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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

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ABSTRACT

The pentasaccharide hapten released from the glycopeptidolipid (GPL) antigen of *M. avium* serovar 26 has been characterized as *O*-(2,4-di-*O*-methyl-α-L-fucopyranosyl)-(1 → 4)-*O*-β-D-glucopyranosyluronic acid-(1 → 4)-*O*-(2-*O*-methyl-α-L-fucopyranosyl)-(1 → 3)-α-L-rhamnopyranosyl-(1 → 2)-6-deoxy-L-talose. The allyl glycosides of the outer glycosyl and glycobiosyl units of this hapten have been synthesized, the latter by a route involving oxidation of the corresponding D-glucopyranose derivative. Conjugation of allyl glycosides to protein by ozonolysis and reductive coupling afforded neoantigens (neo 26-1 and 26-2), both of which interacted with antibodies to *M. avium* serovar 26. The terminal sugar residue of the pentasaccharide hapten of the serovar 25 GPL had been shown to have the *galacto* configuration on the basis of ^{1}H–^{13}C NMR correlation spectroscopy, but absolute configurational assignment for the sugar awaited the synthesis, as for neo 26, of two glycobiosyl NGPs bearing the terminal sugar in the D and L enantiomeric forms, respectively. Only the glycobiosyl NGP bearing the terminal sugar as the D-enantiomer interacted with antibodies to *M. avium* serovar 25, thus providing evidence for the absolute configuration of the sugar, and showing that the complete oligosaccharide hapten has the structure, *O*-(4-acetamido-4,6-dideoxy-2-*O*-methyl-α-D-galactopyranosyl)-(1 → 4)-*O*-β-D-glucopyranosyluronic acid-(1 → 4)-*O*-(2-*O*-methyl-α-L-fucopyranosyl)-(1 → 3)-*O*-α-L-rhamnopyranosyl-(1 → 2)-6-deoxy-L-talose.

INTRODUCTION

Antigenic glycopeptidolipids (GPLs) of the *M. avium* complex are highly characteristic surface components with unusual sugar constituents and they are highly

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specific in their interactions with antibodies to the homologous serovars. The GPLs contain a common *N*-fatty acyl tetrapeptide core with attached oligosaccharide hapten chains comprising an invariant inner disaccharide unit and a variable outer segment of one to three "distinctive" sugar residues. Among the first studied oligosaccharide haptens were those from the GPLs of serovars 9 and 25[1]. Revisions[2] of the originally proposed structure for the serovar 9 hapten led to the recognition of its pentasaccharide nature (**1**), and the most recent studies[3] have confirmed the presence of a terminal 4-*O*-acetyl-2,3-di-*O*-methyl-α-L-fucopyranose residue in which the *O*-acetyl substituent is required for interaction with antibodies to serovar 9. The originally proposed structure for the oligosaccharide hapten of the serovar 25 GPL was also revised and the hapten was shown to be a pentasaccharide bearing a nonreducing terminal 4-acetamido-4,6-dideoxy-2-*O*-methylhexose residue of undefined configuration[2]. ^{1}H NMR spectroscopy showed anomeric protons at δ 5.55 ($J_{1,2}$ 3.5 Hz) and δ 5.25 ($J_{1,2}$ 3.7 Hz) for this residue and that of an internal 2-*O*-methyl-α-L-fucopyranose residue. Further spectroscopic data are reported and chemical synthesis has now furnished evidence for the *fuco* (6-deoxygalacto) configuration for the unknown sugar. The limited quantities of GPL available were insufficient to isolate the sugar in preparative amounts to obtain optical rotational data for comparison with values for a defined enantiomer. Furthermore the susceptibility of 4-acylamino-4-deoxy sugars, cf. *N*-acetylperosamine (4-acetamido-4,6-dideoxy-D-mannose)[4], to acid-catalyzed degradation precluded the preparation of glycosides of chiral alcohols[5]. The assignment of absolute configuration for this sugar residue, and hence for the complete oligosaccharide hapten chain (**2**), therefore awaited immunologically specific recognition by homologous antibodies of a synthetic disaccharide as the epitope.

Herein we report syntheses of neoglycoproteins (NGPs) bearing the terminal disaccharide units of the haptens of GPLs from serovars 25 and 26. For the serovar 25 GPL immunological recognition of the neoantigen has served to define the terminal sugar residue in the pentasaccharide hapten (**2**) as the D-enantiomer. We also report structural studies on the pentasaccharide hapten (**3**) from the serovar 26 GPL and reveal that the outer trisaccharide segment provides a third example of an *O*-α-fucopyranosyl-(1 → 4)-*O*-β-D-glucopyranosyluronic acid-(1 → 4)-α-L-fucopyranose skeleton but with a different array of substituents in the fucose

1 4-Ac-2,3-Me_2-α-L-Fuc*p*-(1 → 4)-β-D-Glc*p*A-(1 → 4)-2,3-Me_2-α-L-Fuc*p*-(1 → 3)-α-L-Rha*p*-(1 → 2)-6-deoxy-L-Tal

2 2-Me-α-D-Fuc*p*4NAc-(1 → 4)-β-D-Glc*p*A-(1 → 4)-2-Me-α-L-Fuc*p*-(1 → 3)-α-L-Rha*p*-(1 → 2)-6-deoxy-L-Tal

3 2,4-Me_2-α-L-Fuc*p*-(1 → 4)-β-D-Glc*p*A-(1 → 4)-2-Me-α-L-Fuc*p*-(1 → 3)-α-L-Rha*p*-(1 → 2)-6-deoxy-L-Tal

residues from those in **1** and **2**. In the light of the apparent stereohomology of the pentasaccharide hapten **2** with **1** and **3** it had seemed most likely that the terminal amino sugar residue in **2** would have the L-configuration.

We have shown previously[6–8] that ozonolysis of allyl glycosides, followed by reductive coupling to ϵ-amino groups of lysine residues in proteins, is a convenient mild procedure for the preparation of neoglycoproteins. The resulting NGPs, which carry serotypically distinctive sugar residues, offer a potential basis for recognition by antibodies and thus for diagnosis of *M. avium* infections. The procedure allows for the retention of *O*-acetyl[3,6] and pyruvic acetal[8] substituents, but the use of allyl glycosidic substituents to be modified for conjugative attachment to protein places some restrictions on the protecting groups which may be used elsewhere. In the case of GPLs related to serovars 9 and 8 the NGPs containing single glycosyl residues interacted with cognate antibodies, but for serovar 4 a glycobiosyl NGP[3,6] was found to be necessary for interaction. The NGP syntheses now reported provide evidence that the minimum size required for the epitopes is variable.

RESULTS AND DISCUSSION

Structural characterization of the pentasaccharide hapten of the GPL of M. avium *serotype 26.*—The oligoglycosyl alditol as released from GPL-26 after β-elimination[2] was purified and GLC analysis of the derived alditol acetates established the presence of residues of 2,4-di-*O*-methylfucose, 2-*O*-methylfucose, glucuronic acid (reduction to glucose), rhamnose, and 6-deoxytalose. FABMS analysis of the oligoglycosylalditol gave molecular ions at m/z 823 (MH^+), 845 (MNa^+), and 861 (MK^+), confirming a molecular mass of 822. ^{1}H NMR spectroscopy of the tetraglycosylalditol revealed resonances at δ 5.25 ($J_{1,2} \sim 3.2$ Hz), 5.10 ($J_{1,2} \sim 3.1$ Hz) and 4.75 (br s), indicative of α-glycosyl residues, and at δ 4.49 ($J_{1,2} \sim 7.2$ Hz), indicating a β-glycosyl residue. Linkage and sequence analysis was effected through per(trideuteriomethyl)ation of the tetraglycosylalditol. Direct hydrolysis of the per-*O*-trideuteriomethylated derivative and conversion into partially methylated alditol acetates for GLC–MS analysis showed the formation of 3-*O*-CD_3-2,4-di-*O*-CH_3-fucitol, 3-*O*-CD_3-2-*O*-CH_3-fucitol, 2,4-di-*O*-CD_3-rhamnitol, and 6-deoxy-1,3,4,5-tetra-*O*-CD_3-talitol. The presence of the uronic acid residue was confirmed by reduction of the per-*O*-CD_3 derivative with $LiAlH_4$ and conversion into alditol acetates including that of 2,3-di-*O*-CD_3-glucitol. The per-*O*-CD_3-oligosaccharide was degraded by treatment with methylsulfinyl carbanion, as described previously[2], and ethylated to give rise to a per-*O*-alkylated diglycosyl alditol in which the original 2-*O*-methylfucose unit was now the terminal sugar; the presence of an OC_2H_5 group at C-4 further corroborated that the glucosyluronic residue was attached at this site. Fig. 1 depicts the origins of fragment ions in the EIMS of the per-*O*-alkylated diglycosyl alditol that led to the above conclusions. In addition, FABMS of the earlier per-*O*-trideuteriomethylated oligosaccharide gave

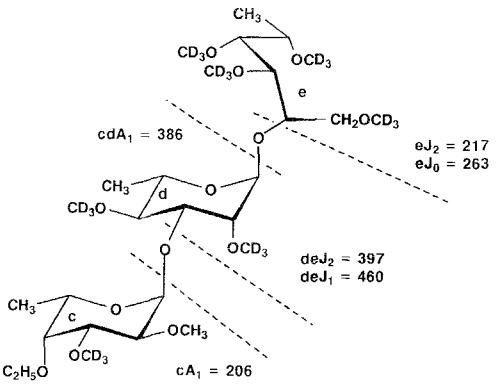


Fig. 1. Fragment ions in EIMS of per-*O*-alkylated diglycosyl alditol formed on base degradation, followed by *O*-ethylation, of per-*O*-trideuteriomethylated tetraglycosyl alditol from *M. avium* serovar 26 GPL. The sugar residues designated c to e arise from the inner segment of the parent oligosaccharide.

fragment ions at *m/z* 192, 419, 776, and 217 which substantiated the sequence of glycosyl residues, together with a molecular ion (MH^+) at *m/z* 1010 that corroborated the proposed structure. The enantiomeric configurations of the glycosyl residues were established by *O*-demethylation of the per-*O*-trideuteriomethylated tetraglycosyl alditol after reduction of the glucuronic acid residue to glucosyl with $LiAlH_4$. The *O*-demethylated product was hydrolyzed and the derived sugars were converted into *O*-trimethylsilyl derivatives of (*S*)- and (*R*)-2-butyl glycosides, which were identified by GLC and shown to originate from L-fucose, L-rhamnose, and D-glucose (from D-glucuronic acid). The proposed structure for the specific hapten of GPL-26 is shown in Fig. 2.

