Effect of Isomers of Swainsonine on Glycosidase Activity and Glycoprotein Processing†

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ABSTRACT: The chemical synthesis of swainsonine [(1*S*,2*R*,8*R*,8α*R*)-trihydroxyindolizidine] from *trans*-1,4-dichloro-2-butene was previously described [Adams, C. E., Walker, F. J., & Sharpless, K. B. (1985) *J. Org. Chem. 50*, 420–424]. A modification of that synthesis provided two other isomers, referred to here as "Glc-swainsonine" [(1*S*,2*S*,8*R*,8α*R*)-trihydroxyindolizidine] and "Ido-swainsonine" [(1*S*,2*S*,8*S*,8α*R*)-trihydroxyindolizidine]. To determine whether these new compounds had biological activity, they were compared to swainsonine as inhibitors of a number of commercially available glycosidases. While swainsonine is a potent inhibitor of jack bean α-mannosidase but does not inhibit other glycosidases, its two isomers were inactive on α-mannosidase but did inhibit other enzymes. Thus, Glc-swainsonine was an inhibitor of the fungal α-glucosidase amyloglucosidase, and this inhibition was of a competitive nature ($K_i = 5 \times 10^{-5}$ M) with respect to the substrate *p*-nitrophenyl α-D-glucopyranoside. This alkaloid also inhibited β-glucosidase, but much less effectively than α-glucosidase. On the other hand, Ido-swainsonine was more effective toward β-glucosidase than toward α-glucosidase, and this inhibition was also of a competitive nature. None of these inhibitors were effective against β-mannosidase or α- or β-galactosidase. Glc-swainsonine was also tested against the glycoprotein processing glycosidases. Surprisingly, in this respect, the alkaloid was like swainsonine in that it inhibited mannosidase II but had no effect or only slight effect on glucosidase I, glucosidase II, and mannosidase I. Glc-swainsonine also inhibited glycoprotein processing in cell culture. The oligosaccharide or oligosaccharides produced in the presence of Glc-swainsonine were hybrid types of structures similar to those seen in the presence of swainsonine and clearly different from oligosaccharides induced by either castanospermine or deoxymannojirimycin. The inhibition of processing and of mannosidase II could not be due to the presence of swainsonine in the Glc-swainsonine preparation, since this material did not inhibit jack bean α-mannosidase, even at 10 μg/mL.

Swainsonine [(1*S*,2*R*,8*R*,8α*R*)-trihydroxyindolizidine] is a plant alkaloid that was shown to be a potent inhibitor of lysosomal and other α-mannosidases (Dorling et al., 1980; Kang & Elbein, 1983). This alkaloid was initially isolated from the wild, toxic Australian plant *Swainsona canescens* (Colgate et al., 1979) and more recently was found in locoweed (i.e., *Astragalus* species) that grows in the southwestern areas of the U.S. (Molyneux and James, 1982; Davis et al., 1984). It has also been found in the fungus *Rhizoctonia leguminicola* (Schneider et al., 1982). It is likely that the toxicosis that results from eating this plant is due to swainsonine (James & Hartley, 1977; Tulsiani & Touster, 1983b) as is the accumulation of high-mannose oligosaccharides in urine and other tissues (Abraham et al., 1983; Sadeh et al., 1983). Swainsonine has also been found to be an inhibitor of glycoprotein processing (Elbein et al., 1981; Kang & Elbein, 1983b; Tulsiani & Touster, 1983b; Gross et al., 1983) by virtue of the fact that it is a potent inhibitor of mannosidase II (Tulsiani & Touster, 1982). Thus, when animal cells are grown in the prsence of swainsonine, they are not able to produce the typical complex types of oligosaccharides on their N-linked glycoproteins since mannosidase II is inhibited. These cells instead produce hybrid types of oligosaccharides.

Since swainsonine is a trihydroxyindolizidine with four asymmetric centers, it seems likely that other alkaloids with different stereochemistry would inhibit other glycosidases. In fact, a precedence for this hypothesis was set by the observation that castanospermine [(1*S*,6*S*,7*R*,8*R*,8α*R*)-tetrahydroxyindolizidine] was a good inhibitor of α- and β-glucosidases (Saul et al., 1983). This alkaloid was also an inhibitor of glycoprotein processing by inhibiting the processing glucosidase I (Pan et al., 1983). In animal cells, castanospermine caused the accumulation of $Glc_3Man_{7-9}(GlcNAc)_2$ structures on the protein.

Recently, the chemical synthesis of swainsonine from the 4-carbon precursor *trans*-1,4-dichloro-2-butene was described (Adams et al., 1985). Utilizing variations of this synthesis, it was possible to synthesize two other isomers of swainsonine designated here as "Glc-swainsonine" [(1*S*,2*S*,8*R*,8α*R*)-trihydroxyindolizidine] and "Ido-swainsonine" [(1*S*,2*S*,8*S*,8α*R*)-trihydroxyindolizidine] (C. E. Adams and K. B. Sharpless, unpublished results). The structures of swainsonine and the two isomers are shown in Figure 1. In this paper, we show that Glc-swainsonine was indeed a good inhibitor of the α-glucosidase amyloglucosidase but this alkaloid did not inhibit α-mannosidase, α- or β-galactosidase, or β-glucosidase. The inhibition of amyloglucosidase was of a competitive nature. On the other hand, Glc-swainsonine was like swainsonine in that it inhibited the glycoprotein processing mannosidase II but did not affect the other processing glycosidases. In terms of in vivo inhibition, Glc-swainsonine also resembled swainsonine in causing the accumulation of hybrid types of oligosaccharides on viral and cellular glycoproteins.

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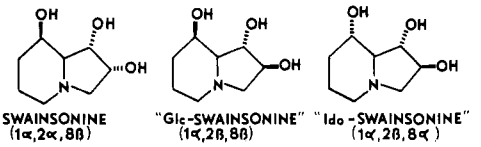

FIGURE 1: Structure of swainsonine and its isomers.

Thus, the alteration in the chirality of the 2-hydroxyl group of the alkaloid did cause it to become an inhibitor of α-glucosidase, but it still retained some of its inhibitory effects on α-mannosidases. Ido-swainsonine was not an inhibitor of any of the processing enzymes, but it did inhibit β-glucosidase to some extent.

These data suggest that the synthesis of isomers of these indolizidine alkaloids that have the proper stereochemistry will provide valuable inhibitors of other glycosidases, and perhaps inhibitors at other steps in the processing pathway. In addition, data on the effects of other inhibitors on susceptible glycosidases should enable us to predict the requirements essential for inhibition of these enzymes.

Experimental Procedures

Materials. [2-^{3}H]Mannose (10 Ci/mmol) and [6-^{3}H]-galactose (14.2 Ci/mmol) were purchased from New England Nuclear or from Pathfinders Laboratories. Minimal essential medium (MEM), calf serum, MEM nonessential amino acids, glutamine, PSN antibiotic mixture, BME vitamin solution, and mycostatin suspension were from Grand Island Biological Co. The following glycosidases were purchased from Sigma Chemical Co.: almond emulsion β-glucosidase, yeast α-glucosidase, fungal amyloglucosidase (an α-glucosidase), bovine liver β-galactosidase, coffee bean α-galactosidase, and jack bean α-mannosidase. These enzymes were assayed with the appropriate *p*-nitrophenyl glycosides, which were also obtained from Sigma Chemical Co. Pronase was from Calbiochem, and endo-β-*N*-acetylglucosaminidase H was from Miles Laboratories. Swainsonine was isolated and purified from locoweed (Davis et al., 1984). Castanospermine was prepared from the seeds of *Castanospermum australe* (Hohenschutz et al., 1981).

