STUDIES ON THIARUBRINE, A NATURALLY OCCURRING DISULFIDE POLYINE

by

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ABSTRACT

Chemical and biological aspects of thiarubrine, a highly antifungal dithiacyclohexadiene polyine, were investigated. A tissue culture system for the production of thiarubrines was developed by culturing hairy roots of *Chaenactis douglasii* induced by *Agrobacterium rhizogenes* strain TR7. One culture line accumulated two times the levels of thiarubrines of nontransformed control root cultures, while maintaining rapid growth. The combination of fast growth and high thiarubrine accumulation could not be duplicated in controls by adding exogenous NAA to the culture medium. Hairy root cultures also produced less thiarubrine B relative to thiarubrine A compared to controls. Thiarubrine synthesis appears to be closely correlated with degree of tissue differentiation; it is suggested that it may be more practical to improve the growth rate of thiarubrine-producing root cultures by transformation rather than seek to induce synthesis in fast-growing suspension cultures.

The biosynthetic relation between thiarubrines and the always co-occurring thiophenes was investigated by performing ^{35}S tracer experiments with *C. douglasii* hairy root cultures. It is possible that the thiophenes are not actively synthesized by the roots but rather are products of thiarubrine decomposition resulting from the extraction procedures and other manipulations of the cultures. The *in vitro* conversion of thiarubrine to thiophene can be induced by light, heat and other agents. No turnover of thiarubrines could be detected in the cultures in late logarithmic or stationary phases of the growth cycle.

Thiarubrines show strong light-independent antibacterial and antifungal activity. The mechanism of action of thiarubrine against *E. coli* and *S. cerevisiae* was investigated using comparative disk bioassays. A very similiar polyine from *Rudbeckia hirta* was as active as thiarubrine in the dark, indicating the central role of the disulfide ring in toxicity of the compounds. Visible light enhanced this activity suggesting that decomposition of the disulfide

ring is important for its antibiotic effects. The photodegradation product, a thiophene, is phototoxic, probably via both type I and type II photosensitization mechanisms.

The root culture extracts of *Rudbeckia hirta* yielded a new isomer of a known dithiacyclohexadiene polyine. MS and NMR analyses confirmed the *cis* configuration of this isomer.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

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GENERAL INTRODUCTION

The great diversity of plant form at the macroscopic level is mirrored at the molecular level by an extraordinary variety of chemical structure. In addition to the compounds required by all plants for normal metabolic functions, thousands of low-molecular weight compounds not directly involved in primary metabolism are found throughout the plant kingdom. This variety of chemicals with no apparent function in the plant is difficult to explain, especially because often these so-called secondary plant metabolites represent a substantial energy investment by the plant. A protective function is ascribed to many of these compounds, and their toxicity to animals, microbes or other plants can often be demonstrated in the laboratory (Swain, 1977; Harborne, 1981). It is difficult, however, to extrapolate from biological activity in the laboratory to an ecological role the field. Moreover, it is not clear why such a variety of products drawn from a number of chemical classes should have evolved to perform essentially the same, protective function.

Whatever their biological roles, secondary plant metabolites have been used by humans for medicinal and spiritual reasons since prehistoric times. Their central role in modern medicine is undisputed; it is estimated that 25% of all pharmaceuticals in use today are derived from higher plants (Hahlbrock, 1986). Thus, for both scientific and practical reasons, secondary plant metabolism is and will continue to be an active area of study.

The research constituting this thesis deals with a secondary plant metabolite belonging to the class of compounds known as polyacetylenes, or polyines. These are common in members of the Campanulaceae, Apiaceae, and Asteraceae and are characterized by one or more C-C triple bonds. Many structural variations, which often include the heteroatoms oxygen, sulfur, or chlorine, are known. However, all are based on C_{13} to C_{18} skeletons synthesised by the

dehydrogenation and desaturation of oleic acid (Bohlmann et al., 1973). Neither the mechanisms nor enzymes of triple bond formation are known.

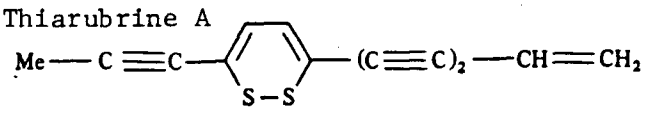
It is the remarkable toxicity, often light-mediated, of many polyines against a range of organisms including insects, nematodes, fungi, bacteria and viruses that has generated interest in these compounds (for a review, see Towers, 1987). Indigenous peoples were aware of such biological effects: for example, the leaves of *Schefflera digitata* which contain the antibiotic polyine falcarindiol were used by the Maoris of New Zealand to treat ringworm (Muir, 1979), and tribes of the Amazon basin still use the polyine-containing extract of the composite *Clibadium sylvestre* to stun and subsequently harvest fish. The Western pharmacopeia, however, does not include any polyines; this may be the result of their chemical instability (Reisch et al., 1973), but perhaps also because of the paucity of information on the effects of many of these compounds.

A particularily interesting group of polyines, on which the present research is focused, are dithiacyclohexadiene polyines. These consist of a disulfide group in a six-membered ring incorporated into an acetylene chain, and are derived from C_{13} polyines by formal addition of $2(H_2S)$. They are distinctly red in color, and were first described by Mortensen et al. (1964), and Bohlmann and Kleine (1965). Two of these dithiacyclohexadiene polyines were isolated from *Chaenactis douglasii* and named thiarubrine A and thiarubrine B (Norton et al., 1985). They were shown to be highly toxic to fungi and bacteria (Towers et al., 1985). In total, four C_{13} dithiacyclohexadiene polyines have been isolated from 18 genera, all in the Asteraceae (Bohlmann and Zdero, 1985). In the course of the present research, a new isomer of a known dithiacyclohexadiene was isolated from *Rudbeckia hirta*. The dithiacyclohexadiene polyines of interest to the present work, together with the co-occurring thiophenes, are shown in Figure 1.

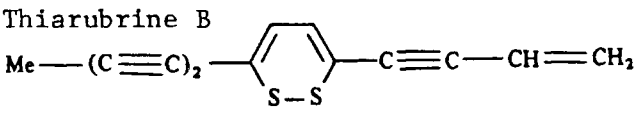
While the biological function of disulfide polyines is not known, the toxicity of thiarubrine points to a defensive role. Medicinal uses have also been documented. Roots of *Chaenactis douglasii* contain very high concentrations of thiarubrines and were used by the natives of southern British Columbia to treat snakebite and stomach ailments (Moerman, 1977).

A

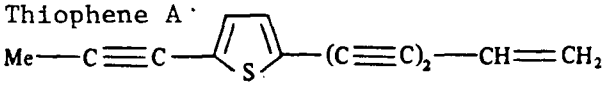
1 Thiarubrine A



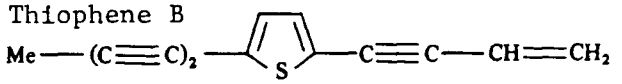
2 Thiarubrine B



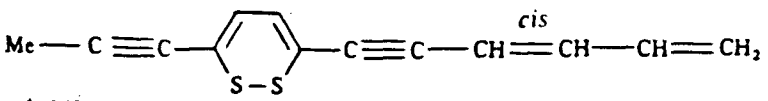
3 Thiophene A



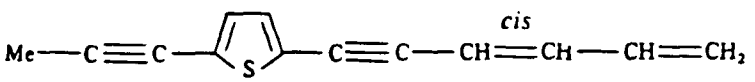
4 Thiophene B



B

5 

6 *trans*

7 

8 *trans*

Figure 1. Thiarubrines and related disulfide polyines and their corresponding thiophenes from *Chaenactis douglasii* (A)and *Rudbeckia hirta* (B)

In Tanzania, the roots of *Aspilia mossambicensis* which contain thiarubrines are prescribed by traditional healers as a tooth-ache remedy. Chimpanzees in East Africa have been observed to swallow leaves of this and other thiarubrine-containing *Aspilia* species in a highly unusual and selective manner suggesting a therapeutic use. (Wrangham and Nishida, 1983).

The biological activity and unusual disulfide structure of thiarubrine raise several questions regarding its synthesis and effect on microorganisms. Some of these are addressed in this thesis by experiments dealing with the accumulation and biosynthesis of thiarubrine in tissue cultures, as well as its mechanism of action.

To investigate the effects of any compound it is necessary to obtain it in sufficient amounts, and one approach is via plant tissue culture. The production of secondary plant metabolites by plant tissue culture for possible commercial exploitation is being investigated by many laboratories. Chapter I describes the development and characterization of a *Chaenactis douglasii* tissue culture system for thiarubrine production using hairy root disease to induce rapid and autonomous root growth in culture. Yields were doubled compared to previous culture systems.

Tissue cultures, being closely defined systems, are useful tools for biosynthetic investigations. The biosynthesis of thiarubrines and related compounds is very poorly understood. Chapter II describes experiments to examine the biogenetic relationship of thiarubrines and the closely related thiophenes which occur with thiarubrines in all plants investigated. Using pulse-chase experiments in *C. douglasii* hairy root cultures, it was determined that the thiophenes are probably not actively synthesized in the plant but rather appear as decomposition products. Furthermore, no measurable turnover of thiarubrines *in vivo* was detected.

Although the biological effects of thiarubrine have been documented (Towers et al., 1985; Hudson et al., 1986a, Towers, 1987), its mechanism of action is unknown. Root cultures of the composite *Rudbeckia hirta* provided a polyine very similiar to thiarubrine but

differing slightly in the acetylenic side chain. This permitted comparative bioassays to be carried out. The discovery that the disulfide ring decomposes under visible light facilitated experiments on the importance of ring decomposition in the toxicity of thiarubrines. The mechanism of action studies are described in Chapter III.

The work on isolating the dithiacyclohexadiene from *R. hirta* resulted in the discovery of a new isomer of both this compound and the corresponding thiophene. Nuclear magnetic resonance and mass spectrometry were used to confirm the structures as described in Chapter IV.

CHAPTER I

THIARUBRINE ACCUMULATION IN HAIRY ROOT CULTURES OF *CHAENACTIS DOUGLASII*

INTRODUCTION

In many plant species, roots are the site of synthesis and accumulation of secondary plant metabolites, yet the production of these often pharmacologically important metabolites has not been studied extensively with root cultures. The roots of *Chaenactis douglasii* (Compositae) accumulate high levels of the antifungal, sulfur-containing polyines thiarubrine A and thiarubrine B (Norton et al., 1985). Neither compound has been detected in callus or suspension culture of *C. douglasii*; certain crown gall tumour cultures, however, synthesize these compounds in differentiated root-like nodules embedded in the callus (Cosio et al., 1986). It is not uncommon to find that secondary plant metabolites found in whole plants and organs are absent from cell cultures, particularly in the case of substances such as polyines which accumulate in resin canals or other specialized structures (Butcher 1976). In those species where products are found in cell cultures, levels of synthesis are unstable and variable, requiring constant selection to maintain high productivity (for a review, see Hahlbrock, 1986). Root cultures of *C. douglasii* consistently produce thiarubrines in similiar quantities as are found in intact plant roots (Cosio et al., 1986). Root tissue cultures are fully differentiated and thus have a number of advantages over cell cultures, such as genetic stability and consistent product synthesis, that make them attractive as culture systems for the production of secondary metabolites. (Lindsey and Yeoman, 1983; D'Amato, 1977).

