Mode of action of maculosin, a host-specific phytotoxin, produced by Alternaria alternata on spotted knapweed (Centaurea maculosa L.)

by Sang Ho Park

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Plant Pathology
Montana State University

Abstract:
Maculosin [diketopiperazine, cyclo (L-Pro-L-Tyr)] is a host-specific phytotoxin produced by Alternaria alternata on spotted knapweed (Centaurea maculosa L.). This study involves its putative receptor, cellular effects and its fate in the host plant. Initially, 14C-maculosin was synthesized by organic methods. The purity and structure were identified by HPLC, NMR and MS.

Host leaves possessed maculosin-binding activity in the cytosolic and membrane fraction and most of the binding activity was recovered from the cytosolic fraction. The binding component was identified as a protein because of its heat-lability and sensitivity to proteases. A 16-fold purification of toxin-binding protein was carried out by ammonium sulfate fractionation, Sephadex G-200 column, and maculosin-affinity chromatography. The affinity column was prepared with epoxy activated Sepharose 6B to which the phenolic group of maculosin was attached. Furthermore, the &aplha;-amino group of tyrosine was coupled to CNBr-activated Sepharose 4B. However, the tyrosine affinity column did not bind any cytosolic proteins. The molecular weight of maculosin-binding protein was estimated as 604 kdaltons and the receptor contained more than one binding protein. One of the maculosin-binding proteins was identified as ribulose 1,5-biphosphate carboxylase.

Metabolites of maculosin were analyzed 5 days after treatment of 14C-maculosin on detached leaves. Maculosin was converted to three polar compounds (M-l, M-2, and M-3). The major metabolite (M-2) was purified and identified as maculosin β-O-D-glucoside. M-3 metabolite was surmised as one of the dipeptides (L-Pro-L-Tyr-OH or L-Tyr-L-Pro-OH), the hydrolyzed products of diketopiperazine (maculosin). The conversion mechanism was enzymatically mediated. The formation of maculosin β-O-glucoside was identified as a biologically inactive intermediate compound which is converted to a methanol insoluble complex in the leaves of spotted knapweed. In oats (monocot), the metabolism of maculosin was the same as in spotted knapweed.

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APPROVAL

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Sang Ho Park

This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

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ABSTRACT

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CHAPTER I

INTRODUCTION

The plant host-parasite interaction resulting in disease development is the result of the interplay of many factors induced or produced by pathogen and the host. Many pathogens causing necrosis of plants often produce disease symptoms by elaborating one or more toxic compounds which are called phytotoxins. Phytotoxins which are involved in disease are classically subdivided into two classes, host-specific and host non-specific.

The term, "host non-specific toxin" implies that the susceptibility of the host plant to a pathogen does not parallel its sensitivity to the toxin. The toxin probably does not play a role in the establishment of the pathogen in the host. However, it is able to induce some characteristics of the disease syndrome and is also known as a virulence factor or secondary determinant of pathogenicity. A great number of non-specific phytotoxins are known which do not reproduce the patterns of resistance and susceptibility of the host to the pathogen. They include all the bacterial phytotoxins known so far and the majority of the fungal toxins (19,40,59).

At present, the host specificity exhibited by a pathogen is the most intriguing problem in plant pathology. It is assumed that many complex physiological and biochemical mechanisms are involved in host specificity. In some cases, the causal fungi recognize their host plants and invade tissues by exuding highly biologically potent chemicals, namely one or more "host-specific toxins." These compounds cause specific cell toxicity in the initial stages of the infection process, but only to the normal hosts of the fungus. It is well known that such toxic substances participate in establishing disease development as a primary determinant of pathogenicity. There are at least fourteen examples in which host specific phytotoxins appear to play a significant role in pathogenicity (52,59,75). Some specific examples are Victoria blight of oats caused by Cochliobolus victoriae in 1947 (39) and Southern leaf blight of corn by Cochliobolus heterostrophus in early 1970 in the USA (54). Commonly, these phytotoxins have been isolated from the fungal genera Alternaria and Helminthosporium (Cochliobolus, Bipolaris) and a few others (31,45).

Along with this important role in disease development in various crops, phytotoxins have been helpful in establishing the molecular basis of disease production and in gaining an understanding of the normal physiology and biochemistry of plants (59).

Fusicoccin is one of the best known phytotoxins which has been used as a molecular probe. It is the major phytotoxic metabolite of Fusicoccum amygdali, the causative agent of peach and almond canker. It acts directly and primarily at the plasma membrane level, where it selectively activates the H^+-ATPase responsible for electrogenic proton extrusion, with consequent influence on the activity of a number of metabolic and physiological processes (36). Furthermore, evidence has been produced that the stimulation of proton extrusion is triggered by a signal that originates from the interaction of fusicoccin with specific receptors in plasma membrane-enriched fractions of a large number of higher plants, and located at the apoplastic side of the plasma membrane (10,16,36).

Green islands, localized areas of chlorophyll retention, are common symptoms in plant tissues infected with certain biotropic fungi. Some phytotoxins can induce such symptoms (49). Gigantenone, a new eremophilane diepoxide, produced by Drechslera gigantea causes the formation of green islands on many graminaceous species tested (29). However, it generally causes necrotic lesions on dicotyledonous species. Gigantenone also induces root formation in mung bean hypocotyls and shows a high level of activity in several plant tissue culture systems (29).

Conventional plant breeding for resistance to pathogens, in many cases, is still too slow to keep pace with the

adaptation of pathogens. Phytotoxins, because of their role in disease development and host selectivity have been proposed as tools for *in vitro* selection (4,35).

Although most well described phytopathogenic microorganisms are parasitic on crop plants, weeds also suffer from various pathogens (43,60,70). There is considerable current interest in the use of plant pathogenic microbes as agents for the control of certain economically important weeds (66). Numerous plant pathogens are being studied throughout the world for possible use as mycoherbicides or biological control agents. *Phytophthora palmivora* and *Colletotrichum gloeopsorioides* f. sp. *aeschynomens* have been developed, registrated as De Vine and Collego, respectively and marketed for the control of strangler (milkweed) vine and northern jointvetch in the United States (66).

In many cases, it is virtually impossible to conceive of the use of a weed pathogen in the field. The application of weedy pathogens may have to do with the longevity of inoculum, the regulatory restriction on the use of the pathogen, the means of application of the pathogen, the favorability of environmental conditions for epidemic spread of pathogens, and the inherent heterogenicity of weeds (7,66,74).

The potential of phytotoxins as herbicides or as a model for new herbicides has been recently suggested (14,18,60). Higher plants and their pathogens have, in all likelihood,

evolved together and in the process may have had considerable biochemical changes. In the recent past, considerable effort has been expended in finding novel and selective phytotoxins with potential use for the control of weeds.

The use of plant phytotoxins in controlling weeds may have several advantages over the use of weed pathogens. The possible use of phytotoxins to create novel models for more effective herbicides is a very promising area of research and may result in herbicides that might enhance effectiveness and environmental safety. For these reasons, phytotoxins may constitute an efficient method of weed control, although to date, none are in commercial use.

Our laboratory has focused on weed pathogens with the hope of finding both novel and more selective chemical control agents. This has led to the isolation of several phytotoxins from weed pathogens (28,60). Some examples of this are tryptophol from Drechslera nodulosum, a pathogen of goose-grass (62); bipolaroxin, a host selective phytotoxin from Bipolaris cynodontis, a pathogen of Bermuda grass (63); and Dihydropyrenophorin from Drechslera avenae, a pathogen of both wild and cultivated oats (61).

This type of research was further extended to the study of spotted knapweed (Centaurea maculosa L.), a member of the family compositae, which is one of the most serious weed problems in Montana. As a fast spreading rangeland weed, this plant was introduced from Europe in the early 1900's and

has widely spread in the northwestern area of the United States and southwest Canada. Since first reported in Montana in 1927, this weed now infests over 4.7 million acres of rangeland, pasture, and roadside (32).

The perennial growth habit, profuse seed production and aggressiveness of spotted knapweed results in its rapid establishment and spread. Initial infestations occur in disturbed areas such as roadsides, trails, construction sites, overgrazed land and waterways (71). Once established it is very competitive, displacing native grasses and forbs, resulting in near-monoculture stands of spotted knapweed. Another factor contributing to its success in North America is the lack of natural enemies. In Europe, its center of origin, spotted knapweed evolved with host-specific insects and pathogens which keep the plant frequency and density at low levels. In North America most of these agents are not present.

After an intensive search through several counties in southern Montana and an examination of thousands of plants in three counties, a fungus was found that causes black necrotic lesions on spotted knapweed. This fungus was further identified as Alternaria alternata. From a cultural filtrate a host specific phytotoxin, maculosin, was isolated (Figure 1) and its structure was confirmed by organic synthesis (56).

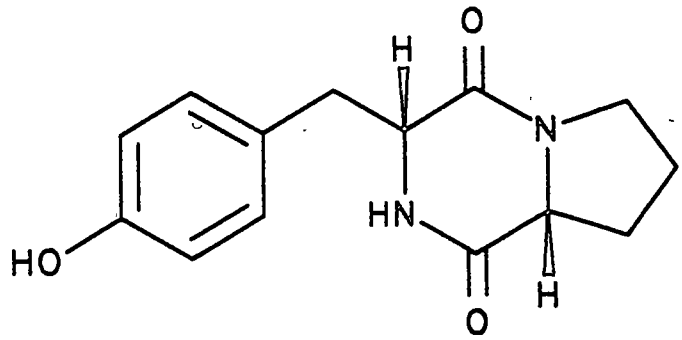


Figure 1. Structure of maculosin, cyclo (L-Pro-L-Tyro).

Fungi belonging to the genus Alternaria are commonly found causing leaf spots and some are known saprophytes (20). Host specific toxins isolated from A. alternata are shown in Table 1. These fungi are not distinguishable based on conidial morphology, but are characterized by the production of a toxin that appears to be responsible for the host range of the fungus and its pathogenicity. Each of these organisms is referred to as a distinct pathotype of A. alternata (Table 1) (44,45).

In the case of A. alternata on spotted knapweed, it produces numerous, non-toxic diketopiperazines (56). However, one termed maculosin [cyclo (L-Pro-L-Tyr)] caused black necrotic lesions on spotted knapweed at 10 μM. Even at 1 mM, maculosin did not affect 19 other grasses and dicots that were tested (56). Thus it is host specific toxin of the diketopiperazines isolated from A. alternata. Maculosin, the most active substance, possesses a phenolic moiety, not

Table 1. Host-specific toxins from A. alternata.

Disease	Pathogen (previous name)	Toxin	Host (pathotype)
Alternaria blotch of apple	A. mali	AM-toxin I, II ,III	Apple
Alternaria leaf spot of Pigeonpea	A. tenuissima	ACT-toxin	Pigeonpea
Alternaria stem canker of tomato	A. alternata f.sp. lycopersici	AL (AAL)-toxin I, II	Tomato
Black leaf spot of strawberry	A. alternata	AF-toxin I, II, III	Strawberry
Black spot of Japanese pear	A. kikuchiana	AK-toxin I, II	Japanese pear
Black spot of rough lemon	A. citri	ACR(L)-toxin	Rough lemon
Brown spot of tangerine	A. citri	ACT-toxin ACTG-toxin	Tangerine
Brown spot of tobacco	A. longipes	AT-toxin	Tobacco

uncommon in phytotoxins. The minor activity of cyclo (L-Pro-L-Tyr), compared to the inactivity of its diastereomer, suggested the importance of structural conformation in its bioactivity.

Although the exact chemistry and host range of maculosin had been established, at the set of this study we had no

information on the factor(s) in the susceptible plant that interact with maculosin and thus no understanding of its mode of action.

In general, studies on the mode of action of the host-specific toxins will play an important role in elucidating the mechanism of host selectivity, providing tools for probing the normal functions of plants. Thus, having the ability to synthesize radiolabelled maculosin, we were in an excellent position to carry out a comprehensive study on its cellular effects, its putative plant receptor(s) and its fate in its host plant. This thesis is a report of such studies.

CHAPTER II

CELLULAR EFFECTS OF MACULOSIN

Introduction

The general biochemical and molecular mechanisms underlying the mode of action of a toxin is of particular interest especially in those cases in which the toxin is specific to a limited host range of certain animals or plants. The hypothesis generally employed to understand this specificity is one of site specific recognition.

Thus the toxin is the ligand and its interaction with a specific receptor is a first site of interaction. This may be on the surface or within the cells such as cytosolic organelles and membranes. For instance, in mammalian cells, workers have shown the value and power of toxins as probes of normal cell function using bacterial toxins in the identification and purification of cellular receptors (69).

In parallel with mammalian toxins, there are also reports of the interaction of phytotoxins with receptors in plant cells (37,59,73). This came with the experimental demonstration of helminthosporoside binding protein in sugar cane in early 1970's (58). Also, fusicoccin, the major phytotoxic metabolite of *Fusicoccum amygdali* binds to the

plasma membrane of a large number of higher plants and it selectively activates the H^+-ATPase responsible for electrogenic proton extrusion (36). The binding of zinniol, a toxin produced by the Alternaria group, to the membranes and chloroplasts stimulates the entry of calcium into plant protoplasts in zinniol sensitive carrot cells (67). Furthermore, tentoxin, a host non-specific phytotoxin, inhibits photophosphorylation on binding to the chloroplast coupling factor CF1 (55).

In the case of maculosin, a host specific toxin of spotted knapweed, if the host plant possesses a specific receptor which has a normal role in the maintenance of cellular function, the binding of the phytotoxin may result in a site specific effect which may modify one or more metabolic processes in the cell. This process would eventually lead to the development of symptoms in knapweed plants affected by the toxin. Also, the interaction of receptor and maculosin may reveal an association of a receptor with the target organelles.

Therefore, the availability of the purified receptor would be crucial in the understanding of host specificity, receptor function, and in characterizing the molecular events occurring at the reactive site in the tissues of spotted knapweed. Thus, in order to assay for a receptor it is critical to have radiolabelled maculosin on hand.

In addition, affinity chromatography has been helpful in the isolation and characterization of receptors from animals and plants (64). After introduction of a ligand to a solid matrix by specific coupling methods, the application of this bioaffinity method is an effective tool in isolating and purifying not only specific receptor molecules but also cell organelles and even cells themselves. For example, the fusicoccin (FC) binding protein was purified by affinity chromatography using FC-linked adipic acid dihydrazide agarose (16), and the nicotine binding protein from rat brain was purified by conjugation of (R,S)-6-(2-hydroxyethyl) nicotine to epoxy-activated Sepharose (1).

This chapter, therefore, elaborates a procedure which allows immobilization of maculosin to a solid matrix. Subsequently, this matrix was used to isolate and characterize those receptors in spotted knapweed which may serve as the binding site for maculosin. Basically two types of affinity columns were constructed. First, epoxy activated Sepharose 6B to which a phenolic group of synthetic maculosin was attached. Secondly, the α-amino group of tyrosine was coupled to Sepharose 4B via CNBr activation to expose the phenoxy group free in the space.

Materials and Methods

Plant Material

Spotted knapweed (*Centaurea maculosa* L.) were individually grown in plastic pots containing a mixture of pasteurized sand, top soil, and peat (1:1:1) in the university plant growth facility with 12 hour light and dark period.

Synthesis of ^{14}C-maculosin

The synthesis of ^{14}C-maculosin followed a two step procedure that involved protection of one of the amino acids with BOC-ON, by the method of Itoh et al. (27). The BOC-protected amino acid was reacted with the methyl ester of the second amino acid, following the method of Nitecki et al., which is known to proceed without racemization (46).

L-tyrosine, 0.23 mmole, mixed with L-[U-^{14}C] tyrosine (75 μCi, specific activity 250 μCi/μmole) was reacted with 0.27 mmole of BOC-ON [2-(tert-butoxycarbonyloxyimino)-2-phenylacetonitrile], which was stirred at 25°C in 7 ml aqueous acetone and 50 μl triethylamine. After two hours the mixture was evaporated and washed with ethyl acetate. The aqueous layer was acidified with 1 N Hcl, then extracted with methylene chloride.

The crude t-boc-L-tyrosine was dissolved in an equimolar mixture of methylene chloride and triethylamine. The L-proline methyl ester hydrochloride was added (0.23 mM),

followed by the addition of ethyl-N-(3-dimethylaminopropyl) carbodiimide hydrochloride (0.23 mM). To prepare the L-proline methyl ester hydrochloride, a L-proline mixture (L-[U-^{14}C] proline 75 μCi, specific activity 250 μCi/μmole) was refluxed with a mixture of thionyl chloride and methanol. The mixture was left at -5°C overnight, then reduced in vacuo. The crude dipeptide was sequentially washed with water, citric acid (1 N), sodium bicarbonate (5 %), water, then evaporated to dryness.

The t-boc-L-tyrosine-L-proline methyl ester was dissolved in 8 ml of formic acid and stirred at room temperature for 2 hours. After removal of excess formic acid, the residue was dissolved in 10 ml of sec-butanol and toluene (vol/vol, 1:1) and refluxed for 2 hours. The structure and purity of the product, maculosin, was confirmed by HPLC, NMR and MS (56). The specific activity of maculosin was 0.11 μCi/μmole.

Localization and Translocation of Maculosin

Spotted knapweed plants, grown for 4 weeks in the green house, were removed from soil, and the roots washed with water to remove debris. Each intact plant was kept in a small vial containing water. ^{14}C-maculosin (0.02 μmole) was overlayered on the leaf with and without a small puncture with a 10 μl Hamilton syringe. For root treatment, ^{14}C-maculosin was adjusted with water to 4.5 mM in a small vial

and the root was dipped into that solution. Each treated plant was maintained in a moist chamber to prevent the evaporation of applied toxin from the leaf. After three days, the droplet of applied ^{14}C-maculosin was carefully removed from the leaf with a syringe and the root was extensively washed with distilled water. The plant was dried with several paper towels and mounted on a sheet of chromatography paper. The distribution of radioactivity in the plant was ascertained by exposure of the plant to autoradiographic film (Kodak Omat) at -70°C for 2 weeks.

To determine the localization of radioactivity, two to three month-old leaves of spotted knapweed were excised and placed in a moist chamber. Wounds were made in the center of each half of the leaf with a 10 μl Hamilton syringe. Each wound was overlayered with 5 μl of 2% ethanol containing 1900 dpm (8 nmole) of ^{14}C-maculosin (specific activity 0.11 μCi/μmole). The treated leaves were kept for three days at 28°C under 12 hours light and dark period. After incubation the leaves were cut into three equal portions; base, middle, and tip, and placed in separate glass tubes. The leaves were then digested with 0.5 ml perchloric acid: hydrogen peroxide (1:2, v/v) and heated at 100°C for 30 min. The digests were solubilized with 10 ml Aquasol and the radioactivity determined.

Fractionation of Maculosin Binding Material

The procedure to isolate the membranes and cytosolic fraction of spotted knapweed was carried out at 0 to 5°C. The leaves (50 g) of three to four month old plants were homogenized in 200 ml of 25 mM Tris (hydroxymethyl)-aminomethane-HCl buffer (pH 7.5) containing 1 mM dithiotreitol (DTT). The homogenate was filtered and strained through 6 layers of cheesecloth and centrifuged at 3000 x g for 20 min. The supernatant was further centrifuged at 100,000 x g for 1 hour. Both the pellet (membrane) and cytosolic fraction were kept at 4°C until used for the binding assay.

