3.07
Alkaloid Glycosidase Inhibitors

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3.07.1   INTRODUCTION

Polyhydroxy alkaloids with glycosidase inhibitory properties have been isolated and identified in the 1980s and 1990s, with few exceptions. Discovery of the indolizidine alkaloids swainsonine[1] and castanospermine,[2] with their potent and specific inhibitory activities towards α-mannosidase and α- and β-glucosidase, respectively, created a recognition that additional nitrogen-containing analogues of simple sugars might have similar properties and stimulated the search for new members of the class. As a result, more than 50 naturally occurring members of the group have been discovered, almost doubling the number discussed in a previous review.[3] Another review has discussed these alkaloids, with particular reference to their ecological significance.[4] Numerous synthetic analogues have been prepared, but the scope of this chapter will be restricted to the chemistry and bioactivity of those alkaloids isolated from natural sources, their glycosidase-inhibitory properties and consequent effects on glycoprotein processing.

## 3.07.2   CHEMISTRY OF ALKALOID GLYCOSIDASE INHIBITORS

3.07.2.1   Structural Classes

The alkaloid glucosidase inhibitors discovered up until 1998 do not conform to a single structural class but do have several features in common, including two or more hydroxyl groups and a nitrogen atom, generally heterocyclic in character. A small group of glycosidase inhibitors isolated from microorganisms also exists, which are structurally more closely related to amino sugars. However, it is possible to integrate the major class of heterocyclic compounds into structural groups based upon five- and six-membered rings, which may also be fused into bicyclic ring systems. Five different subclasses can be defined, from the simple monocyclic examples to the more complex bicyclic rings, as follows. (Commonly used alternative or abbreviated names for individual alkaloids are shown in parentheses.)

3.07.2.1.1   Pyrrolidines

Alkaloids of the pyrrolidine class, with five-membered rings, are exemplified by 2,5-dihydroxy-methyl-3,4-dihydroxypyrrolidine (DMDP), (**1**, R = OH), which is fully (tetra-)substituted at all carbon atom ring positions.[5] The trisubstituted representatives are 6-deoxy-DMDP (**1**, R = H),[6] 1,4-dideoxy-1,4-imino-D-arabinitol (D-AB1) (**2**, R = β-OH),[7] 1,4-dideoxy-1,4-imino-D-ribitol (**2**, R = α-OH),[8] 3,4-dihydroxy-5-hydroxymethyl-1-pyrroline (nectrisine) (**2**, R = β-OH; 1,5-double bond),[9] and *N*-hydroxyethyl-2-hydroxymethyl-3-hydroxypyrrolidine (**3**),[10] the only alkaloid in this group bearing a substituent on the nitrogen atom. Only a single disubstituted member of the group is known, namely 2-hydroxymethyl-3-hydroxypyrrolidine (CYB3) (**2**, R = H).[11]

A pentahydroxy alkaloid, 2,5-dideoxy-2,5-imino-DL-*glycero*-D-*manno*-heptitol (homoDMDP) (**1**, R = CH₂OH) and its 7-apioside (**1**, R = CH₂O-apiose) have been isolated and structurally identified.[12] HomoDMDP is thus the most highly hydroxylated representative of the pyrrolidine class.

(1)   (2)   (3)
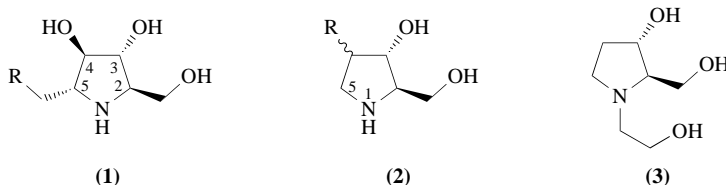
3.07.2.1.2   Piperidines

Alkaloids with six-membered rings of the piperidine class encompass nine members, one of which, 6-deoxyfagomine (**4**, R¹ = H, R² = β-OH),[13] is disubstituted, while two, namely fagomine

(**4**, R^1 = OH, R^2 = β-OH)[14] and 3-*epi*-fagomine (**4**, R^1 = OH, R^2 = α-OH),[8] are trisubstituted. An additional three alkaloids, 1-deoxynojirimycin (DNJ) (**5**, R = α-OH)[15] and its *N*-methyl derivative,[8] and 1-deoxymannojirimycin (DMJ) (**5**, R = β-OH),[16] are tetrasubstituted. The latter has also been found to occur as a series of glycosides, namely: 2-*O*, 3-*O*, and 4-*O*-α-D-glucopyranosides; 2-*O*, 3-*O*, 4-*O*, and 6-*O*-β-D-glucopyranosides; and, 2-*O* and 6-*O*-α-D-galactopyranosides.[8] The remaining four alkaloids are characterized by complete substitution at all carbon atoms, and include the glucose analogue, nojirimycin (**6**, R^1 = α-OH, R^2 = α-OH),[17] the mannose analogue, nojirimycin B (mannojirimycin) (**6**, R^1 = β-OH, R^2 = α-OH),[18] and the galactose analogue, galactostatin (**6**, R^1 = α-OH, R^2 = β-OH).[19] α-Homonojirimycin (HNJ, (**7**)) has a hydroxymethyl group at the 1-position, in place of the hydroxy group found at that position in nojirimycin, and the alkaloid has also been isolated as its 7-*O*-β-D-glucopyranoside.[20]

(**4**)   (**5**)   (**6**)   (**7**)
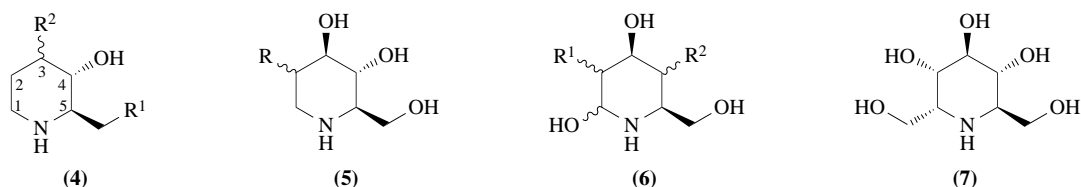
3.07.2.1.3 Pyrrolizidines

The pyrrolizidine alkaloids that are inhibitors of glycosidases may be regarded in a formal structural sense as the result of fusion of two pyrrolidine ring systems, with the common nitrogen atom at the bridgehead. The tetrasubstituted pyrrolizidines, australine (**8**)[21] and alexine (**9**)[22] differ only in the stereochemistry at the bridgehead carbon atom (C-7a), all other substituents having identical configurations. A certain amount of confusion has arisen in the naming of epimers of these compounds because those having a bridgehead configuration identical to that of australine have been classified as 7a-*epi*-alexines. In fact, alexine itself is the only member of this group isolated to date which has an α bridgehead proton.

(**8**)   (**9**)
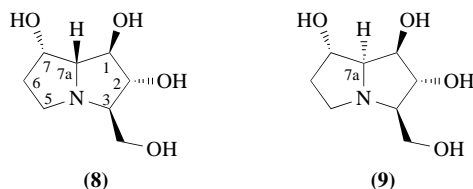
Harris *et al.*[23] have proposed that all alkaloids having the *R* stereochemistry be named as australines and those with the *S* stereochemistry as alexines. Adopting this convention, the three known naturally occurring epimers would therefore be named as follows, (with the alternate name in parentheses): 1-*epi*-australine (1,7a-di-*epi*-alexine) (**8**, 1-OH, α)[24] 3-*epi*-australine (3,7a-di-*epi*-alexine) (**8**, 3-CH$_2$OH, α),[23] and 7-*epi*-australine (7,7a-di-*epi*-alexine) (**8**, 7-OH, β).[24]

A unique tetrasubstituted pyrrolizidine alkaloid is 7a-*epi*-alexaflorine,[25] which also has a 7a-(*R*) bridgehead configuration, consistent with all the other alkaloids except alexine, and may be regarded as an oxidized form of australine. On the basis of its physical properties, including resistance to melting and insolubility in all solvents except for water, together with evidence of a carboxylate ion in its infrared spectrum, this alkaloid was shown to exist in the zwitterionic form (**10**).

(**10**)
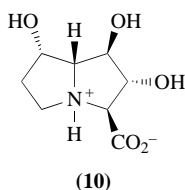
An interesting addition to the class has been casuarine (**11**, R = H),[26] a highly oxygenated penta-substituted pyrrolizidine. This alkaloid has also been found as the 6-glucoside (**11**, R = α-D-glucosyl).[26] The occurrence of several australine/alexine epimers suggests that epimeric forms of casuarine will ultimately be discovered.

(11)
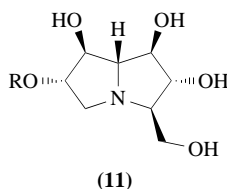
3.07.2.1.4   *Indolizidines*

In an analogous manner to the pyrrolizidine alkaloids, the indolizidine group may be visualized as a pyrrolidine ring fused with a piperidine ring, yielding a bicyclic 5/6 ring system. Seven naturally occurring members have been discovered, the simplest of which are the dihydroxylated alkaloids, lentiginosine (**12**, R = β-OH) and 2-*epi*-lentiginosine (**12**, R = α-OH).[27]

(12)
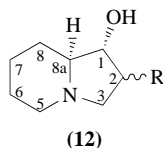
The familiar trihydroxylated alkaloid swainsonine (**13**)[1,28–30] is unique within the indolizidine class as the only member with an 8a-(*R*) bridgehead configuration. A second trihydroxyindolizidine, 7-deoxy-6-*epi*-castanospermine (**14**, R[1] = β-OH, R[2] = H)[31] has the 8a-(*S*) configuration characteristic of the tetrahydroxy alkaloid, castanospermine (**15**),[2] and its epimers 6-*epi*-castanospermine (**14**, R[1] = β-OH, R[2] = β-OH)[32] and 6,7-di-*epi*-castanospermine (**14**, R[1] = β-OH, R[2] = α-OH).[10]

(13)   **(14)**   **(15)**
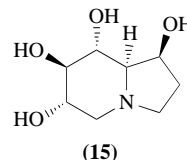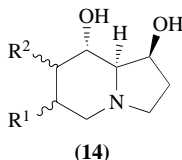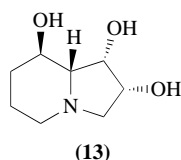
Theoretically, pentahydroxylated indolizidines, corresponding to casuarine, could occur but none have yet been isolated from natural sources.

3.07.2.1.5   *Nortropanes*

The polyhydroxy pyrrolidine, piperidine, pyrrolizidine, and indolizidine groups have been established for some time but the nortropane group is a relatively new addition to the catalog of alkaloid classes with glycosidase-inhibitory properties. Whereas tropane alkaloids are well-known in nature, nortropanes (i.e., compounds in which the nitrogen atom is not methylated) are relatively rare. The nortropane ring system can be conceptualized as a result of fusion of a five-membered pyrrolidine ring with a six-membered piperidine ring, but in contrast to the indolizidines the fusion points are α to the nitrogen atom of each monocyclic system.

The polyhydroxy nortropane group now consists of more individual alkaloids than any of the other classes, and the chemistry of these compounds has been the subject of a review.[33] The alkaloids have been named calystegines after the source of the first member to be isolated, the bindweed *Calystegia sepium*.[34,35] A consistent feature of all calystegines, in addition to the absence of *N*-methylation, is the presence of an α-OH group at the bridgehead junction (C-1) of the bicyclic ring system (i.e., an aminoketal functionality). Three subclasses have been defined, namely calystegines A, B, and C, each of which corresponds to tri-, tetra- and pentahydroxylation, respectively.

Four trihydroxylated alkaloids, calystegines A_3 (**16**, $R^1 = \alpha$-OH, $R^2 = H$),[35] A_5 (**16**, $R_1 = H$, $R^2 = \alpha$-OH),[36] A_6 (**17**),[37] and A_7 (**18**)[13] are known. Although the majority of calystegines bear an equatorial hydroxyl group at the C-3 position, the latter two alkaloids lack this substituent, while calystegine A_6 is unique within the A subgroup in possessing a secondary hydroxyl group on the five-membered ring moiety.

(16)   **(17)**   **(18)**
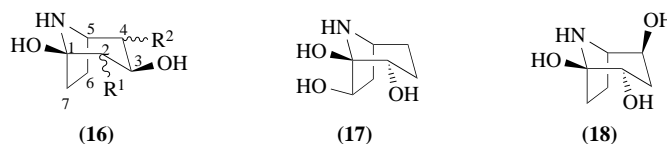
The calystegine B alkaloids consist of five tetrahydroxylated compounds, namely B_1 (**19**),[35] B_2 (**16**, $R^1 = \alpha$-OH, $R^2 = \alpha$-OH),[35] B_3 (**16**, $R^1 = \beta$-OH, $R^2 = \alpha$-OH),[36] B_4 (**16**, $R^1 = \alpha$-OH, $R^2 = \beta$-OH),[38] and B_5 (**20**).[13] Calystegine B_5 is the only alkaloid within this subgroup that does not have a 3-OH substituent. Although both calystegines B_1 and B_5 have secondary hydroxy groups on the five-membered ring moiety, these occur at different positions, namely C-6 and C-7, respectively. The remaining three members, calystegines B_2, B_3 and B_4, differ only in the stereochemistry of the hydroxy groups located at C-2 and C-4 on the six-membered ring; the C-3 hydroxy substituent is β in all three alkaloids. An alkaloid named calystegine N_1,[37] corresponding to calystegine B_2 but with an amino group, rather than a hydroxy group, at the bridgehead C-1 position has also been obtained. However, reactions of nojirimycin derivatives with ammonia-saturated methanol, resulting in replacement of the 2-OH group by an NH_2 substituent,[39] suggest that calystegine N_1 is an artifact of the isolation procedure, which involves elution from an ion-exchange column with dilute ammonium hydroxide.

(19)   **(20)**
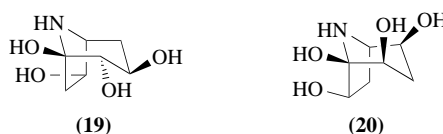
Two pentahydroxylated calystegine C alkaloids are known, having identical substitution patterns, including a hydroxy group at C-6 analogous to calystegine B_1. These alkaloids, calystegines C_1 (**21**, $R = \alpha$-OH)[8] and C_2 (**21**, $R = \beta$-OH),[40] differ only in the stereochemistry of the C-2 hydroxy substituent.