Synthesis of neoglycoproteins.—NGPs related to serovar 26 GPL. In anticipation that an NGP based on the single terminal glycosyl residue of serovar 26 GPL might interact with antibodies to whole cells of the organism, as shown for serovar 9 (ref. 3), allyl 2,4-di-*O*-methyl-α-L-fucopyranoside (**7**) and allyl 3-*O*-acetyl-2,4-di-*O*-methyl-α-L-fucopyranoside (**8**) were conjugated to bovine serum albumin by ozonolysis followed by reductive coupling[9], and the two monoglycosyl NGPs were tested in ELISA against anti-serovar 26 antiserum (Table I). The *O*-acetylated NGP showed only minimal serological activity. However, the non-acetylated NGP (neo 26-1) proved to be a powerful antigen, indicating that, as in the case of many NGPs based on phenolic glycolipid I of *M. leprae*[10], the antibody binding domain resides largely in the terminal *O*-methylated sugar. Interestingly, and disappointingly, this NGP lost its reactivity; when tested 3 years after synthesis it was no longer antigenic. The chemical basis for this loss of activity has not been examined.

Synthesis of a glycobiosyl NGP was also undertaken. For the synthesis of an allyl 4-*O*-glycosyl-β-D-glucopyranosiduronic acid we considered that the required selectivity of substitution could be achieved more readily in the corresponding glucopyranoside, provided that oxidation to the glucuronic acid residue could be

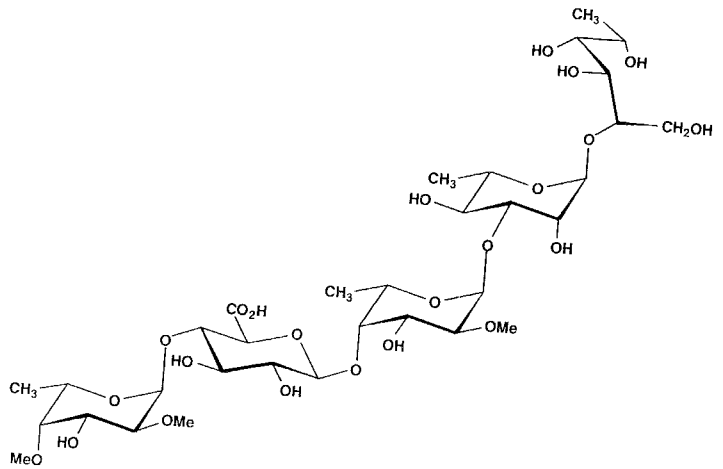


Fig. 2. Structure of hapten liberated as tetraglycosyl alditol by reductive elimination from *M. avium* serovar 26 GPL.

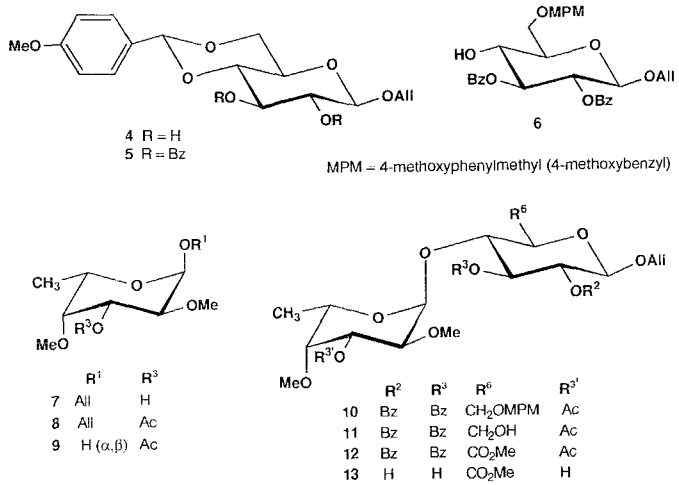
carried out without loss or modification of the 4-*O*-glycosyl substituent and without effect on the allyl glycoside. A similar approach to the synthesis of a pseudoaldobiouronic acid has been reported by Fügedi[11]. Allyl 2,3-di-*O*-benzoyl-6-*O*-[4-methoxybenzyl]-β-D-glucopyranoside (**6**) was obtained by regioselective acetal opening when allyl 2,3-di-*O*-benzoyl-4,6-*O*-(4-*O*-methoxybenzylidene)-β-D-glucopyranoside (**5** from **4**) was treated with acidified sodium cyanoborohydride[12]. Allyl 2,4-di-*O*-methyl-α-L-fucopyranoside (**7**)[6] was acetylated (to **8**), the allyl glycosidic substituent was removed, and the resulting hemiacetal (**9**) was converted, as required, into the corresponding glycosyl chloride. Glycosylation of **6** was per-

TABLE I

Serological activity of the native GPL 26 and the corresponding NGPs against serovar-specific polyclonal rabbit antibodies by ELISA [a]

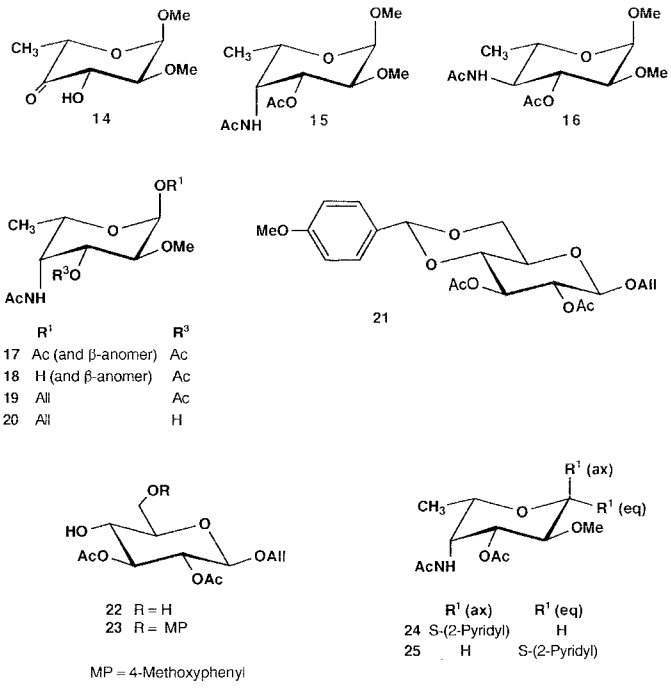
Antigen	A_{490} values at antigen concentrations (μg/mL) listed [b]			
	3.3	1.1	0.37	0.12
Native GPL 26	1.92	0.92	0.36	0.09
Neoantigen	A_{490} values at antigen concentrations (ng/mL) listed [c]			
	60	30	15	7.5
Neo 26-1 (*O*-acetyl)	0.20	0.13	0.06	0.04
Neo 26-1	1.39	1.19	0.56	0.32
Neo 26-2	1.64	1.44	0.83	0.55

[a] ELISA conditions have been described previously[3]. The anti-serovar 26 antibodies were diluted 1 in 250 for reaction against native GPL and all of the serovar 26 NGPs. [b] Concentration of native glycolipid antigens is based on weight. [c] Concentration of neoantigens is given as sugar (glucose) equivalents.



formed with silver triflate as promoter in the presence of tetramethylurea to avoid premature loss of the 4-methoxybenzyl substituent, and afforded as the major product allyl *O*-(3-*O*-acetyl-2,4-di-*O*-methyl-α-L-fucopyranosyl)-(1 → 4)-2,3-di-*O*-benzoyl-6-*O*-(4-methoxybenzyl)-β-D-glucopyranoside (**10**), which was separated from small amounts of the β-L-fucopyranosyl disaccharide. Removal of the 4-methoxybenzyl substituent from **10** on treatment with ceric ammonium nitrate (to **11**) was followed by oxidation with Jones reagent[13] to give the glucuronic acid derivative which was conveniently converted into the methyl ester, methyl *O*-(3-*O*-acetyl-2,4-di-*O*-methyl-α-L-fucopyranosyl)-(1 → 4)-(allyl 2,3-di-*O*-benzoyl-β-D-glucopyranosid)uronate (**12**). Catalytic *O*-deacylation of **12** furnished **13** from which the target disaccharide glycoside was generated in situ by saponification for conversion into the corresponding neoglycoprotein (neo 26-2). This glycobiosyl NGP, when tested in ELISA against anti-serovar polyclonal antibodies (Table I), was found to be equally as active as the monoglycosyl NGP, further corroborating the evidence that most of the activity was resident in the terminal sugar. The glycobiosyl NGP still retained its activity when tested three years after synthesis.

