TOXIC COMPOUNDS IN CYCADS

by

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CONTENTS

PREFACE

The experimental work described in this thesis was carried out in the Department of Chemistry, University of Natal, Durban, from January 1989 to June 1990, under the supervision of Dr Roy Osborne.

These studies represent original work by the author and have not been submitted in any form to another university. Where use was made of the work of others it has been duly acknowledged in the text.
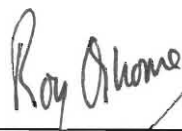
SIGNED : _____

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I hereby certify that the above statement is correct.

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ACKNOWLEDGEMENTS

ABSTRACT

The present-day cycads comprise the diverse, modified, remnants of a much larger group of gymnosperms which flourished in the Mezozoic era. The systematic position that the cycads occupy in the botanical hierarchy is significant in that they represent relatively unchanged survivors from prehistory. The present global complement of 182 species occur in tropical and mild temperate regions in both hemispheres. Despite the density of proliferation of species, about one-half of the extant taxa are considered endangered, vulnerable or rare. Apart from characteristic features such as differences in growth forms, variation in reproductive structures and anatomical details, cycads are distinguished from all other plant groups by the unique phytotoxins, azoxyglycosides, which they possess.

The toxicity of cycads is well-documented in cases which refer to both man and animals. Cycasin , which together with macrozamin represent the major azoxyglycosides occurring in cycads, has been reported to elicit responses similar to those that have been observed during carcinogenicity, mutagenicity and neurotoxicity assays. It has become apparent that the mechanism by which azoxyglycosides manifest their toxicity involves deglucosylation, by enzyme systems, which releases the aglycone, methylazoxymethanol (MAM), and sugar moieties. Metabolic activation of MAM succeeds deglucosylation to generate methylene carbene units ($:CH_2$) which are capable of methylating macromolecules including DNA, RNA and protein.

During this investigation, macrozamin was extracted from seed kernels of *Encephalartos transvenosus* and cycasin was tentatively identified in seed kernels of *Cycas thouarsii*. The hexa-acetate derivative of macrozamin was prepared whereas the tetra-acetate derivative of cycasin was not

secured in a pure form. The spectroscopic techniques employed for identification include UV-absorption, infra-red and nuclear magnetic resonance spectroscopy, all of which are useful for detecting signals which arise as a result of the azoxy function.

A kinetic study was carried out to determine the rate of hydrolysis of macrozamin with 4M sulphuric acid, and to allow calculation of the activation energy for the process. A comparison of the kinetic parameters determined for the above process with those derived for the hydrolysis of cycasin and methylazoxymethanol under similar conditions followed. The rates of hydrolysis increase in the order macrozamin, cycasin, methylazoxymethanol since the molecules contain two, one and zero glycosidic linkages respectively. Additional glycosidic bonds are observed to decrease the rate of reaction. Consequently, activation energies for hydrolysis of the above molecules decrease in the above-stated order.

Macrozamin and cycasin were quantified in cycad material by high performance liquid chromatography (HPLC), gas-liquid chromatography (GLC) and by the chromotropic acid assay. The results of the quantitative analysis has highlighted certain limitations of the methods of detection, and has been found to be applicable to a taxonomic evaluation and a proposal for the biosynthesis of the azoxyglycosides.

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CHAPTER ONE

TOXICITY OF CYCADS

1.1. Introduction

The present-day group of plants known as cycads comprise the diverse, modified, remnants of a much larger group of gymnosperms which flourished in the Mezozoic era, reaching their zenith in the Jurassic period, some 160 million years ago (Arnold, 1953). The exact origin of the cycads is uncertain, but it is believed that the Palaeozoic pteridosperms gave rise to two broad sections within the group: the cycadaleans, ancestors of the extant cycads, and the cycadeoidaleans (Bennettitales), which later became extinct. While it is popularly believed that the extant cycads are relatively unchanged survivors from prehistory, the suggestion of Eckenwalder (1980) that these plants constitute a vigorous and successful group which is still evolving and capable of responding to changing environmental conditions, is probably a better reflection of their botanical status.

In present times distribution of the extant cycads is limited to the tropical and mild temperate regions in both hemispheres. The highest densities in terms of proliferation of species, are found in Mexico (34 species in 3 genera), Queensland, Australia (14 species in 4 genera) and South Africa (33 species in 2 genera) (Stevenson and Osborne, 1990).

The taxonomy within the group is not known with certainty as many species are in the process of being described, and it is anticipated that many more will be added to the present complement of 182 species. The classifications which presently exist include that of Johnson (1959), and those of Stevenson (1981, 1985). The most recent taxonomic proposal by Stevenson (1990) is based largely on cladistic analyses, with ranking according to sub-order, family, sub-family, tribe, sub-tribe, and genus. The three families include: Cycadaceae

which is made up of 1 genus, *Cycas*; Stangeriaceae, which comprises of 2 genera, *Stangeria* and *Bowenia*; Zamiaceae, which contains 8 genera, *Lepidozamia*, *Macrozamia*, *Encephalartos*, *Dioon*, *Microcycas*, *Ceratozamia*, *Zamia* and *Chigua*. Taxonomy within the Cycadales is therefore perceived as a dynamic process which, as with many other plant groups, would require a multi-disciplinary approach to resolve the existing complexities.

Amongst the growth forms of these dioecious cone-bearing plants, are the tall, often branched, arborescent species such as the Australian *Lepidozamia hopei* (stems up to 18m) and the South African *Encephalartos transvenosus* (up to 13m). Subterranean forms are also encountered as in the case of *Encephalartos villosus* and *Zamia pumila* subsp. *pygmaea*. Diversity within the group is further outlined by the unusual cycad *Zamia pseudoparasitica* , which lives as an epiphyte in forest canopies in Costa Rica and Panama. Apart from the differences in growth forms, there is a wide variation in reproductive structures and anatomical details which is beyond the scope of this text.

Cycads have attracted increasing interest not only from a botanical but also from a chemical perspective. This approach has been adopted primarily because of their well-documented toxic potential. Toxicity of the cycads has been attributed to a unique group of phytotoxins, which is the focus of this text.

1.2. Cycad azoxyglycosides

1.2.1. History of cycad toxicity

Plant natural products are divided into primary and secondary compounds (Kingsbury, 1979). Primary compounds include all those that are required for a plant's basic metabolism and secondary compounds are generally all others. Secondary compounds have a host of functions, one of these being that of a defence mechanism.

Phytotoxins, which are secondary compounds, differ with respect to their biological and chemical properties (Hardin and Arena, 1974). Although vertebrates have evolved an array of mechanical and biochemical defences against these toxins, few systems of the vertebrate body are entirely immune to damage by some toxic compound from some plant source. Toxicity, by and large, involves an interrelationship between dosage, absorption, detoxification, and excretion (Kingsbury, 1979). The azoxyglycosides (Figure 1) are the major toxins ubiquitous in and unique to the cycads.

From of old, the cycads have been reputed to contain a toxic ingredient (Whiting, 1963). The botanist, Banks, who accompanied Captain Cook on his voyage to Australia in the 1770's, reported that members of the crew became violently ill and two of the ship's hogs died after eating the nuts of *Cycas media* (Hooker, 1896 as cited by Whiting, 1963). Fitzgerald (1898) observed, during his visit to East Africa, that the starch from local cycad plants caused vomiting and fatal diarrhoea when consumed. Standley (1937) reported that the root of *Zamia furfuracea* was used in Honduras as a poison for criminals and in Costa Rica for both criminals and enemies. The sap from the kernels of *Cycas circinalis* was used for the ritual killing of children in Indonesia (Heyne, 1950 as cited by Whiting, 1963).
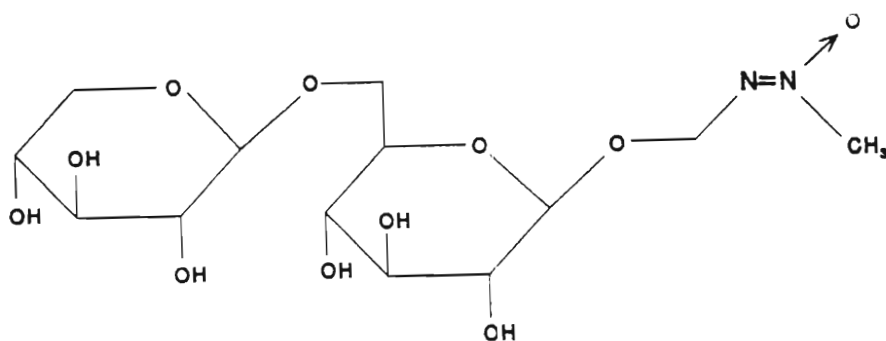
[1] macrozamin

= (methyl-ONN-azoxy) methyl

or methylazoxymethanol β-primeveroside
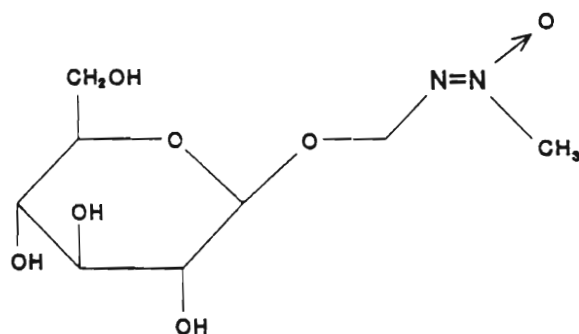
[2] cycasin

= (methyl-ONN-azoxy) methyl

or methylazoxymethanol β-D-glucopyranoside

[2a] neocycasin A = methylazoxymethanol β-laminaribioside

[2b] neocycasin B = methylazoxymethanol β-gentiobioside

[2c] neocycasin C = methylazoxymethanol β-laminaritetraoside

[2d] neocycasin D = methylazoxymethanol β-laminaritrioside

[2e] neocycasin E = methylazoxymethanol β-cellobioside

[2f] neocycasin F = cycasin 6-0-β-laminaribioside

[2g] neocycasin G = cycasin 3-0-β-gentiobioside

NOTE : In [1] and [2] above, the trivial names are followed by a full chemically-descriptive name defined by IUPAC rules, and an abridged alternative. The names β-primeverosyloxyazoxymethane for [1] and β-D-glucosyloxyazoxymethane for [2] are also encountered in the literature.

Figure 1: Cycad azoxyglycosides

The account of Reitz (1929) is probably the first account of the toxicity of cycads occurring in South Africa. It is reported in the account that several members of the Boer commando, including it's leader, General J.C. Smuts, ate cycad seeds from plants growing in the Zuurberg mountains when food supplies dwindled during the South African War (1899-1902) and that the troops were incapacitated for several days. Dyer (1965) is of the opinion that the offending species was *Encephalartos longifolius*. In 1912 two Mpondo lads died at Tabankulu after eating the kernels of *Encephalartos frederici-guilielmi* (Juritz, 1914 as cited by Dyer, 1965). The toxicity of various South African *Encephalartos* species has subsequently been reported. Steyn *et al.* (1948) reported that the outer fleshy layer and/or the megagametophyte of *Encephalartos cycadifolius*, *E.eugene-maraisii*, *E.ferox*, *E.horridus*, *E.lehmannii*, *E.longifolius* and *E.villosus* were either acutely toxic when administered to rabbits or were suspected or known to be toxic when ingested by man. Tustin (1974) found that rats fed with various parts of the female cones of *E.umbeluziensis*, *E.villosus*, *E.lebomboensis* and *E.laevifolius* developed tumors. Similar effects were observed in rats that were fed kernels of *E.lanatus* (Tustin, 1983).

Cycads have been reported to be toxic to livestock (Whiting, 1963). Interest in this area was generated because of the serious economic implications cycad poisoning presented to cattlemen and graziers in tropical and subtropical regions (Whiting, 1963). Grazing by stock on leaves of certain *Macrozamia* species in Australia and *Zamia* in the Americas has resulted in the partial or total paralysis of hind limbs. The animals subsequently perish because of the inability to obtain food and water. Numerous experiments have been conducted on both farm and laboratory animals to ascertain the toxicity of cycads.

1.2.2. Use of cycads as a source of food

Despite the knowledge of the existence of a toxic ingredient
in cycads, they have provided both a staple and an emergency
food supply (Whiting, 1963). Natives of many parts of the
world have used cycads mainly as a source of food starch
(Thieret, 1958). The "arrowroot" starch, generally obtained
from the rhizomes of *Marantha*, can also be extracted from
Zamia roots while "sago" starch, usually derived from the
stem of certain palms, is also harvested from *Cycas*, *Zamia*
and *Macrozamia* plants (Thieret, 1958; Whiting, 1963). Other
vegetative parts of the cycad plant are also reported to be
used for human consumption. The leaves of several *Cycas*
species are used as a vegetable in the Phillipines and
Indonesia while the apparently edible root nodules of *Cycas
revoluta* have been described as a "potato-like" substance
(Thieret, 1958).

1.2.3. Coventional detoxification methods

It is remarkable to note that the natives, who use cycads as
a source of food, appear to have a knowledge of the plants'
toxicity and have therefore, independently, devised
detoxification methods. The records of early travellers in
South Africa (1772-1779) refer to the preparation of bread
from the stems of *Encephalartos* species by the Hottentots. In
the preparation, the pith from the stem was first buried for
up to six weeks (Dyer, 1965).

However, the main focus of cycad toxicity has been on the
large and often abundantly-available seeds. Lamb (1895)
reported that although the children of Australian settlers
experimented with *Macrozamia* nuts, no native ate any until
these had been buried for at least a month. Detoxification
may be aided by fermentation (Nishida, 1936). A common method

of seed detoxification involves the soaking of the sliced seeds in water that is changed regularly, or by steeping the seeds in running water. Roasting of the seeds has also been reported and normally follows soaking. The procedures of soaking in water and roasting are central to the detoxification process.

The azoxyglycosides are readily soluble in water because of the polar hydroxyl groups of the sugar moiety. The toxins therefore diffuse and are washed out because of their solubility in water. Roasting serves both as a means of denaturing any enzymes, which might catalyse the release of methylazoxymethanol (MAM), the toxic component of the azoxyglycosides, from the sugar moiety, and further degrading the azoxyglycosides which decompose when heated. The reliability of the conventional detoxification methods to produce toxin-free material has been tested experimentally. Kobayashi (1972) reported that no cycasin was detected in several samples of home-made "sotetsu miso" (cycad bean paste from *Cycas revoluta*) from the island of Amami-Ohshima. Thus the suggestion of Whiting (1963), that toxicity arising from the use of cycad products is due primarily to insufficient detoxification of the material or ignorance of the toxicity of the plants, is convincing.

1.2.4. <u>Toxic effects of cycads</u>

The adverse effects of cycads when consumed by both humans and animals spurred investigation into the isolation of the toxic principle (section 2.1.). The isolation of the azoxyglycosides, particularly cycasin, from cycad plants further stimulated toxicological investigations. Adverse effects of cycasin to people were suspected when it was observed that the Chamorros of Guam, who consumed cycad products, exhibited a high incidence of amyotrophic lateral sclerosis (ALS) (Laqueur, 1977). Although a clear link

between ingestion of cycasin and ALS has not been established, the possibility of such an association spurred investigation into the biological effects of cycasin (Morgan and Hoffmann, 1983).

In addition to neurotoxic effects, evidence has accumulated on the existence of other toxicologic effects of cycads and cycad products, including carcinogenic, teratogenic, and mutagenic responses. The adverse effects of cycasin have been reported in other living systems. Cycasin inhibited the growth of various micro-organisms; productivities of some fermentation products; and spore formation of molds (Tadera et al., 1987). The toxin also interferred with seed germination and seedling growth of Gramineae, Crucifereae and Fabaceae, and suppressed the formation of α-amylase in rice endosperms (Tadera et al., 1982).

1.2.5. Mechanism of toxicity

Removal of the sugar moiety from cycasin yields the aglycone, MAM, which is responsible for the toxicologic properties of cycasin (Laqueur, 1977). The biological effects of the other azoxyglycosides from cycads are also attributable to the liberation of MAM by the cleavage of the sugar moieties from the parent compounds. Cycasin therefore serves as a model compound for all cycad azoxyglycosides because MAM is the active component (Morgan and Hoffmann, 1983). The more stable acetate derivative of MAM (section 4.8.), which was chemically synthesized by Matsumoto et al. (1965) and Laqueur (1977), rather than MAM derived from cycads, has been used in many studies.

Evidence for the role of MAM as the toxic component of azoxyglycosides was provided by Kobayashi and Matsumoto (1965) who found that cycasin was non-toxic to rats when

injected intraperitoneally but toxic, after a latent period
of twelve hours, when administered orally. They assumed that
cycasin was cleaved by β-glucosidase from the intestinal
microbial flora to release MAM. Consequently, in the same
investigation, Kobayashi and Matsumoto prepared MAM by
enzymatic hydrolysis of cycasin with almond emulsin and
demonstrated that MAM was toxic when injected
intraperitoneally. More recently it has been shown by Ames
(1982) that "fecalase", an enzyme preparation from human
faeces, is also capable of hydrolysing cycasin *in vitro*.

Subsequent to the investigation of Kobayashi and Matsumoto
(1965), evidence was provided by Laqueur *et al.* (1967) to
show that MAM-acetate was toxic to both germ-free rats as
well as normal rats when administered orally or
intraperitoneally. Cycasin, however, proved to be non-toxic
to germ-free rats when administered by either methods
(Laqueur, 1964). Investigation into the activity of the
enzymes of intestinal microbial flora revealed that cycasin
was toxic to germ-free rats that were colonized with
Streptococcus fecalis, which produces β-glucosidase, but non-
toxic to germ-free rats that were colonized with
Lactobaccilus salivarious, which lacks β-glucosidase (Spatz
et al., 1967b).

It also became apparent from further studies that young and
mature animals differ with respect to the enzymatic
deglucosylation of cycasin. Unlike mature animals, neonatal
animals deglucosylate cycasin independently of bacterial
enzymes (Laqueur, 1977). The skin of fetal and neonatal rats,
from 15 days prenatal to 30 days after birth, was reported to
contain β-glucosidase (Spatz, 1968). Although the
deglucosylation of cycasin by bacterial β-glucosidase is
accepted as the principle mechanism by which MAM is released,
it must be noted that the glycosidic bond can also, probably
to a lesser extent, be hydrolytically cleaved by intestinal
digestive juices. In fact, both acid and base can promote the

hydrolysis of the glycosidic bond (sections 3.2. and 3.5.).

Thus, the deglucosylation of cycasin is a prerequisite for the manifestation of its toxicity while MAM, irrespective of the route of administration, is a toxic compound. The relationship between cycasin and MAM was clarified by studies on the carcinogenicity of cycasin, which revealed that cycasin is the toxic component and that MAM is the proximate carcinogen of cycasin (Laqueur, 1977; Laqueur and Spatz, 1975).

After it had been established that the MAM function of cycasin was responsible for its toxicologic effects, investigations were carried out to determine the mechanism by which MAM manifests its toxicity. These investigations were undertaken in lieu of the carcinogenic and mutagenic properties that MAM exhibited.

Miller (1964) proposed that the carcinogenicity of MAM was due to one of its decomposition products, the methylene carbene unit ($:CH_2$), which is a highly reactive methylating species, capable of reacting with cellular macromolecules. Alkylation of macromolecules which include DNA, RNA and protein has been accepted as one of the causes of cancer (Matsumoto and Higa, 1966). Cycasin and MAM have been shown to methylate both DNA and RNA *in vitro* (Matsumoto and Higa, 1966), and *in vivo* (Shank and Magee, 1967; Nagata and Matsumoto, 1969). In both experiments an additional purine base was found and identified as 7-methylguanine.

In addition to these changes in the macromolecules, it was also observed that exposure of rats or mice to MAM-acetate caused decreases in DNA and RNA synthesis (Zedeck *et al.*, 1970), and protein synthesis (Zedeck *et al.*, 1970; Lundeen *et al.*, 1971), particularly in the livers of treated animals.

Miller (1964) based his proposal, that MAM was carcinogenic through its decomposition into methylene diradicals, on both the chemical and biological similarity between cycasin and dimethylnitrosamine (DMN) which was reported by Magee and Barnes (1956, 1962) to have induced hepatic and renal tumors in rats. The proposal of Miller (1964), of a common metabolic pathway for DMN and cycasin, resulting in the formation of methylene diradicals, is depicted in Figure 2.

The synthetic carcinogen dimethylnitrosamine (DMN) is similar to cycasin in its carcinogenic and mutagenic activity. Metabolic activation of DMN and cycasin is an important reaction before these compounds manifest their carcinogenic and mutagenic properties. DMN is demethylated as shown below to the monomethyl derivative in the liver microsomes. Although the monomethyl derivative has not been isolated *in vivo*, it has been made synthetically.

According to recent knowledge, DMN is metabolically activated in animals through enzymatic hydroxylation of the alpha carbon atom leading to demethylation which is catalysed by demethylases (Preussmann and Stewart, 1984). DMN has also been activated *in vitro* to a mutagenic product by a non-enzymatic system called "Undenfriend", a mixture that contains ascorbic acid, EDTA, ferrous ions and molecular oxygen (Malling, 1966; Mayer, 1971). Synthetic monomethylnitrosamine (MMN) is only stable at -70°C; when heated it loses water to form diazomethane. The internal environment of the mammalian body is thus an ideal medium for the dehydration of MMN into diazomethane. The loss of nitrogen from diazomethane then follows to yield reactive methylene diradicals which are capable of methylating DNA, RNA and protein. The pathway of incorporation of methyl groups into macromolecules has been supported by studies which used ^{14}C-labelled DMN (Miller, 1964).

CH₃—N–N=O
CH₃
Dimethylnitrosamine (DMN)

CH₃—N=N–CH₂–O–glucosyl, O
Cycasin

↓ demethylation

↓ deglucosylation by β–glucosidase

CH₃—N–N=O, H
Monomethylnitrosamine (MMN)

CH₃—N=N–CH₂–OH, O
Methylazoxymethanol (MAM)

↓ dehydration –H₂O

↓ removal of formaldehyde –HCHO

CH_2N_2
diazomethane ← –H₂O

CH₃—N=N–H, O
MAM–intermediate

↓ denitrification –N₂

:CH₂
methylene diradical

↓ R–H (R=DNA, RNA, protein)

R–CH₃

Figure 2: Common metabolic pathway for cycasin and DMN
(after Miller, 1964)

Cycasin on the other hand, is metabolically activated through deglucosylation which yields MAM which decomposes into an intermediate form through the loss of formaldehyde as shown above. The loss of water from the intermediate then follows to yield diazomethane and then methylene diradicals through the loss of nitrogen. It was speculated that formaldehyde released during the decomposition of MAM could possibly also contribute to the biological effects of MAM (Miller, 1964).

The deglucosylation of cycasin to yield MAM is required but not necessarily sufficient to explain the mechanism of action of MAM (Morgan and Hoffmann, 1983). Further metabolic modification of MAM may also be toxicologically important; in fact, it has been suggested that MAM may be converted into methylazoxyformaldehyde (MAMAL) which may also react with cellular macromolecules (Grab and Zedeck, 1977; Feinberg and Zedeck, 1980). Thus, the biological activity of MAM can proceed either via MAMAL or through its more direct generation of methylene diradicals as shown in Figure 3.

The alternative pathway for the metabolism of MAM as shown below, involves the oxidation of MAM to the reactive MAMAL by NAD^+-dependent alcohol dehydrogenase. MAMAL may either react directly with macromolecules or be converted back into the MAM intermediate which is generated by the loss of formaldehyde from MAM. Conversion of MAMAL into the MAM intermediate may proceed by cleavage of the aldehyde function either by hydrolysing it into formic acid, or through conjugation with macromolecules.

CYCASIN

| deglucosylation

CH_3
$N=N-CH_2OH$
O

MAM

alcohol dehydrogenase

$NAD^+ \longrightarrow NADH$

CH_3
$N=N-H$
O

MAM-intermediate

¥

CH_3
$N=N-C$
O
O
H

MAMAL

activated to methylene
diradicals (:CH$_2$)

$+ NH_2-R$

CH_3
$N=N-C$
O
NR
H
$+ H_2O$
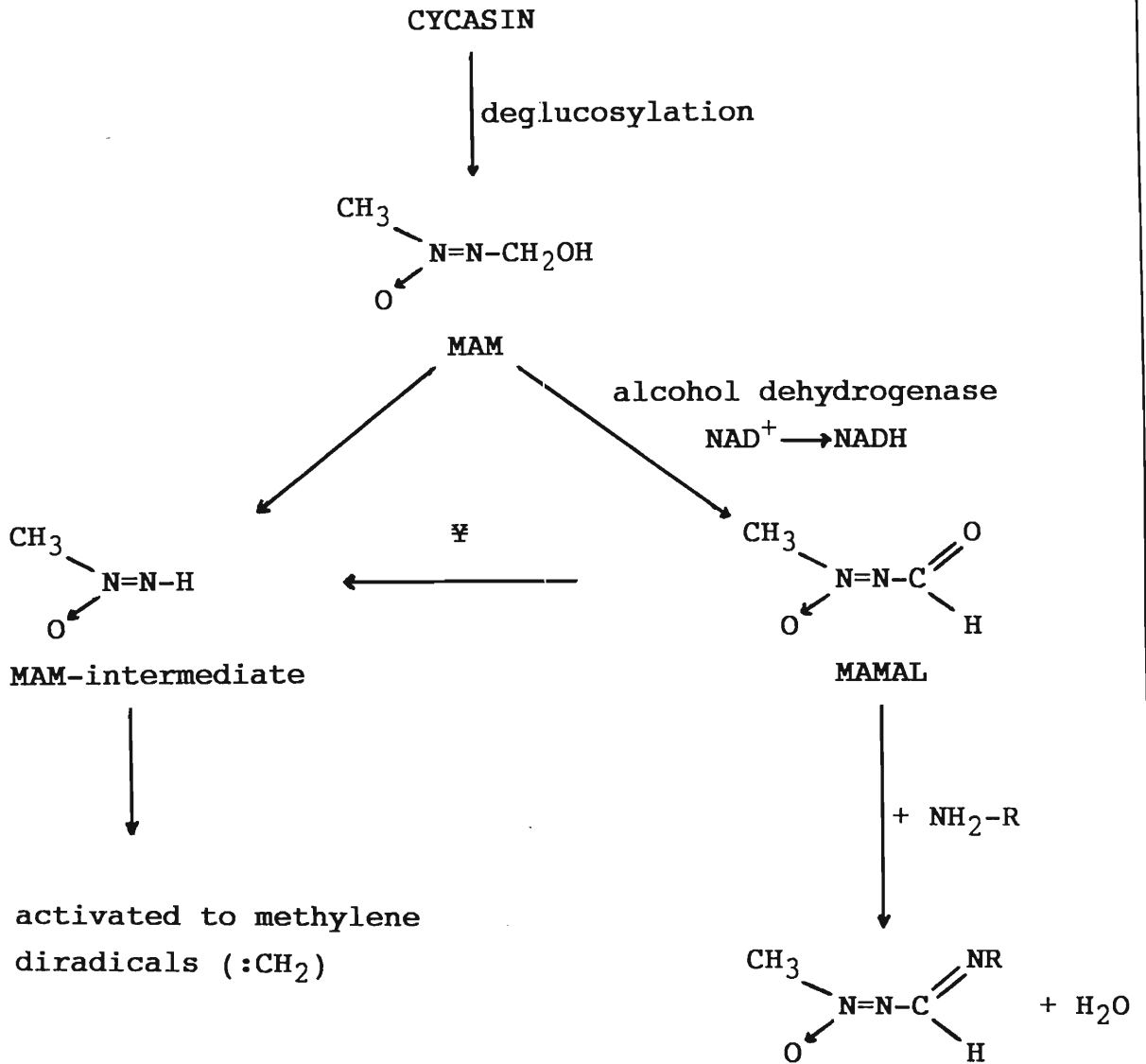
Note: 1. R represents DNA, RNA or protein
2. The cleavage of the aldehyde function from MAMAL in
the reaction denoted by ¥, proceeds in the following
manner:

a. $\sim\!\!C$ $\overset{O}{\underset{H}{}}$ $\xrightarrow{H_2O}$ $HO-C$ $\overset{O}{\underset{H}{}}$

b. $\sim\!\!C$ $\overset{O}{\underset{H}{}}$ $\xrightarrow{NH_2-R}$ $\overset{H}{\underset{R}{N}}-C$ $\overset{O}{\underset{H}{}}$
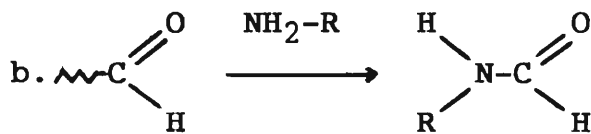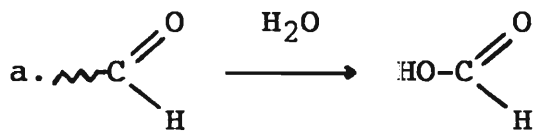
Figure 3: Alternative pathway for MAM metabolism (after
Miller, 1964 and Zedeck *et al.*, 1979)

While the proposal of Miller (1964) affords an explanation for the metabolic activation of azoxyglycosides to yield diazomethane, which is known to be carcinogenic and is used to methylate acidic hydroxy groups, it is questionable in respect of the chemical reactivity of diazomethane. No direct evidence has been presented by Miller to suggest that diazomethane would react in a similar manner in the biological situation as it is known to react chemically. As depicted below, diazomethane (CH_2N_2) is known to decompose to methylene ($:CH_2$) and nitrogen in the presence of UV-light (photolysis) or by heat (thermolysis) (Graham Solomons, 1980).

$$:CH_2 \overset{-}{}\!\!\overset{+}{—N=N:} \quad \xrightarrow[\text{thermolysis}]{\text{photolysis or}} \quad :CH_2 \;+\; :N=N:$$
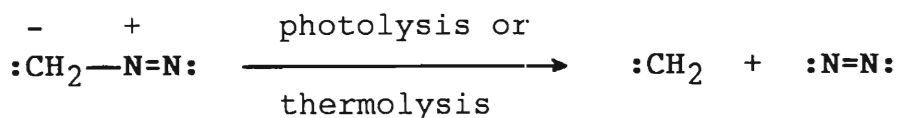
Methylene belongs to the class of compounds referred to as carbenes which are reactive species, all having lifetimes considerably under one second (March, 1977). The reactions of carbenes are especially interesting because, in many instances, the reactions show a remarkable degree of stereospecificity (Kirmse, 1971). The two non-bonded electrons of a carbene may be either paired or unpaired. If they are paired, the species is spectrally a singlet, while two unpaired electrons appear as a triplet. Evidence from experiments in which the addition of carbenes to double bonds was examined has revealed that methylene itself is usually formed as a singlet species which can decay to the triplet state (March, 1977). However, it is possible to prepare triplet $:CH_2$, which resembles a diradical, directly, by a photosensitized decomposition of diazomethane. Methylene ($:CH_2$) is so reactive that it generally reacts as the singlet before it has a chance to decay to the triplet state.

