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Review

The surface glycopeptidolipids of mycobacteria: structures and biological properties

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Abstract. One of the most important opportunistic pathogens associated with acquired immunodeficiency syndrome (AIDS) is the *M. avium* complex. *M. avium* infections are found in up to 70% of individuals in advanced stages of AIDS. It is apparent that *M. avium* can replicate in host macrophages and persist for long periods. This group of mycobacteria are distinguished by the presence of unique, highly antigenic, surface-located lipids known as the glycopeptidolipids (GPLs). The GPLs are the chemical basis of the 31 distinct serovars of the *M. avium* complex, and have also been identified in some other species. The *M. avium* lipids are immunosuppressive and can induce a variety of cytokines that affect general host responses. Despite extensive chemical characterization of the structures of these GPLs, much work is needed to elucidate the molecular mechanism involved in this complex glycosylation pathway and its genetic basis. The challenges for the future lie in explaining the roles of these copious products in the intracellular life and infectivity of mycobacteria. The intention of our review is to offer a concise account of the structures of the *M. avium* lipids, their putative roles in the host responses, bacterial physiology and pathogenesis, particularly in immunocompromised patients such as those infected with human immunodeficiency virus (HIV). Advances in chemical synthesis of the various haptenic oligosaccharides are also given to demonstrate how these have helped to define the immunogenic determinants. We believe that future research should involve the creation of conditional mutants defective in these lipids for both functional and biosynthesis studies which will complement biological assays using chemically defined or modified neoglycoconjugates.

Key words. Glycopeptidolipid (GPL); *Mycobacterium avium* complex (MAC); mycobacteria; haptenic oligosaccharides; neoglycoproteins.

Introduction

Mycobacteria (members of the *Mycobacterium* genus), in the diseases they cause, remain serious problems. With an estimated worldwide incidence of 2.9 million deaths and 8 million new cases per year [1], tuberculosis is the leading cause of death from a single infectious agent. In Africa and the Indian subcontinent, the absolute number of tuberculosis cases has increased steadily over the years. The situation is rapidly worsening in many sub-Saharan African countries because of the increased prevalence of HIV infection, which is the highest risk factor thus far identified for the progression of latent tuberculosis infection to active disease. In addition, coinfection with opportunistic pathogens such as *Mycobacterium avium, Mycobacterium intracellulare, Mycobacterium scrofulaceum* complex (MAC) has been a hallmark for

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HIV-mediated decline in immunocompetency responsible for AIDS [2–4].

Human exposure to MAC is common because the organisms are ubiquitous in the environment. They are readily isolated from water, soil and house dust [5–8], quite resistant to the temperatures at which hot water tanks are maintained and can colonize human respiratory and urinary tracts without causing disease [9]. Although the mode of transmission has yet to be established, likely routes include fine particle aerosol or aspiration [10]. Prior to the AIDS epidemic, most human cases of MAC infections presented as pulmonary diseases. Evidence suggests that prevalence of pulmonary MAC infection (PMAC) has been steadily growing in the United States over the latter half of the 20th century [11]. To some extent, this increased incidence reflects an improved laboratory capacity to distinguish MAC from *M. tuberculosis*, as well as a growing awareness on the part of clinicians of the pathogenic potential of MAC. Today in the United States, disseminated MAC (DMAC) infection is recognized as the most common bacterial infection occurring in patients with AIDS, affecting up to 43% of HIV-seropositive persons [12, 13]. *M. avium* is the etiologic agent in greater that 90% of these patients [14].

Mycobacterial cell wall

In order to reach some understanding of both the pathogenesis and the often intrinsic drug-resistant nature of MAC, it is necessary to first develop an appreciation for the mycobacterial cell wall. Whereas great strides have been made in elucidating the structural and biological characteristics of the mycobacterial cell wall in general, much remains to be determined, especially in the context of MAC. Our current understanding of the architecture of the mycobacterial cell envelope is based on electron microscopy studies which have demonstrated alternating zones of electron density and transparency, pointing to the existence of a substantial lipid layer surrounding the cell [15]. A chemical model was proposed by Minnikin [16], and later studies by Brennan and Nikaido [17] offered biochemical support for his theory. According to this model, the outer half of this lipid layer contains an assortment of extracellular polysaccharides and glycolipids, many of which are species specific. MAC, for example, produces type-specific glycopeptidolipids (GPLs) [18]. The nonspecific lipids are the phthiocerol dimycocerosates, triacylglycerols and a group of acylated trehaloses. Many different versions of the wall model have since been proposed to conceptualize the intermolecular relationship between these surface glycolipids and the underlying mycolylarabinogalactan-peptidoglycan (mAGP) complex that forms the core framework of the mycobacterial cell wall. A simplistic representation is given here (fig. 1) which reflects our own understanding and preference.

Early identification of GPL and other type-specific glycolipids

The glycopepetidolipids or C-mycosides are among the most unique and widely studied of all mycobacterial glycolipids (for extensive reviews and more historical details, see [19, 20]). The early discovery of this class of lipids can be credited to three lines of studies. Smith et al. were the first [18, 21, 22] to identify the GPLs by infrared spectroscopy analysis of chromatographically fractionated ethanol/diethyl ether extracts and named them as the J substances and, later, C-mycosides. An additional component was termed Jab and shown [21] to contain amino acids identical to other J substances but devoid of most of the glycosyl residues. The Jab could be the first notation of the modern-day 'apolar GPLs' or 'nonspecific' GPLs (ns GPLs). In the later part of 1960s, W. B. Schaefer identified antigens which could be used to serotype isolates of the *M. avium* complex [23]. In separate studies Marks et al. described [24–26] a number of lipids which gave distinct patterns on thin-layer chromatography and could be used to group isolates of the *M. avium* complex.

Structurally, we owe our present-day concept of the lipotetrapeptide core of GPLs and the respective attachment sites for the two monosaccharide residues (fig. 2, and later section for more details) to the early work of several French workers during the 1960s [27, 28]. Among the remarkable accomplishments, the three amino acids were established as of the D series, linked by the N-terminal group to 3-hydroxy-C28 fatty acid, and by the C-terminal group to an amino alcohol, alaninol. It was also established that a diacetylated 6-deoxy-L-talose was glycosidically linked to the hydroxyl group of the D-*allo*-threonine, and 2,3,4-tri-*O*-methyl-L-rhamnose to that of alaninol. In the rhamnosyl residue, a 3-*O*-methyl or a 3,4-di-*O*-methyl can replace the 2,3,4-tri-*O*-methyl group. However, it was not until 1979 that Brennan and Goren [29] conclusively demonstrated that the Schaefer antigens and Marks-Jenkins lipids were structurally alike and formally renamed these first as peptidoglycolipids and later, more accurately, as GPLs. It was shown that the molecular basis of serological differences between MAC strains observed by Schaefer [23] was due to the unique variable oligosaccharide sequences [30] elaborated on the 6-dTal. Only these polar variable GPLs were able to confer serospecificity.

The origins of the trehalose-containing lipooligosaccharide antigens (LOSs) of other mycobacterial species also lay in the seminal observations of Schaefer that the products responsible for the specific antigenicity of other atypical mycobacteria were susceptible to alkali treatment, and

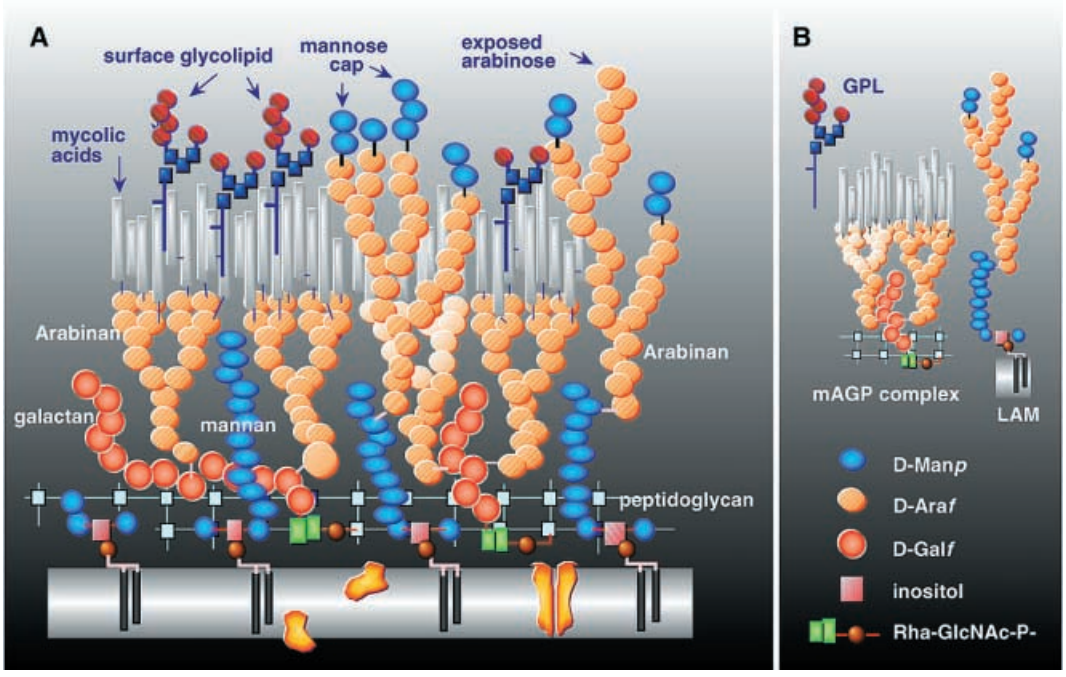


Figure 1. Schematic representation of the mycobacterial cell wall (*A*). The cell wall of *M. tuberculosis* is largely devoid of surface glycolipids, whereas other nontuberculosis mycobacteria express one of the three classes of sero-specific glycolipids, namely the glycopeptidolipid (GPL), lipooligosaccharides (LOS), and phenolic glycolipids (PGL). The three major glyco-constituents of the *M. avium* cell wall are further illustrated in (*B*). mAGP, mycolyl-arabinogalactan-peptidoglycan complex; LAM, lipoarabinomannan. Lipomannan (LM) is related to LAM but lacks the arabinan, whereas the phosphatidylinositol anchor of LAM/LM also exists as phosphatidylinositol mannosides (PIM), e. g. PIM_2 which carries two Man residues on the inositol ring.

thus must differ from the GPLs [31]. Hunter et al. [32] were then able to show that the alkali-labile, highly immunoreactive glycolipids of *Mycobacterium kansasii* are trehalose-containing linear oligosaccharides in which the acyl functions a invariably asymmetrically located on the trehalose unit at one end of the molecule, and specific antigenicity in a combination of unique sugars at the nonreducing end. The first lipoologosaccharides to be described [33, 34] were from *Mycobacterium smegmatis* when the presence of acyltrehaloses bearing O-pyruvated glycosyl substituents were recognized. The phenolic glycolipids (PGLs) were likewise first observed by Smith et al., then referred to as the G substances [35]. Specifically, Ga and Gb were later identified to be mycoside A [22] from *Mycobacterium kansasii* and mycoside B from *Mycobacterium bovis* [36], respectively. The discovery of the PGL I of *Mycobacterium leprae* and the ensuing body of extensive work [37, 38] which demonstrated its role in the immunoreactivity and pathogenesis of leprosy [39] raised the level of general interest in these mycosides.

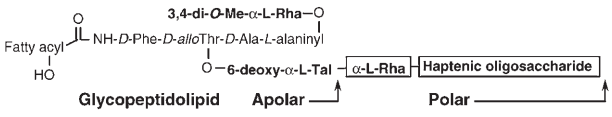


Figure 2. A composite GPL structure from MAC. The apolar GPL lacks further haptenic oligosaccharide extension from the 6-deoxy-Tal (dTal), which could, however, be O-methylated. The first residue attached to the dTal is always α-L-Rha in polar GPLs from all 14 serovars that have been structurally characterized.

In a broader sense, the discovery of the polar, multiglycosylated GPLs was the first step in the recognition of a general phenomenon, namely that most mycobacteria are endowed with large quantities of glycolipids which contain small oligosaccharides of sufficient antigenicity to evoke antibodies of such exquisite specificity as to allow unequivocal identification of species or subspecies. With the current morbidity inflicted by DMAC associated with AIDS, research interest in GPLs has undergone a renaissance. The holy grail now resides not in structural determination for serotyping purpose, but to correlate structural variation with virulence, colony morphotypes, drug resistance and pathogenesis. This review attempts to reflect this progress by charting the course from structural studies and chemical synthesis which form the basis of our recognition of GPLs to more recent dabbling into their genetics and functional correlation.

General approaches in structural determination

Dedicated structural investigation over the last 2 decades has laid the strong foundation on which we stand today in

our quest for the biology of GPLs. Although approaches to the elucidation of glycolipid structures are no different in principle from those used for other groups of complex carbohydrates, several unique features characteristic of GPLs warrant specific attention, and these aspects are discussed below. In general, complete structural elucidation requires acquisition of knowledge on the following features: glycosyl composition, including the anomeric and enantiomeric configurations of glycosyl residues; the sites of linkage between the glycosyl residues; the sequences in which the residues are linked; and the identification and location of short- and long-chain *O*- and *N*-acyl and other noncarbohydrate substituents. Whereas information on these various features has been acquired mainly from analytical chemical manipulations, increasing emphasis is now placed on nuclear magnetic resonance and mass spectroscopic (NMR and MS) data obtained from the examination of the intact molecules and their derivatives.

Glycosyl composition

Compositional analysis of glycolipids isolated from mycobacteria remains the most necessary first step, in light of the widespread occurrence of endogenously methylated glycosyl residues. Acid hydrolysis of the glycolipids, followed by identification of the derived alditol acetates by gas-chromatographic mass spectrometry (GC-MS) using capillary columns, is most appropriate since this method allows for determination of the location of the methyl ether substituents when these are present. To further confirm the identity of the parent sugar, de-*O*-methylation of the unknown sugar may be performed, as with boron tribromide [40]. The absolute enantiomeric configurations of glycosyl residues are determined through the formation, for GC analysis, of equilibrium mixtures of volatile derivatives of glycosides prepared from optically pure 2-butyl or 2-octyl alcohols [41, 42]. Bearing in mind that different derivatives of both enantiomers, such as D- and L-rhamnose in the GPLs from *M. avium* serovars 14 and 20 may be present in the same oligosaccharide chain [43]. In such cases, less direct approaches are required. For example, formation of chiral glycosides of L-rhamnose from the original hapten, but of both D- and L-rhamnose after de-*O*-methylation, showed that the 2-*O*-methylrhamnose was the D enantiomer.

It is also noteworthy that no glycosyl constituents can be liberated without decomposition during conventional hydrolysis with 2 M trifluoroacetic acid [44, 45]. In addition, since uronic acids are rather resistant to hydrolysis, they will be incompletely released, as will the glycosyl units to which they are attached. Thus, for uronic acids, various methods of carboxyl reduction are used in order to generate 6,6-dideuteriohexosyl residues before formation of alditol acetates [44]. The liberation of sugars with branched chains and/or acylamino substituents at C-4, which are decomposed during conventional hydrolysis, may be achieved by modified procedures, including treatment with anhydrous hydrogen fluoride, as was done for the amino sugars in GPLs from *M. avium* serovars 25 [44, 46] and 14 [43], or after base-catalyzed degradation of the terminal uronic acid residue [47] in the GPL from *M. avium* serovar 19 [45]. In the last example, the penultimate 6-deoxy-3-*C*-methyl-2,4-di-*O*-methylmannose residue with a tertiary hydroxyl substituent would be susceptible to degradation during the acid hydrolysis required to cleave the glycosiduronic acid linkage of the terminal unit, and was not detected. Removal of the uronic acid residue from the peralkylated oligosaccharide by base-catalyzed degradation afforded the attenuated triglycosylalditol, from which the branched-chain sugar was liberated without decomposition on controlled hydrolysis [45].

Methylation-linkage analysis

Linkage analysis based on the identification of partially methylated alditol acetates by GC-MS [48] is routinely performed for glycolipids containing endogenous methyl ether using alkylation with trideuteriomethyl iodide. Under the strongly basic conditions used in the Hakomori [49] and Ciucanu and Kerek [50] procedures, base-catalyzed degradation of uronic acid residues by β-elimination [44], which is a potential difficulty, has not been reported. However, base-catalyzed degradation has been carried out on previously permethylated oligosaccharides [44]. Methylation under nonbasic conditions using methyl trifluromethane sulfonate and 2,6-di-*tert*-butylpyridine [51] has also been used to locate of base-labile *O*-acyl substituents. Methylation under nonbasic conditions was followed successively by O-deacylation, ethylation of the exposed hydroxyl groups and GC-MS analysis of the derived partially alkylated alditol acetates [47] in which the location of ethyl groups denote the sites of previous O-acylation.

Sequencing of glycosyl residues and detection of noncarbohydrate substituents by mass spectrometry

Traditional GC-MS in the electron-impact (EI) and chemical ionization modes is still widely used for mono-, di- and triglycosyl derivatives, and many examples are to be found in cited references. EI-MS is also routinely used for identification of all partially alkylated alditol acetates during sugar and linkage analyses. The techniques are, however, rarely adequate for sequencing of higher oligosaccharides or for glycolipids. For these compounds, information on sugar sequences has been derived mostly from fast-atom bombardment mass spectrometry (FAB-MS) [52] and plasma-desorption mass spectrome-

try (PD-MS) with calfornium-252 [53], especially in the positive-ion mode. Although applicable to analysis of native samples, positive-ion FAB-MS analysis is usually more effectively performed on the permethyl and/or peracetyl derivatives of intact GPLs and the released oligoglycosylalditols carrying several methyl ether substituents. Sequence information is readily derived from the characteristic mass increments in the series of glycosyloxonium ions formed on fragmentation, provided that the increments are different, either naturally or by virtue of substituents introduced during derivatization. Unusual mass increments point to the presence of sugars having substituents such as those in the acetamidodideoxy-*O*-methylhexose in *M. avium* serovar 25 [44] and the dideoxy-*C*-methyl-*O*-methylhexose in *M. avium* serovar 19 [45], which had not been detected on standard hydrolysis. PD-MS has also been applied successfully to the detection of readily removable fatty acyl substituents in intact glycolipids and their acylated derivatives.

