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Dissecting glycoprotein biosynthesis by the use of specific inhibitors*

William McDOWELL^{1**} and Ralph T. SCHWARZ²

¹*Institut für Virologie, Justus-Liebig-Universität-Giessen, Frankfurter Strasse 107, D-6300 Giessen, F.R.G., and*

²*Laboratoire de Chimie Biologique, Université des Sciences et Techniques de Lille Flandres-Artois, F-59655 Villeneuve-d'Ascq Cedex, France*

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Summary — It is possible to interfere with different steps in the dolichol pathway of protein glycosylation and in the processing of asparagine-linked oligosaccharides. Thus some clues about the role of protein-bound carbohydrate can be obtained by comparing the biochemical fates and functions of glycosylated proteins with their non-glycosylated counterparts, or with proteins exhibiting differences in the type of oligosaccharide side chains. Cells infected with enveloped viruses are good systems for studying both aspects of protein glycosylation, since they contain a limited number of different glycoproteins, often with well-defined functions. Tunicamycin, an antibiotic, as well as several sugar analogues have been found to act as inhibitors of protein glycosylation by virtue of their anti-viral properties. They interfere with various steps in the dolichol pathway resulting in a lack of functional lipid-linked oligosaccharide precursors.

Compounds that interfere with oligosaccharide trimming represent a second generation of inhibitors of glycosylation. They are glycosidase inhibitors that interfere with the processing glucosidases and mannosidases and, as a result, the conversion of high-mannose into complex-type oligosaccharides is blocked. Depending upon the compound used, glycoproteins contain glycosylated-high-mannose, high-mannose or hybrid oligosaccharide structures instead of complex ones.

The biological consequences of the alterations caused by the inhibitors are manifold: increased susceptibility to proteases, improper protein processing and misfolding of polypeptide chains, loss of biological activity and alteration of the site of virus-budding, to name but a few.

protein glycosylation / oligosaccharide trimming / inhibition / viral glycoproteins

Introduction

Perturbation of biochemical pathways can be brought about in two ways by a genetic approach, through the selection of mutants lacking one or more of the enzymes in a pathway, or through the use of inhibitors that block specific steps in the pathway. Using these approaches, an

ordered sequence of intermediates and reactions that define a particular pathway can be obtained. Both approaches are complimentary and have been used to define the pathways involved in the biosynthesis of the asparagine-linked oligosaccharide side chains of glycoproteins. A discussion of glycosylation mutants is beyond the scope of this article, but recent reviews of this topic

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**Present address: MRC Collaborative Centre, 1–3 Burtonhole Lane, Mill Hill, London NW7 1AD, U.K.

Abbreviations: **GlcNAc**: N-acetyl-D-glucosamine; **Man**: D-mannose; **Glc**: D-glucose; **Dol-P**: dolichol monophosphate; **Dol-PP**: dolichol pyrophosphate; **UDP**: uridine diphosphate; **GDP**: guanosine diphosphate.

[1, 2] can be consulted for more information.

The discovery of compounds that interfere with protein glycosylation resulted in a dramatic increase in the study of the biological role of protein-bound carbohydrate. Many of the compounds were first discovered because of their anti-viral effects, due to the inhibition of glycosylation of virus envelope glycoproteins [3–5]. Virus-infected cells are good systems for studying the biological properties of glycosylation inhibitors and have been useful for establishing the modes of action of many of the inhibitors, as well as investigating the biosynthesis of membrane glycoproteins [3–6].

The first generation of inhibitors, represented by tunicamycin, 2-deoxyglucose, glucosamine and the fluorosugars, block the assembly of the lipid-linked oligosaccharide precursor $\text{Glc}_3\text{Man}_9(\text{GlcNAc})_2\text{-PP-Dol}$ [3–6]. A second generation of glycosylation inhibitors, represented by the 1,5-dideoxy-1,5-imino-D-hexitol analogues of glucose and mannose and the indolizidine alkaloids, interfere with the trimming glycosidases involved in the conversion of the high-mannose structures into those of the complex type [3, 6, 7].

The pathway of protein *N*-glycosylation

It is now well known that the glycosylation of proteins containing oligosaccharides *N*-linked to asparagine proceeds in two stages [8]. Firstly, the precursor oligosaccharide $\text{Glc}_3\text{Man}_9(\text{GlcNAc})_2$ is assembled on a dolichol pyrophosphate (Dol-PP) carrier in the rough endoplasmic reticulum. In the second stage, following its transfer to the nascent polypeptide, the precursor oligosaccharide undergoes a series of processing reactions in the rough endoplasmic reticulum and in the Golgi apparatus, resulting in the formation of the high-mannosidic and complex-type oligosaccharides characteristic of mature asparagine-linked glycoproteins.

The dolichol pathway

The lipid-linked oligosaccharide $\text{Glc}_3\text{Man}_9(\text{GlcNAc})_2\text{-PP-Dol}$ is assembled by a series of reactions constituting the dolichol pathway [8, 9] (Fig. 1). The first step in the pathway is the transfer of GlcNAc-1-P from UDP-GlcNAc to dolichol phosphate (P-Dol) giving GlcNAc-PP-Dol , to which a second GlcNAc residue is transferred from UDP-GlcNAc , forming $(\text{GlcNAc})_2\text{-PP-Dol}$.

This molecule is elongated by the transfer of 5 mannose residues directly from GDP-Man , yielding the $\text{Man}_5(\text{GlcNAc})_2\text{-PP-Dol}$ intermediate [10–13]. Four more mannose residues are subsequently added *via* the intermediate Man-P-Dol [14, 15]. Addition of 3 glucose residues *via* the intermediate Glc-P-Dol completes the assembly of the lipid-linked oligosaccharide $\text{Glc}_3\text{Man}_9(\text{GlcNAc})_2\text{-PP-Dol}$ [16, 17], which is the common precursor of both the high-mannosidic and complex-type oligosaccharide side chains of *N*-linked glycoproteins [18]. Transfer of the completed oligosaccharide to selected asparagine residues contained in the sequon Asn-X-Ser/Thr is a cotranslational event, occurring at the luminal face of the rough endoplasmic reticulum [8, 9, 19–21].

Oligosaccharide trimming

Once the oligosaccharide is protein-bound, it undergoes a sequence of trimming reactions [8, 22] (Fig. 2). The 3 glucose residues are rapidly removed by glucosidases I and II [23, 24], whilst the glycoprotein is still in the endoplasmic reticulum. An endoplasmic reticulum α -mannosidase may initiate mannose trimming at this point by removing one mannose residue before the glycoprotein is transported to the *cis*-compartment of the Golgi apparatus [8, 25]. Subsequent removal of mannose residues by Golgi α -mannosidase I results in the formation of the $\text{Man}_5(\text{GlcNAc})_2$ intermediate [22, 26]. Incomplete removal of mannose at this point yields high-mannose oligosaccharides containing 5–8 mannose residues [8–22]. The Man_5 -intermediate is a substrate for GlcNAc transferase I, which adds a GlcNAc to the free mannose residue linked α -1,3 to the β -mannose residue [8, 22]. Mannosidase II removes two more mannose residues [22, 24]. GlcNAc transferase I and mannosidase II are located in the medial compartment of the Golgi [8]. Addition of further GlcNAc residues and residues of galactose, fucose and sialic acid, catalysed by specific glycosyltransferases in the *trans*-Golgi, completes the assembly of the complex-type oligosaccharides [8, 22, 28, 29].