As an approach to improving the growth and productivity of root cultures, hairy root cultures of *C. douglasii* were investigated, transformed by infection with the causative agent of hairy root disease, *Agrobacterium rhizogenes*. Like its close relative, *Agrobacterium tumefaciens*, this bacterium transfers a segment of DNA from a large plasmid (Ri, or root-inducing plasmid) to a plant cell at a plant wound site. The bacterial DNA is integrated stably into the plant genome (Chilton et al., 1982; Tepfer, 1983), and is inherited along with genomic

DNA (Chantal et al., 1984). The bacterial T-DNA of *A. rhizogenes* apparently encodes auxin biosynthetic genes which are expressed by the transformed plant cell, causing autonomous and prolific root growth at the site of infection (Huffman et al., 1984; White et al., 1985). The resulting hairy roots can be grown axenically in culture and are characterized by high growth rates and increased lateral branching (Tepfer, 1984). Furthermore, hairy root cultures appear to be genetically stable (Hanisch ten Cate et al., 1987).

This chapter describes the establishment of *C. douglasii* hairy root cultures using *A. rhizogenes* strain TR7. One hairy root culture line produced yields of thiarubrines more than twice those of the untransformed controls, and both hairy root culture lines showed a change in the ratio of thiarubrines produced.

MATERIALS AND METHODS

Plant Material and Bacterial Cultures

Chaenactis douglasii (Hook.) H. & A. was collected near Princeton, British Columbia, and Winthrop, Washington State, and grown in a greenhouse until used. Strains of *Agrobacterium rhizogenes* were obtained from Dr. Larry Moore, Department of Plant Pathology, Oregon State University in Corvallis, Oregon. Bacterial cultures were kept on potato dextrose agar at 25 degrees C.

Induction of hairy roots

Whole leaves of *C. douglasii* were surface sterilized with a 5% (v/v) solution of sodium hypochlorite, the cut petioles inoculated with a culture of *A. rhizogenes*, and placed on 0.7% agar in petri plates. Rootlets, appearing at the site of infection after approximately 3 weeks, were excised and transferred to solid Schenk and Hildebrandt (SH) medium (Schenk and Hildebrandt, 1972) containing a combination of vancomycin, carbenicillin, or cefotaxime. After

repeated transfers without visible bacterial growth, the roots were transferred first to solid SH medium without antibiotics, and subsequently to liquid SH medium.

Liquid Root Culture

Hairy root cultures were maintained in liquid SH medium on a rotary shaker (100 rpm) at 25 degrees C in the dark and subcultured every 4 weeks. Nontransformed root cultures were obtained from callus cultures of *C. douglasii* as previously described (Cosio et al., 1986). These were grown in SH medium with 0.3 mg naphthalene acetic acid (NAA) per liter, under the same conditions as hairy root cultures.

Opine Analysis

To confirm the transformed nature of the hairy root cultures, opines from roots were extracted with 1% HCl and analysed by high voltage paper electrophoresis, according to the procedure of Petit et al. (1983) Two microliters of extract was spotted on Whatmann 3MM paper and electrophoresed at 90V/cm for 12 min at pH 9.2 or pH 1.9. Opines were stained with $AgNO_3$ by the procedure of Trevelyan et al. (1950). Authentic opine standards were a gift of Dr. A. Petit, Groupe de Recherche sur les Interactions entre Plantes et Microorganismes, Universite de Paris-Sud, Institut de Microbiologie, Orsay, France.

Analysis of Polyines

Eight to ten grams of fresh material were extracted twice with 30 ml methanol, partitioned twice with 20 ml petroleum ether (30-60), and stored at -20 degrees C as described (Cosio et al.,1986). Polyines were separated on a Varian Micro Pak MCH-10 reverse-phase column using 72% aqueous acetonitrile at a flow rate of 1 ml/min. The components were quantified by their absorption at 340 nm relative to an internal standard of known concentration. The molar absorptivities used were 10,300 for the thiarubrines and 31,500 for the related thiophenes (Bohlmann and Kleine, 1965).

RESULTS

Transformation of C. douglasii

C. douglasii explants were inoculated with a number of strains of *A. rhizogenes*. While all strains tested induced root formation on carrot disks, only two strains were successful in causing hairy root formation on *C. douglasii* (Table 1). One strain, R-1000, resulted in the formation of tumours on *C. douglasii*, but caused hairy roots on carrot. The two hairy root culture lines analysed in all experiments were both induced with strain TR7. Hairy roots appeared about three weeks after inoculation (Figure 2).

Detection of opines by high voltage paper electrophoresis confirmed the transformed nature of the hairy root cultures. Electrophoregrams run at pH 1.9 with extracts of both hairy root culture lines, CD-HR710 and CD-HR211, demonstrated the presence of mannopine but not agropine, whereas the control (CD-N) contained no opines (Figure 3). At pH 9.2, two additional opines, agropinic acid and mannopinic acid, were detected in the hairy root cultures, but were absent in control root culture extracts (data not shown). *A. rhizogenes* strain TR7 is known to encode the synthesis of mannopinic acid, agropinic acid and mannopine, but not agropine (Petit et al., 1983).

Importance of plant origin

Hairy root cultures were established from two natural populations of *C. douglasii*. Although the plants from both sites were morphologically identical, intact plant roots from site 1 (Princeton) typically contained twice as much thiarubrine as those from site 2 (Winthrop) (Table 2). This difference was also visible in the hairy root cultures derived from plants from each of the populations, with CD-HR710 accumulating twice as much thiarubrines as CD-HR211. Such population differences are not uncommon; Zenk et al. (1976) found a large variation in alkaloid content of plants derived from different populations. *C. douglasii* has been shown to be morphologically and cytologically very diverse (Mooring, 1980).

Table 1: Response of *Daucus carota* and *C. douglasii* to inoculation with several strains of *A. rhizogenes*.

Strain	*D. carota*	*C. douglasii*
R-1000	hairy root	callus
A2/83	hairy root	hairy root
A4	hairy root	n.r.[1]
A4/83	hairy root	n.r.
TR7	hairy root	hairy root
15834	hairy root	n.r.
TR105	hairy root	n.r.

[1] no response

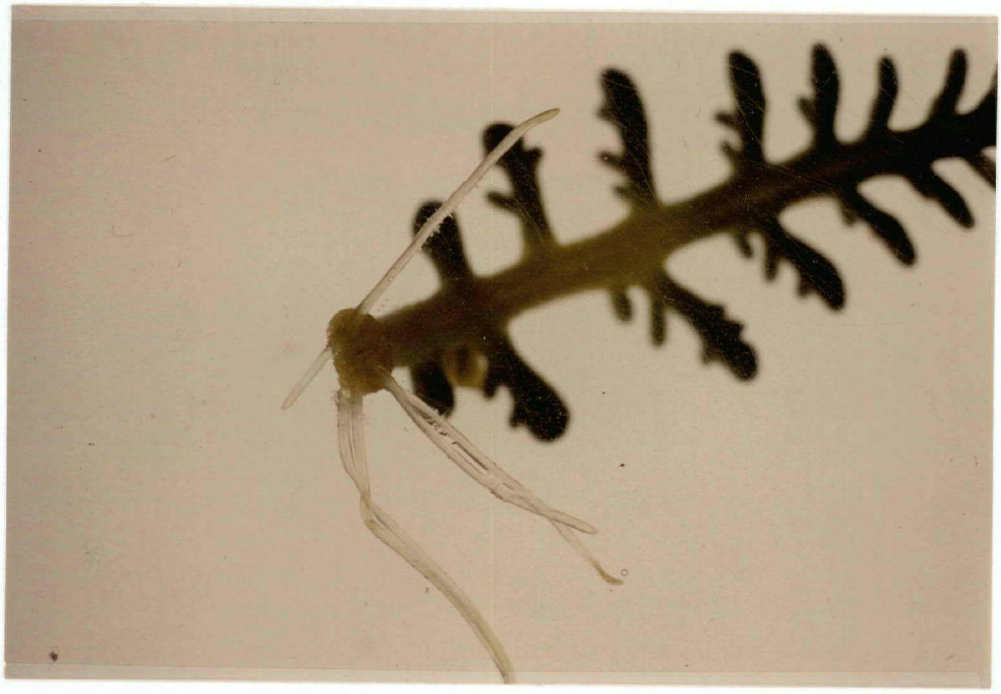


Figure 2: Hairy roots emerging from cut petiole of *C. douglasii* three weeks after inoculation with *A. rhizogenes* strain TR7.

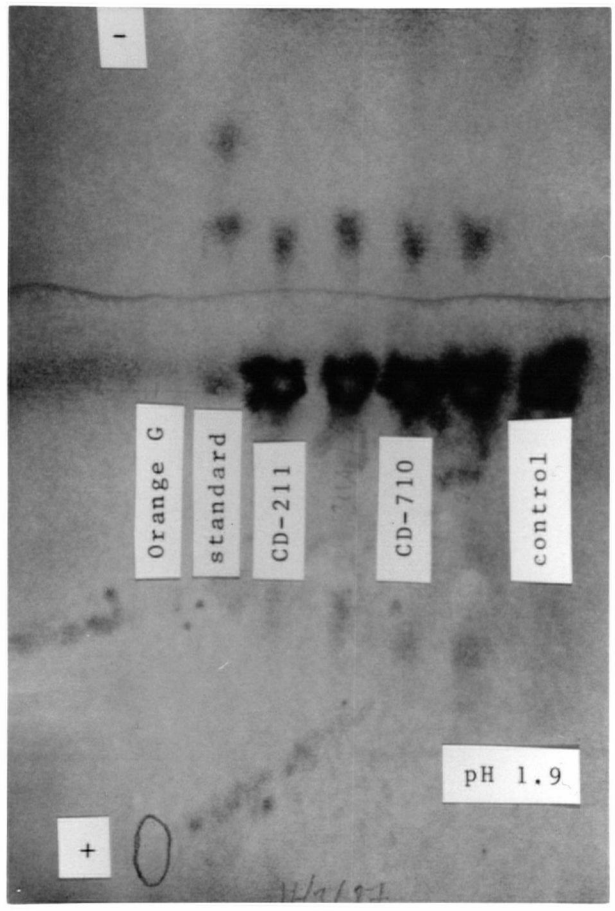


Figure 3: Electropherogram of hairy root and control root culture extracts. Two microliters of extract spotted on Whatmann 3MM paper was electrophoresed with 90 V/cm for 12 min at pH 9.2. The standard contained agropine (I), mannopine (II), agropinic acid (III) and mannopinic acid (IV). Hairy root cultures were induced with the agropine negative strain TR7. The presence of agropinic and mannopinic acid in the extracts is masked by neutral silver nitrate staining material.

Table 2: Thiarubrine content of of *C. douglasii* roots and root cultures from Princeton (site 1) and Winthrop (site 2) populations.

Source	mg/gEDW
Site 1:	
intact plant roots	5.1 (2.1)[1]
hairy root culture (CD-HR710)	9.7 (0.5)
Site 2:	
intact plant roots	1.8 (0.8)
hairy root culture (CD-HR211)	5.0 (1.2)

[1] Values are mean +/- S.D., n=10 (intact plants) or n=4 (root cultures) independent determinations.

Effect of NAA in culture medium

Untransformed roots are able to grow in culture even in the absence of exogenous growth regulators, albeit at a reduced rate. Exogenous auxins, such as NAA, are commonly used to stimulate growth. In order to make meaningful comparisons of thiarubrine content and yield between hairy root cultures and untransformed cultures, an experiment to optimize exogenous auxin levels for the untransformed controls for maximum thiarubrine synthesis was performed. Both hairy root and untransformed cultures were grown on a range of NAA concentrations, and growth and thiarubrine accumulation measured after a 3 week culture period. The results are shown in Figure 4.

Growth of untransformed root cultures, measured as fresh weight (FW), responded positively to an increase of NAA at all concentrations. Thiarubrine yield per flask, however, was maximized at concentrations between 0.3 and 0.5 mg NAA/l. Visual inspection of the cultures indicated a correlation between high auxin levels and callus formation in the culture. This confirmed previous observations that undifferentiated cells do not synthesize acetylenic products readily (Cosio et al., 1986); thus the observed peak in yield can be interpreted as the best compromise between rapid growth and excessive callus formation. Furthermore, maximum thiarubrine content of the cultures, expressed on a mg/g extracted dry weight (EDW) basis, was optimized at 0.3 mg NAA/l; this level of NAA in the medium was considered to be the optimum for untransformed cultures of *C. douglasii* and was used in all further experiments for untransformed control root cultures.