With current advances in MS, it is anticipated that the newer ionization techniques, namely electrospray ionization (ESI) and matrix assisted desorption ionization (MALDI)-MS, will be equally effective and even more sensitive in affording the similar kind of molecular mass information provided in the past by FAB-MS and PD-MS. It should be noted that ESI- and MALDI-MS are considered much softer ionization techniques then FAB-MS, and hence most sequence informative fragment ions can only be produced after collision-induced dissociation (CID) during implementation of various forms of tandem MS. In contrast, FAB-MS analysis of permethyl derivatives of GPLs has afforded very useful fragment ions resulting from cleavage across the tetrapeptide bonds (fig. 3, [54, 55]). Thus, molecular weight information, confirmation of the tetrapeptide sequence, as well as the respective site of attachment of the saccharide appendage could be routinely obtained in one single FAB-MS analysis. This is unlikely to be derived from ESI and MALDI-MS without CID-MS/MS. Fragmentation pathways using these techniques await investigation, and new schemes may have to be drawn. On the other hand, the ability to perform MS^n using an ion-trap analyzer offers potential advances in the precise determination of various substituents.

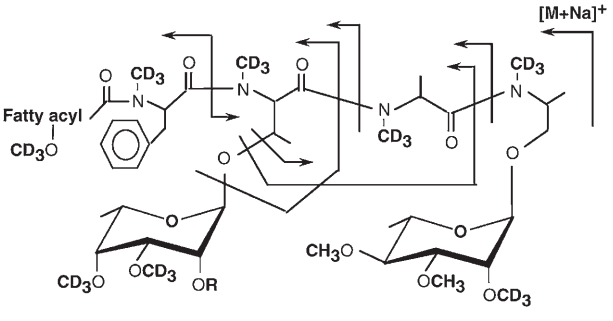


Figure 3. Schematic representation of characteristic cleavages which yield fragment ions in FAB-MS analysis of perdeuteromethylated GPL. Natural *O*-methyl would be retained, if present, giving mass difference of three units. R, haptenic oligosaccharide chain in polar GPL.

NMR and the determination of anomeric and ring configuration of glycosyl residues

Nuclear magnetic resonance spectroscopy (^{1}H and ^{13}C) is the primary tool for determining anomeric configurations. Chemical shifts and $J_{1,2}$ coupling constants in the ^{1}H spectra for the anomeric protons of glycopyranosyl residues of the more common configurations are readily recognized to be of three main types: α-*gluco* and α-*galacto,* β-*gluco* and β-*galacto,* and *manno.* In the last instance, chemical shifts serve to distinguish between α and β anomers. A useful parameter here, and in other cases of ambiguity, is the one-bond heteronuclear C–H coupling constant from C-1 of the individual glycosyl residue [56]. A well-resolved one-dimensional (1D) NMR spectrum may additionally reveal unsuspected structural features, such as noncarbohydrate substituents, whereas two-dimensional (2D) NMR, both homonuclear and heteronuclear correlation spectroscopy (COSY), have been successfully employed to define the complete ring configuration of unusual glycosyl residues. For example, the ^{1}H-NMR spectrum of the oligoglycosylalditol from *M. avium* serovar 25 showed characteristic anomeric protons, including the β-*gluco* configuration of the penultimate glucosyluronic residue [44]. The coupling constants measured in the 1D spectrum for all ring protons of the terminal acetamido sugar indicated that this residue is in the *galacto* configuration. Location of the nitrogen-carrying carbon was established by homo- and heteronuclear (^{13}C–^{1}H) COSY where the CH–NH signal at δ 56.0 was found to correlate with the proton resonance at δ 4.25 in the heteronuclear (^{13}C–^{1}H) spectrum. Other ingenious use of NMR analysis includes two-dimensional ^{1}H-NMR rotating-frame nuclear Overhauser enhancement spectroscopy (ROESY) to detect n.O.e. contacts within the serine-containing GPLs from *Mycobacterium xenopi* [57]. From these experiments it was possible to establish the sequence of the tetrapeptide core, to localize the sites of glycosylation, and to define the locations of different *O*-acyl substituents.

In short, although MS and chemical analysis are sufficient and more suited for high-sensitivity detection of a particular GPL, de novo and complete characterization of novel GPLs could not be completed without resorting to NMR analysis in one form or the other. The most severe limitation to employing NMR as the sole analytical tool often resides in the fact that significantly larger amounts of sample materials are required and signal assignment can sometimes be difficult without complementary input from MS sequencing.

Structural variations in GPLs

The archetypal GPL structure is one that is formulated based on those characterized for MAC serovars, which, as described above, are synonymous with the historical term 'C-mycosides'. Structurally, the GPLs of MAC can be divided into two general classes: the apolar GPLs, expansively synthesized by all serovariants of MAC, and the polar GPLs, which differ between serovars. All GPLs have in common an N-acylated lipopeptide core that bears a rhamnosylated alaninyl C terminus (fig. 2). The chemical difference between the apolar and the polar GPLs lies in the structure of the oligosaccharide attached to the *allo*-threonine residue. In apolar GPLs, this carbohydrate is a 6-deoxytalose (hereafter referred to as dTal) residue, whereas in polar GPLs, this dTal residue is further glycosylated with a haptenic oligosaccharide. Thus, the distinctive features of the MAC polar GPLs are oligosaccharide chains in which the inner disaccharide unit, α-L-Rha*p*-(1$\rightarrow$2)-α-L-dTal*p*, is common to GPL antigens of all serovars examined to date. For serovar 1, this inner core disaccharide unit is not further extended but known to be additionally *O*-acylated, and the *O*-acyl function constitutes part of the serovar-specific epitope [unpublished results]. Several other polar GPLs also carry additional *O*-acyl functions at undefined location, but apart from serovar 1, serovar 9 is the only other instance in which the *O*-acetyl substituent has been identified as an antigenic determinant.

C-mycoside GPLs from MAC

Apolar GPLs

Unlike the polar GPLs which could be defined by seroreactivities and hence immunoassayed and identified, apolar GPLs are generally considered nonantigenic and have received less attention. Pioneering studies by the French [27, 28], followed by the seminal work by Brennan and Goren [29] led to the accepted model for the apolar GPLs in which heterogeneity in *O*-methylation is conspicuous. Thus, the L-Rha*p* attached to alaninol could be either 3,4-di-*O*-methylated or only 3-*O*-methylated; the L-dTal*p* attached to the *allo*Thr could be either 3-*O*-methylated or not *O*-methylated at all. All permutations for the two monosaccharides have been identified, and the heterogeneity pattern could be readily resolved on high-performance thin-layer chromatography (HPTLC). It should be noted, however that, like the polar GPLs, a further level of heterogeneity is commonly imposed by *O*-acylation which is often abrogated during analysis. In addition, application of modern analytical instruments may reveal other novel non-glycosyl substituents not previously appreciated. There is also a lack of detailed comparative analysis among different MAC serovars to ascertain whether a systematic and differential expression pattern exists, as shown recently by a detailed structural investigation into drug resistant isolates [58].

Structurally defined polar GPLs

To date, 14 serovar-specific polar GPLs from MAC, including that of serovar 1, have been structurally characterized [19, 43–46, 59–63], some of which were further confirmed by chemical synthesis. Partially *O*-methylated sugars, especially of 6-deoxyhexoses, are characteristic of all GPLs, but more esoteric glycosyl and non-glycosyl substituents also abound, including uronic acids [44, 45], acetalically linked pyruvic acid moieties, [19] acylaminodideoxyhexoses [60] and branched-chain sugars [45]. No single basis for classification of all the known examples is obvious, but structural similarities between otherwise unrelated GPLs are readily recognized and suggestive of parallel biosynthetic pathways. Assuming that biosynthesis of the oligosaccharide chain is by stepwise transfer from glycosyl esters of nucleoside pyrophosphates to the common α-L-Rha*p*-(1$\rightarrow$2)-α-L-dTal*p* inner unit, the chemical identity of the third glycosyl residue added therefore constitutes a divergent biosynthetic point and offers a provisional basis for structural classification. Under this scenario, three groups can be distinguished (see table 1).

Group 1 GPLs carry haptenic oligosaccharides which emanate via a 2-*O*-Me (or 2,3-di-*O*-Me)-α-L-Fuc*p* residue. Serovar 4 is the most frequently encountered *M. avium* serovar in AIDS patients with disseminated mycobacterioses. Two additional subgroups can be discerned. Serovars 14 and 20 both contain an unusual 2-*O*-Me-D-Rha*p* which is further extended by an *N*-formylkansosamine in the former. Serovars 3, 9, 25 and 26 are related by sharing an α-L-Fuc*p*-β-D-Glc*p*A-α-L-Fuc*p* configurational sequence for the outer trisaccharide units. However, the *galacto* configuration of the 4-acetamido-4,6-dideoxy-2-*O*-methylhexose (FucNAc) from serovar 25 was subsequently shown by chemical synthesis to be α-D and not α-L as expected [46]. This cautions against presumption of structure based on homology in cases where hard data may be difficult to obtain. Group 2 comprises GPLs from serovars 12, 17 and 19 with chains emanating via a further 3-linked α-L-Rha*p* residue. Each carries an unusual substituent, i.e. N-acylated amino sugar in serovars 12 and 17 and branched chain Rha in serovar 19, the absolute configurations of which remain to be established. Group 3 comprises GPLs from serovars 8 and 21, both of which carry a pyruvate acetal on a β-D-Glc*p* and differ only in the presence or absence of a 3-*O*-Me substituent.

GPLs from other MAC serovars

The dominant serovar-specific GPLs from each of serovars 5, 7, 10, 11, 13, 15, 16, 18, 22–24 and 28 have

Table 1. Structurally defined polar GPLs from *M. avium* complex serovars

Serovar	Haptenic oligosaccharide extending from α-L-Rha*p*-(1 → 2)-L-dTal[1]	Ref.
1	Non-extended core; α-L-Rha*p*-(1 → 2)-L-dTal	
	Group 1 : R → α-L-Fuc*p*-(1 → 3)-core	
2	4-*O*-Ac-2,3-di-*O*-Me-α-L-Fuc*p*-(1 → 3)-core	[57]
4	4-*O*-Me-α-L-Rha*p*-(1 → 4)-2-*O*-Me-α-L-Fuc*p*-(1 → 3)-core	[59]
14	4-formamido-4,6-dideoxy-2-*O*-Me-3-*C*-Me-α-L-Man*p*-(1 → 3)-2-*O*-Me-α-D-Rha*p*-(1 → 3)-core 2-*O*-Me-α-L-Fuc*p*-(1 → 3)-core	[39]
20	2-*O*-Me-α-D-Rha*p*-(1 → 3)-2-*O*-Me-α-L-Fuc*p*-(1 → 3)-core	[54]
3	2,3-di-*O*-Me-α-L-Fuc*p*-(1 → 4)-β-D-Glc*p*A-(1 → 4)-2,3-di-*O*-Me-α-L-Fuc*p*-(1 → 3)-core	[58]
9	4-*O*-Ac-2,3-di-*O*-Me-α-L-Fuc*p*-(1 → 4)-β-D-Glc*p*A-(1 → 4)-2,3-di-*O*-Me-α-L-Fuc*p*-(1 → 3)-core	[40]
25	2-*O*-Me-α-D-Fuc*p*NAc-(1 → 4)-β-D-Glc*p*A-(1 → 4)-2-*O*-Me-α-L-Fuc*p*-(1 → 3)-core	[40]
26	2,4-di-*O*-Me-α-L-Fuc*p*-(1 → 4)-β-D-Glc*p*A-(1 → 4)-2-*O*-Me-α-L-Fuc*p*-(1 → 3)-core	[42]
	Group 2 : R → -α-L-Rha*p*-(1 → 3)-core	
12	4-(2-OH)propanamido-4,6-dideoxy-3-*O*-Me-β-D-Glc*p*-(1 → 3)-4-*O*-Me-α-L-Rha*p*-(1 → 3)-α-L-Rha*p*-(1 → 3)-core	[55]
17	3-(3-OH-2-*O*-Me)butanamido-3,6-dideoxy-β-D-Glc*p*-(1 → 3)-4-*O*-Me-α-L-Rha*p*-(1 → 3)-α-L-Rha*p*-(1 → 3)-core	[58]
19	3,4-di-*O*-Me-β-D-Glc*p*A-(1→3)-3-*C*-Me-2,4-di-*O*-Me-α-L-Rha*p*-(1 → 3)-α-LL-Rha*p*-(1 → 3)-core	[41]
	Group 3 : R → -α-D-Glc*p*-(1 → 3)-core	
8	4,6-*O*-(1-carboxyethylidene)-3-*O*-Me-β-D-Glc*p*-(1 → 3)-core	[56]
21	4,6-*O*-(1-carboxyethylidene)-β-D-Glc*p*-(1 → 3)-core	[58]

[1] The haptenic oligosaccharides were mostly characterized as released oligoglycosyl alditol; hence, the reducing terminal dTal was converted to dTalitol, which precluded definition of its anomeric and ring configurations.

been purified and the sugar composition studied [62]. In most cases, the oligoglycosylalditol was not liberated and sequenced. However, based on compositional analysis, it appears that all haptenic oligosaccharides are likewise attached to the *N*-acylpeptide via the →3-α-L-Rha*p*-(1→2)-α-L-dTal*p* inner unit, with the exception of serovars 5, 10 and 11, which are distinguished by the →4)-3-*O*-Me-α-L-Rha*p*-(1→2)-dTal component. Moreover, it was established that the GPLs of serovars 10 and 11 were identical, as were 23 and 24, supporting other evidence that the organisms themselves were identical. The oligosaccharide chain of the GPL from serovar 5 was also distinguished by the presence of glucose and galactose; those of serovars 7, 13, 16 and 28 by new, incompletely characterized amino sugars; that from serovar 15 by 4,6-dideoxy-4-dodecanamido-3-*O*-methylglucopyranose; that from serovar 11 by glucose and 2,3-di-*O*-methylgalactose; that from serovar 18 by 6-*O*-methylglucose and that from serovar 22 by arabinose. Together with those GPLs which were rigorously characterized (table 1), the data therefore support the general idea that MACs distinguish among themselves by structural variations on a common theme, in a manner similar to the serospecific O-antigens of LPS from Gram-negative bacteria.

GPLs from other *Mycobacteria*

C-mycoside and C-mycoside-like GPLs

In addition to those of the *M. avium* serocomplex, C-mycoside GPLs are also found among other mycobacterial species (table 2). Two subclasses could be distinguished. The first group maintains the basic architecture of the GPLs from MAC, namely oligosaccharides are attached to *allo*Thr of the tetrapeptide core with a rhamnosylated alaninyl C terminus (fig. 2). A GPL identical to that of MAC serovar 2 had been isolated previously from strains presumed to be of *Mycobacterium paratuberculosis* [61], although it now appears that these were actually strains of *M. avium* serovar 2. *Mycobacterium simiae* is known to consist of a two-member serocomplex, based on heretofore undefined GPLs. Seroagglutination has indicated that serotype I of *M. simiae* did not differ significantly from the *Mycobacterium habana* strains, originally isolated in Havana from patients with lung disease [64]. Recently, GPLs from *M. habana* strain TMC 5135, which has been used successfully to vaccinate mice against tuberculosis [65] and leprosy [66–68], have been isolated and structurally characterized. Similar to those of *M. avium*, the apolar GPLs contain a 3-*O*-Me-dTal attached to the *allo*Thr and either a 3-*O*-Me-Rha or a 3,4-di-*O*-Me-Rha attached to the alaninol. Three distinct polar GPLs were identified [54]. The oligoglycosyl alditol from the least polar GPL was a pentasaccharide, the terminal Fuc residue of which is further 3-*O*-methylated and 4-*O*-substituted with either an additional 2,4-di-*O*-Me-D-GlcA or a 4-*O*-Me-D-GlcA in the more polar ones. In the same studies, the polar GPLs from *M. simiae* serotype I strain 26110 were shown to be similar but distinct from those of *M. habana*. In particular, composition analysis of the released oligoglycosyl alditols revealed the presence of 3-linked 4-*O*-Me-Rha, 4-linked 2-*O*-Me-Rha, 4-linked 2-*O*-Me-Fuc, 3-linked 6-*O*-Me-Glc, 2-linked dTal and a terminal 3,4-di-*O*-Me-GlcA. It was further shown that the

Table 2. Structurally defined polar GPLs from mycobacteria other than *M. avium* complex