Inhibition of the dolichol pathway

Work conducted in several laboratories including our own has demonstrated that is possible to

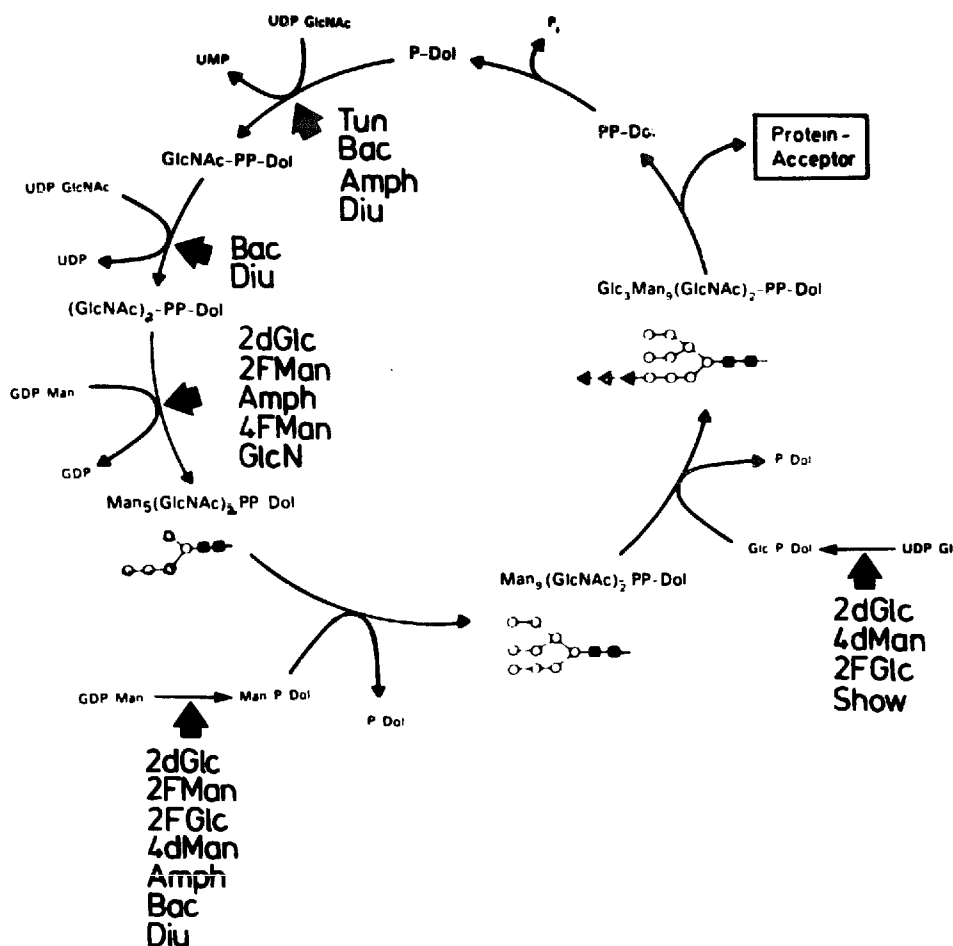


Fig. 1. The dolichol pathway of protein glycosylation. The sites of action of specific inhibitors of lipid-linked oligosaccharide assembly are marked with a thick arrow. Abbreviations used: **Tun**: tunicamycin; **Bac**: bacitracin; **Amph**: amphomycin; **Diu**: diuomycin; **Show**: showdomycin; **2dGlc**: 2-deoxy-D-glucose; **2FMan**: 2-deoxy-2-fluoro-D-mannose; **2FGlc**: 2-deoxy-2-fluoro-D-glucose; **GlcN**: glucosamine; **4dMan**: 4-deoxy-D-mannose; **4FMan**: 4-deoxy-4-fluoro-D-mannose. ■: GlcNAc; ○: Man; ◀: Glc.

interfere with different steps in the dolichol pathway and oligosaccharide processing [3–7, 30]. Initially, inhibitors were discovered that blocked protein glycosylation by interfering with steps in the dolichol pathway [3–5] (Fig. 1).

Antibiotics

The antibiotic tunicamycin is the best well-known inhibitor of this group and there is a wealth of information concerning its mode of action and biological effects in different systems [3, 4, 6, 31]. It inhibits the first step in the dolichol pathway, namely the formation of GlcNAc-PP-Dol from P-Dol and UDP-GlcNAc [32], and appears to be a structural analogue of

UDP-GlcNAc, being composed of uracil, fatty acid, GlcNAc and tunicamine, an unusual 11-carbon aminodeoxydialose [31]. Tunicamycin itself is a generic name for a mixture of closely related structures containing variations in their fatty acid moieties [6].

Several other antibiotics that affect the biosynthesis of the lipid intermediates involved in bacterial cell wall formation interfere with the dolichol pathway [4, 6]. The peptide antibiotics amphomycin, tsushimycin and showdomycin inhibit the formation of dolichol-linked monosaccharides, whereas bacitracin has been found to interfere with several different reactions in the dolichol pathway, depending upon the system used [4, 6]. The elongation of

Man(GlcNAc)₂-PP-Dol (hen oviduct membranes), the formation of GlcNAc-PP-Dol (calf pancreas microsomes) and (GlcNAc)₂-PP-Dol synthesis (yeast membranes) have all been reported to be sensitive to bacitracin [4, 6]. Diumycin was found to inhibit not only Man-P-Dol formation, but also the formation of GlcNAc-PP-Dol and (GlcNAc)₂-PP-Dol, the addition of GlcNAc to GlcNAc-PP-Dol being more sensitive to inhibition than the transfer of GlcNAc-1-P to P-Dol [4, 6].

