THIARUBRINE PRODUCTION IN ROOTS AND ROOT CULTURES OF *AMBROSIA CHAMISSONIS*

by

SHONA MARGARET ELLIS

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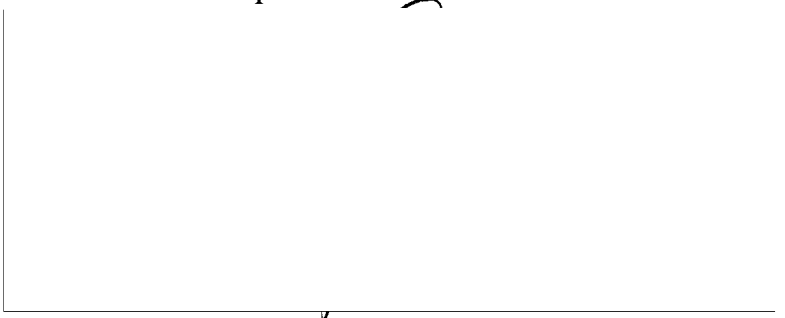

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ABSTRACT

Ambrosia chamissonis (Asteraceae) provides antibiotic sulphur heterocyclic polyynes known as thiarubrines and thiophenes. They occur in all parts of the plant, especially in the roots and can be visualized in hand sections. Methods were developed for their isolation, purification and quantification. Plants were collected from various locations in B. C. and found to have different thiarubrine profiles. A new thiarubrine (3-(1-propynyl)-6-(6-hydroxyhex-3-yn-1ynyl)-1,2-dithiacyclohexadiene) and thiophene (2-(1-propynyl)-6-(6-hydroxyhex-3-yn-1ynyl)-thiophene) were identified from roots and root cultures. Transgenic and non-transgenic root cultures were generated and shown to produce the same compounds as occur in natural roots. Quantitative chemical profiles of the cultured roots matched those of roots which had little secondary growth. Elicitation, with fungal preparations, did not enhance the over-all thiarubrine concentration, but the content of a more fungicidal thiarubrine was enhanced at the expense of a less active, but predominant thiarubrine. Administration of biosynthetic precursors did not have any effect on thiarubrine production. Antibiotic testing indicated that the thiarubrines are toxic against a number of fungi and, to a lesser degree, bacteria.

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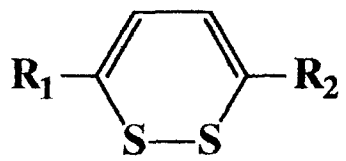
CHAPTER ONE

GENERAL INTRODUCTION

Ambrosia chamissonis grows on sandy maritime beaches along the west coasts of North and South America. Its range extends from Baja California to the Queen Charlotte Islands of British Columbia in North America. In South America it was first reported in Chile in 1892 on Isla de la Mocha (Nakatani *et al.*, 1973). It has spread northward and occupies several hundred miles of coastal Chile. There have been problems with the taxonomy of this species due to morphological variability. Payne (1964) re-evaluated *Ambrosia* and combined a number of species of *Ambrosia* with *Franseria* to form the heteromorphic species *A. chamissonis.* The taxonomy is based on the nature of the spines of the fruiting involucres rather than on leaf morphology. Leaf morphology varies within and between geographic regions. The group of chemicals most extensively studied in this group are the sesquiterpene lactones, but the analyses of these compounds have supplied little chemotaxonomic information (Payne *et al.*, 1973). Like leaf morphology the chemical production of the plant is variable. However, Nakatani *et al.* (1973) point out that the Chilean variety is relatively uniform in both chemistry and morphology. They suggest that this may be due to the introduction of the species and subsequent spread to other sites making this a genetically uniform line. The chemistry and morphology mimic that of the plants found north of San Francisco implying that this may be the origin of the Chilean strain. The only other relatively uniform population is found in southern California and is cut off physically from the rest of the North American population (by extensive cliffs). The genus *Ambrosia,* is considered a highly evolved member of the Family Asteraceae in the tribe Heliantheae and subtribe Ambrosiinae. Bohlmann (1990) points out that the subtribes can be separated based on their main chemical constituents. Information on acetylenes and sesquiterpenes are both useful.



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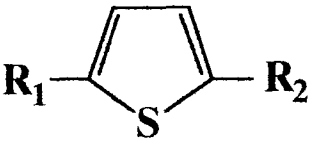

b

Thiarubrines: 1a, 2a, 3a, 4a, 5a, 6a, 7a, 8a.

Thiophenes: 1b, 2b, 3b, 4b, 5b, 6b, 7b, 8b.

	R_1	R_2
1	$CH_3-C\equiv C-$	$-(C\equiv C)_2-CH=CH_2$
2	$CH_3-(C\equiv C)_2-$	$-C\equiv C-CH=CH_2$
3	$CH_3-C\equiv C-$	$-(C\equiv C)_2-CH(O)CH_2$
4	$CH_3-C\equiv C-$	$-(C\equiv C)_2-CHOH-CH_2OH$
5	$CH_3-C\equiv C-$	$-(C\equiv C)_2-CHCl-CH_2OH$
6	$CH_3-C\equiv C-$	$-(C\equiv C)_2-CHOH-CH_2Cl$
7	$HO-CH_2-C\equiv C-$	$-(C\equiv C)_2-CH=CH_2$
8	$CH_3-C\equiv C-$	$-(C\equiv C)_2-CH_2-CH_2OH$