Detergent Solubilization of Membrane Components

The membrane fraction was diluted with the suspension buffer containing 25 mM Tris buffer (pH 7.5) to a protein concentration of 2.2 mg/ml. Each detergent was adjusted to give a final concentration of 0.5% and the membrane proteins extracted under mild conditions with a hand glass homogenizer and the solution kept at 4°C for 2 hours. The solution was centrifuged at 100,000 x g for 1 hour. The supernatant was saved and exhaustively dialyzed in the same buffer whereas the Triton X-100 and digitonin treated fractions were passed through Sephadex G-25 (30 x 1.5 cm) because of their inability to be dialyzed.

Characterization of the Radioactive Component Bound to the Protein

The radioactive fraction of the 10,000 x g supernatant in Sephadex G-25 size exclusion chromatography was pooled and reduced to dryness in vacuo at 35°C. The residue was dissolved in 20 ml of ethanol, further concentrated in vacuo and dissolved in 0.5 ml of ethanol. This was streaked on a normal phase silica gel plate (20 x 5 cm) and developed using a solvent (chloroform:methanol:acetic acid = 14:2:1, v/v/v). The relative R_f region of intact maculosin was scraped and the radioactivity determined.

Preparation of a Biospecific Adsorbent for Maculosin Receptors

Maculosin Affinity Chromatography. Maculosin-Sepharose was prepared by coupling maculosin to epoxy-activated Sepharose 6B (64). Epoxy-activated Sepharose 6B was prepared by reacting it with 1,4-bis-(2,3 epoxy-propyl)-butane giving an uncharged ether linkage between the spacer, gel, and free oxirane group (64). In order to carry out this reaction, Sepharose 6B was washed with distilled water, and the excess water removed by filtration through glass wool. The wet gel (10 ml) was resuspended in 20 ml of 1 M NaOH containing $NaBH_4$ (40 mg) and 10 ml of 1,4-butandiol-diglycidylether. The solution was rotated at 25°C for 10 hours and the reaction mixture was washed with distilled water.

The activated Sepharose was then reacted with 500 mg of

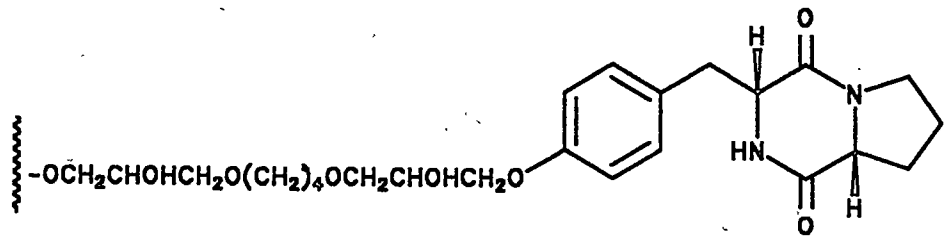
maculosin in NaOH (0.5 M) for 20 hours at 35°C with gentle shaking. The gel was then washed exhaustively with distilled water, followed with bicarbonate buffer (0.1 M, pH 8.0) and acetate buffer (0.1 M, pH 4.0). Then the residual active groups of gel were blocked with 1.0 M ethanolamine overnight. The gel was suspended and washed extensively with 500 ml of 10 mM Tris-HCl buffer pH 7.5 (Figure 2).

Tyrosine Affinity Chromatography. Tyrosine-Sepharose was synthesized by the method of Chan and Takahashi (8). Sepharose 4B was washed with distilled water and decanted. CNBr 1 g, freshly dissolved in 15 ml of distilled water, was added and the pH was adjusted to and maintained at 11.0 by adding 4 M NaOH with continuous stirring. Then 2 g of L-tyrosine was added as a fine powder and the pH was readjusted to 11.0 with 4 M NaOH. The mixture was stirred gently for 16 hours at 4°C. After the reaction was complete, the residual active groups of gel were blocked with 0.2 M glycine. Then the gel was washed with water and equilibrated with 10 mM Tris buffer pH 7.5 (Figure 2).

Preparation of Cytosolic Proteins

The leaves (50 g) of three to four month old plants were homogenized in 200 ml of 25 mM Tris (hydroxymethy)-aminomethane)-HCl buffer pH 7.5 containing 1 mM DTT. The homogenate was filtered through 4 layers of cheesecloth and

COUPLING OF MACULOSIN TO EPOXY-ACTIVATED SEPHAROSE 6B



COUPLING OF TYROSINE TO CNBR-ACTIVATED SEPHAROSE 4B

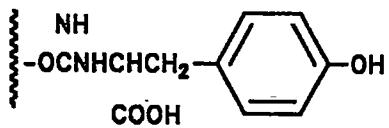


Figure 2. Construction of maculosin-affinity support gel.

centrifuged at 12,000 x g for 30 min and the supernatant was again centrifuged at 100,000 x g for 1 hour. The final supernatant was saved for further purification.

Purification of Cytosolic Maculosin Binding Protein

Solid ammonium sulfate was added slowly to the high-speed supernatant to a 20% concentration. The mixture was stirred for 1 hour and then centrifuged at 12,000 x g for 30 min. The resultant 20% ammonium sulfate supernatant was brought to a final 60% concentration by the addition of solid ammonium sulfate and then stirred for 1 hour. The mixture was again centrifuged at 12,000 x g for 30 min and the precipitate was saved. The precipitate was then resuspended in 10 ml of 10 mM Tris buffer pH 7.5 containing 0.5 mM DTT, and dialyzed against of 4 liters of the same buffer for 12 hours. The dialyzate was centrifuged at 12,000 x g for 30 min and supernatant was applied to a column of Sephadex G-200 (60 x 1.4 cm) pre-equilibrated with 10 mM Tris buffer pH 7.5. The column was eluted with 10 mM Tris buffer at a rate of 15 ml/hour. The eluate was collected in 1.5 ml fractions and monitored at 280 nm with a Beckman spectrophotometer. Fractions were collected at the void volume which contained protein and exhibited binding activity. This pooled void volume fraction was applied to the maculosin affinity column (10 x 1.0 cm) pre-equilibrated with 10 mM Tris buffer. After washing the column extensively with 10 mM Tris buffer, the

protein was eluted with a stepwise gradient (each 30 ml) of 0.0, 0.15, 0.3, 0.6 and 0.9 M NaCl in 10 mM Tris buffer. The eluted proteins were combined, concentrated appropriately with ultrafiltration (Amicon ultrafilter, molecular cutoff 10,000), and dialyzed in 10 mM Tris buffer pH 7.5. The tyrosine affinity column (10 x 1.0 cm) was eluted with a stepwise gradient of salt (NaCl up to 0.9 M) as described above, and a pH stepwise gradient using 30 ml each of 50 mM citrate buffer (pH 3, 4.5, 6) and 50 mM Tris buffer (pH 7, 9).

The purification scheme is presented in Figure 3.

Equilibrium Dialysis

Aliquots (0.5 ml) of the proteins were dialyzed in an Kontron equilibrium dialysis system (a Kontron-Diapak dialysis unit) against 0.5 ml of binding buffer containing a range of different concentrations of ^{14}C-maculosin (30). The dialysis membrane (Spectra/Por, Spectrum, Inc.) had a molecular weight exclusion limit of 8,000. Equilibrium was reached after 10 hours of incubation at 28°C by gentle turning of the dialysis cells. Radioactivity was determined on the contents of each half cell. The total amount of maculosin bound was equal to the difference in radioactivity between each pair of half cells.

Homogenize leaves in Tris buffer

|

Filter through cheesecloth

|

Centrifuge at 12,000 x *g*

|

Save supernatant

|

Ultracentrifuge at 100,000 x *g*

|

Save supernatant

|

20-60% ammonium sulfate fraction

|

Centrifuge at 12,000 x *g*

|

Collect precipitate and dialyze

|

Chromatograph on Sephadex G-200 column

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"Maculosin affinity" chromatography

|

Ultrafiltration and dialysis

|

Purified protein

|

Acrylamide gel electrophoresis

Figure 3. Purification scheme of cytosolic maculosin binding protein.

Protein Determination

Protein was determined by the method of Bradford (3), using Bio-Rad Protein Dye Reagent (Bio-Rad). Bovine serum albumin was used as the standard.

Estimation of Molecular Weight

The molecular weight of maculosin binding protein was determined using a Sepharose 6B column (60 x 1.4 cm) which was calibrated using the following protein standards: thyroglobulin (669,000), apoferritin (443,000), α-amylase (200,000) and bovine serum albumin (66,000).

Radioactivity Determination

All samples were mixed with 10 ml of Aquasol (New England Nuclear Corp.) before counting. Radioactivity measurements were made on a Packard liquid scintillation spectrometer model 2200CA and counts were corrected to disintegrations per minute (dpm).

Electrophoresis

Native Polyacrylamide Gel. Electrophoresis was performed on 7.5% gel according to Davis (15).

Discontinuous Sodium Dodecyl Sulfate (SDS) Polyacrylamide Gel. Electrophoresis was carried out in a 10% gel by the method of Laemmli (33) with modifications. The separating gel contained 10% acrylamide, 0.26% bisacrylamide, 0.1% SDS, 375 mM Tris-HCl, pH 8.8, 0.033% ammonium

persulfate, 0.033% (V/V) N'N'N'N' tetramethyl-ethylenediamine (TEMED). The stacking gel contained 4.0% acrylamide, 0.1% bisacrylamide, 0.1% SDS, 125 mM Tris-HCl, pH 6.8, 0.008% ammonium persulfate, 0.005% TEMED. Samples were mixed at a 1:1 volume ratio with sample buffer containing 125 mM Tris-HCl, pH 6.8, 4.0% SDS, 10% β-mercaptoethanol, 0.01% bromophenol blue and 30% sucrose. Samples were heated for two minutes at 100°C, and then 50 μl aliquots of the protein preparation (35 μg) were applied to the gel. Electrode buffer containing 19 mM glycine, 25 mM Tris-HCl, pH 8.5 and 0.1% SDS was used in both electrode chambers. Electrophoresis was conducted at 150 volts until the bromophenol blue tracking dye reached the bottom of the gel.

Protein was stained with Coomassie Blue (CB) following the procedures of Howard and Traut (25). Gels were soaked 4-10 hours in solution of 50% methanol, 10% acetic acid and 0.25% CB. The gel was then destained with several rinses of a 50% methanol and 10% acetic acid solution.

Double Immunodiffusion

A 1.0% gel was prepared on a glass plate by the method of Ouchterlony (47). The agarose gel was prepared in 50 mM phosphate buffer, pH 7.4 containing 0.03% sodium azide. The plate was kept in a moist chamber at 25°C. The antiserum (20 μl) was pipetted in a center well and cytosolic maculosin binding protein (20 μl, 43 μg) was pipetted into an adjacent

well. The control well contained phosphate buffer. Evidence for a cross reaction was investigated at 36 hours. Antiserum (polyclonal) against ribulose-1,5-biphosphate carboxylase from tobacco plants was provided by Dr. Jensen, University of Arizona.

Measurement of Ribulose-1,5-Biphosphate Carboxylase (RuBPCase) Activity

RuBPCase was prepared and assayed by the method of Chu and Bassham (9) with some modification.

Enzyme Isolation. RuBPCase was extracted from the leaves (10 g) ground in a Sorvall Omnimixer for 5 x 30 seconds with 40 ml of 0.1 M Tris buffer, pH 7.5 containing 1 mM ethylenediaminotetraacetic acid (EDTA), and 10 mM DTT. The homogenate was squeezed through cheesecloth and centrifuged at 12,000 x g for 30 min. The soluble supernatant was fractionated with ammonium sulfate. The protein which precipitated between 35 and 55% saturation with ammonium sulfate was collected by centrifugation and dissolved in a minimum volume of 0.1 M Tris buffer, pH 7.5. The protein solution was dialyzed overnight against 0.1 M Tris buffer, pH 7.5. The dialyzate was centrifuged at 12,000 x g to remove any insoluble material. The solution was layered on a Sephadex G-200 column (60 x 1.4 cm) which had been previously equilibrated with 0.1 M Tris buffer, pH 7.5 containing 5 mM $MgCl_2$. The protein was eluted from the

column with the same buffer and collected from the column in the void volume.

Enzyme Assay. In a final volume of 0.25 ml, the reaction mixture contained the following components at concentrations noted: Tris-HCl, 175 mM; $MgCl_2$, 10 mM; pH 7.8; Ribulose biphosphate (RuBP) 0.08 mM. The concentration of $NaH^{14}CO_3$, maculosin, and enzymes are shown in Table 7 in the results. The assay was incubated at 30°C in a stoppered glass vial and was started by the addition of RuBP. After 10 min incubation in the water bath at 30°C, 0.1 ml of 6 N HCl was added to stop the reaction. The solution was dried at 90°C and the residue was dissolved in 0.2 ml of water. Scintillation fluid (5 ml, Aquasol) was added and the radioactivity determined.

Results

Localization of Maculosin

When ^{14}C-maculosin was applied "in vivo" onto the leaves without a wound, most of the radioactivity was localized at the site of application (Figure 4), whereas some radioactivity was mobilized to the tip of the leaf in which maculosin had been applied to a wound. However, when the root of the plant was dipped into the maculosin solution, most of the radioactivity was acropetally translocated (Figure 4).

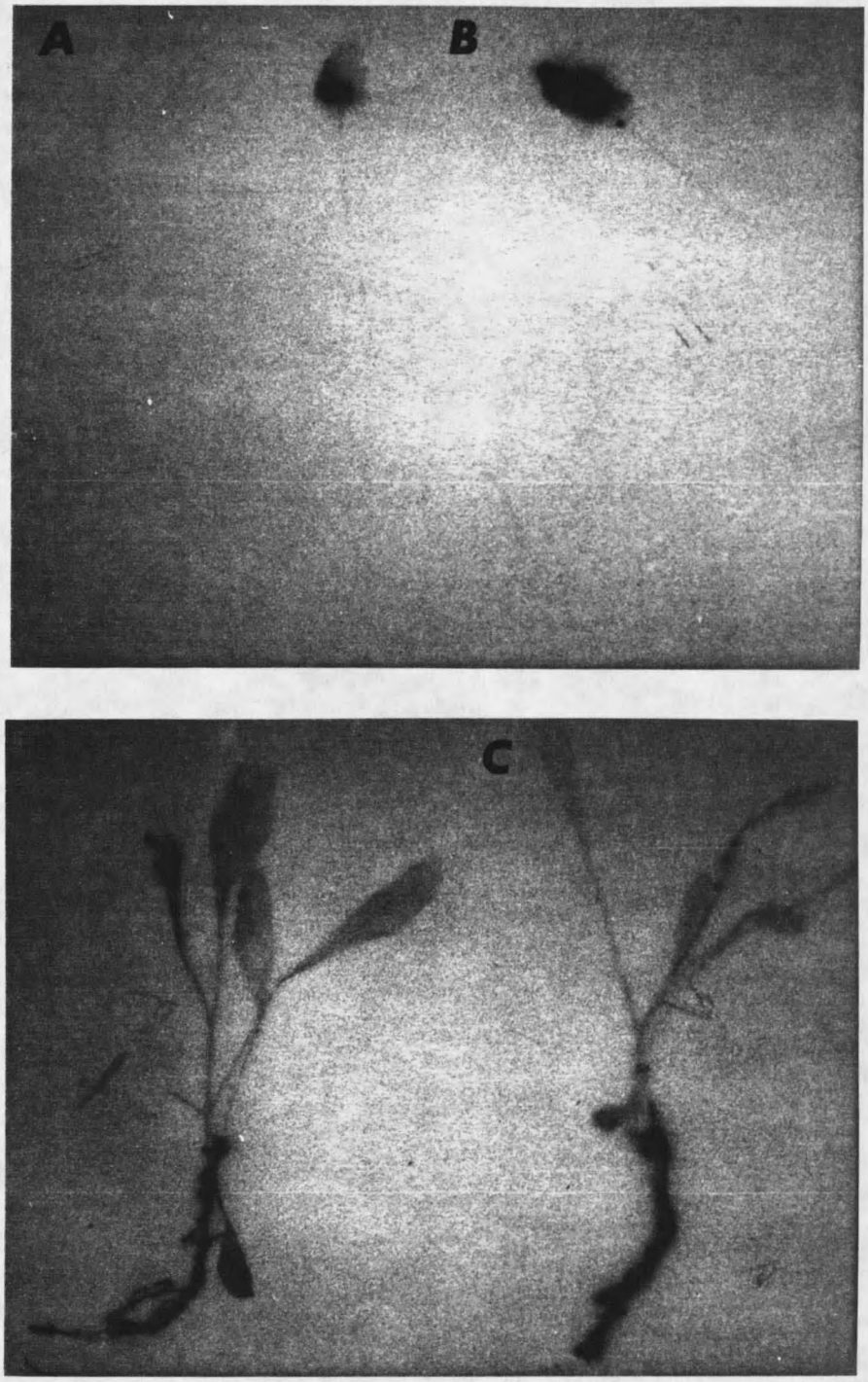

Figure 4. Distribution and translocation of ^{14}C-maculosin on spotted knapweed. Toxin was treated on a leaf without (A) and with (B) a wound, and the root (C).

On detached leaves, ^{14}C-maculosin was applied onto a wound made in the center of each half of the detached leaf (ca. 5.5 cm in length). The leaves were incubated in a moist chamber under intermittent light (6-8 $\mu E/m^2/s$, 12 hr/day) for three days. After incubation, the leaves were segmented into three parts, tip, middle, and base. In the middle part (which was inoculated), there appeared a black necrotic lesion while both the tip and base remained symptomless. The leaf segments were digested and the radioactivity determined. As seen in Table 2, most of radioactivity remained in the center of leaf, in an area approximately 1.8 cm^2. There was very little radioactivity found outside the necrotic area. These data indicated that maculosin applied on the leaves was not mobilized to induce symptom expression.

Table 2. Translocation of maculosin in detached leaves of spotted knapweed. ^{14}C-maculosin (0.02 μmole) was applied to each leaf on a puncture wound. Intermittent light was equivalent to 6-8 $\mu E/m^2/sec$ for 12 hr/day. Values represent the average of three replicates. Plus/minus values represent one S.D.

Leaf segment	DPM
Tip	172 ± 133
Middle	2569 ± 194
Base	46 ± 9

Nature of Toxin Binding Activity

A number of experiments were conducted in order to ascertain the nature of toxin binding activity. The leaf homogenate was centrifuged at 10,000 x g for 30 min and the supernatant was saved. Then 1.5 ml of supernatant (equivalent to 3 mg of protein) was pre-treated as indicated in Table 3. After treatment the solution was mixed with 0.05 μmole of ^{14}C-maculosin (specific activity, 0.11 μCi/μmole) for 1 hour at room temperature. The mixture was passed through a Sephadex G-25 column and the radioactivity was determined.

Table 3. Effect of various treatments on ^{14}C-maculosin binding of leaf extracts. The leaf homogenate was prepared by centrifugation at 10,000 x g for 30 min. The supernatant was incubated with maculosin (0.05 μmole) in 25 mM Tris-HCl buffer (pH 7.5) as previous indicated in the text.