Sugar analogues

Sugar analogues were detected as inhibitors of protein glycosylation by virtue of their anti-viral properties [3-5]. Thus analogues of glucose and mannose, such as 2-deoxy-D-glucose (2DGlc), 2-deoxy-2-fluoro-D-glucose (2FGlc), 2-deoxy-2-fluoro-D-mannose (2FMan) and 2-amino-2-deoxy-D-glucose (glucosamine) were found to interfere with the glycosylation of the viral membrane glycoproteins by inhibiting the assembly

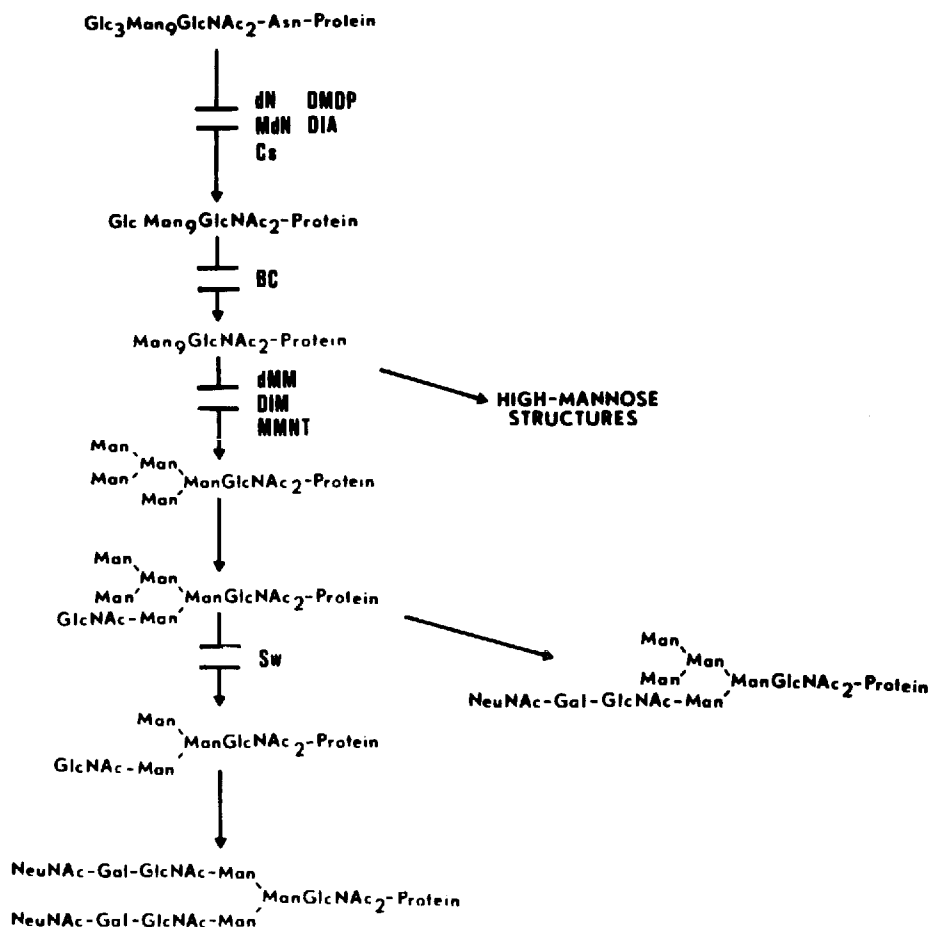


Fig. 2. The processing pathway for the formation of biantennary N-linked oligosaccharides and the sites of action of inhibitors of oligosaccharide trimming. Incomplete mannose trimming yields high-mannose oligosaccharides containing 5-9 mannose residues. In the presence of swainsonine, hybrid oligosaccharides containing high-mannose and sialylated antennae are formed as indicated. Abbreviations used: dN: 1-deoxynojirimycin; MdN: N-methyl-1-deoxynojirimycin; Cs: castanospermine; DMDP: 2,5-dihydroxymethyl-3,4-dihydropyridine; DIA: 1,4-dideoxy-1,4-imino-D-arabinitol; BC: bromoconduritol; dMM: 1-deoxymannojirimycin; DIM: 1,4-dideoxy-1,4-imino-D-mannitol; MMNT: α -D-mannopyranosylmethyl-p-nitrophenyltriazen; Sw: swainsonine.

of the lipid-linked oligosaccharide precursor $\text{Glc}_3\text{Man}_9(\text{GlcNAc})_2\text{-PP-Dol}$. More recently, mannose analogues containing modifications at the C-4 hydroxyl group, 4-deoxy-4-fluoro-D-mannose (4FMan) and 4-deoxy-D-mannose (4dMan) have been synthesized and also found to have similar anti-viral effects [33, 34]. The effects of the aforementioned sugar analogues on the dolichol pathway *in vivo* are listed in Table I.

With the exception of glucosamine, all of the sugar analogues have to be metabolised in order to exert their inhibitory effects [3, 4]. Thus, the respective nucleoside diphosphate esters have been detected in cells treated with the above sugar analogues and, *in vitro*, it is these derivatives that are the actual inhibitory agents. Table II lists the modes of action of these derivatives. The GDP-derivatives of 2dGlc, 2FMan and 4dMan are substrates for the biosynthesis of their respective monosaccharides-P-Dol, since

they are competitive inhibitors of GDP-Man: P-Dol mannosyltransferase [3, 4, 34, 35, 40]. In addition, the formation of 2dGlc-P-Dol, 2FMan-P-Dol or 4dMan-P-Dol from the corresponding labelled nucleotide sugar analogue has been observed *in vitro* [3, 4, 34, 35, 40]. In contrast, 4FMan-P-Dol was not readily synthesised from GDP-4FMan using a chick embryo cell membrane preparation, whereas in BHK cells labelled with $[^{14}\text{C}]4\text{FMan}$, $[^{14}\text{C}]4\text{FMan-P-Dol}$ was one of the metabolites detected [34]. Synthesis was only observed *in vitro* if the membranes had been primed with P-Dol.

3-Deoxy-D-mannose (3dMan) and 6-deoxy-D-mannose (6dMan) are not taken up by cells in tissue culture, however, it was interesting to synthesise the corresponding GDP-derivatives and look at their effects on the dolichol pathway *in vitro*. Both GDP-3dMan and GDP-6dMan were strong inhibitors of Man-P-Dol synthesis and the formation of lipid-linked oligosaccha-

Table I. Inhibition of lipid-linked oligosaccharide formation *in vivo* by sugar analogues.

