THE UNIVERSITY OF ALBERTA

SYNTHETIC ACCEPTORS FOR GLYCOSYLTRANSFERASES

by

SYED HASAN TAHIR

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submitted by SYED HASAN TAHIR

in partial fulfilment of the requirements for the degree of MASTER OF SCIENCE

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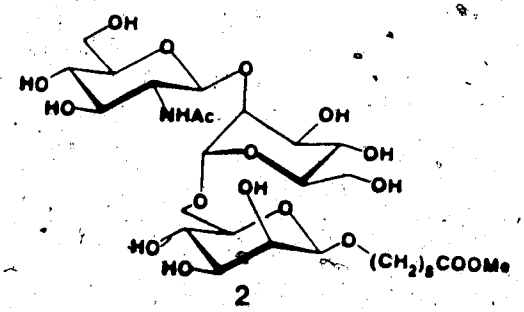
DATE February 13, 1986

ABSTRACT

Oncogenic transformation frequently results in the production of abnormal cell-surface carbohydrates, known as "tumor-associated carbohydrates", and it has been shown that these carbohydrate structures can result from a change in the enzymatic activity of a single glycosyltransferase.

This work was aimed at developing a selective assay for monitoring changes in the intracellular activity of such a known tumor-elevated glycosyltransferase, termed N-acetylglucosaminyl transferase V (GlcNAc transferase V), by using appropriately designed synthetic oligosaccharide substrates. To this end, the trisaccharide β-D-GlcNAc(1→2)α-D-Man(1→6)β-D-Man-O$(CH_2)_8COOCH_3$ (**2**) and the related tetrasaccharide β-D-GlcNAc(1→2)-α-D-Man(1→6)[α-D-Man(1→3)]-β-D-Man-O$(CH_2)_8COOCH_3$ (**3**) have been chemically synthesized as potential selective acceptors for this enzyme.



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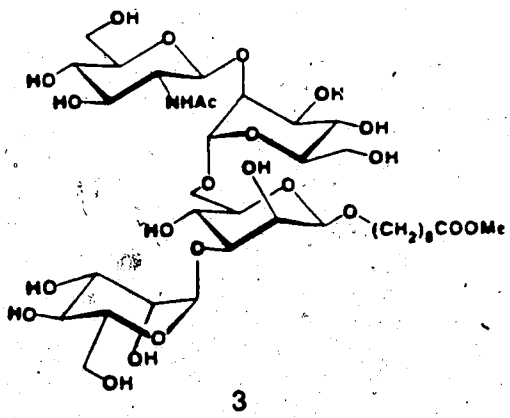

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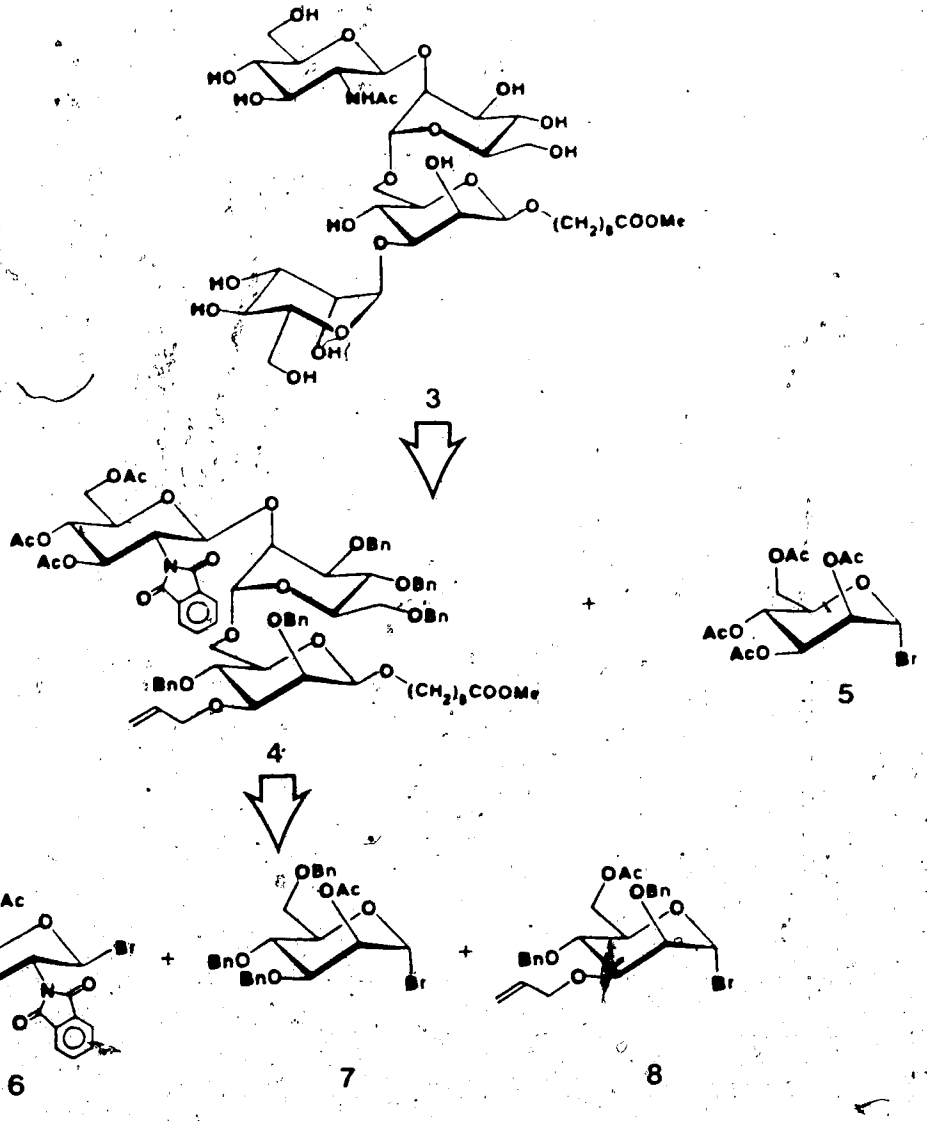
The multi-step syntheses to prepare the oligosaccharides 2 and 3 involved sequential Koenigs Knorr glycosylations of selectively protected carbohydrate derivatives and are summarized in the retrosynthetic scheme shown below:



The trisaccharide 2 was found to be a selective acceptor for GlcNAc transferase V.

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CHAPTER 1

INTRODUCTION

The cell-surface carbohydrates of eukaryotic cells are made up of the sugar chains of glycoproteins and glycolipids and the more loosely associated collagen, heparan sulfate and fibronectin. The covalently attached complex carbohydrates of glycoproteins and glycolipids are usually between two and fifteen sugar units long and typically make up 2-10% of the plasma membrane weight.[1] The carbohydrate chains of these latter glycoconjugates project outward from the cell and, despite their relatively low abundance, it is believed[1] that they almost entirely cover the cell surface and thus form the first layer of interaction with other cells.

Many of the complex carbohydrates appear to have physicochemical functions such as modifying solubility, stabilizing protein conformation and protecting glycoproteins against proteolysis.[2] Current research in the biological sciences is, however, focusing on the possible role of these highly diverse structures as recognition markers which may direct phenomena as diverse as the binding of hormones, toxins, enzymes, viruses and

bacteria to the cell and as "traffic signals" which control the circulation of both glycoproteins and whole cells.[3] Although much of the evidence to support the role of complex carbohydrates in mediating these diverse processes is still largely circumstantial, a handful of structures have been assigned definitive physiological functions. These critical functions include that of β-D-galactose as a recognition marker in the hepatic clearance of serum glycoproteins[4] and of mannose-6-phosphate residues in the targeting of newly-biosynthesized glycoproteins to the lysosomes.[5]

About ten years ago, evidence began to accumulate that the carbohydrate structures of both cell-surface glycoproteins and glycolipids became dramatically altered during both normal and abnormal cellular development.[6-8] Consistent changes in cell-surface carbohydrate structures have now been shown to accompany the development of human melanoma, neuroblastoma and colorectal, gastric and pancreatic carcinoma.[9] The occurrence of large fucosylated highly-branched glycopeptides is in fact one of the most reproducible correlates with the malignant transformation of cells.[10] The functional significance, if any, of these cell-surface structural changes is not at all clear but these aberrant carbohydrate structures are attracting a great deal of clinical interest as potential

tumor markers. The structures of many of these "tumor-associated" carbohydrates have been elucidated in recent years and major research efforts have gone into the production of monoclonal antibodies against many of these structures.[8,11] One of these monoclonal antibodies, termed CA19-9, is already in wide clinical use as a prognostic monitor for colorectal cancer.[12,13]

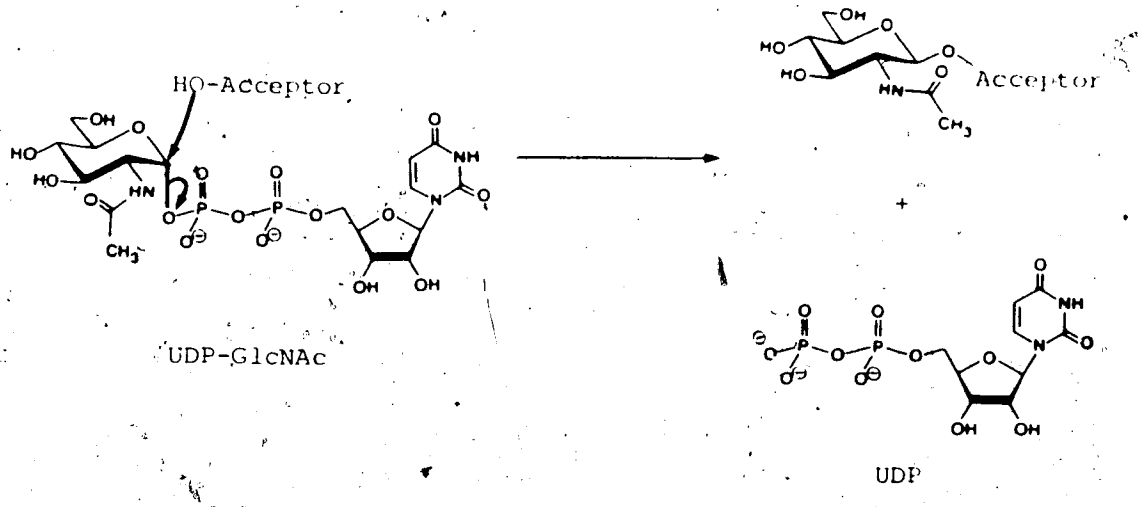
Tumor-associated oligosaccharides are the manifestation of altered carbohydrate biosynthesis and could, in principle, result from any of a large number of cellular irregularities. Glycosylation of proteins is a co- or post-translational modification which requires the sequential action of a series of enzymes (glycosyltransferases) and co-factors (sugar nucleotides) and occurs in the endoplastic reticulum and golgi vesicles.[14] A change in the cellular levels of the glycosyltransferases, the sugar nucleotides or their transport proteins, the biosynthetic precursors of the sugar nucleotides, glycosylhydrolases, or even mild disruptions of the membrane integrities of intracellular organelles could account for the observed changes in cell-surface carbohydrate structures. Because of this tremendous complexity the correlation of the expression of tumor-associated cell-surface carbohydrates with a single transformation-induced molecular event has until very

recently remained elusive.

In 1984, Yamashita et al.[10] compared the carbohydrate structures of the membrane N-linked glycoproteins of baby hamster kidney (BHK) cells and their polyoma transformant (Py-BHK). They found that while the transformed cells produced the same approximately twenty structures as did the normal cells, they produced more of the larger more highly branched oligosaccharides. They proposed that the changes in the relative proportion of the cell-surface oligosaccharides observed on transformation could be explained by the elevation in the activity of a single enzyme, a glycosyltransferase, termed N-Acetyl-β-D-glucosaminyl transferase V (GlcNAc transferase V, GnT V). They subsequently validated this proposal in 1985[15] when they showed that the GnT V activity in Py-BHK cells was in fact elevated two-fold when compared with untransformed cells. This elegant work provided the first demonstration that the changes in cell surface carbohydrates observed on oncogenic transformation could result from a change in the activity of a single glycosyltransferase. Yamashita et al.'s work[10,15] therefore suggests that the activity of a single glycosyltransferase can, in itself, serve as a tumor marker. The detection of a change in a single specific enzymatic activity should be far simpler than the characterization and quantification

of a highly heterogeneous mixture of cell-surface carbohydrate structures produced as a result of this single enzymatic change.

This thesis is concerned with the development of a rapid and convenient assay for measuring the activity of glycosyltransferases, in particular of Yamashita et al.'s elevated GnT V. Glycosyltransferases catalyze the transfer of a glycosyl residue, usually from a sugar nucleotide (termed the donor), to the hydroxyl group of another sugar (termed the acceptor). The sequential action of a large number of such glycosyltransferases is required for the synthesis of complex oligosaccharides. The chemical reaction catalyzed by GlcNAc transferases is the transfer of an N-acetylglucosaminyl residue from uridine-diphospho-N-acetylglucosamine (UDP-GlcNAc) to a hydroxyl group of some acceptor sugar:



The products of the reaction are uridine-diphosphate (UDP) and a β-linked di- or oligosaccharide.

At least 9 different GlcNAc transferases are known to be involved in the biosynthesis of the asparagine-linked oligosaccharides.[3,10,15,16] All of these enzymes use UDP-GlcNAc as the glycosyldonor and the difference between them lies in their specificity for different acceptor structures. A composite structure of the core region of known asparagine-linked oligosaccharides is shown in Figure 1. The oligosaccharides are all attached to protein asparagine residues (N-linked) through a common chitobiose linkage, βDGlcNAc(1→4)βDGlcNAc-Asn. The GlcNAc transferases responsible for generating the diversity of structures observed on cell surface N-linked glycoproteins are labelled GnT I, II, III, IV and V and the GlcNAc residues they transfer are labelled in the same fashion.[15] In the naturally occurring structures other sugar residues, notably D-galactose and L-fucose, are added onto these GlcNAc residues to produce the completed structures. GnT's I-V transfer βDGlcNAc to D-mannose residues, but to different hydroxyl groups of the three different mannose residues. These enzymes are all located in the rough endoplasmic reticulum and in the golgi apparatus of cells where they frequently compete for common glycoprotein substrates.

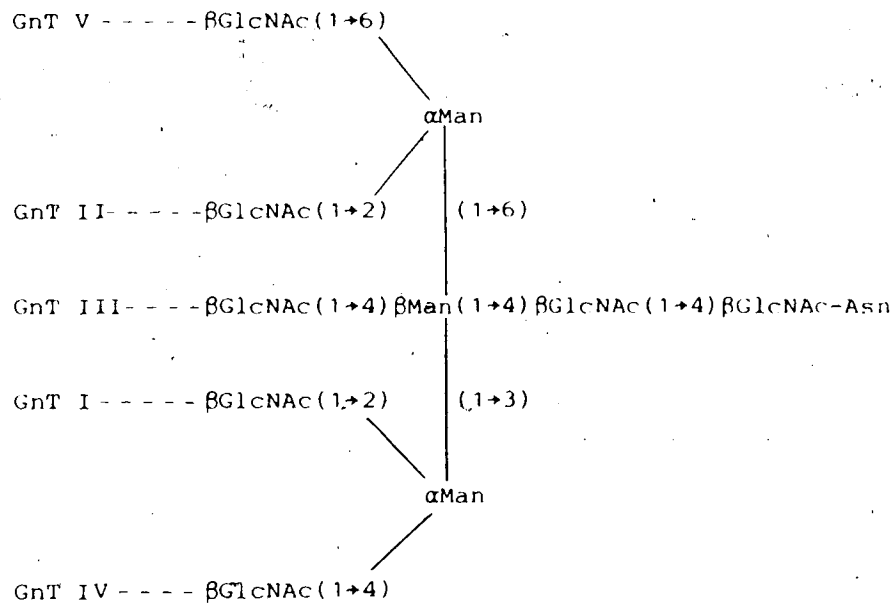


Figure 1. A hypothetical structure showing the βDGlcNAc units added by GlcNAc-transferases I-V (GnT I-V).[15]

A portion of the biosynthetic pathway elucidated by Yamashita et al. for the production of BHK cell-surface glycoproteins is shown in Figure 2. This biosynthetic scheme, reproduced from Yamashita et al.,[15] shows how the product of one glycosyltransferase reaction can frequently be a substrate for several competing glycosyltransferases. The elevation of GnT V in Py-BHK cells results in a shunt in the normal biosynthetic branching indicated by the bold face arrows with the resultant increase in the larger, more highly branched oligosaccharide structures. GnT V transfers βDGlcNAc exclusively to the 6-hydroxyl group of the α(1→6) linked D-mannose unit of the glycopeptide.

Assaying the activity of these various glycosyltransferases, which are present in only minute amounts, invariably involves the measurement of the transfer of a radiolabelled GlcNAc residue from ^{14}C or ^{3}H-UDP-GlcNAc to a suitable acceptor oligosaccharide, followed by isolation and counting of the product.[15,16] The difficulty encountered with assaying the GnT's is that they all use the same sugar nucleotide and several of them may act on any given substrate. As seen in Figure 2, in the biosynthesis of N-linked glycopeptides, GnT V acts on hepta- and octasaccharidic substrates. In their landmark work,[15] Yamashita et al. isolated their substrates from

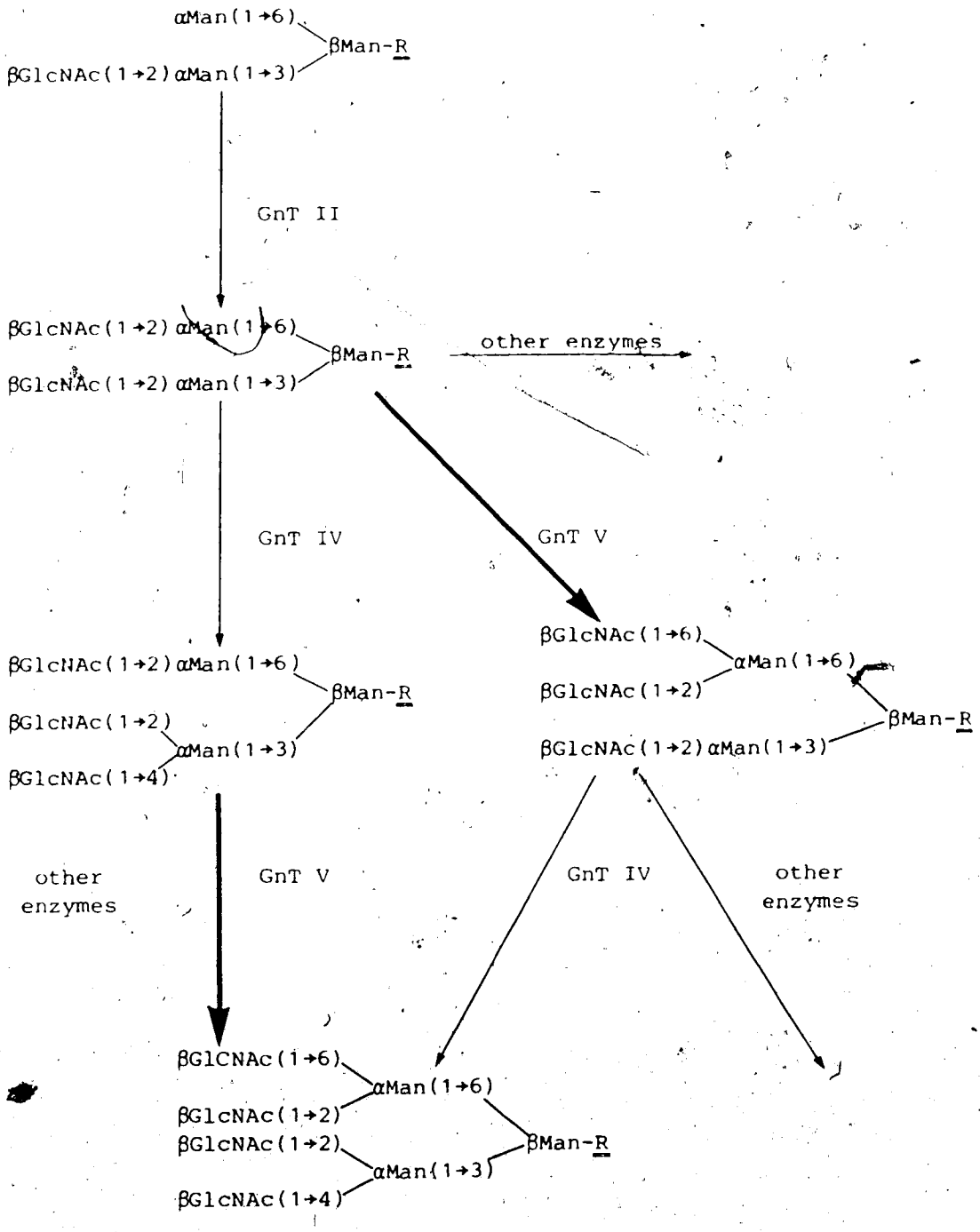


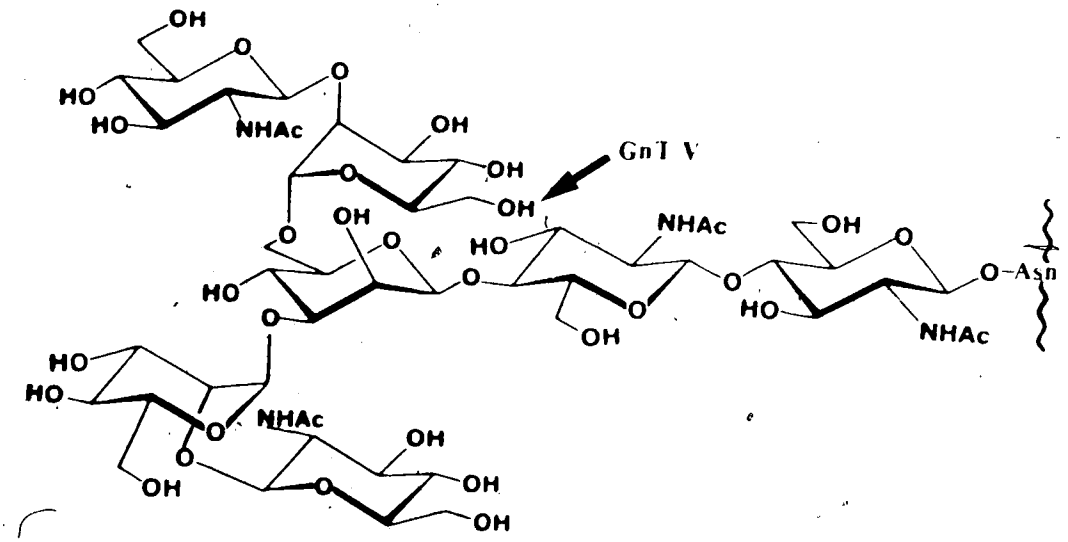
Figure 2. Partial pathway showing the involvement of GlcNAc-transferase V (GnT V) in the biosynthesis of complex sugar chains.[15] R represents βDGlcNAc(1→4)βDGlcNAc-Asn.

the urine of patients with metabolic disorders (mannosidosis, fucosidosis and GM_1-gangliosidosis) and frequently had to further process these structures by removal of outer sugar residues with specific glycosyl hydrolases. Such procedures are not only labor intensive and time consuming but are clearly not general. The amount of material that may be obtained is also limited. Yamashita et al.'s separation of the radioactive products of the enzymatic reactions required high-voltage paper electrophoresis, gel-permeation chromatography, treatment with glycosyl hydrolases and re-chromatography. Both the difficulty and impractability of such glycosyltransferase assays are considered a major obstacle in the study of glycosyltransferase tumor markers.

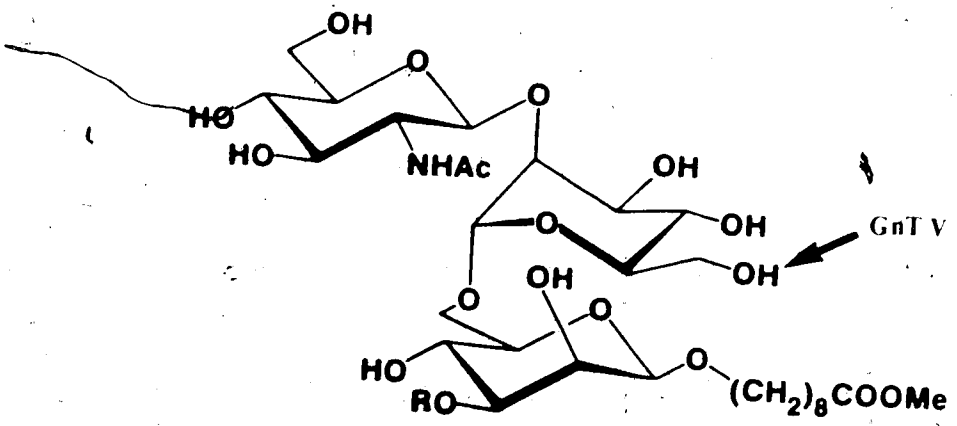
Our approach to this problem was to chemically synthesize oligosaccharides which would, we hoped, be recognized by only a single enzyme thus producing a substrate which could be used in an assay specific for that single glycosyltransferase activity. Such substrates might include oligosaccharides where cross-reacting sugar residues were completely absent or where the interfering hydroxyl groups which might be acted on by other GlcNAc transferases were masked by either O-methylation or deoxygenation. This latter approach has recently[17,18] been successfully applied in the differentiation of two

competing α-L-fucosyltransferases in serum. To be truly useful, such substrates and their glycosylated products should also be amenable to simple rapid isolation from the incubation mixtures of cell extracts or fluids which contain the glycosyltransferase activities being assayed.

In order to test the practicability of this approach we chose to attempt the preparation of substrates selective for Yamashita et al.'s GnT V since this glycosyltransferase was already attracting wide interest as a potential tumor marker. The smallest known substrate for GnT V is the heptasaccharide **1**, shown in Figure 3, which was isolated and used by Yamashita et al.[15] as described above. Recent work,[19-23] from the laboratory of Lemieux and our own, on the molecular basis for the binding of oligosaccharides by monoclonal antibodies and lectins strongly suggested that proteins were not likely to require carbohydrate surfaces much larger than that of a trisaccharide for faithful recognition. We expected that this situation might also hold true for the enzymatic specificity of glycosyltransferases and therefore envisioned the trisaccharide βDGlcNAc(1→2)αDMan(1→6)βDManOR (**2**) as a likely acceptor for GnT V. Our rationale for the choice of **2** can be seen in Figure 3 where the structure of the natural heptasaccharide acceptor **1** is also shown. We set forth,



1: βGlcNAc(1→2)αMan(1→6)
βGlcNAc(1→2)αMan(1→3) → βMan(1→4)βGlcNAc(1→4)βGlcNAc-Asn



2: R=H, βGlcNAc(1→2)αMan(1→6) → βMan-O(CH$_2$)$_8$COOMe

3: R=αMan, βGlcNAc(1→2)αMan(1→6), αMan(1→3) → βMan-O(CH$_2$)$_8$COOMe

Figure 3. A heptasaccharide acceptor (1) for GlcNAc-transferase V (GnT V) compared with the structures of the proposed synthetic acceptors 2 and 3. The bold arrows show the primary hydroxyl group to which GnT V transfers a N-acetyl-β-D-glucosaminyl residue.

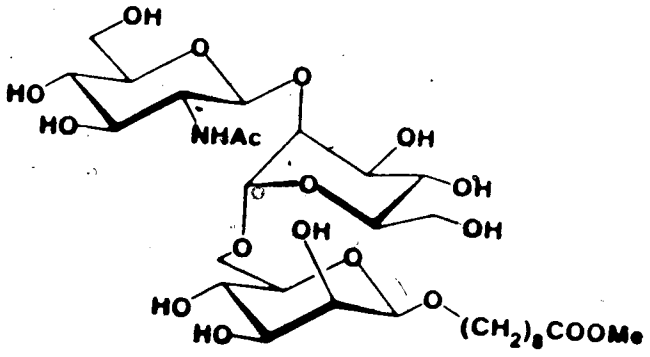
therefore, to synthesize 2, which contains the reactive target hydroxyl group acted on by the enzyme and one sugar residue on either side of the α-D-mannose residue bearing this hydroxyl group. Since we did not know whether this structure incorporated sufficient features for recognition by GnT V, the synthesis was planned in a way that also allowed the preparation of the tetrasaccharide 3 which is closer in structure to the natural acceptor 1. In order to facilitate the isolation of our glycosylated substrates from their enzymatic incubation mixtures, we elected to prepare 2 and 3 as their 8-methoxycarbonyloctyl glycosides[24] since inclusion of this hydrophobic group should facilitate adsorption on reverse phase (C-18) chromatography supports. This "linking arm" might also eventually be used for the attachment of fluorescent, radioactive or enzymatic tags to these potential acceptors to help localize the corresponding enzyme in intracellular organelles or to prepare affinity columns to assist in enzyme isolations.

CHAPTER 2

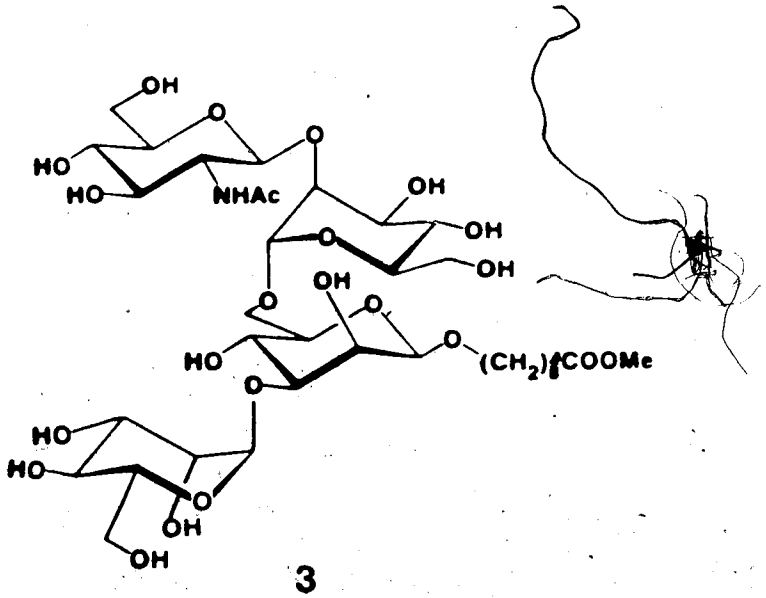
RESULTS AND DISCUSSION

The goal of this thesis project was to synthesize two oligosaccharides, namely 8-methoxycarbonyloctyl 6-O-[2-O-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-α-D-mannopyranosyl]-β-D-mannopyranoside (**2**) and 8-methoxycarbonyloctyl 6-O-[2-O-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-α-D-mannopyranosyl]-3-O-(α-D-mannopyranosyl)-β-D-mannopyranoside (**3**). In order to increase the potential usefulness of the oligosaccharides 2 and 3 we elected to synthesize these structures covalently attached to a linking arm which would allow their subsequent attachment to proteins, solid supports, and fluorescent or radioactive tags. These glycoconjugates might then be used in the intracellular localization of the N-acetylglucosaminyl transferases as well as in their purification.

A retrosynthetic analysis of the target structures 2 and 3 suggested, as the key intermediate, the trisaccharide precursor **4**, which was protected in a manner to allow the selective liberation of the hydroxyl group at C-3 of the β-D-mannopyranosyl residue for subsequent

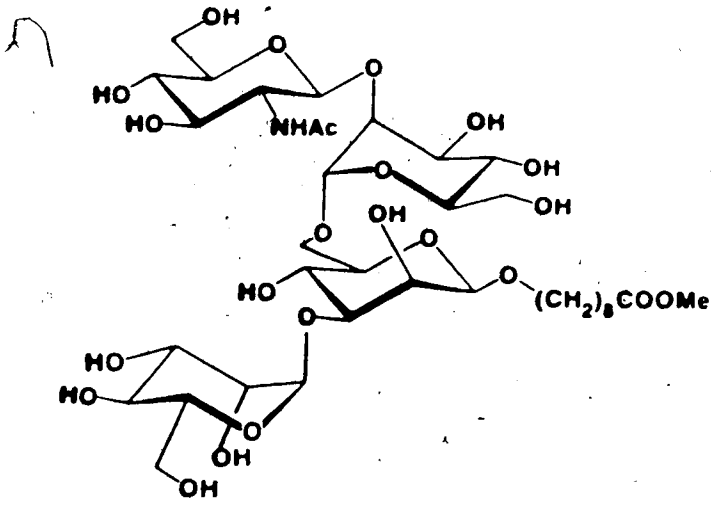


2

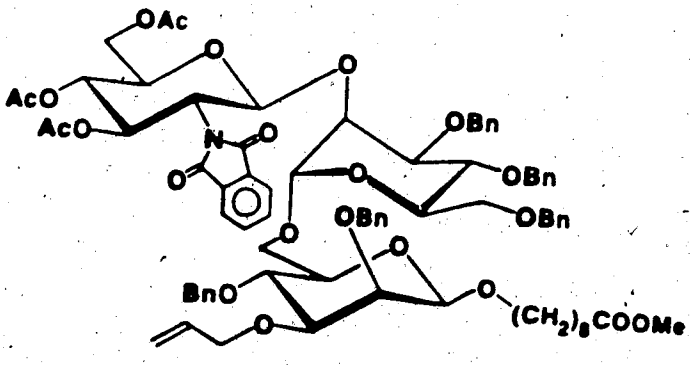


3

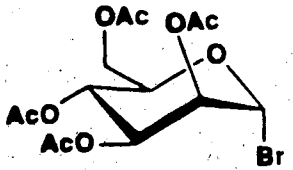
glycosylation by the α-D-mannopyranosyl donor **5**. The key intermediate **4** was, in turn, retrosynthesized into monohexosyl synthons **6**, **7**, and **8**. The two monosaccharide synthons, **6** and **7**, were designed to function as 2-deoxy-2-phthalimido-β-D-glucopyranosyl and α-D-mannopyranosyl


OH
HO
HO
NHAc
O
OH
OH
OH
HO
O
(CH2)8COOMe
HO
HO
OH
OH

3

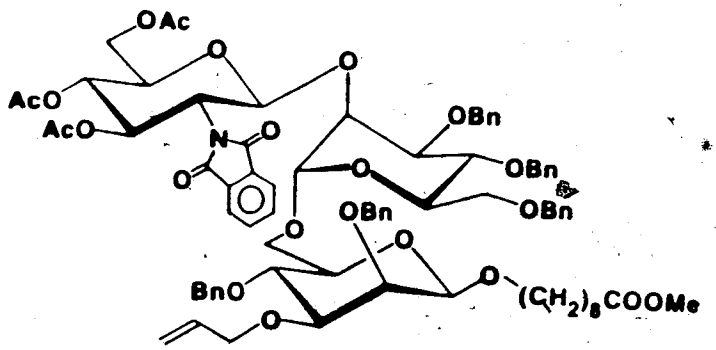

OAc
AcO
AcO
N
O
OBn
OBn
OBn
BnO
(CH2)8COOMe

4


OAc
OAc
AcO
AcO
Br

5

donors respectively. The synthon **8** was properly protected to perform dual functions: first acting as a β-D-manno-pyranosyl donor and then, after selective removal of acetyl group at O-6, as a glycosyl acceptor.



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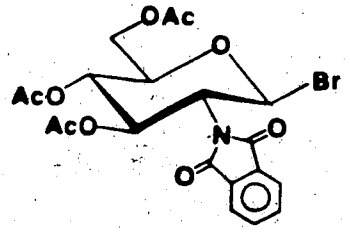
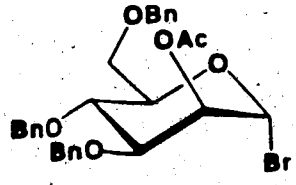


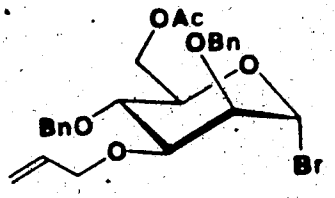
6

+



7

+



8

Of the four monosaccharide synthons (**5**,[25] **6**,[26] **7**,[27] and **8**) thus required, three, namely synthons **5**, **6**, and **7** had already been prepared. Therefore, a synthetic route towards the monosaccharide synthon **8** was first undertaken.

Methyl α-D-mannopyranoside (**9**) was converted to its 4,6-O-benzylidene derivative **10** in 25% yield on treatment with benzaldehyde in the presence of formic acid. This procedure gave only a modest yield, as reported,[28] so our attention was drawn to a recently published method,[29] which involved the reaction of **9** in N,N-dimethylformamide with benzaldehyde dimethylacetal[30] in the presence of tetrafluoroboric acid. This improved procedure provided a 50% yield of the benzylidene derivative **10** and allowed its preparation in sufficient quantities (60 g) to proceed with the preparation of **8**.

Selective 3-O-allylation of **10** was effected by the procedure of Nashed,[31] which involved its reaction with one equivalent of dibutyltin oxide to provide presumably 4,6-O-benzylidene-2,3-O-dibutylstannylene-α-D-mannopyranoside (**11**). Compound **11** was not isolated but was treated with allyl bromide in N,N-dimethylformamide at 100°C to give methyl 3-O-allyl-4,6-O-benzylidene-α-D-mannopyranoside (**12**) in 82% yield. Although **12** appeared

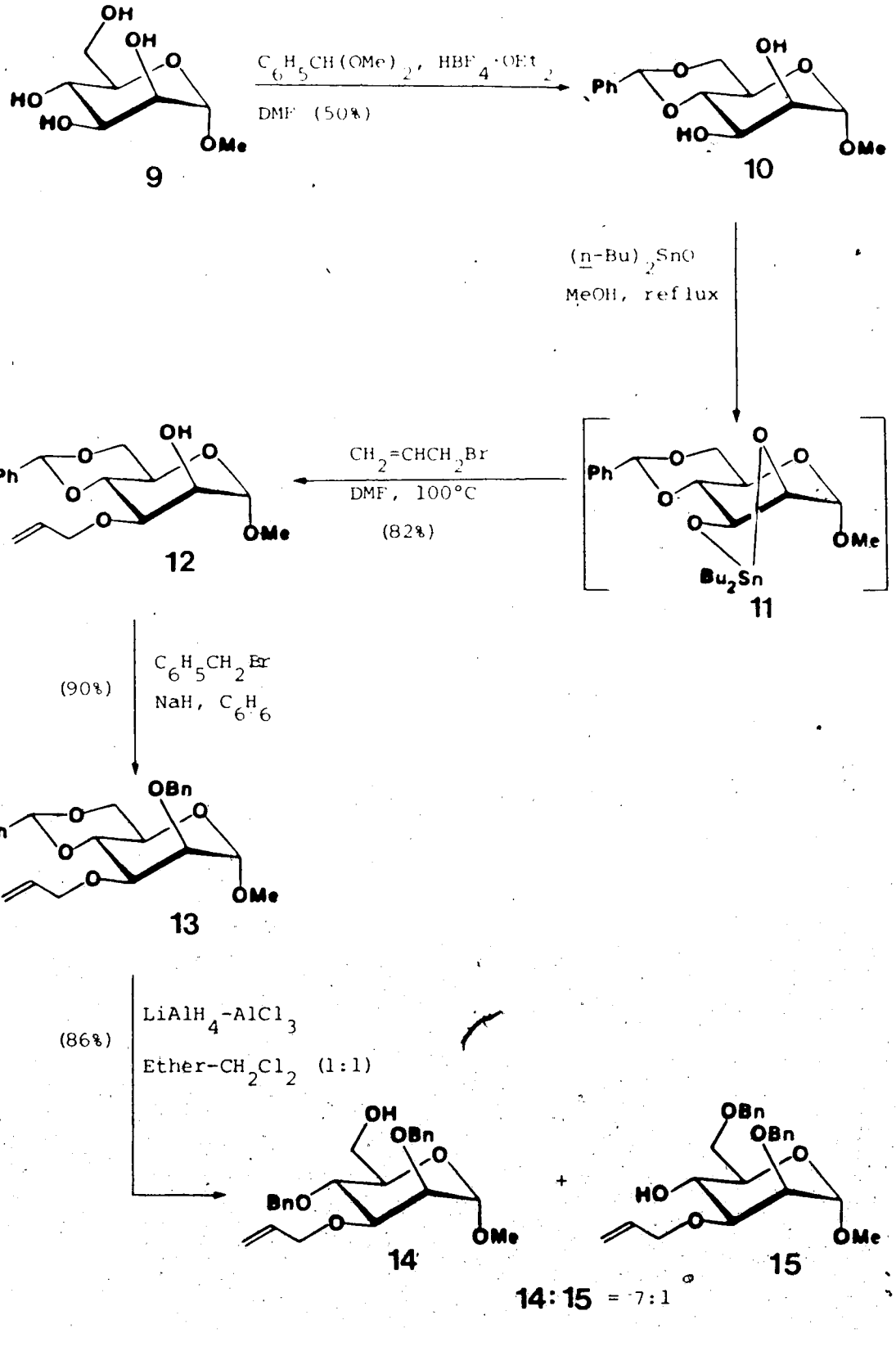
homogeneous by thin layer chromatography (tlc), its optical rotation, $[\alpha]_D^{22}$ + 59.4° (c 1.2, chloroform) differed somewhat from the value reported by Nashed: +63.5° (c 1.5, chloroform). The 400 MHz ^{1}H nmr spectrum, however, showed **12** to be a pure single isomer whose identity could readily be ascertained. The signal for the hydroxylic proton appeared as a doublet ($J_{2,OH}$ = 1.5 Hz) at δ3.045, causing H-2 to produce a broad signal at δ4.033 which collapsed to a doublet of doublets ($J_{2,3}$ = 3.5 Hz and $J_{1,2}$ = 1.5 Hz) on deuterium exchange. The position of free hydroxyl group in **12** was further confirmed by its *in situ* derivatization using trichloroacetyl isocyanate.[32] The ^{1}H nmr spectrum recorded following the addition of trichloroacetyl isocyanate displayed the expected downfield shifted doublet of doublets ($J_{2,3}$ = 3.0 Hz and $J_{1,2}$ = 1.5 Hz) at δ5.349.

Treatment of **12** with benzyl bromide and sodium hydride in benzene[31] furnished the 2-*O*-benzyl derivative **13** in 90% yield. The presence of a benzyl group in **13** was confirmed by the appearance, in the ^{1}H nmr spectrum, of additional signals integrating for five protons in the aromatic region. The signals for the now diastereotopic benzylic protons appeared at δ4.843 and 4.725, each as a one proton AB doublet with a geminal coupling constant of

12.0 Hz.

The reductive cleavage of the benzylidene group in **13** was achieved by refluxing with $LiAlH_4$-$AlCl_3$ in ether dichloromethane (1:1), according to Liptak et al.,[33] to give the 4-O-benzyl (**14**) and 6-O-benzyl (**15**) derivatives, in the ratio of 8:1, in a combined yield of 86%. The structures of the hydrogenolysis products **14** and **15** were assigned on the basis of ^1H nmr data. The ^1H nmr spectrum of the major product **14** showed a doublet of doublets for OH-6 at δ2.046 ($J_{6,OH}$ = 7.0 Hz and $J_{6',OH}$ = 5.5 Hz) as well as a clear signal for one of the H-6's at δ3.840 (ddd, $J_{6,6'}$ = 12.0 Hz, $J_{6,OH}$ = 7.0 Hz, and $J_{5,6}$ = 3.0 Hz). On D_2O exchange, the signal corresponding to OH-6 disappeared and, as expected, the signal for this H-6 simplified to a doublet of doublets, the coupling with hydroxylic proton being absent. The ^1H nmr spectrum of 6-O-benzyl derivative **14**, on the other hand, displayed a doublet for hydroxylic proton at δ2.578 ($J_{4,OH}$ = 2.0 Hz) and a doublet of doublet of doublets for H-4 at δ4.004 ($J_{4,5}$ = 9.0 Hz, $J_{3,4}$ = 9.0 Hz, and $J_{4,OH}$ = 2.0 Hz). After D_2O addition, the 1H nmr spectrum of **14** showed the disappearance of the OH resonance and the simplification of the signal for H-4 to a doublet of doublets.

The 13C nmr data were also in accord with the


OH
OH
HO
HO
OMe
9
$C_6H_5CH(OMe)_2$, $HBF_4 \cdot OEt_2$
DMF (50%)
Ph
HO
10
$(n\text{-Bu})_2SnO$
MeOH, reflux
Bu_2Sn
11
$CH_2=CHCH_2Br$
DMF, 100°C
(82%)
12
(90%)
$C_6H_5CH_2Br$
NaH, C_6H_6
OBn
13
(86%)
$LiAlH_4$-$AlCl_3$
Ether-CH_2Cl_2 (1:1)
BnO
14
15
14:15 = 7:1

structures assigned for **14** and **15**. The well established empirical rule[34] that alkoxylated carbons (ROC) are deshielded by 5-10 ppm compared with the corresponding hydroxylated carbon atoms (HOC) permits a simple verification of the position of the benzyl ethers in **14** and **15**. The major product **14** showed the signal for C-6 at δ62.43 ppm, the normal position for the underivatized hydroxymethyl group carbons of pyranose rings.[34] In **15**, however, this methylene carbon appeared at δ70.66 ppm, confirming the presence of the 6-O-benzyl ether.

Reaction of the 4-O-benzyl compound **14** with acetic anhydride in the presence of pyridine gave the 6-O-acetyl derivative **16** in 98% yield. The downfield shift of the H-6 resonances in the ^{1}H nmr spectrum of **16** due to acetylation of O-6 supported the assigned structure.

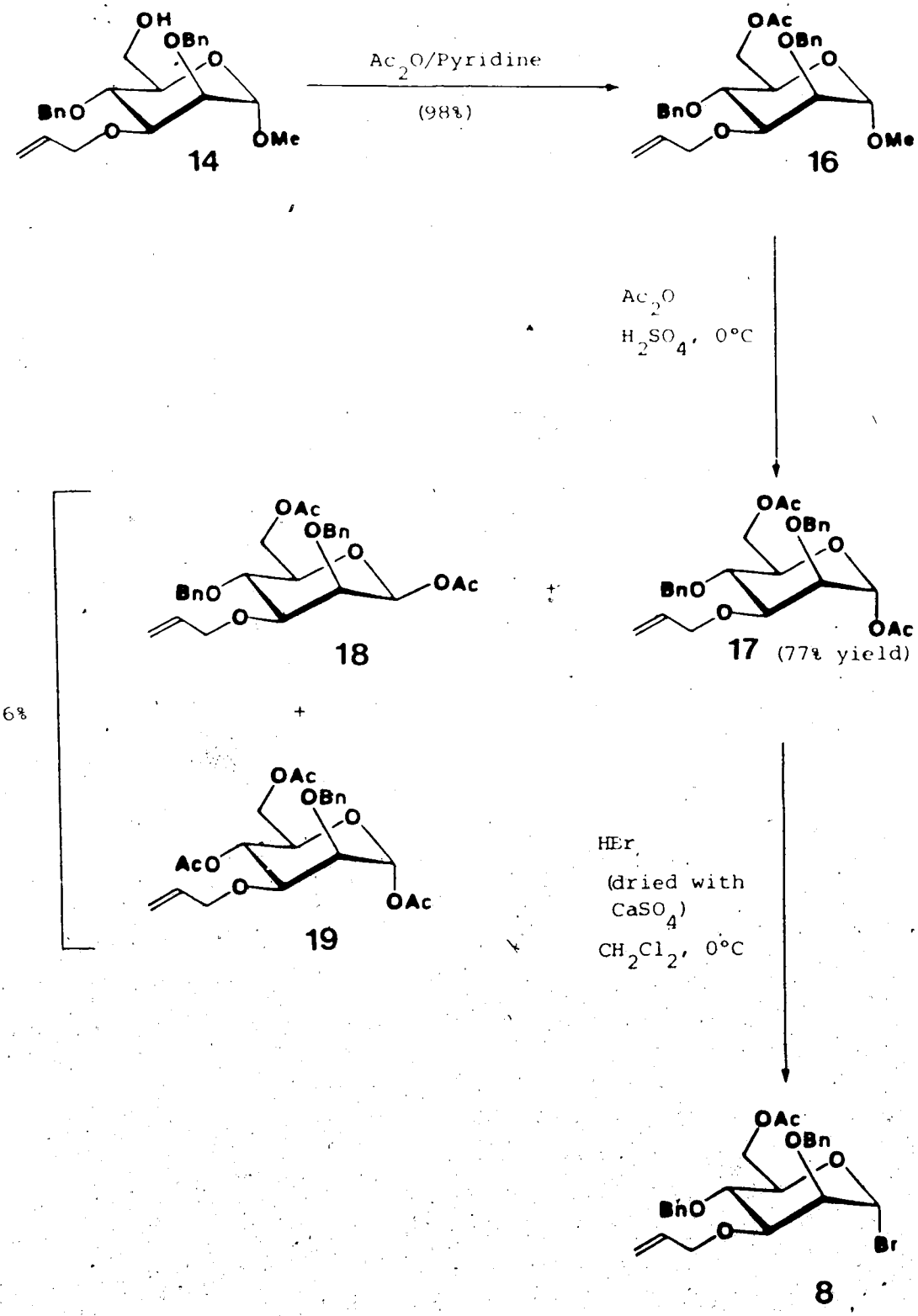
The glycosidic linkage in **16** was acetolyzed[35] using acetic anhydride in the presence of a catalytic amount of sulfuric acid to afford 1,6-di-O-acetyl-3-O-allyl-2,4-di-O-benzyl-α-D-mannopyranose (**17**) in 77% yield. The structure of **17** could be deduced from its ^{1}H nmr spectrum, which included a deshielded signal at δ6.195 (d, $J_{1,2}$ = 2.0 Hz) for H-1 and a six-proton singlet at δ2.055 indicating the presence of two acetyl groups. Further proof for the assigned structure **17** was provided by its

^{13}C nmr spectrum, which contained a shielded signal (as compared with 99.10 ppm for its precursor) at δ91.79 for C-1 and additional signals at δ168.82 and 20.98 for $\underline{O}\underline{C}OCH_3$ and $OCO\underline{C}H_3$ respectively. Along with this major product **17**, the acetolysis reaction also produced two minor products, namely, 1,6-di-O-acetyl-3-O-allyl-2,4-di-O-benzyl-β-D-mannopyranose (**18**) and 1,4,6-tri-O-acetyl-3-O-allyl-2-O-benzyl-α-D-mannopyranose (**19**) in a combined yield of 6%. The anomeric configuration of **18** was evident from the appearance, in its ^1H nmr spectrum, of a doublet ($J_{1,2}$ = 1.0 Hz) for H-1 at δ5.609 (about 0.6 ppm upfield relative to H-1 in the corresponding α-anomer **17**). The presence of shielded signals (as compared with the α-anomer **17**) for H-3 and H-5 at δ3.598 and 3.300 respectively further established[36] the anomeric configuration to be β in **18**. The structure of the other minor product **19** could also be deduced from its ^1H nmr spectral data, which included a doublet ($J_{1,2}$ = 2.0 Hz) at δ6.185 for H-1, indicating the configuration at C-1 to be α, and a deshielded doublet of doublets ($J_{3,4}$ = $J_{4,5}$ = 10.5 Hz) at δ5.431 for H-4 due to the acetylation of O-4, thus confirming the cleavage of the 4-O-benzyl group of **16**.

Reaction of the diacetate **17** in dichloromethane, at

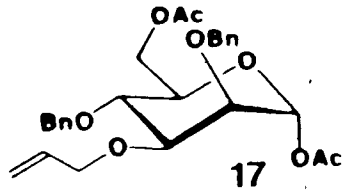
0°C, with hydrogen bromide which was dried by passage through a calcium sulfate column, led to the formation of the glycosyl bromide **8** in essentially quantitative yield. The ^{1}H nmr spectrum of **8** displayed a doublet ($J_{1,2}$ = 1.5) at δ6.444 for H-1 and a singlet at δ2.065, now integrating for only three protons, arising from a single acetyl methyl group. In its ^{13}C nmr spectrum the signal for C-1 appeared at δ87.41, and only one signal each for the carbonyl carbon at δ170.57 and for the acetyl methyl group at δ20.72 were present.

When the diacetate **17** was allowed to react with HBr gas introduced directly from the cylinder without passage through calcium sulfate, the thin layer chromatogram (tlc) of the reaction mixture showed the presence of two products, the minor being the desired bromide **8**. The major product **20** had a slightly lower mobility in tlc. The ^{1}H nmr spectrum of the mixture of **8** and **20** indicated **20** to be the hydrobromination product of **8**, namely, 6-O-acetyl-2,4-di-O-benzyl-3-O-(3-bromopropyl)-α-D-manno-pyranosyl bromide. This structural assignment was made to account for the observation of a triplet for two protons at δ3.688 ($BrCH_2CH_2\underline{CH_2}O$), a multiplet for two protons at δ3.510 for $Br\underline{CH_2}CH_2CH_2O$, and a two proton multiplet at δ2.119 for $BrCH_2\underline{CH_2}CH_2O$. The ratio of **20** to **8** was


OH
OBn
BnO
OMe
14
Ac2O/Pyridine
(98%)
OAc
OBn
BnO
OMe
16
Ac2O
H2SO4, 0°C
OAc
OBn
BnO
OAc
18
OAc
OBn
BnO
OAc
17 (77% yield)
6%
OAc
OBn
AcO
OAc
19
HBr
(dried with
CaSO4)
CH2Cl2, 0°C
OAc
OBn
BnO
Br
8

determined to be 4:1 from this ^1H nmr spectrum.

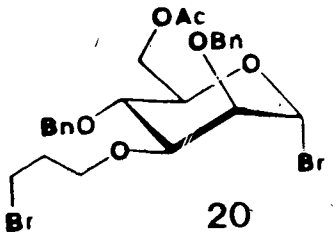
In order to further corroborate both the presence and the position of the bromine atom in the labile anomeric bromide **20** a stable derivative was prepared. Following the separation of **20** from **8** by flash column chromatography,[37] **20** was glycosylated with methanol under the conditions of Garegg *et al.*,[38] using silver carbonate as promoter and dichloromethane as solvent, to provide a mixture of the β- and α-mannosides **21** and **22** (β/α:4/1) in an isolated yield of 91%. The structure of β-anomer **21** was determined from its ^1H and ^{13}C nmr spectral data as well as mass spectral analysis. The ^1H nmr spectrum of the β-anomer **21** showed the presence of a doublet ($J_{1,2}$ = 1.0 Hz) at δ4.308 for H-1 and three two-proton signals: a triplet at 3.484 (J = 7.0 Hz) assigned to $BrCH_2CH_2C\underline{H}_2O$, a multiplet at δ3.41 assigned to $BrC\underline{H}_2CH_2CH_2O$, and another multiplet between δ2.92 and 2.856. The assigned structure **21** was also in agreement with the ^{13}C nmr spectrum, which exhibited a signal for C-1 at δ102.81 and three signals at δ67.00, 32.98, and 30.41 tentatively assigned to $BrCH_2CH_2\underline{C}H_2O$, $Br\underline{C}H_2CH_2CH_2O$, and $BrCH_2\underline{C}H_2CH_2O$, respectively. Unequivocal support for the presence of bromine in **21** (molecular weight 537.447) was provided by the chemical ionization mass spectrum, which showed two



17

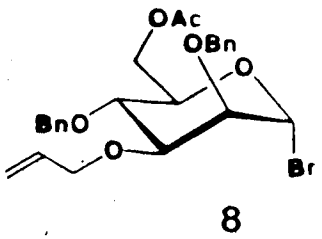
HBr

CH_2Cl_2, 0°C



20

+



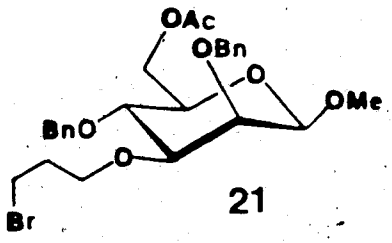
8

(91%)

MeOH

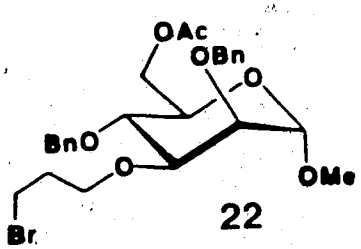
$AgCO_3$, 4 Å molecular sieves

CH_2Cl_2 0°C



21

+



22

21:22 = 4:1

peaks of almost equal intensity at 554 (93.3%) and 556 (100.0%), corresponding to the NH_4^+ adducts containing the ^{79}Br and ^{81}Br isotopes respectively. Furthermore, microanalytical data were in accord with the molecular formula for **21**.

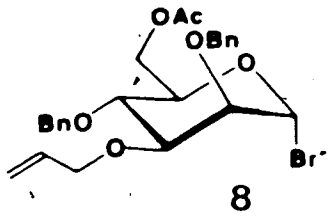
The newly developed method of Paulsen and Lockhoff[39] for the stereoselective synthesis of β-mannosides was used for the preparation of the glycosides **23**β and **23**α. Reaction of the glycosyl bromide **8** and 8-methoxycarbony-octanol in the presence of an insoluble catalyst prepared by precipitation of silver silicate on aluminum oxide, provided a mixture of the β- and α-mannosides, **23** in a combined yield of 56%. All attempts to separate these anomers using chromatography on silica gel, silica gel impregnated with silver nitrate or alumina, were unsuccessful. That **23** was a mixture of the α and β glycosides was evident from the ^1H nmr spectrum which showed a signal at δ4.365 for H-1 of the β-anomer and a doublet ($J_{1,2}$ = 2.0 Hz) at δ4.824 for H-1 of the corresponding α-anomer. The β/α ratio of this mixture, determinted by integration of these signals in the ^1H nmr spectrum of **23**, was found to vary with the temperature of the reaction. At -78°C, the β- and α-anomers were produced in a 6:1 ratio. On the other hand, at the higher

temperatures of -20° and 0° the ratios of β- to α-anomers were 4.3:1 and 3.5:1 respectively. The glycosylation reaction did not go to completion as indicated by the presence of the unreacted glycosyl bromide **8** on tlc. The rate of disappearance of **8** increased with temperature but no improvement in the yield of **23** was observed. Elimination of HBr is likely a major reaction pathway for **8** at these higher temperatures.

Coupling of **8** with 8-methoxycarbonyoctanol[24] was also attempted using freshly prepared silver carbonate in the presence of 4 Å molecular sieves, using dichloromethane as solvent at 0°C, according to the procedure of Garegg *et al.*[38] A mixture of β- and α-anomers (β/α:3/1) was obtained in 54% yield. The reaction was faster under these conditions but provided an overall lower yield than with the silver silicate reaction.

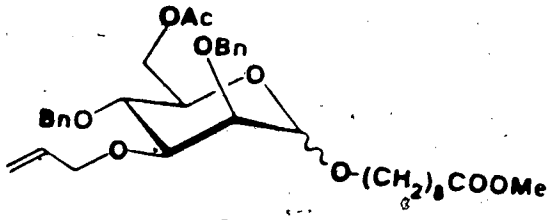
Treatment of the mixture **23** with sodium methoxide in methanol effected the removal of acetyl groups to provide a mixture of **24**β and **25**α which chould now be separated by flash column chromatography on silver nitrate impregnated silica gel. The assignment of structures to the deacetylation products **24** and **25** was made on the basis of their ^{1}H and ^{13}C nmr data.

Appearance, in the ^{1}H nmr spectrum of the major



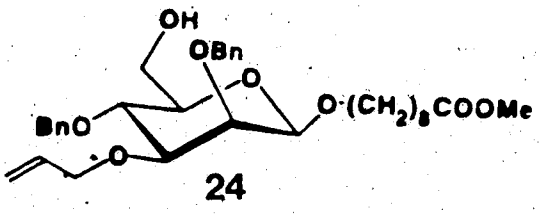
8

(56%) | HO-$(CH_2)_8$COOMe
silver silicate/alumina
CH_2Cl_2, -78°C

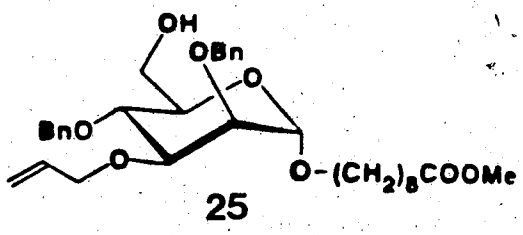


23 (β/α:6/1)

NaOMe/MeOH



24



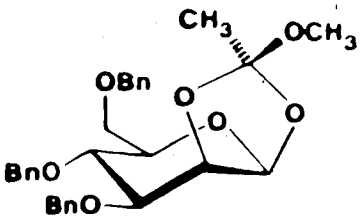
25

product **24**, of a broad signal for one proton at δ4.408 ($J_{1,2}$ ≈ 1 Hz), suggested the anomeric configuration to be β. A one-proton doublet of doublets ($J_{6a,OH}$ = $J_{6b,OH}$ = 6.5 Hz) at δ2.140 for a D_2O-exchangeable hydroxyl proton is also consistent with the structure assigned to **24**. This structural assignment was further supported by the ^{13}C nmr spectrum of **24** where the signal for C-1 at δ98.25 was clearly visible. The β-configuration of the glycosidic linkage in **24** could be unequivocally assigned from its ^{1}H-coupled ^{13}C nmr spectrum where this signal appeared as a doublet, $J_{C-1,H-1}$ = 153.6 Hz, in accord with the empirical rules formulated by Bock and Pedersen[40] for the dependence of the one-bond C-H coupling on the anomeric configuration of pyranosides.

The structure of the minor product **25** was assigned after the observation, in its ^{1}H nmr spectrum, of a doublet for one proton at δ4.790 ($J_{1,2}$ = 2.0 Hz), assigned to H-1, as expected for the corresponding α-anomer. A broad doublet integrating for one proton at δ2.098 for a hydroxylic proton was also present. The ^{13}C nmr spectrum of **25** showed the C-1 signal for C-1 at δ98.25 with $J_{C-1,H-1}$ = 168.2 Hz.

Condensation of the alcohol **24** and 2-O-acetyl-3,4,6-tri-O-benzyl-α-D-mannopyranosyl bromide[27] (**7**), which had been freshly prepared from 3,4,6-tri-O-benzyl-1,2-O-

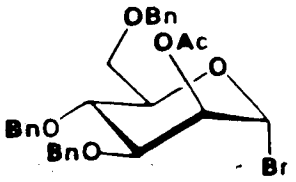
(methoxyethylidene)-β-D-mannopyranose (**26**), under Helferich conditions using mercuric bromide and mercuric cyanide as promoters and acetonitrile as solvent furnished the α-linked disaccharide **27** in 77% yield. The structure of **27** was assigned on the basis of its ^{1}H and ^{13}C nmr spectra. The resonances for two anomeric protons were observed in the ^{1}H nmr spectrum, at δ4.911 ($J_{1',2'}$ = 2.0 Hz) and 4.334 ($J_{1,2}$ ≈ 1 Hz) and were thus assigned to H-1' of the α-mannopyranosyl unit and H-1 of the β-mannopyranosyl unit, respectively. The presence of a deshielded doublet of doublets ($J_{1',2'}$ = 2.0 Hz and $J_{2',3'}$ = 3.0 Hz) at δ5.459 indicated the presence of the expected acetoxy group on C-2' and, by decoupling, thus confirmed the identity of the anomeric doublet at δ4.911. The ^{13}C nmr spectral data were also in agreement with the assigned structure. The configuration of the newly formed glycosidic linkage in **27**, expected to be α due to neighboring group participation of the 2-acetoxy group in the glycosylation reaction, was evident from its ^{1}H-coupled ^{13}C nmr spectrum which showed a doublet each for C-1 and C-1' at δ104.64 ($J_{C-1,H-1}$ = 154.7 Hz) and 97.77 ($J_{C-1',H-1'}$ = 170.4 Hz) respectively. One-bond C-H coupling constants of these magnitudes require the presence of the β and α glycosidic linkages as assigned.



26

HBr , CH_2Cl_2

0°C



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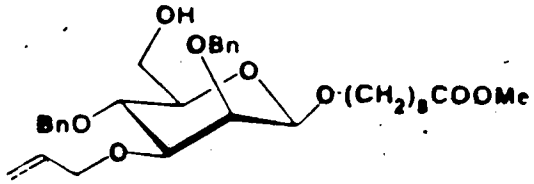


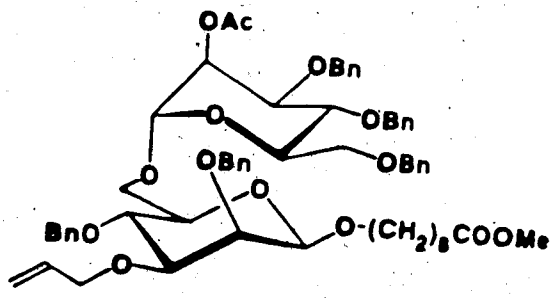
24

(77%)

$HgBr_2$, $Hg(CN)_2$

4 Å molecular sieves

CH_3CN, RT



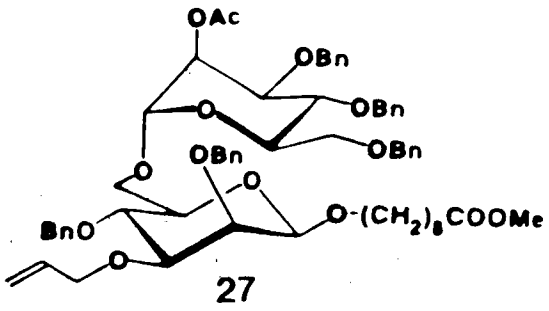
27

Reaction of **27** with sodium methoxide in dry methanol provided the alcohol **28** in quantitative yield. Disappearance, in the ^1H nmr spectrum, of a singlet for three acetyl protons and the upfield shift of the signal for H-2' to δ 4.128 confirmed the removal of acetyl group from O-2' of **27**. Moreover, the presence of a new signal at δ 2.355 (broad singlet), which was exchanged for deuterium by treatment with D_2O, further supported the structure assigned to **28**. The ^{13}C nmr spectrum of **29** showed the expected disappearance of the signals corresponding to carbonyl and methyl carbons.

