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THE INDOLIZIDINE ALKALOIDS, SLAFRAMINE AND SWAINSONINE: Contaminants in Animal Forages

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INTRODUCTION

A long trail of research in both the US and Australia in disparate fields such as plant pathology, toxicology, animal husbandry, and biochemistry is now converging to focus attention on two indolizidine alkaloids, slaframine (I, Figure 1) and swainsonine (II, Figure 1) and the bizarre effects these alkaloids, and related compounds, have in ruminants. Thus swainsonine, a potent α-mannosidase inhibitor (12) isolated from the Australian vetch, *Swainsona*

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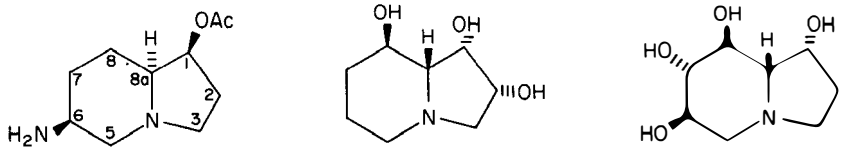
canescens (10), and from the locoweed (38) was ultimatedly linked (27) to the severe pathological sequela that results when ruminants eat the *Swainsona* pea in Australia (13) and the locoweed in the American West (63). The locoweed is viewed by some as the most destructive of American weeds. Whether this pathology can be charged solely to the effects of swainsonine and its α-mannosidase inhibitory action (13, 38, 58) is an extremely interesting question concerning molecular mechanisms of disease. In totally unrelated studies swainsonine (48) and slaframine, a potent parasympathomimetic secretogogue (5), have been identified as products of the lysine metabolism of the mold *Rhizoctonia leguminicola*. This mold parasitizes certain legume forages, particularly red clover, in wide areas of the United States. Hence the effect of these alkaloids, viewed in this instance as mycotoxins when ingested in moldy forages by ruminants, also raises significant questions relating to animal health and perhaps to public health as well (7). These research trails are reviewed herein; some have been previously related (8, 23) and represent a blend of basic and applied work, many aspects of which may be of interest to nutritionists.

SLAFRAMINE

Recognition as a Slobber Factor

The author's laboratory, then in the Department of Dairy Science at the University of Illinois, became involved in this field after a series of severe outbreaks of "the slobbers" in dairy cattle on Illinois farms in the winter of 1959–1960. The cattle were consuming second-cutting red clover hay, and in a similar outbreak of the slobbers in Wisconsin, Smalley et al (52) made the extremely important observation that such forage was infected with the black fungus, *Rhizoctonia leguminicola,* known to cause black patch of red clover (20). It thus appeared that "the slobber factor" might be a mycotoxin produced by *R. leguminicola*. Indeed both the Illinois and Wisconsin groups produced slobbering in dairy cattle by feeding washed *R. leguminicola* mycelium via fistula. Using a guinea pig salivation assay the slobber factor, termed slaframine, (1S, 6S, 8aS)-1-acetoxy-6-aminooctahydroindolizine, was subsequently



I. SLAFRAMINE II. SWAINSONINE III. CASTANOSPERMINE

Figure 1 Indolizidine alkaloids. I: Slaframine, (1S, 6S, 8aS)-1-acetoxyl-6-aminooctahydroindolizine; II: Swainsonine, (1S, 2R, 8R, 8aR) 1,2,8-trihydroxyoctahydroindolizine; III: Castanospermine, 1,6,7,8-tetrahydrooctahydroindolizine.

isolated from *R. leguminicola* mycelium (3, 44) and microgram quantities were shown to produce excessive salivation in laboratory animals, e.g. rats, guinea pigs, and cats.

The slobbers, or excessive salivation, is caused by the consumption of forage (usually red clover, but also white clover, soybean, Kudzu, cow pea, blue lupine, alsike clover, alfalfa, Korean lespedeza, and black medic) that has been infected with *R. leguminicola* (20, 50). Outbreaks are fairly uncommon but have been reported in the northwestern, midwestern, and southeastern United States. The disease, called black patch, is favored by wet weather and high humidity. Following ingestion of forage and after a brief induction period, the ruminant salivates excessively for periods of up to three days. During this period the animal usually goes off feed and dairy cattle suffer a loss of milk production. Because the farmer is then faced with the problem of disposal and replacement of feed, this problem is of some economic consequence as well. Other symptoms that may be associated with slobbering include lacrimation, diarrhea, and frequent urination.

A particularly well-documented series of slobbers in farm animals occurring in Missouri over a ten-year period was compiled by O'Dell et al (41) and is summarized in Table 1. Note that on occasion severe consequences were observed beyond those discussed above, including abortion, bloating, and death. In this regard several investigators have wondered [see (51) and references therein] whether more than one factor may be involved in *R. leguminicola* toxicosis; this question is addressed in this article. All cases of the slobbers that are seen in the field appear to result from consuming *R. leguminicola*–infested forage. In one careful study Hagler & Behlow (26) examined some "slobber hay" that had caused severe slobbering in horses. *R. leguminicola* was the predominant fungus in the hay, and slaframine was isolated directly from the hay, which was estimated to contain 50–100 ppm of the alkaloid.