Characterization of 4-acetamido-4,6-dideoxy-2-O-methylgalactose as the terminal sugar of serovar 25 GPL.—The reported ^{1}H NMR data, $\delta \sim 5.5$ and $J_{1,2}$ 3.6 Hz, for the anomeric proton of the terminal residue in the serovar 25 oligosaccharide[2] pointed to a provisional assignment of the *α-galacto* or *α-gluco* rather than the *α-manno* configuration. More detailed NMR examination of the intact oligosaccharide hapten involving ^{13}C–^{1}H correlation and ^{1}H (2D COSY) spectroscopy permitted an unambiguous assignment of the *galacto* configuration. The location of the nitrogen function on C-4 was established by homo- and hetero-nuclear (^{13}C–^{1}H) COSY. A cross peak to the *C*H–NH signal at δ_C 56 observed at δ 4.25 in the heteronuclear COSY spectrum was assigned to H-4, and this resonance in



the homonuclear COSY showed cross peaks to H-3 (δ 3.42) and H-5 (δ 4.20). Values measured for the coupling constants $J_{1,2}$ (3.6 Hz), $J_{2,3}$ (9.5 Hz), $J_{3,4}$ (3.5 Hz), $J_{4,5}$ (~ 1.5 Hz), and $J_{5,6}$ (6.5 Hz) unequivocally established that the terminal acetamido sugar has the *galacto* configuration. These observations were further substantiated by the identity on GLC–MS of alditol acetates derived from the HF hydrolyzate of the serovar 25 tetraglycosylalditol and from synthetic glycoside **15** described below.

The synthesis of the two 4-acetamido-4,6-dideoxy-2-*O*-methylgalactose enantiomers was based on the approach developed by Tsuda et al.[14,15] for the preparation of 4-amino-4-deoxy-L-arabinose. The dibutylstannylene derivative of methyl 2-*O*-methyl-α-L-fucopyranoside[16] was treated with bromine[17] to yield regioselectively methyl 6-deoxy-2-*O*-methyl-α-L-*xylo*-hexopyranosid-4-ulose (**14**). Reaction of this oxidation product with hydroxylamine gave a mixture of diastereomeric oximes which could be separated chromatographically but equilibrated on standing. Reduction of the oximes gave mixtures of 4-aminohexose derivatives in which the relative proportions of the two isomers were dependent more on the reducing agent than on the configuration of the oxime, the *galacto* isomer predominating on catalytic hydrogenation over platinic oxide whereas reduction with lithium aluminum hydride gave more of the *gluco* isomer. The 4-aminohexose derivatives were converted into methyl 4-acetamido-3-*O*-acetyl-4,6-dideoxy-2-*O*-methyl-α-L-

TABLE II

Serological activity of the native GPL 25 and the corresponding NGPs against serovar-specific polyclonal rabbit antibodies by ELISA [a]

Antigen	A_{490} values at antigen concentrations (μg/mL) listed [b]			
	3.3	1.1	0.37	0.12
Native GPL 25	2.03	1.33	0.73	0.51
Neoantigen	A_{490} values at antigen concentrations (ng/mL) listed [c]			
	60	30	15	7.5
Neo 25-1 (L)	0	0	0	0
Neo 25-2 (L)	0	0	0	0
Neo 25-2 (D)	1.66	0.85	0.44	0.24

[a] ELISA conditions have been described previously[3]. The anti-serovar 25 antibodies were diluted 1 in 400 in all assays. [b] Concentration of native glycolipid antigens is based on weight. [c] Concentration of neoantigens is given as sugar (glucose) equivalents.

galactopyranoside (**15**) and the corresponding L-glucopyranoside (**16**) whose structures were readily established from their ^{1}H NMR spectra *. The methyl glycosides were each converted, by acetolysis followed by reduction with sodium borohydride and acetylation, into corresponding alditol acetates that were clearly separated by GLC.

Synthesis of neoglycoproteins.—NGPs related to serovar 25 GPL. Acetolysis of **15** afforded an anomeric mixture (**17**) of glycosyl acetates from which **18** was obtained on treatment with hydrazine acetate[18]. Reaction of **18** with trichloroacetonitrile in the presence of anhydrous potassium carbonate gave a mixture of trichloroacetimidates from which the β anomer was isolated on chromatography and used directly for conversion into allyl 4-acetamido-3-*O*-acetyl-4,6-dideoxy-2-*O*-methyl-α-L-galactopyranoside **19**, but in less than satisfactory yield. *O*-Deacetylation of **19** furnished the desired allyl glycoside (**20**) for conjugation to bovine serum albumin. The resulting NGP [neo 25-1(L)] failed to interact with polyclonal antibodies to *M. avium* serovar 25 (Table II), indicating that perhaps the D-enantiomer of the terminal 4-acetamido sugar is the true antigenic determinant. However, in order to categorically prove the point, synthesis of the L-glycobiosyl NGP was undertaken.

The synthesis of the allyl glycobioside followed the same general strategy as that employed for the serovar 26 derivative. Allyl 2,3-di-*O*-acetyl-6-*O*-(4-methoxyphenyl)-β-D-glucopyranoside (**23**) was chosen as a more suitable glycosyl acceptor than **6**; it had more readily removable acyl groups and a substituent at O-6 less susceptible to premature loss with traces of acid. Synthesis of **23** from **4** was

* The L-*gluco* derivative **16** gave a satisfactory combustion analysis, but repeated analyses of the L-*galacto* derivative **15**, the D-enantiomer **32**, and the majority of compounds containing residues of these sugars gave analyses low in carbon, although they had been carefully purified by recrystallization for solids, were chromatographically homogeneous, and spectroscopic data showed no evidence for the presence of impurities. Accurate mass determinations left no doubt as to the identity of the compounds.

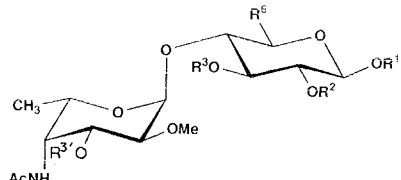
effected by successive acetylation (to **21**), *O*-deacetalation by mild-acid hydrolysis (to **22**), and reaction with triphenylphosphine, diethyl azodicarboxylate, and 4-methoxyphenol[19]. To prepare a glycosyl donor, the conversion of the glycosyl acetates **17** into the corresponding glycosyl chloride by reaction with dichloromethyl methyl ether was attempted, but this was observed to be accompanied by decomposition. When the formation of glycosyl trichloroacetimidate from **18** was performed using DBU (1,8-diazabicyclo[4.5.0]undec-5-ene)[20] as a base, an anomeric mixture was obtained and attempted glycosylation with this mixture resulted in the formation of both α- and β-linked disaccharides. Thus, a change was required from the type of donor employed here in the construction of **13**, and in previous work[6,7].

High stereoselectivity in the coupling step was obtained by use of the recently reported 2-pyridyl thioglycosides[21]. Hemiacetal **18** was converted into a chromatographically separable mixture of the anomers **24** and **25**. Activation of the α-L thioglycoside (**24**) with methyl iodide in the presence of **23** afforded anomerically pure fully protected disaccharide **26** with no trace of the β-linked product. The same observation was made when glycosylation was performed with the anomeric mixture of thioglycosides (**24** and **25**). Treatment of **26** with ceric ammonium nitrate furnished **27**, which was oxidized with the Jones reagent[13], and the derived glucosiduronic acid **28** was immediately converted into the methyl ester **29** for spectroscopic and elemental analysis. A further quantity of **28** was carefully *O*-deacetylated to give the allyl glycoside (**30**) of the target disaccharide for ozonolysis and reductive coupling to yield the NGP **31** [neo 25-2(L)].

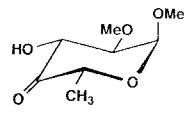
In the light of the failure of this NGP to interact with antibodies to *M. avium* serovar 25, the diastereomeric NGP [neo 25-2(D)] was synthesized by the same general procedure. Crystalline methyl 4-acetamido-3-*O*-acetyl-4,6-dideoxy-2-*O*-methyl-α-D-galactopyranoside (**33**), prepared from glycosidulose **32** as described for the L-enantiomer **15**, was converted into a syrupy mixture of anomeric 2-pyridyl thioglycosides (**34** and **35**). Activation of the thioglycosides with methyl iodide in the presence of **23** gave the fully protected disaccharide **36**. Treatment of **36** with ceric ammonium nitrate (to **37**) was followed by oxidation with Jones reagent[13] to give the protected glycosiduronic acid (**38**). A portion of compound **38** was treated with diazomethane to give the methyl ester (**39**) and the remainder was saponified to give the deprotected allyl glycoside (**40**) of the pseudoaldobiouronic acid. Ozonolysis of this glycoside (**40**) followed by reductive coupling afforded neo 25-2(D) (**41**). This glycobiosyl NGP was indeed highly seroreactive (Table II) demonstrating decisively the biological activity of the D-enantiomer and the high probability that this is the correct configuration of the terminal 4-acetamido sugar.

EXPERIMENTAL

General experimental methods, including specifications for drying solvents, chromatographic procedures, and details of the spectroscopic equipment used,

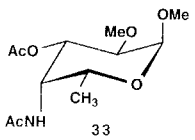


	R^1	R^2	R^3	R^6	$R^{3'}$
26	All	Ac	Ac	CH_2-OMP	Ac
27	All	Ac	Ac	CH_2-OH	Ac
28	All	Ac	Ac	CO_2Me	Ac
29	All	Ac	Ac	CO_2H	Ac
30	All	H	H	CO_2H	H
31	CH_2-CH_2-NH-BSA	H	H	CO_2H	H



32

CH_2CH_2-NH-BSA = linkage to bovine serum albumin via lysine side chains



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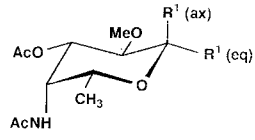
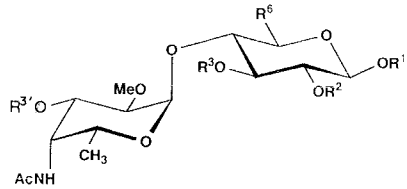


	R^1 (ax)	R^1 (eq)
34	S-(2-Pyridyl)	H
35	H	S-(2-Pyridyl)



	R^1	R^2	R^3	R^6	$R^{3'}$
36	All	Ac	Ac	CH_2-OMP	Ac
37	All	Ac	Ac	CH_2-OH	Ac
38	All	Ac	Ac	CO_2H	Ac
39	All	Ac	Ac	CO_2Me	Ac
40	All	H	H	CO_2H	H
41	CH_2-CH_2-NH-BSA	H	H	CO_2H	H

were described previously[6], and unless otherwise specified, methods for the preparation of glycosyl donors are likewise reported there. Preparative, centrifugally accelerated, radial TLC separations were performed on circular plates coated with silica gel (1 or 4 mm thickness), using a "Chromatotron" Model 7924T (Harrison Research, Palo Alto, CA 94306). Microanalyses were by Guelph Chemical Laboratories, Ltd., Guelph, Ontario, and Canadian Microanalytical Service, Ltd., Delta, British Columbia. Optical rotations were determined at ~20°C for solutions in chloroform unless otherwise indicated.