(21)
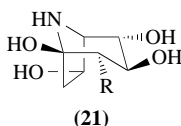
Two additional alkaloids, bearing axially oriented methyl groups on the nitrogen atom, have been isolated and structurally characterized.[13] These compounds, *N*-methylcalystegine B_2 (**22**, $R = H$) and *N*-methylcalystegine C_1 (**22**, $R = OH$) should strictly be classified as tropane alkaloids but the preponderance of polyhydroxy nortropanes isolated to date suggests that these new alkaloids are the result of *N*-methylation of the latter rather than products of the normal biosynthetic route to tropane alkaloids. For the purposes of this chapter they are therefore classified within the nortropane group.

(22)
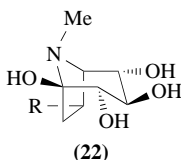
3.07.2.1.6   Miscellaneous glycosidase inhibitors

A few nitrogen-containing glycosidase inhibitors, although they are polyhydroxylated, do not fall readily within the above structural classifications. These include the aminocyclopentanes,

mannostatin A (**23**),[41] and the much more complex glycosylated cyclic urea derivative, trehazolin (**24**),[42] and kifunensine (**25**)[43] and nagstatin (**26**),[44] which may be regarded as highly modified piperidines. All of these compounds are metabolites isolated from various microorganisms.

(23)   (24)   (25)   (26)
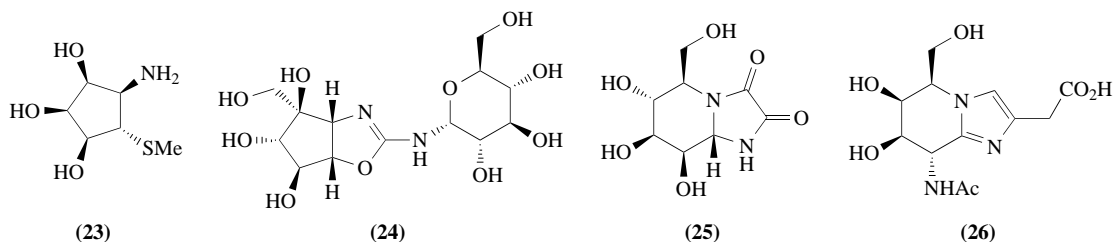
3.07.2.2 Occurrence and Isolation from Natural Sources

3.07.2.2.1 Occurrence

The polyhydroxy alkaloid glycosidase inhibitors have been isolated primarily from plant sources, but also occur in microorganisms and have occasionally been found in insects.[4] The sources of the individual alkaloids are listed in Table 1. Many of the earliest polyhydroxy alkaloids to be discovered, particularly the bicyclic pyrrolizidines and indolizidines, were found in the plant family Leguminosae. This apparent taxonomic relationship has now become far less secure with the isolation of casuarine (**11**) from the Casuarinaceae and Myrtaceae.[26] Moreover, swainsonine (**13**) has been identified as a constituent of several *Ipomoea* species (Convolvulaceae), co-occurring with calystegines.[45] Similarly, the initial isolation of calystegines ((**15**)–(**18**)) from the Convolvulaceae[34,35,46] has now been overshadowed by a much more widespread occurrence in the Solanaceae,[13,36–38,40,47,48] and a limited presence in *Morus* species (Moraceae).[49]

Certain individual alkaloids, predominantly DMDP and swainsonine, have a particularly widespread pattern of occurrence. Thus, DMDP (**1**, R = OH) has been isolated from plants in the families Araceae, Campanulaceae, Euphorbiaceae, Hyacinthaceae, and Leguminosae,[5,19,20,50] as well as from the body of a lepidopteran (*Urania fulgens*),[20] and from a *Streptomyces* species.[51] Similarly, swainsonine (**13**) has also been discovered in two unrelated microorganisms, *Rhizoctonia leguminicola* and *Metarhizium anisopliae*,[29,30] in addition to its quite widespread occurrence in plants.[52] It has been shown that the biosynthetic pathways to swainsonine in the Diablo locoweed, *Astragalus oxyphysus*, and *R. leguminicola* are identical, implying either a direct or indirect relationship between plant and microorganism.[53] Thus, the genetic ability to produce this alkaloid could have been transferred from one to the other in the course of evolution. Alternatively, microorganisms capable of producing the alkaloid may have an endophytic association with the plants. The presence of a calystegine-catabolizing *Rhizobium meliloti* strain in roots of *Calystegia sepium* but not within plants that do not produce calystegines emphasizes the complexity of such interactions.[54] In contrast to the previous examples, castanospermine (**15**) and its epimers (**14**)[2,10,31,32] and the australine/alexine ((**8**)–(**11**))[21–24] alkaloids have so far been restricted to the monotypic *Castanospermum australe* and species of *Alexa*, which are closely related genera in the Leguminosae.

It is apparent from these examples that no consistent conclusions can be drawn regarding the distribution of polyhydroxy alkaloids at the present time. It may be that these natural products are quite widely distributed and many new sources will be discovered in the future. The comparative newness of their discovery relative to many other classes of alkaloids is probably a consequence of their cryptic nature, due to exceptional water solubility and relative insolubility in non-hydroxylic organic solvents.[55] The increasing number, regio- and stereochemical potential for structural variation and significant biological properties of these glycosidase inhibitors will no doubt result in discovery of new members of the known classes. The identification of the nortropane group is also an indicator that new structural groups may yet remain to be discovered.

3.07.2.2.2 Isolation

The hydrophilicity of the polyhydroxy alkaloids renders them incapable of being isolated by conventional extraction and purification methods which involve extraction into nonpolar organic

Table 1 Natural source and enzyme inhibition properties of polyhydroxy alkaloids.

Alkaloid	Natural source	Enzyme inhibited	Ref.
Pyrrolidines			
CYB-3 (**2**, R = H)	*Castanospermum australe* (Leguminosae)	α-Glucosidase (weak)	**56**
6-Deoxy-DMDP (**1**, R = H)	*Angylocalyx pynaertii* (Leguminosae)	β-Mannosidase	**6**
D-AB1 (**2**, R = β-OH)	*Hyacinthoides non-scripta* (Hyacinthaceae) *Angylocalyx* spp. (Leguminosae) *Morus bombycis* (Moraceae) *Arachniodes standishii* (Polypodiaceae)	α-Glucosidase α-D-Arabinosidase	**56,57** **58**
1,4-Dideoxy-1,4-imino-D-ribitol (**2**, R = α-OH)	*Morus alba* (Moraceae)	α-Glucosidase (weak)	**59**
Nectrisine (**2**, R = β-OH; 1,5-double bond)	*Nectria lucida* F-4490 (Ascomycetes)	α-Glucosidase α-Mannosidase	**9** **60**
N-Hydroxyethyl-2-hydroxymethyl-3-hydroxypyrrolidine (**3**)	*Castanospermum australe* (Leguminosae)	Undetermined	
DMDP (**1**, R = OH)	*Aglaonema* spp.; *Nephthytis poissoni* (Araceae). *Omphalea diandra*; *Endospermum* spp. (Euphorbiceae). *Hyacinthoides non-scripta* (Hyacinthaceae). *Derris elliptica*; *Lonchocarpus* spp. (Leguminosae); *Urania fulgens* (Lepidoptera); *Streptomyces* sp. KSC-5791	α- and β-Glucosidase β-Mannosidase Invertase Trehalase	**12,61,62** **63** **50** **51**
HomoDMDP (**1**, R = CH₂OH)	*Hyacinthoides non-scripta* (Hyacinthaceae)	α- and β-Glucosidase	**12**
Piperidines			
6-Deoxyfagomine (**4**, R¹ = H, R² = β-OH)	*Lycium chinense* (Solanaceae)	Undetermined	
Fagomine (**4**, R¹ = OH, R² = β-OH)	*Fagopyrum esculentum* (Fagaceae); *Xanthocercis zambesiaca* (Leguminosae); *Morus* spp. (Moraceae)	β-Galactosidase α-Glucosidase (weak)	**64** **56**
3-*epi*-Fagomine (**4**, R¹ = OH, R² = α-OH)	*Morus alba* (Moraceae)	β-Galactosidase	**64**
1-Deoxynojirimycin (DNJ) (**5**, R = α-OH)	*Morus* spp. (Moraceae); *Bacillus* spp.; *Streptomyces lavandulae*	α- and β-Glucosidase Invertase Trehalase	**15**
N-Methyl-DNJ	*Morus alba* (Moraceae)	α-Glucosidase	**65**
1-Deoxymannojirimycin (DMJ) (**5**, R = β-OH)	*Omphalea diandra* (Euphorbiaceae); *Lonchocarpus* spp. (Leguminosae); *Streptomyces lavandulae*	α-Mannosidase α-Fucosidase	**66,67** **66**
Nojirimycin (**6**, R¹ = α-OH, R² = α-OH)	*Streptomyces* spp.	α- and β-Glucosidase	**68**

Table 1 (continued)

Alkaloid	Natural source	Enzyme inhibited	Ref.
Mannojirimycin (**6**, $R^1 = \beta$-OH, $R^2 = \alpha$-OH)	*Streptomyces lavandulae*	α-Mannosidase	**18**
Galactostatin (**6**, $R^1 = \alpha$-OH, $R^2 = \beta$-OH)	*Streptomyces lydicus*	β-Galactosidase	**69**
α-Homonojirimycin	*Omphalea diandra* (Euphorbiaceae); *Hyacinthoides non-scripta* (Hyacinthaceae); *Urania fulgens* (Lepidoptera)	α-Glucosidase	**56,61,62**
Pyrrolizidines			
Australine (**8**)	*Castanospermum australe* (Leguminosae)	Amyloglucosidase	**21,70**
Alexine (**9**)	*Alexa* spp. (Leguminosae)	Amyloglucosidase Trehalase	**24**
1-*epi*-Australine (**8**, 1-OH, α)	*C. australe*; *Alexa* spp. (Leguminosae)	Amyloglucosidase α-Glucosidase	**24**
3-*epi*-Australine (**8**, 3-CH$_2$OH, α)	*Castanospermum australe* (Leguminosae)	Amyloglucosidase	**23**
7-*epi*-Australine (**8**, 7-OH, β)	*C. australe*; *Alexa* spp. (Leguminosae)	Amyloglucosidase α-Glucosidase	**24**
7a-*epi*-Alexaflorine (**10**)	*Alexa grandiflora* (Leguminosae)	Amyloglucosidase	**25**
Casuarine (**11**, R = H)	*Casuarina equisetifolia* (Casuarinaceae)	Undetermined	
Indolizidines			
Lentiginosine (**12**, R = β-OH)	*Astragalus lentiginosus* (Leguminosae)	Amyloglucosidase	**27**
2-*epi*-Lentiginosine (**12**, R = α-OH)	*Astragalus lentiginosus* (Leguminosae)	None	**27**
Swainsonine (**13**)	*Swainsona* spp.; *Astragalus* spp.; *Oxytropis* spp. (Leguminosae). *Ipomoea* spp. (Convolvulaceae). *Rhizoctonia leguminicola*; *Metarhizium anisopliae*	α-Mannosidase	**71**
7-Deoxy-6-*epi*-castanospermine (**14**, $R^1 = \beta$-OH, $R^2 = $ H)	*Castnospermum australe* (Leguminosae)	Amyloglucosidase	**31**
Castanospermine (**15**)	*C. australe*; *Alexa* spp. (Leguminosae)	α- and β-Glucosidase	**72**
6-*epi*-Castanospermine (**14**, $R^1 = \beta$-OH, $R^2 = \beta$-OH)	*Castanospermum australe* (Leguminosae)	Amyloglucosidase	**32**
6,7-Di-*epi*-castanospermine (**14**, $R^1 = \beta$-OH, $R^2 = \alpha$-OH)	*Castanospermum australe* (Leguminosae)	Amyloglucosidase β-Glucosidase	**10**
Nortropanes			
Calystegine A$_3$ (**16**, $R^1 = \alpha$-OH, $R^2 = $ H)	*Calystegia* spp.; *Convolvulus arvensis*; *Ipomoea* spp. (Convolvulaceae). *Atropa belladonna*; *Datura wrightii*; *Hyoscyamus niger*; *Lycium chinense*; *Mandragora officianarum*; *Physalis alkekengi* var. *francheti*; *Scopolia japonica*; *Solanum* spp. (Solanaceae)	β-Glucosidase Trehalase	**36,46**

Table 1 (continued)

Alkaloid	Natural source	Enzyme inhibited	Ref.
Calystegine A$_5$ (**16**, R^1 = H, R^2 = α-OH)	*Hyoscyamus niger*; *Lycium chinense*; *Physalis alkekengi* var. *francheti*; *Scopolia japonica* (Solanaceae)	None	**36**
Calystegine A$_6$ (**17**)	*Hyoscyamus niger*; *Lycium chinense* (Solanaceae)	Undetermined	
Calystegine A$_7$ (**18**)	*Lycium chinense* (Solanaceae)	Trehalase	**13**
Calystegine B$_1$ (**19**)	*Calystegia sepium*; *Convolvulus arvensis* (Convolvulaceae). *Duboisia leichhardtii*; *Hyoscyamus niger*; *Lycium chinense*; *Mandragora officianarum*; *Physalis alkekengi* var. *francheti*; *Scopolia japonica* (Solanaceae)	β-Galactosidase β-Glucosidase	**36,46**
Calystegine B$_2$ (**16**, R^1 = α-OH, R^2 = α-OH)	*Calystegia* spp.; *Convolvulus arvensis*; *Ipomoea* spp. (Convolvulaceae) *Atropa belladonna*; *Datura wrightii*; *Duboisia leichhardtii*; *Hyoscyamus niger*; *Lycium chinense*; *Mandragora officianarum*; *Physalis alkekengi* var. *francheti*; *Scopolia japonica*; *Solanum* spp. Solanaceae)	α-Galactosidase β-Glucosidase Trehalase	**13** **36,48** **13**
Calystegine B$_3$ (**16**, R^1 = β-OH, R^2 = α-OH)	*Lycium chinense*; *Physalis alkekengi* var. *francheti*; *Scopolia japonica* (Solanaceae)	β-Glucosidase (weak) Trehalase	**13**
Calystegine B$_4$ (**16**, R^1 = α-OH, R^2 = β-OH)	*Duboisia leichhardtii*; *Scopolia japonica* (Solanaceae)	β-Glucosidase Trehalase	**38**
Calystegine B$_5$ (**20**)	*Lycium chinense* (Solanaceae)	Undetermined	
N-Methylcalystegine B$_2$ (**22**, R = H)	*Lycium chinense* (Solanaceae)	α-Galactosidase Trehalase	**13,54**
Calystegine C$_1$ (**21**, R = α-OH)	*Morus alba* (Moraceae) *Duboisia leichhardtii*; *Lycium chinense*; *Scopolia japonica* (Solanaceae)	α-Galactosidase β-Galactosidase β-Glucosidase Trehalase	**13** **8,13,36** **8,13,36** **13**
Calystegine C$_2$ (**21**, R = β-OH)	*Duboisia leichhardtii*; *Lycium chinense* (Solanaceae)	α-Mannosidase	**40**
N-Methylcalystegine C$_1$ (**22**, R = OH)	*Lycium chinense* (Solanaceae)	α-Galactosidase	**13**