Chemical Synthesis and Purity of Swainsonine and Its Isomers. The chemical synthesis of swainsonine from *trans*-1,4-dichloro-2-butene was done as previously described (Adams et al., 1985). Glc-swainsonine was prepared in the same manner as swainsonine except that *cis*-1,4-dichloro-2-butene was used as the starting material rather than the trans isomer. The overall yield for the process was 5% (in 21 steps). Ido-swainsonine was prepared by inversion at C-8 on an intermediate at the latter stages of the Glc-swainsonine synthesis. The purity of synthetic swainsonine and its isomers was determined by 250-mHz ^{2}H NMR. Swainsonine was at least 95% pure [mp 140–142 °C, $[\alpha]^{25}_D$ −73.8° (*c* 0.21, EtOH)]. Glc-swainsonine was better than 90% pure [mp 169.5–172 °C, $[\alpha]^{25}_D$ − 61° (*c* 0.12, absolute EtOH)]. Ido-swainsonine was 95% pure, contaminated with 5% Glc-swainsonine [mp 146.5–149 °C, $[\alpha]^{25}_D$ −17° (*c* 0.16, absolute EtOH)].

Assay of Inhibitory Effects of the Alkaloids on Glycosidases. The reaction mixtures for the assay of the various commercially available glycosidases contained the following components in a final volume of 0.5 mL: 25 mM sodium acetate buffer, pH 5.0, 5 μmol of the appropriate *p*-nitrophenyl glycoside, various amounts of the inhibitor to be tested, and enzyme (usually enough enzyme to give an optical density change of 1.0–2.0 at 410 nm in a 30-min incubation). Each enzyme was assayed at various protein concentrations and for various times to be certain that the measurements were being done under linear conditions of time and protein concentration. Generally, enzyme and inhibitor were mixed in buffer, and the reactions were started by the addition of substrate. After an incubation of 30 min, the reactions were stopped by the addition of 2.5 mL of 0.4 M glycine buffer, pH 10.4, and the yellow color due to the liberated *p*-nitrophenol was measured at 410 nm.

Assay and Inhibition of the Glycoprotein Processing Enzymes. The various glycoprotein processing glycosidases were assayed with the appropriate radioactive oligosaccharides as substrates. Thus, glucosidase I (Szumilo et al., 1986b) was measured by following the release of [^{3}H]glucose from the [^{3}H]glucose-labeled $Glc_3Man_9GlcNAc$, glucosidase II by the release of [^{3}H]glucose from the [^{3}H]glucose-labeled $Glc_2Man_9GlcNAc$, mannosidase I (Szumilo et al., 1986c) by the liberation of [^{3}H]mannose from the [^{3}H]mannose-labeled $Man_9GlcNAc$, and mannosidase II (T. Szumilo and A. D. Elbein, unpublished results) by the release of [^{3}H]mannose from the [^{3}H]mannose-labeled $GlcNAcMan_5GlcNAc$. Since all of these radioactive oligosaccharides bind to columns of concanavalin A–Sepharose, the enzymatic activities can be readily determined by applying the incubation mixtures to small columns of concanavalin A–Sepharose. The oligosaccharide substrates as well as most of the oligosaccharide products bind to these columns whereas the monosaccharides released by the action of the glucosidases emerge in the buffer wash of the columns. Thus, radioactivity in the wash can be determined as a measure of enzymatic activity (Szumilo & Elbein, 1985).

The various radioactive oligosaccharide substrates were synthesized in influenza virus infected MDCK cells as previously described (Pan et al., 1983; Elbein et al., 1985). The methods are briefly described below. Thus, the [^{3}H]-glucose-labeled $Glc_3Man_9GlcNAc$ was prepared by infecting confluent monolayers of MDCK cells with influenza virus and then adding castanospermine, at 200 μg/mL, to inhibit glucosidase I. After allowing the cells to incubate for several hours in the presence of the alkaloid in order to allow it to take effect, [^{3}H]galactose was added to label the viral glycoproteins. The virus was isolated from the medium and digested with Pronase to obtain the radioactive glycopeptides, and these were isolated by chromatography on columns of Bio-Gel P-4. The glycopeptides were then treated with endoglucosaminidase H to release the $Glc_3Man_9GlcNAc$ oligosaccharide, which was further purified on long calibrated columns of Bio-Gel P-4 (Szumilo & Elbein, 1985). The [^{3}H]glucose-labeled $Glc_2Man_9GlcNAc$ was obtained by incubating the $Glc_3Man_9GlcNAc$ structure with a partially purified glucosidase I from mung bean seedlings (Szumilo et al., 1986). The [^{3}H]mannose-labeled $Man_9GlcNAc$ was prepared by incubating influenza virus infected MDCK cells in the presence of deoxymannojirimycin, an inhibitor of mannosidase I, and labeling the viral glycoproteins with [2-^{3}H]mannose (Elbein et al., 1984). The final substrate, $GlcNAcMan_5GlcNAc$, was prepared from [^{3}H]mannose-labeled $Man_9GlcNAc$ by incubating this $Man_9GlcNAc$ with a partially purified mannosidase I from plants (Szumilo et al., 1986c) followed by isolation of the $Man_5GlcNAc$ and incubation of this oligosaccharide with UDP-GlcNAc and a partially purified GlcNAc transferase I from plants (Szumilo et al., 1986a).

Incubation mixtures for the determination of processing glycosidases usually contained 150 mM 4-morpholine-ethanesulfonic acid (MES) buffer, pH 6.5, 0.1% Triton X-100, 2.5 mM ethylenediaminetetraacetic acid (EDTA), 2500–5000 cpm of the radioactive oligosccharide substrate, and various amounts of the glycosidase preparation to be tested, all in a

final volume of 0.2 mL. In some experiments, various amounts of the processing inhibitors were added before the enzyme in order to determine their effects on the different glycosidases. The reactions were initiated by the addition of enzyme, and incubations were done at 37 °C for 15 min to 1 h. At the end of the incubation, the reaction mixtures were deproteinized as described (Szumilo & Elbein, 1985), and the supernatant liquids were applied to columns of concanavalin A–Sepharose to separate the oligosaccharides from the liberated monosaccharides. When processing inhibitors were used, the liberation of monosaccharides was measured by a paper chromatographic assay in order to avoid the possibility that the processing inhibitors might interfere with the binding of oligosaccharides to the lectin columns. The results obtained with the untreated enzyme were the same with either assay method.

