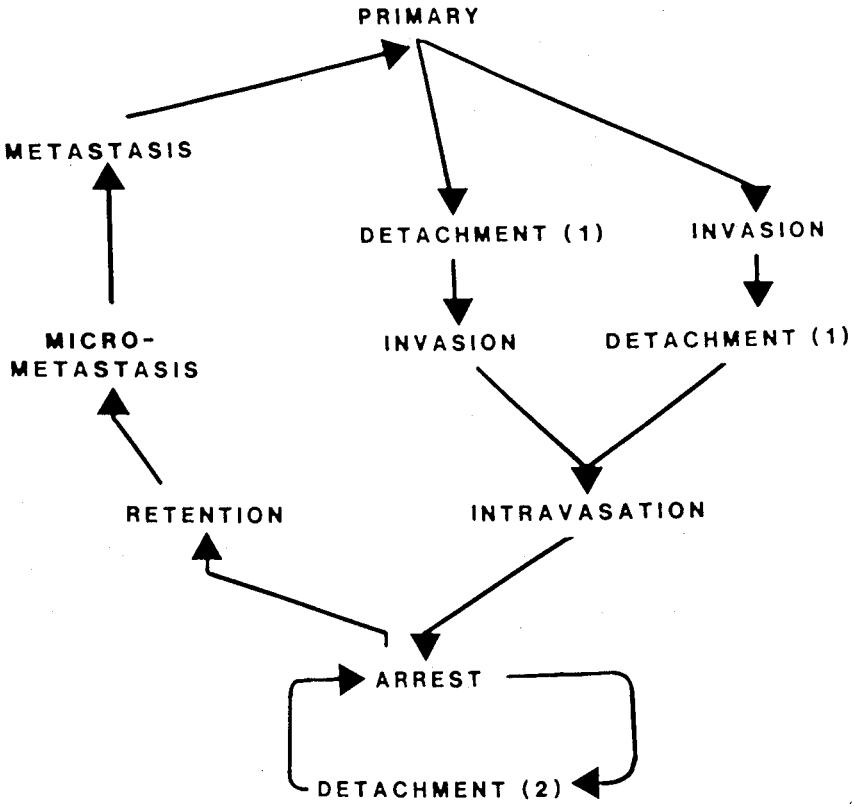


FIG. 11. Various overlapping steps of the metastatic cascade. From Leonard Weiss, *Principles of Metastasis* (Ref. 257); © 1985. Reprinted by permission of Academic Press.

The metastatic process,[257] among many other steps (Fig. 11), is thought to involve the formation of microthrombi composed of tumor cells, leukocytes, and blood platelets. Following their formation in the vasculature, such aggregates would grow to form a metastatic lesion. Adhesion of colon carcinoma cells to vascular endothelial cells has been demonstrated and has been shown to increase upon stimulation of the endothelia by the action of inflammatory mediators.[258] Tumor cell adhesion to endothelia is thought to be mediated by E- and P-selectins and their ligand glycans such as sLe^x and sLe^a expressed on surface mucins of cancer cells.[259,260] Through the use of P-selectin deficient mice that also tolerate human tumor cell lines, Kim *et al.*[261] have demonstrated a role for P-selectin in the promotion of cancerous growth of, and formation of metastatic lesions by, the mucin-producing human carcinoma cell lines[262] LS180 and T-84. Shedding by tumor cells of mucins that carry sLe^x or sLe^a determinants is another mechanism by which carbohydrate–selectin interactions might support the growth and progression of malignant tumors. Conceivably such mucins, by way of their selectin ligand glycans, might bind to selectins on leukocytes or endothelia and thereby interfere with immune responses directed against tumor cells. Tumor-associated mucins have been reported to induce the apoptosis of activated T-lymphocytes; in this manner, the mucins would disrupt the generation of an effective anti-tumor immune response.[263]

IV. Syntheses of Oligosaccharides and Derivatives Corresponding to the Sialyl-Lewis^X (sLe^x) and Sialyl-Lewis^A (sLe^a) Determinants

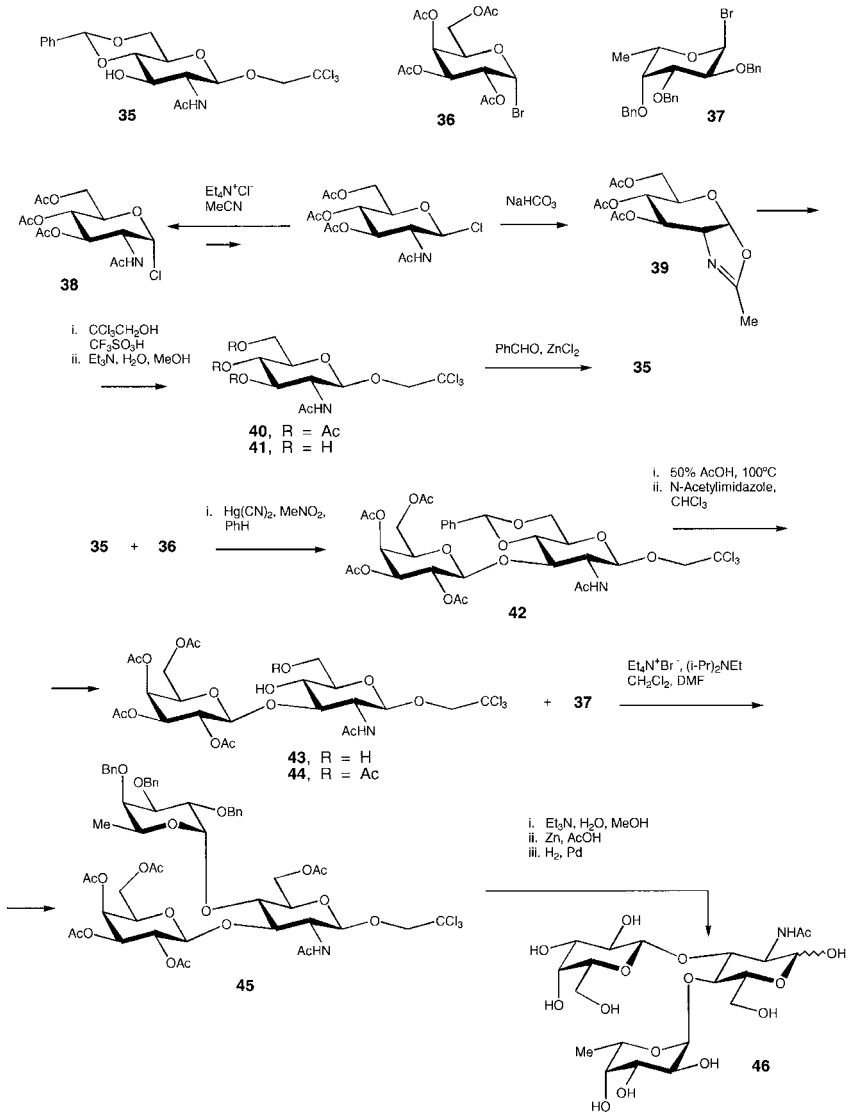
By the early 1970s, the art of oligosaccharide synthesis had reached a remarkable level, so that chemical syntheses of practical amounts of various blood-group-related oligosaccharides became feasible, including the Lewis determinants. In the context of the present article, a brief overview will be given of seminal contributions by the schools of Lemieux,[264,265] Paulsen,[266,267] Ogawa,[268,269] Schmidt,[270] Garegg, and Mukaiyama[271] that constitute the foundations from which several total syntheses of the sLe^x and sLe^a determinants were subsequently developed. General glycosylation methodology has been reviewed,[272] as has been the chemical synthesis of oligosaccharides.[273] Reviews are also available on more specific methods such as the use of thioglycoside,[274] trichloroacetimidate,[275] and isopentenyl[276] glycosyl donors developed by the schools of Garegg, Schmidt, and Fraser-Reid.

Within the present article, a few selected examples are provided of both classical and more recent glycosylation methods applied to the synthesis of sLe^x, sLe^a, or of sulfated Lewis determinants. Recent procedures involve the use of glycal donors as developed by Danishefsky and his associates,[277,278] of glycosyl fluorides according to Mukaiyama[279] and Nicolaou,[280] of sulfoxide donors as reported by Kahne,[281] and enzyme-catalyzed oligosaccharide syntheses as developed by the schools of Whitesides,[389] Wong,[282] and Paulson.[283]

1. Syntheses of Lewis Determinants and Sialyllactosamine Derivatives

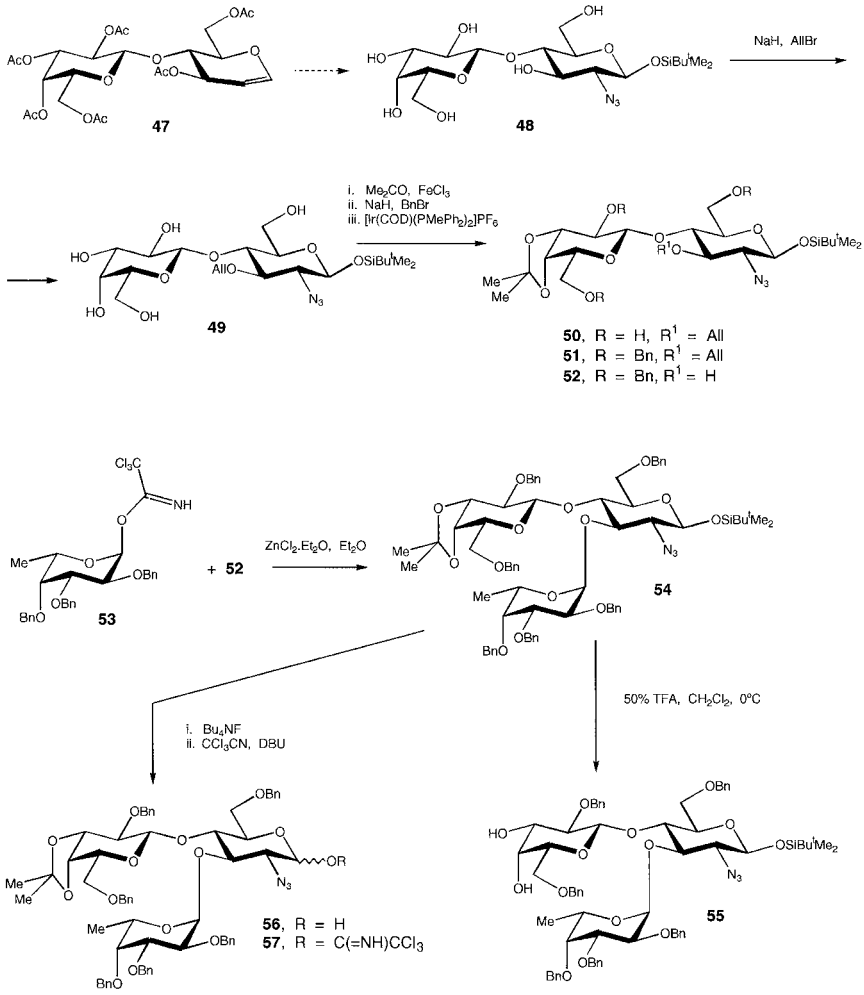
a. Lewis Determinants.—

(i) ***The Lewis[a] Trisaccharide.—***The first organic-chemical syntheses under controlled conditions of complex oligosaccharides of the Landsteiner[284] (ABO) and Lewis[285] blood group systems were reported by Lemieux and Driguez in 1975. Specifically, the blood group B trisaccharide[286] α-D-Gal-(1 $\rightarrow$ 3)-[α-L-Fuc-(1 $\rightarrow$ 2)]-D-Gal and the Le[a] determinant,[287] β-D-Gal-(1 $\rightarrow$ 3)-[α-L-Fuc-(1 $\rightarrow$ 4)]-D-GlcNAc **46** were prepared in high purity and in practical quantities. In many cases, the oligosaccharide determinants were prepared as glycosides of 8-ethoxycarbonyl- or 8-methoxycarbonyl-1-octanol, a form suitable for attachment to macromolecular carriers.[288] The Le[a] determinant **46** was assembled[287] from the GlcNAc, Gal, and Fuc monosaccharide precursors **35, 36,** and **37** (Scheme 6). 2-Acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-glucopyranoside[289] (**38**) was converted into the trichloroethyl β-glycoside **40** by way of the Δ^2-oxazoline derivative[290] **39** (46%). Compound **40** was O-deacetylated (triethylamine in methanol–water) and the crude glycoside **41** treated with benzaldehyde in the presence of anhydrous zinc chloride to afford the 4,6-*O*-benzylidene derivative **35** (78% from **40**). Acceptor **35** was glycosylated with 2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl bromide[291] (**36**) under promotion by mercuric cyanide in 1 : 1 nitromethane–benzene to give the protected disaccharide **42** in 91% yield. Compound **42** was converted into the diol **43** by treatment with 50% acetic acid at 100 °C in practically quantitative yield. Diol **43** was treated with *N*-acetylimidazole[292] in chloroform to regioselectively provide the glycosyl acceptor **44** in 76% yield. Glycosylation of **44** with tri-*O*-benzyl-α-L-fucopyranosyl bromide[293] in the presence of tetraethylammonium bromide and diisopropylethylamine in 5 : 1 dichloromethane–*N*,*N*-dimethylformamide afforded the crystalline Le[a] derivative **45** in 83% yield. The highly successful halide-inversion protocols[294] involve the presence, in the glycosylation mixtures, of a soluble halide salt to produce, from an α-halide donor, small equilibrium concentrations of the highly reactive β-(equatorial) glycosyl halide. The β-halide reacts rapidly with the glycosyl acceptor to form the desired α-(axial) fucoside derivative, because the corresponding transition state is stabilized by the anomeric effect. The transition state leading to the undesired β-(equatorial) glycoside is not comparably stabilized and hence, that reaction is slow. In agreement with the principle of Le Chatelier, practically all of the glycosyl donor is thus converted into the α-fucoside derivative by way of the β-halide. From **45,** the reducing Le[a] trisaccharide **46** was obtained by sequential O deacetylation (triethylamine in methanol–water), treatment with zinc in acetic acid, and catalytic hydrogenation over 5% palladium-on-charcoal. By specific rotation and paper chromatography, **46** was identical with a sample of Le[a] trisaccharide previously prepared from natural sources by Rege *et al.*[295]



SCHEME 6

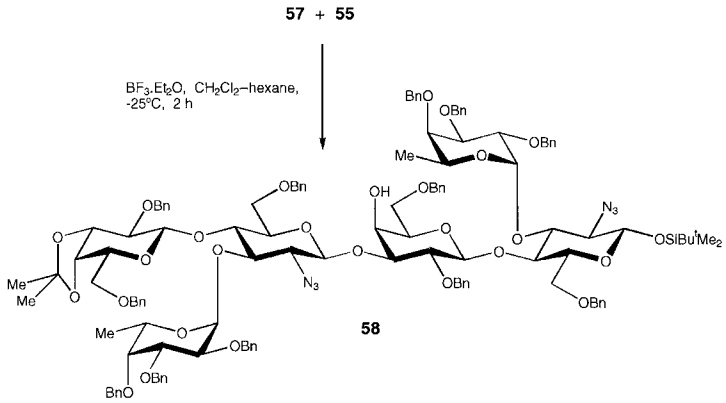
(ii) ***The Dimeric Le^x Determinant.***—An unusual and highly practical approach to the synthesis of a dimeric Le^x glycan has been reported[296] by Schmidt and his associates at the University of Konstanz, Germany. These authors have developed Le^x-related donor and acceptor molecules from lactose and fucose and have assembled them to form the dimeric Le^x synthon **66** (Scheme 10) which is



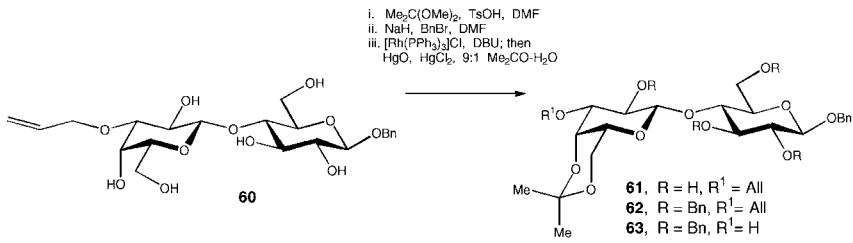
SCHEME 7

suitable for conversion into the corresponding ceramide derivative.[329] Key glycosylation steps involve trichloroacetimidate donors to form a hexasaccharide intermediate **58** from two Le^x trisaccharide derivatives, and the synthesis, from a hexasaccharide donor and a lactose-derived acceptor, of the octasaccharide intermediate **64.** Compound **54** (Scheme 7) is a common intermediate for the preparation of the Le^x donor **57** and the Le^x acceptor **55.** To prepare **54,** the *tert*-butyldimethylsilyl 2-azidolactoside derivative **48** was used as the starting material. Compound **48** is conveniently accessible[296,297] from lactose by way of the

lactal derivative[299] **47.** Treatment of **48** with sodium hydride and a slight excess of allyl bromide in dry *N*,*N*-dimethylformamide (DMF) surprisingly gave a 68% yield of the 3-allyl ether **49,** which was converted into the 3′,4′,-*O*-isopropylidene derivative **50** in 92% yield by the action of acetone in the presence of iron(III) chloride. Compound **50** was per-O-benzylated (benzyl bromide, sodium hydride in DMF, −10 °C) to afford **51** in 83% yield. O Deallylation of **51** required optimization and succeeded by way of isomerization under catalysis by (1,5-cyclooctadiene)bis(methyldiphenylphosphine)iridium hexafluorophosphate[300,301] followed by cleavage of the propenyl glycoside by the action of mercury(II) oxide and mercury(II) chloride to give **52** in 94% yield. Glycosylation (Scheme 7) of acceptor **52** with the fucosyl donor **53** under promotion by zinc chloride etherate required optimization and proceeded in 89% yield in an "inverted" fashion, i.e., when the catalyst was first allowed to form a complex with the acceptor, and the glycosyl donor **53** was added subsequently. The central intermediate **54** was next converted into acceptor **55** (67% yield) by the action of 50% aqueous trifluoroacetic acid in dichloromethane (0 °C, 48 h), a mild hydrolytic reagent that spares the acid-labile glycosidic linkage of the α-fucosyl residue. The reducing trisaccharide derivative **56** ($\alpha:\beta$, 2 : 3) was prepared from **54** by treatment with tetrabutylammonium fluoride in dichloromethane at −15 °C (94%) and was converted into the anomeric mixture of trichloroacetimidates **57** ($\alpha:\beta$, 5 : 1) in 94% yield by the action of trichloroacetonitrile in the presence of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) as base. Glycosylation of Le^x acceptor **55** with Le^x donor **57** (Scheme 8) in an inverted fashion under promotion by boron trifluoride etherate (1 : 1 dichloromethane–hexane, −25 °C, 2 h) afforded the hexasaccharide derivative **58** in 81% yield. The position of the nonreducing Le^x determinant at O-3 of the acceptor Gal residue was demonstrated by NMR spectroscopy following conversion of a small amount of **58** into a 4-trichloroacetylcarbamoyl derivative



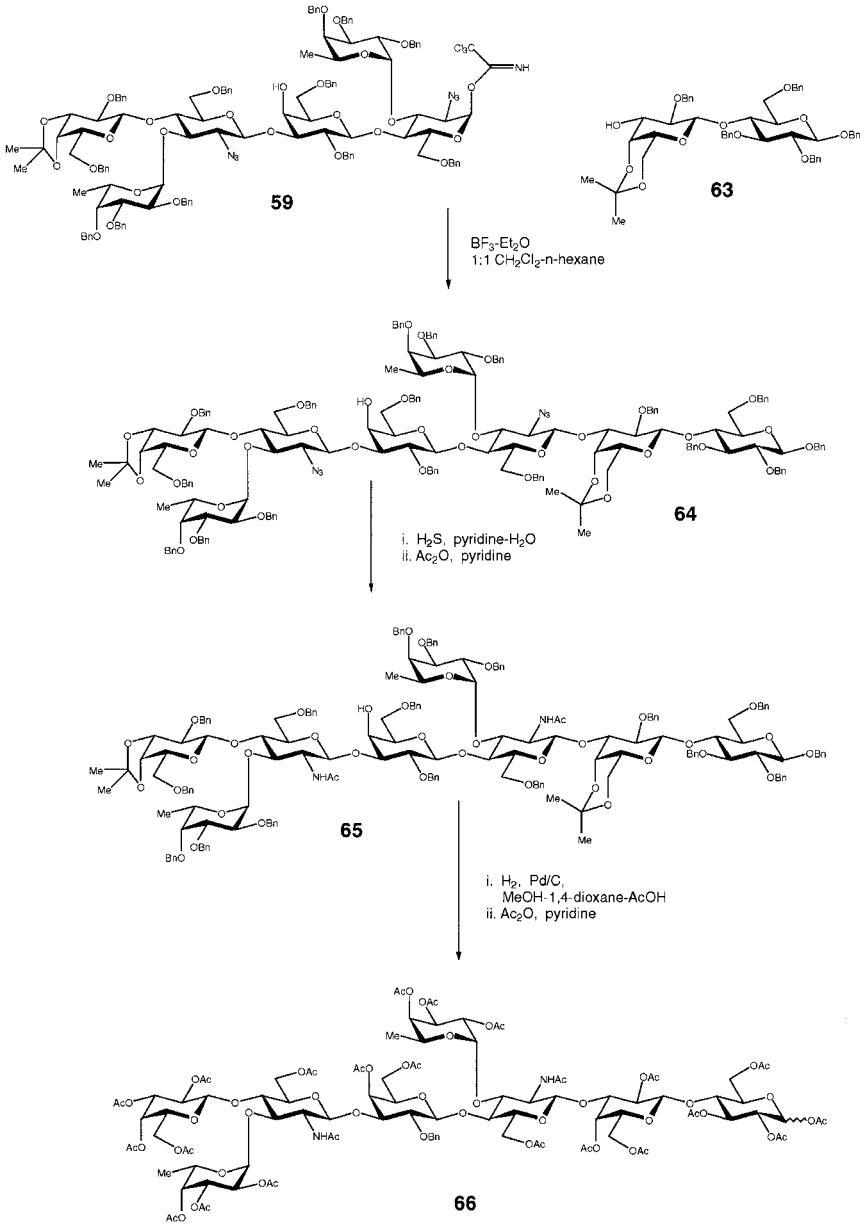
SCHEME 8



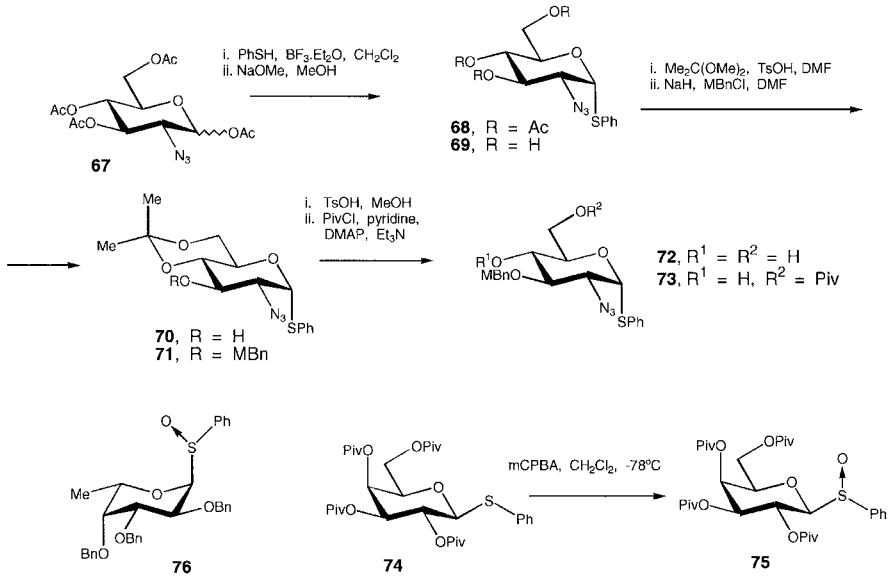
SCHEME 9

by reaction with trichloroacetyl isocyanate.[302] Intermediate **58** was in turn treated with tetrabutylammonium fluoride to afford an anomeric mixture of reducing hexasaccharide derivatives ($\alpha : \beta$, 2 : 3) which reacted with trichloroacetonitrile in the presence of DBU to afford the α-trichloroacetimidate donor **59** in 73% yield. Disaccharide acceptor **63,** required to assemble the octasaccharide target compound **66,** was derived from the lactose derivative **60** as follows[297] (Scheme 9). Treatment with 2,2-dimethoxypropane in dry DMF in the presence of *p*-toluenesulfonic acid gave the 4′,6′,-*O*-isopropylidene derivative **61** in 69% yield. Compound **61** was per-O-benzylated (benzyl bromide, sodium hydride in DMF) to afford **62** (81%). Removal of the allyl ether protecting group of **62** was effected[302] by sequential allyl rearrangement in the presence of Wilkinson's catalyst [tris(triphenylphosphine)rhodium chloride] and DBU, and hydrolysis [mercury(II) oxide, mercury(II) chloride in 9 : 1 acetone–water] to give the lactose acceptor **63** (84%). Compound **63** was glycosylated (Scheme 10) with the hexasaccharide donor **59** to afford a 52% yield of **64** by an inverted procedure under promotion by boron trifluoride etherate in 1 : 1 dichloromethane–*n*-hexane [molecular sieves 4 Å (0.4 nm), $-25\,^{\circ}$C, 30 min]. Also obtained in the glycosylation reaction was a 33% yield of a byproduct wherein the linkage between the dimeric hexasaccharide portion and the Gal residue of the lactose portion is ($1 \rightarrow 6$) rather than ($1 \rightarrow 3$). The authors explained this finding by the rearrangement of acceptor **63** to give a 3′,4′,-*O*-isopropylidene derivative in the presence of the promoter, boron trifluoride etherate. Completing the synthesis, the azido octasaccharide derivative **64** was reduced by treatment with hydrogen sulfide in pyridine–water and the product *N*-acetylated (acetic anhydride–pyridine) to afford the diacetamido derivative **65** in 61% yield. Catalytic hydrogenation over 10% palladium-on-charcoal followed by per-O-acetylation (2 : 1 pyridine–acetic anhydride) then gave **66** as an anomeric mixture ($\alpha : \beta$, 1 : 1) in 73% yield. Compound **66** is suitable for processing into a ceramide derivative[329] by way of selective 1-O-deacetylation[304] (hydrazinium acetate in *N*,*N*-dimethylformamide) and conversion into a trichloroacetimidate donor.

(iii) ***The Lewis^x Determinant.*****—**Kahne and his associates have developed a versatile glycosylation strategy that employs glycosyl sulfoxide donors under

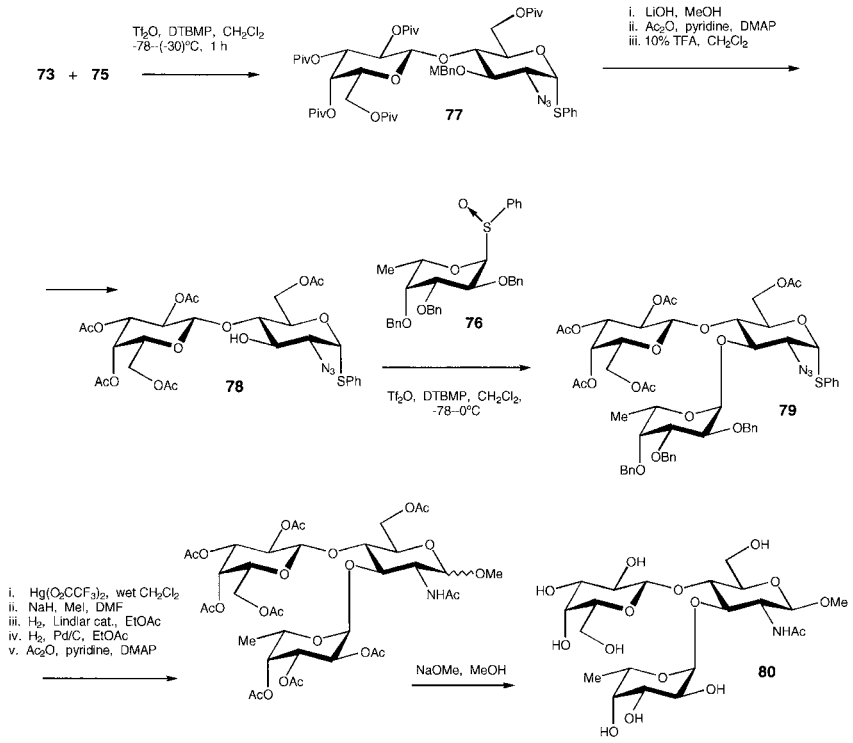

59
63
BF3-Et2O
1:1 CH2Cl2-n-hexane
64
i. H2S, pyridine-H2O
ii. Ac2O, pyridine
65
i. H2, Pd/C,
MeOH-1,4-dioxane-AcOH
ii. Ac2O, pyridine
66

SCHEME 10



SCHEME 11

promotion by triflic anhydride in the presence of 2,6-di-*tert*-butyl-4-methylpyridine as base. The sulfoxide glycosylation reaction has been applied to a multitude of target structures,[305,306] among which the Le^x determinant[307] is briefly mentioned here. The anomeric methyl glycosides of Le^x have been assembled (Scheme 11) from the building blocks **73, 75,** and **76** as precursors of the GlcNAc, Gal, and Fuc residues, respectively. Compound **73** was prepared from 1,3,4,6-tetra-*O*-acetyl-2-azido-2-deoxy-D-glucopyranose[308] (**67**) over six steps in 36% overall yield. Compound **67** was converted into the phenyl thioglycoside **68** by treatment[309] with thiophenol in dichloromethane under catalysis by boron trifluoride etherate. Next, the acetate groups of **68** were removed (sodium methoxide in methanol) to afford **69,** which was treated with acetone dimethyl acetal in *N*,*N*-dimethylformamide in the presence of *p*-toluenesulfonic acid to give the isopropylidene acetal **70.** Compound **70** upon treatment with sodium hydride and *p*-methoxybenzyl chloride in DMF gave the ether **71;** from **71,** the diol **72** was prepared by reacting with *p*-toluenesulfonic acid in methanol. Finally, diol **72** was converted into the 6-*O*-pivaloyl derivative **73** by treatment with pivaloyl chloride in dichloromethane in the presence of 4-dimethylaminopyridine (DMAP) and triethylamine. The Gal precursor **74** was prepared from phenyl 1-thio-β-D-galactopyranoside[309] (pivaloyl chloride, DMAP in pyridine, 24 h reflux) and was converted into the sulfoxide **75** by treatment with *m*-chloroperoxybenzoic acid (*m*CPBA) in dichloromethane at $-78\,^{\circ}C$. Fucose building block **76** was prepared

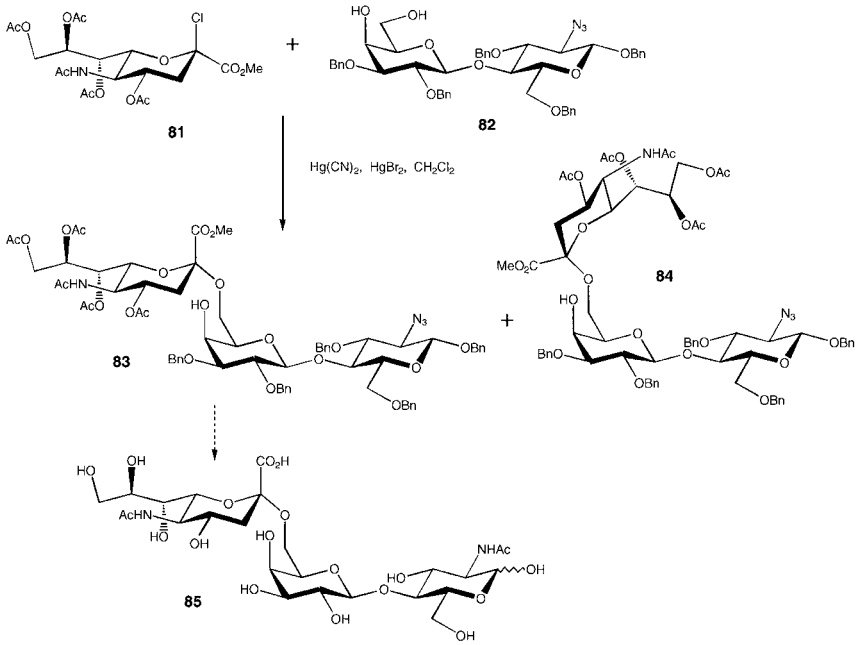


SCHEME 12

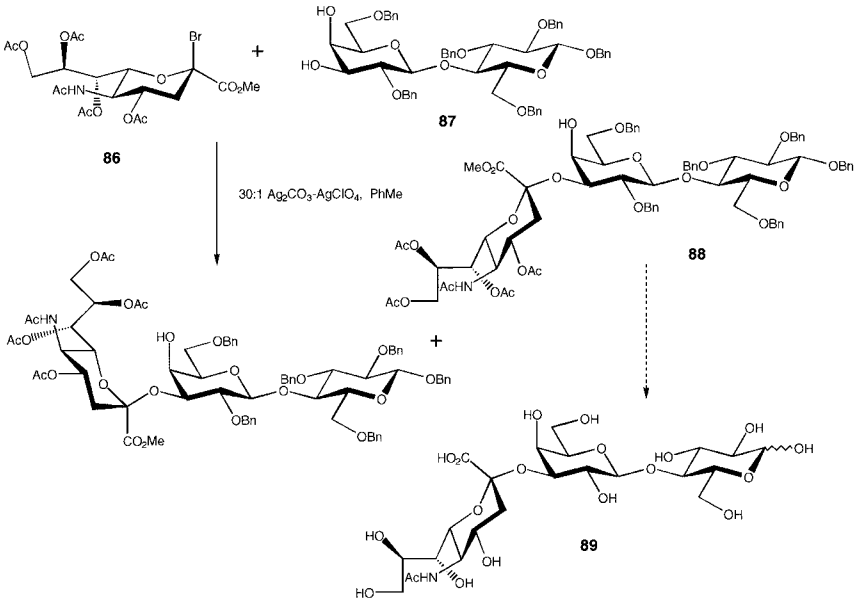
from phenyl 1-thio-L-fucopyranoside[309] by sequential O-benzylation (benzyl chloride, sodium hydride, tetrabutylammonium iodide in DMF, 60 °C, 24 h) and oxidation (*m*CPBA in dichloromethane, −78 °C, 15 min). The GlcNAc acceptor **73** was glycosylated (Scheme 12) in 65% yield with Gal donor **75** under promotion by triflic anhydride in the presence of 2,6-di-*tert*-butyl-4-methylpyridine (DTBMP) in dichloromethane at $-78 \rightarrow -30$ °C, for 1 h. Conversion of the LacNAc derivative **77** into the acceptor **78** was performed by sequential saponification with lithium hydroxide in methanol, O-acetylation (acetic anhydride, pyridine, DMAP), and removal of the *p*-methoxybenzyl ether protecting group by the action of 10% trifluoroacetic acid in dichloromethane (76% from **77**). The LacNAc acceptor **78** was glycosylated with the fucosyl donor **76** to afford the trisaccharide derivative **79** in 83% yield (triflic anhydride, DTBMP, dichloromethane, $-78 \rightarrow 0$ °C, 1.5 h). Thioglycoside **79** may be oxidatively activated to provide a sulfoxide donor of the Le^x determinant. In the present example, the phenyl thio glycoside was hydrolyzed under catalysis by mercury(II) trifluoroacetate (77%) and the resulting anomeric mixture of hemiacetals O-methylated (methyl iodide, sodium

hydride, DMF, room temperature, 1 h; 92%). From the mixture of the methyl α- and β-glycosides, the target structure **80** was prepared as follows. Catalytic hydrogenation over Lindlar catalyst in ethyl acetate provided the acetamido derivative. Subsequently, the benzyl ether protecting groups were cleaved by catalytic hydrogenation over palladium-on-charcoal. The anomeric mixture was then O-acetylated (acetic anhydride, dimethylaminopyridine, triethylamine in dichloromethane) and chromatographed over silica gel (100% ethyl acetate). Finally, the preponderant β-product was O deacetylated by the action of sodium methoxide in methanol to afford the target compound **80** (23% from **79**). In Ref. 307, Kahne cites many previous, alternative syntheses of the Lewis determinants.

b. Sialosyllactosamine Derivatives.—Organic chemical syntheses of sialyloligosaccharides from Neu5Ac halide donors[310] (for example, **81**) by classical, Koenigs–Knorr-type procedures[311] have been attended by low to moderate yields, poor stereoselectivity, and loss of donor due to elimination of hydrogen halide to form Neu5Ac glycal derivatives,[312,313] Paulsen and his associates,[314,315] in the early 1980s, have reported optimized Koenigs–Knorr protocols for linking Neu5Ac residues to position 6′ of *N*-acetyllactosamine [LacNAc, β-D-Gal-(1 $\rightarrow$ 4)-D-GlcNAc] and position 3′ of lactose [β-D-Gal-(1 $\rightarrow$ 4)-D-Glc]. Compound **82** is a suitable acceptor for the preparation of 6′-linked Neu5Ac derivatives and was prepared from hexa-*O*-acetyl-lactal[299] **47** by way of the azidonitration reaction[308] of Lemieux and Ratcliffe.[316] The azido bromide initially obtained was converted into the benzyl β-glycoside which was O deacetylated by the action of sodium methoxide in methanol. The intermediate azido lactose derivative was then converted into acceptor **82** by sequential 4′,6′-benzylidenation, per-O-benzylation, and hydrolysis of the benzylidene acetal. Glycosylation of **82** (Scheme 13) with the Neu5Ac chloride donor[310] **81** [3 : 1 mercury(II) cyanide–mercury(II) bromide, dichloromethane, 20 °C] afforded an anomeric mixture of the α- and β-(2 $\rightarrow$ 6)-linked trisaccharide derivatives **83** and **84,** in 50% yield. Following chromatographic separation, the α anomer **83** is obtained in 22% yield. Removal of the protecting groups from **83** to afford the target structure **84** was performed by sequential treatment with hydrogen sulfide, N-acetylation, sodium methoxide–catalyzed methanolysis, alkaline saponification (to cleave the methyl ester), and catalytic hydrogenation over palladium-on-charcoal. Similarly, the sialyllactose derivative **89** was prepared by Paulsen and von Deessen[315] (Scheme 14) from the Neu5Ac bromide donor **86** and the lactose acceptor **87.** Donor **86** was prepared[317,318] from the Neu5Ac pentaacetyl methyl ester by treatment with titanium tetrabromide. Acceptor **87** was obtained from lactose by way of the benzyl β glycoside, 3′,4′-O-isopropylidenation, per-O-benzylation, and hydrolysis of the isopropylidene acetal. Glycosylation of **87** with donor **86** (30 : 1 silver carbonate–silver perchlorate, toluene, $-5 \rightarrow +5$ °C) afforded a 36% yield of a 1 : 1 mixture of anomers. Following chromatographic separation, a $\sim$16% yield of the α anomer **88** was obtained.



SCHEME 13

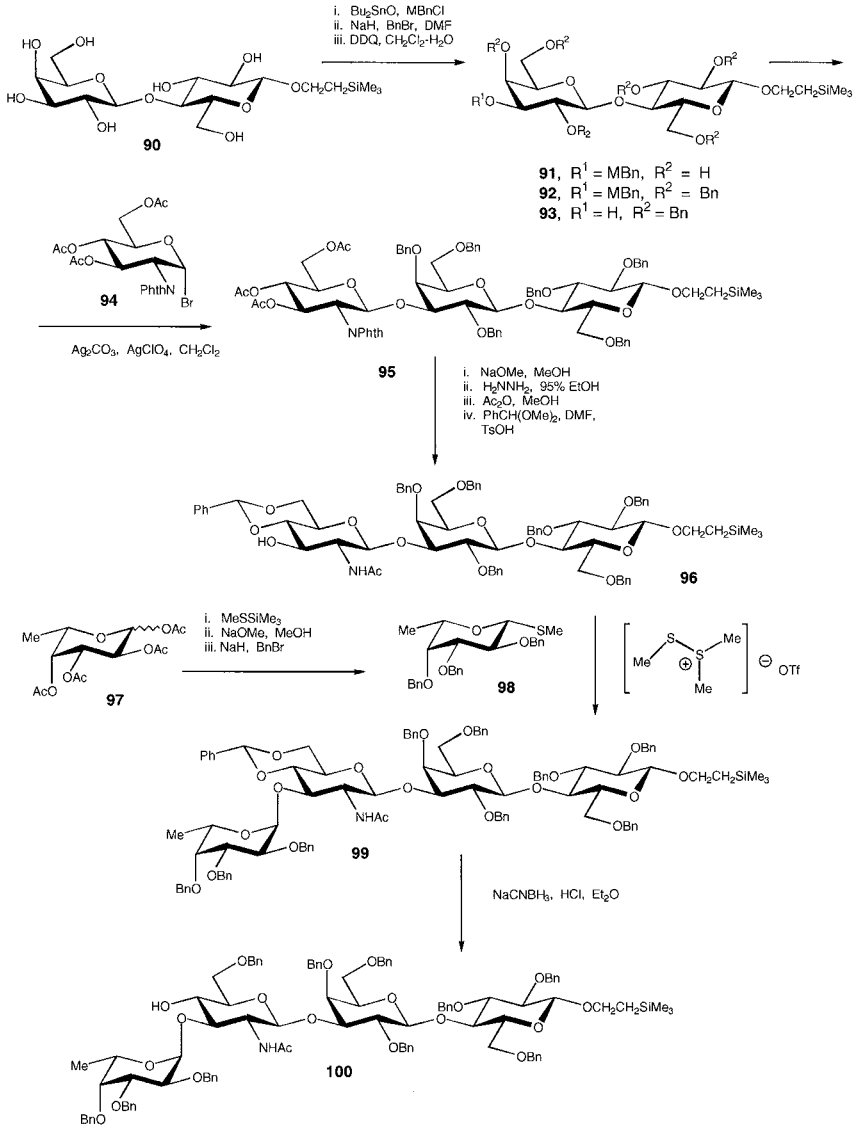


SCHEME 14

2. Syntheses of sLe^x and Related Determinants Based on Organic-Chemical Glycosylations

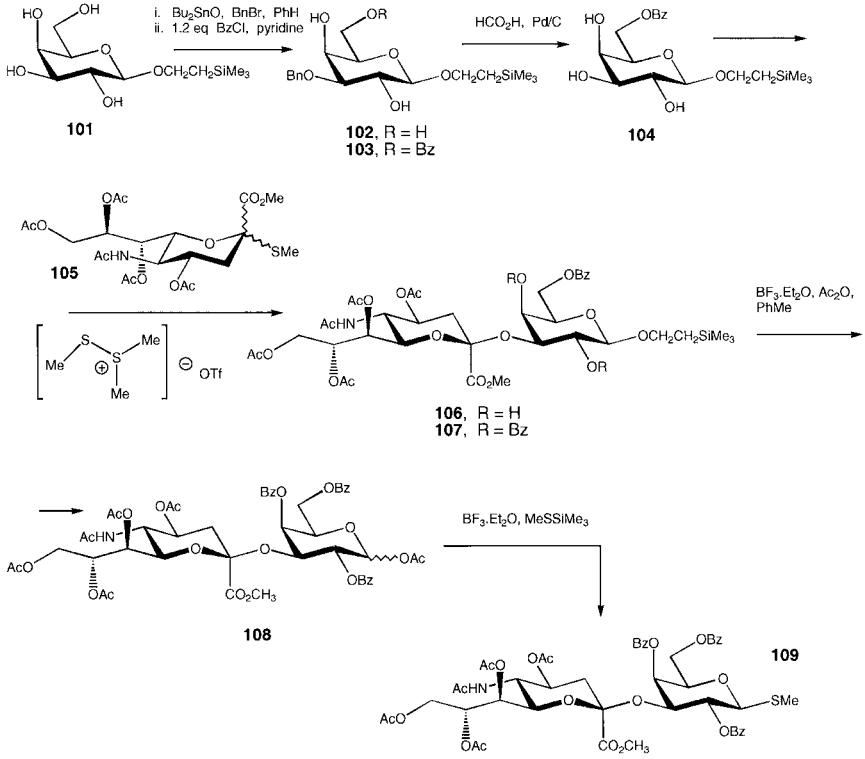
a. Synthesis of a sLe^x Hexasaccharide Ceramide Derivative.—The earliest example of the total synthesis of the sLe^x determinant was provided by Hasegawa and Kiso[320] in their purely organic-chemical synthesis of the hexasaccharide ganglioside derivative α-D-Neu5Ac-(2 → 3)-β-D-Gal-(1 → 4)-[α-L-Fuc-(1 → 3)]-β-D-GlcNAc-(1 → 3)-β-D-Gal-(1 → 4)-β-D-Glc-1,1-ceramide. This work is an extension of the authors' previously reported total syntheses of sialyl lactotetraosylceramide α-D-Neu5Ac-(2 → 3)-β-D-Gal-(1 → 3)-β-D-GlcNAc-(1 → 3)-β-D-Gal-(1 → 4)-β-D-Glc-1,1-ceramide and sialyl neolacto-tetraosylceramide[320] α-D-Neu5Ac-(2 → 3)-β-D-Gal-(1 → 4)-β-D-GlcNAc-(1 → 3)-β-D-Gal-(1 → 4)-β-D-Glc-1,1-ceramide. The backbone pentasaccharide of the neolacto ganglioside corresponds to that of the sLe^x target compound **114;** the task at hand was therefore to modify the earlier synthesis[320] by introducing the O-3″-linked α-fucosyl branch at a suitable stage.

For the assembly of the unbranched ganglioside glycans,[320] Hasegawa and Kiso had adopted a strategy based on block synthons, as follows. Extension of a lactose acceptor block β-D-Gal-(1 → 4)-D-Glc by glycosylation with a GlcNAc donor would afford a trisaccharide acceptor block β-D-GlcNAc-(1 → 3)-β-D-Gal-(1 → 4)-D-Glc. This would be glycosylated with a donor block α-D-Neu5Ac-(2 → 3)-D-Gal to give the desired pentasaccharide derivative (2 + 1+ 2). For the elaboration of the sLe^x structure, the trisaccharide synthon would need to be fucosylated at O-3 of GlcNAc prior to attachment of the α-D-Neu5Ac-(2 → 3)-Gal block. The (trimethylsilyl)ethyl β glycoside of lactose[321,322] **90** (Scheme 15) was selectively converted into the 3′-*p*-methoxybenzyl ether **91** (74%) by treatment with dibutyltin oxide and reaction of the intermediate stannylidene derivative with *p*-methoxybenzyl chloride in the presence of tetrabutylammonium bromide. Compound **91** was per-O-benzylated by the action of sodium hydride and benzyl bromide in *N*,*N*-dimethylformamide to give the fully protected lactose derivative **92** in 79% yield. The acceptor **93** was obtained from **92** in 70% yield upon treatment with 2,3-dichloro-5,6-dicyanobenzoquinone[323] in dichloromethane–water at room temperature. Glycosylation of **93** was performed with the donor 3,4,6-tri-*O*-acetyl-2-deoxy-2-phthalimido-D-glucopyranosyl bromide[324] (**94**) in dichloromethane under promotion by silver carbonate and silver perchlorate to afford the β-D-(1 → 3)-linked trisaccharide derivative **95** in 94% yield. The substituents at the nonreducing GlcNAc residue of **95** were exchanged to provide the *N*-acetyl-4″,6″-*O*-benzylidene acceptor **96** (74% from **95**) by sequential treatment with sodium methoxide in methanol, N-deacylation by the action of hydrazine in 95% ethanol, N-acetylation, and treatment with dimethoxytoluene in *N*,*N*-dimethyl-formamide in the presence of *p*-toluenesulfonic acid. Acceptor **96** was O-fucosylated through the use of the benzylated thiofucoside donor **98** under promotion by dimethyl(methylthio)sulfonium trifluoromethylsulfonate[325,326]



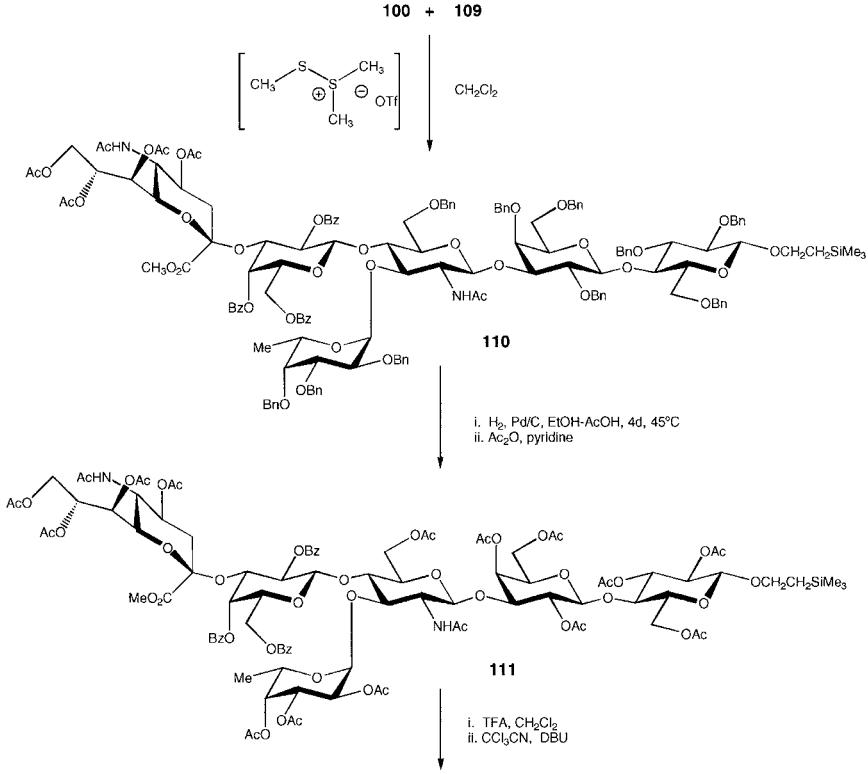
SCHEME 15

(DMTST) to give the tetrasaccharide derivative **99** in 86% yield. Donor **98** was prepared from L-fucose tetraacetate (**97**) by sequential treatments with methylthiotrimethylsilane and sodium methoxide in methanol, followed by O-benzylation by the action of sodium hydride–benzyl bromide. Compound **99** was converted into the glycosyl acceptor **100** (75%) by reductive opening of the benzylidene ring



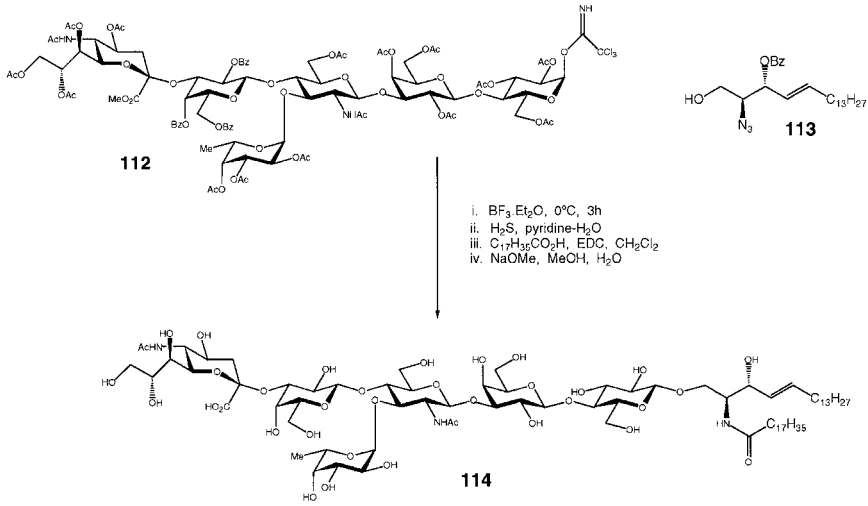
SCHEME 16

by the action of sodium cyanoborohydride–hydrogen chloride in dry ether.[327] The disaccharide block synthon corresponding to the segment α-D-Neu5Ac-(2 $\rightarrow$ 3)-D-Gal[328] was prepared (Scheme 16) from the known[321] (trimethylsilyl)ethyl β-D-galactopyranoside **101.** Regioselective O-benzylation of the corresponding 3,4-*O*-stannylidene acetal with benzyl bromide in benzene afforded the 3-benzyl ether **102** in 77% yield. Compound **102** was treated with benzoyl chloride (1.2 eq.) in pyridine–dichloromethane to give the 6-*O*-benzoyl derivative **103** in 71% yield. The desired glycosyl acceptor **104** was obtained from **103** by hydrogen-transfer reduction over palladium-on-carbon in methanol with the use of formic acid as the hydrogen donor (67%). The Gal triol acceptor **104** was regioselectively 3-O-sialylated by the action of the Neu5Ac thioglycoside donor **105** under promotion by DMTST in 43% yield. The disaccharide derivative **106** was per-O-benzoylated (benzoyl chloride in pyridine) and the resulting compound **107** subjected to acetolysis (boron trifluoride etherate in toluene–acetic anhydride) to afford the glycosyl acetate **108** in high yield. Treatment of **108** with methylthiotrimethylsilane and



SCHEME 17

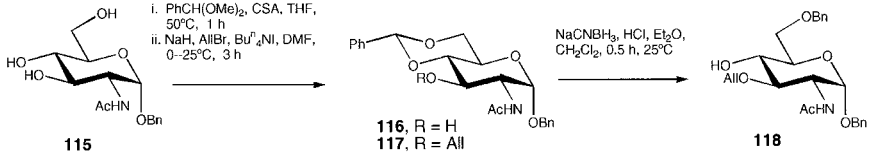
boron trifluoride etherate in dichloromethane provided the desired thioglycoside block donor **109** in 82% yield. The hexasaccharide intermediate **110** was assembled (Scheme 17) by glycosylation of the tetrasaccharide acceptor **100** with the disaccharide thioglycoside donor **109** under promotion by DMTST in dichloromethane in 41% yield. Presumably, the moderate yield of this glycosylation step reflects the low reactivity of OH-4 of GlcNAc in the presence of a 3-linked fucose residue. (compare Section IV.2.f). The hexasaccharide ceramide target structure **114** was finally elaborated from **110** following essentially the strategy developed for syntheses of gangliosides by Schmidt and his associates.[329] Removal of the *O*-benzyl protecting groups was effected by catalytic hydrogenation over palladium-on-carbon in 3 : 1 ethanol–acetic acid (4 d, 45 °C) and was followed by per-O-acetylation (acetic anhydride, pyridine) to afford compound **111** in 81% yield. Treatment of **111** with trifluoroacetic acid in dichloromethane gave an intermediate glucopyranose derivative (94%) which reacted with trichloroacetonitrile in the presence of 1,8-diazabicyclo[5.4.0]undec-7-ene



SCHEME 18

(DBU) to provide a 91% yield of the hexasaccharide-α-trichloroacetimidate donor **112** (Scheme 18). The sphingosine precursor (2*S*,3*R*,4*E*)-2-azido-3-*O*-benzoyl-4-octadecene-1,3-diol (**113**) was glycosylated with donor **112** under promotion by boron trifluoride etherate in dichloromethane in 56% yield. The azido derivative obtained was selectively reduced by the action of hydrogen sulfide in aqueous pyridine to give the corresponding amino compound, which was acylated with the adduct formed from 1-(3-dimethylaminopropyl)-3-ethyl-carbodiimide (EDC) and octadecanoic acid to afford a protected ceramide derivative (81%). From this intermediate, the target compound **114** was obtained in quantitative yield by treatment of with sodium methylate in methanol, followed by addition of water and chromatography on Sephadex-LH 20.

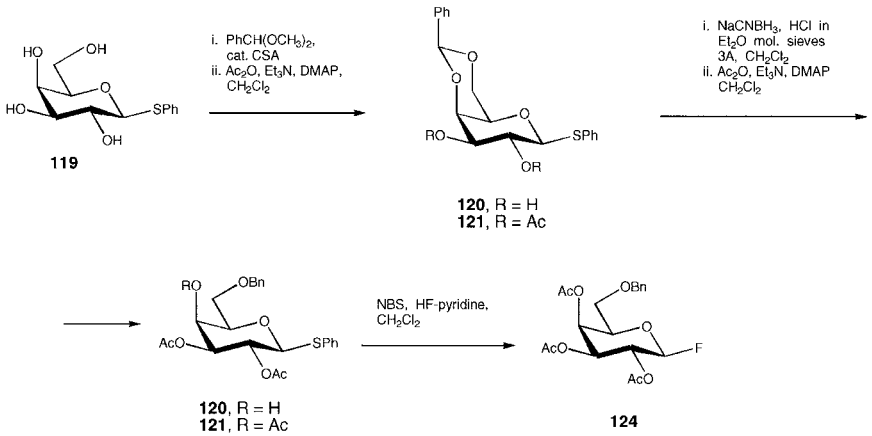
b. Synthesis of a Reducing sLe^x Tetrasaccharide by a Stepwise Protocol.— Nicolaou and his associates[330] synthesized the sLe^x determinant in the form of the reducing tetrasaccharide **136.** Compounds **118, 124, 130,** and **127** served as the monosaccharide precursors of the GlcNAc, Gal, Fuc, and Neu5Ac residues. The GlcNAc precursor **118** was prepared from *N*-acetylglucosamine by the following series of steps in an overall yield of 34% (Scheme 19). Fischer glycosylation of GlcNAc (benzyl alcohol saturated with hydrogen chloride, 100 °C, 1 h) afforded the benzyl α-pyranoside **115,** which was converted into the 4,6-*O*-benzylidene derivative **116** by the action of benzaldehyde dimethyl acetal under catalysis by camphorsulfonic acid in tetrahydrofuran. Compound **116** was treated with sodium hydride and allyl bromide in the presence of a small amount of tetra-*N*-butyl



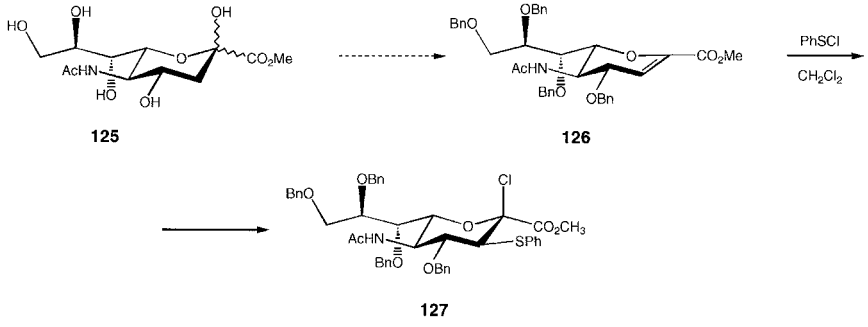
SCHEME 19

iodide in *N*,*N*-dimethylformamide to give the 3-allyl ether **117.** The fully protected derivative **117** was converted into the glycosyl acceptor **118** by treatment with sodium cyanoborohydride in the presence of a saturated solution of hydrogen chloride in ether.

