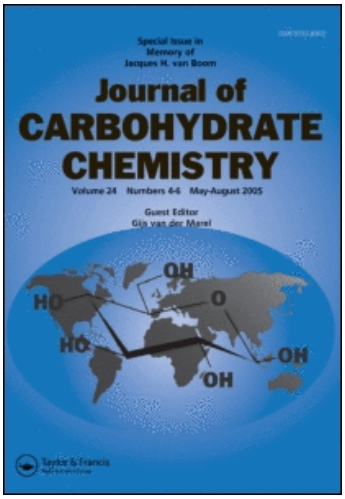

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METABOLIC SUBSTRATE ENGINEERING AS A TOOL FOR GLYCOBIOLOGY

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METABOLIC SUBSTRATE ENGINEERING AS A TOOL FOR GLYCOBIOLOGY*

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INTRODUCTION

The biological importance of oligosaccharides was first recognized in the context of their role in metabolism and energy storage. During the past three decades, it has become apparent that complex oligosaccharides also regulate many of the fundamental processes occurring in a biological system. Of special interest are the oligosaccharides found on the surfaces of cells (Figure 1), which guide their social behavior. Among many other responsibilities, oligosaccharides mediate cell–cell interactions, regulate the serum half-life of glycoproteins, and serve as specific ligands for bacteria, viruses, and parasites.[1]

Progress toward the elucidation of oligosaccharide function has historically been impeded by their structural complexity and heterogeneity on cells.[2] The biosynthesis of glycoconjugates is determined by various factors, including the relative abundance and specificity of glycosyltransferases (the enzymes that build oligosaccharides one saccharide at a time), the spatial localization of these enzymes, the availability of nucleotide sugar donors, and the nature of the underlying substrate. Collectively, these factors result in a diverse array of heterogeneous structures that are difficult to control by using conventional genetic and biochemical tools.

Considerable creativity has been exercised in the development of strategies for modulating oligosaccharide structures on cell surfaces in a well-defined fashion. For example, specific glycosidase and glycosyltransferase inhibitors[3–8] have provided insight into the biosynthesis of glycans as well as their roles in biological processes.

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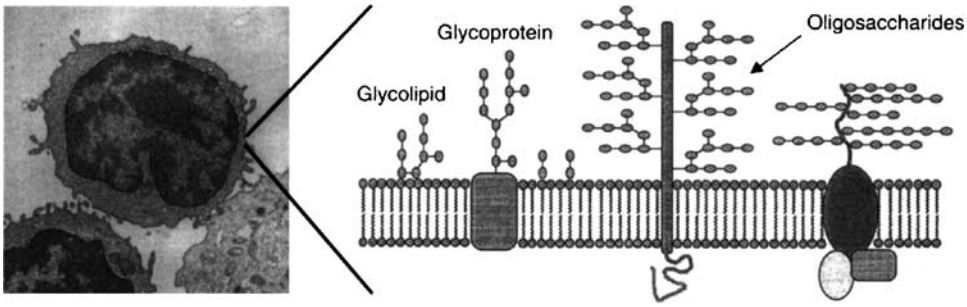

Figure 1. The landscape of the surfaces of cells.

Glycosyltransferases have been used to modify cell surface glycans by exogenous treatment.[9–11] Another approach, the subject of this chapter, is to intercept metabolic pathways with unnatural monosaccharide substrates. If incorporated into cell surface oligosaccharides, modified sugars can aid in the elucidation of biological function (Figure 2). Alternatively, modified substrates might block biosynthetic enzymes, producing phenotypes similar to those induced by glycosyltransferase inhibitors. We summarize here the historical development of metabolic substrate engineering as a tool for glycobiology. This chapter focuses on the journey of carbohydrate precursors and synthetic carbohydrate analogs through the metabolic pathways of the cell and the information that can be gained from investigating such processes.

We begin with an overview of the structures of glycoconjugates on cell surfaces (for more comprehensive reviews, see Refs. 2,12–14, and 24). Next, the pathways of monosaccharide interconversions inside the cell are summarized, providing a framework for opportunities in unnatural monosaccharide metabolism. Specific examples of modified sugars and their effects on cells are then presented. Finally, the biosynthetic pathway of sialic acid is discussed with respect to its utility for cell surface oligosaccharide engineering.

PROTOTYPICAL GLYCOCONJUGATES ON CELL SURFACES

Cell surface glycoconjugates can be grouped into four main classes: N-linked glycoproteins, O-linked glycoproteins, glycosyl phosphatidylinositol (GPI)-anchored proteins, and glycolipids. These are the major cell surface hosts of monosaccharides derived from intracellular metabolism. N-linked glycosylation in eukaryotes is a cotranslational event that occurs in the endoplasmic reticulum (ER).[2,16] It is found almost exclusively on proteins that contain the consensus sequence AsnXaaSer/Thr (where Xaa is any amino acid except Pro). Glycosylation of the asparagine residue in

Figure 2. Metabolic substrate engineering as a strategy for modulating cell surface oligosaccharide structure. Modified metabolic substrates can intercept a biosynthetic pathway in two ways: the pathway might be inhibited, leading to truncated structures on the cell surface, or the modified substrate might be incorporated into oligosaccharides in place of the normal substrate.

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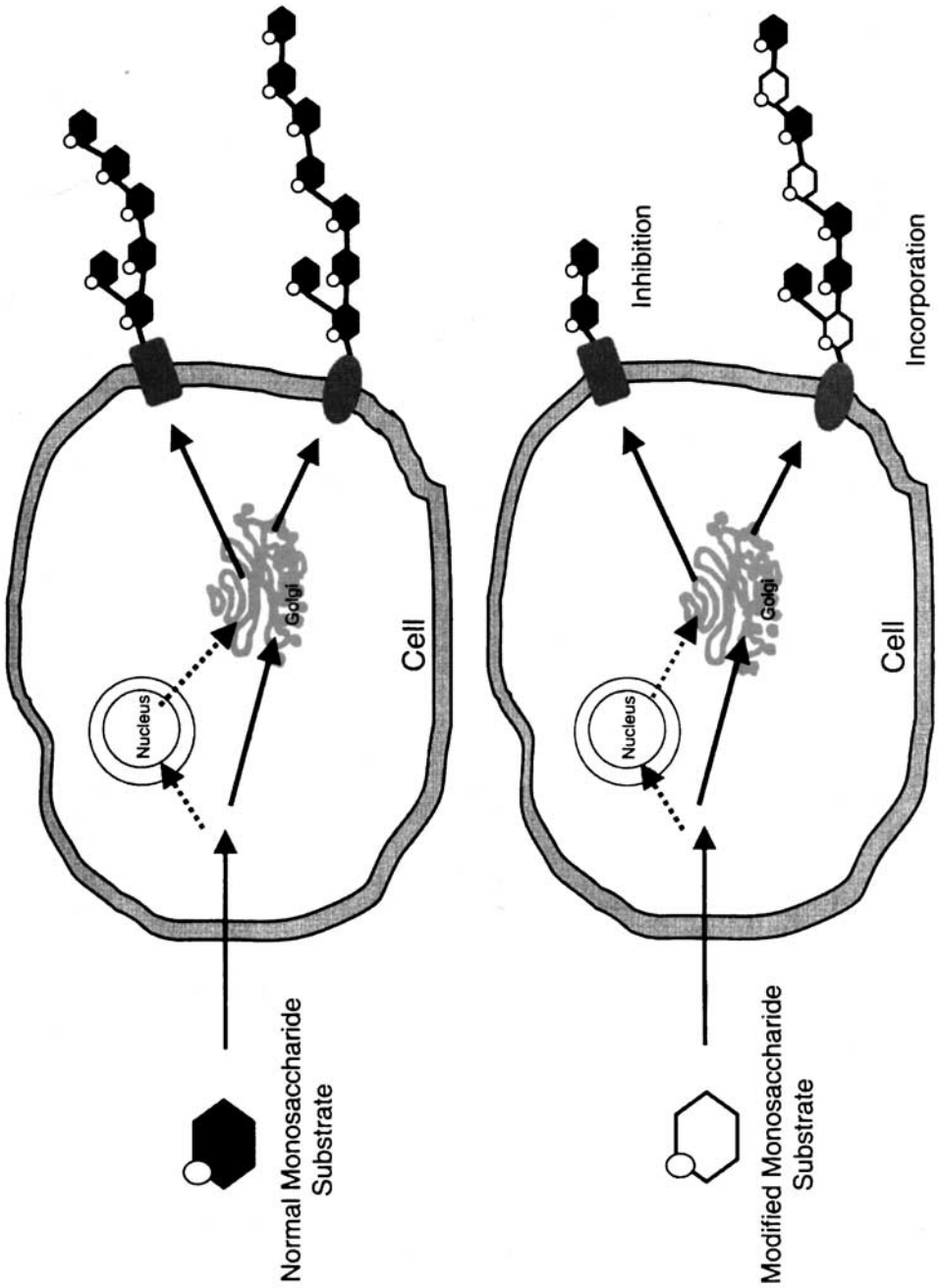

the appropriate sequon is mediated by a membrane-bound enzyme oligosaccharyltransferase. This enzyme transfers an oligosaccharide with the structure $Glc_3Man_9GlcNAc_2$ from a dolichol diphosphate precursor to the side chain of particular asparagine residues in the polypeptide chain (Figure 3). During its journey through the secretory pathway, this tetradecasaccharide is first trimmed and then elaborated to form an N-linked glycan with a high-mannose, hybrid, or complex-type structure.[16,17] Examples of these structures are shown in Figure 4.[25]

Unlike N-glycosylation, O-glycosylation begins with the transfer of a single monosaccharide residue, usually GalNAc, to a serine or threonine residue of the polypeptide chain.[12] The GalNAc residue is then further elongated through stepwise enzymatic modifications by glycosyltransferases, giving rise to specific core structures. To date, eight core structures have been identified by NMR spectroscopy or mass spectrometry (Figure 5).[26–30] These glycans can then be further elaborated by the addition of a sialic acid or Fuc residue, sulfate, methyl, acetyl, or poly-*N*-acetyllactosamine units. A subset of *O*-glycoproteins are the proteoglycans, which present long glycosaminoglycan chains from a peptide-proximal xylose residue (Figure 6).