Reaction of the alcohol **28** with 3,4,6-tri-O-acetyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl bromide[26] (**6**) in dichloromethane using silver zeolite[41] as promoter at room temperature, as described by Schwartz et al.,[42] failed to provide any of the desired trisaccharide **4**. Use of silver triflate/2,4,6-collidine (1.5 molar equivalents each with respect to **6**) at -20°C in nitromethane, according to Lemieux et al.[26] did provide the desired product although the yield of 30% proved unacceptably low. In an attempt to accomplish the preparation of **4** in a more respectable yield, we turned our attention to the conditions described by Paulsen et al.[43] in a recent report. Thus the treatment of **28** with the glycosyl bromide **6** (2 molar equivalents) using silver triflate/2,4,6-collidine (10

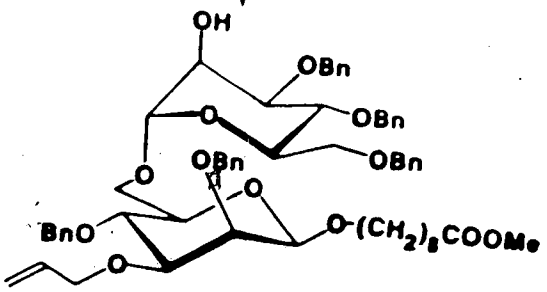
equivalents) using silver triflate/2,4,6-collidine (10 molar equivalents each) in the presence of 4 Å molecular sieves in dichloromethane at -50°C provided the trisaccharide **4** in 76% yield after chromatographic purification. The presence of the 3,4,6-tri-O-acetyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl moiety in **4** was indicated by its ^{1}H nmr spectrum which displayed four deshielded, one-proton signals, characteristic of the newly introduced glucosyl residue: a doublet of doublets at δ5.815 ($J_{3",4"}$ = 9.0 Hz, $J_{2",3"}$ = 10.0 Hz) for H-3", a doublet at δ5.573 ($J_{1",2"}$ = 8.5 Hz) for H-1", a doublet of doublets at δ5.215 ($J_{3",4"}$ = 9.0 Hz, $J_{4",5"}$ = 10.0 Hz) for H-4, and a doublet of doublets at δ4.513 ($J_{2",3"}$ = 10.0 Hz, $J_{1",2"}$ = 8.5 Hz) for H-2. The magnitude of the coupling constant between H-1" and H-2" provides unambiguous proof that these protons are in the trans-diaxial orientation and thus establishes the presence of the β-glucosidic linkage. The ^{13}C nmr spectrum of **4** showed the expected signals at δ170.42, 169.89, and 169.21 for three acetyl carbonyl carbons, and an additional anomeric signal at δ96.48 (C-1") further substantiated the structure assigned to **4**.

Along with the major condensation product **4** a minor product (**29**) with higher R_f, was also produced. This



27

NaOMe/MeOH



28

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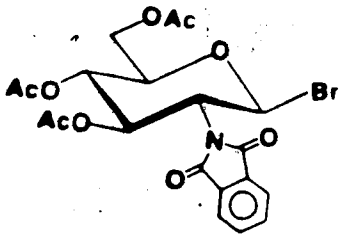
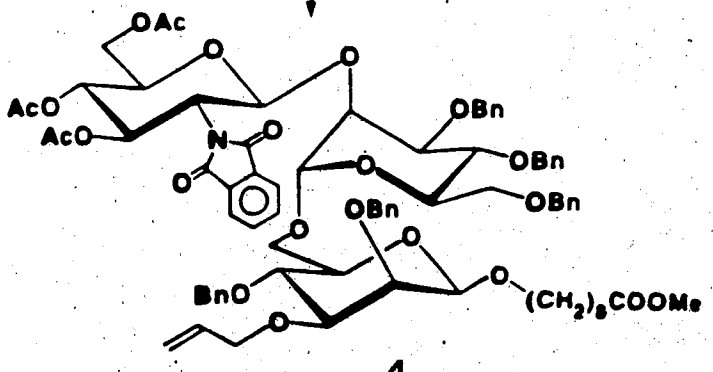


6

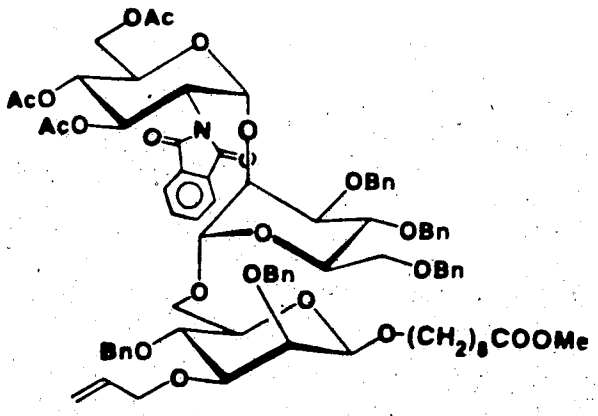
(76%)

CF_3SO_3Ag, sym-collidine
4 Å molecular sieves, CH_2Cl_2
-50°C

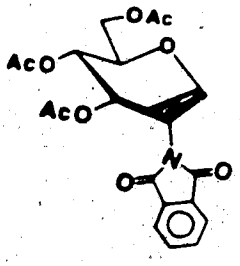


4

minor product, isolated in 6% yield, was not fully characterized but appears to be the α-linked trisaccharide. This structural assignment was made on the basis of its ^{1}H nmr spectrum which included a partially obscured doublet for H-1" at δ5.14 ($J_{1",2"} < 4$ Hz) and a shielded signal (relative to the corresponding β-anomer) for H-3" at δ6.77 ($J_{3",4"} = 9.0$ Hz, $J_{2",3"} = 11.5$ Hz), suggesting the configuration at C-1" to be α. All other signals in the ^{1}H nmr spectrum of **29** were consistent with it being a trisaccharide. As expected, the product of elimination of HBr from the glycosyl bromide **6**, namely 3,4,6-tri-O-acetyl-1,5-anhydro-2-deoxy-2-phthalimido-D-arabino-1-hex-1-enitol (**30**, was also formed in the glycosylation mixture. The glycal **30** had nmr data identical with those previously reported.[24]



29



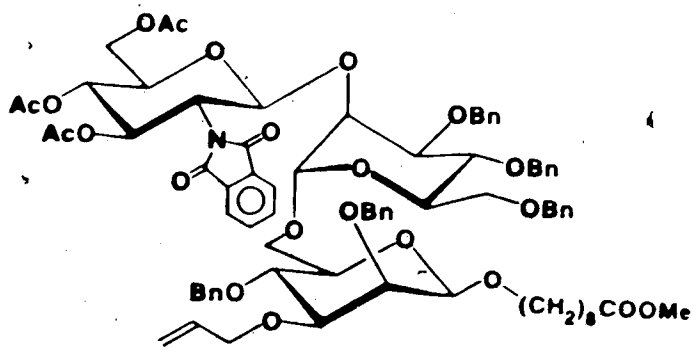
30

Three different methods were examined to effect the removal of the allyl group from **4**. Treatment of **4** with 5% palladium on charcoal,[44] under acidic conditions, furnished the trisaccharide alcohol **31** in only 34% yield. Using palladium(II) chloride as catalyst in acetic acid-water (20:1) according to Ogawa,[45] **31** could be obtained in 90% yield. Finally, deallylation of **4** could also be achieved in two steps: reaction with tris(triphenylphosphine)rhodium(I) chloride as catalyst, according to the procedure developed by Corey and Suggs,[46] effected the isomerization of double bond to give the prop-1-enyl ether, which on treatment[47] with mercuric chloride in the presence of mercuric oxide, underwent hydrolysis to afford the required trisaccharide alcohol **31** in 85% yield. The progress of the isomerization reaction proved difficult to monitor by tlc since the isomerized product (enol ether) and the allyl ether had the same chromatographic mobility. Surprisingly, washing the ether extract with brine acidified to pH 2 failed to accomplish the hydrolysis of the enol ether as had been reported by Corey and Suggs.[46]

The structure of the compound **31** was evident from its ^1H nmr spectrum which was devoid of signals for the protons of the allyl group. The corresponding signals were also absent in the ^{13}C nmr spectrum. Appearance of a

D_2O-exchangeable one-proton doublet at δ2.380 ($J_{3,OH}$ = 10.0 Hz) further supported the assigned structure.

Deacetylation of **31** using sodium methoxide in methanol led to the quantitative formation of **32**, whose identity could easily be ascertained by the absence of acetyl resonances in both its ^1H and ^{13}C nmr spectra. Removal of phthalimido group from **32**, and subsequent N-acetylation of the free amine were performed as described by Bundle and Josephson.[48] Thus, treatment of **32** with hydrazine (8 molar equivalents) in refluxing methanol generated the free amine which was acetylated *in situ* using acetic anhydride in methanol-water (1:1). The N-acetyl derivative **33** was obtained in an overall yield of 60%. No evidence of attack at the 8-methoxycarbonyloctyl ester was obtained. The structure of **33** was supported by the decrease of intensity of the signals from the aromatic protons in ^1H nmr spectrum along with the appearance of two new signals: a three proton singlet arising from the N-acetyl methyl group at δ1.779 and a broad singlet for one D_2O-exchangeable proton at δ5.533 assigned to the amide proton. The ^{13}C nmr spectrum of **33** further supported the assigned structure. The signals at δ168.51, 133.79, 131.84, and 123.23 which were assigned to the carbonyl carbons, two of the tertiary aromatic carbons,

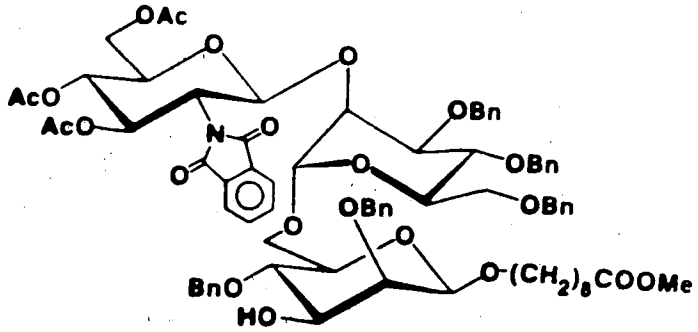


4

(85%)

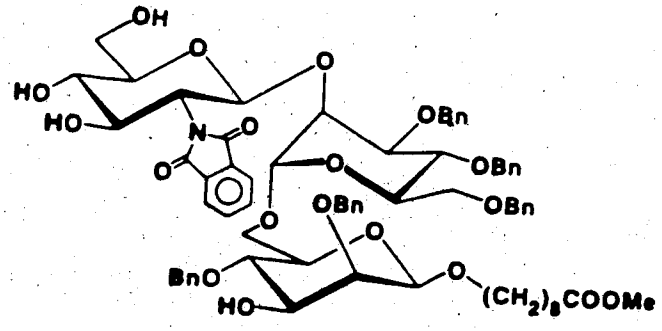
(i) $[(C_6H_5)_3P]_3RhCl$, DABCO
EtOH-benzene-water (7:3:1)

(ii) HgO, $HgCl_2$, Acetone-water (9:1)



31

NaOMe/MeOH

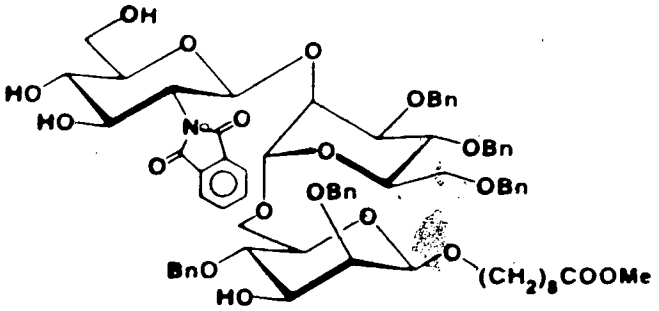


32

the quaternary aromatic carbons, and the remaining two tertiary aromatic carbons of the phthalimido group, respectively in the ^{13}C nmr spectrum of **32**, were absent in the nmr spectrum of **33**. Compound **33**, on the other hand, showed two new resonances at δ172.37 and 23.30 for the carbonyl and the methyl carbons, respectively, of the N-acetyl group.

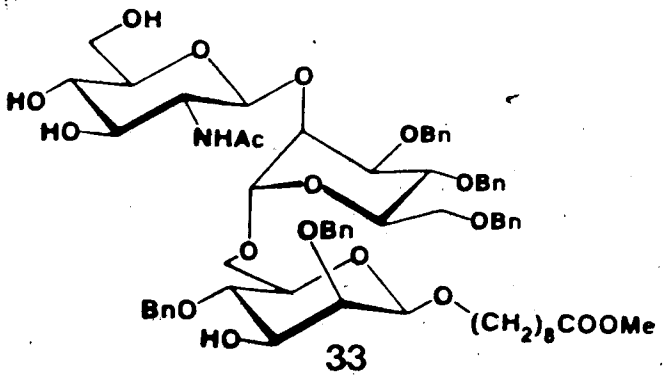
Hydrogenolytic cleavage of the benzyl protecting groups of **33** using 5% palladium-on-charcoal as the catalyst in 98% ethanol furnished the target trisaccharide **2**, which was purified by size-exclusion chromatography on Bio-Gel P-2. Compound **2** was obtained as a white lyophilized powder in 85% yield. The ^{1}H nmr spectrum of **2** showed the expected signals for H-1 at δ4.664 ($J_{1,2}$ < 0.7 Hz), H-1' at δ4.920 ($J_{1',2'}$ = 1.8 Hz) and H-1" at δ4.579 ($J_{1",2"}$ = 8.0 Hz). Other signals were in accord with the structural assignment. The partial 360 MHz ^{1}H nmr spectrum of **2** is reproduced in Figure 4 to show level of anomeric purity of this final product. The ^{1}H-coupled ^{13}C nmr spectrum of **2** again established the configurations at C-1 ($J_{C-1,H-1}$ = 159.4 Hz), C-1' ($J_{C-1',H-1'}$ = 169.5 Hz), and C-1" ($J_{C-1",H-1"}$ = 162.3 Hz) to be β, α, and β respectively.

Having accomplished the synthesis of the target



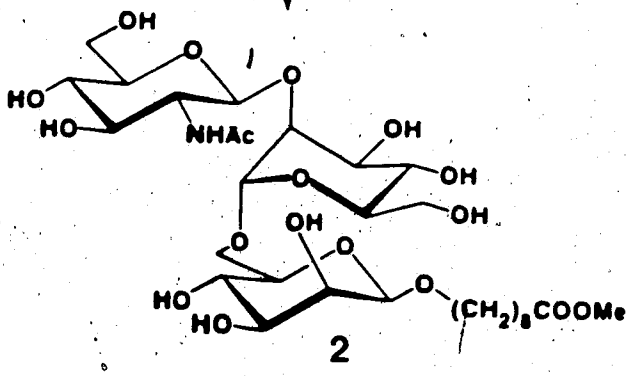
32

(60%) (i) $NH_2NH_2 \cdot H_2O$, MeOH
(ii) Ac_2O, H_2O-MeOH (1:1)



33

(85%) H_2 (1 atm), 5% Pd-C
98% ethanol



2

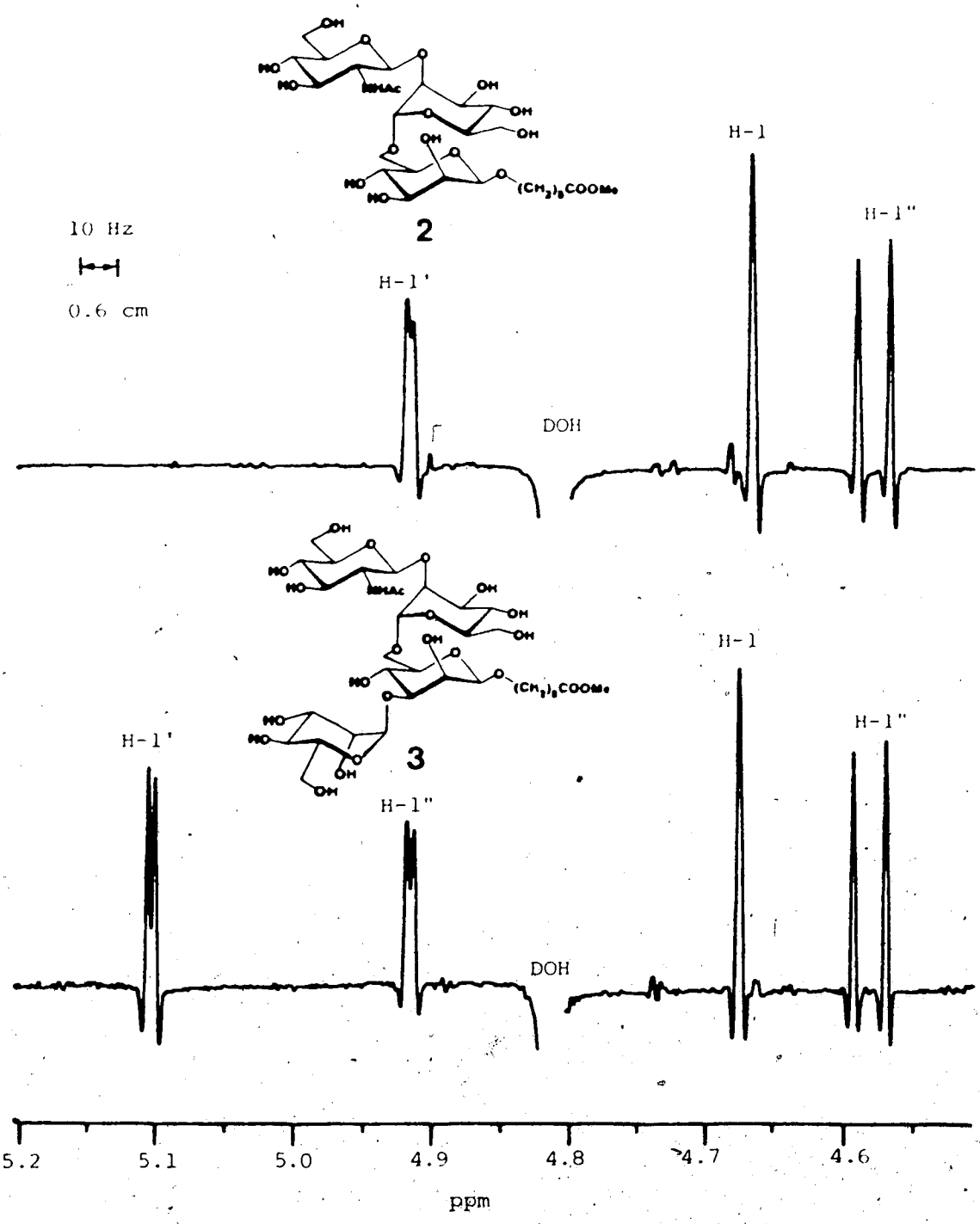
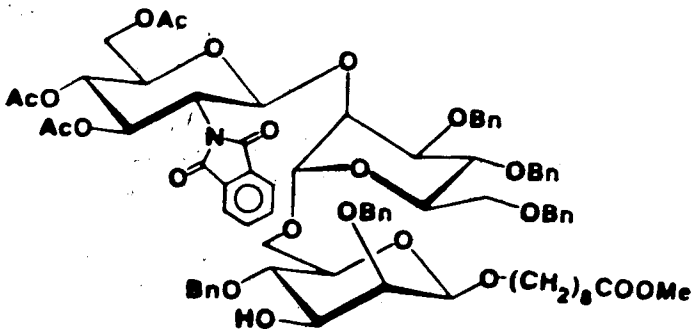


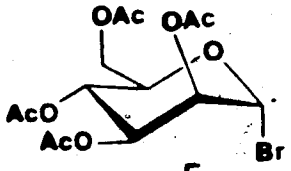
Figure 4. The 360 MHz ^{1}H nmr spectra (only anomeric region shown) of **2** and **3** in D_2O.

trisaccharide 2, the preparation of the tetrasaccharide 3 was undertaken. In order to achieve the coupling of 31 and 2,3,4,6-tetra-O-acetyl-α-D-mannopyranosyl bromide[25] (5), two different promoters were utilized. Under the conditions of Hanessian and Banoub[49] using silver triflate and tetramethylurea as promoter, the glycosylation of 31 with the bromide 5 to provide the tetrasaccharide 34 proceeded in only 38% yield. When the reaction was performed instead in the presence of mercuric bromide and mercuric cyanide in dichloromethane, in the presence of 4 Å molecular sieves, a 65% yield of the condensation product 34 was obtained. The bromide 5 described above was readily available from 1,2,3,4,6-penta-O-acetyl-α/β-D-mannopyranose by treatment with 45% hydrogen bromide in acetic acid.

The ^{1}H nmr data of 34 required the presence of a 2,3,4,6-tetra-O-acetyl-α-D-mannopyranosyl moiety. The signal for the anomeric proton of the newly introduced glycosyl residue appeared at δ5.018 ($J_{1',2'}$ ≈ 2 Hz) and the acetyl region showed the expected seven signals for the methyl acetyl groups. The ^{13}C nmr spectrum showed the presence of four anomeric carbons: δ101.95, $J_{C-1,H-1}$ = 154.1 Hz, 97.64, $J_{C-1'',H-1''}$ = 170.9 Hz, 96.60, $J_{C-1''',H-1'''}$ = 164.16 Hz and the new signal for α-linked mannose at



31



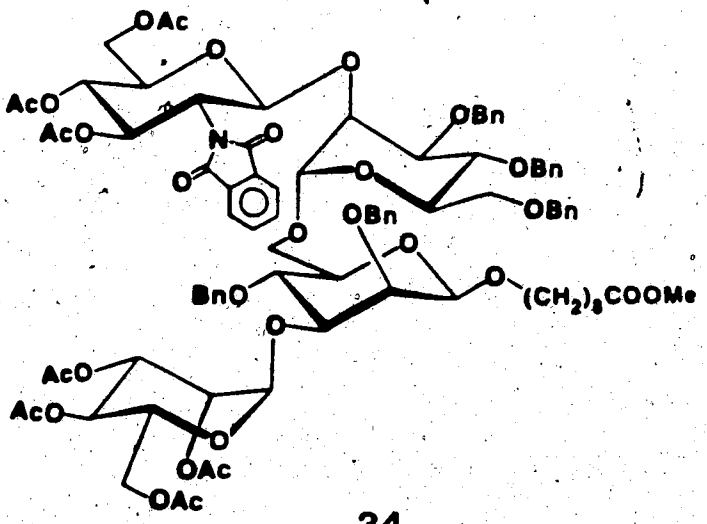
5

(65%)

$HgBr_2$, $Hg(CN)_2$

4 Å molecular sieves

CH_3CN, RT

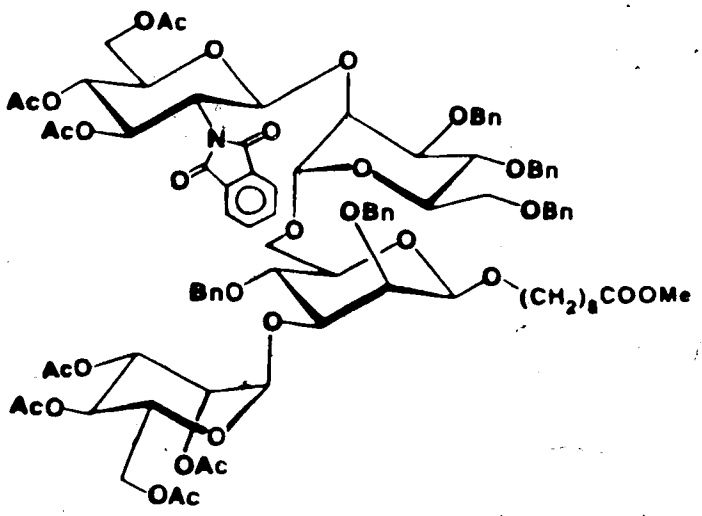


34

δ99.83, $J_{C\text{-}1',H\text{-}1'}$ = 177.8 Hz. Other features of these spectra, described in detail in the experimental section, were in accord with the assigned structures.

Conversion of **34** to **35** was effected in three steps without characterization of the intermediates involved. Deacetylation of **34** with sodium methoxide in methanol gave a white foam which was refluxed with hydrazine in methanol to generate the free amine which, on treatment with acetic anhydride in pyridine, gave the peracetylated product **35** in 63% yield. Attempted N-acetylation of the intermediate free amine, obtained in the second step of the above sequence using acetic anhydride in methanol-water (1:1) gave a very polar hydroxylated derivative which proved difficult to purify by silica gel chromatography. The structure of **35** could readily be deduced from its ^1H nmr spectrum which displayed the presence of only twenty-five aromatic protons and an additional singlet at δ1.705 for three N-acetyl protons. Disappearance of the ^{13}C resonances corresponding to the carbons of the phthalimido group and the presence of two new signals at δ169.54 and 23.27 arising from the N-acetyl group also supported the structure assigned to **35**.

Removal of the O-acetyl and O-benzyl protecting groups of **35** was conveniently accomplished by treatment



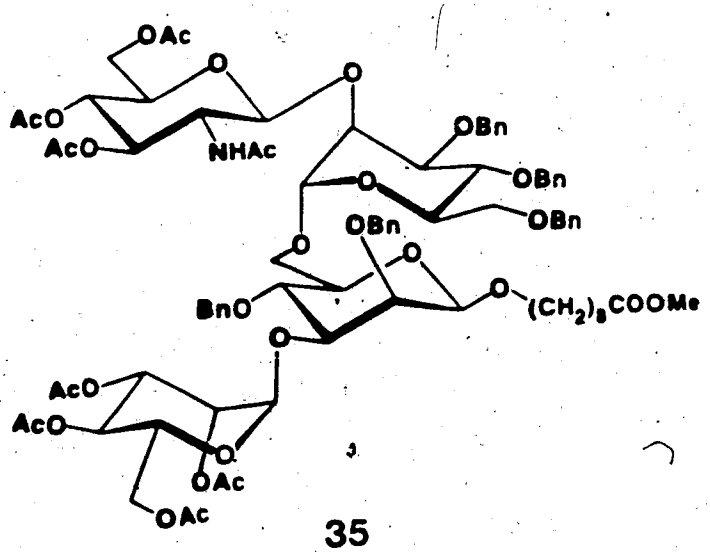
34

(62%)

(i) NaOMe/MeOH

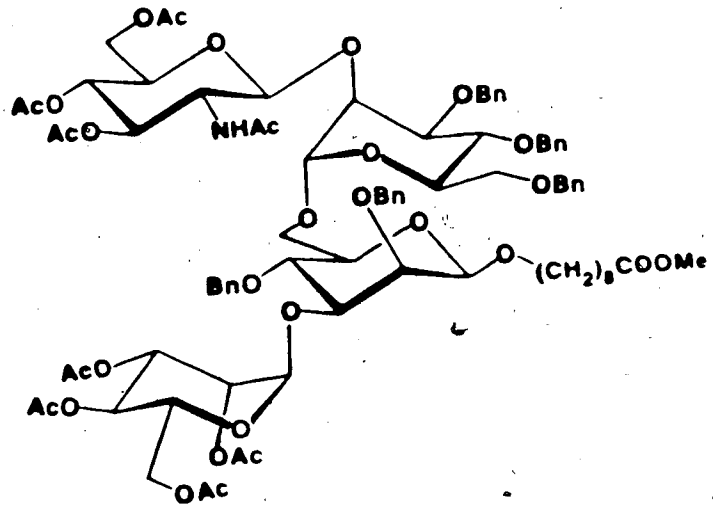
(ii) $NH_2NH_2 \cdot H_2O$, MeOH, reflux

(iii) Ac_2O, Pyridine



35

with sodium methoxide in methanol followed by hydrogenolysis over 5% palladium-on-charcoal. Filtration of the crude product through a column of Bio-Gel P-2 afforded the target tetrasaccharide **3** as a white lyophilized powder in a total yield of 85%. Complete removal of acetyl and benzyl groups was evident from the absence of the corresponding signals in the ^{1}H nmr spectrum of 3. The signals for H-1, H-1', H-1", and H-1"' appeared at δ4.671 ($J_{1,2} < 0.8$ Hz), 5.104 ($J_{1',2'} = 2.0$ Hz), 4.918 ($J_{1",2"} = 1.8$ Hz) and 4.579 ($J_{1"',2"'} = 8.0$ Hz) respectively. The ^{1}H-coupled ^{13}C nmr spectrum of **3** was employed to re-establish the anomeric configurations which were already assigned by the ^{1}H-coupled ^{13}C nmr spectra recorded following the formation of each glycosidic linkage. As anticipated, C-1, C-1', C-1", and C-1"' showed the one-bond C-H couplings of 159.1 Hz (β), 172.00 Hz (α), 169.3 Hz (α), and 159.1 Hz (β), respectively. The partial 360 MHz ^{1}H nmr spectrum of 3 is reproduced in Figure 4 where it is compared with the spectrum of the corresponding trisaccharide **2**.

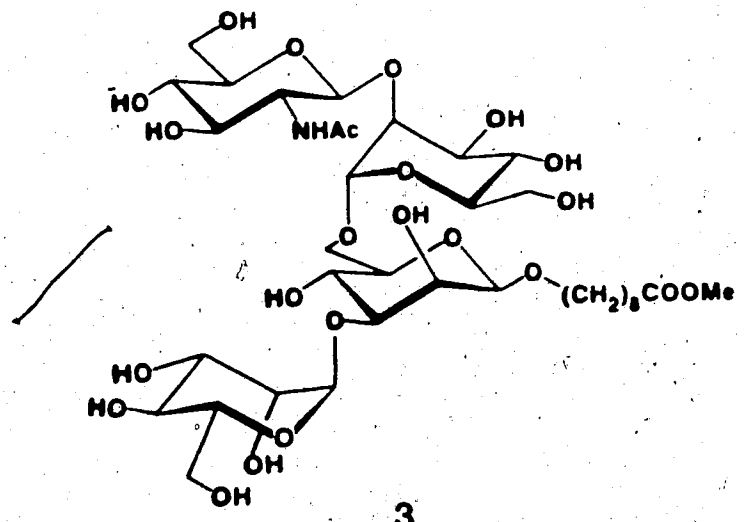


35

(85%)

(i) NaOMe/MeOH

(ii) H_2 (1 atm), 5% Pd-C
98% EtOH



3

CHAPTER 3

TESTING RESULTS

Trisaccharide **2** was found to be an excellent acceptor for GlcNAc-transferase V (GnT V) in experiments performed by Dr. Michael Pierce, Department of Anatomy, University of Miami Medical School. The results of these experiments are included here, briefly, only for the sake of completeness.

In a typical experiment, BHK cells were sonicated in 0.1 M MES [2-(N-morpholino)-ethanesulfonic acid], pH 7.0, and protein was solubilized by addition of Triton X-100 to a 1% solution. The glycosyltransferase assay was performed in a total volume of 20 μL containing 100 μg of the solubilized cellular protein, 1 mM of the acceptor trisaccharide **2**, 1 mM of 2-acetamido-2-deoxy-β-D-gluco-pyranosyl amine (an N-acetyl glucosaminidase inhibitor) and 5 mM UDP-(^{3}H)-GlcNAc. After a 2 hour incubation at 37°, the mixture was diluted with water (100 μL) and passed over a filter supporting Dowex-1-X8 (formate) ion exchange resin to remove almost all of the negatively charged counts. The filtrate was then injected onto a reverse phase (C-18) HPLC column which was eluted

isocratically with 40% aqueous methanol. The chromatogram obtained in this manner is reproduced in Figure 5. In this figure, peak A includes both ^{3}H-UDP-GlcNAc which had passed through the ion exchange resin and, presumably, some of its hydrolytic products including GlcNAc and GlcNAc-1-phosphate. Peak B has a retention volume identical to the acceptor trisaccharide **2** and is absent when this trisaccharide acceptor is omitted from the incubation mixture. The counts produced in this peak increased linearly with time, in the presence of a saturating concentration of acceptor **2** (50-100 nmoles/20 μL), as expected for the enzymatic reaction. The radioactivity in peak B is therefore a measure of the activity of a GlcNAc-transferase.

BHK cells are known to contain only GlcNAc-transferases I, II, IV, V and VI,[15] and all but GnT V require Mn^{++} for activity. The counts in peak B (Figure 5) were not affected by the inclusion of ethylenediamine tetraacetic acid (EDTA) at concentrations known to abolish the activities of these Mn^{++} requiring enzymes. It therefore appears that trisaccharide **2** is indeed a selective substrate for assaying the activity of GnT V. Using acceptor **2** in this assay, the GnT V activity of Rous sarcoma transformed BHK cells was found to be 1.8-2.0 times higher than in the untransformed cells. This

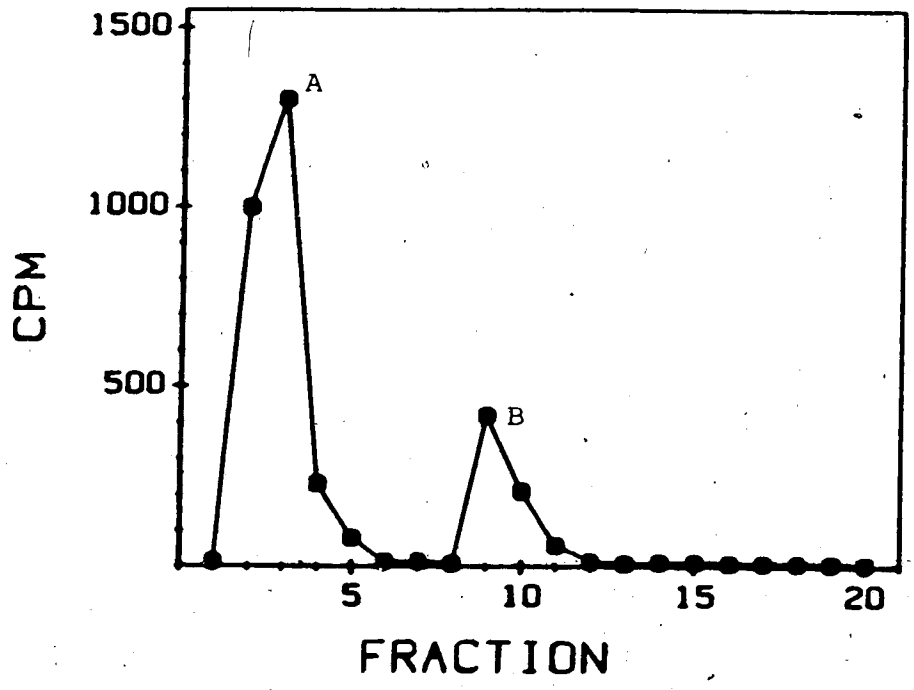


Figure 5. HPLC elution profile of a GlcNAc-transferase assay mixture on a reverse-phase (C-18) column. Peak A includes unreacted UDP-(^{3}H)-GlcNAc and its decomposition products. Peak B is the product of ^{3}H-GlcNAc transfer to the synthetic acceptor trisaccharide **2**.

finding parallels that of Kobata[10,15] for polyoma-transformed BHK cells.

Tetrasaccharide **3** has not yet been tested as an acceptor.

CHAPTER 4

EXPERIMENTAL

General Methods

All solvents and reagents used were reagent grade, and, in cases where further purification was required, standard procedures[50] were followed. All solid reactants for glycosylation were dried overnight over phosphorus pentaoxide in a high vacuum prior to use. Solution transfers where anhydrous conditions were required were done under nitrogen using standard syringe techniques.[51] Molecular sieves were purchased from BDH Chemicals, and the ratio of alcohol to molecular sieves in glycosylation was between 1:5 and 1:10 by weight.

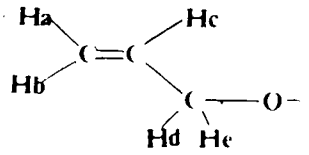
Thin layer chromatograms (tlc) were performed on pre-coated silica gel 60-F254 plates (E. Merck, Darmstadt) and visualized by quenching of fluorescence and/or by charring after spraying with 5% sulfuric acid in ethanol. For flash chromatography[37] 40-63 μm (400-230 mesh) silica gel 60 (E. Merck No. 9385) and distilled solvents were used, and the ratio of silica gel to compound was in the range 50:1-100:1. Skellysolve B refers to hexane supplied by Stanchem, Winnipeg, Manitoba. Solvents were removed on a

rotary evaporator under the vacuum of a water aspirator with bath temperatures of 40° or lower. For gel filtration, Bio-Gel P-2 (200-400 mesh) (Bio-Rad Laboratories, Richmond, California, USA) was used.

Spectral and elemental analyses were performed by the departmental NMR Service Laboratory under the supervision of Dr. T.T. Nakashima and the Analytical Service Laboratory under the supervision of Mr. R. Swindlehurst. Proton nuclear magnetic resonance (^{1}H nmr) spectra were recorded at either 400 MHz (Bruker WH-400) or 360 MHz (Bruker WM-360) with either tetramethylsilane (δ0 in $CDCl_3$) or acetone (δ2.225 in D_2O) as internal standard at ambient temperature. Carbon-13 nuclear magnetic resonance (^{13}C nmr) spectra were recorded at either 100.62 MHz (Bruker WH-400) or 90.56 MHz (Bruker WM-360) with either external tetramethylsilane (δ0 in $CDCl_3$) or external 1,4-dioxane (δ67.4 in D_2O) as reference standard. ^{1}H chemical shifts and coupling constants are reported as if they were first order. Assignments of ^{13}C resonances are tentative. Optical rotations were determined on a Perkin-Elmer 241 polarimeter in a 1 dm cell at ambient temperature (23 ± 1°C).

Protons of the allyl group present in the compounds described in this work were designated as Ha, Hb, Hc, Hd, and He as defined below. These protons showed the same

coupling constants and thus the same multiplicity pattern in all the compounds examined and only the chemical shifts varied. The observed coupling constants were:



Ha, dddd, $J_{a,c}$ = 10.5 Hz

$J_{a,d}$ = 1.5 ± 0.5 Hz

$J_{a,e}$ = 1.5 ± 0.5 Hz

$J_{a,b}$ = 1.5 ± 0.5 Hz

Hb, dddd, $J_{b,c}$ = 17.0 Hz

$J_{b,d}$ = 1.5 ± 0.5 Hz

$J_{b,e}$ = 1.5 ± 0.5 Hz

$J_{a,b}$ = 1.5 ± 0.5 Hz

Hc, dddd, $J_{b,c}$ = 17.0 Hz

$J_{a,c}$ = 10.5 Hz

$J_{c,d}$ = 5.5 Hz

$J_{c,e}$ = 5.5 Hz

Hd, dddd, $J_{d,e}$ = 13.5 Hz

$J_{c,d}$ = 5.5 Hz

$J_{b,d}$ = 1.5 ± 0.5 Hz

$J_{a,d}$ = 1.5 ± 0.5 Hz

He, dddd, $J_{d,e}$ = 13.5 Hz

$J_{c,e}$ = 5.5 Hz

$J_{a,e}$ = 1.5 ± 0.5 Hz

$J_{b,e}$ = 1.5 ± 0.5 Hz

Methyl 3-O-allyl-2-O-benzyl-4,6-O-benzylidene-α-D-manno-pyranoside (13)

To a solution of methyl 3-O-allyl-4,6-O-benzylidene-α-D-mannopyranoside[31] (12) (13.60 g; 42.24 mmol) in dry benzene (500 mL) were added sodium hydride (about 80% dispersion in oil; 2.02 g) and benzyl bromide (7.55 mL; 63.35 mmol). The mixture was refluxed under nitrogen atmosphere for 16 h. After the mixture had cooled to room temperature, the excess of sodium hydride was decomposed by the addition of methanol (250 mL), then water (500 mL) was added. The organic layer was separated, washed with water, dried ($MgSO_4$), filtered, and concentrated. The tlc of the residual oil showed, besides a major spot for the benzylation product, a UV active, fast moving spot which presumably corresponded to unreacted benzyl bromide. Flash chromatography of the oil obtained above using Skellysolve B-ethyl acetate (10:1) as eluent, which provided a good separation on tlc plate (R_f of benzylation product = 0.35), did not result in complete separation.

Purification of the major product could be achieved by gradient flash chromatography employing a mixture of Skellysolve B and ethyl acetate (the ratio of Skellysolve B-ethyl acetate was changed from 50:1 to 2:1 during elution). Finally **13** was obtained as a yellow oil (15.73 g; 90%); $[\alpha]_D$ +33.0° (*c* 1.4, chloroform); R_f 0.35 (Skellysolve B-ethyl acetate, 10:1); ^1H nmr ($CDCl_3$) δ: 7.505-7.259 (10H, aromatic), 5.901 (1H, Hc allyl), 5.609 (s, 1H, $C_6H_5C\underline{H}O_2$), 5.294 (1H, Hb allyl), 5.146 (1H, Ha allyl), 4.843 (d, 1H, J_{gem} = 12.0 Hz, $C_6H_5C\underline{H}HO$), 4.725 (d, 1H, J_{gem} = 12.0 Hz, $C_6H_5C\underline{H}HO$), 4.681 (d, 1H, $J_{1,2}$ = 2.0 Hz, H-1), 4.294-4.208 (2H, Hd allyl [δ4.268] and H-6e [δ4.244, dd, $J_{6a,6e}$ = 10.5 Hz, $J_{5,6a}$ = 4.5 Hz]), 4.185 (dd, 1H, $J_{6a,6e}$ = 10.0 Hz, $J_{5,6a}$ = 10.0 Hz, H-6a), 4.104 (1H, He allyl), 3.885-3.834 (2H, H-3 and H-4), 3.818 (dd, 1H, $J_{2,3}$ = 3.2 Hz, $J_{1,2}$ = 2.0 Hz, H-2), 3.761 (dd, 1H, $J_{4,5}$ = 10.0 Hz, $J_{5,6a}$ = 10.0 Hz, $J_{5,6e}$ = 4.5 Hz, H-5), 3.310 (s, 3H, CH_3O); ^{13}C nmr ($CDCl_3$) δ: 138.18, 137.72 (quat. arom.), 135.0 ($CH_2=\underline{C}HCH_2O$), 128.64, 128.00, 127.87, 127.59, 125.00 (tert. arom.), 116.32 ($\underline{C}H_2=CHCH_2O$), 101.43, 100.52 (C-1 and $C_6H_5\underline{C}HO_2$), 79.09 (C-4), 76.47, 76.03 (C-2 and C-3), 73.56 ($C_6H_5\underline{C}H_2O$), 68.79 ($CH_2=CH\underline{C}H_2O$), 64.03 (C-6), 54.67 (C-5), 29.57 (CH_3O). Anal. calcd. for $C_{24}H_{28}O_6$: C 69.89, H 6.84; found: C 69.67, H 6.94.

Methyl 3-O-allyl-2,4-di-O-benzyl-α-D-mannopyranoside (**14**)

To a stirred solution of **13** (14.27 g; 34.59 mmol) in 1:1 diethyl ether-dichloromethane (300 mL) was added, portionwise, $LiAlH_4$ (3.95 g; 103.9 mmol) and the mixture was slowly heated to the boiling point. To the boiling solution under reflux was added $AlCl_3$ (13.85 g; 103.9 mmol) in diethyl ether (150 mL) over a period of 70 min, after which tlc indicated the absence of starting material. The mixture was cooled. The excess of $LiAlH_4$ was decomposed with ethyl acetate (75 mL), and $Al(OH)_3$ was precipitated by the addition of water (75 mL). After dilution with ether (400 mL), the organic layer was separated from the aqueous layer which was back-extracted with ether (150 mL). The combined ether extracts were washed with water (3 × 150 mL), dried ($MgSO_4$), and concentrated to an oily residue which tlc indicated to be a mixture of two compounds. The separation of these two products was achieved by flash chromatography using Skellysolve B-ethyl acetate (3:1) as eluent. Evaporation of the early fractions provided the minor product **15** (1.69 g) as an oil; $[\alpha]_D$ = -6.25° (c 1.04, chloroform); R_f 0.47 (Skellysolve B-ethyl acetate, 2:1); ^1H nmr ($CDCl_3$) δ: 7.39-7.25 (10H, aromatic), 5.906 (1H, Hc allyl), 5.283 (1H, Hb allyl), 5.185 (1H, Ha allyl), 4.778 (d, 1H, $J_{1,2}$ =

2.0 Hz, H-1), 4.730-4.665 (AB, 2H, J_{gem} = 12.0 Hz, $C_6H_5C\underline{H}_2O$), 4.655-4.583 (AB, 2H, J_{gem} = 12.0 Hz, $C_6H_5C\underline{H}_2O$), 4.070-3.938 (3H, Hd and He allyl overlapping with H-4 [δ4.004, ddd, $J_{3,4}$ = 9.0 Hz, $J_{4,5}$ = 9.0 Hz, $J_{4,OH}$ = 2.0 Hz, simplified to dd with J_{OH} being absent on D_2O exchange]), 3.830-3.704 (4H, H-2, H-5, H-6a, and H-6b), 3.596 (dd, 1H, $J_{3,4}$ = 9.5 Hz, $J_{2,3}$ = 3.0 Hz, H-3), 3.354 (s, 3H, CH_3O), 2.578 (d, 1H, $J_{4,OH}$ = 2.0 Hz, disappeared on D_2O exchange, 4-OH); ^{13}C nmr ($CDCl_3$) δ: 138.40, 138.37 (quat. arom.), 134.76 ($CH_2=\underline{C}HCH_2O$), 128.37, 127.88, 127.66, 127.56 (tert. arom.), 117.22 ($\underline{C}H_2=CHCH_2O$), 99.40 (C-1), 79.41 (C-3), 73.79 (C-2), 73.65, 72.78 ($C_6H_5\underline{C}H_2O$), 71.58 (C-5), 70.73 ($CH_2=CH\underline{C}H_2O$), 70.66 (C-6), 67.96 (C-4), 54.99 (CH_3O). Anal. calcd. for $C_{24}H_{30}O_6$: C 69.55, H 7.30; found: C 69.67, H 7.35.

Evaporation of the later fractions gave the major product **14** (10.78 g) as an oil; $[\alpha]_D$ +48.25° (c 0.97, chloroform); R_f 0.35 (Skellysolve B-ethyl acetate, 2:1); ^{1}H nmr ($CDCl_3$) δ: 7.42-7.24 (10H, aromatic), 5.950 (1H, Hc allyl), 5.326 (1H, Hb allyl), 5.180 (1H, Ha allyl), 4.936 (d, 1H, J_{gem} = 11.0 Hz, $C_6H_5C\underline{H}HO$), 4.813 (d, 1H, J_{gem} = 12.5 Hz, $C_6H_5C\underline{H}HO$), 4.709-4.688 (2H, $C_6H_5C\underline{H}HO$ [δ4.709; d, J_{gem} = 12.5 Hz] overlapping with H-1 [δ4.690, d, $J_{1,2}$ = 2 Hz]), 4.638 (d, 1H, J_{gem} = 11.0 Hz, $C_6H_5C\underline{H}HO$), 4.146-4.064 (2H, Hd and He allyl), 3.913 (dd, 1H, $J_{3,4}$ =

9.5 Hz, $J_{4,5}$ = 9.5 Hz, H-4), 3.840 (ddd, 1H, $J_{6a,6b}$ = 12.0 Hz, $J_{6,OH}$ = 5.5 Hz, $J_{5,6a}$ = 3.0 Hz, D_2O addition resulted in its collapse to dd with $J_{6,OH}$ having disappeared, H-6a), 3.81-3.72 (3H, H-2, H-3, and H-6b), 3.601 (ddd, 1H, $J_{4,5}$ = 9.5 Hz, $J_{5,6a}$ = 3.0 Hz, $J_{5,6b}$ = 5.0 Hz, H-5), 3.298 (s, 3H, CH3O), 2.046 (dd, 1H, J6a,OH = 5.5 Hz, J6b,OH = 7.0 Hz, deuterium exchangeable, 6-OH); ^{13}C nmr ($CDCl_3$) δ: 138.56, 138.37 (quat. arom.), 134.96 (CH_2=$\underline{C}$HCH_2O), 128.36, 128.02, 127.81, 127.67 (tert. arom.), 116.60 ($\underline{C}H_2$=CHCH_2O), 99.47 (C-1), 79.92 (C-3), 75.13 ($C_6H_5\underline{C}H_2O$), 74.88 (C-2 and C-4), 72.99 ($C_6H_5\underline{C}H_2O$), 72.10 (C-5), 71.10 (CH_2=C$\underline{H}CH_2O$), 62.42 (C-6), 54.72 (CH_3O). Anal. calcd. tor $C_{24}H_{30}O_6$: C 69.55, H 7.30; found: C 69.42, H 7.37.

Methyl 6-O-acetyl-3-O-allyl-2,4-di-O-benzyl-α-D-manno-pyranoside (**16**)

To a solution of **14** (10.30 g, 24.85 mmol) in dry pyridine (100 mL) was added acetic anhydride (7.5 mL; 79.54 mmol). The mixture was stirred under nitrogen atmosphere at room temperature overnight. The excess of acetic anhydride was decomposed by dropwise addition of ethanol (5 mL) to the ice cold reaction mixture, and dichloromethane (300 mL) and water (250 mL) were then added. The aqueous layer was separated and back extracted

with dichloromethane (100 mL). The combined dichloromethane extracts were washed with 1 M aqueous HCl and saturated aqueous sodium bicarbonate. The organic phase was dried ($MgSO_4$), filtered, and evaporated under reduced pressure to give **16** (11.06 g; 98%) as a chromatography-pure oil; $[\alpha]_D$ +42.91° (c 1.03, chloroform); R_f 0.45 (Skellysolve B-ethyl acetate, 3:1); ^1H nmr ($CDCl_3$) δ: 7.41-7.25 (10H, aromatic), 5.920 (1H, Hc allyl), 5.309 (1H, Hb allyl), 5.169 (1H, Ha allyl), 4.910 (d, 1H, J_{gem} = 11.0 Hz, $C_6H_5C\underline{H}HO$), 4.778-4.691 (3H, $C_6H_5C\underline{H}_2O$ [δ 4.778-4.691, AB, J_{gem} = 11.0 Hz] overlapping with H-1 [δ 4.718, d, $J_{1,2}$ = 2.0 Hz]), 4.561 (d, 1H, J_{gem} = 11.0 Hz, $C_6H_5C\underline{H}HO$); 4.348-4.268 (2×dd, 2H, $J_{6a,6b}$ = 12.0 Hz, $J_{5,6a}$ = 3.0 Hz, $J_{5,6b}$ = 5.0 Hz, H-6a and H-6b), 4.119-4.025 (2H, Hd and He allyl), 3.844 (dd, 1H, $J_{3,4}$ = 9.0 Hz, $J_{4,5}$ = 9.5 Hz, H-4), 3.781-3.706 (3H, H-3 [δ 3.766, $J_{2,3}$ = 3.5 Hz, $J_{3,4}$ = 9.5 Hz], H-2 [δ 3.743, $J_{1,2}$ = 2.0 Hz, $J_{2,3}$ = 3.5 Hz], and H-5), 3.31 (s, 3H, CH_3O), 2.05 (s, 3H, $OCOCH_3$); ^{13}C nmr ($CDCl_3$) δ: 170.80 $O\underline{C}OCH_3$), 138.34 (quat. arom.), 134.85 ($CH_2=\underline{C}HCH_2O$), 128.36, 128.29, 128.08, 127.70, 127.60 (tert. arom.), 116.70 ($\underline{C}H_2=CHCH_2O$), 99.10 (C-1), 79.89 (C-3), 75.07 ($C_6H_5\underline{C}H_2O$), 74.50, 74.58 (C-2 and C-4), 72.66 ($C_6H_5\underline{C}H_2O$), 70.98 ($CH_2=CH\underline{C}H_2O$), 69.98 (C-5), 63.63 (C-6), 54.73 (CH_3O), 20.81 ($OCO\underline{C}H_3$). Anal. calcd. for $C_{26}H_{32}O_7$: C 68.40, H 7.07; found: C 68.39, H 7.09.

1,6-Di-O-acetyl-3-O-allyl-2,4-di-O-benzyl-α-D-manno-pyranose (**17**)

A solution of concentrated sulfuric acid (0.22 mL) in acetic anhydride (7.68 mL) was added dropwise to a solution of **16** (8.77 g; 19.65 mmol) in acetic anhydride (40 mL) at 0° over 10 min. The mixture was stirred at 0°C for 25 min and at room temperature for 20 min. Then the reaction mixture was poured into dichloromethane (1 L) and ice cold water (1 L) containing sodium bicarbonate, and the resulting mixture was stirred at room temperature for 30 min. The organic and aqueous layers were separated, and the aqueous layer was extracted with dichloromethane (500 mL). The dichloromethane solutions were combined and then washed with saturated aqueous sodium carbonate and water. Finally the organic phase was dried (Na_2SO_4), filtered, and concentrated. The residual syrup was purified by flash chromatography using Skellysolve B-ethyl acetate as eluent, the ratio of Skellysolve B to ethyl acetate being varied from 6:1 to 3:1 during elution. Removal of solvent from the early fractions provided the title compound as an oil (7.35 g; 77% yield); $[\alpha]_D$ +37.85 (c 0.93, chloroform); R_f 0.56 (Skellysolve B-ethyl acetate, 3:1); ^1H nmr ($CDCl_3$) δ: 7.431-7.273 (10H,

aromatic), 6.195 (d, 1H, $J_{1,2}$ = 2.0 Hz, H-1), 5.923 (1H, Hc allyl), 5.315 (1H, Hb allyl), 5.200 (1H, Hc allyl), 4.935 (d, 1H, J_{gem} = 10.5 Hz, $C_6H_5C\underline{H}HO$), 4.840-4.766 (AB, 2H, J_{gem} = 12.0 Hz, $C_6H_5C\underline{H}_2O$), 4.621 (d, 1H, J_{gem} = 10.5 Hz, $C_6H_5C\underline{H}HO$), 4.384-4.310 (2× dd, 2H, H-6a and H-6b), 4.095 (ddd, 2H, CH_2=$CHC\underline{H}_2O$), 3.973 (dd, 1H, $J_{3,4}$ = 10.0 Hz, $J_{4,5}$ = 10.0 Hz, H-4), 3.893 (ddd, 1H, $J_{4,5}$ = 10.0 Hz, $J_{5,6a}$ = 4.0 Hz, $J_{5,6b}$ = 3.0 Hz, H-5), 3.814, 3.785 (2 dd, 2H, $J_{3,4}$ = 10.0 Hz, $J_{2,3}$ = 3.0 Hz, $J_{1,2}$ = 2.0 Hz, H-3 and H-2 respectively), 2.055 (s, 6H, $OCOCH_3$× 2); ^{13}C nmr ($CDCl_3$) δ: 170.82, 168.82 ($O\underline{C}OCH_3$), 138.08, 137.87 (quat. arom.), 134.68 (H_2C=$\underline{C}HCH_2O$), 128.50, 128.40, 128.24, 127.93, 127.88, 127.83 (tert. arom.), 117.04 ($H_2\underline{C}$=$CHCH_2O$), 91.79 (C-1), 79.17 (C-3), 75.36 ($C_6H_5\underline{C}H_2O$), 73,89, 73.39 (C-2 and C-4), 72.53 ($C_6H_5\underline{C}H_2O$), 72.41 (C-5), 71.09 (H_2C=$CH\underline{C}H_2O$), 63.24 (C-6), 20.98, 20.85 ($OCO\underline{C}H_3$). Anal. calcd. for $C_{27}H_{32}O_8$: C 66.93, H 6.66; found: C 66.78, H 6.74.

Evaporation of the subsequent fractions furnished the corresponding β-anomer (**18**) and 1,4,6-tri-O-acetyl-3-O-allyl-2-O-benzyl-α-D-mannopyranose (**19**) in a combined yield of 6%. The ^1H nmr spectra of **18** and **19** were in agreement with the assigned structures.

6-O-Acetyl-3-O-allyl-2,4-di-O-benzyl-α-D-mannopyranosyl bromide (**8**)

Hydrogen bromide gas was bubbled for 30 min through a tube of calcium sulfate into a solution of **17** (5.80 g; 11.97 mmol) in dry dichloromethane (300 mL) at 0°C. The solution was then taken to dryness and the by-product acetic acid removed by evaporation of toluene (100 mL) from the residue (twice). Finally **8** was obtained as an oil, a very small portion of which was purified for elemental and nmr spectral analysis by flash chromatography using Skellysolve B-ethyl acetate (3:1) as eluent; $[\alpha]_D$ +133.08° (c 0.91, chloroform); R_f 0.60 (Skellysolve B-ethyl acetate, 3:1); ^1H nmr ($CDCl_3$) δ: 7.42-7.30 (10H, aromatic), 6.444 (d, 1H, $J_{1,2}$ = 1.5 Hz, H-1), 5.936 (1H, Hc allyl), 5.350 (1H, Hb allyl), 5.233 (1H, Ha allyl), 5.955 (d, 1H, J_{gem} = 10.5 Hz, $C_6H_5C\underline{H}HO$), 4.788-4.718 (AB, 2H, Jgem = 12.5 Hz, $C_6H_5C\underline{H}_2O$), 4.613 (d, 1H, J_{gem} = 10.5 Hz, $C_6H_5C\underline{H}HO$), 4.388-4058 (2H, H-6a and H-6b), 4.243 (dd, 1H, J3,4 = 9.0 Hz, J2,3 = 3.0 Hz, H-3), 4.116 (d, 2H, J = 5 Hz, $CH_2=CHC\underline{H}_2O$), 4.016-3.935 (3H, H-2 [δ4.011, dd, $J_{2,3}$ = 3.0 Hz, $J_{1,2}$ = 1.5 Hz], H-4 and H-5), 2.065 (s, 3H, $OCOCH_3$); 13C nmr ($CDCl_3$) δ: 170.57 ($O\underline{C}OCH_3$), 137.90, 137.55 (quat. arom.), 134.37 ($CH_2=\underline{C}HCH_2O$), 128.63, 128.44, 128.14, 127.97, 127.87,

127.73 (tert. arom.), 117.38 ($\underline{C}H_2$=$CHCH_2O$), 87.41 (C-1), 78.41 (C-3), 78.31, 74.31, 73.57 (C-2, C-4, and C-5), 75.30, 72.89 ($C_6H_5\underline{C}H_2O$), 71.21 (CH_2=$CH\underline{C}H_2O$), 62.39 (C-6), 20.72 ($OCO\underline{C}H_3$). Anal. calcd. for $C_{25}H_{29}O_6Br$: C 59.41, H 5.78, Br 15.81; found: C 59.48, H 5.82, Br 16.17.

8-Methoxycarbonyloctyl 6-O-acetyl-3-O-allyl-2,4-di-O-benzyl-β-D-mannopyranoside (**23**β) and 8-methoxycarbonyloctyl 6-O-acetyl-3-O-allyl-2,4-di-O-benzyl-α-D-mannopyranoside (**23**α)

8-Methoxycarbonyoctanol[24] (7.50 g; 39.89 mmol) and silver silicate/alumina (16.50 g) in dry dichloromethane (100 mL) were stirred at room temperature for 1 h. To the above mixture which was cooled to -78°C was added dropwise, with stirring, a solution of bromosugar **8** (7.44 g; 14.72 mmol) in dry dichloromethane (75 mL) and stirring was continued for 2.5 h at -78°C, and for 10 h at room temperature. The mixture was diluted with dichloromethane (100 mL) and filtered through celite. The filtrate was washed with water, dried (Na_2SO_4), and concentrated to a syrup which was purified by flash chromatography, using Skellysolve B-ethyl acetate (4.5:1) as eluent. A chromatographically inseparable 6:1 mixture of β- and α-mannosides **23** was obtained as an oil (5.14 g; total yield

57%). ^1H nmr ($CDCl_3$) δ: 4.824 (d, $J_{1,2}$ = 1.5 Hz, H-1 of α-anomer), 4.365 (d, $J_{1,2}$ = 0.5 Hz, H-1 of β-anomer), 2.059 (s, $OCOCH_3$ of α-anomer), 2.050 (s, $OCOCH_3$) of β-anomer).

8-Methoxycarbonyloctyl 3-O-allyl-2,4-di-O-benzyl-β-D-mannopyranoside (**24**) and 8-methoxycarbonyloctyl 3-O-allyl-2,4-di-O-benzyl-α-D-mannopyranoside (**25**)

The mixture of α- and β-anomers **23** described above (4.39 g; 7.17 mmol) was dissolved in dry methanol (200 mL) containing a trace of sodium methoxide, and the resulting solution was stirred at room temperature overnight. Neutralization with Amberlite IR-120(H) resin followed by the removal of the resin and evaporation provided a chromatographically pure oily residue (4.10 g) whose tlc on silica gel impregnated with silver nitrate showed it to be a mixture of two compounds. Purification of these two products was accomplished by flash chromatography on silver nitrate-impregnated silica gel using Skellysolve B-ethyl acetate as eluent, the ratio of Skellysolve B to ethyl acetate being decreased from 7:1 to 2:1 during elution. Evaporation of early fractions furnished the α-anomer **25** (0.19 g) as an oil; $[\alpha]_D$ +30.8° (c 0.75, chloroform); ^1H nmr ($CDCl_3$) δ: 7.42-7.27 (10H, aromatic),

5.965 (1H, Hc of allyl), 5.345 (1H, Hb allyl), 5.194 (1H, Ha allyl), 4.945 (d, 1H, J_{gem} = 11.0 Hz, $C_6H_5\underline{C}\underline{H}HO$), 4.843-4.788 (2H, $C_6H_5\underline{C}\underline{H}HO$ [δ 4.828, d, J_{gem} = 12.0 Hz] and H-1 [δ 4.790, d, $J_{1,2}$ = 2.0 Hz], 4.724 (d, 1H, J_{gem} = 12.0 Hz, $C_6H_5\underline{C}\underline{H}HO$), 4.655 (d, 1H, J_{gem} = 11.0 Hz, $C_6H_5\underline{C}\underline{H}HO$), 4.138 (broad d, 2H, Hd and He allyl), 3.923 (dd, 1H, $J_{4,5}$ = 9.5 Hz, $J_{3,4}$ = 9.5 Hz, H-4), 3.870-3.579 (9H, H-2, H-3, H-5, H-6a, H-6b, $O\underline{C}\underline{H}HCH_2$, and OCH_3 [δ 3.665, s]), 3.329 (dt, 1H, J_{gem} = 9.0 Hz, J_{vic} = 6.5 Hz, $O\underline{C}\underline{H}HCH_2$), 2.303 (t, 2H, $\underline{C}\underline{H}_2COOCH_3$, J = 7.5 Hz), 2.098 (broad s, 1H, exchangeable with deuterium, OH), 1.620 (m, 2H, aliphatic), 1.510 (m, 2H, aliphatic), 1.29 (broad s, 8H, remaining aliphatic); ^{13}C nmr ($CDCl_3$) δ: 174.15 ($\underline{C}OOCH_3$), 138.48, 138.43 (quat. arom.), 134.95 ($CH_2=\underline{C}HCH_2O$), 128.35, 128.29, 128.07, 127.72, 127.67, 127.58 (tert. arom.), 116.47 ($\underline{C}H_2=CHCH_2O$), 98.25 (C-1, $J_{C-1,H-1}$ = 168.2 Hz), 79.98 (C-3), 75.18 ($C_6H_5\underline{C}H_2O$), 75.04, 75.01 (C-3 and C-4), 72.88 ($C_6H_5\underline{C}H_2O$), 72.08 (C-5), 71.07 ($CH_2=CH\underline{C}H_2O$), 67.61 ($O\underline{C}H_2CH_2$), 62.46 (C-6), 51.33 ($COO\underline{C}H_3$), 34.01 ($\underline{C}H_2COOCH_3$), 29.32, 29.11, 29.06, 29.01, 25.99, 24.85 (aliphatic). Anal. calcd. for $C_{33}H_{46}O_8$: C 69.45, H 8.12; found: c 69.27, H 8.26.

Further elution provided a mixture of α- and β-anomers (0.9 g) which were in the ratio of 1:3 (α/β) by ^1H nmr.