Figure 1.1 Thiarubrines and Thiophenes characteristic of *Ambrosia chamissonis.*

Members of the Asteraceae characteristically produce polyynes. These acetylenic compounds are also found in the Campanulaceae, Apiaceae, Araliaceae and a number of other plant families as well as in the Basidiomycetes (Bohlmann *et al.*, 1973). A number of sulphur derivatives including dithiacyclohexadiene polyynes and thiophenes are found exclusively in the Asteraceae primarily in the Heliantheae. The dithiacyclohexadiene polyynes, commonly called thiarubrines (Fig. 1.1), are found in a restricted number of genera. For example, *Chaenactis douglasii* and *Eriophyllum lanatum* produce the positional isomers thiarubrine **1a**, 3-(1-propynyl)-6-(5-hexen-3-yn-1-ynyl)-1,2-dithiacyclohexa-3,5-diene and thiarubrine **2a**, 3-(pent-3-yn-1-ynyl)-6-(3-buten-1-ynyl)-1,2-dithiacyclohexa-3,5-diene (Norton *et. al.*, 1985a). These have been trivially called thiarubrines A and B respectively (Norton *et. al.*, 1985a). For simplicity the thiarubrines and thiophenes will be assigned numbers and letters in this paper (Fig. 1.1 and Appendix I). Thiarubrines are designated with the letter "a" and thiophenes with the letter "b". *Rudbeckia hirta* produces geometric isomers of thiarubrine **10a** (Constabel *et al.*, 1988). Table 1.1 summarizes the distribution of the thiarubrines throughout the Asteraceae (the only family they occur in) following the taxonomic scheme of Robinson (1981). The thiarubrines are converted into their corresponding thiophenes via sulphur extrusion by irradiation (Fig. 1.2). Thiarubrine **1a** yields thiophene **1b** (2-(1-propynyl)-5-(5-hexen-3-yn-1-ynyl)-thiophene) and **2a** produces **2b** (2-(pent-3-yn-1ynyl)-5-(3-buten-1-ynyl)-thiophene).

Ambrosia chamissonis has been found to produce the widest array of thiarubrines of any plant analyzed to date(Balza and Towers, 1990, 1993). Thiarubrines **1a** and **2a**, are present in large quantities. Other thiarubrines, based on the thiarubrine **1a** skeleton, have been identified. Thiarubrine **3a**, 3-(1-propynyl)-6-(5,6-epoxyhex-3-yn-1-ynyl)-1,2-dithiacyclohexa-3,5-diene, an epoxide and thiarubrine **4a**, 3-(1-propynyl)-6-(5,6-dihydroxyhex-3-yn-1-ynyl)-1,2-dithiacyclohexa-3,5-diene, a vicinal diol were found to be unique to this group and in relatively high quantity accompanied by their corresponding thiophenes **3b**, 2-(1-propynyl)-5-(5,6-epoxyhex-3-yn-1-ynyl)-thiophene and **4b**, 2-(1-propynyl)-5-(5,6-dihydroxyhex-3-yn-1-ynyl)-

Table 1.1 Distribution of Thiarubrines.

Species	Thiarubrine	Reference
	Tribe Eupatorieae	
Subtribe Ageratinae		
Mikania officinalis	2a	Bohlmann *et al.*, 1973
	Tribe Heliantheae	
Subtribe Ambrosiinae		
Ambrosia artemisifolia	1a	Bohlmann and Kleine, 1965
A. chamissonis	1a	Bohlmann *et al.*, 1977
	2a	Norton *et al.*, 1985a
	3a	Balza *et al.*, 1989
	4a	Balza *et al.*, 1989
	5a	Balza and Towers, 1990
	6a	Balza and Towers, 1990
	7a	Balza and Towers, 1990
	8a	Ellis *et al.*, 1993
	9	Balza and Towers, 1990
A. confertiflora	1a	Lopez *et al.*, 1989
A. cumanensis	1a	Bohlmann *et al.*, 1977
A. eliator	1a	Bohlmann and Kleine, 1965
A. psilostachya	1a	Lopez *et al.*, 1989
A. trifida	1a	Bohlmann and Kleine, 1965
	11a	Lu *et al.*, 1993
A. trifoliata	1a	Bohlmann and Kleine, 1965
Iva xanthifolia	1a	Bohlmann and Kleine, 1965
Subtribe Baeriinae		
Eriophyllum caespitosum	2a	Bohlmann and Kleine, 1965
E. lanatum	1a	Norton *et al.*, 1985a
	2a	Bohlmann *et al.*, 1981
E. staechadifolium	1a	Bohlmann *et al.*, 1981
Lasthenia chrysostoma	12a	Bohlmann and Zdero, 1978
L. coronaria	12a	Bohlmann and Zdero, 1978
Subtribe Chaenactidinae		
Chaenactis douglasii	1a	Norton *et al.*, 1985a
	2a	Norton *et al.*, 1985a
C. glabriuscula	2a	Bohlmann *et al.*, 1973
Palafoxia hookeriana	1a	Bohlmann *et al.*, 1973
P. texana	2a	Bohlmann *et al.*, 1973
Picradeniopsis woodhousei	12a	Bohlmann *et al.*, 1976
Schkuhria abrotanoides	1a	Bohlmann *et al.*, 1973
S. advena	1a	Bohlmann and Kleine, 1965
S. multiflora	1a	Bohlmann, Jakupovic, *et al.*, 1980
S. pinnata	1a	Bohlmann and Zdero, 1977
		Bohlmann and Kleine, 1965
S. senecioides	1a	Bohlmann and Kleine, 1965

Table Cont.