Species/Strain	GPL	Oligosaccharides/Monosaccharide			Ref.
		1. C-mycoside GPLs: Fattyacyl-NH-D-Phe-D-(R_1-*O*-) *allo*Thr-D-Ala-L-alaninol-*O*-R_2			
			R_1	R_2	
M. habana TMC5135	pGPL-I		α-L-Fuc*p*-(1 → 3)-6-*O*-Me-α-D-Glc*p*-(1 → 3)-4-*O*-Me-α-L-Rha*p*-(1 → 3)-α-L-Rha*p*-(1 → 2)-3-*O*-Me-α-L-dTal*p*	3,4-di-*O*-Me-α-L-Rha*p*	[49]
	pGPL-II[a]		2,4-di-*O*-Me-β-D-Glc*p*A-(1 → 4)-3-*O*-Me-α-L-Fuc*p*-(1 → 3)-6-*O*-Me-β-D-Glc*p*-(1 → 3)-4-*O*-Me-α-L-Rha*p*-(1 → 3)-α-L-Rha*p*-(1 → 2)-3-*O*-Me-α-L-dTal*p*	3,4-di-*O*-Me-α-L-Rha*p*	
	pGPL-III		4-*O*-Me-β-D-Glc*p*A-(1 → 4)-3-*O*-Me-α-L-Fuc*p*-(1 → 3)-6-*O*-Me-β-D-Glc*p*-(1 → 3)-4-*O*-Me-α-L-Rha*p*-(1 → 3)-α-L-Rha*p*-(1 → 2)-3-*O*-Me-α-L-dTal*p*	3,4-di-*O*-Me-α-L-Rha*p*	
		2. C-mycoside-like GPLs: Fattyacyl-NH-D-Phe-D-(R_1-*O*-) *allo*Thr-D-Ala-L-alaninol-*O*-R_2			
			R_1	R_2	
M. fortuitum[b] biovar *peregrinum*	GPL I		3-*O*-Me-α-L-Rha*p*	3,4-di-*O*-Me-α-L-Rha*p*-(1 → 2)-3,4-di-*O*-Me-α-L-Rha*p*	[68–71]
	GPL II		3-*O*-Me-α-L-Rha*p*	3-*O*-Me-α-L-Rha*p*-(1 → 2)-3,4-di-*O*-Me-α-L-Rha*p*	
	GPL III		3-*O*-Me-α-L-Rha*p*	α-L-Rha*p*-(1 → 2)-3,4-di-*O*-Me-α-L-Rha*p*	
	GPL IV		3-*O*-Me-α-L-Rha*p*	2-*O*-sulfate-3,4-di-*O*-Me-α-L-Rha*p*	
		3. Non C-mycoside GPLs: Fattyacyl-NH-(R_1-*O*-)-L-Ser-(±*O*-Me)L-Ser- L-Phe-(R_2-*O*-)-D-*allo*Thr-O-Me			
			R_1	R_2	
M. xenopi ATCC 19250	GPL-I		3-*O*-Me-α-L-dTal*p*	α-L-Rha*p*-(1 → 3)-2-*O*-lauryl-α-L-Rha*p*	[73]
	GPL-II		2-*O*-Ac-3-*O*-Me-α-L-dTal*p*	α-L-Rha*p*-(1 → 3)-2-*O*-lauryl-α-L-Rha*p*	
	GPL-III		3-*O*-Me-α-L-dTal*p*	4-*O*-octanoyl/decanoyl-α-L-Rha*p*-(1 → 3)-2-*O*-lauryl-α-L-Rha*p*	
	GPL-IV		2-*O*-Ac-3-*O*-Me-α-L-dTal*p*	4-*O*-octanoyl/decanoyl-α-L-Rha*p*-(1 → 3)-2-*O*-lauryl-α-L-Rha*p*	
NCTC10042[c]	GPL-X-Iib[d]		3-*O*-Me-α-L-dTal*p*	α-L-Rha*p*-(1 → 3)-2-*O*-lauryl-α-L-Rha*p*	[74]
CIPT 14035004	GPL X-I		3-*O*-Me-α-L-dTal*p*	2,3,4-tri-*O*-Me-α-L-dRha*p*-(1 → 3)-2-*O*-lauryl-α-L-dRha*p*-(1 → 3)-α-L-dRha*p*-(1 → 3)-2,4-di-*O*-acetyl/lauryl-6-deoxy-α-L-Glc*p*	[72]

[a] Polar GPLs from *M. simiae* serotype I was shown to be related to pGPL-II but with a terminal 3,4-di-*O*-Me-GlcA.
[b] *M. senegalense* 397 and M263, *M. porcinum* ATCC 33776, *M. peregrinum* ATCC 14467 and *M. fortuitum* ATCC 6841 share a similar set of GPLs with unusual distribution of the disaccharides on the alaninol end. Acetylated forms of the GPLs were also present.
[c] Another GPL from this strain, GPL X-IIa, was implicated to be similar to GPL X Iib but with additional acyl function on the di-rhamnoside.
[d] The structure of this GPL is essentially the same as GPL-I from strain ATCC 19250.

different positions of *O*-Me substitution on the terminal GlcA is sufficient to allow serodifferentiation of *M. habana* from *M. simiae* serotype I.

In addition to those from *M. habana* described above, apolar GPLs similar to those of MAC have also been recently reinvestigated and characterized in *M. smegmatis* mc²155 [69], and *Mycobacterium butyricum* [55]. Both species are not known to express polar GPLs whereas their apolar GPLs appear to be virtually identical in their heterogeneity pattern. The L-dTal*p* attached to *allo*Thr is not O-methylated, whereas the L-Rha*p* attached to alaninol can be either 3,4-di-*O*-methylated or 2,3,4-tri-*O*-methylated. This is in slight contrast to those of *M. habana* and MAC in which the dTal*p* is mostly 3-*O*-methylated, whereas the Rha*p* is either 3-*O*- or 3,4-di-*O*-methylated. Further, heterogeneity resides in the fatty acyl chain lengths as well as the presence on the native peracetylated GPL of either an OH or *O*-Me group, a characteristic feature not reported for *M. habana* [54] or MAC [58]. Otherwise, fatty acyl chain lengths in all four *Mycobacteria* species average at about 30 carbons with variable degree of unsaturation.

The second group of 'C-mycoside-like' GPLs have been isolated from the *Mycobacterium fortuitum* complex. Initially, those from *Mycobacterium chelonae* subsp. *chelonae* and subsp. *abcessus* were identified with an oligosaccharide unit of the apparent structure (incomplete) (3,4-di-*O*-methyl-rhamnose) → (rhamnose) → (6-deoxytalose), whereas those from *M. fortuitum* biovar *peregrinum* were noted to be also of the conventional C-mycoside class [70]. However, later studies by López Marín et al. have provided more definitive structural data. Five different GPLs were isolated from *M. chelonae* and *Mycobacterium abscessus* [71]. The single L-dTal attached to *allo*Thr was shown to contain 2, 1 or no *O*-Ac groups on positions 2 and 3, whereas the L-Rha*p* on the alaninol is mostly 3,4-di-*O*-methylated or 2,3,4-tri-*O*-methylated. In essence, these would conform to the typical apolar C-mycoside GPLs. However, one GPL was characterized to carry a disaccharide α-L-Rha*p*-(1 → 2)-3,4-di-*O*-Me-α-L-Rha*p* on the alaninol, which places it among the 'C-mycoside variants'. In their earlier studies of GPLs from *M. peregrinum*, López Marin et al. [72] were the first to demonstrate the presence of a di-rham-

noside, with variable degree of *O*-methylation, linked to the alaninol, whereas a single sugar, 3-*O*-Me-α-L-Rha*p*, is attached to the threonine (table 2). Interestingly, another GPL with a single 2-*O*-sulfated-3,4-di-*O*-Me-Rha*p* attached to the alaninol and a single 3-*O*-methyl-α-L-Rha*p* attached to the threonine was also identified [73]. Similar structural variations were subsequently identified for GPLs from *Mycobacterium senegalense* and *Mycobacterium porcinum* [74, 75]. Collectively, these works have advanced a common GPL structural model distinct from that of the C-mycoside originally formulated for MAC, although the same tetrapeptide sequence was maintained. It further demonstrated that GPLs are useful markers for studying the taxonomical relationships among mycobacteria. Just as the GPLs of MAC are closely related among one another and to those of *M. habana*, the GPLs from *M. peregrinum*, *M. senegalense* and *M. porcinum* are so similar as to suggest a very close link among the three species which could be further distinguished from *M. fortuitum* and *M. farcinogenes*, or *M. chelonae* and *M. abcessus*, of the same family. Likewise, *M. butyricum* and *M. smegmatis* may be considered as close relatives in sharing the same profile of apolar GPLs, as well as identical pyruvylated acyltrehalose-based LOS [55]. The serotyping specificity and usefulness of GPLs are thus amply documented here and extend equally to the *M. xenopi* serocomplex.

Non-C-mycoside based GPLs from *M. xenopi*

Riviere and Puzo [57, 76] were the first to report on a novel GPL apparently confined to *M. xenopi* isolates which differs markedly from those of the MAC and therefore is classified as a non-C-mycoside GPL. As in the case of the conventional GPLs, *allo*Thr and Phe were present, but serine was also present to give the novel tetrapeptide sequence -L-Ser-L-Ser-L-Phe-D-*allo*Thr-COOMe, whereby the haptenic oligosaccharide chain is O-glycosidically attached to the carboxy-terminal, methyl-esterified *allo*Thr residue. The major structural features of the oligosaccharide, including the sites of attachment of *O*-acyl substituents, were established largely from a combination of pyrolysis EI-MS to give sequence information, and nondegradative NMR spectroscopy, including ^{1}H-^{1}H COSY experiments performed on the native peracetylated GPL for linkage and configurational assignments. The *N*–C_{12} fatty acyl (dodecanoyl) substituent in the lipopeptide core contrasts with the C_{32}–C_{35} acyl substituents in the *M. avium* GPLs, and *O*-acyl (acetyl and dodecanoyl) substituents were also noted on the oligoglycosyl chain of unusual glycosyl constituents. In addition, a single 3-*O*-Me-α-L-dTal substituent was attached to the N-terminal serine residue. Subsequently, Besra et al. [77] completed an independent study of the GPLs from another strain of *M. xenopi* and reported four novel GPL structures based on a similar lipotetrapeptide core, one of which was also identified by Riviere et al. in yet another *M. xenopi* strain [78] (table 2). Like the earlier characterized GPLs, the N-terminal Ser was glycosylated by a single ± (2-*O*-Ac)-3-*O*-Me-α-L-dTal*p* residue, but the C-terminal *allo*threonine methyl ester was glycosylated by a diglycosyl group, 4-*O*-(octanoyl or decanoyl)-α-L-Rha*p*-(1→3)-2-*O*-dodecanoyl-α-L-Rha*p*. Interestingly, the second Ser in the sequence was found to be O-methylated. It was concluded that *M. xenopi*, like *M. avium* and *M. simiae*, is a serocomplex based on serine-containing GPLs rather than the polar C-mycosides. It should be noted that although most polar GPLs were known to carry additional acyl functions, rarely have the actual acyl functions been as rigorously defined as in those of *M. xenopi*. Interested readers are referred to the references cited for the extra efforts taken to preserve the labile acyl function during isolation and subsequent structural studies.

More structures and no functions?

In all likelihood, there could be many more new GPLs among other mycobacterial strains, some of which may be based on C-mycosides and others not. However, the emphasis has now switched not so much to discovery of novel GPLs but to their relevance in drug resistance and pathogenicity and survival. In passing, readers should be reminded that the non-tuberculosis mycobacteria, including MAC, are not normally pathogenic unless the human host is immunocompromized. On the other hand, *Mycobacterium tuberculosis* is not known to synthesize antigenic GPLs. Could it be that a normally functioning immunodefense system is sufficient to encounter the onslaught of these environmental mycobacteria based on recognition of their GPLs, but ineffective in surveying the 'invisible' *M. tuberculosis*? It is, of course, a moot question why MAC should invest in the synthesis of these GPLs in the first place. Could they play an important role in bacterial survival? Could they further modulate our immune response if and when they invade immunocompromised individuals? Do they convey drug resistance and additional virulence? These are pressing questions awaiting answers, and we now have an armamentarium of reagents, both in the form of antigens and antibodies, to test out various ideas.

Synthesis and antigenicity of the haptenic oligosaccharides of GPLs

The synthesis of oligosaccharide haptens related to the glycolipids of different classes was first undertaken in the early 1990s. The aims then were to prepare structurally defined immunoreactive substances in order to delineate

the requisite antigenic determinants interacting with antibodies specific to the particular bacterial species or serotype [79]. The reactivity of the synthetic product against murine monoclonal or rabbit polyclonal antibodies has proved to be a test of structural authenticity and has also demonstrated that the haptenic portion of neoglycoconjugates are located in the distal sugars [79]. The approach, which was evaluated and developed most extensively in relation to the phenolic glycolipid antigen (PGL I) from *M. leprae*, was to synthesize sequentially oligoglycosyl units from the terminal, and presumably the most exposed, region of the glycolipid and to use these in immunoassays to define the minimum size for the epitope. Reducing oligosaccharides were examined first as competitive inhibitors of the interaction between native PGLs and homologous antibodies [80]. Attention was then turned to haptens as glycosides of the correct anomeric configuration and with linker arms attached for conjugation to protein or any other macromolecular carrier to give neoglycoproteins (NGPs).

NGPs based on serovar-specific outer regions

As described, GPLs from serovars of MAC differ from one another in the distinctive outer regions of the oligosaccharide chains external to the (1→3)-α-L-Rha*p*-(1→2)-α-L-dTal*p* core unit (table 1). Aspinall et al. have emphasized the construction of these outer regions for incorporation into NGPs carrying the minimum number of these sugar residues necessary for specific interaction with homologous antibodies. Using the procedure of Bernstein and Hall [81], formylmethyl glycosides generated from ozonolysis of allyl glycosides such as allyl-4-*O*-Ac-2,3-di-*O*-Me-α-L-Fuc*p* (table 3, method 1b) have been successfully conjugated to lysine residues via reductive amination at pH 7.8 with retention of the base-sensitive *O*-acyl substituent [79, 82].

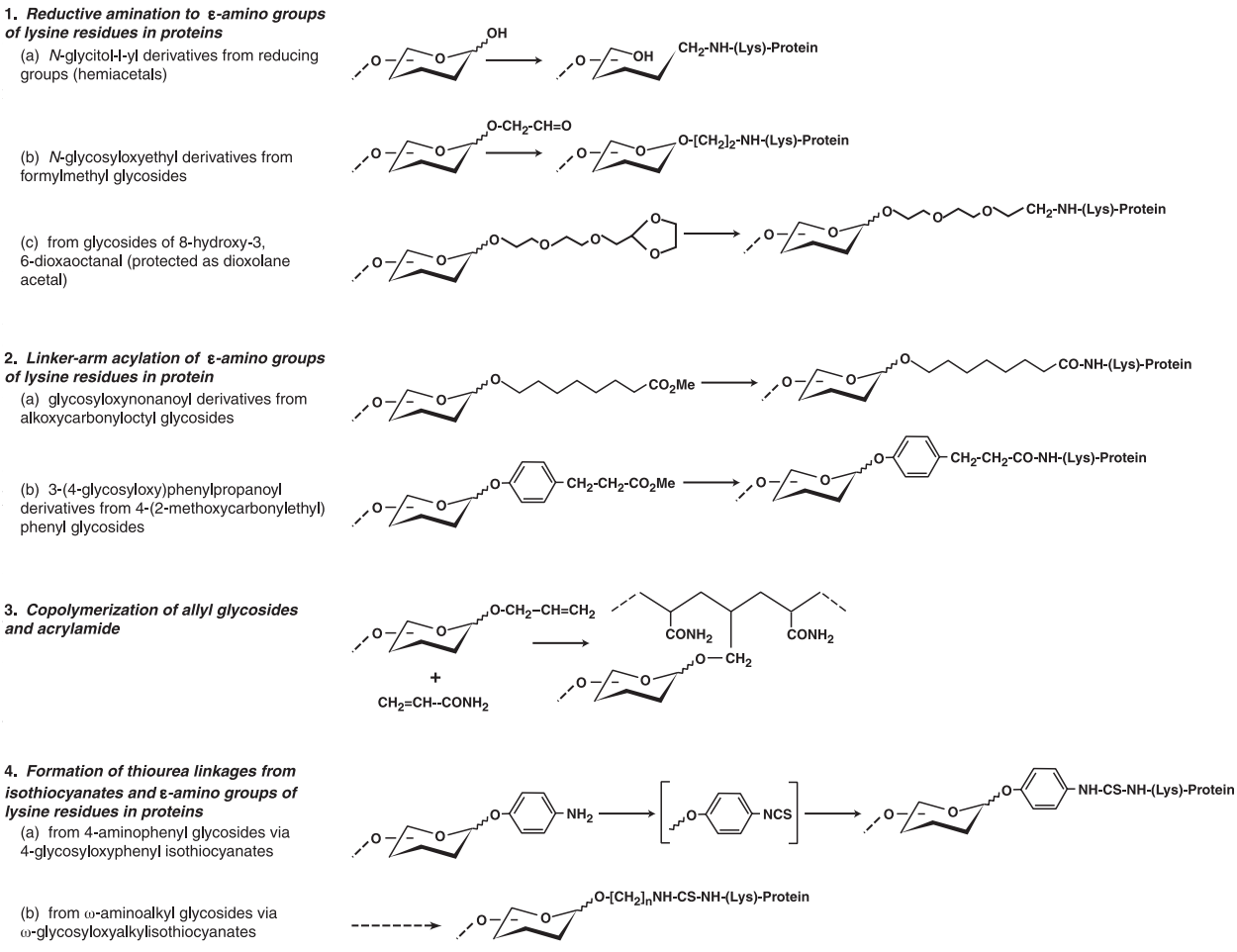
Serovars 4, 8, 20

Monoglycosyl NGPs generated from allyl glycosides were first used to probe the structural determinants of mycobacterial glycolipids by specific interactions [19] between the neoantigens carrying either a 2,3-di-*O*-Me-α-L-Fuc*p* or a 4-*O*-Ac-2,3-di-*O*-Me-α-L-Fuc*p* residue and monoclonal antibodies to *M. avium* serovars 2 and 9, respectively. For most cases, monoglycosyl conjugates failed to interact with homologous antibodies, but all glycobiosyl conjugates examined have proven to be effective. The observation that NGPs bearing the glycobiosyl 4-*O*-Me-α-L-Rha*p*-(1→4)-2-*O*-Me-α-L-Fuc*p* epitope of serovar 4 GPL with *N*-glycosyloxyethyllysine (table 3, method 1b) or *N*-glycosyloxynonanoyllysine (table 3, method 2a) spacers interacted indistinguishably with monoclonal antibody CS-21 raised against whole cells of *M. avium* serovar 4 indicated that epitope recognition was independent of the linker arm [82]. Since the same sugar residue may be found in both terminal and nonterminal positions in GPLs, allyl glycosides have the added advantages of being simple to prepare and suitable for conversion into glycosyl donors as well as acceptors. NGP synthesis from reducing termini of the epitope with allyl, 8-methoxycarbonyloctyl or other glycosides ensures definition of the anomeric configuration of the proximal residue. Improved methods for glycosylation in block synthesis are now available, so that complete oligoglycosyl units may be transferred to the linker arm with high stereoselectivity from donors such as thioglycosides [83]. Synthesis of a monoglycosyl neoantigen related to *M. avium* serovar 8 which involves temporary protection of the allyl glycosidic substituent has been described [84]. Protection was required during the synthesis of a stereochemically defined pyruvate acetal [84], in a reaction involving oxidation [85] by ruthenium tetraoxide of a 3,4-dimethoxyphenylethylidene acetal [86] derivative. For the synthesis of oligosaccharide units containing residues of known 6-deoxyhexoses, standard protecting-group methodology has been employed for the introduction of methyl ether substituents and in the preparation of suitably protected glycosyl acceptors. Syntheses of allyl glycosides of the distal glycobiosyl units of the GPLs from serovars 4 [82] and 20 [59] utilized complementary reactions for regioselective substitutions of the vicinal *cis*-3,4-diol of allyl 2-*O*-Me-α-L-Fuc*p* in preparations of glycosyl acceptors. For the 2-*O*-Me-α-L-Fuc*p* unit of the serovar 20 GPL, regioselective acylation of the axial 4-hydroxyl group was achieved through acid-catalyzed opening of the cyclic orthobenzoate as reviewed in [20]. For the assembly of NGPs related to GPLs containing unusual or hard-to-obtain individual glycosyl or oligoglycosyl units, synthesis can be best achieved through structural modification of more readily available sugars.