Miscellaneous

Alkaloid	Natural source	Enzyme inhibited	Ref.
Mannostatin A (**23**)	*Streptoverticillium verticillus*	α-Mannosidase	**73**
Trehazolin (**24**)	*Micromonospora* sp.	Trehalase	**42**
Kifunensine (**25**)	*Kitasatosporia kifunense*	α-Mannosidase	**74**
Nagstatin (**26**)	*Streptomyces amakusaensis*	β-*N*-Acetyl-glucosaminidase	**44**

solvents and partitioning between aqueous acid and base. Ion-exchange chromatography is therefore generally employed for purification, following extraction from the natural source by water, methanol or ethanol, either alone or in various mixtures. Subsequent separation can be achieved by paper, column, or thin-layer chromatography. The alkaloids are particularly amenable to detection by thin-layer chromatography in association with specific spray reagents, gas chromatography with flame ionization or mass spectrometric detection, and by their glycosidase inhibitory properties. All of these techniques have been reviewed in detail.[75]

Structural determination places a particular reliance on nuclear magnetic resonance spectroscopy which generally permits establishment of the specific ring system present, the substitution pattern, and relative stereochemistry of the hydroxy groups. Mass spectrometry provides similar information, with the exception of stereochemistry. The isolation of increasing numbers of these alkaloids has furnished a spectroscopic database which renders the determination of structures increasingly facile. Determination of the absolute stereochemistry is dependent upon X-ray crystallography, which can be used whenever well-refined crystal data can be obtained, either from the alkaloid itself or a crystalline derivative such as the hydrochloride salt. Alternatively, circular dichroism techniques may be applied, especially the benzoate chirality method.[16] Although this technique may have the most general utility, being independent of the physical state of the alkaloid, it has so far had only very limited application.

3.07.3   GLYCOSIDASE INHIBITION

3.07.3.1   Glycosidase Inhibitory Activity

The inhibitory activity of individual alkaloids may be remarkably specific, as with swainsonine, which inhibits only α-mannosidase and Golgi mannosidase II, or can be more general, showing a spectrum of activity against a series of glycosidases. Additionally, the potency may vary with the source of a particular enzyme, its purity, and the conditions, such as pH, under which the assay is performed. For these reasons the inhibitory properties of individual alkaloids are presented here only in a summary form (Table 1). The inhibition of *N*-linked glycoprotein processing by the most potent and specific of the alkaloids is discussed in detail in Section 3.07.6; particulars regarding other alkaloids should be obtained from the publications referenced in Table 1.

3.07.3.2   Structure–Activity Relationships

Early approaches to correlation of structure of the polyhydroxy alkaloids with their glycosidase inhibitory properties appeared to indicate a rather straightforward relationship.[3] Swainsonine (**13**) was perceived as an aza-analogue of D-mannopyranose, lacking the hydroxymethine group at C-4, but otherwise having the same relative disposition of the remaining hydroxyl groups, which therefore accounted for its ability to inhibit α-mannosidase.[71] The structures of 1-deoxynojirimycin (**5**, R = α-OH) and castanospermine (**15**) correlated even more closely, as monocyclic and bicyclic "aza sugars", with that of glucose, and they inhibited glucosidases as expected. This naive approach had to be reconsidered with the isolation of 6-*epi*-castanospermine (**14**, R^1 = β-OH, R^2 = β-OH) which, in spite of its stereochemical similarity to mannose, failed to inhibit either α- or β-mannosidase but instead proved to be an effective inhibitor of α-glucosidase, with a level of activity only slightly less than that of castanospermine.[32] Numerous additional examples of inhibitory specificities due to both naturally occurring alkaloids and synthetic analogues have further undermined this empirical approach and it is obvious that structure–activity correlations can only be developed with the aid of sophisticated molecular modeling techniques.

Molecular orbital calculations and molecular modeling have been applied to a series of known mannosidase inhibitors and others which were expected to inhibit but failed to do so. The results showed that good inhibitors fit closely with a single low-energy conformer of the mannosyl cation and demonstrated that 6-*epi*-castanospermine did not comply with the structural requirements.[76,77] The electronegative binding groups present in the inhibitor necessary for specificity and activity were established, as were those which were of little significance. Additional studies of this type should provide valuable information regarding the receptor sites on the various enzymes but the inhibition data available is compromised by the variability in enzymes and the conditions under which measurements have been made. A comprehensive screening program using standardized

conditions would provide much more useful information for structure–activity correlations and consequently the design of specific and potent inhibitors.

The crystal structures of glucoamylase and its complex with the inhibitor 1-deoxynojirimycin (**5**, R = α-OH) have recently been reported.[78] This structural data has now been used in a molecular modeling study, using 1-deoxynojirimycin and other deoxynojirimycin derivatives, DMDP (**1**, R = OH), australine (**8**), and castanospermine (**15**), to probe the active site of the enzyme.[79] Preliminary results indicated that binding to specific residues within the active site were essential for inhibitory activity and that the inhibitory potency was dependent upon the number of hydrogen bonds involved in such binding. However, although castanospermine is an excellent inhibitor of the enzyme it lacked these requirements and therefore did not conform to the model. Nevertheless, this approach illustrates the potential value of such methods for understanding enzyme–inhibitor interactions, which should prove useful with increasing refinements in the models and available structural data.

In the absence of more comprehensive molecular modeling studies, the inhibition results obtained have been rationalized on the basis of generally accepted models for glycosidase inhibition. This approach has been developed most effectively for the calystegines, which provide a comprehensive series of structurally related natural polyhydroxy alkaloids. For β-glucosidase inhibition, the model involves the presence of two carboxylic acid groups at the active site of the enzyme, one responsible for generation and the other for stabilization of the glycosyl cation intermediate.[36] It has been speculated that for calystegines B_1 (**19**) and C_1 (**21**, R = α-OH), the *exo* hydroxy group at the 6-position is protonated by the acidic group responsible for catalytic activity within the active site, in an analogous manner to the inhibitor conduritol B epoxide. In contrast, calystegine B_2 (**16**, R^1 = α-OH, R^2 = α-OH), which shows a similar level of inhibitory activity towards β-glucosidase, is supposed to be bound to the glucosyl cation binding site through the hydroxyl group at the 4-position. The essential requirement of equatorial hydroxyl groups at the 2- and 3-positions is in accord with earlier studies of interaction of other inhibitors with β-glucosidase. Thus, the interaction of inhibitory calystegines with glycosidases can be envisioned as binding to the sites determining specificity and to the catalytic center, through specific hydroxyl groups and through the imino group.

The mechanism of galactosidase inhibitory activity is less apparent. Calystegines B_1, B_2 and C_1 are potent inhibitors of either α or β-galactosidase, yet calystegine B_3 (**16**, R^1 = β-OH, R^2 = α-OH), with a much closer configurational similarity to D-galactose than any of the former, has no inhibitory activity against these enzymes, an observation which is reminiscent of the situation with 6-*epi*-castanospermine in the indolizidine alkaloid series. Obviously, a much larger set of natural or synthetic epimers, enantiomers and structural analogues is needed before a complete understanding of structure–activity relationships can be applied to prediction of inhibitory activity. Some progress in this direction has been made through a comparison of glycosidase inhibition by synthetic analogues and derivatives of (+)-calystegine B_2. The nonnatural (−)-enantiomer showed no glycosidase inhibitory properties, whereas *N*-methylation of natural B_2 suppressed inhibition of β-glucosidase while activity towards α-galactosidase was retained.[54]

3.07.3.3 Synthetic Polyhydroxy Alkaloids

In addition to the synthesis of known naturally occurring alkaloids for the purpose of structural confirmation, many epimers, enantiomers, and structural analogues have been prepared. The number of these synthetic alkaloids, particularly those related to swainsonine, castanospermine, and australine, now approaches or perhaps exceeds those isolated from natural sources. The natural product focus of this review does not permit a comprehensive survey of these compounds. Various aspects of the synthetic approaches, either *a priori* syntheses or those routes commencing from carbohydrate-based templates, have been summarized in a number of publications.[80–82] Nonnatural epimers have been prepared by modification of natural alkaloids which are available in large quantities, such as castanospermine,[83] and ring-expanded analogues of pyrrolizidine and indolizidine alkaloids have also been synthesized.[84,85]

It is probable that at least some of the synthetic compounds, especially epimers of known naturally occurring alkaloids, will subsequently be found to occur in nature. In addition, new structural classes have already been generated which might reasonably be expected to be biosynthesized by plants. Predominant among these are polyhydroxy quinolizidine alkaloids, consisting of two six-membered rings fused into a bicyclic system, which are ring-expanded homologues of the indolizidine

alkaloids.[84,86] Although quinolizidine alkaloids are a well-established class of natural products, none have yet been isolated that bear more than two hydroxyl groups. This is probably a consequence of the high water solubility of polyhydroxylated alkaloids which renders them unextractable into the nonhydroxylic solvents normally used for alkaloid purification. The combination of novel natural polyhydroxy alkaloids, together with synthetic analogues tailored to have specific structural features, will ultimately lead to a full comprehension of the interaction of these alkaloids with receptor sites on the enzyme which results in their glycosidase inhibitory properties.

3.07.4 BIOLOGICAL ACTIVITY OF GLYCOSIDASE INHIBITORS

3.07.4.1 Mammalian Toxicity

As might be expected from a class of compounds that inhibits glycosidases and consequently the fundamental cellular function of glycoprotein processing, the polyhydroxy alkaloids exhibit an exceptional diversity of biological activities. Discovery and isolation of many of the alkaloids has been a result of observations of the ultimate clinical effects which result from the consumption by animals of plants containing these bioactive compounds. Predominant among such examples is the occurrence of swainsonine (**13**) in *Swainsona* species (poison peas) of Australia[1] and *Astragalus* and *Oxytropis* species (locoweeds) of North America.[52] The potent α-mannosidase inhibitory activity of swainsonine disrupts glycoprotein processing by mannosidase II in the Golgi, resulting in neuronal vacuolation due to abnormal storage of mannose-rich oligosaccharides, leading to the neurological damage so characteristic of the locoism syndrome. However, the clinical effects are not limited to the nervous system since emaciation, reproductive failure in both males and females, and congestive right-heart failure are also observed. Since the discovery of swainsonine as the causative agent, locoweed poisoning has now been established as a widespread phenomenon, with additional occurrences being reported from South America and many parts of China and Tibet.[87]

Swainsonine has been reported to co-occur with calystegines B_2 (**16**, $R^1 = \alpha\text{-OH}$, $R^2 = \alpha\text{-OH}$) and C_1 (**21**, R = OH) in *Ipomoea* species of Australia which cause poisoning of sheep and cattle,[45] and in *I. carnea*, resulting in toxicity to goats in Mozambique. The clinical signs of poisoning are characterized by the expected neurological damage resulting from swainsonine ingestion but these are exacerbated by muscle-twitching, tremors and epileptiform seizures. Histological examination of tissues showed vacuolation of Purkinje cells in addition to swainsonine-induced cytoplasmic vacuolation of neurons and axonal dystrophy. The calystegines inhibit β-glucosidase and α-galactosidase which would produce phenocopies of the genetic lysosomal storage defects, Gaucher's disease and Fabry's disease, respectively, and the additional syndromes are significant indicators of the latter.

In contrast to the above examples which exhibit a complexity of effects, the alkaloids concentrated in the chestnut-like seeds of *Castanospermum australe* (Black Bean), primarily castanospermine (**15**) and australine (**8**), together with several less potent epimers of both, produce gastrointestinal disturbances in livestock and humans but no discernable neurological damage.[88] This is consistent with the ability of the alkaloids to inhibit α- and β-glucosidase, resulting in a syndrome phenotypic of the genetic defect, Pompe's disease. Although this relationship has not been directly established in field cases of poisoning, rodent feeding experiments with castanospermine resulted in vacuolation of hepatocytes and skeletal myocytes, and glycogen accumulation, consistent with Pompe's disease or type II glycogenesis.[89] Gastrointestinal problems and lethargy have also been observed in livestock grazing bluebells (*Hyacinthoides non-scripta*) in the UK, and the demonstration of the presence of DMDP and homoDMDP in this plant may account for the syndrome.[12]

All of the above poisoning syndromes are relatively obvious once signs develop, although this may take several weeks of consumption of the plant because the alkaloids implicated often are present at very low levels. Nevertheless, they are potent inhibitors and it has been estimated that a swainsonine content of 0.001% of the dry weight of the plant may be sufficient to induce locoism.[87] For those alkaloids which are less active or which are present at extremely low levels, it seems probable that the signs of poisoning would be subclinical, with no overt changes being apparent. In such cases, toxicity may only be manifested as minor digestive disturbances, failure to gain weight and other deviations from optimal health which could be attributed to stress or infectious diseases. The occurrence of various calystegines in human food plants from the family Solanaceae, such as potatoes, eggplant and peppers, could account for a variety of complaints, primarily gastrointestinal, reported in certain individuals consuming these vegetables.[90]

3.07.4.2 Insecticidal Activity

It should be anticipated that compounds capable of inhibiting glycosidases would have an inhibitory effect on digestive enzymes, and defense against herbivorous insects may be one of the roles played by the polyhydroxy alkaloids in plants which contain them. Conversely, it is well established that insects co-evolve with their host plants to circumvent such defenses and utilize the active constituents for their own defense. Such strategies involving specific alkaloids have been demonstrated for several plant–insect relationships.