Production and Analysis of Glycopeptides. The effects of the various indolizidine alkaloids on the structure of the N-linked oligosaccharides of the influenza virus hemagglutinin were studied. In addition, the structures of the glycopeptides and oligosaccharides produced by swainsonine and its isomers were compared to those formed in the presence of naturally occurring swainsonine, castanospermine, and deoxymannojirimycin. In all of these experiments, confluent monolayers of MDCK cells were infected with influenza virus, and 1 h after infection, various amounts of the indicated processing inhibitor were added. The cells were incubated for several hours to allow the inhibitors to take effect, and then [2-^{3}H]-mannose or [6-^{3}H]galactose (about 5 μCi/mL) was added to each flask. The incubations were continued, in the presence of isotope and inhibitor, for an additional 40 h to produce mature virus, and the virus was isolated from the medium by ultracentrifugation. The viral pellets were digested exhaustively with Pronase to obtain the glycopeptide fraction. The cell residues were also saved and digested with Pronase to obtain the cellular glycopeptides.

Glycopeptides were separated on a 1.5 × 150 cm column of Bio-Gel P-4 (200–400 mesh). Since this column did not completely resolve the complex types of glycopeptides from the high-mannose types, the entire glycopeptide peak was pooled, digested with endoglucosaminidase H, and rechromatographed. This treatment liberated the high-mannose and hybrid types of oligosaccharides but left the complex structures intact. The liberated oligosaccharides migrated more slowly on the columns than the original glycopeptides and could therefore be resolved from the complex glycopeptides. Aliquots of every other fraction were removed for the determination of radioactivity, and peak fractions were pooled for structural studies.

Characterization of the Inhibitor-Induced Oligosaccharide. Oligosaccharides released by treatment with endoglucosaminidase H were chromatographed and sized on a 1.5 × 200 cm column of Bio-Gel P-4 (200–400 mesh). The column was standardized with a variety of known oligosaccharides as indicated in the appropriate figures. Oligosaccharides were treated with a number of commercially available glycosidases to determine their susceptibility to these enzymes. Thus, the labeled oligosaccharides were incubated with the following series of enzymes: β-galactosidase, β-galactosidase followed by β-*N*-acetylhexosaminidase, β-galactosidase plus β-*N*-acetylhexosaminidase, and β-galactosidase plus β-*N*-acetylhexosaminidase plus α-mannosidase or α-mannosidase alone. Usually the assays were done in 25 mM sodium acetate buffer, pH 5.0, the radioactive substrate, and 0.1–1 unit of enzyme in a final volume of 0.2 mL. A few drops of toluene were added to retard bacterial growth, and incubations were for 12 h. At the end of this time, a second addition of enzyme(s) was made, and incubations were continued for an additional 12 h. The incubation mixtures were then analyzed by passage over the Bio-Gel P-4 column to determine whether alterations in the size of the oligosaccharide had occurred and whether radioactive monosaccharides had been released. Oligosaccharides and glycopeptides were also examined by lectin affinity chromatography with concanavalin A–Sepharose columns to distinguish tri- and tetraantennary complex chains from biantennary complex structures and from hybrid and high-mannose chains. Columns were prepared and run as described (Cummings & Kornfeld, 1982). On these columns, tri- and tetraantennary complex chains do not bind and emerge in the wash whereas biantennary complex chains bind weakly and are eluted with 10 mM methyl α-glucoside. The hybrid and high-mannose chains bind tightly to the concanavalin A and require 100 mM methyl α-mannoside for elution.

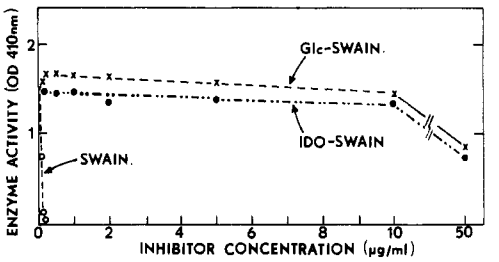

FIGURE 2: Effect of swainsonine and its isomers on jack bean α-mannosidase. Incubations were as described in the text and contained *p*-nitrophenyl α-D-mannopyranoside as substrate. Various amounts of swainsonine, Glc-swainsonine, or Ido-swainsonine were added as indicated in the figure. After an incubation of 30 min, the release of *p*-nitrophenol was measured at 410 nm.

RESULTS

Effect of Swainsonine and Its Isomers on Jack Bean α-Mannosidase. Swainsonine, Glc-swainsonine, and Ido-swainsonine (see Figure 1 for structures) were compared for their ability to inhibit jack bean α-mannosidase as demonstrated in Figure 2. In this experiment, various amounts of each of the inhibitors were tested, and the liberation of *p*-nitrophenol from the *p*-nitrophenyl α-D-mannopyranoside was measured. It can be seen that this enzyme was very sensitive to swainsonine and was almost completely inhibited at less than 200 ng/mL (about 1 μM) of this inhibitor. In addition, the synthetic swainsonine was compared to the naturally occurring swainsonine in terms of their inhibitory capacity, and both were found to inhibit at the same concentrations (data not shown). On the other hand, Glc-swainsonine and Ido-swainsonine were essentially ineffective against the jack bean α-mannosidase, and only slight inhibition was seen with these compounds, even at 50 μg/mL (250 μM), or higher. Thus, epimerization of the hydroxyl group at position 2 of the alkaloid results in a loss in the ability to inhibit jack bean α-mannosidase.

Effect of Swainsonine and Its Isomers on Amyloglucosidase Activity. Amyloglucosidase (glucoamylase; exo-1,4-α-glucosidase) is a fungal α-glucosidase (EC 3.2.1.3) that can be assayed with the *p*-nitrophenyl α-D-glucopyranoside as substrate. Glc-swainsonine was compared to Ido-swainsonine and to swainsonine in terms of the inhibition of this enzyme. Figure 3 shows the inhibition curves obtained with these three alkaloids. It can be seen that the Glc-swainsonine was an effective inhibitor of amyloglucosidase and was clearly the best of the three alkaloids. Thus, 50% inhibition of the enzyme occurred at a Glc-swainsonine concentration of about 10 μg/mL (about 50 μM). On the other hand, Ido-swainsonine was not an inhibitor of this enzyme or at best was a very poor

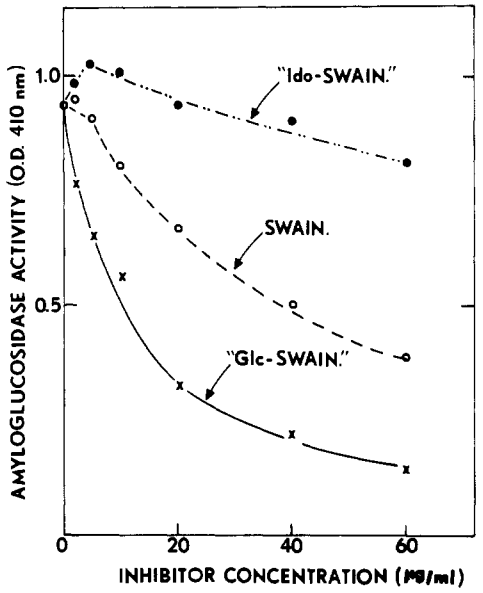

FIGURE 3: Effect of swainsonine and its isomers on amyloglucosidase activity. Incubations were as described in the text and contained *p*-nitrophenyl α-D-glucopyranoside as substrate. Various amounts of the inhibitors were added as indicated in the figure. The release of *p*-nitrophenol was measured at 410 nm.

inhibitor. Strangely enough, the chemically synthesized swainsonine also showed some inhibitory activity against the amyloglucosidase, although it was not nearly as effective as the 2-epimer (i.e., Glc-swainsonine). Since naturally occurring swainsonine does not inhibit the amyloglucosidase, even at 100 μg/mL, it is not clear why the chemically synthesized material shows this activity. It is possible that the synthetic material contains small amounts (5% or less) of another compound that is an inhibitor of amyloglucosidase. If so, this compound must be considerably more active as an inhibitor than is Glc-swainsonine, since it could not represent more than a small percentage of the swainsonine preparation.