The Gal fluoride donor **124** (Scheme 20) was synthesized from the known[309] phenyl thio-β-glycoside **119.** Treatment with benzaldehyde dimethyl acetal in the presence of camphorsulfonic acid in tetrahydrofuran gave the benzylidene derivative **120,** which was converted into the diacetate **121** by the action of acetic anhydride in dichloromethane with triethylamine–4-dimethylaminopyridine as base. Compound **121** was subjected to reductive opening of the benzylidene ring [10 eq. of sodium cyanoborohydride, ethereal hydrogen chloride, molecular sieves 3 Å (0.3 nm) in tetrahydrofuran] to afford the 6-benzyl ether **122.** Another acetylation step analogous to the foregoing 2,3-O-acetylation gave **123,** which was treated with an excess of a solution of hydrogen fluoride in pyridine and 1.5 eq. of *N*-bromosuccinimide in dichloromethane to afford the Gal donor **124** in 37% overall yield from **119.** The Neu5Ac precursor **127** was prepared[330] (Scheme 21) by addition of phenylsulfenyl chloride to the benzylated methyl ester glycal derivative **126** that had been obtained in 32% overall yield from the methyl ester of Neu5Ac **125.** Applications of Neu5Ac derivatives containing the



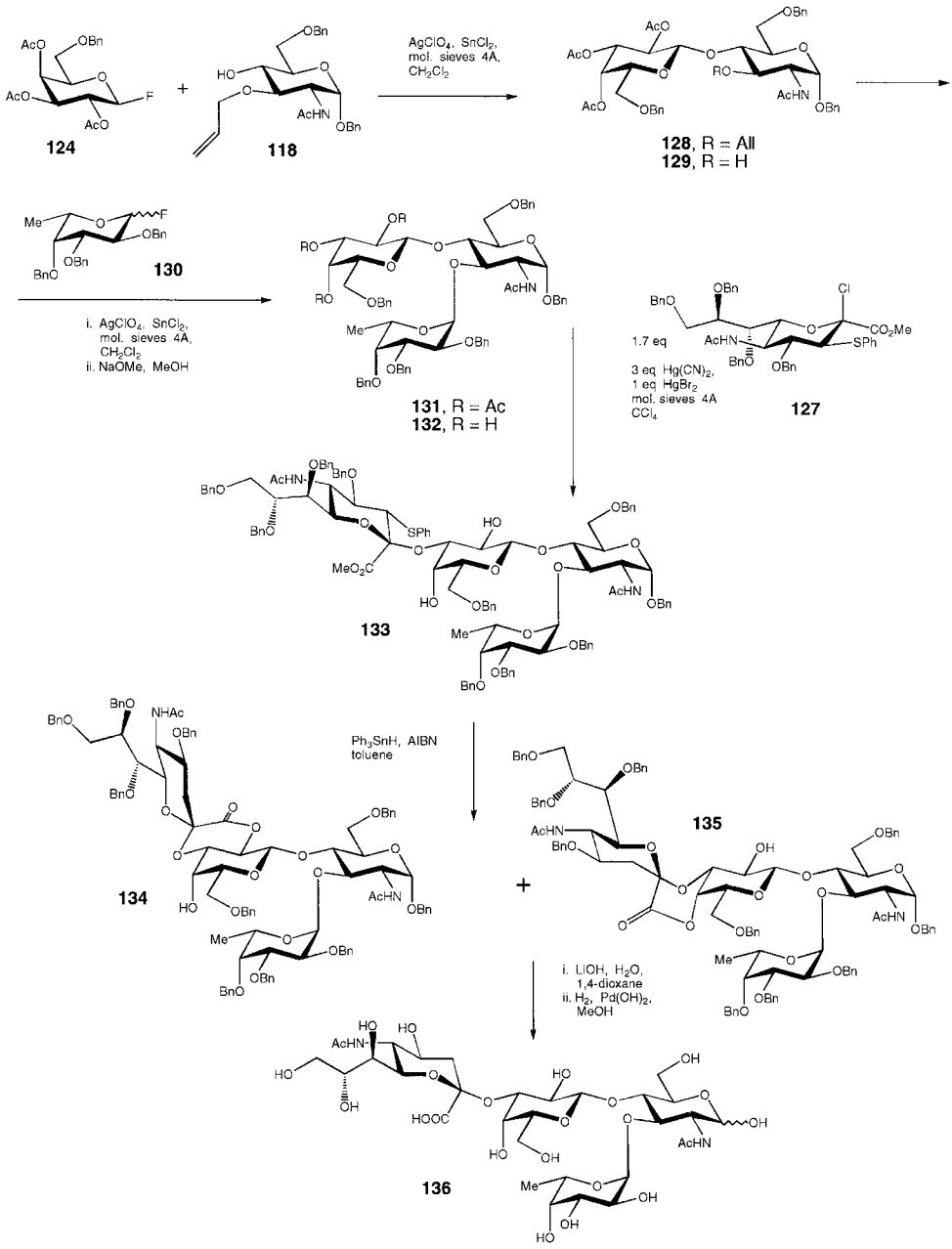
SCHEME 20



SCHEME 21

3-phenylthio or 3-phenylseleno groups for stereoselective syntheses of sialoconjugates have been developed by Ogawa and his associates[332,333] and by Okamoto *et al.*[334]

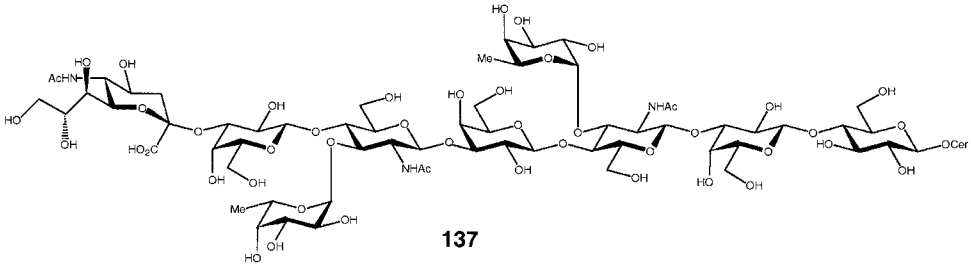
In a step-by-step assembly of the precursors (Scheme 22), the GlcNAc acceptor **118** was first glycosylated with the Gal fluoride donor **124** under a Mukaiyama protocol to produce the *N*-acetyllactosamine derivative **128** in 63% yield [2.5 eq. each of silver perchlorate and stannous chloride, molecular sieves 4 Å (0.4 nm) dichloromethane, 0 °C, 4 h]. Conversion of the allyl ether **128** into the alcohol **129** was effected in 85% yield by sequential treatment with catalytic amounts of tetrakis(triphenylphosphine)ruthenium hydride in ethanol (95 °C) and *p*-toluene sulfonic acid in methanol. Acceptor **129** was next glycosylated with the fucosyl fluoride donor **130** under a Mukaiyama protocol analogous to the step forming the LacNAc derivative **128,** to give **131** in 85% yield. Treatment of the fully protected Le^x derivative **131** with sodium methoxide in methanol afforded the triol **132** in 95% yield. Galactosides with the OH- groups at C-2,-3, and -4 unprotected will function as glycosyl acceptors regioselective for the (1 → 3)-Gal linkage. Sialylation of **132** with the phenylthio chloride donor **127** under promotion by mercuric cyanide–mercuric bromide in carbon tetrachloride afforded the tetrasaccharide derivative **133** in 63% yield based on acceptor **132** consumed. For reductive removal of the auxiliary thioether group, **133** was treated with 5 eq. of triphenyltin hydride in toluene under catalysis by azobis-isobutyronitrile at 130 °C to give a mixture of the deoxynonulopyranosylonic 1′′,2′- and 1′′,4′- interglycosidic lactones **134** and **135** in 77% yield. Under the conditions of these free-radical-catalyzed reductions, minor portions of phenylthio ethers usually remain unchanged; they are chromatographically separated from the desired products and added to subsequent batches for reductive cleavage.[332] The lactones **134** and **135** were subjected to alkali-catalyzed hydrolysis (lithium hydroxide in 1,4-dioxane–water) to give the corresponding carboxylic acid (or its salt) in quantitative yield. Finally, the


124
118
AgClO4, SnCl2, mol. sieves 4A, CH2Cl2
128, R = All
129, R = H
130
i. AgClO4, SnCl2, mol. sieves 4A, CH2Cl2
ii. NaOMe, MeOH
131, R = Ac
132, R = H
1.7 eq
3 eq Hg(CN)2, 1 eq HgBr2, mol. sieves 4A CCl4
127
133
Ph3SnH, AIBN toluene
134
135
i. LiOH, H2O, 1,4-dioxane
ii. H2, Pd(OH)2, MeOH
136

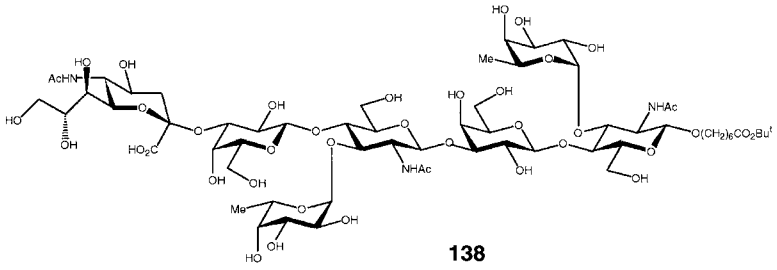
SCHEME 22

persistent benzyl ether protecting groups were removed by catalytic hydrogenation over palladium hydroxide (Pearlman's catalyst) in methanol to give the target compound **136** in 95% yield.

c. Syntheses of Sialyl-di-Le^x Glycans.—The nonaose **137** has been identified as the glycan of a tumor-associated glycolipid by Hakomori and his associates.[335] The glycolipid corresponding to **137** accumulates in human colonic adenocarcinomas. Glycan **137** corresponds to lacto-*N*-fucopentaose, β-D-Gal-(1 $\rightarrow$ 4)-[α-L-Fuc-(1 $\rightarrow$ 3)]-β-D-GlcNAc-(1 $\rightarrow$ 3)-β-D-Gal-(1 $\rightarrow$ 4)-D-Glc, with a sLe^x tetrasaccharide linked β-(1 $\rightarrow$ 3) to the nonreducing Gal unit.

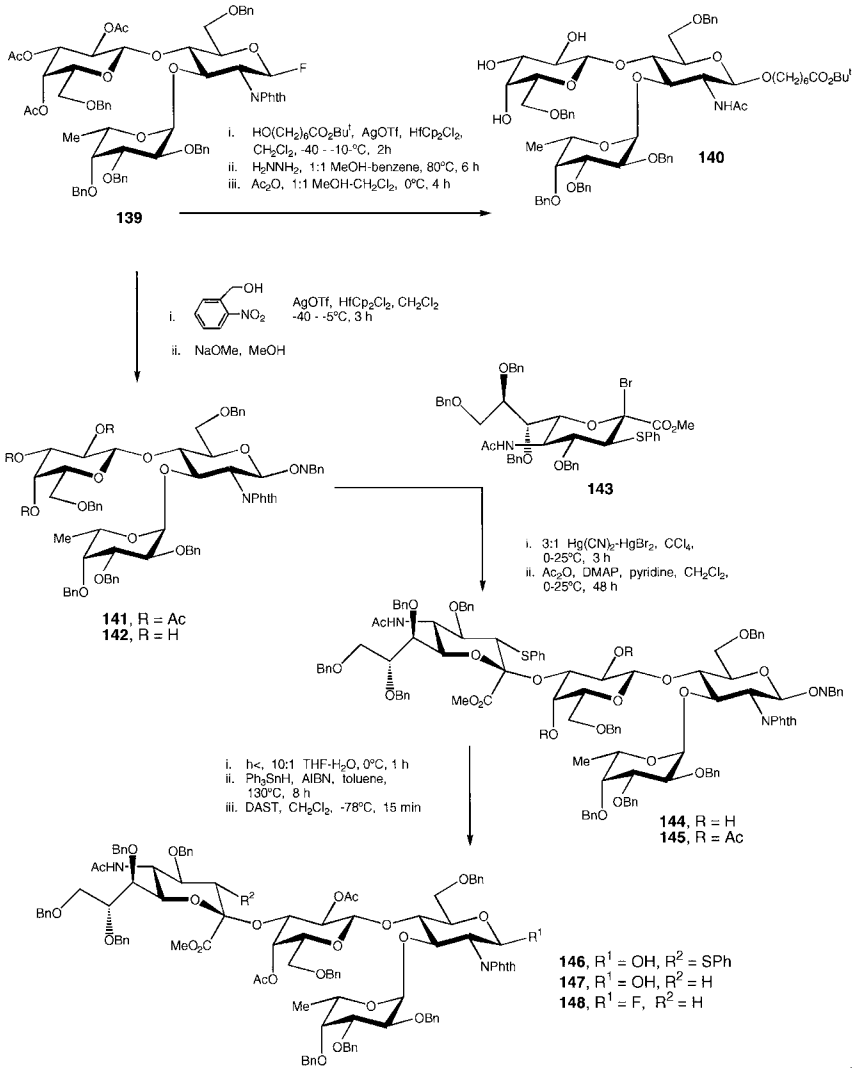


137



138

(i) ***Synthesis of a Sialyl-di-Le^x Heptasaccharide Derivative.—***A model compound corresponding to sialyl-di-Le^x, the nonreducing heptasaccharide segment of glycan **137,** was synthesized by Nicolaou and his co-workers.[336] From a retrosynthetic point of view, the (sialylated di-Le^x) target structure **138** was viewed as a Le^x trisaccharide glycosylated at position 3 of Gal with a sLe^x tetrasaccharide determinant. For its synthesis. Nicolaou ***et al.*** employed a convergent strategy based on a common Le^x trisaccharide precursor, **139.** On the one hand, **139** was used to elaborate a spacer-linked, Le^x trisaccharide derivative **140,** the glycosyl acceptor corresponding to the reducing trisaccharide portion of **138.** On the other hand, **139** was converted into the sLe^x tetrasaccharide donor **148,** the precursor of the

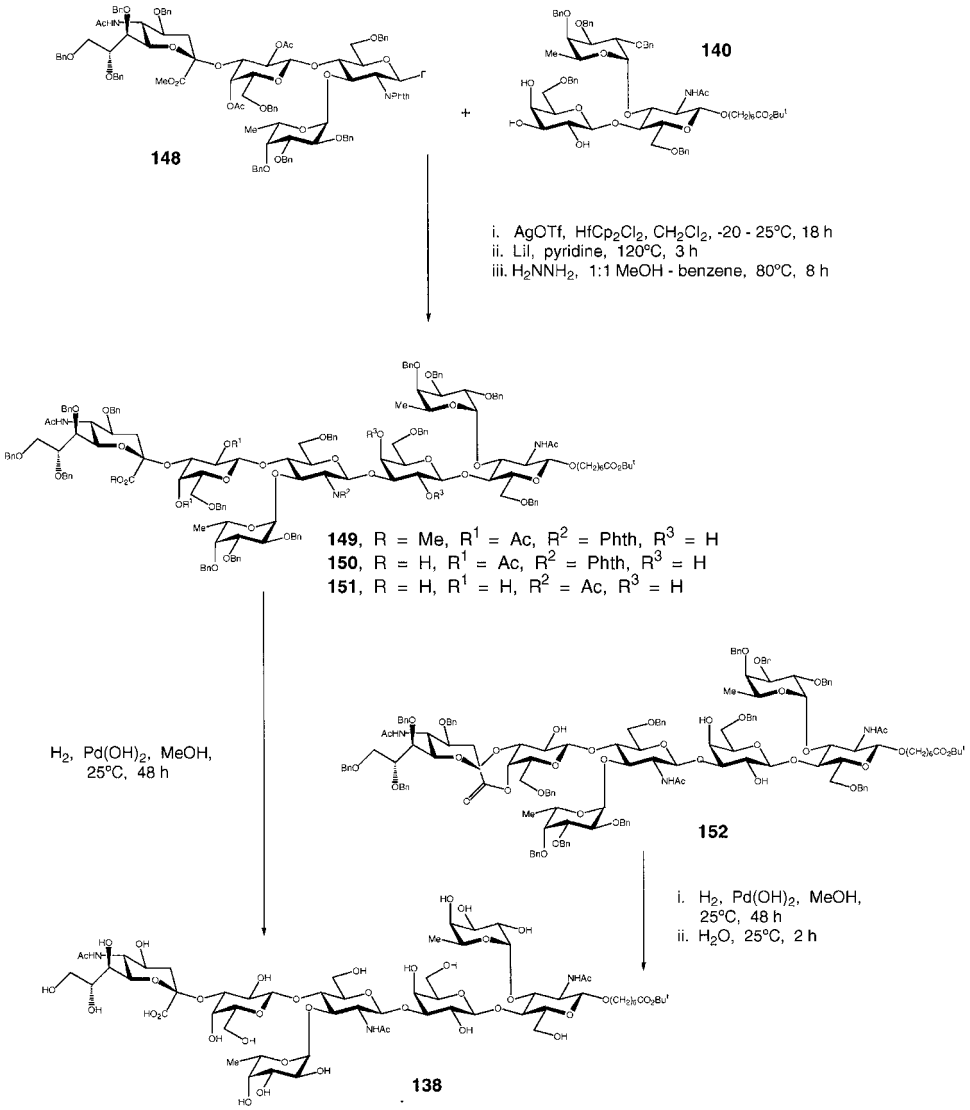


SCHEME 23

nonreducing tetrasaccharide segment of **138.** The phthalimido trisaccharide fluoride donor **139** (Scheme 23) was treated with *tert*-butyl 7-hydroxyheptanoate under Mukaiyama–Suzuki conditions[355,356] to give a spacer-linked Le^x derivative in 87% yield [silver trifluoromethanesulfonate, bis(cyclopentadienyl) hafnium dichloride, and 4 Å (0. 4 nm) molecular sieves in dichloromethane]. Conversion of this adduct into the glycosyl acceptor **140** was effected by hydrazinolytic cleavage of the

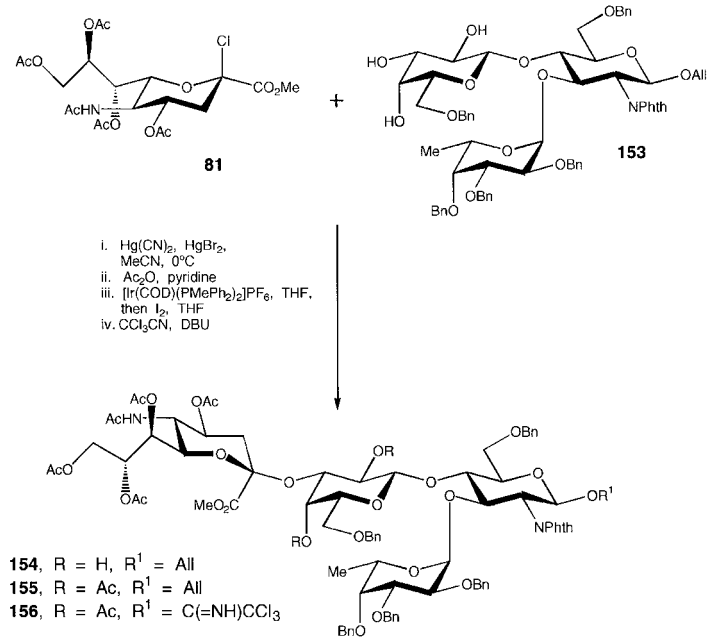
phthalimido and acetate groups, and *N*-acetylation (excess acetic anhydride in 1 : 1 methanol–benzene 83% over two steps). For the synthesis of the sLe^x tetrasaccharide donor **148,** fluoride **139** was converted into the 2-nitrobenzyl glycoside **141** for temporary protection at the anomeric carbon atom of the GlcN residue (Mukaiyama–Suzuki conditions similar to those used for the preparation of the spacer-linked trisaccharide derivative just described, 94% yield). Methanolysis by the action of sodium methoxide in methanol afforded the Le^x acceptor **142** in 98% yield. Sialylation with the benzyl-protected, 2-(phenyl)thio donor **143** occurs regiospecifically at O-3 of Gal to give the sLe^x derivative **144** in 56% yield. The 3-(phenyl)thio substituent of donor **143** is an auxiliary group that directs the incoming acceptor hydroxyl function to the α-face of the anomeric center.[331–333] The sLe^x derivative **144** was *O*-acetylated at C-2 and C-4 of the Gal unit (86%), and the fully protected tetrasaccharide derivative **145** was irradiated in tetrahydrofuran–water (1 h) to remove the temporary anomeric protecting group (82% **146,** 10% starting material recovered). Subsequently, the tetrasaccharide glycose was treated with triphenyltin hydride (azobis-isobutyronitrile, 130 °C, 10 h) to effect the reductive cleavage of the thioether group (**147,** 42% plus 45% recovered starting material). Finally, the reducing sLe^x tetrasaccharide derivative **147** was treated with diethylaminosulfur trifluoride (DAST) in dichloromethane at −78 °C to give the sLe^x fluoride donor **148** in 78% yield. Coupling of **148** with the Le^x acceptor **140** under Mukaiyama–Suzuki conditions[355,356] (Scheme 24) proceeded regioselectively to afford the β-(1 → 3)-linked heptasaccharide diol **149** in 51% yield. The methyl ester group of **149** was first cleaved by the action of lithium iodide in pyridine[343] at 120 °C (80% **150** plus 17% recovered **149**). The hydrazinolysis (hydrazine hydrate in 1 : 1 methanol–benzene of the phthalimide was accompanied by cleavage of the *O*-acetyl groups. Subsequent selective N-acetylation (acetic anhydride in 1 : 1 methanol–dichloromethane gave a 75% yield of a 1 : 1 mixture of heptasaccharide derivatives **151** and **152,** one containing the free carboxylic acid function of the Neu5Ac unit, the other, an interglycosidic 6-ring lactone comprising the Neu5Ac carboxyl group and the (axial) O-4′′′′ of Gal unit d. By catalytic hydrogenation of the mixture over palladium hydroxide in methanol (48 h), all benzyl ether groups were cleaved. The resulting mixture of lactone and carboxylic acid was quantitatively converted into the desired carboxylic acid **138** by keeping it in water at room temperature.

(ii) ***Synthesis of a Nonaosyl Ceramide Derivative with a Sialyl-di-Le^x Sequence.—***The total synthesis of the nonaosyl ceramide derivative **163** was first reported in 1995 by the group of Nunomura in cooperation with Ogawa.[337] Key steps of the synthesis are the glycosylation of the lacto-*N*-fucopentaose acceptor synthon **157** with the sLe^x tetrasaccharide donor **156** (Scheme 26), and the glycosylation of a protected ceramide alcohol with the nonaose donor **162** corresponding to the glycan portion of **163.** The preparation of the lacto-*N*-fucopentaose



SCHEME 24

acceptor synthon **157** had been reported by Ogawa and his co-workers[338] in 1994. For the assembly of the sLe^x tetrasaccharide donor **156** (Scheme 25), the authors employed the Le^x trisaccharide triol **153,** obtained from the known[338] tri-*O*-acetyl derivative by the action of sodium methoxide in methanol. As the sialyl donor, Iida *et al.* employed either the classical peracetylated methyl ester chloride **81**

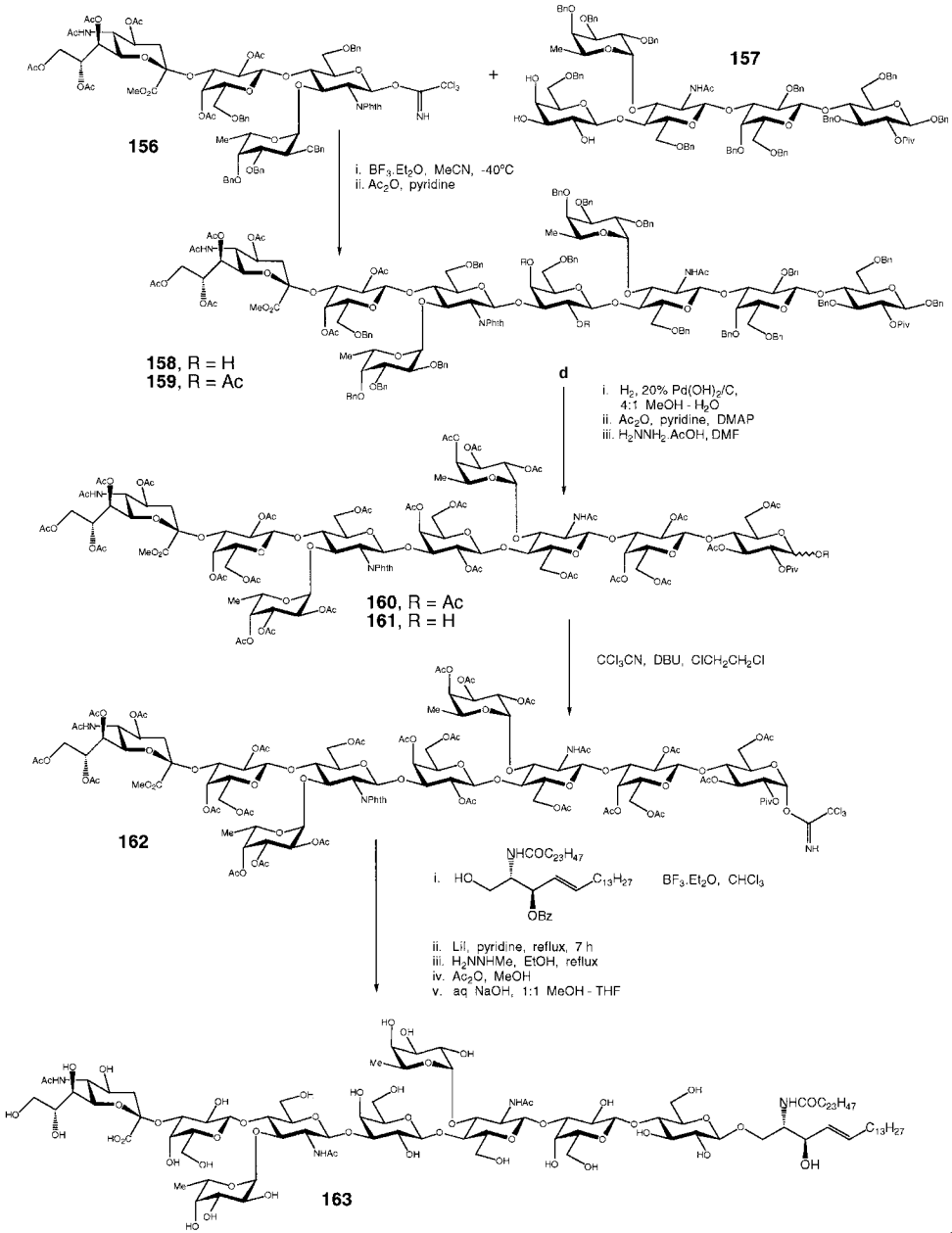


SCHEME 25

(reported by Kuhn and his associates[310] in 1966) under promotion by mercuric cyanide–mercuric bromide (44% yield) or the ketosyl diethyl phosphite first introduced by Schmidt[339] under promotion by trimethylsilyl trifluoromethylsulfonate[339] (Me_3Si triflate) (40%). Following per-*O*-acetylation of the adduct **154,** the regiospecific introduction of the sialyl unit at O-3 of Gal was confirmed from the downfield shifts observed for the ^1H NMR signals corresponding to H-2 and H-4 of the Gal unit. The α-anomeric configuration of the Neu5Ac unit followed from the chemical shift of the Neu5Ac H-4 signal, which is at relatively higher field in the α anomers and at lower fields in the β anomers presumably because of the absence or presence of an inductive deshielding effect of the axially disposed oxygen atom at C-2. The sLe^x derivative **155** was next converted into a glycosyl donor as follows. Allyl rearrangement followed by hydrolysis of the propenyl glycoside by the sequential actions of (1,5-cyclooctadiene)-bis(methyldiphenylphosphine) hexafluorophosphate[300,301] and iodine in aqueous tetrahydrofuran afforded the pyranose derivative in 84% yield. Addition of the hemiacetal to the cyano group of trichloroacetonitrile was catalyzed by 1,8-diazabicyclo[5.4.0]-undecene (DBU) and gave the sLe^x β-trichloroacetimidate donor **156** in 80% yield. The pentasaccharide glycosyl acceptor derivative **157**

corresponds to the lacto-*N*-fucopentaose structure and had been prepared previously[337] by glycosylation of a lactose acceptor block with a Le^x trisaccharide donor synthon. An important feature of pentasaccharide acceptor **157** is the 2-*O*-pivaloyl ester group at O-2 of the reducing glucose unit. Glycosyl donors containing the 2-*O*-pivaloyl in place of the acetyl group have been developed by Ogawa and his school to avoid losses of oligosaccharide donors due to formation of orthoesters during glycosylation reactions.[340,341] The crucial glycosylation of the pentasaccharide acceptor synthon **157** with the sLe^x trichloroacetimidate donor **156** in acetonitrile at $-40\,^{\circ}C$ under promotion by boron trifluoride etherate gave a 52% yield of the nonasaccharide derivative **158.** The β-D-GlcNAc-(1 $\rightarrow$ 3)-D-Gal linkage was formed with a high degree of regio- and stereo-specificity. Acetylation of **158** (acetic anhydride, pyridine, dimethylaminopyridine) afforded **159.** Comparative analysis of the ^{1}HNMR spectrum of **159** revealed that acetylation had occurred at O-2 and O-4 of the Gal unit designated d in Scheme 26, confirming the regioselective joining of the sLe^x tetrasaccharide and lacto-*N*-fucopentaose synthons. The β-anomeric configuration of the GlcN unit followed from the chemical shift and three-bond coupling constant of the signal for H-1*e* (δ 5.346 ppm; $J_{1,2}$ $\sim$8.4 Hz). The nonasaccharide derivative **159** was next subjected to catalytic hydrogenation (20% palladium hydroxide on carbon in 4 : 1 methanol–water) and the polar intermediate per-O-acetylated to afford **160** (47% from **159**). Compound **160** was converted into the reducing pyranose derivative **161** by treatment with hydrazinium acetate[303] in *N*,*N*-dimethylformamide in 98% yield. The α-trichloroacetimidate **162** was obtained by reacting **161** with trichloroacetonitrile in 1,2-dichloroethane in the presence of DBU in 91% yield. The key coupling of nonaoside donor **162** with the ceramide acceptor was performed in chloroform under promotion by boron trifluoride etherate at $-15\,^{\circ}C$ to afford the β-glycoside in 39% yield. The protected glycolipid adduct was then converted into the target structure **163** by the following series of steps. Treatment with a large excess of lithium iodide in dry pyridine under reflux[343] cleaved the methyl ester group at the Neu5Ac residue (93%). To exchange the *N*-phthaloyl for the *N*-acetyl group, the intermediate was treated with methylhydrazine in refluxing ethanol and the free amine reacted with acetic anhydride in methanol. The resulting material was finally saponified by the action of aqueous sodium hydroxide in 1 : 1 methanol–tetrahydrofuran to afford the desired glycolipid **163.**

d. Synthesis of sLe^x by Application of Glycal Chemistry.—In recent years, Danishefsky and his associates at Yale University have successfully explored the utility of glycal chemistry for the synthesis of oligosaccharides.[344] The essential features of their glycosylation reaction are illustrated in Fig. 12 using as an example the construction of a β-GlcNAc linkage. Iodonium ion and a sulfonamide are added to a glycal in a *trans*-antiperiplanar manner to give a


156
157
i. BF3.Et2O, MeCN, -40°C
ii. Ac2O, pyridine
158, R = H
159, R = Ac
d
i. H2, 20% Pd(OH)2/C, 4:1 MeOH - H2O
ii. Ac2O, pyridine, DMAP
iii. H2NNH2.AcOH, DMF
160, R = Ac
161, R = H
CCl3CN, DBU, ClCH2CH2Cl
162
i. BF3.Et2O, CHCl3
ii. LiI, pyridine, reflux, 7 h
iii. H2NNHMe, EtOH, reflux
iv. Ac2O, MeOH
v. aq NaOH, 1:1 MeOH - THF
163

SCHEME 26

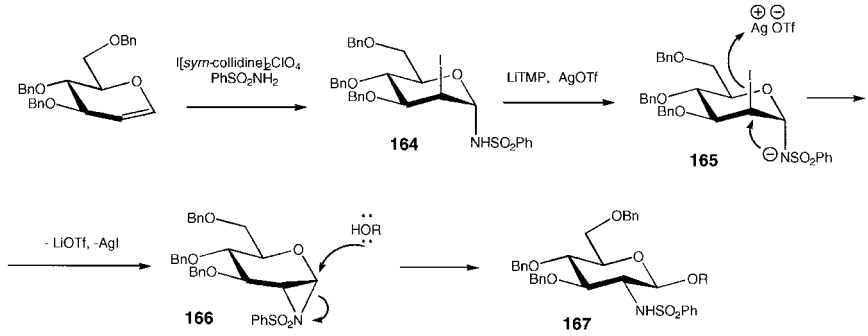
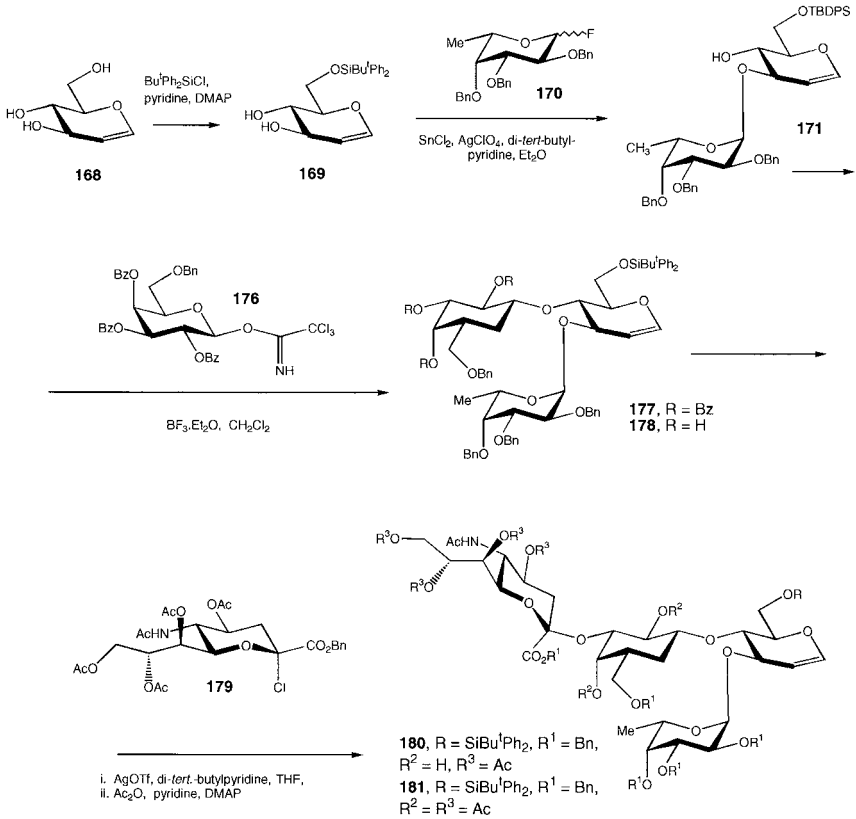


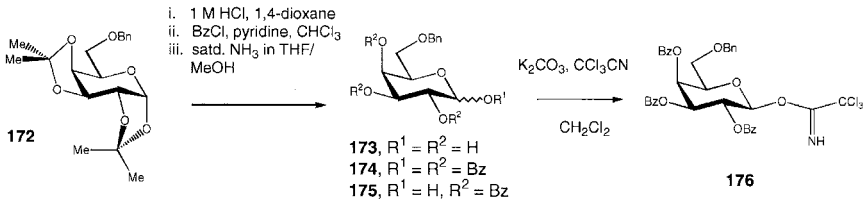
FIG. 12.

2-iodo-2-deoxy sulfonamido glycoside **164.** Treatment of **164** with a strong, sterically hindered base such as lithium 2,2,6,6-tetramethylpiperidide (LiTMP) results in the formation of the sulfonamide anion **165.** In the presence of silver trifluoromethanesulfonate, the anion undergoes neighboring-group displacement of iodide with formation of the sulfonyl aziridine **166.** Glycosylation occurs by nucleophilic attack of a hydroxyl group of the glycosyl acceptor at the anomeric carbon atom, with concomitant opening of the aziridine ring, positioning the 2-sulfonamido substituent trans to the incoming glycosyl unit (**167**). When the glycosyl acceptor is a glycal, the disaccharide glycal formed may in turn be used to construct a disaccharide block donor in an analogous manner. For the construction of glycosidic linkages of sugars other than 2-acetamido sugars, 1,2-oxiranes are used as glycosyl donors; these are prepared, for example, by reacting glycals with 3,3-dimethyldioxirane[344] An alternative synthesis of the reducing sLe^x tetrasaccharide[345,346] serves to illustrate the application of the glycoside synthesis developed by Danishefsky (Scheme 27). D-Glucal **168** was employed as the starting material. Treatment with *tert*-butyldiphenylsilyl (TBDPS) chloride in pyridine in the presence of dimethylaminopyridine afforded the 6-*O*-TBDPS ether **169.** Mukaiyama-type glycosylation[271] of **169** with fucosyl donor fluoride **170** in refluxing ether proceeded regioselectively at the allylic position to give the α-linked disaccharide glycal **171** in 59% yield. The Gal donor **176** was produced from 6-*O*-benzyl-1,2,3,4-di-*O*-isopropylidene-D-galactopyranose (**172**) as follows (Scheme 28). Acid-catalyzed hydrolysis (1 *M* hydrochloric acid in 1,4-dioxane at 100 °C) gave 6-*O*-benzyl-D-galactopyranose (**173**), which was per-O-benzoylated by the action of benzoyl chloride in pyridine to give **174.** Treatment with a saturated solution of ammonia in tetrahydrofuran–methanol converted **174** into the pyranose **175.** The trichloroacetimidate was then obtained by the addition of the hemiacetal **175** to the cyano group of trichloroacetonitrile, catalyzed by potassium carbonate. The β-Gal residue was introduced stereospecifically by glycosylation of **171** with the Gal trichloroacetimidate **176,**



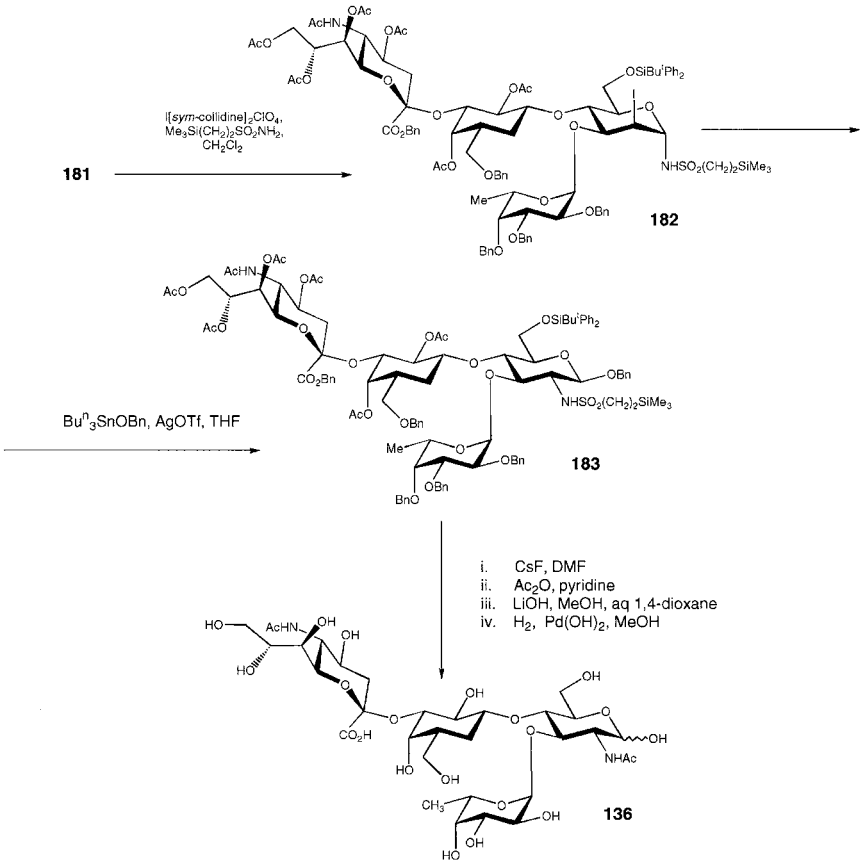
SCHEME 27

under promotion by boron trifluoride etherate in dichloromethane in 75% yield. The branched trisaccharide glycal **177** was converted into the triol **178** by the action of sodium methoxide in methanol. Compound **178** was regio- and stereo-selectively *O*-sialylated using the Neu5Ac benzyl ester halide donor **179** under promotion by silver trifluoromethanesulfonate in the presence of di-*tert*-butylpyridine in



SCHEME 28

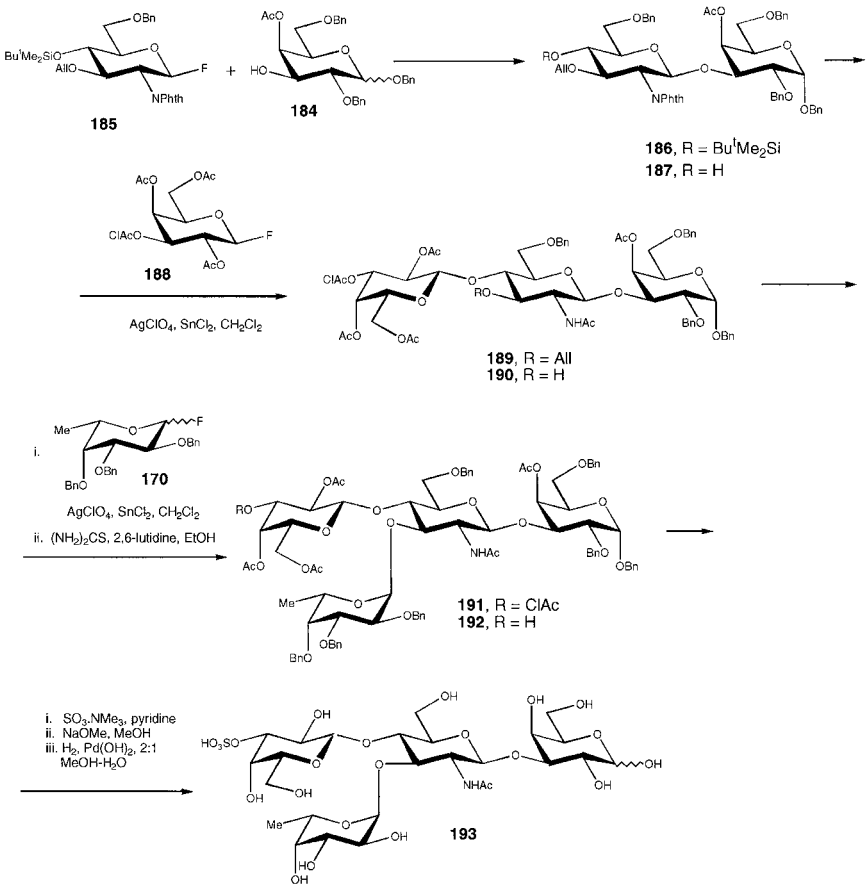
tetrahydrofuran, as reported by the Chembiomed group[347] (compare Section V.7) to give **180;** this was treated with acetic anhydride in pyridine in the presence of 4-dimethylaminopyridine to give the fully protected sLe^x tetrasaccharide glycal **181** (40% isolated yield; 78% based on recovered starting material). Oligosaccharide glycals such as **181** may be converted into the reducing oligosaccharides or may serve as block synthons for the assembly of larger oligosaccharides, glycolipids, or glycopeptides. Thus, the reaction of **181** with iodonium di-*sym*-collidine perchlorate and 2-(trimethylsilyl)ethylsulfonamide gave the iodo sulfonamide **182** in 82% yield (Scheme 29). The reaction of **182** with tributylstannyl *O*-benzyl alkoxide[348] in the presence of silver triflate in tetrahydrofuran gave the tetrasaccharide benzyl glycoside **183** in 64% yield. Removal of the protecting groups required four steps to afford the reducing sLe^x tetrasaccharide **136** in 22% yield from **183**



SCHEME 29

(cesium fluoride in *N*,*N*-dimethylformamide to cleave both the silyl ether and the 2-silylethanesulfonamido groups; acetic anhydride–pyridine; lithium hydroxide in methanol–aqueous 1,4-dioxane; and catalytic hydrogenation over palladium hydroxide in methanol).

e. Syntheses of Sulfated Derivatives of Le^x and Le^a.—Nicolaou and his associates[349] described the syntheses of the sulfated Le^x and Le^a derivatives, **193, 201, 208,** and **211.** Compounds **193** and **211** represent sulfated Le^x and Le^a trisaccharide determinants attached to a reducing-terminal Gal unit. The *N*-phthaloyl fluoride **185,** employed as the precursor[349,330,331] of the central GlcNAc residue of **193,** was designed as a glycosyl donor containing orthogonal,

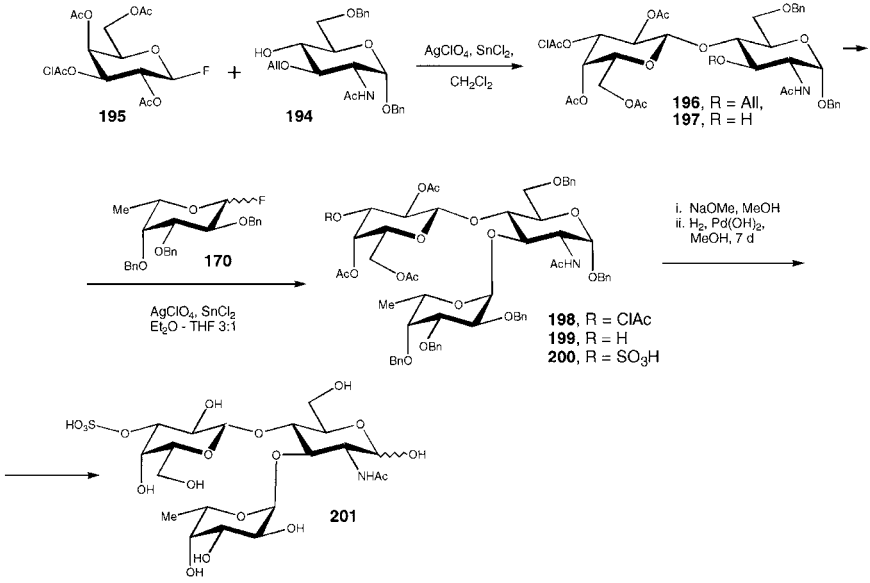


SCHEME 30

temporary allyl, and *tert*-butyldimethylsilyl (tBDMS) protecting groups at C-3 and C-4. Glycosylation of the Gal acceptor **184** with **185** under Mukaiyama conditions[271] (Scheme 30) afforded the β-linked disaccharide derivative **186** in excellent yield and anomeric selectivity. In this procedure, tin perchlorate, generated from silver perchlorate and tin(II) chloride *in situ,* reacts with a glycosyl fluoride to produce a glycosyl cation and a tin fluoride derivative.[273] Compound **186** was converted into the glycosyl acceptor **187** in three steps: treatment with methylhydrazine in refluxing ethanol cleaved both the *N*-phthaloyl auxiliary[324] group and the ester protecting group; the resulting amino alcohol was reacted with acetic anhydride in the presence of triethylamine and 4 dimethylaminophyridine in dichloromethane to give the corresponding *N*,*O*-acetate (80% over two steps); cleavage of the silyl ether group was then effected by the action of tetrabutylammonium fluoride in tetrahydrofuran. Employing again Mukaiyama's conditions,[271] **187** was glycosylated with the galactosyl donor **188,** temporarily protected with a chloroacetyl group at O-3 to allow for the eventual conversion into a sulfate ester (**189,** 75%). Allyl rearrangement of **189** was effected by the action of tetrakis(triphenylphosphine)ruthenium (II) hydride and was followed by hydrolysis of the intermediate propenyl ether (*p*-toluenesulfonic acid) to afford glycosyl acceptor **190** (81%); compound **190** was glycosylated employing fucose fluoride donor **170,** again using the glycosylation conditions of Mukaiyama[271] (**191,** 85%). Compound **191** contains permanent acetate or benzyl ether protecting groups in all positions but C-3″. The chloroacetate **191** was selectively hydrolyzed under catalysis by thiourea[349–353] (**192**) and the resulting alcohol was converted into the 3″-sulfate using the triethylamine–sulfur trioxide complex in pyridine. O-Deacetylation (sodium methoxide in methanol) followed by catalytic hydrogenation over palladium hydroxide in 2 : 1 methanol–water gave the target compound **193** (76% from **192**).

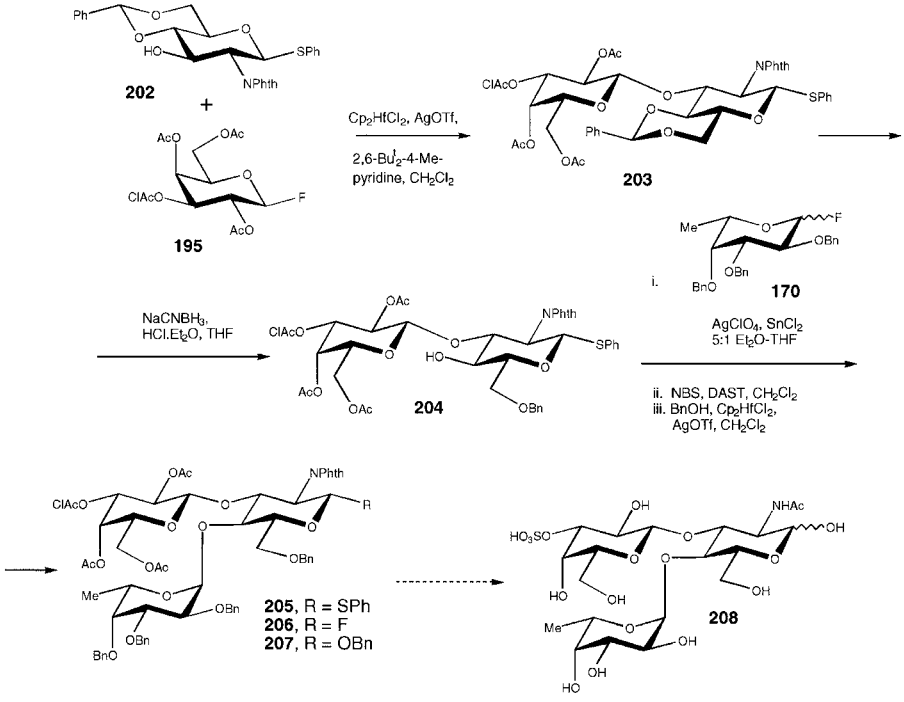
The Le^x trisaccharide **201** was prepared by Nicolaou *et al.* using similar procedures[349] (Scheme 31). However, in this case, the precursor of the reducing GlcNAc unit is the glycosyl acceptor derivative **194.** Glycosylation[271] with the chloroacetylated Gal donor **195** to give **196** (81%), liberation of the 3-OH function of the resulting disaccharide derivative (**197,** 82%), and fucosylation and attachment of the sulfate group as in the synthesis of **193** afforded the protected target structure **200** in 66% yield from **197.** The protecting groups were removed by sequential treatment with sodium methoxide in methanol and catalytic hydrogenation over palladium hydroxide in methanol to give the desired 3′-O-sulfated Le^x derivative **201** (74% from **200**).

For the syntheses of the Le^a-related compounds **208** and **211,** a different strategy was adopted: the precursor of the Le^a trisaccharide determinant, in this case, was designed as a trisaccharide block donor **206;** this would alternatively be converted into benzyl glycoside **207,** precursor of the trisaccharide target structure **208,** or be employed for the glycosylation of the acceptor **209,** precursor of the reducing Gal



SCHEME 31

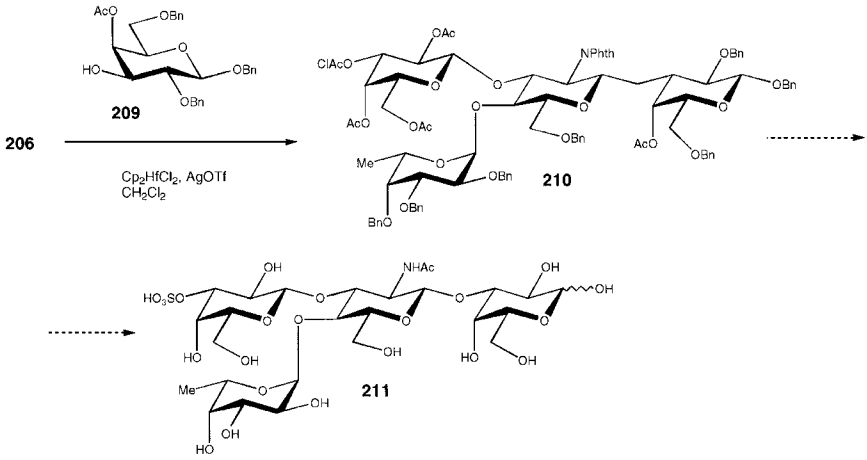
residue of the tetrasaccharide target structure **211.** For the synthesis of **206,** the 4,6-*O*-benzylidene thioglycoside **202** served as the precursor of the GlcNAc residue (Scheme 32). Mukaiyama–Suzuki glycosylation[355,356] of **202** with the galactosyl fluoride **195** as glycosyl donor gave the β-linked disaccharide derivative **203** in good yield and excellent stereoselectivity. In this example of the Suzuki modification, the tin perchlorate derivatives employed as fluoride acceptors under the standard Mukaiyama conditions are replaced by bis(cyclopentadienyl)hafnium triflate. The exclusive formation of the β-product in this reaction is attributed to neighboring-group displacement of fluoride by the carbonyl oxygen atom of the 2-*N*-phthaloyl group. Reductive cleavage[354] of the benzylidene acetal afforded a high yield of the alcohol **204,** which was glycosylated with the fucosyl fluoride **170** to give the trisaccharide thioglycoside derivative **205** in excellent yield. Conversion of **205** into the trisaccharide fluoride donor **206** was performed by treatment with *N*-bromosuccinimide and diethylaminosulfur trifluoride (DAST) in 80% yield. To prepare the Le^a trisaccharide derivative **208,** the trisaccharide fluoride **206** was reacted with benzyl alcohol under Mukaiyama–Suzuki conditions[355,356] to provide the corresponding β-benzyl glycoside **207** in 95% yield. Introduction of the *O*-sulfate group, exchange of the *N*-phthaloyl for the *N*-acetyl group accompanied by O-deacetylation, and removal of the persistent benzyl ether protecting groups afforded the O-sulfated, Le^a-related target structure **208.** Glycosylation of the Gal acceptor **209** with the Le^a donor **206** under Mukaiyama–Suzuki conditions[355,356]



SCHEME 32

gave the tetrasaccharide derivative **210** in good yield and excellent stereoselectivity. Conversion of **210** into the target structure **211** (Scheme 33) proceeded in 27% yield by a sequence analogous to the corresponding steps in the synthesis of **208.**

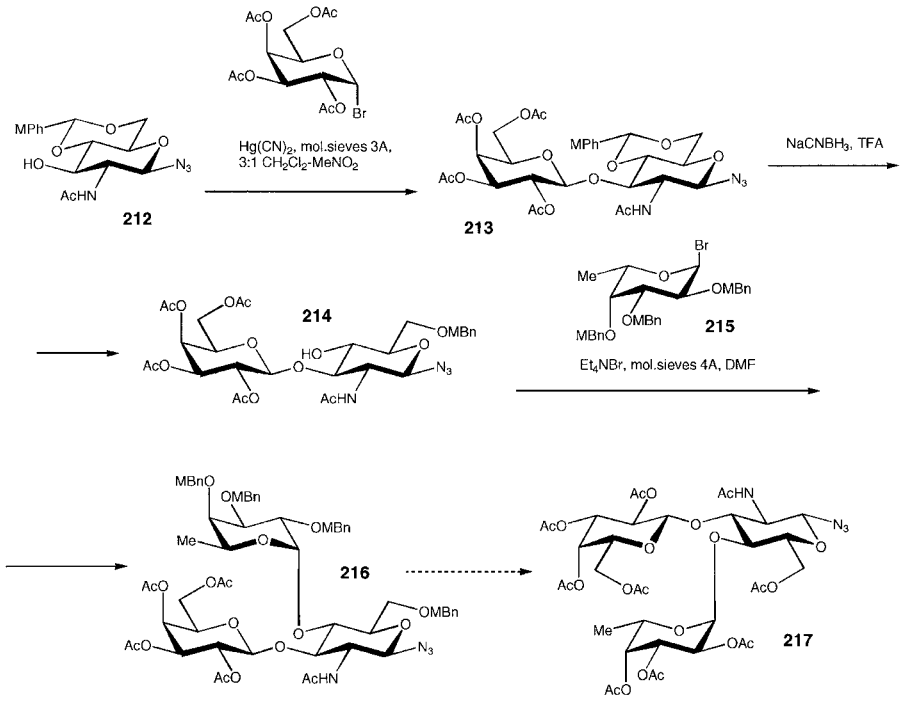
f. Syntheses of Glycopeptides Containing Le^a, Le^x, and sLe^x Determinants.—Oligosaccharide ligands of selectins occur in nature primarily as nonreducing segments of glycoprotein glycans. During initial investigations, the effects exerted by the selectin ligands appeared to be independent of the nature of the glycosyl units that precede the biologically active sLe or sulfated Lewis determinants on the reducing side (compare p. 226). At that time, the protein domains adjacent to the selectin ligands, being even further remote from the interacting selectin determinants, would have been assumed to not specifically participate in the adhesive events, and to serve only as scaffolds for presentation or exposure of the ligand glycans. This view had to be modified with the definition of specific selectin ligand glycans for L-, E-, and P-selectins (Fig. 6), and particularly with the discovery of the sulfated tyrosine consensus sequence that is adjacent to the ligand glycan on PSGL-1 and that is required for binding to P-selectin (compare Section II.4.b). As a result, there is considerable interest in model structures that include peptide



SCHEME 33

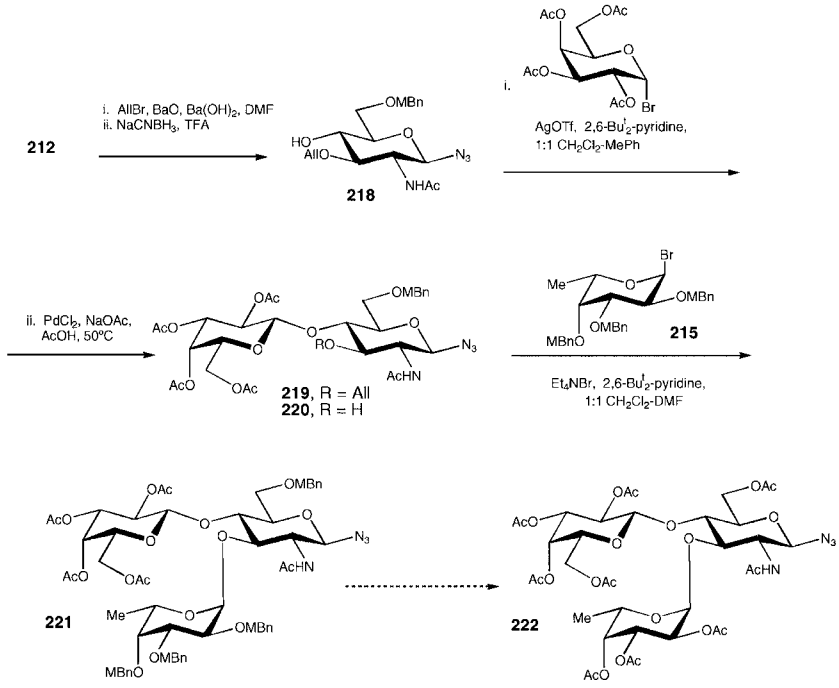
domains adjacent to the selectin ligand glycans. Over the past 20 years, a number of investigators have developed a practicable repertoire of orthogonal protecting group techniques that provide access to a wide variety of model glycopeptides. The field has been expertly and comprehensively reviewed.[357,358] The discussion in the present context will cover only a few examples that are relevant to the chemistry of Lewis and sialyl Lewis antigens as selectin ligand glycans.

The glycosyl azides derived from Le^a, Le^x and sLe^x, **217, 221,** and **229,** have been synthesized by Kunz and his associates and have been utilized in syntheses of N-linked model glycopeptides related to selectin ligands. The β-azido group attached to the reducing, branching GlcNAc residue functions as a persistent anomeric protecting group; following the assembly of the glycan, the glycosyl azide is reduced and the resulting glycosylamine may be acylated, for example, with a precursor of asparagine, the linkage amino acid of N-linked glycoprotein glycans. The amino group may also be used for coupling of the glycan to a variety of carrier molecules that contain activated carboxyl groups or functional groups to which amino groups will add, such as the isothiocyanate group. The β-azide **212** was derived from the acetochloro derivative of GlcNAc,[289] by the following sequence. Phase transfer-catalyzed SN2 reaction[359] with tri-*n*-octylmethylammonium azide[360] was followed by sodium methoxide-catalyzed methanolysis and reaction with 4-methoxybenzaldehyde dimethyl acetal under catalysis by tetrafluoroboric acid in ether. Compound **212** is a precursor common to the Le^a, Le^x, and sLe^x target structures **217, 221,** and **229.** For the synthesis of **217** (Scheme 34), acceptor **212** was treated[361] with "acetobromogalactose" under promotion by mercuric cyanide to give an 81% yield of the disaccharide derivative **213.** Reductive opening of the 4-methoxybenzylidene acetal by the action of sodium cyanoborohydride and



SCHEME 34

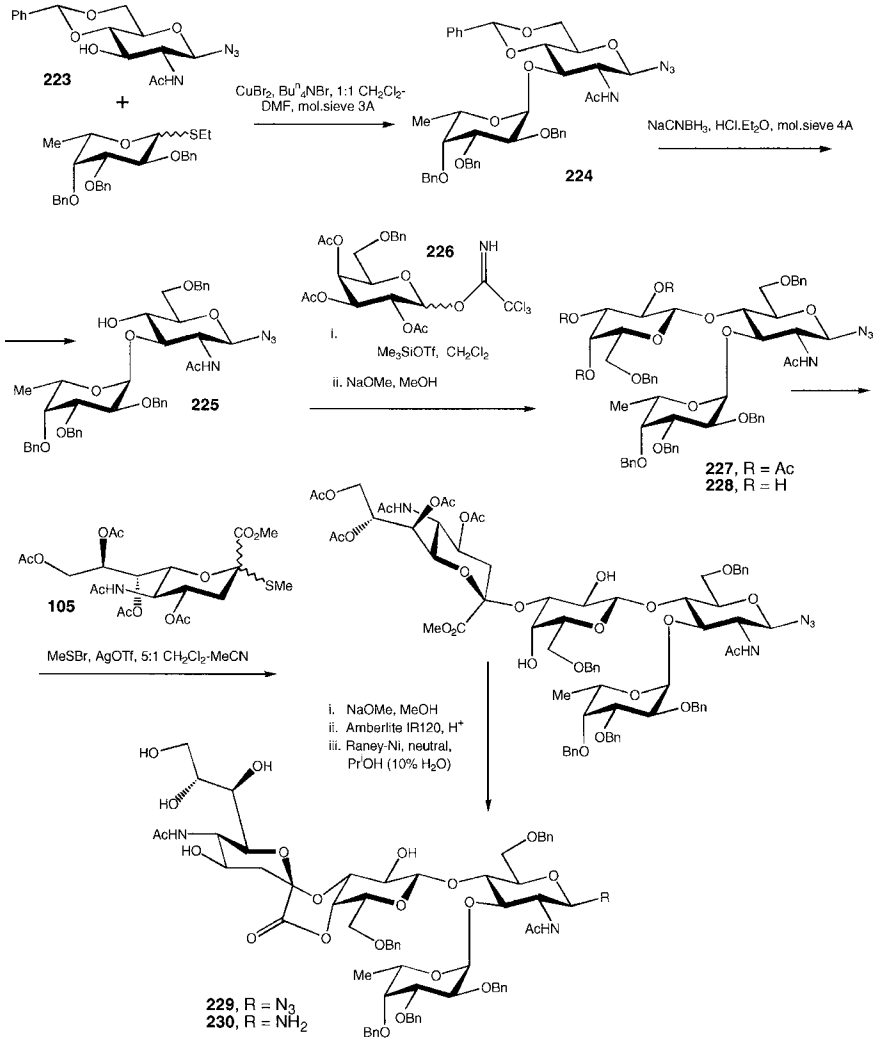
trifluoroacetic acid[363] provided the 6-*O*-(4-methoxybenzylated) glycosyl acceptor, **214,** in 70% yield. Compound **214** was glycosylated[364] with the per-*O*-(4-methoxybenzylated) fucosyl donor **215** under halide ion catalysis[294] to give the Le^a trisaccharide derivative **216** (95%). In an orthogonal reaction sequence preserving the β-anomeric glycosyl azide, catalytic hydrogenation needs to be avoided. Therefore, 4-methoxybenzyl had been chosen as the ether protecting group. This group can be removed under mild hydrolytic conditions following oxidation.[365] Treatment of **216** with ceric ammonium nitrate[362] and subsequent hydrolysis removed the 4-methoxybenzyl protecting groups from the GlcNAc and Fuc residues. The intermediate tetrol was per-O-acetylated (acetic anhydride–pyridine) to afford the ester-protected trisaccharide azide **217.** Compound **217** was hydrogenated over neutral Raney nickel and the resulting glycosylamine attached to a suitable activated derivative of aspartic acid. The resulting aspartic-γ-*N*-glycosylamides are suitable for incorporation into a peptide chain. Under the acidic conditions required for cleavage of *tert*-butyl esters during peptide assembly, the *O*-acyl protecting groups (such as *O*-acetyl) convey additional stability to acid-sensitive glycosidic linkages such as those of α-fucose. This has been explained by charge repulsion



SCHEME 35

due to partial protonation of the ester carbonyl oxygen atoms. Following peptide synthesis, the ester groups are removed by the action of catalytic amounts of sodium methoxide or hydrazine in methanol.