GPI anchors are involved in cell signaling, protein targeting, and protein secretion.[15,23] The addition of a GPI anchor to a polypeptide is a posttranslational event that occurs in the endoplasmic reticulum (ER). The structures of GPI anchors are very complex. The core structure, consisting of ethanolamine, Man residues, GlcN, and phosphatidylinositol, is conserved from protozoan to mammalian organisms (Figure 7). However, the peripheral structures vary through species and cell type. The conserved GPI core can be further modified by the addition to the core mannoses of ethanolamine phosphate residues, GalNAc residues, and Man residues. This entire structure is then bound to a lipid that is embedded in the membrane. Similar to N-glycosylation, GPI modification of proteins involves the transfer of a preassembled precursor, en bloc, to the C-terminus of a target protein by amide bond formation with the ethanolamine group.

Glycolipids present oligosaccharide epitopes immediately proximal to the plasma membrane.[18–22] Most glycolipids can be classified into three major groups: sphingolipids, gangliosides, and glycero- and isoprenol-glycolipids.[31] The biosynthesis of glycolipids proceeds by stepwise addition of monosaccharide units to a lipid carrier in the compartments of the secretory pathway. As in the case of glycoprotein biosynthesis, the transfer of nucleotide-activated monosaccharides is mediated by glycosyltransferases. An example of a ganglioside, GM_3, is shown in Figure 8.[32]

OVERVIEW OF METABOLIC SUBSTRATE ENGINEERING

The complex glycoconjugates expressed on eukaryotic cell surfaces (Figures 3–8) comprise primarily 10 monosaccharides: Glc, Gal, Man, Fuc, GlcN, GlcNAc, GalNAc, Xyl, GlcUA, and sialic acid (Neu5Ac). These sugars can be biosynthesized de novo within a cell, often from Glc; or, in many cases, key metabolic intermediates can be supplied exogenously to a cell. The pathways for these interconversions are summarized in Figure 9.[33–36] If the enzymes in these biosynthetic pathways could tolerate unnatural substrates, subtle modifications, such as replacement of a hydroxyl group with a hydrogen atom or halogen, could be introduced into cell surface glycans.

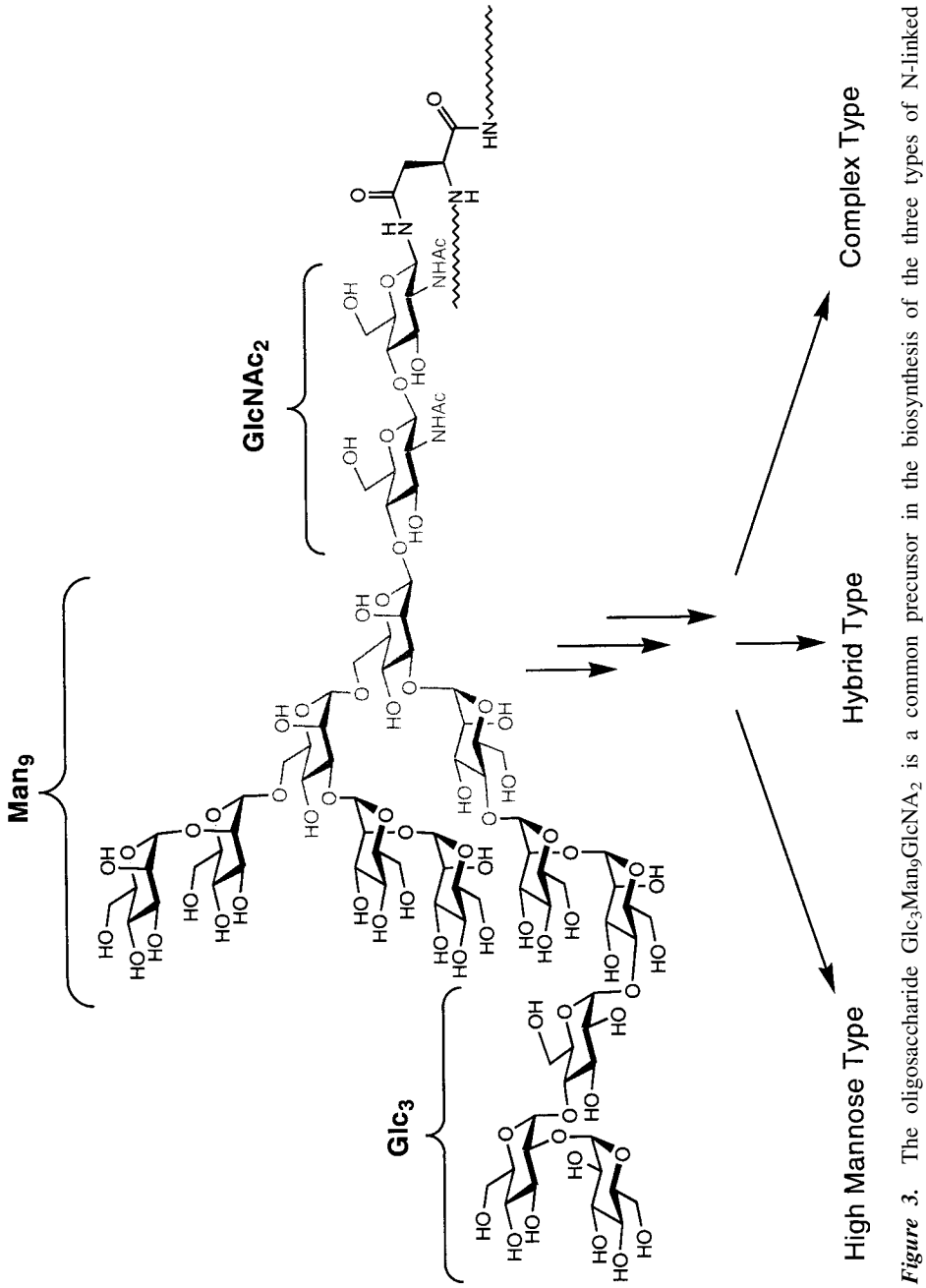

Figure 3. The oligosaccharide $Glc_3Man_9GlcNA_2$ is a common precursor in the biosynthesis of the three types of N-linked oligosaccharides.

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Figure 4. Examples of the three types of N-linked oligosaccharides found in human α-galactosidase A. Their common core structure is shown in gray.

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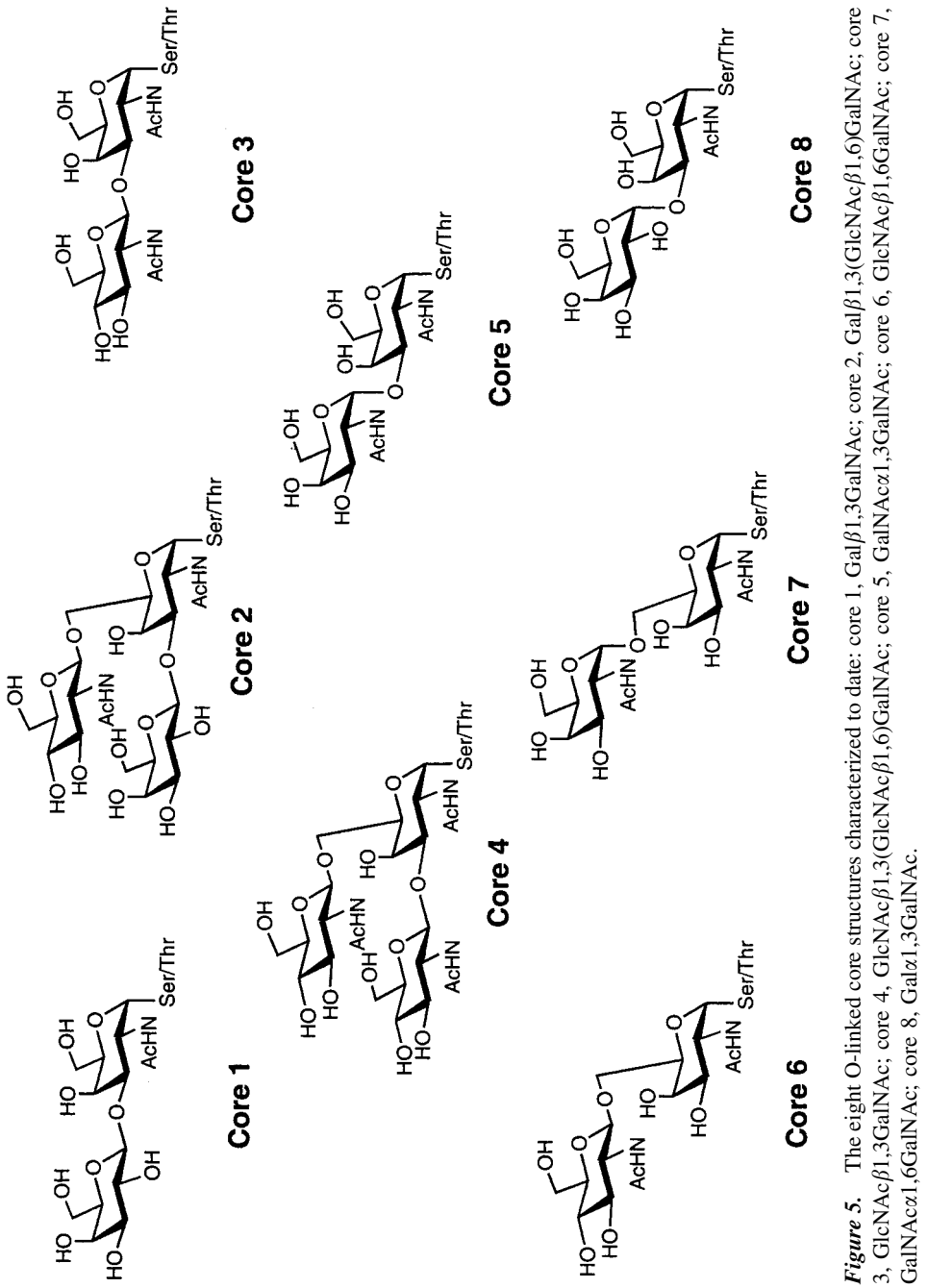

Figure 5. The eight O-linked core structures characterized to date: core 1, Galβ1,3GalNAc; core 2, Galβ1,3(GlcNAcβ1,6)GalNAc; core 3, GlcNAcβ1,3GalNAc; core 4, GlcNAcβ1,3(GlcNAcβ1,6)GalNAc; core 5, GalNAcα1,3GalNAc; core 6, GlcNAcβ1,6GalNAc; core 7, GalNAcα1,6GalNAc; core 8, Galα1,3GalNAc.