Pathological and Other Effects

No in-depth studies of the pathology of excessive salivation in farm animals have yet been done with pure slaframine. Partially purified toxin (1 mg/kg body weight) was lethal to guinea pigs when administered subcutaneously, intraperitoneally, or by force feeding (11). Signs of toxicosis included lacrimination, salivation, frequent defecation, and urination; dyspnea occurred just before death. Suffocation appeared to be the most probable cause of death. Postmortem examination revealed enlarged emphysematous lungs and centrilobular necrosis of the liver. A brief report of Froetschel et al (18) indicates that in 120-day-old broiler chicks, the LD_{50} for slaframine is of the order of 11 mg/kg. In another part of this study, administration of slaframine (1 mg/kg body weight *per os*) was associated with increased growth hormone plasma levels (8–12 hr after dose) by 449–948% ($P < 0.05$). Such findings are exciting and need

Table 1 Incidence of slobbering in Missouri farm animals following ingestion of particular forage (41)

Season grown	County where grown	Type of hay	Symptoms reported
1949	Marion	Red clover–lespedeza mixed with stubble	Cattle slobbered profusely; refused hay
1950	Monroe	Second-cutting red clover	Cattle and horses slobbered and refused feed
1950	Caroll	Second-cutting of red clover with considerable grass	Cattle and horses slobbered profusely
1950	Dade	Red clover mixed with grass, cut from spring seeding	Cattle slobbered, bloated and became stiff in the joints
1950	Schuyler	Second-cutting red clover with very little grass	Cattle slobbered and sheep became sick
1950	Jasper	Red clover with stubble; spring seeding with oats	Cattle slobbered after one feeding, then refused hay
1951	Ray	Red clover, grass, and wheat stubble	Sheep slobbered
1951	Randolph	Red clover and wheat stubble	Cattle, horses and sheep slobbered; mare became sick and aborted
1955	Chariton	Red clover, wheat stubble	Cattle slobbered and refused feed
1955	Calloway	Second-cutting red clover	Slobbering in cattle but most marked in calves and sheep
1955	Oregon	Silage from second-cutting red clover	Cattle slobbered and refused feed
1955	Shelby	Second-cutting red clover	Dairy cattle slobbered and milk production dropped
1957	Livingston	Red clover from spring seeding cut in fall	Cattle slobbered and had diarrhea; one died
1958	Nodaway	Red clover stubble	Cattle slobbered profusely after one feeding; lost weight
1958	Lincoln	Lespedeza-grass	Dairy cattle slobbered (not confirmed in guinea pigs)

confirmation and extension to establish firmly if slaframine is indeed a growth-hormone-releasing agent.

Chemistry and Biosynthesis of Slaframine

The elucidation of the structure of slaframine has been reviewed elsewhere (8). It was originally concluded to be 1-acetoxy-8-aminooctahydroindolizine (4) but subsequent proton magnetic resonance (PMR) spin decoupling experiments led to a reassignment of structure (19) as 1-acetoxy-6-aminooctahydroindolizine (I, Figure 1). The absolute configuration (1S, 6S, 8aS) was deduced

by a combination of PMR and chemical methods. The total synthesis of DL-slaframine has been achieved by several groups [see (46) and references therein]. In the synthesis done by Schneider & Harris (46) a key step is the formation of the octahydroindolizine nucleus, which occurs via a potassium hydride cyclization of *N*-acetyl pipecolate ester. The yield of slaframine obtained from ethyl 5-nitropicolinate was 12%.

The biosynthesis of slaframine in whole cultures or cell-free extracts of *R. leguminicola* from pipecolic acid continues to be actively investigated in the author's laboratory in collaboration with Dr. T. M. Harris at Vanderbilt University. Evidence, principally from radioactive and stable-isotope studies, supports the stereospecific transformations shown in Figure 2. The pathway represents an economy in the marshalling of a few critical nutrients to fashion a complex nitrogen heterocycle possessing three chiral centers. L-lysine formed via the homocitrate-aminoadipate pathway of lysine biosynthesis from acetate, α-ketoglutarate, and glutamate (64) loses its α-amino group to form Δ^1-piperideine-2-carboxylate, which on reduction gives L-pipecolate (IV, Figure 2) (22). Pipecolate and malonate (V) are thought to condense at the thiol ester level to form pipecolylacetate (VI, Figure 2) (9), which on reduction and cyclization would give the stable intermediate, 1-ketooctahydroindolizine (VII, Figure 2) (25). The latter is then stereochemically reduced to the required isomeric form of 1-hydroxyoctahydroindolizine (VIIIa, Figure 2) (25, 47), which is then appropriately functionalized to ultimately yield slaframine (23, 25). This asymmetric synthesis of slaframine is compared below with swainsonine formation also arising from pipecolate metabolism in this fungus.

Physiological Studies

Quantitive studies by Aust (1, 2) of salivary and pancreatic secretions following slaframine administration demonstrated a significant time lag before observation of physiological response. For example, maximum salivary activity in anesthetized cats occurred after about an hour after slaframine administration and then continued up to 6 hr (1). Similarly a substantial delay in pancreatic flow in the sheep, goat, and calf was observed before attaining a maximal response to slaframine (2). Evidence for increased synthesis of digestive enzymes under these latter conditions was suggested by an increase in specific activity of trypsin (goat) and enhanced incorporation of [^{14}C] leucine into pancreatic protein (calf) over controls. The ability of slaframine to stimulate specifically the exocrine glands was indicated by the fact that doses which elicited maximal secretory rates had no effect on heart rate, blood pressure, or respiration.