Evaporation of the later fractions afforded the

desired β-anomer **24** (2.89 g) as an oil; $[\alpha]_D$ -51.33 (c 0.9, chloroform); ^{1}H nmr ($CDCl_3$) δ: 7.49-7.29 (10H, aromatic), 5.893 (1H, Hc allyl), 5.288 (1H, Hb allyl), 5.17 (1H, Ha allyl), 4.969-4.858 (3H, $C_6H_5C\underline{H}_2O$ [δ 4.969-4.800, AB, J_{gem} = 12.5 Hz] and $C_6H_5C\underline{H}HO$ [4.935, d, J_{gem} = 10.5 Hz]), 4.624 (d, 1H, J_{gem} = 10.5 Hz, $C_6H_5C\underline{H}HO$), 4.408 (d, 1H, J = 1 Hz, H-1), 4.024-3.828 (6H, H-2, H-4, H-6a, $OC\underline{H}H\underline{C}H_2$, and $CH_2{=}CHC\underline{H}_2O$), 3.763 (ddd, 1H, $J_{6a,6b}$ = 12.0 Hz, $J_{5,6b}$ = 6.0 Hz, $J_{6b,OH}$ = 6.0 Hz, simplified to dd with $J_{6b,OH}$ being absent on D_2O exchange, H-6b), 3.688 (s, 3H, OCH_3), 3.444-3.386 (2H, H-3 and $OC\underline{H}H\underline{C}H_2$), 3.316 (dt, 1H, $J_{4,5}$ = 9.5 Hz, $J_{5,6b}$ = 6.0 Hz, $J_{5,6a}$ = 3.0 Hz, H-5), 2.330 (t, 2H, $C\underline{H}_2COOCH_3$, J = 7.5 Hz), 2.140 (t, 1H, $J_{6a,OH}$ = 6.0 Hz, $J_{6b,OH}$ = 6.0 Hz, disappeared after D_2O addition, OH), 1.670-1.628 (4H, aliphatic), 1.345 (8H, remaining aliphatic); ^{13}C nmr ($CDCl_3$) δ: 174.50 ($\underline{C}OOCH_3$), 138.66, 138.36 (quat. arom.), 134.70 ($CH_2{=}\underline{C}HCH_2O$), 128.37, 128.28, 128.11, 128.03, 127.73, 127.37 (tert. arom.), 116.75 ($\underline{C}H_2{=}CHCH_2O$), 101.69 (C-1, $J_{C-1,H-1}$ = 153.6 Hz), 82.33 (C-3), 75.77, 74.88, 73.90 (C-2, C-4, and C-5), 75.18, 73.86 ($C_6H_5\underline{C}H_2O$), 70.58, 70.12 ($CH_2{=}CH\underline{C}H_2O$ and $O\underline{C}H_2CH_2$), 62.63 (C-6), 51.36 ($COO\underline{C}H_3$), 34.04 ($\underline{C}H_2COOCH_3$), 29.63, 29.16, 29.04, 26.01, 24.89 (aliphatic). Anal. calcd. for $C_{33}H_{46}O_8$: C 69.45, H 8.12; found: C 69.41, H 8.13.

8-Methoxycarbonyloctyl 6-O-(2-O-acetyl-3,4,6-tri-O-benzyl-α-D-mannopyranosyl)-3-O-allyl-2,4-di-O-benzyl-β-D-mannopyranoside (**27**)

To a solution of **24** (2.89 g; 5.06 mmol) in dry acetonitrile (60 mL) containing 4Å molecular sieves were added, sequentially, mercuric bromide (2.19 g; 6.07 mmol), mercuric cyanide (1.53 g; 6.07 mmol), and a solution of 2-O-acetyl-3,4,6-tri-O-benzyl-α-D-mannopyranosyl bromide[27] (**7**) in dry acetonitrile (25 mL), which had been freshly prepared from 3,4,6-tri-O-benzyl-1,2-O-(methoxyethylidene)-β-D-mannopyranose (**26**) (3.07 g; 6.07 mmol). The reaction mixture was stirred at room temperature for 1 h, and the mixture was then filtered through Celite. Evaporation of the solvent gave an oily residue which was extracted 3 times with dichloromethane. The extracts were combined and washed successively with saturated aqueous potassium chloride, saturated aqueous sodium bicarbonate, water, and brine. The organic layer was dried (Na_2SO_4), filtered, and evaporated to dryness. The resulting oil was purified by flash chromatography using Skellysolve B-ethyl acetate (4:1) as eluent to provide the title compound as a syrup (4.07 g; 77%); $[\alpha]_D$ -6.07° (*c* 1.22,

chloroform); R_f 0.3 (Skellysolve B-ethyl acetate, 3:1); ^1H nmr ($CDCl_3$) δ: 7.47-7.10 (25H, aromatic), 5.86 (1H, Hc allyl), 5.459 (dd, 1H, $J_{2',3'}$ = 3.0 Hz, $J_{1',2'}$ = 2.0 Hz, H-2'), 5.270 (1H, Hb allyl), 5.159 (1H, Ha allyl), 4.980-4.825 (5H, H-1' [δ 4.911] and 4 × $C_6H_5C\underline{H}HO$ [d, J_{gem} = 11.0-12.5 Hz]), 4.680-4.650 (2 d overlapping, 2H, J_{gem} = 10.5 and 12.0 Hz, $C_6H_5C\underline{H}_2O$), 4.513-4.408 (4×d, 4H, J_{gem} = 11.0-12.0 Hz, $C_6H_5C\underline{H}_2O$), 4.334 (s, 1H, H-1), 3.930-3.590 (15H, $COOCH_3$ [δ 3.66], H-2, H-4, H-6a, H-6b, H-3', H-4', H-5', H-6a', H-6b', $CH_2=CHC\underline{H}_2O$ and $OC\underline{H}HCH_2$), 3.410-3.315 (3H, H-3, H-5, and $OC\underline{H}HCH_2O$), 2.283 (t, 2H, J = 7.5 Hz, $C\underline{H}_2COOCH_3$), 2.133 (s, 3H, $OCOCH_3$), 1.620-1.513 (4H, aliphatic), 1.335-1.240 (8H, remaining aliphatic); ^{13}C nmr ($CDCl_3$) δ: 174.08 ($\underline{C}OOCH_3$), 170.12 ($O\underline{C}OCH_3$), 139.00, 138.80, 138.53, 138.46, 138.05 (quat. arom.), 134.79 ($CH_2=\underline{C}HCH_2O$), 128.27, 128.19, 128.13, 128.10, 127.97, 127.77, 127.70, 127.59, 127.54, 127.50, 127.38, 127.29, 127.23 (tert. arom.), 116.67 ($\underline{C}H_2=CHCH_2O$), 101.64 (C-1, $J_{C-1,H-1}$ = 154.7 Hz), 97.77 (C-1', $J_{C-1',H-1'}$ = 170.4 Hz), 82.56 (C-3), 77.94 (C-3'), 74.80, 74.37, 73.91 (C-2, C-4, C-5, and C-4'), 71.36 (C-5'), 68.67 (C-2'), 74.91, 73.71, 73.30, 71.51 ($C_6H_5\underline{C}H_2O$ × 5), 70.43, 69.79 ($CH_2=CH\underline{C}H_2O$ and $O\underline{C}H_2CH_2$), 68.98 (C-6'), 67.06 (C-6), 51.26 ($COO\underline{C}H_3$), 34.05 ($\underline{C}H_2COOCH_3$), 29.65, 29.23, 29.16, 29.07, 26.09, 24.92 (aliphatic), 20.99 ($OCO\underline{C}H_3$). Anal. calcd. for

$C_{62}H_{76}O_{14}$: C 71.24, H 7.33; found: 71.09, H 7.46.

8-Methoxycarbonyloctyl 3-O-allyl-2,4-di-O-benzyl-6-O-(3,4,6-tri-O-benzyl-α-D-mannopyranosyl)-β-D-mannopyranoside (**28**)

The disaccharide **27** (2.88 g; 2.76 mmol) was de-O-acetylated as described for the preparation of **25** and **26**. After removal of the resin by filtration the solvent was evaporated to afford **28** as chromatographically pure oil (2.73 g; 99%); $[\alpha]_D$ +3.13° (c 1.34, chloroform); R_f 0.32 (Skellysolve B-acetone, 3:1); ^1H nmr ($CDCl_3$) δ: 7.48-7.14 (25H, aromatic), 5.883 (1H, Hc allyl), 5.288 (1H, Hb allyl), 5.170 (1H, Ha allyl), 5.258 (broad s, 1H, $J_{1',2'}$ < 2Hz, H-1'), 4.968-4.795 (4×d, 4H, J_{gem} = 11.0 and 12.5 Hz, $C_6H_5\underline{CH_2}O$), 4.644-4.438 (6×d, 6H, J_{gem} = 11.0-12.0 Hz, $C_6H_5\underline{CH_2}O$), 4.335 (s, 1H, $J_{1,2}$ < 1 Hz, H-1), 4.128 (broad s, 1H, H-2'), 4.038-3.593 (15H, OCH_3 [δ3.655, s], H-2, H-4, H-6a, H-6b, H-3', H-4', H-5', H-6a', H-6b', $OCHH\underline{CH_2}$, and $CH_2{=}CH\underline{CH_2}O$), 3.423-3.320 (3H, H-3, H-5, and $OC\underline{H}HCH_2$), 2.355 (broad s, 1H, deuterium-exchangeable, OH), 2.284 (t, 2H, J = 7.5 Hz, $\underline{CH_2}COOCH_3$), 1.59 (4H, aliphatic), 1.28 (8H, remaining aliphatic); ^{13}C nmr ($CDCl_3$) δ: 173.86 ($\underline{C}OOCH_3$), 139.04, 138.73, 138.59, 138.44, 138.03 (quat. arom.), 134.86 ($CH_2{=}\underline{C}HCH_2O$), 128.49, 128.36, 128.29,

128.24, 128.07, 127.99, 127.89, 127.78, 127.62, 127.48, 127.40 (tert. arom.), 116.84 ($\underline{C}H_2$=CH$\underline{C}H_2$O), 101.73 (C-1), 99.77 (C-1'), 82.47 (C-3), 79.69 (C-3'), 75.24, 74.61, 74.36, 74.06 (C-3, C-4, C-5, and C-4'), 75.07, 74.94, 73.88, 73.41, 71.48 ($C_6H_5\underline{C}H_2$O), 71.07 (C-5'), 70.54, 69.88 (CH_2=C$\underline{H}\underline{C}H_2$O and O$\underline{C}H_2\underline{C}H_2$), 69.03 (C-6'), 68.01 (C-2'), 66.65 (C-6), 51.43 (COO$\underline{C}H_3$), 34.12 ($\underline{C}H_2$COOCH$_3$), 29.72, 29.29, 29.25, 29.14, 26.15, 24.99 (aliphatic). Anal. calcd. for $C_{60}H_{74}O_{13}$: C 71.83, H 7.44; found: C 71.59; H 7.31.

8-Methoxycarbonyloctyl 6-O-[2-O-(3,4,6-tri-O-acetyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl)-3,4,6-tri-O-benzyl-α-D-mannopyranosyl]-3-O-allyl-2,4-di-O-benzyl-β-D-mannopyranoside (**4**)

To a solution of **28** (2.73 g; 2.73 mmol) in dry dichloromethane (75 mL) were added silver triflate (7.0 g; 27.25 mmol), sym-collidine (3.6 mL, 27.25 mmol), and 4 Å molecular sieves. To the resulting mixture, cooled to -50°C, was added dropwise a solution of 3,4,6-tri-O-acetyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl bromide[26] (**6**) (2.30 g; 2.60 mmol) in dry dichloromethane (25 mL). The above mixture was stirred at -50°C for 15 min and then allowed to warm to room temperature over a period of 1

h. The tlc of the mixture revealed the presence of unreacted alcohol **28** (~30%). The reaction mixture was again cooled to -50°C and a solution of the bromide **6** (2.30 g; 2.60 mmol) in dry dichloromethane (25 mL) was added dropwise. After stirring at -50°C for 15 min, the mixture was allowed to attain room temperature, with stirring, during 1 h. The tlc now showed the complete disappearance of the alcohol **28**. The mixture was diluted with dichloromethane, and then filtered through Celite. The filtrate was washed sequentially with ice water, ice cold 1N aqueous HCl, and saturated aqueous sodium bicarbonate. The organic phase was dried (Na_2SO_4), filtered, and evaporated. The residual oil was subjected to flash chromatography employing toluene-ethyl acetate (3.5:1) as eluent. The fractions containing the major product were combined and evaporated. The tlc of the oil so obtained, when developed in Skellysolve B-acetone (3:1), also indicated the presence of a minor product. Therefore, the above oil was again chromatographed using Skellysolve B-acetone (3:1) as eluent to provide the α-linked trisaccharide **29** as an oil (0.27 g; 7%). Further elution with the same solvent system gave the title compound as a syrup (2.92 g; 76%); $[\alpha]_D$ +23.04° (*c* 1.02, chloroform); R_f 0.45 (toluene-ethyl acetate, 3:1), 0.17 (Skellysolve B-acetone, 3:1); ^1H nmr ($CDCl_3$) δ: 7.86-7.04

(29H, aromatic), 5.901-5.790 (2H, Hc allyl and H-3" [δ5.815, $J_{3",4"}$ = 9.0 Hz, $J_{2",3"}$ = 11.0 Hz]), 5.573 (d, 1H, $J_{1",2"}$ = 8.5 Hz, H-1"), 5.303-5.143 (3H, H-4" [δ5.215, dd, $J_{4",5"}$ = 10.0 Hz, $J_{3",4"}$ = 9.0 Hz] and Ha and Hb allyl), 4.993 (d, 1H, J_{gem} = 13.0 Hz, $C_6H_5C\underline{H}HO$), 4.890-4.735 (4×d, 4H, J_{gem} = 11.0-12.5 Hz, $C_6H_5C\underline{H}_2O$), 4.676 (d, 1H, $J_{1',2'}$ = 1.5 Hz, H-1'), 4.538-4.459 (2H, H-2" [δ4.513, dd, $J_{2",3"}$ = 11.0 Hz, $J_{1",2"}$ = 8.5 Hz] and $C_6H_5C\underline{H}HO$ [δ4.475, d, J_{gem} = 12.0 Hz]), 4.400 (dd, 1H, $J_{2',3'}$ = 2.5 Hz, $J_{1',2'}$ = 2.5 Hz, H-2'), 4.373-4.303 (4H, H-1 [δ4.344], H-6a", and $C_6H_5C\underline{H}_2O$), 4.233 (dd, 1H, $J_{6a",6b"}$ = 12.5 Hz, $J_{5",6b"}$ = 2.0 Hz, H-6b"), 4.063-3.245 (20H, $OC\underline{H}HCH_2$ [δ4.035, td, J_{gem} = 9.0 Hz, J_{vic} = 6.0 Hz], OCH_3 [δ3.668, s], CH_2=CHC$\underline{H}_2$O, $C_6H_5C\underline{H}_2O$, H-2, H-3, H-4, H-5, H-6a, H-6b, H-3', H-4', H-5', H-6a', H-5", $OC\underline{H}HCH_2$), 2.989 (dd, 1H, $J_{6a',6b'}$ = 11.0 Hz, $J_{5',6b'}$ = 5.5 Hz, H-6b'), 2.31 (t, 2H, $C\underline{H}_2COOCH_3$, J = 7.5 Hz), 2.055 (s, 3H, $OCOCH_3$), 2.024 (s, 3H, $OCOCH_3$), 1.863 (s, 3H, $OCOCH_3$), 1.70-1.59 (4H, aliphatic), 1.44-1.26 (8H, remaining aliphatic); ^{13}C nmr ($CDCl_3$) δ: 173.96 ($\underline{C}OOCH_3$), 170.42, 169.89, 169.21 ($O\underline{C}OCH_3$ and phthalimido carbonyl), 138.69, 138.52, 138.36, 137.80 (benzyl quat. arom.), 134.55 (CH_2=$\underline{C}$HCH_2O), 133.74 (phthalimido tert. arom.), 131.52 (phthalimido quat. arom.), 128.31, 128.19, 128.00, 127.92, 127.86, 127.63, 127.47, 127.43, 127.25, 127.19, 126.99 (benzyl tert.

arom.), 123.16 (phthalimido tert. arom.), 116.52 ($\underline{C}H_2$=$CHCH_2O$), 101.79 (C-1, $J_{C-1,H-1}$ = 152.8 Hz), 97.44 (C-1', $J_{C-1',H-1'}$ = 169.2 Hz), 96.48 (C-1", $J_{C-1",H-1"}$ = 165.1 Hz), 82.64 (C-3), 76.81, 74.54, 74.09, 73.75, 73.49, 72.88, 71.89, 71.45, 70.62, 69.01 (C-2, C-4, C-5, C-2', C-3', C-4', C-5', C-3", C-4", C-5"), 74.64, 73.63, 72.41, 70.33 ($C_6H_5\underline{C}H_2O$), 70.11 (CH_2=$CH\underline{C}H_2O$ and $O\underline{C}H_2CH_2$), 69.40 (C-6'), 66.53 (C-6), 62.17 (C-6"), 54.25 (C-2"), 51.17 ($COO\underline{C}H_3$), 33.86 ($\underline{C}H_2COOCH_3$), 29.47, 29.10, 29.04, 28.90, 25.91, 24.74 (aliphatic), 20.56, 20.43, 20.24 ($OCO\underline{C}H_3$). Anal. calcd. for $C_{80}H_{93}N_1O_{22}$: C 67.64, H 6.60, N 0.99; found: C 67.54, H 6.58, N 0.97.

8-Methoxycarbonyloctyl 6-O-[2-O-3,4,6-tri-O-acetyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl)-3,4,6,-tri-O-benzyl-α-D-mannopyranosyl]-2,4-di-O-benzyl-β-D-mannopyranoside (31)

A solution of **4** (2.79 g; 1.97 mmol), tris(triphenylphosphine)rhodium(I) chloride (129 mg; 0.14 mmol), 1,8-diazabicyclo[2.2.2]octane (58 mg; 0.51 mmol) in ethanol-benzene-water (7:3:1; 100 mL) was heated at reflux for 24 h. The solvent was removed and the residue dissolved in acetone (100 mL) containing a trace amount of mercuric oxide. To this solution was added a solution of

mercuric chloride (3.0 g) in acetone-water (9:1; 50 mL), and the mixture was stirred at room temperature for 45 min. Following evaporation of the solvent, the residue was taken up in dichloromethane (250 mL). The dichloromethane solution was washed with 30% aqueous potassium bromide and water. The organic layer was dried (Na_2SO_4) and evaporated to give an oily residue which was purified by flash chromatography using toluene-ethyl acetate (3.5:1) as eluent. The title compound was obtained as a white foamy solid (2.31 g; 85%); $[\alpha]_D$ -19.07° (c 0.97, chloroform); R_f 0.37 (toluene-ethyl acetate, 3:1); ^1H nmr ($CDCl_3$) δ: 7.88-7.07 (29H, aromatic), 5.84 (dd, 1H, $J_{3",4"}$ = 9.0 Hz, $J_{2",3"}$ = 11.0 Hz, H-3"), 5.61 (d, 1H, $J_{1",2"}$ = 8.5 Hz, H-1"), 5.221 (dd, 1H, $J_{4",5"}$ = 9.0 Hz, $J_{3",4"}$ = 9.0 Hz, H-4"), 5.063 (d, 1H, J_{gem} = 12.0 Hz, $C_6H_5C\underline{H}HO$), 4.853 (d, 1H, J_{gem} = 11.0 Hz, $C_6H_5C\underline{H}HO$), 4.778-4.733 (2×d, 2H, J_{gem} = 11.0 and 12.0 Hz, $C_6H_5C\underline{H}_2O$), 4.673 (d, 1H, $J_{1',2'}$ = 2.0 Hz, H-1'), 4.633 (d, 1H, J_{gem} = 12.0 Hz, $C_6H_5C\underline{H}HO$), 4.543-4.476 (2H, H-2" [δ4.518, dd, $J_{2",3"}$ = 11.0 Hz, $J_{1",2"}$ = 8.5 Hz], $C_6H_5C\underline{H}HO$ [δ4.491, d, J_{gem} = 12.0 Hz]), 4.433 (1H, $J_{1,2}$ < 1 Hz, H-1), 4.385-4.240 (5H, H-2', H-6a", H-6b" and $C_6H_5C\underline{H}_2O$), 4.035-3.935 (4H, $OC\underline{H}HCH_2$, H-5", and $C_6H_5C\underline{H}_2O$), 3.828-3.245 (14H, H-2, H-3, H-4, H-5, H-6a, H-6b, H-3', H-4', H-5', H-6a', $OC\underline{H}HCH_2$ and OCH_3 [δ3.651, s]), 2.998 (dd, 1H,

$J_{6a',6b'}$ = 11.0 Hz, $J_{5',6b'}$ = 5.5 Hz, H-6b'), 2.380 (d, 1H, deuterium-exchangeable, $J_{OH,H-3}$ = 10.0 Hz, OH), 2.290 (t, 2H, $C\underline{H}_2COOCH_3$, J = 7.5 Hz), 2.056,2.050 (2×s, 6H, $OCOCH_3$), 1.860 (s, 3H, $OCOCH_3$), 1.68-1.58 (4H, aliphatic), 1.40-1.26 (8H, remaining aliphatic); ^{13}C nmr ($CDCl_3$) δ: 174.17 ($\underline{C}OOCH_3$), 170.63, 170.12, 169.40 ($O\underline{C}OCH_3$ and phthalimido carbonyl), 138.77, 138.50, 138.37, 138.30, 138.10 (benzyl quat. arom.), 133.95 (phthalimido tert. arom.), 131.82 (phthalimido quat. arom.), 128.56, 128.36, 128.23, 128.13, 128.08, 127.72, 127.62, 127.59, 127.39, 127.21 (benzyl tert. arom.), 123.37 (phthalimido tert. arom.), 101.96 (C-1), 97.25 (C-1'), 96.61 (C-1"), 77.90, 77.22, 76.27, 74.39, 74.34, 74.07, 73.22, 72.12, 71.61, 70.80, 69.14 (C-2, C-3, C-4, C-5, C-2', C-3', C-4', C-5', C-3", C-4" and C-5"), 75.06, 74.83, 74.44, 72.63, 70.49 ($C_6H_5\underline{C}H_2O$), 70.34 ($O\underline{C}H_2CH_2$), 69.50 (C-6'), 66.58 (C-6), 62.33 (C-6"), 54.44 (C-2"), 51.38 ($COO\underline{C}H_3$), 34.05 ($\underline{C}H_2COOCH_3$), 29.61, 29.24, 29.19, 29.08, 26.06, 24.92 (aliphatic), 20.74, 20.62, 20.44 ($OCO\underline{C}H_3$). Anal. calcd. for $C_{77}H_{89}N_1O_{22}$: C 66.99, H 6.50, N 1.01; found: C 66.74, H 6.43, N 0.84.

8-Methoxycarbonyloctyl 6-O-[2-O-(2-deoxy-2-phthalimido-β-D-glucopyranosyl)-3,4,6-tri-O-benzyl-α-D-mannopyranosyl]-2,4-di-O-benzyl-β-D-mannopyranoside (**32**)

To a solution of **31** (0.82 g; 0.59 mmol) in dry methanol (75 mL) was added a trace of solid sodium methoxide. The mixture was stirred at room temperature for 3 h. Neutralization of the mixture with Amberlite IR-120(H^+) resin followed by filtration and evaporation afforded **32** as a white foamy solid in a quantitative yield; $[\alpha]_D$ -31.54° (c 1.3, chloroform); R_f 0.2 (dichloromethane-methanol, 19:1); ^1H nmr ($CDCl_3$) δ: 7.66-7.06 (29H, aromatic), 5.399 (d, 1H, $J_{1",2"}$ = 8.0 Hz, H-1"), 5.035 (d, 1H, J_{gem} = 12.0 Hz, C_6H_5CHHO), 4.824-4.534 (6H, H-1' [δ 4.643] and $C_6H_5CH_2O$ [5×d, J_{gem} = 12.5-11.0 Hz]), 4.408-4.241 (6H, H-1 [δ 4.408], H-2' [δ 4.248, dd, $J_{2',3'}$ = 2 Hz, $J_{1',2'}$ = 2 Hz], H-2", H-3", and $C_6H_5CH_2O$ [J_{gem} = 11.0 Hz]), 4.020-3.208 (21H, H-2, H-3, H-4, H-5, H-6a, H-6b, H-3', H-4', H-5', H-6a', H-4", H-5", H-6a", H-6b", OCH_2CH_2, $C_6H_5CH_2O$ and OCH_3 [δ 3.620, s]), 3.063-2.900 (2H, H-6b' [δ 2.918, dd, $J_{6a',6b'}$ = 10.5 Hz, $J_{5',6b'}$ = 5.5 Hz] and OH [broad s, disappeared on D_2O exchange]), 2.396 (d, 1H, $J_{3,OH}$ = 10.0 Hz, deuterium-exchangeable, 3-OH), 2.269 (t, 2H, J = 8.0 Hz, CH_2COOCH_3), 1.65-1.55 (4H, aliphatic), 1.39-1.26 (m, remaining aliphatic); ^{13}C nmr ($CDCl_3$) δ:

174.43 ($\underline{C}OOCH_3$), 168.51 (phthalimido carbonyl), 138.68, 138.52, 138.35, 138.32, 138.05 (benzyl quat. arom.), 133.79 (phthalimido tert. arom.), 131.84 (phthalimido quat. arom.), 128.58, 128.38, 128.34, 128.26, 128.12, 128.03, 127.80, 127.70, 127.49, 127.44, 127.22 (benzyl tert. arom.), 123.23 (phthalimido tert. arom.), 101.90 (C-1), 97.49 (C-1'), 96.84 (C-1"), 77.69, 77.26, 76.31, 75.65, 74.28, 74.13, 73.38, 72.07, 71.73 (C-2, C-3, C-4, C-5, C-2', C-3', C-4', C-5', C-3", C-4", and C-5"), 74.87, 74.77, 74.48, 72.63, 70.86 ($C_6H_5\underline{C}H_2O$), 70.30 ($O\underline{C}H_2CH_2$), 69.70 (C-6'), 66.65 (C-6), 62.21 (C-6"), 56.54 (C-2"), 51.45 ($COO\underline{C}H_3$), 34.07 ($\underline{C}H_2COOCH_3$), 29.61, 29.17, 29.04, 26.04, 24.88 (aliphatic). Anal. calcd. for $C_{71}H_{83}N_1O_{19}$: C 67.98, H 6.67, N 1.12; found: C 67.43, H 6.60, N 1.10.

8-Methoxycarbonyloctyl 6-O-[2-O-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-3,4,6-tri-O-benzyl-α-D-mannopyranosyl]-2,4-di-O-benzyl-β-D-mannopyranoside (33).

Compound 32 (0.43 g; 0.34 mmol) in methanol (25 mL) was boiled with hydrazine hydrate (0.14 mL of an 85% solution; 2.6 mmol) for 12 h. The tlc showed the presence of the starting material in the reaction mixture. Therefore, more hydrazine hydrate (0.07 mL of an 85% solution, 1.3 mmol) was added and the mixture refluxed for

another 4 h. The solution was evaporated and the residue thoroughly dried to remove traces of hydrazine. The residue was then dissolved in methanol-water (1:1, 15 mL) and acetic anhydride (1 mL) was added. The resulting solution was stirred at room temperature for 2 h. Removal of solvent gave a white solid which was purified by flash chromatography using dichloromethane-methanol (12:1) as eluent. Since the crude product was not completely soluble in the eluent used, it was dissolved using more methanol in the same solvent mixture and then adsorbed on sodium sulfate which was poured onto the top of the silica packing. Pure **33** was obtained as a white foamy solid (0.24 g; 60%); $[\alpha]_D$ -20.46° (c 0.88, chloroform); R_f 0.61 (dichloromethane-methanol, 10:1); ^1H nmr ($CDCl_3$) δ: 7.42-7.16 (25H, aromatic), 5.90-5.65 (broad s, 1H, NH, deuterium-exchangeable), 5.053 (d, 1H, J_{gem} = 12.0 Hz, $C_6H_5C\underline{H}HO$), 4.913-4.825 (3H, $C_6H_5C\underline{H}_2O$ [2×d, J_{gem} = 11.0 Hz] and H-1' [δ4.910, d, $J_{1',2'}$ = 2.0 Hz]), 4.743 (d, 1H, J_{gem} = 12.0 Hz, $C_6H_5C\underline{H}HO$), 4.688-4.574 (3H, H-1" and $C_6H_5C\underline{H}_2O$ [2×d, J_{gem} = 12.0 Hz]), 4.488-4.325 (5H, H-1 [δ4.435] and $C_6H_5C\underline{H}_2O$ [4×d, J_{gem} = 12.0 and 11.0 Hz]), 4.253 (t, 1H, H-2'), 3.995-3.293 (23H, H-2, H-3, H-4, H-5, H-6a, H-6b, H-3', H-4', H-5', H-6a', H-6b', H-2", H-3", H-4", H-5", H-6a", H-6b", OH, $OC\underline{H}_2CH_2$, and OCH_3 [δ3.643, s]), 3.00-2.65 (broad s, 2H, disappeared on D_2O exchange, OH), 2.501

(d, 1H, $J_{3,OH}$ = 9.0 Hz, disappeared on D_2O exchange, 3-OH), 2.276 (t, 2H, J = 7.0 Hz, $\underline{CH_2}COOCH_3$), 1.840 (s, 3H, $NHCOCH_3$), 1.64-1.52 (4H, aliphatic), 1.36-1.22 (8H, remaining aliphatic); ^{13}C nmr ($CDCl_3$) δ: 174.10 ($\underline{COOCH_3}$), 172.37 ($NH\underline{COCH_3}$), 138.60, 138.39, 138.30, 137.93 (quat. arom.), 128.50, 128.34, 128.27, 128.17, 127.93, 127.73, 127.65, 127.58, 127.44 (tert. arom.), 101.90 (C-1'), 99.23 (C-1'), 97.66 (C-1"), 77.62, 76.25, 75.83, 74.58, 74.39, 74.13, 73.71, 73.46, 71.64 (C-2, C-3, C-4, C-5, C-2', C-3', C-4', C-5', C-3", C-4", and C-5"), 74.75, 74.45, 73.20, 71.50, 70.30 ($C_6H_5\underline{CH_2}O$), 68.85 (C-6'), 66.86 (C-6), 62.66 (C-6"), 59.07 (C-2"), 51.26 ($COO\underline{CH_3}$), 33.99 ($\underline{CH_2}COOCH_3$), 29.57, 29.14, 28.99, 25.99, 24.84 (aliphatic), 23.30 ($NHCO\underline{CH_3}$). Anal. calcd. for $C_{65}H_{83}N_1O_{18}$: C 66.94, H 7.17, N 1.20; found: C 66.81, H 7.09, N 1.13.

8-Methoxycarbonyloctyl 6-O-[2-O-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-α-D-mannopyranosyl]-β-D-mannopyranoside (2).

Compound **33** (45 mg; 0.03 mmol) was dissolved in 98% ethanol (9 mL), and 5% palladium-on-charcoal (45 mg) was added. The mixture was stirred under an atmosphere of hydrogen gas for 52 h. The catalyst was removed by

filtration and, after solvent evaporation, the residue was passed through a column of Bio-Gel P2 (2.5 cm × 47 cm) using 10% aqueous ethanol as eluent. The carbohydrate-containing fractions were pooled, concentrated, and lyophilized to provide 2 as a white powder (23.5 mg; 85%); $[\alpha]_D$ -19.34° (c 0.91, water); R_f 0.57 (dichloromethane-methanol-water, 10:6:1); ^1H nmr (D_2O) δ: 4.920 (1H, $J_{1',2'}$ = 1.8 Hz, H-1'), 4.664 (1H, $J_{1,2}$ < 0.7 Hz, H-1), 4.579 (1H, $J_{1",2"}$ = 8.0 Hz, H-1"), 4.129 (dd, 1H, $J_{1',2'}$ = 1.4 Hz, $J_{2',3'}$ = 3.4 Hz, H-2'), 3.988-3.401 [22H, H-2 (δ3.981, $J_{2,3}$ = 3.0 Hz), H-3' (δ3.844, $J_{3',4'}$ = 9.5 Hz, $J_{2',3'}$ = 3.5 Hz), H-2" (δ3.706, $J_{1",2"}$ = 8.0 Hz), H-3, H-4, H-5, H-6a, H-6b, H-4', H-5', H-6a', H-6b', H-3", H-4", H-5", H-5a", H-6b", OCH_2CH_2, and OCH_3 (δ3.690)], 2.388 (t, 2H, J = 7.5 Hz, CH_2COOCH_3), 2.056 (s, 3H, $NHCOCH_3$), 1.68-1.50 (4H, aliphatic), 1.38-1.22 (8H, remaining aliphatic). The above ^1H nmr assignments were confirmed by homonuclear decoupling; ^{13}C nmr (D_2O) δ: 178.58 ($NHCOCH_3$), 175.59 ($COOCH_3$), 100.82 (C-1, $J_{C-1,H-1}$ = 159.4 Hz), 100.47 (C-1", $J_{C-1",H-1"}$ = 162.3 Hz), 97.72 (C-1', $J_{C-1',H-1'}$ = 169.5 Hz), 77.35, 76.75, 75.35, 74.27, 74.10, 73.73, 71.43, 70.92, 70.84, 70.54, 68.20, 67.63 (C-2, C-3, C-4, C-5, C-2', C-3', C-4', C-5', C-3", C-4", C-5", and OCH_2CH_2), 66.99 (C-6), 62.43 (C-6"), 61.55 (C-6'), 56.31 (C-2"), 52.91 ($COOCH_3$), 34.55 (CH_2COOCH_3),

29.46, 29.05, 28.97, 28.93, 25.82, 25.11 (aliphatic), 23.21 ($NHCOCH_3$). Anal. calcd. for $C_{30}H_{53}N_1O_{18}$: C 50.34, H 7.46, N 1.96; found: C 49.27, H 7.26, N 1.73.

8-Methoxycarbonyloctyl 6-O-[2-O-(3,4,6-tri-O-acetyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl)-3,4,6-tri-O-benzyl-α-D-mannopyranosyl]-3-O-(2,3,4,6-tetra-O-acetyl-α-D-mannopyranosyl)-2,4-di-O-benzyl-β-D-mannopyranoside (34)

To a solution of the alcohol **31** (504 mg; 0.371 mmol) in dry acetonitrile (10 mL) containing 4Å molecular sieves were added sequentially mercuric bromide (1.416 g; 3.93 mmol) and mercuric cyanide (988 mg; 3.95 mmol). To the resulting mixture was added a solution of 2,3,4,6-tetra-O-acetyl-α-D-mannopyranosyl bromide[25] (**5**) (0.915 g; 2.23 mmol) in dry acetonitrile (5 mL) in five portions with an interval of 30 min between two additions, and the reaction mixture was stirred for 2 h. Evaporation of the solvent gave an oily residue which was extracted three times with dichloromethane. The organic extracts were combined and washed with saturated aqueous potassium chloride, saturated aqueous sodium bicarbonate, water, and brine. The dried organic layer (Na_2SO_4) was evaporated to give a foamy residue which was purified by flash chromatography using toluene-ethyl acetate (3:1) as eluent. The title

compound was obtained as a white foamy solid (0.405 g; 65%); $[\alpha]_D$ +4.41° (c 0.98, chloroform); R_f 0.25, (toluene-ethyl acetate, 3:1); ^1H nmr ($CDCl_3$) δ: 7.88-7.02 (29H, aromatic), 5.811 (dd, 1H, $J_{3'',4''}$ = 10.0 Hz, $J_{2'',3''}$ = 9.0 Hz, H-3''), 5.574 (d, 1H, $J_{1'',2''}$ = 8.5 Hz, H-1''), 5.391-5.349 (2H, $J_{2',3'}$ = 3.0 Hz, H-2' and H-3'), 5.240-5.094 (4H, H-4', H-4'', $C_6H_5C\underline{H}HO$, and H-1 [δ5.094] $J_{3',4'}$ = $J_{4',5'}$ = 9.5 Hz, $J_{3'',4''}$ = $J_{4'',5''}$ = 9.5 Hz), 4.893-4.750 (3H, J_{gem} = 11.5 Hz and 13.0 Hz, 3×$C_6H_5C\underline{H}HO$), 4.696 (d, 1H, $J_{1'',2''}$ = 1.5 Hz, H-1''), 4.539-4.448 (2H, H-2'' [δ4.515, dd, $J_{1'',2''}$ = 8.5 Hz, $J_{2'',3''}$ = 10.5 Hz] and $C_6H_5C\underline{H}HO$ [J_{gem} = 11.5 Hz]), 4.440-4.284 (6H, H-1 [δ4.440], H-2' [δ4.375, dd, $J_{1'',2''}$ = 2.0 Hz, $J_{2'',3''}$ = 2.0 Hz], H-6a''', and 3×$C_6H_5C\underline{H}HO$ [J_{gem} = 11.0 and 11.5 Hz]), 4.121 (dd, 1H, $J_{6a''',6b'''}$ = 12.0 Hz, $J_{5''',6b'''}$ = 1.5 Hz, H-6b'''), 4.098-3.235 (21H, H-2, H-3, H-4, H-5, H-6a, H-6b, H-5', H-6a', H-6b', H-3'', H-4'', H-5'', H-6a'', H-5''', $OC\underline{H}_2CH_2$, $C_6H_5C\underline{H}_2O$, OCH_3 [δ3.674]), 2.953 (dd, 1H, $J_{6a'',6b''}$ = 10.5 Hz, $J_{5'',6b''}$ = 6.0 Hz, H-6b''), 2.313 (t, 2H, J = 7.5 Hz, $C\underline{H}_2COOCH_3$), 2.059-2.000 (6s, 18H, $OCOCH_3$×6), 1.868 (s, 3H, $OCOCH_3$), 1.74-1.54 (4H, aliphatic), 1.46-1.20 (8H, remaining aliphatic); ^{13}C nmr ($CDCl_3$) δ: 174.11 ($\underline{C}OOCH_3$), 170.58, 170.21, 170.06, 169.65, 169.47, 169.44, 169.35 ($\underline{O}\underline{C}OCH_3$ and phthalimido carbonyl), 138.75, 138.46, 137.73, 137.61 (phthalimido quat. arom.), 133.90 (phthalimido tert.

arom.), 128.82, 128.46, 128.36, 128.15, 128.08, 128.03, 127.87, 127.54, 127.47, 127.36, 127.28, 127.18 (benzyl tert. arom.), 123.31 (phthalimido tert. arom.), 101.95 (C-1, $J_{C-1,H-1}$ = 154.1 Hz), 99.83 (C-1', $J_{C-1',H-1'}$ = 177.8 Hz), 97.62 (C-1", $J_{C-1",H-1"}$ = 170.9 Hz), 96.60 (C-1"', $J_{C-1"',H-1"'}$ = 164.16 Hz), 81.25, 76.29, 74.78, 74.48, 74.17, 72.90, 72.03, 71.80, 70.73, 69.33, 69.15, 68.98, 68.93, 65.94 (C-2, C-3, C-4, C-5, C-2', C-3', C-4', C-5', C-2", C-3", C-4", C-5", C-3"', C-4"', and C-5"'), 74.68, 74.58, 73.71, 72.52, 70.43 ($C_6H_5\underline{C}H_2O$), 70.11 ($O\underline{C}H_2CH_2$), 69.62 (C-6"), 66.29 (C-6), 62.44, 62.32 (C-6' and C-6"'), 54.37 (C-2"'), 51.34 ($COO\underline{C}H_3$), 34.02 ($\underline{C}H_2COOCH_3$), 29.66, 29.27, 29.19, 29.07, 26.10, 24.89 (aliphatic), 20.69, 20.56, 20.40 ($OCO\underline{C}H_3$). Anal. calcd. for $C_{91}H_{107}N_1O_{31}$: C 63.89, H 6.30, N 0.82; found: C 63.45, H 6.31, N 0.82.

8-Methoxycarbonyloctyl 6-O-[2-O-(3,4,6-tri-O-acetyl-2-acetamido-2-deoxy-β-D-glucopyranosyl)-3,4,6-tri-O-benzyl-α-D-mannopyranosyl]-3-O-[2,3,4,6-tetra-O-acetyl-α-D-mannopyranosyl]-2,4-di-O-benzyl-β-D-mannopyranoside (**35**)

Compound **34** (260 mg; 0.15 mmol) was dissolved in dry methanol (15 mL) containing sodium methoxide. The resulting solution was stirred at room temperature for 45

min, and the reaction mixture was neutralized with Amberlite IR-120(H) resin. The resin was removed by filtration and the solvent evaporated to provide a foamy solid which was dissolved in methanol (10 mL). To this solution was added hydrazine hydrate (0.4 mL of an 85% solution, 7.43 mmol) and the mixture was refluxed for 1 h. Removal of solvent gave a white residue. Traces of hydrazine were removed by evaporation of methanol from the product (twice), which was further dried on the vacuum pump. The product was then dissolved in pyridine (3.5 mL) and acetic anhydride (3.5 mL) and stirred overnight at room temperature. Excess acetic anhydride was decomposed by dropwise addition of ethanol to the reaction mixture at 0°C, to which was then added dichloromethane and water. The aqueous layer was separated and back extracted with more dichloromethane and the combined dichloromethane layers were washed with 1 M aqueous HCl and saturated aqueous sodium bicarbonate. The organic phase was dried (Na_2SO_4), filtered, and evaporated to give a foamy solid which was purified by flash chromatography using Skellysolve B-ethyl acetate-ethanol (20:20:1) as eluent. Pure 35 was obtained as a white foamy solid (154 mg; 62%); $[\alpha]_D$ +11.23 (c 1.18, chloroform); R_f 0.3 (Skellysolve B-ethyl acetate-ethanol, 20:20:1); ^1H nmr ($CDCl_3$) δ: 7.70-7.12 (25H, aromatic), 5.613-5.523 (2H, H-3''' [δ5.588] and

THE UNIVERSITY OF ALBERTA

SYNTHETIC ACCEPTORS FOR GLYCOSYLTRANSFERASES

by

SYED HASAN TAHIR

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled SYNTHETIC ACCEPTORS FOR GLYCOSYLTRANSFERASES

submitted by SYED HASAN TAHIR

in partial fulfilment of the requirements for the degree of MASTER OF SCIENCE

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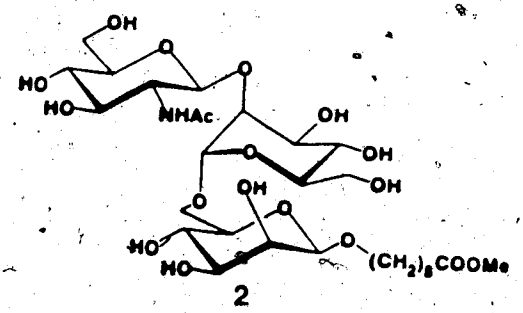
ABSTRACT

Oncogenic transformation frequently results in the production of abnormal cell-surface carbohydrates, known as "tumor-associated carbohydrates", and it has been shown that these carbohydrate structures can result from a change in the enzymatic activity of a single glycosyltransferase.

This work was aimed at developing a selective assay for monitoring changes in the intracellular activity of such a known tumor-elevated glycosyltransferase, termed N-acetylglucosaminyl transferase V (GlcNAc transferase V), by using appropriately designed synthetic oligosaccharide substrates. To this end, the trisaccharide β-D-GlcNAc(1→2)α-D-Man(1→6)β-D-Man-O$(CH_2)_8COOCH_3$ (**2**) and the related tetrasaccharide β-D-GlcNAc(1→2)-α-D-Man(1→6)[α-D-Man(1→3)]-β-D-Man-O$(CH_2)_8COOCH_3$ (**3**) have been chemically synthesized as potential selective acceptors for this enzyme.



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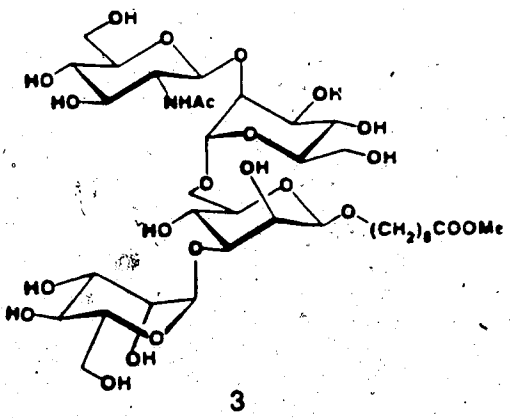

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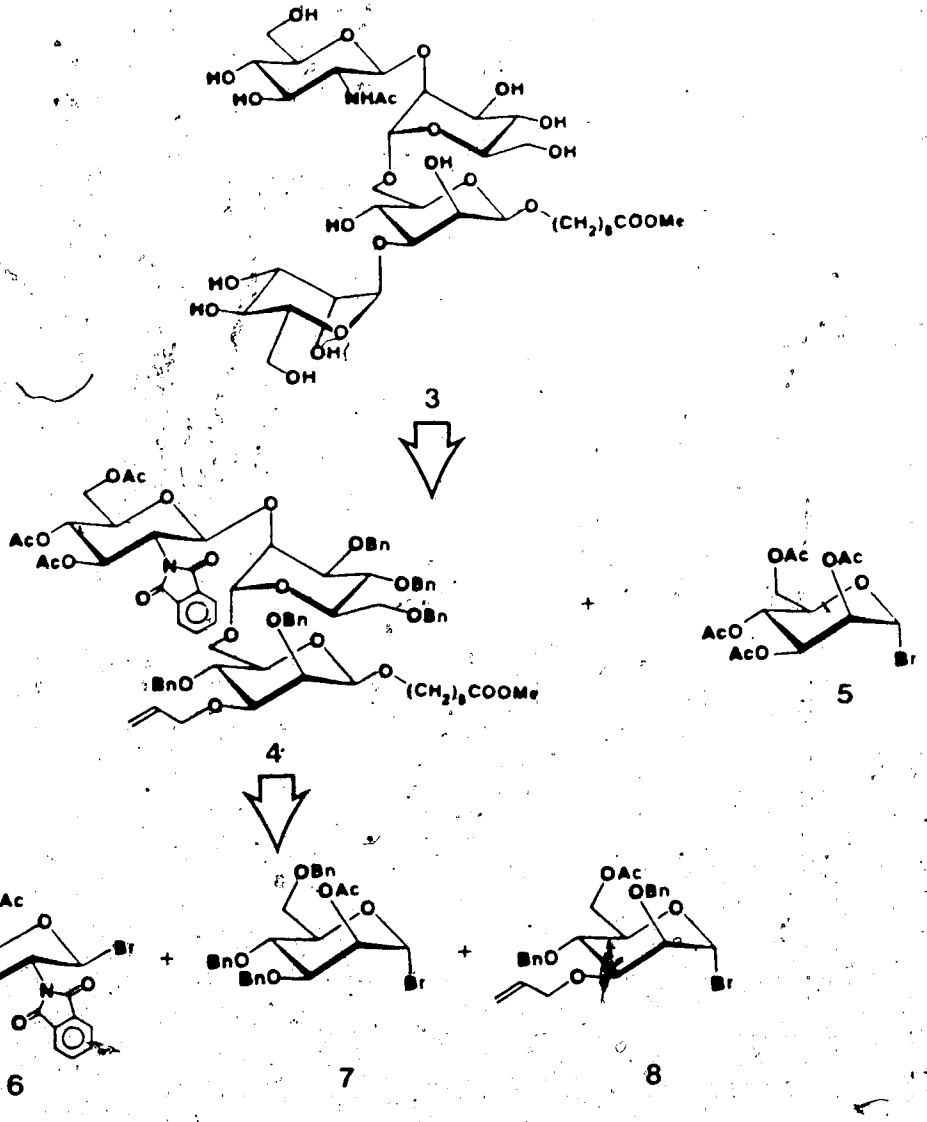
The multi-step syntheses to prepare the oligosaccharides 2 and 3 involved sequential Koenigs Knorr glycosylations of selectively protected carbohydrate derivatives and are summarized in the retrosynthetic scheme shown below:



The trisaccharide 2 was found to be a selective acceptor for GlcNAc transferase V.

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Dr. T.T. Nakashima and his associates for recording high field ^{1}H and ^{13}C nmr spectra;

and finally to Miss A. Wiseman for her skillful assistance in the preparation of this manuscript.

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CHAPTER 1

INTRODUCTION

The cell-surface carbohydrates of eukaryotic cells are made up of the sugar chains of glycoproteins and glycolipids and the more loosely associated collagen, heparan sulfate and fibronectin. The covalently attached complex carbohydrates of glycoproteins and glycolipids are usually between two and fifteen sugar units long and typically make up 2-10% of the plasma membrane weight.[1] The carbohydrate chains of these latter glycoconjugates project outward from the cell and, despite their relatively low abundance, it is believed[1] that they almost entirely cover the cell surface and thus form the first layer of interaction with other cells.

Many of the complex carbohydrates appear to have physicochemical functions such as modifying solubility, stabilizing protein conformation and protecting glycoproteins against proteolysis.[2] Current research in the biological sciences is, however, focusing on the possible role of these highly diverse structures as recognition markers which may direct phenomena as diverse as the binding of hormones, toxins, enzymes, viruses and

bacteria to the cell and as "traffic signals" which control the circulation of both glycoproteins and whole cells.[3] Although much of the evidence to support the role of complex carbohydrates in mediating these diverse processes is still largely circumstantial, a handful of structures have been assigned definitive physiological functions. These critical functions include that of β-D-galactose as a recognition marker in the hepatic clearance of serum glycoproteins[4] and of mannose-6-phosphate residues in the targeting of newly-biosynthesized glycoproteins to the lysosomes.[5]

About ten years ago, evidence began to accumulate that the carbohydrate structures of both cell-surface glycoproteins and glycolipids became dramatically altered during both normal and abnormal cellular development.[6-8] Consistent changes in cell-surface carbohydrate structures have now been shown to accompany the development of human melanoma, neuroblastoma and colorectal, gastric and pancreatic carcinoma.[9] The occurrence of large fucosylated highly-branched glycopeptides is in fact one of the most reproducible correlates with the malignant transformation of cells.[10] The functional significance, if any, of these cell-surface structural changes is not at all clear but these aberrant carbohydrate structures are attracting a great deal of clinical interest as potential

tumor markers. The structures of many of these "tumor-associated" carbohydrates have been elucidated in recent years and major research efforts have gone into the production of monoclonal antibodies against many of these structures.[8,11] One of these monoclonal antibodies, termed CA19-9, is already in wide clinical use as a prognostic monitor for colorectal cancer.[12,13]

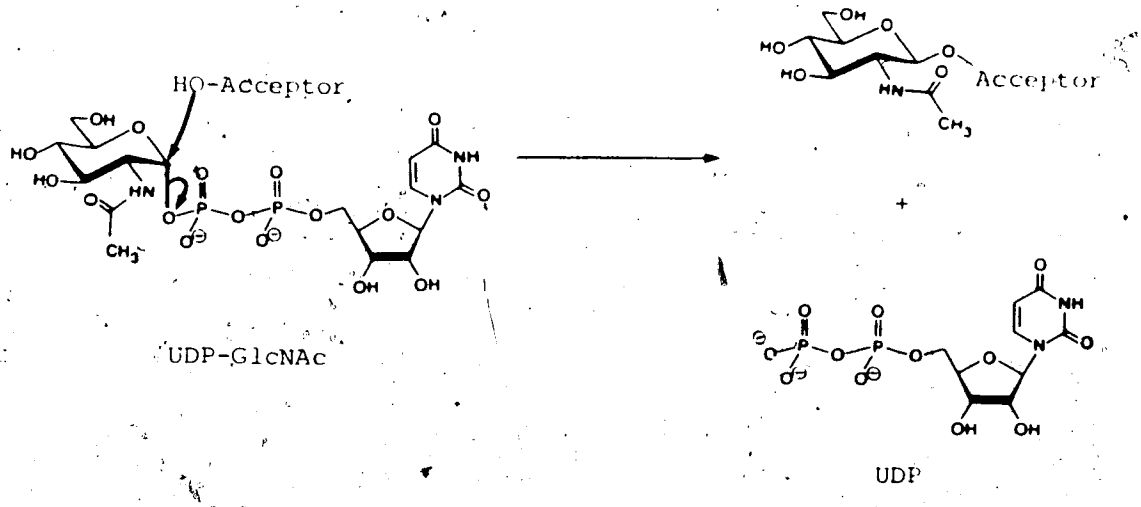
Tumor-associated oligosaccharides are the manifestation of altered carbohydrate biosynthesis and could, in principle, result from any of a large number of cellular irregularities. Glycosylation of proteins is a co- or post-translational modification which requires the sequential action of a series of enzymes (glycosyltransferases) and co-factors (sugar nucleotides) and occurs in the endoplastic reticulum and golgi vesicles.[14] A change in the cellular levels of the glycosyltransferases, the sugar nucleotides or their transport proteins, the biosynthetic precursors of the sugar nucleotides, glycosylhydrolases, or even mild disruptions of the membrane integrities of intracellular organelles could account for the observed changes in cell-surface carbohydrate structures. Because of this tremendous complexity the correlation of the expression of tumor-associated cell-surface carbohydrates with a single transformation-induced molecular event has until very

recently remained elusive.

In 1984, Yamashita et al.[10] compared the carbohydrate structures of the membrane N-linked glycoproteins of baby hamster kidney (BHK) cells and their polyoma transformant (Py-BHK). They found that while the transformed cells produced the same approximately twenty structures as did the normal cells, they produced more of the larger more highly branched oligosaccharides. They proposed that the changes in the relative proportion of the cell-surface oligosaccharides observed on transformation could be explained by the elevation in the activity of a single enzyme, a glycosyltransferase, termed N-Acetyl-β-D-glucosaminyl transferase V (GlcNAc transferase V, GnT V). They subsequently validated this proposal in 1985[15] when they showed that the GnT V activity in Py-BHK cells was in fact elevated two-fold when compared with untransformed cells. This elegant work provided the first demonstration that the changes in cell surface carbohydrates observed on oncogenic transformation could result from a change in the activity of a single glycosyltransferase. Yamashita et al.'s work[10,15] therefore suggests that the activity of a single glycosyltransferase can, in itself, serve as a tumor marker. The detection of a change in a single specific enzymatic activity should be far simpler than the characterization and quantification

of a highly heterogeneous mixture of cell-surface carbohydrate structures produced as a result of this single enzymatic change.

This thesis is concerned with the development of a rapid and convenient assay for measuring the activity of glycosyltransferases, in particular of Yamashita et al.'s elevated GnT V. Glycosyltransferases catalyze the transfer of a glycosyl residue, usually from a sugar nucleotide (termed the donor), to the hydroxyl group of another sugar (termed the acceptor). The sequential action of a large number of such glycosyltransferases is required for the synthesis of complex oligosaccharides. The chemical reaction catalyzed by GlcNAc transferases is the transfer of an N-acetylglucosaminyl residue from uridine-diphospho-N-acetylglucosamine (UDP-GlcNAc) to a hydroxyl group of some acceptor sugar:



The products of the reaction are uridine-diphosphate (UDP) and a β-linked di- or oligosaccharide.

At least 9 different GlcNAc transferases are known to be involved in the biosynthesis of the asparagine-linked oligosaccharides.[3,10,15,16] All of these enzymes use UDP-GlcNAc as the glycosyldonor and the difference between them lies in their specificity for different acceptor structures. A composite structure of the core region of known asparagine-linked oligosaccharides is shown in Figure 1. The oligosaccharides are all attached to protein asparagine residues (N-linked) through a common chitobiose linkage, βDGlcNAc(1→4)βDGlcNAc-Asn. The GlcNAc transferases responsible for generating the diversity of structures observed on cell surface N-linked glycoproteins are labelled GnT I, II, III, IV and V and the GlcNAc residues they transfer are labelled in the same fashion.[15] In the naturally occurring structures other sugar residues, notably D-galactose and L-fucose, are added onto these GlcNAc residues to produce the completed structures. GnT's I-V transfer βDGlcNAc to D-mannose residues, but to different hydroxyl groups of the three different mannose residues. These enzymes are all located in the rough endoplasmic reticulum and in the golgi apparatus of cells where they frequently compete for common glycoprotein substrates.

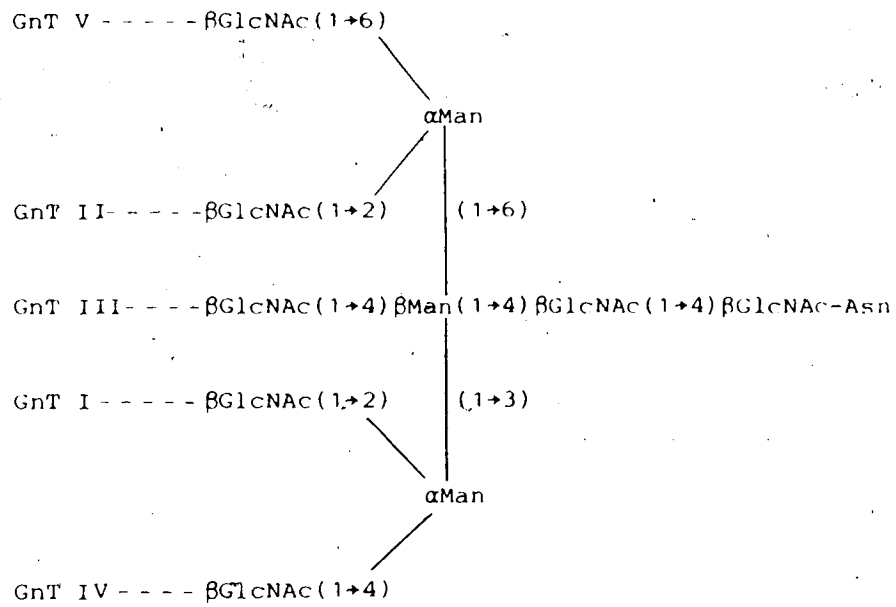


Figure 1. A hypothetical structure showing the βDGlcNAc units added by GlcNAc-transferases I-V (GnT I-V).[15]

A portion of the biosynthetic pathway elucidated by Yamashita *et al.* for the production of BHK cell-surface glycoproteins is shown in Figure 2. This biosynthetic scheme, reproduced from Yamashita *et al.*,[15] shows how the product of one glycosyltransferase reaction can frequently be a substrate for several competing glycosyltransferases. The elevation of GnT V in Py-BHK cells results in a shunt in the normal biosynthetic branching indicated by the bold face arrows with the resultant increase in the larger, more highly branched oligosaccharide structures. GnT V transfers β<u>D</u>GlcNAc exclusively to the 6-hydroxyl group of the α(1→6) linked <u>D</u>-mannose unit of the glycopeptide.

Assaying the activity of these various glycosyltransferases, which are present in only minute amounts, invariably involves the measurement of the transfer of a radiolabelled GlcNAc residue from ^{14}C or ^{3}H-UDP-GlcNAc to a suitable acceptor oligosaccharide, followed by isolation and counting of the product.[15,16] The difficulty encountered with assaying the GnT's is that they all use the same sugar nucleotide and several of them may act on any given substrate. As seen in Figure 2, in the biosynthesis of N-linked glycopeptides, GnT V acts on hepta- and octasaccharidic substrates. In their landmark work,[15] Yamashita *et al.* isolated their substrates from

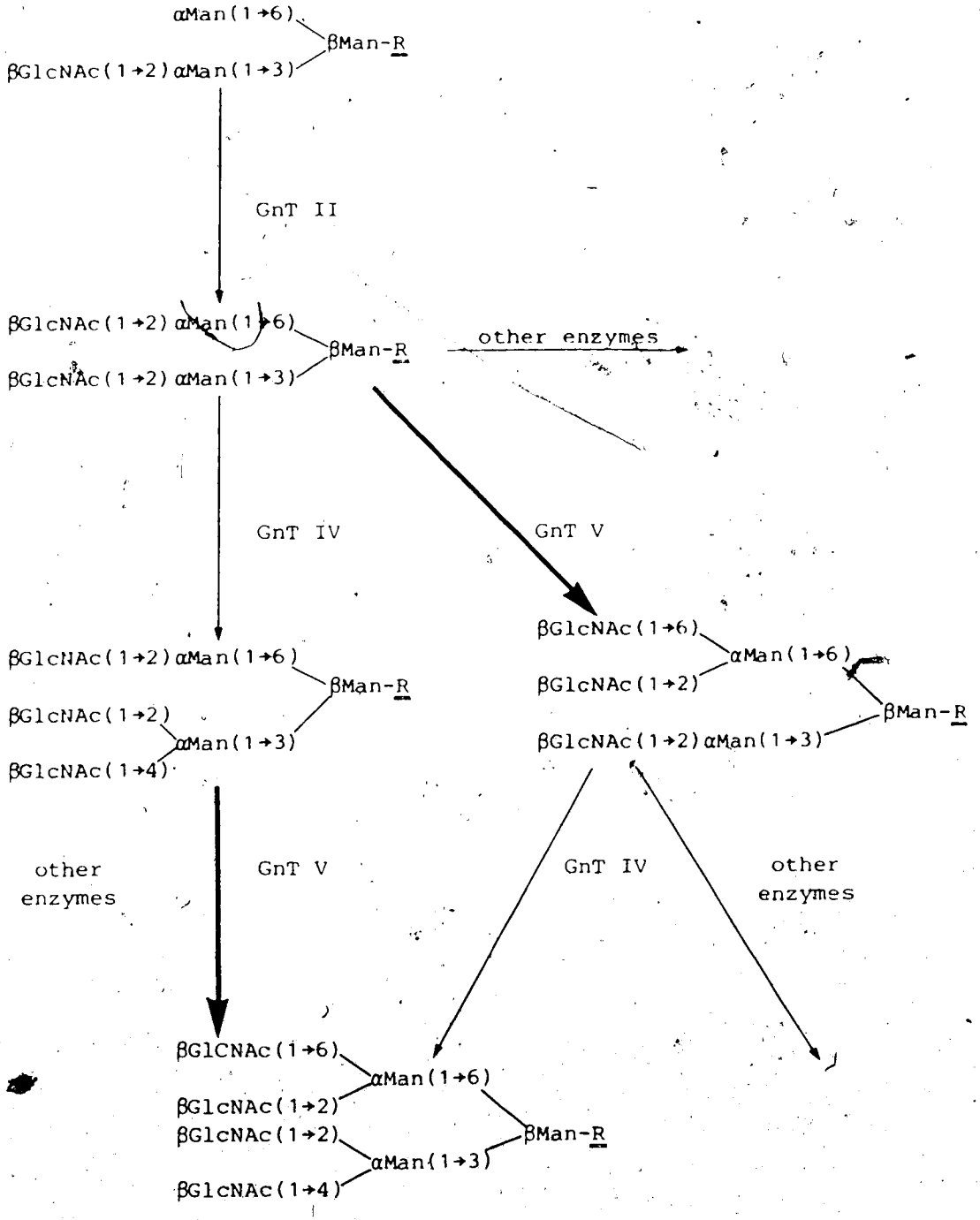


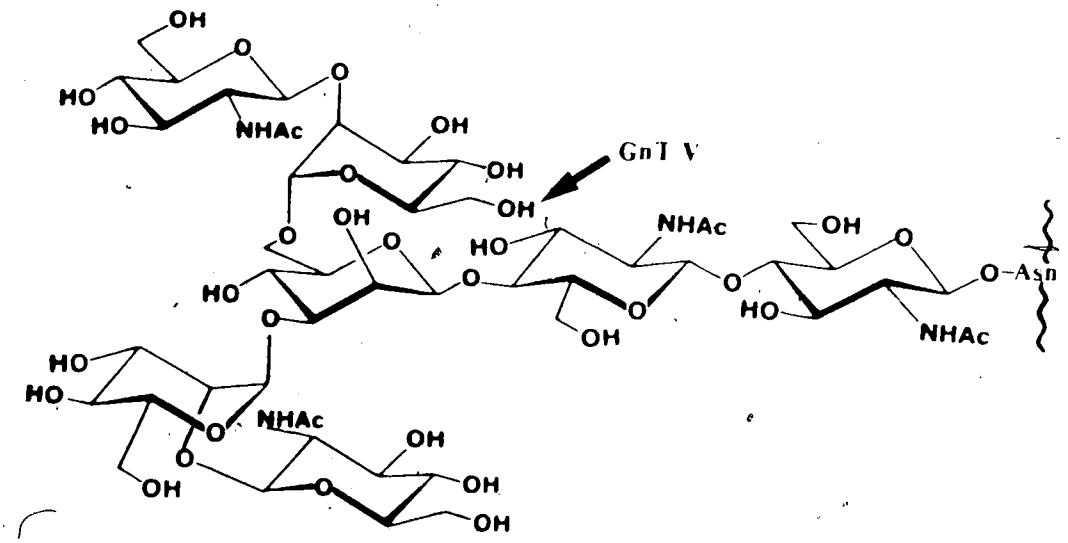
Figure 2. Partial pathway showing the involvement of GlcNAc-transferase V (GnT V) in the biosynthesis of complex sugar chains.[15] R represents βDGlcNAc(1→4)βDGlcNAc-Asn.

the urine of patients with metabolic disorders (mannosidosis, fucosidosis and GM_1-gangliosidosis) and frequently had to further process these structures by removal of outer sugar residues with specific glycosyl hydrolases. Such procedures are not only labor intensive and time consuming but are clearly not general. The amount of material that may be obtained is also limited. Yamashita et al.'s separation of the radioactive products of the enzymatic reactions required high-voltage paper electrophoresis, gel-permeation chromatography, treatment with glycosyl hydrolases and re-chromatography. Both the difficulty and impractability of such glycosyltransferase assays are considered a major obstacle in the study of glycosyltransferase tumor markers.