In contrast, in hairy root cultures, the effect of NAA in the medium was negative at all concentrations. Since these cultures are auxin autonomous, exogenous NAA simply produces an increase of callus growth with a concurrent decrease in thiarubrine accumulation.

Changes in biomass and thiarubrine accumulation with culture age

Figure 5 shows the progression of EDW, thiarubrine content and total thiarubrine yield of two hairy root and an untransformed root culture over a 36-day culture period. All three

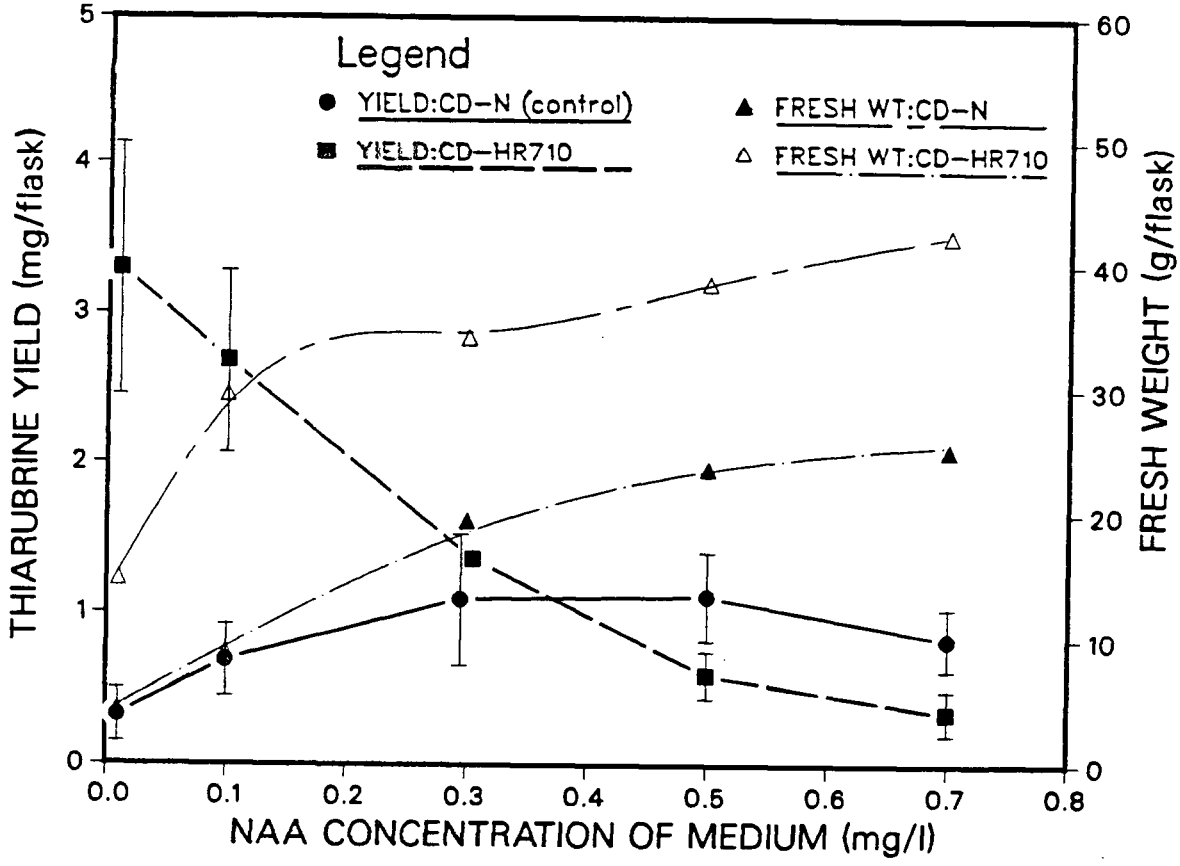


Figure 4: The effect of NAA concentration of culture medium on growth and total thiarubrine yield of hairy root (CD-710) and untransformed root (CD-N) cultures. Cultures were analysed after 3 weeks. Each point is the mean of 4 independent determinations. Bars represent +/- standard deviation. On growth curves they have been omitted for clarity.

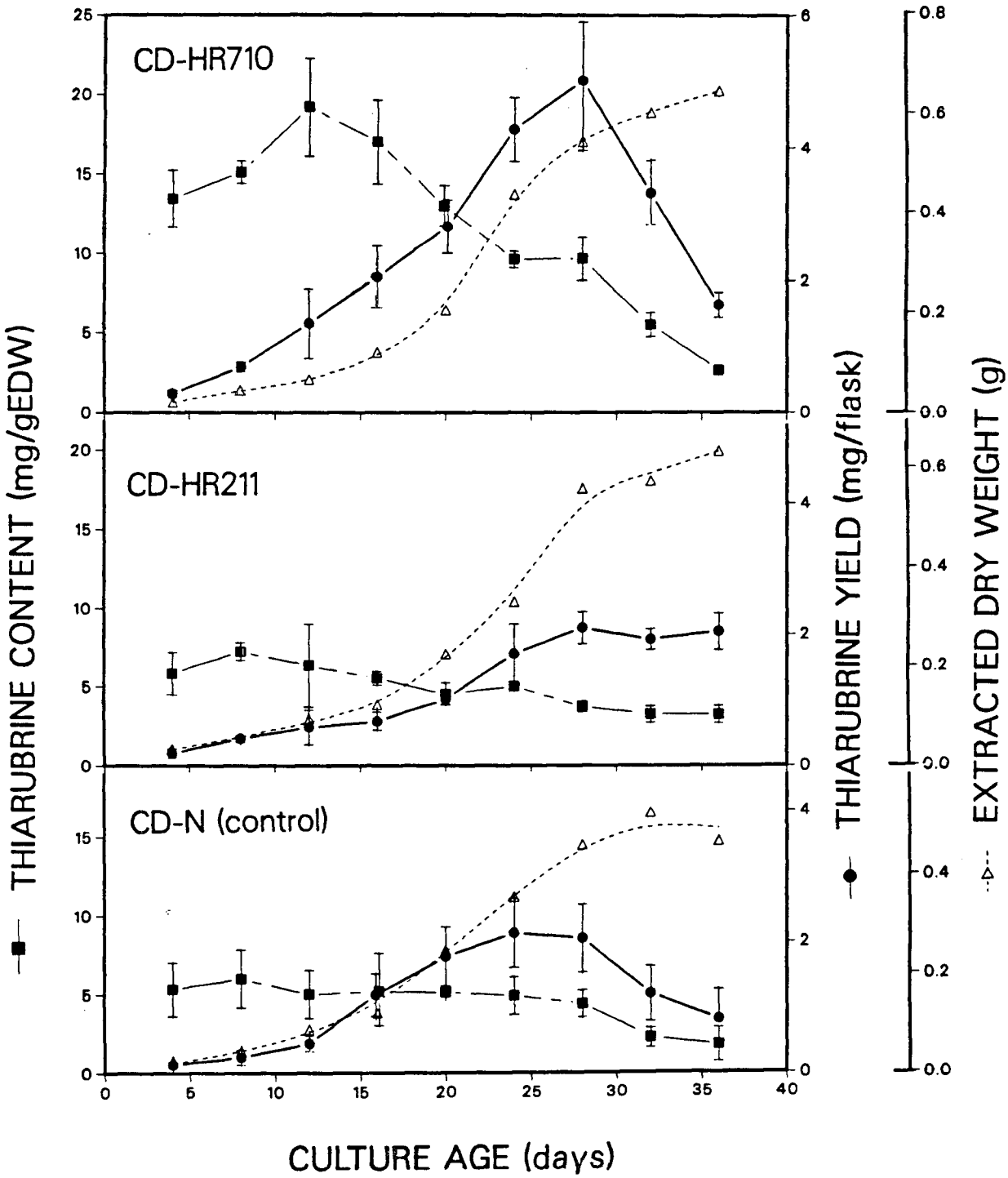


Figure 5: Total thiarubrine content, thiarubrine yield, and EDW of hairy root (CD-HR710, CD-HR211) and untransformed root (CD-N) cultures over a 36-day culture period. Each point is the mean of 4 independent determinations. Bars represent +/- standard deviation. On growth curves they have been omitted for clarity.

culture lines grew at approximately the same rate, as measured by their respective increases in EDW.

Although the two hairy root lines and the control show different maxima, the overall shapes of the growth curves give a similiar pattern. Maximum thiarubrine content (mg/gEDW) is seen very early in the growth cycle. Thiarubrine yield (mg/flask) is maximized much later, in the late logarithmic phase (Day 28), and appears to parallel growth.

While the times of peak product content and yield are similiar in all three culture lines, their maxima are very different. Hairy root line CD-HR710 accumulated twice as much thiarubrines per flask, as well as per gEDW, compared with the untransformed culture line (CD-N). Hairy root line CD-HR211, in contrast, produced levels of thiarubrine simliar to those of CD-N. Both hairy root culture lines contained less callus than the control. The high thiarubrine levels of CD-HR710 are presumably the result of high growth rates, normally achievable in root culture only with exogenous auxin, while keeping callus formation to a minimum. The lower levels of accumulation of thiarubrine in CD-HR211 probably result from the origin of this line in a different population of *C. douglasii* (as discussed above). It was not possible to establish an untransformed control culture from the *C. douglasii* population at site 2.

Types of polyines accumulated

The good separation of the isomeric thiarubrines with HPLC facilitated their separate quantification, so that the molar ratio of the isomers could be calculated. Hairy root cultures accumulate thiarubrines in different ratios than both untransformed root cultures and (untransformed) intact plant roots. This effect is independent of culture age, origin of the plant material, or degree of differentiation (Table 3). Another type of transformed culture of *C. douglasii*, a crown gall tumor line previously established by Cosio et al. (1986) also demonstrates the lower ratio of isomers, which therefore seems to be correlated only with the transformed state.

Table 3: Molar ratio of thiarubrine A / B in transformed and nontransformed roots.

Source	thiarubrine A/ thiarubrine B
Nontransformed:	
intact plant roots (site 1)	5.70 (0.38)[1]
intact plant roots (site 2)	5.71 (0.69)
CD-N (day 12)	4.18 (0.65)
Transformed:	
CD-N (day 24)	4.94 (0.45)
CD-HR710 (day 12)	2.86 (0.42)
CD-HR710 (day 24)	2.69 (0.25)
CD-HR211 (day 12)	2.63 (0.18)
CD-HR211 (day 24)	2.70 (0.33)
tumour culture	3.31 (0.23)[2]

[1] Values are mean +/- standard deviation, n=10 (intact plants) or n=4 (cultures).
[2] Calculated from Cosio et al. (1986).

A feature common to transformed tissue cultures not shared by other culture types is the higher level of endogenous auxin synthesis. Therefore the possibility of a correlation between auxin levels of the medium and the ratio of thiarubrine isomers synthesized was investigated. The ratio of thiarubrine isomers was plotted against the NAA concentration of the culture medium (Table 4). While the untransformed line accumulates thiarubrines in the same ratio independent of NAA concentration, the hairy root line accumulates proportionately less thiarubrine B at higher concentrations of NAA.

DISCUSSION

The results show that hairy root cultures of *C. douglasii* have the potential of significantly improving yields of the antifungal thiarubrines in tissue culture, producing the highest yields of thiarubrines in culture reported to date. The improved yields are due to an increased growth rate without concurrent increases in callus content. A high content of undifferentiated, fast-growing cells is correlated with a loss in biosynthetic capability in *C. douglasii* (Cosio et al., 1986) as well as most other plant species (Lindsey and Yeoman, 1983). Hairy root culture thus represents a different approach to the search for increased yields of secondary metabolites in tissue culture: rather than seeking to induce an undifferentiated, fast-growing culture to synthesize high levels of the product, transformation with *A. rhizogenes* was used to stimulate the growth rate of a root culture known to synthesize substantial amounts of product.