Purification and analysis of the tetraglycosyl alditol isolated from GPL-26.—The purification of the GPL-26, and isolation and purification of the oligosaccharide portion were performed as described in an earlier publication[2]. Detailed structural elucidation involved GLC and GLC–MS (SP 2340 and DB 23 capillary columns), FABMS, ^1H and ^{13}C NMR spectroscopy, and methylation analysis. These procedures have been described[2].

The NGPs synthesized were tested against hyperimmune rabbit sera raised against individual serovars (25 and 26) of the *M. avium* complex.

Neoglycoproteins.—The procedure of Bernstein and Hall[9] was followed as described previously[6–8]. Analysis of carbohydrate content of the NGP products was by the phenol–H_2SO_4 method[22] using the allyl glycoside as reference standard, and all samples showed 25–30 mol of carbohydrate per mol of bovine serum albumin, which has a total of 59 lysine residues.

Immunological procedures.—Details of the application of plate-ELISA to NGPs have been described in detail for other *M. avium*-related NGPs[3] and for NGPs emulating sugar determinants of the *M. leprae* glycolipid[23,24]. Original sources for the preparation of polyclonal rabbit antibodies to whole bacteria have been described by Tsang et al.[25].

Allyl 4,6-O-(4-methoxybenzylidene)-β-D-glucopyranoside (**4**).—A solution of allyl β-D-glucopyranoside[26] (8.45 g), 4-methoxybenzaldehyde dimethyl acetal (11.3 mL), and *p*-toluenesulfonic acid (80 mg) in DMF (70 mL) was stirred overnight at room temperature. The mixture was then heated at 50°C under diminished pressure on an aspirator for 2.5 h. Solid $NaHCO_3$ was added and the solution was concentrated with an oil pump to ~ 20 mL, and this residue was poured into a stirred mixture of ice (25 g), satd aq $NaHCO_3$ (50 mL) and ether (50 mL). The precipitate which formed at the interface was recovered by filtration, washed with light petroleum and water, and dried. Recrystallization from EtOAc–$CHCl_3$ afforded **4** (10.5 g, 76.5%); mp 162.5–163.5°C; $[\alpha]_D$ −53.5° (*c* 1.15). ^{1}H NMR: δ 7.41, 6.88 (2 m, 4 H, Ar-H), 5.48 (s, 1 H, C*H*Ar), 4.43 (d, 1 H, $J_{1,2}$ 7.72 Hz, H-1), and 3.79 (s, 3 H, OCH_3). *Anal.* Calcd for $C_{17}H_{22}O_7$: C, 60.35; H, 6.55. Found: C, 60.11; H, 6.63.

Allyl 2,3-di-O-benzoyl-4,6-O-(4-methoxybenzylidene)-β-D-glucopyranoside (**5**).—Benzoyl chloride (14.7 mL) was added dropwise to **4** (11.55 g) in dry pyridine (75 mL) at 0°C, and the mixture was stirred overnight at room temperature. Water (0.5 mL) was added to destroy excess chloride and the solution was stirred for 1 h, then diluted with CH_2Cl_2, filtered, washed with satd aq $NaHCO_3$ and water, dried and concentrated. Crystallization of the residue from light petroleum–CH_2Cl_2 furnished **5** (15.6 g, 84%); mp 144–145°C; $[\alpha]_D$ −0.8 (*c* 1.3). ^{1}H NMR: δ 7.95, 7.47, 7.34, 6.83 (4 m, 14 H, Ar-H), 5.50, 5.49 (overlapping s, 1 H, C*H*Ar and dd, 1 H, $J_{1,2}$ 7.92, $J_{2,3}$ 9.4 Hz, H-2), 4.84 (d, 1 H, H-1), and 3.75 (s, 3 H, OCH_3). ^{13}C NMR: 165.6, 165.2 (2 × *C*=O), 101.5 (*C*HAr), 100.5 (C-1), 70.3, 68.6 (C-6 and O*C*H_2), and 55.3 (O*C*H_3). *Anal.* Calcd for $C_{31}H_{30}O_9$: C, 68.12; H, 5.53. Found: C, 68.15; H, 5.83.

Allyl 2,3-di-O-benzoyl-6-O-(4-methoxybenzyl)-β-D-glucopyranoside (**6**).—Trifluoroacetic acid (0.42 mL, 5.45 mmol) in dry DMF (3 mL) was added dropwise at 0°C to a stirred mixture of **5** (0.297 g, 5.45 mmol), sodium cyanoborohydride (0.171 g, 2.73 mmol), and 3A molecular sieves in dry DMF (45 mL). After 7 h the mixture was filtered through Celite and the filtrate was poured into ice-cold satd aq $NaHCO_3$ in a separating funnel. The aqueous phase was extracted thrice with CH_2Cl_2 and the combined extracts were washed with aq $NaHCO_3$, dried, and

concentrated. The residue was chromatographed on silica gel with 2:1 hexane–EtOAc to give **6** (185 mg, 62%) as a syrup; $[\alpha]_D$ +52.0° (*c* 1.9). ^{1}H NMR: δ 7.96–6.88 (5 m, 14 H, Ar-H), 4.73 (d, 1 H, $J_{1,2}$ 7.78 Hz, H-1), 4.55 (ABq, 2 H, OCH_2Ar), and 3.80 (s, 3 H, OCH_3). ^{13}C NMR: 167.0, 165.2 (2 *C*=O), 99.8 (C-1), 73.4, 69.9, 68.7 (C-6, OCH_2Ar), and 55.2 (OCH_3). *Anal.* Calcd for $C_{31}H_{32}O_9$: C, 67.87; H, 5.88. Found: C, 67.72; H, 5.94.

*Allyl 3-*O*-acetyl-2,4-di-*O*-methyl-α-*L*-fucopyranoside (**8**).*—Acetylation of **7**[6] (230 mg) with 1:1 acetic anhydride–pyridine (2 mL) with normal work-up afforded **8** (245 mg, 92%); $[\alpha]_D$ −180° (*c* 0.9). ^{1}H NMR: δ 5.17 (dd, 1 H, $J_{2,3}$ 10.59, $J_{3,4}$ 3.10 Hz, H-3), 5.01 (d, 1 H, $J_{1,2}$ 3.68 Hz, H-1), 3.51, 3.45 (2 s, 6 H, OCH_3), 2.12 (s, 3 H, OCOCH_3), and 1.22 (d, 3 H, $J_{5,6}$ 6.56 Hz, H-6). *Anal.* Calcd for $C_{13}H_{22}O_6$: C, 56.92; H, 8.08. Found: C, 56.55; H, 7.85.

*3-*O*-Acetyl-2,4-di-*O*-methyl-α,β-*L*-fucopyranose (**9**).*—A solution of **8** in 7:3:1 EtOH–toluene–water (15 mL) (210 mg) was boiled under reflux with tris(triphenylphosphine)rhodium(I) chloride (7 mg) and 1,4-diazabicyclo[2.2.2]octane (8 mg) for 17 h. The cooled solution was filtered through Celite and the filtrate was concentrated. The residue in acetone (15 mL) containing mercuric oxide (3 mg) was added to mercuric chloride (0.92 g) in 9:1 acetone–water (15 mL), and the mixture was stirred for 2 h at room temperature. The mixture was concentrated, the residue was dissolved in CH_2Cl_2, and the solution was washed with aq 30% KBr, then with water, dried, and concentrated. Chromatography of the residue on silica gel (15:1 CH_2Cl_2–acetone) furnished **9** (1:1 anomeric mixture) as an amorphous powder (160 mg, 89%); $[\alpha]_D$ −102° (*c* 1.1). ^{1}H NMR: δ 5.40 (d, 1 H, $J_{1,2}$ 2.58 Hz, H-1α), 5.15 (dd, 1 H, $J_{2,3}$ 10.34, $J_{3,4}$ 3.05 Hz, H-3α), 4.79 (dd, 1 H, $J_{2,3}$ 10.12, $J_{3,4}$ 3.15 Hz, H-3β), 4.58 (dd, 1 H, $J_{1,2} = J_{2,3} = 7.57$ Hz, H-1β), 4.23 (q, 1 H, $J_{5,6}$ 6.55 Hz, $J_{4,5}$ not observed, H-5α), 3.70 (dd, 1 H, H-2α), 3.65 (dq, 1 H, $J_{4,5}$ 0.95, $J_{5,6}$ 6.45 Hz, H-5β), 3.57 (s, 3 H, OCH_3), 3.52 (s, 6 H, OCH_3), 3.48 (s, 3 H, OCH_3 with H-4α obscured), 3.41 (dd, 1 H, $J_{4,5}$ 0.70, $J_{5,6}$ 3.09 Hz, H-4β), 3.35 (dd, 1 H, H-2β), 3.17 (br s, 1 H, O*H*), 2.12, 2.11 (2 s, 6 H, OCOCH_3), and 1.29, 1.24 (2 d, 6 H, $J_{5,6}$ 6.42, 6.56 Hz, H-6β, H-6α). *Anal.* Calcd for $C_{10}H_{18}O_6$: C, 51.27; H, 8.14. Found: C, 51.36; H, 7.71.