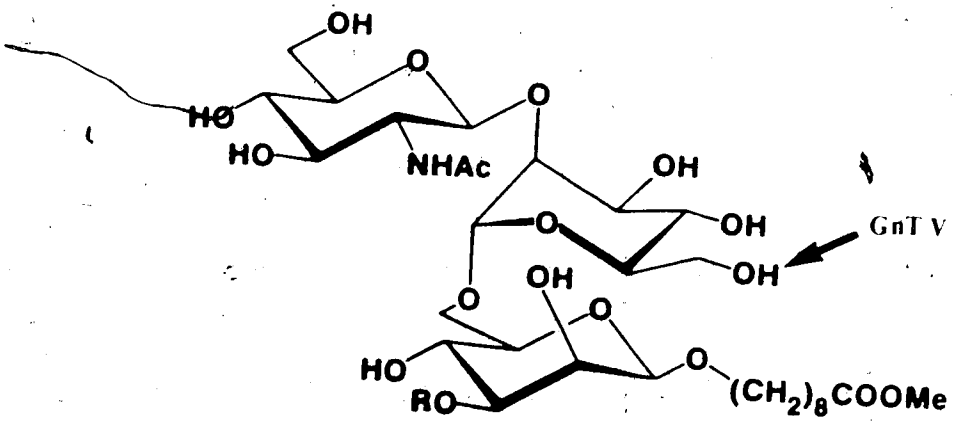
Our approach to this problem was to chemically synthesize oligosaccharides which would, we hoped, be recognized by only a single enzyme thus producing a substrate which could be used in an assay specific for that single glycosyltransferase activity. Such substrates might include oligosaccharides where cross-reacting sugar residues were completely absent or where the interfering hydroxyl groups which might be acted on by other GlcNAc transferases were masked by either O-methylation or deoxygenation. This latter approach has recently[17,18] been successfully applied in the differentiation of two

competing α-L-fucosyltransferases in serum. To be truly useful, such substrates and their glycosylated products should also be amenable to simple rapid isolation from the incubation mixtures of cell extracts or fluids which contain the glycosyltransferase activities being assayed.

In order to test the practicability of this approach we chose to attempt the preparation of substrates selective for Yamashita et al.'s GnT V since this glycosyltransferase was already attracting wide interest as a potential tumor marker. The smallest known substrate for GnT V is the heptasaccharide 1, shown in Figure 3, which was isolated and used by Yamashita et al.[15] as described above. Recent work,[19-23] from the laboratory of Lemieux and our own, on the molecular basis for the binding of oligosaccharides by monoclonal antibodies and lectins strongly suggested that proteins were not likely to require carbohydrate surfaces much larger than that of a trisaccharide for faithful recognition. We expected that this situation might also hold true for the enzymatic specificity of glycosyltransferases and therefore envisioned the trisaccharide βDGlcNAc(1→2)αDMan(1→6)βDManOR (**2**) as a likely acceptor for GnT V. Our rationale for the choice of **2** can be seen in Figure 3 where the structure of the natural heptasaccharide acceptor 1 is also shown. We set forth,



1: βGlcNAc(1→2)αMan(1→6)
βGlcNAc(1→2)αMan(1→3)
βMan(1→4)βGlcNAc(1→4)βGlcNAc-Asn



2: R=H, βGlcNAc(1→2)αMan(1→6)
βMan-O(CH2)8COOMe

3: R=αMan, βGlcNAc(1→2)αMan(1→6)
αMan(1→3)
βMan-O(CH2)8COOMe

Figure 3. A heptasaccharide acceptor (1) for GlcNAc-transferase V (GnT V) compared with the structures of the proposed synthetic acceptors 2 and 3. The bold arrows show the primary hydroxyl group to which GnT V transfers a N-acetyl-β-D-glucosaminyl residue.

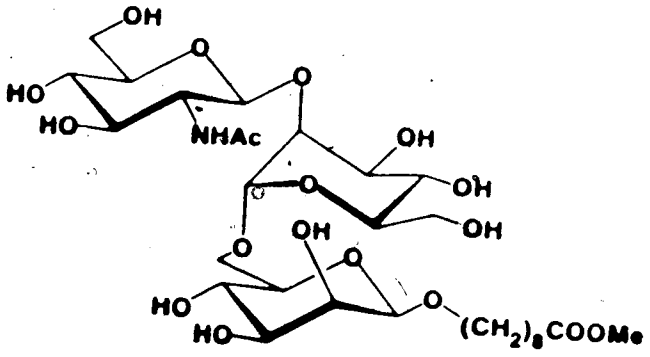
therefore, to synthesize 2, which contains the reactive target hydroxyl group acted on by the enzyme and one sugar residue on either side of the α-D-mannose residue bearing this hydroxyl group. Since we did not know whether this structure incorporated sufficient features for recognition by GnT V, the synthesis was planned in a way that also allowed the preparation of the tetrasaccharide 3 which is closer in structure to the natural acceptor 1. In order to facilitate the isolation of our glycosylated substrates from their enzymatic incubation mixtures, we elected to prepare 2 and 3 as their 8-methoxycarbonyloctyl glycosides[24] since inclusion of this hydrophobic group should facilitate adsorption on reverse phase (C-18) chromatography supports. This "linking arm" might also eventually be used for the attachment of fluorescent, radioactive or enzymatic tags to these potential acceptors to help localize the corresponding enzyme in intracellular organelles or to prepare affinity columns to assist in enzyme isolations.

CHAPTER 2

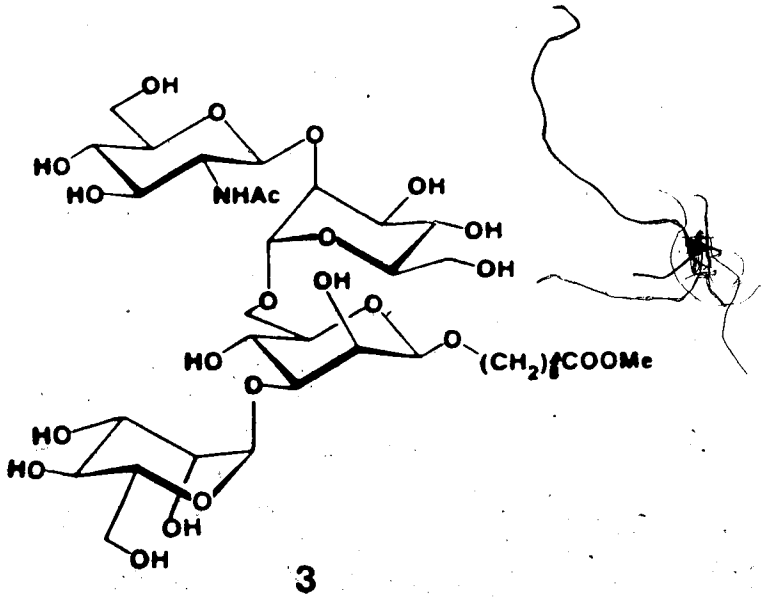
RESULTS AND DISCUSSION

The goal of this thesis project was to synthesize two oligosaccharides, namely 8-methoxycarbonyloctyl 6-O-[2-O-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-α-D-mannopyranosyl]-β-D-mannopyranoside (**2**) and 8-methoxycarbonyloctyl 6-O-[2-O-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-α-D-mannopyranosyl]-3-O-(α-D-mannopyranosyl)-β-D-mannopyranoside (**3**). In order to increase the potential usefulness of the oligosaccharides 2 and 3 we elected to synthesize these structures covalently attached to a linking arm which would allow their subsequent attachment to proteins, solid supports, and fluorescent or radioactive tags. These glycoconjugates might then be used in the intracellular localization of the N-acetylglucosaminyl transferases as well as in their purification.

A retrosynthetic analysis of the target structures **2** and **3** suggested, as the key intermediate, the trisaccharide precursor **4**, which was protected in a manner to allow the selective liberation of the hydroxyl group at C-3 of the β-D-mannopyranosyl residue for subsequent

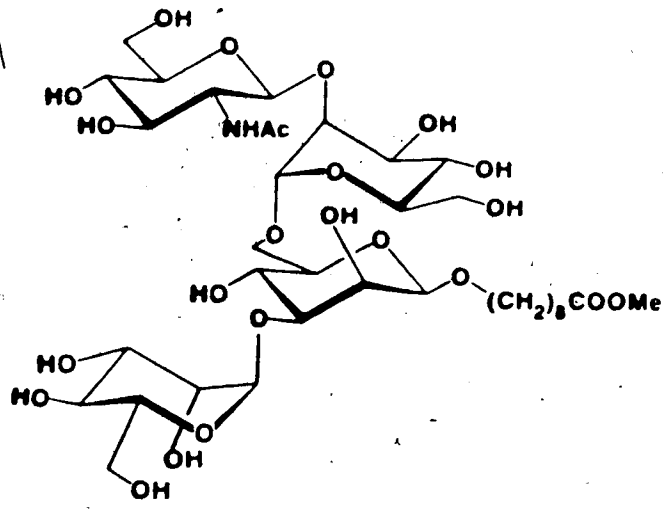


2



3

glycosylation by the α-D-mannopyranosyl donor **5**. The key intermediate **4** was, in turn, retrosynthesized into monohexosyl synthons 6, 7, and 8. The two monosaccharide synthons, 6 and 7, were designed to function as 2-deoxy-2-phthalimido-β-D-glucopyranosyl and α-D-mannopyranosyl



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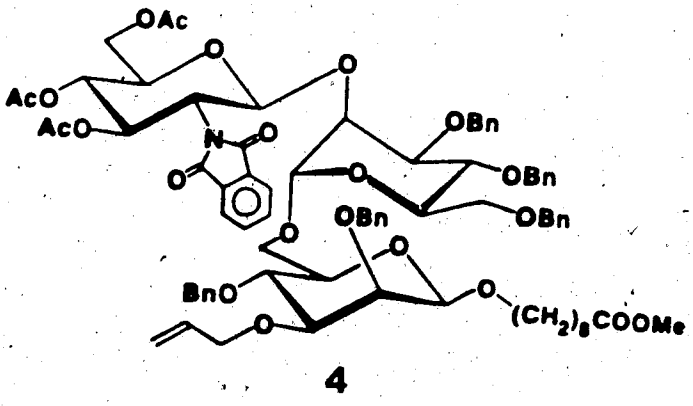

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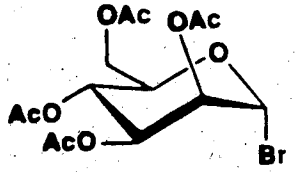
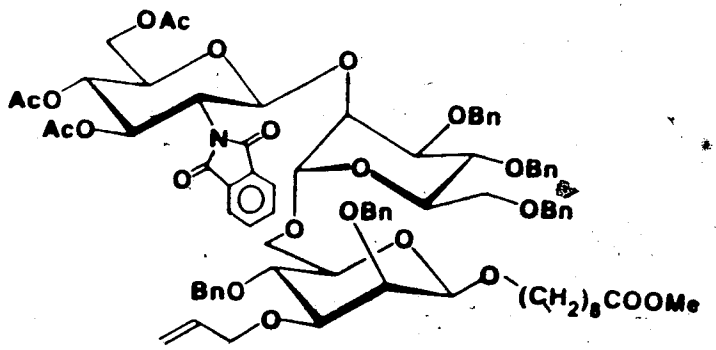
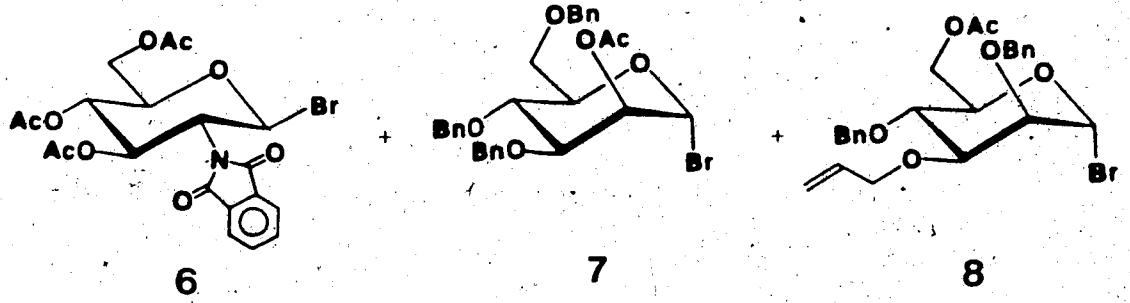


5

donors respectively. The synthon **8** was properly protected to perform dual functions: first acting as a β-D-manno-pyranosyl donor and then, after selective removal of acetyl group at O-6, as a glycosyl acceptor.



4



6 7 8

Of the four monosaccharide synthons (**5**,[25] **6**,[26] **7**,[27] and **8**) thus required, three, namely synthons **5**, **6**, and **7** had already been prepared. Therefore, a synthetic route towards the monosaccharide synthon **8** was first undertaken.

Methyl α-D-mannopyranoside (**9**) was converted to its 4,6-O-benzylidene derivative **10** in 25% yield on treatment with benzaldehyde in the presence of formic acid. This procedure gave only a modest yield, as reported,[28] so our attention was drawn to a recently published method,[29] which involved the reaction of **9** in N,N-dimethylformamide with benzaldehyde dimethylacetal[30] in the presence of tetrafluoroboric acid. This improved procedure provided a 50% yield of the benzylidene derivative **10** and allowed its preparation in sufficient quantities (60 g) to proceed with the preparation of **8**.

Selective 3-O-allylation of **10** was effected by the procedure of Nashed,[31] which involved its reaction with one equivalent of dibutyltin oxide to provide presumably 4,6-O-benzylidene-2,3-O-dibutylstannylene-α-D-mannopyranoside (**11**). Compound **11** was not isolated but was treated with allyl bromide in N,N-dimethylformamide at 100°C to give methyl 3-O-allyl-4,6-O-benzylidene-α-D-mannopyranoside (**12**) in 82% yield. Although **12** appeared

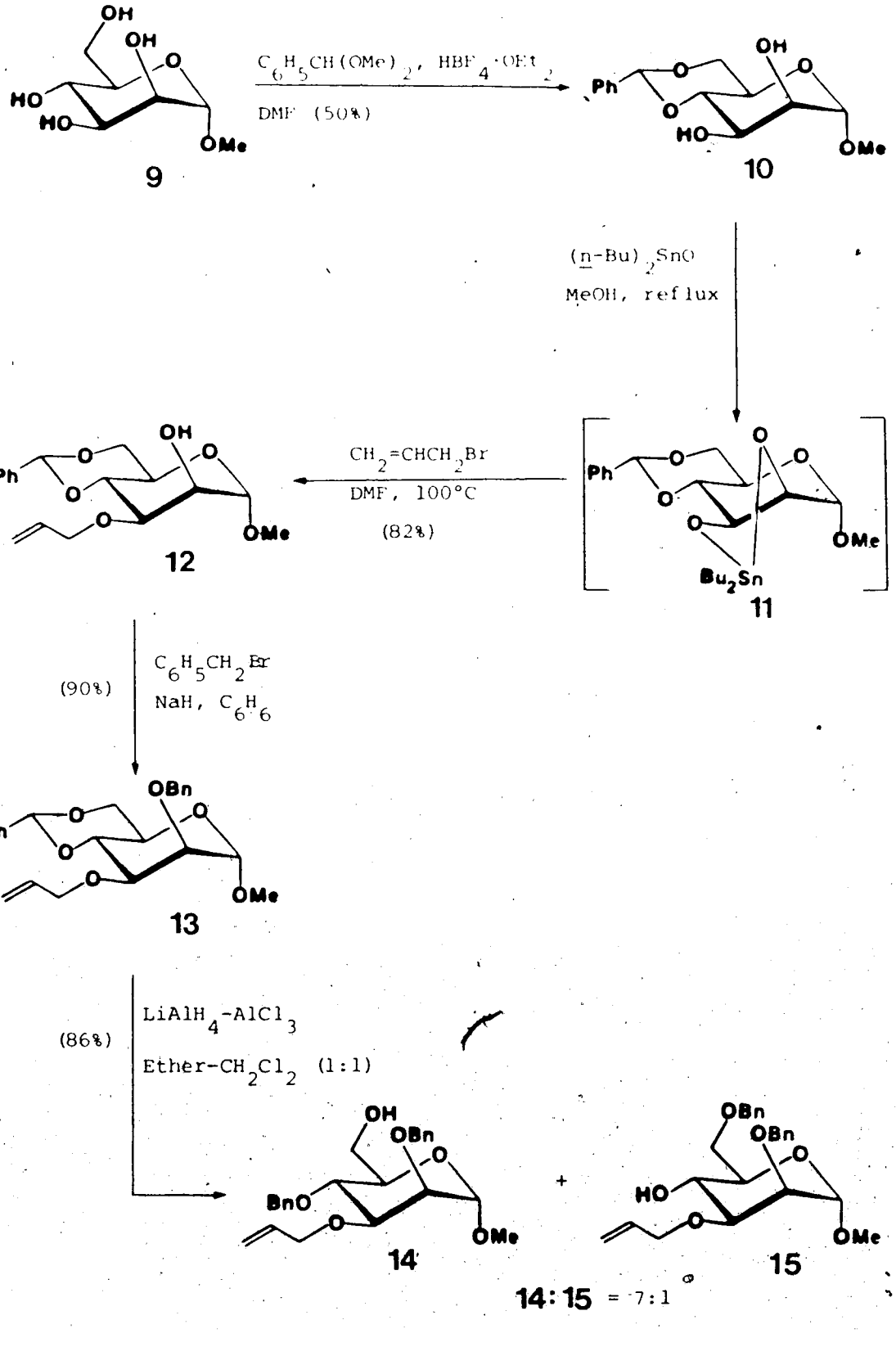
homogeneous by thin layer chromatography (tlc), its optical rotation, $[\alpha]_D^{22}$ + 59.4° (c 1.2, chloroform) differed somewhat from the value reported by Nashed: +63.5°.(c 1.5, chloroform). The 400 MHz ^1H nmr spectrum, however, showed **12** to be a pure single isomer whose identity could readily be ascertained. The signal for the hydroxylic proton appeared as a doublet ($J_{2,OH}$ = 1.5 Hz) at δ3.045, causing H-2 to produce a broad signal at δ4.033 which collapsed to a doublet of doublets ($J_{2,3}$ = 3.5 Hz and $J_{1,2}$ = 1.5 Hz) on deuterium exchange. The position of free hydroxyl group in **12** was further confirmed by its *in situ* derivatization using trichloroacetyl isocyanate.[32] The ^1H nmr spectrum recorded following the addition of trichloroacetyl isocyanate displayed the expected downfield shifted doublet of doublets ($J_{2,3}$ = 3.0 Hz and $J_{1,2}$ = 1.5 Hz) at δ5.349.

Treatment of **12** with benzyl bromide and sodium hydride in benzene[31] furnished the 2-*O*-benzyl derivative **13** in 90% yield. The presence of a benzyl group in **13** was confirmed by the appearance, in the ^1H nmr spectrum, of additional signals integrating for five protons in the aromatic region. The signals for the now diastereotopic benzylic protons appeared at δ4.843 and 4.725, each as a one proton AB doublet with a geminal coupling constant of

12.0 Hz.

The reductive cleavage of the benzylidene group in **13** was achieved by refluxing with $LiAlH_4$-$AlCl_3$ in ether dichloromethane (1:1), according to Liptak et al.,[33] to give the 4-O-benzyl (**14**) and 6-O-benzyl (**15**) derivatives, in the ratio of 8:1, in a combined yield of 86%. The structures of the hydrogenolysis products **14** and **15** were assigned on the basis of ^1H nmr data. The ^1H nmr spectrum of the major product **14** showed a doublet of doublets for OH-6 at δ2.046 ($J_{6,OH}$ = 7.0 Hz and $J_{6',OH}$ = 5.5 Hz) as well as a clear signal for one of the H-6's at δ3.840 (ddd, $J_{6,6'}$ = 12.0 Hz, $J_{6,OH}$ = 7.0 Hz, and $J_{5,6}$ = 3.0 Hz). On D_2O exchange, the signal corresponding to OH-6 disappeared and, as expected, the signal for this H-6 simplified to a doublet of doublets, the coupling with hydroxylic proton being absent. The ^1H nmr spectrum of 6-O-benzyl derivative **14**, on the other hand, displayed a doublet for hydroxylic proton at δ2.578 ($J_{4,OH}$ = 2.0 Hz) and a doublet of doublet of doublets for H-4 at δ4.004 ($J_{4,5}$ = 9.0 Hz, $J_{3,4}$ = 9.0 Hz, and $J_{4,OH}$ = 2.0 Hz). After D_2O addition, the 1H nmr spectrum of **14** showed the disappearance of the OH resonance and the simplification of the signal for H-4 to a doublet of doublets.

The 13C nmr data were also in accord with the


OH
OH
HO
HO
OMe
9
$C_6H_5CH(OMe)_2$, $HBF_4 \cdot OEt_2$
DMF (50%)
Ph
HO
10
$(n\text{-Bu})_2SnO$
MeOH, reflux
Bu_2Sn
11
$CH_2=CHCH_2Br$
DMF, 100°C
(82%)
12
(90%)
$C_6H_5CH_2Br$
NaH, C_6H_6
OBn
13
(86%)
$LiAlH_4$-$AlCl_3$
Ether-CH_2Cl_2 (1:1)
BnO
14
15
14:15 = 7:1

structures assigned for **14** and **15**. The well established empirical rule[34] that alkoxylated carbons (ROC) are deshielded by 5-10 ppm compared with the corresponding hydroxylated carbon atoms (HOC) permits a simple verification of the position of the benzyl ethers in **14** and **15**. The major product **14** showed the signal for C-6 at δ62.43 ppm, the normal position for the underivatized hydroxymethyl group carbons of pyranose rings.[34] In **15**, however, this methylene carbon appeared at δ70.66 ppm, confirming the presence of the 6-O-benzyl ether.

Reaction of the 4-O-benzyl compound **14** with acetic anhydride in the presence of pyridine gave the 6-O-acetyl derivative **16** in 98% yield. The downfield shift of the H-6 resonances in the ^{1}H nmr spectrum of **16** due to acetylation of O-6 supported the assigned structure.

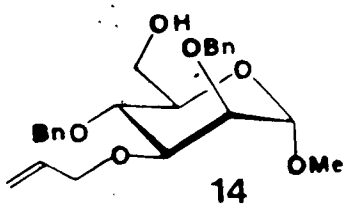
The glycosidic linkage in **16** was acetolyzed[35] using acetic anhydride in the presence of a catalytic amount of sulfuric acid to afford 1,6-di-O-acetyl-3-O-allyl-2,4-di-O-benzyl-α-D-mannopyranose (**17**) in 77% yield. The structure of **17** could be deduced from its ^{1}H nmr spectrum, which included a deshielded signal at δ6.195 (d, $J_{1,2}$ = 2.0 Hz) for H-1 and a six-proton singlet at δ2.055 indicating the presence of two acetyl groups. Further proof for the assigned structure **17** was provided by its

^{13}C nmr spectrum, which contained a shielded signal (as compared with 99.10 ppm for its precursor) at δ91.79 for C-1 and additional signals at δ168.82 and 20.98 for $\underline{O}C\underline{O}CH_3$ and $OCO\underline{C}H_3$ respectively. Along with this major product **17**, the acetolysis reaction also produced two minor products, namely, 1,6-di-O-acetyl-3-O-allyl-2,4-di-O-benzyl-β-D-mannopyranose (**18**) and 1,4,6-tri-O-acetyl-3-O-allyl-2-O-benzyl-α-D-mannopyranose (**19**) in a combined yield of 6%. The anomeric configuration of **18** was evident from the appearance, in its ^1H nmr spectrum, of a doublet ($J_{1,2}$ = 1.0 Hz) for H-1 at δ5.609 (about 0.6 ppm upfield relative to H-1 in the corresponding α-anomer **17**). The presence of shielded signals (as compared with the α-anomer **17**) for H-3 and H-5 at δ3.598 and 3.300 respectively further established[36] the anomeric configuration to be β in **18**. The structure of the other minor product **19** could also be deduced from its ^1H nmr spectral data, which included a doublet ($J_{1,2}$ = 2.0 Hz) at δ6.185 for H-1, indicating the configuration at C-1 to be α, and a deshielded doublet of doublets ($J_{3,4}$ = $J_{4,5}$ = 10.5 Hz) at δ5.431 for H-4 due to the acetylation of O-4, thus confirming the cleavage of the 4-O-benzyl group of **16**.

Reaction of the diacetate **17** in dichloromethane, at

0°C, with hydrogen bromide which was dried by passage through a calcium sulfate column, led to the formation of the glycosyl bromide **8** in essentially quantitative yield. The ^{1}H nmr spectrum of **8** displayed a doublet ($J_{1,2}$ = 1.5) at δ6.444 for H-1 and a singlet at δ2.065, now integrating for only three protons, arising from a single acetyl methyl group. In its ^{13}C nmr spectrum the signal for C-1 appeared at δ87.41, and only one signal each for the carbonyl carbon at δ170.57 and for the acetyl methyl group at δ20.72 were present.

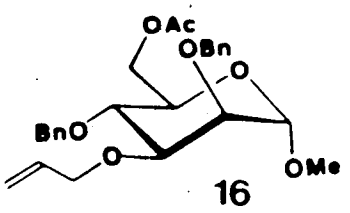
When the diacetate **17** was allowed to react with HBr gas introduced directly from the cylinder without passage through calcium sulfate, the thin layer chromatogram (tlc) of the reaction mixture showed the presence of two products, the minor being the desired bromide **8**. The major product **20** had a slightly lower mobility in tlc. The ^{1}H nmr spectrum of the mixture of **8** and **20** indicated **20** to be the hydrobromination product of **8**, namely, 6-O-acetyl-2,4-di-O-benzyl-3-O-(3-bromopropyl)-α-D-manno-pyranosyl bromide. This structural assignment was made to account for the observation of a triplet for two protons at δ3.688 ($BrCH_2CH_2\underline{CH_2}O$), a multiplet for two protons at δ3.510 for $Br\underline{CH_2}CH_2CH_2O$, and a two proton multiplet at δ2.119 for $BrCH_2\underline{CH_2}CH_2O$. The ratio of **20** to **8** was



14

Ac_2O/Pyridine

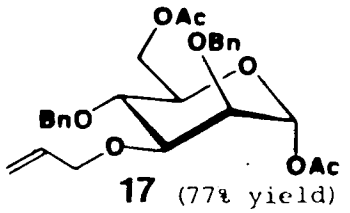
(98%)



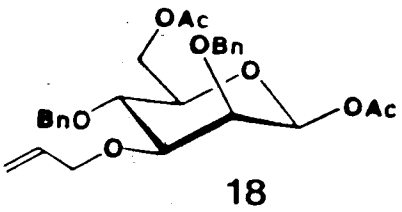
16

Ac_2O

H_2SO_4, 0°C

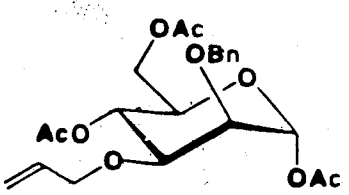


17 (77% yield)



18

+



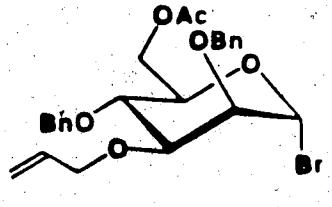
19

6%

HEr

(dried with $CaSO_4$)

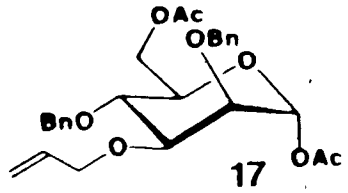
CH_2Cl_2, 0°C



8

determined to be 4:1 from this ^1H nmr spectrum.

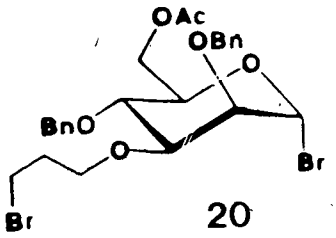
In order to further corroborate both the presence and the position of the bromine atom in the labile anomeric bromide **20** a stable derivative was prepared. Following the separation of **20** from **8** by flash column chromatography,[37] **20** was glycosylated with methanol under the conditions of Garegg et al.,[38] using silver carbonate as promoter and dichloromethane as solvent, to provide a mixture of the β- and α-mannosides **21** and **22** (β/α:4/1) in an isolated yield of 91%. The structure of β-anomer **21** was determined from its ^1H and ^{13}C nmr spectral data as well as mass spectral analysis. The ^1H nmr spectrum of the β-anomer **21** showed the presence of a doublet ($J_{1,2}$ = 1.0 Hz) at δ4.308 for H-1 and three two-proton signals: a triplet at 3.484 (J = 7.0 Hz) assigned to $BrCH_2CH_2C\underline{H}_2O$, a multiplet at δ3.41 assigned to $Br\underline{C}H_2CH_2CH_2O$, and another multiplet between δ2.92 and 2.856. The assigned structure **21** was also in agreement with the ^{13}C nmr spectrum, which exhibited a signal for C-1 at δ102.81 and three signals at δ67.00, 32.98, and 30.41 tentatively assigned to $BrCH_2CH_2\underline{C}H_2O$, $Br\underline{C}H_2CH_2CH_2O$, and $BrCH_2\underline{C}H_2CH_2O$, respectively. Unequivocal support for the presence of bromine in **21** (molecular weight 537.447) was provided by the chemical ionization mass spectrum, which showed two



17

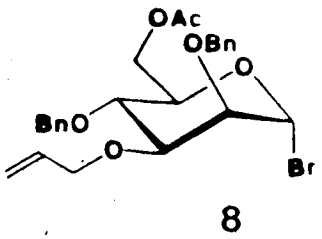
HBr

CH_2Cl_2, 0°C



20

+



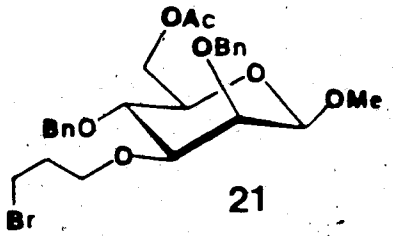
8

(91%)

MeOH

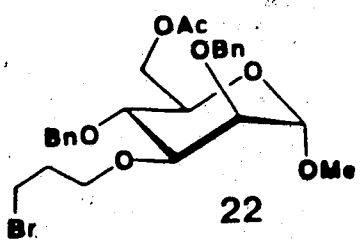
$AgCO_3$, 4 Å molecular sieves

CH_2Cl_2 0°C



21

+



22

21:22 = 4:1

peaks of almost equal intensity at 554 (93.3%) and 556 (100.0%), corresponding to the NH_4^+ adducts containing the ^{79}Br and ^{81}Br isotopes respectively. Furthermore, microanalytical data were in accord with the molecular formula for **21**.

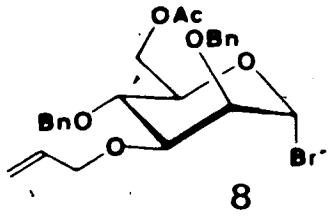
The newly developed method of Paulsen and Lockhoff[39] for the stereoselective synthesis of β-mannosides was used for the preparation of the glycosides **23**β and **23**α. Reaction of the glycosyl bromide **8** and 8-methoxycarbony-octanol in the presence of an insoluble catalyst prepared by precipitation of silver silicate on aluminum oxide, provided a mixture of the β- and α-mannosides, **23** in a combined yield of 56%. All attempts to separate these anomers using chromatography on silica gel, silica gel impregnated with silver nitrate or alumina, were unsuccessful. That **23** was a mixture of the α and β glycosides was evident from the ^1H nmr spectrum which showed a signal at δ4.365 for H-1 of the β-anomer and a doublet ($J_{1,2}$ = 2.0 Hz) at δ4.824 for H-1 of the corresponding α-anomer. The β/α ratio of this mixture, determinted by integration of these signals in the ^1H nmr spectrum of **23**, was found to vary with the temperature of the reaction. At -78°C, the β- and α-anomers were produced in a 6:1 ratio. On the other hand, at the higher

temperatures of -20° and 0° the ratios of β- to α-anomers were 4.3:1 and 3.5:1 respectively. The glycosylation reaction did not go to completion as indicated by the presence of the unreacted glycosyl bromide **8** on tlc. The rate of disappearance of **8** increased with temperature but no improvement in the yield of **23** was observed. Elimination of HBr is likely a major reaction pathway for **8** at these higher temperatures.

Coupling of **8** with 8-methoxycarbonyoctanol[24] was also attempted using freshly prepared silver carbonate in the presence of 4 Å molecular sieves, using dichloromethane as solvent at 0°C, according to the procedure of Garegg *et al.*[38] A mixture of β- and α-anomers (β/α:3/1) was obtained in 54% yield. The reaction was faster under these conditions but provided an overall lower yield than with the silver silicate reaction.

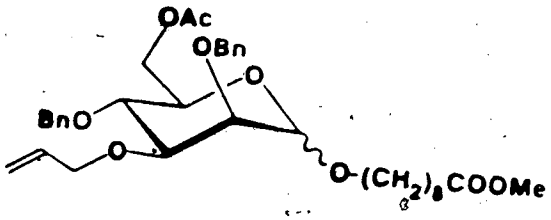
Treatment of the mixture **23** with sodium methoxide in methanol effected the removal of acetyl groups to provide a mixture of **24**β and **25**α which chould now be separated by flash column chromatography on silver nitrate impregnated silica gel. The assignment of structures to the deacetylation products **24** and **25** was made on the basis of their ^{1}H and ^{13}C nmr data.

Appearance, in the ^{1}H nmr spectrum of the major



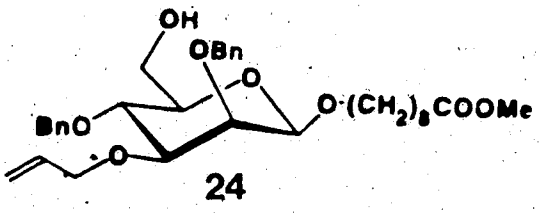
8

(56%) | $HO-(CH_2)_8COOMe$, silver silicate/alumina, CH_2Cl_2, -78°C

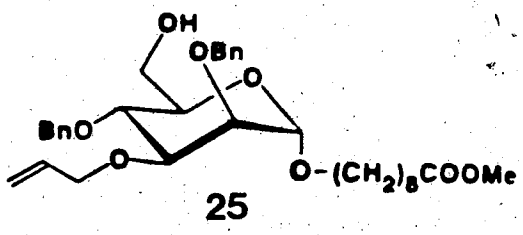


23 (β/α:6/1)

NaOMe/MeOH



24



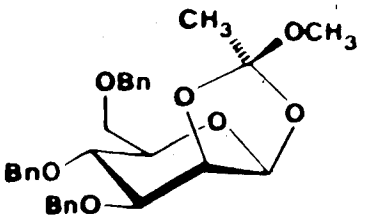
25

product **24**, of a broad signal for one proton at δ4.408 ($J_{1,2}$ ≈ 1 Hz), suggested the anomeric configuration to be β. A one-proton doublet of doublets ($J_{6a,OH}$ = $J_{6b,OH}$ = 6.5 Hz) at δ2.140 for a D_2O-exchangeable hydroxyl proton is also consistent with the structure assigned to **24**. This structural assignment was further supported by the ^{13}C nmr spectrum of **24** where the signal for C-1 at δ98.25 was clearly visible. The β-configuration of the glycosidic linkage in **24** could be unequivocally assigned from its ^{1}H-coupled ^{13}C nmr spectrum where this signal appeared as a doublet, $J_{C-1,H-1}$ = 153.6 Hz, in accord with the empirical rules formulated by Bock and Pedersen[40] for the dependence of the one-bond C-H coupling on the anomeric configuration of pyranosides.

The structure of the minor product **25** was assigned after the observation, in its ^{1}H nmr spectrum, of a doublet for one proton at δ4.790 ($J_{1,2}$ = 2.0 Hz), assigned to H-1, as expected for the corresponding α-anomer. A broad doublet integrating for one proton at δ2.098 for a hydroxylic proton was also present. The ^{13}C nmr spectrum of **25** showed the C-1 signal for C-1 at δ98.25 with $J_{C-1,H-1}$ = 168.2 Hz.

Condensation of the alcohol **24** and 2-O-acetyl-3,4,6-tri-O-benzyl-α-D-mannopyranosyl bromide[27] (**7**), which had been freshly prepared from 3,4,6-tri-O-benzyl-1,2-O-

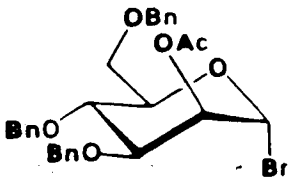
(methoxyethylidene)-β-D-mannopyranose (**26**), under Helferich conditions using mercuric bromide and mercuric cyanide as promoters and acetonitrile as solvent furnished the α-linked disaccharide **27** in 77% yield. The structure of **27** was assigned on the basis of its ^{1}H and ^{13}C nmr spectra. The resonances for two anomeric protons were observed in the ^{1}H nmr spectrum, at δ4.911 ($J_{1',2'}$ = 2.0 Hz) and 4.334 ($J_{1,2}$ ≈ 1 Hz) and were thus assigned to H-1' of the α-mannopyranosyl unit and H-1 of the β-mannopyranosyl unit, respectively. The presence of a deshielded doublet of doublets ($J_{1',2'}$ = 2.0 Hz and $J_{2',3'}$ = 3.0 Hz) at δ5.459 indicated the presence of the expected acetoxy group on C-2' and, by decoupling, thus confirmed the identity of the anomeric doublet at δ4.911. The ^{13}C nmr spectral data were also in agreement with the assigned structure. The configuration of the newly formed glycosidic linkage in **27**, expected to be α due to neighboring group participation of the 2-acetoxy group in the glycosylation reaction, was evident from its ^{1}H-coupled ^{13}C nmr spectrum which showed a doublet each for C-1 and C-1' at δ104.64 ($J_{C-1,H-1}$ = 154.7 Hz) and 97.77 ($J_{C-1',H-1'}$ = 170.4 Hz) respectively. One-bond C-H coupling constants of these magnitudes require the presence of the β and α glycosidic linkages as assigned.



26

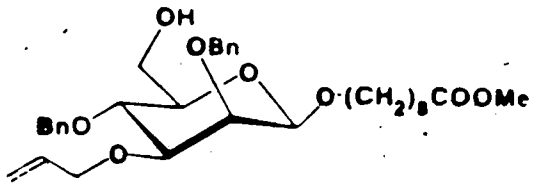
HBr , CH_2Cl_2

0°C



7

+



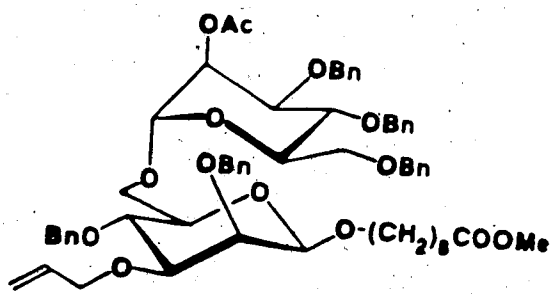
24

(77%)

$HgBr_2$, $Hg(CN)_2$

4 Å molecular sieves

CH_3CN, RT



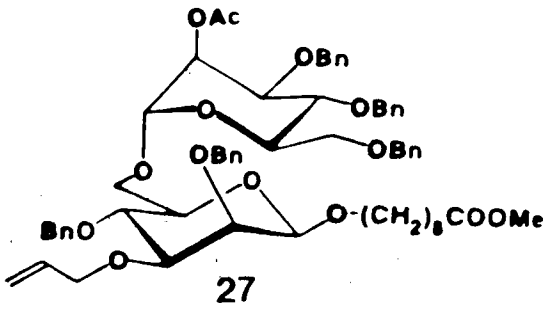
27

Reaction of **27** with sodium methoxide in dry methanol provided the alcohol **28** in quantitative yield. Disappearance, in the ^1H nmr spectrum, of a singlet for three acetyl protons and the upfield shift of the signal for H-2' to δ 4.128 confirmed the removal of acetyl group from O-2' of **27**. Moreover, the presence of a new signal at δ 2.355 (broad singlet), which was exchanged for deuterium by treatment with D_2O, further supported the structure assigned to **28**. The ^{13}C nmr spectrum of **29** showed the expected disappearance of the signals corresponding to carbonyl and methyl carbons.

Reaction of the alcohol **28** with 3,4,6-tri-O-acetyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl bromide[26] (**6**) in dichloromethane using silver zeolite[41] as promoter at room temperature, as described by Schwartz et al.,[42] failed to provide any of the desired trisaccharide **4**. Use of silver triflate/2,4,6-collidine (1.5 molar equivalents each with respect to **6**) at -20°C in nitromethane, according to Lemieux et al.[26] did provide the desired product although the yield of 30% proved unacceptably low. In an attempt to accomplish the preparation of **4** in a more respectable yield, we turned our attention to the conditions described by Paulsen et al.[43] in a recent report. Thus the treatment of **28** with the glycosyl bromide **6** (2 molar equivalents) using silver triflate/2,4,6-collidine (10

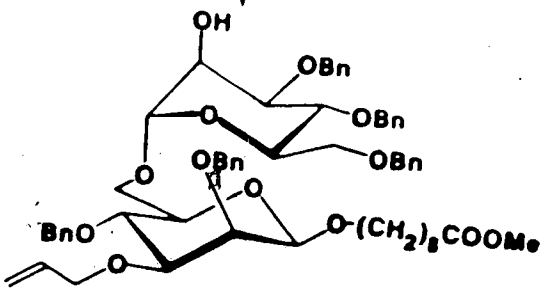
equivalents) using silver triflate/2,4,6-collidine (10 molar equivalents each) in the presence of 4 Å molecular sieves in dichloromethane at -50°C provided the trisaccharide **4** in 76% yield after chromatographic purification. The presence of the 3,4,6-tri-O-acetyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl moiety in **4** was indicated by its ^{1}H nmr spectrum which displayed four deshielded, one-proton signals, characteristic of the newly introduced glucosyl residue: a doublet of doublets at δ5.815 ($J_{3",4"}$ = 9.0 Hz, $J_{2",3"}$ = 10.0 Hz) for H-3", a doublet at δ5.573 ($J_{1",2"}$ = 8.5 Hz) for H-1", a doublet of doublets at δ5.215 ($J_{3",4"}$ = 9.0 Hz, $J_{4",5"}$ = 10.0 Hz) for H-4, and a doublet of doublets at δ4.513 ($J_{2",3"}$ = 10.0 Hz, $J_{1",2"}$ = 8.5 Hz) for H-2. The magnitude of the coupling constant between H-1" and H-2" provides unambiguous proof that these protons are in the trans-diaxial orientation and thus establishes the presence of the β-glucosidic linkage. The ^{13}C nmr spectrum of **4** showed the expected signals at δ170.42, 169.89, and 169.21 for three acetyl carbonyl carbons, and an additional anomeric signal at δ96.48 (C-1") further substantiated the structure assigned to **4**.

Along with the major condensation product **4** a minor product (**29**) with higher R_f, was also produced. This



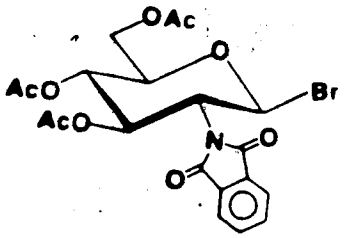
27

NaOMe/MeOH



28

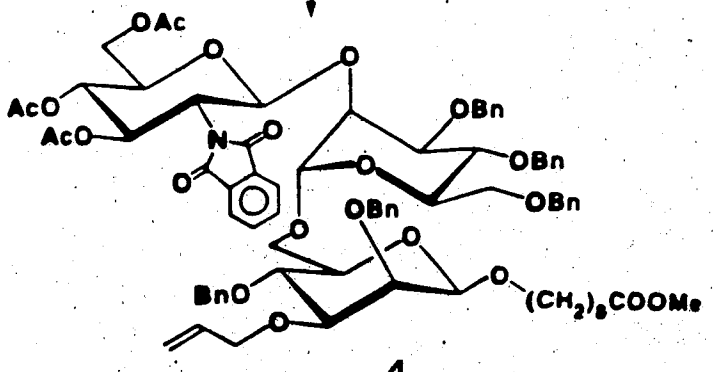
+



6

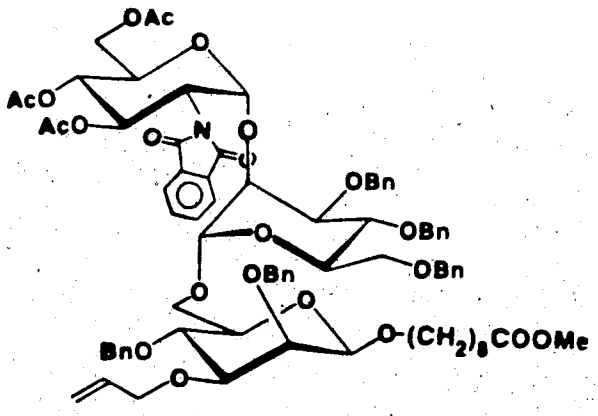
(76%)

CF_3SO_3Ag, sym-collidine
4 Å molecular sieves, CH_2Cl_2
-50°C

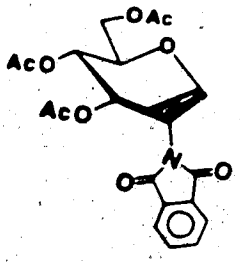


4

minor product, isolated in 6% yield, was not fully characterized but appears to be the α-linked trisaccharide. This structural assignment was made on the basis of its ^{1}H nmr spectrum which included a partially obscured doublet for H-1" at δ5.14 ($J_{1",2"} < 4$ Hz) and a shielded signal (relative to the corresponding β-anomer) for H-3" at δ6.77 ($J_{3",4"} = 9.0$ Hz, $J_{2",3"} = 11.5$ Hz), suggesting the configuration at C-1" to be α. All other signals in the ^{1}H nmr spectrum of **29** were consistent with it being a trisaccharide. As expected, the product of elimination of HBr from the glycosyl bromide **6**, namely 3,4,6-tri-O-acetyl-1,5-anhydro-2-deoxy-2-phthalimido-D-arabino-1-hex-1-enitol (**30**, was also formed in the glycosylation mixture. The glycal **30** had nmr data identical with those previously reported.[24]



29



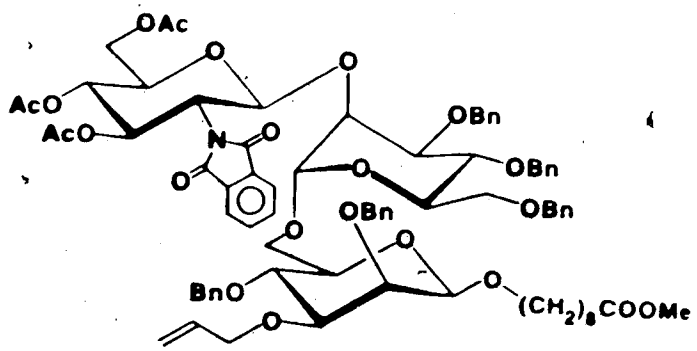
30

Three different methods were examined to effect the removal of the allyl group from **4**. Treatment of **4** with 5% palladium on charcoal,[44] under acidic conditions, furnished the trisaccharide alcohol **31** in only 34% yield. Using palladium(II) chloride as catalyst in acetic acid-water (20:1) according to Ogawa,[45] **31** could be obtained in 90% yield. Finally, deallylation of **4** could also be achieved in two steps: reaction with tris(triphenylphosphine)rhodium(I) chloride as catalyst, according to the procedure developed by Corey and Suggs,[46] effected the isomerization of double bond to give the prop-1-enyl ether, which on treatment[47] with mercuric chloride in the presence of mercuric oxide, underwent hydrolysis to afford the required trisaccharide alcohol **31** in 85% yield. The progress of the isomerization reaction proved difficult to monitor by tlc since the isomerized product (enol ether) and the allyl ether had the same chromatographic mobility. Surprisingly, washing the ether extract with brine acidified to pH 2 failed to accomplish the hydrolysis of the enol ether as had been reported by Corey and Suggs.[46]

The structure of the compound **31** was evident from its ^1H nmr spectrum which was devoid of signals for the protons of the allyl group. The corresponding signals were also absent in the ^{13}C nmr spectrum. Appearance of a

D_2O-exchangeable one-proton doublet at δ2.380 ($J_{3,OH}$ = 10.0 Hz) further supported the assigned structure.

Deacetylation of **31** using sodium methoxide in methanol led to the quantitative formation of **32**, whose identity could easily be ascertained by the absence of acetyl resonances in both its ^1H and ^{13}C nmr spectra. Removal of phthalimido group from **32**, and subsequent N-acetylation of the free amine were performed as described by Bundle and Josephson.[48] Thus, treatment of **32** with hydrazine (8 molar equivalents) in refluxing methanol generated the free amine which was acetylated *in situ* using acetic anhydride in methanol-water (1:1). The N-acetyl derivative **33** was obtained in an overall yield of 60%. No evidence of attack at the 8-methoxycarbonyloctyl ester was obtained. The structure of **33** was supported by the decrease of intensity of the signals from the aromatic protons in ^1H nmr spectrum along with the appearance of two new signals: a three proton singlet arising from the N-acetyl methyl group at δ1.779 and a broad singlet for one D_2O-exchangeable proton at δ5.533 assigned to the amide proton. The ^{13}C nmr spectrum of **33** further supported the assigned structure. The signals at δ168.51, 133.79, 131.84, and 123.23 which were assigned to the carbonyl carbons, two of the tertiary aromatic carbons,

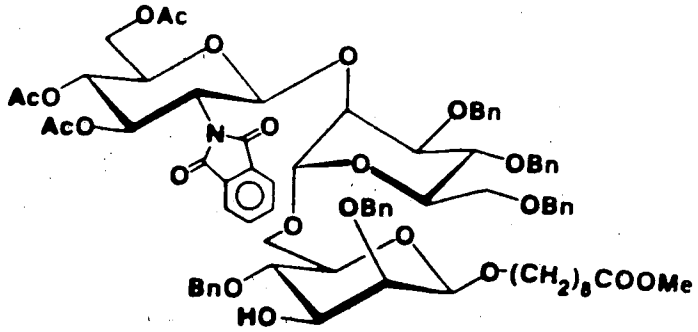


4

(85%)

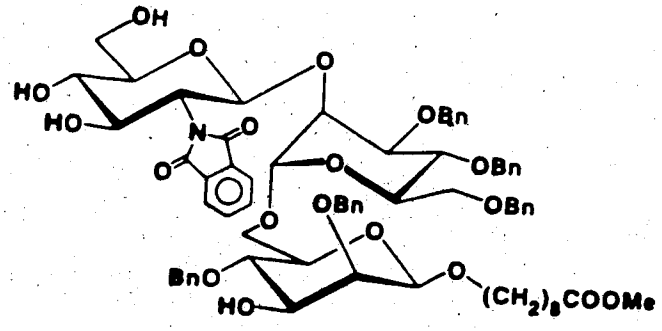
(i) $[(C_6H_5)_3P]_3RhCl$, DABCO
EtOH-benzene-water (7:3:1)

(ii) HgO, $HgCl_2$, Acetone-water (9:1)



31

NaOMe/MeOH

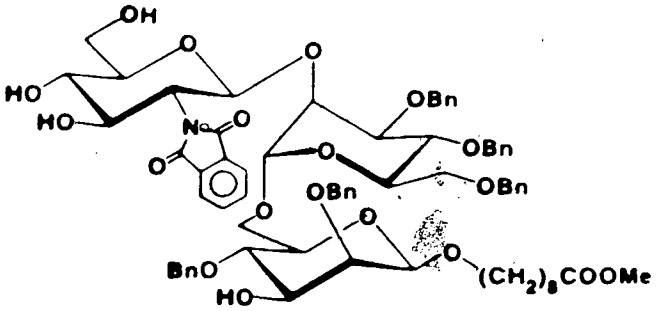


32

the quaternary aromatic carbons, and the remaining two tertiary aromatic carbons of the phthalimido group, respectively in the ^{13}C nmr spectrum of **32**, were absent in the nmr spectrum of **33**. Compound **33**, on the other hand, showed two new resonances at δ172.37 and 23.30 for the carbonyl and the methyl carbons, respectively, of the N-acetyl group.

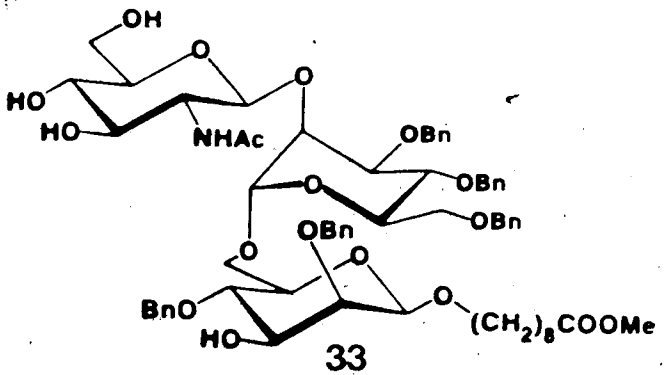
Hydrogenolytic cleavage of the benzyl protecting groups of **33** using 5% palladium-on-charcoal as the catalyst in 98% ethanol furnished the target trisaccharide **2**, which was purified by size-exclusion chromatography on Bio-Gel P-2. Compound **2** was obtained as a white lyophilized powder in 85% yield. The ^{1}H nmr spectrum of **2** showed the expected signals for H-1 at δ4.664 ($J_{1,2}$ < 0.7 Hz), H-1' at δ4.920 ($J_{1',2'}$ = 1.8 Hz) and H-1" at δ4.579 ($J_{1",2"}$ = 8.0 Hz). Other signals were in accord with the structural assignment. The partial 360 MHz ^{1}H nmr spectrum of **2** is reproduced in Figure 4 to show level of anomeric purity of this final product. The ^{1}H-coupled ^{13}C nmr spectrum of **2** again established the configurations at C-1 ($J_{C-1,H-1}$ = 159.4 Hz), C-1' ($J_{C-1',H-1'}$ = 169.5 Hz), and C-1" ($J_{C-1",H-1"}$ = 162.3 Hz) to be β, α, and β respectively.

Having accomplished the synthesis of the target



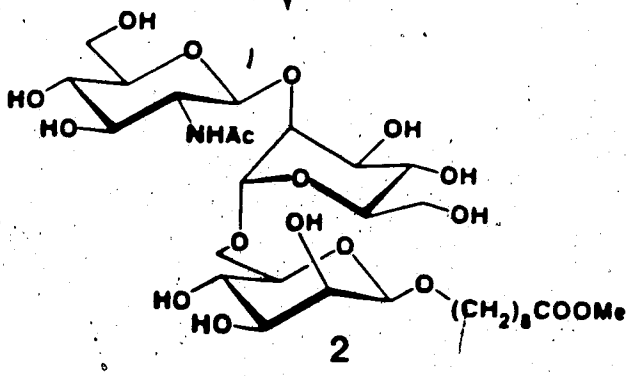
32

(60%) (i) $NH_2NH_2 \cdot H_2O$, MeOH
(ii) Ac_2O, H_2O-MeOH (1:1)



33

(85%) H_2 (1 atm), 5% Pd-C
98% ethanol



2

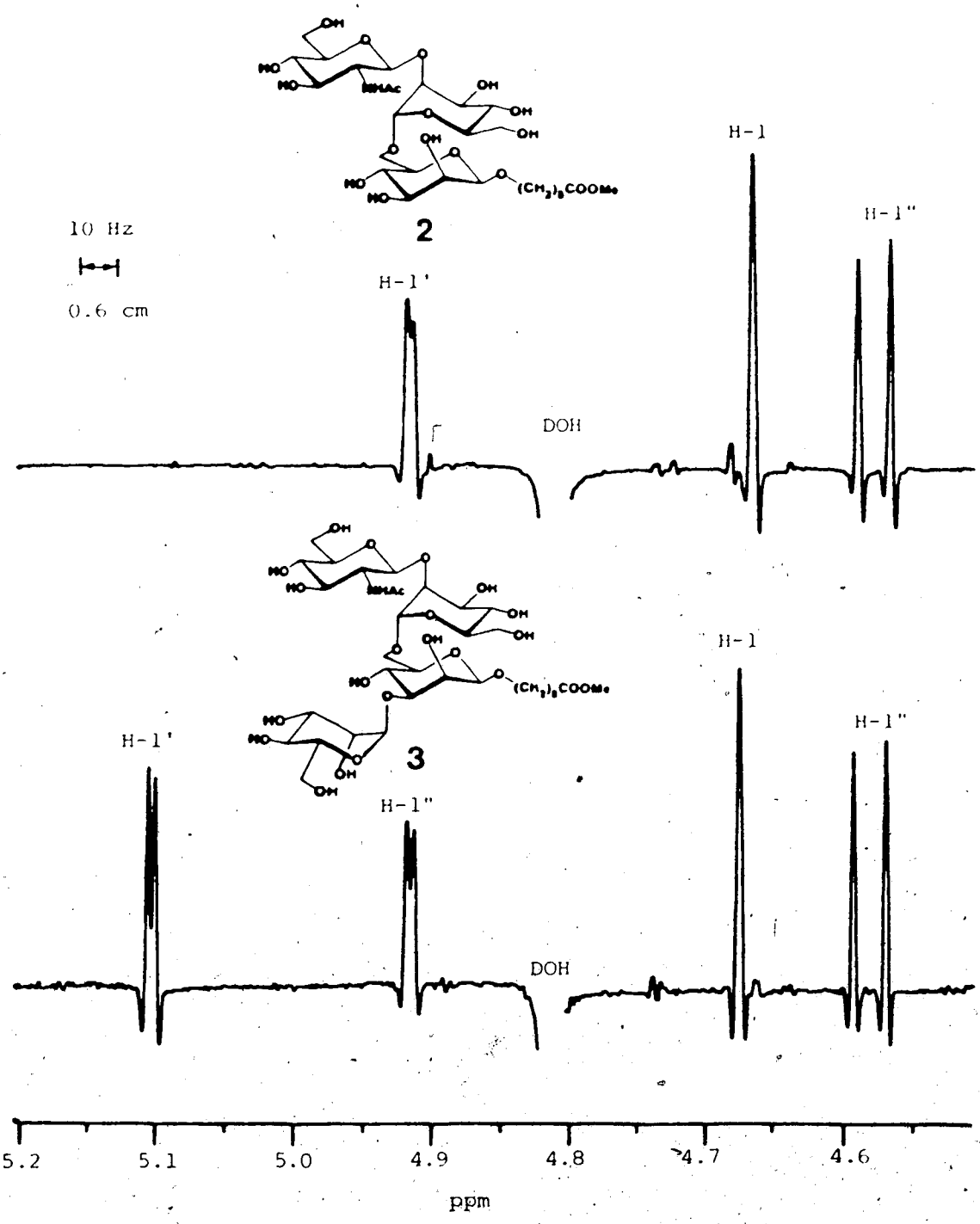
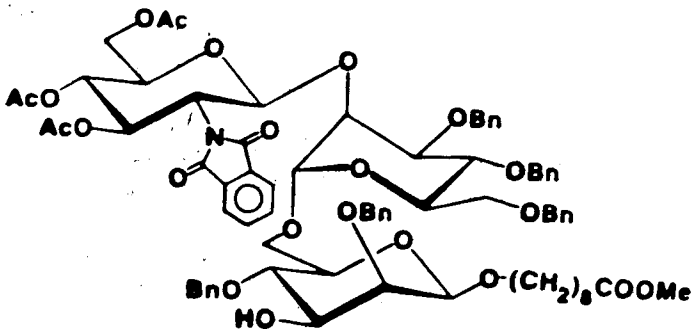


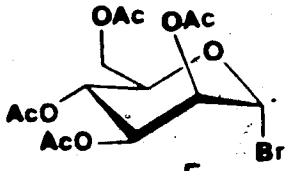
Figure 4. The 360 MHz ^{1}H nmr spectra (only anomeric region shown) of **2** and **3** in D_2O.

trisaccharide 2, the preparation of the tetrasaccharide 3 was undertaken. In order to achieve the coupling of 31 and 2,3,4,6-tetra-O-acetyl-α-D-mannopyranosyl bromide[25] (5), two different promoters were utilized. Under the conditions of Hanessian and Banoub[49] using silver triflate and tetramethylurea as promoter, the glycosylation of 31 with the bromide 5 to provide the tetrasaccharide 34 proceeded in only 38% yield. When the reaction was performed instead in the presence of mercuric bromide and mercuric cyanide in dichloromethane, in the presence of 4 Å molecular sieves, a 65% yield of the condensation product 34 was obtained. The bromide 5 described above was readily available from 1,2,3,4,6-penta-O-acetyl-α/β-D-mannopyranose by treatment with 45% hydrogen bromide in acetic acid.

The ^{1}H nmr data of 34 required the presence of a 2,3,4,6-tetra-O-acetyl-α-D-mannopyranosyl moiety. The signal for the anomeric proton of the newly introduced glycosyl residue appeared at δ5.018 ($J_{1',2'}$ ≈ 2 Hz) and the acetyl region showed the expected seven signals for the methyl acetyl groups. The ^{13}C nmr spectrum showed the presence of four anomeric carbons: δ101.95, $J_{C-1,H-1}$ = 154.1 Hz, 97.64, $J_{C-1'',H-1''}$ = 170.9 Hz, 96.60, $J_{C-1''',H-1'''}$ = 164.16 Hz and the new signal for α-linked mannose at



31



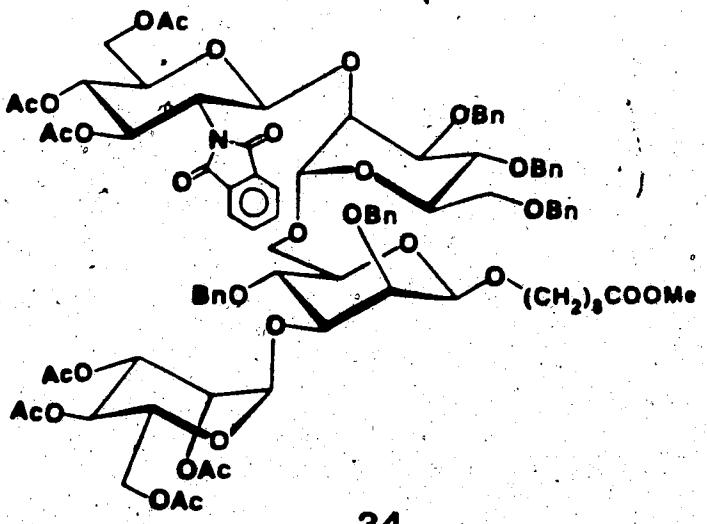
5

(65%)

$HgBr_2$, $Hg(CN)_2$

4 Å molecular sieves

CH_3CN, RT

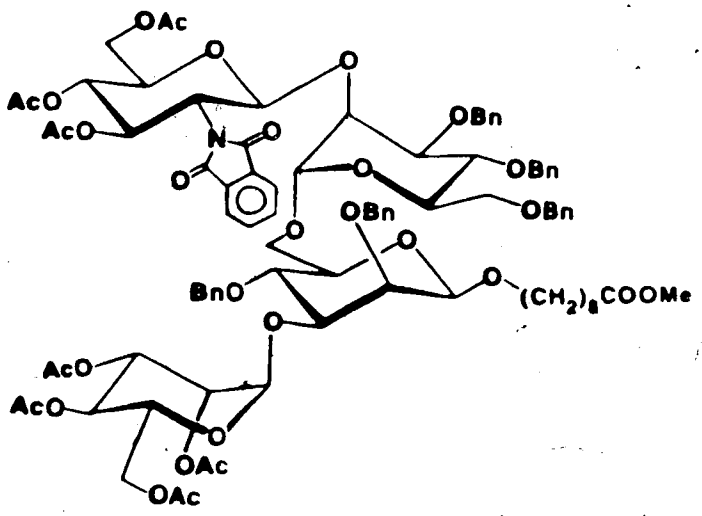


34

δ99.83, $J_{C\text{-}1',H\text{-}1'}$ = 177.8 Hz. Other features of these spectra, described in detail in the experimental section, were in accord with the assigned structures.

Conversion of **34** to **35** was effected in three steps without characterization of the intermediates involved. Deacetylation of **34** with sodium methoxide in methanol gave a white foam which was refluxed with hydrazine in methanol to generate the free amine which, on treatment with acetic anhydride in pyridine, gave the peracetylated product **35** in 63% yield. Attempted N-acetylation of the intermediate free amine, obtained in the second step of the above sequence using acetic anhydride in methanol-water (1:1) gave a very polar hydroxylated derivative which proved difficult to purify by silica gel chromatography. The structure of **35** could readily be deduced from its ^1H nmr spectrum which displayed the presence of only twenty-five aromatic protons and an additional singlet at δ1.705 for three N-acetyl protons. Disappearance of the ^{13}C resonances corresponding to the carbons of the phthalimido group and the presence of two new signals at δ169.54 and 23.27 arising from the N-acetyl group also supported the structure assigned to **35**.