Serovar 14

Bajza and Liptak [87] adopted a route of a thioglycoside donor for the terminal *N*-formyl-α-L-kansosamine residue of assumed α-L configuration in the synthesis of the hapten of the serovar 14 GPL. In a different approach, Aspinall et al. [59] took advantage of the elegant stereoselective syntheses by Giuliano and Kasperowicz [88] of derivatives of the related branched-chain sugars sibirosamine, vinelose and kansosamine. For the conversion of the fully substituted trisaccharide derivative into the allyl glycoside of the distal segment of the serotype 14 GPL, removal of protecting groups, delayed reduction of the azido substituent and subsequent N-formylation were the final stages of the synthesis [59]. The interaction of the derived NGPs with antibodies to whole cells of serovar 14 provided effective confirmation of the assumption concerning the absolute configuration of the *N*-formyl-α-L-kansosamine residue in the GPL.

Table 3. Methods used for conjugation of glycosyl units to protein.



Serovars 9, 25, 26

Of the four GPLs with glycobiosyluronic acid units as distal disaccharide segments, that from serovar 9 is the only case for which an NGP bearing only the terminal sugar residue interacts with antibodies against whole cells of the serovar [79]. Differential substitutions of β-D-Glc*p*A residues were first prepared for glycosylation at *O*-4, with a selectively removable protecting group at *O*-6 for subsequent oxidation to the glycosyluronic acid. For the synthesis of the neoantigen related to serovar 26 bearing a glycobiosyluronic acid unit, allyl 2,3-di-*O*-benzoyl-6-*O*-(4-methoxybenzyl)-β-D-Glc*p* was prepared by regioselective hydride opening of the corresponding 4,6-*O*-alkylidene acetal with acidified sodium cyanoborohydride [89], and glycosylation was effected with 3-*O*-Ac-2,4-di-*O*-Me-α-L-Fuc*p* chloride in the presence of silver triflate. Selective deprotection at *O*-6 followed by oxidation of the resulting disaccharide with Jones's reagent (CrO_3 in acetic acid), which took place without effect on the allyl glycoside substituent; removal of the remaining protecting groups gave the desired allyl glycoside of the glycobiosyluronic acid for conversion into the NGP.

In the absence of evidence for the absolute configuration of the terminal 4-acetamido-4-deoxy-2-*O*-methyl-α-Fuc*p* residue, synthesis of the glycobiosyluronic acid unit of the serovar 25 GPL was undertaken first with the amino sugar as the L enantiomer in light of the apparent stereohomology of the oligosaccharide haptens of the three acidic serovars in which other fucose derivatives were of the L configuration. At the outset there was also uncertainty as to the relative configuration of the amino sugar residue in the tetraglycosylalditol, which showed a coupling constant, $J_{1,2} = 3.6$ Hz, indicative of an α-*galacto* or α-*gluco* configuration. The uncertainty was resolved later from NMR data and by synthesis [46]. Oxidative removal of the 4-methoxyphenyl group in compound **(4-2** in fig. 4**)**, followed by oxidation and deprotection, gave the allyl glycoside of the glycobiosyluronic acid for conversion into neoantigen (**4-4)** bear-

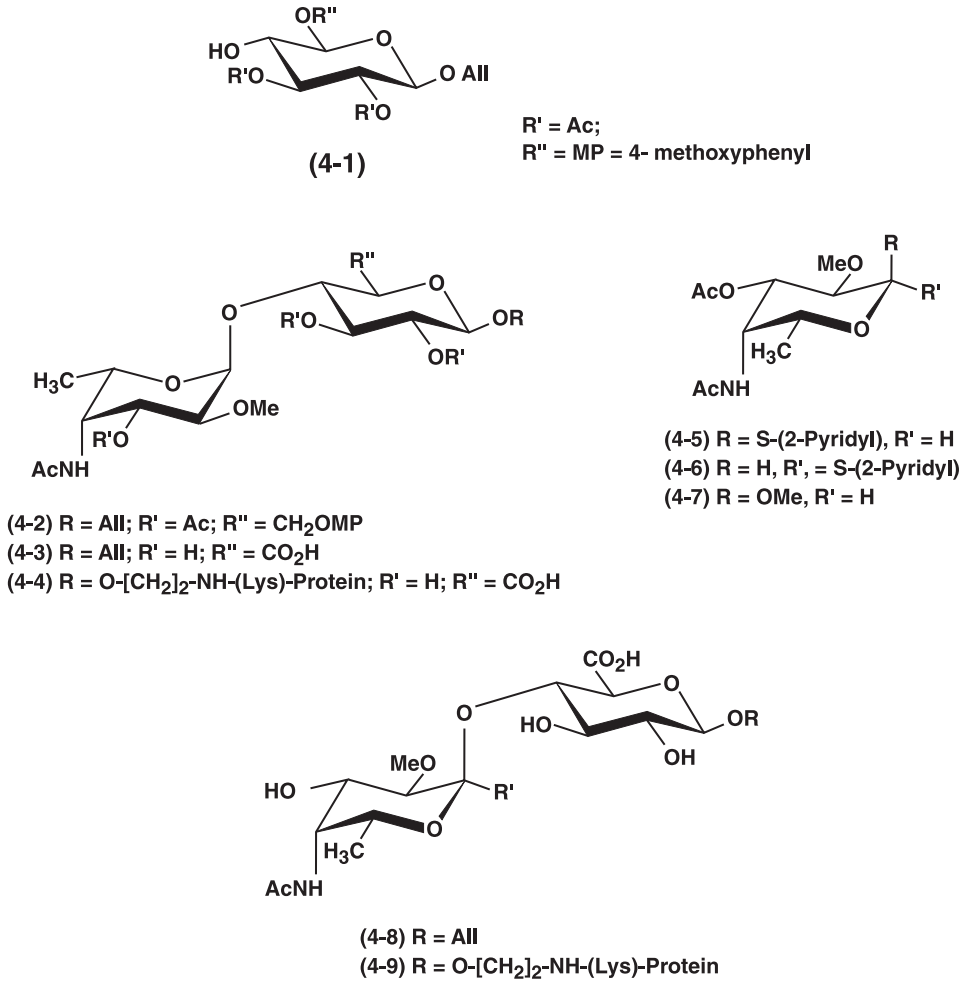


Figure 4. Schematic representation of a few key oligosaccharide synthons, particularly those that were utilized for generation of neo-glycoproteins specific for GPLs of different serovars as indicated in the text. The details of the synthesis and complete reaction schemes are beyond the scope of this review.

ing the 4-acetamido-4-deoxy-2-*O*-Me-α-L-Fuc*p*-(1 $\rightarrow$ 4)-β-D-Glc*p*A epitope. Unexpectedly, this NGP failed to interact with the antibodies to serovar 25. In a parallel synthetic sequence, the allyl glycoside (**4-8**) of the diastereomeric glycobiosyluronic acid was prepared using an anomeric mixture of thioglycosides (**4-5**, **4-6**) formed from the D enantiomer (**4-7**) as glycosyl donor for reaction with the same acceptor (**4-1**). The NGP bearing the 4-acetamido-4-deoxy-2-*O*-Me-α-D-Fuc*p*-(1 $\rightarrow$ 4)-β-D-Glc*p*A epitope (**4-9**) showed a positive interaction with antibodies to serovar 25. These observations provided a striking illustration of immunochemical stereoselectivity for an assignment of absolute configuration that could not be achieved by other means.

Immunoreactivities of NGPs

Table 4 shows the structures of the outer regions of the hapten moieties of the *M. avium* serocomplex, followed by the structures of the glycosyl units in the synthetic NGPs, and the reactivity or lack thereof with the relevant specific antibodies. Antibodies were of two types, polyclonal rabbit antisera and murine monoclonal antibodies (MAbs), raised in most cases against whole cells of the particular serovar, whole lipids or intact (non-deacylated) GPLs. All polyclonal Abs were consistently serovar specific [79, 82] in interacting with the terminal sugar residue in the homologous monoglycosyl and/or glycobiosyl NGCs (table 4). The minimum requirements were for the constitutionally defined terminal sugar inclusive of methyl ether and *O*-acetyl substituents to be of the correct enantiomeric, and presumably also anomeric, configuration. The synthetic NGPs have played a dual role in defining the structural requirements for antigen-antibody interactions. On the one hand, the interactions, or lack thereof, provided supportive or even definitive evidence in confirmation of molecular structure. Additionally, they also define the specificities of antibodies, especially MAbs, where several may be directed to different regions of complex structures. Table 4 summarizes the observed interactions of Abs that relate to the dual role of NGPs.

Table 4. Immunoreactivities of chemically synthesized haptenic oligosaccharides*.

Serovar	Structures of synthesized haptenic oligosaccharides in comparison with those on the GPLs of the respective serovars	Reaction with homologous antibodies
2	4-*O*-Ac-2,3-di-*O*-Me-α-L-Fuc*p*-(1 → 3)-core	+ve
	neo 2-1 2,3-di-*O*-Me-α-L-Fuc*p*-	+ve
	neo 2-2 2,3-di-*O*-Me-α-L-Fuc*p*-(1 → 3)- α-L-Rha*p*-	–ve
	neo 9-1 4-*O*-Ac-2,3-di-*O*-Me-α-L-Fuc*p*-	
4	4-*O*-Me-α-L-Rha*p*-(1 → 4)-2-*O*-Me-α-L-Fuc*p*-(1 → 3)-core	
	neo 4-1 4-*O*-Me-α-L-Rha*p*-	–ve
	neo 4-2 4-*O*-Me-α-L-Rha*p*-(1 → 4)-2-*O*-Me-α-L-Fuc*p*-	+ve
	neo 4-2 4-*O*-Me-α-L-Rha*p*-(1 → 3)-2-*O*-Me-α-L-Fuc*p*-	–ve
9	4-*O*-Ac-2,3-di-*O*-Me-α-L-Fuc*p*-(1 → 4)-β-D-Glc*p*A-(1 → 4)-2,3-di-*O*-Me-α-L-Fuc*p*-(1 → 3)-core	
	neo 9-1 4-*O*-Ac-2,3-di-*O*-Me-α-L-Fuc*p*-	+ve
	neo 2-1 2,3-di-*O*-Me-α-L-Fuc*p*-	–ve
25	2-*O*-Me-α-D-Fuc*p*NAc-(1 → 4)-β-D-Glc*p*A-(1 → 4)-2-*O*-Me-α-L-Fuc*p*-(1 → 3)-core	
	neo 25-1 2-*O*-Me-α-L-Fuc*p*NAc-	–ve
	neo 25-2 2-*O*-Me-α-L-Fuc*p*NAc-(1 → 4)- β-D-Glc*p*A-	–ve
	neo 25-2 2-*O*-Me-α-D-Fuc*p*NAc-(1 → 4)-β-D-Glc*p*A-	+ve
26	2,4-di-*O*-Me-α-L-Fuc*p*-(1 → 4)-β-D-Glc*p*A-(1 → 4)-2-*O*-Me-α-L-Fuc*p*-(1 → 3)-core	
	neo 26-1 2,4-di-*O*-Me-α-L-Fuc*p*-	+ve
	neo 26-1 3-O-Ac-2,4-di-O-methyl-α-L-Fuc*p*-	–ve
	neo 26-2 2,4-di-*O*-Me-α-L-Fuc*p*-(1 → 4)-β-D-Glc*p*A-	+ve
8	4,6-*O*-(1-carboxyethylidene)-3-*O*-Me-β-D-Glc*p*-(1 → 3)-core	
	neo 8-1 4,6-*O*-(1-carboxyethylidene)-3-*O*-Me-β-D-Glc*p*-	+ve
14	4-formamido-4,6-dideoxy-2-*O*-Me-3-*C*-Me-α-L-Man*p*-(1 → 3)-2-*O*-Me-α-D-Rha*p*-(1 → 3)-2-*O*-Me-α-L-Fuc*p*-(1 → 3)-core	
	neo 14-2 4-formamido-4,6-dideoxy-2-*O*-Me-3-*C*-Me-α-L-Man*p*-(1 → 3)-2-*O*-Me-α-D-Rha*p*-	+ve
	neo 14-3 4-formamido-4,6-dideoxy-2-*O*-Me-3-*C*-Me-α-L-Man*p*-(1 → 3)-2-*O*-Me-α-D-Rha*p*-2-*O*-Me-α-L-Fuc*p*-	+ve
20	2-*O*-Me-α-D-Rha*p*-(1 → 3)-2-*O*-Me-α-L-Fuc*p*-(1 → 3)-core	
	neo 20-1 2-*O*-Me-α-D-Rha*p*-	–ve
	neo 20-2 2-*O*-Me-α-D-Rha*p*-(1 → 3)-2-*O*-Me-α-L-Fuc*p*-	+ve

* Only those whose reactivities with existing antibodies have been tested are listed.

The absence of cross-reactions between GPLs of serovars 2 and 9 could be explained when monoglycosyl NGPs with a 2,3-di-*O*-Me-α-L-Fuc*p* residue (neo 2-1) and a 4-*O*-Ac-2,3-di-*O*-Me-α-L-Fuc*p* residue (neo 9-1) were made available. Specific interactions with the homologous polyclonal Abs gave confirmatory evidence [79] for the presence of the latter residue as the terminal unit in the GPL from serovar 9. Conversely, the reactivity of monoglycosyl NGP (neo 26-1) toward homologous anti-serovar 26 rabbit antisera is eliminated on introduction of the 3-*O*-acetyl group. The unpredictable specificity of monoclonal as opposed to polyclonal Abs is illustrated by the interaction of both monoglycosyl neo 2-1 and glycobiosyl neo 2-2 with polyclonal Abs, but of the former only with MAb [82]. A related observation concerning the more precise requirements of Mabs was shown by the recognition of both monoglycosyl NGP 4-1 and glycobiosyl NGP 4-2 by anti-serovar 4 antiserum, but only of the glycobiosyl NGP by anti-GPL 4 MAbs [79, 82]. Two of three MAbs recognizing the GPL of serovar 8 showed an absolute specificity to the 3-*O*-methyl substituent of the terminal 4,6-*O*-(1-carboxyethylidene)-3-*O*-Me-β-D-Glc*p* residue. For one of these MAbs, the monoglycosyl NGP 8-1 provided definitive evidence for the structural identity of the epitope [82]. A third MAb also interacts with the GPL of serovar 21, later examination of which showed the presence of the related nonmethylated, pyruvated terminal sugar.

Synthesis of complete oligosaccharide haptens

Syntheses have been reported of four complete oligoglycosyl haptens from GPLs with attached linker arms bearing functionality for prospective use in conjugation to protein [90]. However, no reports have thus far been published of conjugation to protein of these more complete oligosaccharides or of their immunoreactivity. In addition to the previously outlined strategic considerations, these syntheses have taken advantage of developments in glycosylation methods.