Allyl O*-(3-*O*-acetyl-2,4-di-*O*-methyl-α-*L*-fucopyranosyl)-(1 → 4)-2,3-di-*O*-benzoyl-6-*O*-(4-methoxybenzyl)-β-*D*-glucopyranoside (**10**).*—A mixture of **6** (305 mg, 0.55 mmol), silver trifluoromethanesulfonate (180 mg, 0.70 mmol), tetramethylurea (85 μL, 0.71 mmol), and powdered 4A molecular sieves in dry CH_2Cl_2 was stirred under N_2 for 0.5 h, and then cooled to −78°C. Glycosyl chloride [180 mg, 0.68 mmol, from **9** (160 mg) by reaction with *N*,*N*-dimethyl(chloromethylene)ammonium chloride[27]; δ_H 6.28 (d, 1 H, $J_{1,2}$ 3.78, H-1)] in dry CH_2Cl_2 (3 mL) was stirred under N_2 for 0.5 h, cooled to −78°C, and transferred to the vessel containing the acceptor, using a double-tipped needle. The mixture was stirred overnight and allowed to warm up to room temperature. Further additions of tetramethylurea and silver triflate were made after 20 and 45 h when TLC showed that only small amounts of acceptor and glycosyl halide still remained. These small

amounts of reactants still remained after 80 h, and the solution was filtered through Celite, which was washed with CH_2Cl_2. Combined filtrate and washings were washed with aq $NaHCO_3$ and water, dried, and concentrated. The residue was chromatographed on silica gel (3:1 hexane–EtOAc) to give **10** (189 mg, 43%); $[\alpha]_D$ −29.6° (*c* 0.23). ^{1}H NMR: δ 7.90–6.88 (m, 14 H, Ar-H), 5.65 (dd, 1 H, $J_{2,3}$ 9.58, $J_{3,4}$ 7.91 Hz, H-3), 5.32 (dd, 1 H, $J_{1,2}$ 7.90 Hz, H-2), 5.03 (dd + d, 2 H, $J_{1',2'}$ 4.02 Hz, H-1′ and H-3′), 4.70 (d, 1 H, H-1), 4.57 (ABq, 2 H, OCH_2Ar), 3.81 (s, 3 H, OCH_3), 3.55 (dd, 1 H, $J_{1',2'}$ 3.62, $J_{2',3'}$ 10.66 Hz, H-2′), 3.37, 3.36 (2 s, 6 H, OCH_3), 2.10 (s, 3 H, OCOCH_3), and 0.69 (d, 3 H, $J_{5',6'}$ 6.4 Hz, H-6′). *Anal.* Calcd for $C_{41}H_{48}O_{14}$: C, 64.38; H, 6.32. Found: C, 64.13; H, 6.63.

A minor fraction (24 mg) eluted from the column, which was not examined further, was provisionally identified as the β-linked product since ^{1}H NMR showed signals for the same substituents as **10**: δ 7.94–6.89 (4 m, 14 H, Ar-H), 5.71 (m, 1 H, H-3), 5.31 (dd, 1 H, $J_{1,2}$ 7.86, $J_{2,3}$ 9.97 Hz, H-2), 4.73 (d, 1 H, H-1), 4.57 (ABq, *J* 11.66 Hz, OCH_2Ar), 3.82, 3.44, 3.27 (3 s, 9 H, OCH_3), 2.03 (s, 3 H, OCOCH_3), and 1.22 (d, 3 H, $J_{5,6}$ 6.4 Hz, H-6′).

Allyl O-*(3-*O*-acetyl-2,4-di-*O*-methyl-α-*L*-fucopyranosyl)-(1 → 4)-2,3-di-O-benzoyl-β-*D*-glucopyranoside* (**11**).—Ceric ammonium nitrate (295 mg, 0.54 mmol) was added with stirring to **10** (205 mg, 0.27 mmol) in 9:1 acetonitrile–water (5 mL). After 1 h at room temperature reaction was complete, the solution was diluted with CH_2Cl_2 and washed with aq $NaHCO_3$, the aqueous layer was extracted twice with CH_2Cl_2, and the combined extracts were dried and concentrated. Separation on the Chromatotron with CH_2Cl_2, followed by 19:1 CH_2Cl_2–acetone, afforded **11** (153 mg, 62%); $[\alpha]_D$ −26.7° (*c* 1.2). ^{1}H NMR: δ 7.90–7.32 (3 m, 10 H, Ph-H), 5.09 (dd, 1 H, $J_{2',3'}$ 10.51, $J_{3',4'}$ 3.15 Hz, H-3′), 5.03 (d, 1 H, $J_{1',2'}$ 3.52 Hz, H-1′), 4.74 (d, 1 H, $J_{1,2}$ 7.93 Hz, H-1), 3.50, 3.39 (2 s, 6 H, OCH_3), 2.11 (s, 3 H, OCOCH_3), and 0.86 (d, 3 H, $J_{5',6'}$ 6.47 Hz, H-6′). *Anal.* Calcd for $C_{33}H_{40}O_{13}$: C, 61.48; H, 6.25. Found: C, 61.23; H, 6.14.

Methyl O-*(3-*O*-Acetyl-2,4-di-*O*-methyl-α-*L*-fucopyranosyl)-(1 → 4)-(allyl 2,3-di-O-benzoyl-β-*D*-glucopyranosid)uronate* (**12**).—Jones reagent [0.5 mL, from chromium trioxide (0.25 g) in 3.5 M H_2SO_4 (2 mL)], was added to **11** (11 mg) in acetone (1 mL) at 0°C. The solution was allowed to warm to room temperature and was monitored by TLC. After 1 h, with disappearance of starting material, the reaction was terminated by the addition of 2-propanol, the solution was diluted with water and extracted three times with EtOAc, and the extracts were dried and concentrated. The residue in CH_2Cl_2 was immediately treated with diazomethane in CH_2Cl_2 and, after concentration, the residue was chromatographed on silica gel (2:1 hexane–EtOAc) to yield **12** (8.6 mg); $[\alpha]_D$ −29.7° (*c* 1.0). ^{1}H NMR: δ 7.92–7.33 (3 m, 10 H, Ph-H), 5.67 (dd, 1 H, H-3), 5.37 (dd, 1 H, $J_{1,2}$ 7.57, $J_{2,3}$ 9.20 Hz, H-2), 5.01 (dd + d, 2 H, H-3 and H-1′), 4.80 (d, 1 H, H-1), 3.78 (s, 3 H, ester OCH_3), 3.37, 3.36 (2 s, 6 H, OCH_3), 2.09 (s, 3 H, OCOCH_3), and 0.76 (d, 3 H, $J_{5',6'}$ 6.46 Hz, H-6′). *Anal.* Calcd for $C_{34}H_{40}O_{14}$: C, 60.71; H, 5.99. Found: C, 60.81; H, 6.03.

Methyl O-(2,4-di-O-methyl-α-L-fucopyranosyl)-(1 → 4)-(allyl β-D-glucopyranosid)uronate (**13**).—Methanolic M NaOMe (0.1 mL) was added to **12** (8.6 mg) in dry MeOH (1 mL). After 24 h TLC showed complete disappearance of starting material with one major product and minor slower-moving impurities. The solution was treated with Dowex 50W-X8 (H^+) resin to remove sodium ions, filtered, and concentrated to a syrup which was chromatographed on silica gel (10:1 CH_2Cl_2–MeOH) to furnish **13** (4 mg); mp 127–129°C; $[\alpha]_D$ −112° (*c* 0.1). ^1H NMR: δ 5.09 (d, 1 H, $J_{1',2'}$ 3.67 Hz, H-1'), 4.40 (d, 1 H, $J_{1,2}$ 7.19 Hz, H-1), 3.79 (s, 3 H, ester OCH_3), 3.47, 3.39 (2 s, 6 H, OCH_3), and 1.31 (d, 3 H, $J_{5',6'}$ 6.56 Hz, H-6'). MS: *exact mass* calcd for $C_{18}H_{30}O_{11}$ + H + thioglycerol 531.2111; found: 531.2156.

Methyl 6-deoxy-2-O-methyl-α-L-xylo-hexopyranosid-4-ulose (**14**) *and the D-enantiomer* (**32**).—Methyl 2-*O*-methyl-α-L-fucopyranoside[16] (2.29 g, 11.9 mmol) in benzene (70 mL) containing dibutyltin oxide (6.22 g, 25 mmol) was boiled under reflux for 2 h with removal of water. Bromine was added dropwise to the cooled (0–5°C) solution until a yellow-red colour persisted. The mixture was stirred for 30 min at room temperature and then placed directly onto a column of silica gel in $CHCl_3$. Tin complexes were removed by washing the column with $CHCl_3$ and elution of the column with $CHCl_3$–acetone afforded **14** (1.85 g, 81.5%); $[\alpha]_D$ −177° (*c* 0.88). ^1H NMR: δ 4.97 (d, 1 H, $J_{1,2}$ 3.52 Hz, H-1), 4.55 (dd, 1 H, $J_{2,3}$ 9.88, $J_{3,OH}$ 2.82 Hz, H-3), 4.31 (q, 1 H, $J_{5,6}$ 6.54 Hz, H-5), 3.59, 3.53 (2 s, 6 H, OCH_3), 3.42 (overlapping dd and d, 2 H, H-2 and O*H*), and 1.33 (d, 3 H, H-6). ^{13}C NMR: δ 196.0 (C-4), 97.59 (C-1), 84.31, 75.79, 67.71 (C-2, C-3, C-5), 59.18, 56.08 (OCH_3), and 13.48 (C-6). *Anal.* Calcd for $C_8H_{14}O_5 \cdot H_2O$: C, 48.03; H, 7.75. Found: C, 48.21; H, 7.39.

The D-enantiomer **32** prepared in a similar manner gave spectra identical to those of **14** and had $[\alpha]_D$ +179° (*c* 1.12).