Removal of the O-acetyl and O-benzyl protecting groups of **35** was conveniently accomplished by treatment



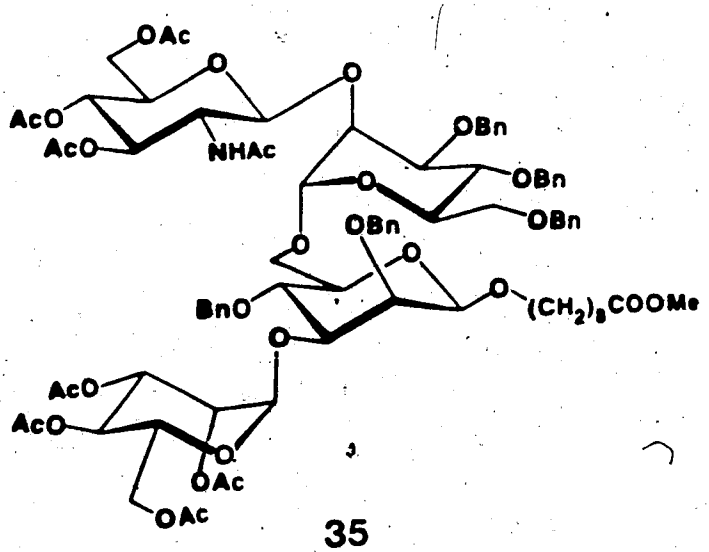
34

(62%)

(i) NaOMe/MeOH

(ii) $NH_2NH_2 \cdot H_2O$, MeOH, reflux

(iii) Ac_2O, Pyridine



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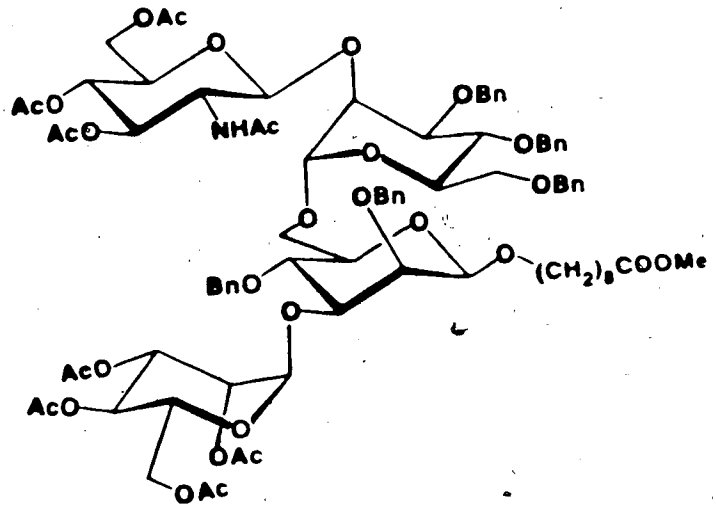
with sodium methoxide in methanol followed by hydrogenolysis over 5% palladium-on-charcoal. Filtration of the crude product through a column of Bio-Gel P-2 afforded the target tetrasaccharide **3** as a white lyophilized powder in a total yield of 85%. Complete removal of acetyl and benzyl groups was evident from the absence of the corresponding signals in the ^{1}H nmr spectrum of 3. The signals for H-1, H-1', H-1", and H-1"' appeared at δ4.671 ($J_{1,2} < 0.8$ Hz), 5.104 ($J_{1',2'} = 2.0$ Hz), 4.918 ($J_{1",2"} = 1.8$ Hz) and 4.579 ($J_{1"',2"'} = 8.0$ Hz) respectively. The ^{1}H-coupled ^{13}C nmr spectrum of **3** was employed to re-establish the anomeric configurations which were already assigned by the ^{1}H-coupled ^{13}C nmr spectra recorded following the formation of each glycosidic linkage. As anticipated, C-1, C-1', C-1", and C-1"' showed the one-bond C-H couplings of 159.1 Hz (β), 172.00 Hz (α), 169.3 Hz (α), and 159.1 Hz (β), respectively. The partial 360 MHz ^{1}H nmr spectrum of 3 is reproduced in Figure 4 where it is compared with the spectrum of the corresponding trisaccharide **2**.



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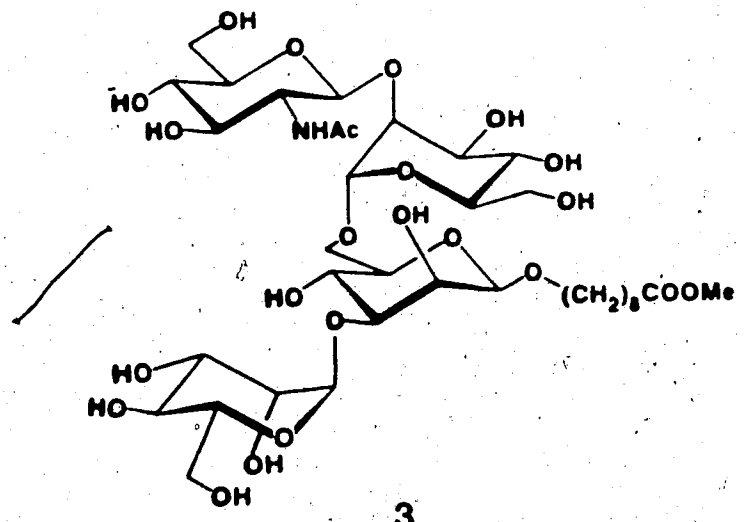
(85%)

(i) NaOMe/MeOH

(ii) H_2 (1 atm), 5% Pd-C
98% EtOH



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CHAPTER 3

TESTING RESULTS

Trisaccharide **2** was found to be an excellent acceptor for GlcNAc-transferase V (GnT V) in experiments performed by Dr. Michael Pierce, Department of Anatomy, University of Miami Medical School. The results of these experiments are included here, briefly, only for the sake of completeness.

In a typical experiment, BHK cells were sonicated in 0.1 M MES [2-(N-morpholino)-ethanesulfonic acid], pH 7.0, and protein was solubilized by addition of Triton X-100 to a 1% solution. The glycosyltransferase assay was performed in a total volume of 20 μL containing 100 μg of the solubilized cellular protein, 1 mM of the acceptor trisaccharide **2**, 1 mM of 2-acetamido-2-deoxy-β-D-gluco-pyranosyl amine (an N-acetyl glucosaminidase inhibitor) and 5 mM UDP-(^{3}H)-GlcNAc. After a 2 hour incubation at 37°, the mixture was diluted with water (100 μL) and passed over a filter supporting Dowex-1-X8 (formate) ion exchange resin to remove almost all of the negatively charged counts. The filtrate was then injected onto a reverse phase (C-18) HPLC column which was eluted

isocratically with 40% aqueous methanol. The chromatogram obtained in this manner is reproduced in Figure 5. In this figure, peak A includes both ^{3}H-UDP-GlcNAc which had passed through the ion exchange resin and, presumably, some of its hydrolytic products including GlcNAc and GlcNAc-1-phosphate. Peak B has a retention volume identical to the acceptor trisaccharide **2** and is absent when this trisaccharide acceptor is omitted from the incubation mixture. The counts produced in this peak increased linearly with time, in the presence of a saturating concentration of acceptor **2** (50-100 nmoles/20 μL), as expected for the enzymatic reaction. The radioactivity in peak B is therefore a measure of the activity of a GlcNAc-transferase.

BHK cells are known to contain only GlcNAc-transferases I, II, IV, V and VI,[15] and all but GnT V require Mn^{++} for activity. The counts in peak B (Figure 5) were not affected by the inclusion of ethylenediamine tetraacetic acid (EDTA) at concentrations known to abolish the activities of these Mn^{++} requiring enzymes. It therefore appears that trisaccharide **2** is indeed a selective substrate for assaying the activity of GnT V. Using acceptor **2** in this assay, the GnT V activity of Rous sarcoma transformed BHK cells was found to be 1.8-2.0 times higher than in the untransformed cells. This

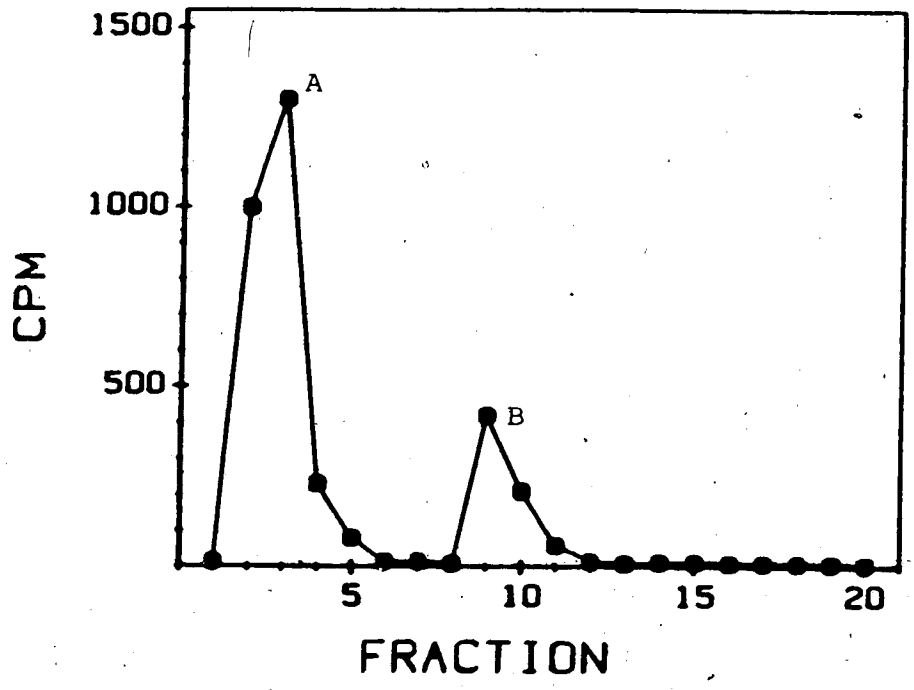


Figure 5. HPLC elution profile of a GlcNAc-transferase assay mixture on a reverse-phase (C-18) column. Peak A includes unreacted UDP-(^{3}H)-GlcNAc and its decomposition products. Peak B is the product of ^{3}H-GlcNAc transfer to the synthetic acceptor trisaccharide **2**.

finding parallels that of Kobata[10,15] for polyoma-transformed BHK cells.

Tetrasaccharide **3** has not yet been tested as an acceptor.

CHAPTER 4

EXPERIMENTAL

General Methods

All solvents and reagents used were reagent grade, and, in cases where further purification was required, standard procedures[50] were followed. All solid reactants for glycosylation were dried overnight over phosphorus pentaoxide in a high vacuum prior to use. Solution transfers where anhydrous conditions were required were done under nitrogen using standard syringe techniques.[51] Molecular sieves were purchased from BDH Chemicals, and the ratio of alcohol to molecular sieves in glycosylation was between 1:5 and 1:10 by weight.

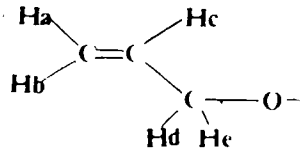
Thin layer chromatograms (tlc) were performed on pre-coated silica gel 60-F254 plates (E. Merck, Darmstadt) and visualized by quenching of fluorescence and/or by charring after spraying with 5% sulfuric acid in ethanol. For flash chromatography[37] 40-63 μm (400-230 mesh) silica gel 60 (E. Merck No. 9385) and distilled solvents were used, and the ratio of silica gel to compound was in the range 50:1-100:1. Skellysolve B refers to hexane supplied by Stanchem, Winnipeg, Manitoba. Solvents were removed on a

rotary evaporator under the vacuum of a water aspirator with bath temperatures of 40° or lower. For gel filtration, Bio-Gel P-2 (200-400 mesh) (Bio-Rad Laboratories, Richmond, California, USA) was used.

Spectral and elemental analyses were performed by the departmental NMR Service Laboratory under the supervision of Dr. T.T. Nakashima and the Analytical Service Laboratory under the supervision of Mr. R. Swindlehurst. Proton nuclear magnetic resonance (^{1}H nmr) spectra were recorded at either 400 MHz (Bruker WH-400) or 360 MHz (Bruker WM-360) with either tetramethylsilane (δ0 in $CDCl_3$) or acetone (δ2.225 in D_2O) as internal standard at ambient temperature. Carbon-13 nuclear magnetic resonance (^{13}C nmr) spectra were recorded at either 100.62 MHz (Bruker WH-400) or 90.56 MHz (Bruker WM-360) with either external tetramethylsilane (δ0 in $CDCl_3$) or external 1,4-dioxane (δ67.4 in D_2O) as reference standard. ^{1}H chemical shifts and coupling constants are reported as if they were first order. Assignments of ^{13}C resonances are tentative. Optical rotations were determined on a Perkin-Elmer 241 polarimeter in a 1 dm cell at ambient temperature (23 ± 1°C).

Protons of the allyl group present in the compounds described in this work were designated as Ha, Hb, Hc, Hd, and He as defined below. These protons showed the same

coupling constants and thus the same multiplicity pattern in all the compounds examined and only the chemical shifts varied. The observed coupling constants were:



Ha, dddd, $J_{a,c}$ = 10.5 Hz

$J_{a,d}$ = 1.5 ± 0.5 Hz

$J_{a,e}$ = 1.5 ± 0.5 Hz

$J_{a,b}$ = 1.5 ± 0.5 Hz

Hb, dddd, $J_{b,c}$ = 17.0 Hz

$J_{b,d}$ = 1.5 ± 0.5 Hz

$J_{b,e}$ = 1.5 ± 0.5 Hz

$J_{a,b}$ = 1.5 ± 0.5 Hz

Hc, dddd, $J_{b,c}$ = 17.0 Hz

$J_{a,c}$ = 10.5 Hz

$J_{c,d}$ = 5.5 Hz

$J_{c,e}$ = 5.5 Hz

Hd, dddd, $J_{d,e}$ = 13.5 Hz

$J_{c,d}$ = 5.5 Hz

$J_{b,d}$ = 1.5 ± 0.5 Hz

$J_{a,d}$ = 1.5 ± 0.5 Hz

He, dddd, $J_{d,e}$ = 13.5 Hz

$J_{c,e}$ = 5.5 Hz

$J_{a,e}$ = 1.5 ± 0.5 Hz

$J_{b,e}$ = 1.5 ± 0.5 Hz

Methyl 3-O-allyl-2-O-benzyl-4,6-O-benzylidene-α-D-manno-pyranoside (13)

To a solution of methyl 3-O-allyl-4,6-O-benzylidene-α-D-mannopyranoside[31] (12) (13.60 g; 42.24 mmol) in dry benzene (500 mL) were added sodium hydride (about 80% dispersion in oil; 2.02 g) and benzyl bromide (7.55 mL; 63.35 mmol). The mixture was refluxed under nitrogen atmosphere for 16 h. After the mixture had cooled to room temperature, the excess of sodium hydride was decomposed by the addition of methanol (250 mL), then water (500 mL) was added. The organic layer was separated, washed with water, dried ($MgSO_4$), filtered, and concentrated. The tlc of the residual oil showed, besides a major spot for the benzylation product, a UV active, fast moving spot which presumably corresponded to unreacted benzyl bromide. Flash chromatography of the oil obtained above using Skellysolve B-ethyl acetate (10:1) as eluent, which provided a good separation on tlc plate (R_f of benzylation product = 0.35), did not result in complete separation.

Purification of the major product could be achieved by gradient flash chromatography employing a mixture of Skellysolve B and ethyl acetate (the ratio of Skellysolve B-ethyl acetate was changed from 50:1 to 2:1 during elution). Finally **13** was obtained as a yellow oil (15.73 g; 90%); $[\alpha]_D$ +33.0° (c 1.4, chloroform); R_f 0.35 (Skellysolve B-ethyl acetate, 10:1); ^1H nmr ($CDCl_3$) δ: 7.505-7.259 (10H, aromatic), 5.901 (1H, Hc allyl), 5.609 (s, 1H, $C_6H_5C\underline{H}O_2$), 5.294 (1H, Hb allyl), 5.146 (1H, Ha allyl), 4.843 (d, 1H, J_{gem} = 12.0 Hz, $C_6H_5C\underline{H}HO$), 4.725 (d, 1H, J_{gem} = 12.0 Hz, $C_6H_5C\underline{H}HO$), 4.681 (d, 1H, $J_{1,2}$ = 2.0 Hz, H-1), 4.294-4.208 (2H, Hd allyl [δ4.268] and H-6e [δ4.244, dd, $J_{6a,6e}$ = 10.5 Hz, $J_{5,6a}$ = 4.5 Hz]), 4.185 (dd, 1H, $J_{6a,6e}$ = 10.0 Hz, $J_{5,6a}$ = 10.0 Hz, H-6a), 4.104 (1H, He allyl), 3.885-3.834 (2H, H-3 and H-4), 3.818 (dd, 1H, $J_{2,3}$ = 3.2 Hz, $J_{1,2}$ = 2.0 Hz, H-2), 3.761 (dd, 1H, $J_{4,5}$ = 10.0 Hz, $J_{5,6a}$ = 10.0 Hz, $J_{5,6e}$ = 4.5 Hz, H-5), 3.310 (s, 3H, CH_3O); ^{13}C nmr ($CDCl_3$) δ: 138.18, 137.72 (quat. arom.), 135.0 ($CH_2=\underline{C}HCH_2O$), 128.64, 128.00, 127.87, 127.59, 125.00 (tert. arom.), 116.32 ($\underline{C}H_2=CHCH_2O$), 101.43, 100.52 (C-1 and $C_6H_5\underline{C}HO_2$), 79.09 (C-4), 76.47, 76.03 (C-2 and C-3), 73.56 ($C_6H_5\underline{C}H_2O$), 68.79 ($CH_2=CH\underline{C}H_2O$), 64.03 (C-6), 54.67 (C-5), 29.57 (CH_3O). Anal. calcd. for $C_{24}H_{28}O_6$: C 69.89, H 6.84; found: C 69.67, H 6.94.

Methyl 3-O-allyl-2,4-di-O-benzyl-α-D-mannopyranoside (**14**)

To a stirred solution of **13** (14.27 g; 34.59 mmol) in 1:1 diethyl ether-dichloromethane (300 mL) was added, portionwise, $LiAlH_4$ (3.95 g; 103.9 mmol) and the mixture was slowly heated to the boiling point. To the boiling solution under reflux was added $AlCl_3$ (13.85 g; 103.9 mmol) in diethyl ether (150 mL) over a period of 70 min, after which tlc indicated the absence of starting material. The mixture was cooled. The excess of $LiAlH_4$ was decomposed with ethyl acetate (75 mL), and $Al(OH)_3$ was precipitated by the addition of water (75 mL). After dilution with ether (400 mL), the organic layer was separated from the aqueous layer which was back-extracted with ether (150 mL). The combined ether extracts were washed with water (3 × 150 mL), dried ($MgSO_4$), and concentrated to an oily residue which tlc indicated to be a mixture of two compounds. The separation of these two products was achieved by flash chromatography using Skellysolve B-ethyl acetate (3:1) as eluent. Evaporation of the early fractions provided the minor product **15** (1.69 g) as an oil; $[\alpha]_D$ = -6.25° (c 1.04, chloroform); R_f 0.47 (Skellysolve B-ethyl acetate, 2:1); ^1H nmr ($CDCl_3$) δ: 7.39-7.25 (10H, aromatic), 5.906 (1H, Hc allyl), 5.283 (1H, Hb allyl), 5.185 (1H, Ha allyl), 4.778 (d, 1H, $J_{1,2}$ =

2.0 Hz, H-1), 4.730-4.665 (AB, 2H, J_{gem} = 12.0 Hz, $C_6H_5C\underline{H}_2O$), 4.655-4.583 (AB, 2H, J_{gem} = 12.0 Hz, $C_6H_5C\underline{H}_2O$), 4.070-3.938 (3H, Hd and He allyl overlapping with H-4 [δ4.004, ddd, $J_{3,4}$ = 9.0 Hz, $J_{4,5}$ = 9.0 Hz, $J_{4,OH}$ = 2.0 Hz, simplified to dd with J_{OH} being absent on D_2O exchange]), 3.830-3.704 (4H, H-2, H-5, H-6a, and H-6b), 3.596 (dd, 1H, $J_{3,4}$ = 9.5 Hz, $J_{2,3}$ = 3.0 Hz, H-3), 3.354 (s, 3H, CH_3O), 2.578 (d, 1H, $J_{4,OH}$ = 2.0 Hz, disappeared on D_2O exchange, 4-OH); ^{13}C nmr ($CDCl_3$) δ: 138.40, 138.37 (quat. arom.), 134.76 ($CH_2=\underline{C}HCH_2O$), 128.37, 127.88, 127.66, 127.56 (tert. arom.), 117.22 ($\underline{C}H_2=CHCH_2O$), 99.40 (C-1), 79.41 (C-3), 73.79 (C-2), 73.65, 72.78 ($C_6H_5\underline{C}H_2O$), 71.58 (C-5), 70.73 ($CH_2=CH\underline{C}H_2O$), 70.66 (C-6), 67.96 (C-4), 54.99 (CH_3O). Anal. calcd. for $C_{24}H_{30}O_6$: C 69.55, H 7.30; found: C 69.67, H 7.35.

Evaporation of the later fractions gave the major product **14** (10.78 g) as an oil; $[\alpha]_D$ +48.25° (c 0.97, chloroform); R_f 0.35 (Skellysolve B-ethyl acetate, 2:1); ^{1}H nmr ($CDCl_3$) δ: 7.42-7.24 (10H, aromatic), 5.950 (1H, Hc allyl), 5.326 (1H, Hb allyl), 5.180 (1H, Ha allyl), 4.936 (d, 1H, J_{gem} = 11.0 Hz, $C_6H_5C\underline{H}HO$), 4.813 (d, 1H, J_{gem} = 12.5 Hz, $C_6H_5C\underline{H}HO$), 4.709-4.688 (2H, $C_6H_5C\underline{H}HO$ [δ4.709; d, J_{gem} = 12.5 Hz] overlapping with H-1 [δ4.690, d, $J_{1,2}$ = 2 Hz]), 4.638 (d, 1H, J_{gem} = 11.0 Hz, $C_6H_5C\underline{H}HO$), 4.146-4.064 (2H, Hd and He allyl), 3.913 (dd, 1H, $J_{3,4}$ =

9.5 Hz, $J_{4,5}$ = 9.5 Hz, H-4), 3.840 (ddd, 1H, $J_{6a,6b}$ = 12.0 Hz, $J_{6,OH}$ = 5.5 Hz, $J_{5,6a}$ = 3.0 Hz, D_2O addition resulted in its collapse to dd with $J_{6,OH}$ having disappeared, H-6a), 3.81-3.72 (3H, H-2, H-3, and H-6b), 3.601 (ddd, 1H, $J_{4,5}$ = 9.5 Hz, $J_{5,6a}$ = 3.0 Hz, $J_{5,6b}$ = 5.0 Hz, H-5), 3.298 (s, 3H, CH3O), 2.046 (dd, 1H, J6a,OH = 5.5 Hz, J6b,OH = 7.0 Hz, deuterium exchangeable, 6-OH); ^{13}C nmr ($CDCl_3$) δ: 138.56, 138.37 (quat. arom.), 134.96 ($CH_2=\underline{C}HCH_2O$), 128.36, 128.02, 127.81, 127.67 (tert. arom.), 116.60 ($\underline{C}H_2=CHCH_2O$), 99.47 (C-1), 79.92 (C-3), 75.13 ($C_6H_5\underline{C}H_2O$), 74.88 (C-2 and C-4), 72.99 ($C_6H_5\underline{C}H_2O$), 72.10 (C-5), 71.10 ($CH_2=CH\underline{C}H_2O$), 62.42 (C-6), 54.72 (CH_3O). Anal. calcd. for $C_{24}H_{30}O_6$: C 69.55, H 7.30; found: C 69.42, H 7.37.

Methyl 6-O-acetyl-3-O-allyl-2,4-di-O-benzyl-α-D-manno-pyranoside (**16**)

To a solution of **14** (10.30 g, 24.85 mmol) in dry pyridine (100 mL) was added acetic anhydride (7.5 mL; 79.54 mmol). The mixture was stirred under nitrogen atmosphere at room temperature overnight. The excess of acetic anhydride was decomposed by dropwise addition of ethanol (5 mL) to the ice cold reaction mixture, and dichloromethane (300 mL) and water (250 mL) were then added. The aqueous layer was separated and back extracted

with dichloromethane (100 mL). The combined dichloromethane extracts were washed with 1 M aqueous HCl and saturated aqueous sodium bicarbonate. The organic phase was dried ($MgSO_4$), filtered, and evaporated under reduced pressure to give **16** (11.06 g; 98%) as a chromatography-pure oil; $[\alpha]_D$ +42.91° (c 1.03, chloroform); R_f 0.45 (Skellysolve B-ethyl acetate, 3:1); ^1H nmr ($CDCl_3$) δ: 7.41-7.25 (10H, aromatic), 5.920 (1H, Hc allyl), 5.309 (1H, Hb allyl), 5.169 (1H, Ha allyl), 4.910 (d, 1H, J_{gem} = 11.0 Hz, $C_6H_5C\underline{H}HO$), 4.778-4.691 (3H, $C_6H_5C\underline{H}_2O$ [δ 4.778-4.691, AB, J_{gem} = 11.0 Hz] overlapping with H-1 [δ 4.718, d, $J_{1,2}$ = 2.0 Hz]), 4.561 (d, 1H, J_{gem} = 11.0 Hz, $C_6H_5C\underline{H}HO$); 4.348-4.268 (2×dd, 2H, $J_{6a,6b}$ = 12.0 Hz, $J_{5,6a}$ = 3.0 Hz, $J_{5,6b}$ = 5.0 Hz, H-6a and H-6b), 4.119-4.025 (2H, Hd and He allyl), 3.844 (dd, 1H, $J_{3,4}$ = 9.0 Hz, $J_{4,5}$ = 9.5 Hz, H-4), 3.781-3.706 (3H, H-3 [δ 3.766, $J_{2,3}$ = 3.5 Hz, $J_{3,4}$ = 9.5 Hz], H-2 [δ 3.743, $J_{1,2}$ = 2.0 Hz, $J_{2,3}$ = 3.5 Hz], and H-5), 3.31 (s, 3H, CH_3O), 2.05 (s, 3H, $OCOCH_3$); ^{13}C nmr ($CDCl_3$) δ: 170.80 $O\underline{C}OCH_3$), 138.34 (quat. arom.), 134.85 ($CH_2=\underline{C}HCH_2O$), 128.36, 128.29, 128.08, 127.70, 127.60 (tert. arom.), 116.70 ($\underline{C}H_2=CHCH_2O$), 99.10 (C-1), 79.89 (C-3), 75.07 ($C_6H_5\underline{C}H_2O$), 74.50, 74.58 (C-2 and C-4), 72.66 ($C_6H_5\underline{C}H_2O$), 70.98 ($CH_2=C\underline{H}CH_2O$), 69.98 (C-5), 63.63 (C-6), 54.73 (CH_3O), 20.81 ($OCO\underline{C}H_3$). Anal. calcd. for $C_{26}H_{32}O_7$: C 68.40, H 7.07; found: C 68.39, H 7.09.

1,6-Di-O-acetyl-3-O-allyl-2,4-di-O-benzyl-α-D-manno-pyranose (**17**)

A solution of concentrated sulfuric acid (0.22 mL) in acetic anhydride (7.68 mL) was added dropwise to a solution of **16** (8.77 g; 19.65 mmol) in acetic anhydride (40 mL) at 0° over 10 min. The mixture was stirred at 0°C for 25 min and at room temperature for 20 min. Then the reaction mixture was poured into dichloromethane (1 L) and ice cold water (1 L) containing sodium bicarbonate, and the resulting mixture was stirred at room temperature for 30 min. The organic and aqueous layers were separated, and the aqueous layer was extracted with dichloromethane (500 mL). The dichloromethane solutions were combined and then washed with saturated aqueous sodium carbonate and water. Finally the organic phase was dried (Na_2SO_4), filtered, and concentrated. The residual syrup was purified by flash chromatography using Skellysolve B-ethyl acetate as eluent, the ratio of Skellysolve B to ethyl acetate being varied from 6:1 to 3:1 during elution. Removal of solvent from the early fractions provided the title compound as an oil (7.35 g; 77% yield); $[\alpha]_D$ +37.85 (c 0.93, chloroform); R_f 0.56 (Skellysolve B-ethyl acetate, 3:1); ^1H nmr ($CDCl_3$) δ: 7.431-7.273 (10H,

aromatic), 6.195 (d, 1H, $J_{1,2}$ = 2.0 Hz, H-1), 5.923 (1H, Hc allyl), 5.315 (1H, Hb allyl), 5.200 (1H, Hc allyl), 4.935 (d, 1H, J_{gem} = 10.5 Hz, $C_6H_5C\underline{H}HO$), 4.840-4.766 (AB, 2H, J_{gem} = 12.0 Hz, $C_6H_5C\underline{H}_2O$), 4.621 (d, 1H, J_{gem} = 10.5 Hz, $C_6H_5C\underline{H}HO$), 4.384-4.310 (2×dd, 2H, H-6a and H-6b), 4.095 (ddd, 2H, $CH_2=CHC\underline{H}_2O$), 3.973 (dd, 1H, $J_{3,4}$ = 10.0 Hz, $J_{4,5}$ = 10.0 Hz, H-4), 3.893 (ddd, 1H, $J_{4,5}$ = 10.0 Hz, $J_{5,6a}$ = 4.0 Hz, $J_{5,6b}$ = 3.0 Hz, H-5), 3.814, 3.785 (2 dd, 2H, $J_{3,4}$ = 10.0 Hz, $J_{2,3}$ = 3.0 Hz, $J_{1,2}$ = 2.0 Hz, H-3 and H-2 respectively), 2.055 (s, 6H, $OCOCH_3$×2); ^{13}C nmr ($CDCl_3$) δ: 170.82, 168.82 ($O\underline{C}OCH_3$), 138.08, 137.87 (quat. arom.), 134.68 ($H_2C=\underline{C}HCH_2O$), 128.50, 128.40, 128.24, 127.93, 127.88, 127.83 (tert. arom.), 117.04 ($H_2\underline{C}=CHCH_2O$), 91.79 (C-1), 79.17 (C-3), 75.36 ($C_6H_5\underline{C}H_2O$), 73,89, 73.39 (C-2 and C-4), 72.53 ($C_6H_5\underline{C}H_2O$), 72.41 (C-5), 71.09 ($H_2C=CH\underline{C}H_2O$), 63.24 (C-6), 20.98, 20.85 ($OCO\underline{C}H_3$). Anal. calcd. for $C_{27}H_{32}O_8$: C 66.93, H 6.66; found: C 66.78, H 6.74.

Evaporation of the subsequent fractions furnished the corresponding β-anomer (**18**) and 1,4,6-tri-O-acetyl-3-O-allyl-2-O-benzyl-α-D-mannopyranose (**19**) in a combined yield of 6%. The ^1H nmr spectra of **18** and **19** were in agreement with the assigned structures.

6-O-Acetyl-3-O-allyl-2,4-di-O-benzyl-α-D-mannopyranosyl bromide (**8**)

Hydrogen bromide gas was bubbled for 30 min through a tube of calcium sulfate into a solution of **17** (5.80 g; 11.97 mmol) in dry dichloromethane (300 mL) at 0°C. The solution was then taken to dryness and the by-product acetic acid removed by evaporation of toluene (100 mL) from the residue (twice). Finally **8** was obtained as an oil, a very small portion of which was purified for elemental and nmr spectral analysis by flash chromatography using Skellysolve B-ethyl acetate (3:1) as eluent; $[\alpha]_D$ +133.08° (c 0.91, chloroform); R_f 0.60 (Skellysolve B-ethyl acetate, 3:1); ^1H nmr ($CDCl_3$) δ: 7.42-7.30 (10H, aromatic), 6.444 (d, 1H, $J_{1,2}$ = 1.5 Hz, H-1), 5.936 (1H, Hc allyl), 5.350 (1H, Hb allyl), 5.233 (1H, Ha allyl), 5.955 (d, 1H, J_{gem} = 10.5 Hz, $C_6H_5C\underline{H}HO$), 4.788-4.718 (AB, 2H, Jgem = 12.5 Hz, $C6H5C\underline{H}_2O$), 4.613 (d, 1H, J_{gem} = 10.5 Hz, $C_6H_5C\underline{H}HO$), 4.388-4058 (2H, H-6a and H-6b), 4.243 (dd, 1H, J3,4 = 9.0 Hz, J2,3 = 3.0 Hz, H-3), 4.116 (d, 2H, J = 5 Hz, $CH_2=CHC\underline{H}_2O$), 4.016-3.935 (3H, H-2 [δ4.011, dd, $J_{2,3}$ = 3.0 Hz, $J_{1,2}$ = 1.5 Hz], H-4 and H-5), 2.065 (s, 3H, $OCOCH_3$); 13C nmr ($CDCl_3$) δ: 170.57 ($O\underline{C}OCH_3$), 137.90, 137.55 (quat. arom.), 134.37 ($CH_2=\underline{C}HCH_2O$), 128.63, 128.44, 128.14, 127.97, 127.87,

127.73 (tert. arom.), 117.38 ($\underline{C}H_2$=$CHCH_2O$), 87.41 (C-1), 78.41 (C-3), 78.31, 74.31, 73.57 (C-2, C-4, and C-5), 75.30, 72.89 ($C_6H_5\underline{C}H_2O$), 71.21 (CH_2=$CH\underline{C}H_2O$), 62.39 (C-6), 20.72 ($OCO\underline{C}H_3$). Anal. calcd. for $C_{25}H_{29}O_6Br$: C 59.41, H 5.78, Br 15.81; found: C 59.48, H 5.82, Br 16.17.

8-Methoxycarbonyloctyl 6-O-acetyl-3-O-allyl-2,4-di-O-benzyl-β-D-mannopyranoside (**23**β) and 8-methoxycarbonyloctyl 6-O-acetyl-3-O-allyl-2,4-di-O-benzyl-α-D-mannopyranoside (**23**α)

8-Methoxycarbonyoctanol[24] (7.50 g; 39.89 mmol) and silver silicate/alumina (16.50 g) in dry dichloromethane (100 mL) were stirred at room temperature for 1 h. To the above mixture which was cooled to -78°C was added dropwise, with stirring, a solution of bromosugar **8** (7.44 g; 14.72 mmol) in dry dichloromethane (75 mL) and stirring was continued for 2.5 h at -78°C, and for 10 h at room temperature. The mixture was diluted with dichloromethane (100 mL) and filtered through celite. The filtrate was washed with water, dried (Na_2SO_4), and concentrated to a syrup which was purified by flash chromatography, using Skellysolve B-ethyl acetate (4.5:1) as eluent. A chromatographically inseparable 6:1 mixture of β- and α-mannosides **23** was obtained as an oil (5.14 g; total yield

57%). ^1H nmr ($CDCl_3$) δ: 4.824 (d, $J_{1,2}$ = 1.5 Hz, H-1 of α-anomer), 4.365 (d, $J_{1,2}$ = 0.5 Hz, H-1 of β-anomer), 2.059 (s, $OCOCH_3$ of α-anomer), 2.050 (s, $OCOCH_3$) of β-anomer).

8-Methoxycarbonyloctyl 3-O-allyl-2,4-di-O-benzyl-β-D-mannopyranoside (**24**) and 8-methoxycarbonyloctyl 3-O-allyl-2,4-di-O-benzyl-α-D-mannopyranoside (**25**)

The mixture of α- and β-anomers **23** described above (4.39 g; 7.17 mmol) was dissolved in dry methanol (200 mL) containing a trace of sodium methoxide, and the resulting solution was stirred at room temperature overnight. Neutralization with Amberlite IR-120(H) resin followed by the removal of the resin and evaporation provided a chromatographically pure oily residue (4.10 g) whose tlc on silica gel impregnated with silver nitrate showed it to be a mixture of two compounds. Purification of these two products was accomplished by flash chromatography on silver nitrate-impregnated silica gel using Skellysolve B-ethyl acetate as eluent, the ratio of Skellysolve B to ethyl acetate being decreased from 7:1 to 2:1 during elution. Evaporation of early fractions furnished the α-anomer **25** (0.19 g) as an oil; $[\alpha]_D$ +30.8° (c 0.75, chloroform); ^1H nmr ($CDCl_3$) δ: 7.42-7.27 (10H, aromatic),

5.965 (1H, Hc of allyl), 5.345 (1H, Hb allyl), 5.194 (1H, Ha allyl), 4.945 (d, 1H, J_{gem} = 11.0 Hz, $C_6H_5\underline{C}\underline{H}HO$), 4.843-4.788 (2H, $C_6H_5\underline{C}\underline{H}HO$ [δ 4.828, d, J_{gem} = 12.0 Hz] and H-1 [δ 4.790, d, $J_{1,2}$ = 2.0 Hz], 4.724 (d, 1H, J_{gem} = 12.0 Hz, $C_6H_5\underline{C}\underline{H}HO$), 4.655 (d, 1H, J_{gem} = 11.0 Hz, $C_6H_5\underline{C}\underline{H}HO$), 4.138 (broad d, 2H, Hd and He allyl), 3.923 (dd, 1H, $J_{4,5}$ = 9.5 Hz, $J_{3,4}$ = 9.5 Hz, H-4), 3.870-3.579 (9H, H-2, H-3, H-5, H-6a, H-6b, $O\underline{C}\underline{H}HCH_2$, and OCH_3 [δ 3.665, s]), 3.329 (dt, 1H, J_{gem} = 9.0 Hz, J_{vic} = 6.5 Hz, $O\underline{C}\underline{H}HCH_2$), 2.303 (t, 2H, $\underline{C}\underline{H}_2COOCH_3$, J = 7.5 Hz), 2.098 (broad s, 1H, exchangeable with deuterium, OH), 1.620 (m, 2H, aliphatic), 1.510 (m, 2H, aliphatic), 1.29 (broad s, 8H, remaining aliphatic); ^{13}C nmr ($CDCl_3$) δ: 174.15 ($\underline{C}OOCH_3$), 138.48, 138.43 (quat. arom.), 134.95 ($CH_2=\underline{C}HCH_2O$), 128.35, 128.29, 128.07, 127.72, 127.67, 127.58 (tert. arom.), 116.47 ($\underline{C}H_2=CHCH_2O$), 98.25 (C-1, $J_{C-1,H-1}$ = 168.2 Hz), 79.98 (C-3), 75.18 ($C_6H_5\underline{C}H_2O$), 75.04, 75.01 (C-3 and C-4), 72.88 ($C_6H_5\underline{C}H_2O$), 72.08 (C-5), 71.07 ($CH_2=CH\underline{C}H_2O$), 67.61 ($O\underline{C}H_2CH_2$), 62.46 (C-6), 51.33 ($COO\underline{C}H_3$), 34.01 ($\underline{C}H_2COOCH_3$), 29.32, 29.11, 29.06, 29.01, 25.99, 24.85 (aliphatic). Anal. calcd. for $C_{33}H_{46}O_8$: C 69.45, H 8.12; found: c 69.27, H 8.26.

Further elution provided a mixture of α- and β-anomers (0.9 g) which were in the ratio of 1:3 (α/β) by ^1H nmr.

Evaporation of the later fractions afforded the

desired β-anomer **24** (2.89 g) as an oil; $[\alpha]_D$ -51.33 (c 0.9, chloroform); ^{1}H nmr ($CDCl_3$) δ: 7.49-7.29 (10H, aromatic), 5.893 (1H, Hc allyl), 5.288 (1H, Hb allyl), 5.17 (1H, Ha allyl), 4.969-4.858 (3H, $C_6H_5C\underline{H}_2O$ [δ 4.969-4.800, AB, J_{gem} = 12.5 Hz] and $C_6H_5C\underline{H}HO$ [4.935, d, J_{gem} = 10.5 Hz]), 4.624 (d, 1H, J_{gem} = 10.5 Hz, $C_6H_5C\underline{H}HO$), 4.408 (d, 1H, J = 1 Hz, H-1), 4.024-3.828 (6H, H-2, H-4, H-6a, $OC\underline{H}H\underline{C}H_2$, and $CH_2{=}CHC\underline{H}_2O$), 3.763 (ddd, 1H, $J_{6a,6b}$ = 12.0 Hz, $J_{5,6b}$ = 6.0 Hz, $J_{6b,OH}$ = 6.0 Hz, simplified to dd with $J_{6b,OH}$ being absent on D_2O exchange, H-6b), 3.688 (s, 3H, OCH_3), 3.444-3.386 (2H, H-3 and $OC\underline{H}H\underline{C}H_2$), 3.316 (dt, 1H, $J_{4,5}$ = 9.5 Hz, $J_{5,6b}$ = 6.0 Hz, $J_{5,6a}$ = 3.0 Hz, H-5), 2.330 (t, 2H, $C\underline{H}_2COOCH_3$, J = 7.5 Hz), 2.140 (t, 1H, $J_{6a,OH}$ = 6.0 Hz, $J_{6b,OH}$ = 6.0 Hz, disappeared after D_2O addition, OH), 1.670-1.628 (4H, aliphatic), 1.345 (8H, remaining aliphatic); ^{13}C nmr ($CDCl_3$) δ: 174.50 ($\underline{C}OOCH_3$), 138.66, 138.36 (quat. arom.), 134.70 ($CH_2{=}\underline{C}HCH_2O$), 128.37, 128.28, 128.11, 128.03, 127.73, 127.37 (tert. arom.), 116.75 ($\underline{C}H_2{=}CHCH_2O$), 101.69 (C-1, $J_{C-1,H-1}$ = 153.6 Hz), 82.33 (C-3), 75.77, 74.88, 73.90 (C-2, C-4, and C-5), 75.18, 73.86 ($C_6H_5\underline{C}H_2O$), 70.58, 70.12 ($CH_2{=}CH\underline{C}H_2O$ and $O\underline{C}H_2CH_2$), 62.63 (C-6), 51.36 ($COO\underline{C}H_3$), 34.04 ($\underline{C}H_2COOCH_3$), 29.63, 29.16, 29.04, 26.01, 24.89 (aliphatic). Anal. calcd. for $C_{33}H_{46}O_8$: C 69.45, H 8.12; found: C 69.41, H 8.13.

8-Methoxycarbonyloctyl 6-O-(2-O-acetyl-3,4,6-tri-O-benzyl-α-D-mannopyranosyl)-3-O-allyl-2,4-di-O-benzyl-β-D-mannopyranoside (**27**)

To a solution of **24** (2.89 g; 5.06 mmol) in dry acetonitrile (60 mL) containing 4Å molecular sieves were added, sequentially, mercuric bromide (2.19 g; 6.07 mmol), mercuric cyanide (1.53 g; 6.07 mmol), and a solution of 2-O-acetyl-3,4,6-tri-O-benzyl-α-D-mannopyranosyl bromide[27] (**7**) in dry acetonitrile (25 mL), which had been freshly prepared from 3,4,6-tri-O-benzyl-1,2-O-(methoxyethylidene)-β-D-mannopyranose (**26**) (3.07 g; 6.07 mmol). The reaction mixture was stirred at room temperature for 1 h, and the mixture was then filtered through Celite. Evaporation of the solvent gave an oily residue which was extracted 3 times with dichloromethane. The extracts were combined and washed successively with saturated aqueous potassium chloride, saturated aqueous sodium bicarbonate, water, and brine. The organic layer was dried (Na_2SO_4), filtered, and evaporated to dryness. The resulting oil was purified by flash chromatography using Skellysolve B-ethyl acetate (4:1) as eluent to provide the title compound as a syrup (4.07 g; 77%); $[\alpha]_D$ -6.07° (*c* 1.22,

chloroform); R_f 0.3 (Skellysolve B-ethyl acetate, 3:1); ^1H nmr ($CDCl_3$) δ: 7.47-7.10 (25H, aromatic), 5.86 (1H, Hc allyl), 5.459 (dd, 1H, $J_{2',3'}$ = 3.0 Hz, $J_{1',2'}$ = 2.0 Hz, H-2'), 5.270 (1H, Hb allyl), 5.159 (1H, Ha allyl), 4.980-4.825 (5H, H-1' [δ 4.911] and 4 × $C_6H_5C\underline{H}HO$ [d, J_{gem} = 11.0-12.5 Hz]), 4.680-4.650 (2 d overlapping, 2H, J_{gem} = 10.5 and 12.0 Hz, $C_6H_5C\underline{H}_2O$), 4.513-4.408 (4×d, 4H, J_{gem} = 11.0-12.0 Hz, $C_6H_5C\underline{H}_2O$), 4.334 (s, 1H, H-1), 3.930-3.590 (15H, $COOCH_3$ [δ 3.66], H-2, H-4, H-6a, H-6b, H-3', H-4', H-5', H-6a', H-6b', $CH_2=CHC\underline{H}_2O$ and $OC\underline{H}HCH_2$), 3.410-3.315 (3H, H-3, H-5, and $OC\underline{H}HCH_2O$), 2.283 (t, 2H, J = 7.5 Hz, $C\underline{H}_2COOCH_3$), 2.133 (s, 3H, $OCOCH_3$), 1.620-1.513 (4H, aliphatic), 1.335-1.240 (8H, remaining aliphatic); ^{13}C nmr ($CDCl_3$) δ: 174.08 ($\underline{C}OOCH_3$), 170.12 ($O\underline{C}OCH_3$), 139.00, 138.80, 138.53, 138.46, 138.05 (quat. arom.), 134.79 ($CH_2=\underline{C}HCH_2O$), 128.27, 128.19, 128.13, 128.10, 127.97, 127.77, 127.70, 127.59, 127.54, 127.50, 127.38, 127.29, 127.23 (tert. arom.), 116.67 ($\underline{C}H_2=CHCH_2O$), 101.64 (C-1, $J_{C-1,H-1}$ = 154.7 Hz), 97.77 (C-1', $J_{C-1',H-1'}$ = 170.4 Hz), 82.56 (C-3), 77.94 (C-3'), 74.80, 74.37, 73.91 (C-2, C-4, C-5, and C-4'), 71.36 (C-5'), 68.67 (C-2'), 74.91, 73.71, 73.30, 71.51 ($C_6H_5\underline{C}H_2O$ × 5), 70.43, 69.79 ($CH_2=CH\underline{C}H_2O$ and $O\underline{C}H_2CH_2$), 68.98 (C-6'), 67.06 (C-6), 51.26 ($COO\underline{C}H_3$), 34.05 ($\underline{C}H_2COOCH_3$), 29.65, 29.23, 29.16, 29.07, 26.09, 24.92 (aliphatic), 20.99 ($OCO\underline{C}H_3$). Anal. calcd. for

$C_{62}H_{76}O_{14}$: C 71.24, H 7.33; found: 71.09, H 7.46.

8-Methoxycarbonyloctyl 3-O-allyl-2,4-di-O-benzyl-6-O-(3,4,6-tri-O-benzyl-α-D-mannopyranosyl)-β-D-mannopyranoside (**28**)

The disaccharide **27** (2.88 g; 2.76 mmol) was de-O-acetylated as described for the preparation of **25** and **26**. After removal of the resin by filtration the solvent was evaporated to afford **28** as chromatographically pure oil (2.73 g; 99%); $[\alpha]_D$ +3.13° (c 1.34, chloroform); R_f 0.32 (Skellysolve B-acetone, 3:1); ^1H nmr ($CDCl_3$) δ: 7.48-7.14 (25H, aromatic), 5.883 (1H, Hc allyl), 5.288 (1H, Hb allyl), 5.170 (1H, Ha allyl), 5.258 (broad s, 1H, $J_{1',2'}$ < 2Hz, H-1'), 4.968-4.795 (4×d, 4H, J_{gem} = 11.0 and 12.5 Hz, $C_6H_5\underline{CH_2}O$), 4.644-4.438 (6×d, 6H, J_{gem} = 11.0-12.0 Hz, $C_6H_5\underline{CH_2}O$), 4.335 (s, 1H, $J_{1,2}$ < 1 Hz, H-1), 4.128 (broad s, 1H, H-2'), 4.038-3.593 (15H, OCH_3 [δ3.655, s], H-2, H-4, H-6a, H-6b, H-3', H-4', H-5', H-6a', H-6b', $OCHH\underline{CH_2}$, and $CH_2{=}CH\underline{CH_2}O$), 3.423-3.320 (3H, H-3, H-5, and $OC\underline{H}HCH_2$), 2.355 (broad s, 1H, deuterium-exchangeable, OH), 2.284 (t, 2H, J = 7.5 Hz, $\underline{CH_2}COOCH_3$), 1.59 (4H, aliphatic), 1.28 (8H, remaining aliphatic); ^{13}C nmr ($CDCl_3$) δ: 173.86 ($\underline{C}OOCH_3$), 139.04, 138.73, 138.59, 138.44, 138.03 (quat. arom.), 134.86 ($CH_2{=}\underline{C}HCH_2O$), 128.49, 128.36, 128.29,

128.24, 128.07, 127.99, 127.89, 127.78, 127.62, 127.48, 127.40 (tert. arom.), 116.84 ($\underline{C}H_2$=CH$\underline{C}H_2$O), 101.73 (C-1), 99.77 (C-1'), 82.47 (C-3), 79.69 (C-3'), 75.24, 74.61, 74.36, 74.06 (C-3, C-4, C-5, and C-4'), 75.07, 74.94, 73.88, 73.41, 71.48 ($C_6H_5\underline{C}H_2$O), 71.07 (C-5'), 70.54, 69.88 (CH_2=C$\underline{H}\underline{C}H_2$O and O$\underline{C}H_2\underline{C}H_2$), 69.03 (C-6'), 68.01 (C-2'), 66.65 (C-6), 51.43 (COO$\underline{C}H_3$), 34.12 ($\underline{C}H_2$COOCH$_3$), 29.72, 29.29, 29.25, 29.14, 26.15, 24.99 (aliphatic). Anal. calcd. for $C_{60}H_{74}O_{13}$: C 71.83, H 7.44; found: C 71.59; H 7.31.

8-Methoxycarbonyloctyl 6-O-[2-O-(3,4,6-tri-O-acetyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl)-3,4,6-tri-O-benzyl-α-D-mannopyranosyl]-3-O-allyl-2,4-di-O-benzyl-β-D-mannopyranoside (**4**)

To a solution of **28** (2.73 g; 2.73 mmol) in dry dichloromethane (75 mL) were added silver triflate (7.0 g; 27.25 mmol), sym-collidine (3.6 mL, 27.25 mmol), and 4 Å molecular sieves. To the resulting mixture, cooled to -50°C, was added dropwise a solution of 3,4,6-tri-O-acetyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl bromide[26] (**6**) (2.30 g; 2.60 mmol) in dry dichloromethane (25 mL). The above mixture was stirred at -50°C for 15 min and then allowed to warm to room temperature over a period of 1

h. The tlc of the mixture revealed the presence of unreacted alcohol **28** (~30%). The reaction mixture was again cooled to -50°C and a solution of the bromide **6** (2.30 g; 2.60 mmol) in dry dichloromethane (25 mL) was added dropwise. After stirring at -50°C for 15 min, the mixture was allowed to attain room temperature, with stirring, during 1 h. The tlc now showed the complete disappearance of the alcohol **28**. The mixture was diluted with dichloromethane, and then filtered through Celite. The filtrate was washed sequentially with ice water, ice cold 1N aqueous HCl, and saturated aqueous sodium bicarbonate. The organic phase was dried (Na_2SO_4), filtered, and evaporated. The residual oil was subjected to flash chromatography employing toluene-ethyl acetate (3.5:1) as eluent. The fractions containing the major product were combined and evaporated. The tlc of the oil so obtained, when developed in Skellysolve B-acetone (3:1), also indicated the presence of a minor product. Therefore, the above oil was again chromatographed using Skellysolve B-acetone (3:1) as eluent to provide the α-linked trisaccharide **29** as an oil (0.27 g; 7%). Further elution with the same solvent system gave the title compound as a syrup (2.92 g; 76%); $[\alpha]_D$ +23.04° (*c* 1.02, chloroform); R_f 0.45 (toluene-ethyl acetate, 3:1), 0.17 (Skellysolve B-acetone, 3:1); ^1H nmr ($CDCl_3$) δ: 7.86-7.04

(29H, aromatic), 5.901-5.790 (2H, Hc allyl and H-3" [δ5.815, $J_{3",4"}$ = 9.0 Hz, $J_{2",3"}$ = 11.0 Hz]), 5.573 (d, 1H, $J_{1",2"}$ = 8.5 Hz, H-1"), 5.303-5.143 (3H, H-4" [δ5.215, dd, $J_{4",5"}$ = 10.0 Hz, $J_{3",4"}$ = 9.0 Hz] and Ha and Hb allyl), 4.993 (d, 1H, J_{gem} = 13.0 Hz, $C_6H_5C\underline{H}HO$), 4.890-4.735 (4×d, 4H, J_{gem} = 11.0-12.5 Hz, $C_6H_5C\underline{H}_2O$), 4.676 (d, 1H, $J_{1',2'}$ = 1.5 Hz, H-1'), 4.538-4.459 (2H, H-2" [δ4.513, dd, $J_{2",3"}$ = 11.0 Hz, $J_{1",2"}$ = 8.5 Hz] and $C_6H_5C\underline{H}HO$ [δ4.475, d, J_{gem} = 12.0 Hz]), 4.400 (dd, 1H, $J_{2',3'}$ = 2.5 Hz, $J_{1',2'}$ = 2.5 Hz, H-2'), 4.373-4.303 (4H, H-1 [δ4.344], H-6a", and $C_6H_5C\underline{H}_2O$), 4.233 (dd, 1H, $J_{6a",6b"}$ = 12.5 Hz, $J_{5",6b"}$ = 2.0 Hz, H-6b"), 4.063-3.245 (20H, $OC\underline{H}HCH_2$ [δ4.035, td, J_{gem} = 9.0 Hz, J_{vic} = 6.0 Hz], OCH_3 [δ3.668, s], CH_2=CHC$\underline{H}_2$O, $C_6H_5C\underline{H}_2O$, H-2, H-3, H-4, H-5, H-6a, H-6b, H-3', H-4', H-5', H-6a', H-5", $OC\underline{H}HCH_2$), 2.989 (dd, 1H, $J_{6a',6b'}$ = 11.0 Hz, $J_{5',6b'}$ = 5.5 Hz, H-6b'), 2.31 (t, 2H, $C\underline{H}_2COOCH_3$, J = 7.5 Hz), 2.055 (s, 3H, $OCOCH_3$), 2.024 (s, 3H, $OCOCH_3$), 1.863 (s, 3H, $OCOCH_3$), 1.70-1.59 (4H, aliphatic), 1.44-1.26 (8H, remaining aliphatic); ^{13}C nmr ($CDCl_3$) δ: 173.96 ($\underline{C}OOCH_3$), 170.42, 169.89, 169.21 ($O\underline{C}OCH_3$ and phthalimido carbonyl), 138.69, 138.52, 138.36, 137.80 (benzyl quat. arom.), 134.55 (CH_2=$\underline{C}$HCH_2O), 133.74 (phthalimido tert. arom.), 131.52 (phthalimido quat. arom.), 128.31, 128.19, 128.00, 127.92, 127.86, 127.63, 127.47, 127.43, 127.25, 127.19, 126.99 (benzyl tert.

arom.), 123.16 (phthalimido tert. arom.), 116.52 ($\underline{C}H_2$=$CHCH_2O$), 101.79 (C-1, $J_{C-1,H-1}$ = 152.8 Hz), 97.44 (C-1', $J_{C-1',H-1'}$ = 169.2 Hz), 96.48 (C-1", $J_{C-1",H-1"}$ = 165.1 Hz), 82.64 (C-3), 76.81, 74.54, 74.09, 73.75, 73.49, 72.88, 71.89, 71.45, 70.62, 69.01 (C-2, C-4, C-5, C-2', C-3', C-4', C-5', C-3", C-4", C-5"), 74.64, 73.63, 72.41, 70.33 ($C_6H_5\underline{C}H_2O$), 70.11 (CH_2=$CH\underline{C}H_2O$ and $O\underline{C}H_2CH_2$), 69.40 (C-6'), 66.53 (C-6), 62.17 (C-6"), 54.25 (C-2"), 51.17 ($COO\underline{C}H_3$), 33.86 ($\underline{C}H_2COOCH_3$), 29.47, 29.10, 29.04, 28.90, 25.91, 24.74 (aliphatic), 20.56, 20.43, 20.24 ($OCO\underline{C}H_3$). Anal. calcd. for $C_{80}H_{93}N_1O_{22}$: C 67.64, H 6.60, N 0.99; found: C 67.54, H 6.58, N 0.97.

8-Methoxycarbonyloctyl 6-O-[2-O-3,4,6-tri-O-acetyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl)-3,4,6,-tri-O-benzyl-α-D-mannopyranosyl]-2,4-di-O-benzyl-β-D-mannopyranoside (31)

A solution of **4** (2.79 g; 1.97 mmol), tris(triphenylphosphine)rhodium(I) chloride (129 mg; 0.14 mmol), 1,8-diazabicyclo[2.2.2]octane (58 mg; 0.51 mmol) in ethanol-benzene-water (7:3:1; 100 mL) was heated at reflux for 24 h. The solvent was removed and the residue dissolved in acetone (100 mL) containing a trace amount of mercuric oxide. To this solution was added a solution of

mercuric chloride (3.0 g) in acetone-water (9:1; 50 mL), and the mixture was stirred at room temperature for 45 min. Following evaporation of the solvent, the residue was taken up in dichloromethane (250 mL). The dichloromethane solution was washed with 30% aqueous potassium bromide and water. The organic layer was dried (Na_2SO_4) and evaporated to give an oily residue which was purified by flash chromatography using toluene-ethyl acetate (3.5:1) as eluent. The title compound was obtained as a white foamy solid (2.31 g; 85%); $[\alpha]_D$ -19.07° (c 0.97, chloroform); R_f 0.37 (toluene-ethyl acetate, 3:1); ^1H nmr ($CDCl_3$) δ: 7.88-7.07 (29H, aromatic), 5.84 (dd, 1H, $J_{3",4"}$ = 9.0 Hz, $J_{2",3"}$ = 11.0 Hz, H-3"), 5.61 (d, 1H, $J_{1",2"}$ = 8.5 Hz, H-1"), 5.221 (dd, 1H, $J_{4",5"}$ = 9.0 Hz, $J_{3",4"}$ = 9.0 Hz, H-4"), 5.063 (d, 1H, J_{gem} = 12.0 Hz, $C_6H_5C\underline{H}HO$), 4.853 (d, 1H, J_{gem} = 11.0 Hz, $C_6H_5C\underline{H}HO$), 4.778-4.733 (2×d, 2H, J_{gem} = 11.0 and 12.0 Hz, $C_6H_5C\underline{H}_2O$), 4.673 (d, 1H, $J_{1',2'}$ = 2.0 Hz, H-1'), 4.633 (d, 1H, J_{gem} = 12.0 Hz, $C_6H_5C\underline{H}HO$), 4.543-4.476 (2H, H-2" [δ4.518, dd, $J_{2",3"}$ = 11.0 Hz, $J_{1",2"}$ = 8.5 Hz], $C_6H_5C\underline{H}HO$ [δ4.491, d, J_{gem} = 12.0 Hz]), 4.433 (1H, $J_{1,2}$ < 1 Hz, H-1), 4.385-4.240 (5H, H-2', H-6a", H-6b" and $C_6H_5C\underline{H}_2O$), 4.035-3.935 (4H, $OC\underline{H}HCH_2$, H-5", and $C_6H_5C\underline{H}_2O$), 3.828-3.245 (14H, H-2, H-3, H-4, H-5, H-6a, H-6b, H-3', H-4', H-5', H-6a', $OC\underline{H}HCH_2$ and OCH_3 [δ3.651, s]), 2.998 (dd, 1H,

$J_{6a',6b'}$ = 11.0 Hz, $J_{5',6b'}$ = 5.5 Hz, H-6b'), 2.380 (d, 1H, deuterium-exchangeable, $J_{OH,H-3}$ = 10.0 Hz, OH), 2.290 (t, 2H, $C\underline{H}_2COOCH_3$, J = 7.5 Hz), 2.056,2.050 (2×s, 6H, $OCOCH_3$), 1.860 (s, 3H, $OCOCH_3$), 1.68-1.58 (4H, aliphatic), 1.40-1.26 (8H, remaining aliphatic); ^{13}C nmr ($CDCl_3$) δ: 174.17 ($\underline{C}OOCH_3$), 170.63, 170.12, 169.40 ($O\underline{C}OCH_3$ and phthalimido carbonyl), 138.77, 138.50, 138.37, 138.30, 138.10 (benzyl quat. arom.), 133.95 (phthalimido tert. arom.), 131.82 (phthalimido quat. arom.), 128.56, 128.36, 128.23, 128.13, 128.08, 127.72, 127.62, 127.59, 127.39, 127.21 (benzyl tert. arom.), 123.37 (phthalimido tert. arom.), 101.96 (C-1), 97.25 (C-1'), 96.61 (C-1"), 77.90, 77.22, 76.27, 74.39, 74.34, 74.07, 73.22, 72.12, 71.61, 70.80, 69.14 (C-2, C-3, C-4, C-5, C-2', C-3', C-4', C-5', C-3", C-4" and C-5"), 75.06, 74.83, 74.44, 72.63, 70.49 ($C_6H_5\underline{C}H_2O$), 70.34 ($O\underline{C}H_2CH_2$), 69.50 (C-6'), 66.58 (C-6), 62.33 (C-6"), 54.44 (C-2"), 51.38 ($COO\underline{C}H_3$), 34.05 ($\underline{C}H_2COOCH_3$), 29.61, 29.24, 29.19, 29.08, 26.06, 24.92 (aliphatic), 20.74, 20.62, 20.44 ($OCO\underline{C}H_3$). Anal. calcd. for $C_{77}H_{89}N_1O_{22}$: C 66.99, H 6.50, N 1.01; found: C 66.74, H 6.43, N 0.84.

8-Methoxycarbonyloctyl 6-O-[2-O-(2-deoxy-2-phthalimido-β-D-glucopyranosyl)-3,4,6-tri-O-benzyl-α-D-mannopyranosyl]-2,4-di-O-benzyl-β-D-mannopyranoside (**32**)

To a solution of **31** (0.82 g; 0.59 mmol) in dry methanol (75 mL) was added a trace of solid sodium methoxide. The mixture was stirred at room temperature for 3 h. Neutralization of the mixture with Amberlite IR-120(H^+) resin followed by filtration and evaporation afforded **32** as a white foamy solid in a quantitative yield; $[\alpha]_D$ -31.54° (c 1.3, chloroform); R_f 0.2 (dichloromethane-methanol, 19:1); ^1H nmr ($CDCl_3$) δ: 7.66-7.06 (29H, aromatic), 5.399 (d, 1H, $J_{1",2"}$ = 8.0 Hz, H-1"), 5.035 (d, 1H, J_{gem} = 12.0 Hz, C_6H_5CHHO), 4.824-4.534 (6H, H-1' [δ 4.643] and $C_6H_5CH_2O$ [5×d, J_{gem} = 12.5-11.0 Hz]), 4.408-4.241 (6H, H-1 [δ 4.408], H-2' [δ 4.248, dd, $J_{2',3'}$ = 2 Hz, $J_{1',2'}$ = 2 Hz], H-2", H-3", and $C_6H_5CH_2O$ [J_{gem} = 11.0 Hz]), 4.020-3.208 (21H, H-2, H-3, H-4, H-5, H-6a, H-6b, H-3', H-4', H-5', H-6a', H-4", H-5", H-6a", H-6b", OCH_2CH_2, $C_6H_5CH_2O$ and OCH_3 [δ 3.620, s]), 3.063-2.900 (2H, H-6b' [δ 2.918, dd, $J_{6a',6b'}$ = 10.5 Hz, $J_{5',6b'}$ = 5.5 Hz] and OH [broad s, disappeared on D_2O exchange]), 2.396 (d, 1H, $J_{3,OH}$ = 10.0 Hz, deuterium-exchangeable, 3-OH), 2.269 (t, 2H, J = 8.0 Hz, CH_2COOCH_3), 1.65-1.55 (4H, aliphatic), 1.39-1.26 (remaining aliphatic); ^{13}C nmr ($CDCl_3$) δ:

174.43 ($\underline{C}OOCH_3$), 168.51 (phthalimido carbonyl), 138.68, 138.52, 138.35, 138.32, 138.05 (benzyl quat. arom.), 133.79 (phthalimido tert. arom.), 131.84 (phthalimido quat. arom.), 128.58, 128.38, 128.34, 128.26, 128.12, 128.03, 127.80, 127.70, 127.49, 127.44, 127.22 (benzyl tert. arom.), 123.23 (phthalimido tert. arom.), 101.90 (C-1), 97.49 (C-1'), 96.84 (C-1"), 77.69, 77.26, 76.31, 75.65, 74.28, 74.13, 73.38, 72.07, 71.73 (C-2, C-3, C-4, C-5, C-2', C-3', C-4', C-5', C-3", C-4", and C-5"), 74.87, 74.77, 74.48, 72.63, 70.86 ($C_6H_5\underline{C}H_2O$), 70.30 ($O\underline{C}H_2CH_2$), 69.70 (C-6'), 66.65 (C-6), 62.21 (C-6"), 56.54 (C-2"), 51.45 ($COO\underline{C}H_3$), 34.07 ($\underline{C}H_2COOCH_3$), 29.61, 29.17, 29.04, 26.04, 24.88 (aliphatic). Anal. calcd. for $C_{71}H_{83}N_1O_{19}$: C 67.98, H 6.67, N 1.12; found: C 67.43, H 6.60, N 1.10.