Other groups have also reported high yields of specific natural products in hairy root cultures (Flores, 1985; Kamada et al., 1986; Hamill et al., 1986; Jung and Tepfer, 1987). In particular Jung and Tepfer (1987) indicate that these improved yields are chiefly due to the high growth rate of hairy root cultures. None of these groups attempted to maximize yields of the nontransformed control cultures using exogenous hormones, making it difficult to estimate the usefulness of their hairy root culture systems. The experiments indicate that the observed

Table 4. The effect of NAA concentration of medium on the ratio of thiarubrine isomers of hairy root (CD-HR710) and normal root (CD-N) cultures.

[NAA] in medium (mg/l)	molar ratio thiarubrine A / B	
	CD-HR710	CD-N
0	2.73 (0.11)[1]	4.81 (0.34)
0.1	3.25 (0.39)	4.88 (0.28)
0.3	3.24 (0.10)	4.81 (0.31)
0.5	3.49 (0.18)	4.52 (0.13)
0.7	3.95 (0.88)	4.55 (0.20)

[1] Values are mean +/- S.D., n=4.

growth-stimulating effect of transformation of *C. douglasii* with *A. rhizogenes* cannot be duplicated by the addition of exogenous NAA to nontransformed root cultures.

The results also show a change in relative quantities of thiarubrine synthesized by root cultures as a result of transformation. Since growth regulators have a profound influence on secondary product synthesis (for a review, see DiCosmo and Towers, 1985), this effect was thought to be due to the different levels of auxins in transformed and nontransformed cultures. However, the reduced ratio of thiarubrine A/ thiarubrine B observed in the transformed cultures could not be obtained in nontransformed root cultures of *C. douglasii* by increasing levels of exogenous NAA in the medium. Thus, it is concluded that hairy root cultures of *C. douglasii* show growth and biosynthetic characteristics which cannot be achieved in nontransformed root cultures by the addition of exogenous NAA. This is consistent with the view that the expression by the plant tissue of bacterial auxin biosynthetic genes contained on the T-DNA is at least partially regulated. Working with *A. tumefaciens*-transformed potato cultures, Burrell et al. (1985) found lower levels of transcription of the T-DNA genes in grafted shoots than in shoots emerging directly from the culture. In the grafted shoots the T-DNA seems to be under some controlling mechanism. Using an auxin transport inhibitor, Quattrocchio et al. (1986) demonstrated that an auxin gradient is established in hairy roots of potato, suggesting that the synthesis of endogenous auxins, though encoded by the bacterial T-DNA, is indeed subject to some degree of regulation by the plant. Clearly, bathing roots in a solution of exogenous auxin cannot duplicate the effects of elevated endogenous auxin synthesis resulting from bacterial transformation.

Tabata (1977) has stressed the importance of time course experiments as an approach to understanding the control of secondary product synthesis in tissue culture. Three basic types of growth curves for cell cultures are described, differing in whether product synthesis occurs parallel to growth, during maximal growth (logarithmic phase), or after growth has stopped (stationary phase). Most commonly, maximum synthesis is observed during late logarithmic growth (Zenk et al., 1976). Not much data are available for root cultures. For all

three root culture lines described in this report, thiarubrine accumulation parallels growth, and maximum product yield is observed close to the point of maximum biomass at the beginning of the stationary phase. This parallel pattern suggests that the biosynthetic pathways are expressed constitutively throughout the culture period, and are not turned on at specific times. The drop in yield of very old cultures is probably the result of a general degeneration of the culture. In root cultures of *Bidens alba*, Norton and Towers (1986) observed a similiar pattern in the accumulation of polyines, although in that system, product accumulation lagged slightly behind biomass production. An apparent lag is also visible in the production of tropane alkaloids and nicotine by root cultures of three species of *Duboisia* (Endo and Yamada, 1985), and may indicate some down-regulation of the biosynthetic pathway. This was not observed in *C. douglasii* root cultures.

It was observed that only two of the seven *A. rhizogenes* strains used to inoculate *C. douglasii* induced root formation; this concurs with the report of Anderson and Moore (1979) which concludes that any given strain *A. rhizogenes* has a relatively restricted host range. The applicability of *A. rhizogenes* transformation to improve secondary product yield in other higher plant species may thus be limited by the host range of this pathogen.

CHAPTER II

^{35}S LABELLING STUDIES OF *CHAENACTIS DOUGLASII* HAIRY ROOT CULTURES

INTRODUCTION

Along with thiarubrines A (**1**) and B (**2**), root extracts of *Chaenactis douglasii* have been found to contain the thiophenes **3** and **4** as minor constituents (Bohlmann and Kleine, 1965; Cosio et al., 1985). These thiophenes are known from many members of the Asteraceae and have been isolated from almost 100 different species. Similiarily, thiophene compounds **7** and **8** co-occur with disulfides **5** and **6** in root extracts of *Rudbeckia hirta*. The biosynthesis of thiarubrines and other polyines is poorly understood; since the thiophenes **3**, **4**, **7**, and **8** are involved in the synthesis of more complex thiophenes (Bohlmann and Zdero, 1985), the possibility of a biosynthetic relation between thiarubrines and thiophenes was investigated. A tissue culture system using hairy roots of *C. douglasii* to produce thiarubrines (Chapter I) provided a well-defined system within which labelling studies could be conducted.

The results demonstrate that in *C. douglasii* thiophenes are not part of the biosynthetic pathway of thiarubrine. It is suggested that they appear only as the breakdown products of the chemically unstable thiarubrines, although other possibilities could not be ruled out. Furthermore, there was no evidence for a significant turnover of thiarubrines *in vivo*.

MATERIALS AND METHODS

Root Cultures

Hairy root cultures were induced on *C. douglasii* with *Agrobacterium rhizogenes* strain TR7 as described in Chapter I. Hairy root cultures were routinely maintained in liquid SH medium (Schenk and Hildebrandt, 1972) on a rotary shaker (100 rpm) at 25 degrees C in the

dark and subcultured every 4 weeks. For some experiments, 1.72 mM $MgSO_4$ was replaced by 0.86 mM $MgSO_4$ + 0.86 mM $MgCl_2$ in the medium (see results).

Radiochemicals

$^{35}SO_4^{-2}$ was obtained from Amersham Radiochemicals as a sodium salt. It was administered to cultures as a filter-sterilized aqueous solution.

Extraction of thiarubrines and thiophenes

Root cultures were rinsed 3x in distilled H_2O, blotted dry, and frozen to -80 degrees. After lyophilizing for 48h, samples were extracted with petroleum ether (30-60) for 24h. The extract was filtered, the filtrate evaporated and the compounds resuspended in HPLC-grade methanol. The polyine components were separated on a Varian Micro Pak MCH-10 reverse-phase column using 72% aqueous acetonitrile at a flow rate of 1 ml/min and were quantified by absorption at 340 nm. An internal standard of known concentration was used to correct for variations in injection volume. The molar absorptivities used for concentration determinations were 10,300 for the thiarubrines and 31,500 for the related thiophenes (Bohlmann and Kleine, 1965). The thiarubrine and thiophene peaks were collected for liquid scintillation counting. All manipulations involving thiarubrines were carried out in dim light to prevent its decomposition.

Liquid scintillation counting

Samples (2 ml) were mixed with 10 ml of a scintillation mixture consisting of 10 mg PPO-BisMSB (ICN Radiochemicals) and 50g naphthalene to 1 l *p*-dioxane. Counts were obtained on a Searle Isocap/300 liquid scintillation counter, and corrected for quenching using the channels method.

Tracer studies

11- to 16-day old root cultures (logarithmic growth phase) were labelled with a filter-sterilized aqueous solution of $Na_2{}^{35}SO_4$ (5-20 uCi). In pulse-chase experiments, the label was diluted out by adding unlabelled $MgSO_4$ (0.080 mmol/flask).

RESULTS

Polyine pattern of C. douglasii *hairy root cultures*

The thiarubrines and thiophenes isolated from intact plant roots and root culture extracts of *C. douglasii* are each found as two isomers, A and B. These are found in a constant ratio (Chapter I), and are therefore analysed together in this study. Earlier experiments had shown thiophenes to be present in *C. douglasii* root extracts at 2-10% of the more abundant thiarubrines (Table 5). In general, thiophenes were present as a greater proportion relative to the thiarubrines early in the culture period. The reason for the large difference in polyine content of intact plant roots from the two populations is not known; however, *Chaenactis* is known to be a variable species complex (Mooring, 1980).

^{35}S incorporation in relation to SO_4^{-2} availability

That roots are able to regulate rates of uptake of specific ions dependent on their availability has been documented (Glass, 1983). Such regulation has been observed with SO_4^{-2} in cultured tobacco cells (Reuveny and Filner, 1977) and whole seedling roots of pea (Deanne-Drummond, 1987). In order to optimize the uptake and incorporation efficiency of ^{35}S into the compounds of interest, cultures of *C. douglasii* roots were grown in SH medium with a range of reduced SO_4^{-2} concentrations. Appropriate amounts of $MgCl_2$ were added to maintain the Mg^{+2} concentration constant. After a 3-day labelling period, thiarubrines were extracted and the radioactivity measured. Results are shown in Figure 6. Cultures that had been grown in SH

Table 5: Polyines in *C. douglasii* root culture and intact plant root extracts (mg/gEDW).

Source	Thiarubrines (1+2)	Thiophenes (3+4)
Root cultures[1]		
12-day-old	6.35 (2.63)[2]	0.66 (0.05)
24-day-old	5.00 (0.22)	0.21 (0.03)
Intact plant roots		
Site 1	5.11 (2.12)	0.36 (0.01)
Site 2	1.80 (0.78)	0.13 (0.04)

[1] Hairy root culture line CD-HR211 (see Chapter II).
[2] Means +/- standard deviation, n=10 (intact plants), or n=4 (cultures).