The hydrolysis of *p*-nitrophenyl α-D-glucopyranoside by the amyloglucosidase was linear with time of incubation and with the amount of enzyme added to the incubation mixtures, up to 100 μg of protein (data not shown). The inhibition of the enzyme by Glc-swainsonine was observed throughout the time course of the reaction, and the extent of inhibition increased with incresing amounts of the inhibitor. Thus, approximately 50% inhibition occurred at all time points when 10 μg of Glc-swainsonine was used, and this increased to 75 or 80% inhibition at 25 μg of inhibitor (data not shown). In order to determine whether the Glc-swainsonine was a competitive inhibitor, substrate concentration curves were done in the absence or presence of different amounts of inhibitor. The formation of *p*-nitrophenol was proportional to the concentration of *p*-nitrophenyl α-D-glucopyranoside up to about 1 mM and then slowly leveled off at higher concentrations of substrate. These data, as well as substrate concentration curves at two different inhibitor concentrations, were plotted according to the method of Lineweaver and Burk. It is clear from the curves shown in Figure 4 that the inhibition is of a competitive nature. The K_i calculated for Glc-swainsonine was about 5×10^{-5} M.

Effect of Swainsonine and Its Isomers on Other Aryl-glycosidases. The three indolizidine alkaloids were also tested as inhibitors of a number of other glycosidases. When tested against almond emulsin β-glucosidase, the Ido-swainsonine was the best inhibitor of this enzyme and showed 50% inhibition at 30–40 μg/mL (about 150–200 μM). On the other hand,

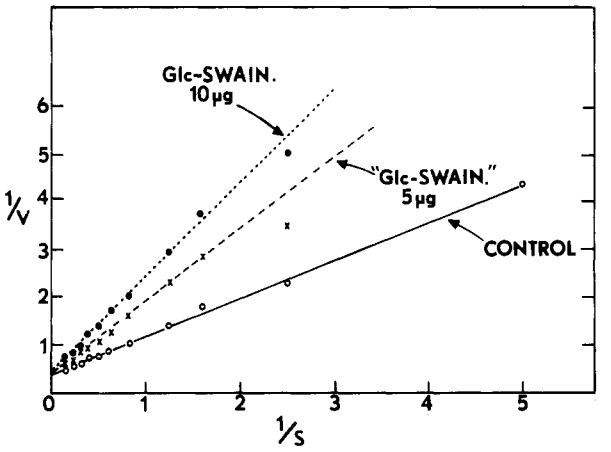

FIGURE 4: Effect of substrate concentration on Glc-swainsonine inhibition of amyloglucosidase. Incubations were as described in the text, except that the amount of *p*-nitrophenyl α-glucoside was varied as indicated. Substrate concentration studies were done in the presence of 5 or 10 μg of Glc-swainsonine. The liberation of *p*-nitrophenol was measured, and the data were plotted by the method of Lineweaver and Burk.

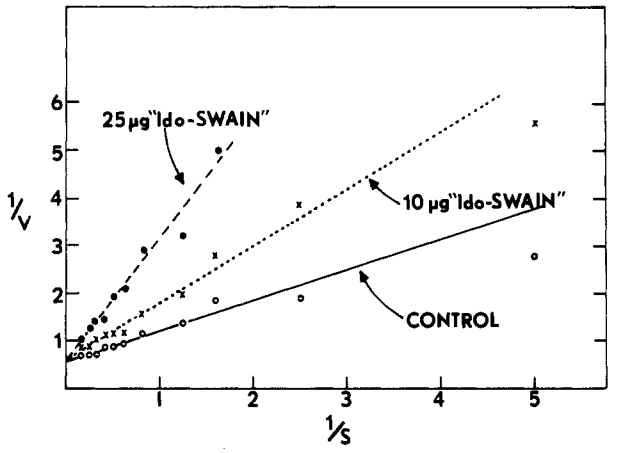

FIGURE 5: Effect of substrate concentration on Ido-swainsonine inhibition of β-glucosidase. Incubations were as described in the text, except that the concentration of *p*-nitrophenyl β-D-glucoside was varied as shown. Concentration curves were done in the presence of 10 or 25 μg of Ido-swainsonine. The release of *p*-nitrophenol was determined at 410 nm, and the data were plotted by the method of Lineweaver and Burk.

Glc-swainsonine was only slightly active toward the β-glucosidase, while swainsonine was completely without effect (data not shown). The inhibition of β-glucosidase by Ido-swainsonine was also of a competitive nature as shown by the Lineweaver–Burk plots presented in Figure 5. The K_i for Ido-swainsonine was estimated to be 5×10^{-4} M.

The three alkaloids were additionally tested as inhibitors of a number of other glycosidases, including coffee bean α-galactosidase, liver β-galactosidase, liver β-*N*-acetylhexosaminidase, and *Aspergillus fumigatus* β-mannosidase. None of the alkaloids were effective inhibitors of any of these glycosidases, even when these compounds were tested at levels of 60 μg/mL (i.e., 300 μM; data not shown).

Effect of Glc-Swainsonine on the Glycoprotein Processing Enzymes. Four of the processing enzymes were partially purified from mung bean seedlings, and these enzymes were used to determine the specificity of the inhibition by Glc-swainsonine. Glc-swainsonine was tested at various concentrations as an inhibitor of glucosidase I and glucosidase II. For comparisons, various amounts of castanospermine were also tested on these enzymes. While castanospermine inhibited both enzymes (50% inhibition of glucosidase I at 2 μM and

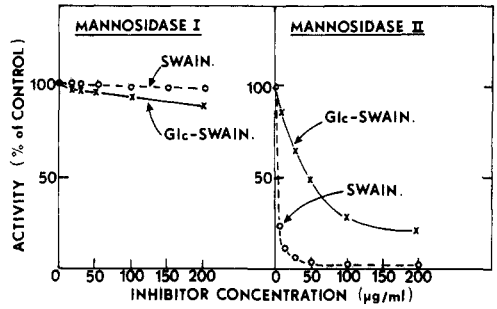

FIGURE 6: Effect of Glc-swainsonine on the glycoprotein processing mannosidase I and mannosidase II. Incubations for mannosidase I (left) contained 2500 cpm of [^{3}H]mannose-labeled $Man_9GlcNAc$, 100 μg of partially purified mannosidase I from mung beans, and various amounts of swainsonine or Glc-swainsonine. After an incubation of 2 h, the release of mannose was measured by the concanavalin A binding assay. In the controls, about 500 cpm of radioactive mannose was released in this incubation. Incubations for mannosidase II (right) contained about 3000 cpm of [^{3}H]mannose-labeled $GlcNAcMan_5GlcNAc$, 200 μg of partially purified mung bean mannosidase II, and various amounts of inhibitor. Mannose released was determined by the concanavalin A binding assay.

of glucosidase II at 50 μM), Glc-swainsonine had almost no effect and gave only 20% inhibition of glucosidase I at 250 μM concentrations (data not shown).