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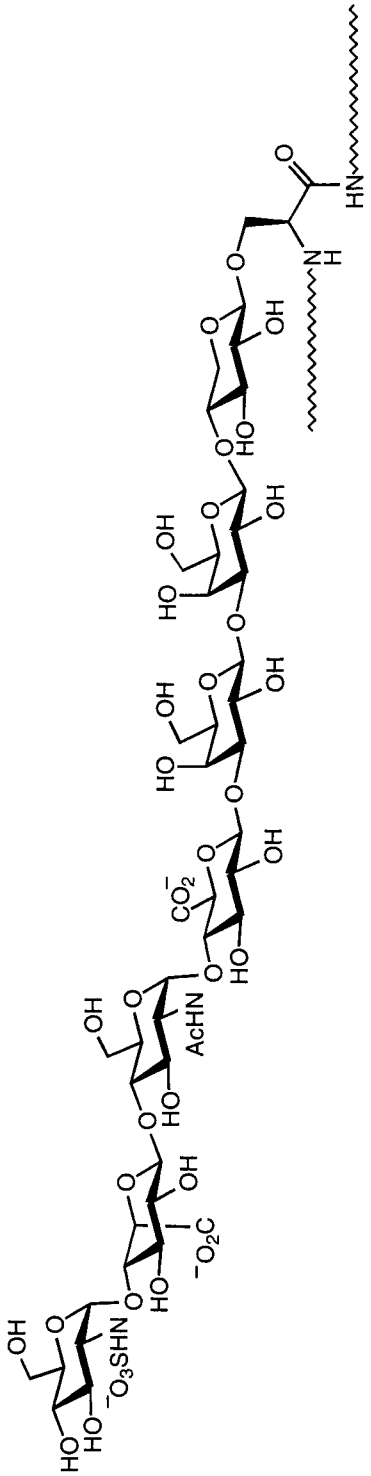

Figure 6. The core region of heparin and heparan sulfate.

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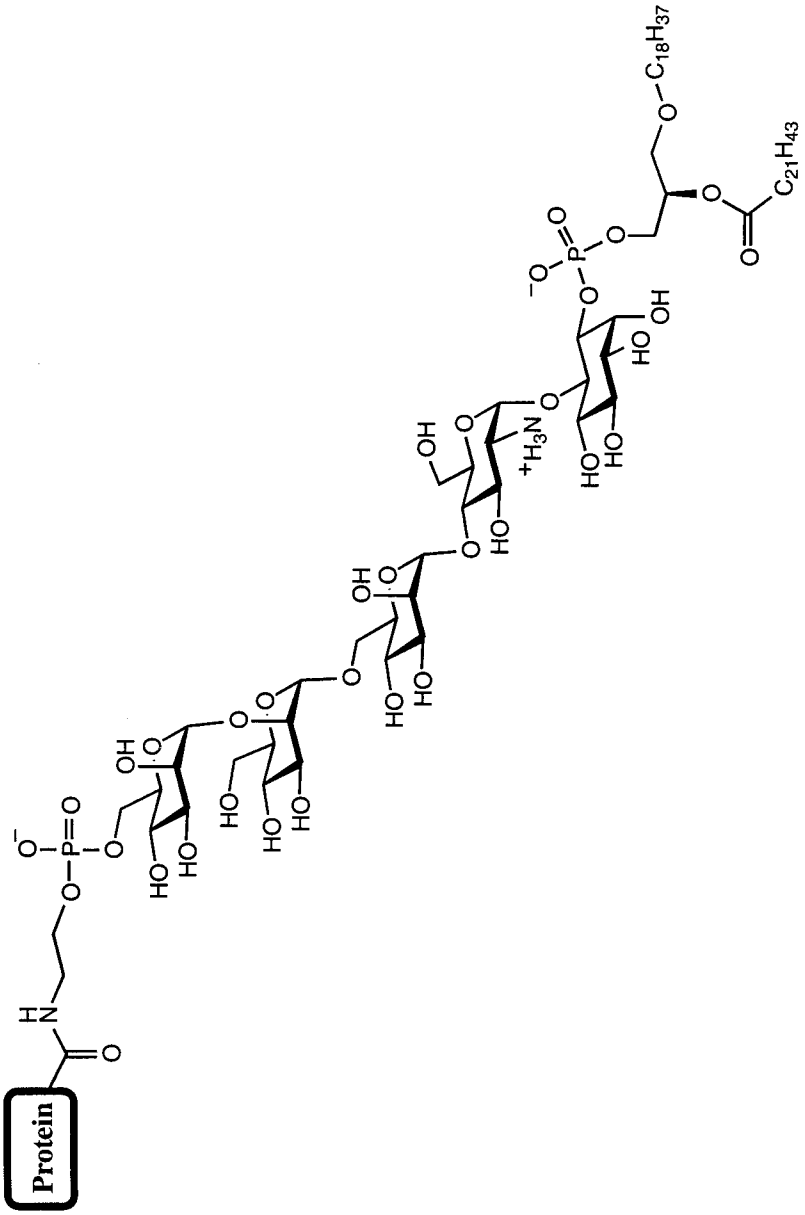

Figure 7. The conserved core structure of GPI anchors found in mammals.

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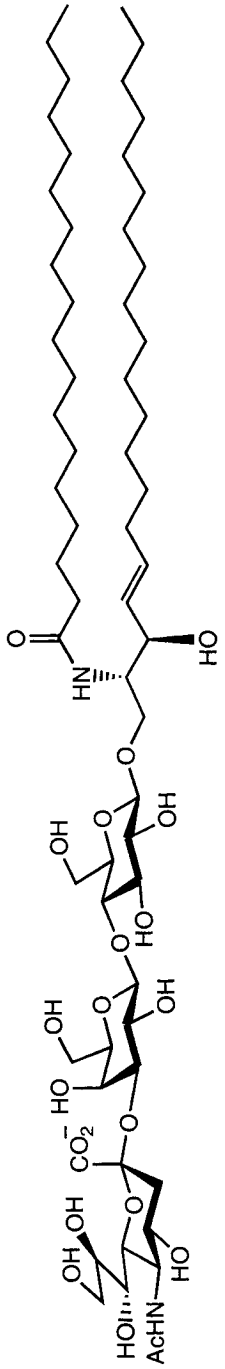


Figure 8. GM_3.

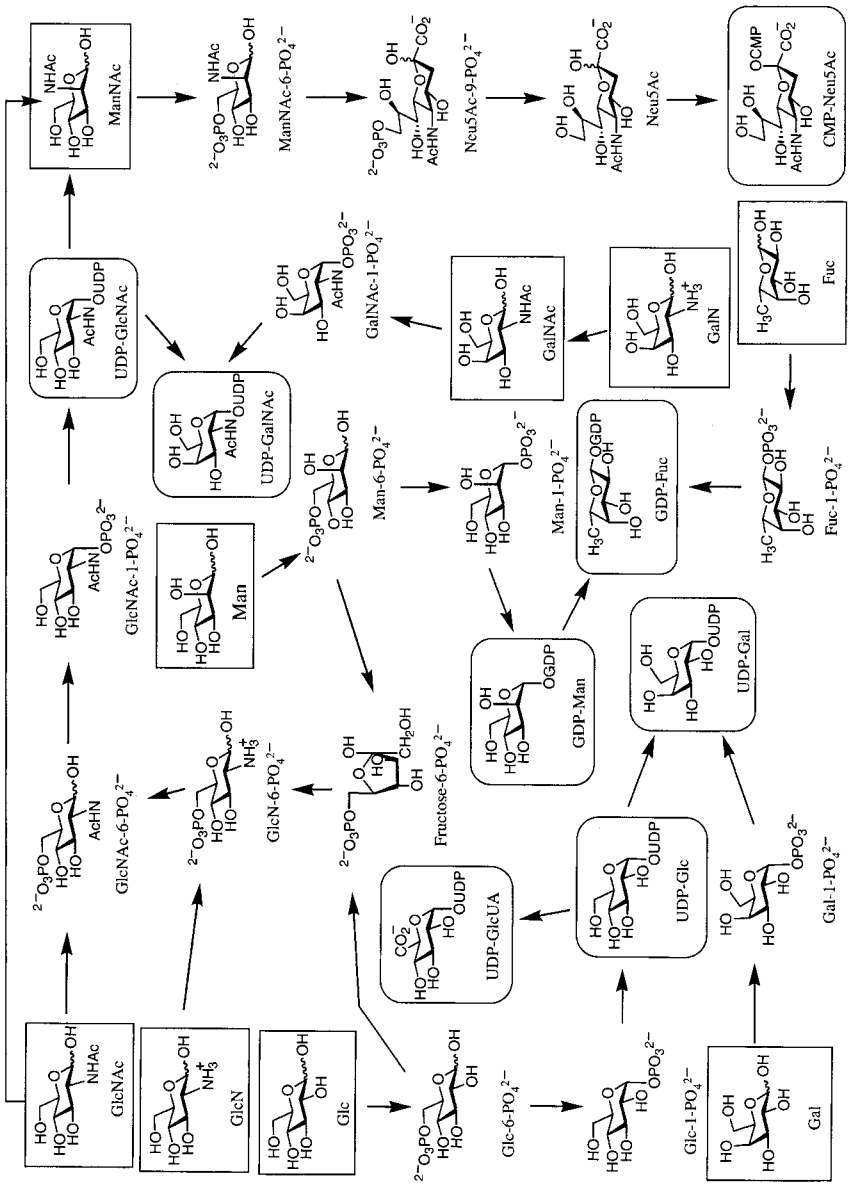


Figure 9. Monosaccharide interconversions in eukaryotic cells. Structures in squared boxes serve as exogenous substrates. Structures in rounded boxes are the activated monosaccharide donors. For simplicity, reactions are shown to progress in the forward direction.

This possibility attracted the attention of many groups interested in understanding how specific carbohydrate structures dictate molecular and cellular interactions.

Some early attempts at metabolic oligosaccharide engineering using halogenated and deoxy sugars resulted in cell death. In retrospect, we now know that some of these compounds are inhibitory of intracellular biosynthetic pathways.[37,38] However, the toxicity of halogenated and deoxy sugars could possibly be exploited therapeutically for antiviral, antibacterial, or anticancer therapy. Examples are presented in detail later in this chapter.