The first synthesis of the complete *M. avium* serovar 4 tetraglycosyl hapten as its methyl glycoside was reported by Gurjar and Viswanadham [91], who used the 2 + 2 block approach in conjugation with high degree of α-se-

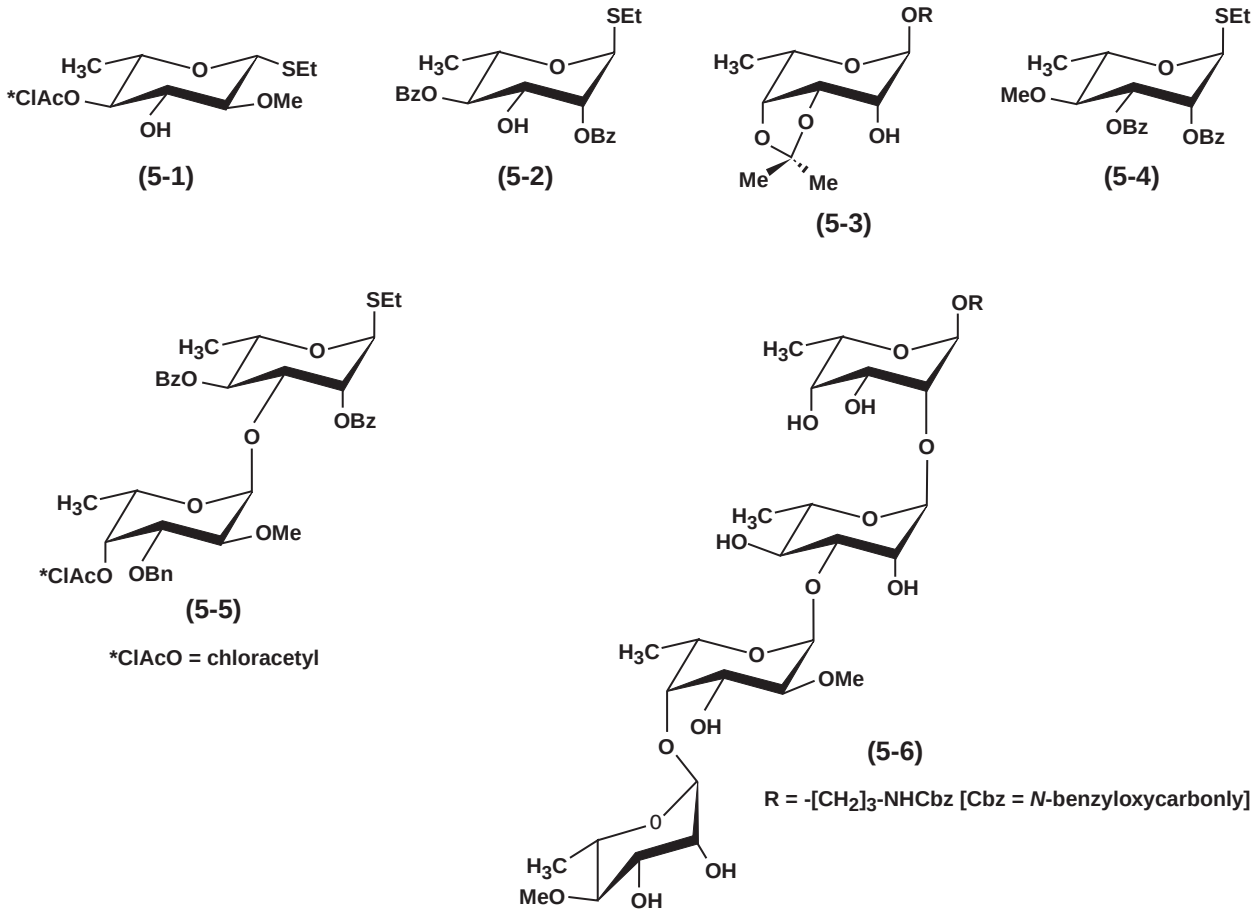


Figure 5. Schematic representation of a few key oligosaccharide synthons, particularly those that were utilized for generation of neo-glycoproteins specific for GPLs of different serovars as indicated in the text. The details of the synthesis and complete reaction schemes are beyond the scope of this review.

lectivity with glycosyl acetates in the presence of boron trifluoride etherate [92]. Using the four key synthons **5-1**, **5-2**, **5-3**, and **5-4**, Zuurmond et al. [93] synthesized the same oligosaccharide with a linker arm (**5-6** in fig. 5). The L-Rha thioglycoside **5-2** provided an excellent example of a synthon having the potential to serve as glycosyl acceptor in reaction with **5-1** as well as glycosyl donor with an iodonium di-*sym*-collidine perchlorate-assisted reaction to give the disaccharide derivative **5-5**. The disaccharide **5-5** with the same functionality, but using *N*-iodosuccinimide and triflic acid as a catalyst, was able to serve as a glycosyl donor in reaction with **5-3** as synthon for the 6-deoxy-L-talose terminus bearing the linker arm. The final assembly of the complete tetrasaccharide **5-6** was achieved with selective removal of the chloroacetyl substituent from the 2-*O*-methyl-L-fucose residue and glycosylation with the 4-*O*-Me-L-Rha synthon **5-4,** followed by removal of protecting groups.

Liptak et al. [94] applied thioglycoside methodology [95] as the principal approach in the synthesis of the complete tetraglycosyl hapten for the *M. avium* serovar 20 GPL.

Synthons **6-1** – **6-4** in figure 6 were prepared for each of the glycosyl residues to serve directly, or after minor modification, as donors or acceptors. In the overall strategy, partial block approaches were used to assemble the inner trisaccharide segment. Removal of the *O*-acetyl group at O-3″ followed sequentially by glycosylation with **6-1**, simultaneous debenzylation and reduction of the nitro group; trifluoroacetylation led to the complete *N*-protected tetraglycosyl hapten with spacer arm **6-5** for potential conjugation to protein. The availability of the D-rhamnose synthon (**6-1**) in a form suitable for selective deprotection at *O*-3, as shown independently in the syntheses of neo 14-2 and neo 14-3 [59], and of a thioglycoside donor aided in the assembly of the kansosamine unit [87].

Ziegler [96] has reported a highly efficient synthesis of the very similar *M. avium* serovar 21 trisaccharide hapten with attached linker arm (**7-7**). The synthetic scheme featured two key operations: (i) the kinetically controlled diastereoselective preparation from D-glucose of the pyruvated glycosyl donor [97] as the trichloroacetimidate **7-1** in figure 7 and (ii) the preparation of L-rhamnose (**7-2**)

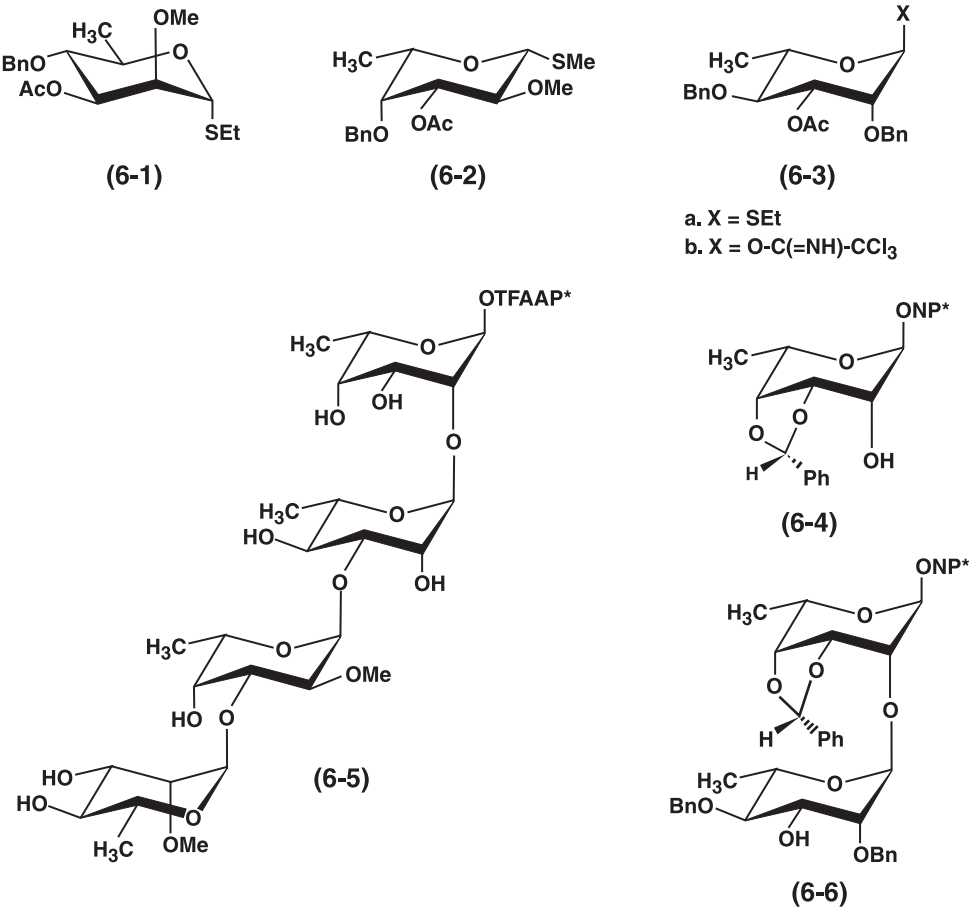


Figure 6. Schematic representation of a few key oligosaccharide synthons, particularly those that were utilized for generation of neo-glycoproteins specific for GPLs of different serovars as indicated in the text. The details of the synthesis and complete reaction schemes are beyond the scope of this review.

and 6-deoxy-L-talose (**7-3**) synthons from a common precursor **7-4**. The outer glycobiosyl unit **7-5** was assembled from **7-1** and **7-2** and then converted into the corresponding glycobiosyl donor **7-6** for condensation with **7-3** to give fully protected trisaccharide hapten (**7-7**) which, after deprotection, gave a water-soluble NGP on reductive cross-linking with protein in the presence of glutardialdehyde. Subsequently, Ziegler [98] reported a similar synthesis of the *M. avium* serovar 8 trisaccharide hapten with an attached linker arm. The synthesis of the unique pentasaccharide of serovar 14 containing the terminal *N*-formylkansosamine unit has also been reported in a recent elegant work [99].

The subsequently discovered different GPL core structures in *M. xenopi* [76, 77] and *M. fortuitum* [72] have attracted attention as feasible synthetic targets [100–102]. Gurjar and Saha [101] have reported the assembly of an *N*-acetyltetrapeptide (fig. 8) bearing the 3-*O*-methyl-6-deoxy-α-L-talopyranosyl substituent at the outer serine residue but lacking the *O*-fatty acylated tetrasaccharide moiety that adorns the threonine residue in the natural GPL.

Biological features of the GPLs

M. tuberculosis, M. leprae and *M. avium* are intracellular parasites able to proliferate inside macrophages, in spite of the antimicrobial activity of the phagocytic cell [103]. Fibrillar, capsule-like structures (the electron-transparent zone, ETZ), often observed in electron micrographs and long considered to be glycolipid in nature, have been implicated in the intracellular survival and persistence of mycobacteria. Draper [104] and later Barrow and Brennan [105] clearly demonstrated that the fibrillar filamentous material mostly comprises GPLs. Application of freeze-fracture electron microscopy to *M. avium* growing within mouse liver macrophages demonstrated progressive accumulation of GPLs around intramacrophagic bacteria, especially in long-term infections [106]. It has been shown further [107], that 10^8–10^{10} organisms can produce ~0.18–18 mg of GPL in vitro. Thus, the ability of GPLs to accumulate in host macrophages has been substantiated by several investigators.

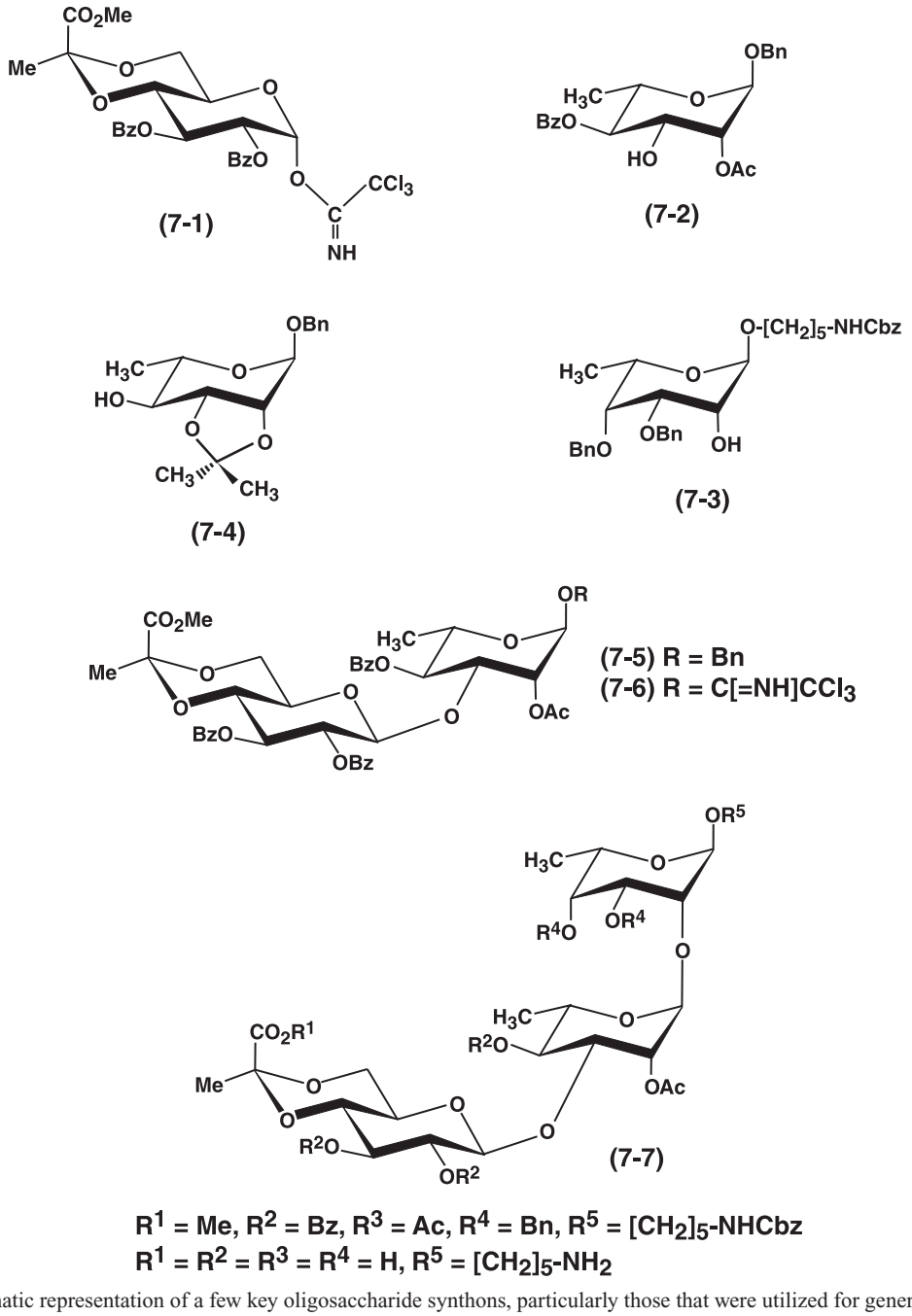


Figure 7. Schematic representation of a few key oligosaccharide synthons, particularly those that were utilized for generation of neo-glycoproteins specific for GPLs of different serovars as indicated in the text. The details of the synthesis and complete reaction schemes are beyond the scope of this review.

Immunomodulatory activities

Control of intracellular pathogens such as *M. avium* by the host depends upon a functional-immune response mediated primarily by two important CD4+ lymphocyte populations of the Th1 phenotype [108]. An important host mechanism that allows CD4+ T cells to mediate antimycobacterial activity is the production of cytokines [109]. Cytokines characteristic for protective Th1-type responses include primarily interleukin (IL)-2 and interferon (IFN)-γ, whereas those characteristic for Th2-type nonprotective responses include IL-4, IL-5 and IL-10 [110]. It has been reported that immunity to *M. avium* is dependent upon the induction of protective CD4+ T cells, the primary products of which are IFN-γ and tumor necrosis factor-α (TNF-α) [108, 111, 112]. More important, the early phase of the host response is CD4+ T cell independent and only shows a CD4+ T-cell-dependent phase of immunity when high bacterial loads are

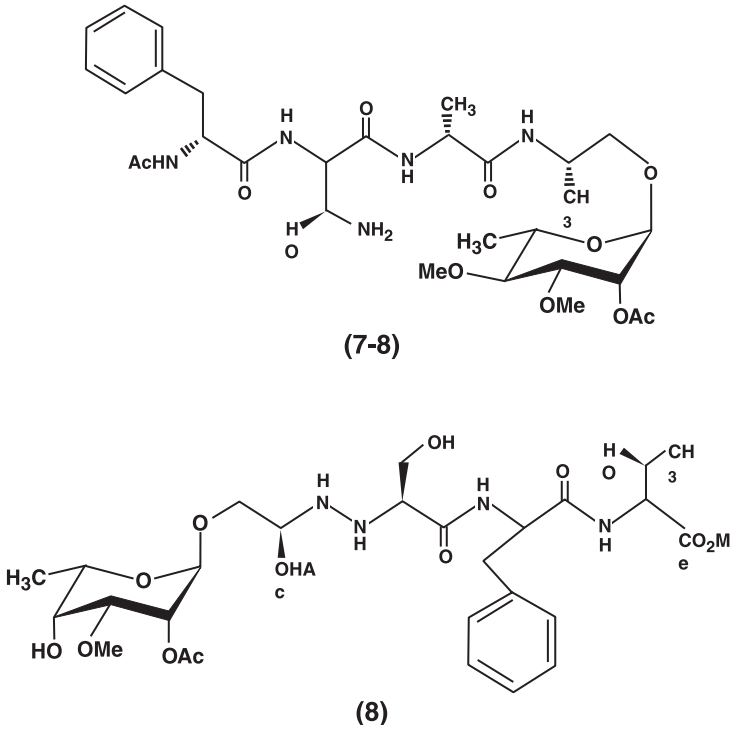


Figure 8. Schematic representation of a few key oligosaccharide synthons, particularly those that were utilized for generation of neo-glycoproteins specific for GPLs of different serovars as indicated in the text. The details of the synthesis and complete reaction schemes are beyond the scope of this review.

achieved [113]. Thus, by using CD4+ T-cell-deficient mice, or CD4+ T-cell-depleted mice [113, 114], it was demonstrated that the dependency for CD4+ T cells does not become apparent until 2–3 months following infection, when *M. avium* loads approach 10^8–10^{10}. In other words, in mice depleted of CD4+ T cells, *M. avium* infection apparently does not compromise a host until late in the disease when high levels of the organism are manifested. This is somewhat similar to what occurs in HIV-infected individuals as the disease progresses through the various stages of CD4+ T cell loss [115]. Although the specific reasons for this dysfunction have not yet been defined, one attractive possibility is that accumulating *M. avium* lipids play an important role in the exacerbation of disease by their ability to induce immunomodulatory components. This could result in an imbalance of the cytokine network and therefore contribute to the overall outcome of the disease process.

Experiments with human peripheral blood monocytes (PBMCs) have established that total extractable lipid and GPLs from *M. avium* can induce TNF-α, IL-6 and IL-1β, cytokines secreted primarily by monocytes. Recent investigations revealed that *M. avium* total lipids have the ability to significantly suppress [116, 117] the secretion of IL-2 and IFN-γ cytokines that are responsible for Th1-type responses. Thus, it has been suggested that as MAC lipids accumulate in chronic infections [118], they interact with the host macrophages initially, and then begin to disperse and affect adjacent cells as the infection progresses. In an immunocompromised individual such as one infected with HIV, this disruption of the cytokine network could be important. Vergne et al. [119] demonstrated the ability of mycobacterial GPLs to become inserted into phospholipid monolayers. From these representative studies, it is possible to formulate a plausible explanation for the ability of *M. avium* lipids to alter host responsiveness by interaction with cell membranes.