8-Methoxycarbonyloctyl 6-O-[2-O-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-3,4,6-tri-O-benzyl-α-D-mannopyranosyl]-2,4-di-O-benzyl-β-D-mannopyranoside (33).

Compound 32 (0.43 g; 0.34 mmol) in methanol (25 mL) was boiled with hydrazine hydrate (0.14 mL of an 85% solution; 2.6 mmol) for 12 h. The tlc showed the presence of the starting material in the reaction mixture. Therefore, more hydrazine hydrate (0.07 mL of an 85% solution, 1.3 mmol) was added and the mixture refluxed for

another 4 h. The solution was evaporated and the residue thoroughly dried to remove traces of hydrazine. The residue was then dissolved in methanol-water (1:1, 15 mL) and acetic anhydride (1 mL) was added. The resulting solution was stirred at room temperature for 2 h. Removal of solvent gave a white solid which was purified by flash chromatography using dichloromethane-methanol (12:1) as eluent. Since the crude product was not completely soluble in the eluent used, it was dissolved using more methanol in the same solvent mixture and then adsorbed on sodium sulfate which was poured onto the top of the silica packing. Pure **33** was obtained as a white foamy solid (0.24 g; 60%); $[\alpha]_D$ -20.46° (c 0.88, chloroform); R_f 0.61 (dichloromethane-methanol, 10:1); ^1H nmr ($CDCl_3$) δ: 7.42-7.16 (25H, aromatic), 5.90-5.65 (broad s, 1H, NH, deuterium-exchangeable), 5.053 (d, 1H, J_{gem} = 12.0 Hz, $C_6H_5C\underline{H}HO$), 4.913-4.825 (3H, $C_6H_5C\underline{H}_2O$ [2×d, J_{gem} = 11.0 Hz] and H-1' [δ4.910, d, $J_{1',2'}$ = 2.0 Hz]), 4.743 (d, 1H, J_{gem} = 12.0 Hz, $C_6H_5C\underline{H}HO$), 4.688-4.574 (3H, H-1" and $C_6H_5C\underline{H}_2O$ [2×d, J_{gem} = 12.0 Hz]), 4.488-4.325 (5H, H-1 [δ4.435] and $C_6H_5C\underline{H}_2O$ [4×d, J_{gem} = 12.0 and 11.0 Hz]), 4.253 (t, 1H, H-2'), 3.995-3.293 (23H, H-2, H-3, H-4, H-5, H-6a, H-6b, H-3', H-4', H-5', H-6a', H-6b', H-2", H-3", H-4", H-5", H-6a", H-6b", OH, $OC\underline{H}_2CH_2$, and OCH_3 [δ3.643, s]), 3.00-2.65 (broad s, 2H, disappeared on D_2O exchange, OH), 2.501

(d, 1H, $J_{3,OH}$ = 9.0 Hz, disappeared on D_2O exchange, 3-OH), 2.276 (t, 2H, J = 7.0 Hz, $\underline{CH_2}COOCH_3$), 1.840 (s, 3H, $NHCOCH_3$), 1.64-1.52 (4H, aliphatic), 1.36-1.22 (8H, remaining aliphatic); ^{13}C nmr ($CDCl_3$) δ: 174.10 ($\underline{C}OOCH_3$), 172.37 ($NH\underline{C}OCH_3$), 138.60, 138.39, 138.30, 137.93 (quat. arom.), 128.50, 128.34, 128.27, 128.17, 127.93, 127.73, 127.65, 127.58, 127.44 (tert. arom.), 101.90 (C-1'), 99.23 (C-1'), 97.66 (C-1"), 77.62, 76.25, 75.83, 74.58, 74.39, 74.13, 73.71, 73.46, 71.64 (C-2, C-3, C-4, C-5, C-2', C-3', C-4', C-5', C-3", C-4", and C-5"), 74.75, 74.45, 73.20, 71.50, 70.30 ($C_6H_5\underline{CH_2}O$), 68.85 (C-6'), 66.86 (C-6), 62.66 (C-6"), 59.07 (C-2"), 51.26 ($COO\underline{CH_3}$), 33.99 ($\underline{CH_2}COOCH_3$), 29.57, 29.11, 28.99, 25.99, 24.84 (aliphatic), 23.30 ($NHCO\underline{CH_3}$). Anal. calcd. for $C_{65}H_{83}N_1O_{18}$: C 66.94, H 7.17, N 1.20; found: C 66.81, H 7.09, N 1.13.

8-Methoxycarbonyloctyl 6-O-[2-O-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-α-D-mannopyranosyl]-β-D-mannopyranoside (2).

Compound **33** (45 mg; 0.03 mmol) was dissolved in 98% ethanol (9 mL), and 5% palladium-on-charcoal (45 mg) was added. The mixture was stirred under an atmosphere of hydrogen gas for 52 h. The catalyst was removed by

filtration and, after solvent evaporation, the residue was passed through a column of Bio-Gel P2 (2.5 cm × 47 cm) using 10% aqueous ethanol as eluent. The carbohydrate-containing fractions were pooled, concentrated, and lyophilized to provide 2 as a white powder (23.5 mg; 85%); $[\alpha]_D$ -19.34° (c 0.91, water); R_f 0.57 (dichloromethane-methanol-water, 10:6:1); ^1H nmr (D_2O) δ: 4.920 (1H, $J_{1',2'}$ = 1.8 Hz, H-1'), 4.664 (1H, $J_{1,2}$ < 0.7 Hz, H-1), 4.579 (1H, $J_{1'',2''}$ = 8.0 Hz, H-1"), 4.129 (dd, 1H, $J_{1',2'}$ = 1.4 Hz, $J_{2',3'}$ = 3.4 Hz, H-2'), 3.988-3.401 [22H, H-2 (δ3.981, $J_{2,3}$ = 3.0 Hz), H-3' (δ3.844, $J_{3',4'}$ = 9.5 Hz, $J_{2',3'}$ = 3.5 Hz), H-2" (δ3.706, $J_{1'',2''}$ = 8.0 Hz), H-3, H-4, H-5, H-6a, H-6b, H-4', H-5', H-6a', H-6b', H-3", H-4", H-5", H-5a", H-6b", $O\underline{CH_2}CH_2$, and OCH_3 (δ3.690)], 2.388 (t, 2H, J = 7.5 Hz, $\underline{CH_2}COOCH_3$), 2.056 (s, 3H, $NHCOCH_3$), 1.68-1.50 (4H, aliphatic), 1.38-1.22 (8H, remaining aliphatic). The above ^1H nmr assignments were confirmed by homonuclear decoupling; ^{13}C nmr (D_2O) δ: 178.58 ($NH\underline{C}OCH_3$), 175.59 ($\underline{C}OOCH_3$), 100.82 (C-1, $J_{C-1,H-1}$ = 159.4 Hz), 100.47 (C-1", $J_{C-1",H-1"}$ = 162.3 Hz), 97.72 (C-1', $J_{C-1',H-1'}$ = 169.5 Hz), 77.35, 76.75, 75.35, 74.27, 74.10, 73.73, 71.43, 70.92, 70.84, 70.54, 68.20, 67.63 (C-2, C-3, C-4, C-5, C-2', C-3', C-4', C-5', C-3", C-4", C-5", and $O\underline{CH_2}CH_2$), 66.99 (C-6), 62.43 (C-6"), 61.55 (C-6'), 56.31 (C-2"), 52.91 ($COO\underline{CH_3}$), 34.55 ($\underline{CH_2}COOCH_3$),

29.46, 29.05, 28.97, 28.93, 25.82, 25.11 (aliphatic), 23.21 ($NHCOCH_3$). Anal. calcd. for $C_{30}H_{53}N_1O_{18}$: C 50.34, H 7.46, N 1.96; found: C 49.27, H 7.26, N 1.73.

8-Methoxycarbonyloctyl 6-O-[2-O-(3,4,6-tri-O-acetyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl)-3,4,6-tri-O-benzyl-α-D-mannopyranosyl]-3-O-(2,3,4,6-tetra-O-acetyl-α-D-mannopyranosyl)-2,4-di-O-benzyl-β-D-mannopyranoside (34)

To a solution of the alcohol **31** (504 mg; 0.371 mmol) in dry acetonitrile (10 mL) containing 4Å molecular sieves were added sequentially mercuric bromide (1.416 g; 3.93 mmol) and mercuric cyanide (988 mg; 3.95 mmol). To the resulting mixture was added a solution of 2,3,4,6-tetra-O-acetyl-α-D-mannopyranosyl bromide[25] (**5**) (0.915 g; 2.23 mmol) in dry acetonitrile (5 mL) in five portions with an interval of 30 min between two additions, and the reaction mixture was stirred for 2 h. Evaporation of the solvent gave an oily residue which was extracted three times with dichloromethane. The organic extracts were combined and washed with saturated aqueous potassium chloride, saturated aqueous sodium bicarbonate, water, and brine. The dried organic layer (Na_2SO_4) was evaporated to give a foamy residue which was purified by flash chromatography using toluene-ethyl acetate (3:1) as eluent. The title

compound was obtained as a white foamy solid (0.405 g; 65%); $[\alpha]_D$ +4.41° (c 0.98, chloroform); R_f 0.25, (toluene-ethyl acetate, 3:1); ^1H nmr ($CDCl_3$) δ: 7.88-7.02 (29H, aromatic), 5.811 (dd, 1H, $J_{3'',4''}$ = 10.0 Hz, $J_{2'',3''}$ = 9.0 Hz, [redacted]), 5.574 (d, 1H, $J_{1'',2''}$ = 8.5 Hz, H-1''), 5.391-5.349 (2H, $J_{2',3'}$ = 3.0 Hz, H-2' and H-3'), 5.240-5.094 (4H, H-4', H-4'', $C_6H_5C\underline{H}HO$, and H-1 [δ5.094] $J_{3',4'}$ = $J_{4',5'}$ = 9.5 Hz; $J_{3'',4''}$ = $J_{4'',5''}$ = 9.5 Hz), 4.893-4.750 (3H, J_{gem} = 11.5 Hz and 13.0 Hz, 3×$C_6H_5C\underline{H}HO$), 4.696 (d, 1H, $J_{1'',2''}$ = 1.5 Hz, H-1''), 4.539-4.448 (2H, H-2'' [δ4.515, dd, $J_{1'',2''}$ = 8.5 Hz, $J_{2'',3''}$ = 10.5 Hz] and $C_6H_5C\underline{H}HO$ [J_{gem} = 11.5 Hz]), 4.440-4.284 (6H, H-1 [δ4.440], H-2' [δ4.375, dd, $J_{1'',2''}$ = 2.0 Hz, $J_{2'',3''}$ = 2.0 Hz], H-6a'', and 3×$C_6H_5C\underline{H}HO$ [J_{gem} = 11.0 and 11.5 Hz]), 4.121 (dd, 1H, $J_{6a'',6b''}$ = 12.0 Hz, $J_{5'',6b''}$ = 1.5 Hz, H-6b''), 4.098-3.235 (21H, H-2, H-3, H-4, H-5, H-6a, H-6b, H-5', H-6a', H-6b', H-3'', H-4'', H-5'', H-6a'', H-5'', $OC\underline{H}_2CH_2$, $C_6H_5C\underline{H}_2O$, OCH_3 [δ3.674]), 2.953 (dd, 1H, $J_{6a'',6b''}$ = 10.5 Hz, $J_{5'',6b''}$ = 6.0 Hz, H-6b''), 2.313 (t, 2H, J = 7.5 Hz, $C\underline{H}_2COOCH_3$), 2.059-2.000 (6s, 18H, $OCOCH_3$×6), 1.868 (s, 3H, $OCOCH_3$), 1.74-1.54 (4H, aliphatic), 1.46-1.20 (8H, remaining aliphatic); ^{13}C nmr ($CDCl_3$) δ: 174.11 ($\underline{C}OOCH_3$), 170.58, 170.21, 170.06, 169.65, 169.47, 169.44, 169.35 ($O\underline{C}OCH_3$ and phthalimido carbonyl), 138.75, 138.46, 137.73, 137.61 (phthalimido quat. arom.), 133.90 (phthalimido tert.

arom.), 128.82, 128.46, 128.36, 128.15, 128.08, 128.03, 127.87, 127.54, 127.47, 127.36, 127.28, 127.18 (benzyl tert. arom.), 123.31 (phthalimido tert. arom.), 101.95 (C-1, $J_{C-1,H-1}$ = 154.1 Hz), 99.83 (C-1', $J_{C-1',H-1'}$ = 177.8 Hz), 97.62 (C-1", $J_{C-1",H-1"}$ = 170.9 Hz), 96.60 (C-1'", $J_{C-1'",H-1'"}$ = 164.16 Hz), 81.25, 76.29, 74.78, 74.48, 74.17, 72.90, 72.03, 71.80, 70.73, 69.33, 69.15, 68.98, 68.93, 65.94 (C-2, C-3, C-4, C-5, C-2', C-3', C-4', C-5', C-2", C-3", C-4", C-5", C-3'", C-4'", and C-5'"), 74.68, 74.58, 73.71, 72.52, 70.43 ($C_6H_5\underline{C}H_2O$), 70.11 ($O\underline{C}H_2CH_2$), 69.62 (C-6"), 66.29 (C-6), 62.44, 62.32 (C-6' and C-6'"), 54.37 (C-2'"), 51.34 ($COO\underline{C}H_3$), 34.02 ($\underline{C}H_2COOCH_3$), 29.66, 29.27, 29.19, 29.07, 26.10, 24.89 (aliphatic), 20.69, 20.56, 20.40 ($OCO\underline{C}H_3$). Anal. calcd. for $C_{91}H_{107}N_1O_{31}$: C 63.89, H 6.30, N 0.82; found: C 63.45, H 6.31, N 0.82.

8-Methoxycarbonyloctyl 6-O-[2-O-(3,4,6-tri-O-acetyl-2-acetamido-2-deoxy-β-D-glucopyranosyl)-3,4,6-tri-O-benzyl-α-D-mannopyranosyl]-3-O-[2,3,4,6-tetra-O-acetyl-α-D-mannopyranosyl]-2,4-di-O-benzyl-β-D-mannopyranoside (35)

Compound **34** (260 mg; 0.15 mmol) was dissolved in dry methanol (15 mL) containing sodium methoxide. The resulting solution was stirred at room temperature for 45

min, and the reaction mixture was neutralized with Amberlite IR-120(H) resin. The resin was removed by filtration and the solvent evaporated to provide a foamy solid which was dissolved in methanol (10 mL). To this solution was added hydrazine hydrate (0.4 mL of an 85% solution, 7.43 mmol) and the mixture was refluxed for 1 h. Removal of solvent gave a white residue. Traces of hydrazine were removed by evaporation of methanol from the product (twice), which was further dried on the vacuum pump. The product was then dissolved in pyridine (3.5 mL) and acetic anhydride (3.5 mL) and stirred overnight at room temperature. Excess acetic anhydride was decomposed by dropwise addition of ethanol to the reaction mixture at 0°C, to which was then added dichloromethane and water. The aqueous layer was separated and back extracted with more dichloromethane and the combined dichloromethane layers were washed with 1 M aqueous HCl and saturated aqueous sodium bicarbonate. The organic phase was dried (Na_2SO_4), filtered, and evaporated to give a foamy solid which was purified by flash chromatography using Skellysolve B-ethyl acetate-ethanol (20:20:1) as eluent. Pure **35** was obtained as a white foamy solid (154 mg; 62%); $[\alpha]_D$ +11.23 (c 1.18, chloroform); R_f 0.3 (Skellysolve B-ethyl acetate-ethanol, 20:20:1); ^1H nmr ($CDCl_3$) δ: 7.70-7.12 (25H, aromatic), 5.613-5.523 (2H, H-3'' [δ5.588] and

THE UNIVERSITY OF ALBERTA

SYNTHETIC ACCEPTORS FOR GLYCOSYLTRANSFERASES

by

SYED HASAN TAHIR

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SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled SYNTHETIC ACCEPTORS FOR GLYCOSYLTRANSFERASES

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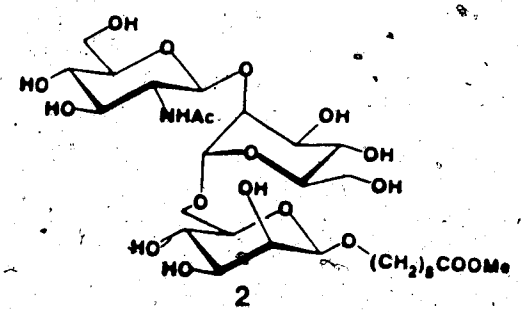
ABSTRACT

Oncogenic transformation frequently results in the production of abnormal cell-surface carbohydrates, known as "tumor-associated carbohydrates", and it has been shown that these carbohydrate structures can result from a change in the enzymatic activity of a single glycosyltransferase.

This work was aimed at developing a selective assay for monitoring changes in the intracellular activity of such a known tumor-elevated glycosyltransferase, termed N-acetylglucosaminyl transferase V (GlcNAc transferase V), by using appropriately designed synthetic oligosaccharide substrates. To this end, the trisaccharide β-D-GlcNAc(1→2)α-D-Man(1→6)β-D-Man-O$(CH_2)_8COOCH_3$ (**2**) and the related tetrasaccharide β-D-GlcNAc(1→2)-α-D-Man(1→6){α-D-Man(1→3)}-β-D-Man-O$(CH_2)_8COOCH_3$ (**3**) have been chemically synthesized as potential selective acceptors for this enzyme.



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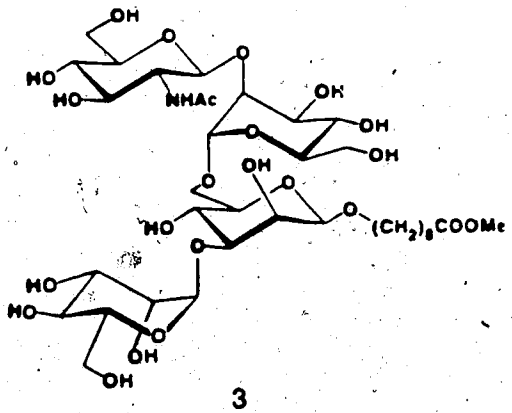

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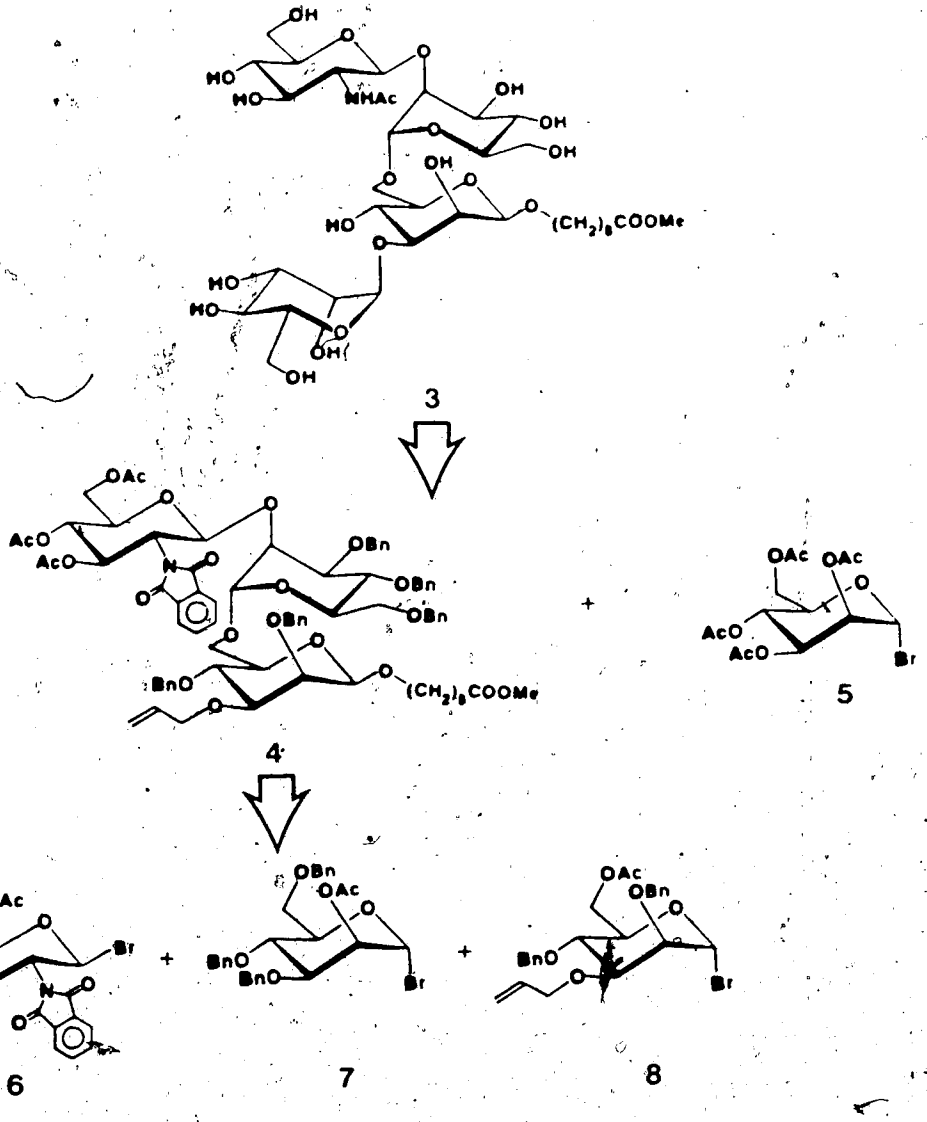
The multi-step syntheses to prepare the oligosaccharides 2 and 3 involved sequential Koenigs Knorr glycosylations of selectively protected carbohydrate derivatives and are summarized in the retrosynthetic scheme shown below:



The trisaccharide 2 was found to be a selective acceptor for GlcNAc transferase V.

ACKNOWLEDGEMENTS

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CHAPTER 1

INTRODUCTION

The cell-surface carbohydrates of eukaryotic cells are made up of the sugar chains of glycoproteins and glycolipids and the more loosely associated collagen, heparan sulfate and fibronectin. The covalently attached complex carbohydrates of glycoproteins and glycolipids are usually between two and fifteen sugar units long and typically make up 2-10% of the plasma membrane weight.[1] The carbohydrate chains of these latter glycoconjugates project outward from the cell and, despite their relatively low abundance, it is believed[1] that they almost entirely cover the cell surface and thus form the first layer of interaction with other cells.

Many of the complex carbohydrates appear to have physicochemical functions such as modifying solubility, stabilizing protein conformation and protecting glycoproteins against proteolysis.[2] Current research in the biological sciences is, however, focusing on the possible role of these highly diverse structures as recognition markers which may direct phenomena as diverse as the binding of hormones, toxins, enzymes, viruses and

bacteria to the cell and as "traffic signals" which control the circulation of both glycoproteins and whole cells.[3] Although much of the evidence to support the role of complex carbohydrates in mediating these diverse processes is still largely circumstantial, a handful of structures have been assigned definitive physiological functions. These critical functions include that of β-D-galactose as a recognition marker in the hepatic clearance of serum glycoproteins[4] and of mannose-6-phosphate residues in the targeting of newly-biosynthesized glycoproteins to the lysosomes.[5]

About ten years ago, evidence began to accumulate that the carbohydrate structures of both cell-surface glycoproteins and glycolipids became dramatically altered during both normal and abnormal cellular development.[6-8] Consistent changes in cell-surface carbohydrate structures have now been shown to accompany the development of human melanoma, neuroblastoma and colorectal, gastric and pancreatic carcinoma.[9] The occurrence of large fucosylated highly-branched glycopeptides is in fact one of the most reproducible correlates with the malignant transformation of cells.[10] The functional significance, if any, of these cell-surface structural changes is not at all clear but these aberrant carbohydrate structures are attracting a great deal of clinical interest as potential

tumor markers. The structures of many of these "tumor-associated" carbohydrates have been elucidated in recent years and major research efforts have gone into the production of monoclonal antibodies against many of these structures.[8,11] One of these monoclonal antibodies, termed CA19-9, is already in wide clinical use as a prognostic monitor for colorectal cancer.[12,13]

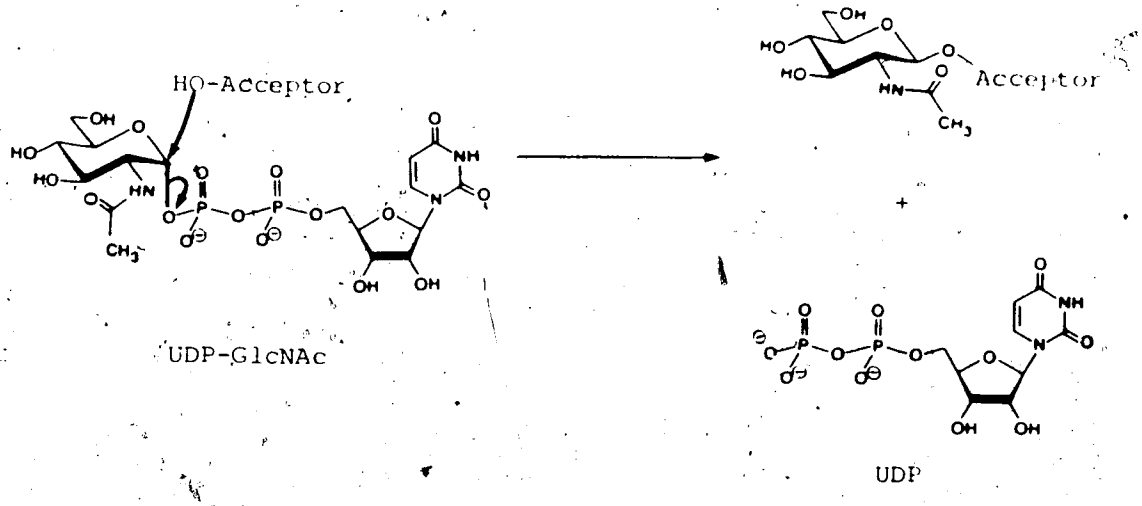
Tumor-associated oligosaccharides are the manifestation of altered carbohydrate biosynthesis and could, in principle, result from any of a large number of cellular irregularities. Glycosylation of proteins is a co- or post-translational modification which requires the sequential action of a series of enzymes (glycosyltransferases) and co-factors (sugar nucleotides) and occurs in the endoplastic reticulum and golgi vesicles.[14] A change in the cellular levels of the glycosyltransferases, the sugar nucleotides or their transport proteins, the biosynthetic precursors of the sugar nucleotides, glycosylhydrolases, or even mild disruptions of the membrane integrities of intracellular organelles could account for the observed changes in cell-surface carbohydrate structures. Because of this tremendous complexity the correlation of the expression of tumor-associated cell-surface carbohydrates with a single transformation-induced molecular event has until very

recently remained elusive.

In 1984, Yamashita et al.[10] compared the carbohydrate structures of the membrane N-linked glycoproteins of baby hamster kidney (BHK) cells and their polyoma transformant (Py-BHK). They found that while the transformed cells produced the same approximately twenty structures as did the normal cells, they produced more of the larger more highly branched oligosaccharides. They proposed that the changes in the relative proportion of the cell-surface oligosaccharides observed on transformation could be explained by the elevation in the activity of a single enzyme, a glycosyltransferase, termed N-Acetyl-β-D-glucosaminyl transferase V (GlcNAc transferase V, GnT V). They subsequently validated this proposal in 1985[15] when they showed that the GnT V activity in Py-BHK cells was in fact elevated two-fold when compared with untransformed cells. This elegant work provided the first demonstration that the changes in cell surface carbohydrates observed on oncogenic transformation could result from a change in the activity of a single glycosyltransferase. Yamashita et al.'s work[10,15] therefore suggests that the activity of a single glycosyltransferase can, in itself, serve as a tumor marker. The detection of a change in a single specific enzymatic activity should be far simpler than the characterization and quantification

of a highly heterogeneous mixture of cell-surface carbohydrate structures produced as a result of this single enzymatic change.

This thesis is concerned with the development of a rapid and convenient assay for measuring the activity of glycosyltransferases, in particular of Yamashita et al.'s elevated GnT V. Glycosyltransferases catalyze the transfer of a glycosyl residue, usually from a sugar nucleotide (termed the donor), to the hydroxyl group of another sugar (termed the acceptor). The sequential action of a large number of such glycosyltransferases is required for the synthesis of complex oligosaccharides. The chemical reaction catalyzed by GlcNAc transferases is the transfer of an N-acetylglucosaminyl residue from uridine-diphospho-N-acetylglucosamine (UDP-GlcNAc) to a hydroxyl group of some acceptor sugar:



The products of the reaction are uridine-diphosphate (UDP) and a β-linked di- or oligosaccharide.

At least 9 different GlcNAc transferases are known to be involved in the biosynthesis of the asparagine-linked oligosaccharides.[3,10,15,16] All of these enzymes use UDP-GlcNAc as the glycosyldonor and the difference between them lies in their specificity for different acceptor structures. A composite structure of the core region of known asparagine-linked oligosaccharides is shown in Figure 1. The oligosaccharides are all attached to protein asparagine residues (N-linked) through a common chitobiose linkage, β D GlcNAc(1→4)β D GlcNAc-Asn. The GlcNAc transferases responsible for generating the diversity of structures observed on cell surface N-linked glycoproteins are labelled GnT I, II, III, IV and V and the GlcNAc residues they transfer are labelled in the same fashion.[15] In the naturally occurring structures other sugar residues, notably D-galactose and L-fucose, are added onto these GlcNAc residues to produce the completed structures. GnT's I-V transfer β D GlcNAc to D-mannose residues, but to different hydroxyl groups of the three different mannose residues. These enzymes are all located in the rough endoplasmic reticulum and in the golgi apparatus of cells where they frequently compete for common glycoprotein substrates.

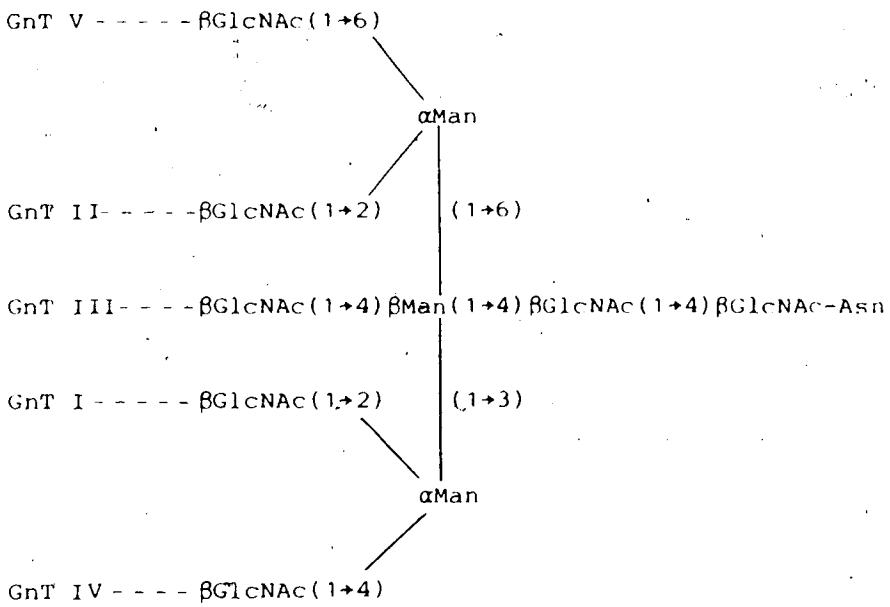


Figure 1. A hypothetical structure showing the βDGlcNAc units added by GlcNAc-transferases I-V (GnT I-V).[15]

A portion of the biosynthetic pathway elucidated by Yamashita *et al.* for the production of BHK cell-surface glycoproteins is shown in Figure 2. This biosynthetic scheme, reproduced from Yamashita *et al.*,[15] shows how the product of one glycosyltransferase reaction can frequently be a substrate for several competing glycosyltransferases. The elevation of GnT V in Py-BHK cells results in a shunt in the normal biosynthetic branching indicated by the bold face arrows with the resultant increase in the larger, more highly branched oligosaccharide structures. GnT V transfers β<u>D</u>GlcNAc exclusively to the 6-hydroxyl group of the α(1→6) linked <u>D</u>-mannose unit of the glycopeptide.

Assaying the activity of these various glycosyltransferases, which are present in only minute amounts, invariably involves the measurement of the transfer of a radiolabelled GlcNAc residue from ^{14}C or ^{3}H-UDP-GlcNAc to a suitable acceptor oligosaccharide, followed by isolation and counting of the product.[15,16] The difficulty encountered with assaying the GnT's is that they all use the same sugar nucleotide and several of them may act on any given substrate. As seen in Figure 2, in the biosynthesis of N-linked glycopeptides, GnT V acts on hepta- and octasaccharidic substrates. In their landmark work,[15] Yamashita *et al.* isolated their substrates from

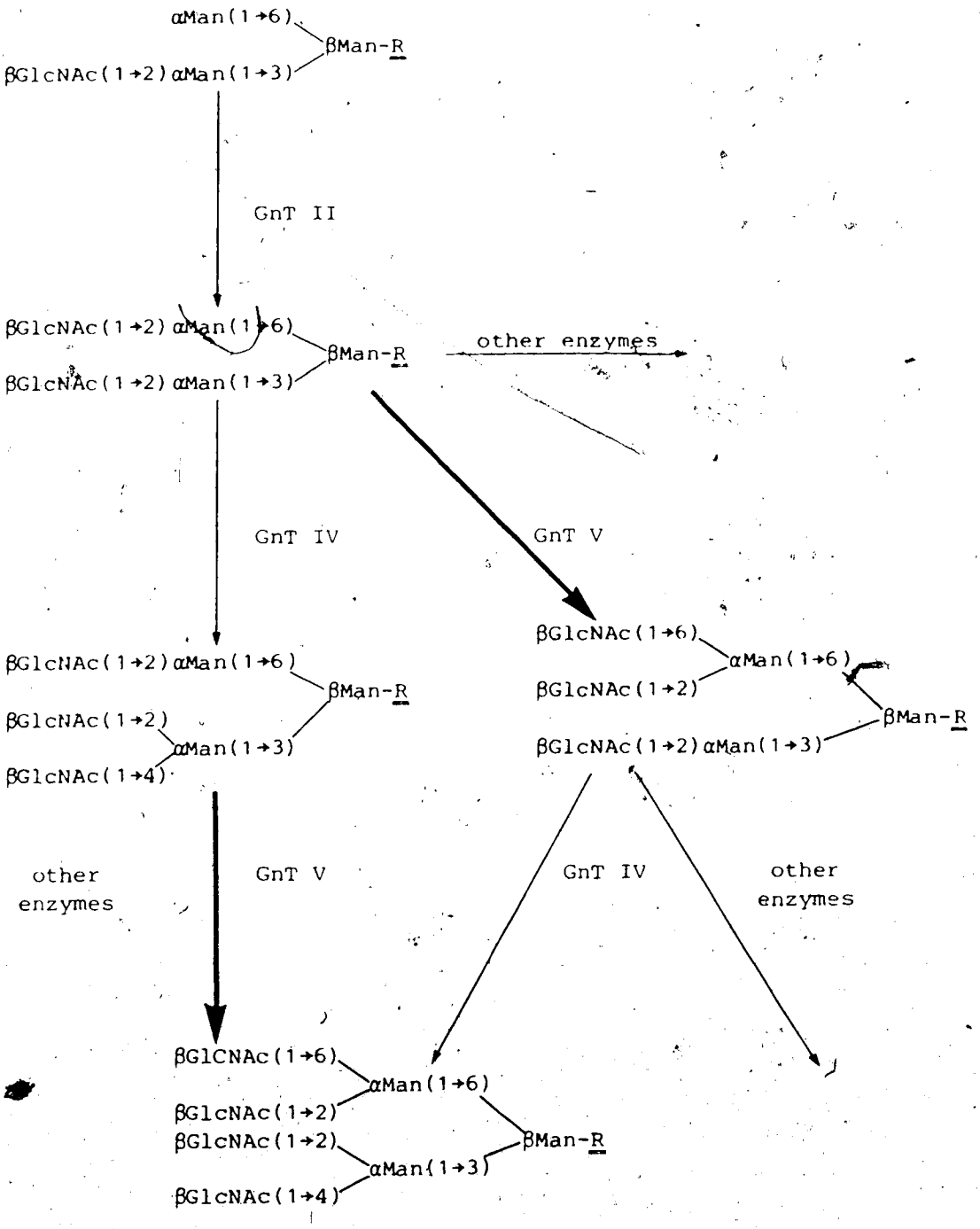


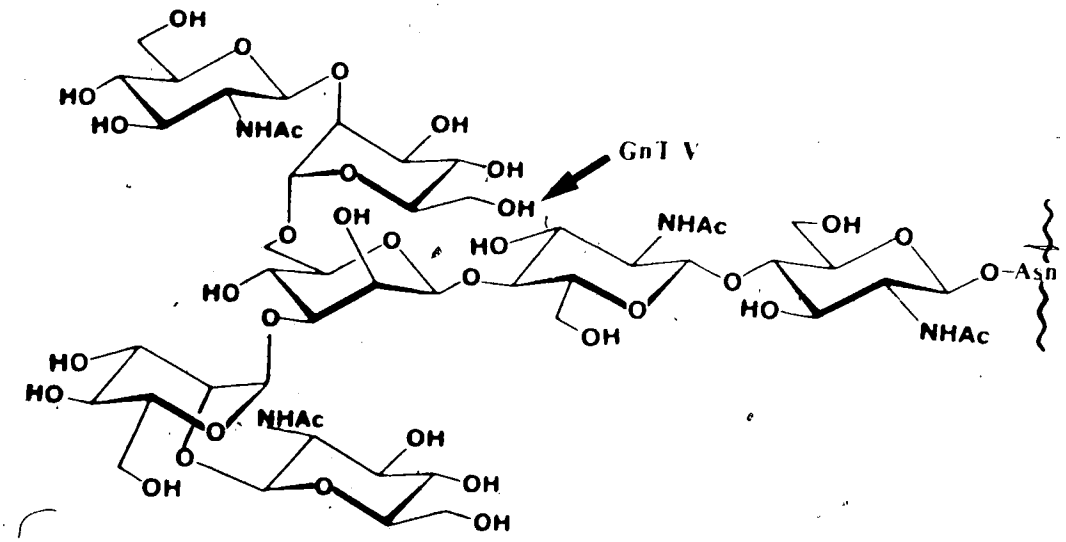
Figure 2. Partial pathway showing the involvement of GlcNAc-transferase V (GnT V) in the biosynthesis of complex sugar chains.[15] R represents βDGlcNAc(1→4)βDGlcNAc-Asn.

the urine of patients with metabolic disorders (mannosidosis, fucosidosis and GM_1-gangliosidosis) and frequently had to further process these structures by removal of outer sugar residues with specific glycosyl hydrolases. Such procedures are not only labor intensive and time consuming but are clearly not general. The amount of material that may be obtained is also limited. Yamashita et al.'s separation of the radioactive products of the enzymatic reactions required high-voltage paper electrophoresis, gel-permeation chromatography, treatment with glycosyl hydrolases and re-chromatography. Both the difficulty and impractability of such glycosyltransferase assays are considered a major obstacle in the study of glycosyltransferase tumor markers.

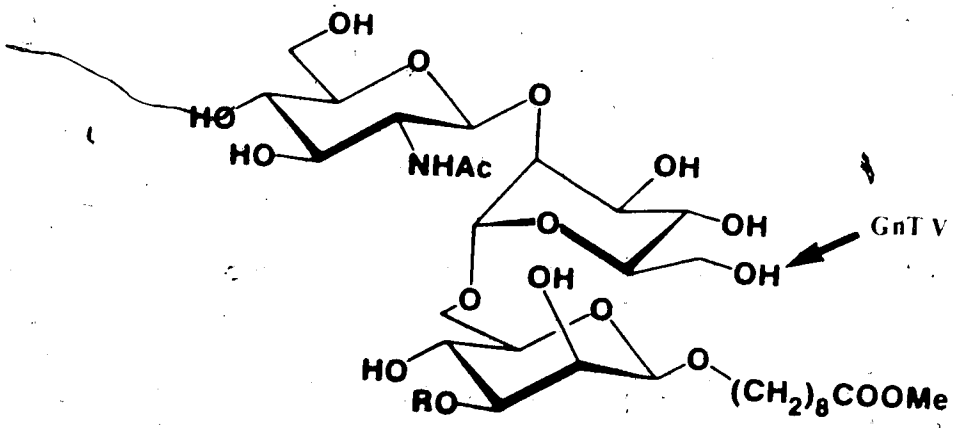
Our approach to this problem was to chemically synthesize oligosaccharides which would, we hoped, be recognized by only a single enzyme thus producing a substrate which could be used in an assay specific for that single glycosyltransferase activity. Such substrates might include oligosaccharides where cross-reacting sugar residues were completely absent or where the interfering hydroxyl groups which might be acted on by other GlcNAc transferases were masked by either O-methylation or deoxygenation. This latter approach has recently[17,18] been successfully applied in the differentiation of two

competing α-L-fucosyltransferases in serum. To be truly useful, such substrates and their glycosylated products should also be amenable to simple rapid isolation from the incubation mixtures of cell extracts or fluids which contain the glycosyltransferase activities being assayed.

In order to test the practicability of this approach we chose to attempt the preparation of substrates selective for Yamashita et al.'s GnT V since this glycosyltransferase was already attracting wide interest as a potential tumor marker. The smallest known substrate for GnT V is the heptasaccharide 1, shown in Figure 3, which was isolated and used by Yamashita et al.[15] as described above. Recent work,[19-23] from the laboratory of Lemieux and our own, on the molecular basis for the binding of oligosaccharides by monoclonal antibodies and lectins strongly suggested that proteins were not likely to require carbohydrate surfaces much larger than that of a trisaccharide for faithful recognition. We expected that this situation might also hold true for the enzymatic specificity of glycosyltransferases and therefore envisioned the trisaccharide βDGlcNAc(1→2)αDMan(1→6)βDManOR (**2**) as a likely acceptor for GnT V. Our rationale for the choice of **2** can be seen in Figure 3 where the structure of the natural heptasaccharide acceptor 1 is also shown. We set forth,



1: βGlcNAc(1→2)αMan(1→6)
βGlcNAc(1→2)αMan(1→3) → βMan(1→4)βGlcNAc(1→4)βGlcNAc-Asn



2: R=H, βGlcNAc(1→2)αMan(1→6) → βMan-O(CH$_2$)$_8$COOMe

3: R=αMan, βGlcNAc(1→2)αMan(1→6)
αMan(1→3) → βMan-O(CH$_2$)$_8$COOMe

Figure 3. A heptasaccharide acceptor (1) for GlcNAc-transferase V (GnT V) compared with the structures of the proposed synthetic acceptors 2 and 3. The bold arrows show the primary hydroxyl group to which GnT V transfers a N-acetyl-β-D-glucosaminyl residue.

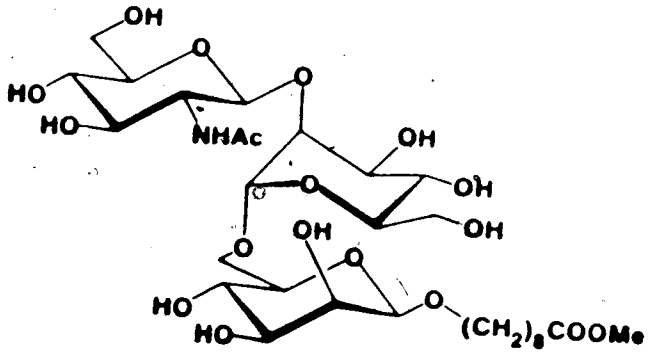
therefore, to synthesize 2, which contains the reactive target hydroxyl group acted on by the enzyme and one sugar residue on either side of the α-D-mannose residue bearing this hydroxyl group. Since we did not know whether this structure incorporated sufficient features for recognition by GnT V, the synthesis was planned in a way that also allowed the preparation of the tetrasaccharide 3 which is closer in structure to the natural acceptor 1. In order to facilitate the isolation of our glycosylated substrates from their enzymatic incubation mixtures, we elected to prepare 2 and 3 as their 8-methoxycarbonyloctyl glycosides[24] since inclusion of this hydrophobic group should facilitate adsorption on reverse phase (C-18) chromatography supports. This "linking arm" might also eventually be used for the attachment of fluorescent, radioactive or enzymatic tags to these potential acceptors to help localize the corresponding enzyme in intracellular organelles or to prepare affinity columns to assist in enzyme isolations.

CHAPTER 2

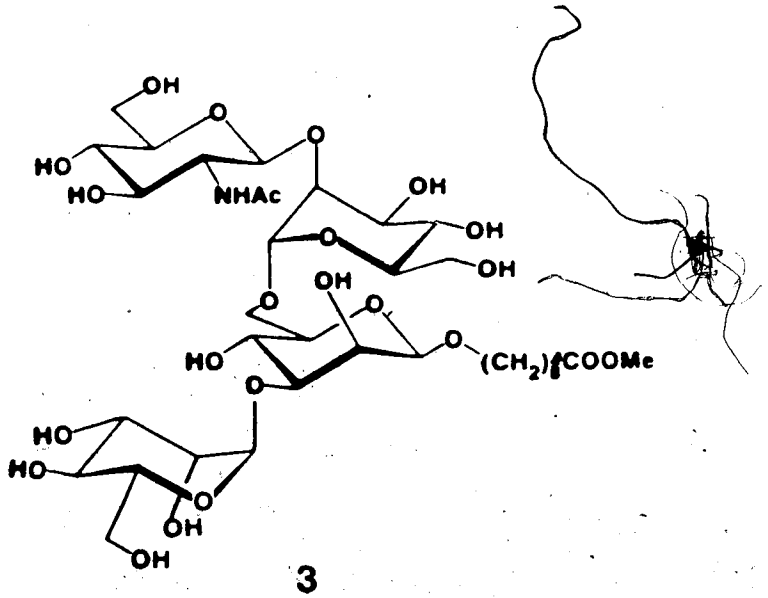
RESULTS AND DISCUSSION

The goal of this thesis project was to synthesize two oligosaccharides, namely 8-methoxycarbonyloctyl 6-O-[2-O-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-α-D-mannopyranosyl]-β-D-mannopyranoside (**2**) and 8-methoxycarbonyloctyl 6-O-[2-O-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-α-D-mannopyranosyl]-3-O-(α-D-mannopyranosyl)-β-D-mannopyranoside (**3**). In order to increase the potential usefulness of the oligosaccharides 2 and 3 we elected to synthesize these structures covalently attached to a linking arm which would allow their subsequent attachment to proteins, solid supports, and fluorescent or radioactive tags. These glycoconjugates might then be used in the intracellular localization of the N-acetylglucosaminyl transferases as well as in their purification.

A retrosynthetic analysis of the target structures 2 and 3 suggested, as the key intermediate, the trisaccharide precursor **4**, which was protected in a manner to allow the selective liberation of the hydroxyl group at C-3 of the β-D-mannopyranosyl residue for subsequent

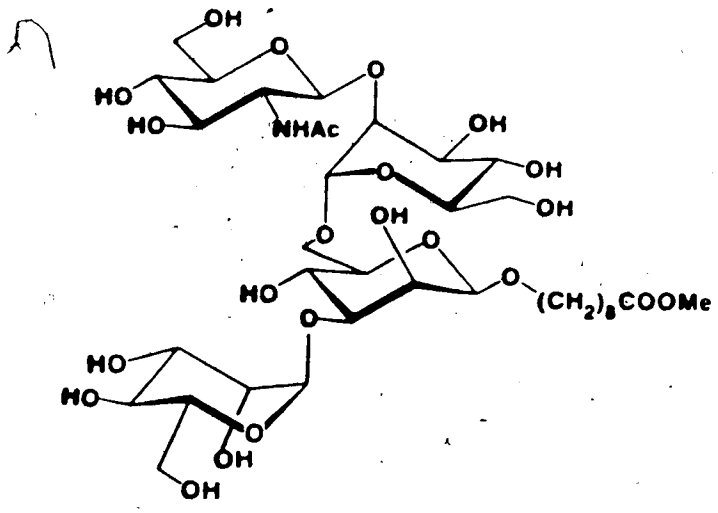


2



3

glycosylation by the α-D-mannopyranosyl donor **5**. The key intermediate **4** was, in turn, retrosynthesized into monohexosyl synthons 6, 7, and 8. The two monosaccharide synthons, 6 and 7, were designed to function as 2-deoxy-2-phthalimido-β-D-glucopyranosyl and α-D-mannopyranosyl



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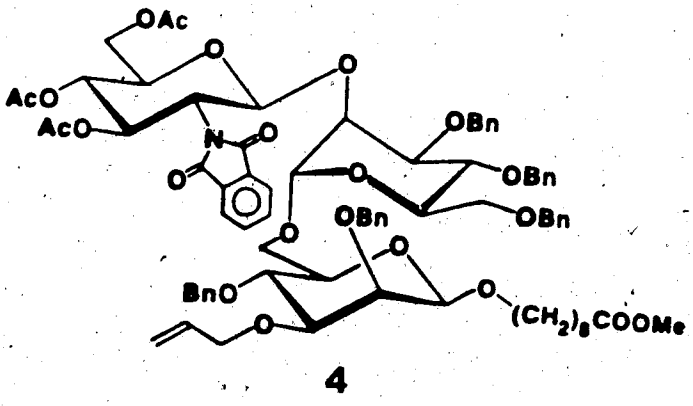

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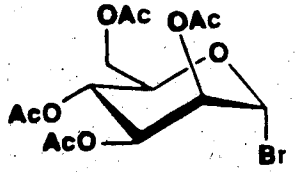
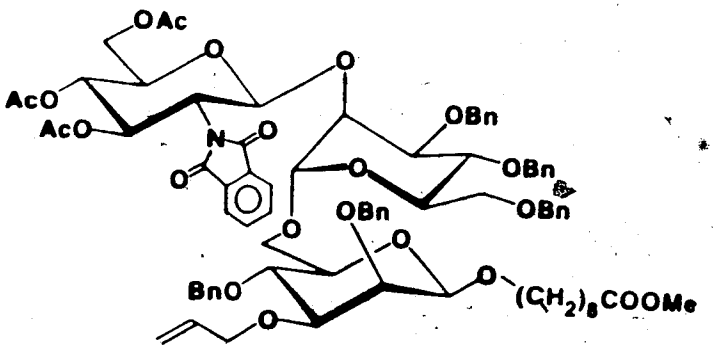


5

donors respectively. The synthon **8** was properly protected to perform dual functions: first acting as a β-D-manno-pyranosyl donor and then, after selective removal of acetyl group at O-6, as a glycosyl acceptor.



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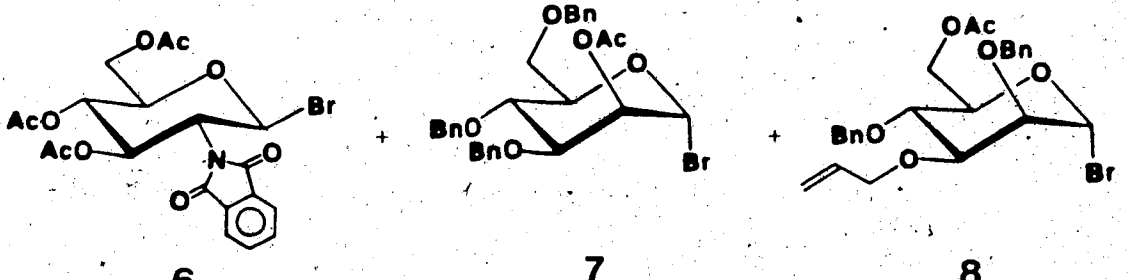


6 7 8

Of the four monosaccharide synthons (**5**,[25] **6**,[26] **7**,[27] and **8**) thus required, three, namely synthons **5**, **6**, and **7** had already been prepared. Therefore, a synthetic route towards the monosaccharide synthon **8** was first undertaken.

Methyl α-D-mannopyranoside (**9**) was converted to its 4,6-O-benzylidene derivative **10** in 25% yield on treatment with benzaldehyde in the presence of formic acid. This procedure gave only a modest yield, as reported,[28] so our attention was drawn to a recently published method,[29] which involved the reaction of **9** in N,N-dimethylformamide with benzaldehyde dimethylacetal[30] in the presence of tetrafluoroboric acid. This improved procedure provided a 50% yield of the benzylidene derivative **10** and allowed its preparation in sufficient quantities (60 g) to proceed with the preparation of **8**.

Selective 3-O-allylation of **10** was effected by the procedure of Nashed,[31] which involved its reaction with one equivalent of dibutyltin oxide to provide presumably 4,6-O-benzylidene-2,3-O-dibutylstannylene-α-D-mannopyranoside (**11**). Compound **11** was not isolated but was treated with allyl bromide in N,N-dimethylformamide at 100°C to give methyl 3-O-allyl-4,6-O-benzylidene-α-D-mannopyranoside (**12**) in 82% yield. Although **12** appeared

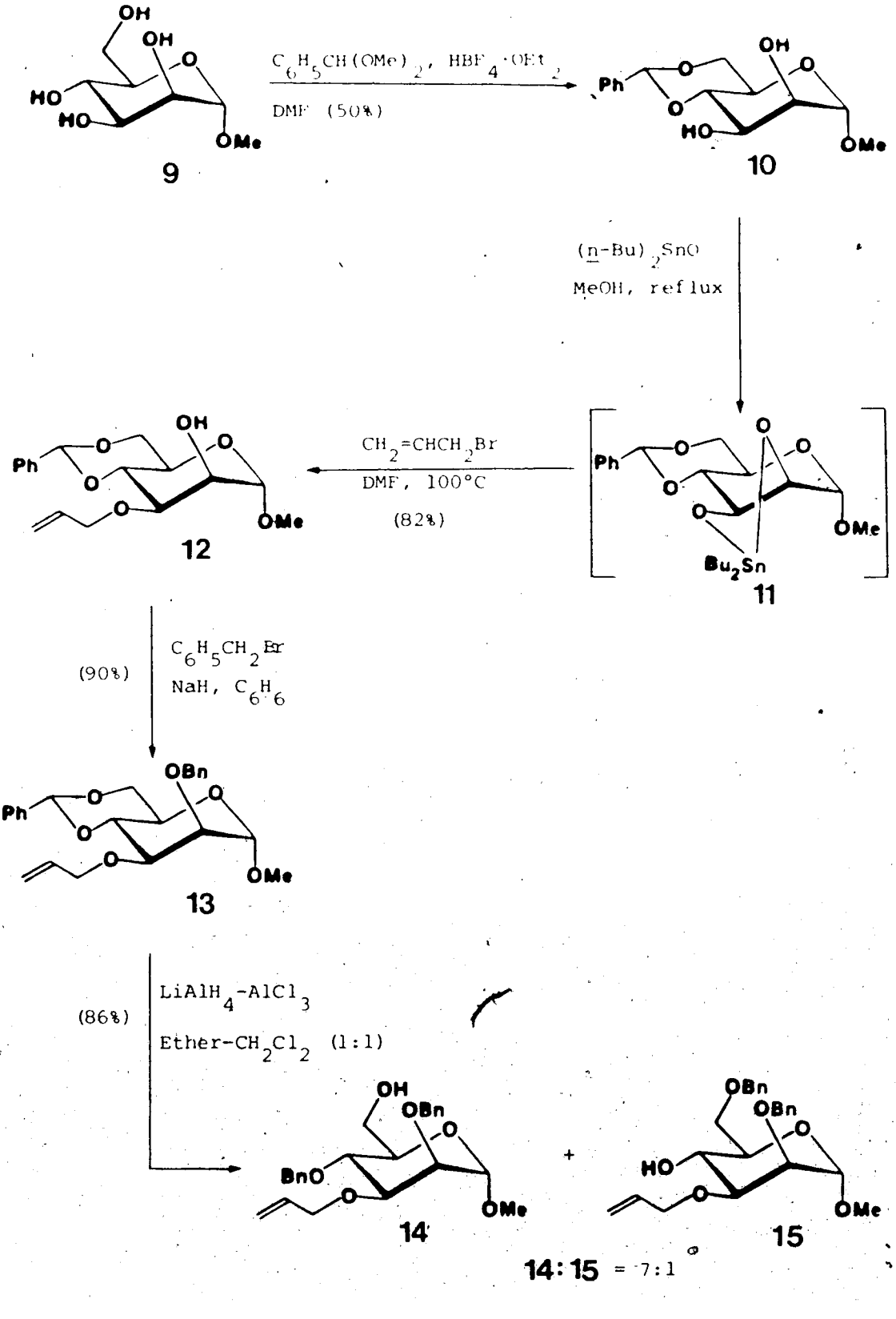
homogeneous by thin layer chromatography (tlc), its optical rotation, $[\alpha]_D^{22}$ + 59.4° (c 1.2, chloroform) differed somewhat from the value reported by Nashed: +63.5° (c 1.5, chloroform). The 400 MHz ^{1}H nmr spectrum, however, showed **12** to be a pure single isomer whose identity could readily be ascertained. The signal for the hydroxylic proton appeared as a doublet ($J_{2,OH}$ = 1.5 Hz) at δ3.045, causing H-2 to produce a broad signal at δ4.033 which collapsed to a doublet of doublets ($J_{2,3}$ = 3.5 Hz and $J_{1,2}$ = 1.5 Hz) on deuterium exchange. The position of free hydroxyl group in **12** was further confirmed by its *in situ* derivatization using trichloroacetyl isocyanate.[32] The ^{1}H nmr spectrum recorded following the addition of trichloroacetyl isocyanate displayed the expected downfield shifted doublet of doublets ($J_{2,3}$ = 3.0 Hz and $J_{1,2}$ = 1.5 Hz) at δ5.349.

Treatment of **12** with benzyl bromide and sodium hydride in benzene[31] furnished the 2-*O*-benzyl derivative **13** in 90% yield. The presence of a benzyl group in **13** was confirmed by the appearance, in the ^{1}H nmr spectrum, of additional signals integrating for five protons in the aromatic region. The signals for the now diastereotopic benzylic protons appeared at δ4.843 and 4.725, each as a one proton AB doublet with a geminal coupling constant of

12.0 Hz.

The reductive cleavage of the benzylidene group in **13** was achieved by refluxing with $LiAlH_4$-$AlCl_3$ in ether dichloromethane (1:1), according to Liptak et al.,[33] to give the 4-O-benzyl (**14**) and 6-O-benzyl (**15**) derivatives, in the ratio of 8:1, in a combined yield of 86%. The structures of the hydrogenolysis products **14** and **15** were assigned on the basis of ^1H nmr data. The ^1H nmr spectrum of the major product **14** showed a doublet of doublets for OH-6 at δ2.046 ($J_{6,OH}$ = 7.0 Hz and $J_{6',OH}$ = 5.5 Hz) as well as a clear signal for one of the H-6's at δ3.840 (ddd, $J_{6,6'}$ = 12.0 Hz, $J_{6,OH}$ = 7.0 Hz, and $J_{5,6}$ = 3.0 Hz). On D_2O exchange, the signal corresponding to OH-6 disappeared and, as expected, the signal for this H-6 simplified to a doublet of doublets, the coupling with hydroxylic proton being absent. The ^1H nmr spectrum of 6-O-benzyl derivative **14**, on the other hand, displayed a doublet for hydroxylic proton at δ2.578 ($J_{4,OH}$ = 2.0 Hz) and a doublet of doublet of doublets for H-4 at δ4.004 ($J_{4,5}$ = 9.0 Hz, $J_{3,4}$ = 9.0 Hz, and $J_{4,OH}$ = 2.0 Hz). After D_2O addition, the 1H nmr spectrum of **14** showed the disappearance of the OH resonance and the simplification of the signal for H-4 to a doublet of doublets.

The 13C nmr data were also in accord with the


OH
OH
HO
HO
OMe
9
$C_6H_5CH(OMe)_2$, $HBF_4 \cdot OEt_2$
DMF (50%)
Ph
OH
HO
OMe
10
$(n\text{-Bu})_2SnO$
MeOH, reflux
$CH_2=CHCH_2Br$
DMF, 100°C
(82%)
Ph
OMe
Bu_2Sn
11
Ph
OH
OMe
12
(90%)
$C_6H_5CH_2Br$
NaH, C_6H_6
OBn
Ph
OMe
13
(86%)
$LiAlH_4$-$AlCl_3$
Ether-CH_2Cl_2 (1:1)
OH
OBn
BnO
OMe
14
+
OBn
OBn
HO
OMe
15
14:15 = 7:1

structures assigned for **14** and **15**. The well established empirical rule[34] that alkoxylated carbons (ROC) are deshielded by 5-10 ppm compared with the corresponding hydroxylated carbon atoms (HOC) permits a simple verification of the position of the benzyl ethers in **14** and **15**. The major product **14** showed the signal for C-6 at δ62.43 ppm, the normal position for the underivatized hydroxymethyl group carbons of pyranose rings.[34] In **15**, however, this methylene carbon appeared at δ70.66 ppm, confirming the presence of the 6-O-benzyl ether.

Reaction of the 4-O-benzyl compound **14** with acetic anhydride in the presence of pyridine gave the 6-O-acetyl derivative **16** in 98% yield. The downfield shift of the H-6 resonances in the ^{1}H nmr spectrum of **16** due to acetylation of O-6 supported the assigned structure.

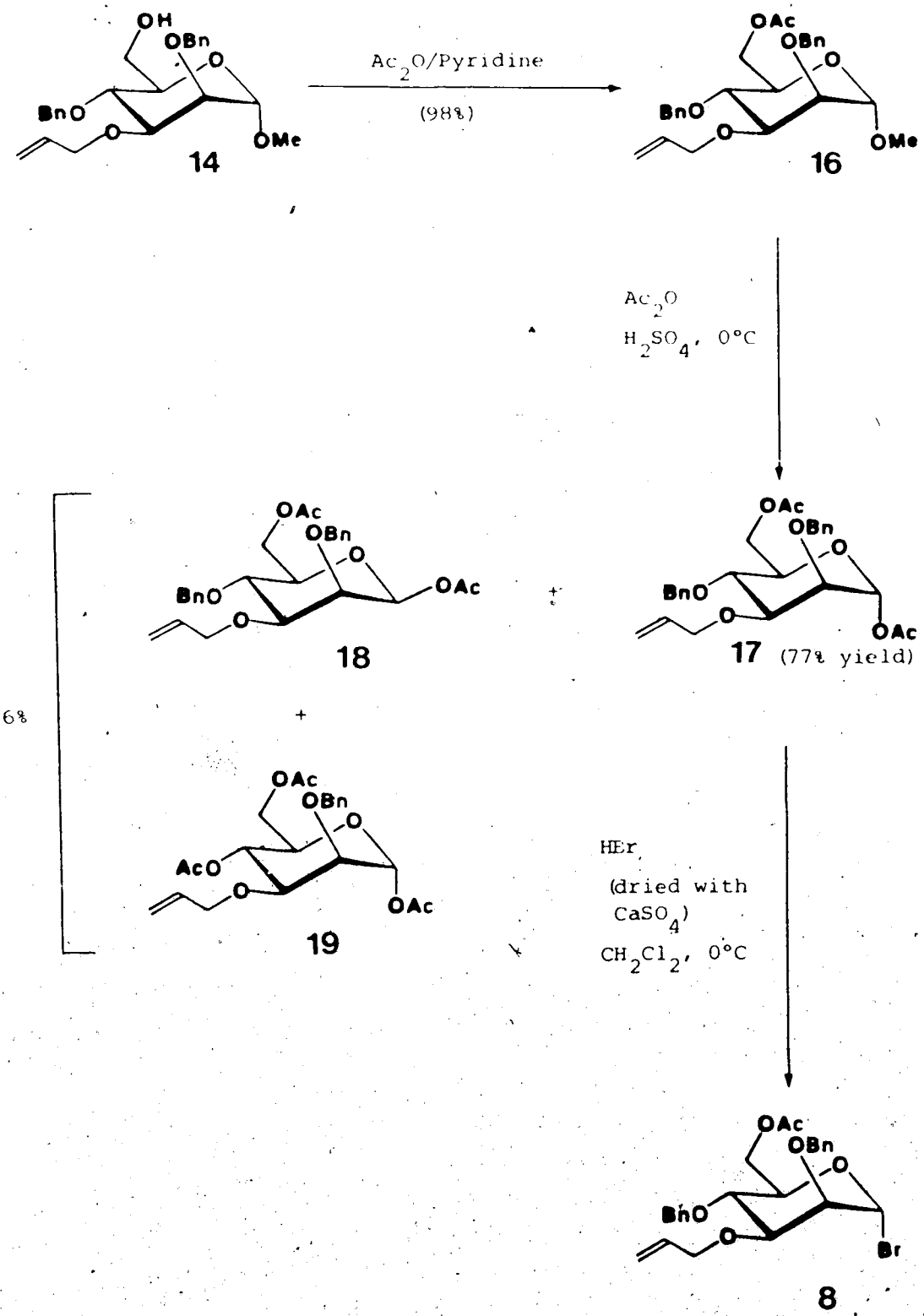
The glycosidic linkage in **16** was acetolyzed[35] using acetic anhydride in the presence of a catalytic amount of sulfuric acid to afford 1,6-di-O-acetyl-3-O-allyl-2,4-di-O-benzyl-α-D-mannopyranose (**17**) in 77% yield. The structure of **17** could be deduced from its ^{1}H nmr spectrum, which included a deshielded signal at δ6.195 (d, $J_{1,2}$ = 2.0 Hz) for H-1 and a six-proton singlet at δ2.055 indicating the presence of two acetyl groups. Further proof for the assigned structure **17** was provided by its

^{13}C nmr spectrum, which contained a shielded signal (as compared with 99.10 ppm for its precursor) at δ91.79 for C-1 and additional signals at δ168.82 and 20.98 for $\underline{O}C\underline{O}CH_3$ and $OCO\underline{C}H_3$ respectively. Along with this major product **17**, the acetolysis reaction also produced two minor products, namely, 1,6-di-O-acetyl-3-O-allyl-2,4-di-O-benzyl-β-D-mannopyranose (**18**) and 1,4,6-tri-O-acetyl-3-O-allyl-2-O-benzyl-α-D-mannopyranose (**19**) in a combined yield of 6%. The anomeric configuration of **18** was evident from the appearance, in its ^1H nmr spectrum, of a doublet ($J_{1,2}$ = 1.0 Hz) for H-1 at δ5.609 (about 0.6 ppm upfield relative to H-1 in the corresponding α-anomer **17**). The presence of shielded signals (as compared with the α-anomer **17**) for H-3 and H-5 at δ3.598 and 3.300 respectively further established[36] the anomeric configuration to be β in **18**. The structure of the other minor product **19** could also be deduced from its ^1H nmr spectral data, which included a doublet ($J_{1,2}$ = 2.0 Hz) at δ6.185 for H-1, indicating the configuration at C-1 to be α, and a deshielded doublet of doublets ($J_{3,4}$ = $J_{4,5}$ = 10.5 Hz) at δ5.431 for H-4 due to the acetylation of O-4, thus confirming the cleavage of the 4-O-benzyl group of **16**.