Species	Thiarubrine	Reference
Subtribe Ecliptinae		
Oyedeae boliviana	2a	Bohlmann and Zdero, 1979
Verbesina alata	2a	Bohlmann *et al.*, 1973
V. boliviana	2a	Bohlmann, Grenz, *et al.*, 1980
V. cinerea	2a	Bohlmann, Grenz, *et al.*, 1980
V. latisquamata	1a	Bohlmann and Lonitz,1978a
V. occidentalis	1a	Bohlmann and Lonitz, 1978b
Zexmenia hispida	2a	Bohlmann and Lonitz, 1978c
Subtribe Melampodiinae		
Melampodium divaricatum	**10a** (*E*)	Bohlmann and Le Van, 1977 Bohlmann and Kleine, 1965
M. longifolium	**10a** (*E*)	Bohlmann and Kleine, 1965
Subtribe Milleriinae		
Milleria quinquefolia	2a	Bohlmann *et al.*, 1973
Subtribe Rudbeckiinae		
R. bicolor	**10a** (*E*)	Bohlmann and Kleine, 1965
R. hirta	**10a**	Bohlmann and Kleine, 1965
R. newmannii	**10a**	Bohlmann *et al.*, 1973
R. speciosa	**10a** (*E*)	Bohlmann and Kleine, 1965
R. sullivantii	**10a**	Bohlmann *et al.*, 1973

* The thiarubrines are present with their corresponding thiophenes

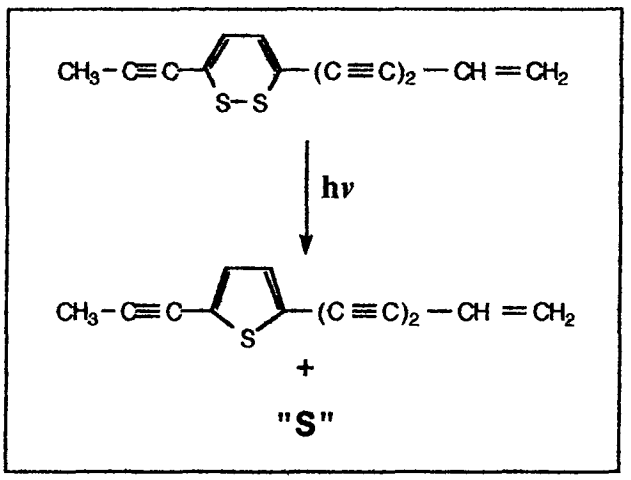

Figure 1.2 Sulphur Extrusion from Thiarubrine **1a** after Irradiation with UV-A Light.

thiophene. Compounds present in lower concentrations included two chlorohydrins **5a** (3-(1-propynyl)-6-(5-chloro-6-hydroxyhex-3-yn-1ynyl)-1,2-dithiacyclohexa-3,5-diene) and **6a** (3-(1-propynyl)-6-(6-chloro-5-hydroxyhex-3-yn-1ynyl)-1,2-dithiacyclohexa-3,5-diene), a primary alcohol **7a** (3-(hydroxyprop-1-ynyl)-6-(5-hexen-3-yn-1-ynyl)-1,2-dithiacylohex-3,5-diene) and their corresponding thiophenes **5b**, **6b** and **7b**. Thiosulphinate **9** (3-(1-propynyl)-6-(5-hexen-3-yn-1-ynyl)-cyclohexa-3,5-diene-1,2-thiosulphinate which exists without a corresponding thiophene.

There is little known about the biosynthesis of the 13 carbon thiarubrines and thiophenes, although it is agreed that they originate from acetylenic precursors. The mechanism of triple bond formation in the generation of the acetylene tridecapentaynene as well as the addition of H_2S, or its biochemical equivalent, to the conjugated triple bonds remain unknown. Tracer studies by Bohlmann *et al.* (1973) indicated that the terminal carbon of a fatty acid is usually lost in the generation of the odd carbon polyynes. This was corroborated by Gomez-Barrios *et al.* (1992) who used ^{13}C-acetate in their tracer work. Thiarubrine **1a** is believed to be the precursor to the other hydroxylated and chlorinated thiarubrines. Even though the thiophenes can be produced by irradiation of the corresponding thiarubrine, there appears to be a separate biosynthetic pathway in these plants as demonstrated in *Chaenactis douglasii* using ^{35}S incorporation (Constabel *et al.*, 1989b). Bohlmann (1973) proposed that the thiarubrines exist in equilibrium with their thioketone isomers which could explain the red coloration, however this explanation does not appear likely (Norton *et al.* 1985). In Bohlmann's comprehensive book, UV-Visible spectra were presented for a large number of polyynes and much can be gleaned about the structures of these compounds by such data.

The function of these chemicals is apparently defensive. Thiarubine **1a** and thiophene **1b**, as well as the *Rudbeckia* thiarubrines and thiophenes, have been tested against the human pathogens *Saccaromyces cerevisiae*, *Escherichia coli*, and *Pseudomonas flavescens* (Constabel and Towers, 1989c). Light (UV-A) was found to be an important factor in the activity of the thiophenes. The thiarubrines, however, have light-independent as well as a light dependent

activity. The process of conversion from the thiarubrine to the thiophene or the activity of the resulting thiophene may be responsible for the toxicity. *P. flavescens* was not affected by any of the compounds at the concentrations tested. Hudson *et al.* (1986) examined the activity of thiarubrines against viruses and found toxicity is light-dependent. The thiophenes are toxic only in the light at higher concentrations. The thiarubrines are toxic to nematodes and to cockroaches (Towers and Abramowski, unpublished).