Castanospermine (**15**) added to an artificial diet is highly inhibitory to feeding by the pea aphid, *Acyrthosiphon pisum*, with a 50% deterrency level of 20 ppm, and a consequent very low survival rate.[91] Although the alkaloid does not inhibit aphid trehalase, it has been shown differentially to inhibit a number of disaccharidases from a wide taxonomic distribution of insects.[92] Castanospermine has also been shown to be an antifeedant compound to the Egyptian cotton leafworm, *Spodoptera littoralis*, as are D-AB1 (**2**, R = β-OH), DMDP (**1**, R = OH), and swainsonine (**13**).[93] DMDP also appears to be a particularly effective feeding deterrent to nymphs of the locusts *Schistocerca gregaria* and *Locusta migratoria*, at levels as low as 0.001% of the body weight.[94] Since these alkaloids inhibit different enzymes, it is difficult to correlate antifeedant activity with inhibition of digestive enzymes alone. It is possible that deterrency may also be a consequence of blocking of the sensory response to glucose.[93]

Insect resistance to the effects of the alkaloids has been observed. Thus, the bruchid beetle *Callosobruchus maculatus*, a feeder on legumes that do not produce DMDP, has a gut α-glucosidase which is 100 times more sensitive to the alkaloid than that of *Ctenocolum tuberculatum*, which has adapted to feed exclusively on DMDP-containing species of the legume subtribe Lonchocarpinae.[4] Among the Lepidoptera, the aposematically-colored moth, *Urania fulgens*, accumulates DMDP and α-homonojirimycin (**7**) from its food plant, the vine *Omphalea diandra*, but does not sequester the other alkaloid present, 1-deoxymannojirimycin (**5**, R = β-OH),[20] while the Death's-Head hawkmoth procures calystegines from its Solanaceous hosts.[47] The mechanism of resistance to the effects of the alkaloids is not understood but it appears likely that those which are accumulated serve a protective role in the insect. In contrast, alkaloids which may be harmful can be specifically excreted. For example, pea aphids feeding upon the spotted locoweed, *Astragalus lentiginosus*, excrete in their honeydew swainsonine (**13**) acquired from the phloem of the plant, while showing no feeding deterrency.[91] Since this plant was colonized opportunistically in the laboratory and is not a normal host for the pea aphid, the implication is that certain insects my have a general ability to compartmentalize and eliminate polyhydroxy alkaloids that might otherwise be harmful.

3.07.4.3 Plant Growth Inhibition

Polyhydroxy alkaloids from several of the structural classes have been shown to be inhibitory to the growth of plants. Particularly noteworthy in this respect is castanospermine (**15**) which has been demonstrated to be a potent root elongation inhibitor of lettuce, *Lactuca sativa*, alfalfa, *Medicago sativa*, barnyard grass, *Echinochloa crusgalli*, and red millet, *Panicum miliaceum*.[95] The alkaloid was much more effective against the dicots, showing 50% inhibition of root length growth at 300 ppb, while the monocots were 1000 times less sensitive. The structurally related indolizidine alkaloid, swainsonine (**13**), failed to exhibit any phytotoxic activity against these species, indicating that the bioactivity is a consequence of α- or β-glucosidase inhibition but not of α-mannosidase inhibition. Nojirimycin (**6**, R[1] = α-OH, R[2] = α-OH) is inhibitory to cell extension of *Pisum sativum* stem segments and of coleoptiles of *Avena* and *Triticum*, induced by auxins. There is considerable evidence that elongation is a consequence of cell-wall loosening due to degradation or depolymerization of xyloglucans by *exo*-β-glucanases and inhibition of these enzymes by the alkaloid could therefore account for the failure of the cells to elongate.[96]

The phytotoxic effects of the polyhydroxy alkaloids may confer a major competitive advantage upon plants which biosynthesize them through the phenomenon of allelopathy. The alkaloids are highly water soluble so that excretion into the surrounding soil or leaching from various parts of the plant can suppress the growth of encroaching species through creation of a zone of inhibition. At the same time, movement of water would transport the compounds through the soil so that concentrations in the vicinity of the secreting plant itself do not attain levels high enough to induce self-inhibition. However, there is some evidence that *Castanospermum australe* seeds may be inhibited from germination by the presence of castanospermine (**15**). Considerable irrigation is required before the seeds commence to sprout and this may be a valuable strategy in the native

environment where rainfall is highly seasonal, enabling germination and rooting to take place only when the rainy season is well-established.

Natural calystegine B_2, that is the (+)-enantiomer (**16**, $R^1 = \alpha$-OH, $R^2 = \alpha$-OH), showed significant inhibition of alfalfa seed germination, and growth and lateral production of roots transformed by *Agrobacterium rhizogenes*, but corresponding effects were not observed with the unnatural (−)-enantiomer.[54] Root length was reduced by 40% after treatment for 43 h with 10 mM (+)-calystegine B_2, while under the same conditions the unnatural (synthetic) alkaloid caused an 18% increase in root length. Such results demonstrate the dependency of bioactivity upon specific structural conformations and stereochemistry.

3.07.4.4   Antimicrobial Activity

There has been little information reported in regard to the effect of polyhydroxy alkaloid inhibitors on growth or function of microorganisms, although nojirimycin (**6**, $R^1 = \alpha$-OH, $R^2 = \alpha$-OH) was discovered as a result of the antimicrobial activity of *Streptomyces nojiriencis*, *S. roseochromogenes*, and *S. lavandulae* against a drug-resistant strain of *Shigella flexneri*.[66] The antibiotic activity of the same alkaloid towards *Xanthomonas oryzae* renders it capable of preventing the bacterial leaf blight of rice.[66]

The calystegines were first isolated from roots of the bindweed, *Calystegia sepium*.[34] Although these alkaloids have now been detected in other plant parts, there appears to be a relatively high abundance in subterranean organs of the Convolvulaceae and Solanaceae and they are therefore believed to be nutritional mediators between such plants and associated rhizosphere bacteria. Over 20% of the bacteria isolated from the rhizospheres of calystegine-producing plants were capable of catabolizing the alkaloids, whereas no bacteria with this ability were obtained from plants which did not elaborate calystegines.[54] In addition, wild-type *Rhizobium meliloti* 41 was capable of using natural (+)-calystegine B_2 (**16**, $R^1 = \alpha$-OH, $R^2 = \alpha$-OH) as an exclusive source of carbon and nitrogen, whereas a catabolism-deficient strain of *R. meliloti* was not. Furthermore, neither organism could utilize the unnatural, synthetic enantiomer, (−)-calystegine B_2. The ability to catabolize such compounds, which at the same time may have antibiotic properties towards other microorganisms, has an obvious competitive advantage for those specific bacteria capable of utilizing them.

3.07.4.5   Therapeutic Activity

The capability of polyhydroxy alkaloids to disrupt the general cellular function of glycoprotein processing leads to the expectation that these compounds should have therapeutic potential for the treatment of various disease states. The significant mammalian toxicity of certain of the alkaloids is an obvious hindrance to their utility. However, this is frequently true of many drug candidates and it is not unreasonable to assume that an appropriate dose–response relationship could be achieved. Moreover, adverse effects, such as the neurological damage caused by swainsonine, often develop quite slowly and appear to be reversible if ingestion of the alkaloid is terminated, as would be the situation with most drug regimens. Investigation of the alkaloids for therapeutic potential has so far concentrated on three major disease states, namely for treatment of cancer and inhibition of metastasis, as antidiabetic drugs, and for antiviral activity.

Swainsonine (**13**) has received particular attention as an antimetastatic agent. *In vivo* experiments with mice have shown that pulmonary colonization is reduced by over 80% if the animals are provided with drinking water containing 3 μg mL^{-1} of swainsonine for 24 h prior to injection with B16-F10 murine melanoma cells.[97] This effect has been shown to be due to enhancement of natural killer T-cells and increased susceptibility of cancerous cells to their effect.[98] The pharmacokinetics of swainsonine in such experiments indicate that the levels of alkaloid and period of administration would not be sufficient to produce neurological damage.[99] It has been suggested that post-operative metastasis of tumor cells in humans could be suppressed by intravenous administration of the alkaloid prior to and following the surgery. Clinical trials in humans with very advanced malignancies showed that lysosomal α-mannosidases and Golgi mannosidase II were inhibited and some improvement in clinical status occurred.[100] Castanospermine has also been reported to suppress metastasis in mice[101] but experiments with this alkaloid have not been as extensive as those with swainsonine.

Castanospermine (**15**) and 1-deoxynojirimycin (**5**, R = α-OH) have been shown to be capable of suppressing the infectivity of a number of retro viruses, including the human immunodeficiency virus (HIV) responsible for AIDS.[102–105] This effect is a consequence of inhibition of glycoprotein processing which results in changes in the structure of the glycoprotein coat of the virus. Cellular recognition of the host is thus prevented and syncytium formation is suppressed. In spite of this significant effect, both of these alkaloids suffer from the disadvantage that they are highly water-soluble and therefore excreted very rapidly. This defect has been overcome by derivatization to give 6-*O*-butyryl-castanospermine and *N*-butyl-deoxynojirimycin,[106,107] and both of these compounds have undergone clinical trials against AIDS in humans, either alone or in combination with AZT. As might be expected, gastrointestinal disturbances have been reported as a significant side effect.

Another structural modification of 1-deoxynojirimycin, the *N*-hydroxyethyl derivative, miglitol, an inhibitor of α-glucosidase, has been clinically evaluated and released as an antidiabetic drug in insulin- and noninsulin-dependent diabetes. The alkaloid was shown potently to inhibit glucose-induced insulin release and also suppressed islet α-glucoside hydrolase activity, thus controlling postprandial glycemia.[108] The structurally related alkaloids, 2-*O*-α-D-galactopyranosyl-DNJ and fagomine, have also been shown to have antihypoglycemic activity in streptozocin-induced diabetic mice but have not been tested in humans.[109]

The ability of polyhydroxy alkaloid glycosidase inhibitors to prevent cellular recognition has resulted in their evaluation for clinical situations where suppression of an immune response would be desirable, or for use against parasitic diseases. Thus, *in vivo* experiments have shown that castanospermine can be used as an immunosuppressive drug, promoting heart and renal allograft survival in rats.[110] Parasitic diseases may also be controlled by altering cellular recognition processes. Castanospermine provides protection against cerebral malaria by preventing adhesion of *Plasmodium falciparum* to infected erythrocytes,[111] while swainsonine inhibits the association of *Trypanosoma cruzi*, the causative agent of Chagas' disease, with host cells by formation of defective mannose-rich oligosaccharides on the cell surface.[112]

There is no doubt that the polyhydroxy alkaloids have considerable potential for treatment of a variety of disease states in humans and animals. The primary challenge in introducing them as commercial drugs is to minimize their toxicity and enhance the specificity of their beneficial effects. Improvement of their pharmacokinetic properties should result in much lower dose rates being necessary so that undesirable side-effects are limited. Increased specificity of action can be achieved by preparation of synthetic derivatives and a comprehensive understanding of structure–activity relationships.

3.07.5 PROCESSING OF *N*-LINKED OLIGOSACCHARIDES

3.07.5.1 Introduction

Glycoproteins are widespread in nature, being found in all eucaryotic cells.[113] They have also been shown to be present in various archaebacteria as well as in some lower bacteria.[114,115] In addition, it has become eminently clear that carbohydrate sequences on glycoproteins, glycolipids, and proteoglycans are critically important as ligands in molecular recognition.[116] At least with regard to the *N*-linked glycoproteins, on which this review focuses, these molecules have been implicated in a number of important physiological functions, especially cell–cell recognition reactions involving such critical phenomena as inflammation,[117] pathogenesis,[118] parasitism,[119] development,[120] cell adhesion,[121] and symbiosis,[122] to mention only a few.

N-linked oligosaccharides are also involved in lysosomal enzyme targeting,[123] in the uptake or removal of glycoproteins from the blood,[124] in protein folding in the endoplasmic reticulum,[125] and in many other physiological phenomena of potential significance.[126,127] Although the carbohydrate portion of the glycoprotein has not been shown to participate in every case of recognition, specific oligosaccharide structures are clearly central to many of these cases. Thus, inhibitors that block specific steps in the assembly of the various *N*-linked oligosaccharides and cause the formation of altered or immature oligosaccharide structures should be valuable tools for probing the role of carbohydrates in glycoprotein function.[128]

Figure 1 shows three representative structures of the *N*-linked oligosaccharides. All of these oligosaccharides have the same core structure shown within the box, and are composed of a branched trimannose structure linked to a disaccharide of GlcNAc (i.e., *N*,*N*′-diacetylchitobiose). The immature or initially synthesized oligosaccharide is a high mannose structure shown in (A), and this

oligosaccharide is the biosynthetic precursor that gives rise to all of the other *N*-linked oligo-saccharides. High-mannose (or oligomannose-type) oligosaccharides are most commonly found in glycoproteins from lower eucaryotes such as fungi and yeast, although a small percentage of the *N*-linked oligosaccharides of animal cell surface proteins are of the high-mannose type.