Specific serotypes such as 1, 4 and 8 can be isolated frequently in humans infected with HIV. The prognosis after infection differs depending on the serotype. Serotype 4 shows unfavorable prognosis, whereas serotype 16 yields rapid recovery. No information has been available to date dealing on the virulence factor of MAC that is directly related with intracellular bactericidal activity. In an effort to test the effect of various GPLs purified from MAC on the phagocytic processes of human PBMCs, GPL-coated heat-killed staphylococcal cells were phagocytosed by PBMCs and the phagosome-lysosome fusion (P-L fusion) was estimated. Strong promotion of phagocytosis and marked inhibition of P-L fusion [120] by serotype 4 GPLs were observed, whereas neither promotion of phagocytosis nor inhibition of P-L fusion in phagocytic cells were shown by serotype 16 GPL. Serotype 8 GPL showed concomitant stimulation of both phagocytosis

and P-L fusion. These effects were interpreted to be due to some unknown interaction between specific carbohydrate chain and organelle membranes. Thus, it appears that GPL components have a variety of biological activities that could influence host responses.

A group of 'polar GPLs' from *M. chelonae* have also been shown to possess biological activity. Although not structurally characterized, these 'polar glycopeptidolipids' were extracted in a manner similar to that used for *M. avium* GPL [121]. In a series of publications, Pilet et al. reported that 'polar glycopeptidolipids' from *M. chelonae* (i) have adjuvant activity with regard to protective effects of an inactivated influenza vaccine [121], (ii) can be used as a hematopoietic growth factor [122–124] and (iii) can increase the resistance of mice against lethal infection with *Candida albicans*, apparently mediated by the ability of GPLs to induce hyperleukocytosis [125].

Postphagocytic events in chronic stages of the disease can only be hypothesized now. Because of their ability to interact with membranes, it is very likely that the GPL and related lipids would eventually interact with immunologically important cells in the vicinity of the macrophage that initially engulfed them. As the lipids begin to accumulate, they might be eliminated from the macrophage by normal processes or released as the result of cell death. Following elimination from host macrophages, the lipids would then most likely interact with other host cells. Further studies will be required to define these parameters in human infection.

Colony morphology, drug resistance and GPL expression profile

It was recognized early on that *M. avium* forms smooth and rough colonies when grown on solid media [126]. Three distinct colony morphologies for MAC have been described: smooth transparent (SmT), smooth opaque (SmO, also referred to as smooth domed) and rough (Rg) [126, 127]. For any given strain of *M. avium*, the colony morphotypes are typically not stable, that is there is a significant rate of conversion of one morphotype to another [128]. Woodley and David showed that the frequency of transition from SmT to SmO is rather high (1 in 5×10^4 cells), whereas the transition of SmO to SmT is low (1 in 1×10^6 cells) [129]. The smooth morphotypes also show transition to the rough morphotype, although the reverse transition does not occur [105].

Although the molecular basis of the SmO and SmT phenotype switch is not known, the SmT variants have generally been considered the more virulent form, in that fresh clinical isolates from AIDS patients with disseminated *M. avium* disease are predominantly of the SmT morphotype [130, 131]. There is also a distinct difference in the types of cytokines produced by monocytes infected with isogenic morphotypes of *M. avium* [132–134]. Thorel et al. [135] showed that SmT and SmO variants differ in the expression of cell surface antigens, but no details were provided as to the nature of these antigens. Variation in colony morphology has also been associated with differences in virulence and drug resistance. The SmT variants are more resistant to antibiotics. Since the GPL composition of both the SmO and SmT variants seems to be identical, it is unlikely that their surface antigens are implicated in the greater propensity of the SmT form to survive in vivo and cause disease. However, it has clearly been demonstrated that the *M. avium* surface components, i.e. GPLs or similar lipids, accumulate in macrophages [136] and survive within the intraphagosomal environment where they are resistant to degradation by lysosomal enzymes [137], lending further support for a role in intracellular survival [106].

In contrast, the Rg mutants of *M. avium* have been shown to be lacking in expression of GPLs [138]. The saprophytic species, *M. smegmatis,* and also *M. avium* are known to be able to translocate on solid surfaces by a flagellum-independent spreading mechanism known as sliding [139], which appears to require the presence of GPLs on the cell surface. Interestingly, rough strains of both species, which lack GPLs, do not exhibit this property of translocation [140]. It was further inferred that motility is likely to play a significant role in the ability of mycobacterium to colonize surfaces in the environment or inside the host [140, 141].

In a recent significant study [58], critical examination of two clinical isolates revealed that their GPL expression profiles differed significantly in that the apolar GPLs were overexpressed in the clinically resistant isolate at the expense of the polar serotype 1 GPL. This polar GPL was the predominant lipid in the susceptible strain (fig. 9).

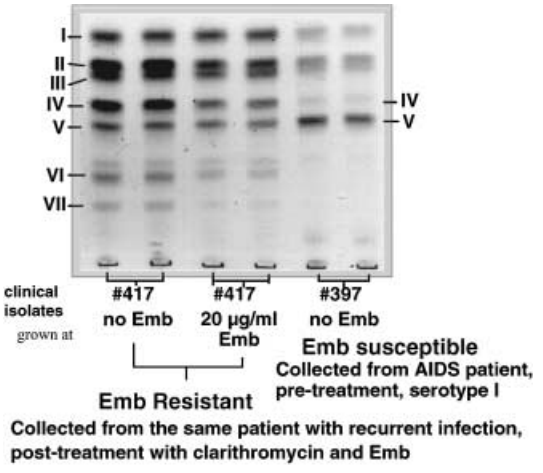


Figure 9. Expression profile of GPLs from drug-resistant (#417) and susceptible (#397) clinical isolates from a patient with dMAC infection. Bands I–VII are numbered based on their mobility on a TLC plate. The solvent system used was $CHCl_3$:CH_3OH:H_2O (30:8:1), and the chromatogram was visualized after spraying and charring with α-naphthol/H_2SO_4.

Table 5 GPLs from *M. avium* clinical isolates #397 and #417.

GPL-Band	Fattyacyl-NH-D-Phe-D-(R_1-*O*-) *allo*Thr-D-Ala-L-alaninol-*O*-R_2	
	R_1	R_2
I	3-*O*-Me-*α*-L-dTal*p*	3,4-di-*O*-Me-*α*-L-Rha*p*
II	*α*-L-dTal*p*	3,4-di-*O*-Me-*α*-L-Rha*p*
III	3-*O*-Me-*α*-L-dTal*p*	3-*O*-Me-*α*-L-Rha*p*
IV	*α*-L-dTal*p*	3-*O*-Me-*α*-L-Rha*p*
V[1]	*α*-L-Rha*p*-(1 → 2)-L-dTal*p*	3,4-di-*O*-Me-*α*-L-Rha*p*
VI[2]	*α*-L-Rha*p*-(1 → 2)-L-dTal*p*	3-*O*-Me-*α*-L-Rha*p*
VII	4-*O*-sulfate-*α*-L-dTal*p*	3-*O*-Me-*α*-L-Rha*p*

[1] GPL found in band V is essentially the characteristic GPL of serovar 1.
[2] Band VI also contains a sulfated GPL related to that found in band VII but carrying an additional *O*-Me group.

Thus, instead of additional rhamnosylation on the dTal appendage to give the serotype-1 specific disaccharide hapten, there was an accumulation of highly *O*-methylated dTal and an unusual 4-*O*-sulfated dTal (table 5). Identification of a sulfate substituent on the dTal made it a novel GPL not previously described. The only other sulfated GPLs found are those from *M. fortuitum* biovar *peregrinum*, characterized by López [73], with sulfated Rha on the alaninol. It should, however, be cautioned that sulfation may be more common than previously appreciated, albeit at a low level. The overexpression of the apolar GPL in this instance has actually facilitated the 'discovery' of an otherwise obscure modification refractory to conventional analysis.

It is not clear whether and how the altered expression of GPLs contributed to the drug resistance of this particular isolate. It also remains to be established whether the phenomenon is relevant to all other MAC serovars whereby their polar GPLs maybe similarly targeted, structurally altered and quantitatively downregulated as a consequence of drug resistance. In all probability, the normally regulated biosynthesis pathways of GPLs may have been perturbed as an indirect consequence of a cellular switch to neutralize the applied drug. It could also be intricately associated with the manifested colony morphotypes and their implicated correlation with drug resistance. In our view, investigation into these moot points at the molecular level should drive the agenda in current and future research of GPL.

Genetics, biosynthesis and mycobacteria genome

In an extensive study, Belisle et al. [142, 143] determined the molecular basis of GPL deficiency among the rough variants. Two types of spontaneous (Rg) mutants were shown to exist in nature. One class (such as Rg-3 and Rg-4) is completely devoid of any semblance of the GPL structure [105]. However, the other (for instance Rg-0 and Rg-1) contained two novel lipopeptides, both devoid of any of the characteristic sugars of the GPLs [142]. The application of GC, FAB-MS, and ^{1}H-NMR demonstrated the structure of one of these as $C_{32:2}$-*β*-hydroxyfatty acyl-D-Phe-D-*allo*Thr-D-Ala-L-alaninol, whereas the other was a minor variation on this structure: $C_{32:1}$-*β*-hydroxyfatty acyl-D-Phe-D-*allo*Thr-D-Ala-L-alaninol [136]. Thus, the rough mutants of *M. avium* differ genetically from the smooth forms due to major deletions of portions of the genes responsible for GPL synthesis [143]. Specifically, the defect in the Rg-0 and Rg-1 mutants, namely those devoid of sugars but retaining the lipopeptide portion, was attributed to the deletion of 21 kb mediated by recombination between repetitive sequences that flank both sides of the 21-kb excised region [144].

Little is known about the biosynthesis of the GPLs. The recognition of two nonglycosylated lipopeptides, obvious core or elemental forms of the GPL antigens, was the culmination of a search begun several years earlier for mutants defective in features of the GPL molecule, particularly glycosyl appendages. Based on the lipopolysaccharide paradigm, it had been suggested that formation of a lipopeptide core was the first phase in the biosynthesis of the GPLs [142]. Two separate means exist in prokaryotes for nonribosomal peptide biosynthesis, the principles of either of which may apply to the short tripeptide-amino alcohol core of the GPLs. The first possibility involves a form of direct synthesis in which the amino acids are added directly to an acceptor through the intervention of adenosine triphosphate (ATP). The muramyl-tetrapeptide unit of the peptidoglycan is synthesized in this way [145]. In the case of the peptide antibiotics, the amino acids are attached through thiol groups to a polyenzyme complex, and the peptide bond is subsequently formed through successive pantetheine-aided transpeptidation-transthiolation steps [146]. If this latter mechanism were to apply to GPL biosynthesis, it is likely that the final step in lipopeptide synthesis would involve transfer of the full peptide unit to the fatty acyl function. David et al. [147, 148] demonstrated that the addition of *m*-fluorophenylalanine to cultures of *M. avium* inhibited GPL biosynthesis and, specifically, the incorporation of radiolabeled amino acids into lipid. They also showed that D-cycloserine inhibited L-Ala racemization to D-Ala, resulting in a 20% inhibition of Ala incorporation into lipid without affecting the incorporation of allothreonine of Phe.

In one recent study, compositional analysis of lipids isolated from five rough colony mutants of *M. smegmatis* has clearly indicated that a defect in GPL synthesis occurred in the assembly of the lipopeptide core. These mutants had transposon insertions in a gene encoding an en-

zyme belonging to the peptide synthetase family. Gene disruption in the wild-type strain yielded a phenotype similar to the transposon mutants. This important study [149] indicated that the conserved lipopeptide core of GPL is synthesized by a peptide synthetase. The involvement of this peptide synthetase in *M. smegmatis* suggests that the lipopeptide backbone of GPLs is assembled by the latter mechanism. Additionally, in this crucial study, the peptide synthetase locus was shown to possess four modules, each encoding an amino acid recognition and adenylation domain. The first three modules also had racemase domains and perhaps could convert the first three amino acids (Phe, Thr, Ala) to the D-isomers consistent with the D-amino acids of the GPL core. The fourth module was devoid of a racemase domain, arguably supporting the fact that the final incorporation was of L-alaninol, which does not require a racemase. Even though these results do favor the direct synthesis route, additional lipopeptide intermediates of this pathway need to be isolated for definitive proof.

From the earlier work of Belisle et al. [150], who actually cloned the genes responsible for the biosynthesis of the oligosaccharide segment of the *M. avium* serovar 2-specific GPL and expressed them in *M. smegmatis* (which naturally contains only the apolar GPLs), it was obvious that the singly glycosylated GPLs are intermediates of the multiglycosylated serovar-specific GPLs. Subsequently, another gene, termed *rtfA*, which encodes for the rhamnosyltransferase essential for the synthesis of serovar-specific oligosaccharides was identified. The *rtfA* gene product demonstrated limited homology to a rhamnosyltransferase (RhlB) involved in rhamnolipid formation in *Pseudomas aeruginosa* [151], and no homology to other bacterial glycosyltransferases [152–154]. Interestingly, homology was observed between RtfA and putative rhamnosyltransferases of *M. leprae* and *M. tuberculosis*, which do not produce GPLs but instead synthesize glycolipids containing rhamnose. The observation that only *rtfA* was required in producing recombinant GPLs in *M. smegmatis* supports the hypothesis that the sugars are added singly and sequentially to the lipopeptide core described above. However, further work is required to determine whether the oligosaccharide hapten of the antigenic GPLs is formed on a lipid carrier and then transferred to the simpler GPL. For instance, is the GPL of serovar 1 (the simplest and shortest among all other serovars) indeed a surrogate intermediate for other serovars like serovar 2 upwards?

More recently, a methyltransferase was identified for one *M. smegmatis* transposon mutant. The mutant was unable to synthesize GPLs and displayed rough colony morphology. The disrupted gene, *mtf1*, shared high homology with several *S*-adenosylmethionine-dependent methyltransferases. It was found that the enzyme encoded by *mtf1* was required to methylate a single rhamnose residue that forms a part of the conserved core GPL structure in *M. smegmatis*. Complementation of the mutant with the wild-type copy of the *mtf1* restored high levels of resynthesis of GPLs containing di- or trimethylated rhamnose. It was further established that disruption of *mtf1* specifically inhibited the addition of *O*-methyl groups to the 3 or 2 position of the rhamnose, ultimately downregulating the overall synthesis of GPLs [69].

The *M. avium* strain 104 is currently being sequenced at the Institute for Genomic Research and funded by the National Institute of Allergy and Infectious Diseases. The size of the genome is predicted to be 4.7 Mb. Much important information will be obtained from the sequence information. We envisage that annotating the *M. avium* genome will provide some information on some of the unique glycosyltransferases and other enzymes implicated, but classical biochemical approaches will still be required to support the biosynthesis of these GPLs. Clearly, isolation and characterization of a variety of deep rough mutants of *M. avium* represent a major development in the goal of elucidating the biosynthetic pathway of the GPLs. These mutants also provide the means to examine the role of GPLs in the disease processes induced by *M. avium*, specifically in eliciting an immunosuppressive response [155].

A chapter can now be closed on the discovery, chemical elucidation and synthesis of the highly unusual glycolipids of *Mycobacterium* spp. The challenges for the future lie in explaining the role of these copious products in the intracellular life and infectivity of mycobacteria, particularly from a biogenesis and genetic basis. Future research will emphasize the creation of conditional mutants defective in these materials which, in turn, will greatly aid elucidation of their roles in bacterial physiology and pathogenesis. The importances of the surface lipids are increasingly becoming apparent with studies involving mutants devoid of some of the lipids. For instance, as shown by Martinez et al. [140], mycobacteria, previously thought to be nonmotile, were shown to be able to spread on the surface of growth medium by a sliding mechanism. The ability to translocate over the surface correlated with the presence of glycopeptidolipids, present in the outermost layer of the cell envelope, and no other extracellular structures such as pili or fimbriae appeared to be involved in this process. This form of motility could play an important role in surface colonization by mycobacteria in the environment as well as in the host.