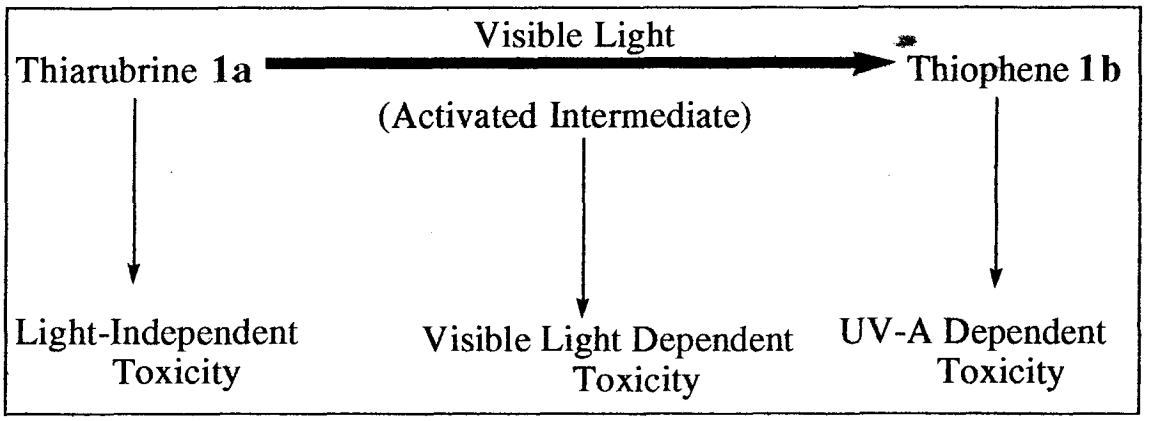

Figure 1.3 Biological Activity of Thiarubrine **1a** (Constabel and Towers, 1989).

Tissue culture has great potential for the study of plant chemistry. To date, however, the utilization of plant cell cultures for large scale production of secondary compounds have not been sucessful in many cases. One of the main reasons for this is that unorganised tissues, such as callus and suspension cultures, which have traditionally been employed, usually do not produce the desired secondary metabolites. On the other hand, organ cultures, such as roots, are good sources of secondary compounds characteristic of the particular tissue. This principle was nicely demonstrated by Cosio *et al.* (1986) who examined crown gall tumour cultures of *Chaenactis douglasii.* The transgenic callus produced and accumulated thiarubrines whereas normal callus did not. Histological inspection of transgenic callus, revealed that they actually resembled roots. Norton *et al.* (1985b) examined the production of thiophenes in crown gall tumours and callus cultures of *Tagetes patula.* The thiophenes were produced at a reduced and inconsistent level. The

tumours apparently had some degree of differentiation into organized tissue where secondary metabolites were synthesized. Callus cultures derived from tumours produced more thiophenes than those which were not transgenic, but the levels were low when compared with the plant roots. Differentiated tissue, such as roots, are relatively easy to culture depending on plant species. Root cultures usually reflect the chemistry of the plant root. Because the thiarubrines are light sensitive, culture conditions as well as extraction procedures must be adapted to accommodate this.

One of the drawbacks of tissue culture is that over time the requirements for growth may change especially with callus and suspension cultures. Root cultures may change their requirement for growth regulators (ie. auxins and/or cytokinins) over time. "Hairy" root cultures do not require plant hormones for their growth. *Agrobacterium rhizogenes* is the causative agent of hairy root disease. This plant pathogen transfers a piece of DNA into the plant cell where it is stably incorporated into the plant nuclear genome. Several genes are involved in this transfer and ultimately cause the proliferation of roots at the infection site. Roots can be induced and maintained in culture.

Hairy root cultures are stable for extended periods of time. Aird *et al.* (1988) examined the chromosome numbe in hairy root cells from various plant species and found the chromosome numbers to be identical to that of the parent plants. Both suspension cultures and callus cultures, exhibit chromosomal variation after numerous subcultures. The production of secondary metabolites in root cultures was shown to be stable over time. There are claims that higher concentrations of secondary metabolites are produced in transgenic cultures than in untransformed root cultures (Constabel and Towers, 1989a). Whether these cultures are intrinsically superior or whether it is the selection of high yielding lines which account for this remains questionable.

Manipulations of culture conditions may also enhance the production of important chemicals, through precursor feeding as well as stimulation of production through the use of elicitors. Since many desirable compounds are defensive in nature, the exposure to a pathogen or a component extracted from a pathogen, may cause the plant tissue to respond by increasing the production of defensive chemicals.

The focus of this research was to continue the examination of the thiarubrines and thiophenes of *Ambrosia chamissonis* as they have medicinal potetnial. Traditionally the roots have been examined as the source of these compounds. In these studies, above ground portions were also investigated. Chemical as well as morphological studies demonstrate the complex nature of the production of these compounds. Production in tissue culture, was examined to determine if cultured plant tissue is an efficient way to produce them. In addition, the activities of the different thiarubrines against numerous microorganisms were examined.

CHAPTER TWO

ROOT CHEMISTRY OF *AMBROSIA CHAMISSONIS*

INTRODUCTION

The genus *Ambrosia* has been re-examined and many species, which have been separated on the basis of leaf morphology, are now believed to belong to one heteromorphic species (Payne *et al.* 1964). No correlation was found between leaf morphology and the sesquiterpene lactone profiles in plants collected from western North America (Payne *et al.* 1973). In British Columbia the Centennial Beach (CB), Tsawassen population is composed of plants with one type of leaf morphology, whereas the North Beach, Queen Charlotte Island (QCI) population is made up of three types (Fig. 1.1). The morphologically distinct plants of QCI may grow within a metre of each other, the most common type (Type 1) being of similar leaf morphology to the Tsawwassen plants. In the autumn there is more variation within the individuals of the QCI plants. The chemistry of the roots from two collection sites were examined.

The roots of species of *Ambrosia* have been examined for their acetylene composition (Bohlmann *et al.*, 1973, Balza *et al.* 1989, 1990). Thiarubrines (**1a** and **2a**) and thiophenes (**1b** and **2b**) were reported as well as pentaynene, a supposed precursor. Balza *et al.* (1989) have identified thiarubrines **1a** and **2a**, as well as others which have the basic structure of thiarubrine **1a** in the roots of *Ambrosia chamissonis* from two populations: Marin County, California and Tsawassen, British Columbia. The vicinal diol, thiarubrine **4a**, as well as the epoxide, thiarubrine **3a**, were identified along with their corresponding thiophenes. Later the structures of others including a primary alcohol (**7a**), two chlorohydrins (**5a** and **6a**), accompanied with their corresponding thiophenes, and a thiosulphinate (**10**) were elucidated (Balza *et al.*, 1990). The low yield of these compounds made it impossible to quantify them accurately, but it was found that the Marin County Collections had a higher level of the chlorohydrins than the Tsawassen collection

which had as its primary minor compounds thiarubrine 7a and thiophene *7b*. It is presumed that the thiarubrines are biosynthetically related although there has been very little investigation on the biosynthesis of these compounds or acetylenic compounds in general.