A

```
Manα1—→2Manα1
                6
                 Manα1
                3        6
Manα1—→2Manα1            Manβ1—→4GlcNAcβ1—→4GlcNAc—→Asn
                        3
Manα1—→2Manα1—→2Manα1
```

B

```
SA—→Gal—→GlcNAc—→Manα1
                      6
                       Manβ1—→4GlcNAcβ1—→4GlcNAc—→Asn
                      3
SA—→Gal—→GlcNAc—→Manα1
```

C

```
Manα1
     6
      Manα1
     3      6
Manα1       Manβ1—→4GlcNAcβ1—→4GlcNAc—→Asn
           3
SA—→Gal—→GlcNAc—→Manα1
```
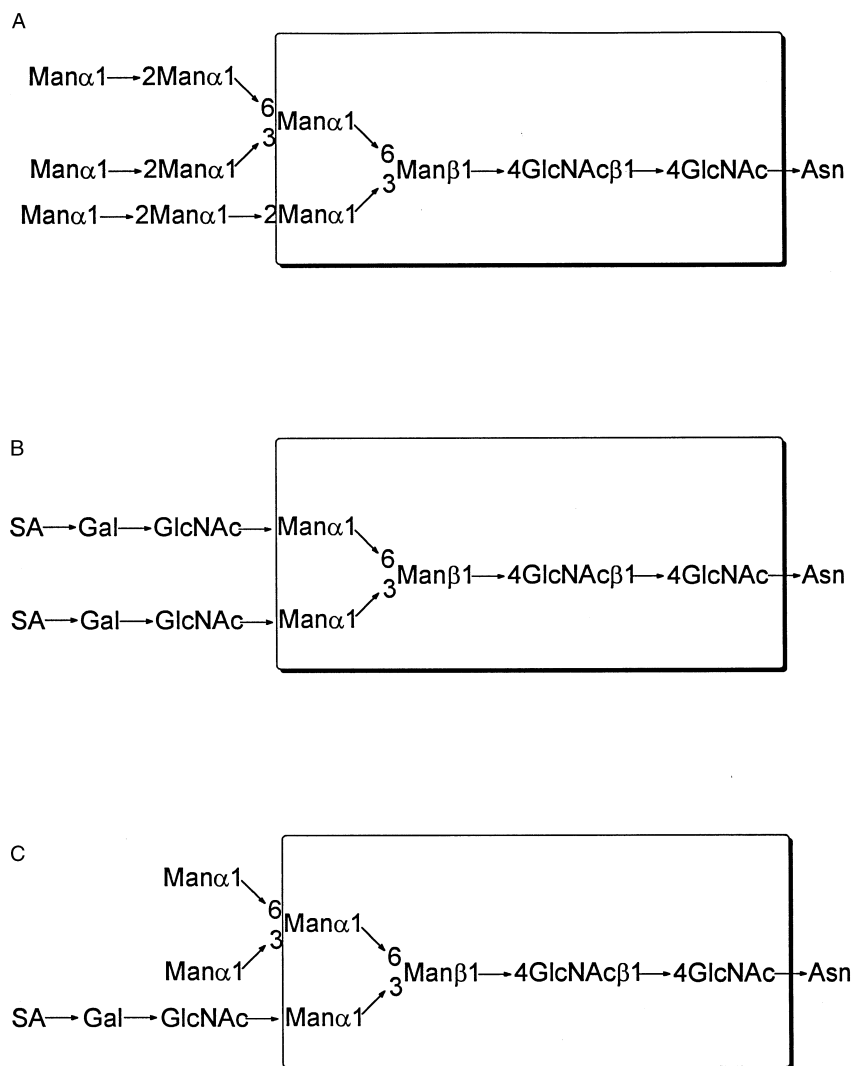
Figure 1 Structural classes of *N*-linked oligosaccharides. A, High-mannose type; B, complex type; and C, hybrid type.

The lower structure (C) of Figure 1 is a hybrid type of oligosaccharide that is produced by partial processing down to the Glc*N*Ac transferase I step, and then addition of various sugars to the 3-linked mannose branch. However, hybrid structures are apparently the result of an absence of mannosidase II action or activity. It is not clear whether hybrid structures are formed normally, but they are found in glycoproteins produced in individuals with HEMPAS disease, a condition where individuals lack mannosidase II activity. Hybrid structures can also be induced by treating cultured cells with swainsonine (**13**). The middle structure (B) in Figure 1 is an example of one type of complex oligosaccharide that is frequently found in cell surface glycoproteins of higher eucaryotes, such as the low density lipoprotein receptor and many other membrane receptors. This particular structure is referred to as a biantennary complex chain, but other complex oligosaccharides may have three of the sialic acid-galactose-Glc*N*Ac chains (triantennary chains), or four of these trisaccharide sequences (tetraantennary chains).

3.07.5.2 Biosynthesis of *N*-Linked Oligosaccharides

The biosynthesis of the *N*-linked oligosaccharide chains involves two rather distinct series of reactions. The first of these pathways gives rise to the precursor, or immature oligosaccharide, which is then transferred cotranslationally to the protein chain while it is being synthesized on membrane-bound polysomes.[129] In contrast, the second series of reactions involves the modification of this precursor oligosaccharide by the removal of some sugars and the addition of others, to give a large number of different oligosaccharide structures.[130] This first pathway requires the participation of a lipid carrier and the involvement of lipid-linked saccharide intermediates. The reactions leading to the production of the final lipid-linked oligosaccharide precursor are presented in Figure 2.
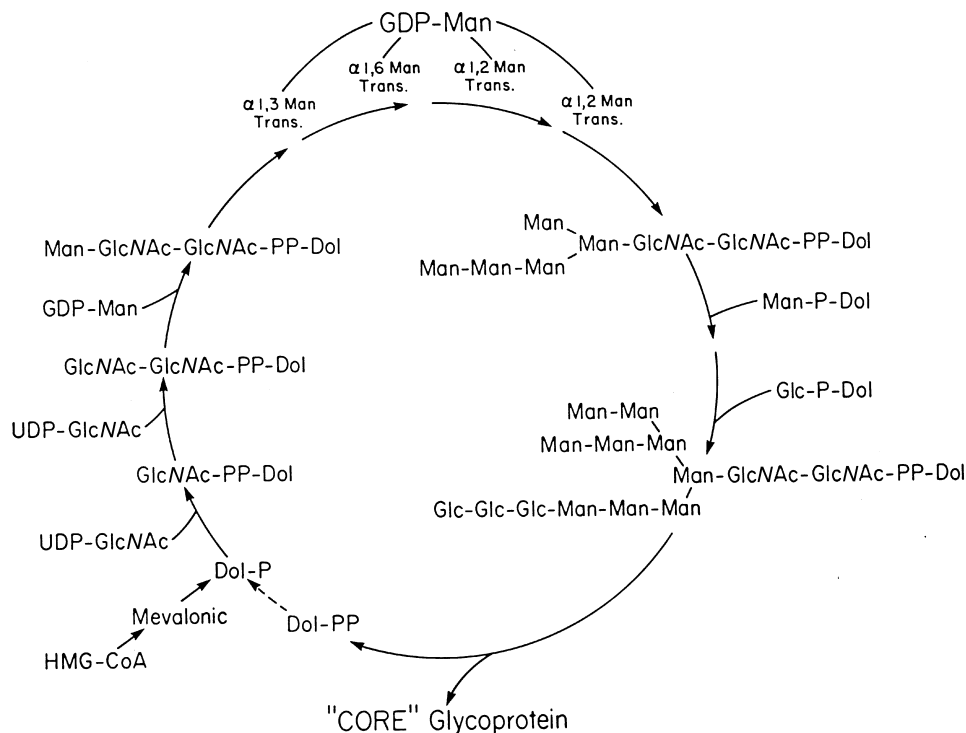

Figure 2 Biosynthetic assembly of the core *N*-linked oligosaccharides.

As shown in Figure 2, the assembly of the *N*-linked oligosaccharide chain is initiated in the endoplasmic reticulum (ER) by the transfer of a GlcNAc-1-P from UDP-GlcNAc to dolichyl-P to form GlcNAc-PP-dolichol.[131] A second GlcNAc is then added, also from UDP-GlcNAc, to produce GlcNAcβ1,4GlcNAc-PP-dolichol.[132] Then, five mannose residues are added, the first in a β1,4 linkage to the terminal GlcNAc, and the next four in α linkages to form the important intermediate, Man$_5$GlcNAc$_2$-PP-dolichol.[133] These first seven reactions are believed to occur on the cytosolic side of the ER membrane, since they involve nucleoside diphosphate sugars as the sugar donors, and these activated sugar donors are biosynthesized in the cytoplasm by soluble sugar nucleotide pyrophosphorylases. It seems likely, therefore, that the sugar acceptor, dolichyl-P, is initially oriented in the ER membrane in such a way that the phosphate group is exposed to the cytoplasm, and is therefore able to accept sugars from the cytosol. After the addition of the first seven sugars to give Man$_5$GlcNAc$_2$-PP-dolichol, this lipid-linked oligosaccharide is believed to undergo a "flip-flop" in the membrane so that the oligosaccharide chain now becomes oriented towards the lumen of the ER.[134]

The assembly of the oligosaccharide is completed by the addition of four more mannose residues and then three glucose units to give a Glc$_3$Man$_9$GlcNAc$_2$-PP-dolichol.[135] These last seven sugars (i.e., four mannose and three glucose units) are all added in the lumen of the ER, and are donated by the activated lipid precursors, mannosyl-P-dolichol and glucosyl-P-dolichol.[136,137] These two sugar donors are synthesized using the sugar nucleotides, GDP-mannose and UDP-glucose, by transfer of the respective sugar to dolichyl-P.[138] The reactions for the synthesis of the activated lipid-linked monosaccharides are proposed to occur on the cytosolic side of the ER membrane and are catalyzed by the enzymes, dol-P-man synthase and dol-P-glc synthase.[139,140]

The final step in this pathway is the transfer of the $Glc_3Man_9GlcNAc_2$ from its lipid carrier to specific asparagine residues on the polysome-bound protein, catalyzed by the enzyme oligosaccharyltransferase.[141,142] The asparagine residue that acts as the acceptor of this oligosaccharide chain must be in the tripeptide consensus sequence, Asn-X-Ser(Thr), where X can be any amino acid except proline, but certain amino acids are favored over others.[143] In addition, the tripeptide sequence must be in a specific conformation or orientation, such as a β-turn of the protein, in order to be glycosylated.[144] In spite of the fact that all of the reactions in this pathway are well known, it is still not clear how the pathway is regulated, nor where the control points are located.

3.07.5.3 Processing of *N*-Linked Oligosaccharides

After the oligosaccharide is transferred to protein and while the protein chain is still being synthesized in the ER, the oligosaccharide begins to undergo a number of processing or trimming reactions. The initial reactions in this second pathway encompass the removal of three glucose residues and up to six mannose residues, but later processing reactions involve the addition of a number of other sugars, principally GlcNAc, galactose, neuraminic acid, L-fucose, and possibly GalNAc.[145] The processing pathway is outlined in Figure 3.
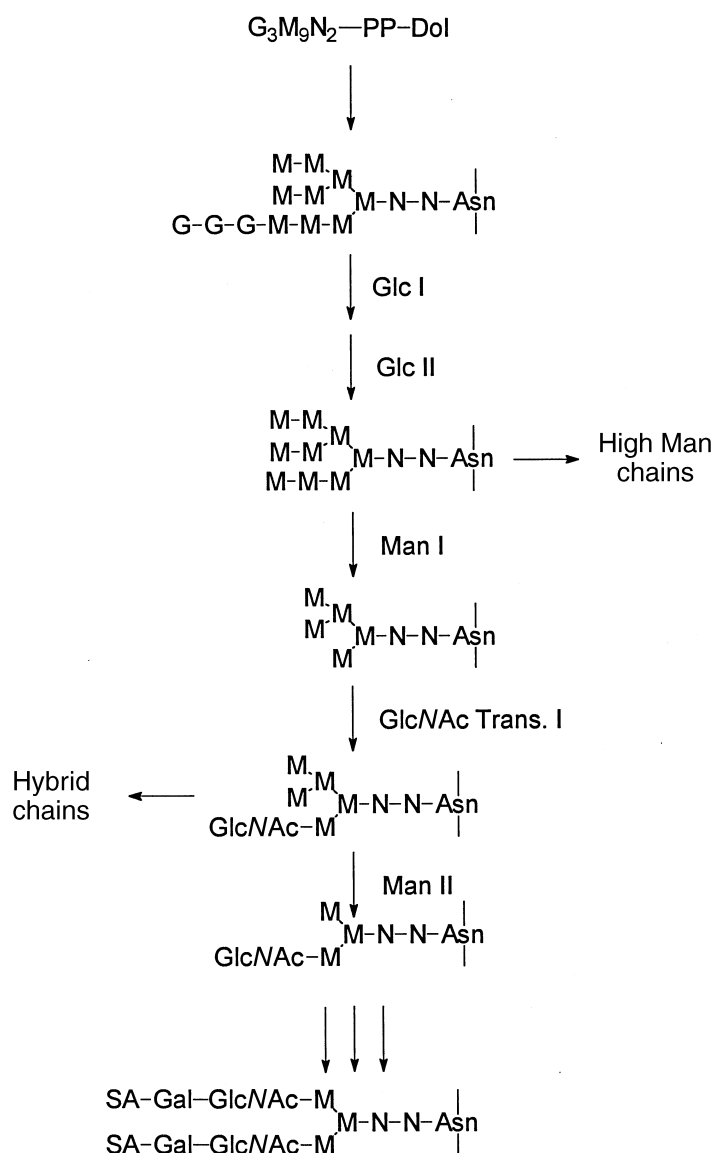

Figure 3 Processing pathway of *N*-linked oligosaccharides.

The first processing step involves a membrane-bound glucosidase, called glucosidase I, which removes the outermost α1,2-linked glucose.[146] This enzyme is quite distinct from the common glycosidases, such as the lysosomal enzymes that are involved in the degradation of polysaccharides, glycolipids and other complex carbohydrates, since those enzymes usually have a pH optimum of around 5, whereas glucosidase I has a pH optimum of about 6.4 to 6.8.[147] In addition, the common glycosidases are only specific for the sugar at the nonreducing terminus and the anomeric configuration of the glycosidic bond, but do not have strong specificity for the group to which this sugar is attached, nor the specific glycosidic linkage if that group is another sugar. Glucosidase I, on the other hand, will only cleave a terminal glucose that is attached in α1,2-linkage to another glucose. Thus, glucosidase I will not work with *p*-nitrophenyl-α-D-glucopyranoside.[148] Finally, these kinds of enzyme can be distinguished by their location; the processing glucosidases are in the ER, while the other hydrolytic α-glucosidases are usually in the lysosomes.