Treatment	Toxin Bound (dpm)	Relative binding activity % of control
None (Control)	2099	100
Heat (85°C / 3 min)	117	6
DNase (1 mg / 30 min / 30°C)	1864	89
RNase (1 mg / 30 min / 30°C)	2011	96
Protease (1 mg / 60 min / 30°C)	839	40

The maculosin binding substance was heat-labile and pretreatment with protease (Bacillus subtilis) largely abolished its binding activity. Incubation with deoxyribonuclease (from beef pancreas), and ribonuclease (from bovine pancreas) had little effect on binding activity (Table 3).

Fractionation of Binding Site

A toxin receptor site may be one or it may be a series of proteins that are widely distributed in the cell such as on the cell surface and/or in the cells (17,23). They are often found in the plasma membrane, cytosol, mitochondria, chloroplasts, nuclear envelope and in the other organelles.

Therefore, the leaf homogenate was mainly divided into two fractions; the membrane and the cytosol. Three to four month old leaves were homogenized in 25 mM Tris buffer (pH 7.5). The homogenate was filtered through cheesecloth and the filtrate was centrifuged at 3,000 x g for 20 min. The supernatant was further centrifuged at 100,000 x g for 1 hour. The pellet was taken up in the same buffer and the supernatant was saved for the determination of binding activity according to the standard equilibrium dialysis method. The results in Table 4 show that most of toxin binding activity was present in the cytosolic fraction and a relatively small amount of binding activity was detected in the native membrane fraction. However, this result did not rule out the possibility of the presence of some binding

Table 4. Distribution of ^{14}C-maculosin binding activity from leaves of spotted knapweed. The leaf homogenate was centrifuged at 3,000 x g for 20 min and 100,000 x g for 1 hr. The binding assay was performed via an equilibrium dialysis (30).

100,000 x g Fraction	Specific binding activity (dpm/mg protein)
Supernatant	698
Precipitant	212

activity being present in the membrane since they were not solubilized by detergents. In fact, more binding activity may be present in the membrane fraction since most of integral proteins are embedded in the lipid bilayer impeding toxin access.

Binding Activity of Various Detergent Treated Membrane Protein

There are many detergents available for use in solubilizing membrane proteins. Furthermore, there are several types of strategies which can be used to purify integral membrane proteins (24). Thus, it is important to choose the appropriate detergent to release the membrane proteins in the cells of spotted knapweed.

The various detergent solubilized proteins possessed binding activities (Table 5). The membrane fraction, when treated with various detergents, showed increased binding

Table 5. ^{14}C-maculosin binding activity of various detergent treated membrane fractions. The crude membrane was prepared by a sequential centrifugation at 3,000 x g for 20 min and 100,000 x g for 1 hour. The pellet was resuspended in 25 mM Tris-HCl buffer (pH 7.5) and made up to 0.5% concentration of each detergent. After mild extraction and incubation for 2 hr at 4°C, the membrane fraction treated with each detergent was centrifuged again at 100,000 x g for 1 hour. The supernatant was collected and detergent removed. The binding activity was measured by an equilibrium dialysis method.

Detergent	Specific binding (dpm/mg protein)
Digitonin	58
Trichloroacetate (sodium salt)	538
Triton X-100	440
Deoxycholate	525
Cholate	476

activity as compared to the non-treated membrane fraction (Table 4) except in the case of the digitonin treatment.

Purification of Cytosolic Maculosin Binding Protein

Because the major binding activity was located in the cytosolic fraction, purification of the activity was started from the 12,000 x g supernatant of homogenized leaves in 25 mM Tris buffer (pH 7.5). The purification scheme is illustrated in Figure 3. After centrifugation, the binding activity was found in the 20 - 60% ammonium sulfate fraction. The elution pattern of the maculosin-bound protein from Sephadex G-200 showed one peak of binding activity

(Figure 5). Elution of the affinity column (maculosin bound onto epoxy-activated Sepharose 6B) reproducibly yielded proteins obtained from the Sephadex G-200 step. After extensively washing the column with elution buffer, the maculosin-binding protein was eluted at 0.3 M NaCl solution, which was sufficient for resolving the maculosin-binding proteins (Figure 6), whereas the other trace proteins could not be detected up to 0.9 NaCl solution.

The protein eluted from Sephadex G-200 column was applied to the tyrosine affinity column. After washing extensively with the same buffer, the column was eluted with a stepwise salt gradient up to 0.9 M NaCl, and a pH gradient (pH 3 - 9). But with these systems, it was found that the tyrosine affinity matrix did not retain any proteins eluted by Sephadex G-200 chromatography. The purification procedure resulted in a 16-fold purification of the maculosin-binding activity on a protein basis (per mg) with an overall yield of 3%. The specific maculosin-binding activity of the final preparation was 43.2 nmole maculosin bound/mg of protein (Table 6).

Characterization of Binding Protein

The purified binding protein from the affinity column was subjected to disc gel electrophoresis using a 10% acrylamide gel in the presence of 0.1% sodium dodecyl sulfate. After electrophoresis, the gel was stained with

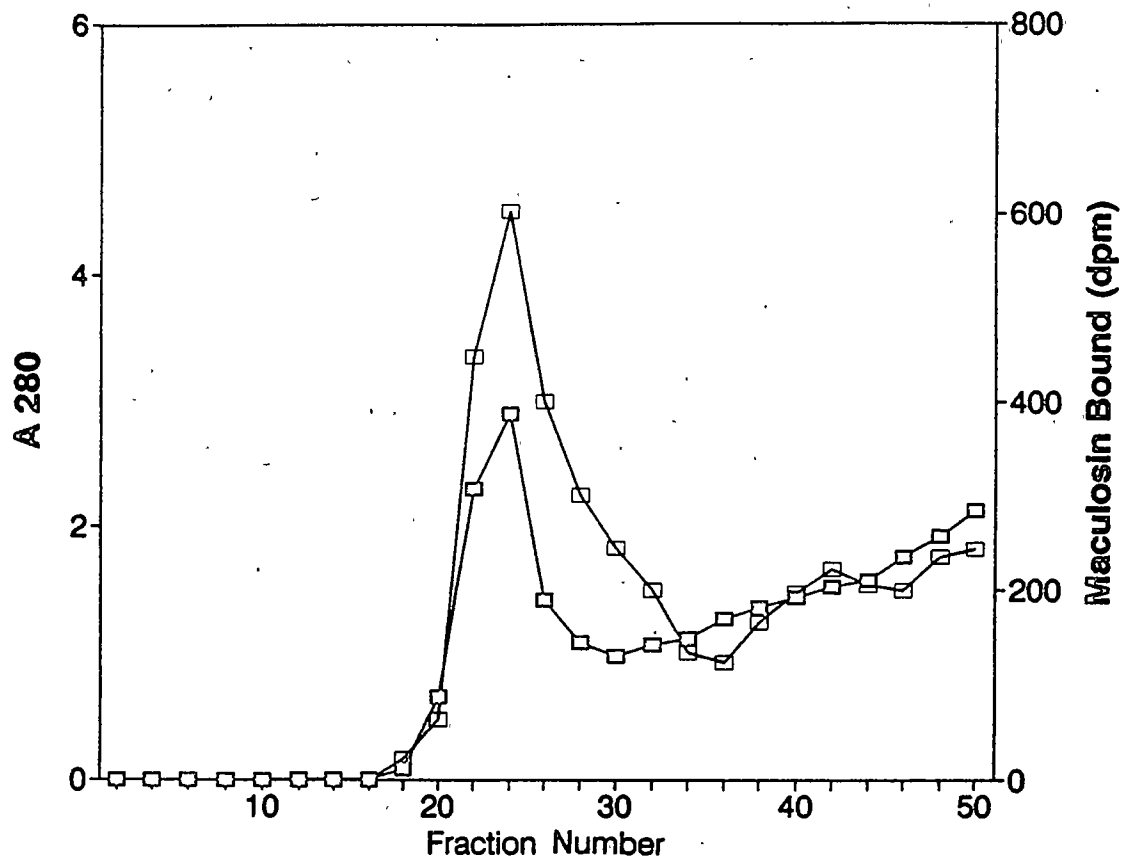

Figure 5. Elution profile of 20-60% ammonium sulfate fraction of cytosolic maculosin binding protein on Sephadex G-200 column. The protein was detected at 280 nm (□) and the radioactivity was counted as dpm (□).

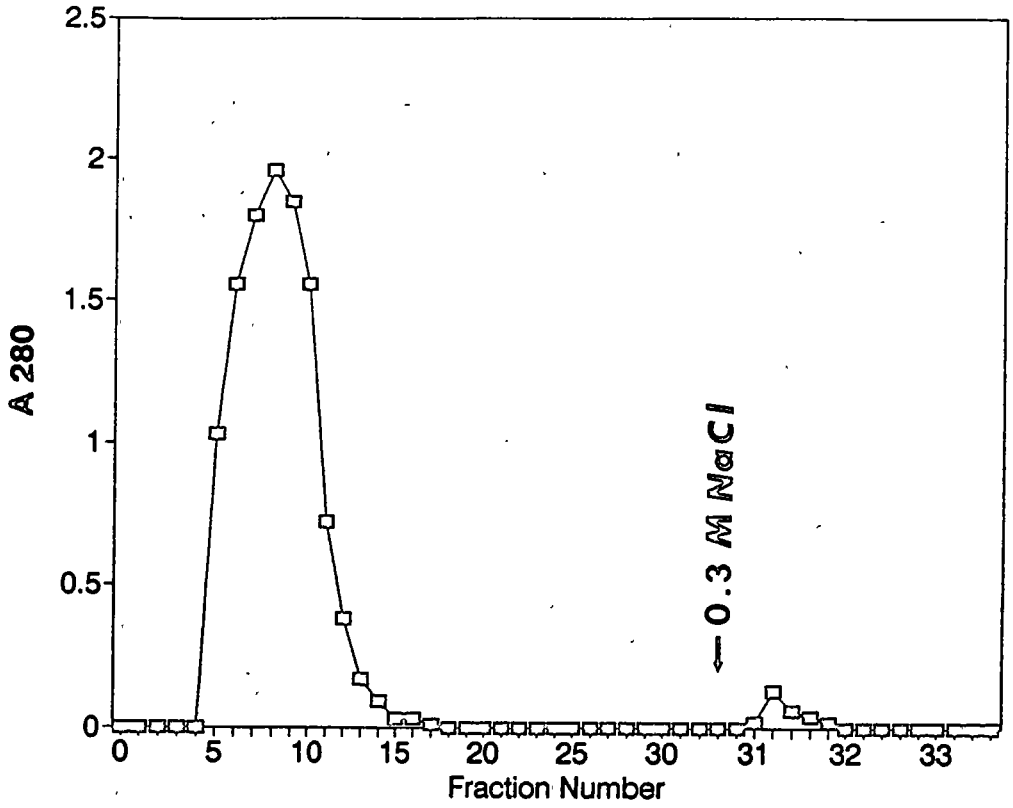

Figure 6. Elution profile of binding protein on maculosin-affinity chromatography. The protein eluted from Sephadex G-200 was applied to the affinity column and eluted with 0.3 M NaCl in 10 mM Tris-buffer (pH 7.5). The protein was detected at 280 nm.

Table 6. Purification of cytosolic maculosin binding protein from the leaves of spotted knapweed

Step	Total activity (nmole bound)	Total protein (mg)	Specific activity (nmole/mg protein)	Yield (%)
12,000 x g supernatant	518	192	2.7	100
100,000 x g supernatant	469	161	2.9	91
20-60% ammonium sulfate	415	67	6.2	80
Sephadex G-200	380	21	19.0	70
Maculosin-affinity column	18	0.41	43.2	3

Figure 7. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of the purified maculosin-binding protein according to Laemmli (33).

Coomassie blue and it showed three closely migrating bands around 60 kdaltons and a weak band near 14 kdaltons (Figures 7, 8).

The molecular weight of the maculosin-binding proteins was estimated by chromatography on a Sepharose 6B column with several proteins of known molecular weight for reference purposes. The binding protein eluted as a aggregated molecular mass of 604 kdaltons (Figure 9). However, non-dissociating 7.5% polyacrylamide gel electrophoresis of the maculosin-binding protein yielded three bands which were of high molecular weight as estimated by their migration position at the top of the gel, and are presumably in a highly aggregated state (Figure 10). Accordingly, there were several proteins adhering to the affinity gel. By its molecular weight, abundance, and the electrophoretic mobility of its subunits, one of the binding proteins was surmised to be ribulose-1,5-biphosphate carboxylase (RuBPCase) which is the most abundant enzyme located in chloroplasts (21,41). Therefore, the affinity purified protein was applied to a double immunodiffusion test employing antibody to RuBPCase from tobacco. Cross reactivity between the purified protein and antiserum of RuBPCase was observed (Figure 11), but when the sample was pre-incubated with 1% SDS in 50 mM Phosphate buffer pH 7.4 at 4°C for 1 hour, the cross reactivity was more intense than the non-treated sample, indicating that this protein was an aggregate.

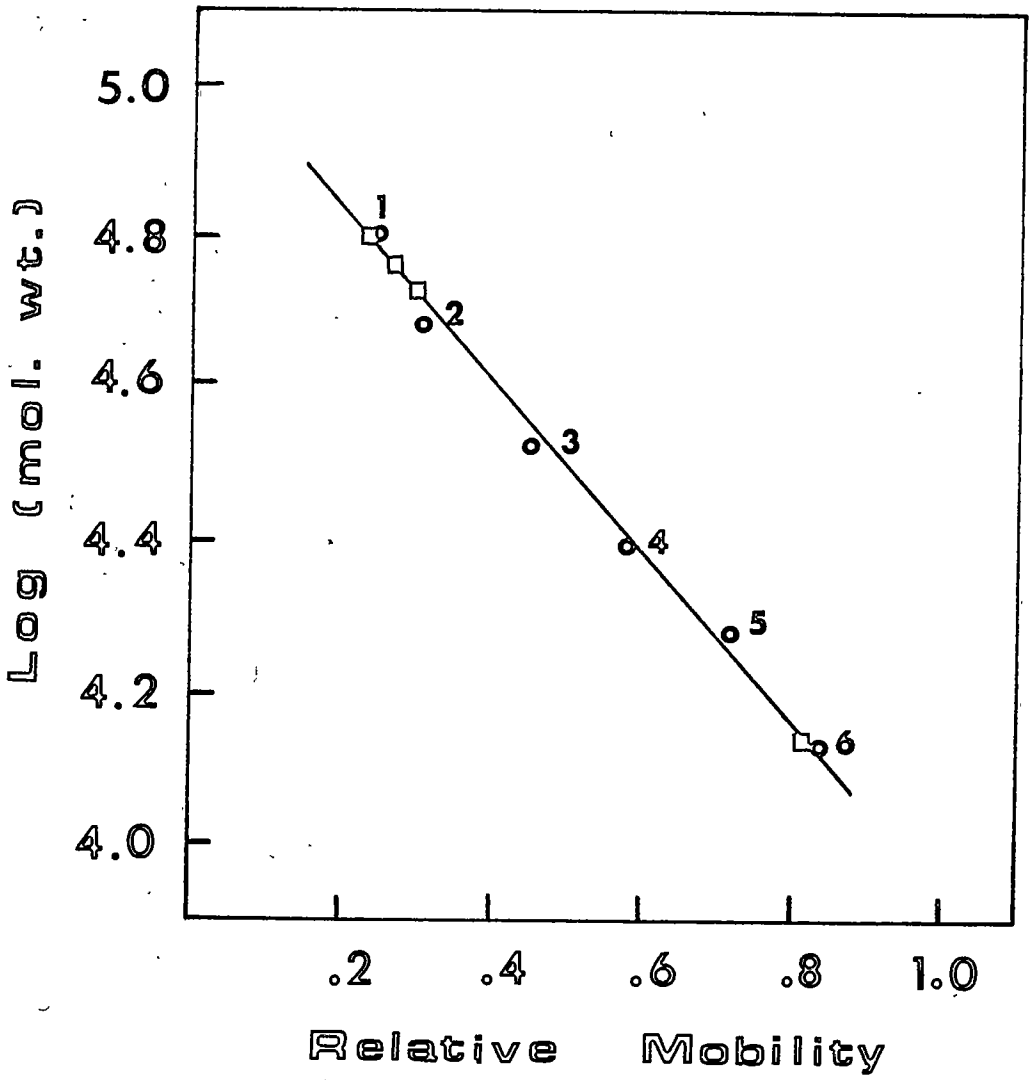

Figure 8. A plot of molecular weights of the purified maculosin binding protein and its subunits (□) by sodium dodecyl sulfate gel electrophoresis. Standard proteins used were: 1. albumin, bovine (66,000), 2. albumin, egg (45,000), 3. pepsin (34,700), 4. trypsinogen (24,000), 5. beta-lactoglobulin (18,400), 6. lysozyme (14,300).

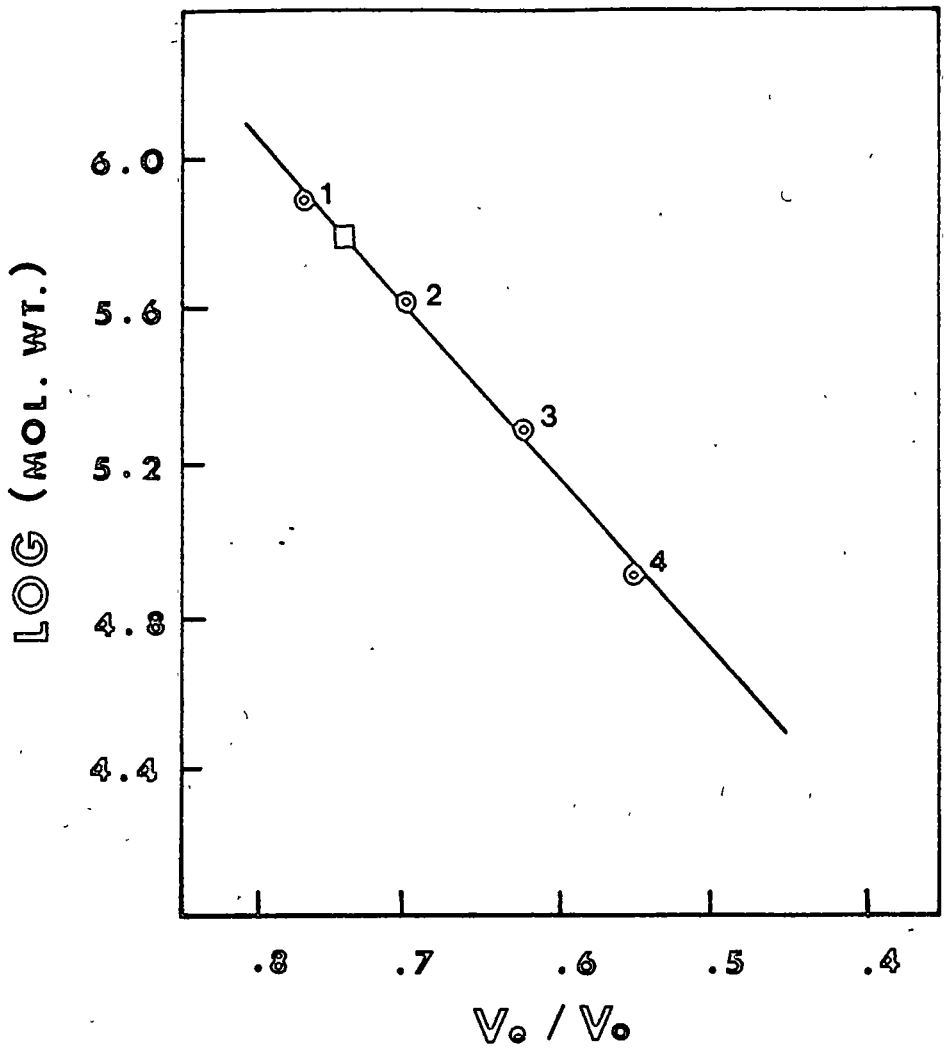

Figure 9. Molecular weight estimation of the purified maculosin-binding protein (□) by chromatography on Sepharose 6B. Standard proteins used were: 1. thyroglobulin (669,000), 2. apoferritin (443,000), 3. alpha-amylase (200,000), 4. bovine serum albumin (66,000).