Sugar analogue	Reactions inhibited
2-Deoxy-D-glucose (2dGlc)	Formation of Man-P-Dol, Glc-P-Dol and GlcNAc-PP-Dol due to the formation of 2dGlc-P-Dol. Mannosylation of $(\text{GlcNAc})_2\text{-PP-Dol}$ and $\text{Man}(\text{GlcNAc})_2\text{-PP-Dol}$ due to the formation of dGlc $(\text{GlcNAc})_2\text{-PP-Dol}$ and dGlc $(\text{GlcNAc})_2\text{-PP-Dol}$. Glucosylation of $\text{Man}_9(\text{GlcNAc})_2\text{-PP-Dol}$ [35-37].
2-Deoxy-2-fluoro-D-glucose (2FGlc)	Formation of Man-P-Dol and Glc-P-Dol. Pool size of UDP-Glc reduced. Major lipid-linked oligosaccharide is $\text{Glc}_3\text{Man}_5(\text{GlcNAc})_2\text{-PP-Dol}$. Proteins are glycosylated by the alternative pathway [38, 39].
2-Deoxy-2-fluoro-D-mannose (2FMan)	Formation of Man-P-Dol and mannosylation of $(\text{GlcNAc})_2\text{-PP-Dol}$ [40].
4-Deoxy-4-fluoro-D-mannose (4FMan)	Depleted pool of $\text{Glc}_3\text{Man}_9(\text{GlcNAc})_2\text{-PP-Dol}$ Dol-P-4FMan detected [33].
4-Deoxy-D-mannose (4dMan)	Formation of Man-P-Dol and Glc-P-Dol. $\text{Man}_9(\text{GlcNAc})_2\text{-PP-Dol}$ is the major lipid-linked oligosaccharide. Possible incorporation of 4dMan into lipid-linked oligosaccharide. Possible incorporation of 4dMan into lipid-linked oligosaccharide [34].
D-Glucosamine (GlcN)	In chick embryo cells lipid-linked oligosaccharide assembly inhibited and $\text{Man}_3(\text{GlcNAc})_2\text{-PP-Dol}$ accumulates [31]. In MDCK cells at low GlcN concentrations (0.5-1.0 mM) $\text{Man}_7(\text{GlcNAc})_2\text{-PP-Dol}$ accumulates. At high concentrations of GlcN (2-10 mM) $\text{Man}_3(\text{GlcNAc})_2\text{-PP-Dol}$ accumulates [42]. GlcN inhibition is reversible [43].
D-Mannosamine (ManN)	Accumulation of $\text{Man}_5(\text{GlcNAc})_2\text{-PP-Dol}$ and $\text{Man}_6(\text{GlcNAc})_2\text{-PP-Dol}$ [44].

Table II. Inhibition of lipid-linked oligosaccharide formation by nucleoside diphosphate esters of sugar analogues.

Sugar nucleotide	Reaction(s) inhibited
GDP-2dGlc	Formation of Man-P-Dol, Glc-P-Dol and GlcNAc-PP-Dol due to trapping of Dol-P as 2dGlc-P-Dol. Elongation of (GlcNAc) ₂ -PP-Dol due to incorporation of 2dGlc instead of Man [35-37].
GDP-2FGlc	No effect on lipid-linked saccharide formation <i>in vitro</i> [38], but formation of retinol phosphate mannose in rat liver microsomes inhibited [45].
GDP-2FMan	Formation of Man-P-Dol, Glc-P-Dol and GlcNAc-PP-Dol due to trapping of Dol-P as 2FMan-P-Dol. Formation of Man(GlcNAc) ₂ -PP-Dol inhibited [40].
GDP-3dMan	Formation of Man-P-Dol, Glc-P-Dol and GlcNAc-PP-Dol due to trapping of Dol-P as 3dMan-P-Dol (W. McDowell and R. T. Schwarz, unpublished data).
GDP-4FMan	Mannosylation of Man(GlcNAc) ₂ -PP-Dol. No effect on dolichol-linked monosaccharide formation [34].
GDP-4dMan	Man-P-Dol, Glc-P-Dol and GlcNAc-PP-Dol formation due to trapping of Dol-P as 4dMan-P-Dol. 4dMan may replace the 5 Man residues of lipid-linked oligosaccharides that come directly from GDP-Man [34].
GDP-6dMan	Formation of Man-P-Dol, Glc-P-Dol and GlcNAc-PP-Dol due to trapping of Dol-P as 6dMan-P-Dol (W. McDowell and R. T. Schwarz, unpublished data).
UDP-2dGlc	Formation of Glc-P-Dol [37].
UDP-2FGlc	Formation of Glc-P-Dol [38].
UDP-2FMan	Formation of Glc-P-Dol [40].
UDP-glucosamine	Formation of Glc-P-Dol due to trapping of Dol-P as GlcN-P-Dol [46].

For the abbreviations of the sugar analogues see Table I.

rides *in vitro* (W. McDowell and R. T. Schwarz, unpublished data). Like the other GDP-sugar analogues, the inhibition of Man-P-Dol synthesis by GDP-3dMan and GDP-6dMan could be reversed by addition of P-Dol, implying that they are competitive inhibitors of GDP-Man: P-Dol mannosyltransferase. The apparent inhibition constants for the GDP-sugar analogues were calculated from Dixon plots (W. McDowell and R. T. Schwarz, unpublished data) and the following order of inhibitor efficiency was found with the apparent K_i between parentheses: GDP-6dMan (0.4 μM) > GDP-3dMan (1.0 μM) > GDP-2dGlc (1.3 μM) > GDP-4dMan (3.1 μM) > GDP-2FMan (15 μM). For compa-

parison, the apparent K_s for GDP-Man was 0.5 μM and the apparent K_i for GDP was 56 μM (W. McDowell and R. T. Schwarz, unpublished data). From these results, it is clear that the hydroxyls at carbons 3 and 6 are not involved in the binding of GDP-Man to the active site, whereas those at carbons 2 and 4 must have some interaction. The high electronegativity of fluorine when present seems to have an adverse effect on the enzyme-substrate interaction, as shown by the fact that the K_i for GDP-2FMan is over 10 times higher than that for GDP-2dGlc. Also, GDP-4dMan is a much better inhibitor of Man-P-Dol formation than GDP-4FMan, which is non-inhibitory up to a

concentration of at least 200 μM [34].

As mentioned above, glucosamine itself, rather than a metabolite, is an inhibitor of protein glycosylation. However, glucosamine in the form of UDP-glucosamine is also an inhibitor [47]. It should be pointed out that UDP-glucosamine is only a product of galactosamine (not glucosamine) metabolism. UDP-glucosamine acts as an analogue of UDP-Glc and, *in vitro*, it inhibits Glc-P-Dol synthesis as a result of the formation of glucosamine-P-Dol, which can no longer be transferred to $\text{Man}_6(\text{GlcNAc})_2\text{-PP-Dol}$, raising the possibility that there may be depletion of P-Dol pools, and consequently an inhibition of protein glycosylation in cells treated with galactosamine [46].

Sugar nucleotides containing modified nucleoside moieties

As indicated above, nucleotide esters of sugar analogues can interfere with the assembly of lipid-linked oligosaccharide and thus protein glycosylation. Little is known about the effects of modification of the nucleoside moiety of sugar nucleotides. To tackle this, two approaches were used [47]. In the first, sugar nucleotides containing 5-fluorouridine were synthesised and, in the second, the ribose of the uridine moiety was periodate oxidised under mild conditions to produce suicide substrates. 5-Fluorouridine (FU)-containing sugar nucleotides, FUDP-Glc and FUDP-GlcNAc were found to be alternative substrates for the formation of Glc-P-Dol and $(\text{GlcNAc})_2\text{-PP-Dol}$, respectively, FUDP-Glc was as good a substrate as UDP-Glc, whereas FUDP-GlcNAc was a poor substrate compared to UDP-GlcNAc [47]. The periodate oxidised sugar nucleotides *o*-UDP-Glc and *o*-UDP-GlcNAc were suicide substrates, inhibiting the formation of Glc-P-Dol and $(\text{GlcNAc})_2\text{-PP-Dol}$ respectively. *o*-UDP-GlcNAc preferentially inhibited the addition of GlcNAc to GlcNAc-PP-Dol [47]. Therefore, on one hand, the introduction of a fluoro group at position 5 on the uracil ring of a UDP-sugar produces an alternative substrate, whereas, on the other hand, chemical modification by periodate oxidation results in an inhibitor.