Synthesis of the per-O-acetylated Le^x azide **221** (Scheme 35) was performed in a similar manner.[366] In this case, the GlcNAc azide **212** was converted into the 3-allyl ether by treatment with allyl bromide in the presence of barium oxide and barium hydroxide in *N*,*N*-dimethylformamide.[367] Opening of the 4-methoxybenzylidene acetal by the action of sodium cyanoborohydride and trifluoroacetic acid afforded alcohol **218,** which was glycosylated using the donor "acetobromogalactose" under promotion by silver triflate in the presence of di-*tert*-butylpyridine in 1 : 1 dichloromethane–toluene in 78% yield. The fully protected disaccharide derivative **219** was subjected to allyl rearrangement by the action of palladium(II) chloride in acetic acid–sodium acetate to afford the intermediate propenyl ether, which was hydrolyzed under the reaction conditions to give the lactosaminyl azide acceptor **220** (55%). Fucosylation in position 3 of the GlcNAc unit of **220** was performed[364] essentially as already described for the Le^a derivative and afforded **221** in 90% yield; following the change of protecting groups as described for **216,** the per-O-acetylated Le^x azide **222** was obtained, suitable for the synthesis of an aspartic acid-γ-carboxamido derivative or a variety of other N-linked conjugates.



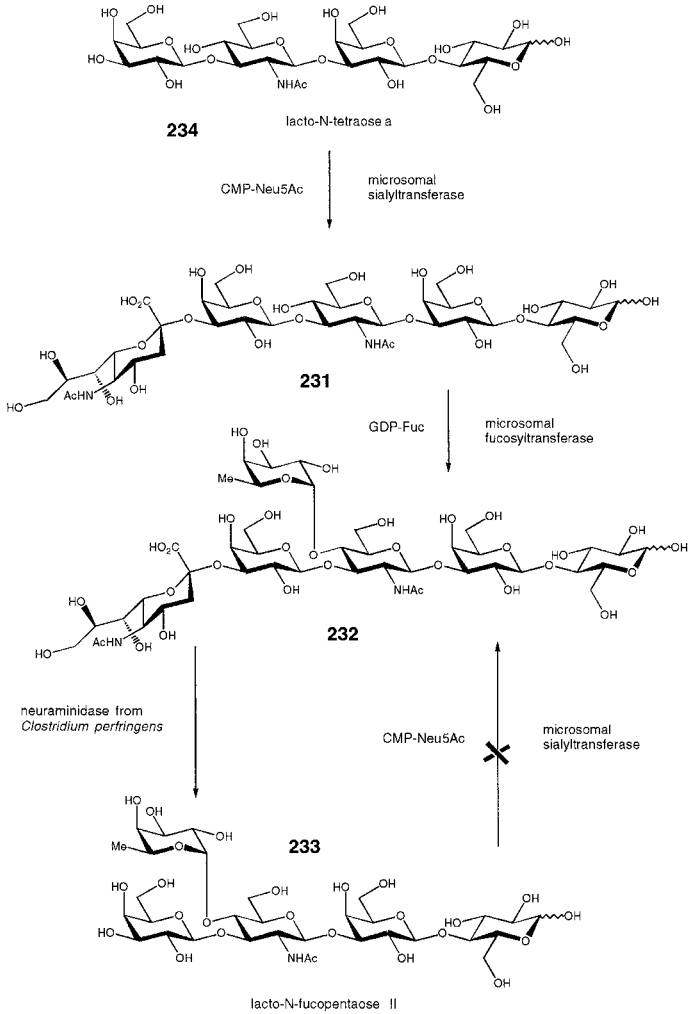
SCHEME 36

For the synthesis[368] of the sLe^x-related glycosyl azide derivative **229** (Scheme 36), the GlcNAc azide **223** was fucosylated with a thioglycoside donor[369] under promotion by copper(II) bromide in the presence of tetrabutylammonium bromide in 1 : 1 dichloromethane–*N*,*N*-dimethylformamide to afford the disaccharide derivative **224** (92%). As the sLe^x-glycosylamine was to be coupled to the carboxyl terminus of the tetrapeptide RGDA in an (unnatural) amide linkage, carbohydrate O-protecting groups compatible with the stepwise assembly of the (glyco)peptide chain were not required and benzyl ether protection was

satisfactory. Reductive opening of the benzylidene acetal (sodium cyanoborohydride and hydrogen chloride–ether) gave glycosyl acceptor **225,** which was glycosylated according to Schmidt's procedure[370] using the trichloroacetimidate galactosyl donor **226** under promotion by trimethylsilyl triflate in dichloromethane (**227**). Because of the steric hindrance provided by the protected fucosyl residue, OH-4 of GlcNAc is but poorly reactive (compare Section IV.2.a): following treatment of compound **227** with sodium methoxide in methanol, the triol **228** was obtained in 44% yield (from **225**). Compound **228,** in accord with several precedents (compare Section IV.2.b) was regio- and stereo-selectively 3-*O*-sialylated by reaction with Hasegawa's Neu5Ac thioglycoside donor **105** under promotion by methyl sulfenyl bromide–silver triflate as recommended by Garegg,[371] the ester protecting groups of the glycosylation product were cleaved (sodium methoxide in methanol with subsequent addition of water) and the resulting sLe^x salt was converted into the interglycosidic lactone **229** (42% from **228**) by treatment with acidic ion-exchange resin (compare structures **134** and **135,** Scheme 22, and structure **152,** Scheme 24). Incorporated into the lactone structure, the carboxyl group of the Neu5Ac residue is conveniently protected during the subsequent manipulations. Compound **229** was reduced by the action of neutral Raney nickel and the resulting glycosylamine **230** was coupled to the free carboxyl end of a protected tetrapeptide derivative that contains *N*-benzyloxycarbonyl and benzyl ester protecting groups.[368] Catalytic hydrogenation over palladium on activated charcoal in 2 : 1 : 1 methanol–1,4-dioxane–acetic acid afforded the lactone–glycopeptide hybrid, which was converted into the desired sLe^x conjugate by treatment with sodium hydroxide in methanol–water (pH 10) followed by neutralization with an acidic ion-exchange resin.

3. Combinations of Organic-Chemical and Enzyme-Catalyzed Methods of Synthesis

The first total synthesis of a sialylated Lewis determinant was reported by Palcic and her colleagues[372] at the University of Alberta, Edmonton. In their biomimetic, combined organic-chemical and enzyme-catalyzed synthesis of the spacer-linked sLe^a pentaasaccharide **237,** these authors followed the pathway of sLe^a biosynthesis previously discovered by Hansson and Zopf[373] (Scheme 37). Hansson and Zopf found that microsomal preparations from the cell line SW 1116 catalyzed the transfer of a fucose residue from GDP-[^{14}C]fucose to position 4 of the GlcNAc residue of sialosylated lacto-*N*-tetraose a [α-D-Neu5Ac*p*-(2 → 3)-β-D-Gal*p*-(1 → 3)-β-D-GlcN*p*Ac-(1 → 3)-β-D-Gal*p*-(1 → 4)-D-Glc, **231**] to form the hexasaccharide α-D-Neu5Ac*p*-(2 → 3)-β-D-Gal*p*-(1 → 3)[α-L-Fuc*p*-(1 → 4)]-β-D-GlcN*p*Ac-(1 → 3)-β-D-Gal*p*-(1 → 4)-D-Glc **232** (Scheme 37). The labeled hexasaccharide was separated from the incubation mixture by adsorption to an affinity column of MAb 19-9 on protein A–Sepharose. Upon treatment

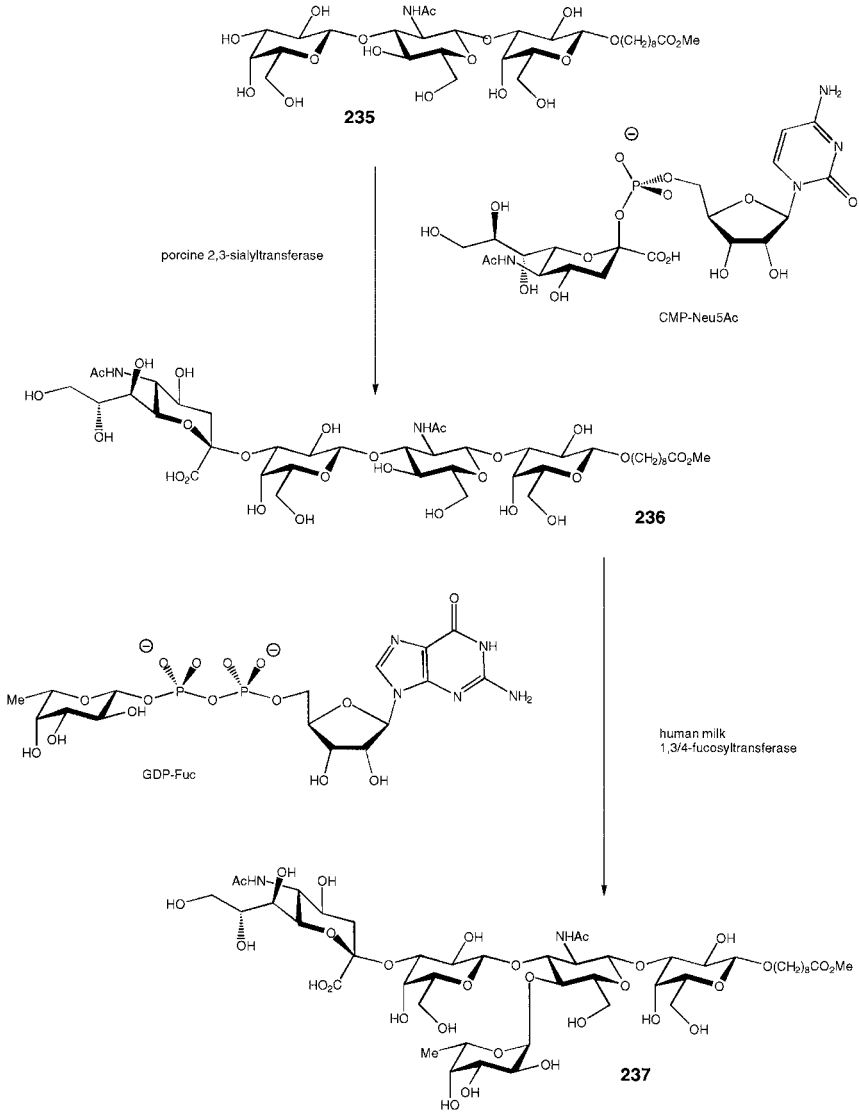


SCHEME 37

of the hexasaccharide with neuraminidase, a radioactive oligosaccharide was formed that was chromatographically indistinguishable, in two systems, from lactoneofucopentaose-II {LNF-II; β-D-Gal*p*-(1 → 3)[α-L-Fuc*p*-(1 → 4)]-β-D-GlcN*p*Ac-(1 → 3)-β-D-Gal*p*-(1 → 4)-D-Glc, **233**}. Similarly, the microsomal preparation catalyzes the transfer of a Neu5Ac residue from CMP-Neu5Ac to position 3 of the nonreducing Gal residue of the tetrasaccharide β-D-Gal*p*-(1 → 3)-β-D-GlcN*p*Ac-(1 → 3)-β-D-Gal*p*-(1 → 4)-D-Glc **234** to give **231.** However, the microsomal preparation would not catalyze the transfer of a Neu5Ac residue from

CMP-Neu5Ac to position 3 of the nonreducing Gal residue of lactoneofucopentaose II (**233**). These findings were interpreted to indicate that, in the biosynthesis of the sLe^a determinant from a type 1 core, the transfer of the Neu5Ac residue precedes, and is a prerequisite for, the transfer of the fucose unit. Holmes, Ostrander, and Hakomori[374] characterized a similar biosynthetic pathway of glycolipids containing the sLe^x determinant in human lung carcinoma PC9 cells. Substrate specificities of the sialyltransferase and fucosyltransferase expressed in these cells were studied by performing the respective glycosyltransferase steps in the presence of CMP-[^{14}C]Neu5Ac or GDP-[^{14}C]fucose. The glycolipid products were then separated by micropreparative thin-layer chromatography (TLC) and their amounts determined by scintillation counting. Thin-layer chromatographic bands containing characteristic determinants such as sLe^x were detected by immunostaining with specific antibodies, for example CSLEX-1 or FH6, and quantitated by autoradiography of bound ^{125}I-protein A. The properties of the α-2,3-sialyltransferase solubilized from PC9 cell membranes are similar to those of other glycolipid sialyltransferases; the enzyme does not catalyze sialylation of fucosylated chains such as those expressing the Le^x determinant. The fucosyltransferase of PC9 cells is highly specific for the type 2 chains of neolactotetraosylceramide (nLc$_4$; β-D-Gal-(1 $\rightarrow$ 4)-β-D-GlcNAc-(1 $\rightarrow$ 3)-β-D-Gal-(1 $\rightarrow$ 4)-β-D-Glc-1,1-ceramide), neolactohexaosylceramide (nLc$_6$; β-D-Gal-(1 $\rightarrow$ 4)-β-D-GlcNAc-(1 $\rightarrow$ 3)-β-D-Gal-(1 $\rightarrow$ 4)-β-D-GlcNAc-(1 $\rightarrow$ 3)-β-D-Gal-(1 $\rightarrow$ 4)-β-D-Glc-1,1-ceramide), and their sialylated forms IV^{3}NeuAcnLc$_4$ and VI^{3}NeuAcnLc$_6$. Fucose is not transferred onto the 6-sialylated derivative IV^{6}NeuAcnLc$_4$, and only one fucose residue is transferred onto VI^{6}NeuAcnLc$_6$ to form VI^{6}NeuAcIII^{3}FucnLc$_6$. Under the conditions studied, this enzyme does not catalyze the transfer of fucose to (the type 1 chain of) lactotetraosylceramide (Lc$_4$) or its sialylated form IV^{3}NeuAcLc$_4$, and is similar to the fucosyltransferase activities found in mutant CHO cells by Campbell and Stanley.[70] In the biosynthesis of sLe^x, as in the case of sLe^a, the transfer of the α-(2 $\rightarrow$ 3)-linked Neu5Ac residue precedes, and is a prerequisite for, the transfer of the fucose residue.[374]

As acceptor substrates of a porcine α-2,3-sialyltransferase (EC 2.4.99.4), Palcic and her associates (Scheme 38) utilized the 8-(methoxycarbonyl)octyl β-glycosides[288] of the trisaccharides β-D-Gal-(1 $\rightarrow$ 3)-β-D-GlcNAc-(1 $\rightarrow$ 3)-D-Gal **235** and β-D-Gal-(1 $\rightarrow$ 3)-β-D-GlcNAc-(1 $\rightarrow$ 6)-D-Gal previously synthesized by organic-chemical methods.[375] In its physiological context, this sialyltransferase[376,377] catalyzes the transfer, from CMP-Neu5Ac, of a sialyl residue to position 3 of the nonreducing Gal residue of mucin determinants such as the T-antigen, β-D-Gal-(1 $\rightarrow$ 3)-α-D-GalNAc. Transfer to the type 1 structure, **235,** occurred with low but sufficient efficacy to yield milligram quantities of sialyl oligosaccharides (**236**). In a second enzyme-catalyzed step, a fucosyl residue was transferred from the biological fucose donor, GDP-fucose, under catalysis by a 1,3/4-fucosyltransferase preparation from human milk,[378–380] to afford the sLe^a



SCHEME 38

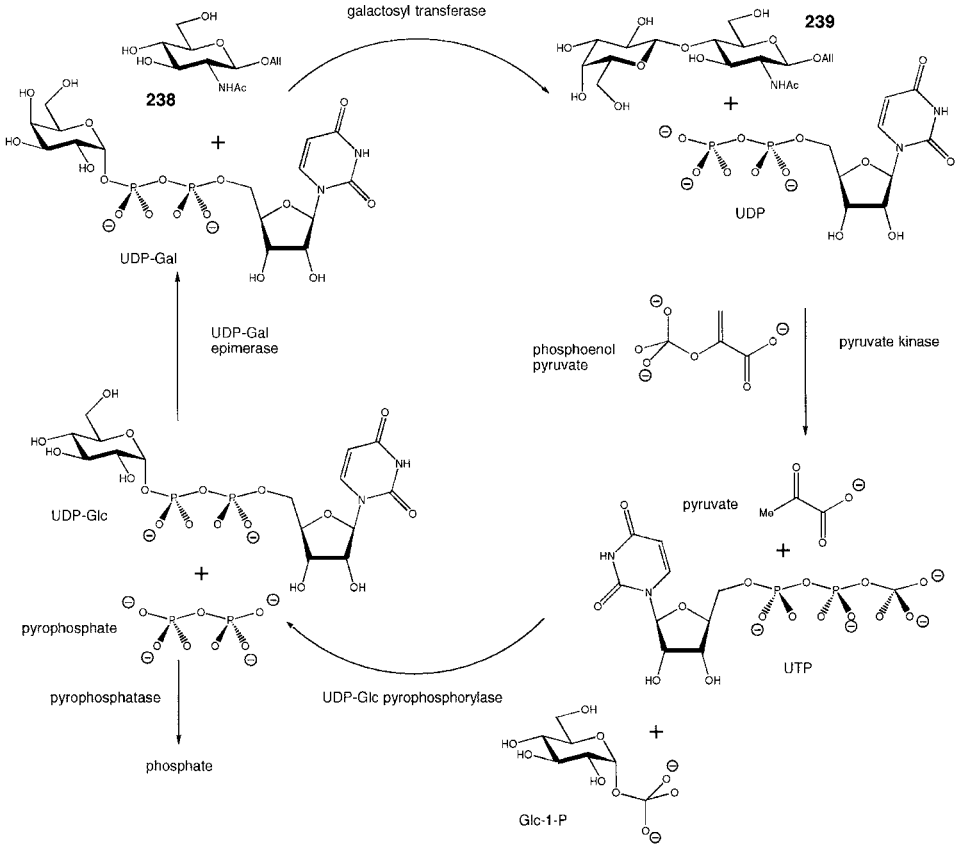
pentasaccharide derivative **237.** Although these initial experiments were conducted on a milligram scale only, they are amenable to scale-up and provided quantities of oligosaccharide derivatives sufficient for the study of antigen–antibody reactions. The hydrophobic spacer arm on compounds **235**–**237** enables their convenient isolation, from aqueous systems, on hydrophobic supports and has been utilized to

advantage in simplified assays of glycosyltransferase activities.[381] By a principle similar to that of Palcic *et al.*, de Vries *et al.*[382] developed an efficient synthesis of sLe^x. These authors used an α-2,3-sialyltransferase (EC 2.4.99.6) from human placenta[383] to catalyze the formation of sialyllactosamine α-D-Neu5Ac-(2 $\rightarrow$ 3)-β-D-Gal-(1 $\rightarrow$ 4)-β-D-GlcNAc from CMP-Neu5Ac and *N*-acetyllactosamine. Although this sialyltransferase is membrane-bound and relatively unstable, it exhibits a pronounced preference for type 2 over type 1 structures[384] and is accompanied by only minor quantities of the (2 $\rightarrow$ 6)-specific sialyltransferase.[385] For the transfer of the fucose residue, de Vries *et al.* obtained optimal results through the use of the plasma type 1,3-fucosyltransferase (Fuc-TV) that had been made available by Lowe in the form of plasmid pcDNA1-Fuc-TV.[386] This enzyme had been previously used to advantage by Ichikawa *et al.* in their synthesis of sialyl Lewis determinants with cofactor regeneration.[390]

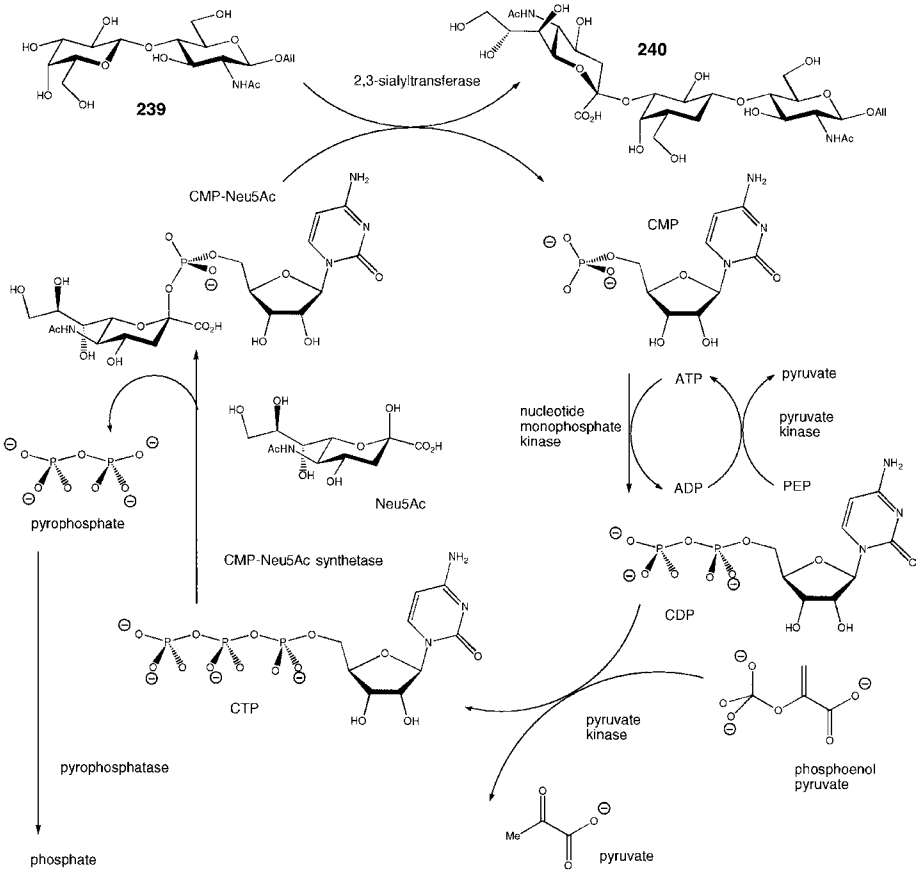
4. Preparations of sLe^x Determinants by Enzyme-Catalyzed Processes with Regeneration of Cofactors

Purely organic-chemical syntheses of complex oligosaccharides have limited suitability for preparations on a kilogram scale as required, for instance, for clinical studies. Primary obstacles are the number of steps required for attachment, exchange, and removal of protecting groups, the frequent lack of crystalline intermediates necessitating large-scale chromatographic separations, and the large quantities of spent organic solvents and toxic catalysts that are difficult to recycle or dispose of. Although protocols of solid-phase oligosaccharide synthesis are expected to ameliorate some of these disadvantages, enzyme-catalyzed processes frequently offer decisive advantages regarding yields, purification of intermediates, and innocuous disposal of by-products. In many cases, substrate specificities of glycosyltransferase enzymes are found to be relaxed, enabling enzyme-catalyzed syntheses of oligosaccharide analogues for structure–activity studies. Examples of this type of work from the Chembiomed and Novartis groups are referred to in Section V of this article. One of the early applications of this principle was developed by the present author together with Macher, Raetz, and Loibner in the synthesis of lipid A analogues catalyzed by lipid-A-synthase.[387,388] Enzyme catalysis of key glycosylation steps often provides excellent to quantitative yields; keeping the numbers of purely organic-chemical steps low, such protocols can be designed to routinely provide target structures on a scale of 50–100 mg, sufficient for many purposes of preclinical pharmaceutical testing. An additional advantage of enzyme-catalyzed syntheses of sLe^x or sLe^a-containing structures lies in their straightforward applicability to di-, tri- and tetra-antennary glycans, structures far too complex for practical syntheses by purely organic-chemical methods. Examples for such preparations are discussed in Section VI.6. Nonetheless, the enzyme-catalyzed approach to oligosaccharide production, at the time of its inception, also presented specific problems. Foremost was the difficulty

in accessing practical quantities of the glycosyltransferase enzymes required as the biological catalysts of glycoside formation. Furthermore, the preparation of the nucleotide sugar cofactors in stoichiometric quantities posed an additional problem of chemical synthesis. Finally, many of the glycosyltransferase reactions are subject to product inhibition by nucleoside phosphates or diphosphates. All three groups of problems have been addressed and essentially solved by the efforts of many investigators, with the most notable contributions provided by the schools of Whitesides[389] and Wong. Indeed, Wong and his associates, among other prominent achievements, developed the first industrial-scale synthesis of sLe^x-containing oligosaccharide derivatives.[390] For example, Ichikawa *et al.* reported on two systems for the enzyme-catalyzed synthesis, with cofactor regeneration, of the allyl β glycoside **239** of *N*-acetyllactosamine (LacNAc). In the first of these (Scheme 39), transfer of a β-Gal residue from the donor UDP-Gal to position 4 of



SCHEME 39



SCHEME 40

the acceptor, allyl 2-acetamido-2-deoxy-β-D-glucopyranoside (**238**), is catalyzed by a 1,4-galactosyltransferase. Spent UDP is phosphorylated to UTP by transfer of a phosphate group from enolpyruvate phosphate under catalysis by pyruvate kinase. Under catalysis by UDP-glucose pyrophosphorylase, UTP reacts with glucose 1-phosphate to form UDP-Glc and inorganic pyrophosphate; the latter byproduct is continuously hydrolyzed by the action of inorganic pyrophosphatase. The UDP-Glc formed is epimerized to UDP-Gal under catalysis by UDP-Gal 4-epimerase to complete the cycle and provide fresh donor for the formation of **239.**

An alternative pathway for the synthesis is based on an analogous transfer of Gal from UDP-Gal and permits the incorporation of Gal analogs or labeled Gal precursors. In this case, a different approach has been used to convert UDP-Glc into UDP-Gal. UDP-Glc reacts with Gal 1-phosphate under catalysis by Gal 1-phosphate uridylyltransferase to form UDP-Gal and Glc 1-phosphate. The Gal

1-phosphate for this reaction is formed together with ADP from Gal and ATP under catalysis by galactokinase. The byproduct ADP accepts a phosphate group from enolpyruvate phosphate under catalysis by pyruvate kinase. Completing the cycle, the byproduct Glc 1-phosphate reacts with UTP under catalysis by UDP-Glc pyrophosphorylase to regenerate UDP-Glc.

Transfer of a Neu5Ac unit onto position 3 of the nonreducing Gal moiety of LacNAc derivative **239** requires the cofactor CMP-Neu5Ac and an α-2,3-sialyltransferase (Scheme 40). In two sequential steps catalyzed by nucleotide monophosphate kinase and pyruvate kinase, spent CMP accepts two phosphate groups from ATP and enolpyruvate phosphate to form CTP. The ADP formed in the first phosphorylation step is converted into ATP by transfer of a phosphate group from enolpyruvate phosphate (PEP) under catalysis by pyruvate kinase. CTP reacts with Neu5Ac under catalysis by CMP-Neu5Ac synthetase to form CMP-Neu5Ac, completing the cofactor regeneration cycle. As with the regeneration of UDP-Gal, the byproduct pyrophosphate is cleaved by the action of inorganic pyrophosphatase.

The allyl β-glycoside of sialyl LacNAc **240** is converted into the sLe^x derivative[390] by the enzyme-catalyzed transfer of a fucose unit from GDP-fucose to position 3 of the GlcNAc unit under catalysis by α-1,3-fucosyltransferase (compare Schemes 37 and 38). The glycosyl donor GDP-fucose may be regenerated by a two-step sequence of enzyme-catalyzed reactions. Under catalysis by pyruvate kinase, spent GDP accepts one phosphate group from enolpyruvate phosphate. The GTP formed reacts with fucose 1-phosphate under catalysis by GDP fucose pyrophosphorylase from porcine thyroid to form GDP-fucose and inorganic pyrophosphate.

V. Approaches to Pharmaceutically Applicable Inhibitors of Selectin–Carbohydrate Interactions

1. A First Set of Structure–Activity Relationships Derived from the Structures of Naturally Occurring Ligand Glycans

a. Screening of Oligosaccharide Determinants from Carbohydrate Libraries.—Tyrrell *et al.* investigated the structural requirements for the carbohydrate ligand of E-selectin.[391] The authors tested a panel of oligosaccharides, synthetically prepared or from natural sources, for their ability to inhibit the binding of E-selectin-transformed COS cells to glycolipids containing the sLe^x epitope, or of radiolabeled HL60 promyelocytic leukemia cells (which are known to express sLe^x) to human umbilical vein endothelial cell (HUVEC) monolayers stimulated with human interleukin-1β. From their data, the authors conclude that the sLe^x determinant and related structures are bound by E-selectin. Substitution of a glucose residue for the reducing GlcNAc residue of sLe^x tetrasaccharide was found to generate a more effective inhibitor of the E-selectin–ligand interaction.

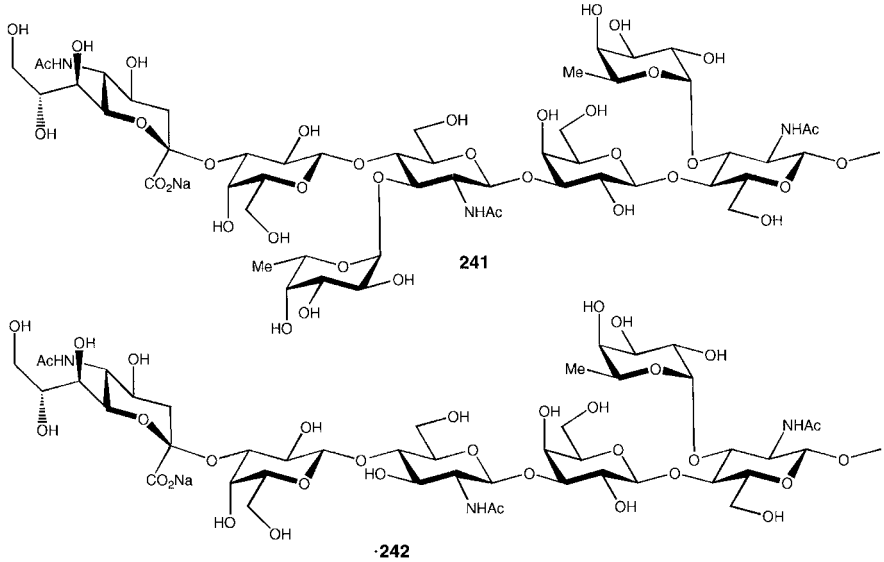


FIG. 13.

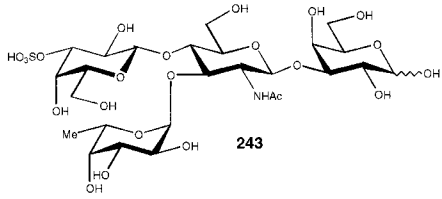
Substitution of the nonreducing, α-(2 → 3)-linked Neu5Ac residue by a (2 → 6)-linked Neu5Ac residue abolishes activity, whereas exchange of an *N*-glycoloyl for the *N*-acetyl group of Neu5Ac does not affect the selectin–ligand interaction. A glycolipid hexasaccharide determinant containing the sLe^a structure linked to a reducing lactose disaccharide partially inhibited E-selectin–ligand interactions; this finding was explained by the authors on the basis of similar epitopes being presented by the sLe^x and sLe^a antigens (compare Fig. 1). The VIM-2 determinant (CD65-antigen), comprising a sialyl-*N*-acetyllactosamine trisaccharide linked to a reducing Le^x trisaccharide, was found only weakly inhibitory compared to sLe^x. Figure 13 shows the VIM-2 (CD65) determinant **242** in relation to the sialyl-di-Le^x determinant **241.** Both determinants have been synthesized by organic-chemical (Section IV. 2) and by combined chemical and enzyme-catalyzed protocols.[392]

Berg *et al.* investigated the specificity of E-selectin binding to a large panel of carbohydrate structures.[182] The assay used is based on cells of the murine pre-B cell line L1-2 transfected with E-selectin cDNA. These cells express functional E-selectin and were studied with respect to their binding to a panel of neoglycoproteins coated on microtiter wells or glass slides (compare Assays, Section II.5.b). Each neoglycoprotein had been constructed so as to present a specific carbohydrate determinant, including sLe^x, sLe^a, Le^x, Le^a, the difucosylated structures Le^b\{α-L-Fuc-(1 → 2)-β-D-Gal-(1 → 3)-[α-L-Fuc-(1 → 4)]-β-D-GlcNac\} and Le^y\{α-L-Fuc-(1 → 2)-β-D-Gal-(1 → 4)-[α-L-Fuc-(1 → 3)]-β-D-GlcNac\}, the Landsteiner (ABH) blood group determinants H type 1

[α-L-Fuc-(1 → 2)-β-D-Gal-(1 → 3)-β-D-GlcNac], H type 2 [α-L-Fuc-(1 → 2)-β-D-Gal-(1 → 4)-β-D-GlcNac], A {α-D-GalNAc-(1 → 3)-[α-L-Fuc-(1 → 2)]-β-D-Gal}, and B {α-D-Gal-(1 → 3)-[α-L-Fuc-(1 → 2)]-β-D-Gal}, the core tetrasaccharides lacto-*N*-tetraose [β-D-Gal-(1 → 3)-β-D-GlcNAc-(1 → 3)-β-D-Gal-(1 → 4)-D-Glc] and lacto-*N*-neotetraose [β-D-Gal-(1 → 4)-β-D-GlcNAc-(1 → 3)-β-D-Gal-(1 → 4)-D-Glc], the corresponding mono- and di-fucosylated pentaoses and hexaoses, some disaccharide determinants such as maltose [α-D-Glc-(1 → 4)-D-Glc], lactose [β-D-Gal-(1 → 4)-D-Glc] and cellobiose [β-D-Glc-(1 → 4)-D-Glc), and a variety of others. Significant binding of the E-selectin cDNA transfectants was observed to the sLe^x and sLe^a-containing matrices only, binding to sLe^a-containing neoglycoproteins being slightly stronger than that to the sLe^x matrix. A monoclonal antibody, HECA-452, which had been identified[393] as recognizing sLe^x and other E-selectin ligand glycans, was also found to bind both sLe^x and sLe^a. Hard-sphere exo-anomeric (HSEA) calculations[394] were performed on these two hexasaccharides. The results indicate that sLe^a and sLe^x epitopes are closely similar except for the inverted position of the GlcNAc residue (Fig. 1). Presumably, the determinants recognized by E-selectin and HECA-452 comprise the NeuAc and Fuc residues, similarly positioned on one face of both sLe^x and sLe^a. Berg *et al.* noted the relevance of their findings to the pathology of cancer, where circulating mucins exposing sLe^a and sLe^x determinants may block selectin-mediated defense mechanisms and thus contribute to the immune suppression observed in cancer patients (compare Section III.4.).

Feizi and her associates[183] studied the binding of ^{3}H-labeled Chinese hamster ovary (CHO) cell transformants, expressing different levels of E-selectin at the cell surface, to a range of neoglycolipids separated on thin-layer chromatography plates or coated on microtiter plates (Section II.5). The cells expressing the highest levels of E-selectin were found to bind moderately to glycolipids containing lacto-*N*-fucopentaose II β-D-Gal-(1 → 3)-[α-L-Fuc-(1 → 4)]-β-D-GlcNAc-(1 → 3)-β-D-Gal-(1 → 4)-β-D-Glc, which contains a nonreducing Le^a determinant, and lacto-*N*-fucopentaose III β-D-Gal-(1 → 4)-[α-L-Fuc-(1 → 3)]-β-D-GlcNAc-(1 → 3)-β-D-Gal-(1 → 4)-β-D-Glc, which contains a nonreducing Le^x determinant. Strong binding of all E-selectin-expressing CHO cells was observed to the glycolipids containing the glycan 3′-sialyl lacto-*N*-fucopentaose II (containing a nonreducing sLe^a determinant) and 3′-sialyl-lacto-*N*-fucopentaose III (containing a nonreducing sLe^x determinant). Significantly, the moderately binding, nonsialylated Lewis glycolipids did not bind CHO transformant cells expressing lower levels of E-selectin. Presumably, the stronger binding of cells to the sialyl Lewis determinants reflects the role of these glycans as physiological ligands of E-selectin.

b. Glycans Containing Nonreducing, 3-Sulfated Gal Residues.—Yuen *et al.*[184] examined a panel of neoglycolipids comprising acidic glycans from an



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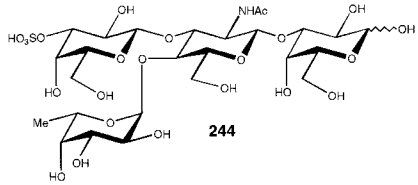

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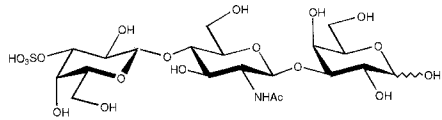

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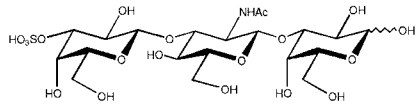

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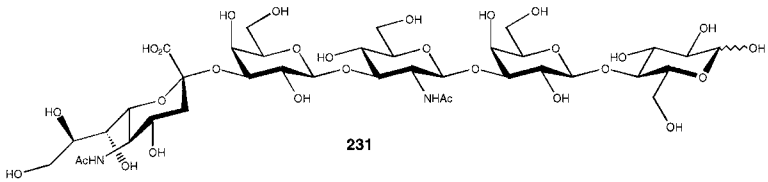

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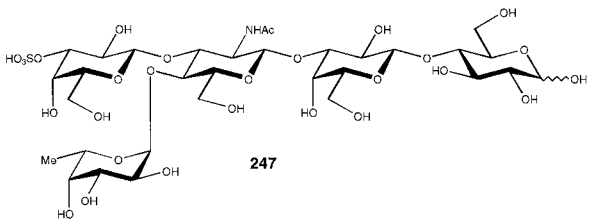

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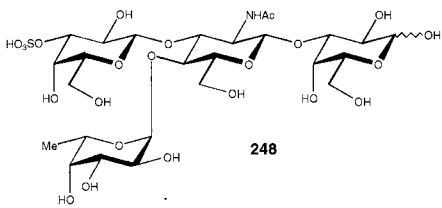
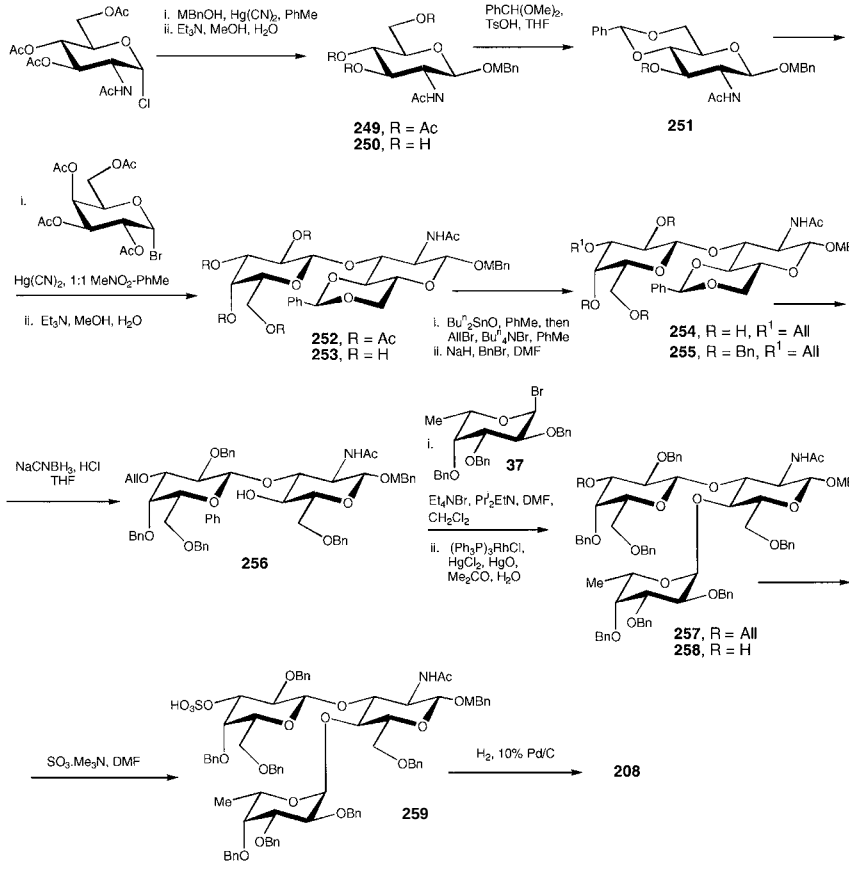


248

ovarian cystadenoma glycoprotein. The glycans were released from the glycoprotein by β-elimination (compare Section II.3.c), attached to L-1,2-hexadecanoyl-*sn*-glycero-3-phosphoethanolamine by reductive amination,[186] separated by thin-layer chromatography, and examined for their ability to bind to CHO cells transfected with E-selectin cDNA. Among several larger, binding neoglycolipids, a fraction was identified, by liquid secondary ion mass-spectral analysis,[188, 395] as a 1 : 1 mixture comprising the 3′-sulfated Le^x and Le^a tetrasaccharide glycans **243** and **244.** This glycolipid mixture was also investigated in conjunction with

L-selectin. Green *et al.* reported[396] that binding of a soluble fusion protein of rat L-selectin and human IgG_1 to glycans **243** and **244** was stronger than binding of that fusion protein, under analogous conditions, to neoglycolipid matrices presenting the sLe^x and sLe^a determinants (Fig. 1; compare Section IV.2). Neoglycolipids comprising the defucosylated glycans **245** and **246** bound L-selectin less strongly than the fucosylated counterparts, but did not bind E-selectin at all. Conversely, L-selectin is not bound by the Le^x and Le^a determinants that weakly bind E-selectin. Among defucosylated structures, **245**/**246** bound L-selectin more strongly than did 3′-sialyl-lacto-*N*-tetraose **231.** Sulfatide (3-O-sulfated galactosylceramide) was found to weakly bind L-selectin, in agreement with previous results obtained by Rosen's group.[397,110] From their binding studies, Green *et al.* concluded[396] that 3-linked sulfate on a nonreducing Gal, GalNAc, or GlcA residue, or on a subterminal Gal residue, importantly contributes to the binding of L-selectin to the respective glycans, and that the contribution of sulfate is more important to the binding of L-selectin than to the binding of E-selectin in the chromatogram binding assay. Yuen *et al.*[185] investigated the binding of [^{3}H]thymidine-labeled CHO cells, transformed with E-selectin cDNA, to a large panel of neoglycolipid matrices prepared with oligosaccharides corresponding to sialyl-Lewis, sulfated Lewis, and Lewis determinants. These were synthetically prepared in the laboratories of Hasegawa and Kiso,[398] Nicolaou (compare Section IV.2) and Lubineau[399] (for example, compounds **114, 193, 201, 247,** and **248**), and some were derived from natural sources. The account by Lubineau and his associates[399] is the first to describe a synthesis of the 3′-sulfated Le^a trisaccharide **208.** Starting from the acetochloro derivative of GlcNAc[289] and 4-methoxybenzyl alcohol (Scheme 4), the authors prepared the glycoside **249** under catalysis by mercury(II) cyanide in toluene (96%). Following the cleavage of the acetate protecting groups by the action of triethylamine in methanol–water, the resulting glycoside **250** was treated with benzaldehyde dimethyl acetal in the presence of *p*-toluenesulfonic acid in tetrahydrofuran to afford the 4,6-benzylidene acetal **251** (93%). Glycosylation of acceptor **251** with "acetobromogalactose" under promotion by mercury(II) cyanide in 1 : 1 nitromethane–toluene afforded the disaccharide derivative **252** in quantitative yield. Compound **252** was O-deacetylated by treatment with triethylamine in methanol–water; the resulting intermediate **253** was regioselectively converted into the 3′-allyl ether **254** by way of the corresponding stannylidene derivative in 77% yield (dibutyltin oxide in toluene; then allyl bromide in the presence of tetrabutylammonium bromide in toluene). Per-O-benzylation (sodium hydride and benzyl bromide in *N,N*-dimethylformamide) gave **255** in 78% yield (65% if N-benzylated material was not recycled). Glycosyl acceptor **256** was then obtained in 81% yield by regioselective opening of the benzylidene acetal by the action of sodium cyanoborohydride and hydrogen chloride in tetrahydrofuran.[327] Compound **256** was fucosylated with per-benzylated fucosyl bromide donor[400] **257** under halide inversion conditions[294] (tetraethylammonium bromide, diisopropylethylamine in dichloromethane–*N,N*-dimethylformamide; compare Section IV.1)

to give **257** in 80% yield. Sequential allyl rearrangement and hydrolysis of the intermediate propenyl ether [tris(triphenylphosphine)rhodium chloride, then mercury(II) chloride, mercury(II) oxide in acetone–water] afforded alcohol **258** in 67% yield (90% with recycling of unchanged starting material). Compound **258** was converted into the 3′-O-sulfated derivative **259** by the action of sulfur trioxide–trimethylamine complex in *N*,*N*-dimethylformamide (82%). Finally, the persistent benzyl ether protecting groups were removed by catalytic hydrogenation over 10% palladium-on-charcoal to give the target structure **208** in 80% yield. Subsequently, Lubineau *et al.* reported[401] on the synthesis of the sulfated Le^a pentasaccharide derivative, **247;** this target structure was prepared by glycosylation of a protected lactose intermediate with a Le^a trisaccharide trichloroacetimidate donor derived from intermediate **257** (Scheme 41). The contributions of Lubineau and his



SCHEME 41

associates to the synthesis of sulfated Le^a and Le^x structures have been reviewed.[402] The total synthesis of a sulfated Le^x pentaosyl ceramide has been reported[403] by Nunomura *et al.* Syntheses of sulfated oligosaccharides have also been discussed in a review by Haque and Ippolito.[404]

The results obtained by Yuen *et al.*[185] indicated that, of the compounds examined by that date, the sulfated Le^a pentasaccharide derivative **247** is the most potent E-selectin ligand glycan, both in terms of binding E-selectin-expressing CHO cells to a neoglycolipid matrix and as a soluble inhibitor of such binding. Sulfated Le^a penta- and tetra-saccharides **247** and **248** had IC_{50} values of 5.6×10^{-8} and 7.0×10^{-8}, respectively, as inhibitors of cell binding to a sLe^a pentasaccharide neoglycolipid matrix. The corresponding sLe^a and sLe^x pentasaccharides were ~5 and 15 times weaker inhibitors, while the sulfated Le^x tetrasaccharide was 100 times weaker. Binding strength was clearly higher with increased oligosaccharide chain length.

c. The Trisaccharide Determinant β-D-GalNAc-(1 → 4)-[α-L-Fuc-(1 → 3)]-β-D-GlcNAc Is an E-Selectin Ligand Glycan.—Grinnell *et al.* discovered that human protein C, an anticoagulant serine protease, inhibits the binding of (the sLe^x expressing) U937 cells to TNF-activated human umbilical vein endothelial cells.[405] This inhibitory effect was shown to be independent of the serine protease activity of protein C and was correlated with the presence, in protein C, of the determinant β-D-GalNAc-(1 → 4)-[α-L-Fuc-(1 → 3)]-β-D-GlcNAc[406] (**260**), designated PC293, an unusual nonreducing-terminal element of N-linked glycans that had been previously identified on human urokinase,[407] on *Schistosoma mansoni* glycoproteins,[408] on a phospholipase from honeybee venom,[409] and on bovine proopiomelanocortin.[410] The PC293 determinant **260** was found to occur on biantennary N-linked glycans of the complex type; the ligand glycans of protein C resemble thus the structures that have been identified by Patel *et al.*[81] as high-affinity ligand glycans binding to E-selectin (Fig. 3). Monovalent model trisaccharides representing the PC293 determinant **260** have been synthesized by the groups of Vliegenthart[411] and Kosma.[412] Matta and his associates[413] have synthesized a model compound representing **260** and an inhibitor **261** corresponding to a core-2 mucin glycan of which the PC293 determinant constitutes one antenna, the sialyl-T-determinant the other (Section VI.8).

d. Segments of the sLe^x Structure That Do Not Contribute to Selectin Binding.—From several studies of the natural ligand glycans of E-selectin, segments of the sLe^x structure have been identified that do not provide specific contributions to E-selectin binding (Fig. 14a–c). Specifically, replacement of the branching GlcNAc residue of sLe^x by Glc, *N*-acetylglucosaminitol, or glucitol does not significantly impair E-selectin binding under static conditions; similarly,

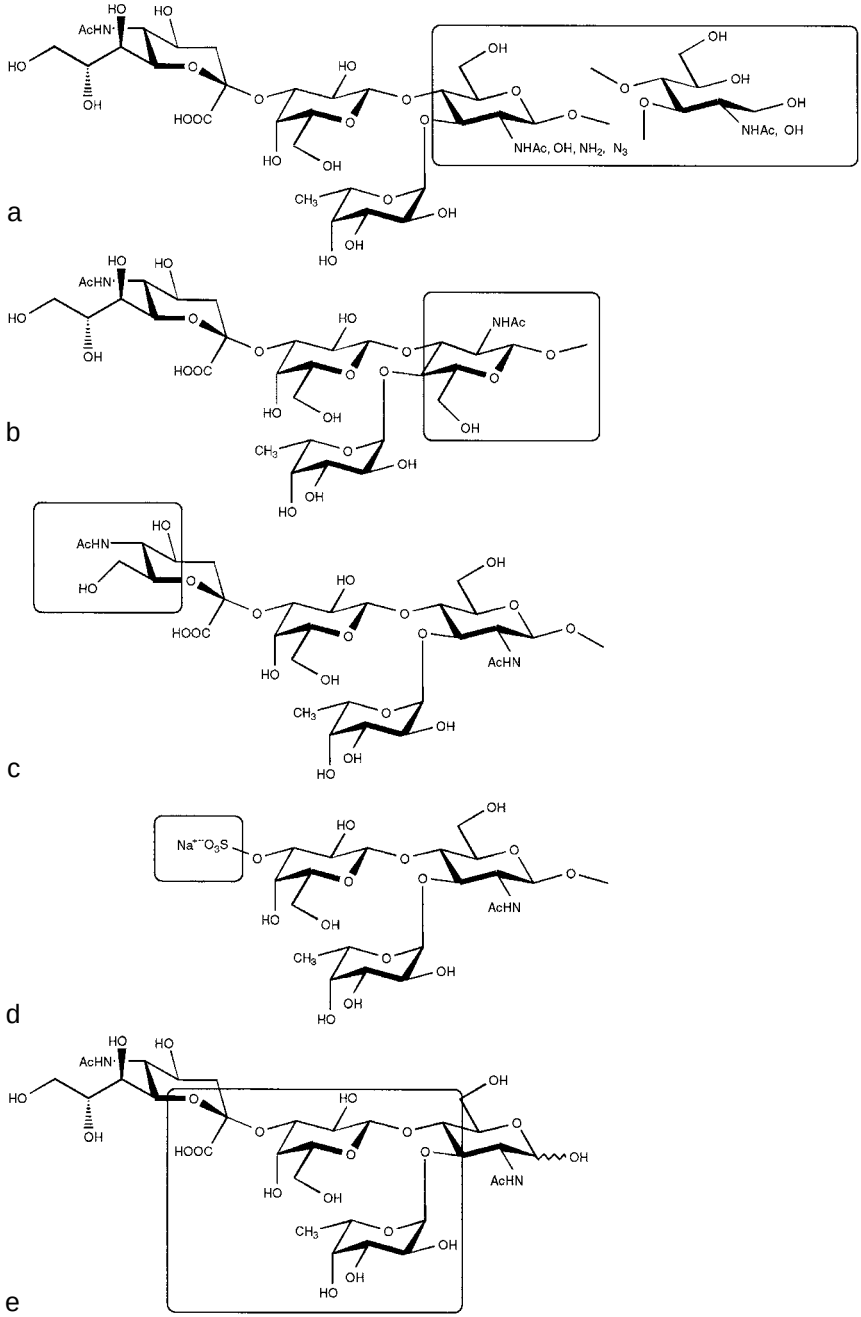
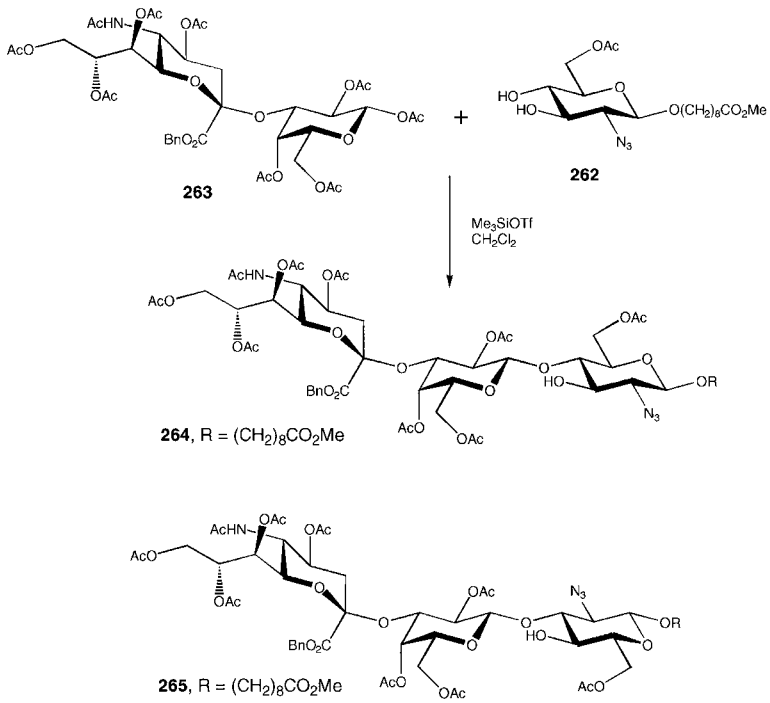


FIG. 14.

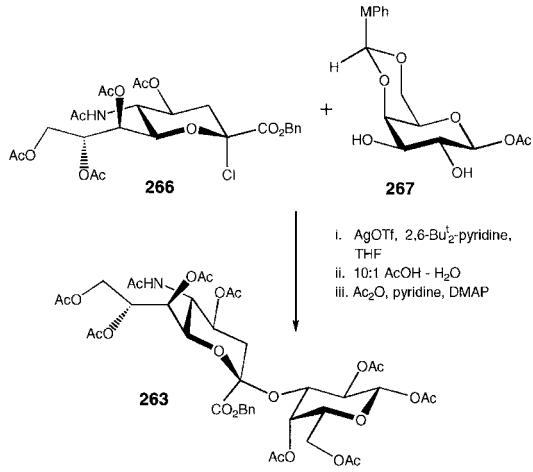
exchanging the points of attachment of the β-D-Gal and the α-L-Fuc residues on the branching GlcNAc residue (change from sLe^x to sLe^a) causes no significant change in binding potency. 3′-Sulfate substituents on Le^x are capable of replacing the entire Neu5Ac residue (Fig. 14d). From such studies with naturally occurring ligand glycans, a limited segment of the sLe^x determinant is identified that is essential for binding to E-selectin under static conditions (enclosed in the rectangle in Fig. 14e).