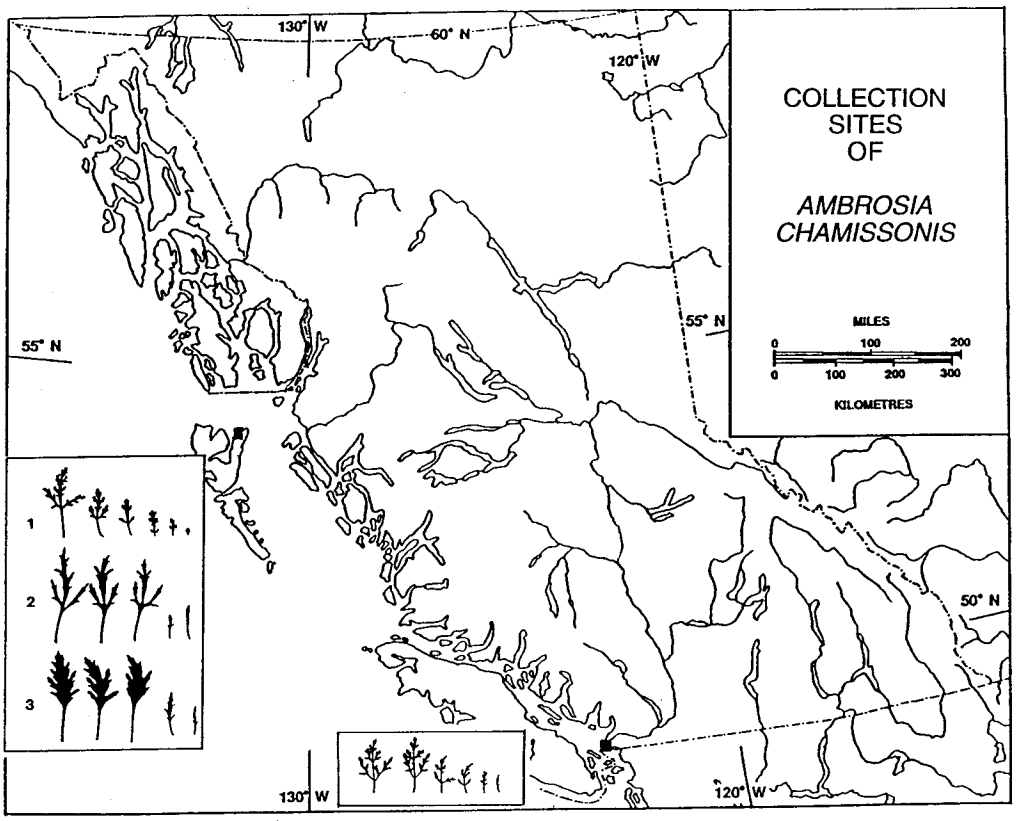

Figure 2.1 Collection Sites, in British Columbia, of *Ambrosia chamissonis*

The thiarubrines have very similar characteristic UV-Visible spectra. On the other hand, the thiophenes with their somewhat similar spectra, have distinguishing peaks (Appendix II). Therefore much can be gleaned from UV-Visible data of the latter. Because the thiarubrines are colored compounds, column chromatography is useful for their isolation. Purification techniques were established for the thiarubrines and thiophenes using column chromatography for preliminary isolation and then HPLC for final purification. HPLC was also employed for the evaluation of crude samples and column fractions. The HPLC was equipped with a photodiode array detector which gives "in flight" UV-Visible spectra. A number of spectroscopic techniques were utilized to

identify the root chemicals: Ultraviolet-Visible spectrophotometry (UV-Vis), Gas Chromatography-Mass Spectrometry (GC-MS) of hydroxylated compounds, and solid probe Mass Spectrometry (MS). Balza *et al.* (1989, 1990) have presented ^{1}H NMR data in the structural identification of the compounds as well as assigned ^{13}C NMR values. These techniques are not required for the known compounds since other spectral data being sufficient.

MATERIALS AND METHODS

A . Establishment of Extraction, Isolation and Purification Procedures for Thiarubrine and Thiophenes

Plant Material

Roots were collected from Centennial Beach, Tsawassen, British Columbia and transported to the University of British Columbia in dark garbage bags to prevent photodegradation. They were immediately frozen to -70°C.

Extraction for Developing Isolation and Purification Techniques

The frozen roots were lyophilized, ground, and extracted over-night in methanol:acetonitrile (7:3). The extract was filtered through Whatman 1 filter paper. Because much of the thiarubrines is lost during the rest of the isolation procedure (Fig. 2.2) quantification was determined at this time. Quantification was accomplished using UV-Visible spectrophotometry. The molar absorptivity (ε) is the same for thiarubrine **1 a** and **2 a** (ε=3000 at 490 nm, ε=10,300 at 340 nm). It is assumed to be the same for the other thiarubrines. Absorbance was measured at 490 nm, using the Beer-Lambert equation the overall thiarubrine content was calculated as follows:

$$\text{conc.(mg/ml)} = \frac{\text{Abs. x mol. wt.}}{\varepsilon}$$

The extract was evaporated *in vacuo* at 28°C to almost dryness and resuspended in acetonitrile. This was filtered through glass wool to remove the precipitate. The sample was roto-evaporated to dryness and resuspended in acetonitrile and filtered. This step was repeated until the solution was clear, ie., no further precipitation occurred.