Froetschel et al (17) considered slaframine's potential as a tool for studying the effects of salivary flow on the rumen environment. Rumen cannulated wethers were fed a pelleted concentrate of moldy ground red clover calculated

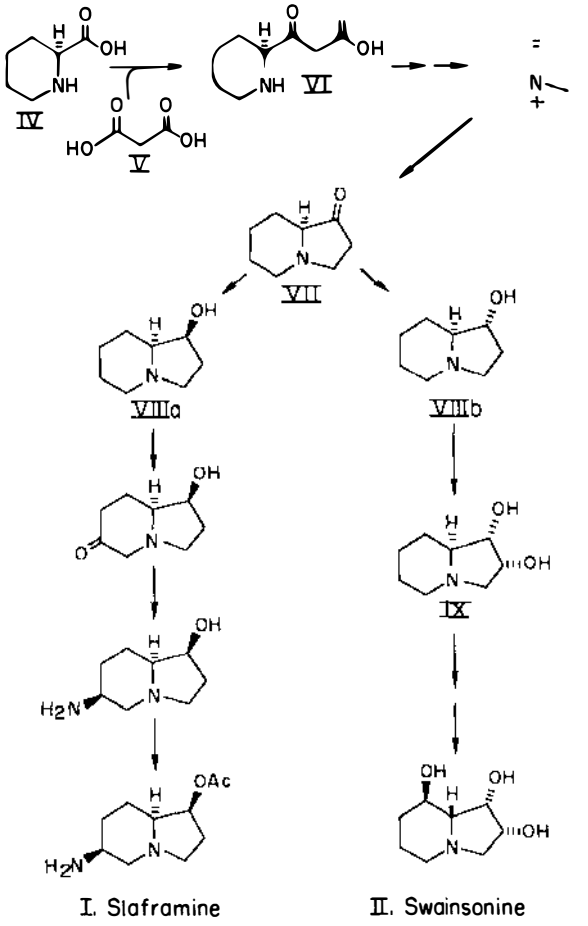


Figure 2 Biogenesis of slaframine and swainsonine in *Rhizoctonia leguminicola*.

to contribute 0.8 mg of slaframine per 100 g of pellets. Such feed pellets fed 12 times daily increased salivary flow and tended to increase rumen fluid dilution rate and acetate/propionate ratio in these animals. Purified slaframine given subcutaneously increased eating salivary flow rate up to 316% in steers. Such observations suggest the interesting possibility that slaframine might be useful in treating digestive disorders associated with the feeding of high grain restricted roughage diets.

Early studies of Aust et al (5) indicted activation of slaframine by the liver prior to action. The portal vein and hepatic artery of rats were clamped for varying times while slaframine was injected directly into the vena cava. The delay in onset of salivation was proportional to the time of isolation of the liver. Subsequently Spike & Aust (53) showed that rat liver microsomes plus

NADPH activated slaframine as demonstrated in a guinea pig ileum contraction assay. Moreover, activation was also achieved with a model system using flavins in the presence of light. Under anaerobic conditions flavin was reduced concomitantly with the production of active metabolite. These results were extended and refined by Guengerich & Aust (21). Products of the photochemical reactions were examined following reduction with $NaBH_4$, from which it was possible to deduce structures and draw conclusions about reactions that were postulated as shown in Figure 3. In the photochemical reaction, I → X, exposure of slaframine to FMN and light results in a nonoxidative deamination and rearrangement to give X, which is without biological activity. The microsomal system involves a four-electron oxidation by FMN, I → XI, to give the inimine XI, followed by hydrolysis giving ammonia and the ketoimine XII thought to be the active slaframine metabolite.

The structural features of XII in common with acetylcholine should be noted, i.e. an alkylated quaternary N atom separated by two carbon atoms to an acetate ester. The ester function is mandatory for parasympathomimetic activity (5). Atropine which blocks the effects of cholinergic compounds also blocks the action of activated slaframine if given *prior* to the metabolite. Thus activated slaframine apparently binds to muscarinic acetylcholine receptors. Such binding must be greater than the binding of atropine since high levels of atropine will not reverse the effect of activated slaframine either in vitro or in vivo.

Certain observations in the foregoing experiments suggested that the mixed function oxidase system might be operational in slaframine activation I → XI (Figure 2). But the insensitivity of the microsomal system to CO and other considerations led to the view that a hepatic microsomal flavoprotein oxidase (43) is most likely the enzyme involved in slaframine activation and that NADPH may be required to stabilize the enzyme.

SWAINSONINE

Swainsonine spp. Toxicosis

Swainsonine, (1S, 2R, 8R, 8aR)-1,2,8-trihydroxyoctahydroindolizine, and its biological action as an α-mannosidase inhibitor were discovered after about 100 years of investigation into the toxicity of the legumes of the genus

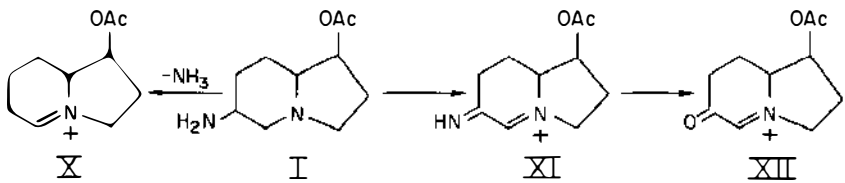


Figure 3 Postulated photochemical and enzymmatic activation of slaframine (21).