Reaction of the diacetate **17** in dichloromethane, at

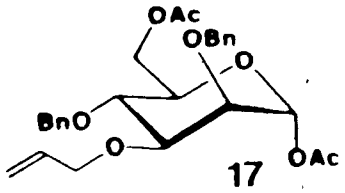
0°C, with hydrogen bromide which was dried by passage through a calcium sulfate column, led to the formation of the glycosyl bromide **8** in essentially quantitative yield. The ^{1}H nmr spectrum of **8** displayed a doublet ($J_{1,2}$ = 1.5) at δ6.444 for H-1 and a singlet at δ2.065, now integrating for only three protons, arising from a single acetyl methyl group. In its ^{13}C nmr spectrum the signal for C-1 appeared at δ87.41, and only one signal each for the carbonyl carbon at δ170.57 and for the acetyl methyl group at δ20.72 were present.

When the diacetate **17** was allowed to react with HBr gas introduced directly from the cylinder without passage through calcium sulfate, the thin layer chromatogram (tlc) of the reaction mixture showed the presence of two products, the minor being the desired bromide **8**. The major product **20** had a slightly lower mobility in tlc. The ^{1}H nmr spectrum of the mixture of **8** and **20** indicated **20** to be the hydrobromination product of **8**, namely, 6-O-acetyl-2,4-di-O-benzyl-3-O-(3-bromopropyl)-α-D-manno-pyranosyl bromide. This structural assignment was made to account for the observation of a triplet for two protons at δ3.688 ($BrCH_2CH_2\underline{CH_2}O$), a multiplet for two protons at δ3.510 for $Br\underline{CH_2}CH_2CH_2O$, and a two proton multiplet at δ2.119 for $BrCH_2\underline{CH_2}CH_2O$. The ratio of **20** to **8** was


OH
OBn
BnO
OMe
14
Ac2O/Pyridine
(98%)
OAc
OBn
BnO
OMe
16
Ac2O
H2SO4, 0°C
OAc
OBn
BnO
OAc
18
OAc
OBn
BnO
OAc
17 (77% yield)
6%
OAc
OBn
AcO
OAc
19
HBr
(dried with
CaSO4)
CH2Cl2, 0°C
OAc
OBn
BnO
Br
8

determined to be 4:1 from this ^1H nmr spectrum.

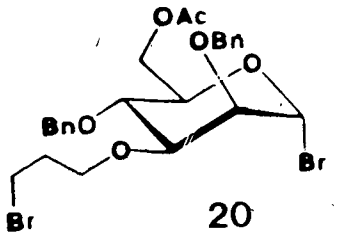
In order to further corroborate both the presence and the position of the bromine atom in the labile anomeric bromide **20** a stable derivative was prepared. Following the separation of **20** from **8** by flash column chromatography,[37] **20** was glycosylated with methanol under the conditions of Garegg et al.,[38] using silver carbonate as promoter and dichloromethane as solvent, to provide a mixture of the β- and α-mannosides **21** and **22** (β/α:4/1) in an isolated yield of 91%. The structure of β-anomer **21** was determined from its ^1H and ^{13}C nmr spectral data as well as mass spectral analysis. The ^1H nmr spectrum of the β-anomer **21** showed the presence of a doublet ($J_{1,2}$ = 1.0 Hz) at δ4.308 for H-1 and three two-proton signals: a triplet at 3.484 (J = 7.0 Hz) assigned to $BrCH_2CH_2C\underline{H}_2O$, a multiplet at δ3.41 assigned to $BrC\underline{H}_2CH_2CH_2O$, and another multiplet between δ2.92 and 2.856. The assigned structure **21** was also in agreement with the ^{13}C nmr spectrum, which exhibited a signal for C-1 at δ102.81 and three signals at δ67.00, 32.98, and 30.41 tentatively assigned to $BrCH_2CH_2\underline{C}H_2O$, $Br\underline{C}H_2CH_2CH_2O$, and $BrCH_2\underline{C}H_2CH_2O$, respectively. Unequivocal support for the presence of bromine in **21** (molecular weight 537.447) was provided by the chemical ionization mass spectrum, which showed two



17

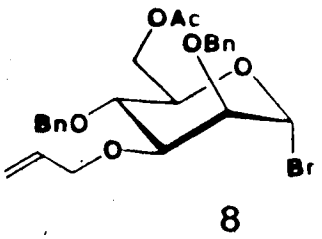
HBr

CH_2Cl_2, 0°C



20

+



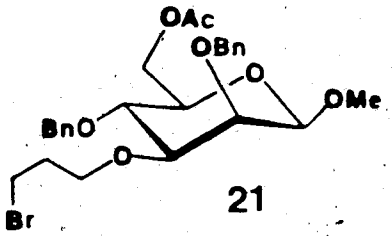
8

(91%)

MeOH

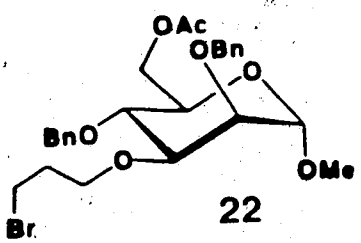
$AgCO_3$, 4 Å molecular sieves

CH_2Cl_2 0°C



21

+



22

21:22 = 4:1

peaks of almost equal intensity at 554 (93.3%) and 556 (100.0%), corresponding to the NH_4^+ adducts containing the ^{79}Br and ^{81}Br isotopes respectively. Furthermore, microanalytical data were in accord with the molecular formula for **21**.

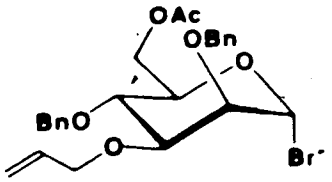
The newly developed method of Paulsen and Lockhoff[39] for the stereoselective synthesis of β-mannosides was used for the preparation of the glycosides **23**β and **23**α. Reaction of the glycosyl bromide **8** and 8-methoxycarbony-octanol in the presence of an insoluble catalyst prepared by precipitation of silver silicate on aluminum oxide, provided a mixture of the β- and α-mannosides, **23** in a combined yield of 56%. All attempts to separate these anomers using chromatography on silica gel, silica gel impregnated with silver nitrate or alumina, were unsuccessful. That **23** was a mixture of the α and β glycosides was evident from the ^1H nmr spectrum which showed a signal at δ4.365 for H-1 of the β-anomer and a doublet ($J_{1,2}$ = 2.0 Hz) at δ4.824 for H-1 of the corresponding α-anomer. The β/α ratio of this mixture, determinted by integration of these signals in the ^1H nmr spectrum of **23**, was found to vary with the temperature of the reaction. At -78°C, the β- and α-anomers were produced in a 6:1 ratio. On the other hand, at the higher

temperatures of -20° and 0° the ratios of β- to α-anomers were 4.3:1 and 3.5:1 respectively. The glycosylation reaction did not go to completion as indicated by the presence of the unreacted glycosyl bromide **8** on tlc. The rate of disappearance of **8** increased with temperature but no improvement in the yield of **23** was observed. Elimination of HBr is likely a major reaction pathway for **8** at these higher temperatures.

Coupling of **8** with 8-methoxycarbonyoctanol[24] was also attempted using freshly prepared silver carbonate in the presence of 4 Å molecular sieves, using dichloromethane as solvent at 0°C, according to the procedure of Garegg et al.[38] A mixture of β- and α-anomers (β/α:3/1) was obtained in 54% yield. The reaction was faster under these conditions but provided an overall lower yield than with the silver silicate reaction.

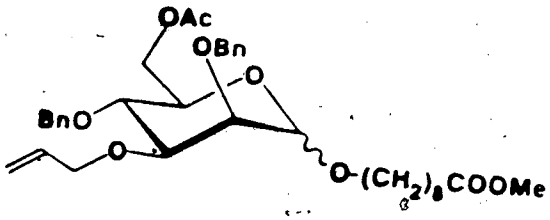
Treatment of the mixture **23** with sodium methoxide in methanol effected the removal of acetyl groups to provide a mixture of **24**β and **25**α which chould now be separated by flash column chromatography on silver nitrate impregnated silica gel. The assignment of structures to the deacetylation products **24** and **25** was made on the basis of their ^{1}H and ^{13}C nmr data.

Appearance, in the ^{1}H nmr spectrum of the major



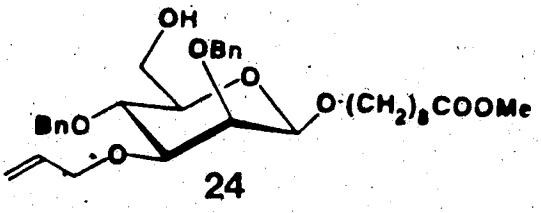
8

(56%) | $HO-(CH_2)_8COOMe$, silver silicate/alumina, CH_2Cl_2, -78°C

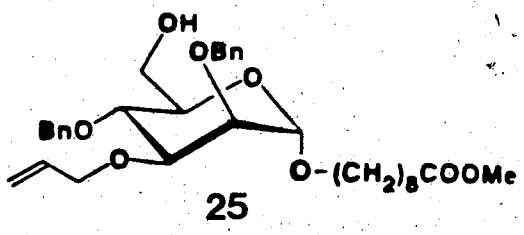


23 (β/α:6/1)

NaOMe/MeOH



24



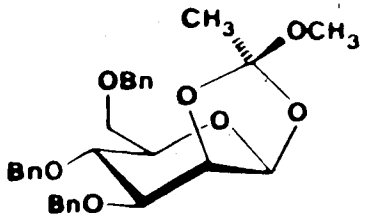
25

product **24**, of a broad signal for one proton at δ4.408 ($J_{1,2}$ ≈ 1 Hz), suggested the anomeric configuration to be β. A one-proton doublet of doublets ($J_{6a,OH}$ = $J_{6b,OH}$ = 6.5 Hz) at δ2.140 for a D_2O-exchangeable hydroxyl proton is also consistent with the structure assigned to **24**. This structural assignment was further supported by the ^{13}C nmr spectrum of **24** where the signal for C-1 at δ98.25 was clearly visible. The β-configuration of the glycosidic linkage in **24** could be unequivocally assigned from its ^{1}H-coupled ^{13}C nmr spectrum where this signal appeared as a doublet, $J_{C-1,H-1}$ = 153.6 Hz, in accord with the empirical rules formulated by Bock and Pedersen[40] for the dependence of the one-bond C-H coupling on the anomeric configuration of pyranosides.

The structure of the minor product **25** was assigned after the observation, in its ^{1}H nmr spectrum, of a doublet for one proton at δ4.790 ($J_{1,2}$ = 2.0 Hz), assigned to H-1, as expected for the corresponding α-anomer. A broad doublet integrating for one proton at δ2.098 for a hydroxylic proton was also present. The ^{13}C nmr spectrum of **25** showed the C-1 signal for C-1 at δ98.25 with $J_{C-1,H-1}$ = 168.2 Hz.

Condensation of the alcohol **24** and 2-O-acetyl-3,4,6-tri-O-benzyl-α-D-mannopyranosyl bromide[27] (**7**), which had been freshly prepared from 3,4,6-tri-O-benzyl-1,2-O-

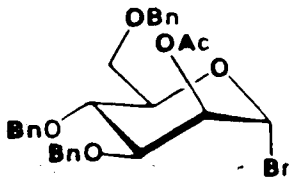
(methoxyethylidene)-β-D-mannopyranose (**26**), under Helferich conditions using mercuric bromide and mercuric cyanide as promoters and acetonitrile as solvent furnished the α-linked disaccharide **27** in 77% yield. The structure of **27** was assigned on the basis of its ^{1}H and ^{13}C nmr spectra. The resonances for two anomeric protons were observed in the ^{1}H nmr spectrum, at δ4.911 ($J_{1',2'}$ = 2.0 Hz) and 4.334 ($J_{1,2}$ ≈ 1 Hz) and were thus assigned to H-1' of the α-mannopyranosyl unit and H-1 of the β-mannopyranosyl unit, respectively. The presence of a deshielded doublet of doublets ($J_{1',2'}$ = 2.0 Hz and $J_{2',3'}$ = 3.0 Hz) at δ5.459 indicated the presence of the expected acetoxy group on C-2' and, by decoupling, thus confirmed the identity of the anomeric doublet at δ4.911. The ^{13}C nmr spectral data were also in agreement with the assigned structure. The configuration of the newly formed glycosidic linkage in **27**, expected to be α due to neighboring group participation of the 2-acetoxy group in the glycosylation reaction, was evident from its ^{1}H-coupled ^{13}C nmr spectrum which showed a doublet each for C-1 and C-1' at δ104.64 ($J_{C-1,H-1}$ = 154.7 Hz) and 97.77 ($J_{C-1',H-1'}$ = 170.4 Hz) respectively. One-bond C-H coupling constants of these magnitudes require the presence of the β and α glycosidic linkages as assigned.



26

HBr , CH_2Cl_2

0°C



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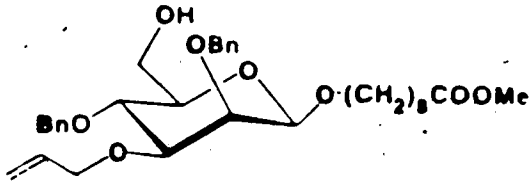


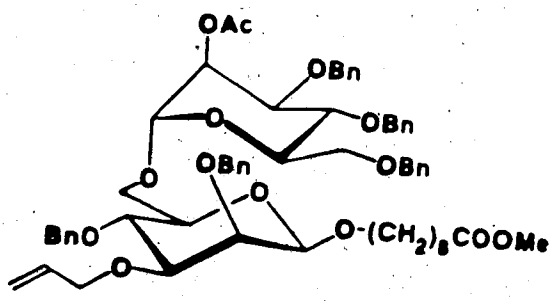
24

(77%)

$HgBr_2$, $Hg(CN)_2$

4 Å molecular sieves

CH_3CN, RT



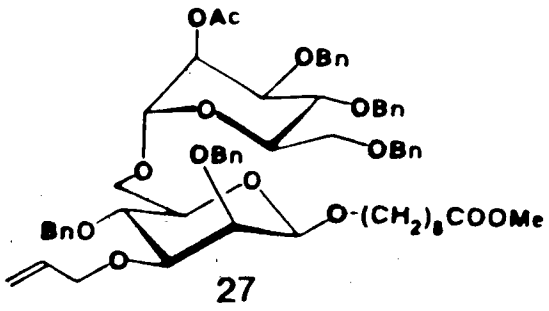
27

Reaction of **27** with sodium methoxide in dry methanol provided the alcohol **28** in quantitative yield. Disappearance, in the ^1H nmr spectrum, of a singlet for three acetyl protons and the upfield shift of the signal for H-2' to δ 4.128 confirmed the removal of acetyl group from O-2' of **27**. Moreover, the presence of a new signal at δ 2.355 (broad singlet), which was exchanged for deuterium by treatment with D_2O, further supported the structure assigned to **28**. The ^{13}C nmr spectrum of **29** showed the expected disappearance of the signals corresponding to carbonyl and methyl carbons.

Reaction of the alcohol **28** with 3,4,6-tri-O-acetyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl bromide[26] (**6**) in dichloromethane using silver zeolite[41] as promoter at room temperature, as described by Schwartz et al.,[42] failed to provide any of the desired trisaccharide **4**. Use of silver triflate/2,4,6-collidine (1.5 molar equivalents each with respect to **6**) at -20°C in nitromethane, according to Lemieux et al.[26] did provide the desired product although the yield of 30% proved unacceptably low. In an attempt to accomplish the preparation of **4** in a more respectable yield, we turned our attention to the conditions described by Paulsen et al.[43] in a recent report. Thus the treatment of **28** with the glycosyl bromide **6** (2 molar equivalents) using silver triflate/2,4,6-collidine (10

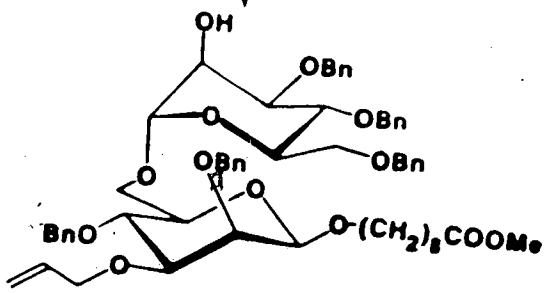
equivalents) using silver triflate/2,4,6-collidine (10 molar equivalents each) in the presence of 4 Å molecular sieves in dichloromethane at -50°C provided the trisaccharide **4** in 76% yield after chromatographic purification. The presence of the 3,4,6-tri-O-acetyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl moiety in **4** was indicated by its ^{1}H nmr spectrum which displayed four deshielded, one-proton signals, characteristic of the newly introduced glucosyl residue: a doublet of doublets at δ5.815 ($J_{3",4"}$ = 9.0 Hz, $J_{2",3"}$ = 10.0 Hz) for H-3", a doublet at δ5.573 ($J_{1",2"}$ = 8.5 Hz) for H-1", a doublet of doublets at δ5.215 ($J_{3",4"}$ = 9.0 Hz, $J_{4",5"}$ = 10.0 Hz) for H-4, and a doublet of doublets at δ4.513 ($J_{2",3"}$ = 10.0 Hz, $J_{1",2"}$ = 8.5 Hz) for H-2. The magnitude of the coupling constant between H-1" and H-2" provides unambiguous proof that these protons are in the trans-diaxial orientation and thus establishes the presence of the β-glucosidic linkage. The ^{13}C nmr spectrum of **4** showed the expected signals at δ170.42, 169.89, and 169.21 for three acetyl carbonyl carbons, and an additional anomeric signal at δ96.48 (C-1") further substantiated the structure assigned to **4**.

Along with the major condensation product **4** a minor product (**29**) with higher R_f, was also produced. This



27

NaOMe/MeOH



28

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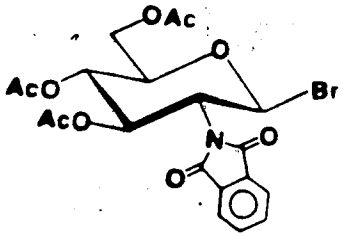
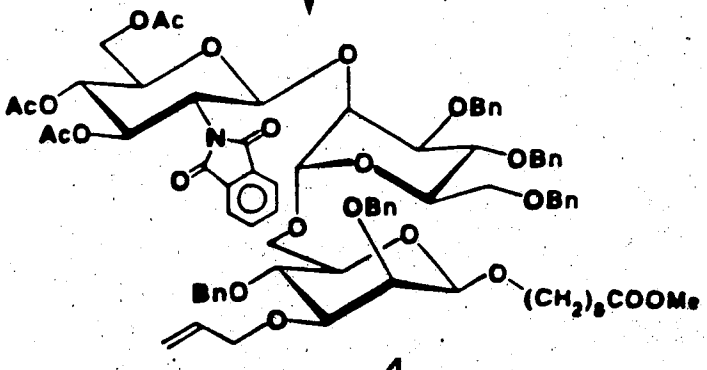


6

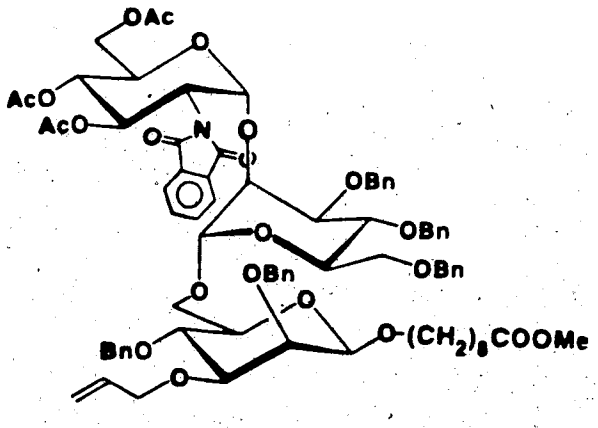
(76%)

CF_3SO_3Ag, sym-collidine
4 Å molecular sieves, CH_2Cl_2
-50°C

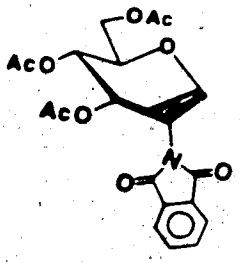


4

minor product, isolated in 6% yield, was not fully characterized but appears to be the α-linked trisaccharide. This structural assignment was made on the basis of its ^{1}H nmr spectrum which included a partially obscured doublet for H-1" at δ5.14 ($J_{1",2"} < 4$ Hz) and a shielded signal (relative to the corresponding β-anomer) for H-3" at δ6.77 ($J_{3",4"} = 9.0$ Hz, $J_{2",3"} = 11.5$ Hz), suggesting the configuration at C-1" to be α. All other signals in the ^{1}H nmr spectrum of **29** were consistent with it being a trisaccharide. As expected, the product of elimination of HBr from the glycosyl bromide **6**, namely 3,4,6-tri-O-acetyl-1,5-anhydro-2-deoxy-2-phthalimido-D-arabino-1-hex-1-enitol (**30**, was also formed in the glycosylation mixture. The glycal **30** had nmr data identical with those previously reported. [24]



29



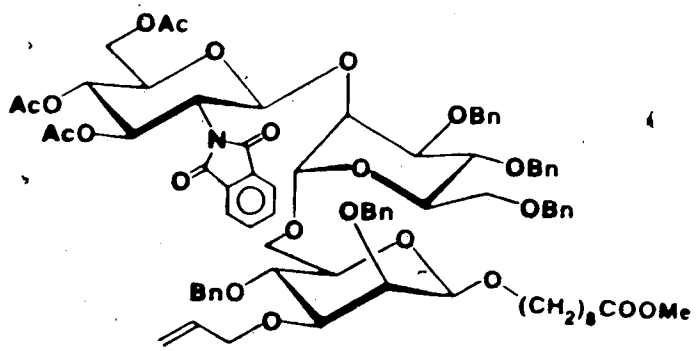
30

Three different methods were examined to effect the removal of the allyl group from **4**. Treatment of **4** with 5% palladium on charcoal,[44] under acidic conditions, furnished the trisaccharide alcohol **31** in only 34% yield. Using palladium(II) chloride as catalyst in acetic acid-water (20:1) according to Ogawa,[45] **31** could be obtained in 90% yield. Finally, deallylation of **4** could also be achieved in two steps: reaction with tris(triphenylphosphine)rhodium(I) chloride as catalyst, according to the procedure developed by Corey and Suggs,[46] effected the isomerization of double bond to give the prop-1-enyl ether, which on treatment[47] with mercuric chloride in the presence of mercuric oxide, underwent hydrolysis to afford the required trisaccharide alcohol **31** in 85% yield. The progress of the isomerization reaction proved difficult to monitor by tlc since the isomerized product (enol ether) and the allyl ether had the same chromatographic mobility. Surprisingly, washing the ether extract with brine acidified to pH 2 failed to accomplish the hydrolysis of the enol ether as had been reported by Corey and Suggs.[46]

The structure of the compound **31** was evident from its ^{1}H nmr spectrum which was devoid of signals for the protons of the allyl group. The corresponding signals were also absent in the ^{13}C nmr spectrum. Appearance of a

D_2O-exchangeable one-proton doublet at δ2.380 ($J_{3,OH}$ = 10.0 Hz) further supported the assigned structure.

Deacetylation of **31** using sodium methoxide in methanol led to the quantitative formation of **32**, whose identity could easily be ascertained by the absence of acetyl resonances in both its ^1H and ^{13}C nmr spectra. Removal of phthalimido group from **32**, and subsequent N-acetylation of the free amine were performed as described by Bundle and Josephson.[48] Thus, treatment of **32** with hydrazine (8 molar equivalents) in refluxing methanol generated the free amine which was acetylated *in situ* using acetic anhydride in methanol-water (1:1). The N-acetyl derivative **33** was obtained in an overall yield of 60%. No evidence of attack at the 8-methoxycarbonyloctyl ester was obtained. The structure of **33** was supported by the decrease of intensity of the signals from the aromatic protons in ^1H nmr spectrum along with the appearance of two new signals: a three proton singlet arising from the N-acetyl methyl group at δ1.779 and a broad singlet for one D_2O-exchangeable proton at δ5.533 assigned to the amide proton. The ^{13}C nmr spectrum of **33** further supported the assigned structure. The signals at δ168.51, 133.79, 131.84, and 123.23 which were assigned to the carbonyl carbons, two of the tertiary aromatic carbons,

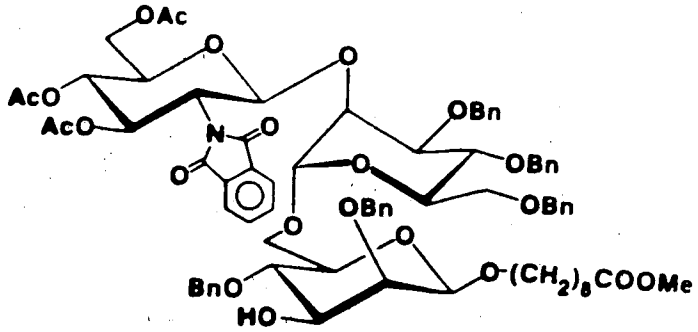


4

(85%)

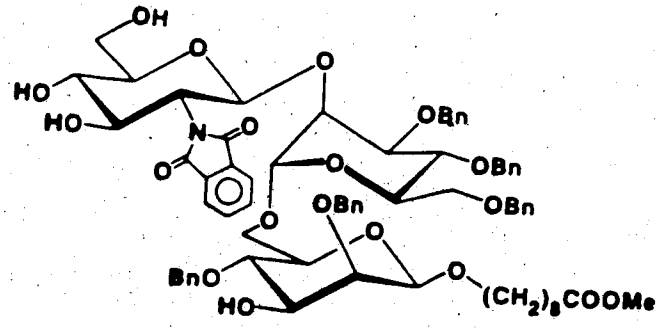
(i) $[(C_6H_5)_3P]_3RhCl$, DABCO
EtOH-benzene-water (7:3:1)

(ii) HgO, $HgCl_2$, Acetone-water (9:1)



31

NaOMe/MeOH

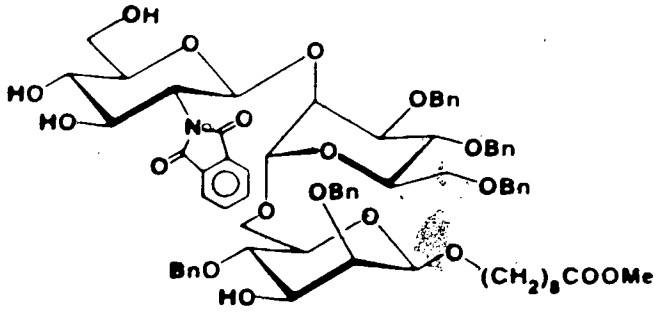


32

the quaternary aromatic carbons, and the remaining two tertiary aromatic carbons of the phthalimido group, respectively in the ^{13}C nmr spectrum of **32**, were absent in the nmr spectrum of **33**. Compound **33**, on the other hand, showed two new resonances at δ172.37 and 23.30 for the carbonyl and the methyl carbons, respectively, of the N-acetyl group.

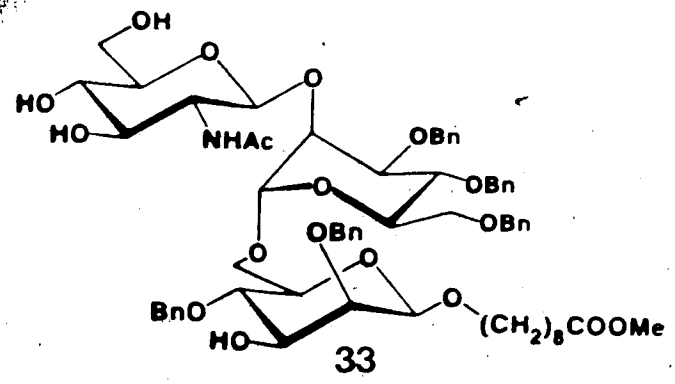
Hydrogenolytic cleavage of the benzyl protecting groups of **33** using 5% palladium-on-charcoal as the catalyst in 98% ethanol furnished the target trisaccharide **2**, which was purified by size-exclusion chromatography on Bio-Gel P-2. Compound **2** was obtained as a white lyophilized powder in 85% yield. The ^{1}H nmr spectrum of **2** showed the expected signals for H-1 at δ4.664 ($J_{1,2}$ < 0.7 Hz), H-1' at δ4.920 ($J_{1',2'}$ = 1.8 Hz) and H-1" at δ4.579 ($J_{1",2"}$ = 8.0 Hz). Other signals were in accord with the structural assignment. The partial 360 MHz ^{1}H nmr spectrum of **2** is reproduced in Figure 4 to show level of anomeric purity of this final product. The ^{1}H-coupled ^{13}C nmr spectrum of **2** again established the configurations at C-1 ($J_{C-1,H-1}$ = 159.4 Hz), C-1' ($J_{C-1',H-1'}$ = 169.5 Hz), and C-1" ($J_{C-1",H-1"}$ = 162.3 Hz) to be β, α, and β respectively.

Having accomplished the synthesis of the target



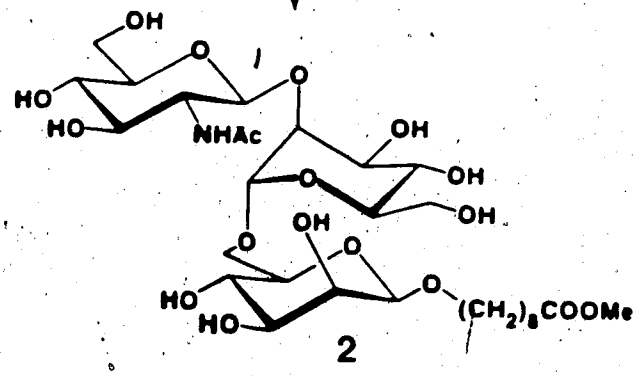
32

(60%) (i) $NH_2NH_2 \cdot H_2O$, MeOH
(ii) Ac_2O, H_2O-MeOH (1:1)



33

(85%) H_2 (1 atm), 5% Pd-C
98% ethanol



2

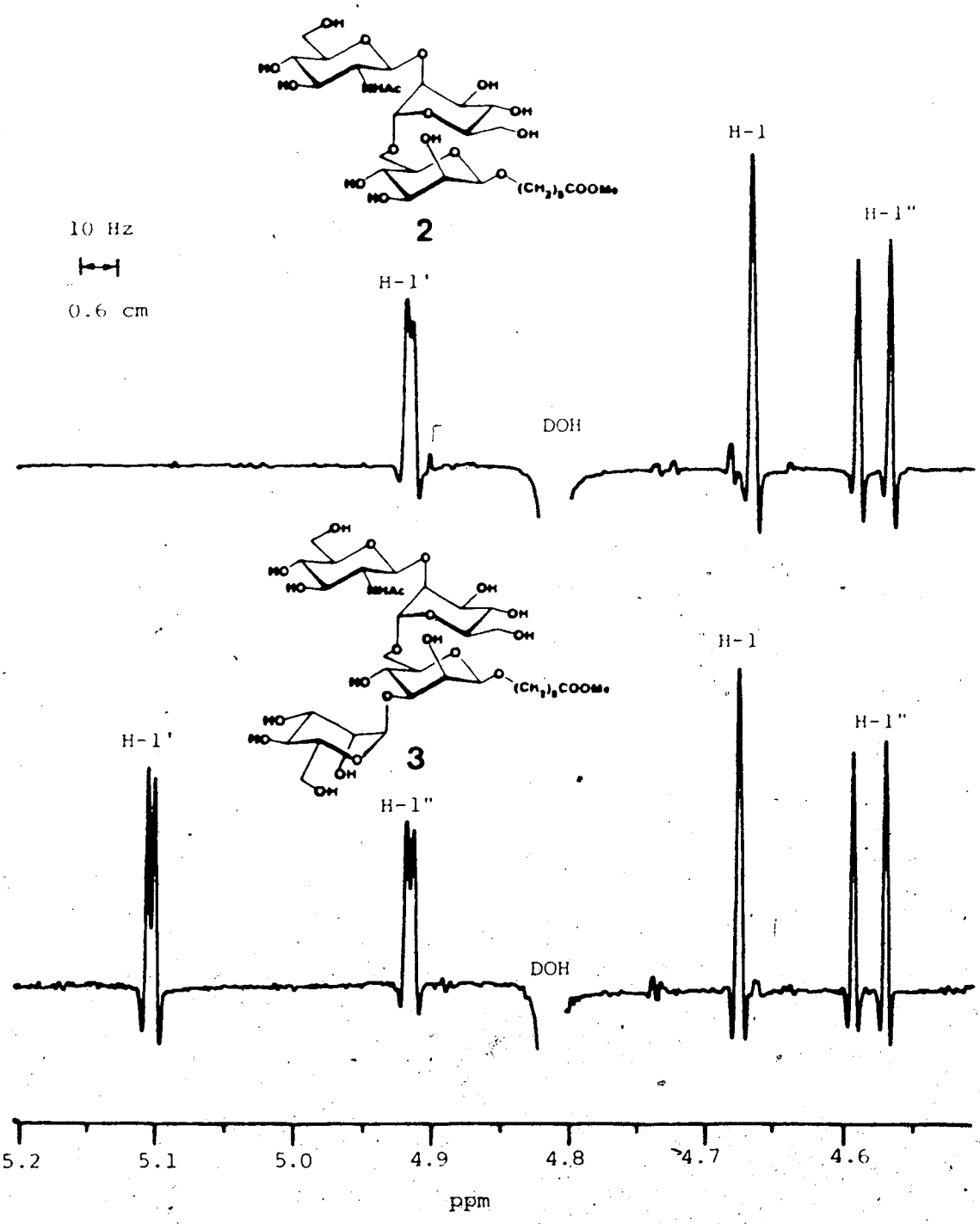
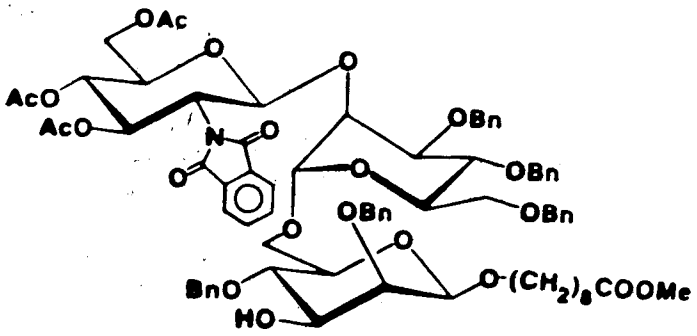


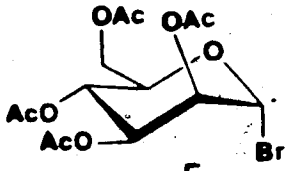
Figure 4. The 360 MHz ^{1}H nmr spectra (only anomeric region shown) of **2** and **3** in D_2O.

trisaccharide 2, the preparation of the tetrasaccharide 3 was undertaken. In order to achieve the coupling of 31 and 2,3,4,6-tetra-O-acetyl-α-D-mannopyranosyl bromide[25] (5), two different promoters were utilized. Under the conditions of Hanessian and Banoub[49] using silver triflate and tetramethylurea as promoter, the glycosylation of 31 with the bromide 5 to provide the tetrasaccharide 34 proceeded in only 38% yield. When the reaction was performed instead in the presence of mercuric bromide and mercuric cyanide in dichloromethane, in the presence of 4 Å molecular sieves, a 65% yield of the condensation product 34 was obtained. The bromide 5 described above was readily available from 1,2,3,4,6-penta-O-acetyl-α/β-D-mannopyranose by treatment with 45% hydrogen bromide in acetic acid.

The ^{1}H nmr data of 34 required the presence of a 2,3,4,6-tetra-O-acetyl-α-D-mannopyranosyl moiety. The signal for the anomeric proton of the newly introduced glycosyl residue appeared at δ5.018 ($J_{1',2'}$ ≈ 2 Hz) and the acetyl region showed the expected seven signals for the methyl acetyl groups. The ^{13}C nmr spectrum showed the presence of four anomeric carbons: δ101.95, $J_{C-1,H-1}$ = 154.1 Hz, 97.64, $J_{C-1'',H-1''}$ = 170.9 Hz, 96.60, $J_{C-1''',H-1'''}$ = 164.16 Hz and the new signal for α-linked mannose at



31



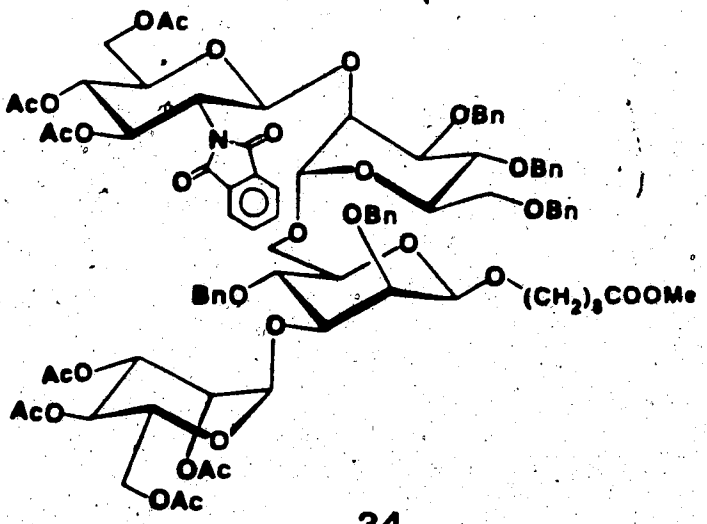
5

(65%)

$HgBr_2$, $Hg(CN)_2$

4 Å molecular sieves

CH_3CN, RT

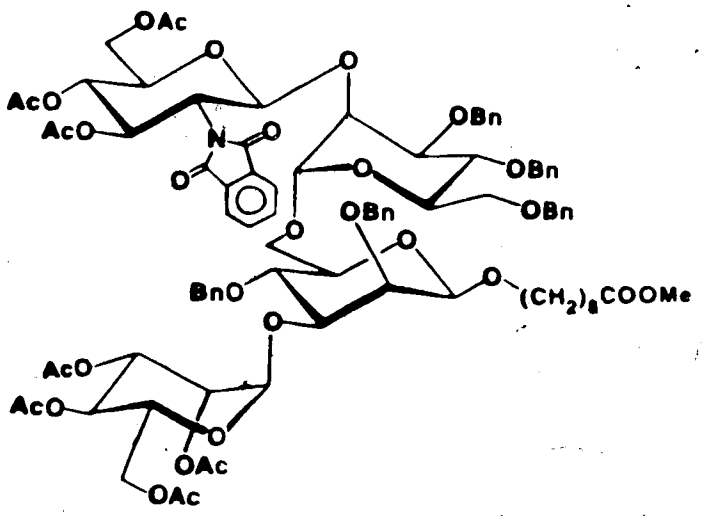


34

δ99.83, $J_{C\text{-}1',H\text{-}1'}$ = 177.8 Hz. Other features of these spectra, described in detail in the experimental section, were in accord with the assigned structures.

Conversion of **34** to **35** was effected in three steps without characterization of the intermediates involved. Deacetylation of **34** with sodium methoxide in methanol gave a white foam which was refluxed with hydrazine in methanol to generate the free amine which, on treatment with acetic anhydride in pyridine, gave the peracetylated product **35** in 63% yield. Attempted N-acetylation of the intermediate free amine, obtained in the second step of the above sequence using acetic anhydride in methanol-water (1:1) gave a very polar hydroxylated derivative which proved difficult to purify by silica gel chromatography. The structure of **35** could readily be deduced from its ^1H nmr spectrum which displayed the presence of only twenty-five aromatic protons and an additional singlet at δ1.705 for three N-acetyl protons. Disappearance of the ^{13}C resonances corresponding to the carbons of the phthalimido group and the presence of two new signals at δ169.54 and 23.27 arising from the N-acetyl group also supported the structure assigned to **35**.

Removal of the O-acetyl and O-benzyl protecting groups of **35** was conveniently accomplished by treatment



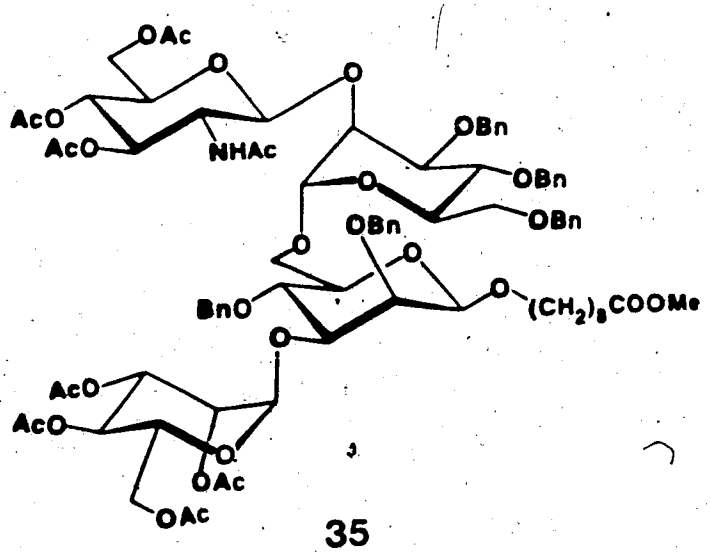
34

(62%)

(i) NaOMe/MeOH

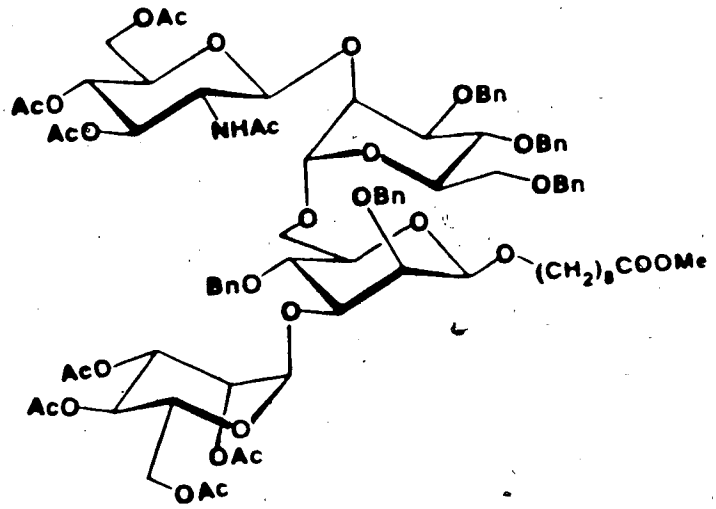
(ii) $NH_2NH_2 \cdot H_2O$, MeOH, reflux

(iii) Ac_2O, Pyridine



35

with sodium methoxide in methanol followed by hydrogenolysis over 5% palladium-on-charcoal. Filtration of the crude product through a column of Bio-Gel P-2 afforded the target tetrasaccharide **3** as a white lyophilized powder in a total yield of 85%. Complete removal of acetyl and benzyl groups was evident from the absence of the corresponding signals in the ^{1}H nmr spectrum of 3. The signals for H-1, H-1', H-1", and H-1"' appeared at δ4.671 ($J_{1,2} < 0.8$ Hz), 5.104 ($J_{1',2'} = 2.0$ Hz), 4.918 ($J_{1",2"} = 1.8$ Hz) and 4.579 ($J_{1"',2"'} = 8.0$ Hz) respectively. The ^{1}H-coupled ^{13}C nmr spectrum of **3** was employed to re-establish the anomeric configurations which were already assigned by the ^{1}H-coupled ^{13}C nmr spectra recorded following the formation of each glycosidic linkage. As anticipated, C-1, C-1', C-1", and C-1"' showed the one-bond C-H couplings of 159.1 Hz (β), 172.00 Hz (α), 169.3 Hz (α), and 159.1 Hz (β), respectively. The partial 360 MHz ^{1}H nmr spectrum of 3 is reproduced in Figure 4 where it is compared with the spectrum of the corresponding trisaccharide **2**.

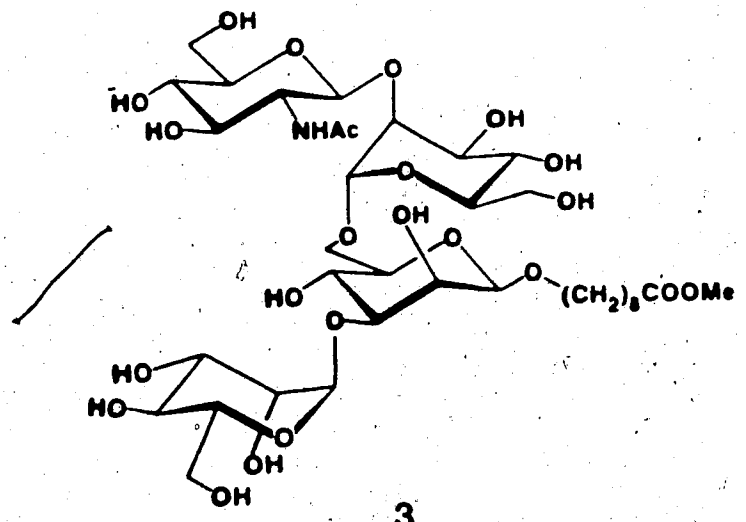


35

(85%)

(i) NaOMe/MeOH

(ii) H_2 (1 atm), 5% Pd-C
98% EtOH



3

CHAPTER 3

TESTING RESULTS

Trisaccharide **2** was found to be an excellent acceptor for GlcNAc-transferase V (GnT V) in experiments performed by Dr. Michael Pierce, Department of Anatomy, University of Miami Medical School. The results of these experiments are included here, briefly, only for the sake of completeness.

In a typical experiment, BHK cells were sonicated in 0.1 M MES [2-(N-morpholino)-ethanesulfonic acid], pH 7.0, and protein was solubilized by addition of Triton X-100 to a 1% solution. The glycosyltransferase assay was performed in a total volume of 20 μL containing 100 μg of the solubilized cellular protein, 1 mM of the acceptor trisaccharide **2**, 1 mM of 2-acetamido-2-deoxy-β-D-gluco-pyranosyl amine (an N-acetyl glucosaminidase inhibitor) and 5 mM UDP-(^{3}H)-GlcNAc. After a 2 hour incubation at 37°, the mixture was diluted with water (100 μL) and passed over a filter supporting Dowex-1-X8 (formate) ion exchange resin to remove almost all of the negatively charged counts. The filtrate was then injected onto a reverse phase (C-18) HPLC column which was eluted

isocratically with 40% aqueous methanol. The chromatogram obtained in this manner is reproduced in Figure 5. In this figure, peak A includes both ^{3}H-UDP-GlcNAc which had passed through the ion exchange resin and, presumably, some of its hydrolytic products including GlcNAc and GlcNAc-1-phosphate. Peak B has a retention volume identical to the acceptor trisaccharide **2** and is absent when this trisaccharide acceptor is omitted from the incubation mixture. The counts produced in this peak increased linearly with time, in the presence of a saturating concentration of acceptor **2** (50-100 nmoles/20 μL), as expected for the enzymatic reaction. The radioactivity in peak B is therefore a measure of the activity of a GlcNAc-transferase.

BHK cells are known to contain only GlcNAc-transferases I, II, IV, V and VI,[15] and all but GnT V require Mn^{++} for activity. The counts in peak B (Figure 5) were not affected by the inclusion of ethylenediamine tetraacetic acid (EDTA) at concentrations known to abolish the activities of these Mn^{++} requiring enzymes. It therefore appears that trisaccharide **2** is indeed a selective substrate for assaying the activity of GnT V. Using acceptor **2** in this assay, the GnT V activity of Rous sarcoma transformed BHK cells was found to be 1.8-2.0 times higher than in the untransformed cells. This

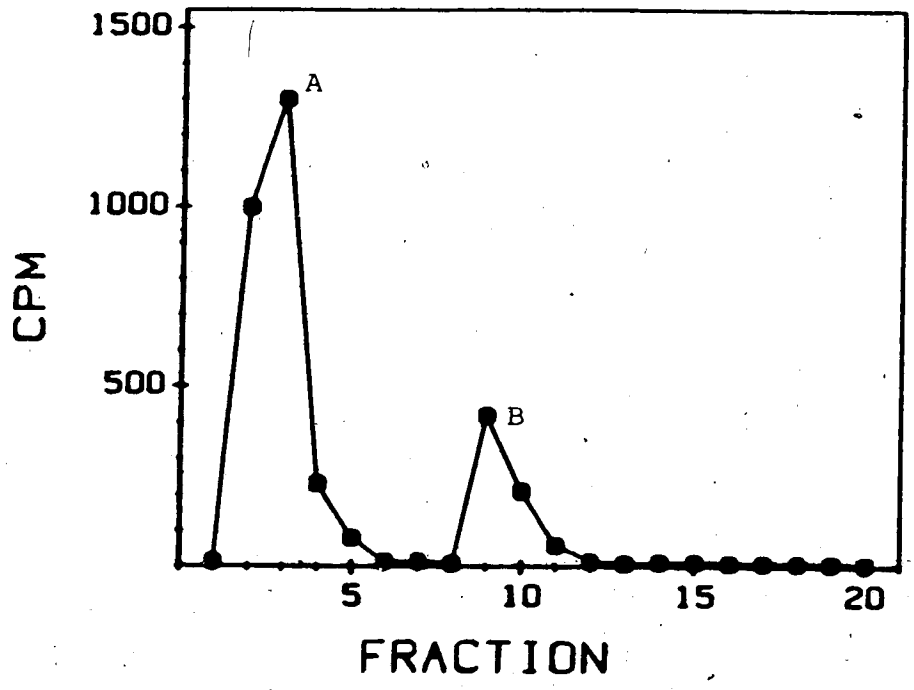


Figure 5. HPLC elution profile of a GlcNAc-transferase assay mixture on a reverse-phase (C-18) column. Peak A includes unreacted UDP-(^{3}H)-GlcNAc and its decomposition products. Peak B is the product of ^{3}H-GlcNAc transfer to the synthetic acceptor trisaccharide **2**.

finding parallels that of Kobata[10,15] for polyoma-transformed BHK cells.

Tetrasaccharide **3** has not yet been tested as an acceptor.

CHAPTER 4

EXPERIMENTAL

General Methods

All solvents and reagents used were reagent grade, and, in cases where further purification was required, standard procedures[50] were followed. All solid reactants for glycosylation were dried overnight over phosphorus pentaoxide in a high vacuum prior to use. Solution transfers where anhydrous conditions were required were done under nitrogen using standard syringe techniques.[51] Molecular sieves were purchased from BDH Chemicals, and the ratio of alcohol to molecular sieves in glycosylation was between 1:5 and 1:10 by weight.

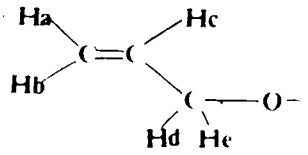
Thin layer chromatograms (tlc) were performed on pre-coated silica gel 60-F254 plates (E. Merck, Darmstadt) and visualized by quenching of fluorescence and/or by charring after spraying with 5% sulfuric acid in ethanol. For flash chromatography[37] 40-63 μm (400-230 mesh) silica gel 60 (E. Merck No. 9385) and distilled solvents were used, and the ratio of silica gel to compound was in the range 50:1-100:1. Skellysolve B refers to hexane supplied by Stanchem, Winnipeg, Manitoba. Solvents were removed on a

rotary evaporator under the vacuum of a water aspirator with bath temperatures of 40° or lower. For gel filtration, Bio-Gel P-2 (200-400 mesh) (Bio-Rad Laboratories, Richmond, California, USA) was used.

Spectral and elemental analyses were performed by the departmental NMR Service Laboratory under the supervision of Dr. T.T. Nakashima and the Analytical Service Laboratory under the supervision of Mr. R. Swindlehurst. Proton nuclear magnetic resonance (^{1}H nmr) spectra were recorded at either 400 MHz (Bruker WH-400) or 360 MHz (Bruker WM-360) with either tetramethylsilane (δ0 in $CDCl_3$) or acetone (δ2.225 in D_2O) as internal standard at ambient temperature. Carbon-13 nuclear magnetic resonance (^{13}C nmr) spectra were recorded at either 100.62 MHz (Bruker WH-400) or 90.56 MHz (Bruker WM-360) with either external tetramethylsilane (δ0 in $CDCl_3$) or external 1,4-dioxane (δ67.4 in D_2O) as reference standard. ^{1}H chemical shifts and coupling constants are reported as if they were first order. Assignments of ^{13}C resonances are tentative. Optical rotations were determined on a Perkin-Elmer 241 polarimeter in a 1 dm cell at ambient temperature (23 ± 1°C).

Protons of the allyl group present in the compounds described in this work were designated as Ha, Hb, Hc, Hd, and He as defined below. These protons showed the same

coupling constants and thus the same multiplicity pattern in all the compounds examined and only the chemical shifts varied. The observed coupling constants were:



Ha, dddd, $J_{a,c}$ = 10.5 Hz

$J_{a,d}$ = 1.5 ± 0.5 Hz

$J_{a,e}$ = 1.5 ± 0.5 Hz

$J_{a,b}$ = 1.5 ± 0.5 Hz

Hb, dddd, $J_{b,c}$ = 17.0 Hz

$J_{b,d}$ = 1.5 ± 0.5 Hz

$J_{b,e}$ = 1.5 ± 0.5 Hz

$J_{a,b}$ = 1.5 ± 0.5 Hz

Hc, dddd, $J_{b,c}$ = 17.0 Hz

$J_{a,c}$ = 10.5 Hz

$J_{c,d}$ = 5.5 Hz

$J_{c,e}$ = 5.5 Hz

Hd, dddd, $J_{d,e}$ = 13.5 Hz

$J_{c,d}$ = 5.5 Hz

$J_{b,d}$ = 1.5 ± 0.5 Hz

$J_{a,d}$ = 1.5 ± 0.5 Hz

He, dddd, $J_{d,e}$ = 13.5 Hz

$J_{c,e}$ = 5.5 Hz

$J_{a,e}$ = 1.5 ± 0.5 Hz

$J_{b,e}$ = 1.5 ± 0.5 Hz

Methyl 3-O-allyl-2-O-benzyl-4,6-O-benzylidene-α-D-manno-pyranoside (13)

To a solution of methyl 3-O-allyl-4,6-O-benzylidene-α-D-mannopyranoside[31] (12) (13.60 g; 42.24 mmol) in dry benzene (500 mL) were added sodium hydride (about 80% dispersion in oil; 2.02 g) and benzyl bromide (7.55 mL; 63.35 mmol). The mixture was refluxed under nitrogen atmosphere for 16 h. After the mixture had cooled to room temperature, the excess of sodium hydride was decomposed by the addition of methanol (250 mL), then water (500 mL) was added. The organic layer was separated, washed with water, dried ($MgSO_4$), filtered, and concentrated. The tlc of the residual oil showed, besides a major spot for the benzylation product, a UV active, fast moving spot which presumably corresponded to unreacted benzyl bromide. Flash chromatography of the oil obtained above using Skellysolve B-ethyl acetate (10:1) as eluent, which provided a good separation on tlc plate (R_f of benzylation product = 0.35), did not result in complete separation.

Purification of the major product could be achieved by gradient flash chromatography employing a mixture of Skellysolve B and ethyl acetate (the ratio of Skellysolve B-ethyl acetate was changed from 50:1 to 2:1 during elution). Finally **13** was obtained as a yellow oil (15.73 g; 90%); $[\alpha]_D$ +33.0° (*c* 1.4, chloroform); R_f 0.35 (Skellysolve B-ethyl acetate, 10:1); ^1H nmr ($CDCl_3$) δ: 7.505-7.259 (10H, aromatic), 5.901 (1H, Hc allyl), 5.609 (s, 1H, $C_6H_5C\underline{H}O_2$), 5.294 (1H, Hb allyl), 5.146 (1H, Ha allyl), 4.843 (d, 1H, J_{gem} = 12.0 Hz, $C_6H_5C\underline{H}HO$), 4.725 (d, 1H, J_{gem} = 12.0 Hz, $C_6H_5C\underline{H}HO$), 4.681 (d, 1H, $J_{1,2}$ = 2.0 Hz, H-1), 4.294-4.208 (2H, Hd allyl [δ4.268] and H-6e [δ4.244, dd, $J_{6a,6e}$ = 10.5 Hz, $J_{5,6a}$ = 4.5 Hz]), 4.185 (dd, 1H, $J_{6a,6e}$ = 10.0 Hz, $J_{5,6a}$ = 10.0 Hz, H-6a), 4.104 (1H, He allyl), 3.885-3.834 (2H, H-3 and H-4), 3.818 (dd, 1H, $J_{2,3}$ = 3.2 Hz, $J_{1,2}$ = 2.0 Hz, H-2), 3.761 (dd, 1H, $J_{4,5}$ = 10.0 Hz, $J_{5,6a}$ = 10.0 Hz, $J_{5,6e}$ = 4.5 Hz, H-5), 3.310 (s, 3H, CH_3O); ^{13}C nmr ($CDCl_3$) δ: 138.18, 137.72 (quat. arom.), 135.0 ($CH_2=\underline{C}HCH_2O$), 128.64, 128.00, 127.87, 127.59, 125.00 (tert. arom.), 116.32 ($\underline{C}H_2=CHCH_2O$), 101.43, 100.52 (C-1 and $C_6H_5\underline{C}HO_2$), 79.09 (C-4), 76.47, 76.03 (C-2 and C-3), 73.56 ($C_6H_5\underline{C}H_2O$), 68.79 ($CH_2=CH\underline{C}H_2O$), 64.03 (C-6), 54.67 (C-5), 29.57 (CH_3O). Anal. calcd. for $C_{24}H_{28}O_6$: C 69.89, H 6.84; found: C 69.67, H 6.94.

Methyl 3-O-allyl-2,4-di-O-benzyl-α-D-mannopyranoside (**14**)

To a stirred solution of **13** (14.27 g; 34.59 mmol) in 1:1 diethyl ether-dichloromethane (300 mL) was added, portionwise, $LiAlH_4$ (3.95 g; 103.9 mmol) and the mixture was slowly heated to the boiling point. To the boiling solution under reflux was added $AlCl_3$ (13.85 g; 103.9 mmol) in diethyl ether (150 mL) over a period of 70 min, after which tlc indicated the absence of starting material. The mixture was cooled. The excess of $LiAlH_4$ was decomposed with ethyl acetate (75 mL), and $Al(OH)_3$ was precipitated by the addition of water (75 mL). After dilution with ether (400 mL), the organic layer was separated from the aqueous layer which was back-extracted with ether (150 mL). The combined ether extracts were washed with water (3 × 150 mL), dried ($MgSO_4$), and concentrated to an oily residue which tlc indicated to be a mixture of two compounds. The separation of these two products was achieved by flash chromatography using Skellysolve B-ethyl acetate (3:1) as eluent. Evaporation of the early fractions provided the minor product **15** (1.69 g) as an oil; $[\alpha]_D$ = -6.25° (c 1.04, chloroform); R_f 0.47 (Skellysolve B-ethyl acetate, 2:1); ^1H nmr ($CDCl_3$) δ: 7.39-7.25 (10H, aromatic), 5.906 (1H, Hc allyl), 5.283 (1H, Hb allyl), 5.185 (1H, Ha allyl), 4.778 (d, 1H, $J_{1,2}$ =

2.0 Hz, H-1), 4.730-4.665 (AB, 2H, J_{gem} = 12.0 Hz, $C_6H_5C\underline{H}_2O$), 4.655-4.583 (AB, 2H, J_{gem} = 12.0 Hz, $C_6H_5C\underline{H}_2O$), 4.070-3.938 (3H, Hd and He allyl overlapping with H-4 [δ4.004, ddd, $J_{3,4}$ = 9.0 Hz, $J_{4,5}$ = 9.0 Hz, $J_{4,OH}$ = 2.0 Hz, simplified to dd with J_{OH} being absent on D_2O exchange]), 3.830-3.704 (4H, H-2, H-5, H-6a, and H-6b), 3.596 (dd, 1H, $J_{3,4}$ = 9.5 Hz, $J_{2,3}$ = 3.0 Hz, H-3), 3.354 (s, 3H, CH_3O), 2.578 (d, 1H, $J_{4,OH}$ = 2.0 Hz, disappeared on D_2O exchange, 4-OH); ^{13}C nmr ($CDCl_3$) δ: 138.40, 138.37 (quat. arom.), 134.76 ($CH_2=\underline{C}HCH_2O$), 128.37, 127.88, 127.66, 127.56 (tert. arom.), 117.22 ($\underline{C}H_2=CHCH_2O$), 99.40 (C-1), 79.41 (C-3), 73.79 (C-2), 73.65, 72.78 ($C_6H_5\underline{C}H_2O$), 71.58 (C-5), 70.73 ($CH_2=CH\underline{C}H_2O$), 70.66 (C-6), 67.96 (C-4), 54.99 (CH_3O). Anal. calcd. for $C_{24}H_{30}O_6$: C 69.55, H 7.30; found: C 69.67, H 7.35.

Evaporation of the later fractions gave the major product **14** (10.78 g) as an oil; $[\alpha]_D$ +48.25° (c 0.97, chloroform); R_f 0.35 (Skellysolve B-ethyl acetate, 2:1); ^{1}H nmr ($CDCl_3$) δ: 7.42-7.24 (10H, aromatic), 5.950 (1H, Hc allyl), 5.326 (1H, Hb allyl), 5.180 (1H, Ha allyl), 4.936 (d, 1H, J_{gem} = 11.0 Hz, $C_6H_5C\underline{H}HO$), 4.813 (d, 1H, J_{gem} = 12.5 Hz, $C_6H_5C\underline{H}HO$), 4.709-4.688 (2H, $C_6H_5C\underline{H}HO$ [δ4.709; d, J_{gem} = 12.5 Hz] overlapping with H-1 [δ4.690, d, $J_{1,2}$ = 2 Hz]), 4.638 (d, 1H, J_{gem} = 11.0 Hz, $C_6H_5C\underline{H}HO$), 4.146-4.064 (2H, Hd and He allyl), 3.913 (dd, 1H, $J_{3,4}$ =

9.5 Hz, $J_{4,5}$ = 9.5 Hz, H-4), 3.840 (ddd, 1H, $J_{6a,6b}$ = 12.0 Hz, $J_{6,OH}$ = 5.5 Hz, $J_{5,6a}$ = 3.0 Hz, D_2O addition resulted in its collapse to dd with $J_{6,OH}$ having disappeared, H-6a), 3.81-3.72 (3H, H-2, H-3, and H-6b), 3.601 (ddd, 1H, $J_{4,5}$ = 9.5 Hz, $J_{5,6a}$ = 3.0 Hz, $J_{5,6b}$ = 5.0 Hz, H-5), 3.298 (s, 3H, CH3O), 2.046 (dd, 1H, J6a,OH = 5.5 Hz, J6b,OH = 7.0 Hz, deuterium exchangeable, 6-OH); ^{13}C nmr ($CDCl_3$) δ: 138.56, 138.37 (quat. arom.), 134.96 (CH_2=$\underline{C}$HCH_2O), 128.36, 128.02, 127.81, 127.67 (tert. arom.), 116.60 ($\underline{C}H_2$=CHCH_2O), 99.47 (C-1), 79.92 (C-3), 75.13 ($C_6H_5\underline{C}H_2O$), 74.88 (C-2 and C-4), 72.99 ($C_6H_5\underline{C}H_2O$), 72.10 (C-5), 71.10 (CH_2=C$\underline{H}CH_2O$), 62.42 (C-6), 54.72 (CH_3O). Anal. calcd. tor $C_{24}H_{30}O_6$: C 69.55, H 7.30; found: C 69.42, H 7.37.