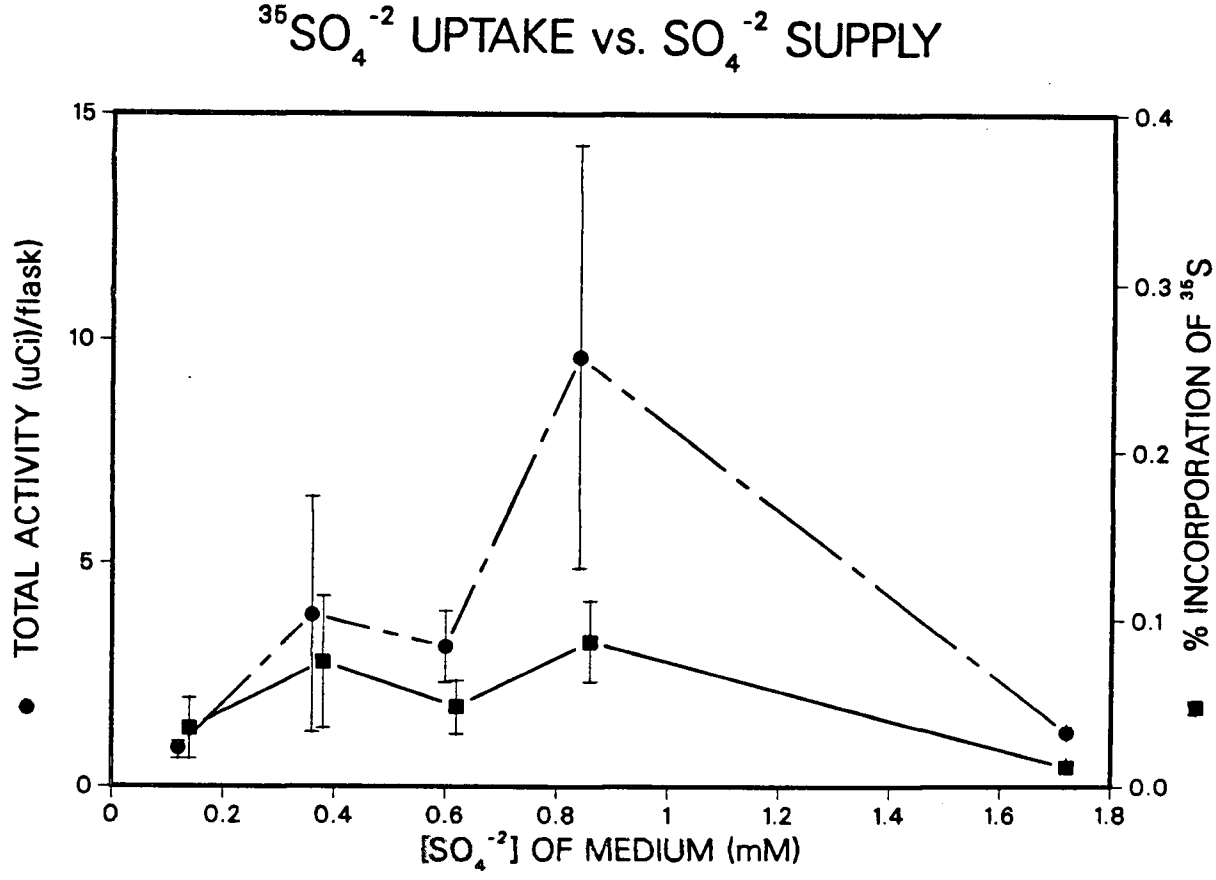


Figure 6: Incorporation of ^{35}S into thiarubrines under various conditions of SO_4^{-2} limitation. Each point is the mean of 4 independent determinations. Bars represent +/- standard deviation. 1.72 mM is the normal concentration of $MgSO_4$ in SH medium.

medium with one half the normal concentration of SO_4^{-2} showed the greatest total activity of thiarubrines. The specific activity was also maximized at this SO_4^{-2} concentration. The increase in specific and total activity was much greater than that expected to result solely from a reduced dilution of the label. Thus the rate SO_4^{-2} uptake had been significantly enhanced by the S-limiting conditions. Thiarubrine synthesis was not inhibited by SO_4^{-2} limitation except at 0.36 mM and 0.12 mM SO_4^{-2}, and growth was not affected except at the lowest SO_4^{-2} concentration (data not shown). For further labelling experiments cultures were grown in SH with 0.86 mM $MgSO_4$ + 0.86 mM $MgCl_2$ rather than only 1.72 mM $MgSO_4$.

Rate of incorporation of ^{35}S into thiarubrine and thiophene

Experiments were carried out to determine how rapidly ^{35}S, supplied as $^{35}SO_4^{-2}$, is incorporated into the polyines of interest. Cultures in the logarithmic growth phase were fed 5 uCi of $^{35}SO_4^{-2}$, and four flasks were extracted and analysed at intervals between 1h and 120h. The results are presented in Table 6. No activity could be detected with a labelling period of 1h or less, and the rate of incorporation decreased after 24h. Based on the above results, a pulse of 24h was chosen for pulse-chase experiments.

Pulse-chase experiments

A 24h $^{35}SO_4^{-2}$ pulse was followed by a chaser of unlabelled $MgSO_4$. Four culture flasks were harvested 0, 2, 4, 8, and 12 days after the end of the labelling period and analysed for thiarubrine and thiophene content and activity. The results are summarized in Figure 7. Thiarubrine yield followed a sigmoidal curve while thiophene increases were less dramatic and more linear. Earlier time-course experiments with hairy root cultures of *C. douglasii* had shown that thiarubrine synthesis parallels the typically sigmoidal pattern of biomass accumulation (Chapter I). For both compounds, the activity curves were very similiar. The specific activities of both compounds increased concurrently after administration of the label. Specific activity of thiarubrine did not increase significantly after day 2, and that of the

Table 6.: Rate of incorporation of ^{35}S into thiarubrines (mCi/mol).

Time(h)	1	3	8	24	120
Specific Activity	0	0.64(0.35)[1]	0.89(0.15)	11.8(2.89)	27.6(2.70)

[1] mean of 4 independent determinations (+/- standard deviation).

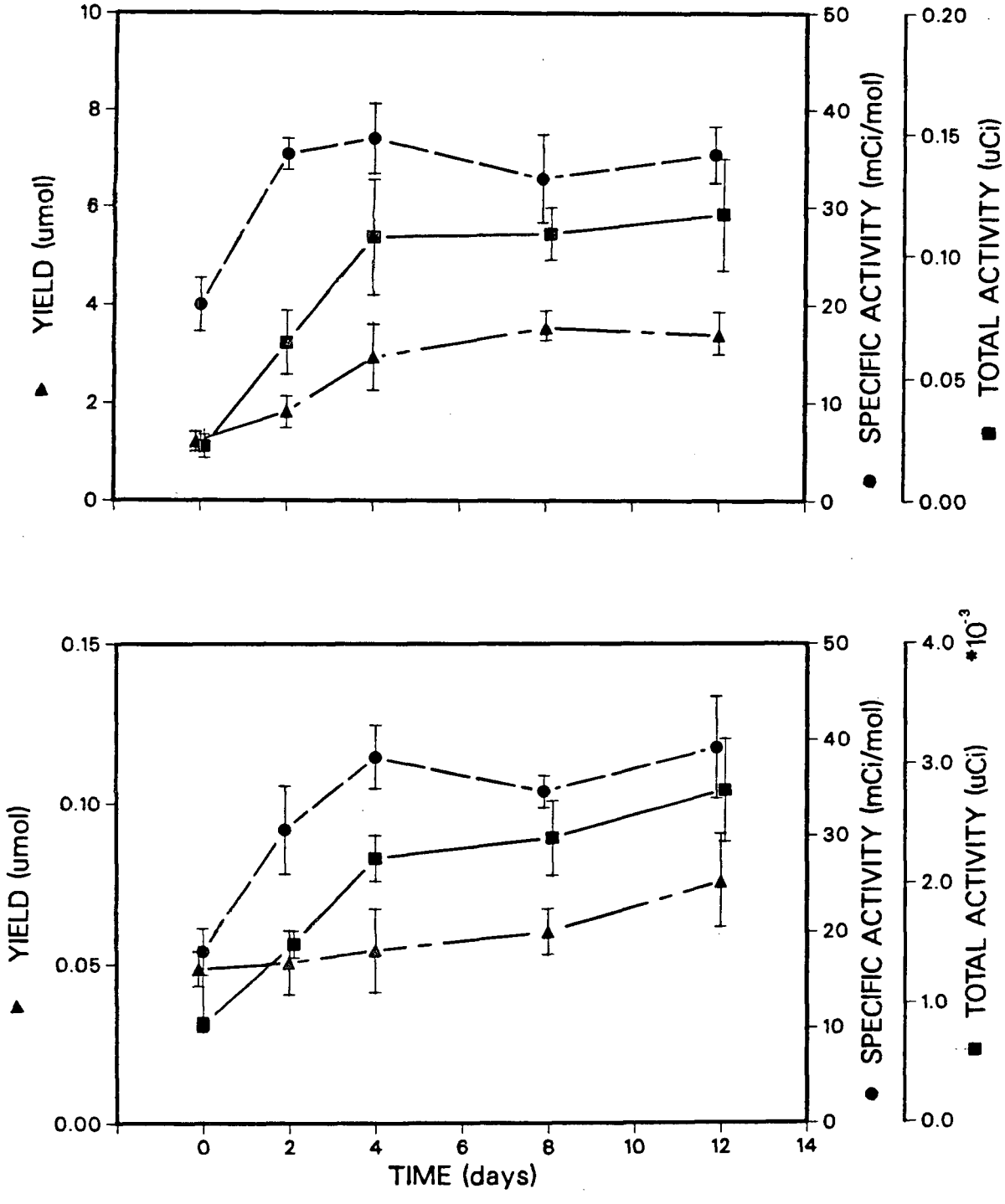


Figure 7: Specific and total activity of thiarubrines (A) and thiophenes (B) over time after pulse-chase labelling with $^{35}SO_4^{-2}$. Each point is the mean of 4 independent determinations. Bars represent +/- standard deviation. The arrows indicate the start of the 24h pulse.

thiophene after day 4. The total activities of both compounds increased steadily to day 4 and then remained constant. Although the $^{35}SO_4^{-2}$ in the medium was diluted by the unlabelled $MgSO_4$ at time 0, ^{35}S continued to be incorporated into the polyines for several days. Apparently sufficient ^{35}S was taken up by the roots in 24h to feed into the biosynthetic pathway for the following 4 days. As a result, the pulse actually received by the thiarubrine pathway spanned more than 24h.

DISCUSSION

The almost identical pattern of ^{35}S incorporation into thiarubrine and thiophene indicated that the label appears in both compounds at the same time, and once incorporated, remains at a constant level in both cases. This is inconsistent with the hypothesis that these compounds are synthesized sequentially as part of the same pathway. Several alternative possibilities could account for the observed pattern. Both compounds could be synthesized in parallel from separate pathways, or in separate reactions from the same precursor. Biosynthetically, the latter is more likely. Furthermore, previous observations indicate that thiarubrine is an unstable molecule and decomposes to the thiophene upon exposure to light, heat, and other agents (Bohlmann and Kleine, 1965). It is therefore also possible that at least some of the thiophenes are not synthesized in the plant but are artifacts of the extraction procedure and other manipulations of the cultures and extracts. All procedures were performed in dim light to minimize possible decomposition; nevertheless, duplicate extractions with an internal standard of HPLC-purified thiarubrine gave decomposition rates of between 2% and 5%, depending on the extraction procedure used. These rates would give concentrations in the range observed for thiophenes. Greater conversion was detected when fresh roots were first extracted into methanol and then partitioned into petroleum ether, rather than being freeze-dried and extracted with petroleum ether directly. While it is felt that decomposition is the most likely

explanation for the origin of thiophenes in these cultures, the evidence presented here cannot exclude other possibilities.

The conclusion that the thiophenes may not be part of the biosynthetic pathway of thiarubrines is surprising in light of the wide distribution of the thiophenes **3** and **4** in the Asteraceae and their documented role as precursors for numerous mono-, bi- and terthienyl polyines (Bohlmann and Zdero, 1985; Bohlmann and Hinz, 1965). No direct precursors are known for thiarubrines, although work by Bohlmann and associates indicates that all C_{13} polyines are ultimately derived from tridecapentaynene. The mechanism of the *in vivo* addition of sulfur is also unresolved. It is likely, though, that different mechanisms give rise to dithio rings and to thiophenes. This is also supported by earlier work in which Bohlmann and Bresinsky (1967) attempted to synthesize six-membered disulfide rings from acetylenes and Na_2S_2. The result, in addition to thiophenes, were five-membered disulfides, suggesting to the authors that it is unlikely that the six-membered dithiacyclohexadienes were intermediates in the pathway to thiophenes .

In recent years it has become increasingly recognized that secondary plant metabolites are not, by definition, inert end products of a biosynthetic pathway, but can be catabolized by the plant itself (Barz and Koster, 1981). Enzymatic breakdown may occur concurrently with biosynthesis of the same product, so that a constant concentration of a metabolite may in effect be the result of a dynamic balance of synthesis and degradation. Thus the rate of turnover of a given product is better indicator of the biosynthetic activity of a plant organ or culture than are concentrations of product. Total activity in pulse-chase experiments is usually used to estimate the turnover time of a given product. (Brown and Wetter, 1972).