Swainsona. There are over 50 such species in Australia, including *S. galegifolia, S. luteola, S. greyana, S. canescens,* and *S. procumbens,* which when consumed by livestock, particularly cattle, sheep, and horses, result in a chronic disease characterized by neurologic disturbances, loss of body weight, and addiction to the plant (27). Hartley (27) described typical symptoms in young beef cattle grazing on *S. galegifola:* there is first a loss of condition, followed by the development of neurologic signs (e.g. staring eyes, head shaking, low head carriage, stiff-clumsy gait, incoordination, and hyperexcitability). Mortality is high in young animals following onset of neurological signs; adult animals may survive as chronic cases for months. The affected animals have a very poor breeding performance; e.g. they may abort or newly born calves may soon die. A most characteristic lesion in *Swainsona* toxicity in cattle, sheep, and horses as seen under the light microscope consists of widespread neurovisceral cytoplasmic "watery" vacuolation. Ultrastructural and biochemical studies suggested that the vacuolar lesion represents a lysosomal storage phenomenon involving either the direct storage of some slowly metabolized molecule from the plant or the induction of endogenous storage by the inhibition of one or more acid hydrolases (32, 33).

Dorling et al (13) followed up on such hypotheses and were able to show that lymph nodes of sheep contained high levels of mannose-rich oligosaccharides, and further that the *Swainsona* plant ingested contained a lysosomal α-mannosidase inhibitor. They concluded that consumption of *Swainsona* induces a lysosomal storage disease, biochemically and morphologically similar to genetically determined mannosidosis in man (40) and cattle (29). It seemed plausible that the neurons of the central nervous system are particularly vulnerable to the harmful effects of mannoside storage, and that such storage associated with cellular vacuolation is brought about by continued exposure to the α-mannosidase inhibitor.

Subsequently the purification and characterization of the α-mannosidase inhibitor in the plant *Swainsona canescens* was accomplished by Colegate, Dorling & Huxtable (10). Employing appropriate spectroscopic techniques, researchers showed the active compound, swainsonine, to be an indolizidinetriol of the structure and stereochemistry of II in Figure 1. At a concentration of 20 μM, swainsonine completely inhibited α-mannosidase activity (pH 4.0) from a variety of mammalian tissue extracts (12). Details of the assay, nature of the inhibition, etc. are discussed below.

Swainsonine and Locoism

It has been known for over a century that in the southwestern US horses, cattle, sheep, and goats are poisoned by eating locoweed. The plant gets its name from the Spanish word that describes the crazy behavior of such animals. Locoweed poisoning was later shown to be associated with the ingestion of certain

members of the genera *Astragalus* and *Oxytropis* (27, 63). Signs of poisoning generally appear after two to three weeks of continuous grazing of the plant. The principal effects on livestock include neurological damage, habituation, emaciation, and reproductive alterations such as abortion and birth defects (63). As an example of the destructive effect of locoweed on livestock, an early USDA bulletin [cited in (38)] describes a loss in 1883 of 25,000 cattle as a result of locoweed poisoning in one area of 35 by 120 miles in southwestern Kansas. Furthermore it is interesting to note a report of more than 80 years ago (37) pointing out similarities in clinical behavior between locoweed-eating sheep in the United States and pea-eating sheep in Australia. Such similarities were brought into clearer focus when it was shown that as in *Swainsona* spp. posioning, the principal lesion in locoism, observed via light and electron microscopy, is neurovisceral cytoplasmic vacuolation of various body tissues. Their sequential development, distribution, and correlation with clinical symptoms is discussed in (63). Finally after a century of searching for the cause(s) of locoism, a USDA group succeeded in isolating swainsonine, together with its N-oxide in a yield of 0.007% from spotted locoweed (38). Molyneux & James suggest (38) that these alkaloids, by virtue of their inhibition of lysosomal α-mannosidase, are the causative agents of locoism in range animals. This view is strengthened in the work of Tulsiani et al (56) wherein swainsonine per se when administered to baby pigs was found to duplicate the effect of locoweed.

Swainsonine and Disruption of Glycoprotein Assembly

The biosynthesis of glycoproteins containing asparagine-linked high mannose and complex oligosaccharides is reviewed elsewhere (31, 35). In brief, as is summarized in Figure 4, the initial *N*-linked oligosaccharides undergo a series of processing reactions whereby sugars are trimmed from high-mannose types of structures. Following such processing other sugars are then added to the oligosaccharide chains ultimately to yield complex types (Figure 4). The events in Figure 4 relative to oligosaccharide transformation can also be expressed employing the following useful convention: $Glc_3Man_9GlcNAc_2 \rightarrow GlcMan_9GlcNAc_2 \rightarrow Man_9GlcNAc_2 \rightarrow Man_5GlcNAc_2 \rightarrow GlcNAcMan_5GlcNAc_2 \rightarrow GlcNAcMan_3GlcNAc_2 \rightarrow \rightarrow \rightarrow$ complex oligosaccharides.