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1 O'Brien R. and Simone P. M. (1999) Tuberculosis Elimination Revisited: Obstacles, Opportunities and a Renewed Commitment, Centers for Disease Control and Prevention, Atlanta, GA
2 Fauci A. S. (1993) Multifactorial nature of human immunodeficiency virus disease: implications in therapy. Science **262:** 1011–1018
3 Klatt E. C., Jensen D. F. and Meyer P. R. (1987) Pathology of *Mycobacterium avium-intracellulare* infection in acquired immunodeficiency syndrome. Hum. Pathol. **18:** 709–714
4 Horsburgh C. R. Jr (1991) *Mycobacterium avium* complex infection in the acquired immunodeficiency syndrome. N. Engl. J. Med. **324:** 1332–1338
5 von Reyn C. F., Waddell R. D. and Eaton T. (1993) Isolation of *M. avium* complex from water in the United States, Finland, Zaire and Kenya. J. Clin. Microbiol. **31:** 3227–3230
6 von Reyn C. F., Maslow J. N., Barber T. W., Falkinham J. O. and Arbeit R. D. (1994) Persistent colonization of potable water as a source of *Mycobacterium avium* infection in AIDS. Lancet **343:** 1137–1141
7 Wolinsky E. and Rynearson T. K. (1968) Mycobacteria in oil and their relation to disease-associated strains. Am. Rev. Respir. Dis. **97:** 1032–1037
8 Yakrus M. A. and Good R. C. (1990) Geographic distribution, frequency, and specimen source of *Mycobacterium avium* complex serotypes isolated from patients with acquired immunodeficiency syndrome. J. Clin. Microbiol. **28:** 926–929
9 du Moulin G. C., Stottmeier K. D., Pelletier P. A., Tsang A.Y. and Hedley-Whyte J. (1988) Concentration of *Mycobacterium avium* by hospital hot water systems. JAMA **260:** 1599–1601
10 Horsburgh C. R. Jr (1996) Epidemeology of *Mycobacterium avium* complex. In: *Mycobacterium avium*-Complex Infection: Progress in Research Treatment, vol. 87, pp. 1–22, Korvick A. J. and Benson C.A. (eds), Marcel Dekker, New York
11 Rusin P. A., Rose J. B., Haaas C. N. and Gerba C. P. (1997) Risk assessment of opportunistic bacterial pathogens in drinking water. In: Reviews of Environmental Contamination and Toxicology, pp. 57–83, Ware G. W., Nigg H. N. and Benevue A. (eds), Springer, New York
12 Young L. S., Inderlied C. B., Berlin O. G. and Gottlieb M. S. (1986) Mycobacterial Infection in AIDS pateints, with an emphasis on the *Mycobacterium avium* complex. Rev. Infect. Dis. **8:** 1024–1033
13 Chaisson R. E., Gallant J. E., Keruly J. C. and Moore R. D. (1998) Impact of opportunistic disease on survival in patients with HIV infection. AIDS **12:** 29–33
14 Yajko D. M., Chin D. P., Gonzalez P. C., Nassos P. S., Hopewell P. C., Reingold A. L. et al. (1995) *Mycobacterium avium* complex in water, food and soil samples collected from the environment of HIV-infected individuals. J. Acquir. Immune Defic. Syndr. Hum. Tetrovirol. **9:** 176–182
15 Draper P. (1982) The anatomy of mycobacteria. In: The Biology of Mycobacteria, vol. 2, pp. 9–52, Ratledge C. and Stanford J. (eds), Academic Press, London
16 Minnikin D. E. (1982) Lipids: complex lipids, their chemistry, biosynthesis and roles. In: The Biology of Mycobacteria, vol. 2, pp. 95–184, Ratledge C. and Stanford J. (eds), Academic Press, London
17 Brennan P. J. and Nikaido H. (1995) The envelope of mycobacteria. Annu. Rev. Biochem. **64:** 29–63
18 Smith D. W., Randall H. M., MacLennan A. P. and Lederer E. (1960) Mycosides: a new class of type specific glycolipids of mycobacteria. Nature **186:** 887–888
19 Brennan P. J. (1988) Mycobacterium and other actinomycetes. In: Mycobacterial Lipids, pp. 203–298, and Ratledge C. and Wilkinson S. G. (eds), Academic Press, London
20 Aspinall G. O., Chatterjee D. and Brennan P. J. (1995) The variable surface glycolipids of mycobacteria: Structures, synthesis of epitopes and biological properties. In: Adv. Carbohydr. Chem. Biochem, vol. 51, pp. 169–242, Horton D. (ed.), Academic Press, Washington, DC
21 Smith D. W., Randall H. M., MacLennan A. P., Putney R. K. and Rao S. V. (1960) Detection of specific lipids in mycobacteria by infrared spectroscopy. J. Bacteriol. **79:** 217–229
22 Smith D. W., Randall H. M., Gastambide-Odier M. M. and Koevoet A. L. (1957) The characterization of mycobacterial strains by composition of their lipid extracts. Ann. N. Y. Acad. Sci. **69:** 145–157
23 Schaefer W. B. (1965) Serologic identification and classification of the atypical mycobacteria by their agglutination. Am. Rev. Respir. Dis. **92:** 85–93
24 Marks J., Jenkins P. A. and Schaefer W. B. (1970) Identification and incidence of a third type of *Mycobacterium avium*. Am. Rev. Respir. Dis. **102:** 499–506
25 Marks J., Jenkins P. A. and Schaefer W. B. (1971) Thin-layer chromatography of mycobacterial lipids as an aid to classification: technical improvements: *Mycobacterium avium, M. intracellulare* (Battey bacilli). Tubercle **52:** 219–225
26 Jenkins P. A., Marks J. and Schaefer W. B. (1971) Lipid chromatography and seroagglutination in the classification of rapidly growing mycobacteria. Am. Rev. Respir. Dis. **103:** 179–187
27 Laneelle G. and Asselineau J. (1968) Structure of a peptidolipid glycoside isolated from a Mycobacterium. Eur. J. Biochem. **5:** 487–491
28 Vilkas E. and Lederer E. (1968) N-methylation of peptides by the method of Hakomori. Structure of mycoside Cbl. Tetrahed. Lett. 1968 **May 26:** 3089–3092
29 Brennan P. J. and Goren M. B. (1979) Structural studies on the type-specific antigens and lipids of the *Mycobacterium avium-Mycobacterium intracellulare Mycobacterium scrofulaceum* complex serocomplex. J. Biol. Chem **254:** 4205–4211
30 Brennan P. J., Mayer H., Aspinall G. O. and Nam Shin J. E. (1981) Structures of the glycopeptidolipid antigens from serovars in the *Mycobacterium avium/Mycobacterium intracellulare/Mycobacterium scrofulaceum* serocomplex. Eur. J. Biochem. **115:** 7–15
31 Schaefer W. B., Wolinsky E., Jenkins P. A. and Marks J. (1973) *Mycobacterium szulgai* – a new pathogen. Serologic identification and report of five new cases. Am. Rev. Respir. Dis. **108:** 1320–1326
32 Hunter S. W., Murphy R. C., Clay K., Goren M. B. and Brennan P. J. (1983) Trehalose-containing lipooligosaccharides. A new class of species-specific antigens from *Mycobacterium*. J. Biol. Chem. **258:** 10481–10487
33 Saadat S. and Ballou C. E. (1983) Pyruvylated glycolipids from *Mycobacterium smegmatis:* structures of two oligosaccharide components. J. Biol. Chem. **258:** 1813–1818
34 Kamisango K., Saadat S., Dell A. and Ballou C. E. (1985) Pyruvylated glycolipids from *Mycobacterium smegmatis*. Nature and location of the lipid components. J. Biol. Chem. **260:** 4117–4121
35 Smith D. W., Harrell W. K. and Randall H. M. (1954) Correlation of biologic properties of strains of Mycobacterium with infrared spectrum III: differentiation of bovine and human varieties of *M. tuberculosis* by means of infra red spectrum. Am. Rev. Tubercl. **69:** 505–510
36 Randall H. M. and Smith D. W. (1953) Infrared spectroscopy in bacteriological research. J. Opt. Soc. Am. **43:** 1086–1092
37 Hunter S. W. and Brennan P. J. (1981) A novel phenolic glycolipid from *Mycobacterium leprae* possibly involved in immunogenicity and pathogenicity. J. Bacteriol. **147:** 728–735
38 Hunter S. W., Fujiwara T. and Brennan P. J. (1982) Structure and antigenicity of the major specific glycolipid antigen of *Mycobacterium leprae*. J. Biol. Chem. **257:** 15072–15078
39 Chan J., Fujiwara T., Brennan P., McNeil M., Turco S. J., Siblle J. C. et al. (1989) Microbial glycolipids: possible virulence

factors that scavenge oxygen radicals. Proc. Natl. Acad. Sci. USA **86:** 2453–2457
40 Hough L. and Theobald R. S. (1963) Dealkylation. In: Methods in Carbohydrate Chemistry, Whistler W. L. and Wolfrom R. S. (eds), vol. 2, pp. 203–206, Acaemic Press, New York
41 Gerwig G. J., Kamerling J. P. and Vliegenthart J. F. G. (1978) Determination of D and L configuration of neutral monosaccharides by high-resolution capillary GLC. Carbohydr. Res. **62:** 349–357
42 Leontein K., Lindberg B. and Lonngren J. (1978) Assignment of absolute configuration of sugars by g.l.c. of their acetylated glycosides formed from chiral alcohols. Carbohydr.Res. **62:** 359–362
43 McNeil M., Gaylord H. and Brennan P. J. (1988) N-formylkansosaminyl-(1→3)-2-O-methyl-D-hamnopyranose: the type-specific determinant of serovariant 14 of the *Mycobacterium avium* complex. Carbohydr. Res **177:** 185–198
44 Chatterjee D., Aspinall G. O. and Brennan P. J. (1987) The presence of novel glucuronic acid-containing, type-specific glycolipid antigens within *Mycobacterium* spp. Revision of earlier structures. J. Biol. Chem. **262:** 3528–3533
45 Chatterjee D., Bozic C. M., Aspinall G. O. and Brennan P. J. (1988) Glucuronic acid- and branched sugar-containing glycolipid antigens of *Mycobacterium avium*. J. Biol. Chem. **263:** 4092–4097
46 Aspinall G. O., Gammon D. W., Sood R. K., Chatterjee D., Rivoire B. and Brennan P. J. (1992) Structures of the glycopeptidolipid antigens of serovars 25 and 26 of the *Mycobacterium avium* serocomplex, synthesis of allyl glycosides of the outer disaccharide units and serology of the derived neoglycoproteins. Carbohydr. Res. **237:** 57–77
47 Lindberg B., Lonngren J. and Thompson J. L. (1973) Degradation of polysaccharides containing uronic acid residues. Carbohydr. Res. **28:** 351–357
48 Bjorndal H., Hellervist C. G., Lindberg B. and Svensson S. (1970) Gas-liquid chromatography and mass spectrometry in methylation analysis of polysaccharides. Angew. Chem. Int. Ed. **9:** 610–619
49 Hakomori S.-I. (1964) A rapid permethylation of glycolipid, and polysaccharide catalysed by methylsulfinyl carbanion in dimethylsulfoxide. J. Biochem. (Tokyo) **55:** 205–208
50 Ciucanu I. and Kerek F. (1984) A simple and rapid method for the permethylation of carbohydrates. Carbohydr. Res. **131:** 209–217
51 Prehm P. (1980) Methylation of carbohydrates by methyl trifluoromethanesulfonate in trimethyl phosphate. Carbohydr. Res **78:** 372–374
52 Dell A. (1987) F.A.B.-mass spectrometry of carbohydrates. In: Adv. Carbohydr. Chem. Biochem, vol. 45, pp. 19–72, Academic Press, Washington, DC
53 Jardine I., Scanlan G., McNeil M. and Brennan P. J. (1989) Plasma desorption mass spectrometric analysis of mycobacterial glycolipids. Anal. Chem. **61:** 416–422
54 Khoo K. H., Chatterjee D., Dell A., Morris H. R., Brennan P. J. and Draper P. (1996) Novel O-methylated terminal glucuronic acid characterizes the polar glycopeptidolipids of *Mycobacterium habana* strain TMC 5135. J. Biol. Chem. **271:** 12333–12342
55 Khoo K. H., Suzuki R., Morris H. R., Dell A., Brennan P. J. and Besra G. S. (1995) Structural definition of the glycopeptidolipids and the pyruvylated, glycosylated acyltrehalose from *Mycobacterium butyricum*. Carbohydr. Res. **276:** 449–455
56 McNeil M., Chatterjee D., Hunter S. W. and Brennan P. J. (1989) Mycobacterial glycolipids: isolation, structures, antigenicity and synthesis of neoantigens. Methods Enzymol. **179:** 215–242
57 Rivière M. and Puzo G. (1992) Use of ^{1}H NMR ROESY for structural determination of *O*-glycosylated amino acids from a serine-containing glycopeptidolipid antigen. Biochem. **31:** 3575–3580
58 Khoo K. H., Jarboe E., Barker A., Torrelles J., Kuo C. W. and Chatterjee D. (1999) Altered expression profile of the surface glycopeptidolipids in drug-resistant clinical isolates of *Mycobacterium avium* complex. J. Biol. Chem. **274:** 9778–9785
59 Aspinall G. O., Khare N. K., Sood R. K., Chatterjee D., Rivoire B. and Brennan P. J. (1991) Structure of the glycopeptidolipid antigen of serovar 20 of the *Mydobacterium avium* serocomplex, synthesis of allyl glycosides of the outer di- and tri-saccharide untis of the antigens of serovars 14 and 20, and serology of the derived neoglycoproteins. Carbohydr. Res. **216:** 357–373
60 Bozic C. M., McNeil M., Chatterjee D., Jardine I. and Brennan P. J. (1988) Further novel amido sugars within the glycopeptidolipid antigens of *Mycobacterium avium*. J. Biol. Chem. **263:** 14984–14991
61 Camphausen R. T., Jones R. L. and Brennan P. J. (1985) A glycolipid antigen specific to *Mycobacterium paratuberculosis*: structure and antigenicity. Proc. Natl. Acad. Sci. USA **82:** 3068–3072
62 Denner J. C. (1991) Identification and Differentiation of Memebers of the *Mycobacterium avium* and Related Complexes, Masters degree thesis, Colorado State University, Fort Collins
63 McNeil M., Tsang A. Y. and Brennan P. J. (1987) Structure and antigenicity of the specific oligosaccharide hapten from the glycopeptidolipid antigen of *Mycobacterium avium* serotype 4, the dominant *Mycobacterium* isolated from patients with acquired immune deficiency syndrome. J. Biol. Chem. **262:** 2630–2635
64 Valdivia-Alvarez J., Suarez-Mendez R. and Echemendia-Font M. (1971) Bol. Hig. Epid. **9:** 65–73
65 Gupta H. P., Singh N. B., Mathur I. S. and Gupta S. K. (1979) *Mycobacterium habana*, a new immunogenic strain in experimental tuberculosis of mice. Indian. J. Exp. Biol. **17:** 1190–1193
66 Singh N. B., Srivastava, Gupta H. P., Sreevatsa and Desikan K. V. (1985) Immunological potential of a cultivable mycobacterial strain *M. habana* against leprosy bacillus in mouse foot pad. Indian J. Lepr. **57:** 278–281
67 Chaturvedi V., Singh N. B. and Sinha S. (1995) Immunoreactive antigens of a candidate leprosy vaccine: *Mycobacterium habana*. Lepr. Rev. **66:** 31–38
68 Singh N. B., Gupta H. P., Srivastava A., Kandpal H. and Srivastava U. M. (1997) Lymphostimulatory and delayed-type hypersensitivity responses to a candidate leprosy vaccine strain: *Mycobacterium habana*. Lepr. Rev. **68:** 125–130
69 Patterson J. H., McConville M. J., Haites R. E., Coppel R. L. and Billman-Jacobe H. (2000) Identification of a methyltransferase from *Mycobacterium smegmatis* involved in glycopeptidolipid synthesis. J. Biol. Chem. **275:** 24900–24906
70 Tsang A. Y., Barr V. L., McClatchy J. K., Goldberg M., Drupa I. and Brennan P. J. (1984) Antigenic relationships of the *Mycobacterium chelonae* complex. Int. J. Syst. Bacteriol. **34:** 35–44
71 López Marín L. M., Gautier N., Lanéelle M.-A., Silvie G. and Daffé M. (1994) Structures of the glycopeptidolipid antigens of *Mycobacterium abcessus* and *Mycobacterium chelonae* and possible chemical basi of serological cross-reactions in the *Mycobacterium fortuitum* complex. Microbiol. **140:** 1109–1118
72 López Marín L. M., Lanéelle M. A., Promé D., Daffé M., Lanéelle G. and Promé J. C. (1991) Glycopeptidolipids from *Mycobacterium fortuitum*: a variant in the structure of C-mycoside. Biochemistry **30:** 10536–10542
73 López Marín L. M., Lanéelle M. A., Promé D., Lanéelle G., Promé J. V. and Daffé M. (1992) Structure of a novel sulfate-containing mycobacterial glycolipid. Biochem. **31:** 11106–11111

74 López Marín L. M., Lanéelle M.-A., Promé D. and Daffé M. (1993) Structures of the glycopeptidolipid antigens of two animal pathogens: *Mycobacterium senegalense* and *Mycobacterium porcinum*. Eur. J. Biochem. **215:** 859–866

75 Besra G. S., Gurcha S. S., Khoo K. H., Morris H. R., Dell A., Hamid M. E. et al. (1994) Characterization of the specific antigenicity of representatives of *M. senegalense* and related bacteria. Zentralbl. Bakteriol. **281:** 415–432

76 Rivière M. and Puzo G. (1991) A new type of serine-containing glycopeptidolipid from *Mycobacterium xenopi*. J. Biol. Chem. **266:** 9057–9063

77 Besra G. S., McNeil M. R., Rivoire B., Khoo K.-H., Morris H. R., Dell A. et al. (1993) Further structural definition of a new family of glycopeptidolipids from *Mycobacterium xenopi*. Biochemistry **32:** 347–355

78 Rivière M., Augé S., Vercauteren J., Wisingerovà E. and Puzo G. (1993) Structure of a novel glycopeptidolipid antigen containing a *O*-methylated serine isolated from *Mycobacterium xenopi* – Complete ^{1}H-NMR and ^{13}C-NMR assignment. Eur. J. Biochem. **214:** 395–403

79 Rivoire B., Ranchoff B., Chatterjee D., Gaylord H., Tsang A., Kolk A. H. J. et al. (1989) Generation of monoclonal antibodies to the specific sugar epitopes of *Mycobacterium avium* complex serovars. Infect. Immun. **57:** 3147–3158

80 Fujiwara T., Hunter S. W., Cho S. N., Aspinall G. O. and Brennan P. J. (1984) Chemical synthesis and serology of disaccharides and trisaccharides of phenolic glycolipid antigens from the leprosy bacillus and preparation of a disaccharide protein conjugate for serodiagnosis of leprosy. Infect. Immun. **43:** 245–252

81 Bernstein M. A. and Hall L. D. (1980) General synthesis of model glycoproteins – coupling of alkenyl-glycosides to proteins, using redutive ozonolysis followed by reductive amination with sodium cyanoborohydride. Carbohydr. Res. **78:** C1–C3

82 Aspinall G. O., Crane A. M., Gammon D. W., Ibrahim I. H., Chatterjee D., Rivoire B. et al. (1991) Synthesis of allyl glycosides for conversion into neoglycoproteins bearing epitopes of mycobacterial glycolipid antigens. Carbohydr. Res. **216:** 337–355

83 Garegg P. J. (1997) Thioglycosides as glycosyl donors in oligosaccharide synthesis. Adv. Carbohydr. Chem. Biochem. **52:** 179–205