The data reported here give no indication of any turnover of thiarubrine in hairy root cultures of *C. douglasii* (Figure 2). The constant total activity between days 4 and 12 demonstrates that during the late logarithmic and stationary phases thiarubrine is removed from active metabolism once synthesized. It should be noted, however, that turnover may be contingent on the growth phase of the culture (Barz, 1977).

Thiarubrine, like many hydrophobic secondary plant metabolites, accumulates extracellularily in the resin canals of the roots (Cosio et al., 1986). As thiarurine A has been shown to be very toxic to fungi, bacteria, and nematodes (Towers et al.,1985; Chapter III), the absence of a turnover of thiarubrine suggests that in the plant it has a protective rather than a physiological function.

CHAPTER III

STUDIES ON THE MECHANISM OF ACTION OF THIARUBRINE A

INTRODUCTION

Many sulfur-containing polyines have been studied intensively since the discovery of their phototoxicity (Gommers, 1972; Camm et al., 1975). Thiarubrines differ in that their antibiotic activity is light-independent; thiarubrine A was shown to be toxic in the dark to *Candida albicans* and *Saccharomyces cerevisiae* as well as a number of bacteria at a concentration of 1 ppm (Towers et al., 1985). In UV-A its antibiotic activity was sometimes enhanced.

UV-A light rapidly converts thiarubrine A (**1**) to thiophene A (**3**). This compound in turn is inactive in the dark but inhibits *C. albicans* and other microorganisms at 0.1 ppm in UV-A. The phototoxicity of thiophene A has made it very difficult to interpret the experiments in which thiarubrine A is exposed to UV-A, as one cannot readily separate its activity from that of its thiophene breakdown product. Thus the observed inhibition of microorganisms by thiarubrine A in UV-A could be due to the compound itself prior to photoconversion, to the phototoxic thiophene A, or to a mechanism of toxicity involving the conversion process itself.

Two approaches were taken to investigate how thiarubrine A affects organisms in the dark and light. The first was to test the importance of the acetylenic side chain for biological activity. Both dark and light activities of thiarubrine A and thiophene A were compared to an analogous pair of compounds (**5** and **7**) isolated from *Rudbeckia hirta*. These differ from thiarubrine A and thiophene A in that one triple bond is substituted by a double bond in the *cis* configuration (Figure 1). Secondly, to test for a possible role of disulfide ring decomposition in the toxicity of thiarubrine A, visible light was used convert thiarubrine A to thiophene A without photosensitizing the thiophene.

The results showed that light-enhanced conversion of thiarubrine A to thiophene A causes toxic effects greater than those resulting from the activity of thiarubrine A alone. This toxicity is independent of the photosensitization of thiophene A observed under UV-A.

The phototoxic effects of thiophene A differ depending on the test organism and most likely involve both photodynamic and non-photodynamic processes.

MATERIALS AND METHODS

Microorganisms

Strains of yeast and bacteria were obtained from the University of British Columbia Culture Collection. Yeast cultures were maintained on potato dextrose agar, and bacteria on Luria broth agar.

Chemicals

Thiarubrine A (Compound **1**) was isolated from hairy root or normal root cultures of *Chaenactis douglasii* grown in liquid culture as described in Chapter I. Compound **5** was isolated from *Rudbeckia hirta* root cultures grown under similiar conditions. These cultures were started from *R. hirta* seed obtained from Richters & Sons Ltd., Goodwood, Ontario. The corresponding thiophenes **3** and **7** were prepared by irradiation of compounds **1** and **5** with UV-A or visible light followed by preparative HPLC on a Varian Mikro-Pak MCH-10 reverse-phase column using 72% aqueous acetonitrile (1.5 ml/min). Compounds were identified using UV, NMR and MS spectra, and were stored in petroleum ether at -20 degrees in the dark.

Toxicity Tests

The inhibitory effect of the compounds was measured using filter paper disk bioassays. The compounds were dissolved in petroleum ether, applied to paper disks and the solvent allowed to evaporate before the disk was placed on a carpet of microorganisms on nutrient agar. Plates were incubated in the dark for 30 minutes to allow the compound to diffuse

from the disk into the agar before exposure to light. UV-A (max 350nm) was provided by 4 black-blue light UV lamps. Plates were exposed for 90 minutes, and then incubated in the dark for 24h. Exceptions are noted.

Visible light without UV was obtained from an incandescent light filtered through 5 cm of water. In visible light experiments, the antibiotic disks were removed from both light and dark treatments after one hour of dark pre-treatment incubation. This was done to ensure that both plates had an equal amount of time for the compound to diffuse into the medium. The effectiveness of a given compound in inhibiting growth of the test organisms was estimated by the diameter of the clear zone in the microbial carpet around the disk. All compounds tested were structurally similiar and were assumed to diffuse into agar at comparable rates. The tests were carried out twice.

RESULTS

Comparative biological activities of two disulfide polyines and their corresponding thiophenes

One of the interesting characteristics of thiarubrine A is its biological activity in the dark as well as light. Earlier results had already indicated that the disulfide ring is necessary for this dark activity (Towers et al., 1985). The data presented here extend this finding to another disulfide compound (**5**). Neither thiophene **3** or **7** was active in the dark, indicating that the disulfide ring was required for light-independent activity (Table 7). The comparable activity of compounds **1** and **5** against *E. coli* and *S. cerevisiae* in the dark demonstrated that the substitution of one of the acetylenic bonds with a double bond did not diminish the light-independent toxicity of thiarubrine A. None of the test compounds inhibited *Pseudomonas flavescens*.

Table 7: Toxicities of Thiarubrine A and related compounds expressed as zones of growth inhibition (mm).

Compounds	Organisms					
	S. cerevisae		*E. coli*		*P .flavescens*	
	UV-A[1]	dark	UV-A	dark	UV-A	dark
1 (thiarubrine A)	8.5[2]	4.7	5.7	2.0	0	0
2 (thiophene A)	4.0	0	4.0	0	n.t.[3]	n.t.
3	7.0	6.3	2.5	2.0	0	0
4	4.0	0	0.5	0	n.t	n.t.

[1] 90 min. UV-A irradiation at 5 W/m^2

[2] Values are means, S.D. $<15\%$.

[3] n.t.= not tested

In UV-A light the thiophenes **3** and **7** showed activity against yeast and *E. coli*. Whereas yeast was equally affected by both compounds, *E. coli* was inhibited more by thiophene **3** than **7**. This suggested that a different mode of thiophene photosensitization is active against yeast than *E. coli*.

Both disulfide compounds showed greater inhibition zones in UV-A than in the dark. Furthermore, they were more toxic under these conditions than their corresponding thiophenes. This led to the hypothesis that the process of disulfide ring decomposition could be important in the toxicity of the disulfide compounds in UV-A.

Disulfide ring decomposition as a mechanism of toxicity

In the course of the experiments it was discovered that very rapid breakdown of thiarubrine to the thiophene occurs in incandescent light as well as in UV-A. Incandescent light, filtered through a layer of water to eliminate possible traces of UV irradiation, causes complete conversion of thiarubrine A to thiophene A in less than 30 minutes. This was monitored by the loss of red color on paper disks and confirmed by HPLC. Under these conditions neither thiophene A nor alpha-terthienyl, included as a control, were photosensitized (Table 8). This allowed us to convert thiarubrine A to thiophene A without photosensitizing the thiophene product; thus it was possible to probe the actual conversion process for antibiotic effects without interference. The disk bioassays were repeated with thiarubrine A and thiophene A, but water-filtered incandescent light was used rather than UV-A. The results are shown in Table 9. Visible light enhanced growth inhibition of thiarubrine A against *E. coli* and *S. cerevisiae*. The thiophene control was not photosensitized under these conditions.

DISCUSSION

Little is known about how polyines affect cells in the dark. Reisch et al. (1973) systematically assayed a series of synthetic polyines for light-independent activity against

Table 8: Toxicities of thiarubrine A and related thiophenes in visible and UV-A light against *E. coli*. Identical results were obtained for *S. cerevisae*.

Compounds	dark	visible light[1]	UV-A[2]
thiarubrine A (**1**)	+	+	+
thiophene A (**3**)	–	–	+
light-treated[3] thiarubrine A	–	–	+
alpha-terthienyl	–	–	+

[1] incandescent irradiation filtered through 5 cm of water ($60W/m^2$).
[2] blue-black light tube irradiation ($5W/m^2$).
[3] 30 min visible light irradiation as above.

Table 9: Toxicity of thiarubrine A and thiophene A in visible light expressed as zones of growth inhibition (mm).

Compounds	Organisms			
	S. cerevisae	*E. coli*		
	light[1]	dark	light	dark
Thiarubrine A	7.5[2](0.9)	5.5 (0.3)	0.8 (0.1)	0.2 (0.2)
Thiophene A	0	0	0	0

[1] 60 min incandescent radiation (see Table 2).
[2] Mean (+/- Standard deviation), n=3.

several bacterial and fungal pathogens. Their results indicate greater antifungal activity with decreasing saturation of the molecule and increasing polarization of the triple bond. The influence of electron-withdrawing substituents such as carbonyl or phenyl groups was interpreted as enhancing the degree of polarization of the triple bonds.

The dark toxicity of thiarubrine A does not follow this pattern. The results indicate that a third triple bond can be replaced by a double bond, and that the resulting decrease in conjugation and increase in saturation of the chain does not reduce the light-independent toxicity of the molecule. Rather, the disulfide ring appears to be of central importance. Exactly how this moiety interacts with living cells is still unclear. The data presented here demonstrate that visible light-induced decomposition of the disulfide ring to the thiophene enhances the toxicity of the compound. Since the breakdown products alone are not toxic at the appropriate concentration, it follows that energy released by the reaction is imparted to one of the products involved, which then interacts with living cells. Singlet sulfur is a possible species, although this has not been demonstrated. Experiments using the singlet oxygen quencher NaN_3 or the singlet oxygen stabilizer D_2O gave ambiguous results due to toxicity of both compounds alone under the conditions employed. Since the disulfide ring is chemically unstable (Bohlmann and Kleine, 1965), ring decomposition may also account for some of the toxic effects of thiarubrine A in the dark. Reextraction and HPLC analysis of the paper disks after a 24h dark incubation period indicated that the disk itself still contained mostly thiarubrine A and very little thiophene A. It is however, difficult to measure the degree of conversion occurring in the agar itself as the compound diffuses.

The phototoxic mechanisms of polyines have been studied more extensively (Marchant and Cooper, 1986). Two types of photosensitization are in competition in polyines. (Foote, 1987; McLachlan et al., 1984; Towers and Champagne, 1986). Type I, also called non-photodynamic, involves the formation of an activated species, probably a free radical. Straight-chain acetylenes are predominantly photosensitized by this mechanism. Type II, or photodynamic, photosensitizers such as alpha-terthienyl transfer energy to molecular oxygen

resulting in singlet oxygen formation. In phenylheptatryine (PHT) both types of photosensitization are in competition (Weir et al., 1984; McRae et al., 1985). Interestingly, McLachlan et al. (1984) found type I photosensitization to be more important against *E. coli* than type II, and type II more effective against yeast than type I.

Thiophenes **3** and **7** show a pattern of toxicity very similiar to that of PHT. Against *E. coli*, the greater phototoxicity of compound **3** compared to compound **7** suggests a non-photodynamic process: in this mechanism, the degree of toxicity is correlated with the number of conjugated triple bonds (Marchant and Cooper, 1986). The observation that yeast was equally inhibited by compound **3** and **7** indicates that against this organism the acetylenic side chain is not as important for phototoxicity as the thienyl ring. Ring moieties are generally important for singlet oxygen generation (McLachlan et al., 1984). The parallels in structure and patterns of phototoxicity of thiophenes **3** and **7** with PHT suggest that in these compounds also competing photodynamic and non-photodynamic mechanisms are involved. This prediction can be tested by performing the experiments under both aerobic and anaerobic conditions.