Glc-swainsonine was also tested as an inhibitor of the plant processing mannosidases as shown in Figure 6. In this experiment, Glc-swainsonine was compared to swainsonine, since the latter alkaloid is an inhibitor of mannosidase II and other α-mannosidases. The curves on the left show that neither swainsonine nor Glc-swainsonine was able to inhibit the processing mannosidase I, even when tested at concentrations of 200 μM (40–50 μg/mL). On the other hand, both of these compounds were inhibitors of mannosidase II as demonstrated by the curves shown on the right of Figure 6. Thus, swainsonine was a very potent inhibitor and showed greater than 50% inhibition of mannosidase II at 1 μM concentrations. Glc-swainsonine also inhibited this enzyme but much less effectively than the swainsonine. In this case, 50% inhibition of the enzyme required about 50 μM concentrations of Glc-swainsonine. This inhibition of mannosidase II (and glycoprotein processing) could not be due to contaminating swainsonine in the Glc-swainsonine since this material did not inhibit jack bean α-mannosidase even at 10 μg/mL (Figure 2). At this level, even 1% contamination by swainsonine should inhibit more than 50%.

Effect of Glc-Swainsonine on Glycoprotein Processing. Since the Glc-swainsonine had no effect on jack bean α-mannosidase but it did inhibit both amyloglucosidase and mannosidase II, it was important to determine whether it would affect glycoprotein processing in cell culture. For these studies, we used influenza virus infected MDCK cells and examined the biosynthesis and processing of the viral hemagglutinin as well as of the glycoproteins remaining within the cells. We have previously used this system to study the site of action of various processing inhibitors (Elbein et al., 1981, 1984; Pan et al., 1983). In the experiments described here, we compared the oligosaccharide structures of glycoproteins biosynthesized in the presence of Glc-swainsonine to those produced in the presence of castanospermine (Pan et al., 1983), deoxymannojirimycin (Elbein et al., 1984), or swainsonine (Kang & Elbein, 1983b; Tulsiani & Touster, 1983b; Gross et al., 1983).

Thus, confluent monolayers of MDCK cells were infected with influenza virus, and various amounts of Glc-swainsonine (or one of the other processing inhibitors) were added to the culture. After several hours to allow the inhibitor to take effect, [2-^{3}H]mannose or [6-^{3}H]galactose was added to the culture to label the viral and cellular glycoproteins. Cultures were incubated for about 40 h (in the presence of inhibitor and isotope) to allow mature virus to be formed, and the viral particles were isolated from the medium by ultracentrifugation. The presence of mature viral particles in the various fractions was determined by the hemagglutination assay. The cell residues were also isolated by low-speed centrifugation, and this material was also examined to determine the structures of the cell-bound glycoproteins.

Both the viral pellets and the cell pellets were digested with Pronase to convert the glycoproteins to glycopeptides, and these glycopeptides were isolated on columns of Bio-Gel P-4. Since these columns did not completely resolve the various types of glycopeptides (i.e., high mannose, hybrid, and complex) from each other, the entire glycopeptide peak was pooled, digested with endoglucosaminidase H, and rechromatographed on the column. Endo H cleaves most high-mannose and hybrid types of glycopeptides between the two internal GlcNAc residues, and therefore, such glycopeptides are converted to oligosaccharides that can easily be resolved from the complex structures.

Figure 7 compares the profiles of glycopeptides and oligosaccharides isolated from the cell residues of control cells to those of cells grown in the presence of swainsonine or Glc-swainsonine. In these experiments, the cellular glycoproteins were labeled with [2-^{3}H]mannose. In the control cells (profile A), two glycopeptide peaks were separated on the column; the first peak (C1) eluted in fractions 30–42 and was resistant to digestion by Endo H, whereas the other peak(s) (C2 and C3) emerged in fractions 43–58 and was (were) sensitive to Endo H. Previous studies have shown that peak C1 is composed of complex types of oligosaccharides while peaks C2 and C3 contains high-mannose oligosaccharides of the $Man_6GlcNAc$ to $Man_9GlcNAc$ type (Kang & Elbein, 1983b; Pan et al., 1983).

The lower two profiles compare the results from cells incubated in the presence of 1 μg/mL swainsonine (profile B) or 200 μg/mL Glc-swainsonine (profile C). It can be seen that the profiles obtained with these two alkaloids were fairly similar to each other and clearly different from that of control cells. In the presence of swainsonine, the glycopeptide peaks were shifted to the right, i.e., to lower molecular weight species. Except for the small peak of radioactivity emerging between fractions 36 and 46 (S1 in profile B), which was resistant to Endo H digestion, the other peaks of radioactivity were sensitive to Endo H treatment. Thus, two large peaks of radioactivity were obtained (S2 and S3) after Endo H digestion; S2 emerged between fractions 47 and 56, while S3 eluted between fractions 57 and 66. The characterization of these oligosaccharide structures is presented below. A summary of these studies identified S1 as complex structures mostly of the biantennary and triantennary type; S2 was composed of hybrid types of oligosaccharides and S3 of high-mannose chains of the $Man_7GlcNAc$ to $Man_8GlcNAc$ type. It should be noted that in the presence of swainsonine high-mannose oligosaccharides were still produced since this alkaloid inhibits at the mannosidase II stage and therefore does not block the normal trimming to high-mannose chains.

The lowest profile (profile C) shows the glycopeptides produced in the presence of Glc-swainsonine. With this inhibitor, the glycopeptide profile was similar to that seen with swainsonine, and the oligosaccharides produced by Endo H were also fairly similar to those observed with swainsonine.