Inhibition of the trimming pathway

The conversion of the glycosylated high-mannose precursor oligosaccharide, once it has been transferred to protein, into oligosaccharides of

the complex-type involves the concerted action of glucosidases and mannosidases [8, 22]. Therefore, it was obvious to search for potential inhibitors of the trimming pathway among known inhibitors of glycosidases and the results of this search have been quite productive with several useful inhibitors being found [7] (Fig. 2). Nevertheless, some of those tested, such as nojirimycin and acarbose, were either not specific inhibitors or had no effect [3].

Swainsonine

The first reported trimming inhibitor was the indolizidine alkaloid swainsonine ($8\alpha\beta$ -indolizidine-1 α ,2 α ,8 β -triol), which was discovered by virtue of its property of inducing a condition identical to that of human mannosidosis in sheep and cattle that had eaten the wild plants *Swainsona canescens* (Australia) or locoweed (U.S.A.) [3, 6]. The alkaloid was isolated from these plants and was found to be a potent inhibitor of lysosomal and jack bean α -mannosidases [3, 6]. Subsequently, it was found to prevent the conversion of high-mannose oligosaccharides into those of the complex-type in various mammalian cell lines, resulting in the formation of hybrid oligosaccharides that were endoglucosaminidase H-sensitive [48]. *In vitro* experiments with Golgi mannosidase I and mannosidase II showed that swainsonine specifically inhibited the latter enzyme [49].

Bromoconduritol and deoxynojirimycin

The glucosidase inhibitors bromoconduritol (6-bromo-3,4,5-trihydroxycyclohex-1-ene) and 1-deoxynojirimycin (1,5-dideoxy-1,5-imino-D-glucitol) were the next trimming inhibitors to be reported [7]. Bromoconduritol is an active site-directed covalent inhibitor of α -glucosidases, being obtained by chemical synthesis from myo-inositol [50]. When incubated with a rat liver glucosidase preparation, bromoconduritol inhibited the release of the innermost glucose residue from the oligosaccharide $\text{Glc}_3\text{Man}_9\text{GlcNAc}$, resulting in the accumulation of $\text{Glc}_1\text{Man}_9\text{GlcNAc}$ [51]. In influenza virus-infected chick embryo cells treated with bromoconduritol, the formation of complex-type oligosaccharides was inhibited and the viral glycoproteins contained high-mannose oligo-saccharides with the composition $\text{Glc}_1\text{Man}_{7-9}(\text{GlcNAc})_2$ [51]. Thus, the *in vitro* and *in vivo* data indicate that bromoconduritol is an inhibitor of glucosidase II, even

though oligosaccharides with 1 Glc residue rather than 2 Glc residues accumulate. A possible explanation for this apparent anomaly could be that bromoconduritol interferes with the binding of oligosaccharide to the active site, after removal of 1 Glc residue from $\text{Glc}_2\text{Man}_9(\text{GlcNAc})_2$, rather than with the catalytic site of the enzyme [3].

1-Deoxynojirimycin is a potent inhibitor of glucosidase I and glucosidase II *in vitro* [3, 6]. The sensitivity of the trimming glucosidases to the drug varies depending upon the enzyme source. Thus, with the enzymes from yeast, 50% inhibition of enzyme activity was obtained with 20 μM for glucosidase I and 5 μM for glucosidase II [52], whereas with calf liver microsomes, 50% inhibition was obtained with 3 μM for glucosidase I and 20 μM for glucosidase II [53]. In intact cells, 1-deoxynojirimycin inhibited the formation of complex-type oligosaccharides [52] and high-mannose oligosaccharides of the composition $\text{Glc}_{1-3}\text{Man}_9(\text{GlcNAc})_2$ accumulated [54]. Oligosaccharides containing 3 glucose residues accounted for only 20% of the mixture, indicating that *in vivo*, whilst 1-deoxynojirimycin interferes with the action of both trimming glucosidases, it preferentially inhibits glucosidase II [54]. Apart from being a trimming inhibitor, 1-deoxynojirimycin may also interfere with the formation of the lipid-linked oligosaccharide precursor $\text{Glc}_3\text{Man}_9(\text{GlcNAc})_2\text{-PP-Dol}$ [3]. It was thought that the inhibitory effect may be related mechanistically to the inhibition of lipid-linked oligosaccharide biosynthesis by glucosamine [3]. Indeed, methylation of the ring nitrogen suppressed the inhibition of lipid-linked oligosaccharide synthesis. The resulting *N*-methyl-1-deoxynojirimycin (MdN) was a more potent inhibitor of glucosidase I than the parent compound [53, 55]. *In vivo* MdN inhibited the formation of complex-type oligosaccharides and high-mannose oligosaccharides of the composition $\text{Glc}_3\text{Man}_{7-9}(\text{GlcNAc})_2$ accumulated [55]. The effects of 1-deoxynojirimycin and MdN on the formation of complex-type oligosaccharides were compared using cultured intestinal epithelial cells. It was found that 5 mM of 1-deoxynojirimycin was required to produce maximal inhibition, whereas only 1 mM of the *N*-methyl-derivative was required to produce the same effect. In addition, in the presence of the *N*-methyl-derivative, 70% of the oligosaccharides contained 3 glucose residues in contrast to 20% in the 1-deoxynojirimycin-treated sample [54]. Therefore, *N*-methyl-1-deoxynojirimycin is the prefer-

red derivative for the inhibition of glucosidase I without the added complication of interfering with lipid-linked oligosaccharide synthesis.

Castanospermine

Castanospermine ($8\alpha\beta$ -indolizidine- $1\alpha,6\beta,7\alpha,8\beta$ -tetrol) was the next inhibitor to appear. Like swainsonine, it is a plant alkaloid and it was isolated from the seeds of *Castanospermum australe*, which are toxic and cause gastrointestinal irritation when eaten by livestock [3, 6, 7]. It was found to be a potent inhibitor of lysosomal enzymes, such as β -glucosidase, β -glucocerebrosidase and α -glucosidase in fibroblast extracts as well as almond emulsin β -glucosidase, but was without effect on yeast α -glucosidase [3, 6]. Treatment of cells with castanospermine inhibited the formation of complex-type oligosaccharides and the glycoproteins were equipped with high-mannose oligosaccharides containing 3 glucose residues [57], similar to *N*-methyl-1-deoxynojirimycin. Castanospermine appears to be non-toxic to the treated cells with no effects on the formation of lipid-linked oligosaccharide or protein synthesis being observed [6]. Thus castanospermine, like *N*-methyl-1-deoxynojirimycin, is a good inhibitor of trimming glucosidase I without having detrimental effects on other cellular functions.