The target molecules of thiarubrine A in dark or light are not known, although it appears that different components are involved in either case. Viruses are unaffected by thiarubrine A unless irradiated with UV-A (Hudson et al., 1986a), and under these conditions membrane-bound viruses are more susceptible than non-membrane viruses (Hudson et al., 1986b). Furthermore, the compound causes leakage of glucose or K^+ from monolamellar vesicles under UV-A, but not in the dark (MacRae, Abramowski, and Towers, unpublished results). Thus it appears that in UV-A, thiarubrine A or its thiophene product acts on membranes; this is in accordance with the proposed target site of other polyines (Hudson et al., 1986b; MacRae et al., 1985). In the dark another target seems to be involved.

It is concluded that thiarubrine A is a complex molecule which can interact with living cells by several different mechanisms (Figure 8). Conversion to thiophene is important in growth inhibition of microbes in visible and UV-A light, and possibly in the dark. UV-A

mediated phototoxicity of the thiophene appears to involve competing photodynamic and non-photodynamic mechanisms.

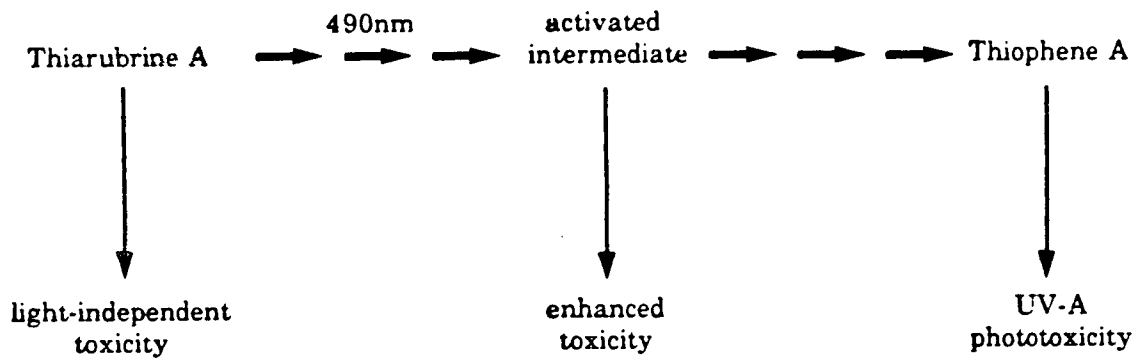


Figure 8: Summary of biological activities of thiarubrine A.

CHAPTER IV

IDENTIFICATION OF A NEW DISULFIDE POLYINE ISOMER FROM *RUDBECKIA HIRTA*

INTRODUCTION

In the course of isolating and identifying the disulfide containing polyine from *Rudbeckia hirta* L. root cultures for the studies described in Chapter III, two additional compounds were observed and separated by HPLC. Although Bohlmann and Kleine (1965) described only the *trans* isomers **6** and **8**, the spectral analyses carried out here indicated that the major components of the material examined are in the *cis* configuration. ^{1}H NMR spectral data of thiarubrine A and B from *Chaenactis douglasii*, are also presented, confirming the stuctures for the isomers thiarubrine A and B given by Norton et al. (1985).

MATERIALS AND METHODS

R. hirta seeds were obtained from Richters & Sons Ltd in Goodwood, Ontario. After surface sterilization with 5% hypochlorite solution, the seeds were germinated on moistened filter paper. The emerging rootlets were excised, transferred to solid Schenk and Hildebrandt (1972) medium (SH), and subsequently to liquid SH medium containing 0.5 mg/l naphthalene acetic acid. Cultures were maintained in 250 ml Erlenmeyer flasks on a rotary shaker (100 rpm) at 25 degrees C in the dark, and were subcultured or harvested after 3 weeks.

Root cultures were blotted dry, homogenized, filtered, and extracted 3x with MeOH. The extract was partitioned 2x into petroleum ether (30-60), concentrated and purified further by column chromatography (silica gel, petroleum ether). Yields were 0.44 mg/g extracted dry weight (EDW) total **5** and **6**, and 0.07 mg/gEDW total **7** and **8**. All procedures were performed in dim light to prevent photodegradation of the compounds. Samples were stored in petroleum ether at -20 degrees C or analysed immediately.

Extracts for HPLC analysis were taken to dryness and resuspended in HPLC grade MeOH. 20ul samples were separated on a Varian 5000 Liquid Chromatograph using an MCH-10 reverse-phase column with MeCN:$H_2$0 (6:4) at a flow rate of 1.5 ml/min. Retention times and integrals were calculated on a Spectra-Physics 4100 Computing Integrator. Compounds **5-8** eluted at R_t=24.6, 23.3, 19.9, and 16.8 respectively.

UV spectra were obtained in petroleum ether with a Philips PU 8820 UV/Visible Spectrophotometer from HPLC- purified samples. Mass spectrometry was carried out on a Finnigan 1020 GC-MS.

Compounds **1** and **2** were isolated and purified from root cultures of *C. douglasii* as described in Chapter I.

RESULTS AND DISCUSSION

HPLC analysis of the petroleum ether extract of *R. hirta* root cultures indicated the presence of four lipophilic compounds. In addition to the previously described **6** and **8** (Bohlmann and Kleine, 1965), the *cis* isomers **5** and **7** were found to be present (Figure 9). The major component of roots grown in liquid culture was **5** (63%), being present at three times the concentration of **6**. Thiophenes **7** and **8** were present in a similiar ratio and together constituted 15% of total acetylenes.

The UV and mass spectral data of **5** and **7** corresponded closely to those of **6** and **8**. Mass spectral analysis gave molecular ions at *m/z* 230 for compounds **5** and **6**, and *m/z* 198 for compounds **7** and **8**. The large numbers of low-molecular weight ions obtained are typical of acetylenes (Bohlmann, 1985). Fragmentation patterns for each pair of geometrical isomers were almost identical, suggesting *e/z* isomers of the same compound. Further evidence came from capillary gas chromatography with a fused silica column of SE 54, which resolved a mixture of **7** and **8** into two peaks of the same molecular ions at *m/z* 198. The observation that **7**

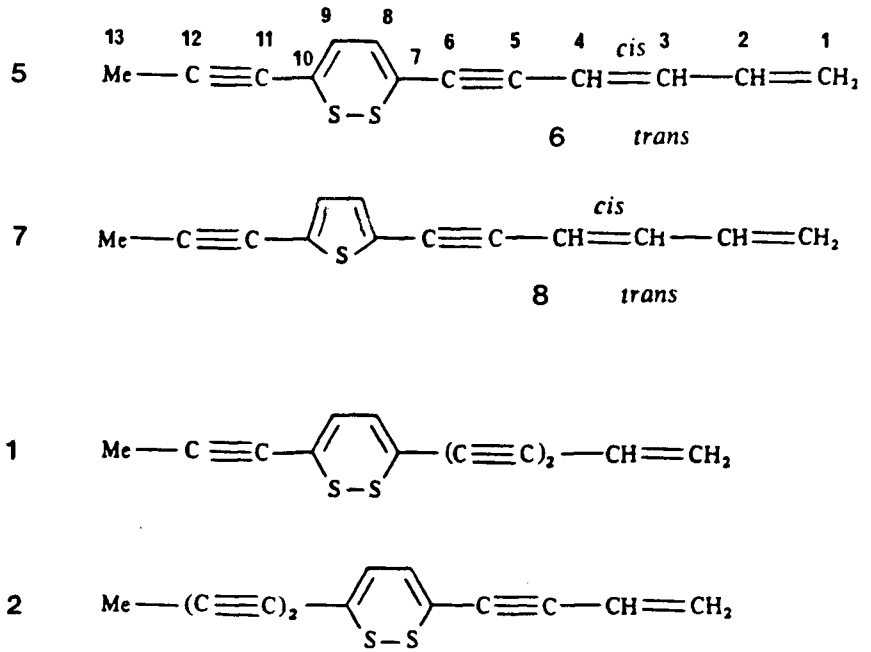


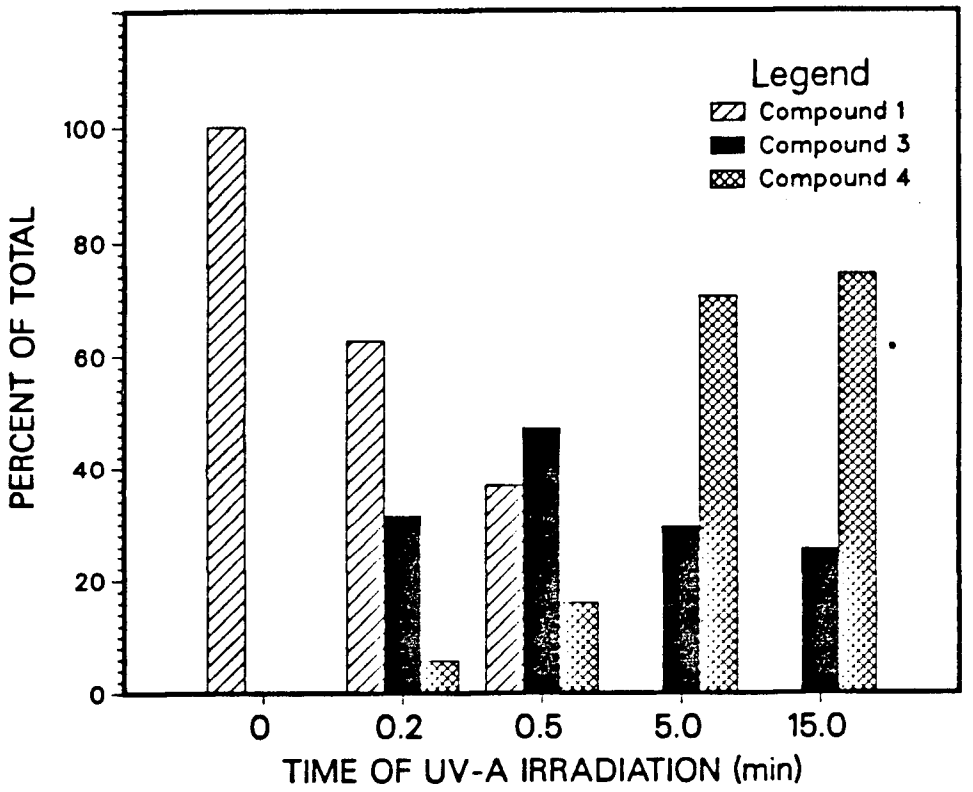
Figure 9: Polyines for which ^{1}H NMR spectra were obtained.

could be converted to **8** by UV light strongly implicated **7** as the *cis* and **8** as the *trans* isomer (Figure 10). GC retention times of 8.9 min for **7** and 8.5 min for **8** supported these assignments.

The isomeric forms of the compounds were definitively identified by ^{1}H NMR (Table 10). The coupling constants for H-3 and H-4, J = 11 in **7** and J = 16 in **8**, confirmed **7** as the *cis* and **8** as the *trans* isomer of this compound. The larger coupling constant in **8** results in the H-3 double doublet signal being resolved into 4 peaks as compared to 3 peaks seen **7**. Signals H-3 and H-4 are shifted upfield in **8** compared to **7**. Compounds **7** and **8** could be generated by decomposing **5** and **6** with incandescent light. The *cis/trans* isomers of **5** and **6** thus followed unequivocally from **7** and **8**.

Differences in the ^{1}H NMR spectrum of the disulfide compound **5** from its corresponding thiophene **7** were seen in a greater downfield shift and a reduced coupling of the ring protons at C-8 and C-9.