Figure 10. Disc gel electrophoresis of the purified maculosin-binding protein according to Davis (15).

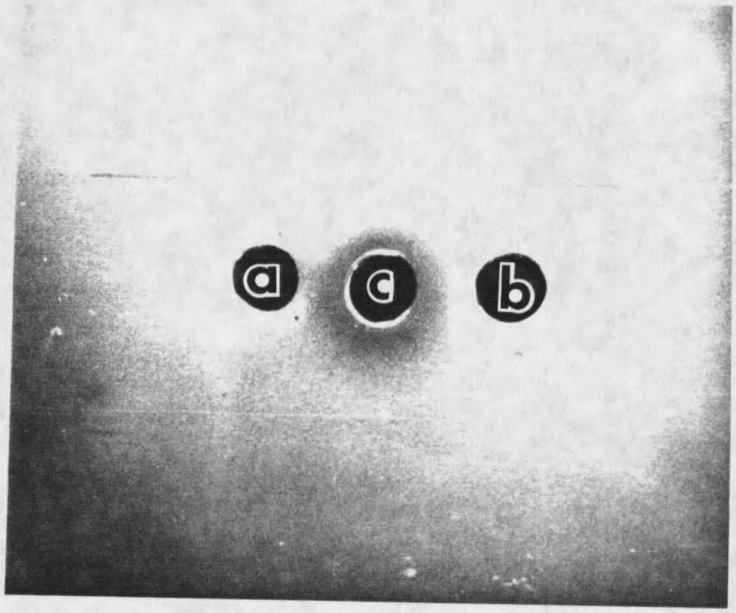

Figure 11. Double immunodiffusion test between the purified maculosin binding protein and the antiserum of ribulose-1,5-diphosphate carboxylase (RuBPcase) from tobacco. The side wells contained (a) phosphate buffer and (b) the purified protein and (c) center well contained the antiserum of RuBPcase.

Activity of Ribulose Biphosphate Carboxylase (RuBPCase)

Before addition of ribulose biphosphate, the reaction mixture containing maculosin was preincubated for 30 min. Activity was expressed as total dpm of fixed ^{14}C-labelled CO_2 per 10 min into acid stable reaction product (carbon 1 of 3-phosphoglycerate). Within 10 minutes, exposure of the spotted knapweed RuBPCase to 0.016 μmole maculosin resulted in 20% inhibition of the enzyme activity as compared to an untreated control preparation (Table 7).

Table 7. Inhibition of ribulose biphosphate carboxylase of spotted knapweed by maculosin. The assay mixture contained protein, 40 μg; RuBP, 0.08 mM; $NaH^{14}CO_3$ (specific activity, 8.4 μCi/umole), 0.07 mM; maculosin (0.15 μmole). Incubation time was 10 min. Values represent the average of six replicates. Plus/minus represent one S.D.

Treatment	^{14}C Total fixed dpm	% of Control
Control (2% ethanol)	269 ± 10	100
Toxin treated	215 ± 9	80

Compared to the activity of spinach RuBPcase (which was purchased from Sigma Co.), the control activity of spotted knapweed was about 3.4 times lower than that of spinach (fixed dpm : 1029 per 50 μg enzyme), while maculosin inhibited about 11% of RuBPCase of spinach in this assay system.

Discussion

The general concept of the mechanism of a host specific phytotoxin implies the interaction of a phytotoxin with some specific macromolecular cellular components. A great deal of evidence has been presented indicating the existence of such receptors in phytotoxin-recognition complex (45,59).

The results of this work suggest that one or more maculosin-binding proteins exist in spotted knapweed in both the cytosolic and membrane fractions (Table 4). The majority of toxin binding activity was recovered from the cytosolic fraction of the 100,000 x g supernatant on the basis of total recovered activity. Even the application of various detergents to the crude membrane faction, the total membrane binding activity remained lower than the cytosolic fraction (Table 5). However, detergent treatment of crude membranes yielded some variation in binding activity (Table 5). For instance, comparable observations made on fusicoccin indicated that binding activity was dependent on the type of detergent, and showed a heterogeneous elution profile by chromatography (10).

A five step procedure starting from the 12,000 x g supernatant was developed to purify cytosolic maculosin binding proteins using an epoxy-activated Sepharose 6B column, where the phenoxy group of maculosin was covalently bound to the Sepharose via an ether linkage bond. The

purified maculosin binding protein obtained by maculosin-affinity chromatography contained three large molecular weight proteins that eluted as an aggregate. This was determined by non-denatured and denatured (SDS) PAGE.

What is the significance of each functional group of maculosin as a recognition factor to protein?

The rationale for selection of maculosin and tyrosine as a ligand for affinity chromatography (Figure 2) was based on previous observations. Even though a number of diketo-piperazines containing proline isolated from the cultural filtrate of A alternata, only (L-Pro-L-Tyr) caused characteristic necrotic symptoms (56). Therefore, this implies that a certain functional group might be necessary for biological activity of maculosin. Maculosin, the most active substance, possesses a phenolic moiety which is very common in phytotoxins (50). Studies of diketopiperazines assumed that the activity was due to its aromatic moiety and its hydroxyl group being accessible for interaction in a biological site. Of two affinity binding columns developed, the maculosin-affinity column retained receptor protein but tyrosine did not, indicating that the possible binding site of maculosin is the diketopiperazine ring containing proline rather than a phenoxy moiety of the molecule. As shown by the metabolism of maculosin in the leaves of spotted knapweed in Chapter 3, the phenoxy group of maculosin (cyclo L-Pro-L-Tyr) was rapidly conjugated to glucose, forming maculosin

β-O-glucoside, not bound to any proteins, indicating that the majority of toxin-binding protein is the portion of diketo-piperazine containing proline moiety. Therefore, this is one explanation why tyrosine affinity chromatography did not bind proteins eluted from Sephadex G-200 column chromatography.

The other interesting characteristic of maculosin-binding proteins is the molecular behavior at low ionic strength by ion-exchange chromatography. Even though the maculosin-binding protein of the cytosolic fraction was retained on DEAE-cellulose, the bound radioactivity was significantly eluted from the column at low ionic strength. However, on the phosphocellulose (hydroxyapatite) column the bound radioactivity retained with the protein (data not shown). These data indicate a certain bonding behavior in the molecular interaction between maculosin and its binding proteins.

The largest amount of total radioactivity bound to the cytosolic proteins were found in the high molecular region on size exclusion chromatography (Figure 9). Sephadex G-75 size exclusion chromatography of the preparation always showed the binding activity retained near the void volume (data not shown). This was similar to the observation reported by Stoddart et al. (57) on the binding of gibberellin A1 in dwarf pea epicotyls. They deduced that while gibberellin A1-binding proteins appeared to have a low specific affinity on a per unit mass basis, the high molecular weights of the

proteins in this fraction dictate a high molar binding activity. By electrophoretic mobility of non-denaturing, and denaturing (SDS treated) PAGE, and its bulk amount of binding activity on chromatographic behavior, one of the maculosin-binding proteins was surmised to be ribulose-1,5-diphosphate carboxylase (RuBPCase) which is one of the most abundant proteins located in the stroma of chloroplasts where it catalyses the carbon dioxide-fixing reaction in photosynthesis in the plant kingdom (21,41). RuBPCase is a high molecular weight soluble protein (just over 500,000 daltons in green plants) and associated with eight large subunits (mol. wt. 5.2-6.0 x 10^4) and eight small subunits (1.2-1.8 x 10^4). Therefore, the double immunodiffusion test was employed. The cross reactivity of purified proteins with tobacco plant antibody showed that one of the binding proteins in the cytosolic fraction is RuBPcase. The visual cross band was more amplified after treatment of 1% SDS because of its aggregated condition (Figure 11). Even though the activity of RuBPCase was inhibited about 20% by maculosin <u>in vitro</u> (Table 7), it could not be determined whether RuBPCase was the actual recognition site of maculosin or if a maculosin caused pleiotropic effects including a reduction in RuBPCase activity.

In addition to the possible role of RuBPCase as one of the maculosin binding proteins, there is good likelihood that other high molecular weight proteins are present (Figure 10),

but not yet characterized. Binding in this fraction therefore could be due to an affinity for one or more of these proteins to the maculosin affinity gel.

Although no direct evidence for a physiological function of maculosin-binding protein has yet been obtained, the protein may play a role in a maculosin mediated cellular physiological processes. If the process of metabolic disorder is mediated by maculosin-receptor complex present in the cytosol of plant leaf, it might be complex mechanism because maculosin needs to transpass at least one or two membranes, the plasma membrane and the chloroplast membrane.

CHAPTER III

FATE OF MACULOSIN

Introduction

For a phytotoxin to cause symptoms in the leaf tissue of a plant, it should reach the sensitive target site(s) and disrupt the normal function, growth and development of cells. If this process is blocked or modified, symptom development will either be delayed or fail.

In many cases, xenobiotics including phytotoxins and herbicides are metabolized after being introduced into plant tissues. This metabolism, whether mediated enzymatically or not, leads to the activation or detoxification of the original bioactive compounds. The basic biochemical metabolic reactions of xenobiotics are oxidation, reduction, hydrolysis, and conjugation (34,51). Much is known about herbicide metabolism in plants, including the nature of transformed products and the alteration of biological activity due to metabolism (12). However, there have been comparatively few attempts to describe the metabolism of phytotoxins in plants. For instance, Tabtoxin, produced by *Pseudomonas tabaci*, appears to require processing before it is toxic. In the diseased plant, non-specific peptidase

cleaves off threonine or serine releasing the biologically active tabtoxinine-β-lactam (68). Conversely, petasol, a toxin produced by Drechslera gigantea, is conjugated with amino acids rendering it inactive (6).

Maculosin is the only known host specific phytotoxin produced by a fungal pathogen, Alternaria alternata on a weed plant, spotted knapweed (Centaurea maculosa L.). Even though this fungus produces numerous, non-toxic diketopiperazines, only maculosin [cyclo (L-Pro-L-Tyr)] causes black necrotic lesions on the leaves of spotted knapweed at 10 μM (56). Maculosin, the most active substance, possesses a phenoxy moiety, suggesting the importance of structural conformation in its bioactivity.

The main objective of this study was to determine the molecular behavior of ^{14}C-maculosin in the leaves of spotted knapweed. TLC and autoradiography were used to analyze leaf extracts and determine the fate of maculosin. A major conversion metabolite was isolated, purified, and tested for biological activity.

Materials and Methods

Plants

All plants used in this study were individually grown in plastic pots containing a mixture of pasteurized sand, top soil, and peat (1:1:1) in the MSU plant growth facility.

Preparation of Maculosin and Dipeptide Methyl Esters

^{14}C-maculosin (specific activity, 0.11 μCi/μmole) was synthesized by the methods previously described in Chapter 2.

Dipeptide methyl esters (L-Pro-L-Tyr-OMe and L-Tyr-L-Pro-OMe) were synthesized by the methods of Itoh et al. (27) and Nitecki et al. (46). The basic procedures are described in Chapter 2. t-Butyloxycarbonylation of an amino acid (starting with 0.5 mmole of each amino acid) was carried out using 10% excess of Boc-On and 50% excess of triethyl amine in 50% aqueous acetone. The t-boc-amino acid was reacted with a second amino acid methyl ester hydrochloride in methylene chloride containing triethylamine and N-ethyl-N'-(3-dimethylaminopropyl)carbodiimid. t-Boc-dipeptide was deblocked by treatment with formic acid at room temperature for 2 hours.

Application of ^{14}C-maculosin

The leaves from three to four month old spotted knapweed plants were excised and placed in a moist chamber. The individual leaves were punctured with a 10 μl Hamilton syringe and overlayered with 5 μg of ^{14}C-maculosin solubilized in 2% methanol. When oats were studied, the apical 5 cm of leaves from 20 day old oat seedlings were treated. After treatment with maculosin, the leaves were incubated for 5 days at 28°C at 12 hour light and dark period.

Chromatography

Thin layer chromatography (TLC) was performed on precoated silica gel G plates (Kiesel gel 60, F254 plates, E. Merk) using the following solvent systems (parts by volume): (A) chloroform:methanol:acetic acid, 14/2/1, (B) n-butanol: methanol:acetic acid, 4/1/1, (C) dichloromethane:methanol: water, 13/5/1. Short UV wavelength light and radioactivity were used to detect the compounds of interest.

Analysis of Metabolites

^{14}C-maculosin treated leaves were placed for 5 days in a moist chamber as previously described. The leaves were then homogenized in 50 ml of methanol in a Sorvall Omnimixer at top speed for 1 min. After filtration through Whatman No. 2 filter paper, the homogenate was concentrated in vacuo at 35°C, and dissolved in 0.5 ml methanol. These metabolites were then analyzed by thin layer chromatography in solvent system A.

Extraction and Purification of the Conversion Products

Procedures for extraction and the separation of maculosin metabolites are summarized in Figure 12. Treated leaf tissues were homogenized for 30 seconds (3 X) with a Sorvall Omnimixer in 200 ml 70% methanol:ethyl acetate (7:3, v/v). The homogenate was filtered through Whatman No 2 filter paper to remove debris, washed with 70% methanol until

Treated leave

|

Grind in 70% methanol:ethyl acetate (7:3, v/v)

|

Filtration

|

Partition in water/ethyl acetate

|

Aqueous phase

|

Extraction with n-butanol

|

Organic phase

|

Sephadex LH-20 column

|

TLC
(chloroform:methanol:acetic acid,
14:2:1, v/v/v)

|

TLC
(n-butanol:methanol:acetic acid,
4:1:1, v/v/v)

|

TLC
(dichloromethane:methanol:water,
13:5:1, v/v/v)

|

Purified major metabolite

Figure 12. Extraction and purification of the major metabolite (M-2).

negligible ^{14}C was detected in the filtrates and concentrated in vacuo at 30°C. The aqueous concentrate was partitioned between water and ethyl acetate. The water fraction was further extracted three times with a half volume of n-butanol. The radioactivity of each extract was determined. The butanol extract was concentrated in vacuo at 30°C with a rotary evaporator and the concentrate was dissolved in 15 ml of 50% methanol. This extract was loaded on a Sephadex LH-20 column (96 x 1.4 cm), and eluted with methanol:water (1:1). Fractions were taken volumetrically (2.3 ml) and monitored at 254 nm on a Beckman spectrophotometer. Each fraction which contained radioactivity was collected, concentrated at 30°C and dissolved in 0.5 ml methanol. These fractions were chromatographed on a silica gel thin layer chromatography plate (20 x 20 cm) using solvent system A, B, and C. Each zone containing radioactivity was scraped from the plate, and the metabolites were removed from the gel with methanol.

Biological Activity

The leaf puncture assay consisted of puncturing a detached leaf (63). The wound was then overlayered with 5 μl of the test solution. Ethanol (2%) was used to dissolve the compounds. The treated leaves were incubated in a moisture chamber for 5 days under 12 hours of light (6-8 $\mu E/m^2/s$) and darkness at 28°C and the development of symptoms was noted. The control in all bioassays was run using 2% ethanol.

Ion-Exchange Properties of Metabolites

^{14}C-maculosin treated leaves were homogenized in 200 ml of water with a Sorvall Omnimixer for 1 min at full speed. The plant homogenate was filtered through several layers of cheesecloth to remove debris. The filtrate was extracted with an equal volume of chloroform. The water soluble metabolites were examined by chromatography on an ion-exchange resin using Dowex-50 and Dowex-1 prepared by the method of Bunkers et al. (5). The Dowex-50 column (15 x 1.5 cm, H^+ form) was activated with 1 N HCl and equilibrated with distilled water. After loading the sample, the column was washed with 3 equal volumes of distilled water. The effluent was collected and saved for further fractionation. The Dowex-50 column was eluted with 4 N ammonium hydroxide. The Dowex-1 column (15 x 1.5 cm, formate form) was activated with 1 M sodium acetate and equilibrated with distilled water. After loading the sample, the column was washed with 3 equal volumes of distilled water and the effluent was collected. The Dowex-1 column was eluted with 6 N formic acid. The radioactive fractions were concentrated in vacuo at 30°C and redissolved in 50% methanol. Each fraction was chromatographed on a Sephadex LH-20 column (96 x 1.4 cm), and eluted with methanol:water (1:1). Fractions were collected volumetrically (2.4 ml) and the radioactivity was determined. Each radioactive fraction was collected, then concentrated at 30°C and chromatographed on TLC in solvent system A.

Translocation of the Major Metabolite

The leaves of spotted knapweed were excised and placed in a moisture chamber. Two wounds were made in the center of each half side of the leaves with a needle puncture. Each wound was overlayered with 5 μl of 2% methanol containing 1634 dpm (0.012 μmole, specific activity 0.064 μCi/μmole) of ^{14}C-labelled major metabolite isolated from the plant. When oats were used, punctures were made in the center of leaves. Each wound was overlayered with 3268 dpm (0.023 μmole) of radioactivity. The treated leaves were kept for 3 days at 28°C under 12 hour light and dark period. After incubation the leaves were cut into 1 cm segments and placed in separate scintillation vials and 0.5 ml perchloric acid:hydrogen peroxide (1:2) digestion solution was added. The vials were taken to 100°C for 30 min. The digests were solubilized in 10 ml Aquasol and radioactivity was determined.

Metabolism of Maculosin and its Major Metabolite as a Function of Time

Each 2000 dpm (0.082 μmole) of ^{14}C-maculosin (total 18,000 dpm) and 1500 dpm (0.01 μmole, specific activity 0.064 μCi/μmole) of ^{14}C labelled major metabolite (total 14,000 dpm) were applied to the puncture wound on leaves with three replicates. The treated leaves were harvested and homogenized in methanol after 0, 1, 3, and 5 days. The homogenate was filtered through a filtration column (6.2 x 1.2 cm, J. T. Baker Inc.) and concentrated in 4 ml methanol. This methanol

fraction was centrifuged to remove the insoluble residue. The supernatant was further dried and redissolved in 300 μl methanol. Each sample was chromatographed on TLC using solvent system A. The R_f of interest was scraped and the radioactivity determined.

Instrumentation

The nuclear magnetic resonance (NMR) spectrum was recorded on a Bruker AC300 spectrometer. Chemical shifts were recorded in ppm units relative to the methyl signal (3.30 ppm) of d_4-methanol. Mass spectra were obtained from a VG instrument 7070 EHF spectrometer. Spectral analysis was kindly performed by Dr. Andrea Stierle, Montana State University.

Determination of Radioactivity

Samples were mixed with 10 ml of Aquasol (New England Nuclear Corp.) before counting. Radioactivity measurements were made on a Packard liquid scintillation spectrometer model 22000CA and counts were corrected to disintegrations per minute (dpm).