2. Molecular Variations at Individual Positions of the Constituent Glycosyl Residues of Sialyl-Lewis^x or Sialyl-Lewis^a

a. Some Variations at the *N*-Acetylglucosamine Residue.—For one of the early structure–activity studies, Venot and his group[414] at Chembiomed Ltd. prepared analogues of sLe^x and sLe^a containing unnatural substituents at C-2 of the GlcNAc residue. In an interesting approach (Scheme 42), the glycosylation of the azido diol acceptor **262** with the Neu5Ac-Gal block donor **263** in the presence of trimethylsilyl trifluoromethylsulfonate[415] simultaneously afforded the type 2 and type 1 trisaccharide derivatives **264** and **265** in 42 and 24% yields, respectively.[416]



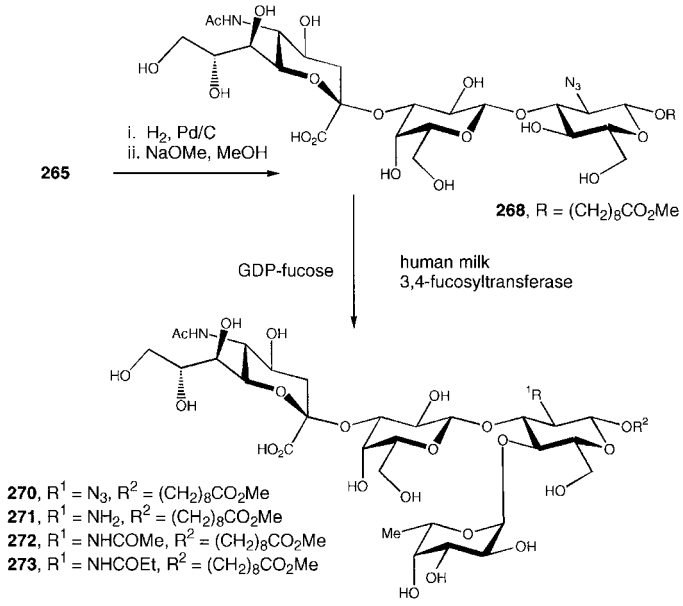
SCHEME 42



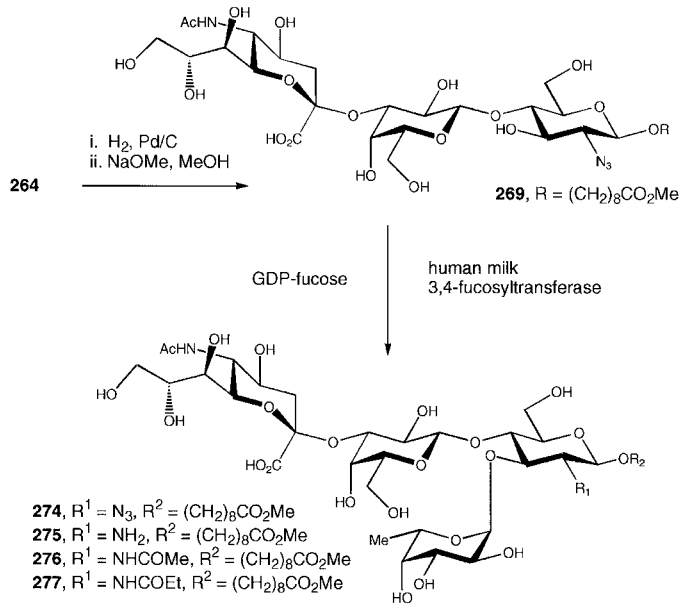
SCHEME 43

The α-Neu5Ac-(2 $\rightarrow$ 3)-D-Gal block **263** was prepared (Scheme 43) from the benzyl ester halide **266** and the galactose-derived diol acceptor **267** under promotion by silver trifluoromethanesulfonate in tetrahydrofuran in the presence of 2,6-di-*tert*-butylpyridine. The crude disaccharide derivative obtained was hydrolyzed in 10 : 1 acetic acid–water, and the resulting 2,4,6-triol peracetylated to provide **263** in an overall yield of 46% (based on **267**). To obtain the benzyl ester halide **266,** Neu5Ac was neutralized with cesium carbonate and the cesium salt stirred with a solution of benzyl bromide in *N*,*N*-dimethylformamide.[417] Treatment of the benzyl ester with acetic anhydride in pyridine afforded the penta-*O*-acetyl derivative,[418] which was allowed to react with acetyl chloride and concentrated hydrochloric acid in dichloromethane to give halide **266.**[419] To prepare **267,** 2,3,4,6-tetra-*O*-benzyl-D-galactopyranose was 1-O-acetylated by way of the corresponding 1,2,3-trialkylpseudourea[420] and the product subjected to hydrogenation over 5% palladium-on-charcoal in acetic acid. The crude 1-acetate was then treated with 4-methoxybenzaldehyde dimethyl acetal in acetonitrile to give **267** ($\sim$46% over three steps). Disaccharide blocks for introduction of the α-Neu5Ac-(2 $\rightarrow$ 3)-β-D-Gal disaccharide determinant have been prepared in the form of the thioglycoside **109** (Ref. 319) and as analogous trichloroacetimidate donors.[421,422]

The type 1 and type 2 trisaccharide derivatives **265** and **264** were each converted into the free azido carboxylic acid in quantitative yields by selective hydrogenation over 5% palladium-on-carbon in ethyl acetate. Zemplén saponification gave the trisaccharide derivatives **268** and **269** (91 and 85%, respectively). Transfer of fucose from GDP-fucose, catalyzed by human milk α-3/4- fucosyltransferases, to the type 1 and type 2 acceptors **268** (Scheme 44) and **269** (Scheme 45) proceeded at rates comparable to those of the natural substrates and afforded tetrasaccharides **270** and **274** in quantitative yields. From the azido tetrasaccharides **270**



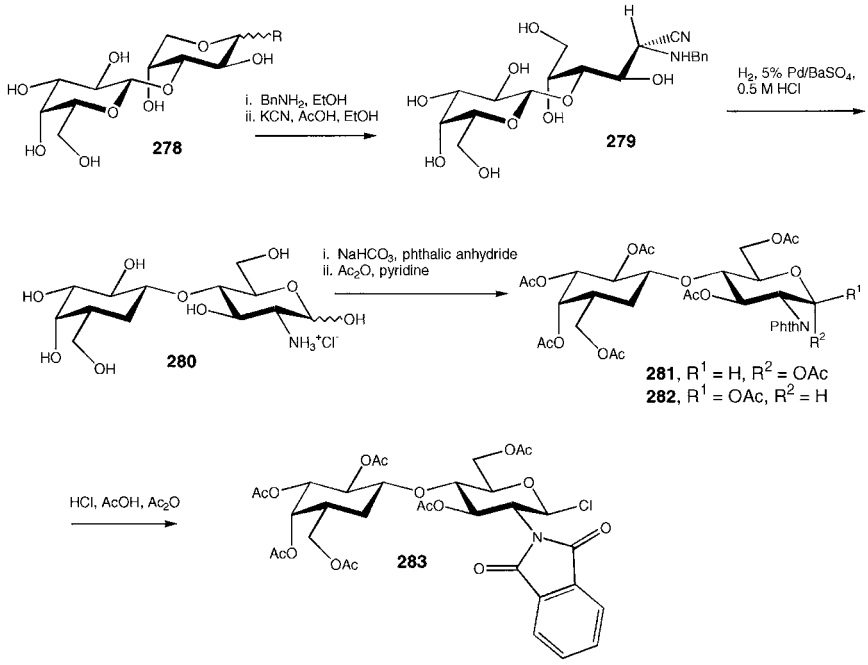
SCHEME 44



SCHEME 45

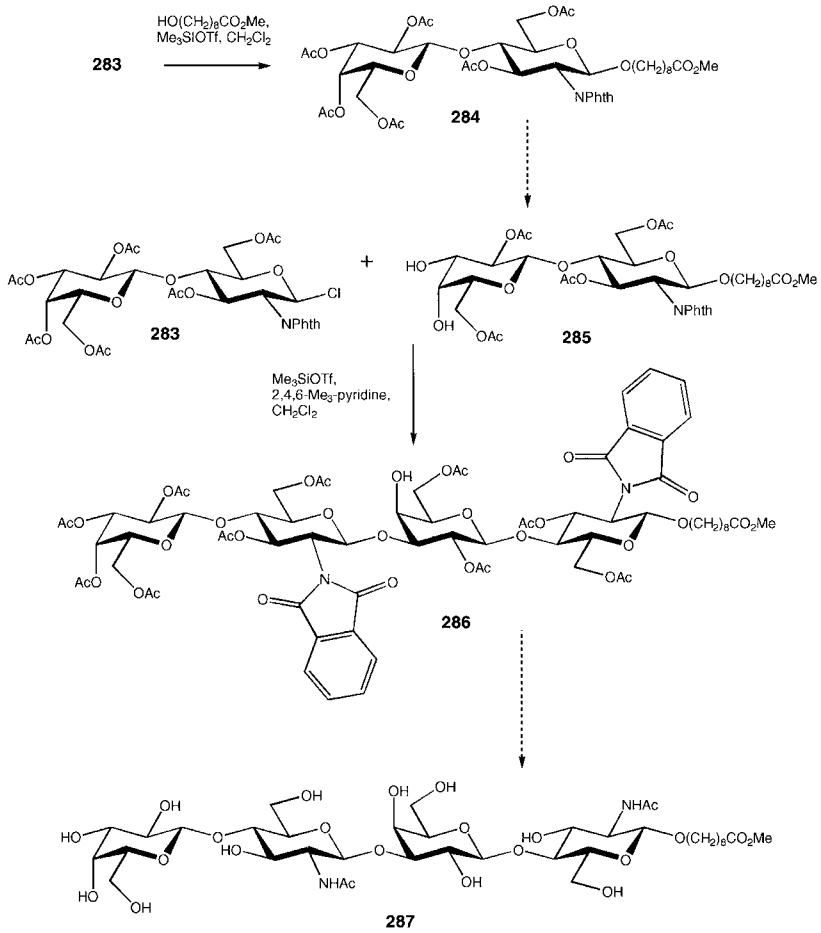
and **274,** the corresponding amino, acetamido, and propionamido derivatives **271** and **275, 272** and **276,** and **273,** and **277** were also prepared following reduction by the action of hydrogen sulfide (Schemes 44 and 45). The type 1 and type 2 tetrasaccharide derivatives **270**–**273** (corresponding to sLe^a) and **274**–**277** (corresponding to sLe^x) were studied in a cell-free selectin-ligand binding assay and in cell adhesion assays by Bevilacqua and his associates (Section II.5). In the cell-free assay, the amino derivative of sLe^a (**271**) had $IC_{50} = 0.02$ m*M* and the corresponding azido derivative **270** had $IC_{50} = 0.06$ m*M*. The sLe^x amino derivative **275** had a value of 0.08. Interestingly, the β-8-(methoxycarbonyl)octyl tetrasaccharide glycosides of sLe^a or sLe^x had approximately double the inhibitory potency of the corresponding reducing tetrasaccharides. This finding was explained by the authors as possibly due to a secondary binding interaction with a hydrophobic area outside the carbohydrate-binding domain (cf. Section VI.5). Similar effects were reported subsequently following binding studies with a sLe^x (2-tetradecyl)hexadecyl glycoside.[423] Previously, enhancement of carbohydrate–receptor binding had been found for hydrophobic aglycons of α-Neu5Ac-(2 → 6)-D-Gal/GalNAc binding to *Sambucas nigra* L. (elderberry) bark lectin by Goldstein[424] and for the binding of Neu5Ac glycosides to influenza A virus neuraminidase by the group of Knowles.[425] Attachment of hydrophobic groups to the glucosamine unit of the sLe^x backbone was subsequently used as a device to create sLe^x analogues with enhanced binding potency[426,423] (cf. Section VI.5). An analogue of SLe^x containing "1-deoxy-GlcNAc" in place of the GlcNAc residue was found ∼20 times more potent than sLe^x as a ligand of P-selectin.[427] Öhrlein and his associates[428] at the CIBA laboratories (now Novartis) showed that glucosamine derivatives substituted with a large variety of N-linked acyl groups function as acceptor substrates of a bovine UDP Gal:N-acetylglucosaminide β-1,4-galactosyltransferase, enabling enzyme-catalyzed syntheses of a library of N-substituted lactosamine analogues. N-Linked groups include formyl, glycoloyl, thioglycoloyl, 3-nitropropanoyl, 3-guanidinopropanoyl, methanesulfonyl, *p*-toluenesulfonyl, urethane, urea, and thiourea groupings, and a range of others. Enzyme-catalyzed syntheses were extended[429] by Öhrlein *et al.* through the use of rat liver α-2,3-sialyltransferase to produce, from the library of lactosamine derivatives, an analogous library of sialyl lactosamine analogues. Finally, the authors used fucosyltransferase VI and GDP-fucose to transform the library of sialyl lactosamine analogues into a library of sLe^x analogues[430] comprising a wide range of unnatural, N-linked groups on the GlcN residue.

b. Some Variations at the Fucose Residue.—The VIM-2 epitope (CD-65 antigen, structure **242**) α-Neu5Ac-(2 → 3)-β-D-Gal-(1 → 4)-β-D-GlcNAc-(1 → 3)-β-D-Gal-(1 → 4)-[α-L-Fuc-(1 → 3)]-β-D-GlcNAc corresponds to a nonreducing sialyl lactosamine trisaccharide attached to a Le^x trisaccharide; it may therefore be considered a sialyl di-Le^x structure without the outer fucose residue. As part of



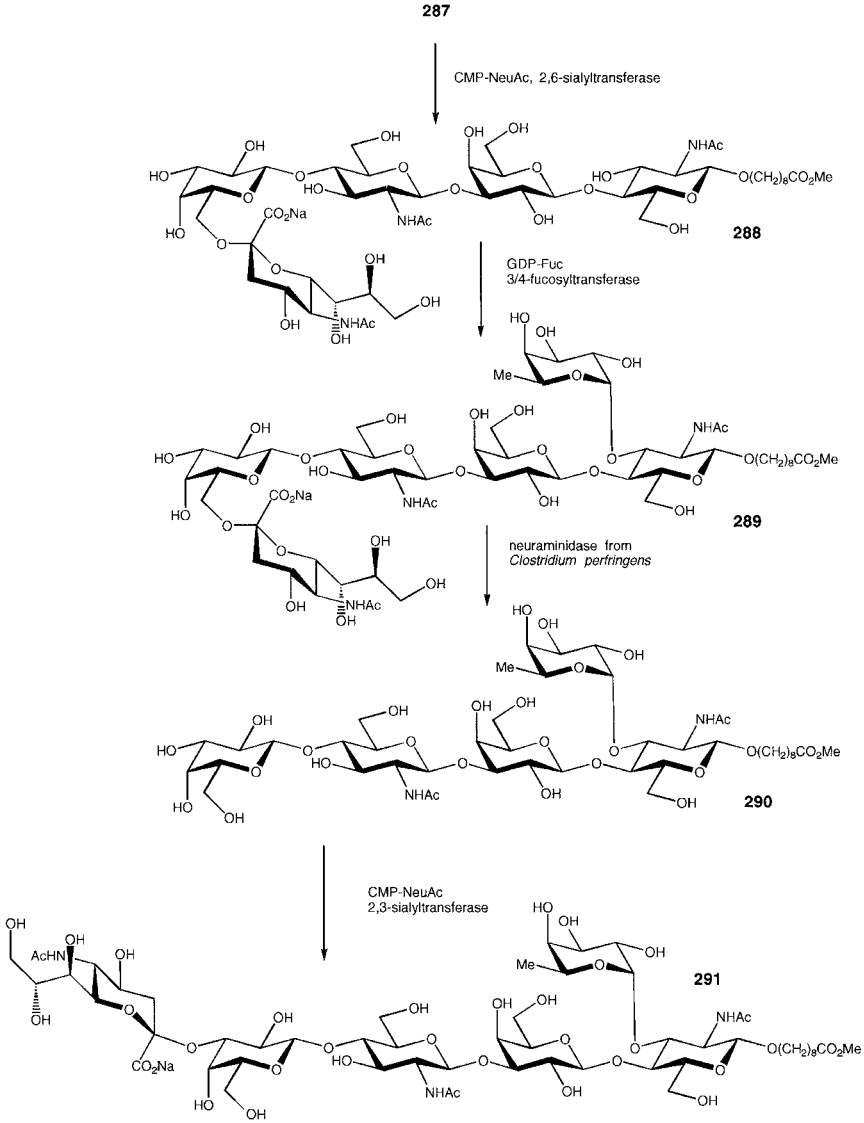
SCHEME 46

their structure–activity studies related to selectin–ligand interactions, a combined organic-chemical and enzyme-catalyzed synthesis of the CD65 determinant was undertaken by the Chembiomed group.[392,432] Suitable intermediates corresponding to the dimeric type-2 tetrasaccharide backbone were synthesized by organic-chemical methods according to the procedure of Alais *et al.*[433,434] Commercially available 3-*O*-β-D-galactopyranosyl-D-arabinose (**278** from lactose; Scheme 46) was treated with benzylamine to afford the corresponding *N*-benzylglycosylamine. Chain extension by the Kiliani reaction with hydrogen cyanide *in situ* gave the 2-benzylamino-2-deoxy-D-gluconononitrile derivative **279,** as previously described by Kuhn and Kirschenlohr[435,436] and Lee and Lee.[437] Compound **279** was subjected to hydrogenation over 5% palladium on barium sulfate in the presence of hydrochloric acid; the lactosamine hydrochloride **280** was neutralized with sodium hydrogen carbonate and converted by combined N-phthaloylation and O-acetylation into a mixture of the anomeric *N*-phthaloyl-1-acetates **281** and **282** (62% from **279**). From the mixture, the bulk of the β anomer **282** was obtained crystalline. The remainder was converted into pure β anomer by treatment with acetic anhydride containing a small amount of perchloric acid. Only the β anomer **282** is useful as a precursor of a halide donor and was converted into the β halide **283** by the action of hydrogen chloride in acetic acid–acetic anhydride in



SCHEME 47

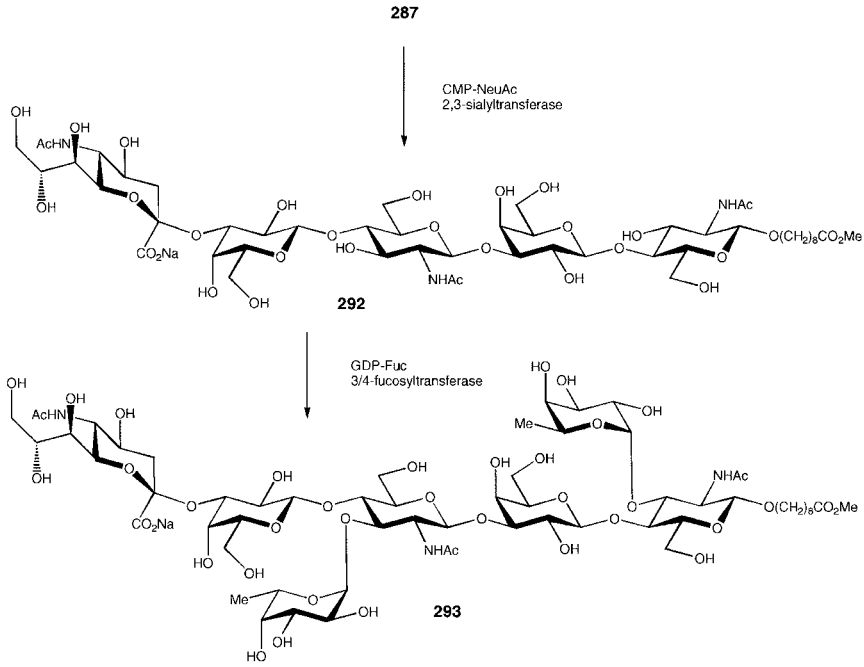
92% yield. The β-halide disaccharide donor **283** was used for the glycosylation of 8-carboxymethyloctanol[288] (Scheme 47) under promotion by trimethylsilyl trifluoromethanesulfonate in dichloromethane to form the spacer-linked precursor of the reducing lactosamine portion of the target tetrasaccharide (**284,** 60%). Intermediate **284** was converted into a glycosyl acceptor **285** by successive treatment with sodium methoxide in methanol–1,4-dioxane, regioselective 3′,4′-O-isopropylidenation under catalysis by *p*-toluenesulfonic acid in acetone,[456] per-O-acetylation (acetic anhydride–pyridine), and acid-catalyzed hydrolysis of the isopropylidene acetal (49% from **284**). The disaccharide diol acceptor **285** was glycosylated with the disaccharide β-chloride donor **283** under promotion by trifluoromethanesulfonic acid–2,4,6-trimethylpyridine in dichloromethane as



SCHEME 48

described by Alais and Veyrières[434] to afford the tetrasaccharide derivative **286.** The unprotected tetrasaccharide glycoside **287,** needed as the starting material for the enzyme-catalyzed part of the synthesis, was obtained from **286** by sequential treatment with hydrazine hydrate and N-acetylation. In the first enzyme-catalyzed step of the synthesis (Scheme 48), the tetrasaccharide derivative **287** was converted

into the 6‴-O-sialylated compound **288** under catalysis by β-D-Gal-(1 → 4)-β-D-GlcNAc-α-2,6-sialyltransferase[431] (EC 2.4.99.6) from rat liver (**291**). The 6‴-linked sialyl residue functions as a protecting group that prevents the transfer of a fucose residue to position 3″ of the chain-internal GlcNAc residue during the subsequent step. Under catalysis by the α-3,4-fucosyltransferase from human milk, only one fucosyl residue is transferred from GDP-fucose onto position 3 of the reducing-terminal GlcNAc residue of pentasaccharide **288** to form the 3-*O*-fucosyl hexasaccharide **289.** The 6‴-linked Neu5Ac unit was removed from **289** under catalysis by immobilized neuraminidase from *Clostridium perfringens*. Finally, a Neu5Ac unit was transferred from CMP-Neu5Ac onto position 3‴ of pentasaccharide **290** under catalysis by the Galβ1,3/4GlcNAc α-2,3-sialyltransferase from rat liver **291.** The tetrasaccharide **287** was also converted, by Venot and his associates, into the "dimeric sLe^x" structure **293** that has been described as a tumor-associated antigen[335] (Scheme 49; compare compounds **137** and **138,** Section IV.2.c). Transfer of a Neu5Ac unit from CMP-NeuAc under catalysis by 2,3-sialyltransferase from rat liver produces the pentasaccharide derivative **292.** Under catalysis by α-1,3/4-fucosyltransferase from human milk, compound **292** accepts two fucose units to afford the heptasaccharide **293** exclusively. A disadvantage of the procedure of Venot *et al.* is the partial hydrolysis of the methyl ester of the Lemieux spacer[288]



SCHEME 49

that occurs especially during prolonged incubations in aqueous systems. This necessitates re-esterification of the respective carboxylic acids by the action of diazomethane in methanol.[432] As reported by Tyrrell *et al.*,[391] the VIM-2 determinant (CD65 antigen) is but a weak inhibitor of E-selectin-ligand binding; this behavior underscores the requirement for the α-(1 $\rightarrow$ 3) or (1 $\rightarrow$ 4)-linked fucose[191] residue as part of the selectin ligand glycan. Brandley *et al.*[438] have performed structure–function studies with analogues of selectin ligand glycans. These authors found that sLe^x glycolipids containing 2-, 3-, and 4-deoxyfucose residues, when coated on microtiter wells, would not bind E- or L-selectin–IgG chimeras. The corresponding P-selectin IgG chimera was bound by the sLe^x glycolipid matrices comprising the 2-deoxy- and 4-deoxyfucose residues. These findings indicated that, for binding of E- or L-selectins, none of the hydroxyl functions of the fucose residue is dispensable, whereas for P-selectin binding, an intact 2,3- or 3,4-diol grouping is sufficient. In a similar investigation, Ramphal *et al.* studied the contributions to E-selectin binding of the hydroxyl groups and methyl group of the fucose residue by comparing the inhibitory potency of the sLe^x–pentasaccharide glycoside, **299,** to that of its analogues **300, 301, 302,** and **303** containing 2-deoxyfucose, 3-deoxyfucose, 4-deoxyfucose, and D-arabinose in place of fucose (Fig. 15).[439] The deoxy fucose residues were introduced by Mukaiyama-type glycosylations of the acceptor **294** with deoxyfucosyl fluoride donors **295, 296, 297,** based largely on previous work by Lindhorst and Thiem,[440,441] and with D-arabinopyranosyl bromide donor **298** according to a halide inversion protocol.[294] In an assay of HL60 cell binding to recombinant soluble E-selectin, analogues **300, 301,** and **302** were not inhibitory up to concentrations of 6.5 m*M*. Compound **299** had IC_{50} 1 m*M,* whereas the arabinose analogue **303** had IC_{50} 5 m*M*. These findings were interpreted to underscore the need for all three hydroxyl functions of the fucose residue during binding of sLe^x to E-selectin, presumably due to their involvement in chelation of the Ca^{2+} ion.

c. Some Modifications at the Galactose Residue.—Stahl *et al.*[442] synthesized sLe^x analogues containing three types of modified Gal residues (Fig. 16). The precursors of these Gal residues had been supplied by Lipták[442] in the form of protected glycosyl acetates. Stahl *et al.* glycosylated a protected disaccharide block acceptor **305,** corresponding to the segment α-L-Fuc-(1 $\rightarrow$ 3)-β-D-GlcNAc, with trichloroacetimidate donors of the Gal analogues (for example, **304;** Scheme 50), then glycosylated the Le^x analogues (for example, **306**) with Neu5Ac donor **105** (Section IV.2.a) under promotion by methylsulfenyl bromide and silver triflate in dichloromethane–acetonitrile at $-70 \rightarrow -40$ °C. Binding of all three sLe^x analogues (**307**–**309**) containing the modified Gal residues was weaker than that of sLe^x.[442] Komba *et al.*[443] prepared modified sLe^x glycolipids **317**–**319** containing modified Gal residues as shown in Fig. 17. These sLe^x analogues were assembled from a protected trisaccharide acceptor **310** corresponding to the segment

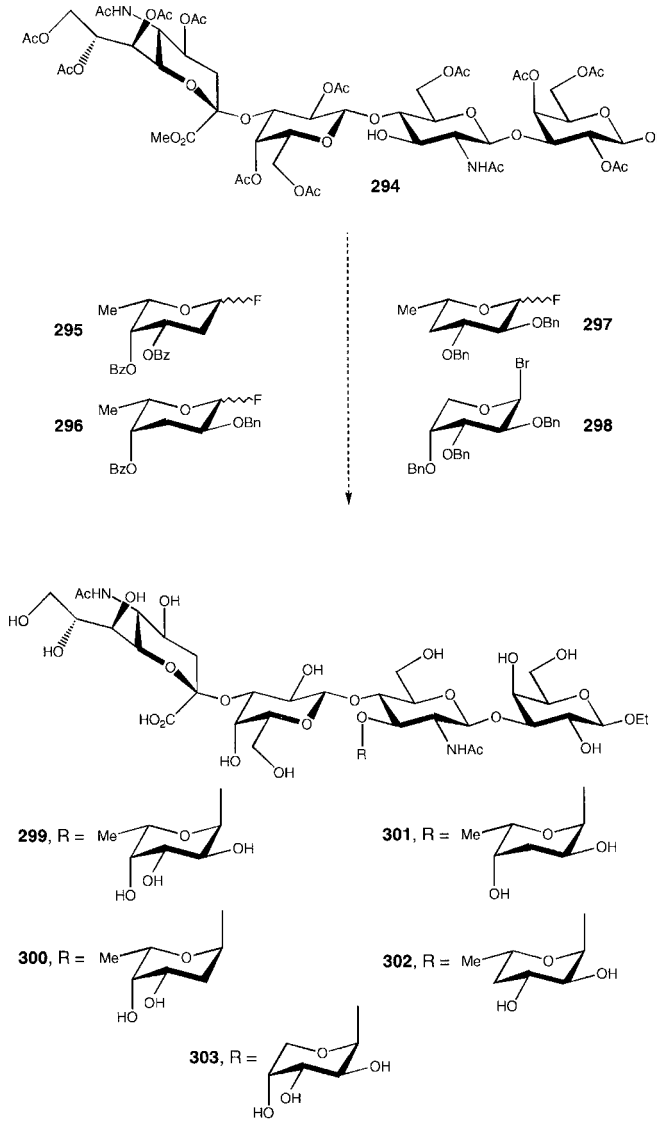


FIG. 15.

α-L-Fuc-(1 $\rightarrow$ 3)-β-D-GlcNAc-(1 $\rightarrow$ 3)-β-D-Gal by way of glycosylation, at O-4 of GlcNAc, with modified α-Neu5Ac-(2 $\rightarrow$ 3)-Gal disaccharide block donors, in analogy to the synthesis of sLe^x outlined in Schemes 15–18 (Refs. 318, 319). For the preparation of analogue **317** (Fig. 17), a disaccharide donor α-Neu5Ac-(2 $\rightarrow$ 3)-D-Fuc was constructed from Neu5Ac and D-fucose.[443] Modified Gal residues of

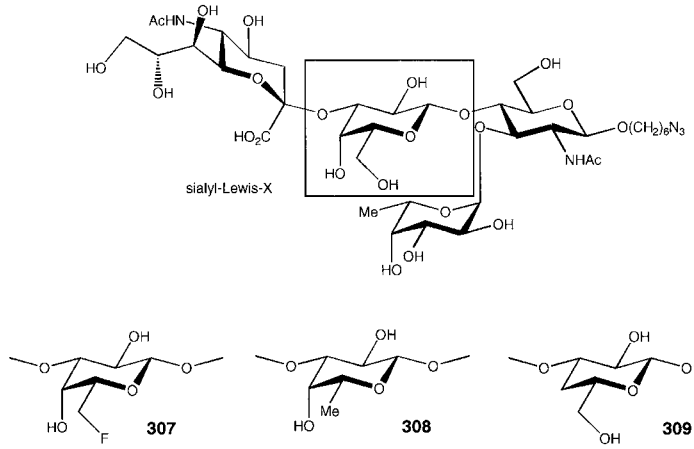
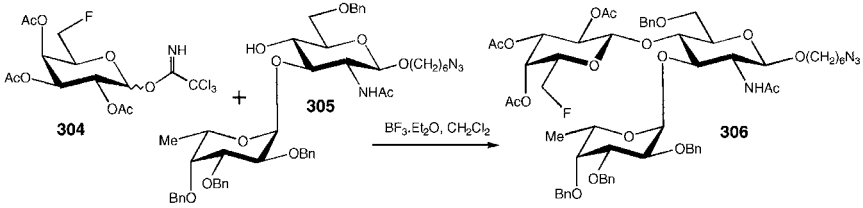


FIG. 16.



SCHEME 50

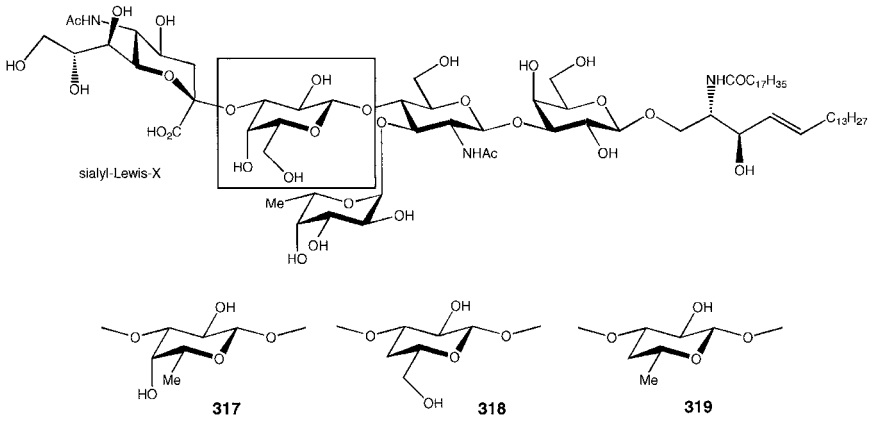
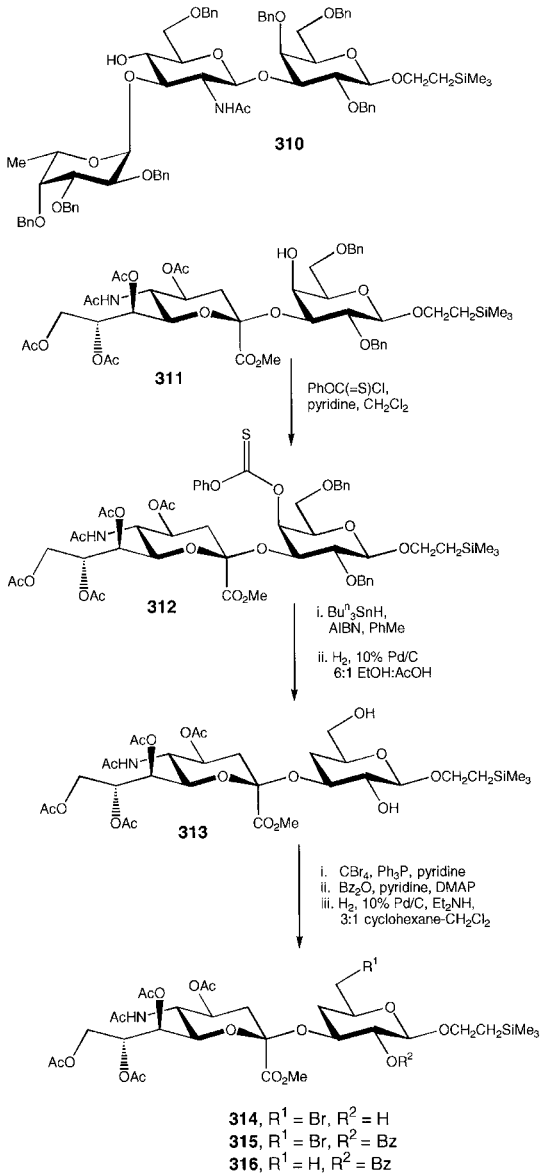


FIG. 17.

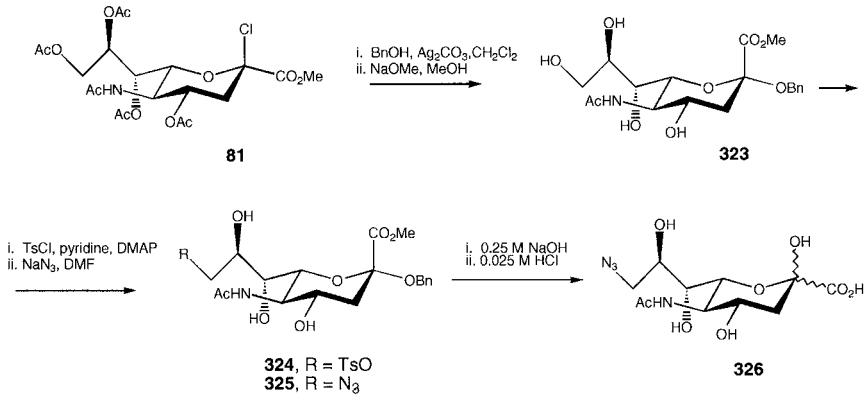
analogues **318** and **319** were introduced via disaccharide block donors **313** and **316** developed from the α-Neu5Ac-(2 $\rightarrow$ 3)-Gal disaccharide synthon **311.** The donor employed for the construction of **318** was obtained (Scheme 51) by way of (phenoxy)thiocarbonylation of OH-4 of Gal (**312**), followed by reduction by the action of tributyltin hydride in the presence of α,α'-azobis-isobutyronitrile to give **313.** From **313,** the disaccharide synthon **316,** comprising the dideoxy modification, was obtained by way of 6-bromination (**314**), 2-O-benzoylation (**315**), and catalytic hydrogenation. Analogues of sLe^x comprising the D-fucose residue **317** or the 4,6-dideoxy modification **319** were not bound by any of the selectins. P-Selectin, but not E- or L-selectin, bound the sLe^x glycolipid **318,** comprising the 4-deoxy modified Gal residue. Together, the results of Stahl *et al.* and of Komba *et al.* indicate a requirement for intact functionality at positions 4 and 6 of the Gal residue of sLe^x for E- and L-selectin–ligand interactions, and a requirement for OH-6 of Gal for the P-selectin–ligand interaction.

d. Some Modifications at the *N*-Acetylneuraminic Acid Residue.—Brandley *et al.* reported[438] that modifications of the glycerol side chain or the *N*-acyl group of the Neu5Ac residue have little effect on the binding of any of the selectins. Thus, binding of all three selectin–Ig chimeras was practically unaffected by exchange of the Neu5Ac residue for a 3-deoxy-D-glycero-D-galacto-2-nonulopyranosylonic (KDN, **320**), 5-acetamido-3,5-dideoxy-L-glycero-D-galacto-2-nonulopyranosylonic (8-epi-Neu5Ac, **321**), or 5-acetamido-3,5-dideoxy-D-galacto-2-octulopyranosylonic acid residue (**322**). Analogues of sLe^x containing the 3-deoxy-2-glyculopyranosylonic acid residues **321** and **322** were synthesized by Yoshida *et al.*[444] by way of disaccharide block synthons analogous to the α-Neu5Ac-(2 $\rightarrow$ 3)-D-Gal block of Hasegawa and Kiso[320] as described in Scheme 16. The Chembiomed group[445] devised methods for enzyme-catalyzed syntheses of sLe^x-related oligosaccharides containing modified Neu5Ac residues. Starting from the classical Neu5Ac halide **81** (Scheme 25; Ref. 310), these authors prepared the benzyl glycoside methyl ester **323** (56% from **81**) as a key intermediate for the synthesis of Neu5Ac analogues (Scheme 52). Compound **323** was regioselectively converted into a 9-*p*-toluenesulfonyl ester **324,** which, upon treatment with sodium azide in *N,N*-dimethylformamide, afforded the 9-azido-9-deoxy derivative **325** (53% from **323**). Analog **326** was obtained from **325** by sequential treatments with sodium hydroxide and dilute hydrochloric acid in 73% yield.[445] Following analogous findings with the 8-azido-8-deoxy derivative of 3-deoxy-2-octulosonic acid (Kdo),[446] the 9-azido-9-deoxy Neu5Ac derivative **326** is one of the early examples[447] of an artificially modified Neu5Ac derivative that is activated by CMP-*N*-acetylneuraminic acid synthetase and transferred, under catalysis by sialyltransferase, to natural acceptor glycans of glycoproteins.[448] In addition to the chemical synthesis of **326** originally performed by Brossmer and Rose,[447] an enzyme-catalyzed synthesis of the analogue was developed by the present author,[447] based on the formation of **326** from 2-acetamido-6-azido-2,6-dideoxy-D-mannose and

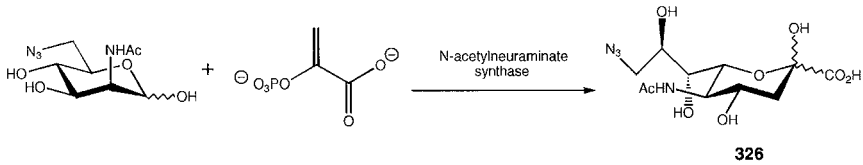


314, R^1 = Br, R^2 = H
315, R^1 = Br, R^2 = Bz
316, R^1 = H, R^2 = Bz

SCHEME 51

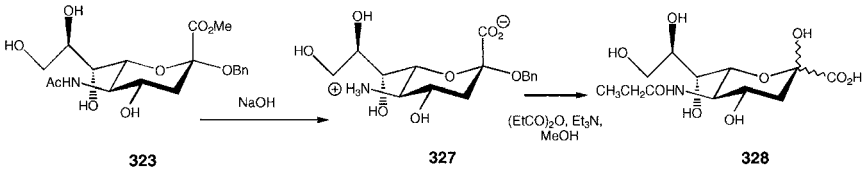


SCHEME 52



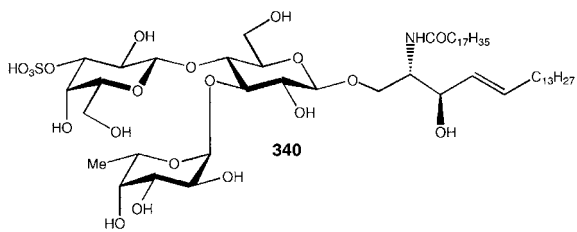
SCHEME 53

enolpyruvate phosphate under catalysis by *N*-acetylneuraminate synthase[450] from *Neisseria meningitidis* 60E (Scheme 53). The initial findings with **326** have later been extended by Brossmer and his associates[451] to include a range of 9-N-substituted Neu5Ac analogues that can be similarly activated and incorporated into cell-surface structures. From intermediate **323,** the amino acid **327** is obtained by treatment with sodium hydroxide (Scheme 54); compound **327** reacts with propionic anhydride to afford, upon catalytic hydrogenation, the *N*-propionyl analogue of Neu5Ac, **328** (72% from **323**). The 8,9-*O*-isopropylidene intermediate **329** was prepared in 88% yield by reacting **323** with 2,2-dimethoxypropane in the presence of *p*-toluenesulfonic acid (Scheme 55). Two additional Neu5Ac analogues were obtained from **329.** Following essentially the procedure of Zbiral *et al.*,[452] the reaction of **329** with *tert*-butyldimethylsilyl chloride regioselectively gave the 4-silyl



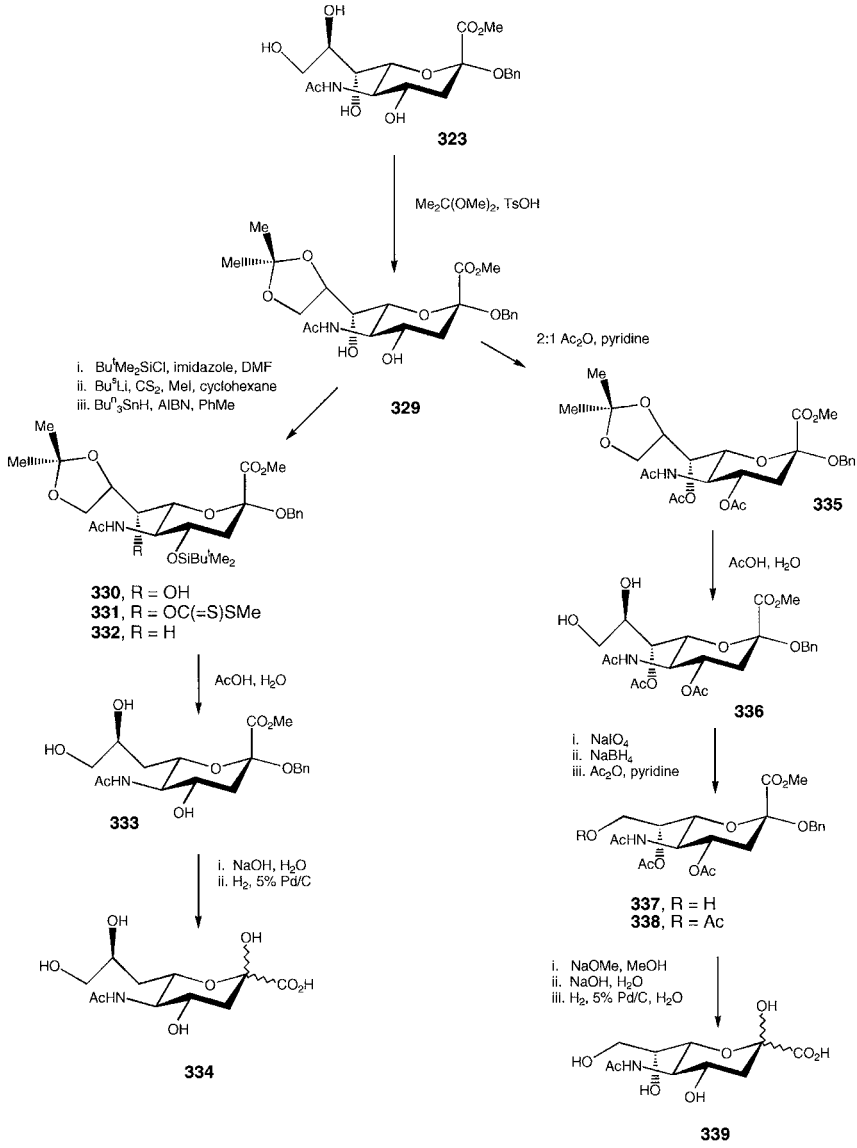
SCHEME 54

ether **330** in 92% yield. Treatment of **330** with *sec*-butyllithium followed by carbon disulfide and methyl iodide gave 7-xanthate **331** (65%) which was converted into the 7-deoxy derivative **332** by treatment with tributyltin hydride and α,α'-azobisisobutyronitrile in 70% yield. Compound **332** was treated with aqueous acetic acid to cleave the isopropylidene acetal and silyl ether groups; the intermediate **333** formed was subjected to saponification and catalytic hydrogenation to give the 7-deoxy-Neu5Ac analogue **334** in 77% yield from **332.** In another application of intermediate **329,** the 4,7-di-*O*-acetyl derivative **335** was obtained in 92% yield by reacting **329** with acetic anhydride in pyridine. Treatment of **335** with aqueous acetic acid gave the diol **336,** which, by sequential reactions with sodium periodate and sodium borohydride, was converted into the C_8-derivative **337,** characterized as the peracetylated compound **338.** Sodium methoxide-catalyzed methanolysis followed by alkaline saponification and catalytic hydrogenation gave the target structure **339** (66% from **335**). Several of the monosaccharide transformations with neu5Ac employed by the Chembiomed group are based on the elegant investigations of Zbiral and his associates at the University of Vienna; their work has been reviewed.[452] Analogues **326, 328, 334,** and **339** were converted, under catalysis by CMP-Neu5Ac synthetase, into the respective CMP-Neu5Ac analogues; using appropriate 2,3- or 2,6-sialyltransferases, the analogues were transferred onto a variety of acceptor glycan derivatives containing different spacer groups.[445] Sialylated analogues of type 1 or type 2 oligosaccharides were then fucosylated, under catalysis by α-1,3/4-fucosyltransferase from human milk, to afford the respective sLe^a or sLe^x analogues. In confirmation of the results obtained by Brandley *et al.*,[438] binding to the selectins was not found significantly affected by the modifications of Neu5Ac analogues **326, 328, 334,** or **339.** Indeed, Brandley *et al.* found that the Le^x trisaccharide derivative **340,** bearing a 3-linked sulfate group on the Gal residue, binds to E-selectin as efficiently as the sLe^x hexasaccharide ceramide derivative **114** (Scheme 18); binding of **340** to L- or P-selectin is stronger than that of **114.** These results confirm the results of Feizi and her associates.[396]



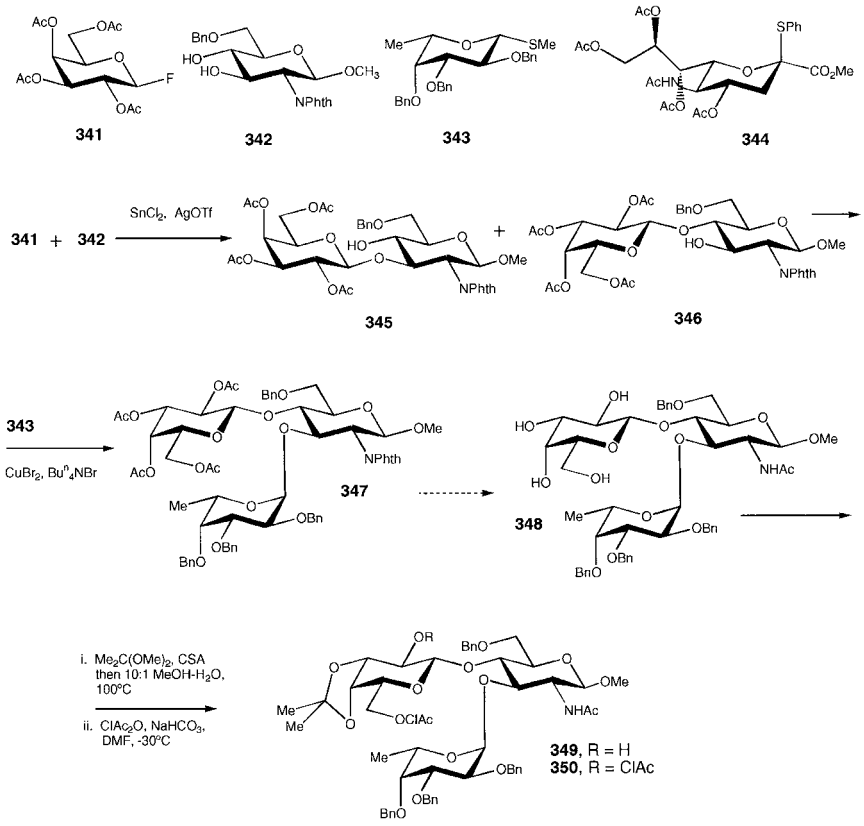
340

e. Sulfated Ligand Glycans of L-Selectin; Role of 6-Sulfation of the GlcNAc or Gal Residues.—Following the discovery of sLe^x-6′-sulfate as a "capping group"



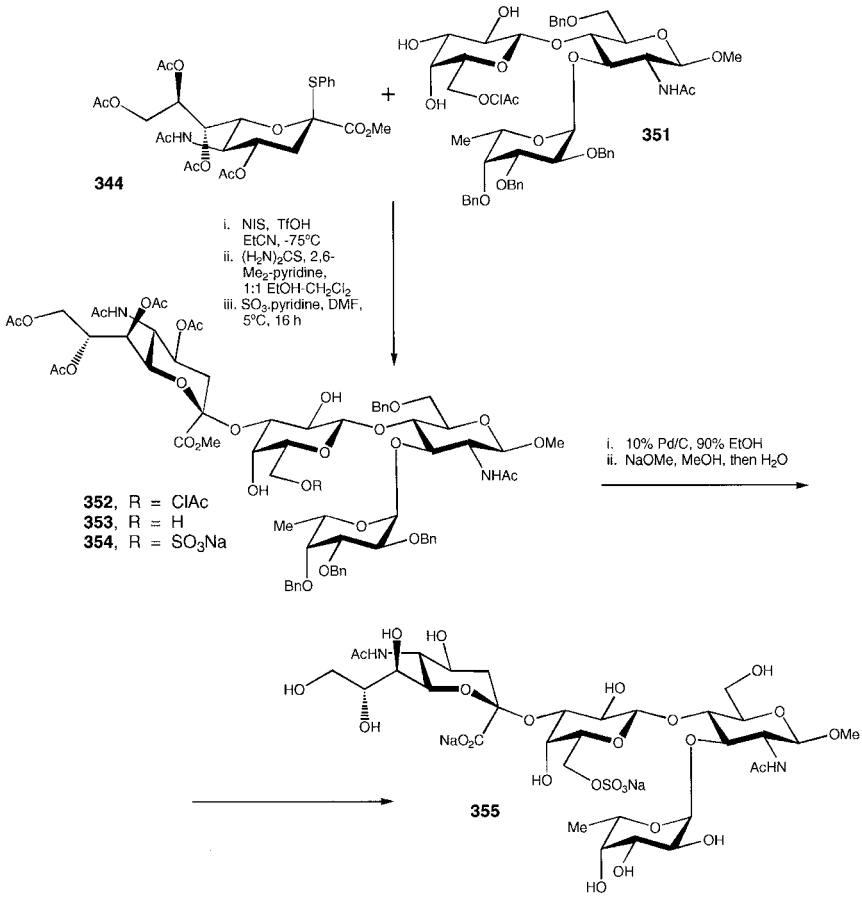
SCHEME 55

of O-linked glycans in GlyCAM-1, syntheses of the sulfated sLe^x structure **26** or its analogues were devised to explore the potential of such compounds as more potent, low molecular weight ligands of L-selectin. Thus, Matta and his associates[454] synthesized the 6′-O-sulfated sLe^x derivative **355** from the protected Gal, GlcN, Fuc, and Neu5Ac monosaccharide derivatives **341, 342, 343,** and **344** (Scheme 56).



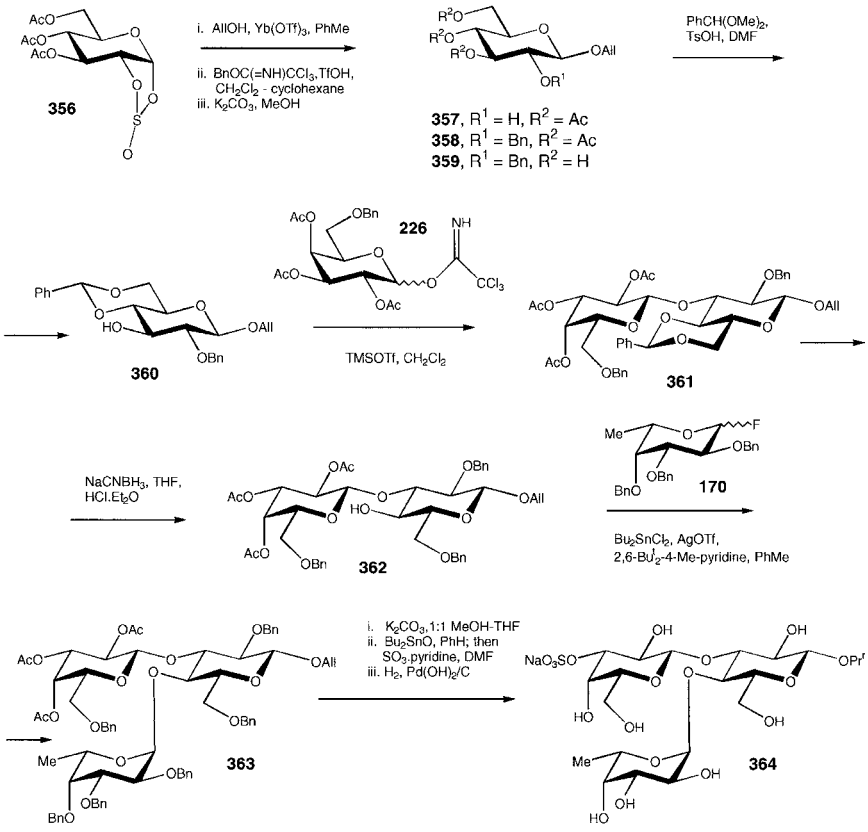
SCHEME 56

Under Mukaiyama's conditions, glycosylation of the GlcN diol acceptor **342** with the Gal fluoride donor **341** afforded both the β-(1 → 3)-linked (type 1, **345,** 17%) and the β-(1 → 4)-linked disaccharide intermediates (type 2, **346,** 52%). Proceeding toward the type 2 (sLe^x-related) target structure, acceptor **346** was glycosylated at the remaining OH-3 of GlcNAc with the fucose thioglycoside donor **343** under promotion by copper bromide according to Ogawa's procedure[455] (**347,** 72%). When **347** was treated with hydrazine hydrate in ethanol followed by acetic anhydride in methanol–triethylamine, all *O*-acetyl groups were removed, and the *N*-phthaloyl group was exchanged for an *N*-acetyl group to give **348.** For the remaining tasks—regioselective α-sialylation at C-3 and O-sulfation at C-6 of Gal—the authors first prepared the 6′-O-chloroacetylated triol acceptor **351:** regioselective O-isopropylidenation of **348** at O-3,4 of Gal (dimethoxypropane under catalysis by camphorsulfonic acid[456]) followed by treatment with chloroacetic anhydride in *N*,*N*-dimethylformamide in the presence of sodium hydrogen



SCHEME 57

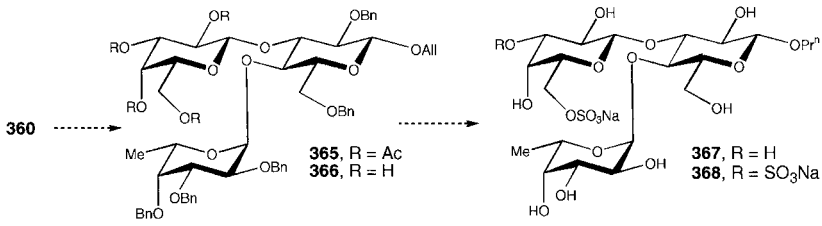
carbonate[457] gave a ∼1 : 1 mixture of the 6′-mono- and 2′,6′-di-chloroacetylated products **349** and **350.** Hydrolytic cleavage of the acetonide **349** gave the triol acceptor **351** (71%) which was O-sialylated at position 3′ with the Neu5Ac thioglycoside donor **344** as described by Marra and Sinaÿ[418] under promotion by *N*-iodosuccinimide and trifluoromethanesulfonic acid[458] in propionitrile in 64% yield (Scheme 57). Lowering of the reaction temperature to −75 °C was required, because of loss of the fucosyl unit at the higher reaction temperatures originally given. Treatment of the tetrasaccharide derivative **352** with thiourea gave the triol **353** (71%), which was regioselectively O-sulfated to afford **354.** The target compound **355** was obtained by sequential catalytic hydrogenation, treatment with sodium methoxide in methanol, and alkali-catalyzed hydrolysis (39% overall from **354**).



SCHEME 58

Kiessling and her associates[459] synthesized the sLe^a analogues **364, 367,** and **368.** These correspond to trisaccharide derivatives analogous to Le^a, substituted with sulfate groups at positions 3 or 6 of the Gal residue, or at both positions. The authors chose to replace the GlcNAc unit with glucose because this modification had previously been shown to result in analogues with enhanced binding strength.[391] As a first step (Scheme 58), the cyclic sulfite **356** (Ref. 460) was converted into the partially protected allyl β-glycoside **357** with allyl alcohol under catalysis by ytterbium tris(trifluoromethanesulfonate) in toluene in 82% yield. Intermediate **357** was 2-O-benzylated by the action of benzyl trichloroacetimidate[461] in the presence of trifluoromethanesulfonic acid in dichloromethane–cyclohexane to afford the acetylated benzyl ether **358** in 97% yield. Compound **358** was deacetylated by the action of potassium carbonate in methanol, and the intermediate triol **359** converted into the 4,6-*O*-benzylidene derivative **360** by treatment with benzaldehyde

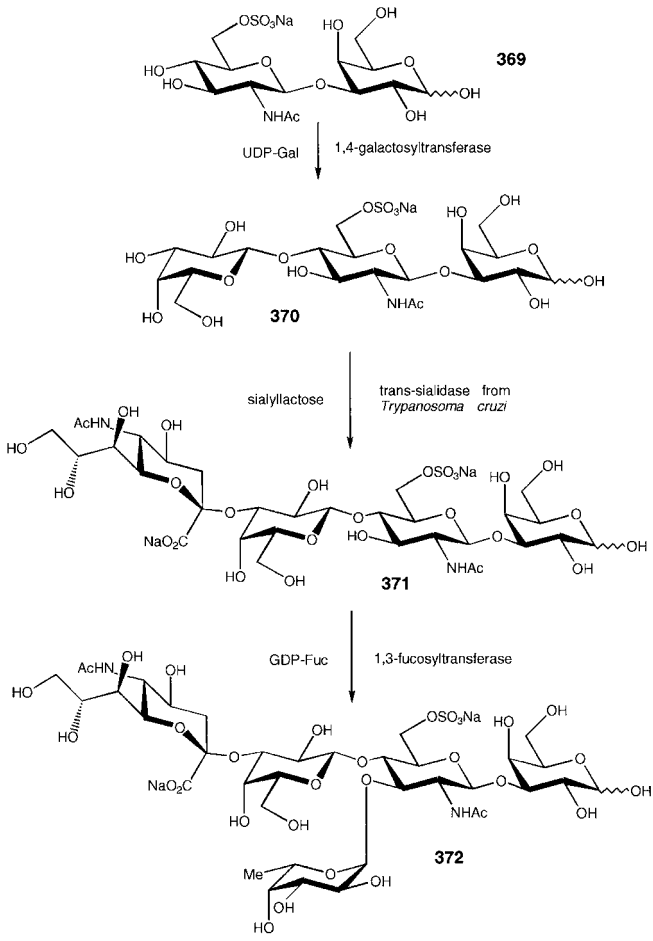
dimethyl acetal in *N*,*N*-dimethylformamide in the presence of *p*-toluenesulfonic acid under diminished pressure (77% from **359**). Glycosylation of **360** with the 6-O-benzylated Gal trichloroacetimidate donor **226** (compare Scheme 36) under promotion by trimethylsilyl trifluoromethanesulfonate in dichloromethane afforded disaccharide derivative **361** in 85% yield. Reductive opening of the benzylidene acetal of the Glc residue of **361** was effected by treatment first with sodium cyanoborohydride in tetrahydrofuran, then hydrogen chloride in ether to give the 6,6′-di-*O*-benzyl disaccharide derivative **362** in 72% yield. Compound **362** was then α-fucosylated, at position 4 of the Glc moiety, according to a Mukaiyama-type protocol[271] by reaction with the benzylated fucosyl fluoride donor **170** under promotion by dibutyltin dichloride–silver trifluoromethanesulfonate in the presence of 2,6-di-*tert*-butyl-4-methylpyridine in toluene to afford the trisaccharide derivative **363** in 90% yield. The acetate protecting groups of **363** were removed by the action of potassium carbonate in 1 : 1 methanol–tetrahydrofuran, and the intermediate trisaccharide triol was converted into its 3′,4′-stannylidene acetal by refluxing with dibutyltin oxide in benzene; treatment of the acetal with sulfur trioxide–pyridine complex in *N*,*N*-dimethylformamide followed by catalytic hydrogenation over palladium hydroxide on charcoal gave the 3′-sulfated target structure **364** in 49% yield from **363.** The analogous 6′-*O*- and 3′,6′-bis-*O*-sulfated target structures **367** and **368** were obtained from **360** by an analogous route (Scheme 59) proceeding by way of galactosylation with a tetra-*O*-acetylgalactopyranosyl trichloroacetimidate donor[275] in the place of **226.** By O-deacetylation of the fully protected trisaccharide derivative **365,** the tetraol **366** was obtained, which served as the intermediate for the preparations of both **367** and **368.** For the preparation of the 6′-O-sulfated product **367,** compound **366** was treated with bis(tributylstannyl) oxide in benzene, and the resulting tributylstannyl ether reacted with sulfur trioxide–pyridine complex in *benzene;* catalytic hydrogenation over palladium hydroxide on charcoal then gave **367** in 59% yield from **366.** Conversely, treatment of the intermediate tributylstannyl ether with sulfur trioxide–pyridine complex in *pyridine,* followed by similar removal of the benzyl ether protecting groups, afforded the 3′,6′-disulfate derivative **368** in 54% yield from **366.** As a consequence of catalytic hydrogenation of the allyl glycosides, target structures **364, 367,** and **368** were all obtained as the *n*-propyl glycosides.



SCHEME 59

In an L-selectin enzyme immunoassay, the O-sulfated Le^a target structures **364, 367,** and **368** had IC_{50} values of 1.5–2.0 m*M*. Unexpectedly, binding of the bis-O-sulfated derivative **368** was not stronger than that of the monosulfates **364** or **367.** Similarly, all three compounds inhibited the binding of GlyCAM-1 to a P-selectin chimera with IC_{50} values of 2–4 m*M*. When examined as an inhibitor of ligand binding to E-selectin, the 3-sulfate **364** ($IC_{50} = 0.14$ m*M*) was superior to the 6′-sulfate **367** ($IC_{50} = 3$ m*M*) or 3′,6′-disulfate **368** ($IC_{50} = 5$ m*M*). Testing of the analogues synthesized by the groups of Matta[454] and of Kiessling[459] indicated that the 6′-(Gal-) sulfated derivatives do not inhibit the interaction of L-selectin with its natural ligands more strongly than sLe^x- or sLe^a-related oligosaccharides without sulfate ester groups. Scudder *et al.*[462] were the first to demonstrate that 6-sulfation on the GlcNAc residue results in analogues with enhanced inhibitory potency. These authors reported on an enzyme-catalyzed synthesis of the pentasaccharide derivative **372,** starting from the 6′-sulfated disaccharide β-D-GlcNAc6S-(1 → 3)-D-Gal **369.*** Compound **369** had been isolated by anion-exchange chromatography from a digest of bovine corneal keratan sulfate with the endo-β-galactosidase from *Bacteroides fragilis*.[463] Despite the presence of the 6′-*O*-sulfate groups, a galactosyl unit was transferred onto **369** from UDP-Gal under catalysis by bovine β-1,4-galactosyltransferase to position 4′ of GlcNAc6S to afford the trisaccharide derivative β-D-Gal-(1 → 4)-β-D-GlcNAc6S-(1 → 3)-D-Gal (**370**) in quantitative yield (Scheme 60). Transfer of an α-sialyl residue from a tenfold excess of sialosyllactose[464] to position 3′′ of **370** was catalyzed by immobilized α-2,3-trans-sialidase from *Trypanosoma cruzi*[464] and gave **371** in ∼90% yield. The trans-sialidase is unique to *T. cruzi*. In the present, preparative application, the enzyme catalyzes the transfer of an α-(2 → 3)-linked sialyl residue from a Gal residue of a sialoglycan [such as α-(2 → 3)-sialyllactose] to the nonreducing β-Gal residue of another glycan to form a new, α-(2 → 3)-linked sialoglycan. In the physiological context, the action of the α-(2 → 3)-trans-sialidase is thought to facilitate the infection of host cells by the parasite *T. cruzi:* sialylation of *T. cruzi* surface glycoprotein glycans is essential for binding to and penetration into host cells. However, the parasite does not possess the enzymes necessary to synthesize sialic acid and the respective biological glycosyl donor, CMP-Neu5Ac. This metabolic deficiency has been overcome by the evolution of the *trans*-sialidase as a specialized neuraminidase that utilizes host sialoglycans as donors in the sialosylation of the parasite's surface glycoprotein glycans.[464] From **371,** the desired pentasaccharide derivative **372** was prepared in quantitative yield by transfer of a fucosyl residue from GDP-fucose to position 3′ of GlcNAc6S under catalysis by recombinant fucosyltransferase V.[465]

* Because of the presence of the (1 → 3)-linked Gal residue, position 6 of the GlcNAc residue is designated 6′ in compound **335;** this needs to be distinguished from position 6′ in sLe^x, which corresponds to C-6 of the Gal residue.