Thus, the carcinogenic and mutagenic properties of MAM are manifested by the metabolically-activated methylene carbene units binding, through covalent interactions, to macromolecules. The carcinogenic potency of an initiating carcinogen can be estimated by the determination of its covalent binding to DNA. Liener (1986) has defined a "covalent binding index" (CBI) which expresses the ratio of a DNA-damage (measured as radioactivity attached to DNA) per applied labelled substance administered to the respective animal. No CBI value is available for cycasin. However, it would be expected to compare with the value of 15 calculated by Liener (1986) for gyromitrin, a potent liver carcinogen, since this compound, like cycasin, is also activated to the same electrophilic methylating species as shown in Figure 4.

Figure 4: Metabolic activation of gyromitrin
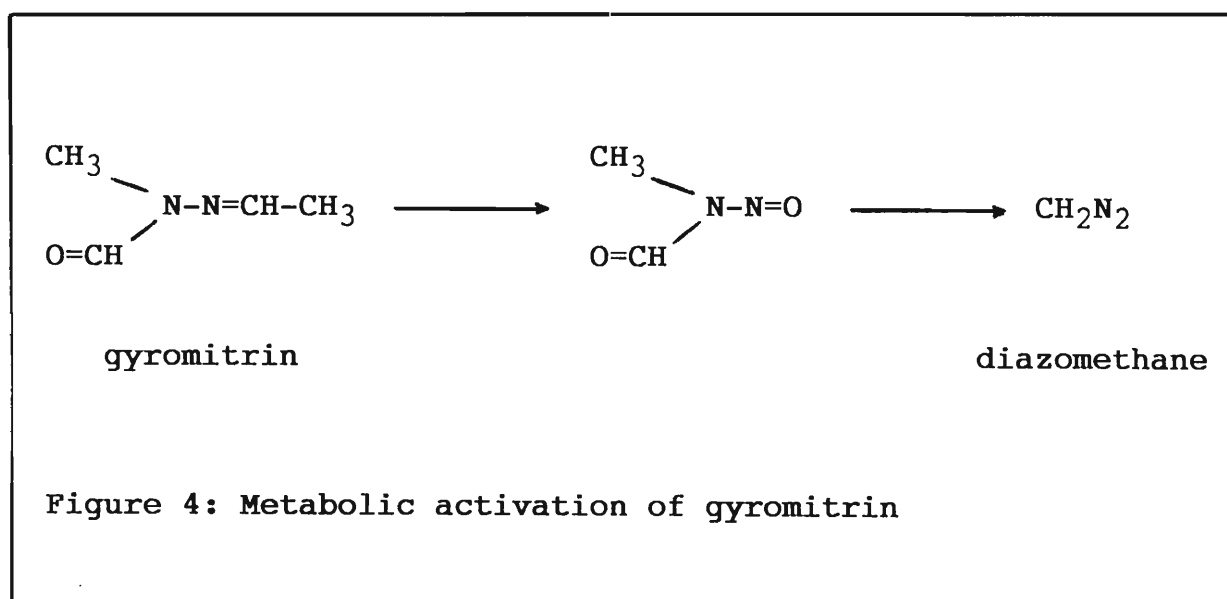
1.2.6. Carcinogenicity

Evidence that crude cycad material was carcinogenic was obtained early in 1962 when rats on a cycad meal diet died because of abdominal tumor masses (Laqueur *et al.*, 1963). Investigation into the carcinogenicity of cycasin followed and it became apparent that whereas cycasin was carcinogenic only after passage through the gastrointestinal tract, its

aglycone MAM induced tumors independent of the route of administration (Laqueur and Matsumoto, 1966; Laqueur et al., 1967).

Sites of predilection for tumor development depended on the duration of feeding. Hepatomas required prolonged administration, whereas renal tumors developed after short periods of feeding, while intestinal neoplasms, which were almost exclusively located in the colon, were least dependent on the duration of exposure (Laqueur, 1964). With respect to dose, it was observed that a single administration of cycasin was sufficient to induce single or multiple tumors in majority of the test animals (Hirono et al., 1968). Investigation into the susceptibility of laboratory animals to cycasin-induced carcinogenicity indicated that in addition to inducing tumors in rats, cycasin also induced tumors in mice (O'Gara, 1964), guinea pigs (Spatz, 1964) and fish (Stanton, 1966).

1.2.7. **Neurotoxicity**

Evidence that cycads were neurotoxic was initially provided by Whiting (1963) who observed a paralytic condition in cattle grazing on land where cycads grew. In addition to this observation Whiting (1964) also reported on the high incidence of amyotrophic lateral sclerosis (ALS) among the Chamorros of Guam who consumed cycad products. Some of the neurological effects observed during experimentation include, demyelination of the spinal column in cattle after ingestion of cycads (Hall et al., 1968), and production of hind-leg paralysis in newborn mice following a single subcutaneous injection of 0.5mg cycasin/g of body weight (Hirono and Shibuya, 1967).

In addition to the above neurological effects, cycasin and

MAM have produced arrest either of normal development or of growth, as well as exaggerated growth responses in the brain and spinal cord of rats (Spatz *et al.*, 1967a). The teratogenicity of MAM was reported by Spatz *et al.* (1967a), who observed malformations of the brain, eyes and extremities in surviving fetuses of hamsters. The most pronounced teratogenic effects of MAM are a consequence of neurotoxicity during brain development (Laqueur, 1977).

1.2.8. Mutagenicity

As mentioned in section 1.2.5., the mutagenicity of cycasin and MAM is manifested through the metabolically-generated methylene carbene unit which is capable of methylating macromolecules. MAM has been reported mutagenic in a variety of genetic toxicological tests. Most evidence indicate that MAM induces base-pair substitution mutations (Smith, 1966; McCann *et al.*, 1975; Jacobs, 1977; Matsushima *et al.*, 1979; Rozenkranz and Poirier, 1979). In addition to point mutations, MAM has been shown to induce mitotic recombinations, sister chromatid exchanges and chromosome aberrations in several test systems (Morgan and Hoffmann, 1983).

1.2.9. Detoxification of cycad azoxyglycosides

Excretion of unmetabolized cycasin seems to be the primary means by which mammals minimize its effects (Morgan and Hoffmann, 1983). Hirono *et al.* (1968) demonstrated that the acute toxic phase of cycasin could be prevented in rats by the use of radioprotective agents. The phenomenon was referred to as the "Radiomimetic Effect" by Teas *et al.* (1965) who demonstrated that exposure of onion seedlings, which lack β-glucosidase activity, to cycasin resulted in as many chromosomal aberrations as could be produced with 200R

of gamma rays. In their investigation, Hirono *et al.* (1968) reported that the test animals could be protected from a lethal dose of cycasin provided that cysteamine or 3-amino-1,2,4-triazole was administered shortly before cycasin. However, all rats which survived beyond six months had tumors. Available evidence suggests that the protective agents decreased the effective dose of cycasin probably by altering the rate of conversion of cycasin to MAM.

Little information is available on detoxification of MAM in mammals, either through conjugation or through the possible oxidation of MAMAL in a reaction involving aldehyde dehydrogenase (Morgan and Hoffmann, 1983). Cycasin is toxic through its conversion into MAM. It was speculated from this knowledge that the reverse phenomenon of conversion of MAM back to cycasin could also be a possible way of detoxifying MAM. In fact, Teas (1967) observed that when larvae of the arctiid moth *Seirarctia echo* were fed MAM in an artificial medium, cycasin was detected in the hemolymph which lacks β-glucosidase activity. Thus, the conversion of MAM to cycasin appears to protect these moths from the toxic effects of the cycad metabolites.

1.3. **Other cycad toxins**

1.3.1. **α-amino-β-methylaminopropionic acid**

The discovery of α-amino-β-methylaminopropionic acid (β-N-methylamino-L-alanine or BMAA, Figure 5, structure 3) bears with it a parallel investigation into the neurological effects of the compound. The neurological disorders in question are amyotrophic lateral sclerosis (ALS), Parkinson's dementia complex (PDC) and Alzheimer's disease, which were observed to occur at extraordinarily high rates among the indigenous Chamorro people of Guam and on other Mariana

islands in the Western Pacific (Kurland, 1988). ALS was prevalent on Guam since the 1800's but the incidence of the disease increased drastically during World War II. Genetic factors as well as viral agents were implicated as possible causes but neither explanation has been borne out (Lewin, 1987). Instead, the belief that environmental agents were linked to the cause of the disease gained impetus (Kurland, 1988).

Cycads were implicated as the causative agent because the seeds of the indigenous *Cycas circinalis* plant formed a staple component of the diet of the islanders whose dependence on the plants increased as a result of food shortages when the Japanese occupied the island during World War II. The azoxyglycoside compounds, cycasin in particular, were rejected as a cause since they repeatedly failed to induce an experimental disorder similar to ALS/PDC.

In their investigation leading to the isolation of BMAA, Vega and Bell (1967) noted that the symptoms of a neurological disorder in cattle, observed by Whiting (1963), involving hindlimb paralysis, resembled those associated with lathyrism. Lathyrism, a neurological disorder observed in man and higher animals, is caused by consumption of seeds of the chickling pea which contains the neurotoxin α-amino-β-oxalylaminopropionic acid (BOAA) (Figure 5, structure 4). Vega and Bell (1967) were of the opinion that BOAA or $\alpha\gamma$-diaminobutyric acid (Figure 5, structure 5), another known plant neurotoxin, or some similar compound was responsible for the neurological effects of cycads. Substantive evidence was provided by the above authors who isolated BMAA from the seeds of *Cycas circinalis*. In a later investigation Vega and Bell (1968) synthesized BMAA and found that the L-isomer was the naturally-occurring compound which was responsible for the biological activity. BMAA has since been found to be widespread in free or bound form in seeds and leaves of *Cycas* but not in any other cycad genera (Dossagi and Bell, 1973).

NH$_2$
|
H$_3$C-HN-H$_2$C——C——COOH
|
H

(3)

O O   NH$_2$
‖ ‖ |
H-C-C-NH-CH$_2$——C——COOH
|
H

(4)

NH$_2$
|
NH$_2$-CH$_2$-CH$_2$——C——COOH
|
H

(5)
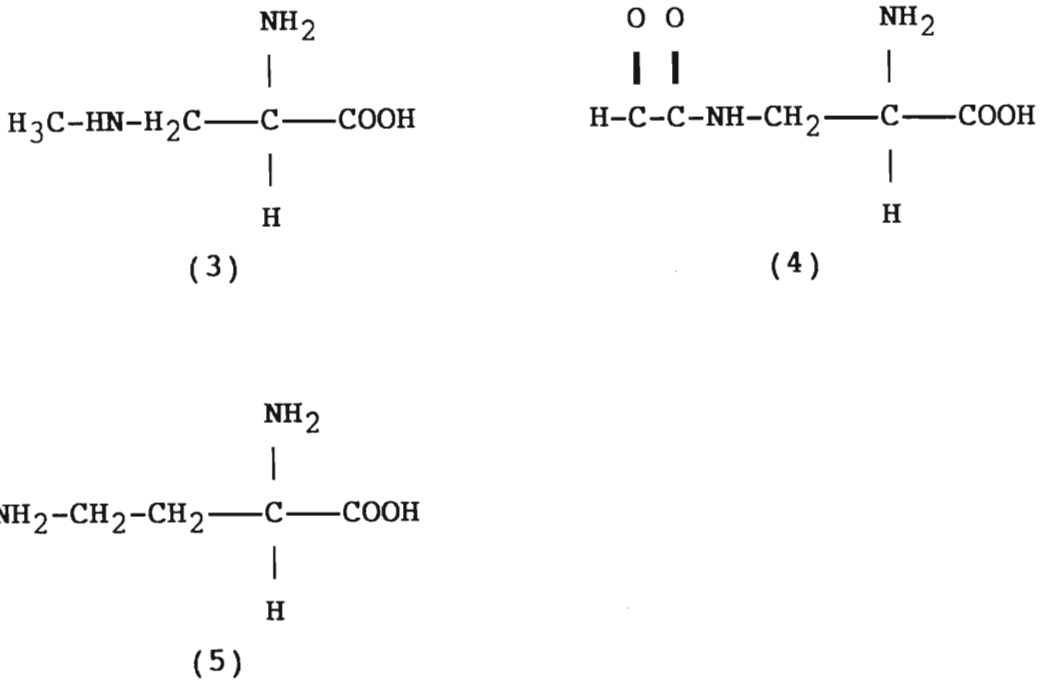
NOTE: (3)= α-amino-β-methylaminopropionic acid

(4)= α-amino-β-oxalylaminopropionic acid

(5)= αγ-diaminobutyric acid

Figure 5: Non-protein amino acid phytotoxins

The isolation of the amino acid therefore served as a tool by which the "Cycad Hypothesis", that cycads were responsible for neurological disease on Guam, could be tested. Dastur (1964) had observed neurological defects in a single rhesus monkey that was fed cycasin-free flour while Vega and Bell (1967) observed that their newly-isolated amino acid was neurotoxic to chicks. However, the hypothesis lost recognition for some time after the 1972 cycad conference at which it was stated that rats given BMAA over a period of 78

days failed to develop any observable neurological changes. However, Spencer and Kurland never disbelieved the hypothesis. Spencer's belief that the amino acid was implicated in the neurological disorders was highlighted in his investigation during which he observed symptoms characteristic of the Guam neurological complex in male *Cynomolgus* monkeys that received varying doses of synthetic BMAA. Kurland (1988) was inspired by Spencer's results to rekindle the initial notion that ALS and PDC on Guam was linked to an environmental neurotoxin.

Spencer *et al.*(1987) speculated that the three diseases which made up the neurological complex on Guam may be elicited by different doses of the cycad toxin. A high level of intoxication leads to ALS while Parkinson's and Alzheimer's disease develop after lower exposures. The damage caused by the diseases were to specific regions of the central nervous system and remained subclinical for several decades but made those affected especially prone to the consequences of age-related neuronal attrition (Calne *et al.*, 1986). This characteristic fitted well with the observation that the diseases were diagnosed twenty years after World War II in Guamanians who had by then either become accultured, due to western influence, or moved to the United States (Lewin, 1987).

The Cycad Hypothesis has not been without controversy. Recently Duncan *et al.* (1988) used a highly specific and sensitive gas-chromatographic/mass spectrometric method to demonstrate that foods prepared from processed cycad flour did not provide neurotoxic levels of BMAA. Low calcium and magnesium levels in water and high aluminium concentrations in soil have also been hypothesized as possible mechanisms for the neurological complex in the Japanese Kii Peninsula, New Guinea and Guam (Gajdusek, 1982). It has also been proposed that motor-system diseases may be related to the toxic potential of endogenous excitatory amino acids

(Plaitakis *et al.*, 1982). Thus, the elucidation of the Guam neurological complex will require a concerted effort from both proponents and opponents of the hypothesis. However, the environmental hypothesis though controversial, is plausible.

1.3.2. Cyclitols

The occurrence of polyhydroxycyclohexanes (cyclitols) is common in the gymnosperms. The compounds myoinositol, pinitol and sequoyitol have been reported to occur in the cycads (Figure 6). Sequoyitol in particular was isolated as an impurity during the extraction of macrozamin from the seeds of *Macrozamia riedlei* (Lythgoe and Riggs, 1949). Sequoyitol, a methyl ether of myoinositol, has also been implicated in neurological disease since it is a derivative of the inositide compounds which are lipid-constituents of the brain. It is speculated that sequoyitol may interfere with the metabolism of these lipids (Matsumoto and Strong, 1963). While short-period feeding at low concentration showed that sequoyitol is not acutely toxic (Matsumoto and Strong, 1963), the effects of long-period and higher concentration feeding experiments must be ascertained before sequoyitol can be eliminated as a factor in the possible relationship of cycad ingestion and the development of neurological disease.
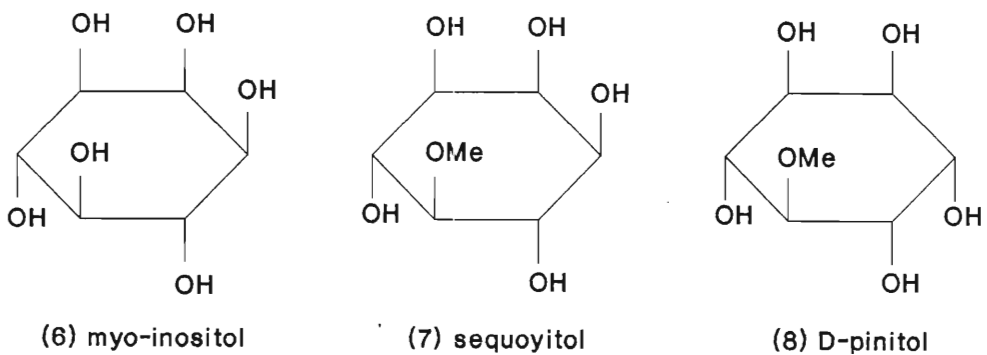
Figure 6: Cyclitols isolated from cycads

1.4. Other phytochemical aspects

1.4.1. Introduction

While the major biochemical interest in cycads has been focussed on the phytotoxins, a number of other phytochemical investigations have been undertaken. Many of these invetigations were used as parameters which were applied to cycad taxonomy. The older work with respect to cycad phytochemistry is summarized in the review of Thieret (1958), which includes a discussion of the somewhat limited uses of cycad material as sources of fibres, gums and oils.

1.4.2. Phenolic acids and biflavonoids

Wallace (1972) found evidence of caffeic, protocatechuic, p-coumaric, p-hydroxybenzoic, ferulic and vanillic acids in all 22 cycad species that were examined. In addition to the above phenolic acids, sinapic acid was identified in *Dioon spinulosum* and *Ceratozamia mexicana* while 2,4-dihydroxybenzoic acid was found in two species of *Encephalartos* and in *Bowenia serrulata*, and syringic acid was tentatively identified in *Ceratozamia mexicana*. The presence of sinapic and syringic acids in cycads indicates that the distribution of these compounds is wider than thought previously (Wallace, 1972). Some of the phenolic compounds which have been been isolated from cycads are shown in Figure 7.

A survey of biflavones in the leaves of 82 cycad species has revealed that the pattern of occurrence of amentoflavone, hinokiflavone, their methyl ethers and other derivatives is consistent within most genera (Dossagi, Mabry and Bell, 1975).
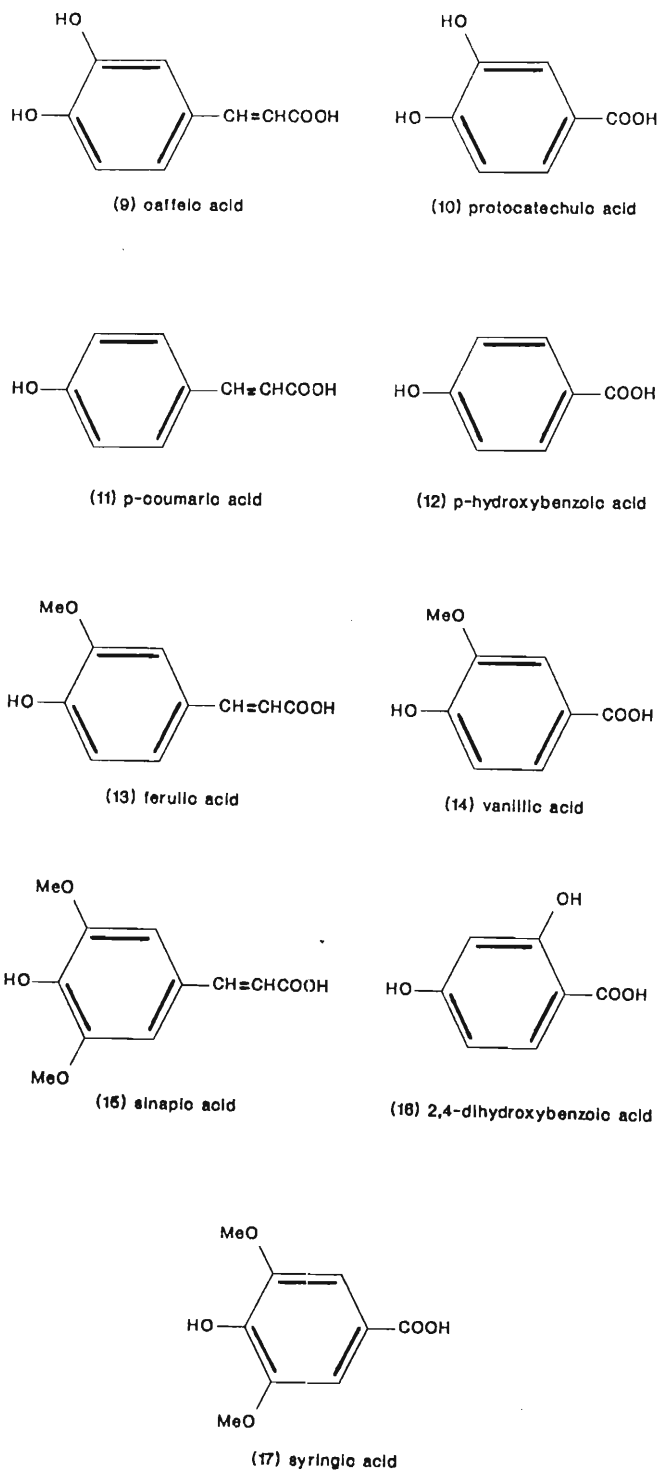
Figure 7: Phenolic compounds isolated from cycads

In an analysis of 8 species , Gadek (1982) found that two representatives from *Macrozamia* *viz*. *M.macdonellii* and *M.communis* are characterized by the occurrence of cupressuflavone, which is unusual outside the gymnosperm families Cupressaceae, Araucariaceae and Podocarpaceae, and amentoflavone derivatives while *Cycas* species contained only the amentoflavone-based compounds. *Encephalartos*, *Lepidozamia* and *Zamia* samples gave only trace amounts of biflavonoids. An unusual feature is the complete absence of biflavonoids in *Stangeria* (Dossagi, Mabry and Bell, 1975). The report of Gadek *et al.* (1984) suggests that biflavonoids in cycad leaves may act as a deterrent to leaf-eating insects and microbial invasion. Biflavonoids isolated from cycads are depicted in Figure 8.

1.4.3. Carotenoids

The highly-coloured seed coats of cycads contain simple carotenoid mixtures (Figure 9). The bright yellow coat of *Cycas revoluta* has zeaxanthin as the major component with smaller amounts of cryptoxanthin and β-carotene (Bouchez *et al.*, 1970). The seed coat of *Zamia* has lycopene as the principal pigment while extracts from *Encephalartos*, *Dioon* and *Macrozamia* contained a mixture of unsubstituted mono- and dihydroxy-β-carotenes (Bauman and Yokoyama, 1976). Semi-β-carotene which was previously found only in the fruits of the citrus relative, *Murraja exotica*, was identified in the leaflets of *Ceratozamia* (Cardini *et al.*, 1987).

1.4.4. Carbohydrates and enzymes

All cycads have a well-developed system of mucilage ducts and excision of the leaf rachis or cone peduncle, or injury to the caudex allow collection of the exudate. This mucilage consists of a complex polysaccharide which may be hydrolysed to its component sugars.
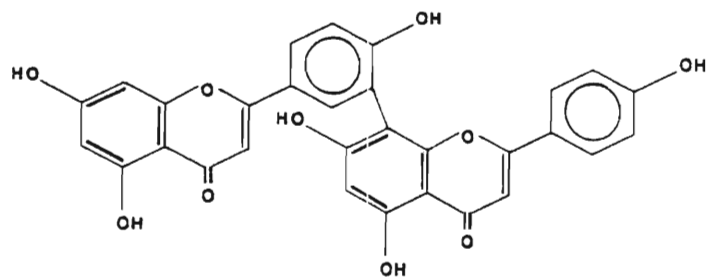
(18) amentoflavone
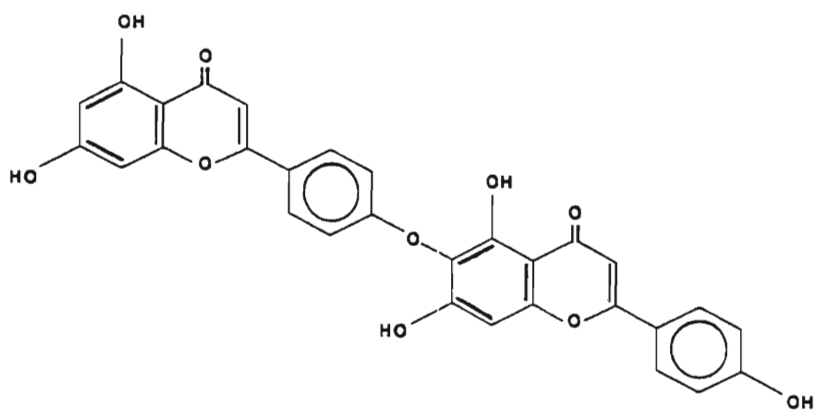
(19) hinokiflavone
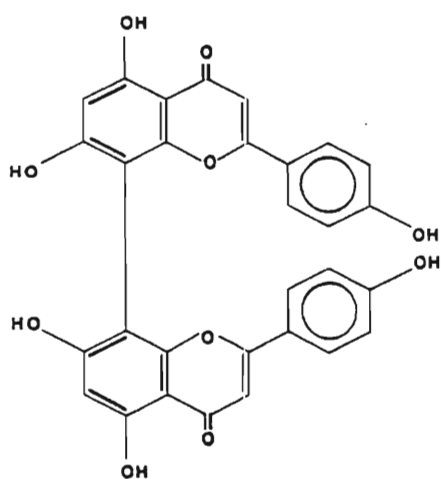
(20) cupressuflavone

Figure 8: Some biflavonoids isolated from cycads

(21) zeaxanthin

(22) cryptoxanthin

(23) β-carotene

(24) lycopene

(25) semi-β-carotenone
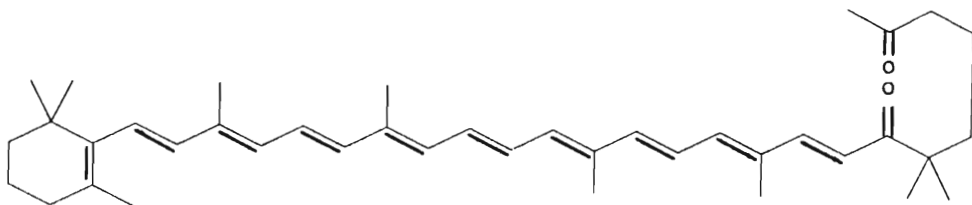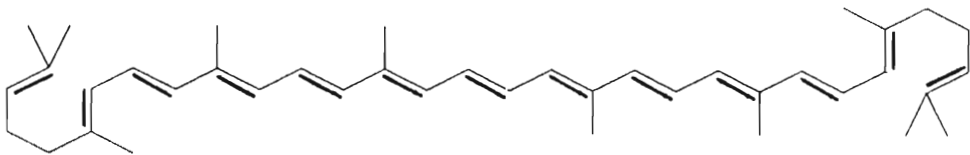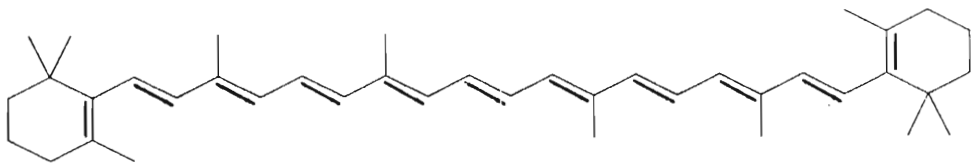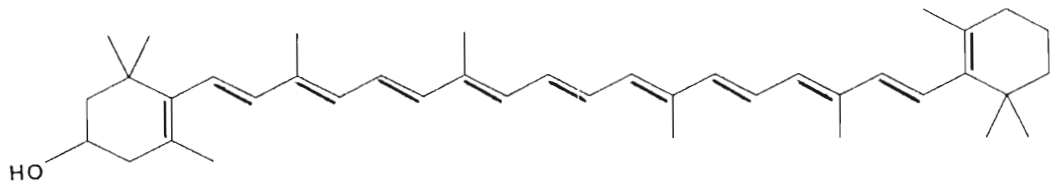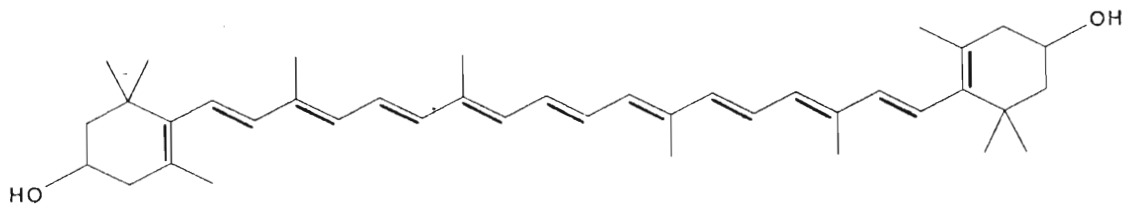
Figure 9: Some carotenoids isolated from cycads

The exudate from a female cone of *Encephalartos longifolius* yielded fucose, rhamnose and 3-0-methylrhamnose, arabinose, xylose, galactose, mannose, and glucuronic acid with its 4-0-methyl ether (Figure 10) (Stephen and de Bruyn, 1967).