Elbein et al (14, 16) were the first to show that swainsonine prevents the formation of complex oligosaccharides of the asparagine-linked class of glycoproteins. Their experiments concerned the effect of swainsonine on the processing of high [^{3}H] mannose glycopeptide by a liver particulate fraction by following the release of [^{3}H] mannose. They concluded (14) that swainsonine inhibits mannosyl cleavage rather than glycosyl cleavage. It has been established that in glycoprotein biosynthesis there are two rat liver Golgi mannosi-

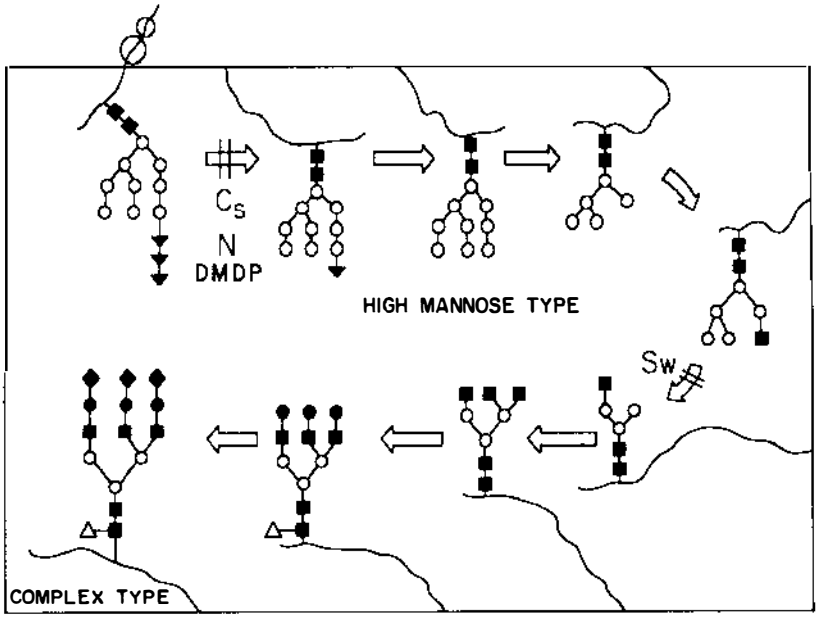


Figure 4 Aspects of biosynthesis of *N*-linked oligosaccharides; sites of inhibition of glycoprotein processing. Closed triangles = glucose, open circles = mannose, closed squares = *N*-acetylglucosamine, closed circles = galactose, open triangles = fucose, diamonds = acetylneuraminic acid. SW = swainsonine, C_s = castanospermine, N = nojirimycin, DMDP = 2,5-dihydroxymethyl 1-3,4-dihydroxypyrrolidine.

dases, IA and IB, specific for α-1,2-mannosyl residues, which convert Man_9 precursors to Man_5 intermediates, i.e. $Man_9GlcNAc_2 \rightarrow Man_5GlcNAc_2$ (54, 59). Following *N*-acetylglucosaminylation, $Man_5GlcNAc_2 \rightarrow GlcNAcMan_5GlcNAc_2$, rat liver Golgi mannosidase II (which is α-1,3 and α-1,6 specific) carries out the following processing: $GlcNAcMan_5GlcNAc_2 \rightarrow GlcNAcMan_3GlcNAc_2$ (59). Tulsiani, Harris & Touster (58) partially purified rat liver Golgi mannosidase IA and IB and mannosidase II enzymes and showed that swainsonine was specific in inhibiting mannosidase II. Thus in a complete system capable of forming $GlcNAcMan_5GlcNAc_2$ and trimming the latter to $GlcNAcMan_3GlcNAc_2$, this processing reaction was blocked by swainsonine as shown in Figure 4.

It will be noted in Figure 4 that both high mannose and complex oligosaccharide types of glycoproteins are formed. Tulsiani & Touster (60) demonstrated that as a consequence of swainsonine inhibition of Golgi mannosidase II, hybrid glycoproteins are formed. Thus in an appropriate human skin fibroblast system, approximately equal amounts of complex and high-mannose glycoproteins were synthesized. But in the presence of swainsonine most of the complex glycoproteins were replaced by hybrid types, the principal oligosac-

charide having the structure shown in Figure 5. The foregoing studies (58, 60) pinpoint a site of swainsonine action in disrupting a step of glycoprotein assembly and should contribute to an understanding of the action of this alkaloid in mammalian mannosidosis.

Mechanism of Swainsonine Inhibition of α-D-Mannosidase

Detailed studies have been made of the nature of swainsonine inhibition of jack bean mannosidase (12, 57), lysosomal α-D-mannosidase of tissue extracts (12, 57), and rat liver Golgi mannosidase II (57). Useful substrates in these studies are 4-methylumbelliferyl α-D-mannopyranoside and *p*-nitrophenyl α-D-mannoside, which on hydrolysis release a fluorophor and chromophor respectively that can be readily measured. Dorling et al (12) found that 20-μM swainsonine completely inhibited α-mannosidase activity (pH 4.0) from a variety of tissues including mouse liver or extracts prepared from kidney, lymph nodes, or liver of sheep, guinea pigs, and rats, and from liver of the lamprey eel. On the other hand α-glucosidase, β-galactosidase, hexosaminidase, and β-glucuronidase from mouse liver were not affected by 200-μM swainsonine. On successive dilution of a jack bean α-mannosidase/swainsonine mixture, α-mannosidase activity increased, which indicated that inhibition by swainsonine is reversible. Such reversibility, however, is complex, and a plot of swainsonine concentration vs enzyme activity revealed sigmoidal kinetics. It was suggested (36) that a glycosyl ion intermediate is formed during hydrolysis of natural substrates by glycosidases, and that inhibition of these enzymes by aldonolactones is mediated by a similar cation. On this basis Dorling et al (12) speculated that the inhibitory action of swainsonine results from structural similarity of its protonated form to the mannosyl cation, as illustrated in Figure 6. The absolute configuration of swainsonine supports this