Although disulfide compound **2** has been described (Bohlmann and Kleine, 1965), its ^{1}H NMR spectrum (Table 10) has previously not been assigned completely. It differs from that of its isomer **1** in greater shielding of C-1 protons most likely due to the extended conjugation resulting from the second triple bond on the proximal side of the ring. Both disulfide ring protons signals are closer together, perhaps reflecting a more symmetrical distribution of conjugated bonds on either side of the ring. These data supplement the evidence for the identification of **1** and **2** from *Chaenactis douglasii* (Norton et al., 1985).



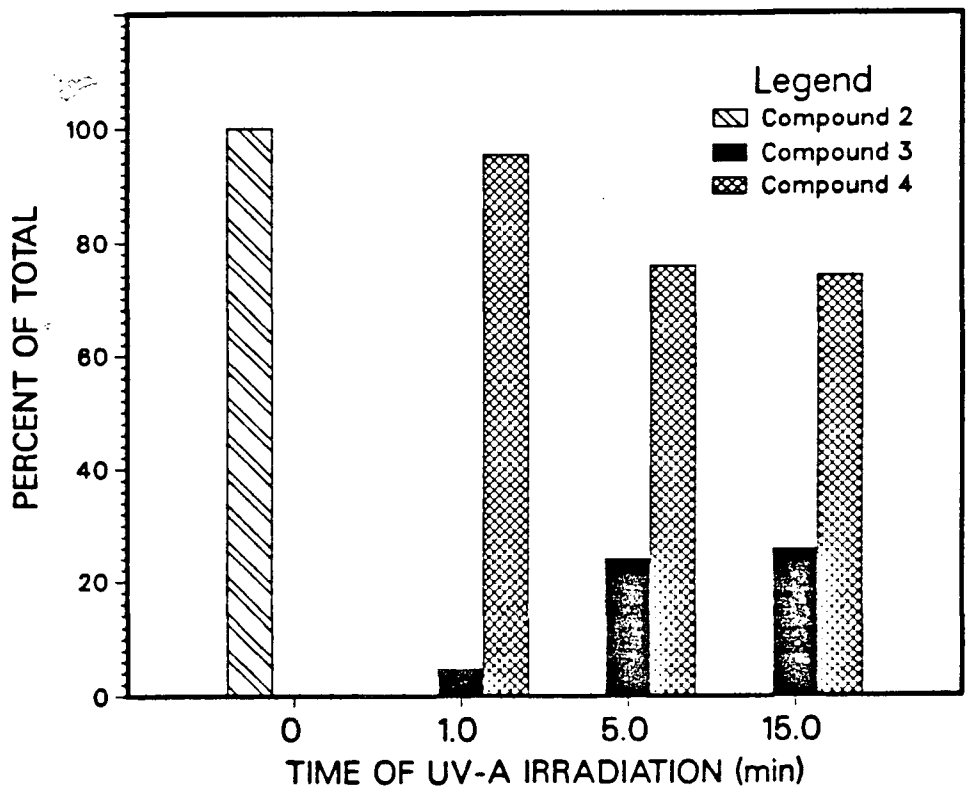


Figure 10: Conversion of **1** and **2** under UV-A (black-blue light tubes, 5W/m^2). Photodecomposition to the thiophenes is followed by photoisomerisation to a *cis/trans* mixture of 1:3 regardless of starting compound.

Table 10. ^{1}H-NMR spectral data of compounds **1**, **2**, **5**, **7**, **8** (400 MHz, CD^3CN, TMS as internal standard).

H	**5**	**7**	**8**	**1**	**2**
1	5.52 *d*	5.49 *d*	5.41 *d*	5.90*dd*	5.77 *dd*
1'	5.40 *d*	5.37 *d*	5.26 *d*	5.78 *dd*	5.67 *dd*
2	6.85 *ddd*	6.90 *ddd*	6.49 *ddd*	6.01 *dd*	6.07 *dd*
3	6.58 *dd*	6.54 *dd*	6.70 *dd*	-	-
4	5.72 *d*	5.73 *d*	5.93 *d*	-	-
8	6.67 *d*	7.11 *d*	7.07 *d*	6.79 *d*	6.68 *d*
9	6.50 *d*	7.03 *d*	7.01 *d*	6.60 *d*	6.78 *d*
13	2.08 *s*	2.09 *s*	2.06 *s*	2.10 *s*	2.20 *s*

J[Hz]: 1',2=11; compounds **1** and **2**: 1,1'=2; 1,2=17.5; 8,9=7; compound **5**: 1,2=17; 2,3=11; 3,4=11; 8,9=7; compound **7**: 1,2=16; 2,3=11; 3,4=11; 8,9=4; compound **8**: 1,2=16; 2,3=11; 3,4=16. 8,9=4.

SUMMARY AND CONCLUSION

This investigation of several aspects of the biology and chemistry of thiarubrine and related polyines is summarized in the following conclusions: 1. Thiarubrine accumulation is doubled in hairy root cultures of *C. douglasii* compared to nontransformed root cultures, and the proportion of thiarubrine A / B is reduced. 2. Thiarubrines are not synthesized sequentially with thiophenes in the same pathway, and are not turned over in mature *C. douglasii* hairy root cultures. 3. The antimicrobial activity of thiarubrine is enhanced by visible light, suggesting ring modification as a mechanism of toxicity. The corresponding thiophenes are probably photosensitzed by both type I and type II mechanisms. 4. *R. hirta* contains the previously unreported *cis* isomer of a known disulfide polyine.

In addition to focusing on the same or very similar secondary plant compounds, the above studies share the use of plant tissue culture throughout, as an experimental system, as an efficient and reliable method of obtaining the polyines of interest, or as a topic of research itself. The work described in this thesis is thus a demonstration that plant tissue culture, more specifically organ culture, can be a valuable tool in the investigation of secondary metabolites. This conclusion is underlined by the experience that *C. douglasii* root cultures in an incubator were easier to propagate and maintain than the intact plants themselves in the greenhouse.

A somewhat personal conclusion, after studying thiarubrine from a number of perspectives, is built on the diversity of aspects investigated: it is because secondary plant chemicals raise questions at a number of levels that they are a stimulating area of research.

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APPENDIX I: SPECTRAL DATA

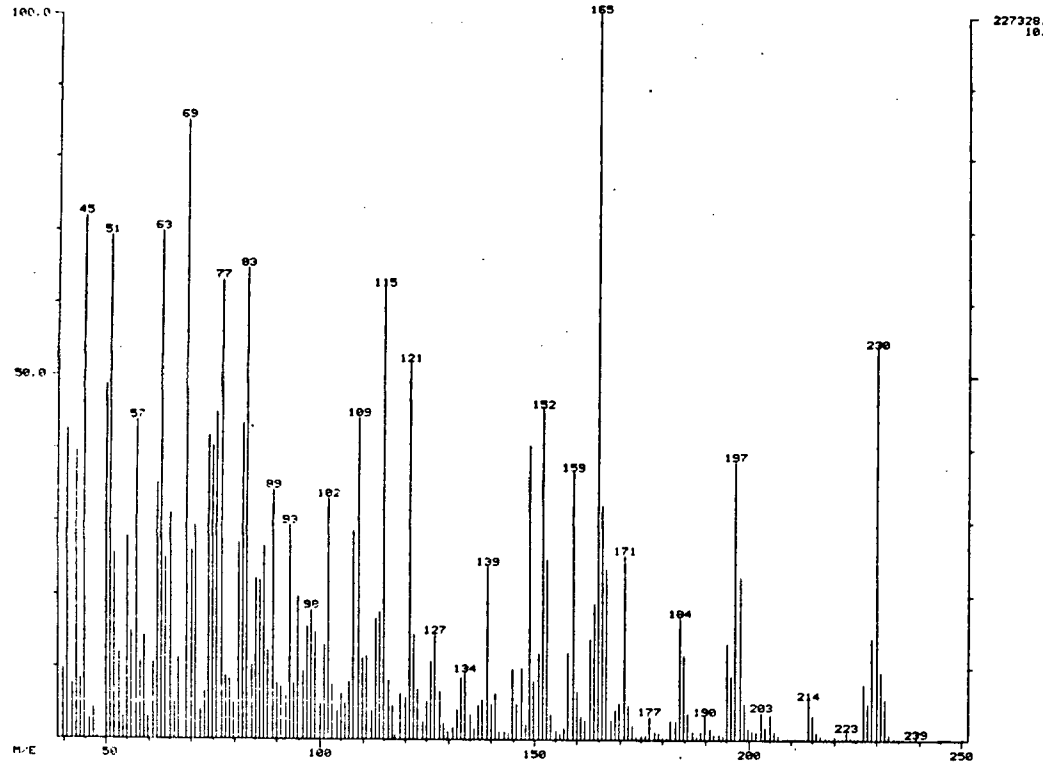
Compound **5**: UV spectral data in petroleum ether (nm): 482, 350, 257; MS *m/z* (rel. int.): 230 (54), 197 (38), 165 (100),69 (85).

Compound **7**: UV spectral data in petroleum ether (nm) 339 248 ; MS m/z (rel. int.): 198 (100), 171 (13), 165 (27), 152 (8).

Spectral data for **6** (UV-max: 478, 349, 258) and **8** (UV-max: 337, 246) matched those reported in the literature (Bohlmann and Kleine, 1965).

Mass spectra for compounds **5** (A) and **7** (B) are shown on following page.

A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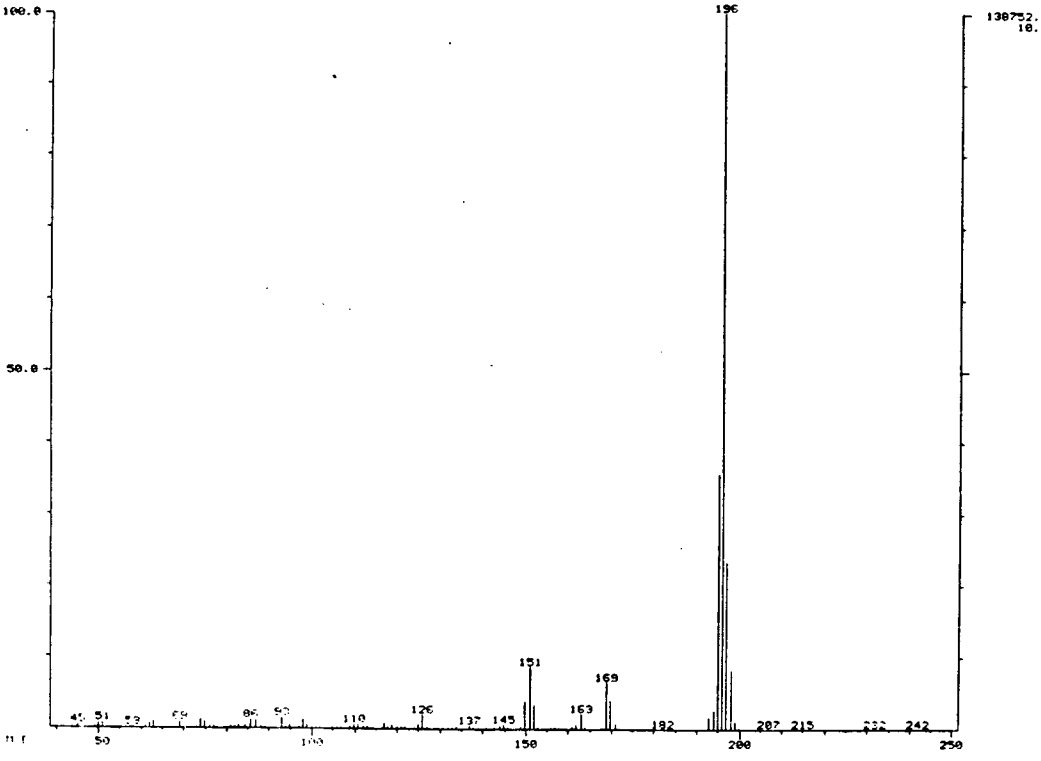
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