De Luca *et al.* (1982) hydrolysed the mucilages from excised leaf raches of 21 cycad species and found that the results were useful taxonomically at the generic level. Arabinose and galactose are the major carbohydrates in the African and Australasian genera while the American genera have higher proportions of fucose and galactose in *Dioon*; rhamnose, fucose and methylrhamnose in *Zamia*; and galactose and methylrhamnose in *Microcycas*, *Lepidozamia* and *Encephalartos* are the only genera which show similar patterns.

Moretti *et al.* (1981b) observed a more-or-less identical monosaccharide pattern in 14 *Encephalartos* species. The structure of the polysaccharide present in the exudates from cones of three species of *Encephalartos*, was elucidated by sequential degredation (Stephens and Stephen, 1988). 3-0-methyl-L-rhamnose occurs in varying amounts which, together with the parent L-rhamnopyranose unit, can comprise more than 20% of the total carbohydrate composition. Another structural feature of the polysaccharide is the high proportion of acidic units, notably D-glucuronic acid and its related 4-methyl ether.

Enzymological techniques have been used to a limited extent in cycad research. Merriam (1974) studied the *Zamia* population in Florida using isoenzyme banding systems and found the peroxidases to provide useful "fingerprints". A study of the peroxidase isoenzyme in the seeds of *Cycas circinalis* showed a difference in the enzyme pattern at different stages during seed maturation (Pena *et al.*, 1983)

(26) β-D-fucose
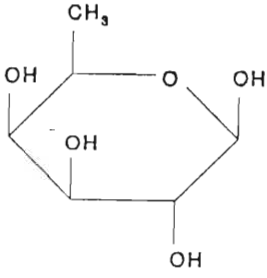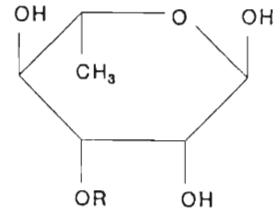
(27) α-L-rhamnose (R=H)

3-O-methyl rhamnose (R=Me)

(28) α-L-arablnose

(29) α-D-xylose
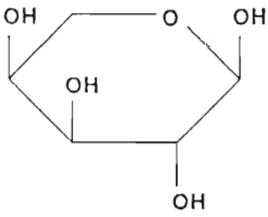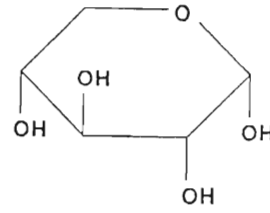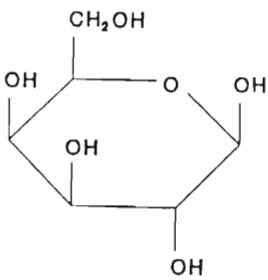
(30) β-D-galactose
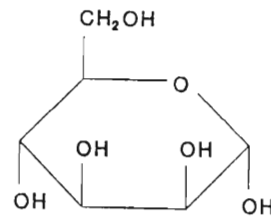
(31) α-D-mannose
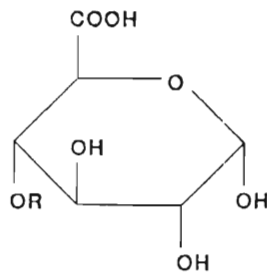
(32) α-D-glucuronlc acid (R=H)

4-O-methyl glucuronic acld (R=Me)

Figure 10: Monosaccharides obtained on hydrolysis of cycad mucilages

1.4.5. Leaf wax hydrocarbons

The large number of reports on the chemical composition of plant epicuticular leaf waxes, particularly with respect to the distribution of n-alkanes, testifies to the usefulness of such data to the chemotaxonomist (Osborne *et al.*, 1989). The above authors used the technique of Gas Chromatography to detect n-alkanes in the epicuticular leaf wax of 42 taxa of *Encephalartos*. The alkanes which all occur in the range from $n\text{-}C_{17}$ to $n\text{-}C_{35}$, were characterized into three chemical groups.

The first group, typified by *E.ferox* and *E.villosus*, has well-defined maxima for the odd carbon-atom-numbered-alkanes $n\text{-}C_{29}$, $n\text{-}C_{31}$ and $n\text{-}C_{33}$, which is the similar pattern in higher plants. The second group shows a skewed-normal n-alkane distribution centred unimodally at *ca* $n\text{-}C_{20}$ and is typified by *E.ghellinckii* and *E.ngoyanus*. This pattern is unusual in higher plants and the validity of the few cases where it has been documented has been questioned (Herbin and Robins, 1969). A bimodal n-alkane distribution with one maximum at or near $n\text{-}C_{20}$ and the other varying between $n\text{-}C_{25}$ and $n\text{-}C_{31}$, typified by *E.altensteinii* and *E.woodii*, characterizes the third and largest group, the pattern of which is uncommon in higher plants.

Cycad leaf wax hydrocarbons provide useful data for taxonomic evaluation since each specimen provides a unique hydrocarbon profile. It is anticipated that the use of such data in conjunction with numerical taxonomy computer programmes could contribute much to the resolution of existing taxonomic problems.

1.4.6. Other compounds

Mention of other compounds isolated from cycads is scattered sparsely in the literature. Takagi and Itabashi (1982) isolated some unusual fatty acids from the seeds of *Cycas revoluta* while Pettit (1982) demonstrated the presence of certain proteins in the pollen tube of *Cycas armstrongii*. The novel storage globulin which was named macrozin, was isolated from the seeds of *Macrozamia communis* by Blagrove *et al.* (1984). The bibliography of Read and Solt (1986) and the review by Hegnauer (1986) are useful reference sources.

CHAPTER TWO

EXTRACTION OF MACROZAMIN AND CYCASIN

2.1. Introduction

Reports of the toxicity of cycads date as far back as the 1700's (section 1.2.1.). Many reports on the toxicity of the plants spurred investigation into isolation of the toxic principle. However, many of such investigations did not meet with much success. The isolation of macrozamin, therefore, from the seeds of the Australian cycad *Macrozamia spiralis*, by Cooper (1940) who subsequently demonstrated that the compound was capable of producing acute neurotoxic symptoms in sheep, was significant in this regard. Macrozamin has since been isolated from *Macrozamia riedlei* (Lythgoe and Riggs, 1949), *Cycas media*, *Bowenia serrulata* and *Macrozamia miquelii* (Riggs, 1954), *Encephalartos hildebrandtii* (Dossagi and Herbin, 1972) and *Encephalartos lanatus* (Altenkirk, 1974), and has been found to be ubiquitous in all cycad genera that have been examined (section 4.7.).

Cycasin, which together with macrozamin represent the major azoxyglycosides in cycads, was first isolated from the Japanese cycad, *Cycas revoluta*, by Nishida *et al.* (1955). The compound has also been isolated from seeds of *Cycas circinalis* (Riggs, 1956), and, like macrozamin, has been found to be characteristic of and exclusive to all genera of cycads (section 4.7.). The neocycasins, which form the group of minor azoxyglycosides, have also been isolated from the Japanese cycad (Nagahama, 1964; Yagi *et al.*, 1985a,b).

The investigation carried out during the course of this work was aimed at extracting and purifying cycasin and macrozamin from a toxin-rich source. Macrozamin was extracted from seeds of *Encephalartos transvenosus*, the "Modjadji palm", the tallest of the South African cycads, reaching heights of up to 13m. Cycasin, on the other hand, was tentatively identified in seeds of the African Cycas, *Cycas thouarsii*. The techniques employed during extraction and identification

of the compounds include column chromatography, ultraviolet absorption spectroscopy, infra-red (IR) spectroscopy and proton nuclear magnetic resonance ([1]Hnmr) spectroscopy.

2.2. <u>Experimental</u>

2.2.1. <u>Materials and method</u>

Thin layer chromatography (TLC) on silica gel plates (Merck Art. 5554) was carried out in a solvent system of butanol, acetone and water (4:5:1). Spots were detected by spraying TLC plates with anisaldehyde spray reagent [H_2SO_4-anisaldehyde-methanol (2:1:100)], and then heating in an oven for 10 minutes.

A carbon column was used for the separation of cycasin and macrozamin from sugars. The column (3cm x 40cm) was prepared according to the method of Whistler and Durso (1950) using equal quantities of charcoal and celite. A cellulose column was used to purify macrozamin and cycasin. The column (3cm x 40cm) was packed dry according to the method of Hough (1949).

Melting points were determined on a Kofler hot stage. UV-absorption spectra were run on a VARIAN DMS 300 spectrophotometer. Infra-red spectra were obtained from a PYE-UNICAM SP3-300 spectrophotometer. Proton nuclear magnetic resonance spectra were recorded using the proton probe of a VARIAN CFT-20 spectrometer at 200MHz.

2.2.2. Isolation of macrozamin

The fractionation scheme designed to separate the water-soluble constituents of *E. transvenosus* and *C. thouarsii*, which is an adaptation of the methods of Riggs (1954) and Dossagi and Herbin (1972), is outlined in Figure 11.

A mass of 1.4kg of megagametophytic material from the seed kernels of *E. transvenosus* was macerated with 1.5 litres of 80% ethanol in a blender and extracted 3 times by stirring for three hours with 1.5 litre portions of 80% ethanol at room temperature. The residue (fraction A) was spread out and dried in a stream of air from a fan to remove ethanol. The combined liquid fraction was then concentrated to a small volume (30ml) under vacuum in a rotary evaporator. To the residual syrup, 500ml of 95% ethanol was added and the mixture allowed to stand overnight at room temperature when starch and protein precipitated (fraction B).

After filtration , the ethanol was evaporated under vacuum. A further 500ml of 95% ethanol was added and the mixture was again allowed to stand overnight when a gummy residue (fraction C) settled on the bottom and sides of the container. The supernatant liquid was decanted and ethanol removed under reduced pressure in a rotary evaporator. Water was added to the syrupy mass which was again concentrated. The residue was dissolved in water and the solution made up to 30ml (fraction D). The solution was then loaded onto the carbon column, which had first been washed with water, and eluted with water.

CYCAD NUTS (Extracted with 80% ethanol)

A———————————————|

CONCENTRATED TO SMALL VOLUME
95% ethanol added

B———————————————|

CONCENTRATED TO SMALL VOLUME (precipitation of
starch and protein with 95% EtOH)

C———————————————|

ETHANOL REMOVED, RESIDUE DISSOLVED IN WATER (D)

CARBON COLUMN

ETHANOLIC ELUATE (F)   AQUEOUS ELUATE (E)

TREATED WITH ACETONE————————————RESIDUE (G)

SUPERNATANT LIQUID

CELLULOSE COLUMN
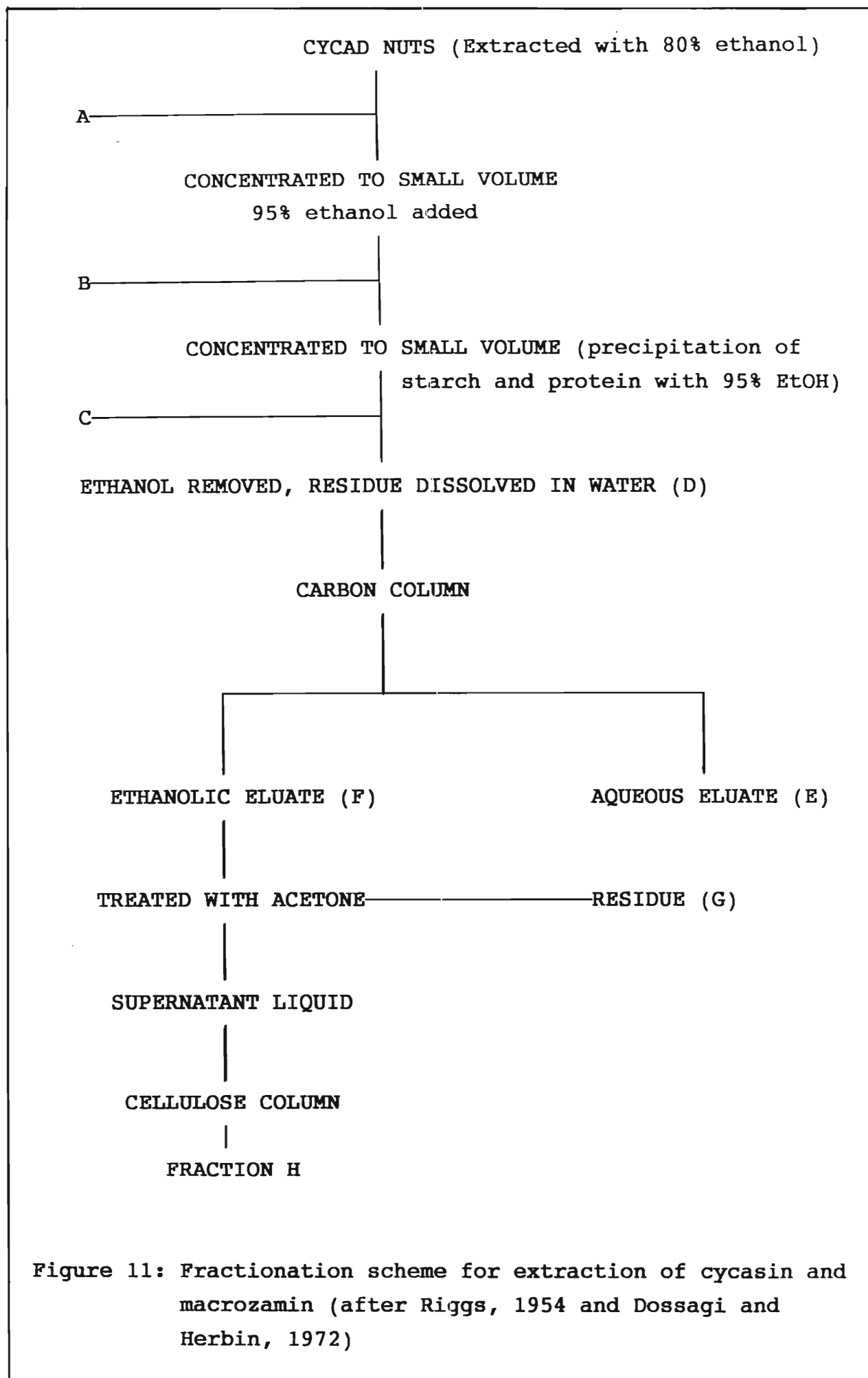
FRACTION H

Figure 11: Fractionation scheme for extraction of cycasin and
macrozamin (after Riggs, 1954 and Dossagi and
Herbin, 1972)

TLC monitoring of this fraction indicated the presence of glucose, fructose and sucrose. The column was then eluted with 200ml each of 10%, 20%, 30%, 40% and 50% ethanol. The ethanol eluates, which showed traces of sugar and a spot (R_f=0.51), were combined and ethanol and water removed by a rotary evaporator to a volume of 15ml. Acetone (100ml) was added to the aqueous solution and the supernatant liquid discarded. The small quantity of residue (fraction G) was extracted 3 times with 15ml portions of acetone-water (4:1).

The combined acetone fraction was then placed on the cellulose column and 30ml fractions were eluted with 80% acetone. Those fractions which showed a single spot at R_f=0.51 were combined and acetone removed by a rotary evaporator. The residue (fraction H) was dissolved in 50% ethanol and allowed to stand in a refrigerator when macrozamin precipitated out as fine white crystals.

2.2.3. Isolation of cycasin

The procedure for the extraction of cycasin from *C.thouarsii* is essentially the same as that outlined for macrozamin with a few modifications. The fresh megagametophytic material from seed kernels (1.5kg) was first macerated in absolute alcohol to denature enzymes and the paste was then dried at 80°C in a draft of air.

The dried paste was then powdered in mortar and extracted twice by stirring at room temperature with 1 litre portions of hexane to remove lipids. Lipids were further removed by twice extracting with 1 litre portions of diethyl ether. The resulting powder was dried in warm conditions to remove traces of ether.

The dried powder was then subjected to the treatment outlined

in Figure 11. Cycasin was not obtained in a pure form despite repeated elution on the charcoal and cellulose columns. Solvent removal from fraction H under reduced pressure provided an off-white, hygroscopic, powder.

2.2.4. Acetylation of macrozamin and cycasin

A quantity of 100mg each of macrozamin and crude cycasin was acetylated by dissolving in 1ml pyridine followed by the addition of 2ml acetic anhydride. The mixture was shaken in a stoppered flask and left standing for 3 days to ensure completion of the reaction which was monitored by TLC. Cold water was then added to the solution to deactivate the residual anhydride and extraction with ethyl acetate followed. The acetate is transferred to the ethyl acetate layer and the solvent was removed under reduced pressure. Methanol was added repeatedly to the mixture obtained from the ethyl acetate extraction, and removed under reduced pressure so that traces of acetic anhydride and pyridine were also drawn off. Hot methanol was then added to the syrup which precipitated the acetate after scratching. Macrozamin acetate is obtained as a white crystalline powder whereas the cycasin acetate was not secured in crystalline form.

2.3. Results and discussion

The melting point of macrozamin was determined as 198-202°C (decomposes) and that for the acetate derivative was 144-146°C. Values of 199-200°C and 144-145°C have been reported for macrozamin and its hexa-acetyl derivative respectively (Lythgoe and Riggs, 1949; Riggs, 1954; Altenkirk, 1974). The crude cycasin fraction had a melting point of 149-150°C and that of its tetra-acetyl derivative was not determined since the compound was not secured in a crystalline state. The melting points of cycasin and its acetyl derivative have been

reported as 154° and 137°C respectively (Riggs, 1956).

The UV-absorption spectrum of macrozamin is a single, broad
band characterized by a maximum at 215nm (logЄ = 3.92) and an
inflexion at 275nm (logЄ = 1.72) (Langley et al., 1951). The
maximum is due to the presence of the azoxy function of the
aglycone, which introduces a chromophoric system in the
molecule, which is responsible for the absorption in the UV-
region of the spectrum. This property of the molecule is
further enhanced by comparison of its UV-absorption spectrum
with those of other synthetic azoxy compounds (Table 1).

Table 1: UV-absorption of selected aliphatic azoxy compounds

COMPOUND	WAVELENGTH (nm)	logЄ
1. ethyl-2-azoxyisobutyrate	223	3.74
2. 2-azoxy-2,5-dimethylhexane	223	3.75
3. 1-azoxy-2,5-dimethylcyclohexane	225	3.88
4. azoxycyclohexane	223.5	3.86
5. macrozamin	215	3.82

Note: a. logЄ is the log extinction coefficient calculated
         from Beer's Law relationship (section 3.3.1).
      b. The following structures are provided:
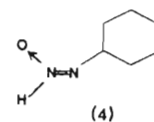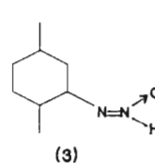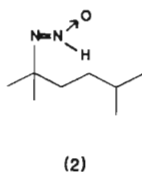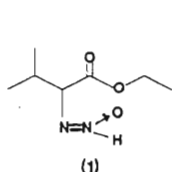
The maxima of the compounds in Table 1 are 8-10nm removed from that of macrozamin, which is well within the range of variation found in compounds with the same unsaturated group but different substituents.

The UV-absorption spectrum obtained for macrozamin (Figure 12) shows a maximum at 215nm (logϵ= 3.82), and an inflexion at 270nm (logϵ= 1.64). The 215nm maximum, which is also characteristic of cycasin, is shifted slightly to 218nm (Figure 13).
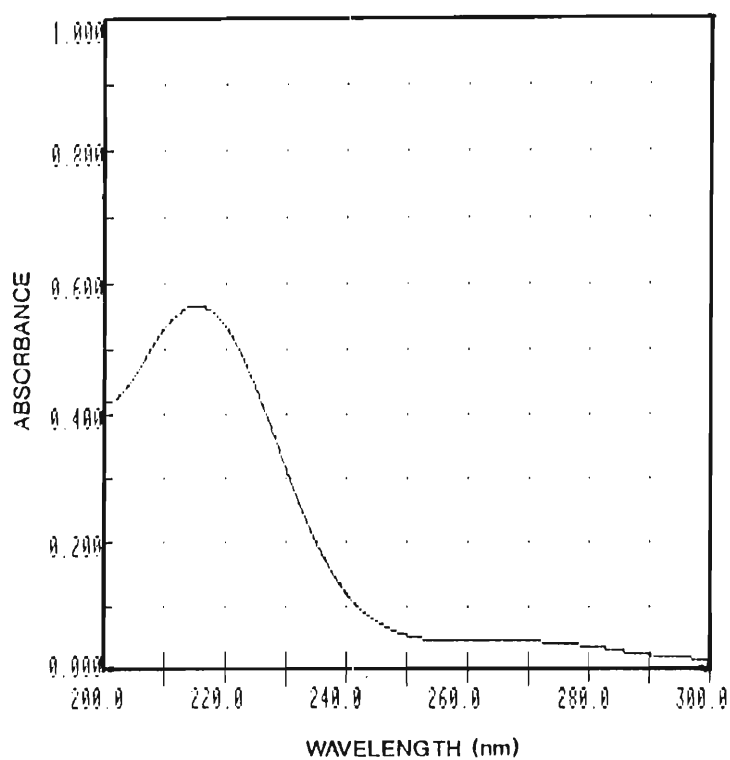
Figure 12: UV-absorption spectrum of macrozamin
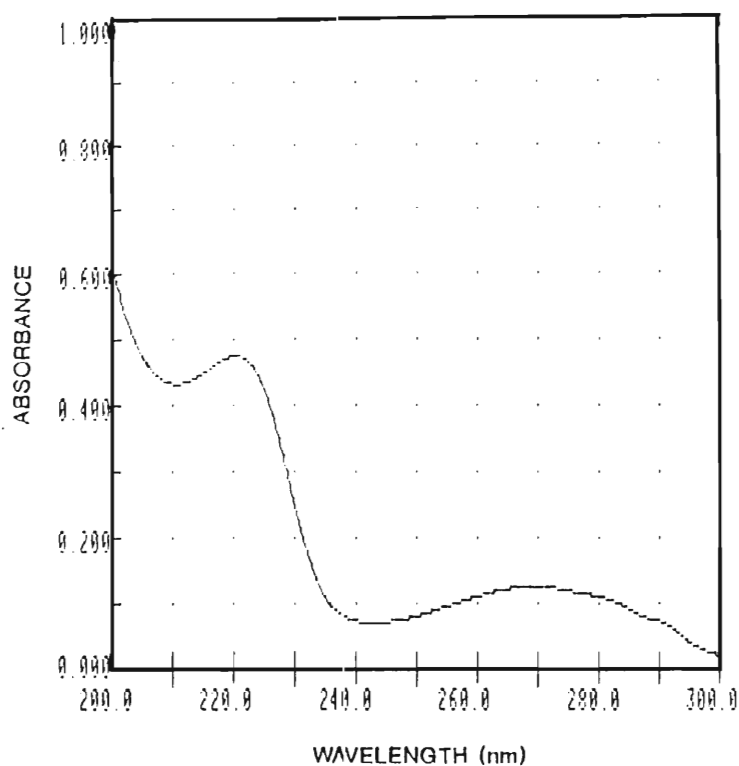
Figure 13: UV-absorption spectrum of cycasin

Langley *et al.*(1951) have reported that the IR-spectrum of macrozamin shows no bands between 1600 and 1800cm^{-1}, which indicates that double bonds involving carbon are absent from the molecule. The band at 1760cm^{-1} in the spectrum of the hexa-acetate is due to the ester carbonyl groups. The characteristic feature of the spectrum of macrozamin and its acetate derivative is the absorption due to the azoxy function. Langley *et al.*(1952) have investigated the infra-red spectra of a number of compounds containing the azoxy group and have shown that the symmetric stretch of this group occurs between 1285-1345cm^{-1} and the asymmetric stretch ranges from 1495 to 1531cm^{-1}. Greene and Hecht(1969) report that typical trans-azoxy compounds show strong infra-red absorption at both 1500cm^{-1} and 1300cm^{-1}.

The spectra of both macrozamin and cycasin show symmetric and asymmetric absorption peaks at 1338 and 1540cm^{-1} respectively (Langley *et al.*, 1952; Kobayashi and Matsumoto, 1954). In addition, the spectrum of macrozamin showed peaks at 1320 and 1440cm^{-1}. Cycasin showed absorptions at 1367 and 1430cm^{-1}. Table 2 is a summary of characteristic absorptions obtained for macrozamin and its hexa-acetate (Figures 14 and 15), and the crude cycasin extract together with its acetate derivative (Figures 16 and 17).

The spectrum of macrozamin also shows a band between 2500 and 4000cm^{-1} for hydroxy functions of the sugar moiety, and the band at 1750cm^{-1} in the spectrum of the hexa-acetate is characteristic of the ester carbonyls. Peaks at 2900cm^{-1} for both macrozamin and its hexa-acetate are due to methyl and methylene groups. These absorptions together with those summarized in Table 2 fit the description of macrozamin and its hexa-acetate respectively as reported by Langley *et al.*, 1951; Kobayashi and Matsumoto, 1954 and Dossagi and Herbin, 1972.

Table 2: Infra-red absorption of macrozamin and crude cycasin

COMPOUND	SYM	ASYM	OTHER	
macrozamin	1329	1529	1371	1425
macrozamin hexa-acetate	1340	1530	1371	1430
cycasin	1380	1515	1380	1480
cycasin tetra-acetate	1370	–	1370	1430

Note: (1) Absorption maxima in table 2 are expressed in units of cm^{-1}.