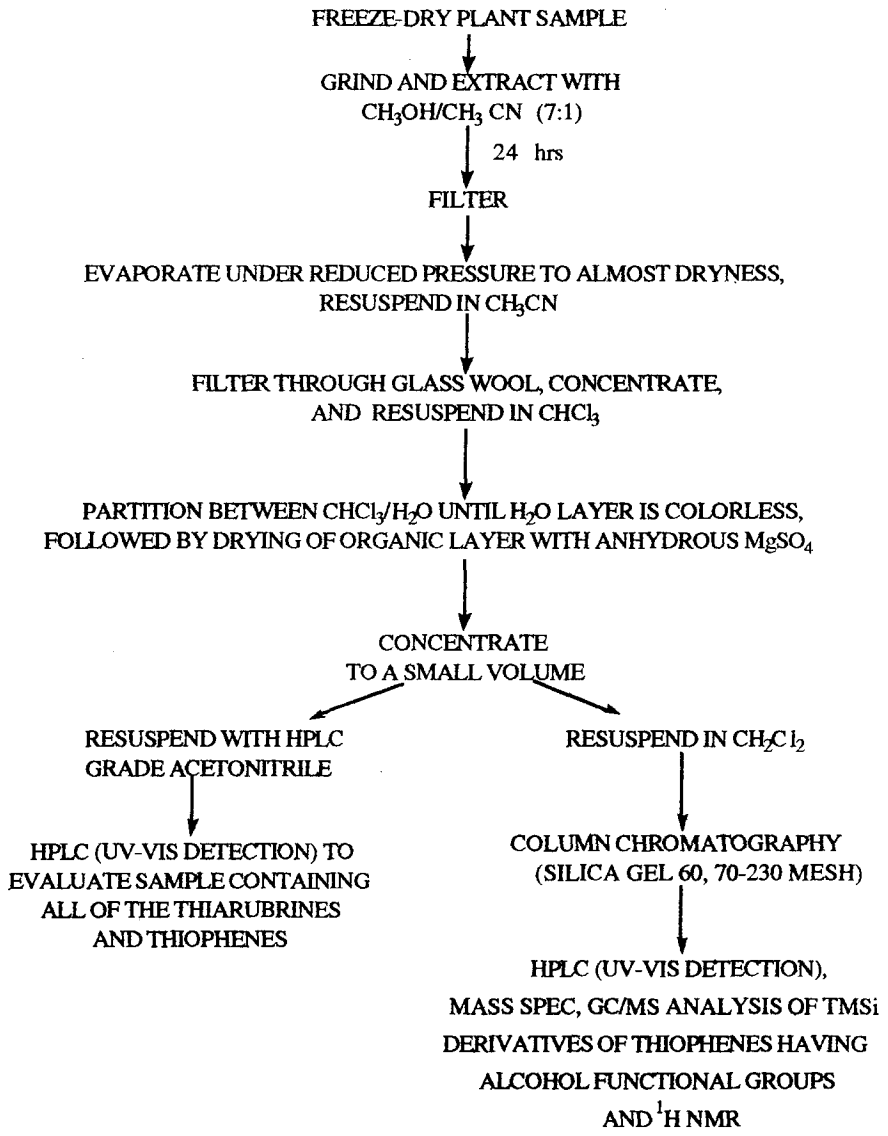

Figure 2.2 Extraction Protocol for Thiarubrines and Thiophenes from Roots of *Ambrosia chamissonis*.

The extract was brought down to a small volume and then partitioned between chloroform and water. The chloroform layer was partitioned repeatedly with water until the aqueous layer was clear. The organic layer was dried with anhydrous $MgSO_4$, concentrated *in vacuo,* resuspended in HPLC grade acetonitrile, and filtered for HPLC. If long term storage was required the sample was suspended in dichloromethane and stored in the dark in the refrigerator. Alternatively, freeze-dried material was extracted initially for 24 hours with petroleum ether to gain the less polar thiarubrines and thiophenes. This was followed by a 24 hour extraction with methanol.

Evaluation of Crude Extracts with HPLC

Analysis by HPLC was done using a Waters HPLC with a photodiode array detector. This was the instrument of choice because "in flight" UV-Visible spectra can be obtained which are useful in the identification of the compounds. The extract was analysed by HPLC using a preparative MCH-10 (C_{18}, 10 µm particles) column (4 x 300 mm), eluted with CH_3CN:H_2O (72:28 and 50:50) at a flow rate of 1 ml/min in order to identify the thiophenes. A portion of the extract was irradiated to convert the thiarubrines to their corresponding thiophenes. Standards of thiophenes **1b**, **2b**, **3b**, **4b**, **5b**, **6b**, and **7b**, were co-injected with the extract to identify the components and infer the structures of the corresponding thiarubrines. The thiosulphinate (**9**) was identified by its characteristic UV-Vis spectrum (Appendix).

Isolation of Thiarubrines and Thiophenes by Thin Layer and Column Chromatography

Thin Layer Chromatography (TLC) was used to monitor extracts and fractions. Aluminum-backed silica plates (Aluminum-backed silica gel_{60} F_{254}, 0.2mm) using two solvent systems: hexane:diethyl ether (9:1), and dichloromethane:acetone (30:1) were employed. Preparative TLC was employed using glass-backed silica gel_{60} F_{254} (2mm) with dichloromethane:acetone (30:1). A silca gel (70-230 mesh) column was prepared with dichloromethane (CH_2Cl_2) and loaded with the crude extract. The column was eluted with CH_2Cl_2. When no more bands were eluted, acetone was added gradually. For large scale

separation a preliminary column (10 cm x 300 cm) was required to remove the more polar components which remained at the top causing poor column performance. This was followed by chromatography of the collected red bands on a longer narrower column (5 cm x 500-1000 cm). In the case of thiarubrine **3a**, isolation was complicated by an accompanying oil, which could be reduced in volume by running the extract through a column numerous times.