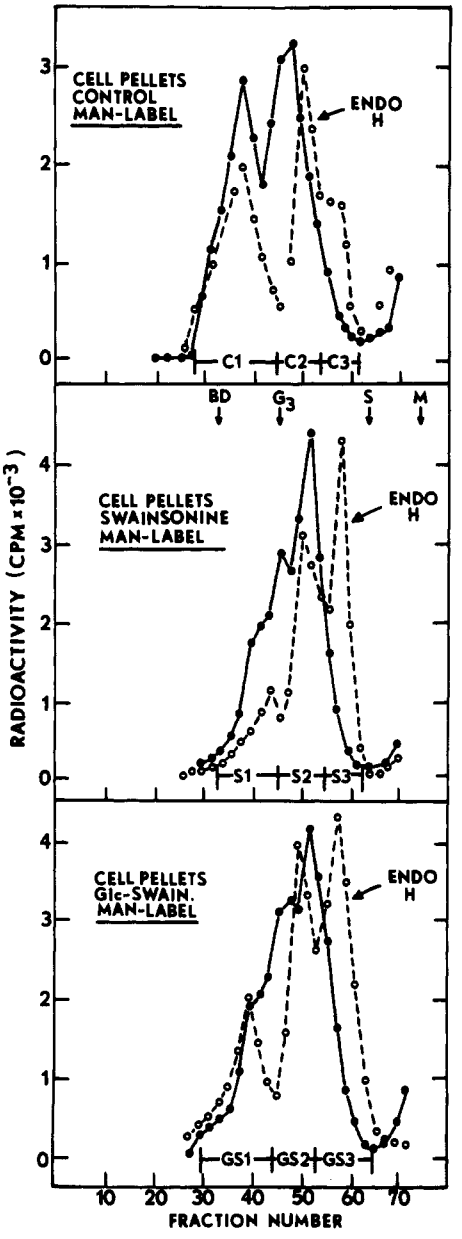

FIGURE 7: Effect of Glc-swainsonine on glycopeptide and oligosaccharide composition of cell-bound glycoproteins. Infected MDCK cells were incubated in the absence (profile A) or in the presence of swainsonine (1 μg/mL) (profile B) or Glc-swainsonine (200 μg/mL) (profile C). After an incubation of 2 h, [2-^{3}H]mannose (10 μCi/mL) was added to each flask. Cells were incubated for 40 h to allow mature influenza virus to form. At the end of this time, the cell residue was isolated by centrifugation at 10000*g* for 20 min, and the supernatant liquids were centrifuged at 100000*g* overnight to obtain the viral pellets. Both cell pellets and viral pellets were digested with Pronase, and the glycopeptides were isolated on the Bio-Gel columns (●). The glycopeptide peaks were pooled, concentrated, and digested with endoglucosaminidase H. These digests were rechromatographed on the columns (○). Aliquots of each fraction were counted to determine their radioactive content.

The major difference between the two profiles (B and C) was the fact that more complex chains (GS1) were produced in the presence of Glc-swainsonine. This is probably not surprising, since other studies (see, for example, Figure 6) have indicated that Glc-swainsonine is much less effective as a inhibitor than is swainsonine. At any rate, the three peaks obtained with Glc-swainsonine (GS1, GS2, GS3) were identical in elution position with those produced in the presence of swainsonine; GS1 was composed of complex oligosaccharides, GS2 of hybrid structures, and GS3 of high-mannose oligosaccharides (see below).

The profiles obtained with Glc-swainsonine were also compared to the profiles obtained when cells were incubated in the presence of castanospermine or deoxymannojirimycin. The profiles produced by these latter two inhibitors were clearly different from those seen in Figure 7. For example, in the presence of sufficient amounts of castanospermine (200 μg/mL), almost all of the radioactive mannose was in a single peak that corresponded in size to a $Hex_{10-11}GlcNAc$ (data not shown). This peak was previously characterized as a $Glc_3Man_{7-8}GlcNAc$ (Pan et al., 1983). In the presence of castanospermine, the high-mannose oligosaccharides seen with swainsonine or Glc-swainsonine were not produced since this inhibitor blocks at the glucosidase I stage. When cells were incubated with deoxymannojirimycin, again almost all of the radioactivity was in a single peak, but this peak was somewhat smaller in molecular weight than the castanospermine peak. It was characterized as a $Man_9GlcNAc$ (data not shown). In this case also, smaller high-mannose chains were not detected (Elbein et al., 1984).

Each of the peaks obtained after Endo H digestion, and shown in Figure 7, were subjected to chromatography on columns of concanavalin A–Sepharose to aid in their characterization. The Endo H resistant peak from cells treated with swainsonine (S1) or Glc-swainsonine (GS1) either did not bind to the concanavalin A or bound very weakly and was displaced with 10 mM methyl α-glucoside. This is consistent behavior for complex types of oligosaccharides. On the other hand, peaks S2, GS2, S3 and GS3, bound tightly to these columns and required 100 mM methyl α-mannoside for elution. This type of binding is indicative of hybrid or high-mannose chains (data not shown). These oligosaccharides were also treated with a variety of enzymes to assist in their characterization. S3 and GS3 were almost completely susceptible to digestion by jack bean α-mannosidase and gave a major peak on Bio-Gel P-4 columns corresponding to free mannose and a small peak corresponding to ManGlcNAc. However, S3 or GS3 was not altered in its mobility by treatment with β-galactosidase or β-*N*-acetylhexosaminidase, either together or sequentially. Thus, these oligosaccharides are of the high-mannose types. On the other hand, S2 and GS2 were only partially susceptible to α-mannosidase, and in each case, this treatment resulted in a peak of free mannose and an oligosaccharide peak that was somewhat smaller in size than the original oligosaccharide. These oligosaccharides were also susceptible to digestion by β-galactosidase, and this enzyme gave a slightly smaller sized oligosaccharide (data not shown). The products produced by β-galactosidase treatment were further analyzed with the galactose-labeled oligosaccharides as described below (see Figure 8).

In order to determine whether the oligosaccharides produced in the presence of Glc-swainsonine contained glucose or galactose residues, the virus-infected MDCK cells were incubated in the presence of swainsonine or Glc-swainsonine and labeled with [6-^{3}H]galactose. Figure 8 compares the glycopeptide profiles of control cells to those of cells grown in the presence of swainsonine (profile B) or Glc-swainsonine (profile C). As in the case of the mannose-labeled glycopeptides, the entire peak was pooled, digested with Endo H, and rechromatographed on the Bio-Gel column.

Much of the galactose incorporated into macromolecules was into Endo H resistant material that emerged in the void volume of these columns. This material is probably mostly proteoglycan since it is not affected by any of the processing inhibitors. In addition, previous studies showed that much of this radioactivity was susceptible to digestion by chrondroi-