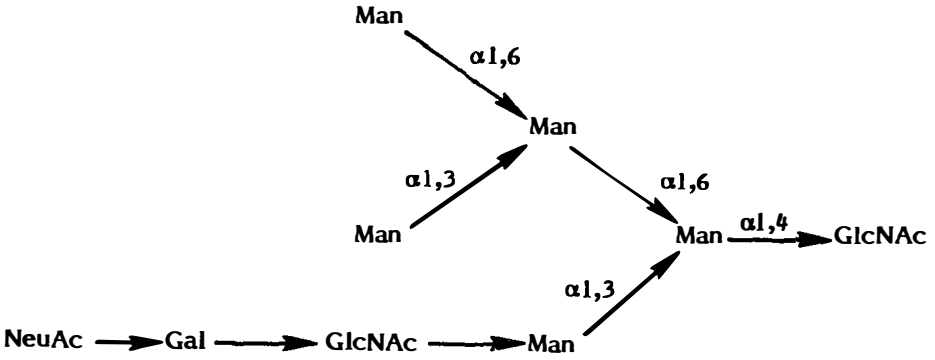


Figure 5 Principal oligosaccharide accumulating in disruption of glycoprotein processing in a skin fibroblast system by swainsonine (60).

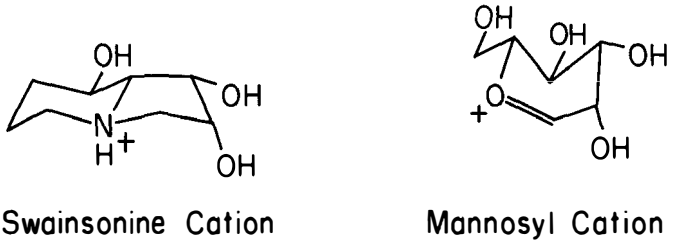


Figure 6 Comparison of swainsonine and mannose cations.

hypothesis; thus C-2, C-1, and C-8a of protonated swainsonine are equivalent to C-2, C-3, and C-5 respectively of the mannosyl cation.

Tulsiani et al (57) extended the above enzyme studies and note that such factors as extent of preincubation of enzyme with inhibitor and concentration of swainsonine have an important bearing on degree of reversibility of the inhibition in the different enzyme systems. From such studies two modes of binding of swainsonine to the liver enzymes were postulated, one being very rapid and irreversible, the other much slower and reversible. From these in vitro studies (12, 57) it is argued (12) that within the lysosome, swainsonine inhibition of α-mannosidase could account for the pathological findings in swainsonine toxicosis.

Effects of Swainsonine on the Rat and the Pig

With the availability of swainsonine from *Rhizoctonia leguminicola* fermentation (48), the alkaloid per se, rather than whole plant or plant extracts, could be administered to animals and swainsonine effects directly studied. In a study (61) where rats received swainsonine in their drinking water (5 μg/ml for 12 weeks), no signs of abnormal behavior were noted. At various times relevant enzymes were studied. The activity of Golgi mannosidase II was markedly decreased (22% of controls), but a surprising finding was that liver acid mannosidase levels increased, and this was true also for brain lysosome mannosidase. In plasma most lysosomal hydrolases increased.

In a collaborative study (56) with the Poisonous Plants Research Laboratory of the USDA in Logan, Utah, it was possible to compare directly swainsonine with locoweed in pigs, a species susceptible to locoism. Hampshire pigs (42–45 kg) kept on a suitable maintenance ration were fed 100 g of *Astragalus lentiginosus* per day, or 100 mg of swainsonine given in the drinking water daily, or served as controls. After 71 days the signs of poisoning typical of locoism were clearly observed in both experimental groups, and microscopic examination of target organs from both of these groups showed characteristic cytoplasmic foamy vacuolation. Enzyme levels in tissues of pigs fed locoweed

or swainsonine were similar to findings in the rat study (61), i.e. liver Golgi mannosidase II was decreased, but most tissue lysosome glycosidases and plasma hydrolases were increased. But an important difference between the rat and the pig, was that in the latter species there were greatly increased levels of oligosaccharides in the tissues. Two of these were isolated from the kidney and brain and shown to have the structures $Man_5GlcNAc_2$ and $Man_4GlcNAc_2$ consistent with the postulated loci of action of swainsonine in the dysfunction of glycoprotein processing (Figure 4). These oligosaccharides are identical with those previously isolated by Sadeh et al (45) from urine of sheep fed locoweed. The results of Tulsiani et al (56) support the conclusion that swainsonine is the principal toxic agent in locoweed and raise interesting questions about molecular mechanisms of disease. Thus the high-mannose oligosaccharides found in the brain of the locoweed- and swainsonine-treated pigs above (56) were not seen in swainsonine-treated rats, which did not develop locoism (61).