Results

Cellular Metabolism

The metabolism of ^{14}C-maculosin was studied in detached leaves. After incubation of maculosin-treated leaves for 5 days, the methanol soluble metabolites were extracted and

analyzed by TLC in solvent system A followed by autoradiography. The autoradiogram showed three metabolites designated as M-1, M-2, and M-3, which were more polar than maculosin, based on their lower R_f values (Figure 13). The upper spot corresponded to the R_f of maculosin. Among metabolites of maculosin, the middle spot (M-2) possessed greater radioactivity than the other two compounds. Therefore, it was designated as the major metabolite. The chromatographic behavior of the other metabolites on TLC is shown in Table 8.

Table 8. R_f of maculosin and its metabolites by thin layer chromatography

Solvent	M-1	M-2	M-3	Maculosin
System A	0.0	0.09	0.22	0.59
System B	-[a]	0.30	0.41	0.64
System C	-[a]	0.55	0.69	0.86

[a]: not determined.
Solvent system A = chloroform:methanol:acetic acid (14:2:1, v/v/v), system B = n-butanol:water:acetic acid (4:1:1, v/v/v), system C = dichloromethane:acetic acid:water (13:5:1, v/v/v).

Properties of Metabolites

All three metabolites were recovered from the water fraction after partitioning between ethyl acetate and water. Therefore, after treatment of ^{14}C-maculosin, the leaves were

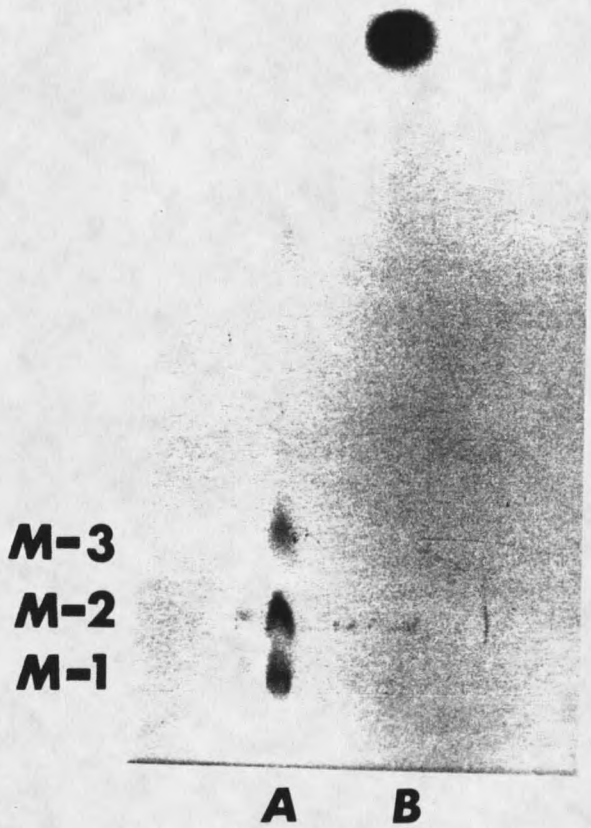

Figure 13. Autoradiographic analysis of ^{14}C-maculosin metabolites from leaves of spotted knapweed. The leaves were extracted with methanol and developed on a normal phase silica plate with a solvent system (chloroform:methanol:acetic acid, 14:2:1, v/v/v). Track: A) crude extract, B) maculosin.

homogenized in 50% methanol and filtered through 1 layer of Whatman No. 2 filter paper. The aliquot was concentrated in vacuo, and dissolved in water. This water fraction was extracted with chloroform to remove maculosin. The water fraction was used to determine the ion-exchange properties and the molecular behavior of all metabolites on a Sephadex LH-20 column (methanol:water, 1:1). While Dowex-50 retained both M-1 and M-3, Dowex-1 retained only M-1. However, the major metabolite (M-2) was not retained either by Dowex-1 or Dowex-50 resin, which suggested the presence of a sugar moiety (Table 9). Also, if one of the amide bonds of the diketopiperazine (maculosin) is cleaved, it would yield a free amine and carboxylic groups, both of which are then free to interact with ion exchange resins. Therefore, these data

Table 9. Ion-exchange properties of water soluble metabolites of ^{14}C-maculosin. The water soluble metabolites were passed through Dowex-50 and Dowex-1 resin. Each fraction containing radioactivity was counted and loaded on a Sephadex LH-20 column, eluting with 50% methanol. Each radioactive fraction was chromatographed on TLC in solvent system A.

Fraction retained	% of Total radioactivity of water soluble metabolites	Retention of metabolite		
		M-1	M-2	M-3
Dowex-50	11	+	-	+
Dowex-1	16	+	-	-
Neutral	73	-	+	-

suggest that M-1 and M-3 might be ring-opened forms of maculosin.

Purification of Metabolites

^{14}C-labeled maculosin treated leaves were extracted by homogenizing with methanol/ethyl acetate/water. This extract was partitioned into aqueous and organic phases using water and ethyl acetate. Both fractions were radioactive, but TLC analysis indicated the conversion products were in the aqueous phase, while most of maculosin was in the ethyl acetate fraction. The aqueous phase was further extracted with n-butanol. Most of the major conversion product was recovered in the butanol phase, which was concentrated in vacuo and dissolved in a small volume of 50% methanol. The insoluble residue was removed by high speed centrifugation. The supernatant liquid was loaded onto a Sephadex LH-20 column pre-equilibrated with 50% methanol. The major metabolite (M-2) had a bed volume of about 107 ml; and that of metabolite-3 was 150 ml. The radioactive fraction was collected and subjected to TLC using solvent system A, B, and C (Table 8).

Identification of the Conversion Products

Employing partition and sequential analysis, the major metabolite (M-2) was purified and identified as maculosin β-O-D-glucoside (Figure 14). The electro-spray mass spectrum

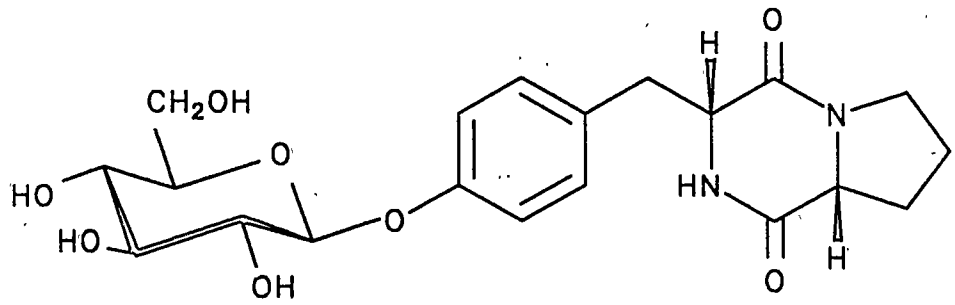

Figure 14. Structure of maculosin β-O-D-glucoside.

showed a pseudo-molecular ion peak at m/z 445 $[M + Na]^+$ (Figure 15). In CI spectroscopy, the compound split into two moieties: the fragmentation pattern of the aglycone moiety (m/z 260) was superimposable upon the CI spectrum of authentic maculosin (56), while a fragment at m/z 180 indicated a sugar (Figure 16). As to the sugar moiety, the presence of glucosyl carbons, was confirmed by the ^{13}C NMR data (Table 10, Figures 17, 18). The ^{13}C NMR spectrum of the metabolite also showed a signal at 102.4 ppm inferring the β-configuration of the glucose. These data indicated that a major metabolite was a glucoside of maculosin. In the ^{1}H NMR spectrum, the aromatic doublets at δ 7.05 and 7.2 were observed down field of the comparable protons in maculosin (Figures 19, 20).

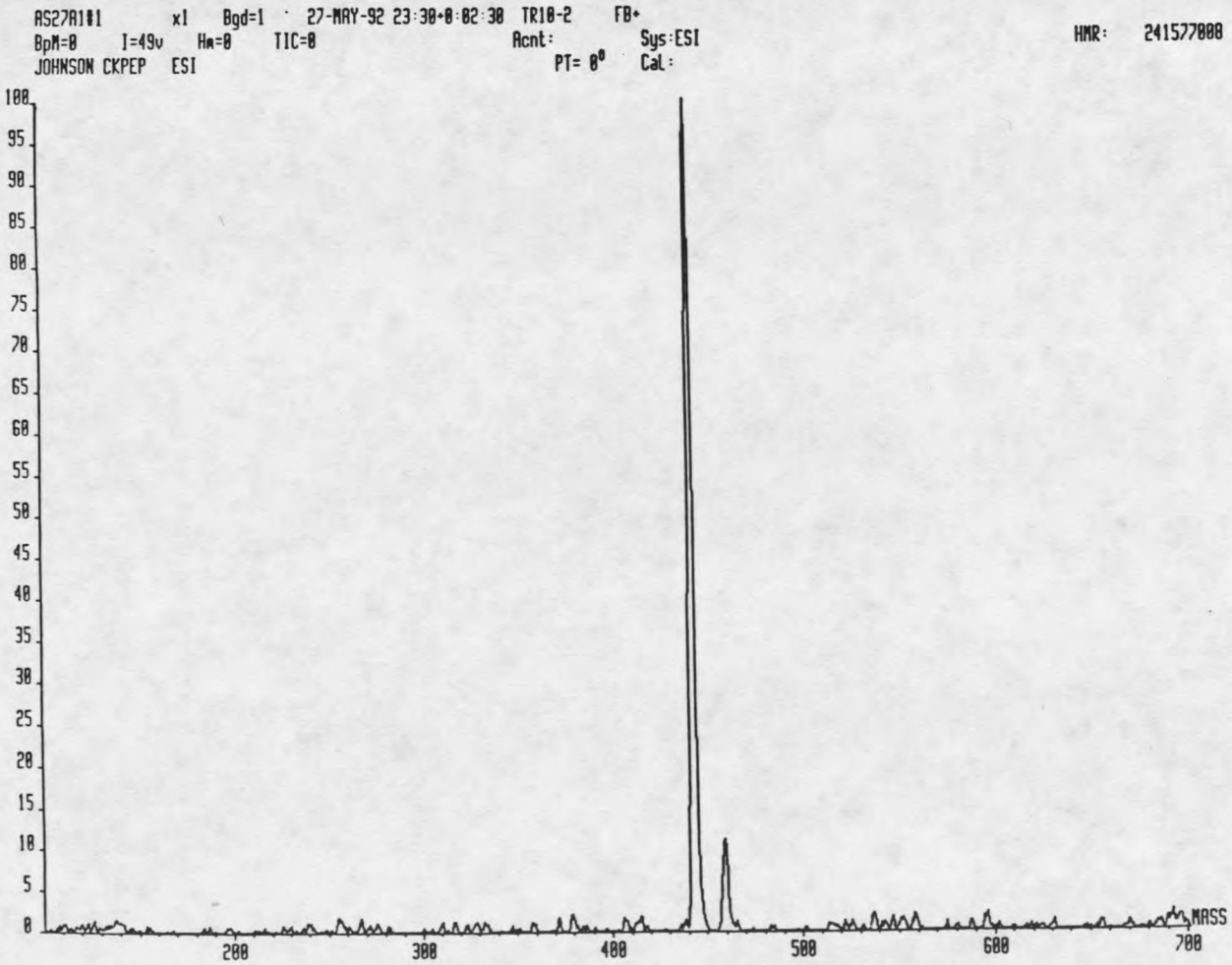

Figure 15. Electro spray mass spectrum of the major metabolite (M-2).

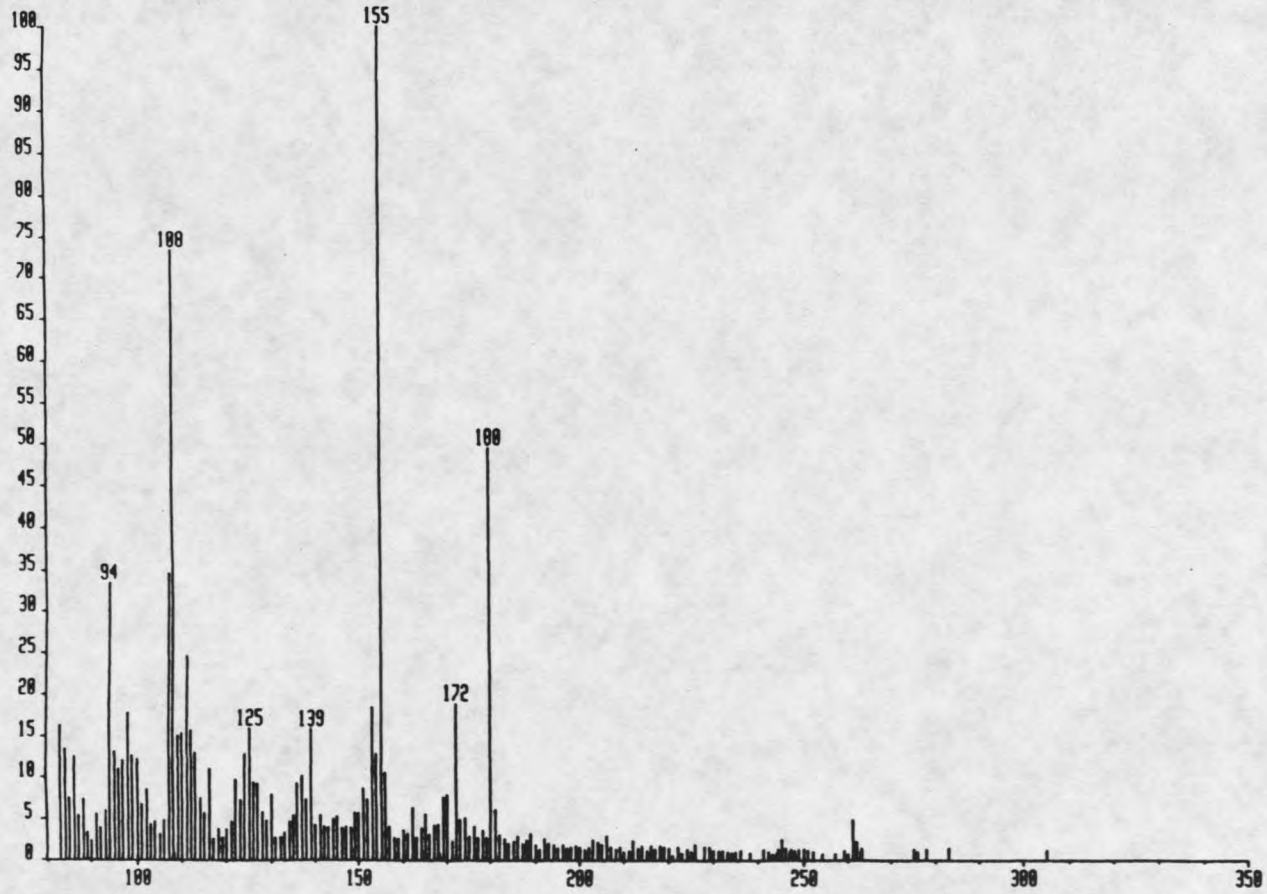

Figure 16. CI mass spectrum of the major metabolite (M-2).

Table 10. ^{13}C-NMR spectral data of maculosin and a major metabolite.

No. of C	Maculosin	Major Metabolite
2	170.8	170.8
3	60.1	60.1
5	167.0	167.0
6	57.9	57.9
7	43.8	43.8
8	22.7	22.7
9	48.5	48.5
10	29.4	29.4
11	127.7	127.7
12,16	132.1	132.1
13,15	157.7	157.7
		Glucose
1′	-	102.4
2′	-	74.9
3′	-	78.1
4′	-	71.4
5′	-	78.0
6′	-	62.2

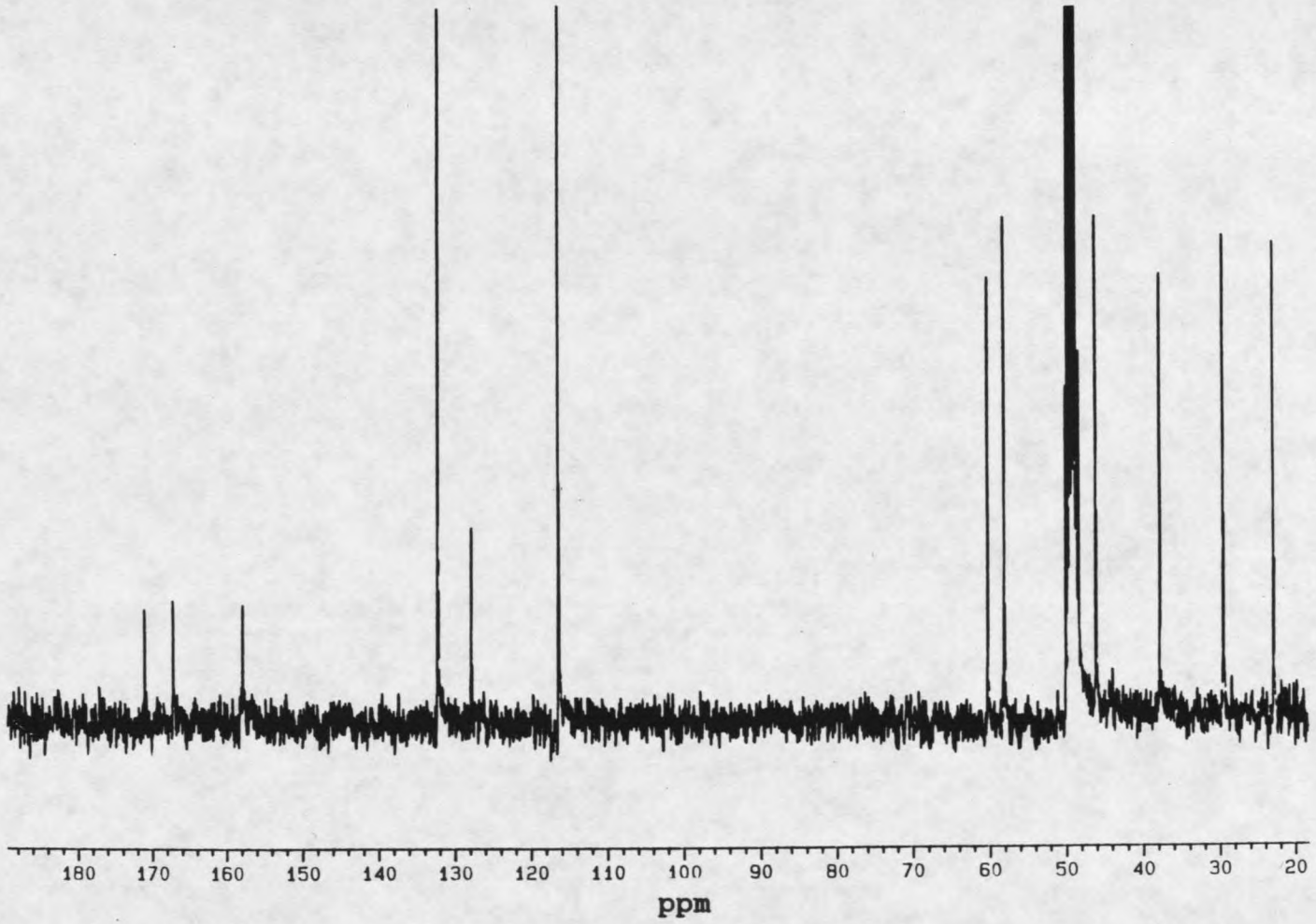

Figure 17. ^{13}C-NMR spectrum of maculosin.