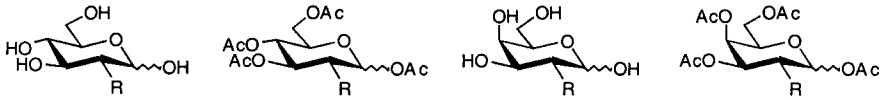
Remarkably, some unnatural monosaccharides were shown to access the metabolic pathways within cells. Some analogs were transformed only to an intermediate stage in the biosynthesis, never reaching the cell surface as a glycoconjugate component. Nevertheless, through the study of the imbalances in metabolic intermediates induced by these compounds, much has been learned about the specificities and roles of the enzymes involved. Perhaps most interesting of all has been a small, but growing number of compounds that successfully navigate glycoconjugate biosynthetic pathways and are presented on the cell surface. Such analogs, discussed in detail later, fall into two major categories. First, deoxy sugars, when incorporated into glycans, can lack the necessary hydroxyl groups required for further elaboration of the glycoconjugate, thereby resulting in cell surfaces deficient in some distal epitopes. The other category of compounds, exemplified by ManNAc derivatives that are converted to cell surface sialic acids, decorate the cell surface with novel structural and functional motifs. A discussion of these will come at the end of this chapter.

Metabolic Studies with Modified Amino Sugars and *N*-Acetamido Sugars

The initiatory metabolic interference studies of the 1970s utilized amino sugars. Early work demonstrated that even the naturally occurring compound GlcN causes specific inhibition of viral glycoprotein synthesis at high concentrations.[39] Removal of exogenous GlcN from cell culture media resulted in a reversal of cytotoxicity. *N*-Bromoacetyl-GlcN and its tetra-*O*-acetyl derivative produced strong in vitro cytotoxicity against both Ehrlich and L1210 leukemia cells at micromolar concentrations (Table 1).[40] Indeed, injection of the same compounds into mice bearing Ehrlich ascites tumors cured 85% of the mice, although successive injections ultimately produced resistance. The molecular basis for this interesting series of results was not explored, but it might reflect the nonspecific alkylating activity of the α-bromoacetamide. Accordingly, the corresponding fluoro and chloro derivatives, which are less reactive alkylating agents, were found to be inactive both in vitro and in vivo, with a toxicity level 10-fold lower than that of the bromo analog (Table 1).

In a separate study, Fondy et al. demonstrated that although the toxicity of fluoroacetamido analogs of GlcNAc and GalNAc was not affected by the presence or absence of *O*-acetyl protecting groups, a fourfold increase in toxicity was observed for tetra-*O*-acetyl *N*-fluoroacetamido-ManN.[41,42] The toxic effects of the chloro- and bromoacetamido analogs of GlcNAc, ManNAc, and GalNAc were more similar to that of the *N*-fluoroacetamido-ManN analog (Table 2). In general, the tetra-*O*-acetylated derivatives were markedly more toxic than their nonacetylated counterparts. Fondy et al.

Table 1. Growth Inhibitory Activity of GlcN and GalN Analogs Against Erlich and L1210 Leukemia Cells



1 **2** **3** **4**

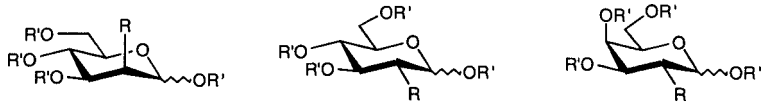
	IC_{50} (μM)			
R	1	**2**	**3**	**4**
NH_3Cl	N/A	N/A	>1000	220
$NHCOCH_2F$	≥100	20	≥100	30–80
$NHCOCH_2Cl$	≥100	30–60	≥100	20–30
$NHCOCH_2Br$	≥100	2–8	≥100	4

postulated that while the bromo and chloro analogs exert their toxic effects as lipophilic alkylating agents, the fluoroacetamido analogs might have a different mechanism of toxicity. Again, the molecular basis of this suggestion was not explored at the time.

The effects of amino sugars on cell viability have been evaluated using bacteria as well. The growth of *Escherichia coli* ML308 and K12 was inhibited by *N*-iodoacetamido-GlcN.[43] Further experiments indicated this compound inhibits the uptake of methyl-α-D-glucoside by the bacterial cells, although complete inhibition was never observed. This effect is not surprising, considering that iodoacetamide reacts with several amino acid residues, such as Cys and His, and also with certain amino groups. It is possible that the iodoacetamide analogs irreversibly alkylated a key glucose transporter.

Korytnyk and coworkers tested several pentaacetylhexosamine analogs for tumor cell toxicity and also evaluated their effects on glycoprotein biosynthesis.[44]

Table 2. Toxicity of ManN, GlcN, and GalN Derivatives to $B6D2F_1$ Mice



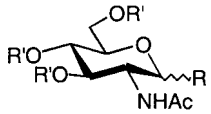
ManN GlcN GalN

		LD_{50} (mmol/kg)		
R	R′	ManN	GlcN	GalN
$NHCOCH_2F$	H	1.6	0.18	0.74
$NHCOCH_2F$	Ac	0.42	0.17	0.98
$NHCOCH_2Cl$	H	>3.7	2.0	1.5
$NHCOCH_2Cl$	Ac	1.6	1.12	0.83
$NHCOCH_2Br$	H	>3.2	2.4	1.7
$NHCOCH_2Br$	Ac	0.36	0.36	0.24

Peracetylated GlcNAc inhibited the incorporation of [^{14}C]GlcN into glycoproteins to a much greater extent than peracetylated GalNAc or ManNAc. However, protein biosynthesis was unaffected, as determined by levels of [^{3}H]leucine incorporation. Interestingly, replacement of the *N*-acetyl group of GlcNAc with a trifluoroacetyl group depressed [^{14}C]GlcN incorporation by 31% within 5 h, while [^{3}H]leucine incorporation was unaffected. Additionally, treatment of P288 cells with 1 mM GlcNAc resulted in no detectable change in ribonucleotide content, while treatment of cells with *N*-trifluoroacetyl-GlcN more than doubled the sugar-nucleotide content in the cell. The difference in the biological effects and metabolism of these compounds remains poorly understood, but a difference in cell permeability may be responsible.

Derivatives in which the anomeric position of GlcNAc was modified were also evaluated as potential metabolic inhibitors of glycoconjugate biosynthesis.[45,46] In these fully acetylated compounds, the hydroxyl group at the anomeric position was replaced by a variety of groups including amino, *N*-chloroacetyl, *N*-bromoacetyl, *N*-iodoacetyl, *N*-trifluoroacetyl, and *N*-trifluoromethanesulfonyl groups. These GlcNAc derivatives were potent growth inhibitors of either mouse mammary adenocarcinoma TA3 or L1210 leukemia cells with IC_{50} values in the micromolar range (Table 3). Toxicity of these compounds might simply reflect their alkylating activity. Further modification of GlcNAc to *S*-glycosyl derivatives revealed that the 1-chloroacetylthio,

Table 3. Growth Inhibitory Activity of GlcNAc Derivatives Displaying Unnatural Anomeric Substituents Against TA3 Mammary Cells



GlcNAc

	IC_{50} (mM)	
R	R′ = Ac	R′ = H
OH	0.27[a]	> 1[a]
NH_2	0.32	> 1
NHAc (β)	0.19	N/A
NHAc (α)	0.22	N/A
N_3	1.0	> 1
$NHCOCH_2Cl$	0.26	> 1
$NHCOCH_2Br$	0.019[a]	0.62[a]
$NHCOCH_2I$	0.026[a]	N/A
$NHCOCF_3$	0.2	N/A
$NHSO_2CF_3$	0.007	N/A
$SCOCH_2Cl$	0.25	N/A
$SCHPh_2$	0.8	N/A
STr	N/A	0.053
$SCPh_2(p\text{-}OMeC_6H_4)$	N/A	0.016

[a]IC_{50} values were determined using L1210 leukemia cells.

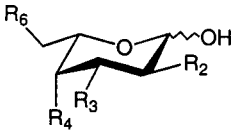
1-*S*-diphenylmethyl, and 1-*S*-trityl, inhibited the growth of TA3 cells at micromolar concentrations (Table 3). In yet another study, a ManN derivative containing a nitrosourea group at C2 was found to have antitumor activity.[47] The compound displayed an IC_{50} value against L1210 leukemia cells in the high micromolar range and increased the life span of leukemic mice by 35%. Although little mechanistic information was gleaned in these early studies, the differential effects of various unnatural substrates suggested possible engagement in metabolic pathways.

Halogenated Monosaccharides

Halogenated compounds have been widely used to study biological processes.[48] The halogens (F, Cl, Br, and I) are readily installed on synthetic analogs and might mimic a polar hydroxyl group. Among the halogens, fluorine has attracted the most attention. A comparison of bond lengths, van der Waals radii, and electronegativities indicates that the C—F bond quite closely resembles the C—OH bond.[49] The similar properties of the fluorine atom and hydroxyl group suggest that substitution might be tolerated by biosynthetic enzymes. The introduction of a fluorohexose or hexosamine analog into a biosynthetic pathway might lead to metabolic incorporation or metabolic disruption. An advantage to the use of fluorosugar analogs is the availability of additional analytical techniques, such as ^{19}F NMR spectroscopy and radio-labeled ^{18}F tracers, that facilitate characterization of downstream metabolic products.[50–53]

Independent studies by Korytnyk and coworkers and May and Sartorelli have probed the effects of halogenated fucose (Fuc) and galactose (Gal) analogs on

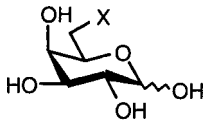
Table 4. Toxicity of Growth Inhibitory Activity of Fuc Derivatives Against L1210 Leukemia Cells



Fuc

R_2	R_3	R_4	R_6	IC_{50} (mM)
H	OH	OH	H	1.0
Cl	OH	OH	H	0.2
Br	OH	OH	H	No effect at 1.0
I	OH	OH	H	0.06
Cl	OAc	OAc	H	0.04
Br	OAc	OAc	H	0.06
OH	OH	OH	F	Slight effect at 1.0
OH	OH	OH	Cl	Slight effect at 1.0
OH	OH	OH	Br	0.4
OH	OH	OH	I	0.18

Table 5. Growth Inhibitory Activity of Various Halogenated Gal Derivatives Against L1210 Leukemia Cells