SCHEME 60

The sLe^x pentasaccharide derivative sulfated in position 6 of the GlcNAc residue (compound **372**) is four times more active than sLe^x tetrasaccharide **136** as an inhibitor of L-selectin binding to a preparation of peripheral node addressins (IC_{50} 0.8 and 3.2 m*M*, respectively; compare Section II.5). As the chain length of inhibitory oligosaccharides influences binding to the selectins, a rigorous comparison would require comparative measurements with the respective pairs of pentasaccharide or tetrasaccharide derivatives. Nonetheless, the data of Scudder *et al.*[462] raise the possibility that sLe^x sulfated at the C-6 position of the GlcNAc unit is a physiological ligand of L-selectin. Subsequent findings of Tsuboi *et al.*[466] appear to conflict with the assessment of Scudder *et al.*[462] The former authors reported on binding studies with E- or L-selectin chimeras and CHO cells displaying either

6-sulfo-sLe^x or 6′-sulfo-sLe^x in the context of artificially modified surface glycans. Cells displaying appropriately modified surfaces were produced by incubation of whole cells with a fucosyl transferase from human milk (EC.) and analogues of GDP-fucose that contain the 6-sulfo-sLe^x, 6′-sulfo-sLe^x, or sLe^x oligosaccharide determinants tethered to C-6 of L-galactose. As discovered by Hindsgaul and his associates,[467] the Lewis fucosyl transferase catalyzes the transfer of the complex, oligosaccharide-modified L-galactose residues from the modified GDP-L-galactose donor to the physiological acceptors of fucose, even when C-6 of the L-galactose is tethered to a relatively large oligosaccharide determinant. When binding to E- or L-selectin matrices of CHO cells thus modified was examined, the cells displaying the tethered ligand α-Neu5Ac-(2 $\rightarrow$ 3)-β-D-Gal6S-(1 $\rightarrow$ 4)-[α-L-Fuc-(1 $\rightarrow$ 3)]-β-D-GlcNAc were bound to L-selectin more strongly than the cells displaying the determinant α-Neu5Ac-(2 $\rightarrow$ 3)-β-D-Gal-(1 $\rightarrow$ 4)-[α-L-Fuc-(1 $\rightarrow$ 3)]-β-D-GlcNAc6S. Although the mode of attachment of the two alternative determinants as chosen by Tsuboi ***et al.*** positions the ligands in an essentially unphysiological context, the discrepancy between the findings of Tsuboi ***et al.*** and those of Scudder ***et al.*** and Kiessling ***et al.*** is difficult to reconcile. Crottet, Kim, and Varki[468] examined the calcium-dependent binding, to an L-selectin affinity column, of mucins or mucin glycans from human colon carcinoma cells and human bronchial mucus. The L-selectin affinity column[469] was prepared by linking an L-selectin–IgG chimera to protein A–Sepharose. The mucins examined contain Neu5Ac, sulfate ester groups, and fucose. The data were taken to indicate that a single, unique oligosaccharide determinant may not be responsible for binding of L-selectin to its ligands. Rather, high-affinity L-selectin ligand sites are presumed to arise when O-linked, sulfated, sialylated, and fucosylated lactosamine-type oligosaccharide chains are present on the polypeptide backbones in uniquely spaced or clustered arrangements.

3. Conformational Analysis by NMR Spectroscopy of Selectin Ligand Oligosaccharides

NMR spectroscopy[470] is a powerful tool for the elucidation of oligosaccharide structures.[470–473] Specific pulse sequences are now available to determine all relevant structural components of an oligosaccharide: the number, identity, and anomeric configurations of the constituent sugar residues, their sequence and linkage sites, and the conformation or three-dimensional structure of the oligosaccharide. An introduction to two-dimensional proton NMR spectroscopy has been provided by Dabrowski.[474] Presently, the conclusions are discussed of some typical NMR studies aimed at defining the configurations of sLe^x or related oligosaccharides in solution or bound to a selectin.

Initial NMR studies of the conformation of the sLe^x determinant in aqueous solution were performed by Bednarski and his co-workers[475] and by Lin ***et al.***[476] Bednarski ***et al.*** synthesized Le^x and sLe^x by a combined organic-chemical and

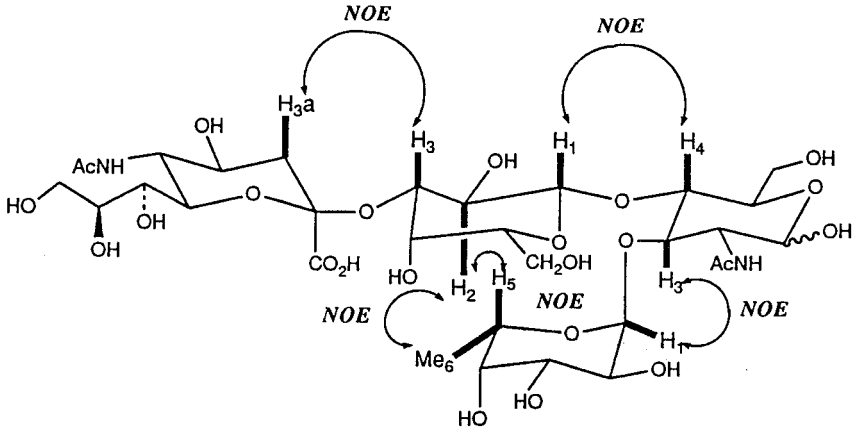


FIG. 18. Nuclear Overhauser effects observed in the ^{1}HNMR-spectra of sLe^{x} reducing tetrasaccharide. Reprinted with permission from G. E. Ball, R. A. O'Neill, J. E. Schultz, J. B. Lowe, B. W. Weston, J. O. Nagy, E. G. Brown, C. J. Hobbs, and M. D. Bednarski, *J. Am. Chem. Soc.* **1992,** *114,* 5449–5451 (Ref. 475); © (1992) Ameican Chemical Society.

enzyme-catalyzed approach (compare Section IV.3). The materials obtained were used for a complete assignment of all ^{1}H- and ^{13}C-chemical shifts and for ROESY (nuclear Overhauser enhancement spectroscopy in rotating frame)[477] and NOESY NMR experiments (nuclear Overhauser enhancement spectroscopy in laboratory frame)[478] to detect interglycosidic nuclear Overhauser effects (nOe). Significant nOe were observed between H-3 of GlcNAc and H-1 of Fuc, H-4 of GlcNAc and H-1 of Gal, H-2 of Gal and H-5 and H-6 of Fuc, and H-3 of Gal and (the axial) H-3a of Neu5Ac. From these data, the authors proposed the conformation of sLe^{x} represented in Fig. 18. Both Le^{x} and the Le^{x} segment of sLe^{x} are present in a folded conformation in which the fucose residue is characteristically tucked underneath the Gal residue.

For the definition of oligosaccharide conformations, the interglycosidic dihedral angles φ and Ψ are commonly used.[479] For the three glycosidic linkages of the sLe^{x} tetrasaccharide, the angles φ and Ψ are defined as the dihedral angles between the following bonds.

α-Neu5*p*Ac-(2 → 3)-β-D-Gal*p*:	φ, C_{1N}-C_{2N}-O_{3G}-C_{3G};	ψ, C_{2N}-O_{3G}-C_{3G}-H_{3G}
β-D-Gal*p*-(1 → 4)-β-D-GlcN*p*Ac:	φ, H_{1G}-C_{1G}-O_{4GN}-C_{4GN};	ψ, C_{1G}-O_{4GN}-C_{4GN}-H_{4GN}
α-L-Fuc*p*-(1 → 3)-β-D-GlcN*p*Ac:	φ, H_{1F}-C_{1F}-O_{3GN}-C_{3GN};	ψ, C_{1F}-O_{3GN}-C_{3GN}-H_{3GN}

Lin *et al.* have reported φ and ψ angles for the sLe^{x} tetrasaccharide, determined with the aid of ROESY NMR measurements and Molecular Mechanics 2 (MM2) calculations,[480,481] as NeuAc, Gal $\{163^{\circ}, -61^{\circ}\}$, Gal, GlcNAc $\{48^{\circ}, 15^{\circ}\}$ and Fuc, GlcNAc $\{22^{\circ}, 30^{\circ}\}$. In a subsequent study from the same laboratory, Ichikawa *et al.*[390] used DQF-COSY (double quantum-filtered correlation spectroscopy)[482] and TOCSY (total correlation spectroscopy)[483] to derive complete ^{1}H and ^{13}C

chemical shift assignments of the sLe^x, sialyl-2,3-*N*-acetyllactosamine, and Le^x structures. For conformational analysis, in addition to NOESY and ROESY, the authors used HMQC (heteronuclear multiple quantum correlation)[484] spectroscopy to provide long-range heteronuclear coupling constants, for example across the glycosidic linkage β-D-Gal-(1 $\rightarrow$ 4)-β-D-GlcNAc. From such data, dihedral angles can be calculated and oligosaccharide conformations estimated by means of a Karplus-type equation.[485] With the aid of GESA (geometry of saccharide)[486] calculations, Ichikawa ***et al.*** confirmed the previously established conformation of the Le^x trisaccharide and the Le^x segment of the sLe^x tetrasaccharide.[390] However, for the α-Neu5Ac-(2 $\rightarrow$ 3)-β-D-Gal linkage, the authors calculated four energy minima corresponding to conformers designated A, B, C, and D, having φ, ψ angles $\{163^\circ, -57^\circ\}$, $\{-170^\circ, -8^\circ\}$, $\{-79^\circ, 7^\circ\}$, and $\{68^\circ, -20^\circ\}$. In 1989, Breg ***et al.,***[495] through the use of nOe data and hard sphere exo-anomeric (HSEA) energy calculations, had already derived three energy minima of the α-Neu5Ac-(2 $\rightarrow$ 3)-linkage in sialyl-*N*-acetyllactosamine, characterized as A (φ, ψ, -70°, 5°), B (-160°, -20°), and C (-95°, -45°). During subsequent studies, several groups have experimentally determined and theoretically calculated the conformational angles φ and ψ for the sLe^x tetrasaccharide. Rutherford ***et al.***[479] studied the internal motions of the reducing sLe^x tetrasaccharide by restrained simulated annealing and restrained molecular dynamics (MD) calculations. While confirming the rigid conformation of the Le^x trisaccharide portion, they observed transitions between predominantly two conformational states of the α-Neu5Ac-(2 $\rightarrow$ 3)-Gal linkage; these correspond to φ,ψ torsion angles of -70°, 5° (conformer A of Breg ***et al.***) and -160°, 20° (conformer B of Breg ***et al.***). From spin-coupling data, the authors concluded that the conformational equilibrium is composed of 75% conformer A and/or C (the closely related C has φ, ψ, -95°, -45°) and 25% conformer B. Cooke ***et al.***[487] observed differences between nOe spectra of sLe^x free in solution or bound to E-selectin, which they interpreted as indicating a change in φ, ψ angles at the α-Neu5Ac-(2 $\rightarrow$ 3)-Gal linkage upon binding of sLe^x to the receptor. Conformational analysis of sLe^x ***in vacuo*** using the SYBYL force field gave two conformational families, essentially identical with respect to the Le^x core but corresponding to conformers B and C of Ichikawa ***et al.*** at the glycosidic linkage α-Neu5Ac-(2 $\rightarrow$ 3)-β-D-Gal. These conformers are the ones designated B and A by Breg ***et al.*** and by Rutherford ***et al.***

Peters and his associates[488] performed comparative NMR measurements of spacer-linked sialyl-Lewis^x tetrasaccharide **276** (Scheme 45) both free and bound to an E-selectin–IgG chimera. Significant differences were observed between interglycosidic nOes in the free oligosaccharide and the corresponding transfer nOes in the bound ligand. With the free sLe^x oligosaccharide a prominent nOe, between H-3_{axial} of the Neu5Ac residue and H-3 of the Gal residue is observed.[475] This is absent in the bound ligand; instead, a transfer nOe (trnOe)[489,490] is observed between H-8 of Neu5Ac and H-3 of Gal. The transfer nOes between the Gal and GlcNAc units in the bound ligand correspond to the nOes seen with

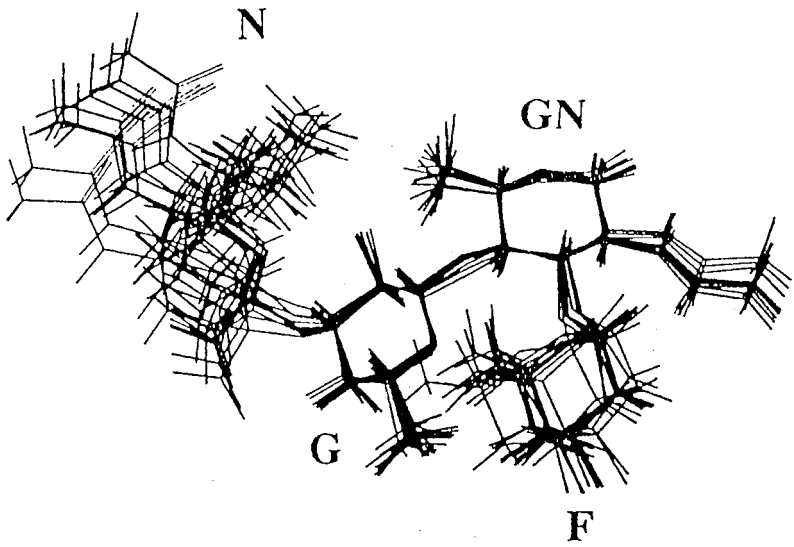


FIG. 19. Conformations of sLe^x bound to E-selectin. Reprinted with permission from K. Scheffler, B. Ernst, A. Katopodis, J. L. Magnani, W. T. Wang, R. Weisemann, and T. Peters, *Angew. Chem.* 107 (1995) 2034–2037 (Ref. 488); © (1995) Wiley-VCH.

the free oligosaccharide. However, the interglycosidic nOe between H-5$_{Fuc}$ and H-2$_{Gal}$, prominent in the free oligosaccharide, corresponds to an extremely weak trnOe in the bound state whereas the trnOe between the *N*-acetyl methyl group at C-2$_{GlcNAc}$ and H-1$_{Fuc}$ is only slightly weaker than the corresponding nOe. The trnOe between the H-6$_{Fuc}$ and H-2$_{Gal}$ is similar to the respective nOe. A transfer nOe is observed between H-5$_{Fuc}$ and H-6$_{Gal}$ in the bound state while a corresponding nOe in the free oligosaccharide is barely detectable. The trnOes of the bound sLe^x oligosaccharide were converted into distance limits that were then applied to select a set of 29 conformers from a complete set of 457, 309 conformers calculated with the aid of a Metropolis–Monte-Carlo simulation at a temperature parameter of 2000 K.[491,492] The conformations deduced in this manner populate only a narrow segment of the conformational space and are therefore considered to reflect the conformation of sLe^x bound to E-selectin. From the resulting model (Fig. 19), the following average φ and ψ angles have been calculated. α-Neu5*p*Ac-(2 $\rightarrow$ 3)-β-D-Gal*p*:$\{-76^\circ \pm 10^\circ,\ 6^\circ \pm 10^\circ\}$, β-D-Gal*p*-(1 $\rightarrow$ 4)-β-D-GlcN*p*Ac:$\{39^\circ \pm 10^\circ,\ 12^\circ \pm 6^\circ\}$, and α-L-Fuc*p*-(1 $\rightarrow$ 3)-β-D-GlcN*p*Ac:$\{38^\circ \pm 7^\circ,\ 26^\circ \pm 6^\circ\}$. The conformation of the Neu5Ac-Gal linkage in the bound ligand corresponds to conformer A of Breg *et al.*

Poppe *et al.*[493] determined the conformation of the sLe^x tetrasaccharide in the free state and bound to E-, P-, and L-selectin–Ig fusion proteins, from NMR measurements of interglycosidic coupling constants and nOe involving hydroxyl protons in slow chemical exchange at low temperatures.[494] From their data,

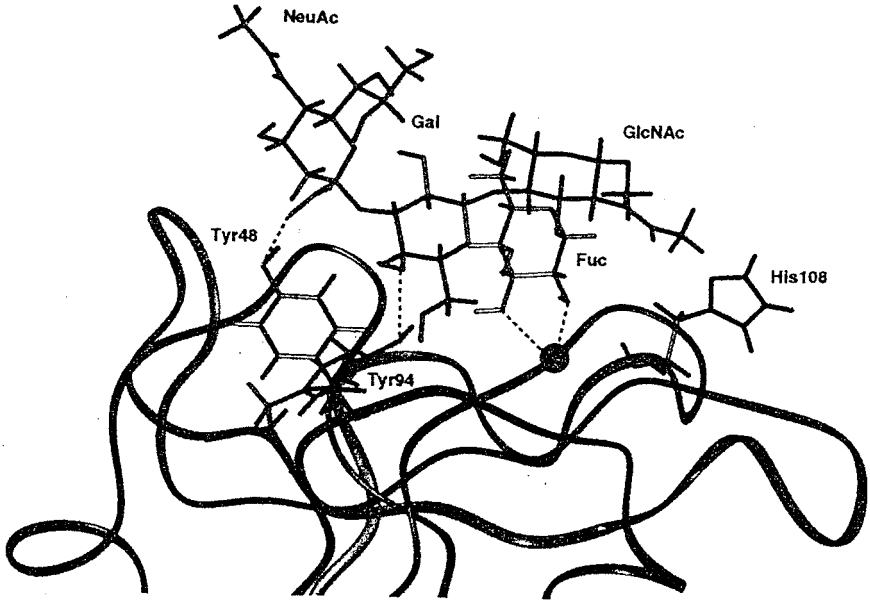


FIG. 20. Stereochemical drawing of the contacts between sLe^x and P-selectin. Reprinted with permission from L. Poppe, G. S. Brown, J. S. Philo, P. V. Nikrad, and B. H. Shah, *J. Am. Chem. Soc.* **1997,** *119,* 1727–1736 (Ref. 493); © (1997) American Chemical Society.

they derived several important conclusions. Of the three calculated[495] conformational minima of the Neu5Ac-(2 → 3)-Gal linkage corresponding to φ,ψ-angles of $\{180^\circ, 0^\circ\}$, $\{-60^\circ, 0^\circ\}$ and $\{-100^\circ, -50^\circ\}$(rounded values of Breg *et al.*[495]), the most populated is $\{-60^\circ, 0^\circ\}$. This conformer is also the one bound to E- and P-selectins, whereas the $\{-100^\circ, -50^\circ\}$ conformer appears to be present in the sLe^x–L-selectin complex. Although Poppe *et al.* agree with Peters in attributing to the bound sLe^x ligand conformation A of Breg *et al.*, they interpret their data to indicate that conformer A is also the one mainly present in solution. As distinct from the interpretation of Peters *et al.*, a conformational transition upon binding is not required according to Poppe *et al.* Furthermore, the data of Poppe *et al.* provide evidence for close contacts [<5 Å (<0.5 nm)] with E- and P-selectin of the H-4 and H-6,6′ of the Gal residue, and of $H\text{-}4_{Gal}$ with L-selectin (Fig. 20). These findings underscore the need for consideration of the contributions of the Gal residue in the design of inhibitors of selectin–ligand interaction. Applications of theoretical methods to the problem of sLe^x conformation have been reviewed by Imberty and Perez[496] and by Imberty.[497] Poveda and Jiménez-Barbero[498] have provided a highly didactic review of NMR studies directed at protein–carbohydrate interactions in solution. NMR and modeling studies of the structure and dynamics of oligosaccharides have been reviewed by Peters and Pinto.[499]

4. Identification of Carbohydrate-Binding Domains through Studies with Peptide Fragments and Mutant Selectins

Erbe and colleagues[500] studied the effects of mutations in the lectin domain of E-selectin on the binding of anti-E-selectin monoclonal antibodies and on binding of E-selectin to immobilized sLe^x glycolipid (Section IV.2). They concluded that Arg-97, Lys-111, Lys-113, and possibly Ser-47 and Tyr-48 would directly function in the binding of sLe^x by E-selectin. Subsequently, following the application of similar techniques to P-selectin, Erbe and co-workers reported[501] that a homologous region of the P-selectin lectin domain is involved in carbohydrate recognition and cell adhesion. Hollenbaugh *et al.*[502] confirmed that Lys-113, Tyr-48, and Tyr-94 are critical for P-selectin binding and reported that the single substitutions Lys113Ala, Tyr48Ala, Tyr48Phe, Tyr94Ala, and Tyr94Phe abolished binding of P-selectin to myeloid cells. Aruffo *et al.* had demonstrated[503] in 1991 that P-selectin binds to sulfatide (β-D-galactopyranosyl-1-ceramide). Bajorath *et al.*, by a combination of computer modeling techniques, site-specific mutagenesis, and ligand and cell binding assays, showed[504] that P-selectin binds to myeloid cells and to sulfatide by means of overlapping but not identical binding sites close to the Ca^{2+} chelation site. Macher and his associates performed structure–activity studies of anti-inflammatory peptides based on a conserved peptide region of the lectin domains of E-, L-, and P-selectin.[505] According to previous findings by the group of Macher, the peptide YYWIGIRK-NH_2 inhibits both myeloid cell adhesion to selectins *in vitro* and neutrophil influx into inflammatory sites *in vivo*.[506] At least one of the Tyr residues at the N terminus of the octapeptide is essential for these activities, but the C-terminal lysine residue was not needed for inhibitory activity. Accordingly, a series of heptapeptides with single amino acid substitutions at each position of the reference peptide, YYWIGIR-NH_2, was synthesized and inhibitory activities were examined in a selectin cell-binding assay. In addition, the ability of the reference heptapeptide and of the control sequence [YY(AIB)IGIR-NH_2] to discriminate between potential synthetic saccharide ligands, including sLe^x, Le^x, and sialyl-*N*-acetyl-lactosamine, was studied using isothermal titration calorimetry. Although many single amino acid substitutions are tolerated in the peptide without complete loss of inhibitory activity, substitution at some positions (such as the Tyr residue) affords inactive compounds. These findings were interpreted by the authors as indicating the importance of these residues in making critical contacts with the appropriate saccharide ligand. Titration calorimetry revealed that the reference peptide does not discriminate between Le^x or sLe^x *in vitro*. The binding activity to these saccharides is ~40-fold higher ($K_D \sim 25\ \mu M$) than that to nonfucosylated trisaccharide, sialyl-*N*-acetyl-lactosamine. The authors concluded that the presence of a sialyl group, per se, is not a requisite for complex formation between the reference peptide and the saccharide ligands. Substitution of single D-amino acid residues at various positions in the reference peptide will diminish

or eliminate the inhibitory properties. Interestingly, the all D-configured peptide (yywigir-NH_2) or the retro-inverso peptide (rigiwyy-NH_2) have greater activity than the all-L-configured reference peptide in the *in vitro* biological assays, and each was an effective inhibitor of neutrophil infiltration in a thioglycolate-induced mouse peritonitis model. These results, combined with the results of titration, were interpreted to indicate that binding between the reference peptide and its saccharide ligand is mediated by the presence of a contiguous, nonpolar surface located essentially at the N terminus of the reference peptide, probably comprising the sequence YYWI. Furthermore, the Trp residue plays a critical role in binding, possibly through formation of a hydrogen bond with a juxtaposed group of the saccharide ligands.

5. X-Ray Crystal-Structure Analyses of E-Selectin, Mannose-Binding Proteins, and Their Selectin-like Mutants

Considerable effort notwithstanding, an X-ray crystal structure analysis of a selectin with a bound oligosaccharide ligand has not been reported to date.* Graves *et al.*[507] have described an X-ray crystal structure analysis, using MAD phasing, of a part structure of E-selectin comprising 157 amino acids of the lectin and adjacent epidermal-growth-factor-like domains. The technique of MAD (multiwavelength anomalous diffraction) phasing requires the substitution of suitable atoms in a macromolecule by relatively heavy atoms and enables an X-ray crystallographic problem hopelessly large for direct phase determination to be solved from the diffraction data of a single crystal. The principle is as follows.[508] In normal Thomson scattering, the electrons are accelerated in direct response to an incident X-ray wave, which in turn produces elastic scattering of the X-rays. When the energy of the X-rays approaches the energy of an electronic transition from a bound atomic orbital, a resonance condition is established that amplifies the acceleration and perturbs the scattering. In standard diffraction experiments, X-rays would have energies on the order of 10,000 eV, corresponding to wavelengths of ~1.0 Å (0.1 nm). For the light atoms of biological macromolecules (C, H, O, N, S, P), such X-rays would not give rise to electronic transitions. However, heavier atoms, such as Zn in a metalloprotein, Hg in a heavy atom derivative of a protein, or Se incorporated in a protein in the form of selenomethionine, would cause "anomalous" resonant scattering. In practice, the distinct scattering from such anomalous atoms can be isolated through appropriate diffraction measurements and used to locate the positions of the anomalous scatterers. Once this substructure is known, its calculated diffraction pattern serves as a set of reference waves by which phases for the reflections of the total diffraction patterns can be determined.

* At the time of completion of this chapter, X-ray crystal structure analyses were reported of E- and P-selectin with bound ligands (Ref. 574).

Graves *et al.*[507] used gadolinium and platinum derivatives for MAD phasing. From their X-ray data, they derived a tentative model of the sLe^x–E-selectin interaction. The sLe^x tetrasaccharide was assumed to bind in the conformation previously determined by NMR studies (Section V.3) and was docked to the selectin by a combination of manual and computational means. According to the model derived, the carbohydrate binding site of E-selectin corresponds to a shallow groove in the vicinity of the Ca^{2+} ion, as suggested by the results of previous loss-of-function mutational studies on E-selectin by Erbe *et al.*,[500,501] and by Quesenberry and Drickamer,[171] and by comparison to the crystal structure of rat mannose binding protein (MBP),[509] a C-type lectin related to the selectins.[35]

The X-ray crystal structure analysis of the oligomannose–MBP complex by Weis, Drickamer, and Hendrickson[509] has provided the first three-dimensional view of a C-type lectin (compare Section II.2) containing a bound oligosaccharide and has revealed that Ca^{2+} forms coordination bonds with the carbohydrate ligand in such complexes. A sketch representing the binding of calcium and Man at calcium binding site 2 of MBP is shown in Fig. 21A. Oxygen atoms from the side chains of amino acids Asn-187, Glu-185, Asn-205, Glu-193, and the main-chain carbonyl oxygen of Asp-206 constitute the base of the pentagonal bipyramid coordination set[510] of Ca^{2+}; one apex of the bipyramid is formed by a carboxylate oxygen atom of Asp-206, the other, by the 3- and 4-OH-groups of a nonreducing Man residue. The oligomannose derivative bound to MBP in the crystals analyzed is the branched oligosaccharide $\underline{\alpha\text{-D-Man}}$-(1 → 2)-$\alpha$-D-Man-(1 → 3)-[$\underline{\alpha\text{-D-Man}}$-(1 → 3)-$\alpha$-D-Man-(1 → 6)-]-$\beta$-D-Man-(1 → 4)-$\beta$-D-GlcNAc-(1 → 4)-$\beta$-D-GlcNAc-1 → N (Asn). Interestingly, in the crystals, both the underlined, nonreducing Man units bind similarly to analogous Ca^{2+} binding sites, thus artificially crosslinking two dimers of the MBP. However, subtle differences in mannose binding are observed and are attributed to the different positions of the two binding, nonreducing Man residues with respect to the other sugar residues of the oligosaccharide.[509] Kogan and his associates[511] derived a molecular model of the E-selectin–ligand interaction combining the NMR data of Cooke *et al.*[487] with the X-ray crystallographic coordinates of free E-selectin as determined by Graves *et al.*,[507] and of mannose-binding protein containing bound oligomannoside as reported by Weis *et al.*[509] On the basis of the combined data, Kogan *et al.* predicted and demonstrated that the exchange of Ala-77 for Lys in E-selectin would provide a mutant selectin capable of binding oligosaccharides containing nonreducing α-D-Man residues. Similarly, substitution of Ala-77 in P-selectin[177] by a lysine residue was reported to change the binding specificity from sLe^x to oligomannoside. On the other hand, the mutational analysis indicated that Lys-113, an amino acid residue of P-selectin implicated in ligand binding, may not be involved in the interaction of the selectin with sLe^x or sulfatide. Blanck *et al.*[170] introduced into the carbohydrate-recognition domain of mannose-binding protein five regions of E-selectin that differ in sequence from the corresponding

regions of MBP. Whereas four of these changes have little effect on ligand binding, insertion of one stretch of positively charged amino acids alters the binding specificity so that a neoglycoprotein is bound that contains sLe^x tetrasaccharide glycans attached to serum albumin. As expected, the mutant MBP also binds to HL60-cells. Ng and Weis reported on X-ray crystal structure analyses of mutant MBPs that contain bound selectin ligand oligosaccharides[512] (Fig. 21B). Although the coordinates confirm the nature of the selectin–ligand interaction as suggested by previous data from X-ray, NMR, and mutational loss-of-function analyses, the carboxyl function of bound sLe^x is not as close to the cluster of three lysine residues Lys-111, Lys-112, and Lys-113 as would be expected if an ion pair were formed between a Neu5Ac carboxylate and one or more ε-ammonium functions of the lysine residues. X-Ray crystal structure analyses have also been performed with crystals containing the 3-sulfate derivatives of Le^x or Le^a (Ref. 512).

It is useful to visualize, in this context, the selectin ligand glycans sLe^x, sLe^a, and 3-sulfate-Le^x or -Le^a in relation to the disaccharide segment comprising the nonreducing and subterminal mannosyl residues (α-D-Man-(1 → 2)-α-D-Man-) of oligomannoside (Fig. 21C): the CHOH groups C-2 and C-3 of the fucosyl residue

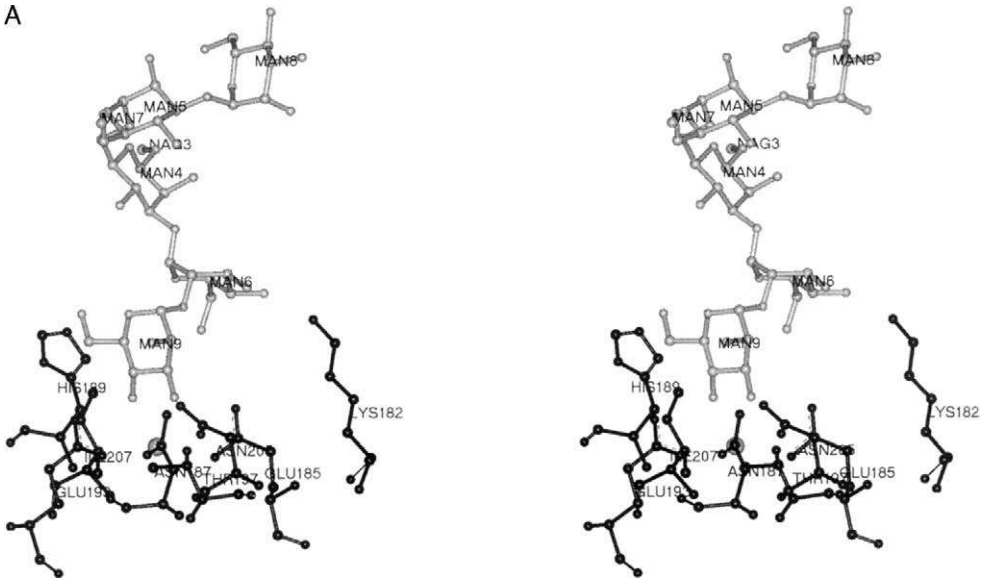


FIG. 21. Stereoscopic drawings of A. the binding of oligomannoside in the binding domain of mannose-binding protein (MBP); B, the sLe^x tetrasaccharide determinant in the binding domain of an MBP mutant; and C, of oligomannoside and the sLe^x tetrasaccharide superimposed in the binding domain of an MBP mutant. According to Weis, Drickamer, and Hendrickson (Ref. 509) and Ng and Weis (Ref. 512). The author thanks Drs. Luckhana Lawtrakul and Peter Wolschann of the Institute of Theoretical Biology and Molecular Structural Biology, University of Vienna, for creating the images of Fig. 21 with the aid of the program WebLab Viewer Pro 4.0 of Molecular Simulations, Inc.

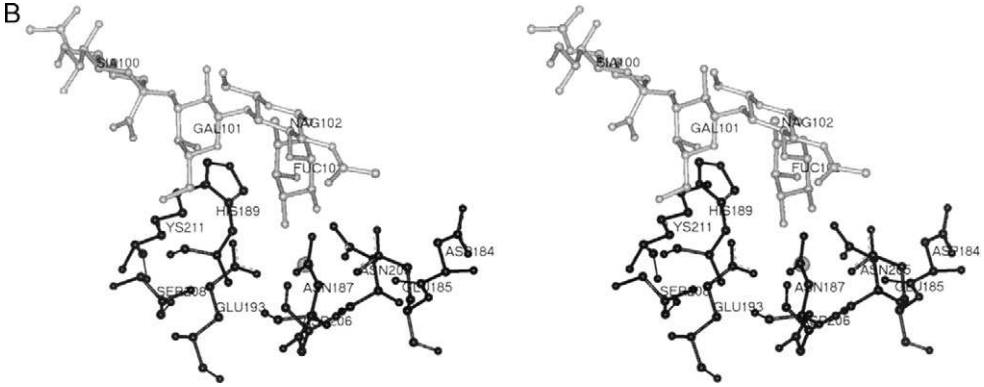


FIG. 21B.

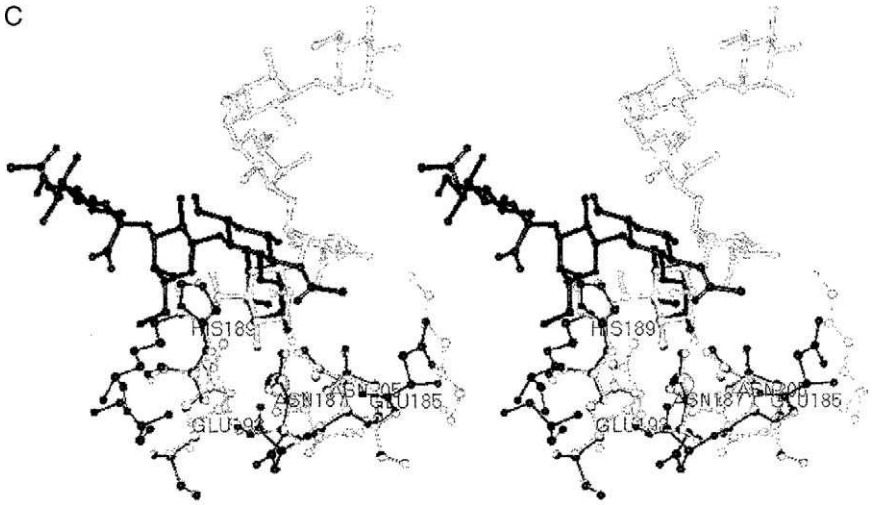


FIG. 21C.

of sLe^x are isosteric with the CHOH groups C-3 and C-4 of the nonreducing α-Man residue. The $^1C_4$ conformation of the L-fucopyranose ring corresponds to a "$^2C_5$" conformation of the D-mannopyranose ring.

Although large segments of the nonreducing Man and Fuc residues are isosteric, the GlcNAc, Gal, and Neu5Ac residues of sLe^x are outside the region considered for binding of the disaccharide segment α-D-Man-(1 $\rightarrow$ 2)-α-D-Man-. The functional groups of the GlcNAc residue make no contribution to binding. Therefore, for the synthesis of pharmaceutically useful derivatives and analogues, the results of these studies have supported a change in paradigm from the ***trisaccharide*** to the ***disaccharide model***. Accordingly, target compounds have been designed and synthesized wherein the Gal residue or its equivalent, with a suitably connected

carboxylate function or its equivalent, would be directly attached to a nonreducing fucose, L-galactose, or D-mannose residue (compare Section VI.3).

6. Formation of Covalent Linkages between L-Selectin and Aldehydo Derivatives of L-Selectin Ligands

Exhaustive oxidation of selectin ligands by the action of periodic acid (50 m*M*) will degrade the glycans, abolishing their ability to bind to selectins.[106] However, as discovered by Norgard *et al.*,[513] treatment with periodic acid under appropriately limiting conditions (2 m*M*) results in oxidative cleavage of only the glycerol side chains of the Neu5Ac residues while the fucose residues remain intact. The resulting aldehydo glycoconjugates, upon reduction with borohydride, afford analogues with 3-deoxyoctulo- or 3-deoxy-heptulopyranosylonic residues in place of the native Neu5Ac residues.[514,515] This type of modification has been applied to prepare substrates for structure–function studies of glycoproteins by Ashwell[516] and by Winzler.[517] Similar treatments applied to Neu5Ac-containing selectin ligands gave the corresponding "truncated" analogues, the binding capacities of which were practically undiminished relative to their native counterparts.[391] When lymph node slices containing L-selectin ligands were subjected to oxidative treatment without the borohydride reduction step, and binding of human peripheral blood leukocytes examined in a Stamper–Woodruff assay,[29] these were surprisingly found to bind more strongly to the oxidized than to native lymph node slices. This finding was interpreted by the authors to indicate the formation of a covalently linked complex, assumed to be a Schiff base, through an amino group of L-selectin and an aldehyde group of the oxidized ligand. This was confirmed by means of an assay based on the interaction of an L-selectin chimera containing a C-terminal domain of the Fc fragment of human IgG2 antibody, with a $^{35}SO_4$-labeled L-selectin ligand preparation that had been enriched by adsorption to a wheat germ agglutinin column.[518] Complexes were allowed to form between the L-selectin chimera and the ligand and were adsorbed on protein A–Sepharose. The resulting adducts were treated with sodium cyanoborohydride, and the whole subjected to centrifugation. When native ligand was used, any complexes formed were dissociated under the conditions of cyanoborohydride treatment, and no radioactivity was found in the pellets after centrifugation. When oxidized ligand was used, radioactivity was found in the pellets, associated with components of higher molecular mass as indicated by SDS–polyacrylamide gel electrophoresis. Presumably, by the action of cyanoborohydride, the Schiff base initially formed was converted into a secondary amine. The radioactive L-selectin ligand was thus bound to the L-selectin chimera by a stable covalent linkage, and the adduct subsequently attached to protein A–Sepharose via the Fc C-terminal domain of the L-selectin chimera.

Whereas Norgard *et al.*, had observed the formation of covalent L-selectin–ligand complexes under static conditions, Puri and Springer[519] have used a dynamic

assay to study the behavior of oxidized selectin ligands during tethering and rolling. Cell suspensions were pumped through a parallel-plate laminar-flow chamber and were observed on the stage of an inverted phase contrast microscope.[193] Of the substrates immobilized on the lower plate, selectins were introduced in a lipid bilayer on glass slides while selectin–IgG chimeras, the L-selectin ligand CD34, or synthetic sialyl Le^x or Le^a hexaosylceramides were adsorbed on polystyrene slides. The partial oxidation of the immobilized selectin ligands was performed by pumping through the chamber a 5 m*M* solution of sodium metaperiodate. Lymphocytes were pumped through the chamber and their adhesive interactions observed before and after oxidation of the substrates. With CD34, oxidation resulted in a more than twofold increase of lymphocyte rolling in a given microscopic field, while the rate of formation of initial rolling attachments was enhanced only 1.2-fold. This was interpreted as indicating that the rate of association (K_{on}) is essentially unaffected while the rate of spontaneous detachment (K_{off}) is decreased following oxidation of the substrate. The number of lymphocytes rolling on CD34 corresponded to control levels when oxidative treatment of the substrate had been followed by reduction with borohydride. No lymphocyte binding occurred after exhaustive oxidation (50 m*M* sodium metaperiodate). In an attempt to reduce the presumed Schiff base to a secondary amine, lymphocytes were first allowed to accumulate on oxidized CD34 and were then exposed to perfusion with 5 m*M* sodium cyanoborohydride. Following this treatment, nearly all cells rolling on the oxidized substrate came to a standstill within 3 min. The arrested cells were not detached by exposure to EDTA and were considered covalently linked to the substrate. In a control experiment, the substrate had been sham-treated without periodate. Under these conditions, subsequent perfusion with cyanoborohydride had no effect on the rolling velocity and all adhering cells were detached by treatment with EDTA.

Ligands for L-selectin are also expressed on human neutrophil leukocytes and the human hematopoietic precursor cell line KG1a. Interaction of the ligands with immobilized L-selectin mediates rolling of these cells. As with the lymphocyte–CD34 interaction, mild periodate treatment of either cell line resulted in better accumulation, slower rolling, and a higher degree of resistance to shear detachment. Similar observations were made when SKW3 T-cells were allowed to roll on the intact or periodate-oxidized glycolipids, sLe^a, or sLe^x glycohexaosylceramides. In appropriate control experiments, hydrolytic removal of the Neu5Ac units catalyzed by neuraminidase gave ligands that did not bind selectin. Similarly, monoclonal antibodies against L-selectin inhibited the interactions.

Selectin–ligand interactions of E- or P-selectin were not enhanced following mild periodate oxidation of the ligands. However, subsequent treatment of rolling cells with cyanoborohydride caused arrest of the cells. Attachment of the cells was not reversed by treatment with EDTA, indicating the formation of covalent bonds. As indicated by Puri and Springer, the phenomena observed are difficult to explain on the basis of Schiff base formation alone.[519]

VI. Design of Inhibitors with Increased Binding Strength, Decreased Molecular Complexity, and Improved Feasibility of Large-Scale Synthesis

Synthetic strategies of remarkable variety have been devised and implemented toward the goal of selectin inhibitors suitable for pharmaceutical development. The high expense of the preparation of sLe^x or related tetrasaccharide derivatives, the relatively poor binding potency of the natural selectin ligand glycans, and the limited potential for sufficient absorption by the oral route of administration have stimulated a search for carbohydrate mimetics that have more favorable binding behavior, are practical synthetically, and are inexpensive to produce. Chronologically, and for purposes of structuring the present review, these have been designated as belonging to the tetrasaccharide, trisaccharide, disaccharide, and monosaccharide paradigms (Fig. 22). Compounds that represent structure variations involving changes of functional groups at individual positions of the sLe^x or sLe^a tetrasaccharides are considered in the preceding Section V.2 of this chapter. As a consequence of previously established structure–activity relationships (Fig. 14, compare Section V), two sets of part structures of the sLe^x or sLe^a tetrasaccharides have been designated. The set of part structures necessary for binding of oligosaccharides to the (E-, P-) selectins comprises the triol grouping at C-2, C-3, and C-4 of the fucose residue, the 1,3-propanediol grouping including C-4 and C-6 of the galactose residue, and the carboxyl group (C-1) of the sialyl residue.

The complementary set, not required for binding to the (E-, P-) selectins, would include all of the functional groups associated with the GlcNAc residue, the 2,3-diol segment including C-2 and C-3 of the galactose residue, and the entire pyranoside ring and glycerol side chain of the Neu5Ac residue.

1. The Tetrasaccharide Model

Discussion under the heading "tetrasaccharide paradigm" will be limited to those compounds comprising a full sialyl residue, or its equivalent other than merely an anionic group, in addition to glycosyl residues or spacer groups corresponding to the Gal, GlcNAc, and Fuc residues.

a. Early Syntheses of sLe^x Analogues.—

(i) ***Use of a Pentaerythritol Scaffold for the Neu5Ac and Fucose Residues.—*** Initial results from cell-biological studies indicated that, for the biosynthesis of functional selectin ligands, expression of a fucosyltransferase is required,[62] and that treatment of cells with neuraminidase would abolish selectin ligand activity. These findings led to a hypothesis according to which the Fuc and Neu5Ac residues are essential, while the GlcNAc and Gal residues function as a scaffold

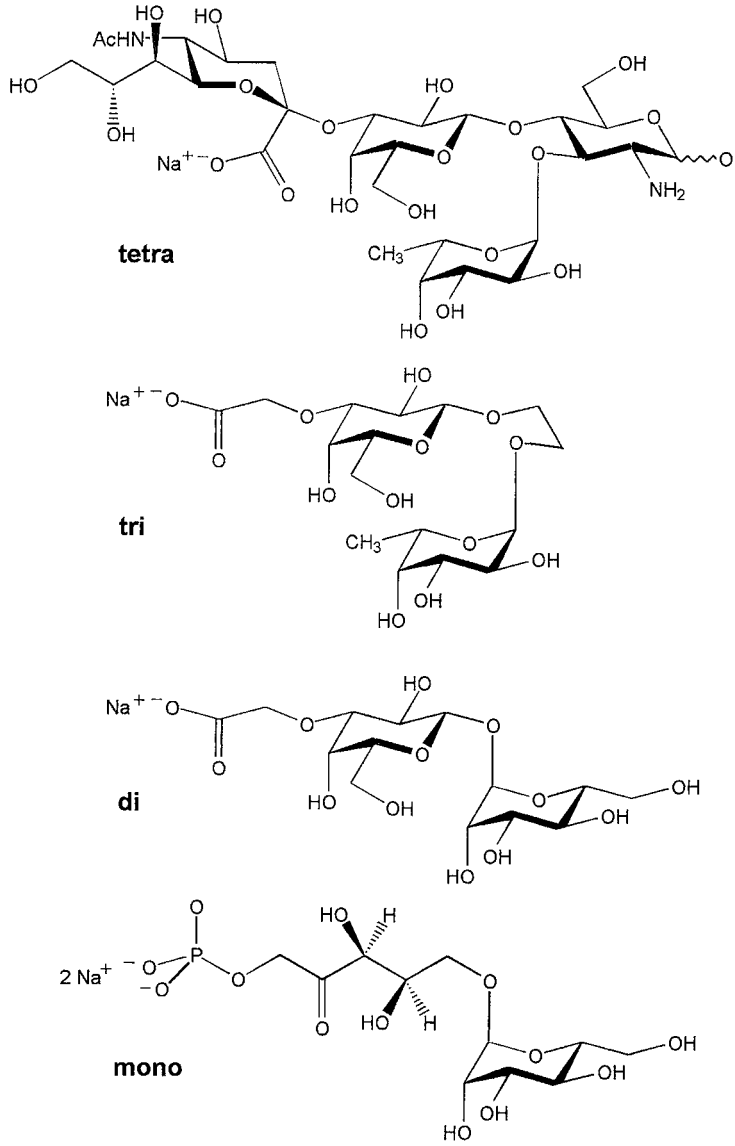


FIG. 22.

that holds the essential residues in place. Accordingly, simplified analogues could be constructed by replacing the Gal and GlcNAc residues with chemical tethers of appropriate length. A model compound **377,** potentially representing the sLe^x tetrasaccharide, has been prepared by Hanessian and Prabhanjan[520] anchoring an α-NeuAc and an α-Fuc residue on a pentaerythritol template (Scheme 61).

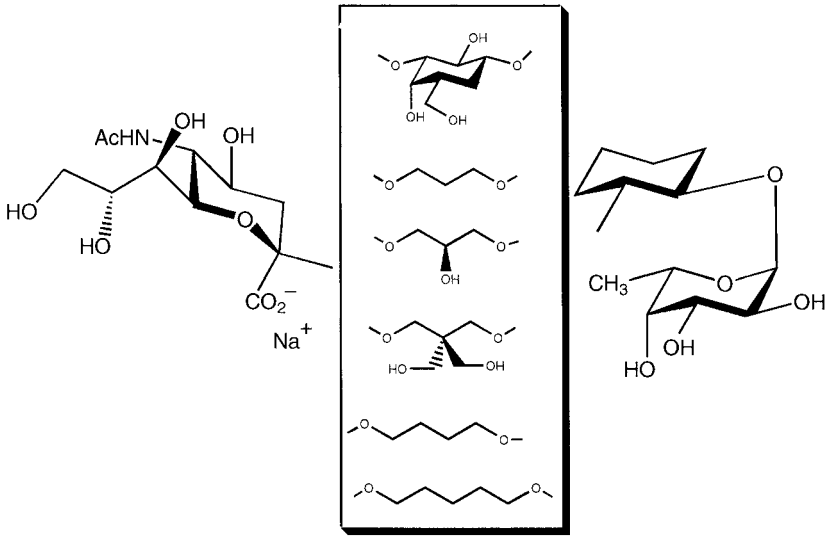
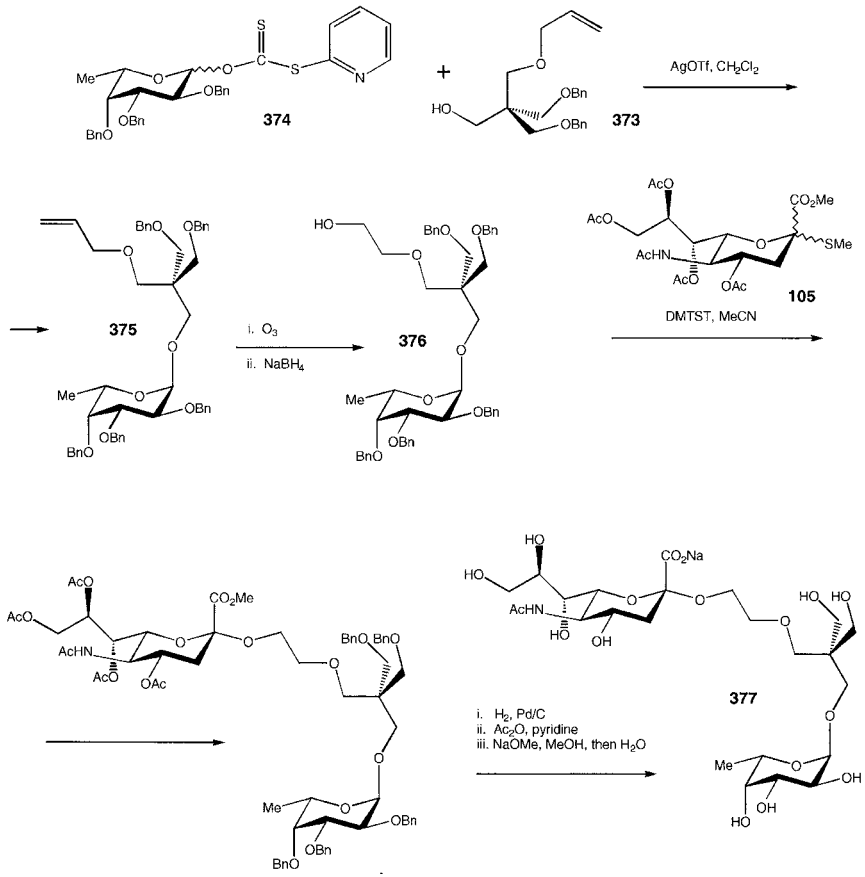


FIG. 23. Replacing the Gal unit by a variety of 1,3-, 1,4-, and 1,5-diols in analogues of the sLe^x tetrasaccharide prepared by Toepfer *et al.* Both anomeric sialosides were examined for binding to E- and P-selectins in each case; cf. Ref. 533.

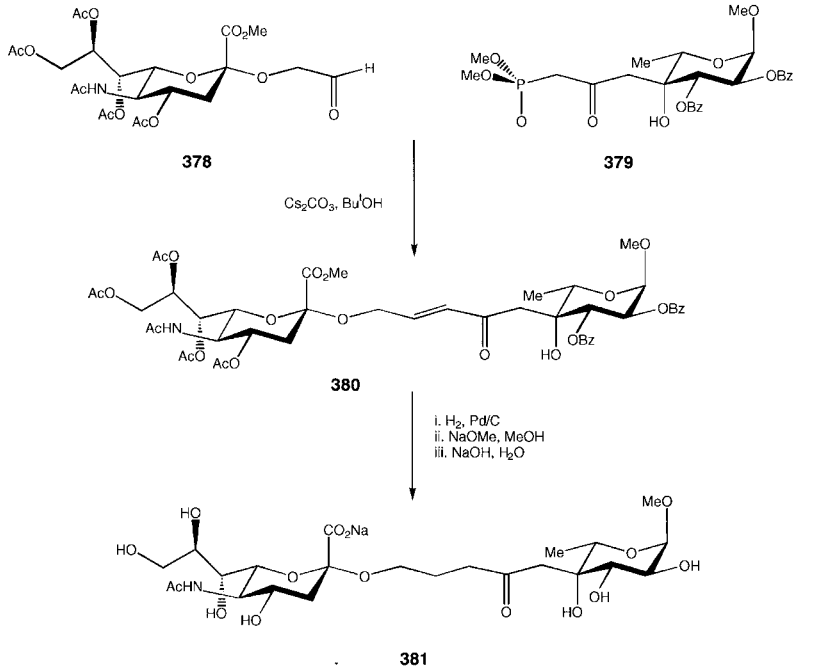
The monoallyl dibenzyl ether derivative of pentaerythritol, **373,** was glycosylated with the anomeric mixture of O-benzylated 2-pyridylthiocarbonate glycosides of L-fucose (**374**) in the presence of silver triflate, to afford an α,β mixture of fucosides in 72% yield. The required α anomer **375** was isolated in 33% yield. Ozonolysis of allyl ether **375** followed by reduction of the intermediate aldehyde by the action of sodium borohydride gave the primary alcohol **376** in 61% yield. Alcohol **376** was α-glycosylated according to Hasegawa's method with the aid of the thioglycoside sialyl donor **105** (Scheme 16) under promotion by dimethyl(methylthio)sulfonium trifluoromethanesulfonate (DMTST) in acetonitrile in 65% yield. Removal of the benzyl ether protecting groups by hydrogenation over palladium-on-carbon, followed by per-O-acetylation, Zemplén saponification, and alkaline hydrolysis of the carboxylate ester afforded the target compound **377** in high yield. However, neither compound **377** nor any of its β-NeuAc/α-Fuc, β-NeuAc/β-Fuc, or α-NeuAc/β-Fuc diastereoisomeric variants showed inhibitory activity at concentrations <5 m*M* (by comparison, sLe^x had an IC_{50} of <0.5 m*M*).

(ii) ***The Neu5Ac and Fucose Residues Tethered by a Pentanone Linker.—*** Allanson *et al.* at the British Biotechnology Company synthesized target structure **381,** which contains the sialic acid and fucose units linked by a pentane-2-one chain.[521] Connectivity to the sialyl residue is through an α-glycosidic linkage. The pentanone tether is attached to C-4 of the fucose unit by an equatorial carbon–carbon bond so that, in **381,** OH-4 is a tertiary alcohol function. The key step



SCHEME 61

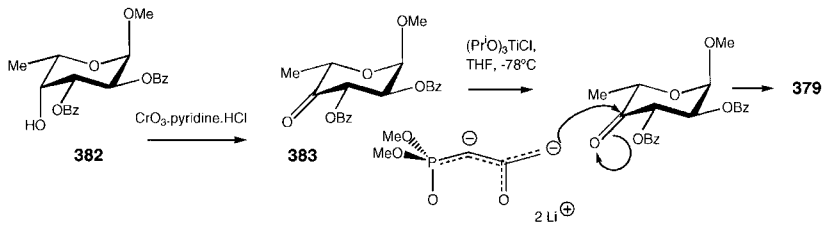
of the synthesis (Scheme 62) is the Wadsworth–Emmons reaction[522] of the sialyl aldehyde **378** with the ketophosphonate **379** derived from fucose. Aldehyde **378** was obtained by ozonolysis of the protected allyl-α-glycoside of NeuAc, which had been prepared previously by Roy.[523] For the Wadsworth–Emmons reaction of **378** with **379,** suitably mild reaction conditions needed to be identified, as both the electrophile and the nucleophile are highly sensitive compounds: an optimal 58% yield was obtained using cesium carbonate in *tert*-butanol.[524] the olefin **380** was converted into the target structure **381** by catalytic hydrogenation over palladium-on-charcoal, treatment of the resulting, ester-protected derivative with sodium methoxide in methanol, and saponification of the carboxylic ester in aqueous alkali. The ketophosphonate **379** was obtained in two steps (Scheme 63) from the known[525] methyl 2,3-di-*O*-benzoyl-α-L-fucopyranoside **382:** oxidation by the



378 **379** **380** **381**

SCHEME 62

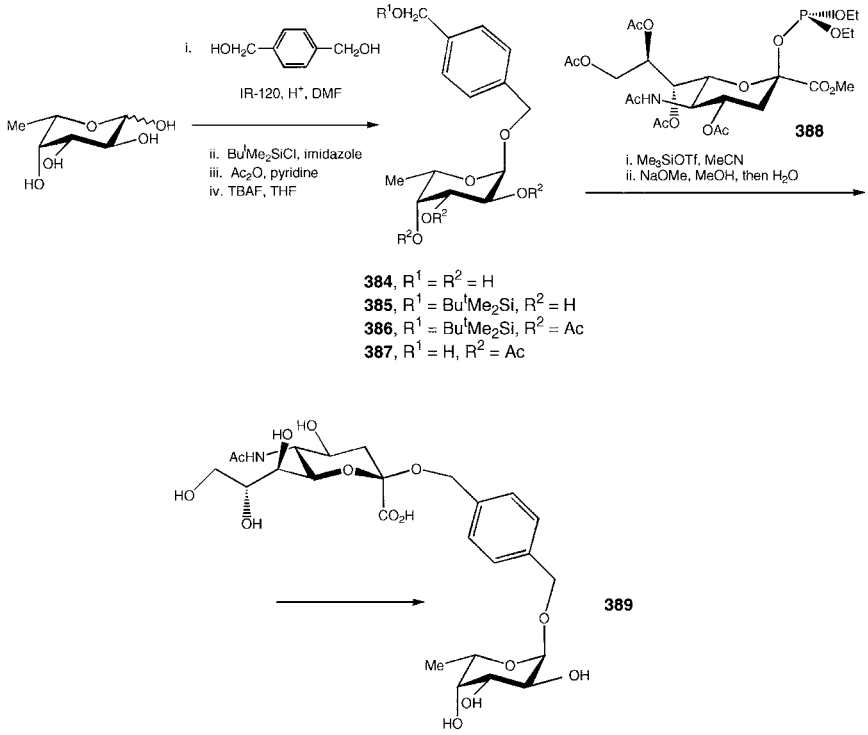
action of pyridinium chlorochromate gave the 4-keto derivative **383** in 80% yield; elaboration of the desired intermediate **379** required much development effort, but was eventually achieved by adding one equivalent of ketone **383** to two equivalents of the lithium dianion of 2-oxopropyl-3-(*O*,*O*-dimethyl)phosphonate[526] in the presence of tris-(2-propoxy) titanium chloride[527] in tetrahydrofuran at −78 °C in 43% yield. In an assay of sLe^x-expressing cells of the cell line U937 binding to activated endothelial cells in culture, the concentration of compound **381** needed to inhibit binding was 25- to 30-fold higher than the concentration of sLe^x required to effect the same extent of inhibition.