(2) SYM and ASYM are abbreviations for symmetric and asymmetric stretching respectively.
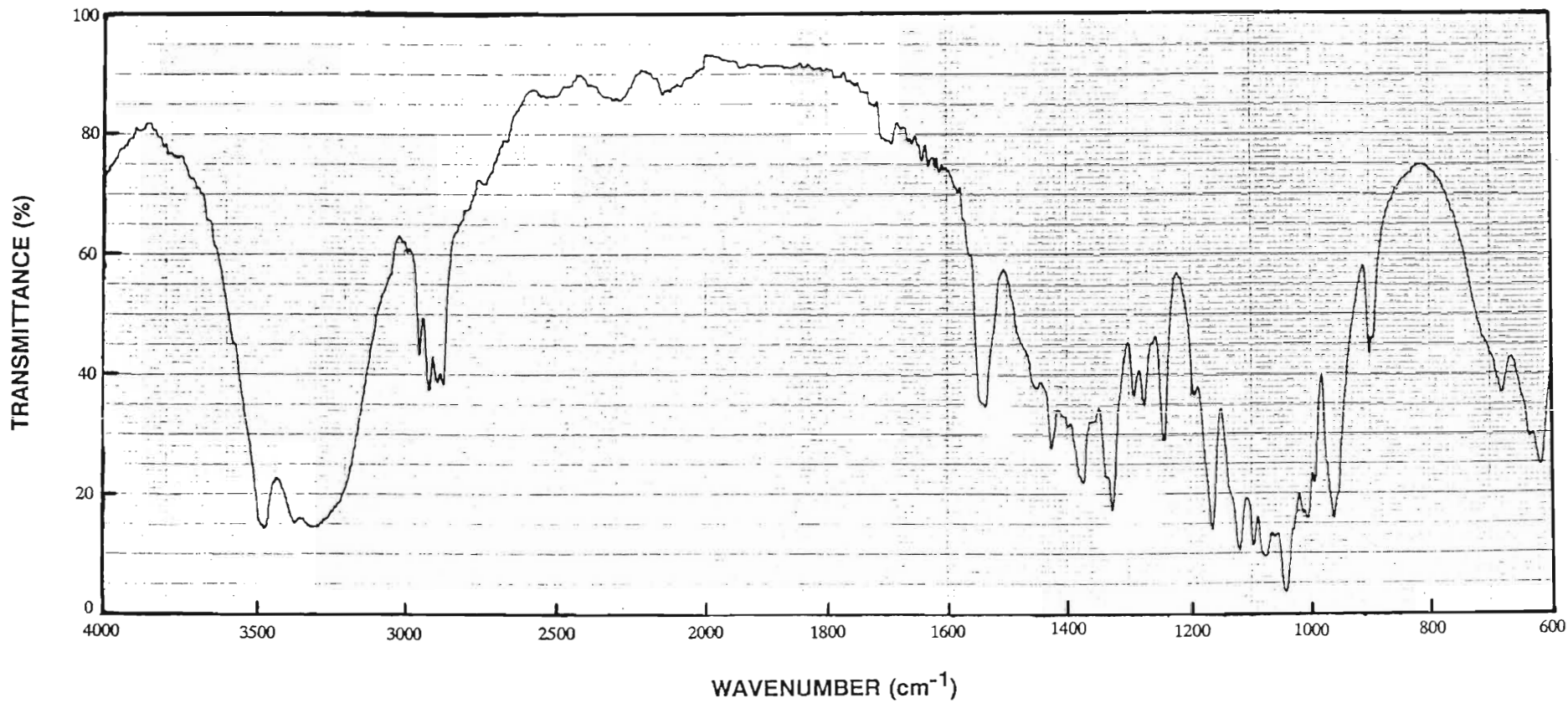
Figure 14: Infra-red spectrum of macrozamin (kBr)
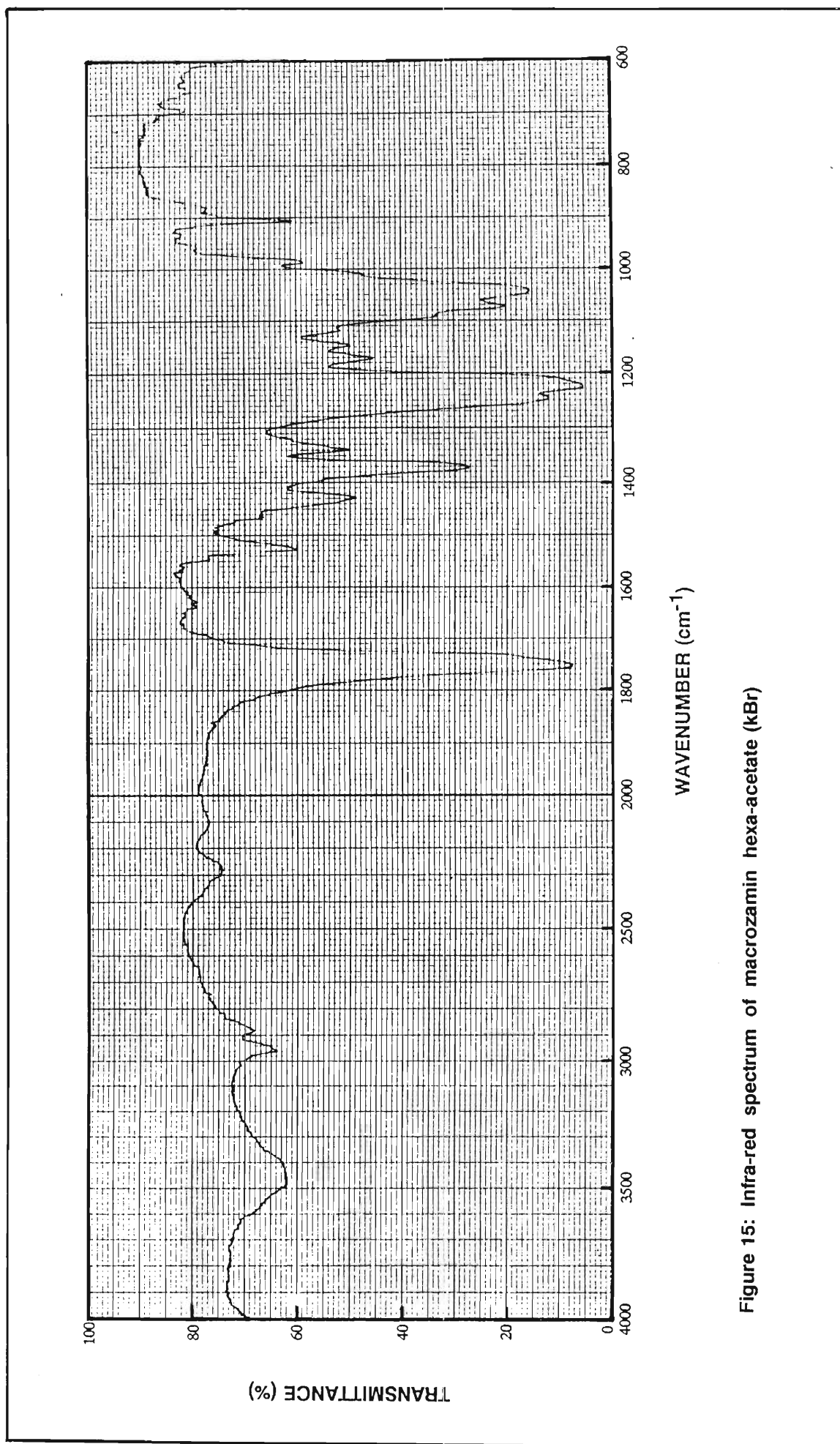
Figure 15: Infra-red spectrum of macrozamin hexa-acetate (kBr)
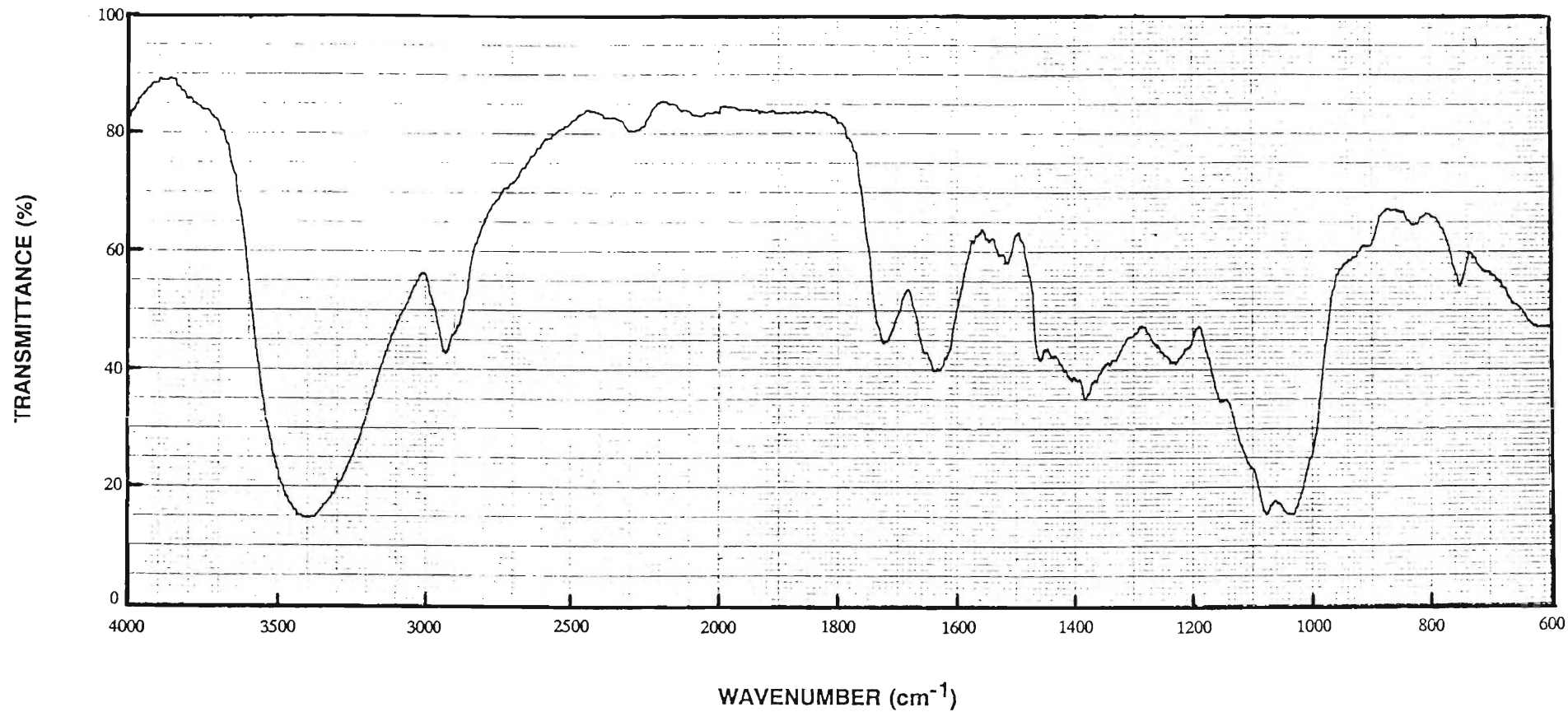
Figure 16: Infra-red spectrum of crude cycasin (kBr)
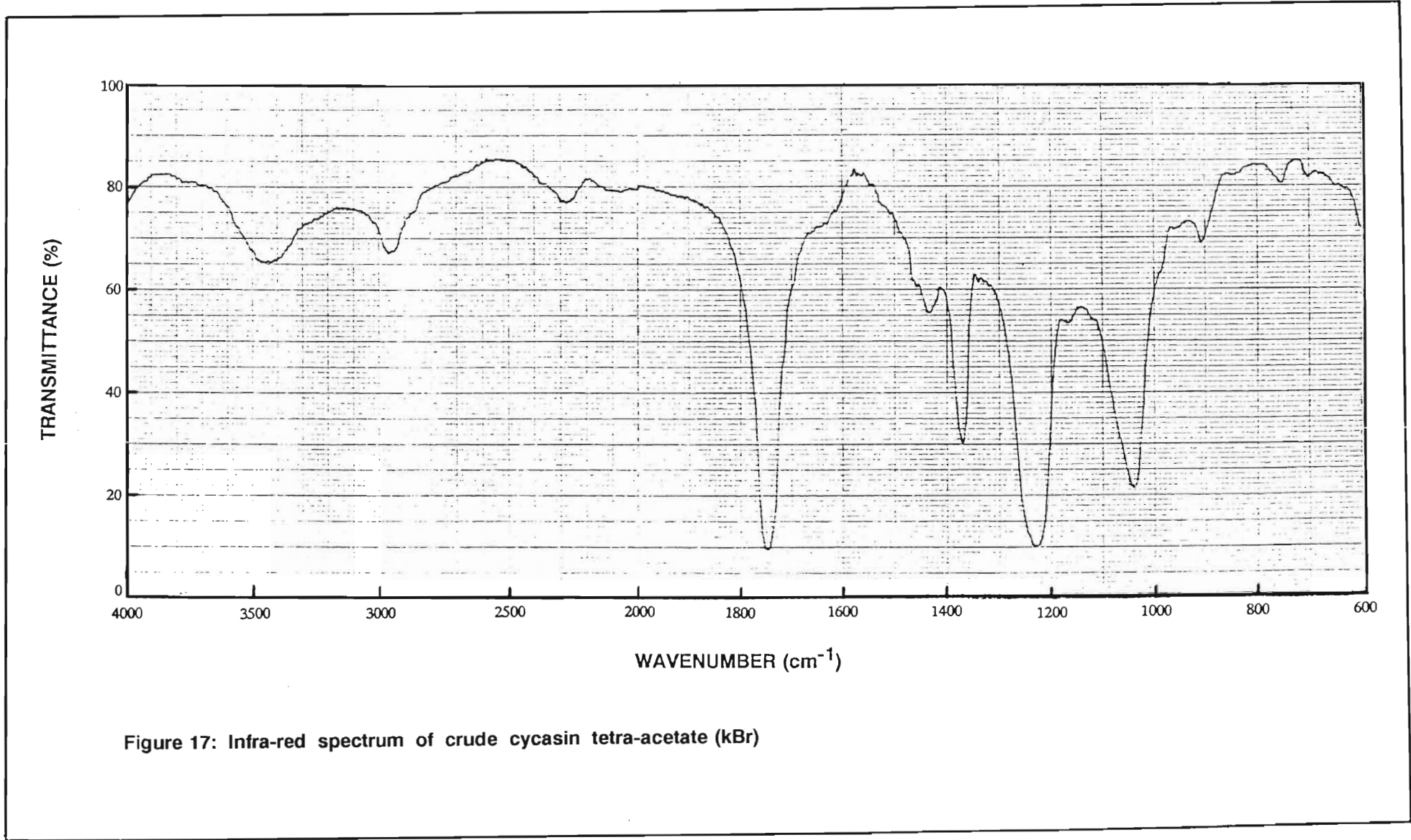
Figure 17: Infra-red spectrum of crude cycasin tetra-acetate (kBr)

The symmetric stretch of crude cycasin and its acetate derivative is far-shifted from the absorption reported at $1338cm^{-1}$. No asymmetric stretch has been detected in the tetra-acetate and the absorption at $1480cm^{-1}$ for cycasin is also shifted from that reported at $1430cm^{-1}$. However, crude cycasin and its tetra-acetate show absorptions characteristic of hydroxy functions and ester carbonyls respectively.

The characteristic feature of nmr spectra of both cycasin and macrozamin are the signals which arise as a result of the azoxy function. Korsch and Riggs (1964) have reported that the methyl and methylene signals due to the aglycone of cycasin are detected as a 1:2:1 triplet at $\delta4.09$ and a quartet at $\delta5.15$ respectively.

The following is a list of the important signals in the spectra of macrozamin (Figure 18), macrozamin hexa-acetate (Figure 19) and crude cycasin (Figure 20):

^{1}Hnmr (macrozamin)

$$\delta_H \quad : \quad \begin{array}{l} 5.20 \text{ quartet } (J=1.8Hz) \\ 4.81 \text{ singlet} \\ 4.65 \text{ doublet } (J=7.8Hz) \\ 4.43 \text{ doublet } (J=7.8Hz) \\ 4.12 \text{ triplet } (J=1.8Hz) \\ 3.92 \text{ multiplet} \\ 3.51 \text{ multiplet} \\ 2.92 \text{ multiplet} \end{array}$$

^{1}Hnmr (macrozamin hexa-acetate)

$$\delta_H \quad : \quad \begin{array}{l} 5.06 \text{ quartet } (J=1.6Hz) \\ 4.75 \text{ doublet } (J=8.0Hz) \\ 4.52 \text{ doublet } (J=7.8Hz) \\ 4.10 \text{ triplet } (J=1.6Hz) \\ 2.05 \text{ multiplet } (6 \times CH_3CO_2-) \end{array}$$

^{1}Hnmr (cycasin)

$$\delta_H \quad : \quad 4.81 \text{ singlet}$$
$$4.73 \text{ doublet (J=1.0Hz)}$$
$$4.52 \text{ doublet (J=8.1Hz)}$$
$$3.78 \text{ multiplet}$$

The quartet at δ5.20 (J=1.8Hz) and triplet at δ4.12 (J=1.8Hz) in the spectrum of macrozamin (Figure 18) are signals of the methylene and methyl groups respectively as a result of long-range coupling across the nitrogen-nitrogen double bond. Doublets at δ4.65 (J=7.8Hz) and δ4.43 (J=7.8Hz) are signals for the anomeric protons of the first and second sugar moieties. The coupling constants for both the anomeric protons (J=7.8Hz) favour a β-configuration (Lemieux, 1964). The multiplet at δ3.92, integrating to two protons, is the signal from the two protons each of carbon number 6 and 11 which appear equivalent since both have oxygen, and a carbon with a single proton as neighbours. The multiplet at δ3.50, integrating to six protons, is the signal for the ring protons of carbons which have hydroxy groups i.e. carbon numbers 2,3,4,8,9,10. The single proton of carbon number 5 is detected as a multiplet at δ2.02. The singlet at δ4.81 in the spectrum of macrozamin is assumed to be the signal for the hydroxy protons since this peak is absent in the spectrum of macrozamin hexa-acetate (Figure 19), but replaced by the multiplet at δ2.02, integrating to 18 protons, which is evidence for the formation of the hexa-acetate.

Assignment of all peaks in the spectrum of crude cycasin (Figure 20) was not possible. The peaks of significance include the doublet at δ4.73 (J=1.0Hz), for the two protons of carbon number 6, doublet at δ4.52 (J=8.2Hz) for the β-anomeric proton of carbon number 1, and the multiplet at δ3.78 for the ring protons.
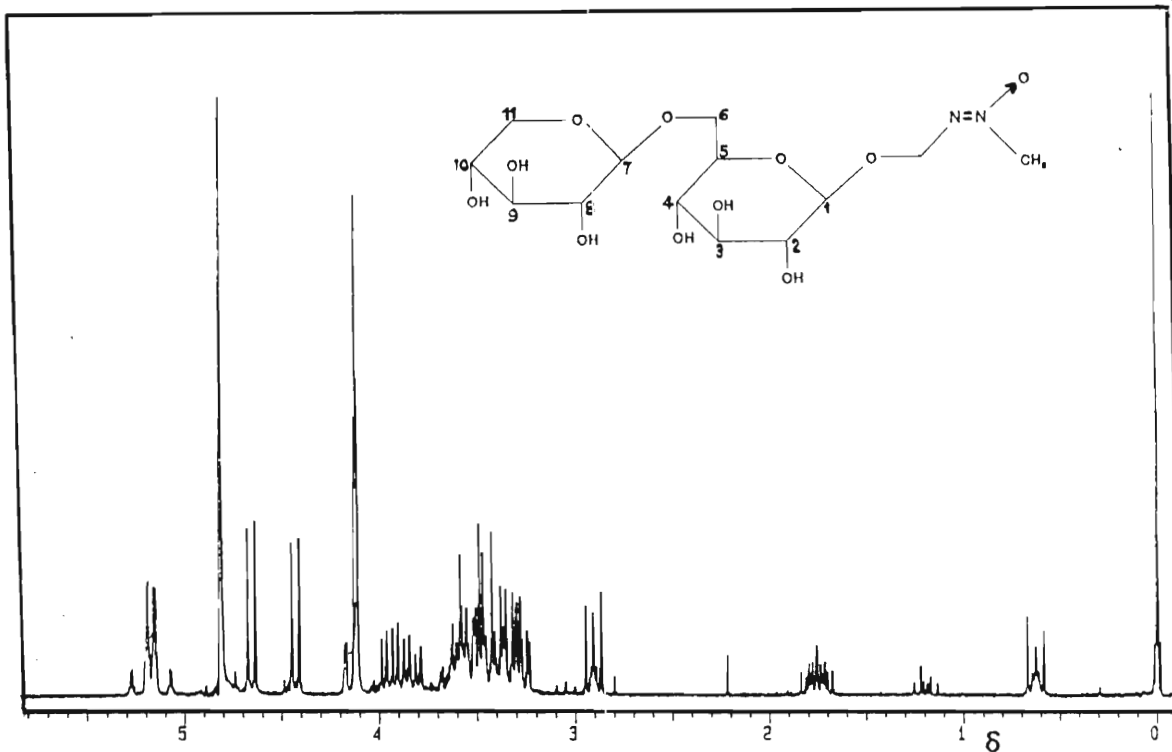
Figure 18: ^{1}H n.m.r. spectrum of macrozamin in D_2O
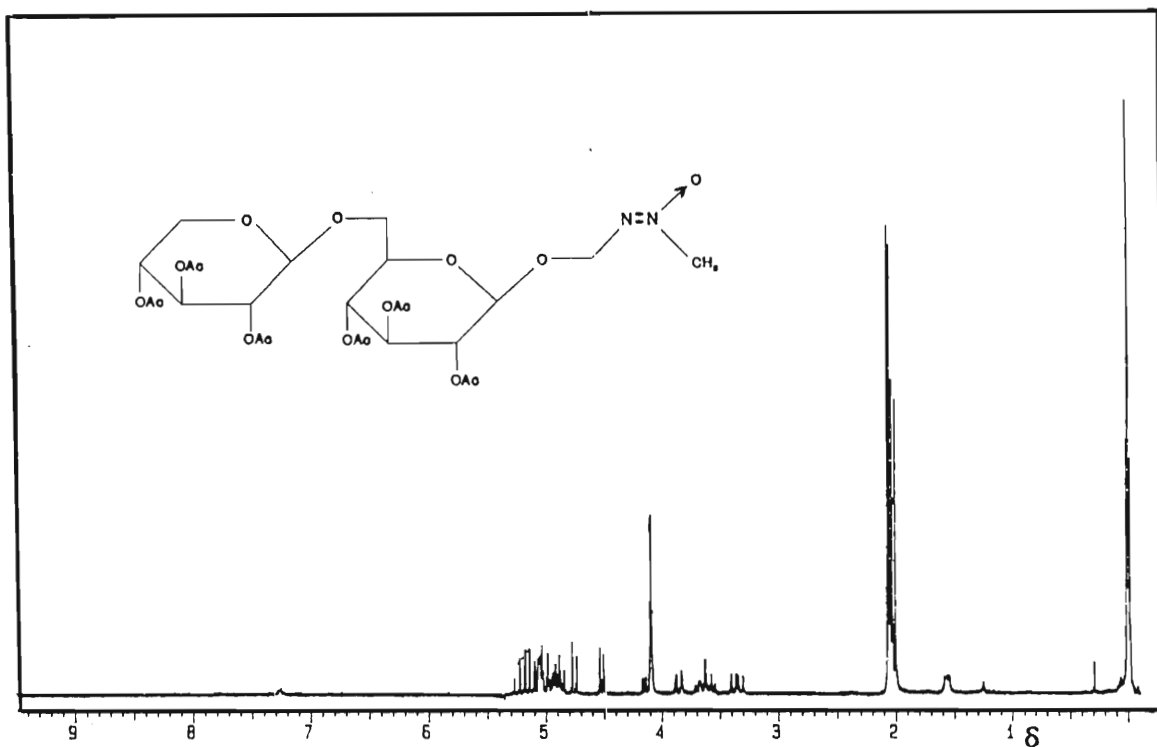
Figure 19: ^{1}H n.m.r. spectrum of macrozamin hexa-acetate in $CDCl_3$
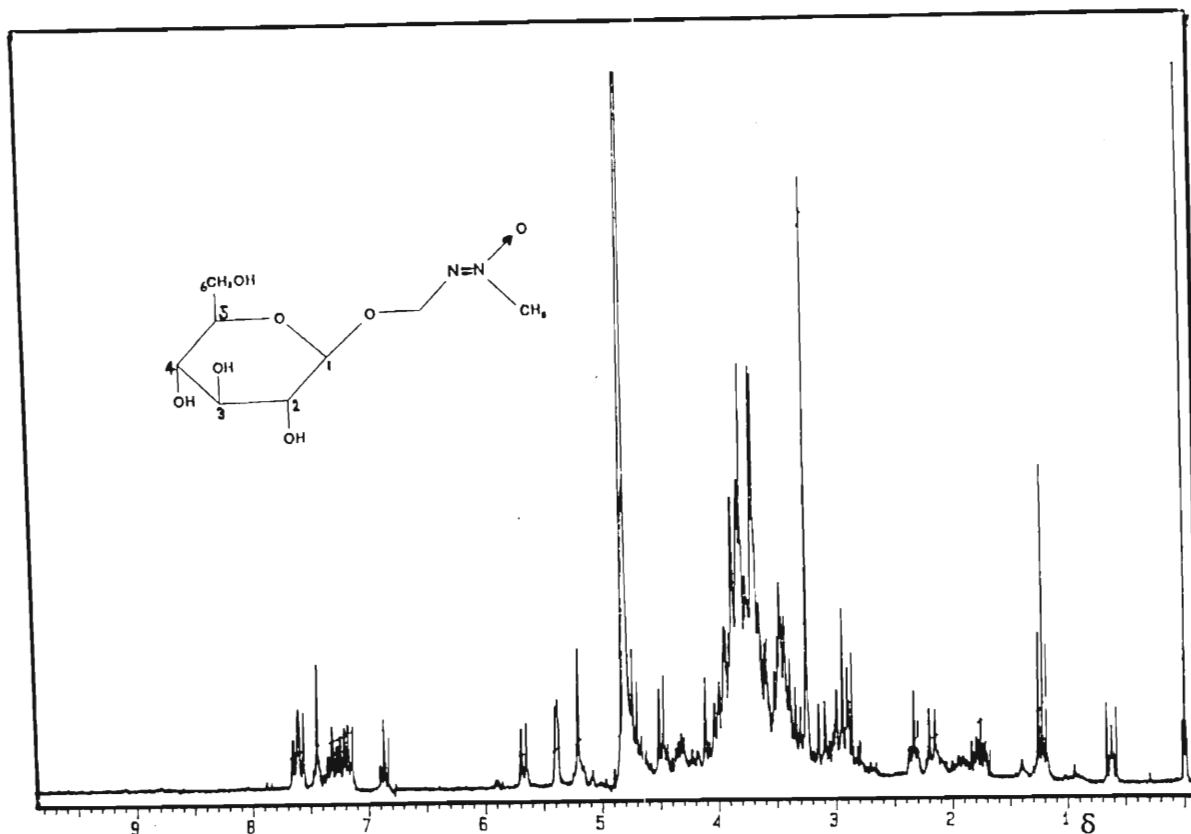
Figure 20: ^{1}Hn.m.r. spectrum of crude cycasin in D_2O

The difficulty experienced in the purification of cycasin is attributable to two possible factors. Firstly, the plant, *Cycas thouarsii*, from which cycasin was extracted may not have been a suitable choice since it is reported that seeds of the above plant contain higher quantities of macrozamin than cycasin (section 4.7). The availabiltiy of local species did not allow extraction from a cycasin-rich source.

Secondly, cycasin and macrozamin were virtually inseparable on columns of charcoal or cellulose. Preparative high performance liquid chromatography (HPLC) may have solved the problem of separation, but such facilities were not available. However, HPLC has been used analytically in the quantitative investigation (section 4.7) from which it has become apparent that the two peaks detected at 215nm for the crude cycasin extract are attributable to cycasin and macrozamin on the basis of their retention times.

CHAPTER THREE

KINETIC CONSIDERATIONS

3.1. Introduction

The azoxyglycosides may be considered from two focal points
of chemistry. Firstly, since the sugar component forms the
structural bulk of the molecule, the compounds may be studied
in terms of carbohydrate chemistry. Secondly, the
methylazoxymethanol function, to which the most important
properties of the molecules have been attributed, forms a
unique branch of nitrogen chemistry. The significant
carbohydrate character of the azoxyglycosides contributes to
the property that they possess to react chemically like
carbohydrates. A typical reaction employed in carbohydrate
analysis is hydrolysis which involves the use of acids, bases
or enzymes. For example, polysaccharides are hydrolysed, and
their constituent sugars identified, using the technique of
sequential degredation.

The hydrolysis reaction served as a useful tool to the
organic chemist in the identification of unknown compounds,
especially glycosides, before the advent of modern
spectroscopic techniques. Lythgoe and Riggs (1949) hydrolysed
macrozamin with hydrochloric acid and subsequently identified
primeverose which was hydrolysed further to glucose and
xylose. Acid hydrolysis of macrozamin also led to the
identification of formaldehyde, nitrogen and methanol from
the aglycone (MAM) (Langley et al., 1951).

3.2. Acid Hydrolysis

The investigation was aimed at determining the rate at which
macrozamin hydrolyses in acid medium. The rate constants
calculated for the different temperatures were used to
calculate the activation energy for the overall process
involving the hydrolysis of macrozamin with 4M sulphuric
acid. Since cycasin was not secured in a pure crystalline

state, no hydrolysis reactions were performed for this compound. However, the data of Matsumoto and Strong (1963) was used to derive the necessary kinetic parameters for cycasin. Methylazoxymethanol was also kinetically analysed using the data of the above authors. A comparison of the kinetic parameters determined for the acid hyrolysis of MAM, cycasin and macrozamin has revealed a characteristic trend in the manner by which these compounds hydrolyse.

The acid hydrolysis of macrozamin involves the cleavage of the glycosidic bond between the aglycone and the sugar components to yield MAM and primeverose. MAM decomposes into one mole each of formaldehyde, methanol and nitrogen while primeverose is hydrolysed further at a second glycosidic bond into glucose and xylose. The steps involved in this reaction are depicted in Figure 21.

A similar reaction is expected for cycasin which contains a glucose molecule bound by a glycosidic linkage to MAM. Acid hydrolysis of cycasin involves the cleavage of the glycosidic bond to yield glucose and methylazoxymethanol which decomposes into the products indicated in Figure 21.
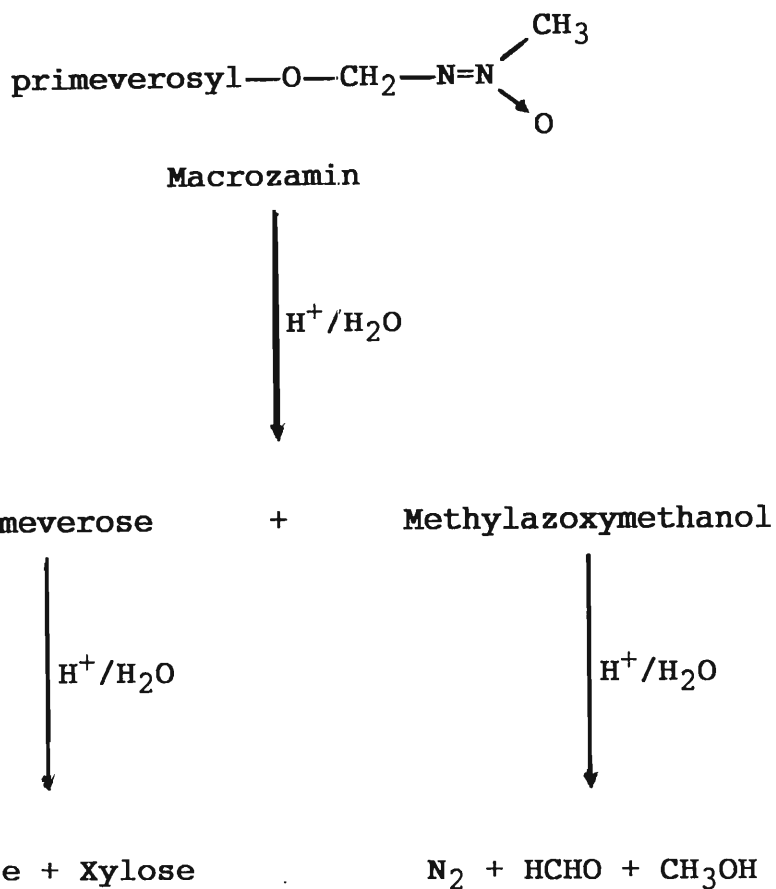
primeverosyl—O—CH$_2$—N=N$\diagdown$$^{CH_3}_{O}$

Macrozamin

$\downarrow$ H$^+$/H$_2$O

Primeverose   +   Methylazoxymethanol

$\downarrow$ H$^+$/H$_2$O $\downarrow$ H$^+$/H$_2$O

Glucose + Xylose   N$_2$ + HCHO + CH$_3$OH

NOTE: a) Acid is denoted by H$^+$.

b) The hydrolysis products of Methylazoxymethanol
   are nitrogen (N$_2$), formaldehyde (HCHO) and
   methanol (CH$_3$OH).