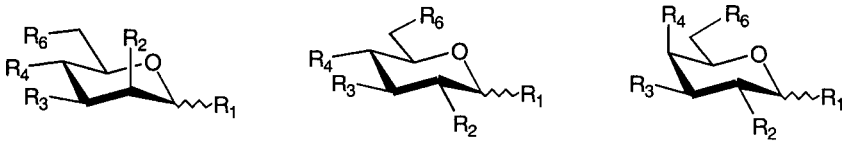
Gal

X	IC_{50} (mM)
F	> 1
Cl	1.0
Br	0.13
I	> 1
Ms	0.075

glycoconjugate biosynthesis.[54–56] Synthetic analogs bearing fluoro, chloro, bromo, or iodo groups at the 2- or 5-positions of Fuc, and the 6-position of Gal were shown to have inhibitory effects on the incorporation of the corresponding natural sugars, [^{3}H]Fuc or [^{3}H]Gal, into glycoproteins (Tables 4 and 5). The growth of murine L1210 leukemia cells was inhibited by 2-iodo-Fuc with an IC_{50} value of 0.06 mM (Table 4). Similarly, when mice with L1210 ascites tumors were injected with either 6-fluoro-Gal (Table 5) or 2-bromo-Fuc (Table 4), their life span was prolonged by 32 and 25%, respectively. The 6-methanesulfonyl (Ms) analog of Gal had similar toxicity against L1210 leukemia cells. Other halogenated derivatives of Fuc such as 2-bromo-Fuc, 6-chloro-Fuc, and 6-bromo-Fuc were much less toxic, with very little activity at concentrations above 1 mM (Table 4). The 6-halogenated analogs of Gal had inhibitory effects similar to those of their enantiomers, the corresponding 6-halogenated-Fuc analogs (Table 5). Some halogenated analogs displayed cell-type-specific effects: 6-fluoro-Fuc did not inhibit the growth of L1210 leukemia cells at 1 mM, although it did reduce the incorporation of [^{3}H]Fuc by 91% in human mammary tumor cells. Other compounds, such as 6-fluoro-Gal, although appearing non-toxic at 1 mM, had significant inhibitory effects toward the incorporation of [^{3}H]Gal in P2888 murine lymphoma cells.[55] Westwood and coworkers have investigated the antitumor activity of fluorohexoses in vivo. From a panel of 6-halogenated Gal and Glc analogs, only 6-fluoro-Glc exhibited significant antitumor activity, blocking the growth of R-1 lymphoma up to 90% in mice.[57]

Fluorohexosamine analogs that have been used in metabolic studies include 6-fluoro-ManNAc, 6-fluoro-ManN, methyl 3-fluoro-ManNAc, 3-fluoro-GlcNAc, 4-fluoro-GlcNAc, and 4-fluoro-GalNAc.[58–60] Many of these compounds were evaluated in both acetylated and nonacetylated forms. Among several 6-fluoro analogs tested in murine L1210 leukemic cells, fully acetylated 6-fluoro-ManNAc had an IC_{50} value of 100 μM, 6-fluoro-ManN had an IC_{50} value of 1 mM, and 6-fluoro-ManNAc was inactive (Table 6). The 3-fluoro analogs both inhibited cell growth at micromolar

Table 6. Growth Inhibitory Activity of Various ManNAc, GlcNAc, and GalNAc Derivatives Against L1210 Leukemia Cells



					IC_{50} (mM)		
R_1	R_2	R_3	R_4	R_6	ManN	GlcN	GalN
OH	NHAc	OH	OH	F	Inactive	N/A	N/A
OAc	NHAc	OAc	OAc	F	0.1	N/A	N/A
OH	NH_3Cl	OH	OH	F	1.0	N/A	N/A
OMe (α)	NHAc	F	OAc	OAc	0.043	N/A	N/A
OAc	NHAc	F	OAc	OAc	N/A	0.027	N/A
OAc	NHAc	OH	F	OH	N/A	Inactive	N/A
OH	NHAc	OAc	F	OAc	N/A	0.034	0.035
OAc	NHAc	OAc	F	F	N/A	N/A	0.024

concentrations and reduced tumor growth by 50%. The 3-fluoro GlcNAc derivative also appeared to inhibit glycoconjugate biosynthesis. In the presence of the 3-fluoro analog, incorporation of [^{3}H]GlcN was reduced by 41% and [^{3}H]thymidine by only 7%. This indicates that 3-fluoro-GlcNAc competes in the pathway as GlcNAc itself. Contrary to the results in vitro, the 3-fluoro derivatives were relatively inactive in vivo, increasing life span by only 14% at 100 mg/kg. 4-Fluoro-GalNAc, on the other hand, exhibited antitumor activity, increasing the life span of mice by 68% when administered a dosage of 50 mg kg^{-1} d^{-1} for 5 consecutive days. Since GalNAc is primarily contained in O-linked structures (Figure 5) this might reflect an important role for O-linked glycoproteins in tumor growth.

Despite the diverse behavior exhibited by the various halogenated Fuc and Gal derivatives, two clear trends emerged. First, the incorporation of ^{3}H-labeled natural sugar analogs decreased with the increasing size of the halogen atom on the derivatized counterpart, and this was accompanied by an increase in cytotoxicity. This pair of effects suggests that more sterically demanding substituents are not tolerated by biosynthetic enzymes, thus minimizing biosynthetic competition with nagive substrates. Second, acetylated versions of all the derivatives were much more toxic than their analogous nonacetylated counterparts. This increase in toxicity could be attributed to the greater cellular permeability of these, and in general, any acetylated sugars.[61] Once inside a cell, esterases enzymatically remove the acetyl groups to produce a free monosaccharide that has full biological activity. Some reports suggest that partially deacetylated sugars can also enter the biosynthetic pathways.[62] Depending on the degree of deacetylation, these sugars may not be recognized by all biosynthetic enzymes for metabolism to cell surface glycans, thereby perhaps also contributing to the inhibitory and toxic effects of these compounds.

Other studies addressed the conversion of fluorosugars to metabolic products, and ultimately to cell surface glycoconjugates. This section discusses the various fluorinated analogs that have been used in incorporation studies and the effects they had on the cell compared with their nonfluorinated counterparts. An analog of GlcNAc with a single fluorine atom on the *N*-acetamido group has been shown to be incorporated into glycosaminoglycans.[63] Glycosidase digestion of macromolecules isolated from rabbit tracheal explants that were incubated with radiolabeled *N*-fluoroacetyl-GlcN indicated the presence of these compounds within hyaluronic acid. These findings indicate that *N*-fluoroacetyl-GlcN traverses a pathway similar to GlcNAc and does not block the biosynthetic enzymes. Furthermore, cultivation of mouse embryo cells with *N*-fluoroacetyl-GlcN caused a 50% inhibition in the conversion of GlcNAc to UDP-GlcNAc, perhaps the result of alternate substrate competition.[64]

Unlike the metabolism of the previous fluoro analogs, 4-fluoro-Man inhibits the synthesis of glycoproteins found on the vesicular stomatitis virus, probably through disruption of the biosynthesis of $Glc_3Man_9GlcNAc_2$-PP-Dol.[65] At a concentration of 1 mM, 4-fluoro-Man specifically inhibited protein glycosylation of BHK21 cells infected with the virus. When the concentration was raised to 10 mM, general protein synthesis was also affected. It is possible that 4-fluoro-Man was incorporated into the oligosaccharide-Dol substrate and acted as a chain terminator, blocking further elaboration. Indeed, in *Saccharomyces cerevisiae* S288C, 4-fluoro-Man is converted to the 1,6-bisphosphate, 6-phosphate, and GDP analogs.[65] Furthermore, 4-fluoro-Man was transferred by mannosyltransferase into the cell wall polysaccharide. The 3-fluoro analog, on the other hand, was transported into the cells at a lower rate and was not incorporated into cell wall glycoproteins. Although conversion of 3-fluoro-Man to its GDP derivative was much less efficient than the 4-fluoro analog, similar metabolites were isolated from the cells.

3-Fluoro-Glc is an inhibitor of glucose and galactose metabolism in yeast.[67] This analog also interferes with polysaccharide synthesis, as well as serving as a phosphate trap, as indicated by significant decreases in intracellular levels of phosphoglyceric acids, ATP, and UDP-Glc.[68] Exposure to 2-fluoro-Glc has a drastic effect on *S. cerevisiae*.[69] This toxic compound is a potent inhibitor of cell wall glucan biosynthesis and leads to cell wall degradation within a matter of hours. Similarly, Datema and Schwarz showed that 2-fluoro-Glc and 2-fluoro-Man inhibit formation of lipid-linked oligosaccharides in vivo.[70]

2-Fluoro-Man is a more potent antiviral agent that 2-fluoro-Glc in many virus-host cell systems.[71] Moreover, addition of exogenous Man, but not Glc, to culture media containing the fluorosugars, restored virus multiplication. A look at the metabolism of these analogs revealed that they are both activated by GTP or UTP, respectively, to yield their corresponding GDP or UDP derivatives.[72] 2-Fluoro-Man and 2-fluoro-Glc are incorporated into glycoproteins in both yeast and chicken embryo cells.[71] 2-Fluoro-Glc and 2-fluoro-Man were also found to be interconverted in vitro, presumably by epimerization. Furthermore, 2-fluoro-Glc appears to be converted to 2-fluoro-Gal. Therefore, these fluorinated sugars have multiple avenues for incorporation into glycoproteins.

The metabolic fates of 2-fluoro-Gal have also received attention. Several groups have used ^{19}F NMR spectroscopy and positron emission tomography (PET) with an ^{18}F label to track the substrate. These workers have determined that 2-fluoro-Gal is

converted to 2-fluoro-Glc and also to phosphorylated and nucleotide-bound 2-fluoro-Gal analogs.[51–53,71] Thus, it is likely that 2-fluoro-Gal can be directly incorporated into glycoconjugates.

6-Fluoro derivatives of several hexoses have been investigated and found to have biological effects that significantly differed from the 2-fluoro derivatives mentioned earlier. In particular, 6-fluoro-Gal inhibited the incorporation of [^{3}H]Gal, but not of Man, Glc, or leucine into L1210 leukemic cells. Electron microscope autoradiography was used to show that this analog is activated to form the nucleotide analog and then localized to the Golgi compartment.[73,74] Moreover, upon treatment with 6-fluoro-Gal, cells showed a reduction in available sites for external modification using an α2,6-sialyltransferase, providing evidence that the analog was incorporated into cell surface glycoconjugates.

Because of its importance in tumor glycosylation and blood antigen structures, Fuc has attracted attention as a target for metabolic structural modification.[75] 2-Fluoro-Fuc was shown to be incorporated into glycoproteins in mouse fibroblasts.[76] Specific localization of 6-fluoro-Fuc in the Golgi apparatus, nuclear membrane, plasma membrane, and cytoplasm of human mammary tumor cells indicated glycoprotein incorporation there as well.[54] Likewise, the metabolites GDP-6-fluoro-Fuc and 6-fluoro-Fuc-1-phosphate have been directly observed in these cells.