Glucosidase I is the enzyme that initiates the trimming or maturation of the *N*-linked oligosaccharide chains and therefore may play a key role in controlling the rate of transport or exit of newly formed glycoproteins from the ER to the Golgi apparatus. This enzyme has been purified from a number of sources, including calf[149] and porcine[150] liver, and bovine mammary glands,[151] as well as plants (mung bean seedlings)[148] and yeast (*Saccharomyces cerevisiae*).[152] The pig liver glucosidase I was cloned from a human hippocampus cDNA library and expressed in COS 1 cells. The expressed enzyme had a molecular mass of 95 kDa and was degraded by endoglucosaminidase H (Endo H) to a 93 kDa form, indicating that the enzyme has a high-mannose oligosaccharide at the asparagine 655 glycosylation site.[153] The hydrophobicity profile of the enzyme and the fact that trypsin treatment of microsomes released a 4 kDa fragment, support the view that the glucosidase I is a transmembrane glycoprotein containing a short cytoplasmic domain of about 37 amino acids, followed by a transmembrane domain and a large C-terminal catalytic domain on the luminal side of the ER membrane.[153]

A yeast mutant gls1, has been isolated that is lacking glucosidase I and produces glycoproteins with $Glc_3Man_9GlcNAc_2$ structures.[154] This alteration in the normal structure of the oligosaccharides on these yeast proteins has no effect on their secretion. A CHO mutant that is missing glucosidase I was also isolated by virtue of its resistance to the toxic effects of the lectin L-PHA. The mutation in these cells, called Lec 23, has profound effects on the secretion or targeting of glycoproteins.[155]

A second glucosidase, located in the lumen of the ER and called glucosidase II, removes the other two α1,3-linked glucoses to give a $Man_9GlcNAc_2$-protein. Interestingly, this enzyme removes the outermost α1,3-linked glucose quite rapidly ($t_{1/2} = 5$ min), whereas removal of the innermost α1,3-linked glucose is considerably slower ($t_{1/2} = 20$–30 min).[156] Those earlier observations on the activity of this enzyme correlate well with the more recently described role of this enzyme in protein folding. That is, a single α1,3-linked glucose on the high-mannose chain functions as a recognition site to bind a chaperone to those proteins that are improperly folded or denatured, and that chaperone expedites or assists their proper folding.

Thus, it has been shown that the ER contains a protein called calnexin that functions to help newly synthesized membrane proteins fold into their proper conformation, a step that is apparently necessary for many of these proteins to be transported to the Golgi apparatus at the proper rate.[125] Calnexin is a lectin that recognizes a single α1,3-linked glucose on the high mannose chains of unfolded or denatured proteins.[157] Since glucosidase II acts fairly slowly on the final α1,3-linked glucose, there must be a time period when the glycoprotein has only a single glucose on its oligosaccharide. This glucose on the high-mannose chains of unfolded proteins is the recognition site for calnexin to bind to those proteins that have not yet assumed their proper conformation.[158–160]

The ER also contains a safety mechanism to assure that unfolded or improperly folded glycoproteins can interact with this chaperone to obtain the conformation that is required for exit from the ER into the Golgi apparatus. Thus, an unusual glucosyltransferase that is localized in the ER functions to transfer a glucose from UDP-glucose to high mannose chains on denatured, but not on native, glycoproteins.[161] Once this glucose has been added, calnexin can recognize and assist this protein in its proper folding and transfer to the Golgi.[162] As a result, a glycoprotein that has had all of its three glucose residues removed by glucosidase I and II but has failed to fold into the proper conformation can be reglucosylated by this novel enzyme, and this signal then allows the protein another opportunity to interact with calnexin and fold properly. This mechanism, involving the removal of glucoses by the glucosidases and reglucosylation by the glucosyltransferase, is postulated to be part of a unique "glycoprotein-specific folding and quality control mechanism" in the ER that allows this organelle to control and pass properly folded glycoproteins on to the next step in transport and processing.

Glucosidase II has a fairly high pH optimum of about 6.5 to 7.0, but also hydrolyzes *p*-nitrophenyl-α-D-glucoside.[163] On the other hand, the enzyme does appear to be fairly specific for the α1,3-linked glucose since hydrolysis of $Glc_2Man_9GlcNAc_2$ is inhibited by nigerose, an α1,3-linked disaccharide of glucose, but not by the corresponding α1,2-, α1,4-, or α1,6-linked disaccharides of glucose.[164] The enzyme from pig kidney was shown to have a subunit molecular mass of 100 kDa and to contain a high-mannose oligosaccharide,[165] while the enzyme from mung bean seedlings had two 110 kDa subunits as well as high-mannose oligosaccharides,[166] although in some other animal systems, glucosidase II subunits were reported to have molecular masses of 65 kDa.[167,168] This enzyme has been reported to be located in the rough and smooth ER of pig hepatocytes[169] but has also been located in post-Golgi structures in tubular cells of pig kidney.[170] The cDNA for glucosidase II was cloned using degenerate oligonucleotides based on the amino acid sequences derived from a purified pig liver glucosidase II. A 3.9 kb cDNA was isolated with an open reading frame of 2.9 kb. The amino acid sequence did not contain any known ER retention signals or any hydrophobic regions that might represent transmembrane domains, but it did contain a single *N*-linked oligosaccharide consensus site near the amino terminus.[171]

The processing glucosidases can best be assayed, *in vitro*, using the radiolabeled oligosaccharide substrates, $[^3H]Glc_3Man_9GlcNAc$ and $[^3H]Glc_2Man_9GlcNAc$. These substrates are readily prepared in cultured animal cells infected with an enveloped virus, such as influenza virus, that has an *N*-linked glycoprotein coat. Thus, MDCK cells are infected with influenza virus, and progeny virus are produced in these cells in the presence of a glucosidase or mannosidase processing inhibitor to prevent the removal of those specific sugars.[172] For example, if the virus is grown in the presence of castanospermine (**15**), the oligosaccharide chains on its envelope glycoproteins will be mostly of the $Glc_3Man_9GlcNAc_2$ structure, whereas if the virus is grown in the presence of deoxymannojirimycin (**5**, R = β-OH) or kifunensine (**25**), it would have mostly $Man_9GlcNAc_2$ structures.[173] The oligosaccharides are radiolabeled by growing the virus in the presence of either $[^3H]$galactose to label the three glucose residues of the oligosaccharides, or in $[2\text{-}^3H]$mannose to label the nine mannose units. The virus-infected MDCK cells are incubated for 40 h to allow the virus to replicate and lyse the cells and the virus particles are isolated from the culture medium by ultracentrifugation. The viral pellet is then treated exhaustively with pronase to digest the proteins and the resulting glycopeptides are isolated by gel filtration. These glycopeptides are then incubated with Endo H (i.e., endoglucosaminidase H) to cleave the high-mannose and glucose-containing high-mannose glycopeptides, and the resulting oligosaccharides, having a single GlcNAc at the reducing end, are isolated by gel filtration on columns of Biogel P-4.[174]

Once the two glucosidases have removed all three glucoses from the *N*-linked oligosaccharide as shown in Figure 3, a number of α-mannosidases can remove one or more of the four α1,2-linked mannose residues to ultimately give a $Man_5GlcNAc_2$-protein (i.e., Manα1,3(Manα1,6)Manα1,6 [Manα1,3]Manβ1,4GlcNAcβ1,4GlcNAc-protein).[175] There are believed to be at least three different α1,2-mannosidases involved in the conversion of $Man_9GlcNAc_2$ to $Man_5GlcNAc_2$; an ER α-mannosidase, a Golgi Man_9-mannosidase, and a Golgi mannosidase I.[176] These enzymes differ in a number of properties including their substrate specificity, their sensitivity to various mannosidase inhibitors, and their intracellular location. The ER mannosidase presumably removes only a single mannose to generate a unique and specific $Man_8GlcNAc_2$ structure. This enzyme is reported to cleave the α1,2-mannosidic linkage in $Man_9GlcNAc_2$ that is normally resistant to hydrolysis by the Golgi Man_9-mannosidase.[177] However, a soluble form of the ER α-mannosidase has been shown to exhibit rather low specificity, in that it can release several different α1,2-linked mannose residues from the $Man_9GlcNAc$ substrate. These mannoses are removed in a random fashion so that three different $Man_8GlcNAc$ structures are produced, as well as a number of $Man_7GlcNAc$ isomers.[178] The discrepancy in specificity between the ER mannosidase and the soluble mannosidase reported in these two studies may be due to the effects of the protein itself on substrate specificity (i.e., the ER α-mannosidase may act differently in its specificity on the free oligosaccharide) compared with the protein-bound oligosaccharide.

The Man_9-mannosidase, at least the enzyme from pig liver, cleaves both free and peptide-bound $Man_9GlcNAc_2$ to give a specific $Man_6GlcNAc_2$ isomer.[179] Thus, the ER mannosidase and the Man_9-mannosidase may be complementary to each other. Another α1,2-mannosidase, isolated from rat liver Golgi and requiring Ca^{2+}, apparently cleaves each of the four α1,2-mannoses in the $Man_9GlcNAc_2$ at a comparable rate, indicating that it alone could produce the $Man_5(GlcNAc)_2$ that is involved in the formation of complex types of oligosaccharides.[180,181] The exact function of these different α-mannosidases is not currently known. The fact that each of these enzymes removes α1,2-linkages, and that there is considerable redundancy in their action, indicates that each has a

specific role in the processing, and perhaps the targeting pathway, and that they may function to produce oligosaccharides with specific signals for particular roles in the cell.

In addition to these *exo*-α1,2-mannosidases, some animal cells and tissues contain an *endo*-α1,2-mannosidase that cleaves the glucose branch of the $Glc_{3-1}Man_9GlcNAc_2$ between the two terminal mannoses to release a Glc_3Man, Glc_2Man or Glc_1Man from the oligosaccharide and leave a $Man_8GlcNAc_2$-protein.[182] This enzyme presumably prefers oligosaccharides with a single glucose on the high-mannose chain and may represent an alternate route to that utilizing glucosidase I and glucosidase II. Nevertheless, the specific role of this interesting enzyme in the processing pathway is still not clear; it may represent a new targeting route in some cells.

The cDNA encoding an endoplasmic reticulum α-mannosidase was isolated from a rat liver gt11 library. Two degenerate oligonucleotides were prepared based on the amino acid sequences obtained from the purified enzyme. These oligonucleotides were used as primers in PCR with liver cDNA as the template to generate an unambiguous cDNA probe. The 524 base-pair cDNA fragment was then used to isolate cDNA clones by hybridization. Two overlapping clones were used to construct a full length cDNA of 3392 bases which encoded an open reading frame of 1040 amino acids and a 116 kDa protein that contained six of the known peptide sequences. No signal sequence or membrane spanning domains were found in the amino acid sequence. Northern blots of various animal tissues using the cDNA as a probe revealed that a 3.5 kb mRNA was present in all tissues examined, but was enriched in adrenal glands and testis and was less abundant in spleen, intestine, and muscle. The rat liver ER α-mannosidase bears striking homology to the vacuolar α-mannosidase from *Saccharomyces cerevisiae*.[183]

The Man_9-mannosidase was also cloned in gt10, using a mixed pig liver cDNA library. Three isolated clones allowed the construction of a 2731 base-pair full length cDNA. This cDNA construct contained an open reading frame of 1977 bp and encoded a 73 kDa protein of 659 amino acids. The 73 kDa active enzyme expressed in COS cells had the same substrate specificity, sensitivity to inhibitors and metal ion requirements as a previously isolated 49 kDa active fragment. Structural and hydrophobicity analysis of the coding region as well as other studies indicated that this enzyme is a nonglycosylated, type II transmembrane protein with a 48 residue cytosolic tail, followed by a 22 amino acid membrane anchor, a luminal 100 residue stem and a 49 kDa C-terminal catalytic domain.[184] Immunofluorescence studies indicted that the pig liver enzyme expressed in COS cells resides in the ER. On the other hand, the human kidney enzyme expressed in COS cells was localized in the Golgi apparatus.[185] The authors speculate that localization is likely to be sequence dependent.

After removal of the four α1,2-linked mannose units, the $Man_5GlcNAc_2$-protein is a substrate for GlcNAc transferase I, a glycosyltransferase in the medial Golgi stacks, that transfers a GlcNAc from UDP-GlcNAc to the mannose on the α1,3-branch to give $GlcNAc$-$Man_5GlcNAc_2$-protein.[186,187] This enzyme was purified to homogeneity from various sources and shown to be a type II integral membrane protein. The enzyme is specific for the Manα1,3,Manβ1,4GlcNAc arm of the *N*-glycan core, and transfers a GlcNAc in β1-2-linkage to the terminal 1,3-linked mannose.[188,189] This reaction is necessary before mannosidase II can remove the α1,3 and α1,6 mannoses from the Manα1,6 arm to give the trimannose structure. The gene for this enzyme was disrupted by homologous recombination in embryonic stem cells and transmitted to the germ line. Mice lacking GlcNAc transferase I activity did not survive to term, and biochemical and morphological analysis of embryos showed that they were developmentally retarded especially in regard to neural tissue.[190]

Once the GlcNAc has been added to the 3-linked mannose, mannosidase II can remove the two mannoses that are linked to the α1,6-linked mannose branch. The result of this reaction is a $GlcNAc\beta1,2Man\alpha1,3(Man\alpha1,6)Man\beta1,4GlcNAc\beta1,4GlcNAc$-protein.[191] Mannosidase II has been purified to homogeneity from rat liver[192] and mung bean seedlings.[193] The animal enzyme and the plant enzyme had apparent molecular masses of about 125 kDa on SDS gels, and both enzymes appeared to be glycoproteins.[192,193] However, the primary sequence of the murine mannosidase II derived from cloning studies predicted a molecular mass of 132 kDa for the deglycosylated enzyme.[194] This discrepancy may be explained by anomalous migration on SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) by the deglycosylated or glycosylated protein, since the glycosylated enzyme migrates as a 124 kDa protein.

The full length mannosidase II cDNA has been isolated from a 3T3 cDNA library. The murine enzyme is a type II transmembrane glycoprotein with a cytoplasmic tail of five amino acids, a single transmembrane domain, and a luminally oriented catalytic domain.[194] The cDNA was overexpressed in COS cells, resulting in the appearance of immunoreactive material in a perinuclear membrane array indicating Golgi localization. The human α-mannosidase cDNA has also been isolated and this gene was mapped to chromosome 5.[194] Although the enzyme has been located in the Golgi apparatus, its "subGolgi" location depends on the cell type.[195] Thus, in exocrine pancreatic cells,

hepatocytes, and intestinal goblet cells, the enzyme is found in the medial to trans Golgi. But in CHO cells, it was restricted to the medial Golgi.[195] Thus, in some cells, mannosidase I and mannosidase II appear to colocalize in the same region of the Golgi.