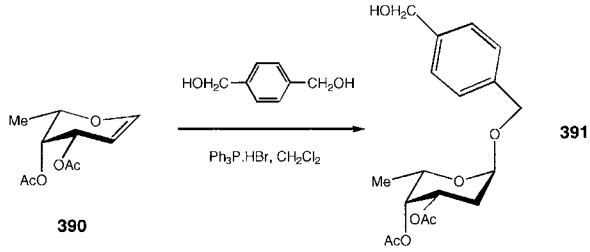
382 **383**

SCHEME 63

(iii) ***The Neu5Ac and Fucose Residues Linked by an Aromatic Spacer.—*** In an approach based on similar considerations, Kaila, Yu, and Xiang[528] at the Genetics Institute replaced the Gal-GlcNAc disaccharide segment by a 4-(oxymethyl)benzyloxy group so as to position the sialic acid and fucose residues in a spacial arrangement similar to that seen in sLe^x. Molecular modelling had been used to design target structure **389** and similar compounds wherein the distance between the glycosidic oxygen atoms of the sialyl and fucosyl residues is ~6.8 Å (0.68 nm), in agreement with that measured for the model of the sLe^x oligosaccharide. Target structure **389** was prepared in six steps starting with a Fischer glycosylation of L-fucose (Scheme 64). The free sugar was heated with excess 4-(hydroxymethyl)benzyl alcohol in *N*,*N*-dimethylformamide in the presence of Amberlite IR-120 (H^+ form) cation-exchange resin to afford a 1.3 : 1 α,β-anomeric mixture of fucosides (54%), from which the required α anomer **384** was isolated in crystalline form. Benzyl alcohol **384** was regioselectively converted into *tert*-butyldimethylsilyl ether **385** by the action of *tert*-butyldimethylchlorosilane in *N*,*N*-dimethylformamide in the presence of imidazole. The secondary hydroxyl



SCHEME 64



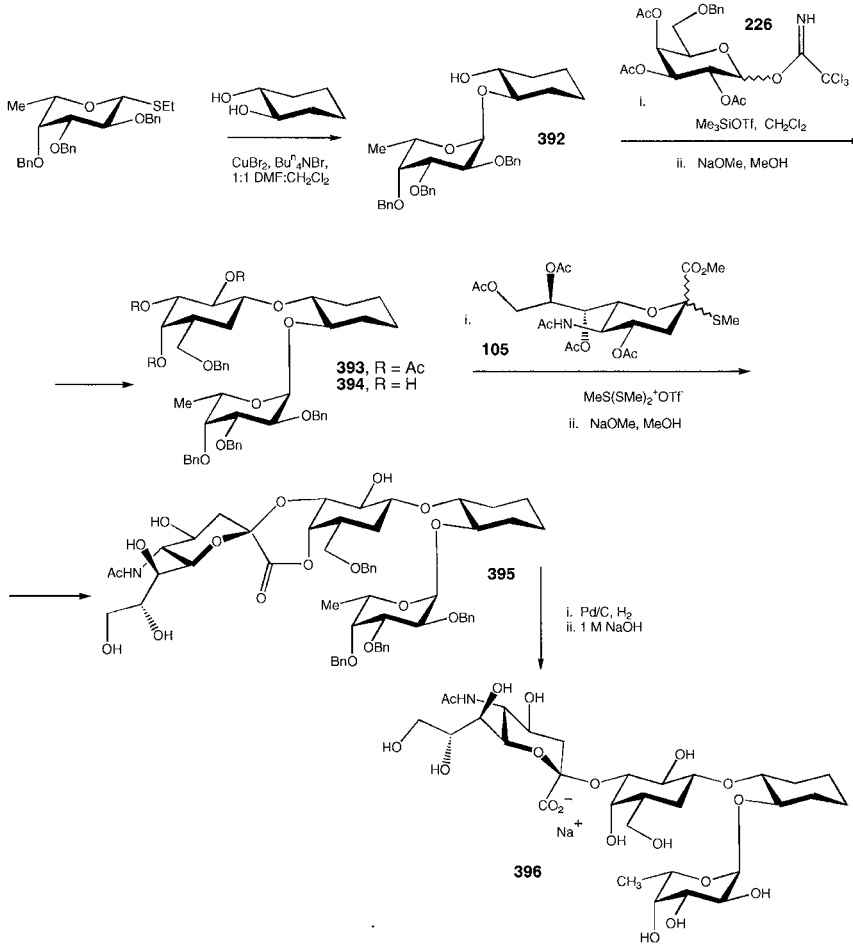
SCHEME 65

functions of the saccharide were acetylated (acetic anhydride in pyridine, **386**), and the silyl ether cleaved using tetrabutylammonium fluoride in tetrahydrofuran, to give the intermediate benzyl alcohol **387** (42% overall from **384**). Conversion of **387** into the desired sialyl derivative was performed by application of Schmidt's phosphite procedure[529]: the reaction of **387** with the NeuAc phosphite derivative **388** in acetonitrile in the presence of trimethylsilyl triflate at $-40\ ^{\circ}C$ gave a 50% yield of anomeric sialosides ($\alpha:\beta$, 1 : 1). The acetate and methyl ester protecting groups were removed by treatment with sodium methoxide in methanol and alkali-catalyzed hydrolysis. From the unprotected mixture of anomers, the target structure **389** was isolated by chromatography on silica gel. In an assay that measures the binding of E-selectin to recombinant human α-1 acid glycoprotein (a glycoprotein that contains five sLe^x glycan chains per molecule), compound **389** and its 2-deoxyfucose and 3-(oxymethyl)benzyloxy analogues were $\sim$20 times less active than sLe^x, a value remarkably similar to that found for compound **381** already discussed. The 3-(oxymethyl)benzyloxy analogue of **389** was prepared by analogous procedures, using 3-(hydroxymethyl)benzyl alcohol in the place of 4-(hydroxymethyl)benzyl alcohol. Similarly, the 2-deoxyfucose analog of **389** was obtained. In that case, however, the benzyl alcohol intermediate **391** was prepared (Scheme 65) by the reaction of 3,4-di-*O*-acetyl-L-fucal (**390**) with 4-(hydroxymethyl)benzyl alcohol in the presence of catalytic amounts of triphenylphosphine hydrobromide in dichloromethane[530,531] in 74% yield. The starting fucal derivative **390** was synthesized from L-fucose in three steps according to a procedure by Iselin and Reichstein.[532]

b. Replacing the GlcNAc Residue of sLe^x with a Simple Diol.—

(i) ***(R,R)-1,2-Dihydroxycyclohexane.—***The pseudotetrasaccharide **396** is an analogue of sLe^x in which the GlcNAc residue is replaced by (*R,R*)-1,2-dihydroxycyclohexane. Toepfer ***et al.***[533] at Hoechst Research and Technology have designed analogue **396** on the premise that sLe^x and sLe^a bind to E-selectin with roughly the same binding constant[2,182] and that therefore the functional groups of

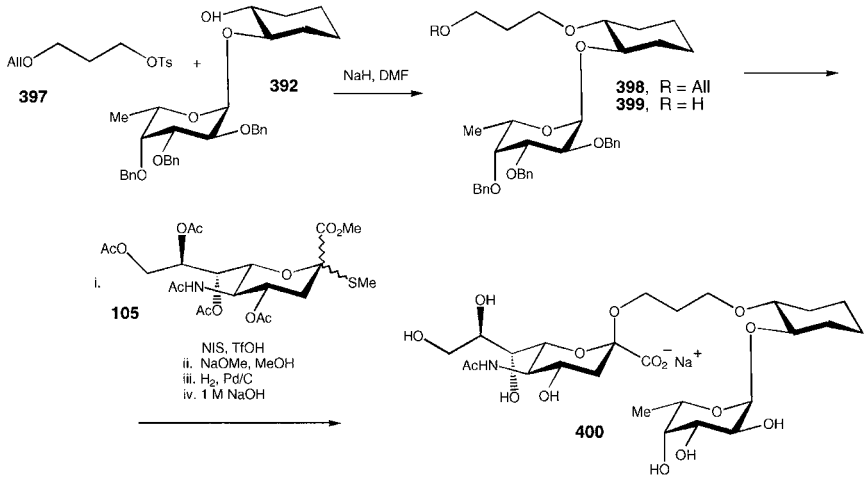
the GlcNAc residue would not make specific contributions to the binding of the sLe^x or sLe^a tetrasaccharides (compare Fig. 23). As a first step, one of the hydroxyl functions of (*R*,*R*)-1,2-dihydroxycyclohexane was fucosylated (Scheme 66) under promotion by copper bromide, with the use of a thioglycoside donor, as described by Ogawa *et al.*[534] (**392,** 81%). The trichloroacetimidate Gal donor **226,** previously reported by Schmidt and his associates,[535] was employed to introduce the Gal residue, affording **393** in 75% yield. With O-6 of the Gal residue in **393** permanently protected by a benzyl group, the acetyl protecting groups were removed by Zemplén saponification, and the resulting 2,3,4-triol **394** was regioselectively



SCHEME 66

3-O-sialylated under a thioglycoside protocol of Hasegawa and Kiso.[536] Zemplén saponification of the protected pseudotetrasaccharide derivative gave the interglycosidic lactone **395** (56% from **393**), which was finally converted into the target structure **396** by sequential catalytic hydrogenation over palladium-on-charcoal and alkali-catalyzed hydrolysis (86%). Compound **396** has IC_{50} values of 0.4 and 0.6 m*M* in receptor assays of oligosaccharide binding to E-selectin and P-selectin, respectively (sLe^x, 1 and 2 m*M*).

In another series of sLe^x/sLe^a analogues prepared by Toepfer *et al.*, the sLe^x/sLe^a tetrasaccharide model was further simplified by replacing the GlcNAc residue with (*R,R*)-1,2-dihydroxycyclohexane and the Gal residue with a variety of symmetrical diols: 1,3-propanediol, glycerol, pentaerythritol (compare Section VI.1.a), 1,4-butanediol, and 1,5-pentanediol (Fig. 23). Typically, one of the hydroxyl groups of the diols was temporarily protected in the form of an allyl ether grouping, to be liberated for O-sialylation following the formation of the ether linkage to the cyclohexane moiety of pseudo-disaccharide **392;** the other hydroxyl group was transformed into a (tosylate or triflate) leaving group suitable for the formation of the ether linkage. Additional hydroxyl groups of the spacer units, if any, were permanently protected in the form of *O*-benzyl ether groups. For example, 1,3-propanediol was mono-O-allylated by the action of allyl bromide in the presence of potassium carbonate and tetrabutylammonium bromide, and the allyl ether reacted with *p*-toluenesulfonyl chloride in pyridine to afford 1-*p*-tolylsulfonyloxy-3-allyloxypropane **397** in 54% yield from the starting diol. Compound **397** was reacted (Scheme 67) with the fucosyloxy-cyclohexanol derivative **392** under promotion by sodium hydride in *N,N*-dimethylformamide to yield the 1,3-diether

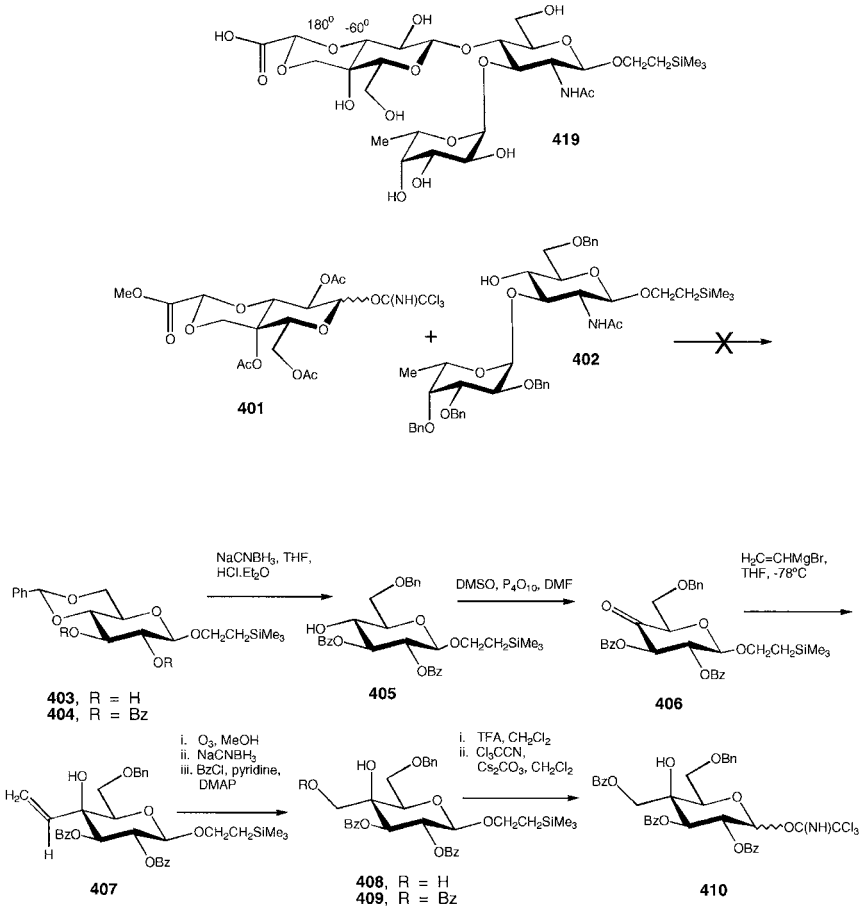


SCHEME 67

derivative **398** (92%). Conversion of **398** into the alcohol **399** was effected by sequential isomerization [tetrakis(triphenylphosphine)rhodium chloride][301] and hydrolysis of the intermediate propenyl ether in 64% yield. Alcohol **399** was O-sialylated through the use of the thioglycoside sialyl donor **105** under promotion by *N*-iodosuccinimide–triflic acid to afford an anomeric mixture of sialosides in 76% yield ($\alpha : \beta$, $\sim$4 : 3). Following chromatographic separation of the anomers, the protected target structure and its β anomer were subjected to sequential catalytic hydrogenation, Zemplén saponification, and alkali-catalyzed hydrolysis to give the candidate inhibitors in 86% yield. Interestingly, the (unnatural) sialyl β anomer **400** had IC_{50} values of 1.6 m*M* and 1.0 m*M* against E-selectin and P-selectin, respectively. IC_{50} values were generally higher when the propanediol spacer molecules substituting for Gal contained additional hydroxyl groups. Spacer groups derived from 1,4-butanediol or 1,5-pentanediol gave unsatisfactory results. Generally, however, P-selectin–ligand binding was more efficiently inhibited than E-selectin binding by the target structures comprising spacer molecules in the place of the Gal residue.[533] The finding of inhibitory activity of the β anomer **400** corroborates the theory that, of the NeuAc residue, only the carboxyl group contributes to binding at the receptor; therefore, the results of Toepfer *et al.* support the considerations that have led to the adoption of the trisaccharide model of selectin–ligand binding (see Section VI.2.). The work at Hoechst Research and Technology on sLe^x glycomimetics has been reviewed by Kretzschmar.[537]

c. An Analogue with a Conformationally Fixed Carboxyl Group.—Thoma *et al.*[538] synthesized the target structure **419,** a compound that contains a conformationally fixed carboxyl group attached to the Gal unit in the form of a fused glyoxylic acid acetal. The angles φ' and ψ' in **419** (180° and −60°) are similar to the φ and ψ angles (167 and −63°) determined initially[475,476] by NMR spectroscopy for the preferred conformation of the Neu5Ac glycosidic linkage in sLe^x in aqueous solution. Should the conformation of the Neu5Ac glycosidic linkage in the selectin-bound sLe^x determinant correspond to the preferred conformation in solution, the authors expected an enhancement of selectin binding of **419** relative to sLe^x, due to decreased loss of entropy upon binding to the receptor.

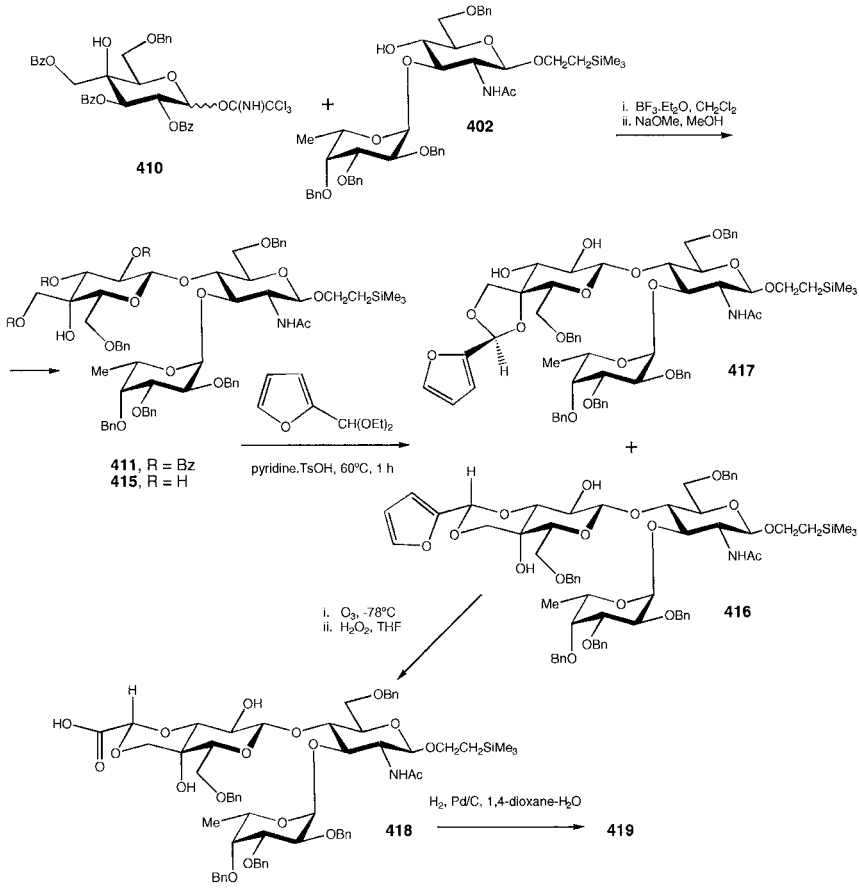
As an initial approach to the synthesis of **419,** Thoma *et al.* attempted to glycosylate the α-L-Fuc-(1 $\rightarrow$ 3)-β-D-GlcNAc disaccharide acceptor **402** with a galactose-derived donor **401** that would comprise the glyoxylic acid acetal modification as designed. However, failure attended several attempts at glycosylation of disaccharide or GlcNAc monosaccharide acceptors with bicyclic donors such as **401.** The target compound **419** was then prepared by glycosylation of acceptor **402** with the conformationally less restricted Gal donor derivative **410.** Synthesis of **410** started with the known trimethylsilylethyl 4,6-*O*-benzylidene-β-D-glucopyranoside (**403**), which was converted into the 2,3-di-*O*-benzoyl derivative **404** (benzoyl chloride, pyridine, 4-dimethylaminopyridine, 96%; Scheme 68). Treatment of **404** in tetrahydrofuran with a solution of hydrogen chloride in ether



SCHEME 68

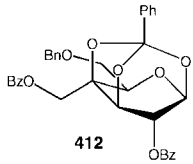
in the presence of sodium cyanoborohydride[327] resulted in reductive cleavage of the cyclic acetal to afford the benzyl ether **405** in 93% yield. Alcohol **405** was oxidized to the 4-keto derivative **406** by the action of dimethyl sulfoxide under catalysis by tetraphosphorus decaoxide. The reaction of vinyl magnesium bromide with the ketone **406** in tetrahydrofuran at $-78\ ^{\circ}C$ gave the galacto tertiary alcohol, **407,** exclusively. The galacto configuration of **407** follows from the nOes observed between the proximal alkenic hydrogen atom and (the axial) H-3 and H-5 of **407.** Ozonolysis of alkene **407,** followed by sodium borohydride reduction of the intermediate aldehyde, afforded the diol **408,** which was esterified at the primary position by the action of benzoyl chloride in pyridine to afford **409** (70% from **405**). The trimethylsilylethyl glycoside **409** was cleaved (1 : 1 trifluoroacetic acid–dichloromethane, 0 °C), and the intermediate hemiacetal treated

with trichloroacetonitrile in the presence of cesium carbonate in dichloromethane to give the trichloroacetimidate donor **410** (81% from **409**). Glycosylation of the α-L-Fuc-(1 $\rightarrow$ 3)-β-D-GlcNAc disaccharide acceptor **402** with donor **410** under carefully optimized conditions [boron trifluoride etherate in dichloromethane, 4 Å (0.4 nm) molecular sieves; Scheme 69] afforded a moderate yield of the trisaccharide derivative **411** (55% with respect to acceptor **402** consumed). In addition, the side products **412, 413,** and **414** were formed during the glycosylation step.[538] The temporary ester protecting groups of **411** were removed (sodium methoxide in methanol) to give **415** (84%). Upon treatment of tetraol **415** with 2-furaldehyde diethyl acetal in the presence of pyridinium *p*-toluenesulfonate, a mixture was produced of the desired, fused six-membered ring acetal **416** (33%) and the five-membered ring spiroacetal **417** (41%). Acetal **416** was subjected to

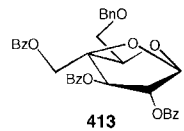


SCHEME 69

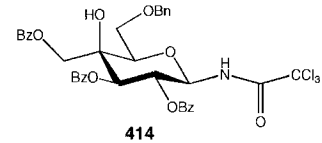
ozonolysis in dichloromethane, and the mixture treated with hydrogen peroxide in tetrahydrofuran to give benzyl-protected **418** (66%). Catalytic hydrogenation over 10% palladium-on-charcoal in 2 : 1 1,4-dioxane–water finally gave **419** in 94% yield. In a competitive E-selectin binding assay,[182] compound **419** was found to be inactive up to 10 m*M* concentration. This finding was taken by the authors to support the conclusions from NMR experiments,[488] which had indicated that the conformation of the α-Neu5Ac-(2 $\rightarrow$ 3)-D-Gal glycosidic linkage assumed during binding to the selectin differs from the preferred conformation in solution. However, subsequent NMR studies by Poppe *et al.* have been interpreted to indicate that the conformer assumed by Ball *et al.,* by Lin *et al.,* and by Scheffler *et al.* to preponderate in solution (φ, ψ, 167°, −63°), while giving rise to a large nOe, is present in only minor proportions. According to Poppe *et al.,* model compound **419** is therefore reflective neither of the conformer bound to E- or P-selectins nor of the one preponderant in solution.



412

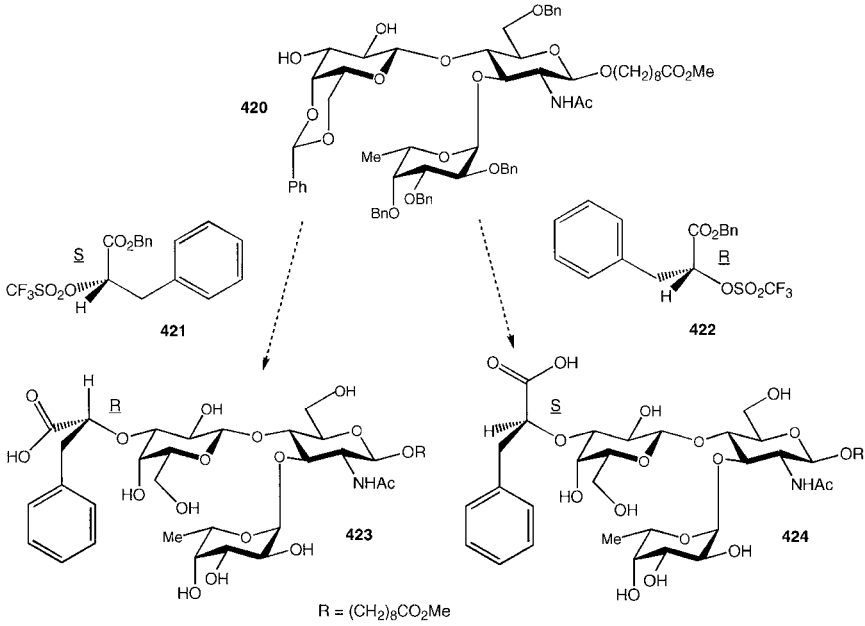


413



414

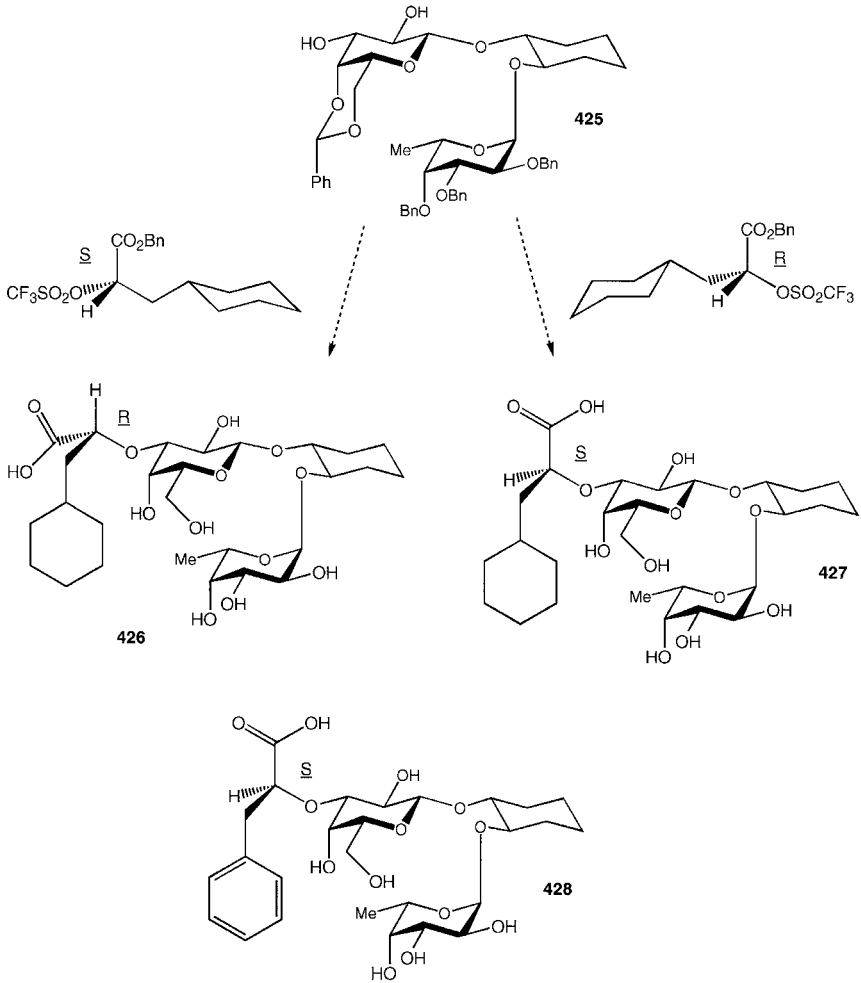
d. Derivatives of Glycolic and Lactic Acids as Substitutes for the Neu5Ac Residue.—Kolb and Ernst have designed a series of sLe^x derivatives that contain *O*-glycolyl, *O*-lactyl, *O*-phenyllactyl, and *O*-cyclohexyllactyl ether groups in position 3 of the Gal residue.[539] Formation of the lactyl ether derivatives was achieved by an SN2 reaction of the Le^x-related nucleophiles **420** or **425** with trifluoromethylsulfonyl esters such as **421** and **422,** described by Degerbeck *et al.*[540] The Le^x nucleophile diol **420** was prepared by benzylidenation of a precursor synthesized by the Chembiomed group.[541,542] As in many similar intermediates, OH-3 of **420** is the only reactive hydroxyl function; in addition, **420** is a glycoside of the hydrophobic aglycon, methyl 9-hydroxynonanoate, a spacer originally designed by Lemieux and his associates for linking to carriers of biologically active oligosaccharides and their derivatives.[288] For example, formation of the (*R*) and (*S*)-phenyllactyl ether derivatives **423** and **424** from **420** and the (*S*)- and (*R*)-benzyl 3-phenyl-2-(trifluoromethylsulfonyloxy)propanoates **421** and **422** is illustrated in Scheme 70. Compound **420** was converted into a 2,3-*O*-stannylidene derivative, which was reacted with excess **421** or **422** to give intermediate (*S*)-phenyllactyl ethers from which the target compounds **423** or **424** were obtained by catalytic hydrogenation in good overall yield. Similarly, Scheme 71 illustrates the preparation of the diastereoisomeric cyclohexyllactyl derivatives **426** and **427** from the Le^x-related precursor **425,** which contains an (*R*,*R*)-1,2-cyclohexanediol grouping



SCHEME 70

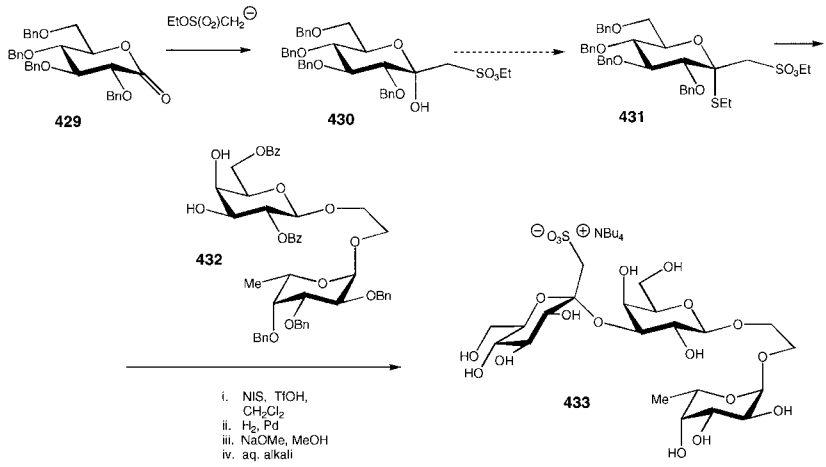
in the place of the GlcNAc residue. Of the series of substituted lactyl ether derivatives prepared by Kolb and Ernst, only certain (*S*)-lactyl diastereoisomers are biologically active. Among the compounds derived from **420,** the (*S*)-lactyl and the (*S*)-phenyllactyl derivatives were 4 times and 2.6 times less active than sLe^x. Of the series containing (*R,R*)-cyclohexanediol in place of the GlcNAc unit, the glycolyl ether derivative was 4.5 times less active than sLe^x. However, the corresponding (*S*)-cyclohexyllactyl and (*S*)-phenyllactyl derivatives **427** and **428** were 3 times and 10 times more potent than spacer-linked sLe^x. Based on Monte Carlo (jumping between wells)/stochastic dynamics calculations, Kolb and Ernst have developed a molecular modeling tool that enables to predict that the (*S*)-phenyllactyl or -cyclohexyllactyl diastereoisomers populate conformational energy minima that correspond to the conformation of bound sLe^x, and are therefore more potent ligands of selectins.[539]

e. Carbohydrate Sulfonic Acids as Substitutes of the Neu5Ac Residue.— Recently, carbohydrate-derived sulfonic acids have been used to substitute for the Neu5Ac residue of sLe^x. Lipták and his associates[543] prepared sulfonomethyl analogues of aldos-2-ulosonic acids by treating per-*O*-benzyl aldonolactones with the carbanion derived from ethyl methanesulfonate (Scheme 72). The heptulose derivative **430,** obtained from 2,3,4,6-tetra-*O*-benzylglucono-1,5-lactone (**429**), was



SCHEME 71

converted into a thioglycoside donor **431,** which was used to glycosylate the Le^x acceptor analogue **432** under promotion by *N*-iodosuccinimide–trifluoromethanesulfonic acid in dichloromethane. The product **433** of this glycosylation reaction contains the sulfonic acid unit in the α-(axial) anomeric configuration, whereas the Neu5Ac unit of the natural sLe^x structure is attached by an α-equatorial linkage. Appropriate binding data will be required to show whether the sulfonate group of analogue **433** is suitably positioned to provide the binding strength required of pharmaceutically applicable inhibitors.

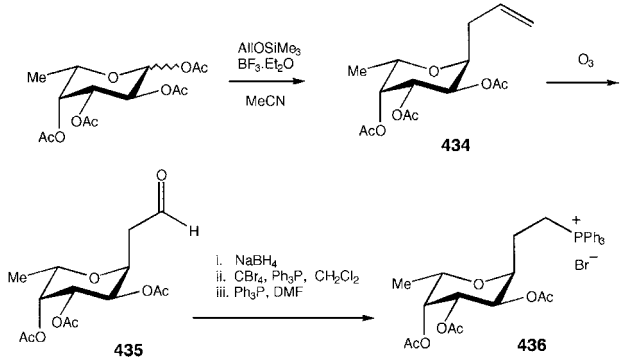


SCHEME 72

2. The Trisaccharide Model

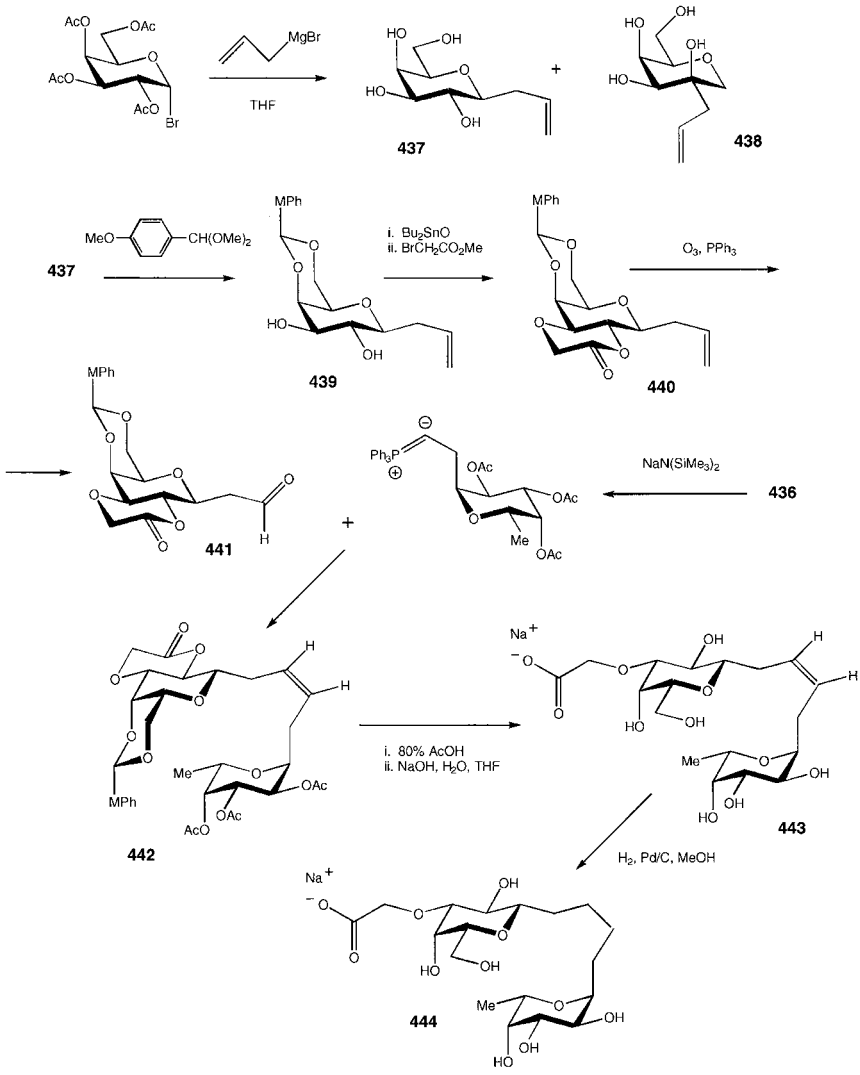
Results of several of the searches for natural selectin ligands, of structure–activity studies, and of investigations directed at the development of ligand–selectin binding inhibitors (Section V) had indicated that, of the Neu5Ac residue in sLe^x or sLe^a, only the carboxyl group or an equivalent, negatively charged group is required for binding. As a consequence, a substantial number of candidate selectin inhibitors have been synthesized analogous to the Le^x or Le^a trisaccharides and containing a small, anionic functional group to represent the carboxyl group of the NeuAc residue. Here, such compounds are considered under the "trisaccharide paradigm."

a. *C*-Glycosylic Synthons, Representing the Fucose and Galactose Residues, Joined by Way of a Wittig Alkene Synthesis.—Based on the previously established structure–activity relationships outlined in Section V, Wong and his associates[544] designed the target structures **443** and **444** that contain the β-Gal and α-Fuc residues *C*-glycosylically linked to a spacer group representing the GlcNAc residue. The Neu5Ac residue is replaced by a 3-*O*-glycolic acid ether grouping linked to the Gal residue. To obtain the nucleophilic reactant of the Wittig alkene synthesis, 1,2,3,4-tetra-*O*-acetyl-L-fucopyranose (Scheme 73) was treated with allyltrimethylsilane and boron trifluoride etherate in dry acetonitrile at room temperature to afford the *C*-glycosyl compound **434** in excellent yield and 10 : 1 anomeric selectivity.[545] Compound **434** was subjected to ozonolysis, the resulting aldehyde **435** reduced to the primary alcohol by the action of sodium borohydride, the alcohol converted into the bromide (carbon tetrabromide–triphenylphosphine), and the bromide treated with triphenylphosphine to afford



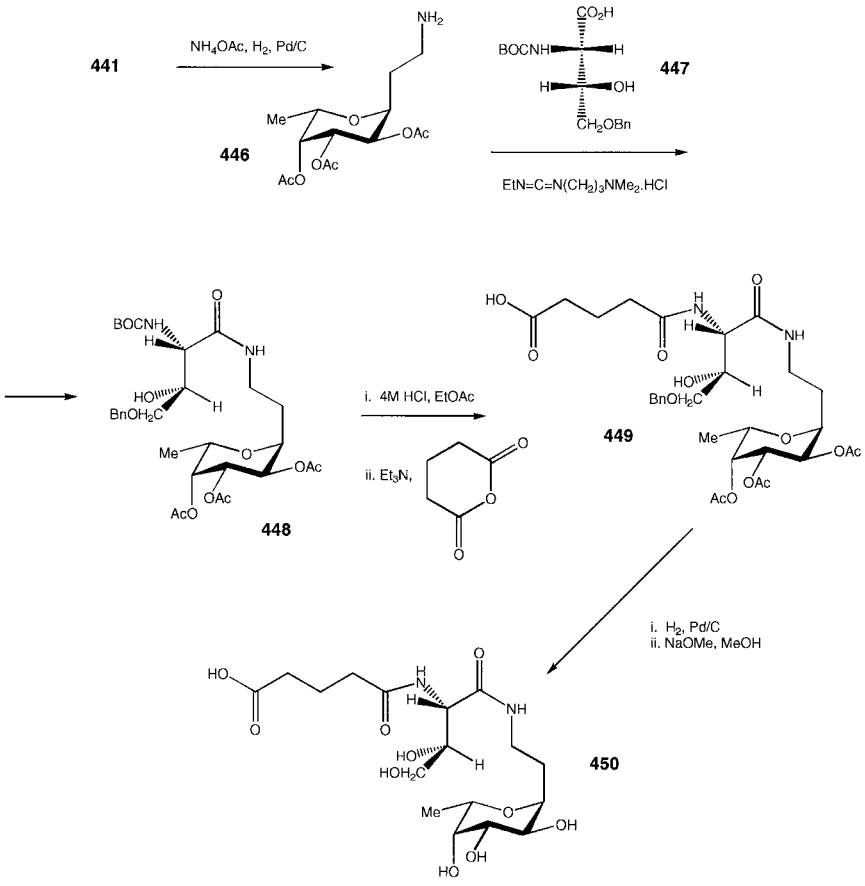
SCHEME 73

the phosphonium salt **436** (48% from **434**). To prepare the β-*C*-glycosyl aldehyde **441,** the synthon representing the β-Gal residue, "acetobromogalactose," was treated with allylmagnesium bromide in tetrahydrofuran to afford a 3 : 1 mixture of the desired β-*C*-galactopyranosyl derivative **437** (60%) and the tertiary alcohol **438** as an unexpected side product (Scheme 74). Compound **437** was treated with *p*-methoxybenzaldehyde dimethyl acetal in the presence of *p*-toluenesulfonic acid to afford the 4,6-*O*-*p*-methoxybenzylidene derivative **439** (90%). Attachment of the glycolic acid ether group was effected by way of the 2,3-*O*-(dibutyl)stannylidene derivative, obtained by refluxing **439** with dibutyl tin oxide in toluene. The stannylidene acetal was then treated with methyl bromoacetate in toluene at reflux in the presence of tetrabutylammonium iodide to give the lactone ether **440.** Compound **440** was subjected to ozonolysis, the primary ozonide being decomposed by the action of triphenylphosphine, to afford aldehyde **441** (60% from **438**). The Wittig reaction of **441** with the ylide obtained by treatment of phosphonium salt **436** with the sodium salt of hexamethyldisilazane in tetrahydrofuran at $-78\ ^{\circ}C$ afforded a 60% yield of the alkene **442.** The acetal and ester protecting groups were sequentially removed from **442** by the actions of aqueous acetic acid and sodium hydroxide in aqueous tetrahydrofuran to give the target structure **443** (80% from **442**). Olefin **443** was converted into the additional target structure **444** by catalytic hydrogenation over palladium-on-carbon in methanol in quantitative yield. The IC_{50} values for compounds **443** and **444** as inhibitors of the binding of sLe^x glycoconjugate to immobilized E-selectin are 15 and 20 m*M*, respectively; by comparison, sLe^x oligosaccharide has an IC_{50} of 1 m*M* in this assay. Interestingly, the analogue **445** (Scheme 76), produced by O-glycosylation of an ethylene glycol spacer, had an IC_{50} of 1.5 m*M*. These findings were taken to indicate that binding of conformationally flexible candidate inhibitors such as **444** entails a relatively large loss of entropy, making these compounds less potent ligands of the selectins.



SCHEME 74

b. Substituting a Dihydroxy Amino Acid for the Gal Residue.—The intermediate aldehyde **435** was utilized, in addition, for the synthesis[544] of the amide-linked target structure **450** (Scheme 75). Catalytic hydrogenation of **435** in the presence of ammonium acetate gave the primary amine **446** (50% from **435**). Subsequently, compound **446** was condensed with **447,** a derivative of (2*S*,3*S*)-2-amino-3,4-dihydroxybutanoic acid, to afford amide **448** in 82% yield.

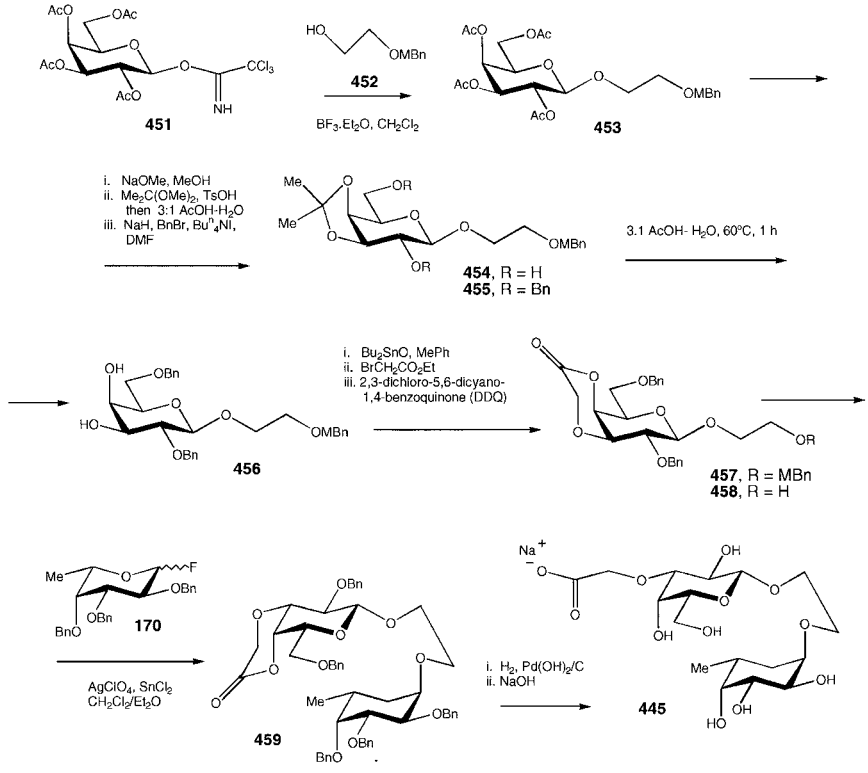


SCHEME 75

1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) was used as the condensing agent for the reaction. The N-protecting *tert*-butoxycarbonyl group was removed from **448** by the action of 4 *M* hydrochloric acid in admixture with ethyl acetate to afford a secondary amine that, upon reacting with glutaric anhydride in the presence of triethylamine, gave the glutaric half-amide derivative **449** (80% from **448**). Sequential catalytic hydrogenation and Zemplén transesterification gave the target structure **450** (62% from **449;** IC_{50} 1.3 m*M* as determined in the assay mentioned in the previous paragraph). The inhibitory activity of **450** is comparable to that of sLe^x, a finding attributed by the authors to the presence, in **450,** of all the essential functional groups required for binding of sLe^x to E-selectin: hydroxyl functions 2, 3, and 4 of Fuc in the form of the *C*-glycosylic fucosyl residue; hydroxyl functions 4 and 6 of the Gal residue in the form of hydroxyl

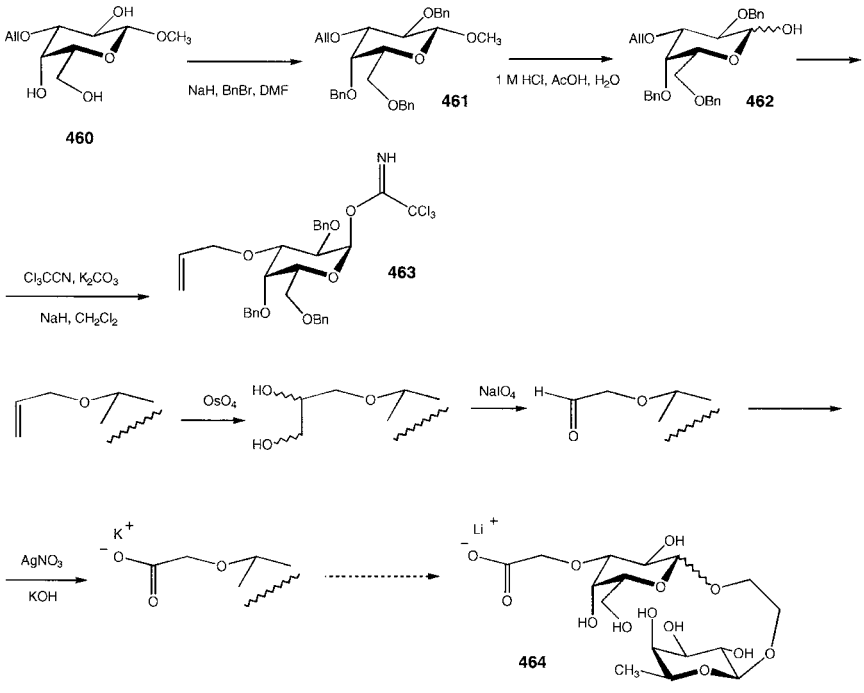
groups 3 and 4 of the (2*S*,3*S*)-2-amino-3,4-dihydroxy-butanoic acid residue; and the carboxyl group of the Neu5Ac residue in the form of the free carboxyl function of the glutaric half amide.

c. Substituting an Ethylene Glycol Spacer for the GlcNAc Residue.—In the target structure **445,** the reducing, branching GlcNAc unit of sLe^x or sLe^a is replaced by an ethylene glycol spacer. For the synthesis of **445** by Wong and his associates,[544] the primary alcohol **452** was first prepared by reacting the (dibutyl)stannylidene derivative of ethylene glycol with 4-methoxybenzyl bromide. Alcohol **452** was glycosylated using as galactosyl donor the trichloroacetimidate **451** (Ref. 275; Scheme 76) under catalysis by boron trifluoride etherate in 78% yield. The tetraacetylated β-galactoside **453** was subjected to Zemplén saponification and the resulting glycoside reacted with 2,2-dimethoxypropane in the presence of *p*-toluenesulfonic acid, then dilute acetic acid,[456] to afford the 3,4-*O*-isopropylidene derivative **454** (79% from **453**). Compound **454** was O-benzylated in positions 2 and 6, and the isopropylidene acetal **455** hydrolyzed to give the



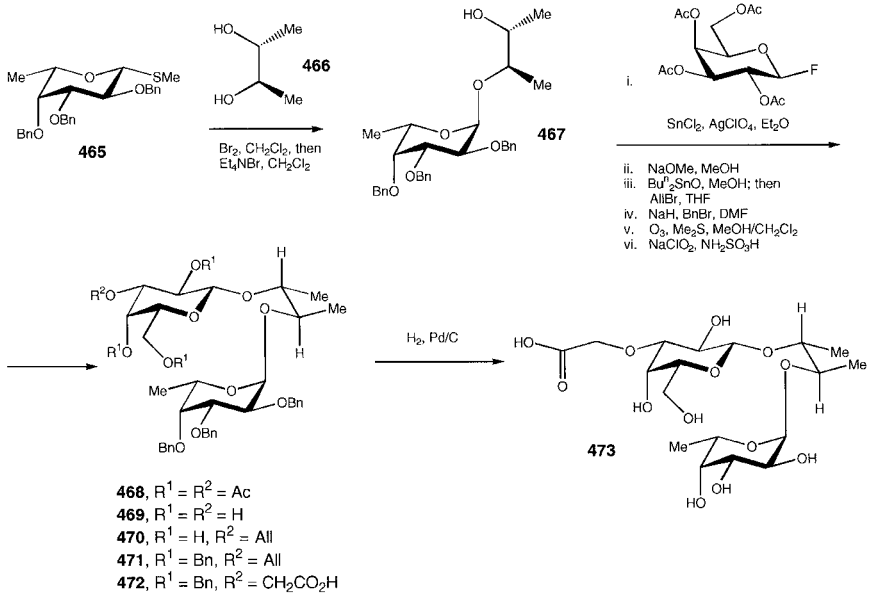
SCHEME 76

3,4-diol **456** (84% from **454**). Treatment of **456** with dibutyltin oxide in toluene afforded the stannylidene acetal, which was reacted with ethyl bromoacetate in the presence of tetrabutylammonium iodide to give the ether lactone **457** in 82% yield. The 4-methoxybenzyl ether protecting group was removed from **457** by the action of 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ; **458,** 88%). Alcohol **458** was glycosylated under Nicolaou's conditions of the Mukaiyama glycosylation reaction (silver perchlorate, tin(II) chloride in dichloromethane–ether) using as fucosyl donor the benzylated fluoride **170.** Donor **170** had been obtained in 85% yield by treating the known 2,3,4-tri-*O*-benzyl-L-fucopyranose with diethylaminosulfur trifluoride (DAST). The desired β-D-Gal-α-L-Fuc diastereoisomer **459** was obtained as the major component of a 3.3 : 1 mixture with its β-D-Gal-β-L-Fuc anomer. The anomeric product mixture was inseparable by thin-layer chromatography (TLC), but was successfully separated by high-performance liquid chromatography (HPLC) on a silica support. To remove the protecting groups, **459** was hydrogenated over palladium hydroxide on carbon, and the resulting mixture of methyl ester and carboxylic acid treated with alkali to afford the target compound **445** in near-quantitative yield from **459.** In one of the earlier studies aimed at the preparation of inhibitors of the sLe^x–E-selectin interaction, Ragan and Cooper[546] developed an approach to the synthesis of **445** (Scheme 77). However,



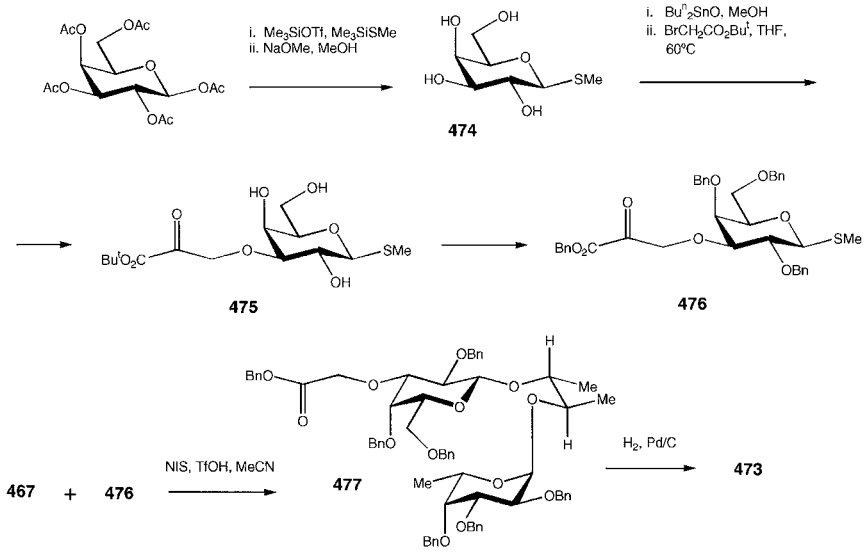
SCHEME 77

their preparation **464** contains the fucose unit in the unnatural β-anomeric configuration and constitutes an anomeric mixture of galactosides. Not surprisingly, **464** was found to inhibit the binding of HL60 cells to E-selectin only at concentrations 40–45 fold higher than those of sLe^x tetrasaccharide. The authors used the synthon **463** to introduce the modified Gal residue into **464.** Compound **463** was prepared from methyl β-D-galactopyranoside in four steps: the starting material was selectively converted into the 3-allyl ether **460** via the 3,4-*O*-stannylidene derivative (87%). Compound **460** was O-benzylated (sodium hydride and benzyl bromide in *N*,*N*-dimethylformamide, 84% of **461**). Glycoside **461** was subjected to acid-catalyzed hydrolysis (1 *M* hydrochloric acid in aqueous acetic acid, 100 °C, 62%), and the protected pyranose **462** converted[547] into the trichloroacetimidate **463** in quantitative yield. Following transfer of the modified Gal residue from donor **463** to the ethylene glycol spacer, the 3-allyl ether group was converted into the glycolic acid ether group by sequential dihydroxylation of the double bond (osmium tetraoxide, *N*-methylmorpholine-*N*-oxide in aqueous tetrahydrofuran), oxidative cleavage of the diol by the action of periodic acid, and oxidation of the glycolic aldehyde ether to the carboxylic acid by treatment with alkaline silver nitrate in aqueous tetrahydrofuran (Scheme 77). Another pseudotrisaccharide target structure, compound **473,** was synthesized by Prodger *et al.*[548]; this candidate inhibitor contains the 3-*O*-glycolic acid ether group to substitute for the Neu5Ac residue, and (*R*,*R*)-2,3-butanediol **466** to provide the spacer or scaffolding function of the GlcNAc residue of sLe^x. In their initial approach, the authors started by fucosylating one of the hydroxyl groups of the diol **466** using as fucosyl donor the known[549] thioglycoside **465** (Scheme 78). According to the glycosidation protocol chosen, the thioglycoside donor is first converted, by treatment with bromine, into an anomeric mixture of glycosyl bromides; under the "halide inversion" conditions of Lemieux,[294] the kinetically controlled glycosylation reaction provides for a 78% yield of fucoside **467,** having exclusively the α-(L-) anomeric configuration. The glycosylation of the hydroxyl group of **467** through the use of acetylated galactosyl donors presented considerable difficulty; an optimal yield of **468** (21%) was obtained under Mukaiyama conditions.[271] The use of (the relatively unreactive or "disarmed"[550]) acetylated Gal donors was dictated by the authors' strategy: temporary (ester) protection of the Gal residue would enable the eventual manipulations of that residue, required for the introduction of the 3-glycolic acid ether group, without affecting the (permanently protected) α-L-fucose residue. Zemplén saponification of **468** gave the tetraol **469** in 93% yield. Compound **469** was regioselectively converted into the 3-allyl ether **470** (65%) by treatment with allyl bromide of an intermediate 3,4-stannylidene acetal in tetrahydrofuran. Conventional O-benzylation of **470** (sodium hydride, benzyl bromide in *N*,*N*-dimethylformamide) gave the fully protected allyl ether derivative **471** in 73% yield. For the conversion of the allyl ether group into the *O*-glycolic acid ether group, compound **471** was subjected to ozonolysis with reductive workup (dimethyl sulfide), and the intermediate



SCHEME 78

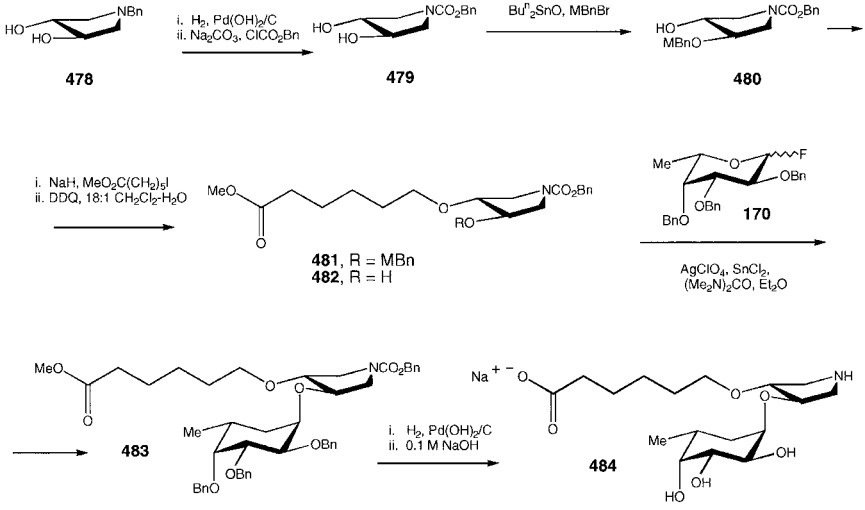
aldehyde oxidized by the action of sodium chlorite in the presence of amidosulfonic acid to afford the carboxylic acid **472** (35% from **471**). Catalytic hydrogenation of **472** over palladium-on-charcoal gave the target structure **473** in 85% yield. As an alternative to this linear route, an alternative, convergent synthesis of **473** was devised by Prodger and his associates,[548] avoiding both the poor yields of the galactosylation step and the disadvantageously late introduction and processing of the 3-allyl ether group. In this route (Scheme 79), thioglycoside **474** was used as the synthon corresponding to the Gal residue. To prepare **474,** galactose pentaacetate was treated with trimethylsilyl triflate and (methylthio)trimethylsilane to afford the O-acetylated β-thiogalactoside; Zemplén saponification gave the free thioglycoside **474** (60% from galactose pentaacetate). The 3-*O*-glycolic acid substituent was then regioselectively introduced by way of the 3,4-stannylidene acetal; however, rather than proceeding through the allyl ether intermediate, the authors used *tert*-butyl bromoacetate as the electrophilic reactant of the etherification reaction (**475;** 65% from **474**). O-Benzylation of compound **475** (sodium hydride, benzyl bromide in *N*,*N*-dimethylformamide) proceeded readily but was accompanied by saponification of the *tert*-butyl ester. The carboxylic acid derivative was then in turn subjected to the same O-benzylation conditions to afford the benzyl ester **476** (36% from **475**). The intermediate alcohol **467** (Scheme 78) was next glycosylated, **476** being used as the Gal donor. In a kinetically controlled glycosylation reaction as described by R. R. Schmidt and his associates[551] (*N*-iodosuccinimide,



SCHEME 79

triflic acid, acetonitrile), a 57% yield of the desired compound **477** was obtained (α anomer, 15%). Catalytic hydrogenation of **477** over palladium-on-charcoal gave the target compound **473** in 70% yield.

d. Substituting a Dihydroxypyrrolidine Ring for the GlcNAc Residue.—In the target structure **484,** the (*R*,*R*)-3,4-dihydroxypyrrolidine ring serves as a substitute of the GlcNAc unit of sLe^x or sLe^a. For the synthesis of **484** described by Huang and Wong,[552] *N*-benzyl-(*R*,*R*)-3,4-dihydroxypyrrolidine **478,** prepared according to the literature[553,554] from L-threaric (D-tartaric) acid, was used as the starting material. First, compound **478** was converted into the *N*-benzyloxycarbonyl derivative **479** by sequential catalytic hydrogenation (in the presence of acetic acid) and treatment with benzyl chloroformate (79% from **478;** Scheme 80). The diol **479** was refluxed with dibutyltin oxide in toluene, and the stannylidene acetal treated with 4-methoxybenzyl bromide in the presence of tetrabutyl ammonium iodide to give the mono-(4-methoxy)benzyl ether **480** (62%). The 5-(methoxy)carbonyl pentyl ether derivative **481** was obtained from **480** by treatment with methyl 6-iodohexanoate (prepared from the commercially available 6-hydroxyhexanoic acid) in the presence of sodium hydride in 56% yield. Treatment of **481** with 2,3-dichloro-5,6-dicyano-1,4-quinone in dichloromethane–water afforded the ether–alcohol **482,** which was glycosylated (**483,** 64% yield from **481**) using the tri-*O*-benzyl fucosyl fluoride donor **170** under Mukaiyama conditions.[279] Catalytic hydrogenation of **483** over palladium hydroxide in the presence of acetic acid

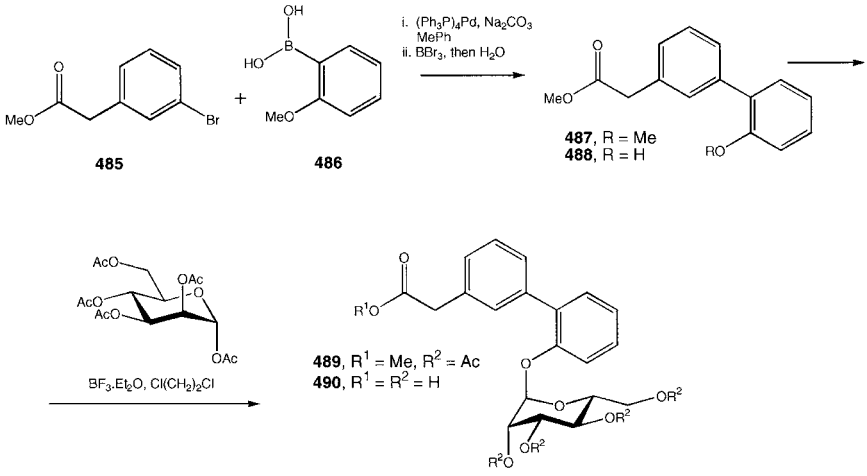


SCHEME 80

removed the *O*-benzyl and *N*-benzyloxycarbonyl protecting groups, and subsequent treatment with 0.1 *M* sodium hydroxide gave the target compound **484.** The IC_{50} value of **484** in a standard assay was found to be ~12.5 times that of sLe^x tetrasaccharide, or 5 times that of Le^x-3-sulfate under analogous conditions. The lower activity of **484** was attributed by the authors to the lack of a functional equivalent of the 4,6-diol grouping of the Gal residue of sLe^x.

3. The Disaccharide Model

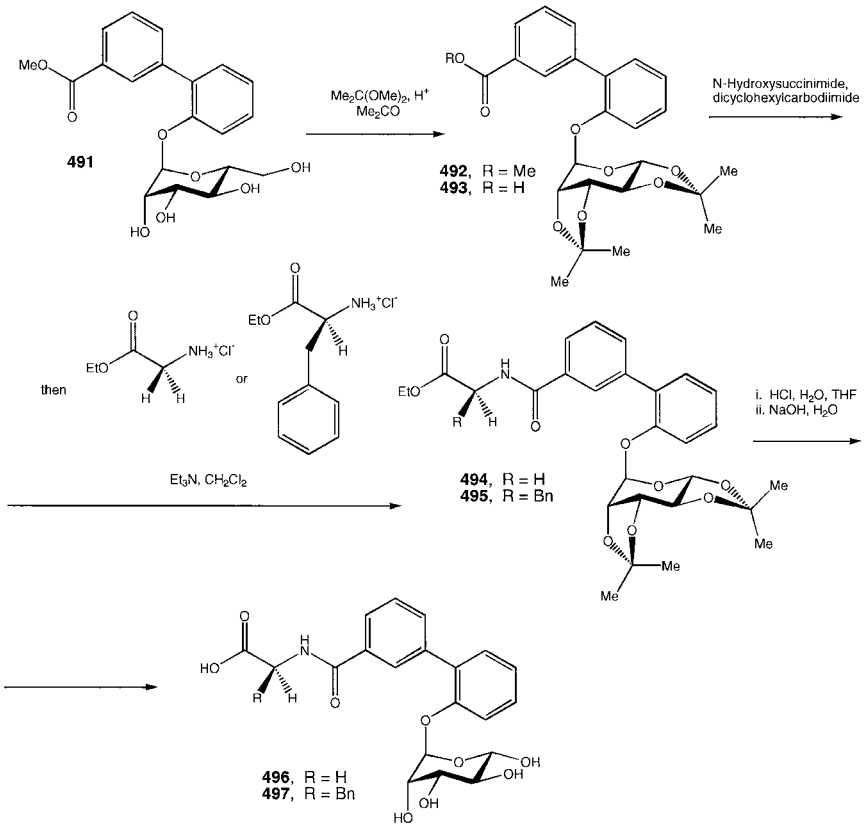
Several candidate inhibitors have been designed according to the disaccharide paradigm, an attractive concept that derives from the homology between the selectins and rat mannose binding protein (MBP). However, the isosteric relationship between the L-fucose and D-mannose residues as visualized in Fig. 21 is not the only one conceivable. Rather, CHOH groups C-2, C-3 and C-4 of the L-fucose residue of sLe^x may be considered isosteric with CHOH groups C-4, C-3 and C-2 of an α-mannose residue.[555] With regard to sLe^x analogues of decreased complexity, the disaccharide model suggests that α-mannopyranose could substitute for the fucose residue of sLe^x and could be linked directly to the Gal residue or its equivalent. The GlcNAc residue or the corresponding spacer groups could be altogether omitted. As in the trisaccharide model, the contribution of the Neu5Ac residue would be provided by a negatively charged group attached directly to the Gal residue or its equivalent.