Deoxymannojirimycin

The success of the derivatives of nojirimycin as glucosidase trimming inhibitors led to the synthesis [57] and testing [58] of the mannose analogue of 1-deoxynojirimycin, 1-deoxymannojirimycin (1,5-dideoxy-1,5-imino-D-mannitol, dMM) as an inhibitor of trimming mannosidases. It was shown to be an inhibitor of Golgi mannosidase I [58], and, in cells treated with the drug, the conversion of high-mannose oligosaccharides into those of the complex-type was blocked [59–61]. The major oligosaccharide present on the glycoproteins was $\text{Man}_9(\text{GlcNAc})_2$, confirming that Golgi mannosidase I is the sensitive enzyme [59–61]. dMM is a competitive inhibitor of mannosidase I having a K_i of 2 μM for the rat liver enzyme [62]. The rat liver endoplasmic reticulum α -mannosidase was not sensitive to the drug [62]. One disadvantage of the use of dMM as a trimming inhibitor lies in the fact that it can also act as an inhibitor of the formation of lipid-linked oligosaccharides [59] (A. Tlusty, W. McDowell and R.T. Schwarz, unpublished

data). In chick embryo cells, for instance, the inhibition of lipid-linked oligosaccharide synthesis is drastic (90% inhibition with 1 mM), whereas with BHK or MDCK cells there is little or no inhibition at concentrations of dMM sufficient for the suppression of mannosidase I activity [59, 60]. As with 1-deoxynojirimycin, *N*-methylation suppressed the inhibition of lipid-linked oligosaccharide, but the resulting *N*-methyl-1-deoxymannojirimycin was not a more potent mannosidase inhibitor [41].

Polyhydroxylated piperidines and pyrrolidines

The aforementioned inhibitors have all, in the meantime, become commercially available. Nevertheless, there is considerable interest in the development of novel compounds exhibiting different specificities and modes of action towards glycosidases. One such group of compounds are the polyhydroxylated piperidines and pyrrolidines which are potential glycosidase inhibitors due to their structural resemblance to pyranose sugars. Compounds belonging to this group have been isolated from plants and found to be potent inhibitors of glycosidases [3, 6]. Chemical synthesis has made them available in amounts suitable for testing as inhibitors of glycoprotein oligosaccharide processing [70].

2*R*,5*R*-Dihydroxymethyl 3*R*, 4*R*-dihydropyrrolidine (DMDP) was the first of this new type to be used as a trimming inhibitor. It has been isolated from the leaves of *Derris elliptica* and seeds of *Lonchocarpus sericeus*, and its synthesis from glucose has been reported [63]. DMDP is a potent inhibitor of both α - and β -glucosidases with the concentrations required to give 50% inhibition about 60 times and 10 times lower, respectively, than those of 1-deoxynojirimycin [64]. However, there have been conflicting reports concerning the effects of DMDP on oligosaccharide processing *in vivo*. It was first reported [65] with influenza virus-infected MDCK cells that DMDP inhibited the trimming glucosidase I, since in treated cells the conversion of high-mannose oligosaccharides into complex-type oligosaccharides was inhibited and oligosaccharides of the composition $\text{Glc}_3\text{Man}_{8,9}(\text{Glc-NAc})_2$ accumulated on the viral glycoproteins. In a subsequent study [66], using cultured intestinal epithelial cells, the formation of complex-type side chains was also inhibited but a mixture of high-mannose oligosaccharides was found with the major oligosaccharides

having the composition $\text{Man}_{7-9}(\text{GlcNAc})_2$. Thus, in intestinal cells, the main effect of DMDP seemed to be directed towards the trimming mannosidases [66]. The situation with influenza virus-infected chick embryo cells differed yet again. Under appropriate conditions, glucosylated high-mannose oligosaccharides of the composition $\text{Glc}_3\text{Man}_{8,9}(\text{Glc-NAc})_2$ were present on the viral glycoproteins formed in the presence of DMDP but the predominant structures were $\text{Man}_{8,9}(\text{GlcNAc})_2$ [67]. It is not clear why the effects of DMDP on oligosaccharide processing in MDCK cells, intestinal epithelial cells and chick embryo cells should be so different but may be related to protonation of the ring nitrogen as a result of changes in pH during uptake into the cell and transport to, and penetration through the endoplasmic reticulum membrane, since the non-protonated form of DMDP is the active inhibitor [3].

An analogous compound, 1,4-dideoxy-1,4-imino-*D*-mannitol (DIM) was designed as an α -mannosidase inhibitor and synthesised chemically from benzyl- α -*D*-mannopyranoside [68]. It is as effective an inhibitor of jack bean α -mannosidase as swainsonine, exhibiting 50% inhibition at 0.5 μM . Lysosomal α -mannosidase was also sensitive to inhibition, but a much higher concentration was needed for 50% inhibition. In both cases, the inhibition was competitive and, as with DMDP, the non-protonated form is the more effective inhibitor [68, 69]. In influenza virus-infected MDCK cells treated with DIM [69], the synthesis of complex-type oligosaccharides was inhibited maximally by about 75%. The major high-mannose oligosaccharide that accumulated had the composition $\text{Man}_9(\text{GlcNAc})_2$, indicating that DIM inhibits mannosidase I. In addition, some of the oligosaccharides synthesised in the presence of DIM were of the hybrid type, suggesting that DIM also exerts an effect on mannosidase II. Thus, in intact cells, DIM acts by inhibiting both mannosidase I and mannosidase II, although it is a less effective inhibitor of the formation of complex-type oligosaccharides than swainsonine [69].

Another chemically synthesised iminopentitol is 1,4-dideoxy-1,4-imino-*D*-arabinitol (DIA), which was subsequently found to be identical to an alkaloid extracted from the legume *Angylocalyx boutiqueanus* [70]. DIA is a potent competitive inhibitor of yeast α -glucosidase (50% inhibition with 0.18 μM). It also had some effect on almond emulsin β -glucosidase and jack bean α -mannosidase but the concentrations required for

50% inhibition were 1000-fold higher than that for yeast α -glucosidase [70]. Treatment of influenza virus-infected chick embryo cells with DIA in medium containing 10 mM glucose resulted in an inhibition of the conversion of high-mannose oligosaccharides into those of the complex-type but no glucosylated high-mannose oligosaccharides accumulated [67]. However, when the glucose in the medium was reduced to 2 mM, then glucosylated high-mannose oligosaccharides with the composition $\text{Glc}_3\text{Man}_{8,9}(\text{GlcNAc})_2$ accumulated, suggesting that DIA has inhibited glucosidase I [67].