Figure 21: Acid Hydrolysis of Macrozamin
           (after Langley *et al.*,1951)

3.3. Kinetic analysis

3.3.1. Beer's Law relationship

The hydrolysis of macrozamin is followed by observing the progressive reduction of the absorbance at 215nm, which is characteristic of the azoxy function, during the reaction. The change in absorbance at 215nm as a function of time is therefore indicative of the rate of hydrolysis. Any rate is given by the change in a measurable quantity with time, and the rate of a chemical reaction is expressed in terms of the change in concentration of a reactant in a given time. Concentration can be substituted by absorbance when the spectrophotometric method is used to determine the rate of the reaction, provided the solution obeys Beer's Law. Beer's Law relationship is expressed in the following manner:

$$A = \epsilon c l \qquad\qquad\qquad (1)$$

where: A= Absorbance
ϵ= Extinction coefficient (l/mol/m)
c= concentration (mol/l)
l= path length of solution cell (0.01m)

Since the extinction coefficient of macrozamin and the path length of the cell are constant, absorbance is proportional to concentration. The absorbance at 215nm of the macrozamin solution was observed to increase with an increase in concentration.

3.3.2. Determination of rate constants

Consider the reaction:

$A \longrightarrow Products$ where A could represent macrozamin

Let a be the initial concentration of A and let x be the decrease in concentration of A in time t. The concentration of A at time t is therefore $a-x$. The rate of reaction is given by:

$$-d[A]/dt = -d[a-x]/dt = dx/dt \qquad (2)$$

The differential rate equation, $-d[A]/dt = k_r[A]$ where k_r is the rate constant, can therefore be written as:

$$dx/dt = k_r[a-x]$$
or
$$dx/[a-x] = k_r dt \qquad (3)$$

Integration of equation 3 gives:

$$-ln[a-x] = k_r t + constant$$

Since at $t=0$, $x=0$, the $constant$ is equal to $-ln\ a$, so that substitution in equation 3 gives:

$$k_r t = ln(a/a-x)$$
or
$$k_r = 1/t\ ln(a/a-x) \qquad (4)$$

Using logarithms to the base 10,

$$k_r = 2.303/t \, log_{10}(a/a-x) \qquad (5)$$

Equation 5 can be rearranged to give:

$$log_{10}(a-x) = log_{10}a - (k_r t/2.303) \qquad (6)$$

This equation is analogous to the straight line graph equation, $y = mx + c$. A plot of $log_{10}(a-x)$ against t will be linear with slope equal to $-k_r/2.303$. If the rate data obtained gives a linear plot the reaction is first order, and the rate constant (k_r) is obtained from the slope.

Since the solution obeys Beer's Law, concentration is substituted by absorbance. Let the absorbance be A_o at the start of the reaction and A_t at time t. Therefore, A_o is proportional to the initial concentration of reactants a and A_t is proportional to the concentration of reactants $a-x$ at time t. Therefore equation 5 takes the form:

$$k_r = 2.303/t \, logA_o/A_t \qquad (7)$$

Equation 5 is rearranged to give:

$$log_{10}[A_t] = log[A_o] - k_r t/2.303 \qquad (8)$$

A plot of $log[A_t]$ against t is linear with slope equal to $-k_r/2.303$.

3.3.3. Dependance of rate on tempertaure

The rate equation of the form,

$$rate = k_r [A]^{n1} [B]^{n2} \qquad (9)$$

expresses the dependance of reaction rate on the concentration of the reactants. However, the rate of a reaction varies greatly with temperature, since for a typical process the rate can double or treble for a rise in temperature of 10°C. In equation 9, the concentration terms and the order are not sensitive to changes in temperature, and it is the rate constant k_r which is the temperature-dependant term. The rate constant k_r varies with temperature according to the relationship:

$$log_{10}k_r = b - a/T \qquad (10)$$

where a and b are constants and T is the absolute or thermodynamic temperature. It was shown by van't Hoff and Arrhenius that the theoretical basis for this law is the relationship between the equilibrium constant K_C and temperature known as the van't Hoff isochore,

$$dlnK_C/dT = \triangle E/RT^2 \qquad (11)$$

where K_C is the equlibrium constant in terms of concentration, $\triangle E$ is the energy change and R is the gas constant with a value of 8.314J/K/mol.
Consider a reaction:

$$A + B \overset{k_1}{\underset{k_{-1}}{\rightleftharpoons}} C + D$$

The rate of the forward reaction is $k_1[A][B]$ and the rate of the reverse reaction is $k_{-1}[C][D]$, where k_1 and k_{-1} are the rate constants for the forward and reverse reactions respectively.

At equilibrium ,

$$k_1[A][B] = k_{-1}[C][D]$$

and the equlibrium constant is given by:

$$K_C = [C][D]/[A][B] = k_1/k_{-1} \tag{12}$$

Equation 11 therefore takes the form of:

$$dlnk_1/dT - dlnk_{-1}/dT = \triangle E/RT^2 \tag{13}$$

and can be expressed as two equations,

$$dlnk_1/dT = E^{\mathbf{x}}{}_1 + I$$
and
$$dlnk_{-1}/dT = E^{\mathbf{x}}{}_{-1} + I$$

where $\triangle E = E^{\mathbf{x}}{}_1 - E^{\mathbf{x}}{}_{-1}$ and I is an integration constant.

Arrhenius found that for a number of reactions, I was equal to zero and formulated his law as:

$$dlnk_r/dT = E^{\neq}/RT^2 \qquad (14)$$

where k_r is the rate constant and $E^{\neq}$ is the activation energy. The Arrhenius equation can be expressed as:

$$k_r = Aexp(-E^{\neq}/RT) \qquad (15)$$

where A is a constant known as the frequency factor which has the same dimensions as the rate constant and is related to the frequency of collisions between reactant molecules. It can be seen that the rate constant for any reaction depends on two factors:

a) The frequency of collisions between reactant molecules.
b) The value of the activation energy.

3.3.4. Determination of activation energy

The logarithmic form of the Arrhenius equation is given by:

$$lnk_r = lnA - E^{\neq}/RT \qquad (16)$$
or
$$log_{10}k_r = log_{10}A - E^{\neq}/2.303RT \qquad (17)$$

The activation energy of a reaction can therefore be determined if the rate constant is measured at a number of different temperatures. A plot of $log_{10}k_r$ against $1/T$ is linear if equation 17 is obeyed, and the slope is equal to $-E^{\neq}/2.303R$.

3.4. Experimental

A 10ml sample of macrozamin solution (0.118mg/ml) was pipetted into a 100ml volumetric flask together with 50ml of water. A 10ml aliquot of 4M sulphuric acid was added to the solution which was then made up to volume with water. An aliquot (*ca.* 3ml) was taken immediately and its UV-absorbance measured at 215nm against a blank of 0.4M sulphuric acid. The flask was then stoppered and immersed in a water bath and similar aliquots were taken at 20 minute intervals, cooled and UV-absorbances obtained at 215nm. For the kinetic investigation, hydrolyses were carried out at 50°, 60°, 70°, 80°, 90° and 100°C in the manner described above.

3.5. Results and discussion

The 215nm absorbance of macrozamin, which is characteristic of the azoxy function, decreases with increasing reaction time when hydrolysed in a boiling water bath with 4M sulphuric acid (Figure 22). Applying Beer's Law relationship to this reaction, it is inferred that the amount of azoxy function is proportionally decreasing. This characteristic fits well with the observation that the azoxy function once hydrolysed off the sugar moiety decomposes spontaneously into one mole each of formaldehyde, nitrogen and methanol.

The absorbances obtained at 215nm with increasing reaction time are an indication of the amount of intact or unhydrolysed macrozamin still present in the system, while the difference between any two reaction times is indicative of the amount of hydrolysed macrozamin. The quantities of the decomposition products of MAM *viz*. formaldehyde, methanol and nitrogen; and the amounts of primeverose and xylose and glucose would be expected to increase as more molecules of macrozamin are hydrolysed. Complete hydrolysis is attained when no absorbance is detected at 215nm.

Figure 22: Hydrolysis of macrozamin with 4M sulphuric acid
in a boiling water bath

NOTE: Numbering on the spectrum is as follows:
1 – macrozamin in 4M sulphuric acid
2 – absorbance after 20 minutes
3 – absorbance after 40 minutes
4 – absorbance after 60 minutes

The absorption spectra for macrozamin when hydrolysed at 50°, 60°, 70°, 80°, 90° and 100°C with 4M sulphuric acid are depicted in Figures 23-28, which indicate that while the distances between reaction times are less-pronounced in the temperature range 50°-70°C, they become more defined between 80° and 100°C. Plots of log [absorbance] against reaction time for each temperature are illustrated in Figure 29 which indicate that slope is gentle or nearly-absent between 50° and 70°C but becomes more pronounced between 80° and 100°C. Increasing reaction rates for each temperature is reflected by increasing inclination of slope. The slopes of the graphs in Figure 29 were calculated using the formula:

$$slope = dy/dx$$

The rate constants (k_r) for each temperature state were then calculated from the slopes using the equation:

$$slope = -k_r/2.303$$

The rate constants increase with every 10°C rise in temperature (Table 3). This suggests that the MAM function once hydrolysed off the sugar moiety decomposes more rapidly at higher temperatures. A plot of log [rate constant] against reciprocal Kelvin temperature (Figure 30) was obtained from the data in Table 3. The activation energy ($E^{\neq}$) for acid hydrolysis of macrozamin with 4M sulphuric acid was calculated from the slope of the graph using the equation:

$$slope = -E^{\neq}/2.303R$$

Figure 23: UV-monitoring of acid hydrolysis of macrozamin at 50°C

Figure 24: UV-monitoring of acid hydrolysis of macrozamin at 60°C

Figure 25: UV-monitoring of acid hydrolysis of macrozamin at 70°C

Figure 26: UV-monitoring of acid hydrolysis of macrozamin at 80°C

Figure 27: UV-monitoring of acid hydrolysis of macrozamin at 90°C

Figure 28: UV-monitoring of acid hydrolysis of macrozamin at 100°C

Figure 29: Macrozamin hydrolysis (50-100°C), change in absorbance with time

Table 3: Rate constants obtained for macrozamin hydrolysis

TEMP (°C)	TEMP (K)	$1/T$ (K^{-1})	RATE CONSTANT(min^{-1})	$logk_r$
50	323	310×10^{-5}	9.06×10^{-5}	-4.04
60	333	300×10^{-5}	3.44×10^{-4}	-3.46
70	343	292×10^{-5}	3.92×10^{-4}	-3.41
80	353	283×10^{-5}	1.82×10^{-3}	-2.74
90	363	276×10^{-5}	3.46×10^{-3}	-2.46
100	373	268×10^{-5}	6.91×10^{-3}	-2.16

Figure 30: Change in the rate of hydrolysis of macrozamin with temperature

As mentioned earlier no hydrolysis reactions were carried out on cycasin. Useful information was extracted from the work of Matsumoto and Strong (1963) who, in an attempt to observe the disappearance of the 215nm absorbance, hydrolysed cycasin with 4M sulphuric acid but did not pursue any kinetic analyses. The author has used this data as a foundation from which a kinetic scheme for acid hydrolysis of cycasin has been derived.

Absorbance values for each 15 minute interval were obtained from Figure 31 by extrapolation onto the absorbancy axis, and the log of these values were plotted against reaction times for the process at 100°C (Figure 32).The rate constant (k_r) calculated for the process at 100°C from the slope of the graph had a value of 1.15×10^{-2} min^{-1} and was about twice the value of the rate constant calculated for macrozamin (k_r=

6.91×10^{-3} min^{-1}) under similar conditions. The factor of two in the rate at which cycasin hydrolyses more rapidly is accounted for by the presence of the extra sugar moiety (xylose) in macrozamin. In other words, the disaccharide moiety of macrozamin hydrolyses less rapidly than the monosaccharide unit of cycasin since the restriction imposed on the process, through steric interactions, by the single glucose molecule in cycasin is not as large as that provided by the disaccharide (primeverose) in macrozamin. Furthermore, the fact that the primeverose unit is hydrolysed further into xylose and glucose by the acid , may also be considered as a rate-decreasing factor.

Figure 31 : Hydrolysis of cycasin at 100°C with 4M sulphuric acid, carried out by Matsumoto and Strong (1963)

NOTE : The letters A-D in the spectrum represent the following:

A- absorbance of cycasin in 4M sulphuric acid

B- absorbance after 15 minutes

C- absorbance after 30 minutes

D- absorbance after 60 minutes

Figure 32: Cycasin hydrolysis (100°C), change in absorbance with time

Avery (1976) observed that for a typical kinetically-monitored process, the rate doubles or trebles for every 10°C rise in temperature. This was observed to be valid for acid hydrolysis of macrozamin , the rates of which, on average, are doubled for every 10°C rise in temperature (Table 3). Since the hydrolysis reaction for both cycasin and macrozamin involves the cleavage of the MAM function from the sugar unit, the above principle was applied to cycasin hydrolysis to predict a doubling of rate constants with a 10°C rise in temperature.

The predicted rate constants for cycasin hydrolysis at 50°, 60°, 70°, 80° and 90°C using the rate constant calculated for it at 100°C are depicted in Table 4. A plot of log [rate constant] against the reciprocal Kelvin temperature was obtained (Figure 33), the slope of which was used to calculate the activation energy for the process. The

predicted activation energy for the acid hydrolysis of cycasin is $71.80kJ.mol^{-1}$ and that calculated for macrozamin is $86.16kJ.mol^{-1}$.

Table 4: Rate constants predicted for cycasin hydrolysis

TEMP (°C)	TEMP (K)	1/T (K^{-1})	RATE CONSTANT(min^{-1})	logk$_r$
50	323	310×10^{-5}	3.59×10^{-4}	-3.45
60	333	300×10^{-5}	7.19×10^{-4}	-3.14
70	343	292×10^{-5}	1.44×10^{-3}	-2.84
80	353	283×10^{-5}	2.89×10^{-3}	-2.54
90	363	276×10^{-5}	5.75×10^{-3}	-2.24
100	373	268×10^{-5}	1.15×10^{-2}	-1.94

Figure 33: Change in the rate of hydrolysis of cycasin with temperature

The activation energy is also considered as a potential energy barrier which molecules only with sufficient energy can reach. Thus, the smaller the barrier (the smaller the activation energy), the greater the number of activated molecules, and the faster is the rate of reaction. Hence, cycasin with the smaller activation energy hydrolyses faster. The rate constants calculated at 100°C for cycasin and macrozamin (0.015 and 0.00691 min^{-1} respectively), enhances the observation that more cycasin molecules than macrozamin molecules are hydrolysed per minute at a given temperature.

The MAM function once hydrolysed off the glycoside decomposes probably because of the active media it is released into. The stability of MAM may be greatly reduced in acidic or basic media and may be compounded by any temperature increments. Less-drastic conditions may allow MAM to be secured in the free state. In fact, Matsumoto and Strong (1963) demonstrated that the alcohol could be secured in the free state during enzymatic hydrolysis of cycasin. They suggest further that this is a naturally-occurring event and that the enzyme β-glucosidase which is capable of carrying out this reaction, and which also occurs naturally in the plant was responsible for the lower than expected yield of cycasin from the seeds of *Cycas circinalis*.

MAM is relatively unstable and would be expected to hydrolyse more rapidly than cycasin or macrozamin since it is not limited by the rate of cleavage of any glycosidic bonds. Matsumoto and Strong (1963) observed that MAM is completely hydrolysed in ½-hour with 4M sulphuric acid at 100°C, which is evidenced by the absence of any 215nm absorbance after this period. A kinetic analysis similar to that performed for cycasin was also carried out for MAM using the data of Matsumoto and Strong (1963).

The rate data (Table 5) obtained from the UV-absorption spectrum of acid-hydrolysed MAM was used to construct the log [rate constant] vs reciprocal Kelvin temperature plot (Figure 34), the slope of which yielded a value of $65.10 kJ.mol^{-1}$ for the activation energy for the process. This predicted value is, as anticipated, lower than the value obtained for either cycasin ($E^{\neq}= 71.80 kJ.mol^{-1}$) or macrozamin ($E^{\neq}= 86.16 kJ.mol^{-1}$) since MAM, being more unstable than cycasin or macrozamin, hydrolyses more rapidly. MAM is also not hindered by any rate-decreasing glycosidic bonds.

Table 5: Rate constants predicted for MAM hydrolysis

TEMP (°C)	TEMP (K)	1/T (K^{-1})	RATE CONSTANT(min^{-1})	$logk_r$
50	323	310×10^{-5}	1.41×10^{-3}	-2.85
60	333	300×10^{-5}	2.81×10^{-3}	-2.55
70	343	292×10^{-5}	5.63×10^{-3}	-2.25
80	353	283×10^{-5}	1.13×10^{-2}	-1.95
90	363	276×10^{-5}	2.25×10^{-2}	-1.65
100	373	268×10^{-5}	4.50×10^{-2}	-1.35

Log [Rate constant]

Figure 34: Change in the rate of hydrolysis of MAM with temperature

Thus, the rates for acid hydrolysis at a given temperature increase in the order macrozamin, cycasin, methylazoxymethanol because these molecules contain two, one and zero glycosidic bonds respectively. Consequently, activation energies for the process decrease in the order stated above. The pattern for hydrolysis of MAM, cycasin and macrozamin, that glycosidic bonds decrease the rates of reactions, could be extended to include other azoxyglycosides *viz.* the neocycasins which include trisaccharide compounds. These compounds which contain additional glycosidic bonds would be expected to have lower rate constants and higher activation energies than either cycasin or macrozamin.

Base hydrolysis of macrozamin and cycasin occurs more rapidly than the acid reaction since it was observed that when macrozamin is hydrolysed with 4M sodium hydroxide at 50°C, no

absorbance at 215nm is detected after 20 minutes. A kinetic analysis of the base reaction was, therefore, not pursued. Although it would have been possible to carry out the analysis at a weaker base strength, this was not attempted since the additional concentration factor which the process introduces would not warrant comparison with the acid reaction which proceeded optimally with 4M sulphuric acid.

In addition to formaldehyde, methanol and nitrogen which are detected as the breakdown products of MAM, cyanide is also produced during base hydrolysis (Langley *et al.*, 1951; Kobayashi and Matsumoto, 1965). The fact that azoxyglycosides release cyanide during base hydrolysis is the reason that they are also regarded as pseudocyanogenetic glycosides. Thus, for a given concentration, higher rate constants and lower activation energies is expected for base hydrolysis than the acid reaction at the same concentration.

Enzymatic hydrolysis of cycasin has been caried out with β-glucosidase which is specific for cleavage of β-glucosidic linkages (Kobayashi and Matsumoto, 1965). The rate of hydrolysis is dependant on both the enzyme and cycasin concentrations. The enzyme β-primeverosidase which is specific for macrozamin (Yagi and Tadera, 1987), would be expected to react in a similar manner towards macrozamin.

Investigation into the kinetics of hydrolysis of azoxyglycosides is an interesting prospect for further research. Kinetic data for hydrolysis of macrozamin and cycasin by acid, base and enzyme may be useful in understanding the manner in which the toxicity of the metabolites, which arise as a result of the hydrolysis of the compounds, is manifested.

CHAPTER FOUR

QUANTIFICATION OF CYCAD AZOXYGLYCOSIDES

4.1. **Introduction**

Quantification of the azoxyglycosides, particularly cycasin and macrozamin, has received increasing attention primarily because of the toxic properties of these molecules. The results of such investigations are of ecological interest in that cycad material is often eaten (De Luca *et al.*, 1980). In particular, analytical results have proved to be useful for studies on cattle poisoning caused by ingestion of cycad leaves in the southwestern islands of Japan (Yagi *et al.*, 1983). Quantitative studies may also be useful in the elucidation of the biosynthetic pathway of the azoxyglycosides and may contribute much to the understanding of the taxonomy within the group.

The techniques used for azoxyglycoside analysis have become increasingly sophisticated with the advancement of technology. However, the simple methods initially developed for qualitative detection and quantitative estimation of cycasin and macrozamin still remain useful. For example Nishida *et al.* (1955a, 1955b) extracted and estimated cycasin in seed kernels of *Cycas revoluta* by employing column chromatography, ion-exchange resins and activated charcoal, and by semi-quantitative paper chromatography and colorimetry. Quantitative estimations have also been made by polarography (Nishida *et al.*, 1956). In addition, cycasin has been analysed by biological assay *via* growth rates or lesions in rats (Campbell *et al.*, 1966).

For optimum accuracy and practicality, azoxyglycoside determination requires a rapid, specific and sensitive method. In this regard, the techniques of gas liquid chromatography (GLC) and high pressure liquid chromatography (HPLC) have been found to be useful. Both cycasin and macrozamin have been quantified in different cycad species by either GLC or HPLC. Wells *et al.*(1968) analysed cycad flour

for cycasin by gas chromatography of the trimethylsilyl derivative while de Luca *et al.*(1980) used the same technique to detect the presence of cycasin in 17 cycad species. The distribution and quantitative estimation of macrozamin in seeds of Australasian representatives of *Cycas*, *Bowenia*, *Lepidozamia* and *Macrozamia* have also been described using a similar method (Moretti *et al.* 1981a). Cycasin and macrozamin content of various cycad species has been determined by HPLC (Yagi *et al.*, 1980, 1983; Yagi and Tadera, 1987).

In this investigation both GLC and HPLC techniques have been used for the quantitative analysis of cycasin and macrozamin. In addition, a colorimetric-spectrophotometric method has been adapted and developed for azoxyglycoside analysis. A comparison of the results obtained by the three methods has highlighted both similarities and discrepancies. Information derived from this work and from investigations of others has been used to propose a biosynthetic pathway for the azoxyglycosides.

4.2. Colorimetric method (Chromotropic acid assay)

Determination of the azoxyglycoside content of cycad material by the chromotropic acid (CTA) assay is based on the fact that cycad toxins release formaldehyde when heated and hydrolysed in acid medium. When formaldehyde is heated with chromotropic acid (4,5-dihydroxy-2,7-naphthalene disulphonic acid) in sulphuric acid solution, a violet-pink colour develops. The colour reaction is due to the formation of a formaldehyde-chromotropic acid complex which has maximum absorbance at 570nm.

Although the chemistry of the colour reaction is not known with certainty, evidence derived from the fact that aromatic hydroxy compounds condense with formaldehyde to yield

colourless hydroxydiphenylmethanes, has led to a proposed mechanism for the reaction (MacFadyen, 1945). The initial step of the colour reaction consists of a condensation of the phenolic chromotropic acid with formaldehyde and this is followed by an oxidation to a p-quinoidal compound (Figure 35).

Sulphuric acid participates in both phases of the reaction. Firstly, sulphuric acid hydrolyses the glycosidic bonds of azoxyglycosides to release the sugar moieties and MAM which decomposes into formaldehyde, methanol and nitrogen when heated. In the second phase, it functions as a dehydrant to bring about the condensation and also serves as oxidant and is reduced to sulphurous acid. Thus, a measurement of the absorbance at 570nm is related to the molar formaldehyde content which is representative of the molar azoxyglycoside content since one mole of formaldehyde is released per mole of azoxyglycoside.

4.3. HPLC method

High performance liquid chromatography (HPLC) is defined by Snyder and Kirkland (1974) as "an automated, high-pressure liquid chromatography in columns, with a capability for the high-resolution separation of a wide range of sample types, within a few minutes to perhaps an hour". High-performance liquid chromatography has many advantages over other methods of carbohydrate analysis. As compared to paper chromatography and open-column chromatography, it is much faster, more sensitive, and more suitable for routine quantitative analysis (McGinnis and Fang, 1980).

Figure 35 : Proposed scheme for the chromotropic acid reaction
(after MacFadyen, 1945)

While GLC methods for carbohydrate analysis are more sensitive, both HPLC and GLC procedures are approximately equivalent in terms of actual separation, in precision and accuracy, and in their potential for automation . However, the HPLC procedure for carbohydrate analysis is much faster and more suitable for routine analysis since the carbohydrates in aqueous solutions can be determined without derivitization or potential sample loss and with a minimum of sample preparation.

Detection of cycasin and macrozamin by HPLC is facilitated by the presence of the azoxy function in these molecules. UV-detection of the compounds are possible since the azoxy function absorbs strongly at 215nm.

4.4. GLC method

GLC is preferred to HPLC in a number of instances. It is a more sensitive technique, allowing the analysis of sub-nanomolar amounts of carbohydrates, and is generally less prone to interference (e.g. from salts and protein). Detection is usually by means of a flame ionization detector (FID) which responds to all carbohydrate-related molecules over an extremely wide linear range. GLC separation is dependent upon the differential extractive distillation of the components in the mixture. It is fundamental to the technique, therefore, that volatile derivatives of the carbohydrates are prepared.

The trimethylsilyl (TMS) derivitization technique for carbohydrate analysis has been adapted for azoxyglycoside analysis (Wells et al., 1968). This technique was employed in this investigation. The reaction involves derivitization of the hydroxyl groups with trimethylsilyl substituents. The trimethylsilyl derivative decreases the polarity and boiling

points of the molecules, thus allowing differential extractive distillation.

4.5. Internal standards

Internal standards are generally used for HPLC and GLC analyses since it is difficult to inject a reproducible proportion of a sample into the chromatograph. Standards may be any similar compound which is not already present in the mixture to be analysed and which is clearly separable from the other components. α-naphthol was chosen as a suitable internal standard for both the HPLC and GLC analyses since it is a synthetic compound with a conjugated system which makes UV-detection of the compound possible. In addition, the single hydroxy function is easily derivitized by trimethylsilylation for GLC application.

4.6. Experimental

4.6.1. Extraction of plant material

A weighed amount of approximately 0.5g of fresh plant material was macerated in 50% ethanol and made up to volume with 50% ethanol in a 25ml volumetric flask. The solution was sedimented overnight to give the primary extract.

4.6.2. Chromotropic acid assay

1.00ml of primary extract was diluted to 10ml with 50% ethanol to give the secondary extract. 3.0ml of chromotropic acid reagent (0.2g chromotropic acid dissolved in 20ml water and diluted to 100ml with 12.5M sulphuric acid) was added to

300μl of secondary extract and the mixture was heated for 30 minutes in a boiling water bath. Absorbances of the cooled solutions were measured at 570nm against blanks which contained 300μl secondary extract together with 3.0ml of 12.5M sulphuric acid.

The formaldehyde content of a commercial solution was assayed titrimetrically as follows: 5.00ml of the formaldehyde solution was diluted to 50ml with water. 20ml of the dilute solution was used for analysis, to which 30ml of 3% hydrogen peroxide (neutral to phenolphthalein) and 30ml of 1M sodium hydroxide were added. Evolution of a gas together with heat generation was observed after a short while, and the reaction was completed by boiling the solution for 10 minutes. The cooled solution was then back-titrated with 1M hydrochloric acid with phenolphthalein indicator. The amount of alkali consumed gives the formaldehyde content according to the equation:

$$2CH_2O + H_2O_2 + 2NaOH \longrightarrow 2HCOONa + H_2 + 2H_2O$$

The commercial formaldehyde solution, assayed as above, was diluted to give 0.15, 0.30, 0.45, 0.60 and 0.75μmol/ml solutions. 0.5ml of each of the solutions was then carried through the chromotropic acid procedure after the addition of 5ml chromotropic acid reagent. Absorbances at 570nm were read against blanks which contained 0.5ml of the respective standard together with 5ml of 12.5M sulphuric acid. Absorbances at 570nm for each standard are plotted against concentration (in μmol/ml) of the standard (Figure 36). Formaldehyde content of extracts was obtained from the calibration curve.
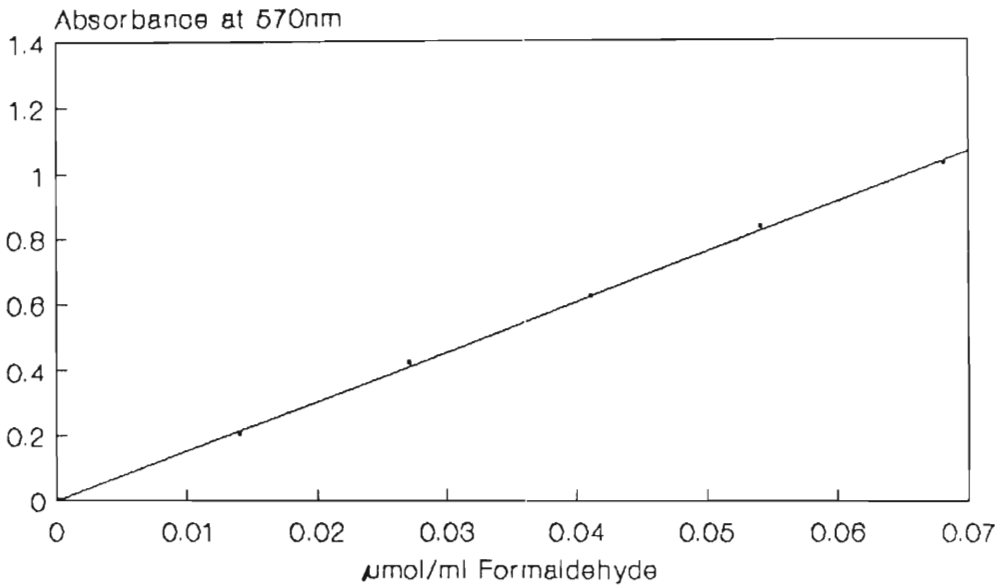
Figure 36: Formaldehyde calibration curve (by CTA assay)

4.6.3. HPLC method

The primary extract, 1.00ml, together with 1.0ml of 0.04% α-
naphthol were diluted to 10ml with methanol and the resulting
solution filtered through a 0.45 micron nylon mesh unit. A
10μl aliquot of the filtered solution was injected directly
into the HPLC machine comprising a Spectra Physics SP8700
solvent delivery system coupled to a SP8440 UV/VIS detector.
The column used was an analytical C-18 reverse phase HPLC
column and helium-degassed HPLC grade 80% methanol was used
as the eluting solvent. Cycasin and macrozamin were
quantified by measurement of the strength of absorbance at
215nm.