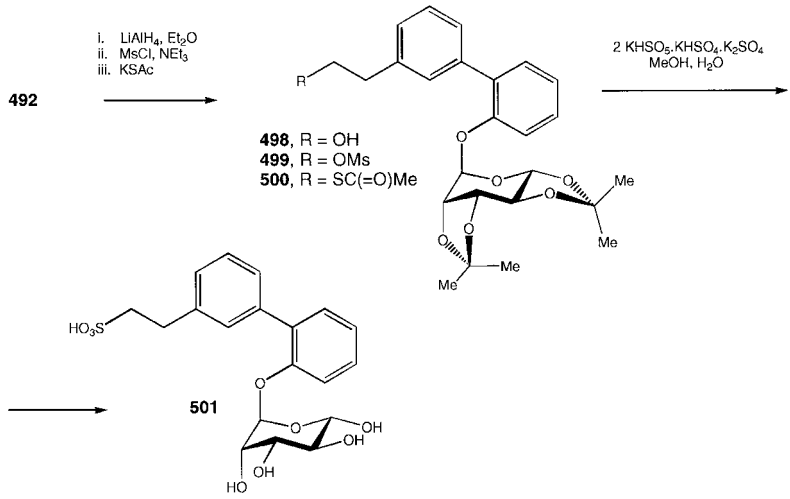
SCHEME 81

a. Analogues Derived from (α-D-Mannopyranosyloxy)biphenyl-Substituted Carboxylic Acids.—An extensive series of (α-D-mannopyranosyloxy) biphenyl-substituted carboxylic acids have been prepared by Kogan and his associates at the Texas Biotechnology Corporation.[555] In the design of Kogan *et al.*, the biphenyl system provides a spacer function equivalent to the Gal residue of sLe^x, presumably to hold the carboxylate group in the position requried for interaction with Arg-97 of E-selectin. Compound **490** (Scheme 81) is a typical example of this type of biphenyl analogue. The key step in the synthesis of **490** and of related derivatives is the palladium(0)-mediated, Suzuki coupling[556] of suitably substituted 2-methoxyphenylboronic acids with halide derivatives of phenylacetic acids, benzoic acids, phenoxyacetic acids, and the like. Thus, for the synthesis of **490,** anisole was converted into 2-methoxyphenylboronic acid (**486**) by treatment with *n*-butyllithium and trimethyl borate in tetrahydrofuran. Compound **486** was coupled with methyl 3-bromophenylacetate (**485**) in the presence of tetrakis(triphenylphosphine)palladium(0) and sodium carbonate in toluene. The resulting biphenyl ether derivative **487** was hydrolyzed by the action of boron tribromide–water in dichloromethane to afford the phenol **488** in 66% yield. Glycosylation of **488** with the donor α-D-mannose pentaacetate in the presence of boron trifluoride etherate in 1,2-dichloroethane gave the α-mannoside **489** in 91% yield. Following saponification of **489** by the action of lithium hydroxide, the crystalline carboxylic acid **490** was obtained in 47% yield. Compound **490** had IC_{50} values of 2, 2.6, and 1 m*M* (sLe^x, 3, 2, and 2 m*M*) when tested in an *in vitro* model of HL-60 cell binding to human E-, P-, or L-selectin fusion proteins linked to magnetic beads[177] (cf. Section II.5.b.iii).

In addition, the authors examined a number of compounds comprising additional substituents on the glycosylated aromatic ring; all of these were less active than **490.** Also explored was the effect of the distance between the aromatic ring and the carboxylate function in compound **490.** In agreement with their model studies, Kogan *et al.* found the phenylacetic acid type to be the most active. Another series of compounds were prepared by linking amino acids, in amide linkage, to the carboxyl group of benzoic acid-type analogues of **490** (Scheme 82). For this purpose, intermediate **489** was subjected to Zemplén saponification, and the resulting tetraol **491** converted into the 2,3; 4,6-diisopropylidene acetal **492.** Alkali-catalyzed saponification of afforded the carboxylic acid **493,** which was converted into the amide acetal esters **494** or **495,** using the ethyl ester hydrochlorides of glycine or phenylalanine. Target compounds **496** and **497** were obtained by sequential acid- and alkali-catalyzed hydrolysis; in an E-selectin cell binding



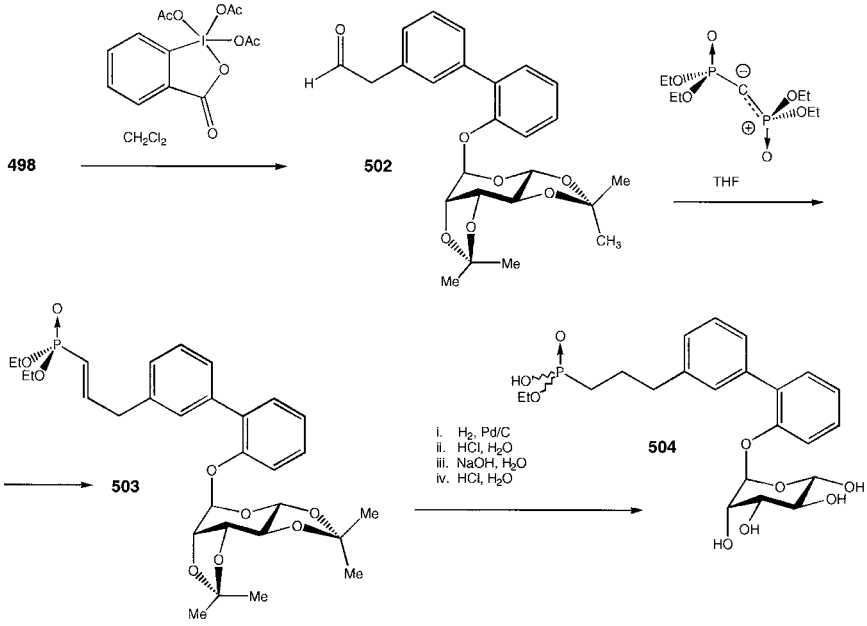
SCHEME 82



SCHEME 83

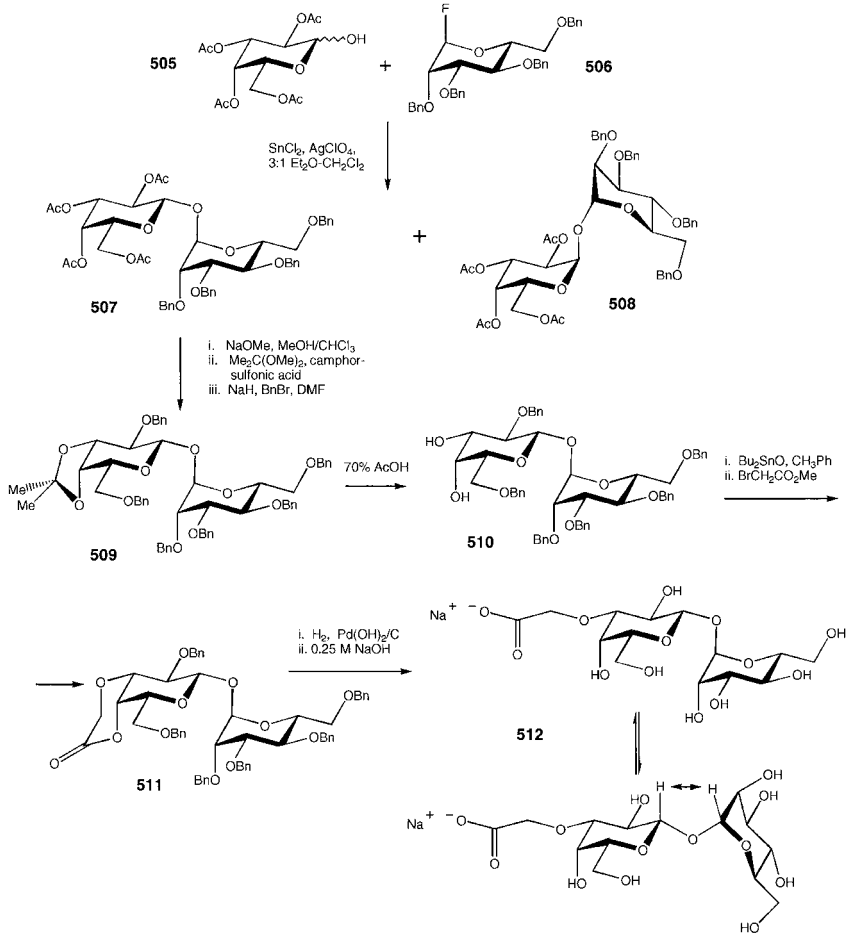
assay, they had IC_{50} values of 4.6 and 3.8 m*M*, respectively, but were inactive in the corresponding P- or L-selectin binding assays. Analogues have also been prepared that contain alkylsulfonate or alkylphosphonate groups to represent the carboxylate group of Neu5Ac. Thus, intermediate **492** (Scheme 82) was reduced by the action of lithium aluminum hydride to afford the ethanol derivative **498** in 94% yield (Scheme 83). Alcohol **498** was converted, in 90% yield, into the methanesulfonate **499,** which was treated with potassium thioacetate to give the xanthate **500** (80%). Conversion of **500** into the required target structure **501** was effected by sequential oxidation by the action of Oxone (2 $KHSO_5$. $KHSO_4$. K_2SO_4) and acid-catalyzed hydrolysis (38%). The intermediate ethanol derivative **498** was also oxidized to the aldehyde **502** by the action of Dess–Martin periodinane in 57% yield (Scheme 84). Aldehyde **502** was subjected to Wadsworth–Emmons reaction with the ylid produced by treatment of tetraethylmethylene diphosphonate with the potassium salt of hexamethyldisilazane, to afford the propenyl phosphonate **503** in 63% yield. Sequential acid- and alkali-catalyzed hydrolysis gave the phosphonic acid ethyl half-ester **504** in 40% yield. Both the sulfonic acid and phosphonic acid half ester derivatives **501** and **504** were less potent inhibitors of selectin–ligand interaction than the related carboxylic acid derivatives.

b. Design and Syntheses of Candidate Inhibitors Derived from the Sequence β-D-Gal-(1 → 1)-α-D-Man.—In another application of the disaccharide model, Hiruma *et al.*[557] designed compound **512,** in which an α-D-mannopyranosyl group is linked to the β-anomeric hydroxyl group of galactopyranose. In a first attempt at the synthesis of **512,** a tetra-*O*-acetylgalactose trichloroacetimidate donor



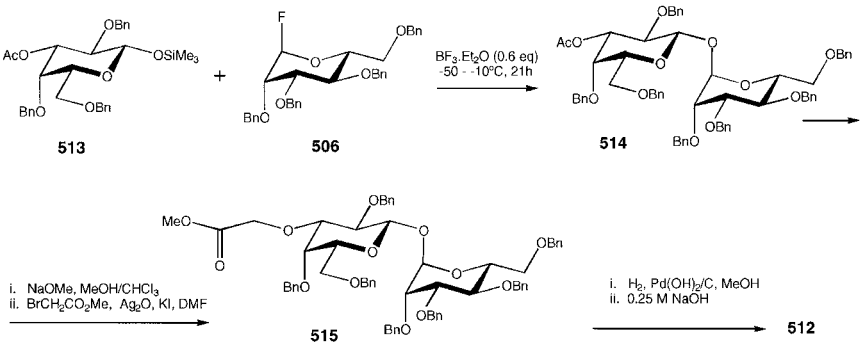
SCHEME 84

with tetra-O-benzylated or tetra-O-acetylated mannopyranose acceptors under promotion by trimethylsilyl triflate or boron trifluoride etherate had given unexpected products, such as mannopyranosyl-mannopyranosides or 2-O-mannosylated galactopyranose derivatives. When 2,3,4,6-tetra-*O*-benzyl-α-D-mannopyranosyl fluoride **506** as the glycosyl donor was allowed to react (Scheme 85) with the acceptor, 2,3,4,6-tetra-*O*-acetyl-D-galactopyranose (**505**) under Mukaiyama conditions[271] (stannous chloride–silver perchlorate), a mixture of β- and α-D-galactopyranosyl-α-D-mannopyranosides (**507** and **508**) was obtained. The required disaccharide intermediate **507** was separated and subjected to Zemplén deacetylation; the resulting tetraol was treated with 2,2-dimethoxypropane in the presence of D,L-camphorsulfonic acid to afford the 3,4-*O*-isopropylidene derivative which was O-benzylated under standard conditions to give the hexa-*O*-benzyl ether acetal **509** (27% from **507**). Hydrolytic treatment of **509** (70% acetic acid) gave the 3,4-diol **510** in 88% yield. Introduction of the glycolic acid ether group was performed by sequential conversion of **510** into the 3,4-stannylidene acetal and treatment of the acetal with methyl bromoacetate in the presence of tetrabutylammonium iodide for an 80% yield of the lactone **511.** Catalytic hydrogenation over palladium hydroxide on charcoal, followed by alkali-catalyzed hydrolysis of the intermediate hexahydroxy lactone, gave the target compound **512** in 89% yield from **511.** The IC_{50} of compound **512** was determined as 0.1 m*M* in the cell-free assay of



SCHEME 85

Weitz-Schmidt *et al.*[166] (sLe^x, 0.5 m*M*). At a concentration of 3 m*M*, **512** showed 77 and 71% inhibition of ligand binding to P- and L-selectins, respectively (3 m*M* sLe^x, 0 and 50%). The observation of nuclear Overhauser effects (nOe) between Man-H-1 and Gal-H-2, and between Man-H-2 and Gal-H-1 in the ^1H NMR spectrum of **512,** indicates the nonreducing disaccharide to be present in a conformation that places the Man residue within the space occupied by the Fuc residue of sLe^x or sLe^a, and the Gal residue roughly within the space occupied by the Gal residue of sLe^x or sLe^a. However, an nOe was also observed between Man-H-1 and Gal-H-1, suggestive of the presence, in equilibrium, of a second conformer of **512** (see Scheme 85). The authors suggested that a derivative of **512,** lacking the freedom

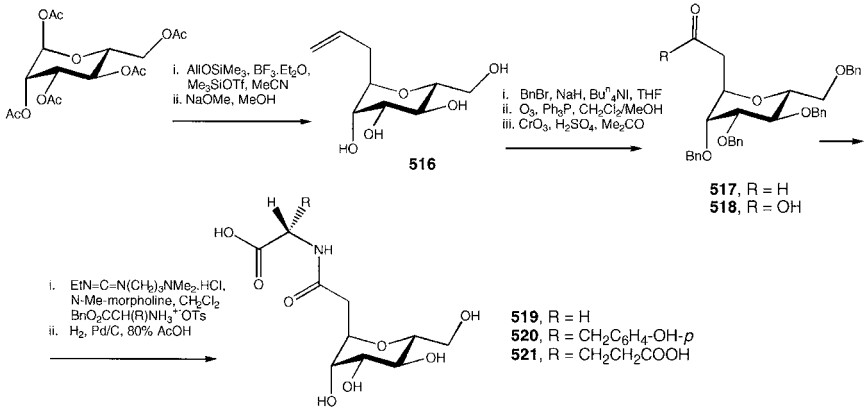


SCHEME 86

of rotation about the glycosidic linkage, might be even more potent than **512** as an inhibitor of ligand–selectin binding.

As in the work of Prodger *et al.*,[548] unsatisfactory overall yields resulted from the introduction and processing of the glycolic acid ether group late in the linear reaction sequence. Using an approach similar to the previous work,[548] Wong and his associates improved this aspect by employing, for a convergent synthesis of **512,** the trimethylsilyl galactoside donor **513,** containing persistent (*O*-benzyl) protecting groups at positions 2, 4, and 6, and a temporary *O*-acetyl protecting group at position 3 (Scheme 86). The reaction of **513** with the fluoride donor **506** under promotion by boron trifluoride etherate at a reaction temperature of $-50\ ^{\circ}C$ slowly rising to $-10\ ^{\circ}C$ afforded an anomeric mixture of the β-D-Gal-$(1\rightarrow 1)$-α-D-Man and α-D-Gal-$(1\rightarrow 1)$-α-D-Man nonreducing disaccharide derivatives (β anomer **514,** 68%; α anomer, 16%). Compound **514** was subjected to Zemplén saponification (95%), and the resulting alcohol converted into the protected glycolyl ether derivative **515** with methyl bromoacetate in *N*,*N*-dimethylformamide under promotion by silver oxide and potassium iodide in 43% yield. The protecting groups of **515** were removed by sequential catalytic hydrogenation over palladium hydroxide on charcoal and alkali-catalyzed hydrolysis of the methyl ester to afford **512** in 83% yield (from **515**).

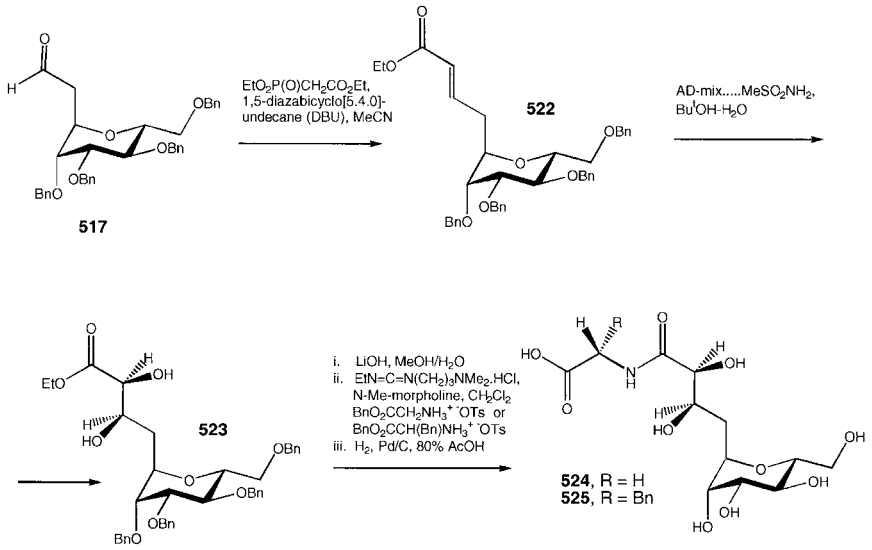
c. C-Mannose Derivatives Containing Amino Acids as Substitutes of the Gal Residue.—A series of candidate inhibitors have been derived from the *C*-mannopyranosyl alkene **516** (Scheme 87) by Wong and his associates.[558] In all target structures of that series, *C*-glycosylically linked α-mannopyranose serves as the substitute for the fucose residue of sLe^x. In compounds **519, 520,** and **521,** the galactose unit has been replaced by the amide-linked spacer amino acids glycine, tyrosine, and glutamic acid, respectively, with the 1-carboxyl groups of the amino acids serving as the substitutes for the Neu5Ac residue.



SCHEME 87

The reaction of mannose pentaacetate with allytrimethylsilane in acetonitrile in the presence of trimethylsilyl trifluoromethanesulfonate and boron trifluoride etherate[559] gave a crude *C*-allyl glycoside, which was subjected to Zemplén deacetylation to afford the α-*C*-glycosyl tetraol **516** in 76% yield and 8 : 1 stereoselectivity. This reaction had been previously applied by Wong and his associates for the synthesis of a corresponding derivative (**434**) of L-fucose (compare Scheme 73). Compound **516** was per-O-benzylated, the resulting benzyl ether subjected to ozonolysis, and the intermediate aldehyde derivative **517** oxidized by the action of chromium trioxide and sulfuric acid in acetone (Jones' reagent) to give carboxylic acid **518** (83% from **516**). Acid **518** was converted into the protected amino acid amides of glycine, tyrosine, or glutamic acid using the respective *p*-toluenesulfonic acid salts of the amino acid benzyl esters. Catalytic hydrogenation over palladium-on-charcoal in 80% acetic acid then afforded the target compounds **519, 520,** and **521** (63, 60, and 62% from **518**). Compounds **519** and **520** have only modest inhibitory activity in an E-selectin binding assay,[166] whereas the dicarboxylic acid **521** is fivefold more potent than sLe^x (IC_{50}, 0.1 m*M*). The biological activity of **521,** in spite of the lack of hydroxyl groups to represent the 4,6-diol of the Gal unit, may be due to the formation of an ion pair between the second carboxyl function of **521** and a positively charged group on the selectin.

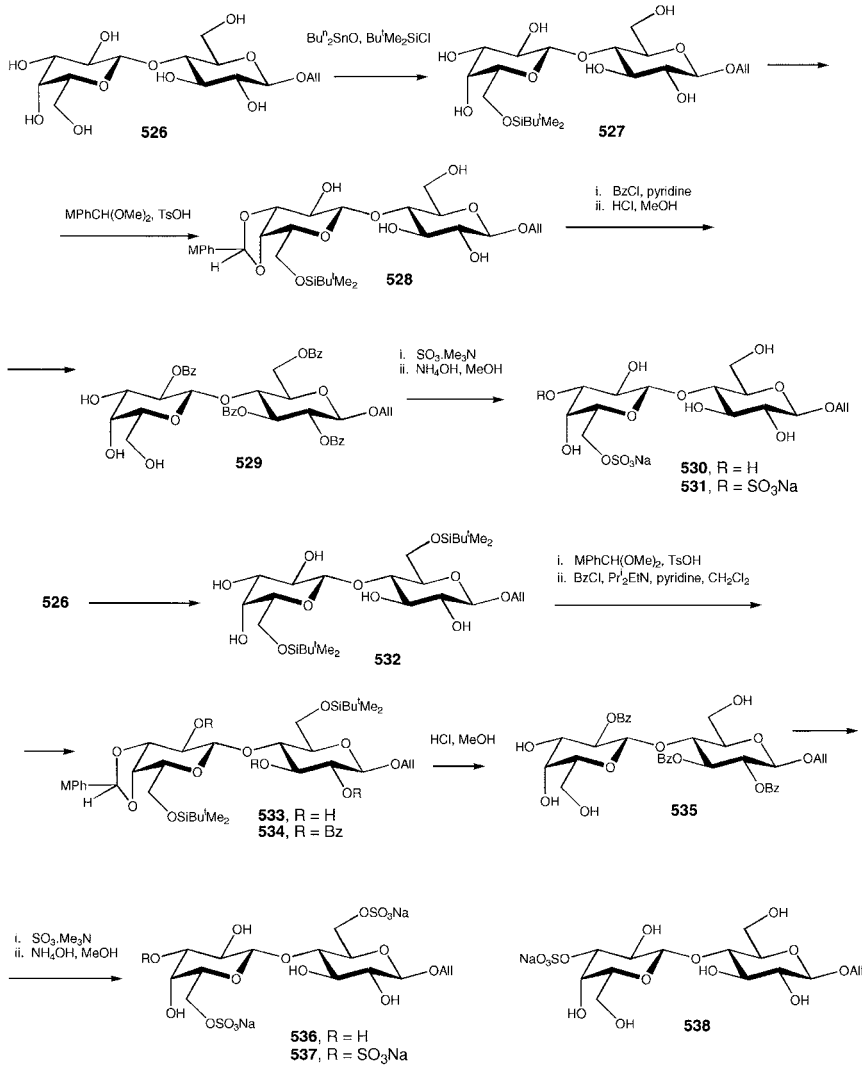
The *O*-benzylated aldehyde **517** was also converted[558] into the α,β-unsaturated ester **522** through Wadsworth–Emmons reaction[522] with ethyl 2-(diethoxyphosphono)acetate[560] in excellent yield (Scheme 88). The intermediate alkene **522** was subjected to Sharpless asymmetric dihydroxylation[561,562] to afford the diol ester **523** in excellent yield and with a diastereoselectivity in excess of 95 : 5. Subsequent to alkali-catalyzed hydrolysis of **523,** the carboxylic acid obtained was condensed with the *p*-toluenesulfonate salt of glycine benzyl ester or phenylalanine benzyl ester, by the action of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC), to afford the benzyl-protected amide derivatives; catalytic



SCHEME 88

hydrogenation over palladium-on-charcoal in 80% acetic acid then afforded the glycine derivative **524** and the phenylalanine derivative **525** (both in 84% yield from **523**). Compound **524** had an IC_{50} of 0.16 m*M* and is thus threefold more potent than sLe^x; compound **525** was found inactive, a result attributed by the authors to the presence of the aromatic ring.

d. Sulfated Analogues of the Disaccharide β-D-Gal-(1 → 4)-β-D-GlcNAc: Candidate Inhibitors of L-Selectin-Ligand Interaction.—A different kind of disaccharide model, based on structural variation of the sulfated disaccharide segment β-D-Gal-(1 → 4)-β-D-GlcNAc, was explored by Bertozzi and her associates[563] in a quest for pharmaceutically applicable inhibitors of L-selectin–ligand interaction. These authors synthesized compounds **530, 531, 535,** and **536,** *O*-sulfate derivatives of allyl β-lactoside,[564] which inhibit the binding of L-selectin to the physiological ligand GlyCAM-1. For the synthesis of compounds **530** and **531,** the allyl β-glycoside of lactose (**526,** Scheme 89) was first treated with dibutyltin oxide and *tert*-butyldimethylchlorosilane to afford, surprisingly,[565] the 6′-monosilylated derivative **527** in 71% yield. In principle, this reaction would be expected to furnish the 3′-O-silylated derivative; presumably, the formation of that product is sterically hindered and the 6′-O-silylated derivative is formed by way of kinetic concentrations of the 4′,6′′-stannylidene acetal.[565] Silyl ether **527** was converted into the 3′,4′-*O*-*p*-methoxybenzylidene derivative **528** by the action of *p*-methoxybenzaldehyde dimethyl acetal under catalysis by *p*-toluenesulfonic acid in (71% of a 3 : 1 mixture of diasteroisomers). Intermediate **528** was



SCHEME 89

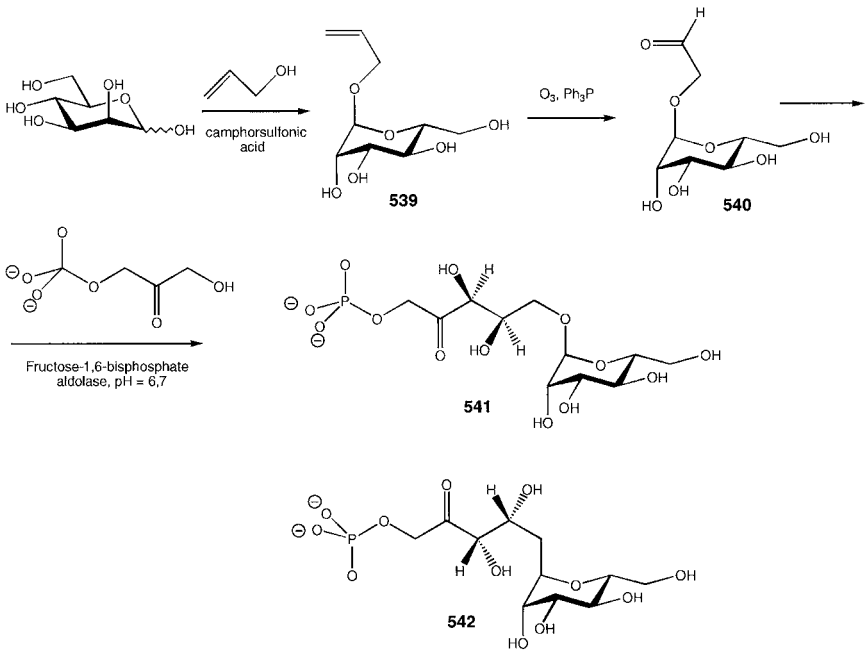
per-O-benzoylated (benzoyl chloride in pyridine) and the resulting, fully protected derivative subjected to hydrogen chloride-catalyzed methanolysis to provide the triol **529** in 67% yield. Compound **529** was next treated with 2 equivalents of sulfur trioxide–trimethylamine complex in *N,N*-dimethylformamide at room temperature, formation of the monosulfated product being monitored with the aid of HPLC. The product was purified by chromatography, heated with aqueous ammonia in

refluxing methanol, and converted into the sodium salt over a cation-exchange resin to provide **530** (55%). Alternatively, O-sulfation of **529** was conducted with 3 equivalents of sulfur trioxide–trimethylamine complex at 37 °C, and the resulting material elaborated into the disulfated product **531** essentially as described for **530.** Candidate inhibitors **536** and **537,** O-sulfated on both the Gal and Glc residues, were prepared from **526** by way of the bis-silyl ether **532.** The reaction of **526** with 2.2 equivalents of *tert*-butyldimethylchlorosilane in the presence of diisopropylethylamine and dimethylaminopyridine in *N*,*N*-dimethylformamide gave **532** in 43% yield. Compound **532** was converted into the 3′,4′-*O*-*p*-methoxybenzylidene derivative **533** by treatment with *p*-methoxybenzaldehyde dimethyl acetal and a catalytic quantity of *p*-toluenesulfonic acid in *N*,*N*-dimethylformamide (44%). Intermediate **533** was per-O-benzoylated (benzoyl chloride, diisopropylethylamine, and pyridine in dichloromethane) to afford **534** in quantitative yield. The silyl ether and *p*-methoxybenzylidene groups of **534** were cleaved by hydrogen chloride-catalyzed methanolysis to provide 2′,2,3-tri-*O*-benzoyl-lactoside **535** in 48% yield. Treatment of **535** with excess sulfur trioxide–trimethylamine complex in *N*,*N*-dimethylformamide at 37 °C gave a mixture of di-, tri- and tetra-O-sulfated products. Further processing of these materials was performed in analogy to that leading to compounds **530** and **531,** whereby the di- and tri-O-sulfated derivatives were separated by chromatography. Finally, compound **536** was obtained in 60% and **537** in 28% yield (from **535**). Of the lactose derivatives synthesized in that series, the 6′,6-disulfated analogue **536** was found to be the most potent inhibitor of L-selectin–GlyCAM-1-interaction. In spite of the lack of the fucose residue, **536** was more than twice as potent as sLe^x or sLe^a, twofold more potent than the 3′,6′-disulfated derivative **531,** and 3–5 times more potent than the monosulfated derivatives **530** and **538.** Surprisingly, trisulfation as in compound **537** did not increase the inhibitory activity of these lactose derivatives. Thus, the 6,6′-disulfate **536** inhibits L-selectin binding with greater potency than sLe^x-β-OMe, yet can be synthesized on a large scale in only a few steps from lactose, an inexpensive disaccharide.

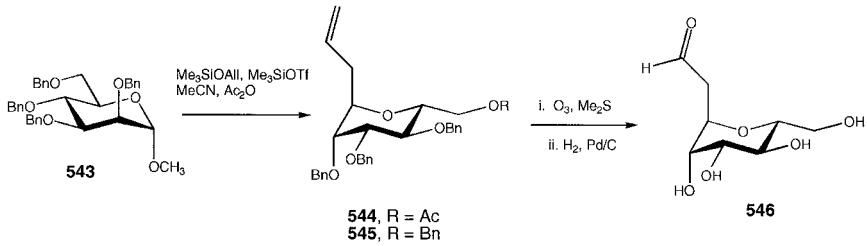
4. The Monosaccharide Model

The efforts to design selectin inhibitors with increased binding strength, decreased molecular complexity, and improved feasibility of large-scale synthesis have gained considerable momentum through the construction of selectin ligands according to the disaccharide paradigm, containing α-D-mannopyranosyl residues in the place of the α-L-fucopyranosyl groups present in sLe^x or sLe^a. As exemplified in the previous section, the GlcNAc residue of sLe^x presumably makes no contribution to binding, whereas of the Neu5Ac residue, only the carboxyl group (or an equivalent anionic functionality) is required for attachment of a ligand to E- or P-selectin.

Several observations suggest that only OH-4 and OH-6 of the Gal residue are required for efficient binding of sLe^x or sLe^a analogues to selectins. Several types of effective selectin ligand-binding inhibitors have been prepared wherein the hydroxyl groups of the Gal residue are replaced by a 1,2-diol (for example, compound **524**). Certain inhibitors are totally lacking functional groups corresponding to those of the Gal residue (for example, compound **490**). For these reasons, a radical extension of the disaccharide paradigm would be the *monosaccharide paradigm.* According to this model, only one monosaccharide unit (the fucose residue or its mannose equivalent) of sLe^x or sLe^a is fully attached to the receptor, much in the way saccharides will bind to many plant lectins. An α-Man residue advantageously replaces the fucose unit, because its anomeric carbon atom is closer to the locations of the Gal and Neu5Ac residues. Functional groups representing the carboxyl group contributed by the Neu5Ac residue and the diol grouping of the Gal residue can both be included as part of an acyclic polyhydroxyalkyl chain that is α-glycosidically attached to the Man residue and terminates in a carboxyl group or other anionic functionality. Target structure **541,** designed and synthesized by Wong and his associates,[566] can be viewed as a straightforward and elegant application of the monosaccharide paradigm. For the synthesis of **541,** D-mannose was converted (Scheme 90) into allyl α-D-mannopyranoside (**539**) by Fischer glycosidation in allyl alcohol in the presence of camphorsulfonic acid (76%). Ozonolysis of



SCHEME 90



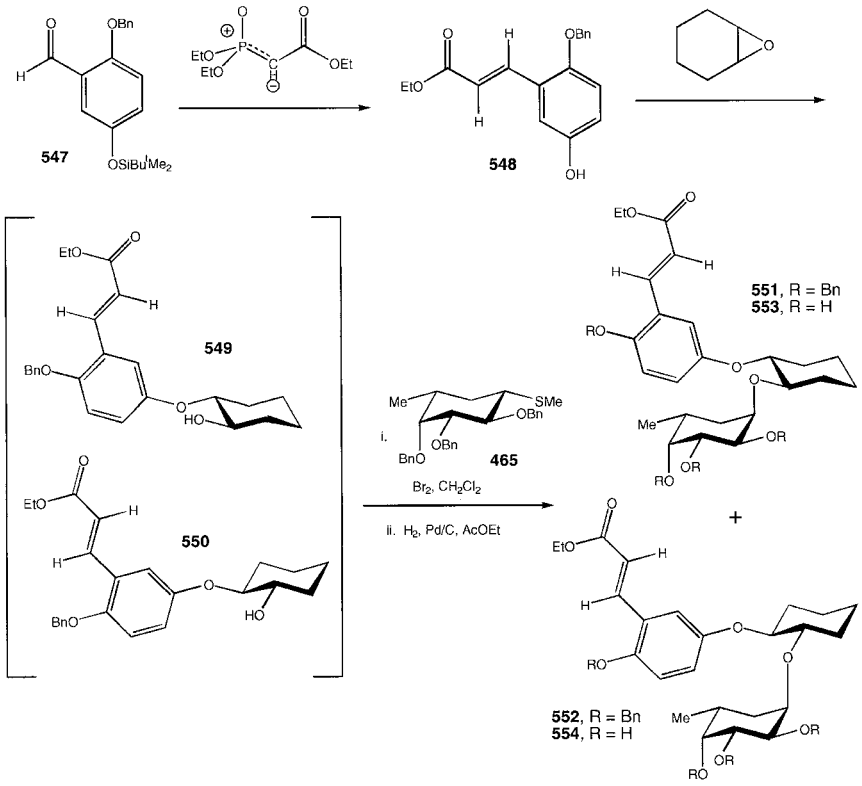
SCHEME 91

539 in 1 : 4 dichloromethane–methanol afforded the glycolaldehyde ether **540,** which reacted with dihydroxyacetone phosphate under catalysis by fructose-1,6-bisphosphate aldolase at pH = 6,7 and 25 °C to give the target compound **541** in 35% yield. In this enzyme-catalyzed reaction, two chiral centers having the D-*threo* configuration are generated from the hydroxymethyl group (C-3) of dihydroxyacetone phosphate and the aldehyde carbon atom.[567–569] The IC_{50} values of **541,** determined in a cell-free assay of polymeric sLe^a interacting with selectins coated on microtiter plates,[166] are 0.1, 6×10^{-4}, and 9.5×10^{-2} m*M* against E-, P-, and L-selectins, respectively. Interestingly, for the related *C*-mannosyl derivative **542,** the corresponding values are 0.8, 5×10^{-3}, and 4×10^{-2} m*M*. Note, however, that in compound **542,** the aglycon chain is shortened by one methylene group (or ether oxygen atom), resulting presumably in less than ideal distances, at least for P-selectin binding, between the mannopyranosyl unit and the diol or phosphate groups. Compound **542** was prepared from methyl 2,3,4,6-tetra-*O*-benzyl-α-D-mannopyranoside (**543**). Similar to an analogous conversion with fucose, compound **543** was treated with allyltrimethylsilane (Scheme 91), then acetic anhydride in acetonitrile in the presence of trimethylsilyl triflate to afford the 6-O-acetylated *C*-allyl derivative **544** in 83% yield. Zemplén saponification of **544** gave the 6-hydroxy derivative which was O-benzylated in high yield (benzyl bromide, sodium hydride in tetrahydrofuran; **545**). The per-O-benzylated *C*-glycosyl derivative **545** was subjected to ozonolysis, and the intermediate benzyl ether aldehyde hydrogenated over palladium-on-charcoal in 3 : 1 tetrahydrofuran–water to afford the aldehyde **546** in 98% yield. The enzyme-catalyzed aldol reaction of **546** was performed as described for the synthesis of **541.**

5. Enhancement of Binding Strength through Additional Hydrophobic Substituents

The attachment of hydrophobic groups to selectin-binding scaffolds is an important approach to candidate inhibitors with increased potency and improved pharmaceutical properties. This strategy has been implemented by a number of authors; in the following, three selected examples are presented.

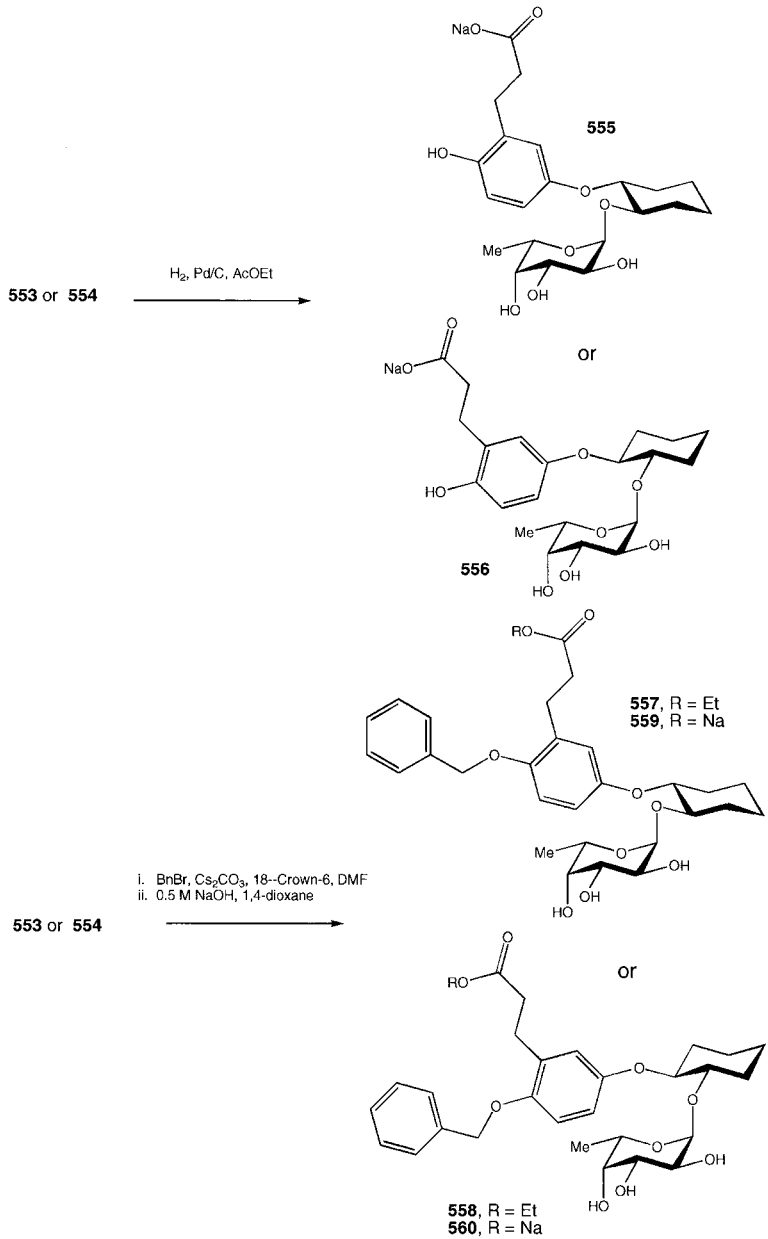
a. Candidate E-Selectin Inhibitors Designed on an Aryl-Cyclohexyl Ether Scaffold.—The group of Liu *et al.*[570] of the Roche Research Center at Nutley designed and synthesized a series of candidate inhibitors of E-selectin binding. These molecules, of which **560** was found to be the most effective inhibitor, are more hydrophobic in character than most other antagonists synthesized. An aryl-cyclohexyl ether serves as a spacer unit to replace the Gal-GlcNAc disaccharide segment. The α-fucose residue is attached to the cyclohexane ring by an *O*-glycosidic linkage, 1,2-trans to the phenoxyl group, essentially as in the series of compounds prepared by Toepfer *et al.*[533] (Section VI.1.b). For the synthesis of **560,** 2,5-dihydroxybenzaldehyde was selectively converted into the 5-*tert*-butyldimethylsilyl ether (88%), which was then O-benzylated at the remaining 2-position to afford the protected aldehyde **547** in 90% yield. Horner–Emmons reaction in *N*,*N*-dimethylformamide of **547** with the ylide derived by the action of potassium hydride from ethyl 2-(*O*,*O*-diethylphosphono)acetate (Scheme 92)



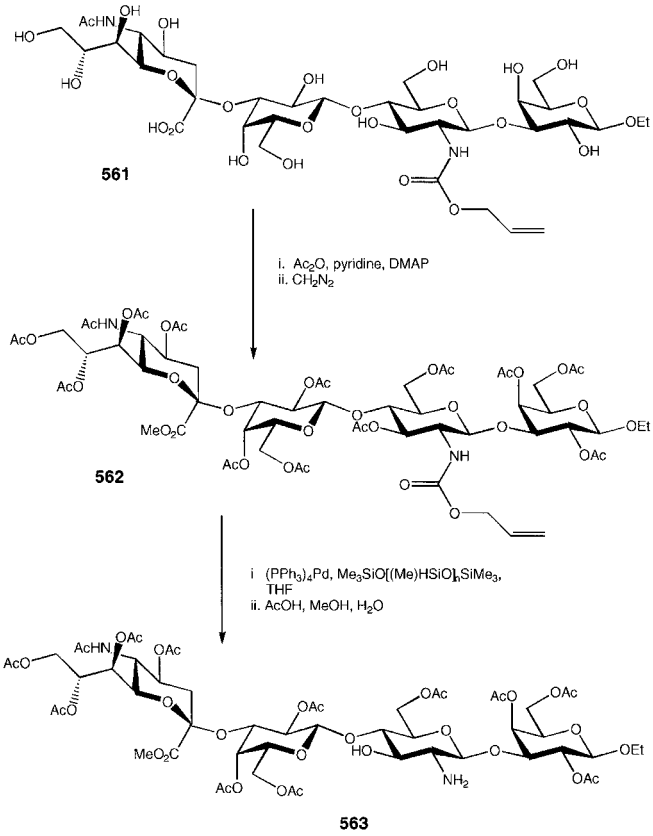
SCHEME 92

proceeded with cleavage of the silyl ether group and afforded the phenolic unsaturated ester **548** in 80% yield. Heating of **548** with excess cyclohexene oxide in the presence of potassium carbonate in *N*,*N*-dimethylformamide gave a racemic mixture of aryl cyclohexyl ethers, **549** and **550,** in 90% yield. Enantiomers **549** and **550** were O-fucosylated according to a procedure of Ogawa,[571] developed on the basis of Lemieux' halide inversion principle,[294] through the use of a bromide fucosyl donor derived from thioglycoside **465,** to afford the chromatographically separable diastereoisomers **551** (*R*,*R*) and **552** (*S*,*S*) in 44 and 38% yields, respectively. By catalytic hydrogenation of **551** or **552** over palladium-on-charcoal in ethyl acetate, the double bond was saturated and all benzyl ether groups cleaved to give **553** or **554** (92% yield in both cases). The phenolic esters **553** or **554** (Scheme 93) were either directly converted, by alkali-catalyzed hydrolysis, into those target structures lacking the benzyl phenyl ether grouping, compounds **555** or **556,** or were O-benzylated under suitably mild conditions (benzyl bromide, cesium carbonate, 18-crown-6, in *N*,*N*-dimethylformamide) to afford the diastereoisomeric phenylbenzyl ethers, **557** or **558** (82% in both cases). Alkali-catalyzed hydrolysis of **557** or **558** then gave the target compounds **559** or **560** in fair yields. Notably, however, the absolute configuration of the two chiral centers of the 1,2-cyclohexanediol in the more active derivative, **560** (IC_{50}, 0.87 m*M*), is (1*S*,2*S*), whereas the absolute configuration of the equivalent segment in sLe^x or sLe^a would be (1*R*,2*R*). The (1*R*, 2*R*)-diastereoisomer **559** has an IC_{50}-value of 3.3 m*M*. This indicates that the aryl-cyclohexyl ether segment serves to provide a particular distance between the fucose unit on one hand, and the carboxyl group and the benzyl group on the other, without specifically contributing to binding. Compound **560** does not contain polar groups equivalent to the 4,6-diol of the Gal residue of sLe^x. The carboxyl group representing the contribution by the Neu5Ac residue is attached to the aromatic ring by an ethylene spacer. Interestingly, derivatives **555** and **556,** containing a hydroxyl group in the place of the phenoxyl group of **558,** are inactive. This may be interpreted as indicating the presence, on the receptor, of a hydrophobic segment, capable of interacting with a hydrophobic group on the ligand, located in the vicinity of the carboxyl group.

b. Identification of a New Binding Site for N-Acyl Aromatic Glucosamine Substituents of sLe^x.—De Frees *et al.* have designed the pentasaccharide sLe^x analogues **565, 567,** and **569** containing *N*-naphthoyl-, *N*-benzoyl, or *N*-(cyclohexyl)carbonyl substituents in the place of the *N*-acetyl group of GlcNAc.[572] The synthesis of these target structures represents an interesting combination of chemical and enzyme-catalyzed steps. Starting from an *N*-allyloxycarbonyl-modified derivative of the disaccharide β-D-GlcN-(1 $\rightarrow$ 3)-β-D-Gal, the tetrasaccharide ethyl β-glycoside **561** was first assembled by sequential galactosyl and sialyl transfers from UDP-Gal and CMP-Neu5Ac, catalyzed by 1,4-galactosyltransferase and 2,3-sialyltransferase (cf. Section IV.2). Compound **561**

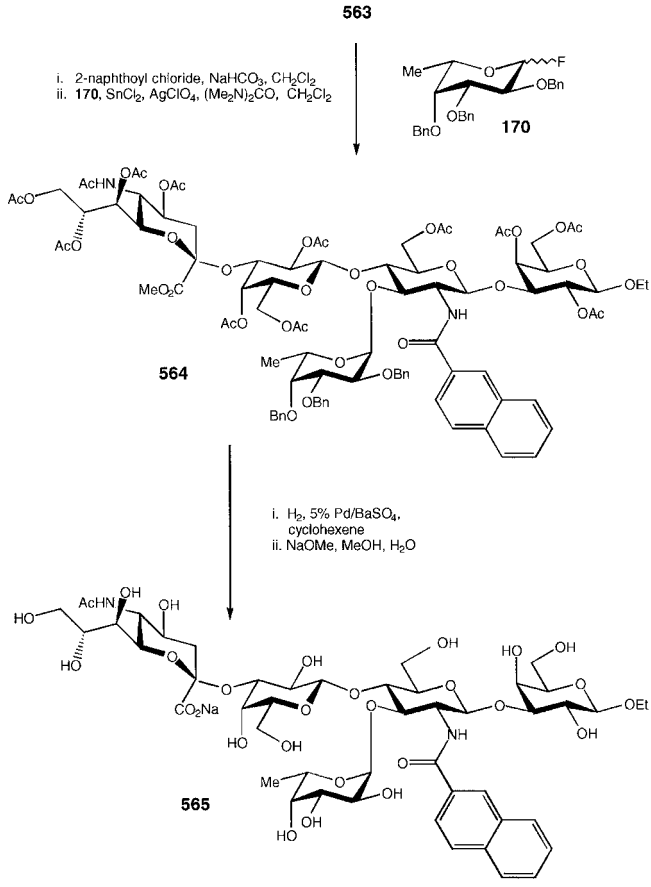

555
556
557, R = Et
559, R = Na
558, R = Et
560, R = Na
or
or
553 or 554
H2, Pd/C, AcOEt
i. BnBr, Cs2CO3, 18--Crown-6, DMF
ii. 0.5 M NaOH, 1,4-dioxane
553 or 554

SCHEME 93



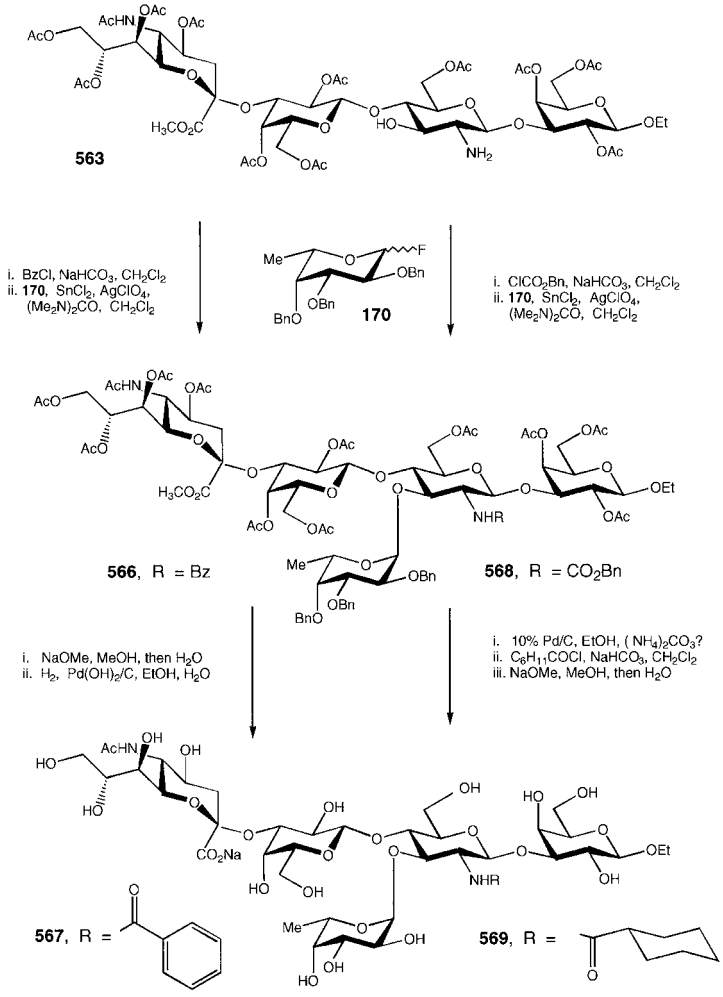
SCHEME 94

was then protected[573] (Scheme 94) by sequential per-O-acetylation and treatment with diazomethane to afford **562.** Elegant, simultaneous removal of the *N*-allyloxycarbonyl- and the neighboring 3-*O*-acetyl groups of the GlcN unit was achieved by sequential treatment of tetrasaccharide derivative **562** with tetrakis(triphenylphosphine)palladium and poly(methylhydrosiloxane) in tetrahydrofuran, and 1 molar equivalent of acetic acid in methanol–water to afford the amino alcohol **563** in 49% yield. Compound **563** was then N-naphthoylated (2-naphthoyl chloride in dichloromethane in the presence of sodium hydrogencarbonate) and the amide derivative converted (Scheme 95) into the 3-*O*-α-fucoside **564** by a Mukaiyama-type glycosylation[279] in 44% yield from **563** [tri-*O*-benzyl-L-fucosyl fluoride (**170**), stannous chloride, silver perchlorate, tetramethylurea in dichloromethane]. The protecting groups of **564** were then removed by sequential catalytic phase-transfer hydrogenation, Zemplén saponification, and



SCHEME 95

alkali-catalyzed hydrolysis to afford the target compound **565** (71% from **564**). The *N*-benzoyl derivative **567** was similarly prepared by way of the *N*-benzoyl intermediate **566** (Scheme 96). For the synthesis of the *N*-(cyclohexyl)carbonyl analogue **569,** amino alcohol **563** was first converted into the *N*-benzyloxycarbonyl derivative (benzyl chloroformate, sodium hydrogencarbonate, dichloromethane, 65%). The N-protected alcohol was then O-fucosylated as already described for target structure **565** to afford **568** in 73% yield. Catalytic hydrogenation (10% palladium-on-charcoal, ammonium carbonate in refluxing ethanol) removed both the *O*-benzyl protecting groups of the fucose unit and the *N*-Cbz group to afford a partially protected aminopentasaccharide intermediate in 95% yield. Treatment with cyclohexylcarbonyl chloride in the presence of sodium hydrogencarbonate, followed by Zemplén saponification and alkali-catalyzed hydrolysis, then gave the

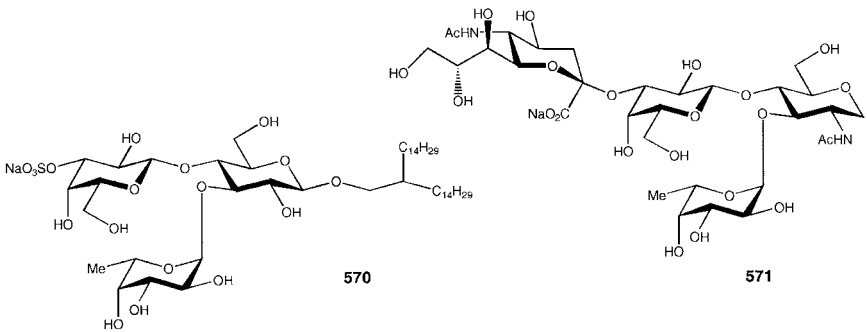


SCHEME 96

target compound **569** (93%). The *N*-naphthoyl derivative **565** was found to have an IC_{50} value of 0.08 m*M* (sLe^x, 1.0 m*M*), and the *N*-benzoyl derivative **567** had IC_{50} 0.3 m*M*. DeFrees *et al.* considered two alternative explanations for these findings. The first of these is that changes in oligosaccharide conformation might be induced by the presence of the aromatic rings, resulting in more tightly binding conformers; according to the other hypothesis, a suitably located hydrophobic region on the selectin could interact with the aromatic groups to provide a binding contribution in addition to the interactions that constitute the binding of the physiological

ligands. Conformational analysis by NMR spectroscopy of compound **565** revealed no difference between the oligosaccharide conformation of **565** and that of the physiological *N*-acetyl derivatives. The enhanced E-selectin binding of **565** was therefore attributed by the authors to a hydrophobic interaction between the aromatic groups and an undefined region of the receptor. Notably, the aromatic nature of the *N*-acyl groups in **565** and **567** appears to be necessary for enhanced binding: replacement of the *N*-acetyl group by the *N*-cyclohexylcarbonyl group resulted in a compound (**569**) three times less potent than sLe^x. Also, structures containing fatty acyl amido groups of various chain lengths did not bind to E-selectin more strongly than the physiological *N*-acetyl derivatives.[572]

c. Analogues Comprising a Branched Long-Chain Alkyl Aglycon and a 1-Deoxy GlcNAc Residue.—The 3′-sulfated Le^x derivative **570,** designated GSC-150, comprises a glucose residue in place of the GlcNAc residue, and a (2-tetradecyl)hexadecyl aglycon.[423] Compound **570** has IC_{50} values of 0.28, 0.10, and 0.03 m*M* against E-, P-, and L-selectins, respectively; the values for sLe^x are 0.60, >1.0, and >1.0 m*M*. By means of a mathematical molecular dynamics simulation, Tsujishita *et al.* supported their interpretation that each of the branched alkyl chains extend on the surface of E-selectin and interact with two hydrophobic portions of that protein. Ohmoto *et al.*[181] reported that compound **571,** a sLe^x analog comprising a 1-deoxy-GlcNAc residue, is up to 20 times more potent an inhibitor of P- and L-selectin binding than the sLe^x tetrasaccharide; apparently, this enhancement of binding potency is attributable to the conversion of the GlcNAc residue from a polar into a more hydrophobic segment of the ligand glycan.

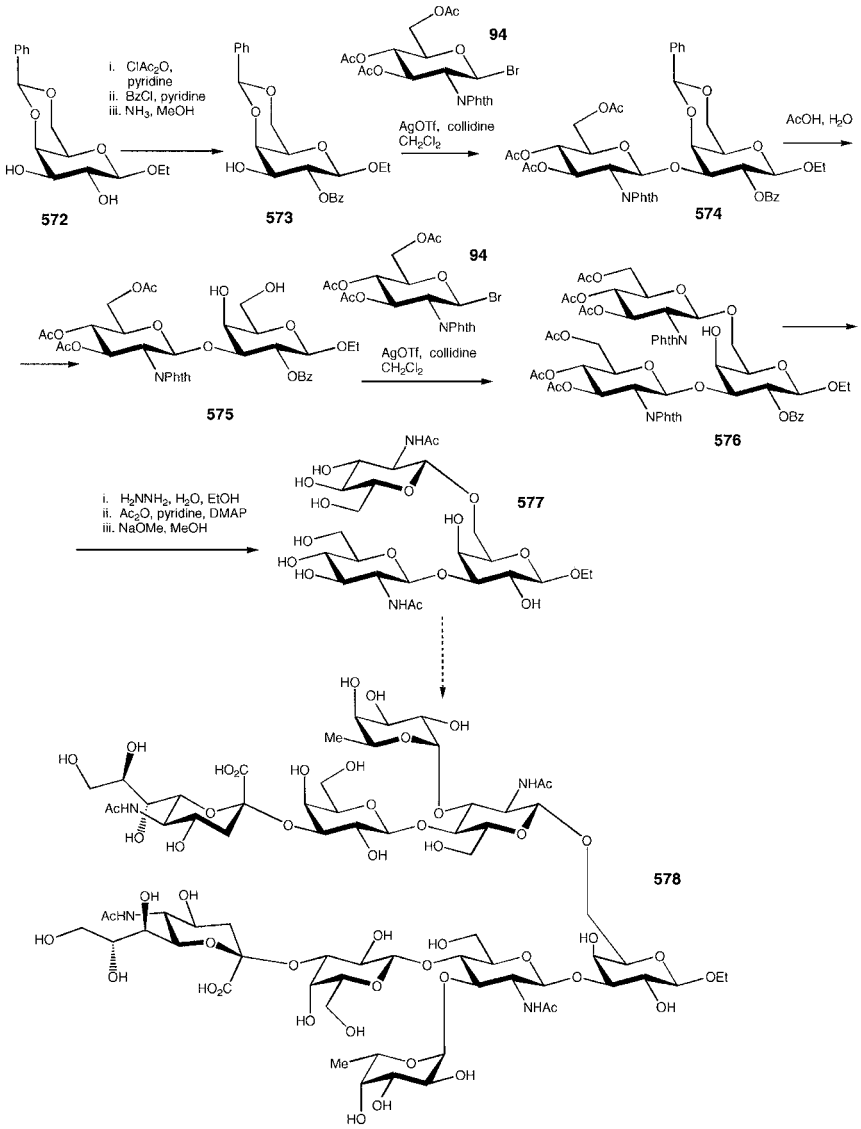


570 **571**

6. Enhancement of Binding Strength through Dimerization or Polymerization

a. Bivalent Selectin Ligands Constructed on a Branching Galactose Residue.—De Frees *et al.* reported[175] on syntheses of bivalent sLe^x derivatives (for example, **578**) by a combined organic-chemical and enzymatic strategy that

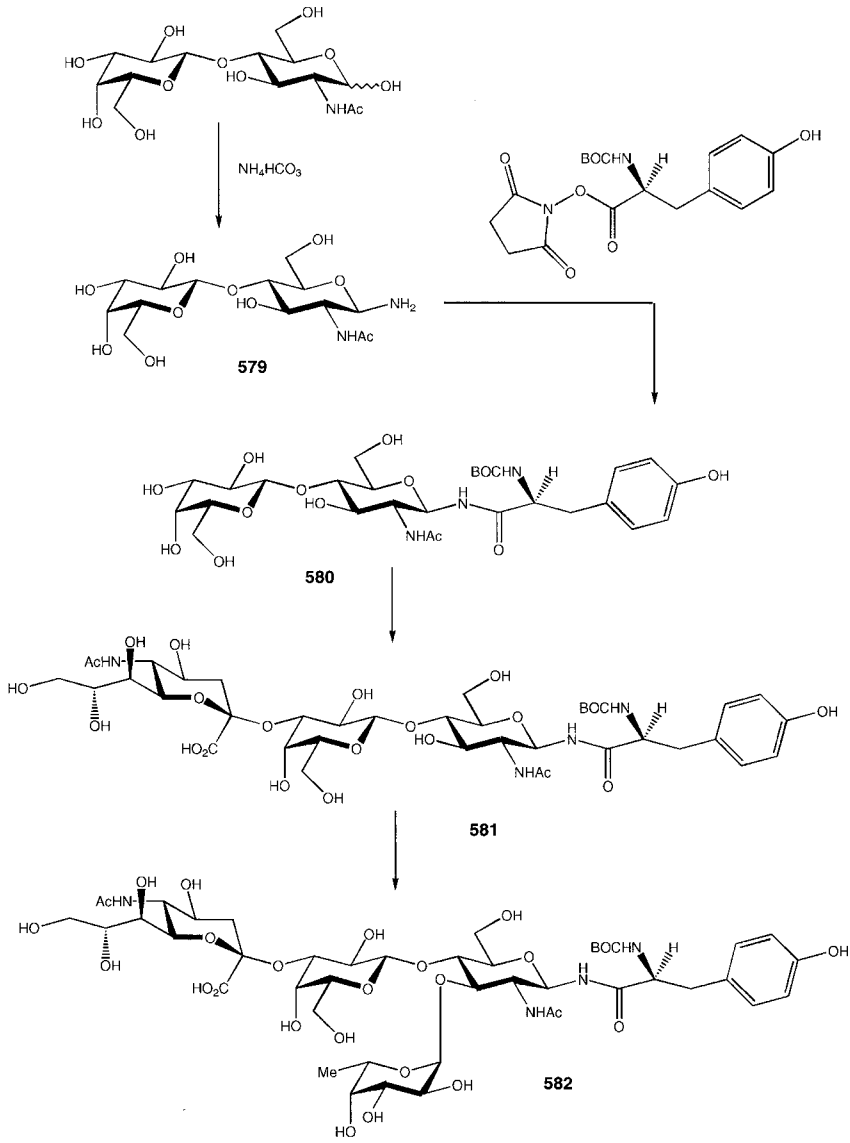
constitutes an extension of the approach that Palcic *et al.*[372] had chosen for the preparation of the sLe^a determinant: the organic-chemical synthesis of the ethyl glycoside of a "core" branched trisaccharide β-D-GlcNAc-(1 → 6)-[β-D-GlcNAc-(1 → 3)]-β-D-Gal (**577**) was followed by three steps, catalyzed by Gal-, Neu5Ac-, and Fuc-transferases, to produce the divalent sLe^x ligand **578** (Scheme 97).