The results obtained with DMDP, DIM and DIA have demonstrated that nitrogen analogues of furanoses can be effective glycosidase inhibitors. The polyhydroxylated pyrrolidines and piperidines are relatively easy to synthesise and no doubt an array of such compounds inhibiting a variety of different glycosidases will be most useful for investigating structure-function relationships in oligosaccharides as well as to experimentally induce conditions that can be studied as models of the genetically inherited disorders of metabolism.

Glycosylmethyl-p-nitrophenyltriazenes

Most of the aforementioned inhibitors interact with their respective sensitive enzymes in a competitive manner. Suicide substrates offer an alternative means of inhibition by covalent modification of the active site of an enzyme. Glycosylmethyl-*p*-nitrophenyltriazenes were recognised as being potential suicide substrates for glycosidases [71]. The β -D-galactopyranosyl- and β -D-glucopyranosyl-derivatives inactivate bacterial and lysosomal β -galactosidase and lysosomal β -glucosidase [71, 72]. On the basis of these findings, α -D-mannopyranosylmethyl-*p*-nitrophenyltriene (MMNT) was synthesised and tested as a mannosidase inhibitor [73, 74]. Lysosomal and jack bean α -mannosidases were inactivated as was Golgi mannosidase I. Golgi mannosidase II and endoplasmic reticulum α -mannosidase were resistant. 50% inhibition of mannosidase I was obtained with 0.18 mM MMNT [73]. In hepatocytes treated with MMNT, the α 1-acid glycoprotein was glycosylated with high-mannose oligosaccharides of the composition $\text{Man}_{7-9}(\text{GlcNAc})_2$ [75]. Influenza virus glycoproteins were equipped mainly with high-mannose oligosaccharides of the composition $\text{Man}_{7,8}(\text{GlcNAc})_2$, when synthesised in infected chick embryo cells treated with MMNT

[73]. Thus, *in vivo*, MMNT appears to interfere with Golgi mannosidase I activity.

Biological effects of trimming inhibitors on viral systems

Inhibition of protein glycosylation can have diverse biological effects depending upon the glycoprotein and cell type under investigation [3, 6, 7]. Through the use of trimming inhibitors, it has been possible to further investigate how specific changes in oligosaccharide composition affect intracellular transport, polypeptide folding and virus maturation as well as the relative importance of high-mannose and complex-type oligosaccharides. Although the effects of trimming inhibitors have been studied in many systems, virus-infected cells have provided some interesting biological phenomena, some of which are listed in Table III.

As shown in Table III, the formation of some influenza viruses and Rous sarcoma virus is not affected by inhibitors of glucosidase or mannosidase trimming. One exception is the fowl plaque virus strain of influenza virus, the haemagglutinin of which is metabolically unstable when oligosaccharide trimming has been inhibited with the glucosidase II inhibitor bromoconduritol, and oligosaccharides with the composition $\text{Glc}_1\text{Man}_{7-9}(\text{GlcNAc})_2$ are present on the glycoprotein. The differences between fowl plaque and the other strains of influenza virus (PR8, NWS, virus N) that have been used in the examination of the modes of action of trimming inhibitors are probably related to the cleavability of their haemagglutinins. Fowl plaque virus contains a cleaved haemagglutinin in which the 2 subunits, HA1 and HA2, linked by disulphide bonds are present, whereas influenza viruses PR8, NWS and N contain an uncleaved haemagglutinin [3]. Apparently uncleaved haemagglutinin is metabolically stable independent of oligosaccharide composition. Cleavage of haemagglutinin containing $\text{Glc}_3\text{Man}_{7-9}(\text{GlcNAc})_2$ oligosaccharides yields HA1 and HA2 subunits resistant to degradation by protease, whereas cleavage of haemagglutinin containing $\text{Glc}_1\text{Man}_{7-9}(\text{GlcNAc})_2$ yields products that are susceptible to proteolytic degradation, probably as a result of misfolding.

An essential role for glucose trimming in the establishment of a functional conformation for some viral glycoproteins has come from observations made not only with influenza virus but also

Table III. Effects of inhibitors of oligosaccharide processing on some viral systems.

Virus	Glycoprotein	Inhibitor	Effects
Influenza (FPV)	haemagglutinin	BC	Formation of infectious particles inhibited. HA1 + HA2 susceptible to proteolytic degradation [51, 76].
Influenza (PR8)	haemagglutinin	BC	No effect on virus particle formation [76].
Influenza (all strains)	haemagglutinin	MdN, dN, Cs, DMDP	No effect on virus particle formation. Sulphation in NWS-infected MDCK cells inhibited by Cs [55, 56, 65, 77, 78].
Influenza (all strains)	haemagglutinin	dMM, Sw, DIM	No effect on virus particle formation [59, 69, 79].
Influenza (FPV, PR8)	haemagglutinin neuraminidase	MMNT	Virus particle formation inhibited by 0.5–2 mM MMNT. Activity of haemagglutinin and neuraminidase unaffected by 0.5–1 mM MMNT. ^a
VSV (San Juan)	G-protein	dN, Cs	Virus particle formation inhibited, but effect is temperature-sensitive. Transport of G-protein to cell surface unaffected [80].
VSV (Orsay)	G-protein	dN, Cs, Sw	No effect on virus particle formation [80, 81].
VSV (Indiana)	G-protein	dN, dMM	No effect on virus particle formation or on surface expression of G-protein [77].
Sindbis	E1, E2	BC, dN, Cs, MdN, DIA	Virus particle formation inhibited, but effect temperature-sensitive (dN, Cs, MdN). Cleavage of pE2 to E2 blocked. Cell surface expression of pE2 [50, 67, 76, 82].
Sindbis	E1, E2	dMM	Virus buds from internal membranes [50].
Sindbis	E1, E2	Sw	No effect on virus particle formation [50].
Coronavirus (MHV)	E2	MdN, Cs	Decreased virus particle formation. Cleavage of E2 delayed. E2 accumulates intracellularly. Cell fusion retarded [83].
Coronavirus (MHV)	E2	dMM	Effects not as great as with MdN or Cs [83].
Rous sarcoma	gp85 / 35	MdN, BC, dMM, Sw	No effect on virus particle formation. Cleavage of the precursor gpR92 ^{env} not affected [84, 85].
Friend mink cell focus-inducing murine leukemia	PrENV	dN	Decreased incorporation of env protein into particles. Decreased virus formation. Transport and proteolytic cleavage of env protein affected [86].
Feline sarcoma	gp140v-fms	MdN, Cs	Growth of transformed cells becomes serum dependent. Poor growth in soft agar. Transformed properties of the cells altered [87, 88].
Avian erythroblastosis	v-erb	dN, DMDP, Sw	Transformed state of cells maintained [89].
HIV	gp120	dN, Cs	Infectivity of particles reduced. Inhibition of syncytium formation. No effect on cleavage of the precursor gp160. gp120 still expressed at cell surface [90, 91].
HIV	gp120	dMM	No effect on virus infectivity or syncytium formation [90].