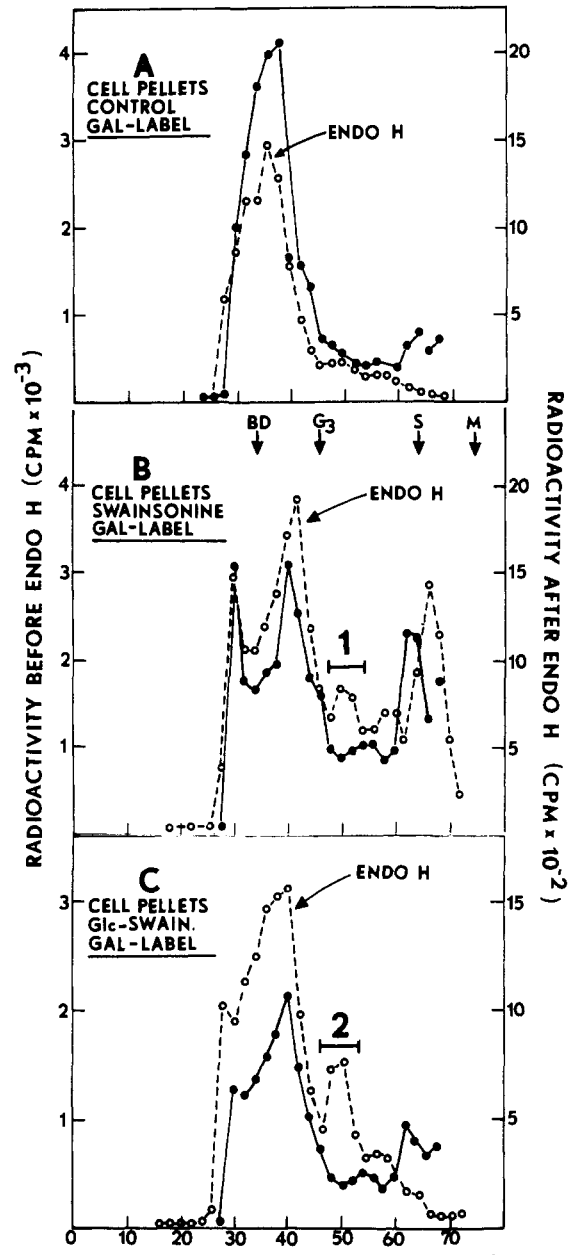

FIGURE 8: Effect of Glc-swainsonine on incorporation of [^{3}H]galactose into cell-bound glycoproteins. Infected MDCK cells were incubated with inhibitors as described in Figure 7, except that [6-^{3}H]galactose (5 μCi/mL) was added to each flask to label the glycoproteins. Cell-bound and viral glycoproteins were digested with Pronase and then with endoglucosaminidase H as described in Figure 7.

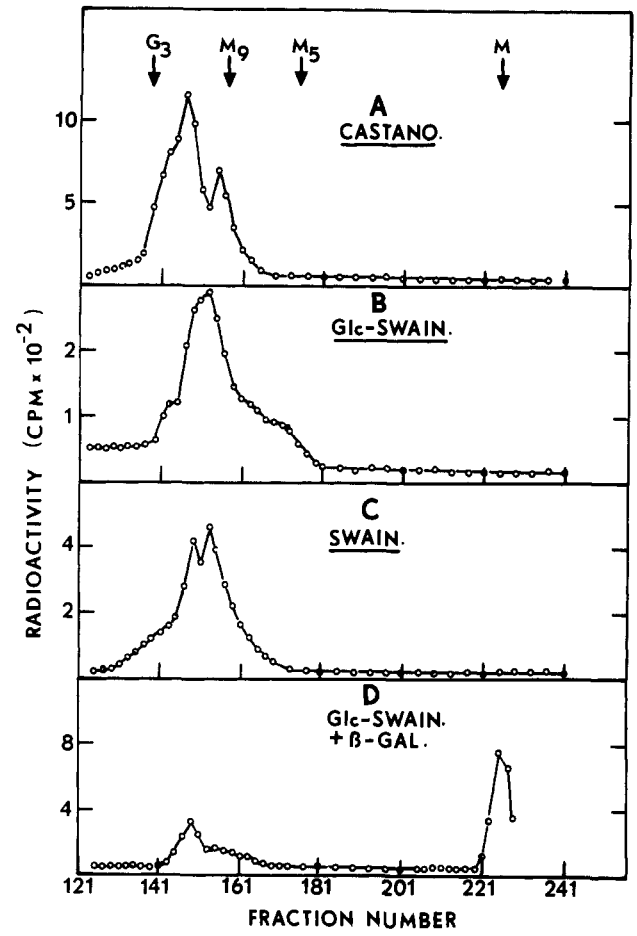

FIGURE 9: Determination of size of oligosaccharides produced in the presence of swainsonine and Glc-swainsonine—effect of β-galactosidase. The galactose-labeled oligosaccharides produced in the presence of swainsonine (profile C) or Glc-swainsonine (profile B) were chromatographed on a calibrated column of Bio-Gel P-4 (1 × 200 cm, 200–400 mesh). For purposes of comparison, the glucose-labeled oligosaccharide formed in the presence of castanospermine was also run on this column. Aliquots of every other fraction were removed for the determination of radioactivity. In the lower profile (D), the Glc-swainsonine oligosaccharide (fractions 147–159) was treated with liver β-galactosidase, and the digest was chromatographed on the calibrated column. Standards shown by the arrows are $Glc_3Man_9GlcNAc$ (G_3), $Man_9GlcNAc$ (M_9), $Man_5GlcNAc$ (M_5), and mannose (M).

tinase ABC and could be precipitated with cetylpyridinium chloride (Merkle et al., 1985). Although there were differences in the amount of radioactive galactose incorporated into material emerging in the void volume of swainsonine- or Glc-swainsonine-incubated cells, the reason for these differences is not known. The void volume material has not been further characterized. Of considerable interest is the fact that, in the presence of swainsonine (profile B) or Glc-swainsonine (profile C), a new labeled peak was produced in the presence of Endo H that eluted from the columns in the same position as S2 and GS2 from the mannose-labeled, alkaloid-treated cells (i.e., the hybrid structures). This new peak is designated 1 in profile B and 2 in profile C of Figure 8. Each of these peaks was pooled and chromatographed on a long, calibrated column of Bio-Gel P-4 as shown in Figure 9. For purposes of comparison, the oligosaccharide produced in the presence of castanospermine was also run (profile A). The major peak formed in castanospermine emerged after the $Glc_3Man_9GlcNAc$ standard and is mostly a $Glc_3Man_7GlcNAc$. On the other hand, the major peak produced in the presence of swainsonine or Glc-swainsonine was smaller than the castanospermine peak and migrated near the $Man_9GlcNAc$ standard. In the presence of swainsonine, there appeared to be two peaks that probably represent hybrid chains with one or two GlcNAc residues. These two peaks elute in the same position as the broad single peak from Glc-swainsonine-incubated cells, suggesting heterogeneity of hybrid structures in both cases. The lowest profile of Figure 9 shows that most of the radioactivity in the Glc-swainsonine oligosaccharide (and also in the swainsonine oligosaccharide) was released as monosaccharide upon digestion with β-galactosidase. This radioactivity migrated with authentic galactose upon paper chromatography. On the other hand, no radioactivity was released when these oligosaccharides were treated with the plant processing glucosidase I and/or glucosidase II, although these latter two enzymes did remove the galactose label from the castanospermine-induced oligosaccharide (as glucose). Finally, the swainsonine- and Glc-swainsonine-induced oligosaccharides were converted to slightly smaller sized oligo-

saccharides by treatment with α-mannosidase, and these oligosaccharides moved just ahead of the $Man_9GlcNAc$ standard. However, no radioactivity was released by this enzyme.

The data on the characterization of the mannose-labeled or galactose-labeled oligosaccharides produced in the presence of Glc-swainsonine indicate that these are hybrid types of oligosaccharides that contain one or two GalGlcNAc branches. Similar oligosaccharides are produced in the presence of swainsonine. It should also be pointed out that the data shown here were from the cell-bound glycoproteins. However, the glycoproteins associated with the virus were also characterized in the same way. The results with the viral glycoproteins were essentially the same as those shown. That is, both swainsonine and Glc-swainsonine caused a great reduction in the amount of complex chains, and instead, hybrid types of oligosaccharides were formed. However, in both control virus and the alkaloid-treated virus, there was a much smaller proportion of high-mannose chains formed. Nevertheless, these experiments indicate that Glc-swainsonine like swainsonine inhibits mannosidase II in cell culture and causes the accumulation of hybrid structures.