Purification and Identification of Compounds

Purification was accomplished by preparative HPLC using a Varian 5000 HPLC with a MCH-10 (8 mm x 300 mm) reverse phase steel column, eluted isocratically with H_2O:CH_3CN at 3 ml/min. The solvent mix was adjusted for each compound.

Confirmation of the identities of the compounds was carried out by Mass Spectroscopy. The isolated hydroxylated thiophenes were converted to the trimethylsilyl (TMS) ethers by drying the sample (~1 mg) and then adding 10 ml of dry pyridine and 10 ml of N,O-bis-trimethylsilyltrifluoroacetamide (BSTFA) at room temperature. GC-MS analysis was carried out on the TMS-derivatives using an automated Finnigan 1020 GC-MS equipped with a fused-silica capillary column (30 mm x 0.25 mm) of SE-54 and operated in the EI mode (70 eV) with an ion-source temperature of 95°C. Solid probe Mass Spectroscopy was also carried out with an automated Finnigan 1020 GC-MS. The thiarubrines were identified by solid probe MS instead of GC/MS because they are unstable at the high temperature required.

B . Extraction in Spring and Autumn of Roots from Two Collecting Sites

Plant Collections

Collections were made from Centennial Beach, Tsawassen, British Columbia (Sept., June) and North Beach (at the end of Cemetery Road) in the Queen Charlotte Islands, British Columbia (Sept., June) . Three entire plants were taken from each site. Only one plant which had a different leaf morphology (Type 2) was removed from North Beach due to a limited population of the variety. All plants were placed in dark green garbage bags to prevent photodegradation of the

compounds. Five grams of ground freeze dried material was extracted, prepared and analysed by HPLC as already described.

RESULTS

The tap roots from plants growing on Centennial Beach have well-developed periderm, with succulent lateral secondary roots lacking periderm. The larger tap roots may be hollow due to necrosis (Figure 2.3). Using longitudinal sections of the outer bark there are circular pockets whereas closer to the center exhibit longitudinal strands, which are not necessarily connected. The thiarubrines are located within canals in the outermost cortical region and immature periderm Figures 2.3 and 2.4).

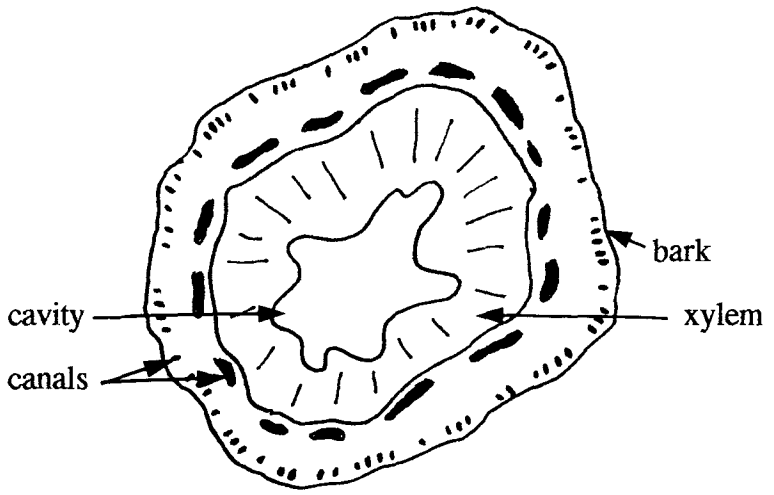

Figure 2.3 Localization of Thiarubrine Canals in woody roots of *Ambrosia chamissonis* (Cross-Section).

Reverse phase HPLC (CH_3CN:H_2O, 72:28 and 50:50) resolved the thiarubrines and thiophenes as shown in Figure 2.5. HPLC runs were made at 490 nm as well as at 340 nm (Figure 2.6) confirming that the compounds absorbing at the former wavelength were thiarubrines thus justifying the calculations made for the quantification at this wavelength. "In flight" UV-Visible spectra indicated the peaks of the thiarubrines and the thiophenes, as well as another which

Visible spectra indicated the peaks of the thiarubrines and the thiophenes, as well as another which represented an unknown polyyne, which will be referred to as "Unknown Polyyne #1", (Appendix III). When the solvent system was changed to a 1:1 mixture the thiarubrines and the thiophenes with polarity intermediate to thiarubrine **4a** and thiophene, **3b**, were resolved. Thiarubrines **1a** and **2a** and thiophenes **1b** and **2b** are not included in the figure because of their long retention times and the fact that their peaks become flattened and resolution is poor. When peak enhancement was performed using standards of the thiophenes it became apparent that a hitherto unknown thiophene with a polarity intermediate to that of the primary alcohol and the diol was present (Figure 2.5). The identification of this new compound and its corresponding thiarubrine (**8b** and **8a** respectively) will be presented in Chapter 3.