A standard 1mg/ml macrozamin solution was diluted to 5, 10, 15, 20, 25 and 30μg/ml solutions each of which contained 40μg of α-naphthol internal standard. The cycasin standard was a 0.1712mg/ml solution which was diluted to 6.85, 13.7, 20.55, 27.4, 34.25 and 41.1μg/ml solutions each of which contained 40μg of α-naphthol. 10μl of each cycasin and macrozamin standard was injected. Peak area ratios (toxin : α-naphthol) were plotted against the microgram amount of cycasin or macrozamin injected (Figure 37). Macrozamin and cycasin content of the extracts were derived from the calibration curve, and expressed as micromole toxin per gram fresh weight.
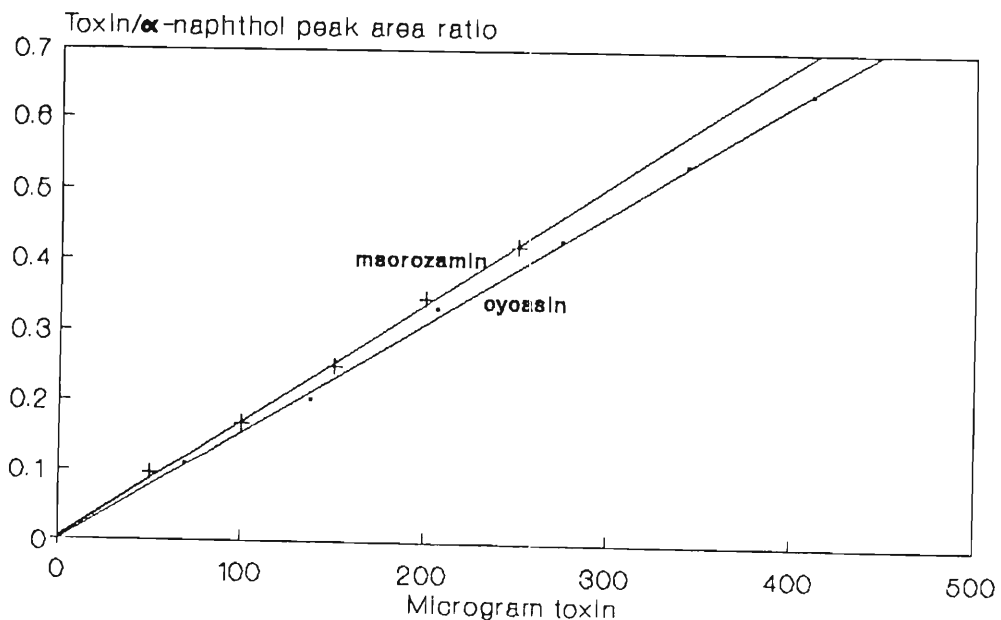
Figure 37: Calibration curve for cycasin and macrozamin (by HPLC)

4.6.4. <u>GLC method</u>

A volume of 10ml of primary extract together with 10ml of 0.1% α-naphthol were evaporated to dryness in silylation vials in a vacuum desiccator so that the dried extracts contained approximately 200mg plant material and 1mg α-naphthol. Silylation reactions were carried out on all dried samples prior to GLC analysis. Anhydrous conditions were required for the silylation reaction since water destroys the silylation reagent. The reaction was carried out by shaking each sample with trimethylsilylation (TMS) reagent which was a mixture of trimethylchlorosilane, hexamethyldisilizane and pyridine in a ratio of 1:3:9. A 3μl aliquot of each derivitized sample was injected into a Varian 6000 gas chromatograph provided with a flame ionization detector (FID) for peak detection, a 5% phenylmethylsilicone fused silica capillary column as the support medium and nitrogen as the carrier gas. Operating conditions were found to be optimized during an isothermal run at 240°C, with the injector and detector temperatures set at 250°C and 280°C respectively.

Quantities of 1.00mg of cycasin and macrozamin standards were analysed with α-naphthol after silylation. The average relative response factors for both cycasin and macrozamin, in relation to α-naphthol, were determined by repeated injection of these compounds and were used in the quantitative estimation by comparison with the response factors determined for each specimen.

4.7. Results and discussion

Chromatograms obtained for standard cycasin and macrozamin by HPLC analysis are depicted in Figures 38 and 39. The chromatogram for cycasin also depicts the macrozamin impurity whereas that for macrozamin indicates a relatively pure sample. Both chromatograms show the α-naphthol peak. Chromatograms obtained for the two compounds by GLC analysis are depicted in Figures 40 and 41, which also indicate that the cycasin sample contains impurity whereas the macrozamin sample is relatively pure.

The retention time for α-naphthol by GLC analysis preceeds that for either cycasin or macrozamin since the mono-TMS derivative of α-naphthol boils at a lower temperature than the tetra or hexa-TMS derivatives of cycasin and macrozamin respectively. On the other hand, the conjugated structure of α-naphthol causes its retention time to exceed that of cycasin and macrozamin during HPLC monitoring.

Results obtained for the quantitative analysis are displayed in Table 6. Generally, it was found that cycasin and macrozamin are present in detectable amounts in both vegetative and reproductive material with no apparent difference between male and female species. A discussion of the quantities of toxin detected by the three methods in the plant structures and the significance of the presence of toxin will first ensue before any discrepancies are explained. All figures listed in Table 1 for the quantitative estimation, together with those cited from the literature, in the discussion, are expressed as micromole toxin per gram fresh weight.
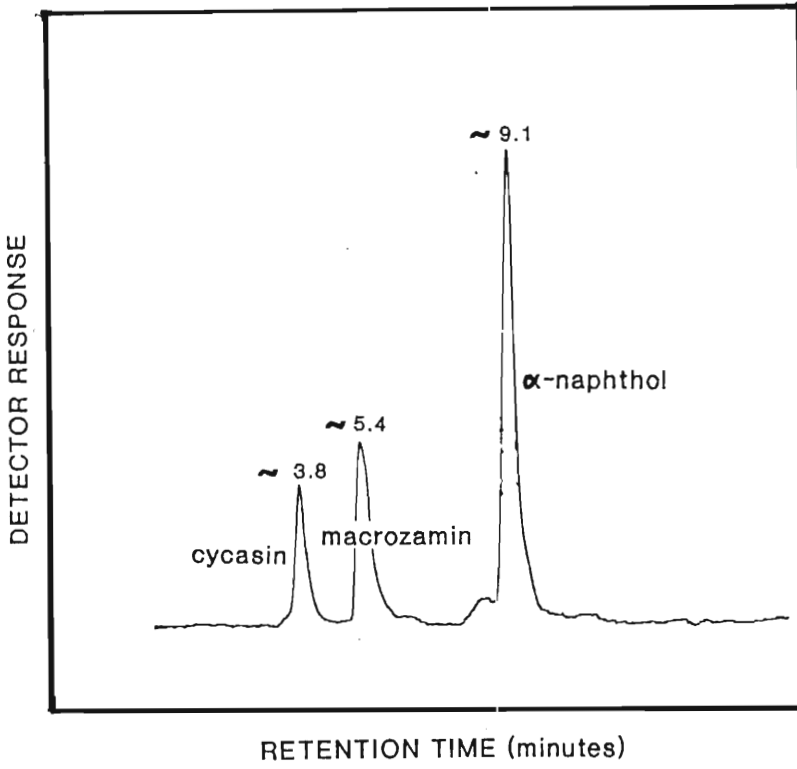
Figure 38: HPLC chromatogram for cycasin
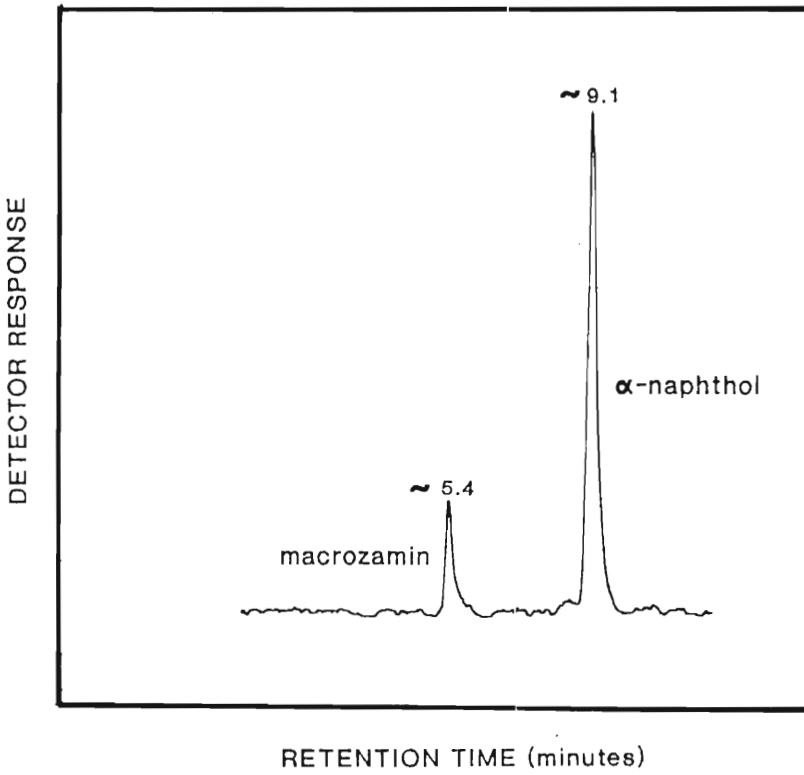
Figure 39: HPLC chromatogram for macrozamin
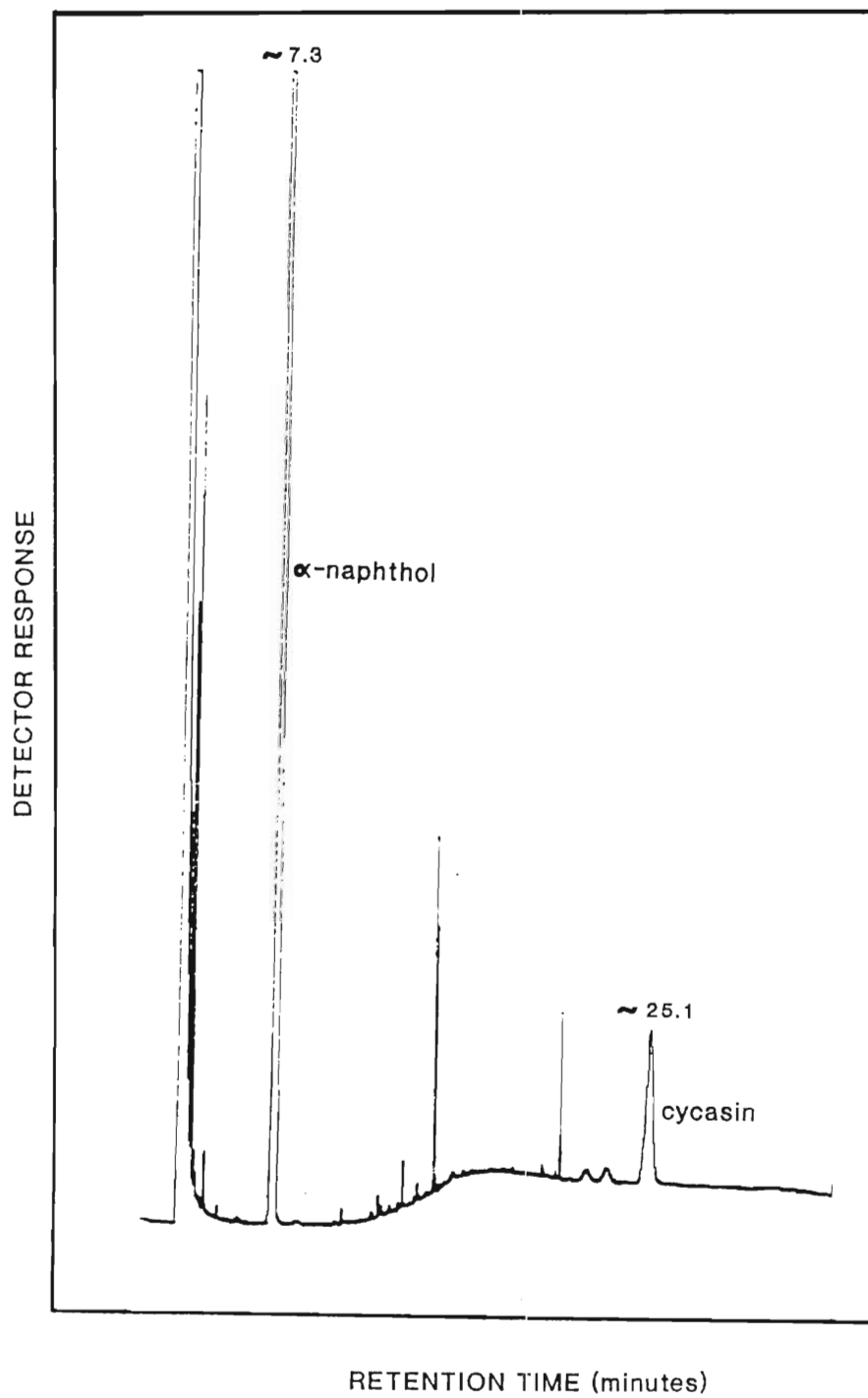
Figure 40: GLC chromatogram for cycasin

DETECTOR RESPONSE

~ 7.3

α-naphthol

~ 25.8

macrozamin

RETENTION TIME (minutes)
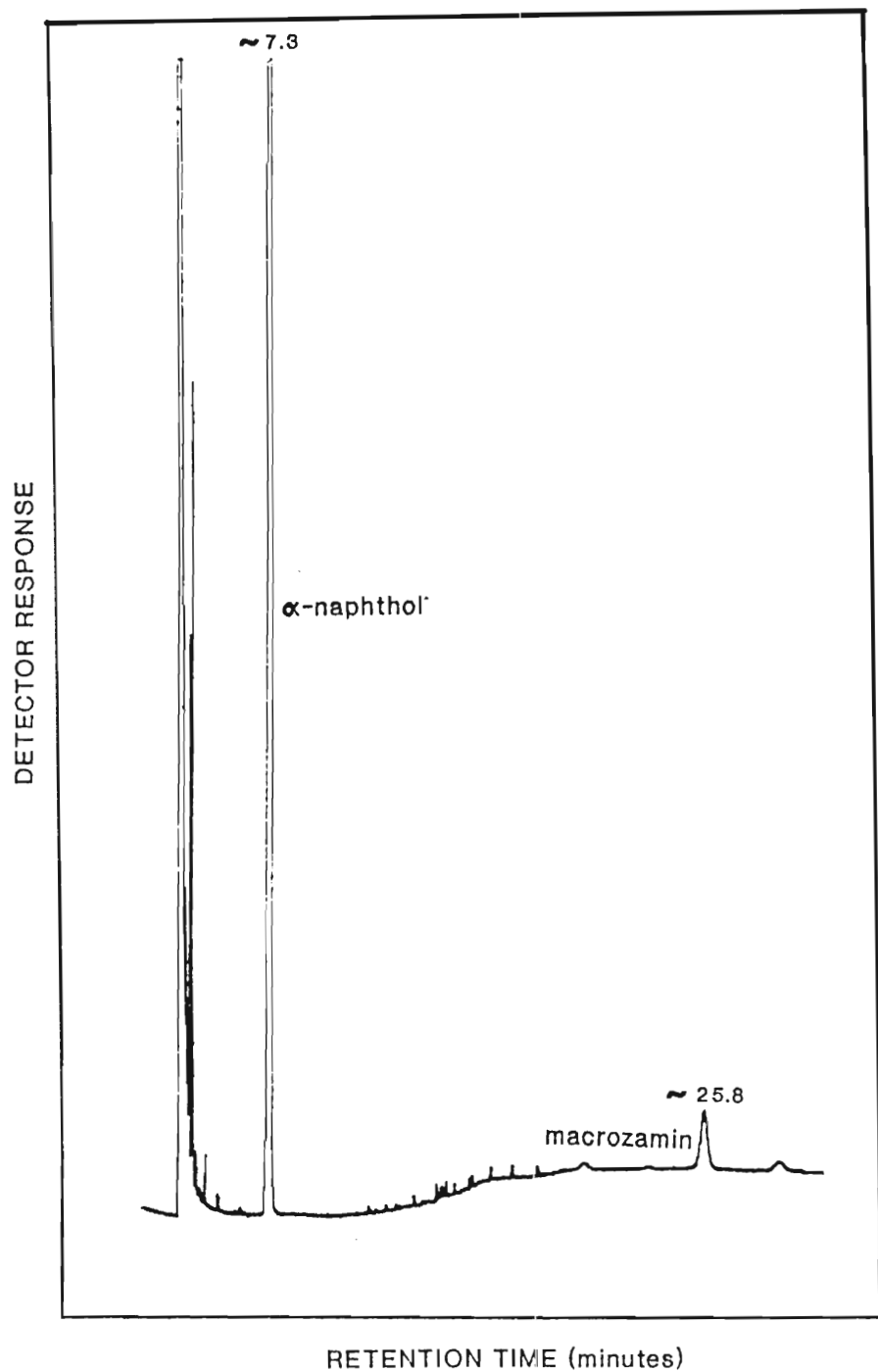
Figure 41: GLC chromatogram for macrozamin

Table 6: Quantification of macrozamin and cycasin in cycad
tissue by HPLC, GLC and chromotropic acid assay

	SPECIES	PLANT PART	CTA	HPLC	GLC
F	C.thouarsii	coralloid root	26.0	18.99	–
M	E.aemulans	"	26.5	43.7	–
F	E.aemulans	"	23.2	45.9	–
M	E.msinga	"	33.0	17.97	–
M	E.natalens	"	30.0	43.2	–
F	E.villosus	"	33.1	20.6	7.3
M	C.revoluta	mature leaf	30.4	140.9	–
F	C.revoluta	"	26.3	62.4	–
M	C.thouarsii	"	28.7	217.4	–
F	C.thouarsii	"	41.3	519.6	1.93
F	E.inopinus	"	8.70	7.25	–
F	E.natalens	"	11.5	243.2	–
F	E.transven	"	12.3	176.3	14.3
M	E.villosus	"	21.9	128.2	–
M	S.eriopus	"	50.9	225.7	–
F	S.eriopus	"	47.5	135.7	–
M	Z.furfurac	"	23.0	117.6	–
M	C.thouarsii	juvenile leaf	59.9	69.7	–
F	C.thouarsii	"	49.8	73.5	–
M	E.villosus	"	30.1	115.5	–
M	Z.furfurac	"	23.3	43.8	–
F	C.revoluta	cone scale	7.80	68.6	–
M	C.thouarsii	"	17.9	61.3	–
F	E.bubalinus	"	9.70	38.8	–
F	E.inopinus	"	7.50	59.99	–
M	E.natalens	"	15.0	31.6	–
F	E.natalens	"	22.3	39.8	–
M	E.villosus	"	18.8	116.9	–
M	S.eriopus	"	38.1	94.0	–
F	S.eriopus	"	48.1	40.8	0.60

Table 6 (contd.)

	SPECIES	PLANT PART	CTA	HPLC	GLC
M	C.thouarsii	cone axis	23.3	37.5	–
M	E.natalens	"	17.8	19.0	–
M	E.villosus	"	20.4	45.1	–
F	C.revoluta	sarcotesta	9.2	79.3	–
F	C.thouarsii	"	38.2	86.7	23.0
F	E.bubalinus	"	29.5	32.7	–
F	E.inopinus	"	19.8	64.6	–
F	E.natalens	"	28.6	55.0	–
F	S.eriopus	"	111.8	53.99	0
F	C.revoluta	kernel	39.3	59.4	–
F	C.thouarsii	"	105.5	55.6	2.14
F	E.bubalinus	"	24.8	51.8	–
F	E.inopinus	"	17.4	39.6	–
F	E.msinga	"	53.3	60.9	–
F	E.natalens	"	24.0	38.5	–
F	S.eriopus	"	214.8	121.1	–
M	C.thouarsii	pollen	50.7	55.3	0
M	E.lebomboen	"	25.9	102.2	0.18
M	E.natalens	"	20.9	44.7	–
M	E.transven	"	37.3	41.3	0
M	E.villosus	"	14.7	11.95	–
M	Z.furfurac	"	33.0	147.7	–

Table 6 (contd.)

Note: (1) Male and female specimens are denoted by the
          letters M and F respectively.

      (2) CTA results are expressed as μmol formaldehyde/gram
          fresh weight.

      (3) HPLC and GLC results are expressed as μmol (cycasin
          + macrozamin)/gram fresh weight.

      (4) The following abbreviations have been used:

          a. E.natalens for E.natalensis.

          b. E.transven for E.transvenosus.

          c. Z.furfurac for Z.furfuraceae.

          d. E.lebomboen for E.lebomboensis.

The amount of total toxin detected by the CTA method in the
coralloid roots indicates a degree of consistency between
species examined while cycasin and macrozamin content of the
same samples (from HPLC) are spread out over a wider range.
However, the results obtained for the coralloid roots by the
CTA and HPLC methods are generally most closely correlated
compared to results obtained by the two methods for other
structures.

The presence of azoxyglycoside toxins within coralloid roots
may be of some significance. Coralloid roots, which have been
found in all cycads examined so far (Lindblad, 1990), play an
important role as far as nitrogen metabolism is concerned
since they serve as hosts to cyanobacterial symbionts which
are responsible for fixing nitrogen. Grobbelaar (*pers. comm*)
has observed that the nitrogenase enzyme, which carries out
the six electron reduction of nitrogen to ammonia, is
inhibited in certain instances. Firstly, macerated coralloid
roots do not show any nitrogenase activity despite the fact
that they still contain many unbroken cyanobacterial

filaments. Secondly, cyanobacteria which have leached into culture media from sliced coralloid roots also lack nitrogenase activity.

Grobbelaar is of the opinion that either a prefabricated nitrogenase inhibitor is released by the wounded root or that wounding of the root stimulates it to produce a nitrogenase inhibitor. Evidence for the speculation that azoxy toxins may be involved in nitrogenase inhibition is derived from the fact that the inhibitor is deactivated both by adsorption onto activated charcoal and by heat. These two properties are characteristic of cycasin and macrozamin both of which were purified by adsorption onto charcoal (section 2.2.2.), and were found to be heat labile (section 3.5.). Investigation is required to establish whether this phenomenon is indeed true. The results of such studies would also shed light on the mechanism by which inhibition occurs. Yagi *et al.* (1983) detected an amount of 14.4µmol toxin/g in coralloid roots of *C.revoluta* which correlates well with the figure of 18.99 obtained during this investigation from HPLC analysis of coralloid roots of the female *C.thouarsii* plant of the same genus. Macrozamin and cycasin have also been found in tuberous roots of *C.revoluta* in which an amount of 8.5µmol toxin/g was determined by HPLC (Yagi and Tadera, 1987).

Largest discrepancies in results between HPLC and CTA methods were observed during analysis of leaf material. Except for the leaves of the female *Encephalartos inopinus* plant, which yielded closely-correlated values by the two methods, all other leaf specimens were found to contain grossly higher proportions of cycasin and macrozamin, as indicated by HPLC results, than anticipated from both CTA data and from the range of values obtained for leaf samples by others. Yagi and Tadera (1987) found an average value of 49.4µmol/g fresh weight for combined cycasin and macrozamin contents of leaves of *Cycas revoluta* by HPLC. The figure of 140.9µmol/g obtained by HPLC for male leaves of *C.revoluta* will have to be

regarded with some scepticism on the basis of the data of the above authors. However, the results for both male and female leaves of the same species, with exception of the above-mentioned specimen, obtained by either HPLC or CTA assay, correlate well with the figure of 49.4μmol/g. The figure of 14.3μmol/g (cycasin + macrozamin) obtained by GLC of leaves of the female *E.transvenosus* plant correlates well with the value obtained for the same specimen by the CTA method.

Juvenile leaves were generally observed to contain higher quantities of toxin than the corresponding mature specimen. Although the significance of this difference in toxin levels is not known, it is speculated that the presence of higher quantities of toxin may have an anti-predator function in the developing leaf which is the most vulnerable stage. Yagi *et al.* (1983) monitored toxin levels in developing leaves of *C.revoluta* over a period of 8 months and found that whereas macrozamin was detectable only at the young stage, cycasin content increased gradually after shoot elongation had ceased and showed a maximal value after two months. Rothschild *et al.* (1986) have also made a similar observation in *Zamia floridana*, juvenile leaves of which were found to contain higher quantities of cycasin than mature specimens.

All parts of both male and female cones tested were found to contain toxin. Results by the CTA method for cone scales were generally the lowest range of figures obtained of all structures. Cone axes of the male specimens examined also exhibited low quantities of toxin, and the results of the two methods of quantification are more-or-less correlated.

Despite popular belief that sarcotesta does not contain toxins (Giddy, 1984), the presence of toxin within the sarcotesta may bear relation to cases of cycad poisoning. The sarcotesta, which comprises the fleshy outer covering of cycad seeds, has been observed to be eaten by both man and

animals including monkeys, baboons and some species of birds (Giddy, 1984). Detection and quantification of azoxyglycosides in these structures may be useful to medical personnel in cases of cycad poisoning, and may possibly serve as a means by which the uninformed may be made aware of offending species.

The presence of toxin within cycad kernels has been well-documented. Not only have cycad kernels been used in many toxicity investigations, but they are the plant parts which appear to contain a rich source of the compounds since all azoxyglycoside compounds extracted for structure elucidation are derived from cycad kernels. It has also become apparent from other quantitative investigations that highest toxin concentrations are localized within these kernels. Siniscalco Gigliano (1990) has reported from GLC analyses that seed kernels of the species *Bowenia spectabilis* contain the highest macrozamin content (131.25µmol/g), and a smaller cycasin quantity (16.7µmol/g), of all species examined to date. A similar pattern of higher quantities of macrozamin than cycasin has been found in *Bowenia serrulata*, *Macrozamia miquelii*, *M.moorei* and all other specimens examined with the exception of *C.revoluta* and *C.lane-poolei* in which cycasin quantities are higher (Siniscalco Gigliano, 1990).

The results obtained by both CTA assay and HPLC indicate high values for kernels, compared to other structures, but not as high as that anticipated. For a given species, the observation is best represented by *Stangeria eriopus*, the kernels of which were found to contain, by both methods, higher proportions of toxin than the other structures of the same plant. Values of 122.4 and 0.794µmol/g have been reported for macrozamin and cycasin respectively in kernels of *S.eriopus* (Siniscalco Gigliano, 1990). Figures of 72.7 and 48.4µmol/g of macrozamin and cycasin respectively were detected by HPLC in kernels of the same species during this investigation. Whereas total macrozamin and cycasin

quantities (121.1μmol/g) detected in kernels of *S.eriopus* correlate well with those reported by the above author, the amount of macrozamin is considerably lower while cycasin content is grossly higher than that reported. The cycasin content of kernels of *S.eriopus* is therefore reported with reservation especially on the basis that the highest detected value of cycasin (28.6μmol/g), which has been reported for *C.lane-poolei* (Siniscalco Gigliano, 1990), is about one-half the value (48.4μmol/g) reported in this investigation. CTA result for the same specimen expresses the theoretical assumption, that the assay should yield higher values for toxin quantification than HPLC, but the magnitude of the difference is too great.

Total macrozamin and cycasin content in kernels of *C.revoluta* and *C.thouarsii* have been reported as 17.9 and 10.5μmol/g fresh weight respectively (Siniscalco Gigliano, 1990). Both these values are far lower than the values obtained for the above specimens by HPLC or CTA assay. While individual cycasin and macrozamin quantities detected in the two *Cycas* species are far higher than those reported by the above author, the results obtained by HPLC demonstrate the reported pattern of a higher quantity of cycasin than macrozamin in *C.revoluta* and the reverse situation in *C.thouarsii*. The figure of 2.14μmol/g obtained by GLC analysis of kernels of *C.thouarsii* comprises of 0.52μmol/g cycasin and 1.62μmol/g macrozamin. Siniscalco Gigliano (1990) has reported that the same specimen contained 2.4 and 8.1μmol/g cycasin and macrozamin respectively. Despite the discrepancy, the GLC result is the better approximation of the toxin content than either of the two other methods of detection.

Macrozamin and cycasin were detected in pollen samples of all three genera examined. The detection of macrozamin and cycasin in pollen samples is a significant contribution to azoxyglycoside quantification since no reports exist of analysis of these plant components. Results obtained for

pollen samples of *Cycas* and two members of the genus *Encephalartos* exhibit values which are comparable by CTA and HPLC methods. The presence of azoxyglycoside compounds in both male and female reproductive tissue may be a significant feature of the reproductive biology of the cycads.

Results obtained in this investigation by HPLC and CTA assay are generally much higher than those anticipated. This assumption is made on the basis that seed kernels, which were both assumed and reported to contain highest toxin levels, were found to contain far greater quantities of toxin than the corresponding literature reference. It is assumed, therefore, that the discrepancy in toxin levels between values reported for kernels in this investigation and those extracted from the literature is laterally applicable to other plant parts that were examined.

In particular, results obtained by the HPLC method were found to be grossly high in many specimens. For example, values of 519.6 and 243.2μmol/g, which are total cycasin and macrozamin values, obtained for analysis of leaf specimens of female *C.thouarsii* and *E.natalensis* plants respectively, are far greater than the corresponding CTA result. Such results are therefore reported with reservation.