Methyl 6-O-acetyl-3-O-allyl-2,4-di-O-benzyl-α-D-manno-pyranoside (**16**)

To a solution of **14** (10.30 g, 24.85 mmol) in dry pyridine (100 mL) was added acetic anhydride (7.5 mL; 79.54 mmol). The mixture was stirred under nitrogen atmosphere at room temperature overnight. The excess of acetic anhydride was decomposed by dropwise addition of ethanol (5 mL) to the ice cold reaction mixture, and dichloromethane (300 mL) and water (250 mL) were then added. The aqueous layer was separated and back extracted

with dichloromethane (100 mL). The combined dichloromethane extracts were washed with 1 M aqueous HCl and saturated aqueous sodium bicarbonate. The organic phase was dried ($MgSO_4$), filtered, and evaporated under reduced pressure to give **16** (11.06 g; 98%) as a chromatography-pure oil; $[\alpha]_D$ +42.91° (c 1.03, chloroform); R_f 0.45 (Skellysolve B-ethyl acetate, 3:1); ^1H nmr ($CDCl_3$) δ: 7.41-7.25 (10H, aromatic), 5.920 (1H, Hc allyl), 5.309 (1H, Hb allyl), 5.169 (1H, Ha allyl), 4.910 (d, 1H, J_{gem} = 11.0 Hz, $C_6H_5C\underline{H}HO$), 4.778-4.691 (3H, $C_6H_5C\underline{H}_2O$ [δ 4.778-4.691, AB, J_{gem} = 11.0 Hz] overlapping with H-1 [δ 4.718, d, $J_{1,2}$ = 2.0 Hz]), 4.561 (d, 1H, J_{gem} = 11.0 Hz, $C_6H_5C\underline{H}HO$); 4.348-4.268 (2×dd, 2H, $J_{6a,6b}$ = 12.0 Hz, $J_{5,6a}$ = 3.0 Hz, $J_{5,6b}$ = 5.0 Hz, H-6a and H-6b), 4.119-4.025 (2H, Hd and He allyl), 3.844 (dd, 1H, $J_{3,4}$ = 9.0 Hz, $J_{4,5}$ = 9.5 Hz, H-4), 3.781-3.706 (3H, H-3 [δ 3.766, $J_{2,3}$ = 3.5 Hz, $J_{3,4}$ = 9.5 Hz], H-2 [δ 3.743, $J_{1,2}$ = 2.0 Hz, $J_{2,3}$ = 3.5 Hz], and H-5), 3.31 (s, 3H, CH_3O), 2.05 (s, 3H, $OCOCH_3$); ^{13}C nmr ($CDCl_3$) δ: 170.80 $O\underline{C}OCH_3$), 138.34 (quat. arom.), 134.85 ($CH_2=\underline{C}HCH_2O$), 128.36, 128.29, 128.08, 127.70, 127.60 (tert. arom.), 116.70 ($\underline{C}H_2=CHCH_2O$), 99.10 (C-1), 79.89 (C-3), 75.07 ($C_6H_5\underline{C}H_2O$), 74.50, 74.58 (C-2 and C-4), 72.66 ($C_6H_5\underline{C}H_2O$), 70.98 ($CH_2=CH\underline{C}H_2O$), 69.98 (C-5), 63.63 (C-6), 54.73 (CH_3O), 20.81 ($OCO\underline{C}H_3$). Anal. calcd. for $C_{26}H_{32}O_7$: C 68.40, H 7.07; found: C 68.39, H 7.09.

1,6-Di-O-acetyl-3-O-allyl-2,4-di-O-benzyl-α-D-manno-pyranose (**17**)

A solution of concentrated sulfuric acid (0.22 mL) in acetic anhydride (7.68 mL) was added dropwise to a solution of **16** (8.77 g; 19.65 mmol) in acetic anhydride (40 mL) at 0° over 10 min. The mixture was stirred at 0°C for 25 min and at room temperature for 20 min. Then the reaction mixture was poured into dichloromethane (1 L) and ice cold water (1 L) containing sodium bicarbonate, and the resulting mixture was stirred at room temperature for 30 min. The organic and aqueous layers were separated, and the aqueous layer was extracted with dichloromethane (500 mL). The dichloromethane solutions were combined and then washed with saturated aqueous sodium carbonate and water. Finally the organic phase was dried (Na_2SO_4), filtered, and concentrated. The residual syrup was purified by flash chromatography using Skellysolve B-ethyl acetate as eluent, the ratio of Skellysolve B to ethyl acetate being varied from 6:1 to 3:1 during elution. Removal of solvent from the early fractions provided the title compound as an oil (7.35 g; 77% yield); $[\alpha]_D$ +37.85 (c 0.93, chloroform); R_f 0.56 (Skellysolve B-ethyl acetate, 3:1); ^1H nmr ($CDCl_3$) δ: 7.431-7.273 (10H,

aromatic), 6.195 (d, 1H, $J_{1,2}$ = 2.0 Hz, H-1), 5.923 (1H, Hc allyl), 5.315 (1H, Hb allyl), 5.200 (1H, Hc allyl), 4.935 (d, 1H, J_{gem} = 10.5 Hz, $C_6H_5C\underline{H}HO$), 4.840-4.766 (AB, 2H, J_{gem} = 12.0 Hz, $C_6H_5C\underline{H}_2O$), 4.621 (d, 1H, J_{gem} = 10.5 Hz, $C_6H_5C\underline{H}HO$), 4.384-4.310 (2× dd, 2H, H-6a and H-6b), 4.095 (ddd, 2H, CH_2=$CHC\underline{H}_2O$), 3.973 (dd, 1H, $J_{3,4}$ = 10.0 Hz, $J_{4,5}$ = 10.0 Hz, H-4), 3.893 (ddd, 1H, $J_{4,5}$ = 10.0 Hz, $J_{5,6a}$ = 4.0 Hz, $J_{5,6b}$ = 3.0 Hz, H-5), 3.814, 3.785 (2 dd, 2H, $J_{3,4}$ = 10.0 Hz, $J_{2,3}$ = 3.0 Hz, $J_{1,2}$ = 2.0 Hz, H-3 and H-2 respectively), 2.055 (s, 6H, $OCOCH_3$× 2); ^{13}C nmr ($CDCl_3$) δ: 170.82, 168.82 ($O\underline{C}OCH_3$), 138.08, 137.87 (quat. arom.), 134.68 (H_2C=$\underline{C}HCH_2O$), 128.50, 128.40, 128.24, 127.93, 127.88, 127.83 (tert. arom.), 117.04 ($H_2\underline{C}$=$CHCH_2O$), 91.79 (C-1), 79.17 (C-3), 75.36 ($C_6H_5\underline{C}H_2O$), 73,89, 73.39 (C-2 and C-4), 72.53 ($C_6H_5\underline{C}H_2O$), 72.41 (C-5), 71.09 (H_2C=$CH\underline{C}H_2O$), 63.24 (C-6), 20.98, 20.85 ($OCO\underline{C}H_3$). Anal. calcd. for $C_{27}H_{32}O_8$: C 66.93, H 6.66; found: C 66.78, H 6.74.

Evaporation of the subsequent fractions furnished the corresponding β-anomer (**18**) and 1,4,6-tri-O-acetyl-3-O-allyl-2-O-benzyl-α-D-mannopyranose (**19**) in a combined yield of 6%. The ^1H nmr spectra of **18** and **19** were in agreement with the assigned structures.

6-O-Acetyl-3-O-allyl-2,4-di-O-benzyl-α-D-mannopyranosyl bromide (**8**)

Hydrogen bromide gas was bubbled for 30 min through a tube of calcium sulfate into a solution of **17** (5.80 g; 11.97 mmol) in dry dichloromethane (300 mL) at 0°C. The solution was then taken to dryness and the by-product acetic acid removed by evaporation of toluene (100 mL) from the residue (twice). Finally **8** was obtained as an oil, a very small portion of which was purified for elemental and nmr spectral analysis by flash chromatography using Skellysolve B-ethyl acetate (3:1) as eluent; $[\alpha]_D$ +133.08° (c 0.91, chloroform); R_f 0.60 (Skellysolve B-ethyl acetate, 3:1); ^1H nmr ($CDCl_3$) δ: 7.42-7.30 (10H, aromatic), 6.444 (d, 1H, $J_{1,2}$ = 1.5 Hz, H-1), 5.936 (1H, Hc allyl), 5.350 (1H, Hb allyl), 5.233 (1H, Ha allyl), 5.955 (d, 1H, J_{gem} = 10.5 Hz, $C_6H_5C\underline{H}HO$), 4.788-4.718 (AB, 2H, Jgem = 12.5 Hz, $C6H5C\underline{H}_2O$), 4.613 (d, 1H, J_{gem} = 10.5 Hz, $C_6H_5C\underline{H}HO$), 4.388-4058 (2H, H-6a and H-6b), 4.243 (dd, 1H, J3,4 = 9.0 Hz, J2,3 = 3.0 Hz, H-3), 4.116 (d, 2H, J = 5 Hz, $CH_2=CHC\underline{H}_2O$), 4.016-3.935 (3H, H-2 [δ4.011, dd, $J_{2,3}$ = 3.0 Hz, $J_{1,2}$ = 1.5 Hz], H-4 and H-5), 2.065 (s, 3H, $OCOCH_3$); 13C nmr ($CDCl_3$) δ: 170.57 ($O\underline{C}OCH_3$), 137.90, 137.55 (quat. arom.), 134.37 ($CH_2=\underline{C}HCH_2O$), 128.63, 128.44, 128.14, 127.97, 127.87,

127.73 (tert. arom.), 117.38 ($\underline{C}H_2$=$CHCH_2O$), 87.41 (C-1), 78.41 (C-3), 78.31, 74.31, 73.57 (C-2, C-4, and C-5), 75.30, 72.89 ($C_6H_5\underline{C}H_2O$), 71.21 (CH_2=$CH\underline{C}H_2O$), 62.39 (C-6), 20.72 ($OCO\underline{C}H_3$). Anal. calcd. for $C_{25}H_{29}O_6Br$: C 59.41, H 5.78, Br 15.81; found: C 59.48, H 5.82, Br 16.17.

8-Methoxycarbonyloctyl 6-O-acetyl-3-O-allyl-2,4-di-O-benzyl-β-D-mannopyranoside (**23**β) and 8-methoxycarbonyloctyl 6-O-acetyl-3-O-allyl-2,4-di-O-benzyl-α-D-mannopyranoside (**23**α)

8-Methoxycarbonyoctanol[24] (7.50 g; 39.89 mmol) and silver silicate/alumina (16.50 g) in dry dichloromethane (100 mL) were stirred at room temperature for 1 h. To the above mixture which was cooled to -78°C was added dropwise, with stirring, a solution of bromosugar **8** (7.44 g; 14.72 mmol) in dry dichloromethane (75 mL) and stirring was continued for 2.5 h at -78°C, and for 10 h at room temperature. The mixture was diluted with dichloromethane (100 mL) and filtered through celite. The filtrate was washed with water, dried (Na_2SO_4), and concentrated to a syrup which was purified by flash chromatography, using Skellysolve B-ethyl acetate (4.5:1) as eluent. A chromatographically inseparable 6:1 mixture of β- and α-mannosides **23** was obtained as an oil (5.14 g; total yield

57%). ^1H nmr ($CDCl_3$) δ: 4.824 (d, $J_{1,2}$ = 1.5 Hz, H-1 of α-anomer), 4.365 (d, $J_{1,2}$ = 0.5 Hz, H-1 of β-anomer), 2.059 (s, $OCOCH_3$ of α-anomer), 2.050 (s, $OCOCH_3$) of β-anomer).

8-Methoxycarbonyloctyl 3-O-allyl-2,4-di-O-benzyl-β-D-mannopyranoside (**24**) and 8-methoxycarbonyloctyl 3-O-allyl-2,4-di-O-benzyl-α-D-mannopyranoside (**25**)

The mixture of α- and β-anomers **23** described above (4.39 g; 7.17 mmol) was dissolved in dry methanol (200 mL) containing a trace of sodium methoxide, and the resulting solution was stirred at room temperature overnight. Neutralization with Amberlite IR-120(H) resin followed by the removal of the resin and evaporation provided a chromatographically pure oily residue (4.10 g) whose tlc on silica gel impregnated with silver nitrate showed it to be a mixture of two compounds. Purification of these two products was accomplished by flash chromatography on silver nitrate-impregnated silica gel using Skellysolve B-ethyl acetate as eluent, the ratio of Skellysolve B to ethyl acetate being decreased from 7:1 to 2:1 during elution. Evaporation of early fractions furnished the α-anomer **25** (0.19 g) as an oil; $[\alpha]_D$ +30.8° (c 0.75, chloroform); ^1H nmr ($CDCl_3$) δ: 7.42-7.27 (10H, aromatic),

5.965 (1H, Hc of allyl), 5.345 (1H, Hb allyl), 5.194 (1H, Ha allyl), 4.945 (d, 1H, J_{gem} = 11.0 Hz, $C_6H_5\underline{C}\underline{H}HO$), 4.843-4.788 (2H, $C_6H_5\underline{C}\underline{H}HO$ [δ 4.828, d, J_{gem} = 12.0 Hz] and H-1 [δ 4.790, d, $J_{1,2}$ = 2.0 Hz], 4.724 (d, 1H, J_{gem} = 12.0 Hz, $C_6H_5\underline{C}\underline{H}HO$), 4.655 (d, 1H, J_{gem} = 11.0 Hz, $C_6H_5\underline{C}\underline{H}HO$), 4.138 (broad d, 2H, Hd and He allyl), 3.923 (dd, 1H, $J_{4,5}$ = 9.5 Hz, $J_{3,4}$ = 9.5 Hz, H-4), 3.870-3.579 (9H, H-2, H-3, H-5, H-6a, H-6b, $O\underline{C}\underline{H}HCH_2$, and OCH_3 [δ 3.665, s]), 3.329 (dt, 1H, J_{gem} = 9.0 Hz, J_{vic} = 6.5 Hz, $O\underline{C}\underline{H}HCH_2$), 2.303 (t, 2H, $\underline{C}\underline{H}_2COOCH_3$, J = 7.5 Hz), 2.098 (broad s, 1H, exchangeable with deuterium, OH), 1.620 (m, 2H, aliphatic), 1.510 (m, 2H, aliphatic), 1.29 (broad s, 8H, remaining aliphatic); ^{13}C nmr ($CDCl_3$) δ: 174.15 ($\underline{C}OOCH_3$), 138.48, 138.43 (quat. arom.), 134.95 ($CH_2=\underline{C}HCH_2O$), 128.35, 128.29, 128.07, 127.72, 127.67, 127.58 (tert. arom.), 116.47 ($\underline{C}H_2=CHCH_2O$), 98.25 (C-1, $J_{C-1,H-1}$ = 168.2 Hz), 79.98 (C-3), 75.18 ($C_6H_5\underline{C}H_2O$), 75.04, 75.01 (C-3 and C-4), 72.88 ($C_6H_5\underline{C}H_2O$), 72.08 (C-5), 71.07 ($CH_2=CH\underline{C}H_2O$), 67.61 ($O\underline{C}H_2CH_2$), 62.46 (C-6), 51.33 ($COO\underline{C}H_3$), 34.01 ($\underline{C}H_2COOCH_3$), 29.32, 29.11, 29.06, 29.01, 25.99, 24.85 (aliphatic). Anal. calcd. for $C_{33}H_{46}O_8$: C 69.45, H 8.12; found: c 69.27, H 8.26.

Further elution provided a mixture of α- and β-anomers (0.9 g) which were in the ratio of 1:3 (α/β) by ^1H nmr.

Evaporation of the later fractions afforded the

desired β-anomer **24** (2.89 g) as an oil; $[\alpha]_D$ -51.33 (c 0.9, chloroform); ^{1}H nmr ($CDCl_3$) δ: 7.49-7.29 (10H, aromatic), 5.893 (1H, Hc allyl), 5.288 (1H, Hb allyl), 5.17 (1H, Ha allyl), 4.969-4.858 (3H, $C_6H_5C\underline{H}_2O$ [δ 4.969-4.800, AB, J_{gem} = 12.5 Hz] and $C_6H_5C\underline{H}HO$ [4.935, d, J_{gem} = 10.5 Hz]), 4.624 (d, 1H, J_{gem} = 10.5 Hz, $C_6H_5C\underline{H}HO$), 4.408 (d, 1H, J = 1 Hz, H-1), 4.024-3.828 (6H, H-2, H-4, H-6a, $OC\underline{H}H\underline{C}H_2$, and $CH_2{=}CHC\underline{H}_2O$), 3.763 (ddd, 1H, $J_{6a,6b}$ = 12.0 Hz, $J_{5,6b}$ = 6.0 Hz, $J_{6b,OH}$ = 6.0 Hz, simplified to dd with $J_{6b,OH}$ being absent on D_2O exchange, H-6b), 3.688 (s, 3H, OCH_3), 3.444-3.386 (2H, H-3 and $OC\underline{H}H\underline{C}H_2$), 3.316 (dt, 1H, $J_{4,5}$ = 9.5 Hz, $J_{5,6b}$ = 6.0 Hz, $J_{5,6a}$ = 3.0 Hz, H-5), 2.330 (t, 2H, $C\underline{H}_2COOCH_3$, J = 7.5 Hz), 2.140 (t, 1H, $J_{6a,OH}$ = 6.0 Hz, $J_{6b,OH}$ = 6.0 Hz, disappeared after D_2O addition, OH), 1.670-1.628 (4H, aliphatic), 1.345 (8H, remaining aliphatic); ^{13}C nmr ($CDCl_3$) δ: 174.50 ($\underline{C}OOCH_3$), 138.66, 138.36 (quat. arom.), 134.70 ($CH_2{=}\underline{C}HCH_2O$), 128.37, 128.28, 128.11, 128.03, 127.73, 127.37 (tert. arom.), 116.75 ($\underline{C}H_2{=}CHCH_2O$), 101.69 (C-1, $J_{C-1,H-1}$ = 153.6 Hz), 82.33 (C-3), 75.77, 74.88, 73.90 (C-2, C-4, and C-5), 75.18, 73.86 ($C_6H_5\underline{C}H_2O$), 70.58, 70.12 ($CH_2{=}CH\underline{C}H_2O$ and $O\underline{C}H_2CH_2$), 62.63 (C-6), 51.36 ($COO\underline{C}H_3$), 34.04 ($\underline{C}H_2COOCH_3$), 29.63, 29.16, 29.04, 26.01, 24.89 (aliphatic). Anal. calcd. for $C_{33}H_{46}O_8$: C 69.45, H 8.12; found: C 69.41, H 8.13.

8-Methoxycarbonyloctyl 6-O-(2-O-acetyl-3,4,6-tri-O-benzyl-α-D-mannopyranosyl)-3-O-allyl-2,4-di-O-benzyl-β-D-mannopyranoside (**27**)

To a solution of **24** (2.89 g; 5.06 mmol) in dry acetonitrile (60 mL) containing 4Å molecular sieves were added, sequentially, mercuric bromide (2.19 g; 6.07 mmol), mercuric cyanide (1.53 g; 6.07 mmol), and a solution of 2-O-acetyl-3,4,6-tri-O-benzyl-α-D-mannopyranosyl bromide[27] (**7**) in dry acetonitrile (25 mL), which had been freshly prepared from 3,4,6-tri-O-benzyl-1,2-O-(methoxyethylidene)-β-D-mannopyranose (**26**) (3.07 g; 6.07 mmol). The reaction mixture was stirred at room temperature for 1 h, and the mixture was then filtered through Celite. Evaporation of the solvent gave an oily residue which was extracted 3 times with dichloromethane. The extracts were combined and washed successively with saturated aqueous potassium chloride, saturated aqueous sodium bicarbonate, water, and brine. The organic layer was dried (Na_2SO_4), filtered, and evaporated to dryness. The resulting oil was purified by flash chromatography using Skellysolve B-ethyl acetate (4:1) as eluent to provide the title compound as a syrup (4.07 g; 77%); $[\alpha]_D$ -6.07° (*c* 1.22,

chloroform); R_f 0.3 (Skellysolve B-ethyl acetate, 3:1); ^1H nmr ($CDCl_3$) δ: 7.47-7.10 (25H, aromatic), 5.86 (1H, Hc allyl), 5.459 (dd, 1H, $J_{2',3'}$ = 3.0 Hz, $J_{1',2'}$ = 2.0 Hz, H-2'), 5.270 (1H, Hb allyl), 5.159 (1H, Ha allyl), 4.980-4.825 (5H, H-1' [δ 4.911] and 4 × $C_6H_5C\underline{H}HO$ [d, J_{gem} = 11.0-12.5 Hz]), 4.680-4.650 (2 d overlapping, 2H, J_{gem} = 10.5 and 12.0 Hz, $C_6H_5C\underline{H}_2O$), 4.513-4.408 (4×d, 4H, J_{gem} = 11.0-12.0 Hz, $C_6H_5C\underline{H}_2O$), 4.334 (s, 1H, H-1), 3.930-3.590 (15H, $COOCH_3$ [δ 3.66], H-2, H-4, H-6a, H-6b, H-3', H-4', H-5', H-6a', H-6b', $CH_2{=}CHC\underline{H}_2O$ and $OC\underline{H}HCH_2$), 3.410-3.315 (3H, H-3, H-5, and $OC\underline{H}HCH_2O$), 2.283 (t, 2H, J = 7.5 Hz, $C\underline{H}_2COOCH_3$), 2.133 (s, 3H, $OCOCH_3$), 1.620-1.513 (4H, aliphatic), 1.335-1.240 (8H, remaining aliphatic); ^{13}C nmr ($CDCl_3$) δ: 174.08 ($\underline{C}OOCH_3$), 170.12 ($O\underline{C}OCH_3$), 139.00, 138.80, 138.53, 138.46, 138.05 (quat. arom.), 134.79 ($CH_2{=}\underline{C}HCH_2O$), 128.27, 128.19, 128.13, 128.10, 127.97, 127.77, 127.70, 127.59, 127.54, 127.50, 127.38, 127.29, 127.23 (tert. arom.), 116.67 ($\underline{C}H_2{=}CHCH_2O$), 101.64 (C-1, $J_{C-1,H-1}$ = 154.7 Hz), 97.77 (C-1', $J_{C-1',H-1'}$ = 170.4 Hz), 82.56 (C-3), 77.94 (C-3'), 74.80, 74.37, 73.91 (C-2, C-4, C-5, and C-4'), 71.36 (C-5'), 68.67 (C-2'), 74.91, 73.71, 73.30, 71.51 ($C_6H_5\underline{C}H_2O$ × 5), 70.43, 69.79 ($CH_2{=}CH\underline{C}H_2O$ and $O\underline{C}H_2CH_2$), 68.98 (C-6'), 67.06 (C-6), 51.26 ($COO\underline{C}H_3$), 34.05 ($\underline{C}H_2COOCH_3$), 29.65, 29.23, 29.16, 29.07, 26.09, 24.92 (aliphatic), 20.99 ($OCO\underline{C}H_3$). Anal. calcd. for

$C_{62}H_{76}O_{14}$: C 71.24, H 7.33; found: 71.09, H 7.46.

8-Methoxycarbonyloctyl 3-O-allyl-2,4-di-O-benzyl-6-O-(3,4,6-tri-O-benzyl-α-D-mannopyranosyl)-β-D-mannopyranoside (**28**)

The disaccharide **27** (2.88 g; 2.76 mmol) was de-O-acetylated as described for the preparation of **25** and **26**. After removal of the resin by filtration the solvent was evaporated to afford **28** as chromatographically pure oil (2.73 g; 99%); $[\alpha]_D$ +3.13° (c 1.34, chloroform); R_f 0.32 (Skellysolve B-acetone, 3:1); ^1H nmr ($CDCl_3$) δ: 7.48-7.14 (25H, aromatic), 5.883 (1H, Hc allyl), 5.288 (1H, Hb allyl), 5.170 (1H, Ha allyl), 5.258 (broad s, 1H, $J_{1',2'}$ < 2Hz, H-1'), 4.968-4.795 (4×d, 4H, J_{gem} = 11.0 and 12.5 Hz, $C_6H_5\underline{CH_2}O$), 4.644-4.438 (6×d, 6H, J_{gem} = 11.0-12.0 Hz, $C_6H_5\underline{CH_2}O$), 4.335 (s, 1H, $J_{1,2}$ < 1 Hz, H-1), 4.128 (broad s, 1H, H-2'), 4.038-3.593 (15H, OCH_3 [δ3.655, s], H-2, H-4, H-6a, H-6b, H-3', H-4', H-5', H-6a', H-6b', $OCHH\underline{CH_2}$, and $CH_2{=}CH\underline{CH_2}O$), 3.423-3.320 (3H, H-3, H-5, and $OC\underline{H}HCH_2$), 2.355 (broad s, 1H, deuterium-exchangeable, OH), 2.284 (t, 2H, J = 7.5 Hz, $\underline{CH_2}COOCH_3$), 1.59 (4H, aliphatic), 1.28 (8H, remaining aliphatic); ^{13}C nmr ($CDCl_3$) δ: 173.86 ($\underline{C}OOCH_3$), 139.04, 138.73, 138.59, 138.44, 138.03 (quat. arom.), 134.86 ($CH_2{=}\underline{C}HCH_2O$), 128.49, 128.36, 128.29,

128.24, 128.07, 127.99, 127.89, 127.78, 127.62, 127.48, 127.40 (tert. arom.), 116.84 ($\underline{C}H_2$=CH$\underline{C}H_2$O), 101.73 (C-1), 99.77 (C-1'), 82.47 (C-3), 79.69 (C-3'), 75.24, 74.61, 74.36, 74.06 (C-3, C-4, C-5, and C-4'), 75.07, 74.94, 73.88, 73.41, 71.48 ($C_6H_5\underline{C}H_2$O), 71.07 (C-5'), 70.54, 69,88 (CH_2=C$\underline{H}\underline{C}H_2$O and O$\underline{C}H_2\underline{C}H_2$), 69.03 (C-6'), 68.01 (C-2'), 66.65 (C-6), 51.43 (COO$\underline{C}H_3$), 34.12 ($\underline{C}H_2$COOCH$_3$), 29.72, 29.29, 29.25, 29.14, 26.15, 24.99 (aliphatic). Anal. calcd. for $C_{60}H_{74}O_{13}$: C 71.83, H 7.44; found: C 71.59; H 7.31.

8-Methoxycarbonyloctyl 6-O-[2-O-(3,4,6-tri-O-acetyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl)-3,4,6-tri-O-benzyl-α-D-mannopyranosyl]-3-O-allyl-2,4-di-O-benzyl-β-D-mannopyranoside (**4**)

To a solution of **28** (2.73 g; 2.73 mmol) in dry dichloromethane (75 mL) were added silver triflate (7.0 g; 27.25 mmol), sym-collidine (3.6 mL, 27.25 mmol), and 4 Å molecular sieves. To the resulting mixture, cooled to -50°C, was added dropwise a solution of 3,4,6-tri-O-acetyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl bromide[26] (**6**) (2.30 g; 2.60 mmol) in dry dichloromethane (25 mL). The above mixture was stirred at -50°C for 15 min and then allowed to warm to room temperature over a period of 1

h. The tlc of the mixture revealed the presence of unreacted alcohol **28** (~30%). The reaction mixture was again cooled to -50°C and a solution of the bromide **6** (2.30 g; 2.60 mmol) in dry dichloromethane (25 mL) was added dropwise. After stirring at -50°C for 15 min, the mixture was allowed to attain room temperature, with stirring, during 1 h. The tlc now showed the complete disappearance of the alcohol **28**. The mixture was diluted with dichloromethane, and then filtered through Celite. The filtrate was washed sequentially with ice water, ice cold 1N aqueous HCl, and saturated aqueous sodium bicarbonate. The organic phase was dried (Na_2SO_4), filtered, and evaporated. The residual oil was subjected to flash chromatography employing toluene-ethyl acetate (3.5:1) as eluent. The fractions containing the major product were combined and evaporated. The tlc of the oil so obtained, when developed in Skellysolve B-acetone (3:1), also indicated the presence of a minor product. Therefore, the above oil was again chromatographed using Skellysolve B-acetone (3:1) as eluent to provide the α-linked trisaccharide **29** as an oil (0.27 g; 7%). Further elution with the same solvent system gave the title compound as a syrup (2.92 g; 76%); $[\alpha]_D$ +23.04° (*c* 1.02, chloroform); R_f 0.45 (toluene-ethyl acetate, 3:1), 0.17 (Skellysolve B-acetone, 3:1); ^1H nmr ($CDCl_3$) δ: 7.86-7.04

(29H, aromatic), 5.901-5.790 (2H, Hc allyl and H-3" [δ5.815, $J_{3",4"}$ = 9.0 Hz, $J_{2",3"}$ = 11.0 Hz]), 5.573 (d, 1H, $J_{1",2"}$ = 8.5 Hz, H-1"), 5.303-5.143 (3H, H-4" [δ5.215, dd, $J_{4",5"}$ = 10.0 Hz, $J_{3",4"}$ = 9.0 Hz] and Ha and Hb allyl), 4.993 (d, 1H, J_{gem} = 13.0 Hz, $C_6H_5C\underline{H}HO$), 4.890-4.735 (4×d, 4H, J_{gem} = 11.0-12.5 Hz, $C_6H_5C\underline{H}_2O$), 4.676 (d, 1H, $J_{1',2'}$ = 1.5 Hz, H-1'), 4.538-4.459 (2H, H-2" [δ4.513, dd, $J_{2",3"}$ = 11.0 Hz, $J_{1",2"}$ = 8.5 Hz] and $C_6H_5C\underline{H}HO$ [δ4.475, d, J_{gem} = 12.0 Hz]), 4.400 (dd, 1H, $J_{2',3'}$ = 2.5 Hz, $J_{1',2'}$ = 2.5 Hz, H-2'), 4.373-4.303 (4H, H-1 [δ4.344], H-6a", and $C_6H_5C\underline{H}_2O$), 4.233 (dd, 1H, $J_{6a",6b"}$ = 12.5 Hz, $J_{5",6b"}$ = 2.0 Hz, H-6b"), 4.063-3.245 (20H, $OC\underline{H}HCH_2$ [δ4.035, td, J_{gem} = 9.0 Hz, J_{vic} = 6.0 Hz], OCH_3 [δ3.668, s], CH_2=CHC$\underline{H}_2$O, $C_6H_5C\underline{H}_2O$, H-2, H-3, H-4, H-5, H-6a, H-6b, H-3', H-4', H-5', H-6a', H-5", $OC\underline{H}HCH_2$), 2.989 (dd, 1H, $J_{6a',6b'}$ = 11.0 Hz, $J_{5',6b'}$ = 5.5 Hz, H-6b'), 2.31 (t, 2H, $C\underline{H}_2COOCH_3$, J = 7.5 Hz), 2.055 (s, 3H, $OCOCH_3$), 2.024 (s, 3H, $OCOCH_3$), 1.863 (s, 3H, $OCOCH_3$), 1.70-1.59 (4H, aliphatic), 1.44-1.26 (8H, remaining aliphatic); ^{13}C nmr ($CDCl_3$) δ: 173.96 ($\underline{C}OOCH_3$), 170.42, 169.89, 169.21 ($O\underline{C}OCH_3$ and phthalimido carbonyl), 138.69, 138.52, 138.36, 137.80 (benzyl quat. arom.), 134.55 (CH_2=$\underline{C}$HCH_2O), 133.74 (phthalimido tert. arom.), 131.52 (phthalimido quat. arom.), 128.31, 128.19, 128.00, 127.92, 127.86, 127.63, 127.47, 127.43, 127.25, 127.19, 126.99 (benzyl tert.

arom.), 123.16 (phthalimido tert. arom.), 116.52 ($\underline{C}H_2$=CHCH_2O), 101.79 (C-1, $J_{C-1,H-1}$ = 152.8 Hz), 97.44 (C-1', $J_{C-1',H-1'}$ = 169.2 Hz), 96.48 (C-1", $J_{C-1",H-1"}$ = 165.1 Hz), 82.64 (C-3), 76.81, 74.54, 74.09, 73.75, 73.49, 72.88, 71.89, 71.45, 70.62, 69.01 (C-2, C-4, C-5, C-2', C-3', C-4', C-5', C-3", C-4", C-5"), 74.64, 73.63, 72.41, 70.33 ($C_6H_5\underline{C}H_2O$), 70.11 (CH_2=CH$\underline{C}H_2O$ and O$\underline{C}H_2CH_2$), 69.40 (C-6'), 66.53 (C-6), 62.17 (C-6"), 54.25 (C-2"), 51.17 (COO$\underline{C}H_3$), 33.86 ($\underline{C}H_2COOCH_3$), 29.47, 29.10, 29.04, 28.90, 25.91, 24.74 (aliphatic), 20.56, 20.43, 20.24 (OCO$\underline{C}H_3$). Anal. calcd. for $C_{80}H_{93}N_1O_{22}$: C 67.64, H 6.60, N 0.99; found: C 67.54, H 6.58, N 0.97.

8-Methoxycarbonyloctyl 6-O-[2-O-3,4,6-tri-O-acetyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl)-3,4,6,-tri-O-benzyl-α-D-mannopyranosyl]-2,4-di-O-benzyl-β-D-mannopyranoside (31)

A solution of **4** (2.79 g; 1.97 mmol), tris(triphenylphosphine)rhodium(I) chloride (129 mg; 0.14 mmol), 1,8-diazabicyclo[2.2.2]octane (58 mg; 0.51 mmol) in ethanol-benzene-water (7:3:1; 100 mL) was heated at reflux for 24 h. The solvent was removed and the residue dissolved in acetone (100 mL) containing a trace amount of mercuric oxide. To this solution was added a solution of

mercuric chloride (3.0 g) in acetone-water (9:1; 50 mL), and the mixture was stirred at room temperature for 45 min. Following evaporation of the solvent, the residue was taken up in dichloromethane (250 mL). The dichloromethane solution was washed with 30% aqueous potassium bromide and water. The organic layer was dried (Na_2SO_4) and evaporated to give an oily residue which was purified by flash chromatography using toluene-ethyl acetate (3.5:1) as eluent. The title compound was obtained as a white foamy solid (2.31 g; 85%); $[\alpha]_D$ -19.07° (c 0.97, chloroform); R_f 0.37 (toluene-ethyl acetate, 3:1); ^1H nmr ($CDCl_3$) δ: 7.88-7.07 (29H, aromatic), 5.84 (dd, 1H, $J_{3",4"}$ = 9.0 Hz, $J_{2",3"}$ = 11.0 Hz, H-3"), 5.61 (d, 1H, $J_{1",2"}$ = 8.5 Hz, H-1"), 5.221 (dd, 1H, $J_{4",5"}$ = 9.0 Hz, $J_{3",4"}$ = 9.0 Hz, H-4"), 5.063 (d, 1H, J_{gem} = 12.0 Hz, $C_6H_5C\underline{H}HO$), 4.853 (d, 1H, J_{gem} = 11.0 Hz, $C_6H_5C\underline{H}HO$), 4.778-4.733 (2×d, 2H, J_{gem} = 11.0 and 12.0 Hz, $C_6H_5C\underline{H}_2O$), 4.673 (d, 1H, $J_{1',2'}$ = 2.0 Hz, H-1'), 4.633 (d, 1H, J_{gem} = 12.0 Hz, $C_6H_5C\underline{H}HO$), 4.543-4.476 (2H, H-2" [δ4.518, dd, $J_{2",3"}$ = 11.0 Hz, $J_{1",2"}$ = 8.5 Hz], $C_6H_5C\underline{H}HO$ [δ4.491, d, J_{gem} = 12.0 Hz]), 4.433 (1H, $J_{1,2}$ < 1 Hz, H-1), 4.385-4.240 (5H, H-2', H-6a", H-6b" and $C_6H_5C\underline{H}_2O$), 4.035-3.935 (4H, $OC\underline{H}HCH_2$, H-5", and $C_6H_5C\underline{H}_2O$), 3.828-3.245 (14H, H-2, H-3, H-4, H-5, H-6a, H-6b, H-3', H-4', H-5', H-6a', $OC\underline{H}HCH_2$ and OCH_3 [δ3.651, s]), 2.998 (dd, 1H,

$J_{6a',6b'}$ = 11.0 Hz, $J_{5',6b'}$ = 5.5 Hz, H-6b'), 2.380 (d, 1H, deuterium-exchangeable, $J_{OH,H-3}$ = 10.0 Hz, OH), 2.290 (t, 2H, $C\underline{H}_2COOCH_3$, J = 7.5 Hz), 2.056,2.050 (2×s, 6H, $OCOCH_3$), 1.860 (s, 3H, $OCOCH_3$), 1.68-1.58 (4H, aliphatic), 1.40-1.26 (8H, remaining aliphatic); ^{13}C nmr ($CDCl_3$) δ: 174.17 ($\underline{C}OOCH_3$), 170.63, 170.12, 169.40 ($O\underline{C}OCH_3$ and phthalimido carbonyl), 138.77, 138.50, 138.37, 138.30, 138.10 (benzyl quat. arom.), 133.95 (phthalimido tert. arom.), 131.82 (phthalimido quat. arom.), 128.56, 128.36, 128.23, 128.13, 128.08, 127.72, 127.62, 127.59, 127.39, 127.21 (benzyl tert. arom.), 123.37 (phthalimido tert. arom.), 101.96 (C-1), 97.25 (C-1'), 96.61 (C-1"), 77.90, 77.22, 76.27, 74.39, 74.34, 74.07, 73.22, 72.12, 71.61, 70.80, 69.14 (C-2, C-3, C-4, C-5, C-2', C-3', C-4', C-5', C-3", C-4" and C-5"), 75.06, 74.83, 74.44, 72.63, 70.49 ($C_6H_5\underline{C}H_2O$), 70.34 ($O\underline{C}H_2CH_2$), 69.50 (C-6'), 66.58 (C-6), 62.33 (C-6"), 54.44 (C-2"), 51.38 ($COO\underline{C}H_3$), 34.05 ($\underline{C}H_2COOCH_3$), 29.61, 29.24, 29.19, 29.08, 26.06, 24.92 (aliphatic), 20.74, 20.62, 20.44 ($OCO\underline{C}H_3$). Anal. calcd. for $C_{77}H_{89}N_1O_{22}$: C 66.99, H 6.50, N 1.01; found: C 66.74, H 6.43, N 0.84.

8-Methoxycarbonyloctyl 6-O-[2-O-(2-deoxy-2-phthalimido-β-D-glucopyranosyl)-3,4,6-tri-O-benzyl-α-D-mannopyranosyl]-2,4-di-O-benzyl-β-D-mannopyranoside (**32**)

To a solution of **31** (0.82 g; 0.59 mmol) in dry methanol (75 mL) was added a trace of solid sodium methoxide. The mixture was stirred at room temperature for 3 h. Neutralization of the mixture with Amberlite IR-120(H^+) resin followed by filtration and evaporation afforded **32** as a white foamy solid in a quantitative yield; $[\alpha]_D$ -31.54° (c 1.3, chloroform); R_f 0.2 (dichloromethane-methanol, 19:1); ^1H nmr ($CDCl_3$) δ: 7.66-7.06 (29H, aromatic), 5.399 (d, 1H, $J_{1",2"}$ = 8.0 Hz, H-1"), 5.035 (d, 1H, J_{gem} = 12.0 Hz, C_6H_5CHHO), 4.824-4.534 (6H, H-1' [δ 4.643] and $C_6H_5CH_2O$ [5×d, J_{gem} = 12.5-11.0 Hz]), 4.408-4.241 (6H, H-1 [δ 4.408], H-2' [δ 4.248, dd, $J_{2',3'}$ = 2 Hz, $J_{1',2'}$ = 2 Hz], H-2", H-3", and $C_6H_5CH_2O$ [J_{gem} = 11.0 Hz]), 4.020-3.208 (21H, H-2, H-3, H-4, H-5, H-6a, H-6b, H-3', H-4', H-5', H-6a', H-4", H-5", H-6a", H-6b", OCH_2CH_2, $C_6H_5CH_2O$ and OCH_3 [δ 3.620, s]), 3.063-2.900 (2H, H-6b' [δ 2.918, dd, $J_{6a',6b'}$ = 10.5 Hz, $J_{5',6b'}$ = 5.5 Hz] and OH [broad s, disappeared on D_2O exchange]), 2.396 (d, 1H, $J_{3,OH}$ = 10.0 Hz, deuterium-exchangeable, 3-OH), 2.269 (t, 2H, J = 8.0 Hz, CH_2COOCH_3), 1.65-1.55 (4H, aliphatic), 1.39-1.26 (remaining aliphatic); ^{13}C nmr ($CDCl_3$) δ:

174.43 ($\underline{C}OOCH_3$), 168.51 (phthalimido carbonyl), 138.68, 138.52, 138.35, 138.32, 138.05 (benzyl quat. arom.), 133.79 (phthalimido tert. arom.), 131.84 (phthalimido quat. arom.), 128.58, 128.38, 128.34, 128.26, 128.12, 128.03, 127.80, 127.70, 127.49, 127.44, 127.22 (benzyl tert. arom.), 123.23 (phthalimido tert. arom.), 101.90 (C-1), 97.49 (C-1'), 96.84 (C-1"), 77.69, 77.26, 76.31, 75.65, 74.28, 74.13, 73.38, 72.07, 71.73 (C-2, C-3, C-4, C-5, C-2', C-3', C-4', C-5', C-3", C-4", and C-5"), 74.87, 74.77, 74.48, 72.63, 70.86 ($C_6H_5\underline{C}H_2O$), 70.30 ($O\underline{C}H_2CH_2$), 69.70 (C-6'), 66.65 (C-6), 62.21 (C-6"), 56.54 (C-2"), 51.45 ($COO\underline{C}H_3$), 34.07 ($\underline{C}H_2COOCH_3$), 29.61, 29.17, 29.04, 26.04, 24.88 (aliphatic). Anal. calcd. for $C_{71}H_{83}N_1O_{19}$: C 67.98, H 6.67, N 1.12; found: C 67.43, H 6.60, N 1.10.

8-Methoxycarbonyloctyl 6-O-[2-O-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-3,4,6-tri-O-benzyl-α-D-mannopyranosyl]-2,4-di-O-benzyl-β-D-mannopyranoside (33).

Compound 32 (0.43 g; 0.34 mmol) in methanol (25 mL) was boiled with hydrazine hydrate (0.14 mL of an 85% solution; 2.6 mmol) for 12 h. The tlc showed the presence of the starting material in the reaction mixture. Therefore, more hydrazine hydrate (0.07 mL of an 85% solution, 1.3 mmol) was added and the mixture refluxed for

another 4 h. The solution was evaporated and the residue thoroughly dried to remove traces of hydrazine. The residue was then dissolved in methanol-water (1:1, 15 mL) and acetic anhydride (1 mL) was added. The resulting solution was stirred at room temperature for 2 h. Removal of solvent gave a white solid which was purified by flash chromatography using dichloromethane-methanol (12:1) as eluent. Since the crude product was not completely soluble in the eluent used, it was dissolved using more methanol in the same solvent mixture and then adsorbed on sodium sulfate which was poured onto the top of the silica packing. Pure **33** was obtained as a white foamy solid (0.24 g; 60%); $[\alpha]_D$ -20.46° (c 0.88, chloroform); R_f 0.61 (dichloromethane-methanol, 10:1); ^1H nmr ($CDCl_3$) δ: 7.42-7.16 (25H, aromatic), 5.90-5.65 (broad s, 1H, NH, deuterium-exchangeable), 5.053 (d, 1H, J_{gem} = 12.0 Hz, $C_6H_5C\underline{H}HO$), 4.913-4.825 (3H, $C_6H_5C\underline{H}_2O$ [2×d, J_{gem} = 11.0 Hz] and H-1' [δ4.910, d, $J_{1',2'}$ = 2.0 Hz]), 4.743 (d, 1H, J_{gem} = 12.0 Hz, $C_6H_5C\underline{H}HO$), 4.688-4.574 (3H, H-1" and $C_6H_5C\underline{H}_2O$ [2×d, J_{gem} = 12.0 Hz]), 4.488-4.325 (5H, H-1 [δ4.435] and $C_6H_5C\underline{H}_2O$ [4×d, J_{gem} = 12.0 and 11.0 Hz]), 4.253 (t, 1H, H-2'), 3.995-3.293 (23H, H-2, H-3, H-4, H-5, H-6a, H-6b, H-3', H-4', H-5', H-6a', H-6b', H-2", H-3", H-4", H-5", H-6a", H-6b", OH, $OC\underline{H}_2CH_2$, and OCH_3 [δ3.643, s]), 3.00-2.65 (broad s, 2H, disappeared on D_2O exchange, OH), 2.501

(d, 1H, $J_{3,OH}$ = 9.0 Hz, disappeared on D_2O exchange, 3-OH), 2.276 (t, 2H, J = 7.0 Hz, $\underline{CH_2}COOCH_3$), 1.840 (s, 3H, $NHCOCH_3$), 1.64-1.52 (4H, aliphatic), 1.36-1.22 (8H, remaining aliphatic); ^{13}C nmr ($CDCl_3$) δ: 174.10 ($\underline{C}OOCH_3$), 172.37 ($NH\underline{C}OCH_3$), 138.60, 138.39, 138.30, 137.93 (quat. arom.), 128.50, 128.34, 128.27, 128.17, 127.93, 127.73, 127.65, 127.58, 127.44 (tert. arom.), 101.90 (C-1'), 99.23 (C-1'), 97.66 (C-1"), 77.62, 76.25, 75.83, 74.58, 74.39, 74.13, 73.71, 73.46, 71.64 (C-2, C-3, C-4, C-5, C-2', C-3', C-4', C-5', C-3", C-4", and C-5"), 74.75, 74.45, 73.20, 71.50, 70.30 ($C_6H_5\underline{CH_2}O$), 68.85 (C-6'), 66.86 (C-6), 62.66 (C-6"), 59.07 (C-2"), 51.26 ($COO\underline{CH_3}$), 33.99 ($\underline{CH_2}COOCH_3$), 29.57, 29.11, 28.99, 25.99, 24.84 (aliphatic), 23.30 ($NHCO\underline{CH_3}$). Anal. calcd. for $C_{65}H_{83}N_1O_{18}$: C 66.94, H 7.17, N 1.20; found: C 66.81, H 7.09, N 1.13.

8-Methoxycarbonyloctyl 6-O-[2-O-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-α-D-mannopyranosyl]-β-D-mannopyranoside (2).

Compound **33** (45 mg; 0.03 mmol) was dissolved in 98% ethanol (9 mL), and 5% palladium-on-charcoal (45 mg) was added. The mixture was stirred under an atmosphere of hydrogen gas for 52 h. The catalyst was removed by

filtration and, after solvent evaporation, the residue was passed through a column of Bio-Gel P2 (2.5 cm × 47 cm) using 10% aqueous ethanol as eluent. The carbohydrate-containing fractions were pooled, concentrated, and lyophilized to provide 2 as a white powder (23.5 mg; 85%); $[\alpha]_D$ -19.34° (c 0.91, water); R_f 0.57 (dichloromethane-methanol-water, 10:6:1); ^1H nmr (D_2O) δ: 4.920 (1H, $J_{1',2'}$ = 1.8 Hz, H-1'), 4.664 (1H, $J_{1,2}$ < 0.7 Hz, H-1), 4.579 (1H, $J_{1'',2''}$ = 8.0 Hz, H-1"), 4.129 (dd, 1H, $J_{1',2'}$ = 1.4 Hz, $J_{2',3'}$ = 3.4 Hz, H-2'), 3.988-3.401 [22H, H-2 (δ3.981, $J_{2,3}$ = 3.0 Hz), H-3' (δ3.844, $J_{3',4'}$ = 9.5 Hz, $J_{2',3'}$ = 3.5 Hz), H-2" (δ3.706, $J_{1'',2''}$ = 8.0 Hz), H-3, H-4, H-5, H-6a, H-6b, H-4', H-5', H-6a', H-6b', H-3", H-4", H-5", H-5a", H-6b", $O\underline{CH_2}CH_2$, and OCH_3 (δ3.690)], 2.388 (t, 2H, J = 7.5 Hz, $\underline{CH_2}COOCH_3$), 2.056 (s, 3H, $NHCOCH_3$), 1.68-1.50 (4H, aliphatic), 1.38-1.22 (8H, remaining aliphatic). The above ^1H nmr assignments were confirmed by homonuclear decoupling; ^{13}C nmr (D_2O) δ: 178.58 ($NH\underline{C}OCH_3$), 175.59 ($\underline{C}OOCH_3$), 100.82 (C-1, $J_{C-1,H-1}$ = 159.4 Hz), 100.47 (C-1", $J_{C-1",H-1"}$ = 162.3 Hz), 97.72 (C-1', $J_{C-1',H-1'}$ = 169.5 Hz), 77.35, 76.75, 75.35, 74.27, 74.10, 73.73, 71.43, 70.92, 70.84, 70.54, 68.20, 67.63 (C-2, C-3, C-4, C-5, C-2', C-3', C-4', C-5', C-3", C-4", C-5", and $O\underline{CH_2}CH_2$), 66.99 (C-6), 62.43 (C-6"), 61.55 (C-6'), 56.31 (C-2"), 52.91 ($COO\underline{CH_3}$), 34.55 ($\underline{CH_2}COOCH_3$),

29.46, 29.05, 28.97, 28.93, 25.82, 25.11 (aliphatic), 23.21 ($NHCOCH_3$). Anal. calcd. for $C_{30}H_{53}N_1O_{18}$: C 50.34, H 7.46, N 1.96; found: C 49.27, H 7.26, N 1.73.

8-Methoxycarbonyloctyl 6-O-[2-O-(3,4,6-tri-O-acetyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl)-3,4,6-tri-O-benzyl-α-D-mannopyranosyl]-3-O-(2,3,4,6-tetra-O-acetyl-α-D-mannopyranosyl)-2,4-di-O-benzyl-β-D-mannopyranoside (34)

To a solution of the alcohol **31** (504 mg; 0.371 mmol) in dry acetonitrile (10 mL) containing 4Å molecular sieves were added sequentially mercuric bromide (1.416 g; 3.93 mmol) and mercuric cyanide (988 mg; 3.95 mmol). To the resulting mixture was added a solution of 2,3,4,6-tetra-O-acetyl-α-D-mannopyranosyl bromide[25] (**5**) (0.915 g; 2.23 mmol) in dry acetonitrile (5 mL) in five portions with an interval of 30 min between two additions, and the reaction mixture was stirred for 2 h. Evaporation of the solvent gave an oily residue which was extracted three times with dichloromethane. The organic extracts were combined and washed with saturated aqueous potassium chloride, saturated aqueous sodium bicarbonate, water, and brine. The dried organic layer (Na_2SO_4) was evaporated to give a foamy residue which was purified by flash chromatography using toluene-ethyl acetate (3:1) as eluent. The title

compound was obtained as a white foamy solid (0.405 g; 65%); $[\alpha]_D$ +4.41° (c 0.98, chloroform); R_f 0.25, (toluene-ethyl acetate, 3:1); ^1H nmr ($CDCl_3$) δ: 7.88-7.02 (29H, aromatic), 5.811 (dd, 1H, $J_{3'',4''}$ = 10.0 Hz, $J_{2'',3''}$ = 9.0 Hz, [redacted]), 5.574 (d, 1H, $J_{1'',2''}$ = 8.5 Hz, H-1''), 5.391-5.349 (2H, $J_{2',3'}$ = 3.0 Hz, H-2' and H-3'), 5.240-5.094 (4H, H-4', H-4'', $C_6H_5C\underline{H}HO$, and H-1 [δ5.094] $J_{3',4'}$ = $J_{4',5'}$ = 9.5 Hz; $J_{3'',4''}$ = $J_{4'',5''}$ = 9.5 Hz), 4.893-4.750 (3H, J_{gem} = 11.5 Hz and 13.0 Hz, 3×$C_6H_5C\underline{H}HO$), 4.696 (d, 1H, $J_{1'',2''}$ = 1.5 Hz, H-1''), 4.539-4.448 (2H, H-2'' [δ4.515, dd, $J_{1'',2''}$ = 8.5 Hz, $J_{2'',3''}$ = 10.5 Hz] and $C_6H_5C\underline{H}HO$ [J_{gem} = 11.5 Hz]), 4.440-4.284 (6H, H-1 [δ4.440], H-2' [δ4.375, dd, $J_{1'',2''}$ = 2.0 Hz, $J_{2'',3''}$ = 2.0 Hz], H-6a'', and 3×$C_6H_5C\underline{H}HO$ [J_{gem} = 11.0 and 11.5 Hz]), 4.121 (dd, 1H, $J_{6a'',6b''}$ = 12.0 Hz, $J_{5'',6b''}$ = 1.5 Hz, H-6b''), 4.098-3.235 (21H, H-2, H-3, H-4, H-5, H-6a, H-6b, H-5', H-6a', H-6b', H-3'', H-4'', H-5'', H-6a'', H-5'', $OC\underline{H}_2CH_2$, $C_6H_5C\underline{H}_2O$, OCH_3 [δ3.674]), 2.953 (dd, 1H, $J_{6a'',6b''}$ = 10.5 Hz, $J_{5'',6b''}$ = 6.0 Hz, H-6b''), 2.313 (t, 2H, J = 7.5 Hz, $C\underline{H}_2COOCH_3$), 2.059-2.000 (6s, 18H, $OCOCH_3$×6), 1.868 (s, 3H, $OCOCH_3$), 1.74-1.54 (4H, aliphatic), 1.46-1.20 (8H, remaining aliphatic); ^{13}C nmr ($CDCl_3$) δ: 174.11 ($\underline{C}OOCH_3$), 170.58, 170.21, 170.06, 169.65, 169.47, 169.44, 169.35 ($O\underline{C}OCH_3$ and phthalimido carbonyl), 138.75, 138.46, 137.73, 137.61 (phthalimido quat. arom.), 133.90 (phthalimido tert.

arom.), 128.82, 128.46, 128.36, 128.15, 128.08, 128.03, 127.87, 127.54, 127.47, 127.36, 127.28, 127.18 (benzyl tert. arom.), 123.31 (phthalimido tert. arom.), 101.95 (C-1, $J_{C-1,H-1}$ = 154.1 Hz), 99.83 (C-1', $J_{C-1',H-1'}$ = 177.8 Hz), 97.62 (C-1", $J_{C-1",H-1"}$ = 170.9 Hz), 96.60 (C-1'", $J_{C-1'",H-1'"}$ = 164.16 Hz), 81.25, 76.29, 74.78, 74.48, 74.17, 72.90, 72.03, 71.80, 70.73, 69.33, 69.15, 68.98, 68.93, 65.94 (C-2, C-3, C-4, C-5, C-2', C-3', C-4', C-5', C-2", C-3", C-4", C-5", C-3'", C-4'", and C-5'"), 74.68, 74.58, 73.71, 72.52, 70.43 ($C_6H_5\underline{C}H_2O$), 70.11 ($O\underline{C}H_2CH_2$), 69.62 (C-6"), 66.29 (C-6), 62.44, 62.32 (C-6' and C-6'"), 54.37 (C-2'"), 51.34 ($COO\underline{C}H_3$), 34.02 ($\underline{C}H_2COOCH_3$), 29.66, 29.27, 29.19, 29.07, 26.10, 24.89 (aliphatic), 20.69, 20.56, 20.40 ($OCO\underline{C}H_3$). Anal. calcd. for $C_{91}H_{107}N_1O_{31}$: C 63.89, H 6.30, N 0.82; found: C 63.45, H 6.31, N 0.82.

8-Methoxycarbonyloctyl 6-O-[2-O-(3,4,6-tri-O-acetyl-2-acetamido-2-deoxy-β-D-glucopyranosyl)-3,4,6-tri-O-benzyl-α-D-mannopyranosyl]-3-O-[2,3,4,6-tetra-O-acetyl-α-D-mannopyranosyl]-2,4-di-O-benzyl-β-D-mannopyranoside (35)

Compound **34** (260 mg; 0.15 mmol) was dissolved in dry methanol (15 mL) containing sodium methoxide. The resulting solution was stirred at room temperature for 45

min, and the reaction mixture was neutralized with Amberlite IR-120(H) resin. The resin was removed by filtration and the solvent evaporated to provide a foamy solid which was dissolved in methanol (10 mL). To this solution was added hydrazine hydrate (0.4 mL of an 85% solution, 7.43 mmol) and the mixture was refluxed for 1 h. Removal of solvent gave a white residue. Traces of hydrazine were removed by evaporation of methanol from the product (twice), which was further dried on the vacuum pump. The product was then dissolved in pyridine (3.5 mL) and acetic anhydride (3.5 mL) and stirred overnight at room temperature. Excess acetic anhydride was decomposed by dropwise addition of ethanol to the reaction mixture at 0°C, to which was then added dichloromethane and water. The aqueous layer was separated and back extracted with more dichloromethane and the combined dichloromethane layers were washed with 1 M aqueous HCl and saturated aqueous sodium bicarbonate. The organic phase was dried (Na_2SO_4), filtered, and evaporated to give a foamy solid which was purified by flash chromatography using Skellysolve B-ethyl acetate-ethanol (20:20:1) as eluent. Pure **35** was obtained as a white foamy solid (154 mg; 62%); $[\alpha]_D$ +11.23 (c 1.18, chloroform); R_f 0.3 (Skellysolve B-ethyl acetate-ethanol, 20:20:1); ^1H nmr ($CDCl_3$) δ: 7.70-7.12 (25H, aromatic), 5.613-5.523 (2H, H-3'' [δ5.588] and

NH [δ5.533, d, J = 7.5 Hz]), 5.405-5.340 (2H, H-2' [δ5.399, dd, $J_{2',3'}$ = 3.5 Hz, $J_{1',2'}$ = 1.5 Hz] and H-3' [δ5.358, dd, $J_{3',4'}$ = 10.0 Hz, $J_{2',3'}$ = 3.5 Hz]), 5.213-4.990 (5H, H-4' [δ5.188, dd, $J_{4',5'}$ = 10.0 Hz, $J_{3',4'}$ = 10.0 Hz], $C_6H_5C\underline{H}HO$ [δ5.133, d, J_{gem} = 13.0 Hz], H-1' [δ5.100], H-1''' [δ5.074, d, $J_{1''',2'''}$ = 8.5 Hz], and H-4''' [δ5.015, dd, $J_{4''',5'''}$ = 10.0 Hz, $J_{3''',4'''}$ = 10.0 Hz]), 4.905-4.765 (4H, H-1'' [δ4.850, d, $J_{1'',2''}$ = 2.5 Hz] and 3×$C_6H_5C\underline{H}HO$ [J_{gem} = 11.5-13.0 Hz]), 4.575-4.530 (2H, J_{gem} = 11.5 Hz and 12.0 Hz, $C_6H_5C\underline{H}_2O$), 4.466-4.228 (6H, H-1 [δ4.466], 3×$C_6H_5C\underline{H}HO$ [J_{gem} = 12.0 Hz], H-2'' [δ4.288, broad s], H-6a''' [δ4.250, dd, $J_{6a''',6b'''}$ = 12.5 Hz, $J_{5''',6a'''}$ = 5.0 Hz]), 4.119-3.300 (13H, H-6b''' [δ4.100, dd, $J_{6a'''6b'''}$ = 12.0 Hz, $J_{5''',6b'''}$ = 2.0 Hz], H-2, H-3, H-4, H-5, H-6a, H-6b, H-5', H-6a', H-6b', H-3'', H-4'', H-5'', H-6a'', H-6b'', H-2''', H-5''', $OC\underline{H}_2CH_2$, and OCH_3 [δ3.665]), 2.305 (t, 2H, $C\underline{H}_2COOCH_3$, J = 7.5 Hz), 2.048-1.993 (21H, 7×$OCOCH_3$), 1.779 (s, 3H, $NHCOC\underline{H}_3$), 1.67-1.52 (4H, aliphatic), 1.40-1.14 (8H, remaining aliphatic); ^{13}C nmr ($CDCl_3$) δ: 174.22 ($\underline{C}OOCH_3$), 171.03, 170.70, 170.33, 170.15, 169.84, 169.59, 165.54 ($O\underline{C}OCH_3$ and $NH\underline{C}OCH_3$), 138.71, 138.46, 137.91, 137.73 (quat. arom.), 128.55, 128.50, 128.45, 128.37, 128.27, 128.22, 127.94, 127.87, 127.72, 127.61, 127.51, 127.41, 127.34 (tert. arom.), 101.90 (C-1), 99.83 (C-1'), 98.35 (C-1''), 98.09 (C-1'''), 81.09, 76.97, 76.52, 74.93,

74.48, 74.37, 73.57, 72.02, 71.61, 69.33, 69.20, 69.11, 68.90, 65.88 (C-2, C-3, C-4, C-5, C-2', C-3', C-4', C-5', C-2", C-3", C-4", C-5", C-3"', C-4"', and C-5"'), 74.93, 74.66, 73.74, 73.03, 70.77 ($C_6H_5\underline{C}H_2O$), 70.59 ($O\underline{C}H_2CH_2$), 69.27 (C-6"), 66.37 (C-6), 62.42 (C-6' and C-6"'), 55.90 (C-2"'), 51.45 ($COO\underline{C}H_3$), 34.07 ($\underline{C}H_2COOCH_3$), 29.66, 29.33, 29.25, 29.14, 26.08, 24.94 (aliphatic), 23.27 ($NHCO\underline{C}H_3$), 20.76, 20.68 ($OCO\underline{C}H_3$). Anal. calcd. for $C_{85}H_{107}N_1O_{30}$: C 62.91, H 6.65, N 0.86; found: C 63.05, H 6.67, N 0.83.

8-Methoxycarbonyloctyl 6-O-[2-O-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-α-D-mannopyranosyl]-3-O-(α-D-mannopyranosyl)-β-D-mannopyranoside (**3**)

A solution of **35** (111 mg; 0.07 mmol) in dry methanol (6 mL) containing sodium methoxide was stirred at room temperature for 1 h. Neutralization of the mixture with Amberlite IR-120(H) resin followed by removal of the resin by filtration and evaporation of the solvent provided the deacetylated product as a foamy solid (89 mg) which was dissolved in 98% ethanol (17 mL) and hydrogenolyzed over 5% palladium-on-charcoal (180 mg) at atmospheric pressure for 5 days. Following filtration through Celite the filtrate was concentrated to an oily residue which was

passed through a column of Bio-Gel P2 (2.5 cm × 47 cm) using 10% aqueous ethanol as eluent. The carbohydrate-containing fractions were combined and evaporated. Pure 3 was obtained following lyophilization as a white amorphous solid (51 mg; 85%); $[\alpha]_D$ +6.48° (c 0.91, water); R_f 0.28 (dichloromethane-methanol-water, 10:6:1); ^1H nmr (D_2O) δ: 5.104 (1H, $J_{1',2'}$ = 2.0 Hz, H-1'), 4.918 (1H, $J_{1'',2''}$ = 1.8 Hz, H-1"), 4.671 (1H, $J_{1,2}$ < 0.8 Hz, H-1), 4.579 (d, 1H, $J_{1''',2'''}$ = 8.0 Hz, H-1'"), 4.139 (2H, H-2 and H-2"), 4.065 (dd, 1H, $J_{2',3'}$ = 3.0 Hz, $J_{1'',2''}$ = 1.5 Hz, H-2'), 4.018-3.445 (26H, H-3' [δ 3.891, $J_{3',4'}$ = 10.0 Hz, $J_{2',3'}$ = 3.0 Hz], H-3" [δ 3.843, $J_{3'',4''}$ = 9.5 Hz, $J_{2'',3''}$ = 3.0 Hz], H-3 [δ 3.728, $J_{3,4}$ = 9.5 Hz], H-2'" [δ 3.705], OCH_3 [δ 3.688, s], H-4, H-5, H-6a, H-6b, H-4', H-5', H-6a', H-6b', H-4", H-5", H-6a", H-6b", H-3'", H-4'", H-5'", H-6a'", H-6b'", and OCH_2CH_2), 2.388 (t, 2H, CH_2COOCH_3, J = 7.5 Hz), 2.055 (s, 3H, $NHCOCH_3$), 1.66-1.50 (4H, aliphatic), 1.40-1.20 (8H, remaining aliphatic). The above ^1H nmr assignments were confirmed by homonuclear decoupling; ^{13}C nmr (D_2O) δ: 178.76 ($NHCOCH_3$), 175.66 ($COOCH_3$), 103.22 (C-1', $J_{C-1',H-1'}$ = 172.0 Hz), 100.69 (C-1, $J_{C-1,H-1}$ = 159.1 Hz), 100.40 (C-1'", $J_{C-1''',H-1'''}$ = 159.1 Hz), 97.61 (C-1", $J_{C-1'',H-1''}$ = 169.3 Hz), 81.65, 77.14, 76.70, 75.12, 74.24, 74.18, 73.70, 71.24, 71.16, 70.94, 70.80, 70.49, 68.17, 67.67, 66.63 (C-2, C-3, C-4, C-5, C-2', C-3', C-4', C-5',

C-2‴, C-3", C-4", C-5"; C-3‴, C-4‴, and C-5‴), 70.99 ($O\underline{C}H_2CH_2$), 66.63 (C-6), 62.49, 61.86, 61.51 (C-6', C-6", and C-6‴), 56.25 (C-2‴), 52.95 ($COO\underline{C}H_3$), 34.59 ($\underline{C}H_2COOCH_3$), 29.50, 29.11, 29.07, 28.99, 25.86, 25.15 (aliphatic), 23.22 ($NHCO\underline{C}H_3$).

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