Deoxy Sugars as Inhibitors of Biosynthesis

The Glc and Man analog 2-deoxy-D-glucose has been shown to be a general inhibitor of cellular metabolism. Early work by Woodward et al. showed that this deoxy sugar is a strong inhibitor of both the growth of yeast cells and their fermentation of Glc.[77] It blocks glycoconjugate biosynthesis and has antiviral activity.[39,78–83] Since 2-deoxy-Glc uses the same transport system as Glc, it might simply block Glc uptake, depriving cells of a key metabolic substrate.[84–87] This has been directly demonstrated at high concentrations in yeast, chick fibroblasts, and Ehrlich ascites carcinoma cells.[88,89] However, at lower concentrations, 2-deoxy-Glc is taken up and converted to its UDP and GDP analogs. Incorporation into cell wall glucans in yeast and glycoconjugates in mammalian cells has been demonstrated.[81,90–94]

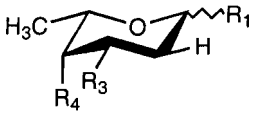
Within glycoconjugates, 2-deoxy-Glc might serve as a chain terminator for further elongation. Studies of altered cell surface architecture induced by treatment of 2-deoxy-Glc have been performed.[90] Lectin binding studies suggest that 2-deoxy-Glc actually replaces Man, not Glc. Agglutination of cells by the lectins *Ricinus communis* (RCA) and *Phaseolus vulgaris* (PHA),[78] both of which bind to Gal and GalNAc residues, is significantly decreased during incubation with 2-deoxy-Glc. This result indicates that the underlying mannosyl residues are unable to support further chain elongation to include elaboration with Gal or GalNAc, residues necessary for binding to these lectins. Another possibility is that the underlying Man residues are simply missing. However, binding of concanavalin A (ConA),[78,95] a Man-specific lectin, increases on cells incubated with 2-deoxy-Glc, suggesting that Man residues are present, perhaps as terminal residues if the 2-deoxy modification prevents further elongation. Other studies provide more direct evidence that 2-deoxy-Glc functions as a Man

substitute rather than a Glc substitute in cells. The addition of low doses of Man, but not Glc, to cells grown with 2-deoxy-Glc can reverse the effects of this antimetabolite.[96,97] Addition of Man to cells grown with 2-deoxy-Glc restores natural lectin agglutinating properties, further confirming that the compound functions as a Man mimic.

Similar to 2-deoxy-Glc, it has been established that 2-deoxy-Gal is incorporated into glycoproteins in both rat and human cells.[98–100] When rats to which 2-deoxy-Gal had been administered were injected with [^{3}H]Fuc, a significant decrease in Fuc incorporation was observed. This result suggested that the missing 2-OH group on Gal prevented 2-fucosylation, and this was confirmed by treatment with an α1,2-fucosidase. Interestingly, analysis with α1,3- and α1,4-fucosidases revealed a corresponding increase in α1,3/α1,4-fucosylation. Incubation of AS-30D. rat ascites hepatoma cells with 2-deoxy-[1-^{14}C]Gal[101] or the administration of this compound to rats[102,103] resulted in a large accumulation of 2-deoxy-Gal-1-phosphate. This metabolite comprised 78% of the total radioactivity detected in the liver, with the remaining metabolites being 20% UDP-2-deoxy-Gal and 0.7% UDP-2-deoxy-Glc.[104] The formation of the uridylate intermediates occurred only after high levels of 2-deoxy-Gal-1-phosphate had been achieved. The accumulation of UDP-2-deoxy-Gal-1-phosphate was attributed to the limited epimerization of UDP-2-deoxy-Gal-1-phosphate to UDP-2-deoxy-Glc-1-phosphate. This phenotype is characteristic of uridylyltransferase-deficient cells and tissues induced by Gal and may serve as an excellent model for studying the cellular injury in galactosemia.

2-Deoxy-Fuc, a residue found to be a constituent of several natural products, was tested for inhibition and cytotoxicity properties in L1210 leukemia, mouse mammary adenocarcinoma (TA3), and P288 leukemia cells (Table 7).[105] This compound had no significant effect on the growth of any of the cell lines tested. However, the fully acetylated version of this substrate was active as a growth inhibitor, and it exhibited significant influence on glycoprotein biosynthesis in cultured P288 leukemia cells. The methyl glycoside of the fully acetylated 2-deoxy-Fuc had an even greater effect on cell viability, with an IC_{50} of 500 μM in L1210 leukemia cells; cell growth was inhibited to

Table 7. Growth Inhibitory Activity of 2-Deoxy Fuc Analogs Against L1210 Leukemia Cells



2-deoxy Fuc

R_1	R_3	R_4	IC_{50} (mM)
OH	OH	OH	> 1
OMe	OH	OH	> 1
OMe	OAc	OAc	0.5
OAc	OAc	OAc	> 1[a]

[a]IC_{50} value was determined using TA3 mammary cells.

43% of control cells. Furthermore, this compound decreased cellular incorporation of [^{3}H]GlcN and [^{3}H]leucine by 20 and 9%, respectively.

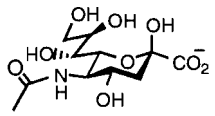
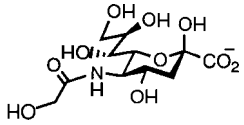
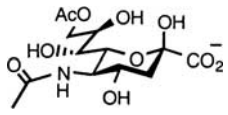
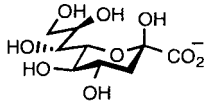
In summary, much early work with subtly modified monosaccharide substrates suggested the capacity to alter cell surface glycosylation through metabolic processes. Direct application of the technique to the study of cell surface carbohydrate recognition was realized later, mostly using sialic acids, as summarized in Section IV.

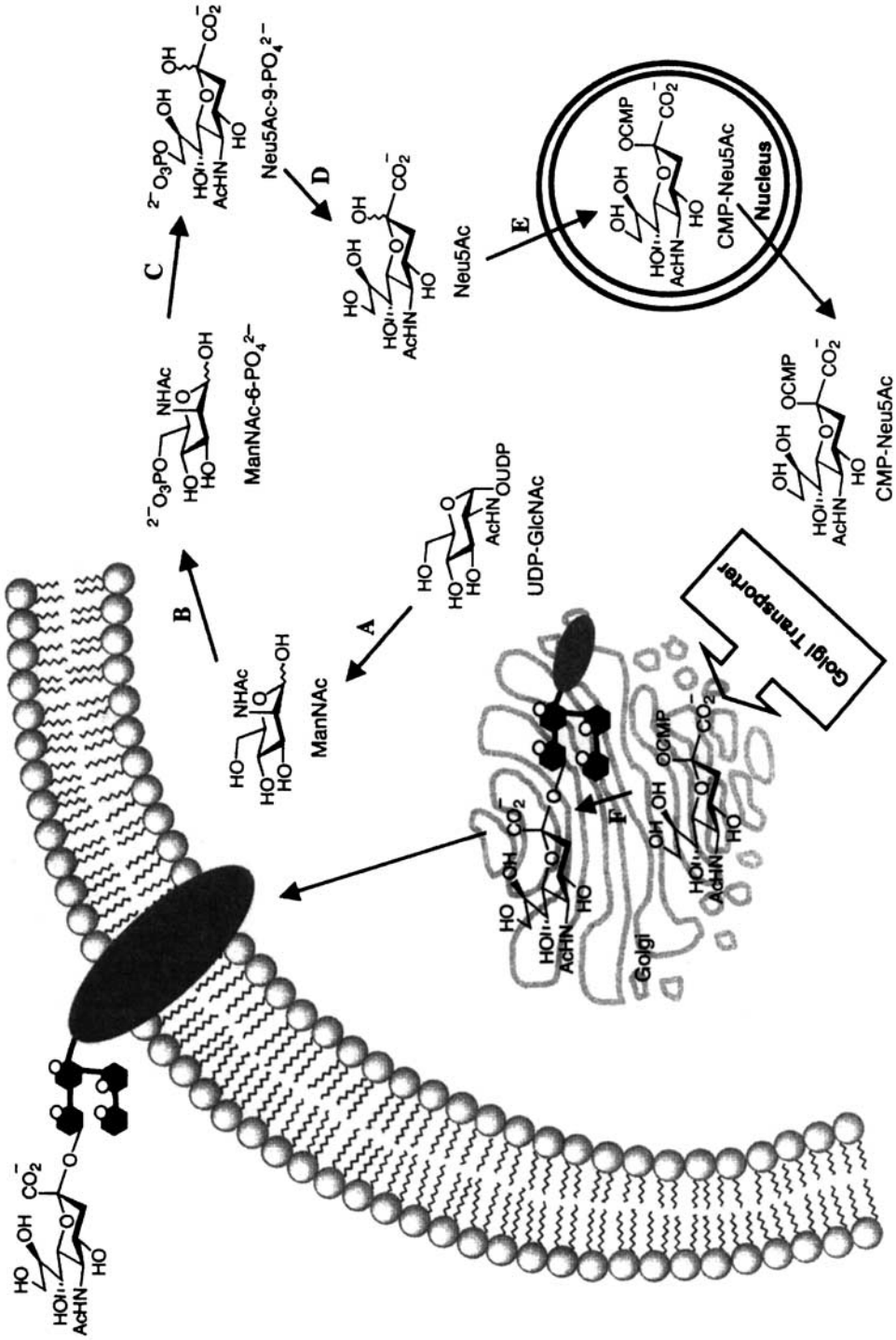
INTRODUCTION TO SIALIC ACIDS

Sialic acid is a unique monosaccharide typically found at the nonreducing end of oligosaccharides. Sialic acids serve as modulators for cell adhesion,[106–108] ligand determinants for specific viral and bacterial receptors, and epitopes for lectin binding.[109,110] They can also mask underlying antigenic sites.[111,112] The metastatic potential of tumor cells has been positively correlated with the total sialic acid content of the cell.[113] Their biological significance suggests that manipulation of the sialic acid content of complex oligosaccharides, as well as modification of the sialic acid structure, may provide insight into the molecular basis of cell surface interactions. The remainder of this chapter focuses on the biosynthesis of sialic acids and incorporation of unnatural analogs into cell surface glycoconjugates.