Use has been made of a variable wavelength UV-detector for detection of the azoxy function at 215nm. The 215nm band is a sensitive region of the spectrum since many conjugated systems also absorb at this wavelength. Phenolic acids and biflavonoids are of particular interest with this regard since many representatives of both these class of compounds have conjugated structures, and many phenolic acids and biflavonoid compounds have been reported to occur in cycad material (Wallace, 1972; Dossagi *et al.*, 1975). It is possible that co-elution of such compounds with cycasin and macrozamin may have occurred which has resulted in elevated

figures for the azoxy toxins.

Higher toxin concentrations were anticipated from the CTA assay than either HPLC or GLC methods since the assay expresses total azoxyglycoside content in the form of released formaldehyde. However, results obtained by the assay were also higher, but not as high as in HPLC, than the available literature values. Discrepancy in results could be explained in terms of the fact that some of the detected formaldehyde could have arisen from sources other than azoxyglycosides. In addition, the pure enzyme emulsin β-glucosidase, which is present in the seed kernels, was found to give a colour reaction identical to that of formaldehyde on treatment with chromotropic acid (Dastur and Palekar, 1966). Also, it is reported that acid digestion of pentosans yields furfuraldehyde which also reacts with chromotropic acid to give the characteristic colour reaction (Feigl, 1956).

Discrepancy in results obtained by the three methods could also be explained in terms of a number of other factors. Enzymatic hydrolysis of cycasin and macrozamin has been reported to reduce yields of the compounds during quantitative analyses (Dastur and Palekar, 1966; Yagi et al., 1980; Yagi and Tadera, 1987). Procedures have been developed which aimed at minimizing or eradicating the element of hydrolysis by enzymes during extraction. The effect of boiling material before or during extraction has been explored (Dastur and Palekar, 1966), and has been found to be useful in denaturing cycad emulsin, but has also been observed to cause some decomposition of azoxyglycosides which are heat-labile.

Low temperature extraction has been reported to greatly minimize the effects of enzymes (Yagi et al., 1980, 1983; Yagi and Tadera, 1987). Chemical denaturation of cycad

emulsin by alcohol has been reputed to be an effective means of reducing the effect of the enzyme, but may not be sufficient to ensure a reaction that is totally devoid of any enzymatic interactions. Yagi and Tadera (1987), have expressed success with this regard by a procedure which employed both alcohol and freezing temperature during extraction. While chemical degredation of cycad enzymes by alcohol has been employed during this investigation, the effect of temperature was not considered and may have had some bearing on the results obtained.

It has been reported that a reliable estimate of azoxyglycosides can be made, despite the reducing effects of cycad enzymes, by measuring the breakdown products of the hydrolysis reaction. The hydrolysis reaction generates methylazoxymethanol (MAM) which is degraded further to methanol, formaldehyde and nitrogen. Yagi *et al.* (1980) used HPLC to quantify cycasin in seeds of *C.revoluta* by measuring the absorbance of the compound at 215nm. In addition, free MAM was also detected at 215nm, and formaldehyde was detected at 225nm by conversion into its semicarbazone. Both MAM and formaldehyde contributed to the value for the total cycasin content of the specimen.

Other factors which may be implicated in the discrepancy between values reported in the literature and those obtained in this investigation include environmental parameters such as variation in soil type and soil composition, altitude, habitat and climatic conditions. The above factors may interact with physiological and biochemical processes some of which may play an important role in the expression of the azoxy toxins. In addition, no systematic survey of such evidence exists to suggest that the quantities of toxin detected are species-specific.

Cycasin is characteristic of and exclusive to the cycads,

being present in 10 genera of the group that were examined, but absent from all other gymnosperm taxa (De luca *et al.*, 1980). Macrozamin has also been found to be ubiquitous in the 10 genera examined (Moretti *et al.*, 1983). It has become apparent from the above investigations that macrozamin is generally more abundant than cycasin and occurs in quantities that vary between 5.2μmol/g in *C.cairnsiana* and 131.3μmol/g in *Bowenia spectabilis*, while cycasin varies between very low quantities, in most of the species examined, to 28.6μmol/g in *C.lane-poolei*. While cycasin concentration is not significant taxonomically, macrozamin concentration does show differences at the generic level (Siniscalco Gigliano, 1990). The results of this investigation are not suitable for use in a taxonomic evaluation. However, results for mature leaves by the CTA assay exhibit a characteristic pattern of toxin distribution in which total toxin content of representatives of the 4 cycad genera examined increase in the order *Encephalartos*, *Zamia*, *Cycas*, *Stangeria*.

An offshoot of the quantitative analysis of azoxy toxins in cycad material has been the investigation to ascertain whether the compounds could be detected in insect predators. Two local species of predators which became available during the course of this work include the leopard magpie moth (*Zerenopsis leopardina*) and the so-called "cycad weevil" (*Antliarhinus zamiae*). The leopard moth appears in summer during which eggs are laid on cycad leaf surfaces and larvae, which develop very rapidly, have been observed to devour newly sprouted leaves, leaving only bare rachises. It is believed that the pupae bore down into the cycad plant where they remain until the following summer when the cycle is repeated (Osborne, *pers. comm.*). Potentially more damaging is the cycad weevil, female specimens of which are recognized by the elongated rostrum or "snout" which is used to drill through shells of cycad nuts in which eggs are laid (Goode, 1989). The larvae feed on the flesh of the seeds, and heavy infestation can destroy an entire seed-crop.

Amounts of 103.9 and 26.8μmol/g of cycasin and macrozamin respectively were found during this investigation by HPLC analysis of larvae of the leopard moth removed from *Encephalartos villosus* plants. The toxin value for the larvae represents 3.65% of fresh body weight whereas cycasin and macrozamin formed 9.85% of the fresh body weight of the adult moth. The distribution pattern of higher levels of toxin in the adult than in larvae of the leopard moth appears to be similar to the pattern observed in the Lycaenid butterfly (*Eumaeus atala florida*), which feeds on leaves of *Zamia floridana*, adults of which were found to contain more cycasin than the larvae or pupae (Rothschild *et al.*, 1986). Quantities of toxin detected in the cycad weevil were estimated as 71.4μmol/g cycasin and 47.7μmol/g macrozamin which together represent 3.63% of fresh body weight. The presence of toxin within the insects may serve as a deterrent to potential predators. It has been observed that there are no predators for the leopard moth (Osborne, *pers. comm.*).

The means by which the toxins have remained innocuous to the insects is probably the point of real fascination. Teas (1967) observed that larvae of the arctiid moth *Seirarctia echo*, which feed on *Zamia floridana*, exhibited measurable quantities of cycasin. It was also shown that the gut of the above insect contains β-glucosidase, and it was suggested that MAM intoxication is avoided by glucosylation or reglucosylation of liberated MAM to cycasin. This may also be the means by which the leopard moth and cycad weevil are protected from the toxic effects of cycasin metabolites. No information is available on detoxification of macrozamin, but enzymatic means is probably the most-likely mechanism. The ability that the insects possess to cope with toxins, together with other characteristic features, indicates a long association with the plant host.

The three methods employed in the quantitative analysis are useful in their own right. The CTA assay is relevant to

azoxyglycoside analysis since it is specific for and sensitive to formaldehyde, a breakdown product of the hydrolysis of azoxy compounds. The assay is probably best suited for analysis of structures which do not contain pigments when the colour reaction is clearly visible. Decolorization or removal of pigments such as chlorophyll and carotenoids prior to analysis would help much in the examination of structures such as leaves and sarcotestae.

HPLC analysis by use of a variable wavelength UV-detector is useful for detection of azoxyglycosides at 215nm which is the wavelength of maximum absorption of the compounds. However, the fact that other compounds also absorb at this wavelength cannot be ignored. A system has to be devised which would ensure that the 215nm absorption is as a result of the azoxy function alone.

GLC analysis of trimethylsilyl derivatives of cycasin and macrozamin is possible since the molecules possess the essential hydroxy groups. Values obtained by GLC analysis are more comparable to reported values than those obtained by either of the two other methods, which suggests that the technique requires greater attention. Available resources did not allow analysis of all specimens during this investigation.

While Australasian and American species have been used frequently in assays for detection and quantitative estimation of cycad toxins, reference to analysis of African species is lacking. The investigation carried out during the course of this work is significant, therefore, in this respect. While the results obtained may not be applied in the manner as have been reported, they may serve as a foundation for further investigation. Consequently, it can be stated without uncertainty that investigations of such nature should form an integral part of on-going research.

4.8. Implications to the biosynthesis of azoxyglycosides

The azoxy group, in fact, is very rare in organisms and it is known only in three other naturally-occurring compounds which have been isolated from two bacterial *Streptomyces* species (Shoental, 1969), and one species of fungus, *Calvatia lilacina* (Gasco *et al.*, 1974). The presence of azoxyglycosides, which have as yet been detected only in the cycads, is the central phytochemical aspect which sets these plants apart from all other plant groups. The striking feature of this group of toxins is the azoxy function and its presence in the cycads is the first real indication that biological systems are capable of synthesizing and metabolizing such compounds. A biosynthetic consideration for these compounds should therefore have the azoxy function as its focus. The exploration of the biosynthetic pathway for these unique compounds would contribute to the understanding of the properties which they exhibit, especially their well-documented toxic potential.

A survey of the literature reveals that the biosynthetic pathway of the azoxyglycosides is not known. While quantitative studies which employed either or a combination of chromatographic, spectrophotometric and analytical techniques have revealed the presence of the compounds in varying concentrations in the plant organs, the site of the biosynthesis has not been established. However, since the properties of the molecule arise from the azoxy function, it could be speculated that synthesis may be initiated in an organ that is capable of metabolizing molecular nitrogen. The roots of plants which fix nitrogen are the starting-centres for the biological nitrogen cycle. It is in cycad "coralloid" roots with their associated cyanobacterial symbionts that molecular nitrogen, derived mostly from the atmosphere, is reduced to a form that can be further metabolized by the plant. The fixation step involves the six-electron reduction of nitrogen to ammonia by the enzyme nitrogenase (Bray, 1983)

as shown below.

$$N_2 + 6H^+ + 6e^- \xrightarrow{\text{Nitrogenase}} 2NH_3$$
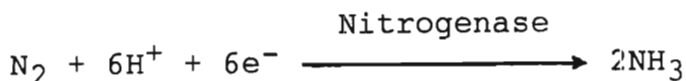
Bray notes further that free intermediates of the reductive process e.g. hydrazine (N_2H_4) or diimide (N_2H_2) have not been detected, but that it has been possible to demonstrate the presence of an enzyme-bound dinitrogen hydride intermediate only when the enzyme is reducing nitrogen.

The role of the dinitrogen hydride intermediate has not been established but it could be speculated that it may serve as the starting material for the biosynthesis of MAM from which all azoxyglycoside compounds arise. Evidence for the possible role of the intermediate as starting material is derived from an investigation into the chemical synthesis of MAM-acetate. The acetate derivative of MAM was synthesized from 1,2-dimethylhydrazine by oxidation of the compound to azoxymethane, followed by bromination in the allylic position and subsequent conversion to the acetate (Matsumoto et al., 1965; Laqueur, 1977), as depicted in Figure 42.

NOTE: a. The compounds numbered 1, 2 and 3 are 1,2-dimethylhydrazine, azoxymethane and MAM-acetate respectively.

b. The reaction steps have the following notation:

a- oxidation

b- bromination

c- acetylation

Figure 42: Chemical Synthesis of MAM-acetate (after Matsumoto et al., 1965 and Laqueur, 1977)
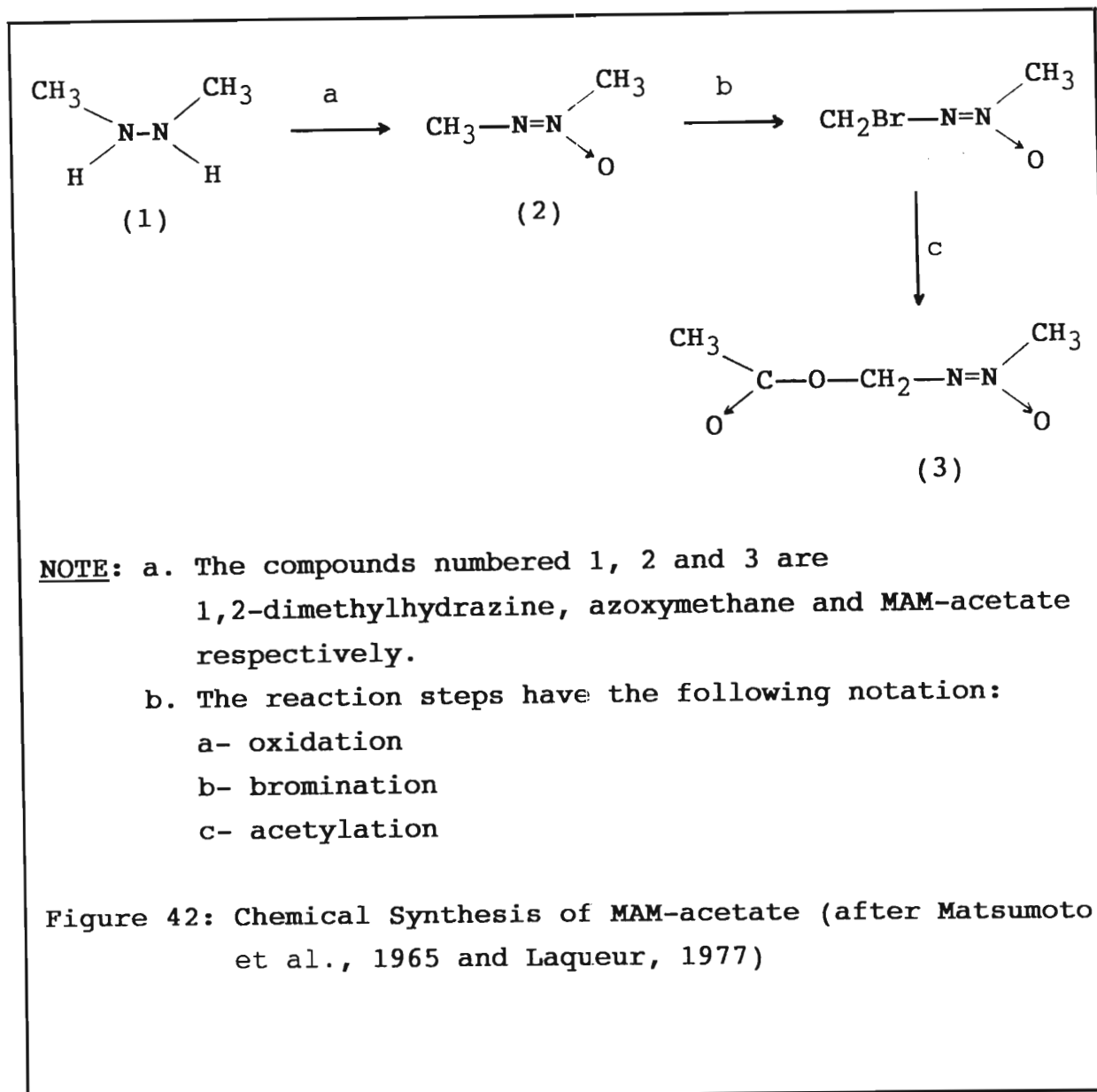
The above series of reactions allows speculation of the existence, in the cycad plant, of a dinitrogen hydride intermediate, chemically similar to 1,2-dimethylhydrazine, which leads to the production of MAM. The biosynthetic process would obviously differ from that presented in the synthetic reaction above and may involve complex enzymatic processes.

The biosynthetic process for the formation of MAM from a free intermediate of the reductive reaction in the nitrogen cycle should therefore be exclusive to the cycads. However, the reactions preceeding the formation of MAM are not exclusive

to the cycads since they form an integral part of the convential nitrogen cycle. Thus the biosynthetic process discussed above sets the cycads apart as a group of plants which have possibly evolved the ability to incorporate both chemical and enzymatic reactions, into the nitrogen cycle, which lead to the formation of MAM.

The formation of azoxy compounds by reactions other than those involving the nitrogen cycle may also be a possible means by which azoxy toxins arise. Fiala *et al.* (1978) have speculated that azoxy products might arise from the coupling of N-hydroxy and C-nitroso intermediates during the metabolic oxidation of aliphatic amines. In the investigation to test their hypothesis that aliphatic azoxy compounds could be formed from the corresponding amines, Fiala *et al.* (1981a) found that azoxy-2-phenylethane was formed from ^{14}C-labelled phenylethylamine that was incubated with rabbit liver microsomes, manganese ions (Mn^{2+}) and a NADPH-generating system as shown below.

Ph—CH$_2$—CH$_2$—NH$_2$ ⟶ Ph—CH$_2$—CH$_2$ / N=N / \ O ↖ CH$_2$—CH$_2$—Ph

phenylethylamine azoxy-2-phenylethane
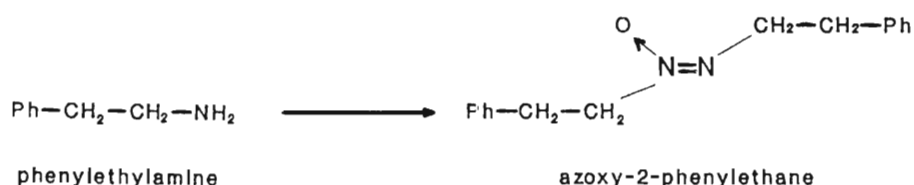
In an analogous experiment, Fiala *et al.* (1981b) found that azoxycyclohexane was detected as an *in vitro* metabolite of cyclohexylamine as depicted below. That this is the similar phenomenon in cycads, for the formation of MAM from the corresponding aliphatic amine, remains to be investigated. However, the investigation of the above authors represents the first demonstration of the formation of aliphatic azoxy compounds in a biological system.

cyclohexylamine ⟶ azoxycyclohexane
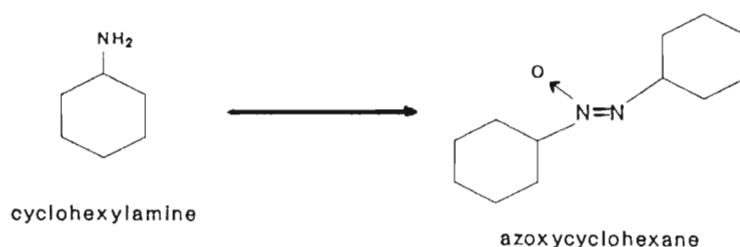
Enzymatic conversion of MAM to the azoxyglycosides has been accepted as the mechanism for the last reaction in the biosynthetic pathway. Tadera *et al.* (1985) discovered the presence of the enzyme UDP-glucose:methylazoxymethanol glucosyltransferase which catalyses the transfer of glucose from a UDP-glucose substrate to MAM in the leaves of *Cycas revoluta*. This reaction affords a reasonable mechanism for the formation of cycasin. Since the cycasin structure is common to all known azoxyglycoside compounds, it probably serves as a precursor for other azoxyglycosides.

Macrozamin, like cycasin, is a major azoxyglycoside and could arise by the addition of xylose to cycasin. However, it has been suggested from evidence that macrozamin is ubiquitous in the 10 genera examined, in which it occurs in larger quantities than cycasin, that the compound may represent the most primitive MAM glycoside (Siniscalco Gigliano, 1990). In addition, evidence derived from recent work by Yagi and Tadera (1987) suggests that macrozamin may be formed directly from MAM. The above authors found that the enzyme β-primeverosidase, a glucosidase which occurs in the leaves of *Cycas revoluta*, was capable of hydrolysing primeveroside from macrozamin. The presence of β-primeverosidase in the leaves of the plants raises the possibility of existence of a reciprocal transferase enzyme, which catalyses the transfer of primeverose to MAM, analogous to the glucosyltransferase enzyme. The neocycasins which include both disaccharide and trisaccharide azoxy compounds have cycasin as a common entity, and form either by the addition of sugars onto the cycasin skeleton or from a mixture containing both cycasin and macrozamin, in an enzymatic reaction involving transglucosylation (Nagahama, 1964; Yagi *et al.*, 1985a, b).

Although the site of the biosynthesis cannot be pinpointed, it may be speculated that the process is initiated in the roots because of the availability of metabolizing molecular nitrogen. The dinitrogen hydride intermediate of the

reductive reaction in the nitrogen cycle may be converted to MAM in some other organ. The process would require transportation. Pate (1990) has observed the transfer of certain compounds containing fixed nitrogen to the host cycad through the xylem during the assimilation of nitrogen within the coralloid roots of *Macrozamia riedlei*. MAM is then converted to the different azoxyglycosides via an enzymatic reaction by a transglucosylation mechanism. The proposed biosynthetic pathway for the azoxyglycosides is depicted in Figure 43.

There are some aspects of the proposed pathway which require comment. The glucosyl transferase enzyme has been detected only in the leaves of *Cycas revoluta* and would therefore require an investigation to establish whether the enzyme is present in other species which contain cycasin. The investigation should also aim at determining whether the enzyme is present in plant organs other than leaves. The results of such studies would shed some light to the understanding of the site of biosynthesis.

Quantitative studies reveal that highest toxin concentrations occur in the seed kernels (section 4.7.). Since coning is an intermittent event, while biosynthesis of azoxyglycosides is not necessarily season-dependant, it is speculated that seed kernels are not hosts to any active component of the biosynthetic process. However, the significant amount of toxin in cycad seeds represents the reservoir which would not only become part of the phytochemical make-up of the developing plant, but may ensure that seeds survive by serving as a deterrent to potential predators.

nitrogen cycle

NITROGEN FIXATION

DINITROGEN HYDRIDE INTERMEDIATE (N_2H_x)

MAM

glucose   transferase   primeverose
                   enzymes

CYCASIN   MACROZAMIN

transglucosylation

OTHER AZOXYGLYCOSIDES
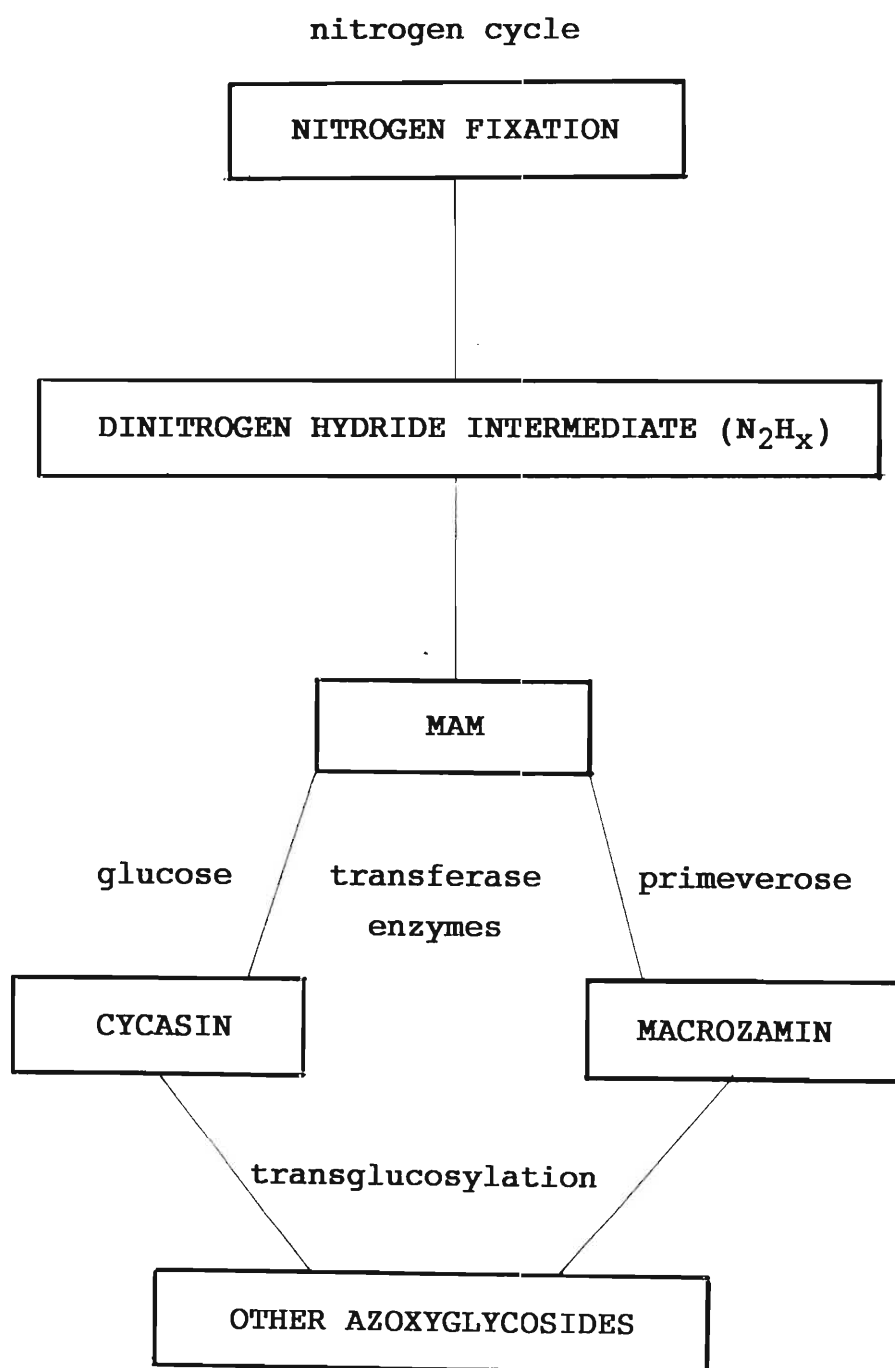
Figure 43: Proposed biosynthetic pathway for the
azoxyglycosides

Since they perform a host of synthetic functions, it is speculated that the leaves are the most probable site at which synthesis may continue after initiation in the roots. The presence of the necessary transferase enzymes in the leaves of cycads would also complement this suggestion. The innovative work of Fiala *et al.* will have to be extended to include plant systems to ascertain whether the phenomenon of formation of aliphatic azoxy compounds from the corresponding amines is applicable to such systems.

Azoxyglycosides represent the products of a primitive biochemical pathway devoted to nitrogen storage (Siniscalco Gigliano, 1990). Cycads retained these products probabaly for their toxic effects, which may have played an important ecological role during the long evolutionary history of the plants. Elucidation of the biochemical pathway for the compounds would be useful in understanding the systematic position of the cycads in the botanical hierarchy and their evolutionary history.

CHAPTER 5

SUMMARY AND CONCLUSIONS

Toxic compounds generally serve as defence mechanisms in organisms. The presence of azoxyglycoside compounds in cycads may have played an important role in ensuring the survival of the plants during their long evolutionary history. Naturally-occurring compounds that contain the azoxy function are rare. Toxicity of the azoxyglycosides has been attributed to the aglycone, methylazoxymethanol (MAM). Metabolic activation of cycasin involves deglucosyltion by β-glucosidase which releases glucose and MAM which is further activated, via diazomethane, to generate methylene units ($:CH_2$). These ionic species are reactive intermediates which are capable of methylating macromolecules which include DNA, RNA and protein. The phenomenon of alkylation of macromolecules has been accepted as one of the causes of cancer.

The principle of extraction of cycasin and macrozamin is based on the fact that the sugar components form the structural bulk of these molecules and as such they react like carbohydrates. Identification of cycasin and macrozamin by spectroscopic techniques is based on characteristic signals which arise as a result of the azoxy function. UV-detection of the compounds is characterized by the absorbance at 215nm. Signals which arise as a result of the the azoxy function are also a characteristic feature of spectra obtained by infra-red and nuclear magnetic resonance spectroscopy.

The low yield of cycasin is due to the choice of species, *Cycas thouarsii*, from which the compound was extracted. *Cycas revoluta* would have provided a richer source of cycasin, but no local plants were available. It has become apparent from the low yields that were retrieved for both cycasin and

macrozamin that larger quantities of material are required
for extraction. Extraction and identification of cycasin and
macrozamin is relevant not only in terms of structure
elucidation but also useful for kinetic studies and
quantitative estimations. In this respect the investigation
is significant since no local source of the compounds exist.

Hydrolysis of azoxyglycosides proceeds by use of acid, base
or enzyme. Spectrophotometric measurement of the progressive
reduction of the absorbance at 215nm is useful for monitoring
the course of the hydrolysis reaction. The rate of hydrolysis
of macrozamin with 4M sulphuric acid increases with every
10°C rise in temperature in the range 50-100°C. Cycasin
hydrolyses at a greater rate than macrozamin since it
contains a single glycosidic linkage. A second glycosidic
linkage in macrozamin is observed to decrease the rate of
hydrolysis of the compound. Methylazoxymethanol hydrolyses
more rapidly than either cycasin or macrozamin since it is
not inhibited by the rate of cleavage of any glycosidic
bonds. The activation energies, which are calculated from the
rate constants, increase in the order methylazoxymethanol,
cycasin, macrozamin. Base hydrolysis proceeds more rapidly
than the acid reaction under similar conditions. Cycasin and
macrozamin are also hydrolysed by β-glucosidase and β-
primeverosidase respectively.

Hydrolysis of macrozamin and cycasin is viewed not only as a
kinetic exercise, but may bear relation to the manner by
which the toxicity of the compounds is manifested. Hydrolysis
of the compounds is a prerequisite for their toxicity to be
manifested. Thus, alignment of parameters during kinetic
experimentation with those that occur naturally during
toxicity assays may be useful in understanding the toxic
properties of the azoxy compounds. A comparative study of
hydrolysis of methylazoxymethanol, cycasin and macrozamin by
acid, base and enzyme is an interesting prospect for further
investigation.