84 Aspinall G. O., Ibrahim I. H. and Khare N. K. (1990) A stereoselective synthesis of pyruvic 4,6-acetals of D-hexopyranose residues. Carbohydr. Res. **200:** 247–256

85 Carlsen P. H. J., Katsuki T., Martin V. S. and Sharpless K. B. (1981) A greatly improved procedure for ruthenium tetraoxide catalyzed oxidations of organic-compounds. J. Org. Chem. **46:** 3937–3938

86 Liptak A. and Fugedi P. (1983) Formation, structure and synthetic use of glucopyranoside and galactopyranoside acetophenone acetals with 1,3-dioxane rings. Angew. Chem. Int. Ed. **22:** 255–256

87 Bajza I. and Liptak A. (1990) Synthesis of phenyl 4-(N-benzylformamido)-4,6-dideoxy-3-C-methyl-2-O-methyl-1-thio-α-L-mannopyranoside, an N-formylkansosamine glycosyl donor. Carbohydr. Res. **205:** 435–439

88 Giuliano R. M. and Kasperowicz S. (1988) Synthesis of branched-chain sugars – stereoselective route to sibirosamine, kansosamine and vineloe from a common precursor. Carbohydr. Res. **183:** 277–285

89 Johansson R. and Samuelsson B. (1984) regioselective reductive ring-opening of 4-methoxybenzylidene acetals of hexopyranosides – access to a novel protecting group strategy. J. Chem. Soc.-Perkin Trans. 1: 2371–2374

90 Liptak A., Borbas A. and Bajza I. (1994) Synthesis of carbohydrate-containing surface antigens of mycobacteria. Med. Res. Rev. **14:** 307–352

91 Gurjar M. K. and Viswanadham G. (1991) A stereoconvergent synthesis of the oligosaccharide segment of glycopeptidolipid antigen of *Mycobacterium avium* serotype-4 – a dominant serovariant observed in patients with acquired immune-deficiency syndrome. Tetrahedr. Lett. **32:** 6191–6194

92 Gurjar M. K. and Viswanadham G. (1991) Synthesis of Me α-L-Rhap-(1-])-2-O-Me-α-L-Rhap and Me 2,3,4-Tri-O-Me-α-L-Fucp-(1-]3)-α-L-Rhap-(1-]3)-2-O-Me- α-L-Rhap – oligosaccharide segments of phenolic glycolipids in *Mycobacterium bovis* Bcg and tuberculosis strain Canetti. J. Carbohydr. Chem. **10:** 481–485

93 Zuurmond H. M., Veeneman G. H., Vandermarel G. A. and Vanboom J. H. (1993) Iodonium ion-assisted synthesis of a haptenic tetrasaccharide fragment corresponding to the inner cell wall glycopeptidolipid of *Mycobacterium avium* serotype-4. Carbohydr. Res. **241:** 153–164

94 Kerekgyarto J., Szurmai Z. and Liptak A. (1993) Synthesis of P-trifluoroacetamidophenyl 6-deoxy-2-O-(3-0-[2-0-methyl-3-0-(2-0-methyl-α-D-rhamnopyranosyl)-α-L-fucopyranosyl]-α-L-Rhamnopyranosyl)-α-L-talopyranoside – a spacer armed tetrasaccharide glycopeptidolipid antigen of *Mycobacterium-Avium* serovar 20. Carbohydr. Res. **245:** 65–80

95 Fugedi P., Garegg P. J., Lonn H. and Norberg T. (1987) Thioglycosides as glycosylating agents in oligosaccharide synthesis. Glycoconj. J. **4:** 97–108

96 Ziegler T. (1992) Synthesis of the species-specific *Mycobacterium avium* trisaccharide (serovar-21) for the preparation of a neoglycoprotein for immunological studies. Angew. Chem. Int. Ed. **31:** 1358–1360

97 Ziegler T. and Lau R. (1995) Intramolecular glycosylation of prearranged glycosides. A novel tool for controlling the reactivity and anomeric selectivity of glycosylations. Tetrahedr. Lett. **36:** 1417–1420-

98 Ziegler T. (1994) Synthesis of 5-aminopentyl monosaccharide to trisaccharide haptens related to the species-specific glycopeptidolipids of *Mycobacterium avium intracellulare* serovar-8 and serovar-21. Carbohydr. Res. **253:** 151–166

99 Bajza I., Kover K. E. and Liptak A. (1998) Synthesis of p-trifluoroacetamidophenyl(4,6-dideoxy-4-formamido-3-*C*-methyl-2-*O*-methyl-α-L-mannopyranosyl)-(1-3)-(2-)-methyl-α-D-rhamnopyranosyl-(1-3)-(2-*O*-methyl-α-L-fucopyranosyl)-(1-3)-(α-L-rhamnopyranosyl)-(1-2)-6-deoxy-α-L-talopyranoside: a spacer armed pentasaccharide glycopeptidolipid antigen of *Mycobacterium avium* serovar 14. Carbohydr. Res. **308:** 247–258

100 Gurjar M. K. and Mainkar A. S. (1992) Synthesis of methyl 3-O-[3-O-(2,3,4-Tri-O-methyl-α-L-rhamnopyranosyl)-α-L-rhamnopyranosyl]-α-L-rhamnopyranoside – the outer trisaccharide unit of a unique *Mycobacterium xenopi* glycopeptidolipid. Tetrahedron **48:** 6729–6738

101 Gurjar M. K. and Saha U. K. (1992) Synthesis of the glycopeptide O-(3,4-Di-O-methyl-2-O-[3,4-Di-O-methyl-α-L-rhamnopyranosyl]-α-L-rhamnopyranosyl)-L-alaninol – an unusual part structure in the glycopeptidolipid of *Mycobacterium fortuitum*. Tetrahedron **48:** 4039–4044

102 Gurjar M. K. and Saha U. K. (1992) Synthesis of a new serine containing glycotetrapeptide from *Mycobacterium xenopi* glycopeptidolipid: an unusual structural variant in *Mycobacterium* genus. Tetrahedr. Lett. **33:** 4979–4982

103 Stokes R. W. and Collins F. M. (1988) Growth of *Mycobacterium avium* in activated macrophages harvested from inbred mice with differing innate susceptibilities to mycobacterial infection. Infect. Immun. **56:** 2250–2254

104 Draper P. (1974) The mycoside capsule of *Mycobacterium avium*. J. Gen. Microbiol **83:** 431–433

105 Barrow W. W. and Brennan P. J. (1982) Isolation in high frequency of rough variants of *Mycobacterium intracellulare* lacking C-mycoside glycopeptidolipid antigens. J. Bacteriol **150:** 381–384

106 Rulong S., Aguas A. P., de Silva P. P. and Silva M. T. (1991) Intramacrophagic *Mycobacterium avium* bacilli are coated by a multiple lamallar structure: freeze fracture analysis of infected mouse liver. Infect. Immun. **59:** 3895–3902

107 Barrow W. W., Davis T. L., Wright E. L., Labrousse V., Bachelet M. and Rastogi N. (1995) Immunomodulatory spectrum of lipids associated with *Mycobacterium avium* serovar 8. Infect. Immun. **63:** 126–133

108 Daugelat S. and Kaufmann S. H. E. (1996) Role of Th1 and Th2 cells in bacterial infections. Chem. Immunol. **63:** 66–97

109 Toosi Z. (1996) Cytokine circuits in tuberculosis. Infect. Agents. Dis. **5:** 98–107

110 Romagniaini S. (1991) Human Th1 and Th2 subsets: doubt no more. Immunol. Today **12:** 256–297

111 Appelberg R. and Pedros J. (1992) Induction nd expression of protective T cells during *Mycobacterium avium* infections in mice. Clin. Exp. Immunol. **87:** 379–395

112 Castro A. G., Minóprio P. and Appelberg R. (1995) The relative impact of bacterial virulence and host genetic background on cytokine expression during *Mycobacterium avium* infection of mice. Immunol. **85:** 556–561

113 Orme I. M., Roberts A. D., Furney S. K. and Skinner P. S. (1994) Animal and cell-culture models for the study of mycobacterial infections and treatment. Eur. J. Clin. Microbiol. Infect. Dis. **13:** 994–999

114 Appelberg R., Castro A. G., Pedrosa A. G., Silva R. A., Orme I. and Pinoprio P. (1994) Role of gamma interferon and tumor necrosis factor alpha during T-cell independent and dependent phases of *Mycobacterium avium* infection. Infect. Immun. **62:** 3962–3971

115 Levy J. A. (1993) Pathogenesis of human immunodeficiency virus infection. Microbiol. Rev. **57:** 183–289

116 Horgen L., Barrow E. L., Barrow W. W. and Rastogi N. (2000) Exposure of human peripheral blood mononuclear cells to total lipids and serovar-specific glycopeptidolipids from *Mycobacterium avium* serovars 4 and 8 results in inhibition of TH1-type responses. Microb. Pathog. **29:** 9–16

117 Pourshafie M. R., Sonnenfeld G. and Barrow W. W. (1999) Immunological and ultrastructural disruptions of T lymphocytes following exposure to the glycopeptidolipid isolated from the *Mycobacterium avium* complex. Scand. J. Immunol. **49:** 405–410

118 Barrow W. W. (1997) Processing of mycobacterial lipids and effects on host responsiveness. Front. Biosci. **2:** d387–400

119 Vergne I., Prats M., Tocanne J.-F. and Lanéelle G. (1995) Mycobacterial glycopeptidolipid interactions with membranes: a monolayer study. FEBS Lett. **375:** 254–258.

120 Takegaki Y. (2000) Effect of serotype specific glycopeptidolipid (GPL) isolated from *Mycobacterium avium* complex (MAC) on phagocytosis and phagosome-lysosome fusion of human peripheral blood monocytes. Kekkaku **75:** 9–18

121 Gjata B., Hannoun C., Boulouis H. J., Neway T. and Pilet C. (1994) Adjuvant activity of polar glycopeptidolipids of *Mycobacterium chelonae* (pGPL-Mc) on the immunogenic and protective effects of an inactivated influenza vaccine. C. R. Acad. Sci. III **317:** 257–263

122 de Souza Matos D. C., Marcovistz R., Neway T., Vieira da Silva A. M., Alves E. N. and Pilet C. (2000) Immunostimulatory effects of polar glycopeptidolipids of *Mycobacterium chelonae* for inactivated rabies vaccine. Vaccine **18:** 2125–2131

123 Vincent-Naulleau S., Neway T., Thibault D., Barrat F., Boulouis H. J. and Pilet C. (1995) Effects of polar glycopeptidolipids of *Mycobacterium chelonae* (pGPL-Mc) on granulomacrophage progenitors. Res. Immunol. **146:** 363–371

124 Vincent-Naulleau S., Thibault D., Neway T. and Pilet C. (1997) Stimulatory effects of polar glycopeptidolipids of *Mycobacterium chelonae* on murine haematopoietic stem cells and megakaryocyte progenitors. Res. Immunol. **148:** 127–136

125 Lagrange P. H., Fourgeaud M., Neway T. and Pilet C. (1995) Mycobacterial polar glycopeptidolipids enhance resistance to experimental murine candidiasis. C. R. Acad. Sci. III **318:** 359–365

126 Fregnan G. B. and Smith D. W. (1962) Description of various colony forms of mycobacteria. J. Bacteriol. **83:** 819–827

127 Vestal A. L. and Kubica G. P. (1966) Differential colony characteristics of mycobacteria on Middlebrook and Cohn 7H10 agar base medium. Am. Rev. Resp. Dis. **94:** 247–252

128 MaCarthy C. M. (1970) Spontaneous and induced mutations in *Mycobacterium avium*. Infect. Immun. **2:** 223–228

129 Woodley C. L. and David H. L. (1976) Effects of temperature on the rate of the transparent to opaque colony type transition in *Mycobacterium avium*. Antimicrob. Agents. Chemother. **9:** 113–119

130 Meylan P. R., Richman D. D. and Kornbluth R. S. (1990) Characterization and growth in human macrophages of *Mycobacterium avium* complex strains isolated from the blood of patients with acquired immunodeficiency syndrome. Infect. Immun. **58:** 2564–2568

131 Crawford J. T. and Bates J. H. (1986) Analysis of plasmids in *Mycobacterium avium intracellulare* isolates from persons with acquired immunodeficiency syndrome. Am. Rev. Respir. Dis **134:** 659–661

132 Shiratsuchi H., Toossi Z., Mettler M. A. and Ellner J. J. (1993) Colonial morphotype as a determinant of cytokine expression by human monocytes infected with *Mycobacterium avium*. J. Immunol. **150:** 2945–2954

133 Cooper A. M., Appelberg R. and Orme I. M. (1998) Immunopathogenesis of *Mycobacterium avium* infection. Front. Biosci. **3:** e141–148

134 Furney S. K., Skinner P. S., Roberts A. D., Appelberg R. and Orme I. M. (1992) Capacity of *Mycobacterium avium* isolates to grow well or poorly in murine macrophages resides in their ability to induce secretion of tumor necrosis factor. Infect. Immun. **60:** 4410–4413

135 Thorel M. and David H. L. (1984) Specific surface antigens of SmT variants of *Mycobacterium avium*. Infect. Immun. **43:** 438–439

136 Barksdale L. and Kim K. S. (1977) *Mycobacterium*. Bacteriol. Rev. **41:** 217–232

137 Tereletsky M. J. and Barrow W. W. (1983) Postphagocytic detection of glycopeptidolipids associated with the superficial L1 layer of *Mycobacterium intracellulare*. Infect. Immun. **41:** 1312–1321

138 Belisle J. T. and Brennan P. J. (1994) Molecular basis of colony morphology in *Mycobacterium avium*. Res. Microbiol. **145:** 237–242

139 Henrichsen J. (1972) Bacterial surface translocation: a survey and classification. Bacteriol. Rev. **36:** 478–503

140 Martinez A., Torello S. and Kolter R. (1999) Sliding motility in mycobacteria. J. Bacteriol **181:** 7331–7338

141 Recht J., Martinez A., Torello S. and Kolter R. (2000) Genetic analysis of sliding motility in *Mycobacterium smegmatis*. J. Bacteriol. **182:** 4348–4351.

142 Belisle J. T., McNeil M. R., Chatterjee D., Inamine J. M. and Brennan P. J. (1993) Expression of the core lipopeptide of the glycopeptidolipid surface antigens in rough mutants of *Mycobacterium avium*. J. Biol. Chem. **268:** 10510–10516

143 Belisle J. T., Klaczkiewicz K., Brennan P. J., Jacobs W. R. Jr and Inamine J. M. (1993) Rough morphological variants of *Mycobacterium avium*. Characterization of genomic deletions resulting in the loss of glycopeptidolipid expression. J. Biol. Chem. **268:** 10517–10523

144 Eckstein T. M., Inamine J. M., Lambert M. L. and Belisle J. T. (2000) A genetic mechanism for deletion of the ser2 gene cluster and formation of rough morphological variants of *Mycobacterium avium* J. Bacteriol. **182:** 6177–6182

145 Ghuysen J. M. (1977) Biosynthesis and assembly of bacterial cell walls. In: Cell Surface Reviews, pp. 463–595, Poste G. and Nicholson G. L. (eds), North-Holland, Amsterdam
146 Perlman D. and Bodanszky M. (1971) Biosynthesis of peptide antibiotics. Ann. Rev. Biochem. **40:** 449–464
147 David H. L., Rastogi N., Clavel-Seres S. and Clement F. (1987) Studies on clofazimine resistance in mycobacteria: is the inability to isolate drug-resistance mutants related to its mode of action? Zentralbl. Bakteriol. Mikrobiol. Hyg. [A] **266:** 292–304
148 Rastogi N., Goh K. S. and David H. L. (1988) Activity of five fluoroquinolones against *Mycobacterium avium-intracellulare* complex and *M. xenopi*. Ann. Inst. Pasteur Microbiol. **139:** 233–237
149 Billman-Jacobe H., McConville M. J., Haites R. E., Kovacevic S. and Coppel R. L. (1999) Identification of a peptide synthetase involved in the biosynthesis of glycopeptidolipids of *Mycobacterium smegmatis*. Mol. Microbiol. **33:** 1244–1253
150 Belisle J. T., Pascopella L., Inamine J. M., Brennan P. J. and Jacobs W. R. Jr (1991) Isolation and expression of a gene cluster responsible for biosynthesis of the glycopeptidolipid antigens of *Mycobacterium avium*. J. Bacteriol. **173:** 6991–6997
151 Oschsner U. A., Flechter A. and Reiser J. (1994) Isolation, characterization and expression in *Escherichia Coli* of the *Pseudomonas aeruginos*a *rhlAB* genes encoding a rhamnosyltransferase involved in the rhamnolipid biosurfactant synthesis. J. Biol. Chem. **269:** 19787–19795
152 Liu D., Haase A. M., Lindqvist L., Lindberg A. A. and Reeves P. R. (1993) Glycosyl transferases of O-antigen biosynthesis in *Salmonella enterica*: identification and characterization of transferase genes of groups B, C2 and E1. J. Bacteriol. **175:** 3408–3413
153 Mitchison M., Bulach D. M., Vinh T., Rajakumar K., Faine S. and Adler B. (1997) Identification and characterization of the dTDP-rhamnose biosynthesis and transfer genes of the lipopolysaccharide-related rfb locus in *Leptospira interrogans* serovar Copenhageni. J. Bacteriol. **179:** 1262–1267
154 Stevenson G., Neal B., Liu D., Hobbs M., Packer N. H., Batley M. et al. (1994) Structure of the O antigen of *Escherichia coli* K-12 and the sequence of its *rfb* gene cluster. J. Bacteriol. **176:** 4144–4156
155 Tassell S. K., Pourshafie M., Wright E. L., Richmond M. G. and Barrow W. W. (1992) Modified lymphocyte response to mitogens induced by the lipopeptide fragment derived from *Mycobacterium avium* serovar-specific glycopeptidolipids. Infect. Immun. **60:** 706–711