Sialic acid is a generic term used to represent a family of over 30 neuraminic acid derivatives.[114–116] The most abundant sialic acids are *N*-acetylneuraminic acid (Neu5Ac) and *N*-glycolylneuraminic acid (Neu5Gc). These sialic acids, in addition to a few other commonly found sialic acids, are shown in Table 8. While both eukaryotes and prokaryotes possess similar sialic acids, there exists some variation in the biosynthetic pathway leading to the expression of these compounds on the cell surface.

Table 8. Some Commonly Found Sialic Acids

Common Name	Abbreviation	Structure
N-Acetylneuraminic acid	Neu5Ac	
N-Glycolylneuraminic acid	Neu5Gc	
9-*O*-Acetyl-*N*-acetylneuraminic acid	Neu5,9Ac$_2$	
2-Keto-3-deoxynononic acid	Kdn	



The biosynthetic pathway of Neu5Ac in mammals is shown in Figure 10. In mammalian cells, exogenous ManNAc and Neu5Ac can be taken up, metabolized through the sialic acid biosynthetic pathway, and then expressed on the cell surface as sialosides.[117] Thus, cell surface glycoconjugates could be altered if unnatural ManNAc or sialic acid analogs exogenously introduced to the cell were successfully converted into sialoglycoconjugates.

The Unnatural Substrate Tolerance of the Enzymes Involved in Sialic Acid Biosynthesis

The possibility that sialic acid biosynthesis might be amenable to interception with unnatural substrates was first suggested by studies of the individual enzymes in the pathway. Several groups have analyzed the tolerance of various enzymes for unnatural substrates, revealing sites on metabolic intermediates at which functional group substitutions might be tolerated. Of the enzymes involved in sialic acid biosynthesis, the two that have attracted the most attention are CMP-Neu5Ac-synthetase and various sialyltransferases. Both bacterial and mammalian homologs from these two enzyme families have been characterized.

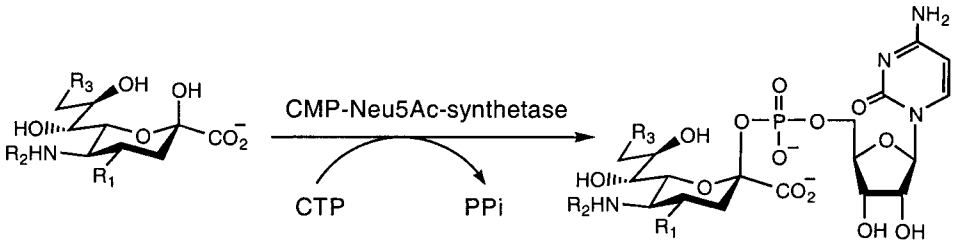
CMP-Neu5Ac-synthetase catalyzes the condensation of free Neu5Ac with CTP to make CMP-Neu5Ac. The enzymes from bovine brain and rat liver have been investigated with respect to their activity with unnatural sialic acids.[118] Sialic acids modified at C5 and C9 have been extensively studied; several of these are accepted by the enzyme and are successfully converted into the corresponding CMP-sialic acid analogs in yields of 40–90%[119] (Table 9). The efficiency of the reaction depended on the analog. While the enzyme tolerates rather large substituents at C9, structural perturbation at C5 must be conservative. Examples of acceptable modifications are shown in Table 9.[119–124] The 4-deoxy derivative of Neu5Ac is also a substrate for CMP-Neu5Ac synthetase.[125] The large variety of compounds that have been successfully converted into their CMP analogs indicate that bovine CMP-Neu5Ac-synthetase might be useful for in vitro enzymatic synthesis of unnatural CMP–sialic acid analogs.

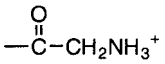
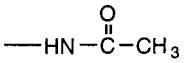
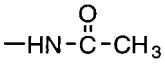
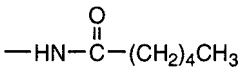
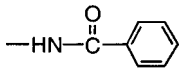
In eukaryotes, sialyltransferases are located in the Golgi compartments.[126,127] They transfer a sialic acid residue from CMP–sialic acid to a nonreducing Gal-, GalNAc-, GlcNAc-, or sialic acid residue via an α-glycosidic linkage. The possible linkages include α2,3, α2,6, or α2,8, and each is the product of a different sialyltransferase.

Sialyltransferases from rat and human liver have been studied extensively.[128] Similar to CMP-Neu5Ac-synthetase, the broad specificity of sialyltransferases has made them useful for the synthesis of neoglycoconjugates containing a variety of sialic acid analogs. Examples of CMP-sialic acid analogs that have been successfully converted

Figure 10. The sialoside biosynthetic pathway in eukaryotic cells: A, UDP-GlcNAc-2-epimerase; B, ManNAc-6-kinase; C, Neu5Ac-9-PO_4^{2-} synthase; D, Neu5Ac-9-PO_4^{2-} phosphatase; E, CMP-Neu5Ac-synthetase; F, sialyltransferase. Enzymes A and B function together as a bifunctional enzyme.

Table 9. A Limited Number of Sialic Acid Derivatives Are Recognized as Substrates for CMP–Sialic Acid Synthetase from Bovine Brain and Rat Liver



R_1	R_2	R_3
H	Ac	OH
OH		OH
OH		OH
OH		OH
OH	Ac	N_3
OH	Ac	NH_3^+
OH	Ac	
OH	Ac	
OH	Ac	
OH	Ac	
OH	Ac	SHgMe

into glycoconjugates by sialyltransferases are shown in Table 10.[118–120,125,128–131] Even perturbations as large as a fluorescein group at C5 or C9 are tolerated by some sialyltransferases, allowing one to install fluorescent probes in cell surface glycoconjugates.[132,133] This approach has provided a method for kinetic analysis of the rates of different sialyltransferases within cells.[134,135] Furthermore, the C9 fluorescein derivative has been used to localize sialyltransferases to the Golgi compartments in rat liver.[127] Although alterations at C5 and C9 can reduce efficiency of sialyltransferases, the reactions occur at a reasonable rate and are therefore useful for studying cellular processes.

Sialic acid biosynthesis in bacteria is less complex than in mammals. There are only three enzymes involved in Neu5Ac biosynthesis; they are the Neu5Ac–synthase, CMP-Neu5Ac-synthetase, and sialyltransferase. Although these enzymes were cloned only within the past 10 years, their straightforward overexpression in bacteria has allowed extensive studies to be performed.

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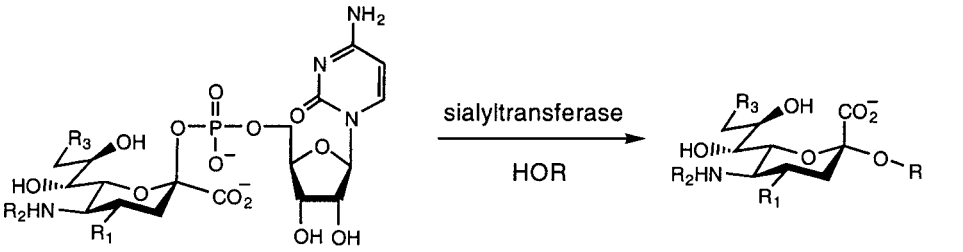
Table 10. A Limited Number of CMP–Sialic Acid Derivatives Are Accepted as Substrates by α2,3- and α2,6-Sialyltransferases from Bovine and Human Liver



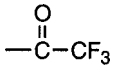
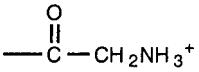
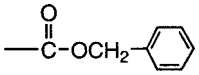
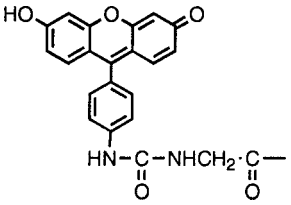
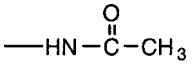
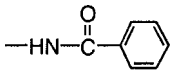
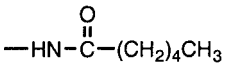
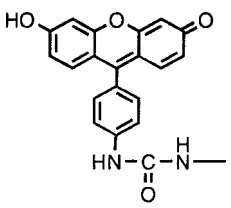
R_1	R_2	R_3
H	Ac	OH
OH	Ac	OAc
OH	Gc	OH
OH		OH
OH		OH
OH		OH
OH		OH
OH		OH
OH	Ac	F
OH	Ac	N_3
OH	Ac	NH_3^+
OH	Ac	
OH	Ac	
OH	Ac	
OH	Ac	

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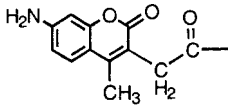
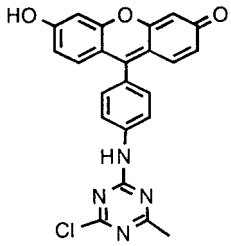
Table 10. Continued

R_1	R_2	R_3
OH	Ac	
OH	Ac	

It was not until 4 years ago that the first bactrial Neu5Ac-synthase was cloned.[136] This enzyme, derived from *E. coli* K1, is unlike the mammalian enzyme in that it does not accept the ManNAc-6-PO_4^{2-} as a substrate. Rather, it condenses ManNAc with phosphoenolpyruvate (PEP) to produce Neu5Ac. Furthermore, 6-N_3 ManNAc can be converted to 9-N_3 Neu5Ac, although only with 59% efficiency compared to the native substrate.[137] The *E. coli* synthase has also been shown to accept ManN as a substrate, albeit with only a minimal production of the sialic acid analog. This lack of specificity was also seen with the purified Neu5Ac-synthase from *Neisseria meningitidis*.[138] Since its initial identification in *E. coli*, the Neu5Ac-synthase gene has also been cloned from numerous microbial organisms including *Helicobacter pylori*. The availability of these enzymes promises to facilitate the enzymatic synthesis of sialic acid analogs.

In contrast to the eukaryotic CMP-Neu5Ac-synthetases, the bacterial counterparts have not been extensively characterized. Therefore, it would be interesting to see whether the bacterial enzymes exhibit the same broad substrate specificity. Thus far, only 9-N_3 and 9-NH_3^+ modified sialic acids have been tested with the *E. coli* and *N. meningitidis* CMP-Neu5Ac-synthetase.[138,139] While the 9-N_3 analog was similar to Neu5Ac as a substrate for the enzyme, the 9-NH_3^+ derivative was a poor substrate. Unfortunately, many of the sialic acid analogs that were active as substrates for the mammalian enzyme have not yet been examined by means of bacterial enzymes. A few C5-modified analogs have been tested, namely, *N*-propanoyl sialic acid (SiaProp), *N*-glycolylneuraminic acid (Neu5Gc), and *N*-carbomethoxyneuraminic acid (Neu5CMe), and these were tolerated by the enzyme. 5-Azidoneuraminic acid was not a substrate for the enzyme, and *N*-carbobenzyloxyneuraminic acid (Neu5Cbz) coupled at a much slower rate than Neu5Ac.[140] While it would appear that the bacterial enzyme is more restrictive in its substrate specificity, it does show some tolerance for unnatural C5 and C9 substituted sialic acid derivatives.