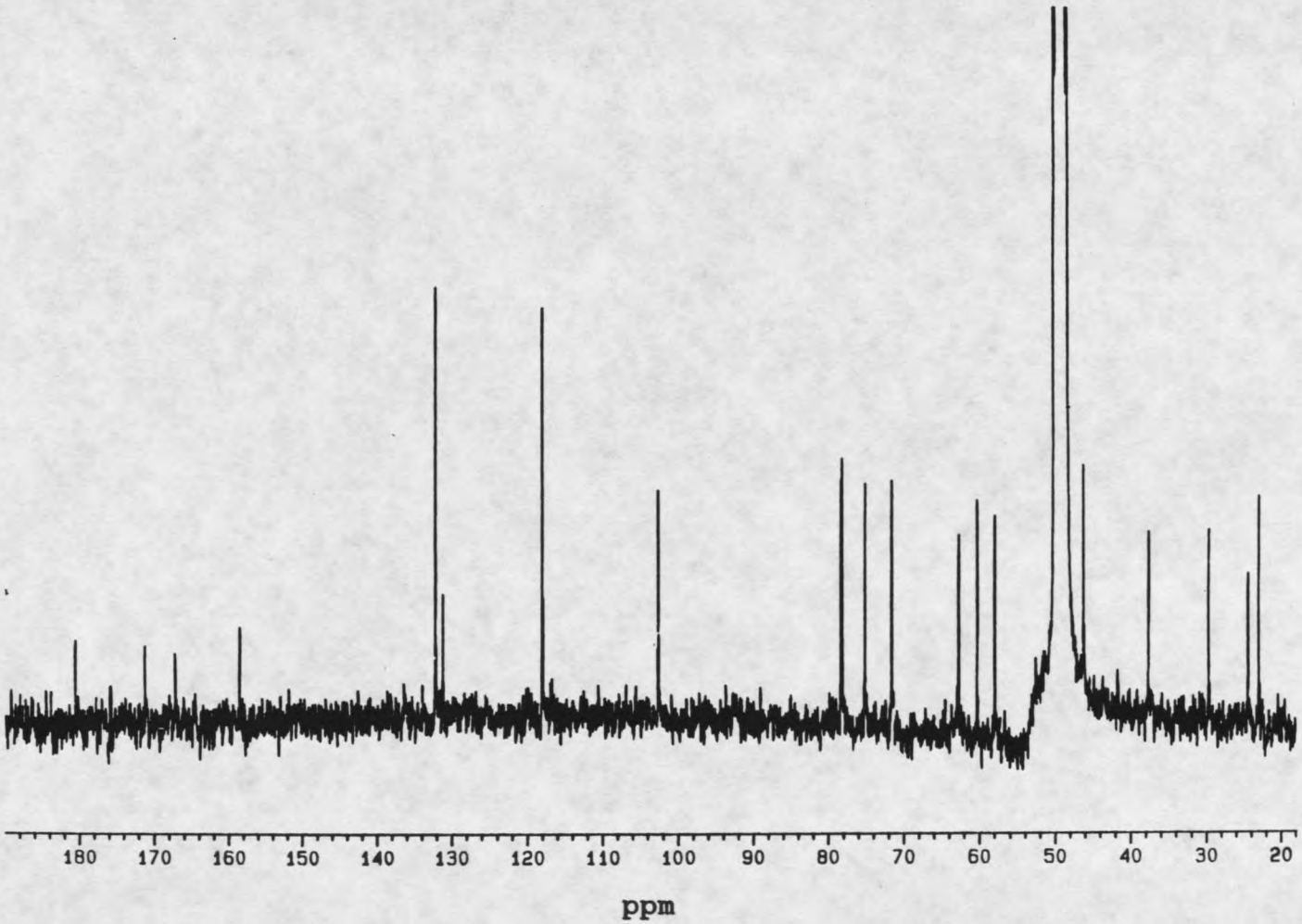

Figure 18. ^{13}C-NMR spectrum of the major metabolite (M-2).

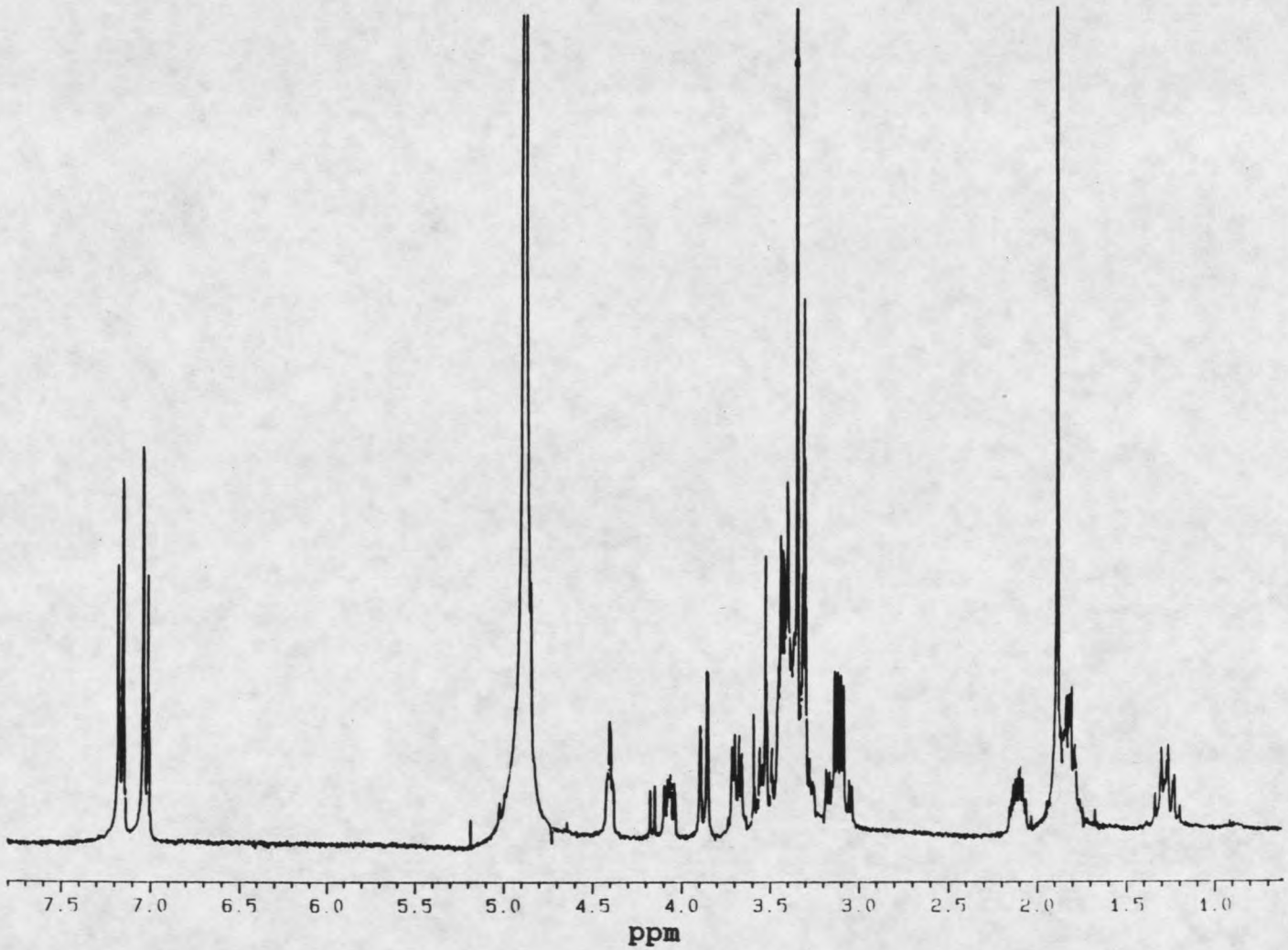

Figure 19. ^{1}H-NMR spectrum of the major metabolite (M-2) in d-methanol.

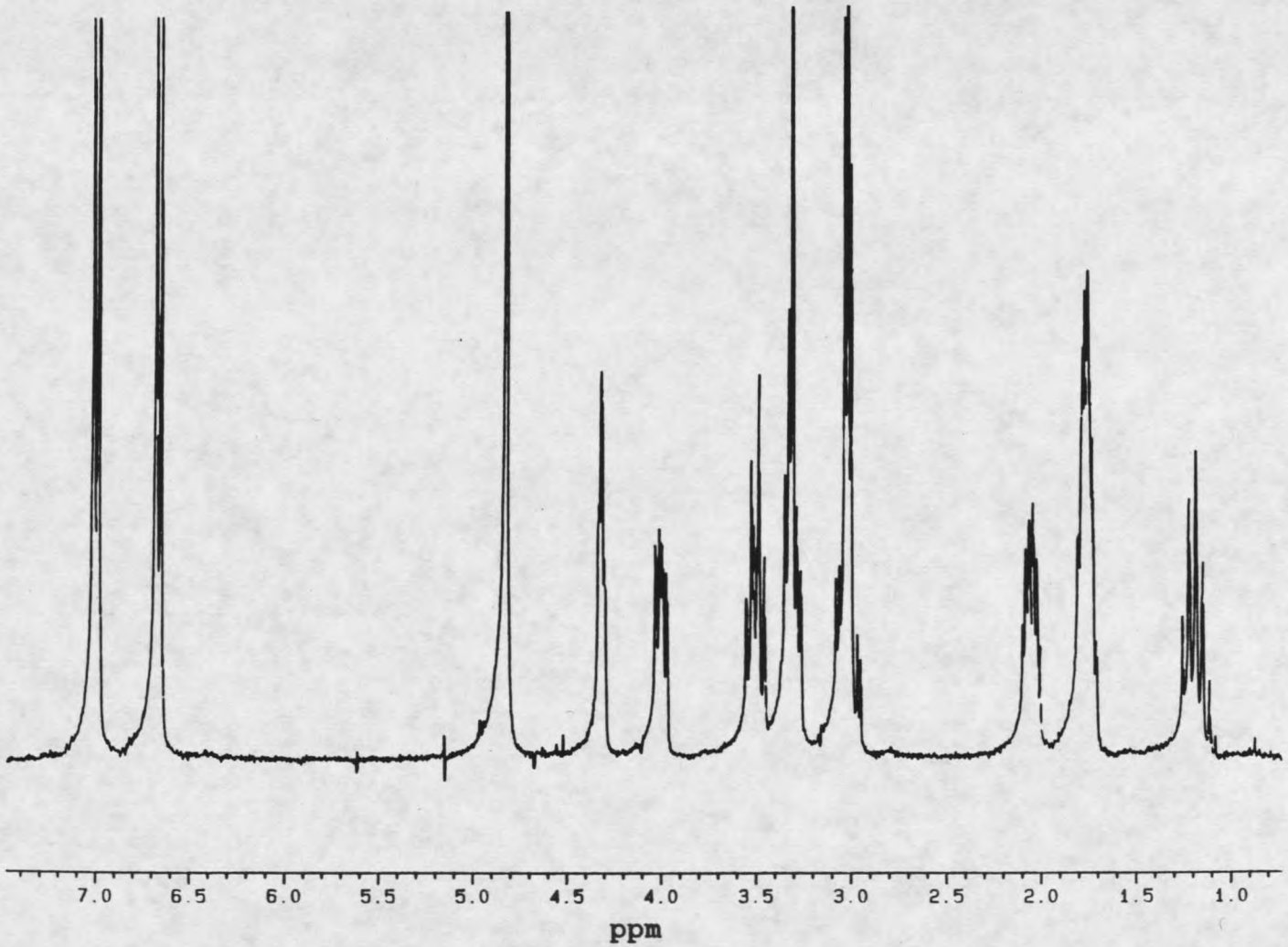

Figure 20. ^{1}H-NMR spectrum of maculosin in d-methanol.

Attention will be addressed to metabolite-3 (M-3). During the purification of M-3 employing partition and thin layer chromatography, the total amounts of this metabolite were continuously reduced as manifested by a continuous loss in the recovered radioactivity. Therefore, it was difficult to obtain sufficient amounts of this metabolite for spectral analysis.

If one of the amide bonds of a diketopiperazine (maculosin) is hydrolyzed, it can yield a dipeptide which contains both a free amine and a carboxylic group. There are reports that dipeptides are unstable and readily cyclized to yield diketopiperazines because of the susceptibility of the carbonyl group to the attack of an amine group (2,23,42). Therefore, the partially purified metabolite-3 (12,000 dpm) was refluxed in a solution 10 ml of toluene and sec-butanol (1:1, v/v) for 2 hours (this is a synthetic method to cyclize the dipeptide to make diketopiperazine as described in Chapter 2) and chromatographed on TLC in solvent system A. More than 95% of the radioactivity was recovered at the R_f of standard maculosin rather than the R_f of the metabolite-3.

When maculosin was stored in 2% methanol for 4 months at 4°C, it was surprisingly converted to a compound which had the same R_f as metabolite-3 (Figure 21). Therefore, the structure of this metabolite was surmised as L-Tyr-L-Pro-OH or L-Pro-L-Tyr-OH because there are two possible cleavage sites of the diketopiperazine which would produce two

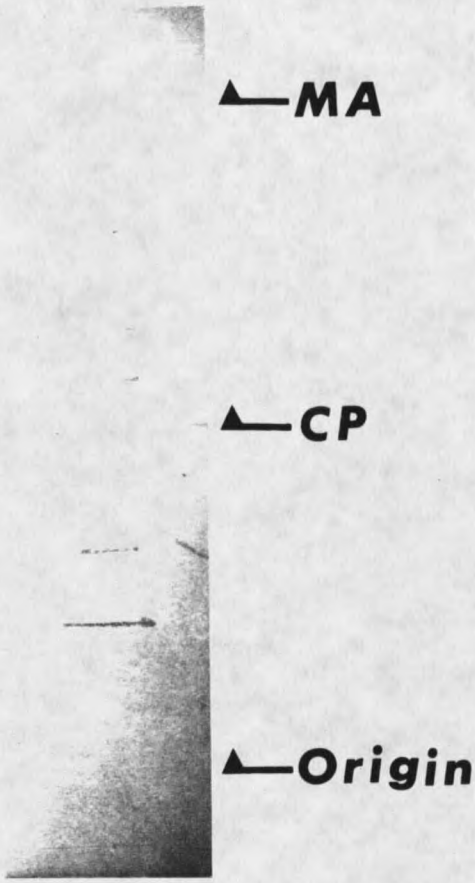

Figure 21. Non-enzymatic conversion of maculosin in 2% methanol at 4°C for four months. Autoradiographic analysis was performed on a normal phase silica plate with a solvent system (chloroform: methanol:acetic acid, 14:2:1, v/v/v). MA: maculosin, CP: conversion product.

different compounds. Once the amide bond is hydrolyzed there is also the possibility of methyl esterification of the carboxylic acid moiety by the reaction with methanol, which is continuously used in various purification procedures in this experiment. Also, there are reports that the carboxylic groups of herbicides are esterified by methanol (12).

Therefore, the two kinds of dipeptide methyl esters (L-Tyr-L-Pro-OMe and L-Pro-L-Tyr) were synthesized. Their ^{1}HMR spectra are shown in Figures 22 and 23. Comparing their chromatogaphic behavior on TLC, it is noted that both these dipeptides closely migrated at the R_f of metabolite-3 and both of these dipeptides were easily converted to a compound at the same R_f of intact maculosin. This compound was scraped, eluted with methanol and again co-chromatographed with maculosin on TLC. By measuring the R_f and homogenicity of a spot developed on TLC, it was identified as maculosin, which indicated the cyclization of dipeptides. However, the rate of cyclization of L-Tyr-L-Pro-OMe was faster than that of L-Pro-L-Tyr-OMe on the basis of the intensity of the maculosin spot on TLC because L-Tyr-L-Pro-OMe has a primary amine while L-Pro-L-Tyr has a secondary amine. Therefore, it was not ruled out that the metabolite-3 might be one of the methyl esters of the suggested dipeptide.

Biological Activity

Maculosin β-O-glucoside was applied to the leaves of spotted knapweed. At 1 mM concentration, maculosin β-O-glucoside did not induce any symptoms on the leaves of spotted knapweed and did not have any selectivity to the other plants tested in contrast to maculosin (Table 11).

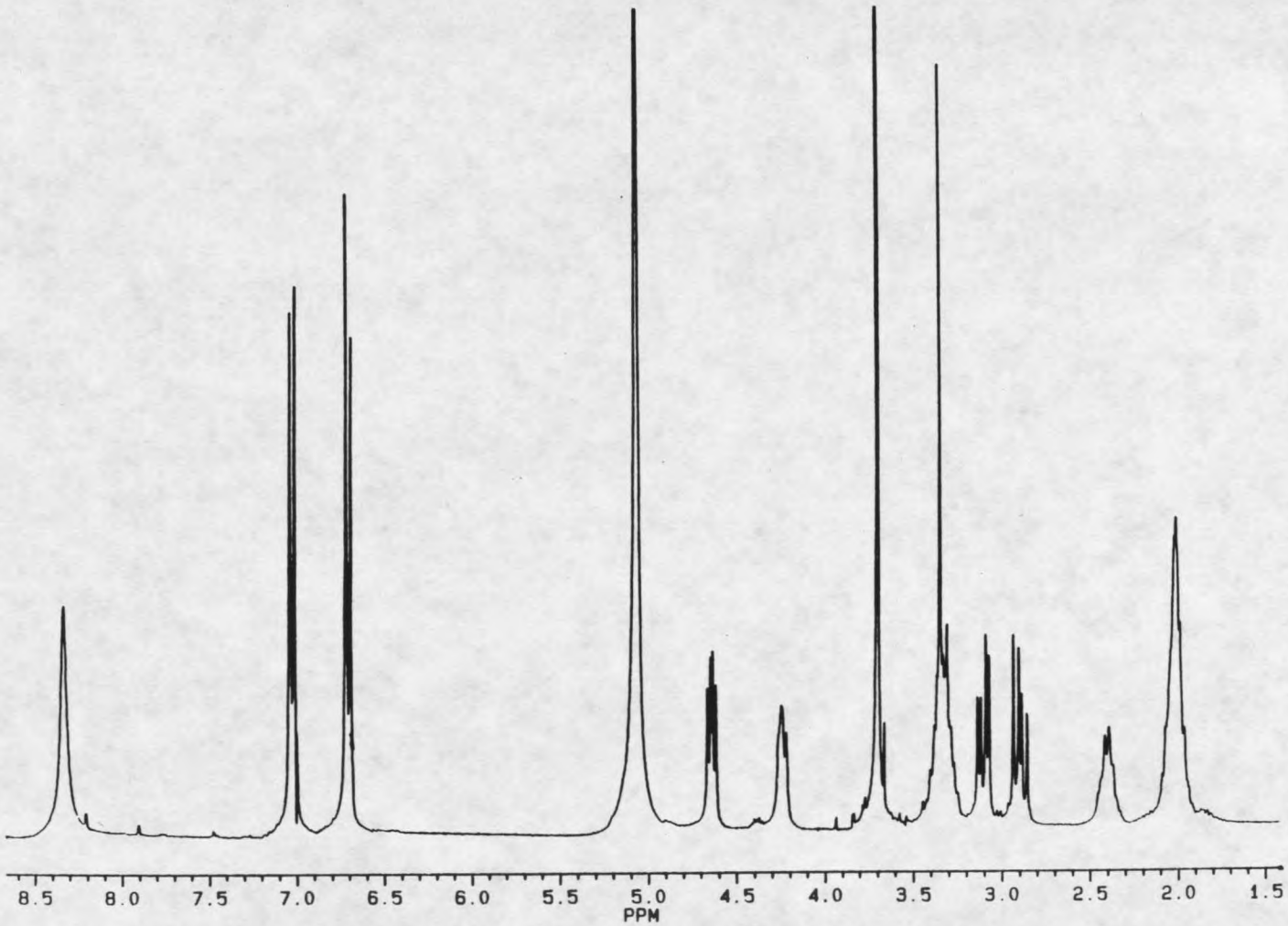

Figure 22. ^{1}H-NMR spectrum of L-Pro-L-Tyr-OMe.