A Lysine Metabolite in Rhizoctonia leguminicola

During studies on the biogenesis of slaframine from [^{3}H] pipecolic acid in *Rhizoctonia leguminicola* (24), it was noted that a second somewhat less basic radioactive compound, termed RL 173, was also produced in addition to [^{3}H] slaframine. Moreover, other radioactive precursors of slaframine (e.g. [1-^{14}C] lysine and [6-^{14}C] lysine) also labeled RL 173. Initially this compound was thought to be a pyrindine derivative in nature (24), but as more material became available for study and with increased sophistication of NMR spectrometry, it was finally concluded that RL 173 was identical in structure with swainsonine (48).

Present methods for producing slaframine and swainsonine are briefly summarized. The alkaloids are extracted from *R. leguminicola* mycelium with ethanol. After removal of the ethanol in vacuo and solution of the residue in water, slaframine can be extracted from the organic aqueous layer with methylene chloride at pH 10 and purified as the dipicrate salt. Swainsonine in the water layer can be purified via cation exchange chromatography (pH gradient, $7 \rightarrow 10$). Those fractions with α-mannosidase inhibitory activity are combined, concentrated to dryness, taken up in water, and continuously extracted with butanol. On evaporation of the butanol extract, swainsonine can be obtained by sublimation of the residue. Yields of the alkaloids vary and may range from 3–5 mg/g dry mycelium (slaframine) and from 3–10 mg/g dry mycelium (swainsonine).

As inferred from the above discussion, it became apparent that slaframine and swainsonine have common biogenetic precursors in lysine and pipecolic acid. The latter is used in the formation of 1-ketooctahydroindolizine (VII, Figure 2), which is then reduced from both the α and β faces to give the

respective 1-hydroxyoctahydroindolizines, VIIIa and VIIIb, leading ultimately to slaframine and swainsonine (47). Another interesting feature in the stereochemistry of slaframine and swainsonine is that the L-configuration of the α-carbon atom of pipecolate is retained with respect to the 8a H atom throughout the entire sequence of transformations leading to slaframine, but it is epimerized at some stage in swainsonine biosynthesis (Figure 2) (47). It is now established (28) that 1,2-dihydroxyoctahydroindolizine (structure IX, Figure 2) is a swainsonine intermediate and that hydroxylation at C-8 and epimerization at C-8a are the final steps in swainsonine biogenesis in *R. leguminicola* (28).

In summary, Figure 2 presents the biosynthesis of slaframine and swainsonine deriving from lysine metabolism. Following formation of 1-ketooctahydroindolizine (VII, Figure 2), the pathway branches to accommodate specific stereochemical steps and functionalization of the indolizidine ring germane to the formation of the individual alkaloids with their uniquely different physiological actions. It will be interesting to learn if any of the features of the regulation of branched pathways of amino acid biosynthesis in bacteria (62) apply in the present instance to a branched pathway of secondary amino acid metabolites in a fungal system. Presumably swainsonine present in *Swainsona* spp. and the locoweed (which grow under harsh, dry conditions not conducive to fungal growth) is, in these instances, a product of metabolism of these higher plants.

Swainsonine Considered as a Mycotoxin

Mention was made earlier of the direct isolation of slaframine from red clover hay contaminated with *R. leguminicola* (26). Such forage had caused a serious outbreak of slobbers in horses. Swainsonine has since also been identified in this same slobber hay (7) at an estimated level of 2.5 ppm. It now becomes pertinent to question if certain of the "slobber syndrome" seen sporadically in wide areas of the United States, and attributed allegedly to slaframine, may be due in part to swainsonine ingestion as well? And if so is swainsonine found in the fluids and tissues of livestock consuming such forage? To what degree, if any, could this constitute a public health problem? Clearly such questions will demand answers and will stimulate research in these areas. For example it is important to establish if swainsonine is toxic to man. The occurrence of mannosidosis of genetic origin has been documented in children (40).

It may be significant that certain of the slobber hay syndromes listed in the documentation of Table 1 are serious and have been noted in *Swainsona* toxicosis and in locoism, i.e. weight loss, abortions, and death. Of course the forages ingested in these severe instances could have contained toxicants other than slaframine and swainsonine. It may be relevant to note a report of James & Hartley (34) that calves, lambs, and kittens given milk from cows fed

locoweed developed microscopic lesions typical of locoweed poisoning. In preliminary work (6) 1 g of swainsonine was administered intravenously to each of two dairy cattle and blood and milk samples monitored at intervals for swainsonine content. Swainsonine was rapidly cleared from the blood (6 hr), was detected very quickly in the milk (30 min), but then fell rapidly and could not be detected after 23 hr.

In his review on field and storage conditions for the production of mycotoxins in the United States, Tuite points out (55) that mycotoxin problems in the United States are ill-defined and improperly assessed because of the lack of extensive surveys and inadequate knowledge of the effects of mycotoxins on animal health. The mycotoxicoses discussed in the review included aflatoxicosis, ochratoxicosis, paspalum staggers, ergotism, fusariotoxicoses, swine refusal, hyperestrogenism, and slobbers. It is interesting to note that the section on slobbers was limited to 12 lines! One would hope that this means slobbers is a minor problem of mycotoxicoses, but the recent research developments discussed herein suggesting that swainsonine may contribute to slobbers mycotoxicosis call for renewed investigation.