α-Mannosidase II activity has been demonstrated in all mammalian tissues that have been examined. However, the level of the enzyme is very low in brain.[196] Interestingly enough, this tissue has been found to have an alternate hydrolytic enzyme that has α1,2, α1,3, and α1,6-mannosidase activity and can cleave $Man_9GlcNAc_2$ down to $Man_3GlcNAc_2$.[197] This enzyme is clearly distinct from mannosidase II in terms of its substrate specificity and its reaction to various mannosidase inhibitors (see Section 3.07.6.3). Its specific role in glycoprotein processing is still to be determined.

A lack of mannosidase II has also been observed in HEMPAS disease, a hereditary affliction that is characterized by altered expression of one or several of the glycoprotein processing enzymes.[198] One form of the disease results from a deficiency in mRNA expression of α-mannosidase II. Lymphocytes derived from patients having this defect contain less than 10% of control mannosidase II levels, and their glycoproteins contain mostly hybrid types of oligosaccharides.[199]

The catalytic domain of the murine mannosidase II cDNA shows a considerable amount of similarity in sequence to the lysomal α-mannosidase cloned from the slime mold, *Dictyostelium discoideum*.[200] Nevertheless, these two enzymes have considerable differences in pH optimum, substrate specificity, and localization within the cell. Based on the sequence similarity, it has been proposed that the two enzymes were derived from the duplication and divergence of a primordial α-mannosidase gene with later acquisition of localization information and substrate specificity. A lesser degree of sequence similarity was observed between murine α-mannosidase II and the endoplasmic reticulum α-mannosidase or its cytoplasmic homologue, or the yeast vacuolar α-mannosidase.[201]

Following the action of the various glycosidases in the trimming part of the pathway, a number of glycosyltransferases act on the $GlcNAcMan_3GlcNAc_2$-protein to produce the complex types of *N*-linked oligosaccharides. Thus, in the trans-Golgi apparatus, there are a number of GlcNAc transferases, galactosyltransferases, fucosyltransferases, and sialyltransferases, that can add these sugars to the *N*-linked chains to give a great diversity of complex chains, having biantennary, triantennary, or tetraantennary structures. Many of these enzymes have been well characterized and a number of the genes for these important proteins have now been cloned.[202] Although there are not any good inhibitors of these enzymes currently available, the search for, or the chemical synthesis of, such compounds should be a rewarding future goal.

3.07.6 INHIBITORS OF *N*-LINKED GLYCOPROTEIN PROCESSING

3.07.6.1 Introduction

A number of low molecular mass compounds have been isolated from natural sources, or synthesized chemically, that specifically inhibit the glycosidases in the trimming pathway. These inhibitors have become valuable tools to use in biological systems to determine the role of *N*-linked oligosaccharide processing on the function of various membrane or secretory glycoproteins. The inhibitors are of special interest since they are small molecules which are able to permeate most cells and therefore can be used with intact cells and tissues to study "*in vivo*" situations. In addition, these inhibitors have been very useful in distinguishing the various processing enzymes from each other. The best example is shown in Table 2 where it is clear that the many different α-mannosidases have very different sensitivities to the various mannosidase inhibitors.[128,173] The remaining sections of this chapter describe the chemistry and biological activities of the various classes of alkaloidal and alkaloidal-like compounds that function as inhibitors of *N*-linked oligosaccharide processing.

A number of naturally occurring, sugar-like compounds, in which the ring oxygen is replaced by a nitrogen, have been isolated and are described in Section 3.07.2. Many of these alkaloids have been shown to be potent inhibitors of various glycosidases. The nitrogen in the ring apparently mimics the catalytic intermediate in the reaction (i.e., an oxycarbanion intermediate) but these compounds are still specifically recognized and bound to the active site of a particular glycosidase because of the resemblance in chirality to specific sugars like D-glucose and D-mannose. Thus, they function as valuable inhibitors of glycosidases, such as those that are involved in glycoprotein processing.

Table 2 Effect of processing inhibitors on various α-mannosidases.

Enzyme	Alkaloid				
	Swainsonine (μM)	*Deoxymannojirimycin* (μM)	*Kifunensine* (μM)	*Mannostatin* (μM)	*Mannoamidrazone* (μM)
ER-Man-ase				?	0.5–1
M_9N-Man-ase (ER)		5–7	?	?	?
Man-ase IA (Golgi)		1–2	?	?	?
Man-ase I (Mung bean)		40–50	0.02–0.05		4
Man-ase II (Rat liver)	0.2			?	?
Man-ase II (Mung bean)	0.09			0.09	0.1

3.07.6.2 Glucosidase Inhibitors

Castanospermine (**15**), as indicated earlier, is an indolizidine alkaloid that was first isolated from the seeds of the Australian tree, *Castanospermum australe*.[2] The initial studies on the effect of this compound in biological systems demonstrated that it was a reasonably potent inhibitor of β-glucosidase.[72] Later studies also showed that castanospermine inhibited a number of isolated α-glucosidases, including the glycoprotein processing enzymes, glucosidase I and glucosidase II, sucrase, maltase and lysosomal α-glucosidase.[203] Since this compound is such a potent inhibitor of intestinal maltase and sucrose, it prevents the degradation of the disaccharides sucrose and maltose, and therefore blocks the normal digestion of starch and sucrose. As a result, the seeds of *Castanospermum australe* are toxic to animals and cause severe diarrhea and other gastrointestinal upsets.[89] In addition, when castanospermine is fed to mice over a four or five day period, it inhibits the lysosomal α-glucosidase and causes the accumulation of partially degraded glycogen particles within the lysosomes (i.e., a situation similar to that which occurs in Pompe's disease, a genetic disease where afflicted individuals are lacking the lysosomal α-glucosidase).[204]

When various cultured animal cells are grown in the presence of castanospermine, the processing of the *N*-linked oligosaccharides is blocked at the first step (i.e., glucosidase I), and the asparagine-linked glycoproteins have mostly oligosaccharides with $Glc_3Man_{9-7}GlcNAc_2$ structures.[205] However, in some cells there is an endomannosidase in the Golgi that can release a $Glc_{1-3}\alpha1,3Man$ from glucose-containing *N*-linked oligosaccharides.[206] Although this enzyme prefers to act on the mono-glucosylated oligosaccharide and release the disaccharide Glcα1,3Man, it can apparently also cleave the oligosaccharide containing three glucose residues. Thus, cells that contain this enzyme may be able to get around a castanospermine block. As mentioned above, the role of the endomannosidase in glycoprotein processing is not yet understood.

There are other glucosidase inhibitors that act at the level of glucosidase I and have similar effects to that of castanospermine but may have somewhat different levels of activity, or different specificities. These include 1-deoxynojirimycin (**5**, R = α-OH), which is a polyhydroxylated piperidine analogue that corresponds to D-glucopyranose, but has a nitrogen in the ring. This compound also inhibits α- and β-glucosidases.[207] Another inhibitor is the pyrrolidine alkaloid, 2,5-dihydroxymethyl-3,4-dihydroxypyrrolidine (DMDP) (**1**, R = OH).[208] The latter compound is much less effective than the above two inhibitors, which suggests that a six-membered ring structure is preferred for inhibitory activity. Nevertheless, DMDP does inhibit α- and β-glucosidase.[209]

The effect of preventing the removal of the glucose residues from the *N*-linked oligosaccharides on the targeting of the glycoproteins can be quite dramatic. Thus, when the hepatocyte cell line, Hep-G2, was incubated for various times in the presence of 1-deoxynojirimycin, the rate of secretion of the serum protein, α_1-antitrypsin, was greatly diminished, while the rate of secretion of other serum *N*-linked glycoproteins, such as ceruloplasmin and the C-3 component of the complement, were only marginally affected.[210] Cell fractionation studies indicated that the antitrypsin had accumu-

lated or was held up in the ER–Golgi compartment, suggesting that the presence of glucose on the oligosaccharides might retard the movement of those proteins from the ER to, or through, the Golgi apparatus. Similar results were obtained when the biosynthesis and targeting of the low density lipoprotein receptor of fibroblasts and smooth muscle cells were examined, in the absence and presence of castanospermine. In these studies, it could be shown that cells grown in the presence of the inhibitor had only about one-half the number of receptor molecules at their cell surface, and therefore bound much less ^{125}I-LDL. However, these inhibited cells still had the same total number of LDL receptor molecules in the cells. The missing receptor molecules were found to be located in the ER or Golgi, based on cell fractionation studies.[211]

An interesting study was done in IM-9 lymphocytes where castanospermine was used to examine the role of oligosaccharide processing in the biosynthesis and targeting of the insulin receptor. Cells treated with castanospermine had a 50% decrease in the number of insulin receptors at the cell surface, as demonstrated by the binding of ^{125}I-insulin. The studies showed that removal of glucose residues from the *N*-linked glycoprotein was not necessary for the cleavage of the insulin proreceptor, that is for the maturation of the receptor. However, as shown in other systems, the presence of glucose apparently slowed the transport of this glycoprotein out of the ER to the Golgi, resulting in a decrease in the number of receptor molecules at the cell surface.[212]

In the case of the E_2 glycoprotein of coronavirus, both castanospermine and deoxynojirimycin caused a significant drop by log2 in the formation of virus, and also a dramatic inhibition in the appearance of E_2 glycoprotein at the cell surface. Significantly, the E_2 that was formed in the presence of the glucosidase inhibitors was still acylated with fatty acids as was the control viral E_2. However, the drug-induced E_2 accumulated in an intracellular compartment that was not definitively identified, but was probably the ER.[213]

Another study dealing with the sodium channel of rat brain neurons also showed that addition of palmitic acid to this protein was not prevented by the processing inhibitors.[214] The sodium channel is composed of α- and β-subunits that form a complex during maturation of the channel. The α-subunit undergoes post-translational modification by the addition of a palmitate, and the incorporation of this fatty acid into the glycoproteins was prevented by tunicamycin, a glycosylation inhibitor that completely prevents formation of *N*-linked oligosaccharides. On the other hand, castanospermine prevented processing of the oligosaccharide chains and the addition of sialic acids, but had no effect on the addition of palmitic acid. This alkaloid also did not affect the covalent assembly of the α- and β-subunits or the biological function of the channel.[214] Thus, the oligosaccharide is apparently necessary for palmitate addition, but the specific structure of the oligosaccharide (i.e., high-mannose or complex) is presumably not critical for the addition of palmitate groups.

GP120 is the envelope protein of HIV, the AIDS associated virus, and this protein is a glycoprotein with many oligosaccharide chains. These oligosaccharides are involved in the recognition and mechanism of attachment of HIV to the CD4 receptor on T lymphocytes and other susceptible cells. GP120 interacts with target molecules on the susceptible cells to cause the fusion of the cells with the formation of syncytia, which are necessary for viral formation and infectivity. The glucosidase inhibitors, 1-deoxynojirimycin (DNJ) and castanospermine, caused a significant decrease in the formation of new virus and in syncytium formation.[102,104,215] As a result of these interesting results, these inhibitors have been tested in human clinical trials as potential antiAIDS drugs. Although the results have not been published, one reported side effect in humans was the occurrence of diarrhea and other gastrointestinal problems in individuals taking these compounds. As shown in Table 1, there are a number of other compounds in addition to castanospermine and (DNJ) that are also inhibitors of glucosidases and glycoprotein processing. One such compound is the pyrrolidine alkaloid DMDP (**1**, R = OH), which occurs in several different plant families. When placed in a medium of cultured animal cells, DMDP inhibits the same step and gives the same oligosaccharide structure (i.e., Glc$_3$Man$_{9-7}$Glc*N*Ac$_2$) as do castanospermine and DNJ.[208] However, DMDP is much less effective than these other inhibitors and therefore considerably higher concentrations are necessary in the medium. The fact that a five-membered ring structure can show glycosidase activity against enzymes that act on hexopyranosides is significant and would certainly warrant modeling studies of this structure in comparison to the indolizidine and piperidine alkaloids. Several other unusual structures that show increased selectivity towards the two processing glucosidases (i.e., glucosidase I and glucosidase II) are discussed below.

Australine (**8**) is a tetrahydroxypyrrolizidine alkaloid that was found in the same seeds that contain castanospermine, namely *Castanospermum australe*.[21] However, australine is present in the seeds in much lower amounts than is castanospermine. This compound is a good inhibitor of fungal amyloglucosidase, but it also inhibits the processing glucosidase I. However, in contrast to the other

glucosidase I inhibitors discussed above which are also fairly effective against glucosidase II, australine is a very poor inhibitor of glucosidase II.[70] Thus, australine is the first glucosidase inhibitor to distinguish between these two processing enzymes. Nevertheless, the key affect of australine in cell culture is to block glucosidase I and cause the accumulation of glycoproteins having Glc_3Man_9 GlcNAc_2 structures. Additional compounds such as australine, and especially ones with more potent activity, will be useful tools to help understand the differences between glucosidase I and glucosidase II inhibitors.

Another interesting glucosidase inhibitor is 2,6-diamino-2,6-imino-7-*O*-(*β*-D-glucopyranosyl)-D-*glycero*-L-guloheptitol (MDL 25 637). This compound, referred to in the following discussion as MDL, was synthesized chemically to resemble a disaccharide that would function as a transition state analogue of the intestinal enzyme, sucrase.[216] As anticipated, MDL did inhibit rat intestinal maltase, sucrase, isomaltase, glucoamylase, and trehalase when present in micromolar amounts. Most interesting was the observation that MDL also showed specificity for the glucosidases but in the opposite manner to that of australine. Thus, MDL was much more effective against glucosidase II than it was against glucosidase I.[217] In cell culture, MDL was quite different from the other glucosidase inhibitors in that it caused the accumulation of glycoproteins having Glc_2Man_9 (GlcNAc)_2 structures. However, the overall effects of MDL on glycoprotein function in cell culture are likely to be similar to those observed with castanospermine and other inhibitors of glucosidase I.