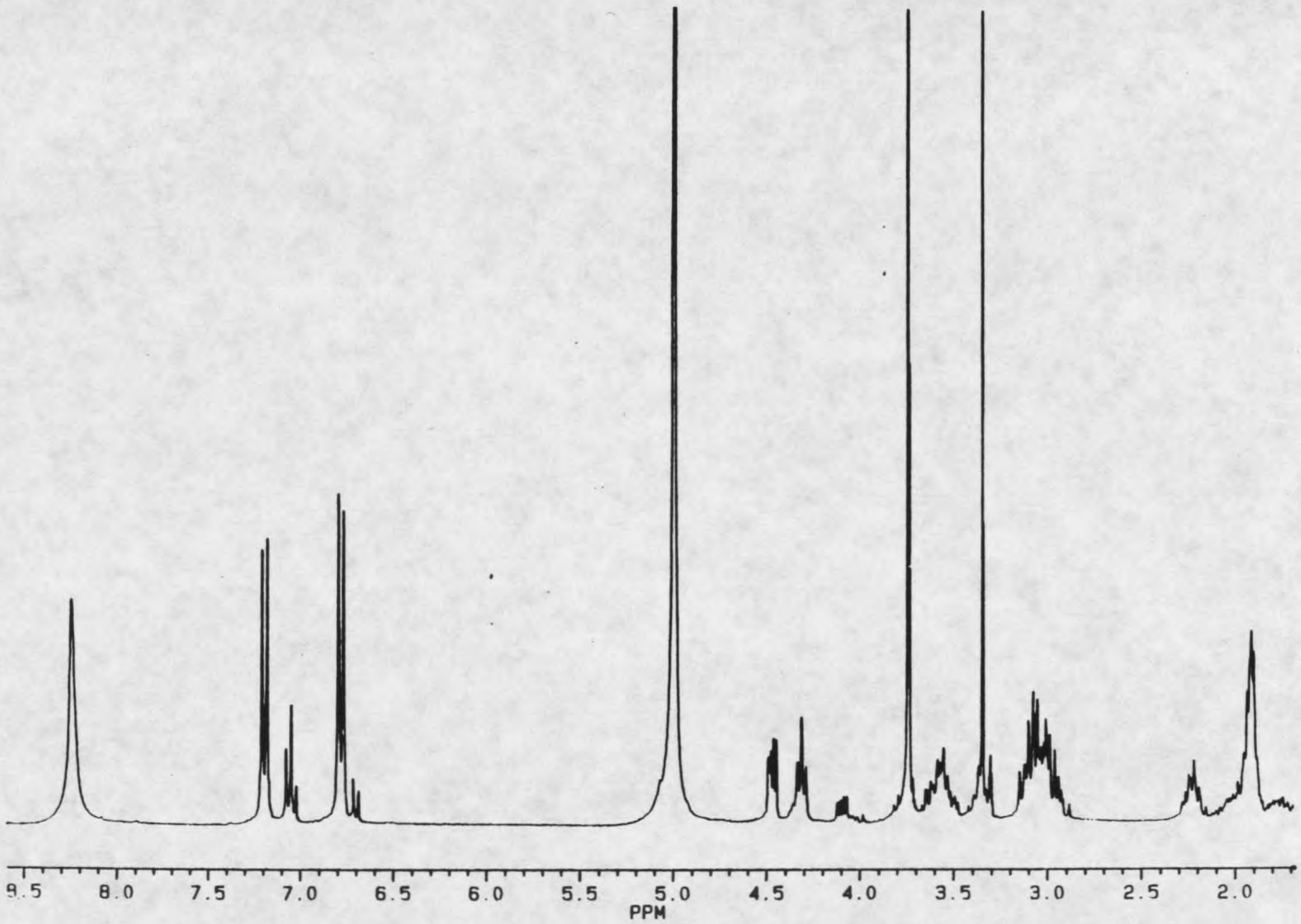

Figure 23. ^{1}H-NMR spectrum of L-Tyr-L-Pro-OMe in d-methanol.

Table 11. Effect of maculosin and maculosin β-O-glucoside on different plant species using the leaf puncture assay.

	Maculosin		Maculosin-O glucoside	
Concentration (mM)	1.0	0.1	1.0	0.1
Dicots				
Spotted Knapweed (*Centaurea maculosa*)	++[a]	+	-	-
Diffusa Knapweed (*Centaurea diffusa*)	-	-	-	-
Mung bean (*Phaseolus aureus*)	-	-	-	-
Alfalfa (*Medicago sativa*)	-	-	-	-
Squash (*Cucurbita maxima*)	-	-	-	-
Banana (*Musa acuminata*)	-	-	-	-
Dancyl Tangerine (*Citrus reticulata*)	-	-	-	-
Monocots				
Park oat (*Avena sativa*)	-	-	-	-
Barley (*Hordeum vulgare*)	-	-	-	-
Wheat (*Triticum aestivum*)	-	-	-	-
Rice (*Oryzae sativa*)	-	-	-	-
Corn (*Zea mays*)	-	-	-	-
Nutsedge (*Cyperus rotundus*)	-	-	-	-

[a] ++: necrotic lesion more than 3 mm; +:necrotic lesion 0.5-3 mm; -: no lesion or less than 0.5 mm.

Translocation

The ^{14}C-major metabolite isolated from the leaves was applied to spotted knapweed and oat. Two punctures were made in the center of both sides of spotted knapweed leaves and one in the center of oat leaves. The leaves were inoculated and sampled as previously indicated. Most of the radioactivity of the major metabolite remained in the middle part of leaves of spotted knapweed and oat, while little radioactivity moved to the tip region of the leaf (Table 12).

Table 12. Translocation of maculosin β-O-glucoside in detached leaves of spotted knapweed and oat. ^{14}C-maculosin β-O-glucoside (0.023 μmole) was applied to each leaf. Intermittent light was equivalent to 6-8 $\mu E/m^2/sec$ for 12 hr/day. Values represent the average of three replicates. Plus/minus values represent one S.D.

Leaf segment	DPM	
	Spotted knapweed	Oat
Tip	58 ± 5	32 ± 11
Middle	1701 ± 214	1726 ± 77
Base	49 ± 17	25 ± 17

Enzymatic Formation of Conversion Products

To determine whether the formation of maculosin metabolites is enzymatically mediated, the leaves were boiled in water for three minutes at 85°C. The heated leaves were dried between paper towels to remove excess water. Maculosin was applied to the leaves and its fate was determined over time. As shown in Figure 24, while the

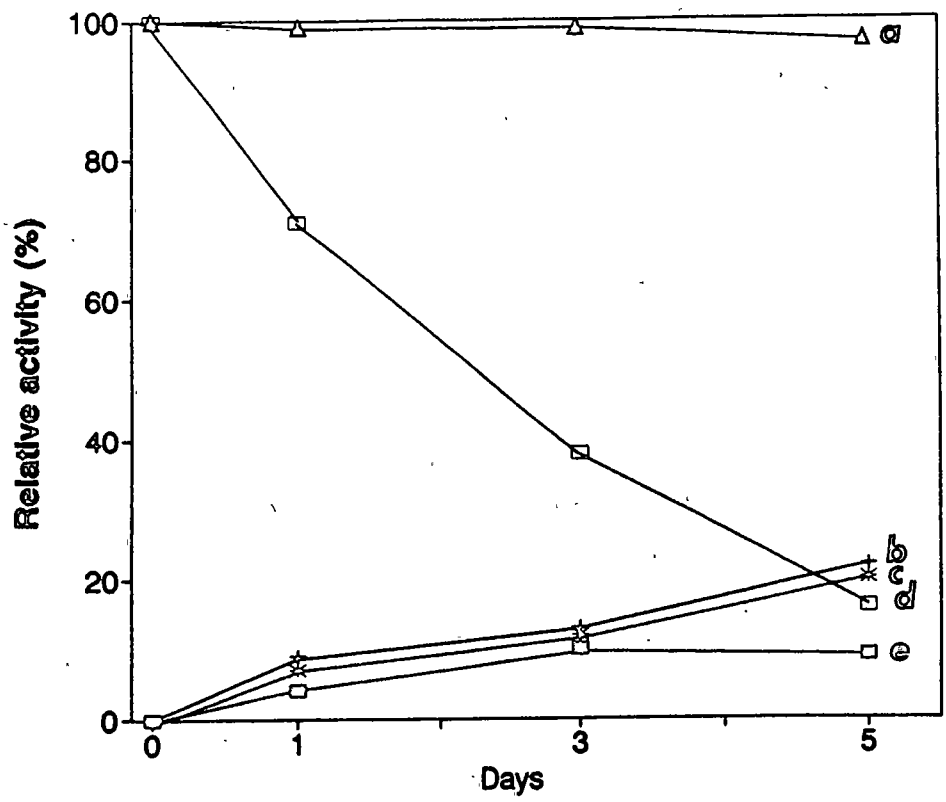

Figure 24. Metabolism of maculosin in the leaves of spotted knapweed over time. M-1 metabolite (e), M-2 metabolite (b), and M-3 metabolite (c) were respectively formed after treatment of maculosin (d) in the non-treated leaves. Only maculosin (a) appeared in the heat treated leaves. The leaves were extracted with methanol and developed on a normal phase silica plate with a solvent system (chloroform:methanol:acetic acid, 14:2:1, v/v/v).

recovery of maculosin was not changed and no metabolites appeared in the heat treated leaves, three metabolites were constantly formed in the non-heat treated leaves. Thus these data suggest that maculosin is enzymatically metabolized, producing at least three metabolites in the leaves which is consistent with Figure 13 showing the conversion products.

Metabolism of Maculosin and its Major Metabolite as a Function of Time

Among the metabolites, the major metabolite (maculosin β-O-glucoside) was always produced as a dominant product which may play some role in regulating the concentration of the other metabolites and maculosin (Figure 13). Therefore, the purified major metabolite (M-2, maculosin β-O-glucoside) was applied to the leaves and its behavior investigated over time. After application of this metabolite, methanol soluble radioactivity was analyzed by TLC in solvent system A. Over 5 days, this glucoside was also converted to the other metabolites. At 1 day, it yielded maculosin and the formation of maculosin rapidly decreased at 3 and 5 days (Figure 25). When maculosin β-O-glucoside was applied to the leaves, the glucoside was converted to a methanol insoluble complex up to 90% of applied radioactivity after 5 days Subsequently, this glucoside was converted to a methanol insoluble complex with up to 90% of applied radioactivity, whereas 70% of the radioactivity was recovered from the leaves treated with maculosin (Figure 26).

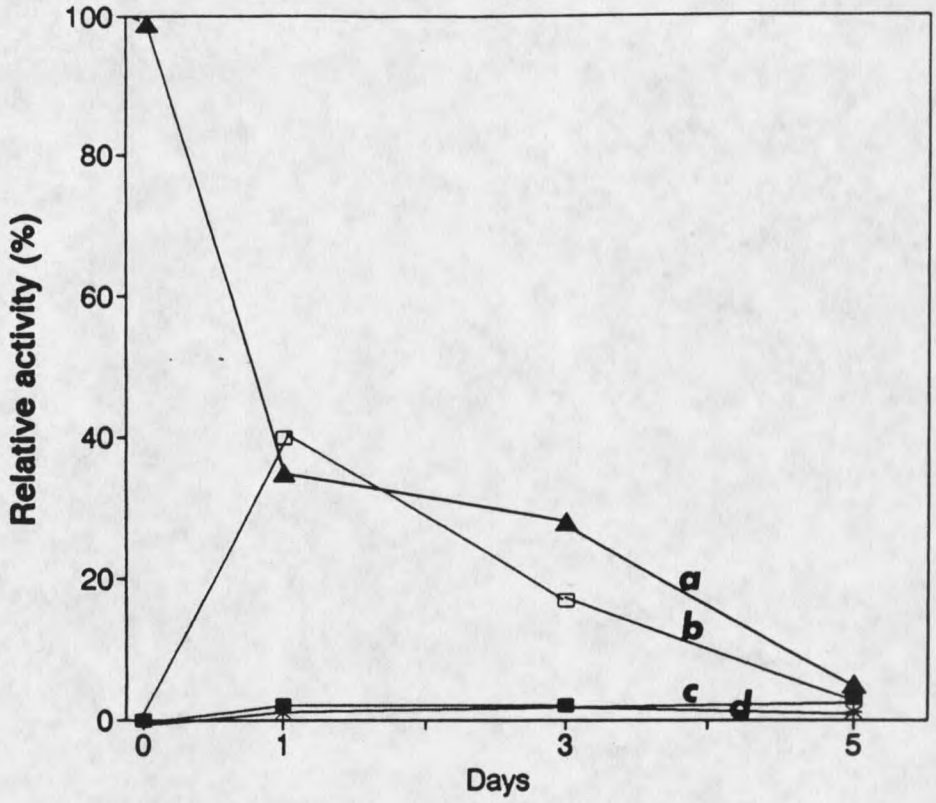

Figure 25. Metabolism of maculosin β-O-glucoside in leaves of spotted knapweed. M-1 metabolite (d), M-3 metabolite (c), and maculosin (b) were respectively formed after treatment of maculosin β-O-glucoside (a) in the leaves. The leaves were extracted with methanol and developed on a normal phase silica plate with a solvent system (chloroform:methanol:acetic acid, 14:2:1, v/v/v).

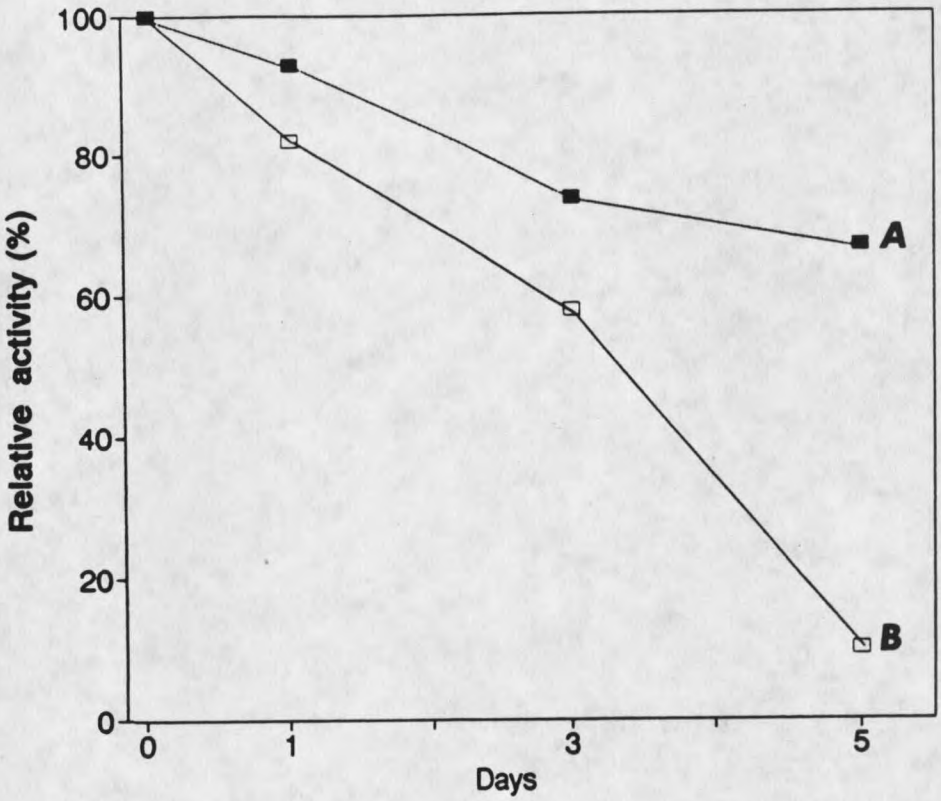

Figure 26. Recovery of methanol soluble radioactivity in the leaves of spotted knapweed after treatment of maculosin (A) and maculosin β-O-glucoside (B) over time. The leaves were extracted with methanol and developed on a normal phase silica plate with a solvent system (chloroform: methanol:acetic acid, 14:2:1, v/v/v).

Metabolism of Maculosin in Monocot

Oat is a monocot and insensitive to maculosin (56). Therefore, maculosin treatment was applied to Park oat leaves to determine if it is metabolized in the same manner as on spotted knapweed. After treatment with ^{14}C-maculosin and incubation for 5 days, the leaves were extracted with methanol and subjected to TLC followed by autoradiography. The autoradiograph in Figure 27 shows that the crude extract had the identical pattern of radioactivity as that corresponding to that of maculosin treated spotted knapweed. The intensity and size of these metabolites indicated that the metabolism of maculosin in oats would be approximately the same as in spotted knapweed.

Discussion

In previous studies, maculosin-binding receptors were mainly found in the cytosolic fraction of the leaf, and these were identified as high molecular weight proteins of spotted knapweed. One of these proteins was surmised to be involved in maculosin induced symptom development in the plant. Therefore, the fate of maculosin was investigated in this study to determine how maculosin behaves in the leaf tissue to induce disease symptoms *in vivo*.

Maculosin was metabolized to a minimum of three polar compounds, as demonstrated by their behavior in normal phase TLC (Figure 14). Unlike maculosin, these metabolites turned

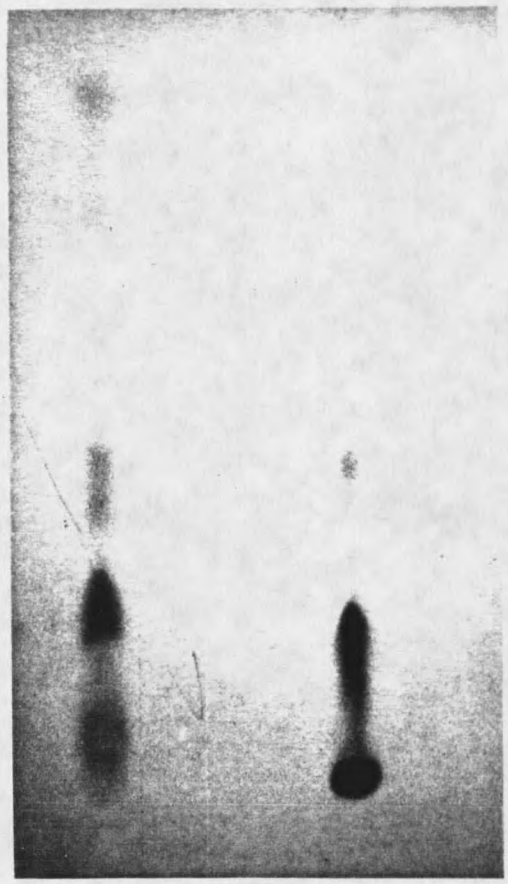

Figure 27. Autoradiographic analysis of the ^{14}C-maculosin metabolites from leaves of oats. The leaves were extracted with methanol and developed on a normal phase silica plate with a solvent system (chloroform:methanol:acetic acid, 14:2:1, v/v/v). (A) Spotted Knapweed, (B) Oat.

out to be water soluble compounds. The major metabolite (M-2) was identified as maculosin β-O-D-glucoside (Figure 14) by spectral methods. ^{1}H-NMR and ^{13}C-NMR of a metabolite showed that the metabolite contained a glucose and that the aglycone was intact maculosin. Without methylation, glucose-conjugated maculosin was applied to chemical ionization mass spectrometry (CI), which generally affords a high intensity of molecular ions and fragment ions in the high mass range (65). Although oligosaccharides need to be derivatized to be volatilized prior to ionization, sugars can be analyzed by CI (65). As shown in Figure 16, the fragmentation pattern of this metabolite closely resembled that of glucose and maculosin. Following derivatization with trimethysilane, it was determined that a glucose was conjugated to maculosin (Personal communication with Dr. Albersheim, University of Georgia). Also, electro spraying MS showed m/z 445 $[M + Na]^{+}$ which is consistent with a maculosin-glucoside.