Methyl 4-acetamido-3-O-acetyl-4,6-dideoxy-2-O-methyl-α-L-galactopyranoside (**15**), *the D-enantiomer* (**33**), *and methyl 4-acetamido-3-O-acetyl-4,6-dideoxy-2-O-methyl-α-L-glucopyranoside* (**16**).—Hydroxylamine hydrochloride (1.81 g, 26.1 mmol) was added to a stirred solution of **14** (1.65 g, 8.7 mmol) in 1:1 pyridine–EtOH (15 mL) and the solution was kept at room temperature. After 2 h TLC (2:1 benzene–acetone) showed the absence of starting material and the formation of two products of lower mobility which were detected by spraying with cupric acetate. The solution was poured into ice–water and extracted 4 times with EtOAc. The combined extracts were dried, concentrated, and chromatographed on silica gel using 19:1 CH_2Cl_2–MeOH to give a syrupy mixture of stereoisomeric oximes (1.725 g, 96%), which were chromatographically separable and gave distinct but similar ^1H and ^{13}C NMR spectra. The oximes rapidly equilibrated on standing, and since catalytic reduction or treatment with lithium aluminum hydride, followed by acetylation, gave **15** and **16** in the same proportions, the originally separated mixture was used without further purification. Oximes (1.52 g, 7.4 mmol) in glacial acetic acid (30 mL) were hydrogenated over platinic oxide (Adams' catalyst, 0.6 g) for 1 h. The solution was filtered, the filtrate was concentrated, and the residual

mixture of amines was immediately dissolved in 1:1 pyridine–acetic anhydride and kept overnight at room temperature. Ice and water were added, the solution was stirred for 1 h before extraction with $CHCl_3$, and the dried extracts were concentrated. The mixture of products was chromatographed on silica gel (benzene–acetone mixtures) to give **15** (1.10 g, 54%) and **16** (0.25 g, 12%). Compound **15** was recrystallized from CH_2Cl_2–hexane and had mp 124°C, and $[\alpha]_D$ −142° (*c* 0.53). ^{1}H NMR: δ 5.82 (d, 1 H, $J_{NH,4}$ 9.45 Hz, N*H*COCH$_3$), 5.15 (dd, 1 H, $J_{2,3}$ 10.63, $J_{3,4}$ 4.15 Hz, H-3), 4.86 (d, 1 H, $J_{1,2}$ 3.81 Hz, H-1), 4.47 (ddd, 1 H, $J_{4,5}$ 1.62 Hz, H-4), 4.17 (qd, 1 H, $J_{5,6}$ 6.44 Hz, H-5), 3.46 (s, 3 H, OC*H*$_3$), 3.42 (s and m, 4 H, OC*H*$_3$ and H-2), 2.07 (s, 3 H, OCOC*H*$_3$), 2.02 (s, 3 H, NHCOC*H*$_3$), and 1.13 (d, 3 H, $J_{5,6}$ 6.53 Hz, H-6). MS: *exact mass* calcd for $C_{12}H_{21}NO_6$ + H: 363.1655; found: 363.1647.

Compound **16** was recrystallized from diisopropyl ether and had mp 179–180°C, and $[\alpha]_D$ −198° (*c* 0.96). ^{1}H NMR: δ 5.45 (d, 1 H, $J_{NH,4}$ 9.23 Hz, N*H*COCH$_3$), 5.14 (dd, 1 H, $J_{2,3}$ 10.1, $J_{3,4}$ 10.1 Hz, H-3), 4.84 (d, 1 H, $J_{1,2}$ 3.52 Hz, H-1), 3.90 (ddd, 1 H, $J_{4,5}$ 10.15 Hz, H-4), 3.46 and 3.42 (2 s, 6 H, OC*H*$_3$), 3.41 (dd, 1 H, H-2), 2.06 (s, 3 H, OCOC*H*$_3$), 1.93 (s, 3 H, NHCOC*H*$_3$), and 1.21 (d, 3 H, $J_{5,6}$ 6.24 Hz, H-6). *Anal.* Calcd for $C_{12}H_{21}NO_6$: C, 52.36; H, 7.38; N, 5.08. Found: C, 52.08; H, 7.87; N, 4.95.

Samples (5 mg) of **15** and **16** were each acetolyzed in 5:1 acetic anhydride–trifluoroacetic acid for 18 h at room temperature and solutions were concentrated. The resulting anomeric mixtures of glycosyl acetates were reduced with sodium borohydride in 3:1 MeOH–water for 2 h and, after removal of sodium ions and boric acid, the alditols were acetylated. Glycosides **15** and **16** each furnished single partially methylated alditol acetates, respectively 4-acetamido-3,5-di-*O*-acetyl-4,6-dideoxy-2-*O*-methyl-L-galactitol and 4-acetamido-3,5-di-*O*-acetyl-4,6-dideoxy-2-*O*-methyl-L-glucitol [retention times 14.76 and 19.91 min on a DB 225 column at 210°C], the former being inseparable from the derivative formed from the sugar liberated from the serovar 25 GPL. Examination by GLC–MS showed that each derivative had M^+ at *m/z* 347 and gave characteristic fragment ions at *m/z* 274, 260, 200, 158, 117, and 98. In synthesis in the D-series **32** was converted, as for **15**, into **33** which was spectroscopically indistinguishable from **15** and had mp 120–121°C, and $[\alpha]_D$ +144° (*c* 1.0).

*4-Acetamido-3-*O*-acetyl-4,6-dideoxy-2-*O*-methyl-*L*-galactose (**18**) via the anomeric glycosyl acetates (**17**).*—A solution of **15** (561 mg) in 100:40:1 acetic anhydride–acetic acid–H_2SO_4 (10 mL) was kept at room temperature for 20 h. The solution was poured into aq 10% K_2CO_3 (50 mL) at 0°C, stirred for 30 min and extracted with CH_2Cl_2 (2 × 75 mL). The dried extract was concentrated to a syrup which was separated using the Chromatotron with benzene–acetone mixtures to yield anomeric glycosyl acetates (**17**) (492 mg, 80.7%) ($\alpha:\beta > 3:1$). ^{1}H NMR: δ 6.35 (d, $J_{1,2}$ 3.90 Hz, H-1α), 5.91 (d, $J_{NH,4}$ 9.60 Hz, N*H*α), 5.75 (d, $J_{NH,4}$ 9.49 Hz, N-H*β*), 5.54 (d, $J_{1,2}$ 8.10 Hz, H-1*β*), 5.13 (dd, $J_{2,3}$ 10.64, $J_{3,4}$ 4.07 Hz, H-3α), 4.90 (dd, $J_{2,3}$ 10.15, $J_{3,4}$ 4.22 Hz, H-3*β*), 4.53 (ddd, $J_{4,5}$ 1.62 Hz, H-4α), 4.46 (ddd, $J_{4,5}$ 1.23 Hz, H-4*β*), 4.29 (m, $J_{4,5}$ 1.50 Hz, H-5α), 3.90 (m, H-5*β*), 3.51 (dd, H-2α), 3.42 (s, 3 H,

OCH_3), 3.30 (dd, H-2β), 2.15, 2.08, 2.04 (3 s, 9 H, $NHCOCH_3$ and $OCOCH_3\alpha$), 2.17, 2.07, 2.05 (3 s, 9 H, $NHCOCH_3$ and $OCOCH_3\beta$), 1.19 (d, $J_{5,6}$ 6.39 Hz, H-6β), and 1.13 (d, $J_{5,6}$ 6.46 Hz, H-6α). *Anal.* Calcd for $C_{13}H_{21}NO_6$: C, 51.48; H, 6.98; N, 4.62. Found: C, 51.48; H, 7.05; N, 4.63.

Glycosyl acetates **17** (446 mg) in DMF (5 mL) were treated with hydrazine acetate (162 mg) for 5 h at room temperature at which time TLC showed that most of the starting material had disappeared with formation of **18** with minor amounts of products of lower mobility. The solution was diluted with EtOAc, the extract was washed with satd aq NaCl, the aqueous washings were extracted twice with EtOAc, and the combined organic extracts were dried and concentrated. Separation of the residual syrup on the Chromatotron with dichloromethane–MeOH mixtures furnished **18** as a mixture of anomers (291 mg, 75.9%). ^{1}H NMR: δ 5.37 (d, $J_{1,2}$ 3.83 Hz, H-1α), 4.67 (d, $J_{1,2}$ 7.59 Hz, H-1β). *Anal.* Calcd for $C_{11}H_{19}NO_6$: C, 50.57; H, 7.33; N, 5.36. Found: C, 50.94; H, 6.91; N, 5.07.

*Allyl 4-acetamido-3-*O*-acetyl-4,6-dideoxy-2-*O*-methyl-α-*L*-galactopyranoside* (**19**). —A solution of **18** (153 mg) in trichloroacetonitrile (0.5 mL) and DMF (2 mL) was stirred with powdered 4A molecular sieves under Ar at room temperature. Freshly baked finely ground K_2CO_3 was added and after 2 h TLC showed complete disappearance of starting material. The mixture was filtered through Celite, which was washed with CH_2Cl_2, and the combined filtrate and washings were washed with aq $NaHCO_3$, dried, and concentrated. The residue was chromatographed on silica gel (ether–acetonitrile mixtures) to give samples of α-imidate (41 mg, 17%) and β-imidate (92 mg, 38%). The ^{1}H NMR spectrum of the β anomer showed: δ 8.72 (s, 1 H, N*H*), 5.75 (d, 1 H, $J_{NH,4}$ 8.10 Hz, N*H*$COCH_3$), 5.70 (d, 1 H, $J_{1,2}$ 8.01 Hz, H-1), 3.54 (s, 3 H, OCH_3), 2.09, 2.05 (2 s, 6 H, $OCOCH_3$, $NHCOCH_3$), and 1.23 (d, 3 H, $J_{5,6}$ 6.4 Hz, H-6).