Discussion

Several indolizidine alkaloids, and other structurally related compounds, have been shown to be specific inhibitors of glycosidases. For example, castanospermine (Saul et al., 1983) and deoxynojirimycin (Saunier et al., 1982) are inhibitors of α-glucosidases and also inhibit the glycoprotein processing enzyme glucosidase I (and also glucosidase II). Deoxymannojirimycin is an inhibitor of the processing mannosidase I (Fuhrmann et al., 1984), while swainsonine inhibits various aryl-α-mannosidases (Dorling et al., 1980; Kang & Elbein, 1983a) as well as the glycoprotein processing enzyme mannosidase II (Tulsiani & Touster, 1982, 1983; Kang & Elbein, 1983b; Gross et al., 1983). Presumably, the inhibition by these various compounds is due to the presence of a nitrogen in the ring in place of the oxygen and also to the proper stereochemistry of the hydroxyl groups. However, at this time there is not enough information available to know what the requirements are that cause a given structure to inhibit a specific glycosidase. Thus, it would be of considerable value to have a number of isomers of each of these inhibitors in order to determine if, and how, the specificity of inhibition is altered with changes in chirality. That is, would an isomer of swainsonine or castanospermine inhibit a different glycosidase than that inhibited by the parent compound and if so which glycosidase? Once enough information of this type has been accumulated, it should be possible to make models in order to predict the necessary structural requirements for inhibition of specific enzymes.

In this paper, an attempt is made to relate changes in structure of swainsonine to inhibition of specific glycosidases. In these studies, two isomers of swainsonine that were synthesized chemically were tested to determine whether they inhibited any of the commercially available glycosidases. Thus, while swainsonine [(1*S*,2*R*,8*R*,8α*R*)-trihydroxyindolizidine] inhibits jack bean α-mannosidase, its 2-epimer [(1*S*,2*S*,8*R*,8α*R*)-trihydroxyindolizidine] inhibits α-glucosidase but not α-mannosidase. Although Glc-swainsonine is not nearly as potent an inhibitor of α-glucosidase as swainsonine is of α-mannosidase, its altered activity would suggest that the 2-hydroxyl group of the alkaloid is analogous to the 2-hydroxyl group of the sugar substrate for the enzyme.

However, Glc-swainsonine proved to be similar to swainsonine in terms of its effect on the processing glycosidases. Thus, rather than inhibiting one or both of the glucosidases (i.e., glucosidase I or glucosidase II), Glc-swainsonine proved to be an inhibitor of mannosidase II. This is the same enzyme that is inhibited by the parent compound, swainsonine, and like this alkaloid, Glc-swainsonine was inactive on mannosidase I. In addition, in cell culture Glc-swainsonine caused the accumulation of hybrid types of oligosaccharides on the N-linked glycoproteins that were identical in structure with those caused by swainsonine. In both of these cases, Glc-swainsonine was much less effective than was swainsonine, since it required much higher concentrations for inhibition. These data indicate that the structural requirements necessary for inhibition of the arylglycosidases appear to be different from those necessary for the processing glycosidases, and it is not possible with the data available to predict the necessary structures.

A similar dilema in terms of predictability exists for an isomer of castanospermine. Recently, we isolated the 6-epimer of castanospermine from extracts of the seeds of *Castanospermum australe* (unpublished observations). This alkaloid would be expected to be an inhibitor of mannosidases, since the 6-hydroxyl of the alkaloid should be analogous to the 2-hydroxyl of the sugar (i.e., the glucose–mannose pair). Neither castanospermine nor 6-epicastanospermine was an inhibitor of the α- or β-arylmannosidases, nor did they inhibit the α- or β-arylgalactosidase. However, while castanospermine inhibits both α- and β-glucosidase, the 6-epicastanospermine only inhibits the α-glucosidase. The 6-epicastanospermine is also like castanospermine in that it inhibits the glycoprotein processing glucosidase I. Thus, in this case, epimerization of the 6-hydroxyl group of the alkaloid results in a loss of the ability to inhibit β-glucosidase.

The above studies indicate that additional information is required before we can understand the requirements for glycosidase inhibition. Since methods are now becoming available for the synthesis and/or modification of swainsonine, castanospermine, and other inhibitors, it should be possible to test a number of compounds with altered chirality and determine which glycosidases are affected.

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In Vivo Effects of Photosynthesis Inhibitors in *Synechococcus* As Determined by ^{31}P NMR Spectroscopy†

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ABSTRACT: Phosphorus-31 nuclear magnetic resonance spectra were obtained from darkened cells of the unicellular cyanobacterium *Synechococcus* sp. Resonance peaks were assigned to intracellular pools of sugar–phosphates, inorganic phosphate (P_i), nucleotides, and polyphosphate. An internal pH of 7.2 was estimated from the chemical shift of the P_i resonance. Cells were then illuminated at 1600 $\mu E \cdot m^{-2} \cdot s^{-1}$ photosynthetically active radiation by a fiber optic cable immersed in the cell sample. Spectra obtained after approximately 15 min of illumination showed an increase in nucleotide pools and an increase in the cytoplasmic pH to 7.6. In the presence of 0.3 mM dinitrophenol (DNP), an uncoupler of phosphorylation, spectra of illuminated cells showed an immediate decline in nucleotide pools while sugar–phosphate levels remained constant. Addition of the photosystem II (PS II) electron-transport inhibitor 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) (7.2 μM) did not affect nucleotide levels in the cells during the time course of the experiment (15–30 min). However, an abrupt rise in the resonance in the sugar–phosphate region was noted. Spectra of DCMU-treated cell extracts indicated that one metabolite was principally responsible for the change in pool size. The metabolite was identified as 3-phosphoglyceric acid. Spectra of illuminated cells were also obtained in the presence of the natural herbicide cyanobacterin. Cyanobacterin inhibits electron transport in PS II. The mechanism by which the inhibitor acts is unknown. Unlike results obtained with DNP or DCMU, spectra of cyanobacterin-treated cells showed no major changes in nucleotide or sugar–phosphate resonances. A slow decline in cytoplasmic pH was seen in the presence of cyanobacterin, indicating that the natural product affects the proton pumping mechanism in PS II.

Recently, whole cell and tissue nuclear magnetic resonance techniques have been used to study various metabolic processes in plants. For example, intracellular pH changes in cytoplasm and vacuoles have been estimated in maize (Roberts et al., 1980) and other tissues grown in culture (Martin et al., 1982). In addition, the concentrations of various phosphorylated metabolites in plant cells have been estimated by ^{31}P NMR (Mitsumori et al., 1985). Other nuclei such as ^{13}C and ^{15}N have also been used as tracers in studies in plant metabolism [see Roberts (1984) for a review].

However, the major physiological process which occurs in plants is photosynthesis. NMR could be used to study in vivo pH and metabolite changes in plant tissues after the dark to light transition. The method should be useful in confirming previous in vitro studies and could be extended to the investigation of metabolite pools and herbicide activity during photosynthetic electron transport and carbon dioxide fixation. A few reports on photosynthesis using NMR methods have been published, but several technical problems limit the usefulness of the technique. Since NMR is relatively insensitive, thick cell suspensions are required to obtain reproducible signals. Providing enough light and carbon dioxide to cells

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