SCHEME 97

For the synthesis of **577,** ethyl-4,6-*O*-benzylidene-β-D-galactopyranoside (**572**) was converted into the 2-*O*-benzoyl derivative **573** (48% overall) by sequential 3-O-chloroacetylation and benzoylation in pyridine followed by selective ammonolysis of the 3-chloroacetate ester by the action of ammonia in methanol at -30 °C. Acceptor **573** was β-glycosylated in 86% yield using the donor 2-deoxy-2-phthalimido-2,4,6-tri-*O*-acetyl-β-D-glucopyranosyl bromide (**94**) in dichloromethane under promotion by silver triflate and collidine at -20 °C. Treatment with aqueous acetic acid of the disaccharide derivative **574** afforded the acceptor diol **575** (80%), which was selectively 6-O-glycosylated in 86% yield under conditions analogous to those employed for the glycosylation of acceptor **573.** The *N*-phthaloyl groups of trisaccharide derivative **576** were removed by the action of hydrazine in ethanol–water, and the intermediate diamino derivative was peracetylated (acetic anhydride, pyridine, 4-dimethylaminopyridine) to afford, after treatment with sodium methoxide in methanol, the trisaccharide glycoside **577** (70%), suitable as a glycosyl acceptor for the subsequent enzyme-catalyzed glycosylation step. To both GlcNAc residues of **577,** β-(1 $\rightarrow$ 4)-linked Gal residues were transferred from UDP-galactose under catalysis by galactosyltransferase (EC 2.4.1.22). The UDP-Gal was generated *in situ* from UDP-glucose under catalysis by uridine-5′-diphosphogalactose 4″-epimerase (EC 5.1.3.2). Alkaline phosphatase (EC 3.1.3.1) was added to the reaction mixture to catalyze the hydrolysis of the uridine-5′-diphosphate (UDP) formed; this measure prevents UDP from inhibiting the enzyme-catalyzed galactosylation reaction.[575] The two LacNAc branches of the pentasaccharide were then further extended by transfer of one Neu5Ac residue each to the nonreducing Gal residues from CMP-Neu5Ac under catalysis by the N-type α-2,3-sialyltransferase (EC 2.4.99.6). Finally, the sialyl-LacNAc derivative was converted into the sLe^x ligand **578** by the transfer from GDP-fucose of one α-L-fucose residue each to position 3 of the GlcNAc residues, under catalysis by fucosyltransferase V. In an assay of HL60-cell binding to immobilized soluble E-selectin, the (3 $\rightarrow$ 6)-linked sLe^x divalent conjugate **578** was the most potent ($IC_{50} \sim 0.2$ m*M*) of a series of similar inhibitors that each contained two sLe^x determinants linked to positions 2,3,4,6, and 2,6 of ethyl β-D-galactopyranoside. The IC_{50} values of these molecules ranged from 0.2 to 0.45 m*M*. The corresponding monovalent tetrasaccharide sLe^x derivative had $IC_{50} \sim 1.2$ m*M* in this assay. The IC_{50} values of divalent derivatives that contained two sLe^x determinants linked by a 1,4-butanediol or 1,5-pentanediol linker did not exceed those of the corresponding monovalent derivatives.

b. Organ Uptake of Mono-, Di-, Tri-, and Tetra-valent sLe^x Conjugates in Mice.—Rice and his associates have reported on an extensive study in mice of the binding specificity of ligand glycan derivatives to LPS-induced E-selectin.[576] To assess the valency of sLe^x determinants optimal for selectin binding, the authors prepared a set of monovalent, divalent, trivalent, and tetravalent sLe^x glycoconjugates (**582**–**585**) derived from *N*-acetyl-lactosamine (LacNAc) and from



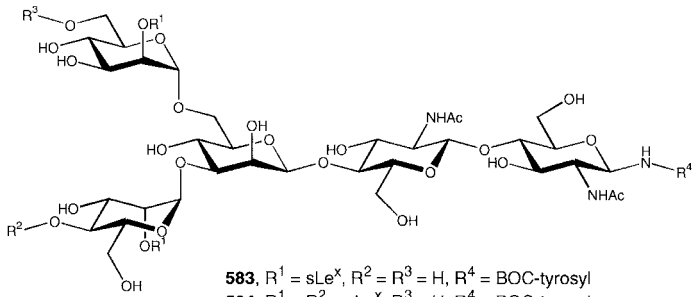
SCHEME 98

biantennary, triantennary, and tetraantennary N-linked glycoprotein glycans.[577] As a monovalent ligand, the *N*-glycosyl-*tert*-butoxycarbonyl-(BOC-)tyrosylamide derivative of sLe^x **582** was prepared[578] from *N*-acetyl-lactosamine [LacNAc, β-D-Gal-(1 $\rightarrow$ 4)-D-GlcNAc] as follows (Scheme 98). Treatment of LacNAc with ammonium hydrogencarbonate[579,580] afforded an equilibrium mixture of the

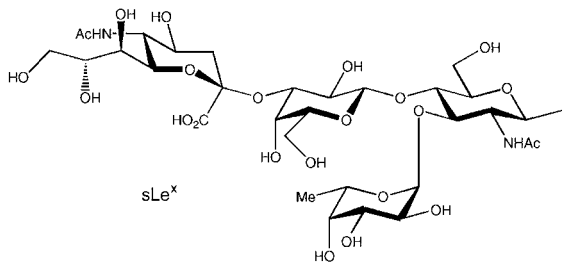
glycosylamine **579** and its hydrogencarbonate salt. Complete conversion into the free glycosylamine and removal of carbon dioxide and water was achieved by repeated lyophilization of the mixture. Glycosylamine **579** was then treated with a hundredfold excess of the hydroxysuccinimide ester of BOC-L-tyrosine to form the *N*-glycosyl-BOC-tyrosylamide derivative **580,** which was purified by semipreparative C_8-reversed phase HPLC. Compound **580** was then converted into the monovalent target structure **582** by two enzyme-catalyzed steps. First, a Neu5Ac residue was transferred onto **580** from CMP-*N*-acetylneuraminic acid (CMP-Neu5Ac) under catalysis by recombinant α-2,3-sialyltransferase (EC 2.3.99.6) that had been contributed by Paulson.[431] The resulting sialoconjugate **581** was then incubated with GDP-fucose in the presence of α-3,4-fucosyltransferase partially purified from human milk to give the desired sLe^x derivative **582.** For the preparation of compounds **583** and **584,** the required N-glycans were isolated from bovine fetuin.[578] The disulfide bonds of fetuin were reduced, and the glycoprotein was digested by the action of trypsin. The resulting peptides were hydrolytically cleaved under catalysis by N-glycosidase F (EC 3.2.2.18) to afford a mixture of the reducing glycoprotein N-glycans. The mixture was de-sialylated by the action of neuraminidase from *Clostridium perfringens* (EC 3.2.1.18). The asialooligosaccharides were then converted into the *N*-glycosyl-BOC-tyrosinamide derivatives, separated by HPLC, sialylated, and fucosylated as already described for the synthesis of the monoantennary sLe^x derivative **582.** For the synthesis of the tetraantennary probe **585,** a suitable *N*-glycan was isolated from human serum orosomucoid[582] and was converted into **585** by procedures similar to those just outlined. The glycoconjugates produced by transformation of naturally occurring glycans have several important advantages: the β-glycosylamide linkage preserves the reducing-terminal β-D-GlcNAc residue in a form similar to its natural linkage to asparagine residues of glycoproteins; the presence of the relatively nonpolar BOC-tyrosyl residues renders the natural glycan mixtures separable by conventional, semipreparative reversed phase chromatography; the glycoconjugates may be photometrically detected by UV absorption at 280 nm; the *N*-BOC protecting group may be removed by the action of trifluoroacetic acid without affecting the integrity of the glycan chains, to afford an amino function for coupling to macromolecular carriers; the free amino group may be additionally utilized to remove the tyrosyl residue by Edman degradation, regenerating the reducing glycans, for example after chromatographic separation; finally, the tyrosine hydroxyl group provides the option of radioiodination to obtain ^{125}I-labeled probes for biological studies.

This latter method was applied by Rice's group to study the biodistribution in mice of the ^{125}I-labeled[583,584] set of N-linked glycan derivatives **582**–**585.** On the one hand, targeting of the conjugates to organs of normal mice was compared to targeting following application of lipopolysaccharide (LPS) which had been previously shown[585] to induce the systemic expression of E-selectin in mice. On the other hand, the biodistribution of conjugates comprising sLe^x determinants was

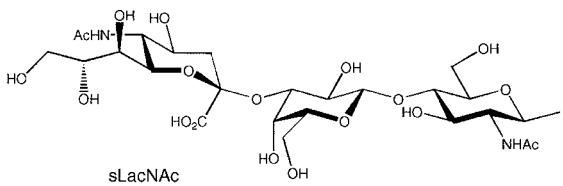
compared to the behavior of similar conjugates containing sialyl-lactosamine determinants [α-Neu5Ac-(2 → 3)-β-D-Gal-(1 → 4)-β-D-GlcNAc]. Conjugates **586**–**588** differ from **583**–**585** only by the absence of the α-(1 → 3)-linked fucose residues that had previously been demonstrated to be essential for binding of ligand glycans to E-selectin *in vitro*.[62] Preliminary experiments by Thomas *et al.* had indicated[576] that the kidney is the preferred target for E-selectin-specific binding of ligand glycans following stimulation with LPS. When the targeting efficiency was compared of compounds **582**–**585,** the di- and tri-valent conjugates **583** and **584** were found to specifically bind to E-selectin. Binding of the sialyl LacNAc analogues **586** and **587** was significantly lower. In the absence of stimulation by LPS, no significant differences were observed between organ targeting of the sLe^x



583, R^1 = sLe^x, R^2 = R^3 = H, R^4 = BOC-tyrosyl
584, R^1 = R^2 = sLe^x, R^3 = H, R^4 = BOC-tyrosyl
585, R^1 = R^2 = R^3 = sLe^x, R^4 = BOC-tyrosyl
586, R^1 = sLacNAc, R^2 = R^3 = H, R^4 = BOC-tyrosyl
587, R^1 = R^2 = sLacNAc, R^3 = H, R^4 = BOC-tyrosyl
588, R^1 = R^2 = R^3 = sLacNAc, R = BOC-tyrosyl



sLe^x

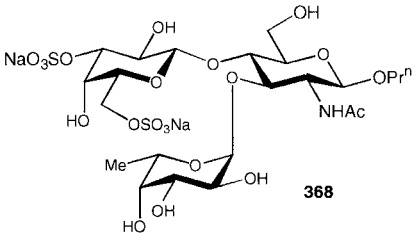


sLacNAc

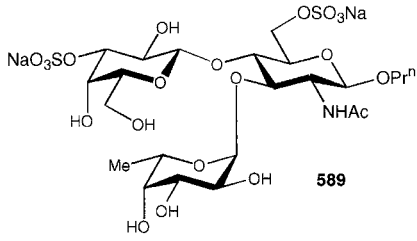
or sialyl LacNAc derivatives. The low targeting efficiency of the mono- and tetravalent derivatives **582** and **585** was similar in normal and LPS-treated mice. The data were interpreted to indicate that the biantennary arrangement of sLe^x determinants in probes **583** and **584** reflects the nature of the physiological ligands of E-selectin (cf. Section II, Fig. 6). In view of this, the binding potency of the monovalent probe **582** would be too low for that compound to accumulate in mouse organs while the fourth sLe^x determinant present in the tetraantennary probe **585** would impede E-selectin binding because of steric hindrance. Mono-, di-, or tetra-valent sLe^x conjugates synthesized by the group of Renkonen[586–588] have been shown in Stamper–Woodruff assays[29] to inhibit the adhesion of lymphocytes to vascular endothelia of allografts during heart transplant rejection in rats.

c. Inhibition in Static or Rolling Assays by L-Selectin Ligands in Monomeric or Polymeric Forms.—Sanders *et al.*[196] studied the inhibitory potency of disulfated saccharide derivatives in monomeric form (**368** and **589**) or as pendant oligosaccharide determinants in the context of polymers **590** and **591** produced by ring-opening metathesis polymerization.[589] The inhibition of L-selectin–ligand interactions was examined both under static conditions and in a flow chamber assay. Under static conditions, candidate inhibitors were tested for their ability to inhibit the binding of L-selectin–IgG-coated beads to a heparin–albumin matrix. In the dynamic assay, rolling on GlyCAM-1 was assessed of cells of the human-L-selectin-transfected mouse pre-B cell line 300.19. In a previous study, compounds **589** and **368** had been found to inhibit the static interaction between L-selectin and GlyCAM-1 with IC_{50} values of 1 and 3 m*M*, respectively. In the static heparin-binding L-selectin assay, the reference compound sLe^x had IC_{50} ~3 m*M*, whereas the neoglycopolymers **590** and **591** had IC_{50} 0.039 and 0.035 m*M*. The superior inhibitory potency of the polymers is in agreement with other, previous findings. However, the lack of a significant difference in the IC_{50} values of **590** and **591** indicated that, under equilibrium conditions, the position of the sulfate groups on the carbohydrate backbone is less important than the presence of multiple, pendant anionic oligosaccharide determinants on the polymers. In the rolling assay performed by Sanders *et al.*, the reference compound sLe^x had IC_{50} ~2.5 m*M*, a value in agreement with data obtained in static cell-adhesion assays. However, the disulfated monovalent compounds **589** and **368** did not interfere at all with L-selectin-mediated rolling on GlyCAM-1. From these data, the authors concluded that inhibitory properties determined in static assays do not necessarily correlate with data measured under conditions of flow. When the polymeric glycoconjugates **590** and **591** were examined as inhibitors of L-selectin-mediated rolling on GlyCAM-1, **591** had IC_{50} ~0.001 m*M* and is thus ~1000-fold more effective than sLe^x. By contrast, polymer **590,** representing the 6′-sulfated L-selectin ligands, showed no inhibition of rolling up to saccharide concentrations of 5 m*M*. Unexpected on the basis of the data obtained in the static assay, this result led the authors

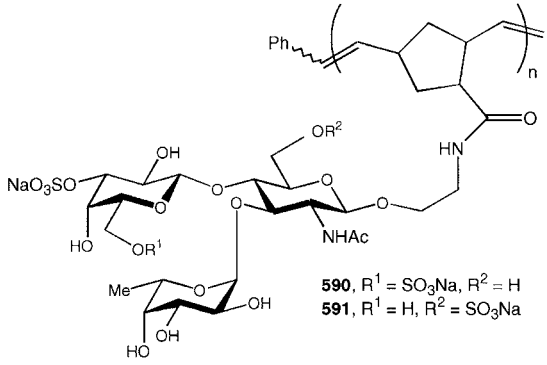
to conclude that the sulfation pattern of the L-selectin ligand glycans is critical with respect to their inhibitory potency under conditions of flow. Presumably, the poor activity of polymer **590** is due to unfavorable kinetic properties that are not reflected in the static assay where measurements are performed under conditions of equilibrium.



368



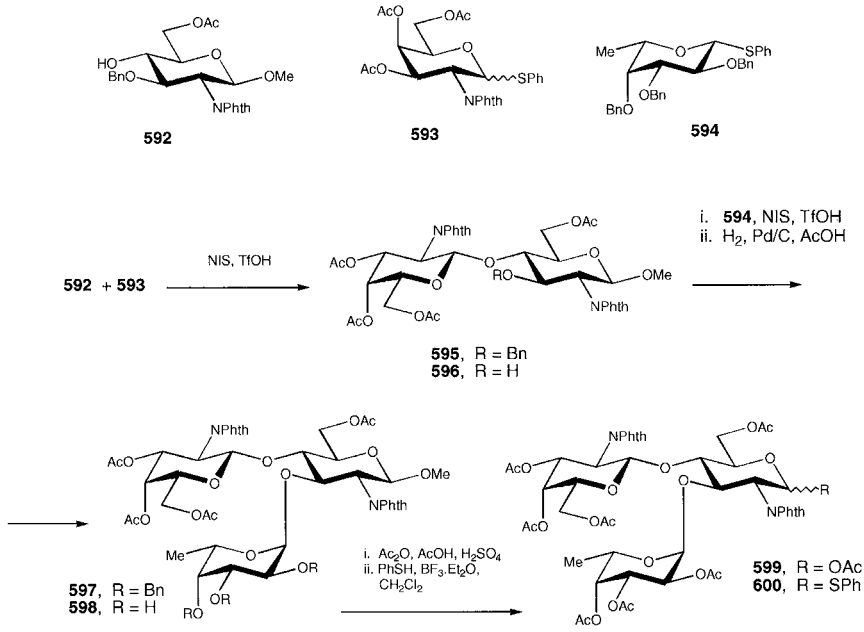
589



590, $R^1 = SO_3Na$, $R^2 = H$
591, $R^1 = H$, $R^2 = SO_3Na$

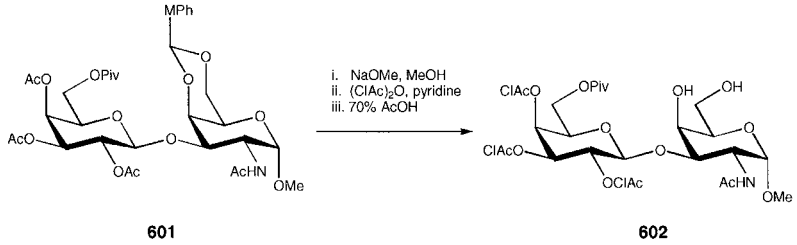
7. Candidate Inhibitors Modeled on Core-2 Mucin Glycans

Matta and his associates[590] have synthesized compound **606** among other inhibitors modeled on core-2 mucin glycans. For the synthesis of **606,** trisaccharide donor **600,** corresponding to a Le^x determinant containing GalNAc in the place of Gal, was combined with the disaccharide acceptor **602,** representing the T-antigen segment β-D-Gal-(1 $\rightarrow$ 3)-α-D-GalNAc. Key glycosylation steps were designed to involve thioglycoside donors activated with *N*-iodosuccinimide[591] (NIS) and trifluoromethanesulfonic acid[592] (triflic acid). Trisaccharide donor **600** was prepared (Scheme 99) from the monosaccharide precursors **592, 593,** and **594** representing the GlcNAc, GalNAc, and Fuc residues. GlcNAc acceptor **592** was obtained by regioselective acylation of the known[593] methyl 3-*O*-benzyl-2-deoxy-2-phthalimido-β-D-glucopyranoside (acetyl chloride in pyridine–dichloromethane).



SCHEME 99

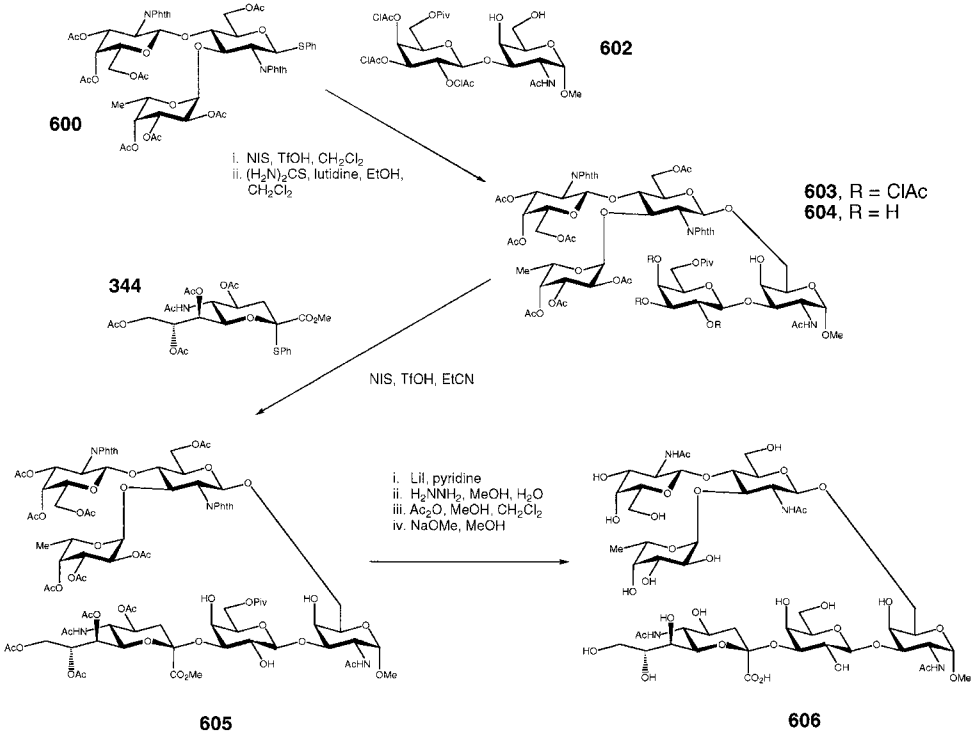
The GalNAc donor **593** was prepared by treatment of the known[594] 1,3,4,6-tetra-*O*-acetyl-2-deoxy-2-phthalimido-D-galactopyranose with thiophenol and boron trifluoride etherate in dichloromethane.[595] Glycosylation of the acceptor **592** with GalNAc donor **593** (NIS, triflic acid) afforded the disaccharide derivative **595** in 52% yield. Compound **595** was converted into the acceptor **596** by catalytic hydrogenation over 10% palladium-on-charcoal (62%). Acceptor **596** was glycosylated with the fucosyl donor **594** (NIS, triflic acid) to give the fully protected trisaccharide derivative **597** in 68% yield. Intermediate **597** was facultatively converted into the GalNAc-Le^x determinant (see Section VI.8) or processed as follows to provide **600** as a key donor for the assembly of the core-2-related selectin ligand, **606.** Catalytic hydrogenation of **597** over 10% palladium-on-charcoal in glacial acetic acid provided **598,** which was subjected to acetolysis (acetic anhydride–acetic acid, catalyzed by sulfuric acid, 5 °C, 16 h) to afford an anomeric mixture of acetates **599** (76% from **597**). This mixture was treated with thiophenol in dichloromethane in the presence of boron trifluoride etherate (30 °C, 5 h) to give the thioglycoside donor synthon **600** (49%). For the preparation of the T-disaccharide acceptor **602** (Scheme 100), compound **601** was subjected to partial, sodium methoxide-catalyzed methanolysis to afford a triol derivative, which was per-monochloroacetylated to give an intermediate from which acceptor



601 **602**

SCHEME 100

602 was obtained by treatment with 70% acetic acid. The Neu5Ac residue was then attached, in an α-(2 $\rightarrow$ 3) linkage, to the nonreducing Gal residue of the T-antigen branch. Glycosylation of **602** with trisaccharide donor **600** (NIS, triflic acid; Scheme 101) gave **603,** which was treated with thiourea and lutidine (1 : 1 ethanol–dichloromethane, 80 °C, 6 h) to give the partially protected pentasaccharide derivative **604** in 37% yield. As documented by previous precedents, triol

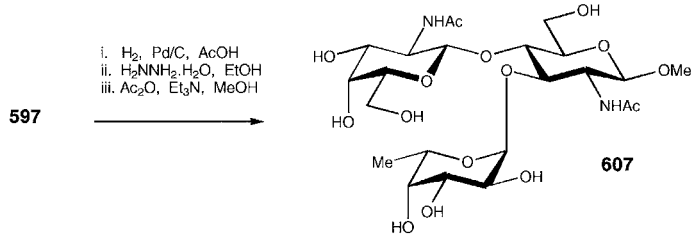


SCHEME 101

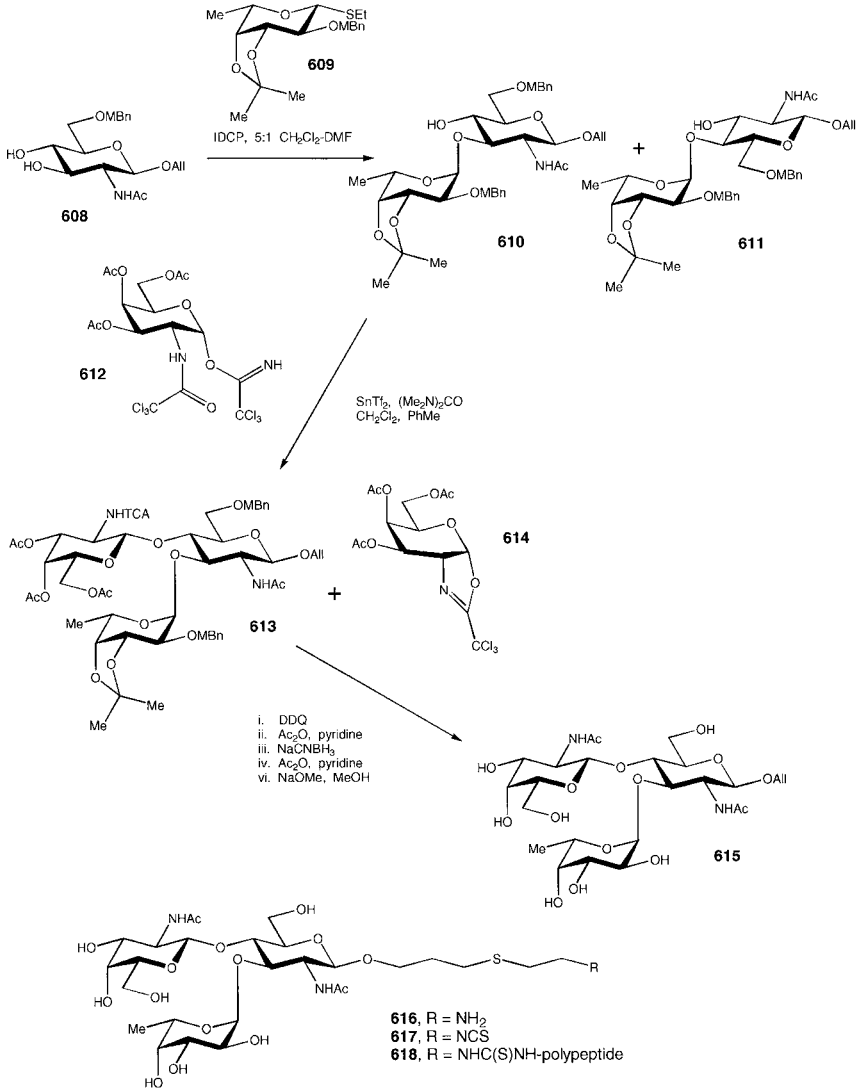
604 is a specific acceptor in which only Gal OH-3 is reactive (the primary hydroxyl function being esterified, OH-2 unreactive because of steric hindrance, and OH-4 unreactive, under these conditions, as a consequence of its axial orientation). Glycosylation of acceptor **604** with sialyl donor **344** afforded the hexasaccharide derivative **605** in 47% yield [NIS, triflic acid, propionitrile, molecular sieves 4 Å (0.4 nm), −65 °C]. Removal of the protecting groups of **605** was accomplished in four steps. Treatment with lithium iodide in pyridine converted methyl ester **605** into the free carboxylic acid; next, the phthalimido groups were cleaved by the action of hydrazine hydrate in methanol; treatment of the product with acetic anhydride in methanol–dichloromethane provided all nitrogen functions in the form of acetamido groups; finally, sodium methoxide-catalyzed methanolysis afforded the hexasaccharide target structure **606** (24% from **605**). The biantennary candidate inhibitor **606** had IC_{50} values of ∼0.09 and ∼0.1 m*M* in an ELISA competition assay against recombinant P- and L-selectins, respectively; by comparison, sLe^x had IC_{50} 0.5 and 0.6 m*M* in this assay.

8. Candidate Inhibitors Modeled on GalNAc-Le^x

For conversion into the trisaccharide target structure **607,** intermediate **597** was subjected to catalytic hydrogenation over palladium-on-charcoal in acetic acid (Scheme 102); the resulting product was heated with hydrazine hydrate in ethanol (100 °C, 16 h) to cleave the *N*-phthaloyl and *O*-acetyl groups. The amino derivative thus obtained was converted into the GalNAc-Le^x derivative **607** by treatment with acetic anhydride in methanol in the presence of triethylamine. Compound **607** had IC_{50} values of 0.5, 0.4, and 0.3 m*M* in an EIA assay of recombinant E-, P-, and L-selectins binding to immobilized sLe^x; by comparison, the analogous sLe^x derivative had IC_{50} 0.55, 0.5, and 0.6 m*M* in the same system.[590] This finding confirms the equivalence of monovalent GalNAc-Le^x with sLe^x (in static assays) as previously reported[596] by Grinnell *et al.* The GalNAc-Le^x determinant **607** has been synthesized by the Utrecht group[597] by transfer of a fucose residue from GDP-fucose to the disaccharide derivative β-D-GalNAc-(1 → 4)-β-D-GlcNAc-OMe, catalyzed by α-1,3/4-fucosyltransferase from human milk. An alternative chemical synthesis



SCHEME 102



SCHEME 103

of the allyl glycoside **615** corresponding to **607** has been reported by Kosma and his associates.[598] These authors glycosylated the GlcNAc acceptor derivative **608** (Scheme 103) with the fucose thioglycoside donor[599] **609** [iodonium dicollidine perchlorate, 5 : 1 dichloromethane–*N*,*N*-dimethylformamide, molecular sieves 4 Å (0.4 nm), room temperature, 40 h] to obtain a mixture of disaccharide derivatives

610 (49%) and **611** that could be processed further to the type 2 (Le^x) or type 1 (Le^a) analogues. Glycosylation of **610** with the *N*-trichloroacetyl-trichloroacetimidate donor[600,601] **612** required optimization and succeeded under promotion by tin(II) triflate in the presence of *N*,*N*,*N*′,*N*′-tetramethylurea in dichloromethane–toluene to afford **613** (41%) accompanied by oxazoline **614** and unreacted glycosyl acceptor. Trisaccharide derivative **613** was converted into the desired allyl β-glycoside of GalNAc-Le^x **615** by sequential treatment with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ), 3-O-acetylation, hydrolysis of the isopropylidene acetal (90% trifluoroacetic acid), reduction of the trichloroacetamide by the action of sodium cyanoborohydride, per-O-acetylation, and treatment with sodium methoxide in methanol. For elaboration of a neoglycoprotein suitable for use as an artificial antigen, compound **615** was reacted with cysteamine to produce the primary amine **616,** which was treated with thiophosgene to form the isothiocyanate **617.** Coupling of **617** to bovine serum albumin occurs by addition of the ε-amino group of a lysine residue to the –N=C double bond with formation of a neoglycoprotein (**618**) that contains the oligosaccharide determinant bound by a thiourea linkage.

9. Inhibition of Selectin–Ligand Interactions: Alternatives to Small Molecules

a. Soluble Selectins and Soluble Selectin Ligands.—

(i) ***Soluble Selectins.—***The Biogen group[602] has reported on the construction of a truncated cDNA for E-selectin by insertion of a stop codon close to the boundary between the sixth complement regulatory consensus repeat and the transmembrane domain. This cDNA encodes a functional protein termed rsE-selectin that has been expressed in CHO cells and purified to homogeneity on a column of MAb BB11 linked to proteinA–Sepharose. When immobilized on plastic surfaces, rsE-selectin binds only HL60 cells and other cells known to bind E-selectin expressed on human endothelial cells. However, rsE-selectin is only a weak inhibitor of E-selectin-mediated cell adhesion. The adhesion of activated neutrophils to endothelium can be prevented by a soluble version of P-selectin.[603] Shedding of L-selectin occurs during the rolling process; the shed L-selectin has been considered to inhibit further L-selectin-mediated adhesive interaction.[604]

(ii) ***Soluble Selectin Ligands.—***Takada *et al.* demonstrated[605] that soluble P-selectin glycoprotein ligand (sPSGL) prevents the early manifestations of ischemia-reperfusion injury associated with renal transplantation in the rat. When sPSGL was administered, E-selectin mRNA remained at baseline levels, no infiltrating leukocytes were found in the injured kidneys, and secretion of several inflammatory mediators was markedly inhibited. Administration of the soluble

ligand was considered a promising intervention with the potential to reduce later deleterious effects that would threaten the survival of the renal allografts.

b. Antibodies against Selectins and Their Ligands.—

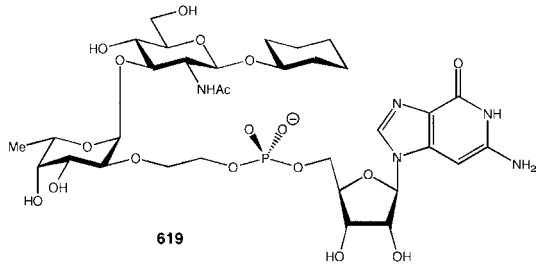
(i) *Anti-Selectin Antibodies.*—The anti-E-selectin MAb 1.2B6 has been described by Groves *et al.*[228] Functional inhibition with monoclonal antibodies of L-selectin or P-selectin only partially reduced (ca. 50%) the reperfusion—induced tissue injury in the cat myocardium.[606,607] P-Selectin antibody did not provide any protection against reperfusion of postischemic skeletal muscle, whereas an L-selectin antibody was only moderately effective in this tissue.[608]

(ii) *Anti-Ligand Antibodies.*—The monoclonal antibody MECA-79 preferentially reacts with the selectin-binding high-endothelial venules of mouse peripheral lymph nodes, inhibits L-selectin-dependent lymphocyte binding to HEV *in vitro* and *in vivo,* and immunoprecipitates HEV molecules that bind lymphocytes and L-selectin transfectants or L-selectin immunoglobulin chimaeric proteins.[609,153,111,156] The monoclonal antibody HECA452 has been described as recognizing carbohydrate epitopes that can be recognized by E-selectin and that are related to but not identical with sLe^x (Refs. 182, 393, 610). HECA 452 stains high endothelial venules in tonsils, appendix, and lymph nodes and binds to vessels with the morphologic appearance of HEV in areas of extensive lymphoid infiltration in autommune thyroiditis, for example, Graves' and Hashimoto's diseases, and in the gut of patients with Crohn's disease. Several groups of investigators have raised monoclonal antibodies against sialylated Lewis determinants; examples are the anti-sLe^x MAbs SNH3 (Ref. 611), CSLEX-1 (Ref. 75), FH6 (Ref. 612), and the anti-sLe^a-MAb CA 19–9 (Ref. 2).

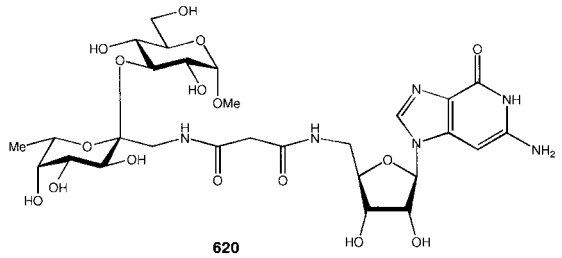
c. Selectin-Immunoglobulin Fusion Proteins.—Watson, Fennie, and Lasky[613] reported that administration to mice of an L-selectin–IgG chimera significantly decreased neutrophil influx into peritoneum following experimental inflammation by the action of thioglycolate; the authors concluded that this type of soluble selectin fusion protein could be a candidate antiinflammatory therapeutic.

d. Inhibitors of Ligand Biosynthesis.—Hakomori and his associates[614] found that the expression of sLe^x and sLe^a on the surface of HL60, Colo205, and U937 tumor cells is strongly inhibited following culturing of these cells for 72 h in the presence of 2 m*M* benzyl 2-acetamido-2-deoxy-α-D-galactopyranoside. As demonstrated by Kim *et al.,*[615] this artificial substrate competes with the α-GalNAc residues linked to serine or threonine that function as glycosyl acceptors during the biosynthesis of O-linked glycoprotein glycans. Decreases in, or the absence of, expression of sLe^x or sLe^a were observed by flow cytometry using the anti-sLe^x MAb SNH3 (Ref. 611) or the anti-sLe^a MAb CA-19-9 in conjunction with

a $F(ab')_2$ anti-mouse IgG. As a positive control, the increased formation of polypeptide linked α-D-GalNAc monosaccharide residues was demonstrated with the aid of the GalNAc specific lectin from *Helix pomatia*. Furthermore, the formation was observed of mucin-type oligosaccharides containing benzyl α-D-GalNAc as the reducing terminus. Inhibitors of N-linked glycosylation[616] such as castanospermine, swainsonine, or deoxymannonojirimycin did not cause any reduction in the expression of sLe^x or sLe^a determinants. The group of van Boom[617] synthesized a series of carbohydrate derivatives designed to constitute transition-state inhibitors of the fucosyl transfer reaction that results in the formation of the sLe^x determinant from GDP-fucose and precursor glycoprotein glycans (compare Scheme 38). Compounds **619** and **620** are examples of the target structures synthesized.



619



620

e. DNA Aptamers.—With the use of the polymerase chain reaction (PCR), oligonucleotides termed aptamers may be synthetically evolved and exponentially enriched that bind specifically to a given protein.[618] Applications of the aptamer technique have been reviewed by Gold.[619] Varki and his associates[620] have produced aptamers that function as calcium-dependent antagonists of L-selectin–ligand binding. Hicke *et al.* have subsequently prepared superior L-selectin-binding aptamers[621] that bind to L-selectin 10^5–10^6 times more strongly than sLe^x and block the trafficking of human lymphocytes to peripheral lymphoid tissue in severe combined immune deficiency (SCID) mice. Typically,[621] a starting pool of 10^{15} random-sequence single-stranded DNA molecules is incubated with

an L-selectin–Ig chimera bound to protein A–Sepharose beads. The beads are removed and washed extensively, and the bound oligosaccharides are eluted with EDTA solution and subjected to PCR amplification. Following separation of the DNA strands, the cycle is repeated. After 15 iterations, the DNA aptamers prepared in the work of Hicke *et al.* were found to bind to the L-selectin–Ig chimera with a $K_D = 0.9 \pm 0.1$ n*M* compared to >5 μM for the initial pool.

f. Antisense Oligonucleotides.—Bennett *et al.*[622] reported on the preparation and biological activities of phosphorothioate oligonucleotides 18 to 21 bases long, designed to hybridize to the mRNAs of E-selectin, ICAM-1, and VCAM-1. Oligonucleotides designed to hybridize to the 3′-untranslated regions of the respective mRNAs caused decreases of the corresponding mRNA levels. E-selectin antisense oligonucleotides inhibited cell surface expression of E-selectin and caused diminished adhesion of HL-60 cells to TNF-activated HUVEC.

VII. Conclusions: Prospects, Alternatives, and Opportunities

1. Prospects of Small-Molecule Inhibitors Derived from Sialyl-Lewis^x

With the use of their flow chamber model, Lawrence and Springer[194] have demonstrated that, under flow, tethering and rolling of leukocytes mediated by selectin–ligand interactions is a necessary, preliminary step prior to the firm adhesion of these cells mediated by the interaction of β_2-integrin with ICAM-1. These findings have strengthened the pharmacological hypothesis which states that inhibitors of selectin–ligand interactions would block the subsequent cell adhesion events preceding inflammation and would thus be prospective antiinflammatory agents. Nonetheless, earlier reviewers[623] have expressed caution regarding the choice of selectin–ligand interactions as a target for therapeutic intervention in inflammation. Among other issues, they have questioned whether univalent inhibitors with relatively low binding affinity will be effective at reasonable concentrations for antagonizing the multivalent interactions associated with cell adhesion. Kubes *et al.*[624] also question the prospects of developing clinically useful antiinflammatory agents from inhibitors of selectin–ligand binding. These authors used intravital microscopy of cat mesentery to observe that experimentally induced ischemia followed by reperfusion caused a strong increase in rolling and adhesion of leukocytes. Leukocyte rolling was reduced by more than 90% following administration of a high dose of fucoidan (25 mg per kg body weight) and was still reduced by 60% using a dose of 1 mg/kg. However, although the higher dose significantly reduced the integrin-mediated leukocyte adhesion, the levels of cell adhesion remained unaffected at the lower dose. Despite the 60% reduction in rolling, the remaining rolling cells were capable of firm adhesion via β_2-integrin. Cell adhesion was even stronger in blood vessels in which shear rates were reduced

by 30–50%. A reduction in rolling of 60% was also achieved using anti-P-selectin or anti-L-selectin antibodies, or a combination of both, but this reduction, as with the lower dose of fucoidan, did not result in diminished cell adhesion. Kubes *et al.* concluded that, at the onset of an inflammatory condition, there is an excessive number of rolling leukocytes of which only a small proportion eventually adhere via β_2-integrin. Therefore, a form of therapy that targets leukocyte rolling would require greater than 90% efficacy to reduce leukocyte adhesion by 50%. Another comment on tethering and rolling as an antiinflammatory target has been presented by Ward.[625] This author points out that, during inflammation in a number of organs of rabbits, neutrophil accumulation appears to develop around capillaries rather than larger vessels such as postcapillary venules. Because of the smaller dimensions of capillaries, firm adhesion of activated neutrophils to endothelial cells may be able to proceed without prior tethering and rolling. It remains to be seen whether these observations are paralleled in humans and, if so, which of the pathologically relevant phenomena depend on tethering and rolling and which do not.

On the other hand, prior to the report of Kubes *et al.*,[624] Cytel Corporation in San Diego had launched an ambitious program directed at the development of selectin ligand glycan derivatives as antiinflammatory agents. The company pioneered the pharmaceutical application of an sLe^x oligosaccharide derivative, Cylexin (CY 1503) in several clinical indications.[626] In a pre-clinical setting, the suitability of sLe^x-related oligosaccharide derivatives as therapeutics against the acute inflammatory sequelae of occlusion–reperfusion had been demonstrated through the successful outcomes of several types of animal experiment. Mulligan *et al.*[627] demonstrated that the spacer-linked sLe^x tetrasaccharide or pentasaccharide derivatives **621** or **622** (Fig. 24) dramatically reduced acute lung injury resulting from intravenous infusion of cobra venom factor into rats. This type of injury is caused by oxygen radicals produced by neutrophils that extravasate into lung tissue following adhesion by a P-selectin-dependent mechanism. Over a dose range of 50–500 μg of **622,** the parameters of lung permeability, hemorrhage, and content of myeloperoxidase, a marker for neutrophils in lung tissue, were reduced by 67, 47, and 49%. By contrast, the analogous sialosyllactosamine derivatives **623** and **624** were ineffective. Lefer *et al.*[628] similarly demonstrated that CY 1503 (Cylexin) **622** significantly reduces the degree of injury to the canine heart associated with coronary artery ischemia and reperfusion. Injection of a single bolus (5 mg/kg body weight) of CY-1503 5 min before reperfusion resulted in less creatine kinase released by the ischemic heart muscle, attenuation of necrosis by 65% within the area at risk, reduction by 63% of PMN accumulation within the necrotic zone, and virtually unchanged vasodilation of the left circumflex coronary artery in response to acetylcholine or nitroglycerin. Murohara *et al.*[629] studied the efficacy of a stealth liposome formulation[630–632] of Cylexin in protection of the feline heart against reperfusion injury following myocardial ischemia. The liposomal dosage form required

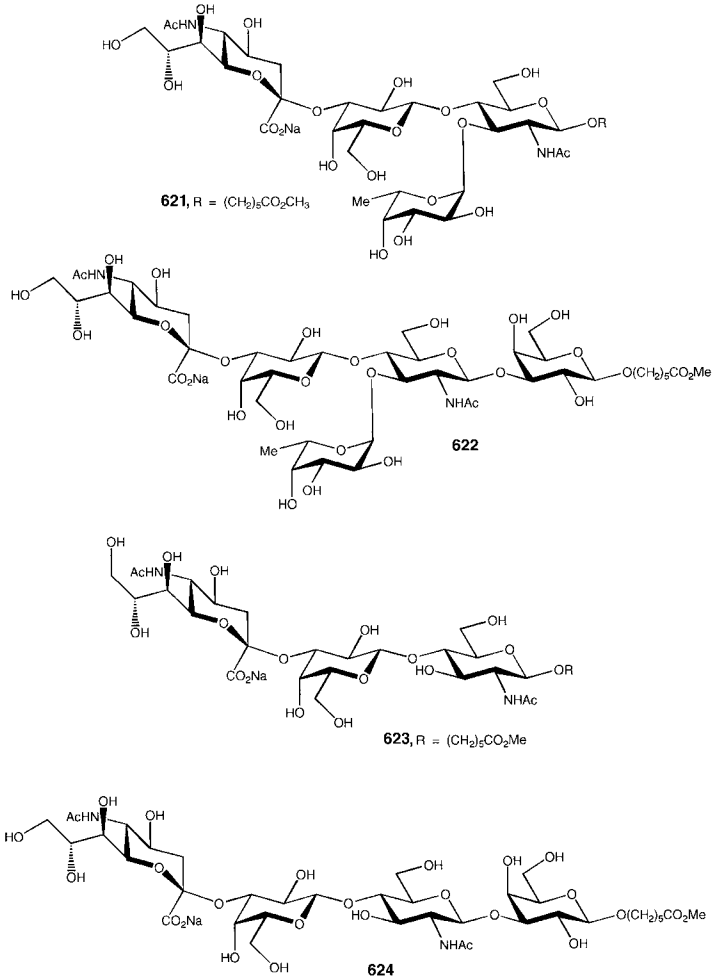


FIG. 24. Structures of oligosaccharide derivatives examined by Mulligan *et al.* for inhibition of neutrophil extravasation in rats (Ref. 627). Compound 622 (Cylexin-TM) was later tested as a candidate antiinflammatory therapeutic in humans.

only 4% of the amount of active ingredient used in a previous study[633] to provide significantly lowered myocardial necrosis, plasma creatine kinase, and PMN accumulation in the ischemic heart muscle. Whereas free Cylexin, in the blood of cats, has a half-life of less than 20 min,[633] stealth liposomes have half-lives on the order of 12 h.[630–632] Fulfilling a further requirement for the clinical application of Cylexin, by developing a process of enzyme-catalyzed sLe^x synthesis[390] with cofactor regeneration, the Cytel investigators have elegantly solved the formidable

manufacturing problem posed by the need to provide kilogram amounts of the highly pure sLe^x oligosaccharide ingredient. In 1997, Cylexin™ was "in phase II clinical trials to evaluate its ability to mitigate reperfusion injury which often follows cardiopulmonary bypass used in surgery. These trials are referred to as CALYPSO trials. Cylexin™ is also being studied relative to its use as an aid in improving the post-operative course of surgery to correct congenital heart defects in newborn infants . . . . [Source: Business Wire—02/18/97.]"[634] In April of 1999, it was reported[635] that ". . . Disappointing results of the Phase II/III clinical trial of Cylexin, Cytel's cell adhesion inhibitor compound, led to the termination of the cell adhesion therapeutic business." This setback was widely recorded and led to the termination of programs directed at carbohydrate-based cell-adhesion inhibitors elsewhere in the pharmaceutical industry. At the same time, successful clinical studies had been completed in similar indications with inhibitors of platelet integrin binding, so that interest was redirected toward this new class of therapeutics.

2. Antagonists of Integrin Binding as Inhibitors of Cell Adhesion

a. Antibodies Directed against β_2-Integrin.—Prevention of neutrophil adhesion (neutralization of β_2-integrin by anti-β_2-integrin antibodies) consistently prevented reperfusion-induced tissue injury by more than 85% in a number of animal models.[636–638] However, successful clinical application of such antibodies has not been reported.

b. Antibodies Directed against the Platelet Integrin $\alpha_{IIb}\beta_3$.—Aggregation of blood platelets is a key event in thrombosis of coronary arteries.[639] The principal mechanism of platelet aggregation involves the interaction of the platelet integrin $\alpha_{IIb}\beta_3$ (glycoprotein IIb/IIIa) with fibrinogen.[640] The first anti-integrin receptor therapeutic to be approved for human use in the United States is ReoPro developed by Centocor (*Abciximab;* the Fab fragment of the mouse–human chimeric monoclonal antibody 7E3, directed against the platelet glycoprotein GPIIb/IIIa [$\alpha_{IIb}\beta_3$]). This antagonist of a platelet surface receptor was found safe and effective in reducing the risk of ischemic complications after percutaneous coronary intervention (PCI; angioplasty or atherectomy).[640]

c. Low-Molecular Mass Anti-Integrin Receptor Therapeutics.—Eptifibatide (Integrilin, COR Therapeutics, South San Francisco), is a synthetic cyclic heptapeptide and functions as a selective high-affinity inhibitor of the platelet glycoprotein IIb/IIIa receptor.[643] It produces dose-dependent *ex vivo* inhibition of platelet aggregation and reduces the frequency of acute ischemic complications of percutaneous coronary revascularization. Several interesting low-molecular-mass antagonists have been discovered of the interaction between the $\alpha_4\beta_1$ integrin (very late antigen 4 or VLA-4) and its counter-receptor VCAM-1. The N-substituted

3,4-diamino-3-cyclobutene-1,2-dione derivatives patented for American Home Products Corp. constitute examples of such compounds.[641]

3. Discovery of New Mechanisms of Therapeutic Action Targeting Protein–Carbohydrate Interactions

a. Induced Shedding of L-Selectin.—Kiessling and her associates[642] have reported on a new class of multivalent selectin ligands termed "neoglycopolymers," which promote the proteolytic cleavage of L-selectin and its shedding from the surface of human neutrophils (compare Section VI.6). Their experiment represents a comparison, by flow cytometry, of the L-selectin expressed on the surface of human neutrophil leukocytes exposed to equimolar solutions of an L-selectin ligand glycan derivative in monovalent or polyvalent forms. Thus, one batch of cells was incubated in a 4 m*M* solution of the monovalent L-selectin ligand, 3′,6-disulfo-Le^x (**589**), the other, in an equimolar (per saccharide) solution of a multivalent array of 3′,6-disulfo-Le^x (**591**) attached as pendant groups to a neoglycopolymer produced by ring-opening metathesis polymerization.[589] Exposure of the neutrophils to the neoglycopolymer resulted in loss of L-selectin from the cell surface in a dose-dependent manner. The L-selectin shedded was detected in the supernatants of the polymer-treated cells. In contrast, no release of L-selectin was found with neutrophils exposed to the monovalent ligand. In a control experiment, Kiessling *et al.* examined the neoglycopolymer-induced shedding of L-selectin in comparison to the shedding of L-selectin that follows activation of cells by phorbol esters or chemotactic peptides. While promoting the shedding of L-selectin, such activators elicit a strong increase of cell surface expression of CD11b/CD18 (Mac-1), the β_2-integrin that mediates the subsequent step of firm neutrophil adhesion to endothelial cells.[644] As shown by flow cytometry, the surface expression of CD11b/CD18 on human neutrophils was not changed upon incubation with the 3′,6-disulfo-Le^x neoglycopolymer. This finding indicates that the mechanism by which the neoglycopolymer induces shedding of L-selectin from these cells is distinct from that operative during cellular activation by phorbol esters or chemotactic peptides. Agents such as the neoglycopolymers described by Kiessling *et al.* constitute a new approach to immune suppression; not only would specific shedding of L-selectin from neutrophils decrease the number of cells proceeding to firm adhesion and extravasation, but the shed L-selectin would also block L-selectin ligands on other leukocytes and thus provide additional inhibition of adhesive processes.

b. Antiinflammatory Potentials of Heparan Sulfates and Heparins.—Recent results[645] by the groups of Linhardt and Varki indicate that components of heparan sulfate and heparin preparations are bound by L- and P-selectins but not by E-selectin, as determined by selectin affinity chromatography. Affinity columns were prepared by incubation of protein A–Sepharose with the respective selectin–Ig chimeras. High molecular mass heparan sulfate or heparin fractions (tetradecasaccharides or larger) were bound to the selectins much more strongly than

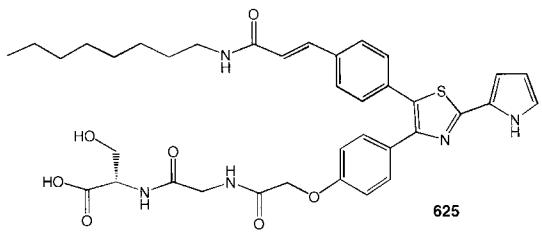
low molecular mass heparin fragments. Undegraded heparin from porcine intestinal mucosa, a material that is similar to heparin preparations in clinical use as anticoagulants, was shown to inhibit the binding of HL60 cells to L- or P-selectins with IC_{50} values ~12 or 50-fold lower than the recommended therapeutic dose. Since several of the clinical indications considered for selectin inhibition (for example, coronary infarct and stroke) are routinely treated with heparin as anticoagulant, Koenig *et al.* have suggested[645] that at least part of the clinical benefit of heparin might be due to blocking of P- or L-selectins. Since low-molecular-mass heparin fragments showed much weaker selectin binding, the authors also suggested that the conceivable antiinflammatory benefits of heparin therapy would not be available when therapeutic regimens involving unfractionated heparin are replaced by those based on low-molecular-mass heparin fragments. During the work of Koenig *et al.*, regions of the heparin molecule binding to L-selectin were characterized as including more highly sulfated and epimerized segments as well as a higher incidence of free amino groups. Because of the heterogeneity of the naturally derived materials and the low content of individual components, a more detailed structural characterization of the L-selectin binding structures has not been performed. However, the chemistry[646,647] developed by the groups of Petitou and van Boeckel for the synthesis of antithrombotic oligosaccharides[648] derived from heparin should provide interesting libraries for the study of selectin-binding sites.

Several recent discoveries are pertinent to the study of protein–carbohydrate interactions with a view to the design of new therapeutics. Bundle and his associates[649] at the University of Alberta, Edmonton, have designed and prepared multivalent carbohydrate ligands termed STARFISH that neutralize Shiga-like toxins of *Escherichia coli* O57:H7. Shiga-like toxins can cause serious clinical complications in humans following ingestion of spoiled hamburgers. Varki and his co-workers[650] are studying the properties and biological significance of Siglecs, a family of sialic acid-binding lectins belonging to the immunoglobulin superfamily. Brewer and his associates[651]have discussed multivalent protein–carbohydrate interactions of galectins. These lectins form supramolecular assemblies and function in apoptosis (programmed cell death).

4. Outlook

In spite of recent unfavorable clinical developments, pessimism is unjustified with respect to the more general field of rationally designed inhibitors of protein–carbohydrate interaction. The unsuccessful outcome of the Cylexin clinical studies should be taken to indicate that monovalent oligosaccharide derivatives are ineffective as therapeutics in the acute inflammatory conditions studied. However, bright and tempting prospects still exist, possibly in different indications, for more potent derivatives with better pharmaceutical properties. Thus, a fresh approach to selectin inhibitor design has been initiated[652] by Rao and his colleagues of the Glycomed group (now Ligand Pharmaceuticals) in Alameda, California. These

authors used conformational energy computations, high-field NMR, and structure–function studies to define distance parameters of critical functional groups of sLe^x. The sLe^x pharmacophore obtained was used to search a three-dimensional data base of chemical structures, and compounds with a similar spatial relationship of functional groups were tested as inhibitors of selectin binding. The triterpene derivative, glycyrrhizin, was found to fulfil the requirements and to inhibit selectin binding to sLe^x ***in vitro***. Subsequently, different monosaccharides were substituted for the glucuronic acid residues of glycyrrhizin, with the L-fucose derivative found to be the most active ***in vitro*** and ***in vivo***. Finally, a *C*-fucosyl derivative was synthesized and identified as an effective selectin blocker with anti-inflammatory activity. As indicated by the work of Rao ***et al.,*** development of a range of therapeutically useful compounds from inhibitors of selectin–ligand interactions may well require departure from the focus on the intrinsically polar carbohydrate derivatives. Although suitable drug leads might not arise from isosterically substituting nonpolar groups such as aromatic or cyclohexane rings for the individual glycosyl residues of the sLe^x determinant, a more promising approach might be to devise alternative scaffolds that are only partially coextensive with carbohydrate determinants but provide strong intrinsic receptor binding. Conceivably, such lead structures can be identified by exploring sites within or adjacent to the carbohydrate-recognition domains: identified by rational design, combinatorial synthesis, or high-throughput screening of natural products, suitable lead structures will be complementary to, rather than isosteric with, the selectin ligand glycans. The secondary binding sites discovered by studies with hydrophobic substituents on the sLe^x scaffold (Section VI.5) could well serve as one of several starting points for the discovery of such new lead structures. Similarly, a combinatorial chemistry approach based on the Ugi reaction has been pursued in a search for pharmaceutically interesting derivatives.[653] Ontogen Corp. and NV Organon have recently discovered *N*-[2-[4-[5-[4-(3-octylamino-3-oxo-1-propenyl)phenyl]-2-(1-*H*-pyrrol-2-yl)thiazol-4-yl]phenoxy]acetyl]glycyl-L-serine (**625**), one of several selective P- and L-selectin inhibitors[654] ($IC_{50} = 0.18$ and 0.16 μM, respectively, vs > 500 μM for E-selectin in human ELISA assays). At 30 μM concentration, compound **625** inhibits HL60 cell adhesion to P-selectin by 95%. An example for comparative exploration of structure–activity relationships among different receptor-binding scaffolds is provided by the studies of Schreiber and his associates[655] on the interaction of cyclosporin A, FK 506, and rapamycin with



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immunosuppressant-binding proteins (immunophilins) of T-lymphocytes. Recent work from the present author's laboratory[656] has been directed at new strategies for chemical exploration of carbohydrate-binding domains; for example, the CBDs of model plant lectins are being studied, using as probes a range of sulfur-containing analogues of T-disaccharide, β-D-Gal-(1 $\rightarrow$ 3)-α-D-GalNAc. While the nonreducing Gal residue of this disaccharide is essential for binding to the lectins, the GalNAc residue is only partially in contact with the CBD and will tolerate a substantial degree of chemical modification. Replacement of certain hydroxyl groups of the GalNAc residue by thiol groups provides scaffolds that retain the ability to bind to the lectin yet can be conveniently derivatized, in a combinatorial approach, to probe the immediate environment of the CBD with hydrophobic groups attached to the scaffold by thioether linkages. A similar approach to selectin-binding derivatives could exploit structural differences outside the CBDs and possibly provide selectin-specific inhibitors.

Once appropriate drug scaffolds have been identified, the choice of drug leads will require increased consideration of the constraints imposed by routine clinical use of drugs in the respective indications.[537] Aside from safety and effective receptor blocking, such compounds are preferably administered by the oral route and need to display consistent, high bioavailability. The molecular properties of drug candidates that fulfill some of these demands have led Lipinski and his associates[657] to formulate the "rule of 5," which predicts that—aside from efficacy—poor absorption or permeation of a drug candidate compound is more likely when there are more than five H-bond donors, ten H-bond acceptors, the molecular mass is greater than 500, and the calculated log *P* (the octanol/water partition coefficient) is greater than 5. From these criteria, it follows that innovative lead structures will likely be derived from scaffolds other than sLe^x or sLe^a. On the other hand, such new structures will offer wider opportunities for molecular variation by combinatorial approaches to achieve candidate drugs that combine strong receptor binding with desirable pharmacological properties. Finally, as concluded by Albelda and Buck[203] in their review of 1994, detailed knowledge is rapidly increasing of the precise combinations of selectins, integrins, and chemoattractants participating in specific disease states (compare Section III). In their view, this knowledge could be used to design anti-adhesive therapeutics. Especially, rational combinations of agents inhibiting selectin, integrin, and chemoattractant binding could conceivably lead to new therapeutics of superior efficacy and specificity.

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