A compound named trehazolin (**24**) was isolated as a trehalase inhibitor and has also been tested as an inhibitor of the processing glucosidases.[218] This compound inhibited glucosidase I quite well, but was a very poor inhibitor of glucosidase II.[219] The isolation and demonstration that structures like australine, MDL or trehazolin do exist, and that these compounds have selective actions against the processing glucosidases should stimulate the search for more and better inhibitors. Such inhibitors will be useful tools for additional studies on the role of carbohydrate and especially of the glucose residues in the function and localization of *N*-linked glycoproteins.

In the last few years, it has become clear why and how inhibitors of glucosidase I cause many *N*-linked glycoproteins to accumulate in the ER. Helenius[220] as well as other investigators have elegantly shown that the ER has a "protein correction and folding system" that helps newly synthesized ER proteins fold into the proper conformation that is necessary for transport to the Golgi apparatus. This system involves the action of a chaperone (i.e., a protein that helps other proteins fold). The chaperone, named calnexin, is also a lectin that recognizes a monoglucosylated high-mannose oligosaccharide on the unfolded glycoprotein. In the presence of castanospermine or other glucosidase I inhibitors, the first glucose cannot be removed, and therefore the unfolded protein cannot be recognized by calnexin and cannot be helped to fold. Most proteins will fold on their own given enough time, but the folding of some may be very slow and interaction with calnexin can help speed up this process. Thus, proteins like the LDL (low density lipoprotein) receptor, or the insulin receptor, or $α_1$-antitrypsin, are transported to the Golgi at a much slower rate in the presence of glucosidase inhibitors because of the inability of calnexin to bind to the protein.

3.07.6.3 Mannosidase Inhibitors

A number of α-mannosidase inhibitors have been identified from natural sources or synthesized chemically. In addition to their use as tools to examine the role of mannose oligosaccharides in the function of *N*-linked glycoproteins, they have also been valuable in distinguishing the various α-mannosidase activities from each other.

The first glycoprotein processing inhibitor to be reported was the indolizidine alkaloid, swainsonine (**13**),[1] an inhibitor of mannosidase II.[221] This compound was initially shown to be an inhibitor of the lysosomal α-mannosidase and to cause symptoms of the lysosomal storage disease α-mannosidosis when administered to animals.[71] Thus, swainsonine was essentially the prototype which chemists could use to design other glycosidase inhibitors. That is, based on the structures of swainsonine, castanospermine and 1-deoxynojirimycin, it appeared evident that a useful glycosidase inhibitor should have the following characteristics:

 (i) a ring structure, probably of the pyranose type, with nitrogen replacing the heterocyclic oxygen;
(ii) a number (unknown at the time and still not certain) of hydroxyl groups; and
(iii) stereochemistry of the hydroxyl groups matching that of the sugar for which the glycosidase to be inhibited is specific.

In this section on mannosidase inhibitors, they will be discussed in the order in which they act in the glycoprotein processing pathway (Figure 2), rather than in order of their historical identification.

Based on the fact that 1-deoxynojirimycin (DNJ) (**5**, R = α-OH) was a good inhibitor of α-glucosidases, it was reasonable to assume that a related structure, but with mannose chirality, would be an inhibitor of α-mannosidases. The 2-epimer of DNJ, namely 1-deoxymannojirimycin (DMJ) (**5**, R = β-OH) was synthesized chemically and was indeed found to be a potent inhibitor of the glycoprotein processing mannosidase I.[222,223] Most interestingly, DMJ did not inhibit jack bean or lysosomal α-mannosidase, nor did it inhibit mannosidase II. Those observations on the selective specificity of DMJ demonstrate that it is dangerous to screen for new glycosidase inhibitors by using the commonly occurring aryl-glycosidases (i.e., α- and β-glucosidase, galactosidase, or mannosidase) to test for the inhibitory activity. That is, if the goal is to find a new glycoprotein processing inhibitor, such as an inhibitor of ER α-mannosidase, then one would desire a specific inhibitor that does not work on Golgi mannosidase I or mannosidase II, or jack bean or lysosomal α-mannosidase. Thus, if one used the enzymes that hydrolyze aryl-mannosides (such as *p*-nitrophenyl–D-manno-pyranoside) to screen for such a compound, the screens would obviously be negative and any potential inhibitor would be discarded.

In the period since deoxymannojirimycin was synthesized and shown to be a specific inhibitor of Golgi mannosidase I, a number of other neutral α-mannosidase activities have been reported in animal cells. These enzymes have all been discussed in Section 3.07.5.3 on glycoprotein processing, although it is still not clear what role, if any, some of them play in the trimming of *N*-linked oligosaccharides. As also indicated earlier, these enzymes have different substrate specificities from mannosidase I, and thus many of them are resistant to inhibition by DMJ. As these new mannosidases are purified and separated from each other, and from other competing activities, and as rapid assays for measuring their activities become available, it will be easier to identify or synthesize specific new inhibitors for each of these enzymes. Nevertheless, at this time, a number of α-mannosidase inhibitors have been identified and the activities of these various compounds on different α-mannosidases are presented in Table 2.

In animal cells, DMJ inhibited the Golgi mannosidase IA/B and caused the accumulation of glycoproteins having a high mannose oligosaccharide, mostly of the Man$_9$GlcNAc$_2$ structure.[224] In contrast to the effect of the glucose analogue DNJ, which prevented the secretion of IgD and IgM by cells in culture, DMJ had no effect.[225] As suggested above, this effect of DNJ is due to the function of calnexin on protein folding and its interaction with glucose. However, once the protein has folded and the glucoses are removed, the protein is treated normally with respect to targeting, regardless of whether it has a high mannose or modified chain.

In one interesting study, DMJ was used as a tool to determine whether glycoproteins were recycled through the Golgi during the endocytic process. In this experiment, membrane glycoproteins were synthesized in CHO cells in the presence of DMJ to inhibit mannose trimming, together with [2-^{3}H]mannose to label the *N*-linked glycoproteins. After an appropriate incubation, the medium was changed to remove inhibitor and label and the cells were incubated for additional times. During this second period, the oligosaccharide structure of the transferrin receptor was determined under conditions where it would undergo endocytosis. Before the chase, the oligosaccharide structure of the transferrin receptor was of the high mannose type, but during the chase period, a small percentage of the recycled receptor molecules underwent processing and gave complex types of structure. These studies indicated that some endocytosed glycoproteins do recycle through the Golgi compartments and may undergo oligosaccharide processing.[226] However, the amount of glycoprotein molecules that were actually modified in this experiment was small, indicating that recycling through the Golgi is probably not a major route.

UT-1 cells were used to examine the role of the ER α-mannosidase in glycoprotein targeting and function. UT-1 cells are cells that overexpress HMG CoA reductase, a glycoprotein enzyme that resides in the ER of the cell. The oligosaccharide chains of this protein are of the high mannose type and mostly Man$_8$GlcNAc$_2$ and Man$_6$GlcNAc$_2$ structures. Since previous studies had shown that the ER mannosidase is not inhibited by DMJ, this inhibitor was used to determine whether the initial trimming of mannoses involved the ER mannosidase. In these studies, the HMG CoA reductase produced in the presence of DMJ had mostly Man$_8$GlcNAc$_2$ structures and the smaller oligosaccharides were not found, indicating that the ER enzyme was involved in the removal of the first mannose, but other mannoses were trimmed by DMJ-sensitive mannosidase(s).[227]

DIM (1,4-dideoxy-1,4-imino-D-mannitol) is another inhibitor that was synthesized from benzyl-α-D-mannopyranose and shown to be a good inhibitor of jack bean α-mannosidase.[228] It also inhibited glycoprotein processing in cultured MDCK cells, and gave rise to glycoproteins having mostly Man$_9$GlcNAc$_2$ structures suggesting that it inhibited the Golgi α-mannosidase I.[229] In keeping

with these observations, *in vitro* studies with a partially purified preparation of mannosidase I showed that DIM did inhibit release of [^{3}H]mannose from [^{3}H]Man$_9$GlcNAc.[229] However, DIM is not nearly as effective an inhibitor of α-mannosidases as is either swainsonine or kifunensine (see below). On the other hand, DIM is of considerable interest as an inhibitor since:

(i) it has a furanose rather than a pyranose ring structure, and
(ii) it is synthesized chemically and therefore can be produced in large amounts and readily modified to produce various structural analogues.

It is not clear whether this compound also inhibits the ER mannosidase since this activity may not be present in MDCK cells.

Kifunensine (**25**) is an alkaloid produced by the actinomycete, *Kitasatosporia kifunense*, and it corresponds in structure to the cyclic oxamide derivative of 1-amino-DMJ.[43] This alkaloid is a very weak inhibitor of jack bean α-mannosidase, as is DMJ, but is a strong inhibitor of the Golgi mannosidase I ($IC_{50} = 2$ to 5×10^{-8} M). This inhibition is almost 100 times higher than the inhibition of mannosidase I by DMJ. Interestingly, kifunensine had no effect on either the ER mannosidase or on mannosidase II.[74] Influenza virus-infected MDCK cells incubated in the presence of kifunensine produced influenza virus particles in which the envelope glycoproteins had N-linked oligosaccharides mostly having Man$_9$GlcNAc$_2$ structures. This is the same effect as that seen in the presence of DMJ. However, kifunensine was much more effective in causing this change in structure and only 1/50 as much of this inhibitor was needed compared with DMJ.[74]

A compound that mimics the mannopyranosyl cation, the intermediate proposed as being involved in the enzymatic hydrolysis of α-mannopyranosides, was synthesized chemically and named mannonolactam amidrazone.[230] This compound not only inhibited Golgi mannosidase I with an IC_{50} of 4 μM, and mannosidase II with an IC_{50} of 100 nM, but was also a potent inhibitor of ER α-mannosidase (IC_{50} of 1 μM).[231] Furthermore, the compound also inhibited the aryl-α-mannosidase (IC_{50} of 400 nM) and the aryl-β-mannosidase (IC_{50} of 150 μM), although it clearly preferred α-linkages. In cell culture studies, mannonolactam amidrazone gave rise to glycoproteins with the same type of high mannose oligosaccharide as seen with DMJ and kifunensine. Thus inhibition of Golgi mannosidase I (and/or ER mannosidase) appears to prevent trimming of most if not all mannose residues.[231] The designers of this compound[230] hypothesize that the reason that it is so effective as a general mannosidase inhibitor is that it is the first analogue of mannose that mimics the true half-chair conformation of the cationic intermediate that is believed to be involved in catalysis of the α-mannosides. Mannonolactam should serve as a model for the synthesis of more specific mannosidase inhibitors.

As mentioned earlier, the first processing inhibitor to be described was the indolizidine alkaloid, swainsonine (**13**).[1] In early studies, swainsonine was added to the culture media of MDCK cells infected with influenza virus, and these cultures were labeled by the addition of [2-^{3}H]mannose. This inhibitor caused a significant inhibition in the amount of mannose-labeled, Endo H-resistant oligosaccharides (i.e., complex oligosaccharides) and a great increase in the amount of mannose-labeled Endo H-sensitive structures. These latter oligosaccharides were shown to be hybrid types of oligosaccharides.[221,232] However, the change in the structure of the viral oligosaccharides from complex to hybrid types did not affect the production, maturation or release of the influenza virus particles.

These early studies did not identify the specific site of swainsonine inhibition, but later *in vitro* studies with the purified α-mannosidases demonstrated that swainsonine specifically inhibited mannosidase II, and was inactive towards mannosidase I.[233] In keeping with this site of action, swainsonine caused the formation of hybrid structures when it was added to the medium of cultured animal cells producing VSV glycoproteins (i.e., G protein),[234] fibronectin,[235] and BHK cell surface glycoproteins.[236] In most studies where swainsonine was used to determine the effect of changes in oligosaccharide structure on glycoprotein function, this inhibitor had little effect on functional aspects of the proteins in question, although it did cause alterations in structure to hybrid chains. The inhibitor did prevent the receptor-mediated uptake of mannose-terminated glycoproteins by macrophages. This inhibition was probably due to the formation of hybrid structures on the macrophage surface which could then react with and bind the mannose receptors.[237]

Swainsonine proved to be a valuable tool in determining the sequence of addition of certain sugars during the assembly of the N-linked oligosaccharides. Thus, the addition of L-fucose or sulfate to the influenza viral protein was studied in the presence of various processing inhibitors. When the glycoproteins were produced in the presence of castanospermine or DMJ, there was no [^{3}H]fucose[238] or [^{35}S]sulfate[239] associated with the glycoproteins, suggesting that fucose and sulfate

were added after the mannosidase I step in processing. However, in the presence of swainsonine, the glycoproteins contained both L-fucose and sulfate indicating that the transferases that added these groups worked after the GlcNAc transferase I processing step. These results agree with the reported acceptor oligosaccharide specificity (i.e., GlcNAc-Man$_5$GlcNAc$_2$ of the fucosyltransferase and the sulfotransferase.

In some studies, swainsonine did cause a loss in the function of specific proteins. Thus, glucocorticoid stimulation of resorptive cells, involving the attachment of osteoblasts to bone, is inhibited by swainsonine.[240] Treatment of either the parasite, *Trypanosoma cruzi*, or the macrophages with swainsonine inhibits the interaction of these cells with each other.[112] This alkaloid also caused a dramatic decline in the ability of B16 melanoma cells to colonize the lungs of experimental animals.[241] As a result of these and similar studies, swainsonine has been undergoing tests and consideration as a drug to treat certain types of cancers. These are only a few of the many studies that have been done with this interesting compound. Many of these other studies are summarized in a review.[209]

Another inhibitor of mannosidase II, named mannostatin (**23**), was isolated from the fungus, *Streptoverticillium verticillus*.[41] This compound is of special interest because it has a very unusual structure with an exocyclic nitrogen, a five-membered ring, and a thiomethyl group, but is still a glycosidase inhibitor. Mannostatin was found to be a potent inhibitor of jack bean α-mannosidase as well as mannosidase II ($IC_{50} = 100$ nM). In cell culture studies, mannostatin caused the formation of the same types of hybrid oligosaccharides as are formed in the presence of swainsonine.[73] Interestingly, acetylation of the amino group of mannostatin resulted in loss of mannosidase activity. While this compound does not have any functional advantage over swainsonine as an inhibitor, it is of considerable interest, since it adds a great deal of additional structural information to our understanding of the requirements necessary for a compound to be a glycosidase inhibitor.

3.07.7   REFERENCES

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