The β-imidate (37 mg) was used directly by stirring with allyl alcohol (0.2 mL) and oven-dried, powdered 4A molecular sieves in dry CH_2Cl_2 (10 mL) under an N_2 atmosphere for 1 h at room temperature. Boron trifluoride etherate ($\sim$ 10 μL) was added and the mixture was stirred for 1 h at which time complete reaction of imidate had occurred. Solid $NaHCO_3$ was added and the mixture was stirred for 1 h before filtering through Celite. The filtrate and CH_2Cl_2 washings were concentrated and the residue in 9:1 CH_2Cl_2–acetone was purified by passage through a short silica gel column to yield **19** (12 mg). ^{1}H NMR: δ 5.93 (m, 1 H, C*H*=CH_2), 5.64 (d, 1 H, $J_{NH,4}$ 9.7 Hz, N*H*$COCH_3$), 5.30 (m, 2 H, CH=CH_2), 5.18 (dd, 1 H, $J_{3,4}$ 4.2, $J_{2,3}$ 10.7 Hz, H-3), 4.22–4.06 (m, 3 H, H-5 and OCH_2CH=CH_2), 3.44 (s, 3 H, OCH_3), 3.41 (dd, 1 H, H-2), 2.06, 2.02 (2 s, 6 H, $OCOCH_3$, $NHCOCH_3$), and 1.12 (d, 3 H, $J_{5,6}$ 6.5 Hz, H-6). *Anal.* Calcd for $C_{14}H_{23}NO_6$: C, 55.80; H, 7.69; N, 4.65. Found: C, 55.38; H, 7.66; N, 4.74.

O-Deacetylation of **19**.—Treatment of **19** with methanolic NaOMe furnished allyl 4-acetamido-4,6-dideoxy-2-*O*-methyl-α-L-galactopyranoside (**20**) (8 mg). ^{1}H NMR: δ 5.78 (d, 1 H, $J_{NH,4}$ 8.5 Hz, N*H*$COCH_3$), 5.00 (d, 1 H, $J_{1,2}$ 3.6 Hz, H-1),

3.48 (s, 3 H, OCH_3), 2.10 (s, 3 H, NHCOCH_3), and 1.15 (d, 3 H, $J_{5,6}$ 6.5 Hz, H-6). This product was used directly for conjugation to bovine serum albumin.

*Allyl 2,3-di-*O*-acetyl-4,6-*O*-(4-methoxybenzylidene)-β-*D*-glucopyranoside* (**21**).—A solution of **4** (4.59 g, 13.5 mmol) in 2:1 acetic anhydride–pyridine (215 mL) was stirred at room temperature for 5 h. The mixture was concentrated, dissolved in CH_2Cl_2, washed with water, dried, and again concentrated to give **21** (5.67 g, 99%) as a solid which was crystallized from $CHCl_3$–hexane and had mp 206–207°C, $[\alpha]_D$ −93° (*c* 1.0). ^{1}H NMR: δ 7.35, 6.87 (2 d, 4 H, Ar-H), 5.45 (s, 1 H, acetalic C*H*), 4.63 (d, 1 H, $J_{1,2}$ 7.83 Hz, H-1), 3.79 (s, 3 H, OCH_3), 2.05, and 2.03 (2 s, 6 H, OCOCH_3). *Anal.* Calcd for $C_{21}H_{26}O_9$: C, 59.70; H, 6.20. Found: C, 59.37; H, 6.20.

*Allyl 2,3-di-*O*-acetyl-β-*D*-glucopyranoside* (**22**).—A solution of **21** (5.21 g, 12.3 mmol) in 4:1 acetic acid–water (150 mL) was stirred at room temperature for 2.5 h and the solution was concentrated. The residue was chromatographed on silica gel (1:1 then 1:5 light petroleum–$CHCl_3$) to give **22** as a syrup (3.7 g, 98%); $[\alpha]_D$ −58.5° (*c* 1.5). ^{1}H NMR: δ 4.56 (d, 1 H, $J_{1,2}$ 7.6 Hz, H-1), 2.10, and 2.06 (2 s, 6 H, OCOCH_3). *Anal.* Calcd for $C_{13}H_{20}O_8$: C, 51.31; H, 6.62. Found: C, 51.46; H, 6.59.

*Allyl 2,3-di-*O*-acetyl-6-*O*-(4-methoxyphenyl)-β-*D*-glucopyranoside* (**23**).—A solution of **22** (1.56 g, 5.1 mmol), triphenylphosphine (1.75 g, 6.7 mmol), diethyl azodicarboxylate (1.05 mL, 6.7 mmol) and 4-methoxyphenol (1.91 g, 15.4 mmol) in dry oxolane (30 mL) was boiled under reflux for 1 h. The solvent was evaporated and the product was chromatographed on silica gel (2:1 light petroleum–EtOAc) to furnish **23** as a syrup (1.36 g, 65%); $[\alpha]_D$ −40.3° (*c* 0.9). ^{1}H NMR: δ 6.9–6.8 (m, 4 H, Ar-H), 4.56 (d, 1 H, $J_{1,2}$ 7.5 Hz, H-1), 3.76 (s, 3 H, OCH_3), 2.10, and 2.05 (2 s, each 3 H, 2 OCOCH_3). *Anal.* Calcd for $C_{20}H_{26}O_9$: C, 58.53; H, 6.38. Found: C, 58.21; H, 6.34.

*2-Pyridyl 4-acetamido-3-*O*-acetyl-4,6-dideoxy-2-*O*-methyl-1-thio-α-* (**24**) *and -β-*L*-galactopyranoside* (**25**), *and the* D*-enantiomers* (**34** *and* **35**).—A solution of **18** (562 mg, 2.15 mmol), 2,2′-dipyridyl disulfide (Aldrithiol, 591 mg), and tributylphosphine (0.67 mL) in dry CH_2Cl_2 (25 mL) was stirred overnight under N_2 at room temperature. After removal of solvent the residue was chromatographed on silica gel (10:1 ether–acetone) to give **24** (348 mg, 46%) and **25** (110 mg, 15%). Compound **24** had mp 81–82°C; $[\alpha]_D$ −258° (*c* 1.28). ^{1}H NMR: δ 8.49–7.03 (m, 4 H, Ar-H), 6.83 (d, 1 H, $J_{1,2}$ 5.91 Hz, H-1), 5.65 (d, 1 H, $J_{4,NH}$, 9.5 Hz, N*H*), 3.45 (s, 3 H, OCH_3), 2.05, 2.04 (2 s, 6 H, NHCOCH_3, OCOCH_3), and 1.09 (d, 3 H, $J_{5,6}$ 6.4 Hz, H-6). *Anal.* Calcd for $C_{16}H_{22}N_2O_5S$: C, 54.22; H, 6.25; N, 7.90. Found: C, 54.38; H, 6.19; N, 7.80.

Compound **25** had mp 70–72°C, $[\alpha]_D$ +17° (*c* 0.95). ^{1}H NMR: δ 8.49–7.07 (m, 4 H, Ar-H), 6.20 (d, 1 H, $J_{4,NH}$ 9.8 Hz, N*H*), 5.14 (d, 1 H, $J_{1,2}$ 9.9 Hz, H-1), 3.49 (s, 3 H, OCH_3), 2.12, 2.06 (2 s, 6 H, NHCOCH_3, OCOCH_3), and 1.19 (d, 3 H, $J_{5,6}$ 6.4 Hz, H-6). *Anal.* Calcd for $C_{16}H_{22}N_2O_5S$: C, 54.22; H, 6.25; N, 7.90. Found:C, 54.28; H, 6.32; N, 7.41.

In the D-series thioglycosides **34** and **35** were prepared as an anomeric mixture and used without separation.

Allyl O-(4-acetamido-3-O-acetyl-4,6-dideoxy-2-O-methyl-α-L-galactopyranosyl)-(1 → 4)-2,3-di-O-acetyl-6-O-(4-methoxyphenyl)-β-D-glucopyranoside (**26**).—Methyl iodide (1 mL) was added to a stirred solution of **24** (220 mg, 0.62 mmol) and **23** (273 mg, 0.66 mmol) in dry CH_2Cl_2 (10 mL) in the presence of 4A molecular sieves and under N_2, and the mixture was kept at 50°C for 48 h. TLC showed incomplete consumption of **24**, further MeI (1 mL) was added, and reaction was extended for another 24 h. The reaction mixture was filtered through Celite, the combined filtrate and washings were concentrated, and the residue was chromatographed on silica gel (4:1 $CHCl_3$–acetone) to give **26** (336 mg, 83%) as a solid which was recrystallized from CH_2Cl_2–ether and had mp 235–236°C, $[\alpha]_D$ −83° (*c* 1.07). ^{1}H NMR: δ 6.87–6.80 (m, 4 H, Ar-H), 5.43 (d, 1 H, $J_{4',NH}$ 9.7 Hz, N*H*), 4.97 (d, 1 H, $J_{1',2'}$ 3.5 Hz, H-1′), 4.58 (d, 1 H, $J_{1,2}$ 8.0 Hz, H-1), 3.76, 3.14 (2 s, 6 H, OCH_3), 2.03, 1.99 (2 s, 12 H, NHCOCH_3, OCOCH_3), and 1.04 (d, 3 H, $J_{5',6'}$ 6.4 Hz, H-6′). *Anal.* Calcd for $C_{31}H_{43}NO_{14}$: C, 56.96; H, 6.63; N, 2.14. Found: C, 56.69; H, 6.64; N, 2.16.

Allyl O-(4-acetamido-3-O-acetyl-4,6-dideoxy-2-O-methyl-α-L-galactopyranosyl)-(1 → 4)-2,3-di-O-acetyl-β-D-glucopyranoside (**27**).—A solution of **26** (288 mg, 0.44 mmol) and ceric ammonium nitrate (621 mg) in 4:1 acetonitrile–water (10 mL) was stirred at room temperature for 10 min. The solution was partitioned between brine and EtOAc, the organic layer was washed twice with aq $NaHCO_3$, dried, concentrated, and chromatographed on silica gel (2:1 CH_2Cl_2–acetone) to give **27** (239 mg, 99%) as a solid which after recrystallization from ether had mp 172–173°C, $[\alpha]_D$ −134° (*c* 1.0). ^{1}H NMR: δ 5.50 (d, 1 H, $J_{4',NH}$ 9.4 Hz, N*H*), 5.08–5.03 (unresolved m, 2 H, H-3′,1′), 3.52 (s, 3 H, OCH_3), 2.06, 2.03, 2.02 (3 s, 12 H, NHCOCH_3, OCOCH_3), and 1.04 (d, 3 H, $J_{5',6'}$ 6.4 Hz, H-6′). *Anal.* Calcd for $C_{24}H_{37}NO_{13}$: C, 52.64; H, 6.81; N, 2.55. Found: C, 52.28; H, 6.75; N, 2.59.