^aW. McDowell, A. Tlustý, R. Roh, J.N. BeMiller, J.A. Bohn, R.W. Meyers and R.T. Schwarz, submitted for publication.
Abbreviations: BC: bromoconduritol; MdN: *N*-methyl-1-deoxynojirimycin; dN: 1-deoxynojirimycin; Cs: castanospermine; DMDP: 2,5-dihydroxymethyl-3,4-dihydroxypyrrolidine; dMM: 1-deoxymannojirimycin; Sw: swainsonine; DIM: 1,4-dideoxy-1,4-imino-*D*-mannitol; MMNT: α -*D*-mannopyranosylmethyl-*p*-nitrophenyltriazene; DIA: 1,4-dideoxy-1,4-imino-*D*-arabinitol; FPV: fowl plaque virus; MHV: mouse hepatitis virus; HIV: human immunodeficiency virus; VSV: vesicular stomatitis virus.

Sindbis virus, vesicular stomatitis virus and mouse hepatitis virus (Table III). These results corroborate observations made in other non-viral systems dealing with the secretion or surface expression of glycoproteins [3, 6, 8, 30] and a consensus suggests that glucose trimming may be important in the initial stages of protein folding, once the polypeptide chain has been released from the ribosome. Mannose trimming, on the other hand, does not appear to be as important, although with the mannosidase inhibitors, 1-deoxymannojirimycin or swainsonine, the inhibition of complex-type oligosaccharide formation is better than with any of the glucosidase inhibitors [66], and it is usual for some mannose trimming to take place even when glucose trimming has been inhibited (*cf.* the oligosaccharides formed in the presence of bromoconduritol, MdN or castanospermine). The incomplete inhibition of complex-type oligosaccharide formation has been attributed to the presence of a Golgi endo- α -D-mannosidase that can remove a glucosylmannose disaccharide yielding $\text{Man}_8(\text{GlcNAc})_2$ [92] and which provides an alternative route for oligosaccharide processing to the sequential action of glucosidase II and mannosidase I [92]. The initial stages of mannose trimming are important in the Sindbis virus-BHK system for determining the final destination of viral envelope glycoproteins, since treatment with the mannosidase inhibitor 1-deoxymannojirimycin resulted in budding of the virus from internal membranes [60]. This effect was also observed in cells treated with monensin [93] and in a mutant cell line [94]. The observations concerning the biological effects of trimming inhibitors confirm that *N*-linked oligosaccharides can serve a structural role at the time of polypeptide folding and are in agreement with the idea [95] that the existence of a single pathway for oligosaccharide processing demands that the precursor oligosaccharide meet the structural requirements of the most sensitive glycoproteins.

The results of the treatment of cells transformed by retroviruses with trimming inhibitors (Table III) add weight to the notion that every system has to be examined in its own right and that no generalisations can be made concerning the effects of inhibitors of oligosaccharide processing. Thus, on one hand, avian retroviruses, such as Rous sarcoma virus [84, 85] and avian erythroblastosis virus [89], appear to be unaffected by changes in the oligosaccharide composition of their envelope glycoprotein or their onco-

gene product, whereas the formation of Friend mink cell focus-inducing murine leukaemia virus [86] and the transforming properties of the feline sarcoma virus *v-fms* oncogene product [87, 88] are affected. Of current topical interest is the observation that the virus responsible for causing the disease AIDS, human immunodeficiency virus (HIV), appears to be sensitive to the effects of glucosidase trimming inhibitors [90, 91]. In the presence of castanospermine or 1-deoxynojirimycin, both envelope glycoprotein-mediated syncytium formation and HIV infection of CD4^+ T-cells were inhibited [90, 91]. It is thought that perturbation of the carbohydrate side-chains of the HIV envelope glycoprotein results in a lower affinity of gp120 for the CD4 receptor of CD4^+ T-cells [90]. The decrease in syncytium formation was attributed to inhibition of processing of the precursor protein gp160, resulting in decreased cell surface expression of the mature envelope glycoprotein gp120. In addition, steps involved in membrane fusion after binding of CD4 antigen may be affected [91]. The anti-viral effects exhibited by the glucosidase trimming inhibitors suggest that modification of the glycosylation state of the HIV envelope glycoprotein may be of use in the treatment of HIV infections [90, 91].

The observation that inhibitors of protein glycosylation and oligosaccharide trimming have anti-viral effects leads to the question as to whether they could be used as chemotherapeutic agents. Certainly many of the trimming inhibitors, which appear to be non-toxic, have the potential for use in anti-viral chemotherapy [3], as indicated by the findings with HIV, but inhibitors of lipid-linked oligosaccharide assembly, such as tunicamycin, are too toxic. In fact, glycosylation inhibitors have already been tried as anti-viral agents against localised herpes virus infections. 2-Deoxyglucose was used to treat herpes simplex virus-induced keratitis in rabbits [96] and human genital herpes simplex virus infections [97]. However, the significance of these results was questioned [98] and further studies failed to show any effect of deoxyglucose, which discouraged further work on the treatment of herpes infections with glycosylation inhibitors [3]. In addition to their potential anti-viral properties, inhibitors of oligosaccharide trimming may be useful as immunomodulators for the treatment of cancerous tumours. Tumour invasion and metastasis are presumably mediated *via* interactions between oligosaccharides located on cell surfaces. It would therefore be

expected that changes in the oligosaccharide composition at the cell surface may alter the binding of tumour cells and thus the spread of the cancer. Swainsonine has been found to be useful as an immunomodulator. Thus treatment of B16-F10 murine lymphoma cells with swainsonine inhibited pulmonary colonisation [100] and, in immunodeficient mice, the growth of a sarcoma was inhibited and metastasis of the B6 melanoma in the lung was reduced [101].

The ideal anti-viral agent acts only in virus infected cells, thus minimising toxicity problems. In this vein, anti-herpetic drugs, such as acyclovir (9-(2-hydroxyethoxymethyl)-guanine), are activated in virus-infected cells by a virus-encoded thymidine kinase, producing inhibitors of DNA polymerase [99]. In an analogous manner, it may be possible to develop virus-specific inhibitors of glycosyltransferases. Although the synthesis of viral DNA is undoubtedly the prime target for anti-viral chemotherapy with nucleoside analogues, these compounds may also interfere with other processes, such as glycosylation of viral glycoproteins, through the formation of sugar nucleotide analogues that interfere with the glycosyltransferases involved in the glycosylation pathways. Indeed, the result that (*E*)-5-(2-bromovinyl)-2'-deoxyuridine inhibits galactosylation of *N*- and *O*-linked oligosaccharides of herpes simplex virus-1 glycoproteins [102] indicates the potential of nucleoside analogues as glycosylation inhibitors.

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