CONCLUDING REMARKS

The realization that *Rhizocotonia leguminicola*–infested red clover hay contains *both* slaframine and swainsonine, and the fact that both of these alkaloids can now be produced in pure form via *R. leguminicola* fermentation (48), call for careful investigation of the effects of these alkaloids, both separately and in combination, relative to slobbers mycotoxicosis. If it can be shown for example that much of the toxicity of "slobber hay", e.g. as documented in Table 1, is due to swainsonine, it is possible that advantage can be taken of the properties of slaframine concerned with stimulation of digestive secretions (17). Adequate quantities of saliva are essential for proper digestive function in ruminants because of its contribution to the physical and chemical environment of the rumen. Hence stimulation of salivary secretion with slaframine in cattle receiving restricted roughage diets could be one approach to alleviate problems associated with reduced salivation (17).

Slaframine, in common with many xenobiotics, requires bioactivation for parasympathomimetic activity. The structural analogy of the postulated active form, XII in Figure 3, to acetylcholine is intriguing in considering structure-function relationships of this novel indolizidine derivative. Swainsonine apparently does not require bioactivation, although swainsonine *N*-oxide has been recognized as a swainsonine metabolite (38). The discovery that swainsonine (*a*) is a potent α-mannosidase inhibitor, (*b*) can be viewed as an analogue of mannose (Figure 6), and (*c*) that under carefully defined conditions is a

competitive inhibitor of certain α-mannosidases provides insights into a new class of natural antimetabolites.

In this regard, current research [reviewed briefly in (49)] is revealing a number of additional natural products that inhibit processing of *N*-linked oligosaccharides (Figure 4). Of particular interest to indolizidine chemistry is the discovery of the alkaloid castanospermine, 1,6,7,8-tetrahydroxyoctahydroindolizine (III, Figure 1), isolated from the toxic seeds of the Australian legume *Castanospermum Australe* (30). The seeds of this species, frequently eaten by livestock, can cause severe gastrointestinal irritation and sometimes death. Recent studies show that castanospermine prevents formation of complex types of oligosaccharides by inhibiting glucosidase I in influenza viral hemagglutin (42). The antibiotic nojirimycin (see Figure 7), viewed as a 5-amino analogue of glucose, also inhibits glucosyl trimming reactions (39). It is fully analogous to glucose, having equivalent chiral centers at C-2, C-3, C-4, and C-5. Lastly, a pyrrolidine alkaloid, 2,5-dihydroxymethyl-3,4-dihydroxypyrrolidine (DMDP, Figure 7), present in the closely related plants *Derris elliptica* and *Lonchocarpu sericeus*, also inhibits glycoprotein processing (15). DMDP may be viewed as an analogue of β-D-fructofuranose. The common site of action of castanospermine, nojirimycin, and DMDP as inhibitors of trimming reactions in glycoprotein processing is shown in Figure 4. It is interesting to note that the piperidine ring of nojirimycin and the pyrrolidine ring of DMDP are moieties of the indolizidine ring of swainsonine and castanospermine; and the stereochemistry of the hydroxyl substitutents in all of these *N*-heterocycles contributes importantly to their specific loci of inhibition. Inhibition of protein glycosylation has diverse biological effects, depending on the cell type and glycoprotein under investigation (49), and the aforementioned indolizidines, piperidine, and pyrrolidine derivatives constitute important new tools in glycoprotein research. *Swainsona* toxicosis and presumably locoism appear to produce a phenocopy of mannosidosis of human (40) and of Angus cattle (29). Thus swainsonine offers the possibility of inducing a lysosomal storage disease in animals and should be useful in answering questions concerning the pathogenesis of storage-mediated tissue injury in vivo.

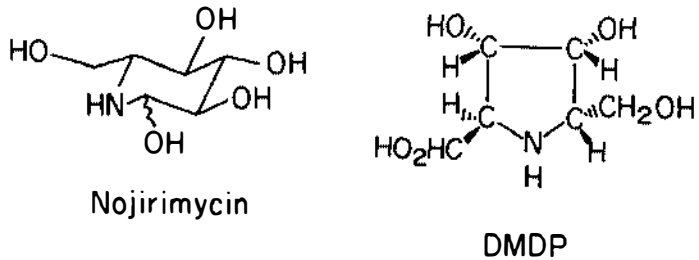


Figure 7 Nojirimycin and 2,5-dihydroxymethyl-3,4-dihydropyrrolidine (DMDP): inhibitors of glycoprotein processing.

The long trail of research described herein was based initially on observations of *Swainsona* toxicosis by Australian herdsmen, of locoism by Southwestern cattle ranchers, and of the slobbers by Midwestern dairy farmers; it ultimately led to the discovery of a unique class of physiological compounds, namely the indolizidine alkaloids swainsonine and slaframine. The addition of castanospermine (III, Figure 1) to this series further heightens interest in this class of *N*-heterocycles. This story is another illustration of the successful application of contemporary science to the solution of long-standing agricultural problems.

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