Sialylated glycoconjugates found in bacteria often mimic oligosaccharides found on the surface of mammalian cells.[141–143] The similarity in structure between the glycoconjugates of mammalian and bacterial cells might reflect a mechanism by which

bacterial cells evade the host immune response. Given the similarity of bacterial and mammalian sialoglycoconjugate structures, the bacterial sialyltransferases might have substrate recognition properties similar to those of their mammalian counterparts. Unfortunately, few bacterial sialyltransferases have been cloned or characterized.[144] However, it is known that similar to mammalian sialyltransferases, bacterial sialyltransferases are membrane-associated proteins that are responsible for terminating glycans by transferring a sialic acid residue to a terminal GlcNAc, GalNAc, or Gal residue. Like their mammalian counterparts, they too are capable of producing α2,3-, α2,6-, or α2,8-linked sialosides. An α2,6-sialyltransferase isolated from the bacterium *Photobacterium damsela* is capable of sialylating 3′-sialylLacNAc oligosaccharides, generating a rare 3′,6′-disialylated structure.[145] Bacterial sialyltransferases are also capable of forming α2,9-linked structures. The α2,8- and α2,9-linked sialic acids are generally found as homopolymers that make up the capsule of pathogenic bacteria like *N. meningitidis*, *E. coli* K1, and *E. coli* Bos 12.

Of the few bacterial sialyltransferases cloned, even fewer have been studied with respect to donor-substrate specificity. CMP-Neu5Ac, CMP-Neu5Gc, CMP-SiaProp, and CMP-9-fluoro-Neu5Ac have been tested as substrates for the *N. meningitidis* α2,3-sialyltransferase.[146–148] While these substrates were effectively recognized, the small panel does not provide enough information to generalize the specificity of the enzyme. If the enzyme is to be useful in chemoenzymatic synthesis or in studying cellular processes, knowledge of its specificity with a broader panel of substrates would be useful.

Metabolic Conversion of ManNAc Analogs to Cell Surface Sialosides

The notion that unnatural analogs of ManNAc might be metabolized to sialic acids in living cells was first explored in the 1970s. Initial studies of the sialic acid biosynthetic pathway focused on the inhibitory properties of metabolic precursors.[62,149,150] It was discovered that some ManNAc analogs, while exhibiting a degree of toxicity to the cells, competed with native ManNAc and were incorporated into the sialic acid biosynthetic pathway. This finding, along with the earlier knowledge that cells can take up exogenous ManNAc and convert it into sialic acid,[117,151] opened up many possibilities for cell surface modification. Figure 11 shows the different ManNAc analogs that have been successfully metabolized by cells and converted into cell surface sialosides. For example, peracetylated ***N***-trifluoroacetyl ManN, although toxic to cells at high micromolar concentrations, competed with ManNAc in glycoconjugate biosynthesis.[62]

The expression of metabolically modified sialic acids on the cell surface has been used to modulate several biological processes. Schmidt et al. showed that *N*-propanoylmannosamine (ManProp), which is metabolized to SiaProp, stimulates proliferation in specific cells found in the rat central nervous system (CNS).[152] Incubation of cells from neonatal rat brain with ManProp successfully stimulated proliferation of astrocytes and microglia, but not of oligodendrocyte progenitor cells in culture. The basis for this proliferation is unclear, since all cells examined converted ManProp into cell surface SiaProp. Similarly, human diploid fibroblast cells grown with ManProp, *N*-butanoylmannosamine (ManBut), or *N*-pentanoylmannosamine (ManPent) lost their sensitivity to contact inhibition of growth.[153]

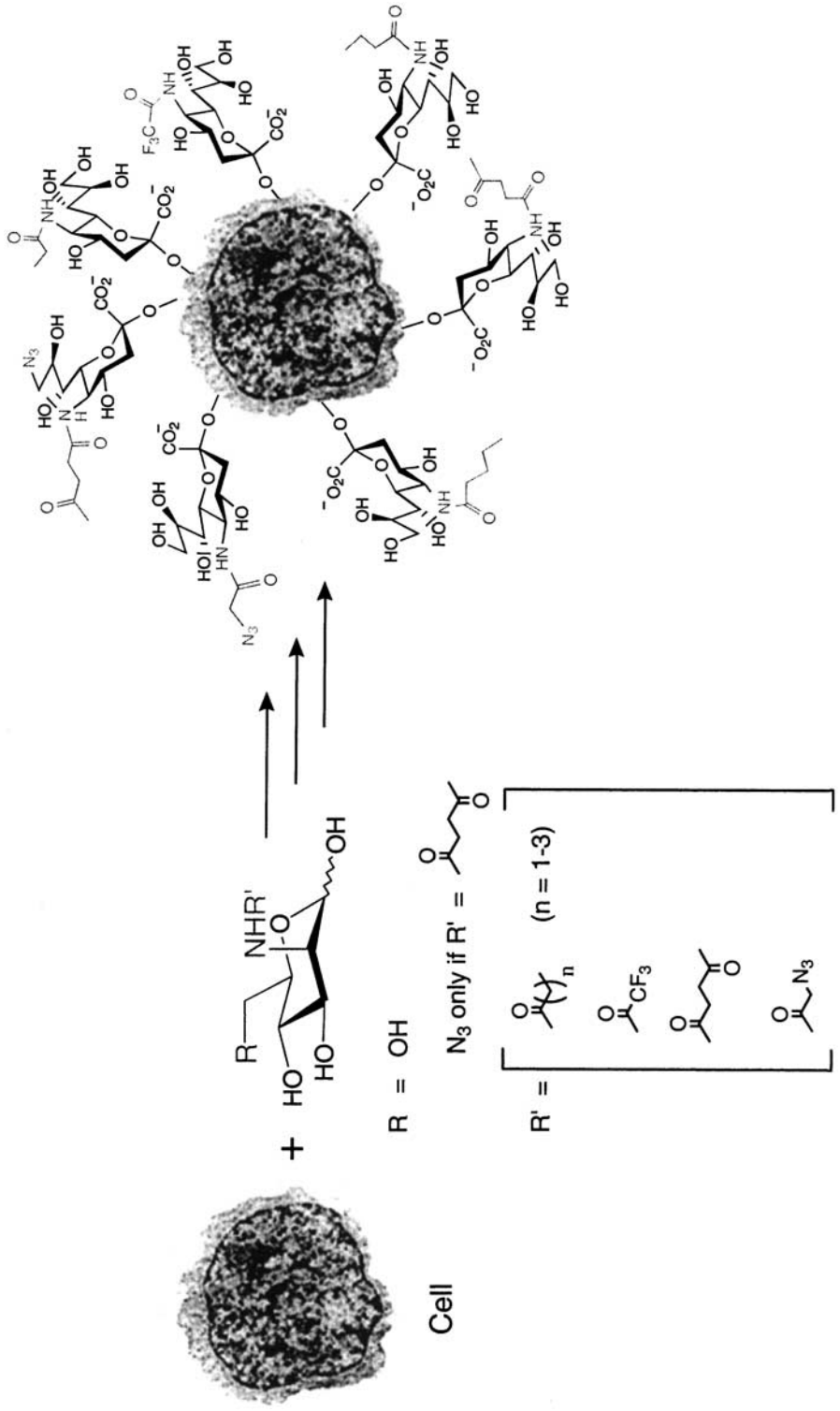


Figure 11. ManNAc derivatives that are metabolically converted to the corresponding cell surface sialic acid analogs in mammalian cells.

Virus–receptor interactions have also been shown to be affected by these ManN derivatives.[154,155] Treatment of human B-lymphoma BJA-B cells or African green monkey kidney epithelium cells with either ManProp, ManBut, or ManPent resulted in structural modification of about 50% of total cell surface sialic acids. Polyoma viruses, which use sialic acids as ligands for binding prior to infection, show either reduced or enhanced ability to infect cells carrying these modified sialic acids, depending on the ManN analog used. The expression of modified sialic acids might therefore be a new mechanism to prevent viral infection.

An analog similar to ManPent, but containing a ketone group on the *N*-acyl side chain, has been synthesized as a sialic acid precursor.[156] This derivative, called *N*-levulinoylmannosamine (ManLev), is converted to the corresponding sialic acid (SiaLev), delivering reactive ketone groups to cell surface glycoconjugates. The biosynthesis of *N*-levulinoyl sialic acid has been demonstrated in a variety of human cell lines (e.g., Jurkat, HeLa, and HL-60 cells). Scatchard analysis of ManLev-treated cells indicated the presence of at least several million derivatized sialic acid residues on the cell surface.[157] The expression of SiaLev on the cell surface introduces an orthogonal reactive group, the ketone, into cell surface glycans. The ketone group, which can undergo a number of coupling reactions under physiological conditions, can be used to introduce new recognition epitopes onto the cell surface, a method called ''glycoform remodeling.''[157] In this fashion, large biomolecules can be displayed on cell surfaces by using chemical methods rather than conventional genetics tools. For example, it has been demonstrated that aminooxy- and hydrazide-functionalized oligosaccharides can be chemoselectively reacted with SiaLev to further elongate the oligosaccharide chain. Modified sialic acids bearing ketones have been exploited in new approaches to selective drug delivery[156] and for targeting of diagnostic agents.[158] Other functionalized *N*-acyl side chains on ManN, such as the *N*-azido acetyl group, are also tolerated by the biosynthetic enzymes.[159] Discovery of this property opens up new opportunities for modifying sialic acids and reacting them with exogenous agents.

CONCLUSION

The field of metabolic substrate engineering has evolved from initial cytotoxicity studies using unnatural sugars, which produced empirical observations, to cell surface engineering using chemically defined epitopes. The transformation of this rapidly growing field is the result of molecular cloning efforts of the past decades, which have enabled detailed studies of enzyme specificities. Moreover, analytical tools such as mass spectrometry and HPLC have advanced to the point that characterization of unnatural metabolites within complex glycoconjugates is now possible. With the availability of powerful genetic and analytic methods, it should be possible to apply metabolic substrate engineering more broadly to fundamental studies of glycobiology.

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