Methyl O-(4-acetamido-3-O-acetyl-4,6-dideoxy-2-O-methyl-α-L-galactopyranosyl)-(1 → 4)-(allyl 2,3-di-O-acetyl-β-D-glucopyranosid)uronate (**28**).—Chromium trioxide (190 mg) in 3.5 M H_2SO_4 (1.5 mL) was added with stirring to an ice-cooled solution of **27** (30 mg, 0.54 mmol) in acetone (2.5 mL), the mixture was stirred at room temperature for 3.5 h when reaction was terminated by the addition of 2-propanol, water was added, and the mixture was extracted with EtOAc. The dried organic extract was concentrated, the residue was treated immediately with diazomethane in CH_2Cl_2 and, after concentration, was chromatographed on silica gel (2:1 $CHCl_3$–acetone) to yield **28** (13 mg, 42%); mp 83–85°C; $[\alpha]_D$ −92° (*c* 1.2). ^{1}H NMR: δ 5.46 (d, 1 H, $J_{4',NH}$ 9.7 Hz, N*H*), 4.99 (d, 1 H, $J_{1',2'}$ 3.2 Hz, H-1′), 4.61 (d, 1 H, $J_{1,2}$ 7.7 Hz, H-1), 3.74 (s, 3 H, CO_2CH_3), 3.35 (s, 3 H, OCH_3), 2.04, 2.03, 2.00 (3 s, 12 H, NHCOCH_3, OCOCH_3), and 1.03 (d, 3 H, $J_{5',6'}$ 6.4 Hz, H-6′). *Anal.* Calcd for $C_{25}H_{37}NO_{14}$: C, 52.17; H, 6.48; N, 2.43. Found: C, 52.24; H, 6.76; N, 2.32.

Oxidation of **27** *with subsequent O-deacetylation.*—Treatment of a further portion of **27** with Jones reagent, as just described, followed by chromatographic separation on silica gel (4:3 acetone–2-propanol) afforded **29** (44%) as a solid; mp

235°C (dec); $[\alpha]_D$ −105° (*c* 1.0). ^{1}H NMR: δ 5.30–4.91 (m, 6 H, H-2,3,1′,3′, and CH_2), 4.69 (d, 1 H, $J_{1,2}$ 7.4 Hz, H-1), 3.40 (s, 3 H, OCH_3), 2.06, 2.05, 2.04, 2.02 (4 s, 12 H, NHCOCH_3, OCOCH_3), and 1.06 (d, 3 H, $J_{5',6'}$ 6.4 Hz, H-6′).

Catalytic *O*-deacetylation of **29** with methanolic NaOMe, followed by removal of sodium ions with cation-exchanger and concentration furnished *O*-(4-acetamido-4,6-dideoxy-2-*O*-methyl-α-D-galactopyranosyl)-(1 → 4)-(allyl β-D-glucopyranosid)uronic acid (**30**); mp 106–108°C; $[\alpha]_D$ −118° (*c* 1.09, MeOH). ^{1}H NMR (MeOH-d_4): δ 7.86 (d, 1 H, $J_{4',NH}$ 9.9 Hz, N*H*), 4.79 (d, 1 H, $J_{1',2'}$ 3.79 Hz, H-1′), 4.30 (d, 1 H, $J_{1,2}$ 7.5 Hz, H-1), 3.40 (s, 3 H, OCH_3), 1.96 (s, 3 H, NHCOCH_3), and 0.97 (d, 3 H, $J_{5',6'}$ 6.17 Hz, H-6′).

Allyl O-*(4-acetamido-3-*O*-acetyl-4,6-dideoxy-2-*O*-methyl-α-D-galactopyranosyl)-(1 → 4)-2,3-di-*O*-acetyl-6-*O*-(4-methoxyphenyl)-β-D-glucopyranoside* (**36**).—Methyl iodide (2 mL) was added to a stirred solution of a mixture of **34** and **35** (240 mg, 0.6 mmol) and **23** (363 mg, 0.88 mmol) in dry CH_2Cl_2 (20 mL) in the presence of 4A molecular sieves, and under N_2, and the mixture was kept at 50°C for 48 h. Work up as for **26** afforded **36** as a foam (323 mg, 73%) which was recrystallized from CH_2Cl_2–ether, and had mp 170–171°C, $[\alpha]_D$ +50° (*c* 0.5). ^{1}H NMR: δ 6.88–6.50 (m, 4 H, Ar-H), 4.58 (d, 1 H, $J_{1,2}$ 8.81 Hz, H-1), 3.76, 3.41 (2 s, 6 H, OCH_3), 2.05, 2.04, 2.01, 2.00 (4 s, 12 H, NHCOCH_3, OCOCH_3), and 0.80 (d, 3 H, $J_{5',6'}$ 6.5 Hz, H-6′); MS: *exact mass* calcd for $C_{31}H_{43}NO_{14}$ + H: 654.2646; found: 654.2712. *Anal.* Calcd for $C_{31}H_{43}NO_{14}$: C, 56.96; H, 6.63; N, 2.14. Found: C, 56.25; H, 6.61; N, 2.12.

Allyl 4-O-*(4-acetamido-3-*O*-acetyl-4,6-dideoxy-2-*O*-methyl-α-D-galactopyranosyl)-(1 → 4)-2,3-di-*O*-acetyl-β-D-glucopyranoside* (**37**).—A solution of **36** (300 mg, 0.45 mmol) and ceric ammonium nitrate (754 mg) in 4:1 acetonitrile–water (10 mL) was stirred at room temperature for 15 min. The mixture, worked up as for **27**, gave **37** as a solid (220 mg, 88%) which, after recrystallization from CH_2Cl_2–ether, had mp 113–115°C, $[\alpha]_D$ +31.3° (*c* 0.74). ^{1}H NMR: δ 5.62 (d, 1 H, $J_{4,NH}$ 9.44 Hz, N*H*), 4.56 (d, 1 H, $J_{1,2}$ 8.0 Hz, H-1); 3.40 (s, 3 H, OCH_3), 2.04, 2.02, 2.00 (3 s, 12 H, NHCOCH_3, OCOCH_3), and 1.13 (d, 3 H, $J_{5',6'}$ 6.53 Hz, H-6′). MS: *exact mass* calcd for $C_{24}H_{37}NO_{13}$ + H: 548.2343; found: 548.2336.

Methyl O-*(4-Acetamido-3-*O*-acetyl-4,6-dideoxy-2-*O*-methyl-α-D-galactopyranosyl)-(1 → 4)-(allyl 2,3-di-*O*-acetyl-β-D-glucopyranosid)uronate* (**39**).—Oxidation of **37** (75 mg, 0.13 mmol) in acetone (7 mL) with chromium trioxide (502 mg) in 3.5 M H_2SO_4 (4 mL) followed by esterification of the product with diazomethane, as described for **28**, furnished **39** (28 mg, 35%) which, after recrystallization from ether, had mp 183–185°C, $[\alpha]_D$ +29° (*c* 0.5). ^{1}H NMR: δ 5.09 (d, 1 H, $J_{1',2'}$ 4.0 Hz, H-1′), 4.60 (d, 1 H, $J_{1,2}$ 7.5 Hz, H-1), 3.80, 3.39 (2 s, 6 H, OCH_3), 3.30 (dd, 1 H, $J_{2',3'}$ 10.74 Hz, H-2′), 2.04, 2.03, 2.00 (3 s, 12 H, NHCOCH_3, OCOCH_3), and 1.07 (d, 3 H, $J_{5',6'}$ 6.47 Hz, H-6′). MS: *exact mass* calcd for $C_{25}H_{37}NO_{14}$ + H: 576.2292; found: 576.2274. *Anal.* Calcd for $C_{25}H_{37}NO_{14}$: C, 52.17; H, 6.48; N, 2.43. Found: C, 51.45; H, 6.35; N, 2.43.

O-*(4-Acetamido-4,6-dideoxy-2-*O*-methyl-α-D-galactopyranosyl)-(1 → 4)-(allyl β-*

D-*glucopyranosid)uronic acid* (**40**).—Oxidation of **37** as just described followed by chromatographic separation on silica gel (4:3 acetone–2-propanol) afforded **38** (42%) as a solid which, after recrystallization from CH_2Cl_2–ether, had mp 140–142°C, $[\alpha]_D$ +42° (*c* 0.7, MeOH). ^{1}H NMR: δ 4.64 (d, 1 H, $J_{1,2}$ 7.4 Hz, H-1), 3.41 (s, 3 H, OCH_3), 2.05, 2.00 (2 s, 12 H, $NHCOCH_3$, $OCOCH_3$), and 1.06 (s, 3 H, $J_{5',6'}$ 6.14 Hz, H-6′).

Catalytic *O*-deacetylation of **38** with methanolic NaOMe, followed by removal of sodium ions with cation-exchanger and concentration afforded **40** as a solid, mp 116–118°C; $[\alpha]_D$ +70.7° (*c* 0.6, MeOH). ^{1}H NMR (D_2O): δ 5.66 (d, 1 H, $J_{1',2'}$ 3.97 Hz, H-1′), 4.58 (d, 1 H, $J_{1,2}$ 7.95 Hz, H-1), 3.51 (s, 3 H, OCH_3), 2.09 (s, 3 H, $NHCOCH_3$), and 1.07 (d, 1 H, $J_{5',6'}$ 6.45 Hz, H-6′). MS: *exact mass* calcd for $C_{18}H_{29}NO_{11}$ + H: 436.1825; found: 436.1815.

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