Conjugation is not unique to maculosin. It has been reported for other biologically important compounds such as auxin (11) and many herbicides (12,51). These bioactive compounds have been shown to be conjugated to amino acids, sugars and glutathione. These conjugates are usually not biologically active. They are the products of deactivation or detoxification reactions, which result in the loss of specific receptor interaction and facile elimination from the cell.

In the case of herbicides, glucose-conjugation is an important factor in regulating pesticide selectivity in plants by converting lipophilic parent herbicides into more polar, water soluble metabolites (12). The mechanism of glucose-conjugation is mediated by an enzyme, glucosyltransferase (48). This enzyme is very specific and its best substrate is UDP-glucose (12,48). If glucose-conjugation occurs with the active herbicide and yields a biologically inactive conjugate, this is considered to be direct detoxification. But, if the metabolite is biologically active, glucose-conjugation may influence herbicide selectivity by acting as a regulatory mechanism for controlling the levels of toxic metabolites (12,51).

From a pathological perspective in knapweed, attention should be addressed to the major metabolite because of its predominant production among several metabolites. When maculosin β-O-glucoside was applied to leaves, it was surprisingly converted to maculosin and other metabolites over time (Figure 25). The formation of maculosin from maculosin-glucoside may be an enzymatic process that is probably mediated by β-glucosidase. β-Glucosidases are generally loosely attached to the cell wall or are located primarily in the cellular spaces of the intact leaf (12).

The significant difference in the molecular behavior between maculosin and maculosin β-O-glucoside was defined by the amount of recovered ^{14}C in the methanol soluble fraction

from the treated leaves. When maculosin was applied to the leaves, about 70% of the radioactivity was recovered in the methanol fraction, but when maculosin β-O-glucose was applied, most of the radioactivity remained in the water soluble fraction (Figure 26).

If the xenobiotic contains amide bonds, it could be hydrolyzed by an appropriate deamidase (dipeptidase) to the corresponding amine and carboxylic acid (13). Therefore, even though spectral data are lacking, M-3 metabolite was surmised as one of the dipeptides (L-Pro-L-Tyr-OH or L-Tyr-L-Pro-OH), the hydrolyzed product of the diketopiperazine moiety of maculosin (Figure 28). On the basis of experimental observations: (i) this metabolite was retained on Dowex-50 which indicates an exposed free amine group of the dipeptide resulting from hydrolysis of diketopiperazine; (ii) molecular behavior, a similar retention volume to maculosin, on Sephadex LH-20 column chromatography; (iii) the continuous advent of radioactivity at the same R_f of maculosin during the purification of this metabolite. There are several reports of instability of dipeptides which artificially form diketopiperazines (2,22,42); (iv) the presence of enzymes that hydrolyse cyclized peptides (2,22) because, in heat treated leaves, this metabolite was not detected (Figure 24); (v) most of the radioactivity was reversibly recovered from the same R_f of standard maculosin after the cyclization process of this metabolite obtained

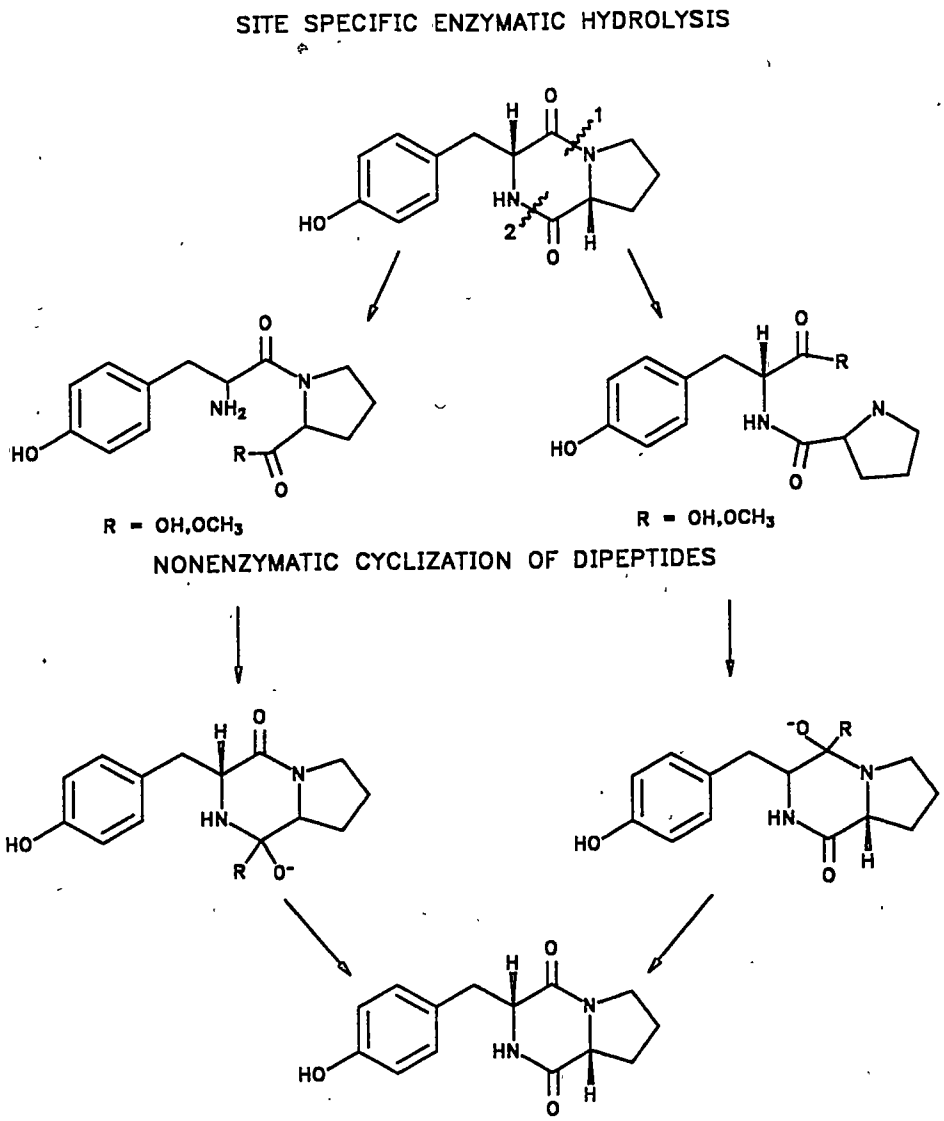

Figure 28. The possible mechanism of maculosin hydrolysis, yielding dipeptides and cyclization of dipeptides.

from leaves; (vi) Storage of intact maculosin in water at 4°C for four months yielded a product with the same R_f (Figure 21); (vii) the synthetic dipeptides, acid and methyl ester of Tyr-Pro and Pro-Tyr, have similar chromatographic behaviors on TLC and these are non-enzymatically cyclized to yield maculosin (Table 8).

M-1 metabolite seems to be heterogeneous. If maculosin is hydrolyzed to either Pro-Tyr-OH or Tyr-Pro-OH (the carboxylic group can be substituted by a methyl ester because of the nucleophilicity of methanol), it can be hydrolyzed to tyrosine and proline respectively by an enzyme (dipeptide hydrolase). Even though these results are fragmentary, some explanation might be: (i) these metabolites are partially solubilized in methanol insoluble heterogeneous polymers via covalent coupling of the glucose moiety of maculosin glucoside, (ii) it would be an amino acid product, tyrosine (insoluble in water) and/or proline. If maculosin is further hydrolyzed by dipeptidase, it yields tyrosine and proline which are not mobile (R_f 0.0) on TLC in solvent system A.

To learn if these maculosin metabolites are unique to spotted knapweed or if other plants could also metabolize maculosin, oat leaves (monocot) were treated with maculosin because oats are insensitive to maculosin. As shown in Figure 27, oats metabolized maculosin to the same metabolites as did knapweed. This result is not surprising because many xenobiotics are metabolized in the same manner in both

susceptible and resistant plants (53,72). In the case of HC-toxin produced by Cochliobolus carbonum, when HC-toxin was introduced to maize leaf segments, leaf segments from both resistant and susceptible maize inactivated toxin equally well over time (38). Even though the metabolites appear to be qualitatively similar between spotted knapweed and oat, the difference of quantity and rate of metabolism need to be further examined to explain the specificity of maculosin between the different tissue types.

The possible interrelationship of maculosin, maculosin β-O-glucoside, hydrolyzed diketopiperazine and methanol-insoluble or bound residues in the maculosin metabolism is summarized in Figure 29. The biosynthesis of maculosin β-O-glucoside seems to be a significant maculosin detoxification step which may result in the majority of maculosin being immobilized in methanol-insoluble compounds via covalent coupling of glucosyl moiety of maculosin β-O-glucoside. It is well known that glucose conjugates of plant growth regulators may serve as growth regulator reserves and provide a possible mechanism for the bioregulation of growth regulator activity (11). If maculosin β-O-glucoside functions in a similar manner, both maculosin and maculosin β-O-glucoside could be in vivo considered as phytotoxic or potentially as phytotoxic compounds. However, in this experiment, maculosin β-O-glucoside did not either cause symptoms on spotted knapweed or have selectivity to the other

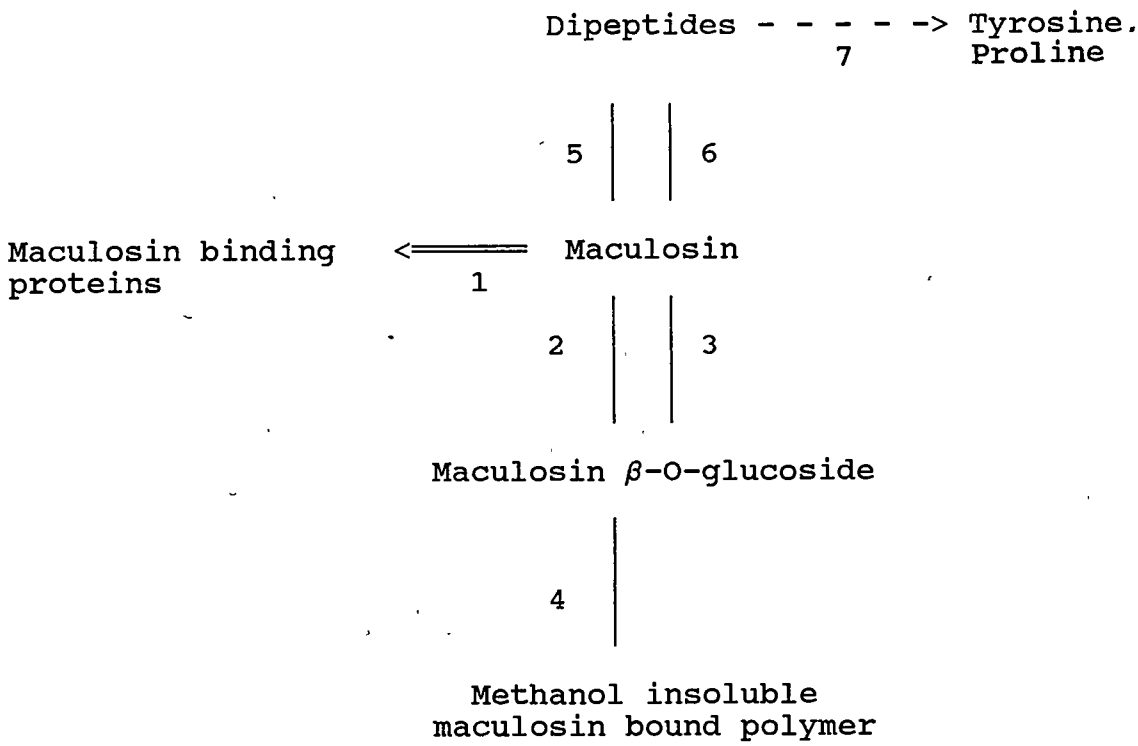

Figure 29. Proposed metabolic pathway of maculosin on the leaves of spotted knapweed.
(1) Binding of maculosin to the cytosolic proteins, (2) glucose conjugation by UDP-glucosyltransferase, (3) hydrolysis of glucose by β-glucosidase, (4) conjugation of maculosin-glucoside to methanol insoluble polymers, (5) enzymatic hydrolysis of one of amide bond of the diketopiperazine, (6) non-enzymatic cyclization of dipeptide, and (7) hydrolysis of dipeptides by amidopeptidase.

plants tested. As shown in Table 11, the reason maculosin β-O-glucoside does not induce symptom is attributed to the formation of a methanol-insoluble complex even though maculosin appeared at 1 day in the leaves applied with maculosin β-O-glucoside (Figure 25, 26). If glycosidase is kinetically dominant over glucosyltransferase, the biosynthesis of maculosin β-O-glucoside would be reduced and would effectively compete for the available maculosin.

However the real mechanism remains an open-question. First, critical biochemical differences in the rate and extent of glucosyltransferase (k^1), β-glucosidase (k^{-1}), diketopiperazinase (k^2), cyclization (k^{-2}) and dipeptidase (k^3) may regulate maculosin phytotoxicity. Secondly, compartmentalization (subcellular localization) of each metabolite would play an important role in producing a sufficient amount of maculosin to induce symptoms. Methanol-insoluble or bound maculosin-glucoside residues may provide an effective means for the reduction of maculosin concentration in vivo. If maculosin β-O-glycosides are especially deposited in the vacuoles like the other xenobiotics, it also significantly reduces the available maculosin. Thirdly, the rate of binding of toxin per unit of cell would be critical during the process of conjugation. Fourthly, the dipeptides, hydrolyzed products of diketopiperazine would be another determinant to provide maculosin if they are again cyclized non-enzymatically.

If this model is correct, it suggests that intact maculosin is a main symptom induction molecule. However, the model suggested here does leave room for the further investigation of the enzymatic alteration of maculosin, ultimately resulting in metabolic chaos at the cellular level causing symptom development.

CHAPTER IV

SUMMARY

In the last decade, there are at least fourteen examples in which host specific phytotoxins from crops have been isolated and identified with the help of modern analytical techniques. However, maculosin [the diketopiperazine, cyclo (L-Pro-L-Tyr)], is the first compound to be isolated, characterized, and synthesized that has the properties of a host specific phytotoxin produced by a fungus, Alternaria alternata on a weed plant, spotted knapweed (Centaurea maculosa L.).

Even though there are growing views of the host specific phytotoxins as the molecular probe and pathogenicity determinant, the molecular mechanism of host specific phytotoxins is not well understood. Therefore, this study was carried out to investigate the mode of action of maculosin in spotted knapweed.

When ^{14}C-maculosin was in vivo applied onto the leaves of spotted knapweed without a wound, most of the radioactivity was localized at the site of application, whereas some radioactivity was mobilized to the tip of the leaf when maculosin had been applied to a wound. However, when the root of the plant was dipped into the maculosin

solution, most of the radioactivity was acropetally translocated.

The results of this work suggest that one or more maculosin-binding proteins exist in spotted knapweed in both the cytosolic and membrane fractions. However, the majority of toxin binding activity was recovered from the cytosolic fraction. Even with the application of various detergents to the crude membrane fraction, the specific binding activity was lower than the cytosolic fraction.

A procedure starting from the 12,000 x g supernatant has been developed to purify the cytosolic maculosin-binding proteins using maculosin-affinity chromatography. The affinity column was prepared with epoxy activated Sepharose 6B column to which the phenolic group of maculosin was attached. The purified maculosin-binding protein obtained through maculosin affinity chromatography showed a 16-fold purification that contained three large molecular weight proteins and these eluted as an aggregate. However, tyrosine-affinity column, the alpha amino group of tyrosine was coupled to Sepharose 4B via CNBr activation to expose the phenoxy group free in the space, did not bind any cytosolic proteins.

Based on electrophoretic mobility of non-denatured, and denatured (SDS treated) polyacrylamide gel electrophoresis and its bulk amount of binding activity on chromatographic behavior, one of the maculosin-binding proteins was surmised

to be ribulose-1,5-biphosphate carboxylase (RuBPCase) which is one of the most abundant proteins located in chloroplasts in the plant kingdom. The cross reactivity of purified proteins with antibody of RuBPCase from tobacco showed that one of the binding proteins in the cytosolic fraction is RuBPCase.

In the study of the fate of maculosin, it was found that maculosin was enzymatically metabolized to a minimum of three polar compounds (M-1, M-2, and M-3) based on their chromatographic behavior in normal phase TLC. Unlike maculosin, these metabolites turned out to be water soluble compounds. While Dowex-50 retained both M-1 and M-3, Dowex-1 retained only M-1. However, the major metabolite (M-2) was not retained either on Dowex-1 or Dowex-50, which suggested the presence of a sugar moiety.

The major metabolite (M-2) was purified and identified as maculosin β-O-D-glucoside by spectral analysis. Even though the spectral analysis was lacking, the minor metabolite (M-3) was surmised to be one of the possible dipeptides which might be the hydrolyzed product of diketopiperazine (maculosin).

Because of its predominant production among several metabolites, the molecular behavior of maculosin β-O-glucoside was investigated. When maculosin β-O-glucoside was applied on the leaves, it was converted to maculosin and other metabolites over time. One day after inoculation,

maculosin β-O-glucoside yielded maculosin and the formation of maculosin rapidly decreased. Subsequently, maculosin β-O-glucoside was converted to a methanol insoluble complex with up to 90% of applied radioactivity being counted after 5 days treatment, whereas 70% of the radioactivity was recovered from the leaves treated with maculosin.

When maculosin β-O-glucoside was applied on detached leaves, most of the radioactivity remained in the middle part of leaves of spotted knapweed and oat, with a small amount of radioactivity moving to the tip region of the leaf.

To learn if metabolites are unique to spotted knapweed or if other plants could also metabolize maculosin, oat leaves were treated with maculosin because oats are insensitive to maculosin. Oats metabolized maculosin to the same metabolites as did knapweed.

In terms of bioactivity, maculosin β-O-glucoside did not induce any symptoms on the leaves of spotted knapweed and did not have any selectivity to the other plants tested.

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