Cycasin and macrozamin occur in detectable amounts in various tissue of both male and female cycad plants. The chromotropic acid assay for formaldehyde determination is useful for total azoxyglycoside analysis since one mole of formaldehyde is released per mole of azoxglycoside hydrolysed. However, the fact that formaldehyde could have arisen from sources other than azoxy compounds cannot be ignored. HPLC analysis by use of a variable wavelength UV-detector is facilitated by the presence of the azoxy function, which absorbs at 215nm, in cycasin and macrozamin. The 215nm absorption band is a sensitive region of the spectrum since molecules containing conjugated systems also absorb at this wavelength.

GLC analysis by the trimethylsilylation derivitization technique is applicable since both macrozamin and cycasin contain hydroxy groups. Results of specimens which were analysed by GLC generally occur within the range of figures that have been reported for cycasin and macrozamin. Toxin values for cycad tissue have been found to be applicable taxonomically. Thus, toxin profiles, like hydrocarbon profiles, for various cycad species may in future be included as a parameter in the taxonomic evaluation.

A knowledge of the biosynthetic pathway for the azoxyglycosides may be useful in understanding the properties of the molecules and their evolutionary history. Monitoring of toxin levels in the different organs of the cycad plant together with experiments involving the use of radioactive nitrogen, which is fundamental to the azoxy function, and following its course through the plant, may be useful in the elucidation of the biosynthetic processes.

REFERENCES

ALTENKIRK, B. 1974. Occurrence of macrozamin in the seeds of *Encephalartos transvenosus* and *E. lanatus. Lloydia* 37: 636-637.

AMES, B.N. 1982. Mutagens, carcinogens, and anti-carcinogens. In *Genetic Toxicology and Agricultural Perspectives*, ed. Fleck, R.A. and Hollaender, A., pp. 499-508, New York : Plenum.

ARNOLD, C.A. 1953. Origins and relationships of the cycads. *Phytomorphology* 3: 51-65.

AVERY, H.E. 1976. Basic Reaction Kinetics and Mechanisms. Second edition. London: Macmillan.

BAUMAN, A.J. and YOKOHAMA, H. 1976. Seed coat carotenoids of the cycad genera *Dioon*, *Encephalartos*, *Macrozamia* and *Zamia* : evolutionary significance. *Biochemical Systematics and Ecology* 4: 73-74.

BLAGROVE, R.J., LILLEY, G.G. and HIGGINS, A. 1984. *Australian Journal of Plant Physiology* 11: 69-78.

BOUCHEZ, A.J., ARPIN, N., DERUAZ, D. and GUILLLUY, R. 1970. Chemotaxonomy of vascular plants. XX. Chemical study of *Cycas revoluta*; seed pigments. *Plantes Medicinales et Phytotherapie* 4: 117-125.

BRAY, C.M. 1983. Nitrogen Metabolism in Plants. First edition London and New York: Longman.

CALNE, D.B., EISEN, A., McGEER, E. and SPENCER, P.S. 1986. *Lancet* pp.1967-1968.

CAMPBELL, M.E., MICKELSON, O. YANG, M.G., LAQUEUR, G.L. and KERESZTESY, J.C. 1966. Effects of age, strain and diet on the response of rats to ingestion of *Cycas revoluta. Journal of Nutrition* 88: 115-124.

CARDINI, F., GINANNESCHI, M., SELVA, A. and CHELLI, M. 1987. Semi-β-carotenone from leaves of two cycads. *Phytochemistry* 26: 2029-2031.

COOPER, J.M. 1940. Isolation of a toxic principle from the seeds of *Macrozamia spiralis*. *Proceedings of the Royal Society of New South Wales* 74: 450-454.

DASTUR, D.K. 1964. Proccedings of the third conference on the toxicity of cycads. *Federation Proceedings* 23: 1368.

DASTUR, D.K. and PALEKAR, R.S. 1966. Effect of boiling and storing on cycasin content of *Cycas circinalis* L. *Nature* 210: 841-843.

DE LUCA, P., MORETTI, A., SABATO, S. and SINISCALCO GIGLIANO, G. 1980. The ubiquity of cycasin in cycads. *Phytochemistry* 19: 2230-2231.

DE LUCA, P., MORETTI, A., SABATO, S. and SINISCALCO GIGLIANO, G. 1982. A comparative study of cycad mucilages. *Phytochemistry* 21: 1609-1611.

DOSSAGI, S.F. and HERBIN, G.A. 1972. Occurrence of macrozamin in the seeds of *Encephalartos hildebrandtii*. *Federation Proceedings* 31: 1470-1472.

DOSSAGI, S.F. and BELL, E.A. 1973. Distribution of α-amino-β-methylaminopropionic acid in *Cycas*. *Phytochemistry* 12: 143-144.

DOSSAGI, S.F., MABRY, T.J. and BELL, E.A. 1975. Biflavonoids of the Cycadales. *Biochemical Systematics and Ecology* 2: 171-175.

DUNCAN, M.W., KOPIN, I.J., LAVINE, L., GARRUTO, R. and MARKEY, S.P. 1988. The putative neurotoxin BMAA in cycad-derived foods is an unlikely cause of ALS-PD. *Lancet* pp. 631-632.

DYER, R.A. 1965. The Cycads of Southern Africa. *Bothalia* 8: 405-515.

ECKENWALDER, J.E. 1980. Cycads: the prime of their lives. *Fairchild Tropical Garden Bulletin* 35: 11-19.

FEIGL, F. 1956. Spot Tests in Organic Analysis. Fifth edition Amsterdam: Elsevier Publishing Company.

FEINBERG, A. and ZEDECK, M.S. 1980. Production of a highly reactive alkylating agent from the organospecific carcinogen methylazoxymethanol by alcohol dehydrogenase. *Cancer Research* 40: 4446-4450.

FIALA, E.S., KULAKIS, C., CHRISTIANSEN, G. and WEISBURGER,J.H 1978. Inhibition of the metabolism of the colon carcinogen, azoxymethane, by pyrazole. *Cancer Research* 38: 4515-4521.

FIALA, E.S., KOHL, N.E., HECHT, S.S., YANG, J.J. and SHIMADA, T. 1981a. The formation of azoxy-2-phenylethane during the biological oxidation of phenylethylamine. *Carcinogenesis* 2: 165-173.

FIALA, E.S., KOHL, N.E., HECHT, S.S., YANG, J.J. and SHIMADA, T. 1981b. Formatiom of mutagenic aliphatic azoxy compounds from the corresponding amines by rabbit liver microsomes. *Proceedinds of the American Association of Cancer research and the American Association of Clinical Oncology* 22: 373.

FITZGERALD, W.W.A. 1898. Travels in the coastlands of British East Africa and the islands of Zanzibar and Pemba. London: Faber and Faber.

GADEK, P.A. 1982. Biflavonoids from the seed testa of Cycadales. *Phytochemistry* 21: 889-890.

GADEK, P.A., QUINN, C.J. and ASHFORD, A.E. 1984. Localization of the biflavonoid fraction in plant leaves, with special reference to *Agathis robusta*. *Australian Journal of Botany* 32: 15-32.

GAJDUSEK, D.C. 1982. An update on longterm *in vivo* and *in vitro* studies designed to identify the cause of amyotrophic lateral sclerosis, Parkinsonism-dementia, and Parkinson's disease. In *Human Motor Neuron Disease*, ed. Rowland , L.P. First edition, pp.343-353. New York: Raven Press.

GASCO, A., SERAFINO, A. MORTARINI, V. and MENZIANI, E. 1974. An antibacterial and antifungal compound from *Calvatia lilacina. Tetrahedron Letters*, pp.3431-3432.

GIDDY, C. 1984. Cycads of South Africa. Second revised edition. Cape Town: Struik.

GOODE, D. 1989. Cycads of Africa. First edition. Cape Town: Struik.

GRAB, D.J., and ZEDECK, M.S. 1977. Organ-specific effects of the carcinogen methylazoxymethanol related to metabolism of nicotinamide adenine dinucleotide-dependent dehydrogenase. *Cancer Research* 37: 4182-4189.

GRAHAM SOLOMONS, T.W. 1980. Organic Chemistry. Second edition, pp.341-343. New York: Wiley and sons.

GREENE, F.D. and HECHT, S.S. 1969. Cyclic azoxy compounds: relation of structural consideration to N.M.R. spectra. *Tetrahedron Letters*, pp.575-578.

GROBBELAAR, N. 1989. Personal communication. University of Pretoria, South Africa.

HALL, W. T. and McGAVIN, M. D. 1968. Cinical and neuropathological changes in cattle eating the leaves of *Macrozamia lucida* and *Bowenia serrulata*. *Pathologia Veterinaria* 5: 26-34.

HARDIN, J.W. and ARENA, J.M. 1974. Human Poisoning from Native and Cultivated Plants. Second edition. North Carolina: Duke University Press.

HEGNAUER, R. 1986. Chemotaxonomie der Pflanzen. Band 7, pp. 462-474. Basel: Birkhäuser Verlag.

HERBIN, G.A. and ROBINS, P.A. 1969. Plant cuticular waxes. VI Patterns of variation and development in leaf wax alkanes. *Phytochemistry* 8: 1985-1998.

HIRONO, I., LAQUEUR, G.L. and SPATZ, M. 1968. Tumor induction in Fischer and Osborne-Mendel rats by a single administration of cycasin. *Journal of the National Cancer Institute* 40: 1003-1010.

HIRONO, I. and SHIBUYA, C.H. 1967. Induction of a neurological disorder by cycasin in rats. *Nature* 216: 1311-1312.

HOUGH, L.J., JONES, K.N. and WADMAN, W.H. 1949. Quantitative analysis of mixtures of sugars by the method of partition on chromatography. Part IV. The separation of the sugars and their methylated derivatives on columns of cellulose. *Journal of the Chemical Society*, pp.2511-2516.

JACOBS, M.M. 1977. Inhibitory effects of selenium on 1,2-dimethylhydrazine and methylazoxymethanol colon carcinogenesis. *Cancer* 40: 2557-2564.

JOHNSON, L.A.S. 1959. The families of cycads and the Zamiaceae of Australia. *Proceedings of the Linnaen Society of New South Wales* 68: 64-117.

KINGSBURY, J.M. 1979. The problem of poisonous plants. In *Toxic Plants*. New York: Columbia University Press.

KIRMSE, W. 1971. Carbene Chemistry. Second edition, pp.18-21. New York and London: Academic Press.

KOBAYASHI, A. 1972. Cycasin in cycad materials used in Japan. *Federation Proceedings* 31: 1476-1477.

KOBAYASHI, A. and MATSUMOTO, H. 1954. Methylazoxymethanol the aglycone of cycasin. *Federation Proceedings* 23: 1354.

KOBAYASHI, A. and MATSUMOTO, H. 1965. Studies on methylazoxymethanol, the aglycone of cycasin. *Archives of Biochemistry and Biophysics* 110: 373-380.

KOBAYASHI, A., TADERA, K. and YAGI, F. 1980. Natural Toxins. First edition, pp.647-657. London and New York: Pergamon.

KORSCH, B.H. and RIGGS, N.V. 1964. Proton magnetic resonance spectra of aliphatic azoxy compounds and the structure of cycasin. *Tetrahedron Letters*, pp.523-525.

KURLAND, L.T. 1988. Amyotrophic lateral sclerosis and Parkinson's disease complex on Guam linked to an environmental neurotoxin. *Trends in Neuroscience* 11: 51-54.

LAMB, S. 1895. Plants injurious to stock. *Agricultural Gazette of New South Wales* 6: 505.

LANGLEY, B.W., LYTHGOE, B. and RAYNER, L.S. 1952. Macrozamin. Part III. Azoxymethane and some simple analogues. *Journal of the Chemical Society*, pp.4191-4198

LANGLEY, B.W., LYTHGOE, B. and RIGGS, N.V. 1951. Macrozamin. Part II. The aliphatic azoxy structure of the aglycone part. *Journal of the Chemical Society*, pp.2309-2316.

LAQUEUR, G.L. 1964. Carcinogenic effects of cycad meal and cycasin, methylazoxymethanol glycoside, in rats and effects of cycasin in germ-free rats. *Federation Proceedings* 23: 1368-1387.

LAQUEUR, G.L. 1977. Oncogenicity of cycads and its implications. *Advances in Modern Toxicology and Environmental Cancer* 3: 231-261.

LAQUEUR, G.L. and MATSUMOTO, H. 1966. Neoplasms in female Fischer rats following intraperitoneal injection of methylazoxymethanol. *Journal of the National Cancer Institute* 37: 217-232.

LAQUEUR, G.L., McDANIEL, E.G. and MATSUMOTO, H. 1967. Tumor induction in germfree rats with methylazoxymethanol (MAM) and MAM-acetate. *Journal of the National Cancer Institute* 39: 355-371.

LAQUEUR, G.L., MICKELSEN, O., WHITING, M.G. and KURLAND, L.T. 1963. Carcinogenic properties of nuts from *Cycas circinalis* L.indigenous to Guam. *Journal of the National Cancer Institute* 31: 919-951.

LAQUEUR, G.L. and SPATZ, M. 1975. Oncogenicity of cycasin and methylazoxymethanol. *Monograph on Cancer Research* 17: 189-204.

LEMIEUX, R.U. 1964. Chemical synthesis of glucosides. *Chemistry in Canada* 16: 14-18.

LEWIN, R. 1987. Environmental hypothesis for brain diseases strengthened by new data. *Science* 237: 483-484.

LIENER, I.E. 1986. Natural Toxins: Animal, Plant, and Microbial. First edition. Oxford: Oxford Science Publications.

LINDBLAD, P. 1990. Nitrogen and carbon metabolism in coralloid roots of cycads. *Memoirs of the New York Botanical Garden* 57: 104-113.

LUNDEEN, P.B., BANKS, G.S. and RUDDON, R.W. 1971. Effects of the carcinogen methylazoxymethanol acetate on protein synthesis and drug metabolism in rat livers. *Biochemical Pharmacology* 20: 2522-2527.

LYTHGOE, B. and RIGGS, N.V. 1949. Macrozamin. Part I. The identity of the carbohydrate component. *Journal of the Chemical Society*, pp.2716-2718.

MACFADYEN, D.A. 1945. Estimation of formaldehyde in biological mixtures. *Journal of Biological Chemistry* 58: 107-133.

MAGEE, P.N. and BARNES, J.M. 1956. The production of malignant primary hepatic tumors in the rat by feeding dimethylnitrosamine. *British Journal of Cancer* 10: 114-122.

MAGEE, P.N. and BARNES, J.M. 1962. Induction of kidney tumors in the rat with dimethylnitrosamine. *Journal of Pathology and Bacteriology* 84: 19-31.

MALLING, H.V. 1966. Mutagenicity of two potent carcinogens, dimethylnitrosamine and diethylnitrosamine in *Neurospora crassa*. *Mutation Research* 3: 537-540.

MARCH, J. 1977. Advanced Organic Chemistry. Second edition, pp.178-180. New York: McGraw-Hill.

MATSUMOTO, H. and HIGA, H. H. 1966. Studies on methylazoxymethanol, the aglycone of cycasin:methylation of nucleic acids *in vitro*. *Biochemistry Journal* 98: 20c-25c.

MATSUMOTO, H., NAGAHAMA, T. and LARSON, H.O. 1965. Studies on methylazoxymethanol, the aglycone of cycasin: a synthesis of methylazoxymethanol acetate. *Biochemistry Journal* 95: 13c-14c.

MATSUMOTO, H. and STRONG, F.M. 1963. The occurrence of methylazoxymethanol in *Cycas circinalis*. *Archives of Biochemistry and Biophysics* 101: 299-310.

MATSUSHIMA, T., MATSUMOTO, H., SHIRAI, A., SAWAMURA, M. and SUGIMURA, T. 1979. Mutagenicity of the naturally-occurring carcinogen, cycasin and synthetic methylazoxymethanol and synthetic methylazoxymethanol conjugates in *Salmonella typhimurium*. *Cancer Research* 39: 3780-3782.

MAYER, V.M. 1971. Mutagenicity of dimethylnitrosamine and diethylnitrosamine for *Saccharomyces* in an *in vitro* hydroxylating system. *Molecular and General Genetics* 112: 289-294.

McCANN, J.E., CHOI, E., YANG, E. and AMES, B.N. 1975. Detection of carcinogens as mutagens in the Salmonella/microsome: assay of 300 chemicals. *Proceedings of the National Academy of Science* 72: 5135-5139.

McGINNIS, G.D. and FANG, P. 1980. High Performance Liquid Chromatography. In *Methods in Carbohydrate Chemistry*, ed Whistler, R.L. and BeMiller, J.N. Vol. VII, New York: Academic Press.

MERRIAM, A.D. 1974. An electrophoretic and chromatographic investigation of Florida coastal *Zamia* populations. M.A. thesis, University of South Florida.

MILLER, J.A. 1964. Comments on the chemistry of cycads. *Federation Proceedings* 23: 1361-1362.

MORETTI, A., SABATO, S. and SINISCALCO GIGLIANO, G. 1981a. Distribution of macrozamin in Australasian cycads. *Phytochemistry* 20: 1415-1416.

MORETTI, A., SABATO, S. and SINISCALCO GIGLIANO, G. 1981b. Monosaccharide composition of the mucilages in *Encephalartos* Lehm. (Zamiaceae). Giornale Botanico Italiano 115: 291-297.

MORETTI, A., SABATO, S. and SINISCALCO GIGLIANO, G. 1983. Taxonomic significance of methylazoxymethanol glycosides in the cycads. *Phytochemistry* 22: 115-118.

MORGAN, R.W. and HOFFMANN, G.R. 1983. Cycasin and its mutagenic metabolites. *Mutation Research* 114: 19-58.

NAGAHAMA, T. 1964. Neocycasins, new glycosides of cycads. *Bulletin of the Faculty of Agriculture, Kagoshima University* 14: 1-50.

NAGATA, Y. and MATSUMOTO, H. 1969. Studies on methylazoxymethanol: methylation of nucleic acids in the fetal rat brain. *Federation Proceedings* 132: 383-385.

NISHIDA, K. 1936. Biochemical studies on "sotetsu", the Japanese cycad. VI. Removal of poisonous constituents in the seeds and the nutritive value of the seeds. *Journal of the Agricultural Chemical Society of Japan* 12: 1106-1116.

NISHIDA, K., KOBAYASHI, A. and NAGAHAMA, T. 1955a. Cycasin, a toxic glycoside of *Cycas revoluta* Thun. I. Isolation and structure of cycasin. *Bulletin of the Agricultural Chemical Society of Japan* 19: 77-84.

NISHIDA, K., KOBAYASHI, A. and NAGAHAMA, T. 1955b. Cycasin, a toxic glycoside of *Cycas revoluta* Thun. II. Hydrolysis of cycasin with cycad emulsin. *Bulletin of the Agricultural Chemical Society of Japan* 19: 172-177.

NISHIDA, K., KOBAYASHI, A. and NAGAHAMA, T. 1956. Cycasin, a toxic glycoside of *Cycas revoluta* Thun. V. Quantitative determination of cycasin in cycad seeds. *Bulletin of the Agricultural Chemical Society of Japan* 20: 74-76.

O'GARA, R.W., BROWN, J.M. and WHITING, M.G. 1964. Induction of hepatic and renal tumors by topical application of aqueous extract of cycad nut to artificial skin ulcers in mice. *Federation Proceedings* 23: 1383.

OSBORNE, R. 1986. Cycad research in the 80's. *Encephalartos* 6:26-34.

OSBORNE, R. 1989. Focus on...*Encephalartos transvenosus*. *Encephalartos* 20: 10-18.

OSBORNE, R. 1989. Personal communication. University of Natal Durban, South Africa.

OSBORNE, R., GROBBELAAR, N. and VORSTER, P. 1988. South African cycad research: progress and prospects. *South African Journal of Science* 84: 891-896.

OSBORNE, R., SALATINO, M.L.F. and SALATINO, A. 1989. Alkanes of foliar epicuticular waxes of the genus *Encephalartos*. *Phytochemistry* 28: 3027-3030.

PATE, J.S. 1990. Nitrogen fixation in cycads with special reference to *Macrozamia riedlei*. *Proceedings of the Second International Conference on Cycad Biology*. Queensland, Australia (In print).

PENA, E., GRILLO, E. and PEREZ, D. 1983. Peroxidases in *Cycas circinalis* L. 1: Studies on the seed. *Revista del Jardin Botanico Nacional (Havana)* 4: 117-132.

PETTIT, J.M. 1982. Ultrastructural and immunocytochemical demonstration of gametophytic proteins in the pollen tube wall of the primitive gymnosperm *Cycas*. *Journal of Cell Science* 57: 189-214.

PLAITAKIS, A., BERL, S. and YAHR, M.D. 1982. Abnormal glutamate metabolism in an adult - onset degenerative neurological disorder. *Science* 216: 193.

PREUSSMANN, R. and STEWART, B.W. 1984. N-nitroso carcinogens in the environment. In *Chemical Carcinogens*, ed. Searle, C.S. Second edition, Vol. 2, pp.643-828. Washington, DC: Academic Press.

READ, R.W. and SOLT, M.L. 1986. Bibliography of the living cycads. *Lyonia* 2: 33-200.

REITZ, D. 1929. Commando: A Journal of the Boer War. Second revised edition. London: Faber and Faber.

RIGGS, N.V. 1954. The occurrence of macrozamin in the seeds of cycads. *Australian Journal of Chemistry* 7: 123-124.

RIGGS, N.V. 1956. Glucosylazoxymethane, a constituent of the seeds of *Cycas circinalis* L. *Chemistry and Industry* 8: 926.

ROSENKRANZ, H.S. and POIRIER, L.A. 1979. Evaluation of the mutagenicity and DNA-modifying activity of carcinogens and non-carcinogens in microbial systems. *Journal of the Cancer Institute* 62: 873-892.

ROTHSCHILD, M., NASH, R.J. and BELL, E.A. 1986. Cycasin in the endangered butterfly *Eumaeus atala florida*. *Phytochemistry* 25: 1853-1854.

SHANK, R.C. and MAGEE, P.N. 1967. Similarities between the biochemical actions of cycasin and dimethylnitrosamine. *Biochemistry Journal* 105: 521-527.

SHOENTAL, R. 1969. Carcinogenic action of elaiomycin in rats *Nature* 221: 765-766.

SINISCALCO GIGLIANO, G. 1990. Chemotaxonomic significance of MAM glycosides and mucilages in cycads. *Memoirs of the New York Botanical Garden* 57: 123-131.

SMITH, D.W.E. 1966. Mutagenicity of cycasin aglycone, a naturally-occurring carcinogen. *Science* 152: 1273-1274.

SNYDER, L.R. and KIRKLAND, J.J. 1974. *Introduction to Modern Liquid Chromatography*. New York: John Wiley.

SPATZ, M. 1964. Effects of cycad meal and cycasin on histochemically demonstrable liver phosphatases and non-specific esterases. *Federation Proceedings* 23: 1381-1382.

SPATZ, M. 1968. Hydrolysis of cycasin by β-glucosidase in skin of newborn rats. *Proceedings of the Society for Experimental Biology and Medicine* 128: 1005-1008.

SPATZ, M., DOUGHERTY, W.J. and SMITH, D.W.E. 1967a. Teratogenic effects of methylazoxymethanol. *Federation Proceedings* 124: 476-478.

SPATZ, M., SMITH, D.W.E., McDANIEL, E.G. and LAQUEUR, G.L. 1967b. Role of intestinal micro-organisms in determining cycasin toxicity. *Proceedings of the Society for Experimental Biology and Medicine* 124: 691-697.

SPENCER, P.S., NUNN, P.B., HUGON, J., LUDOLPH, A.C., ROSS, S.M., ROY, D.N. and ROBERTSON, R.C. 1987. Guam amyotrophic lateral sclerosis-Parkinsonism-dementia linked to an environmental neurotoxin. *Science* 11: 51-54

STANDLEY, P.C. 1937. Flora of Costa Rica. Part I. Field Museum of Natural History. *Chicago Botanical Series* 18: Publications 391 and 392.

STANTON, M.F. 1966. Hepatic neoplasm of aquarium fish exposed to *Cycas circinalis*. *Federation Proceedings* 25: 661.

STEPHEN, A.M. and DE BRUYN, D.C. 1967. The gum exudate from *Encephalartos longifolius* Lehm. (female) (family Cycadaceae). *Carbohydrate Research* 5: 256-265.

STEPHENS, D.C. and STEPHEN, A.M. 1988. Exudates from *Encephalartos* cones as chemical taxonomic markers. *South African Journal of Science* 84: 263-266.

STEVENSON, D.W. 1981. Observation on ptyxis, phenology and trichomes in the Cycadales and their systematic implications. *American Journal of Botany* 68: 1104-1114.

STEVENSON, D.W. 1985. A proposed classification of the Cycadales. *American Journal of Botany* 72, Abstract No. 489, pp.971-972.

STEVENSON, D.W. and OSBORNE, R. 1990. The World List of Cycads. *Proceedings of the Second International Conference on Cycad Biology*. Queensland, Australia (In print).

STEYN, D.G., VAN DER WALT, S.J. and VERDOORN, I.C. 1948. The seeds of some species of *Encephalartos* (cycads):- a report on their toxicity. *South African Medical Journal* 22: 758-760.

TADERA, K., TERAZONO, H. and YAGI, F. 1987. Effects of cycasin on various micro-organisms. *Memoirs of the Faculty of Agriculture, Kagoshima University* 23: 71-80.

TADERA, K., YAGI, F., ARIMA, M. and KOBAYASHI, A. 1985. Formation of cycasin from methylazoxymethanol by UDP-glucosyltransferase from leaves of Japanese cycad. *Agricultural Biological Chemistry* 49: 2827-2828.

TADERA, K., YAGI, F., MINEMOTO, S. and KOBAYASHI, A. 1982. Inhibitory effects of cycasin on germination growth and α-amylase biosynthesis in rice plants. *Agricultural Biological Chemistry* 46: 1453-1456.

TAKAGI, T. and ITABASHI, Y. 1982. cis-5-Olefinic unusual fatty acids in seed lipids of Gymnospermae and their distribution in triacylglycerols. *Lipids* 17: 716-723.

TEAS, H.J. 1967. Cycasin synthesis in *Seirarctia echo* (Lepidoptera) larvae fed methylazoxymethanol. *Biochemical and Biophysical Research Communications* 26: 686-690.

TEAS, H.J., SOX, H.J. and SOX, K. 1965. Cycasin: radiomimetic effect. *Science* 149: 541-542.

THIERET, J.W. 1958. Economic botany of the cycads. *Economic Botany* 12: 3-41.

TUSTIN, R.C. 1974. Toxicity and carcinogenicity of some South African cycad (Encephalartos) species. *South African Medical Journal* 48: 2369-2372.

TUSTIN, R.C. 1983. Notes on the toxicity and carcinogenicity of some South African cycad spesies with special reference to that of *Encephalartos lanatus*. *Journal of the South African Veterinary Association* 54: 33-42.

VEGA, A. and BELL, E.A. 1967. α-amino-β-methylaminopropionic acid, a new amino acid from seeds of *Cycas circinalis*. *Phytochemistry* 6: 759-762.

VEGA, A. and BELL, E.A. 1968. The preparation of L- and D-α-amino-β-methylaminopropionic acids and the identification of the compound isolated from *Cycas circinalis* as the L-isomer. *Phytochemistry* 7: 1885-1887.

VORSTER, P. and VORSTER, E. 1986. Focus on...*Cycas thouarsii*. *Encephalartos* 6: 3-10.

WALLACE, J.W. 1972. A survey for benzoic and cinnamic acids of the Cycadaceae. *American Journal of Botany* 59: 1-4.

WELLS, W.W., YANG, M.G., BOLZER, W. and MICKELSEN, O. 1968. Gas-chromatographic analysis of cycasin in cycad flour. *Analytical Biochemistry* 25: 325-329.

WHISTLER, R.L. and DURSO, D.F. 1950. Chromatographic separation of sugars on charcoal. *Journal of the American Chemical Society* 72: 677-679.

WHITING, M.G. 1963. Toxicity of cycads. *Economic Botany* 17: 270-302.

WHITING, M.G. 1964. Food practices in ALS foci in Japan, the Marianas and New Guinea. *Federation Proceedings* 23: 1343-1345.

YAGI, F. and TADERA, K. 1987. Azoxyglycoside contents in seeds of several cycad species and various parts of Japanese cycad. *Agricultural Biological Chemistry* 51: 1719-1721.

YAGI, F., TADERA, K. and KOBAYASHI, A. 1980. Simultaneous determination of cycasin, methylazoxymethanol and formaldehyde by high performance liquid chromatography. *Agricultural Biological Chemistry* 44: 1423-1425.

YAGI, F., TADERA, K. and KOBAYASHI, A. 1983. Simultaneous determination of azoxyglycosides by high performance liquid chromatography and their contents in growing leaves of Japanese cycad. *Agricultural Biological Chemistry* 47: 137-139.

YAGI, F., TADERA, K. and KOBAYASHI, A. 1985a. A new azoxyglycoside containing isomaltose : neocycasin Bα. *Agricultural Biological Chemistry* 49: 1531-1532.

YAGI, F., TADERA, K. and KOBAYASHI, A. 1985b. Formation of three new trisaccharide-azoxyglycosides by transglucosylation by cycad β-glucosidase. *Agricultural Biological Chemistry* 49: 2985-2990.

ZEDECK, M.S., FRANK, N. and WIESSLER, M. 1979. Metabolism of colon carcinogen methylazoxymethanol acetate. *Frontiers of Gastrointestinal Research* 4: 32-37.

ZEDECK, M.S., STERNBERG, S.S., POYNTER, R.W. and McGOWAN, J. 1970. Biochemical and pathological effects of methylazoxymethanol acetate, a potent carcinogen. *Cancer Research* 30: 801-812.