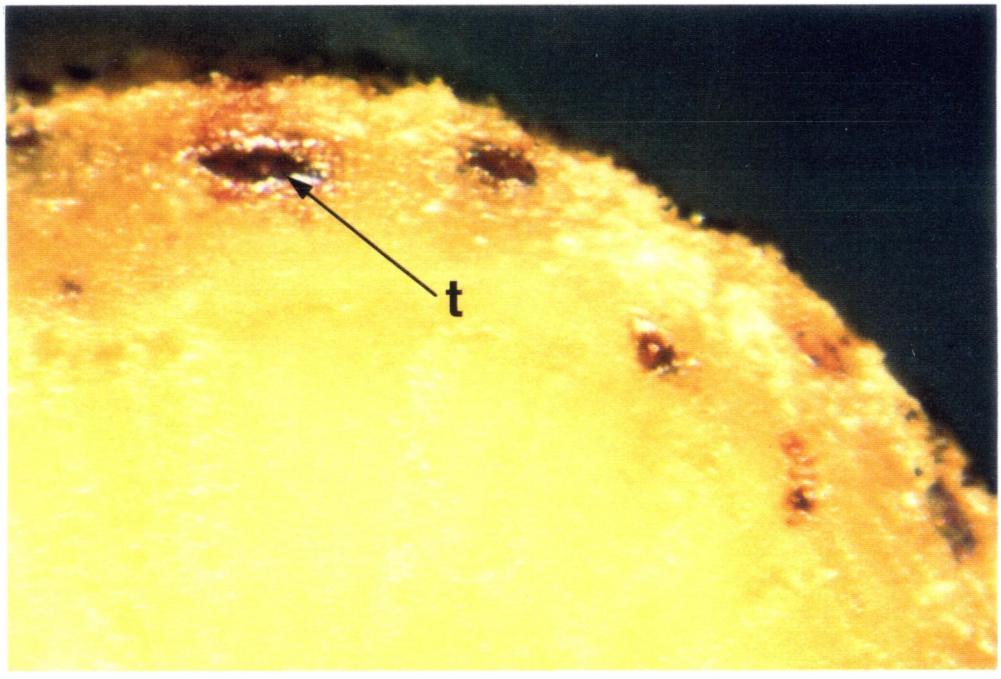

Figure 2.4 Localization of Thiarubrine Canals in Non-Woody (Succulent) Roots of *Ambrosia chamissonis*. (t = thiarubrine canal)

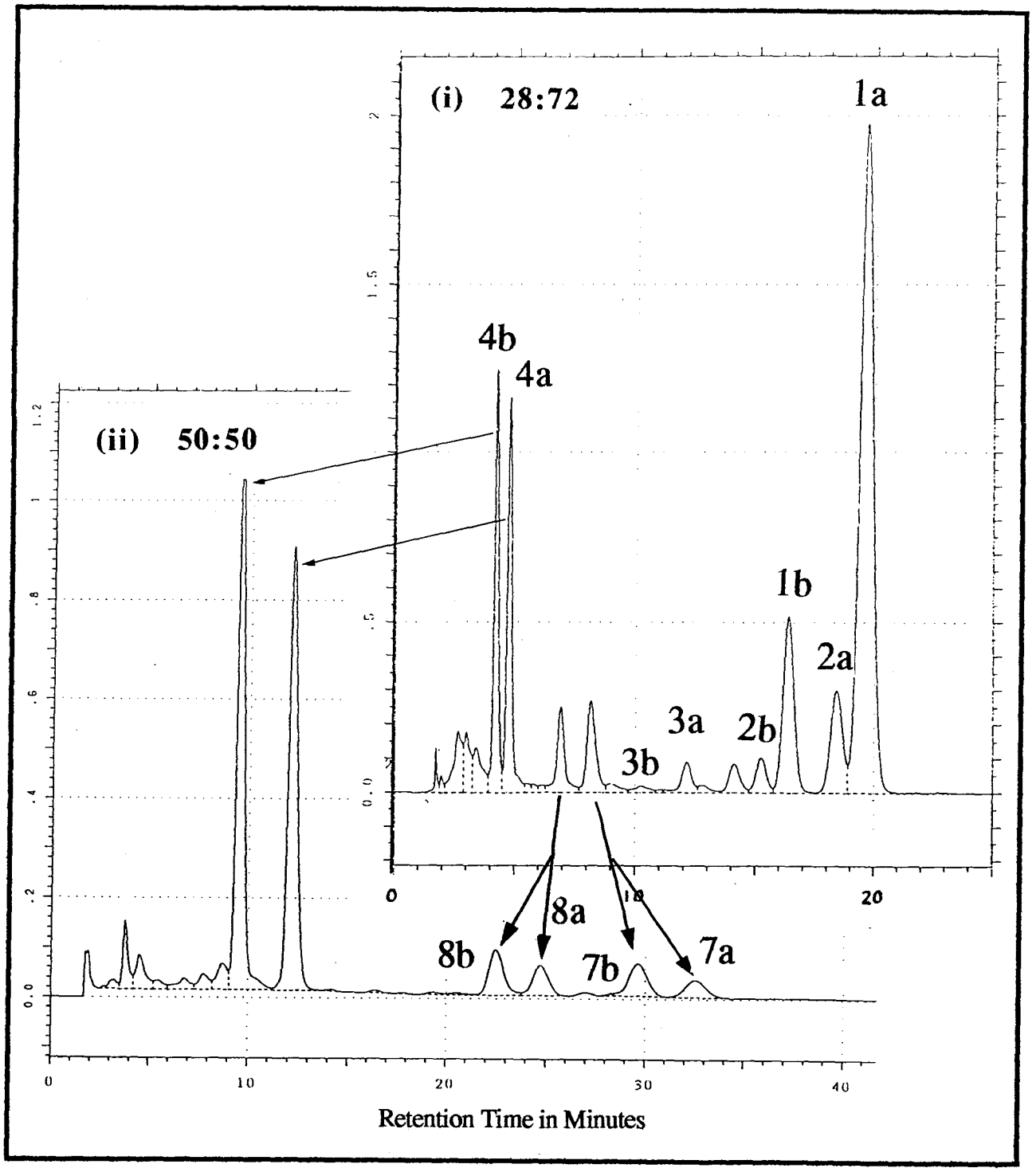

Figure 2.5 HPLC Trace at a Flow Rate of 1 ml/min at (i) 28:72 (H_2O:CH_3CN) and (ii) 50:50 (H_2O:CH_3CN).

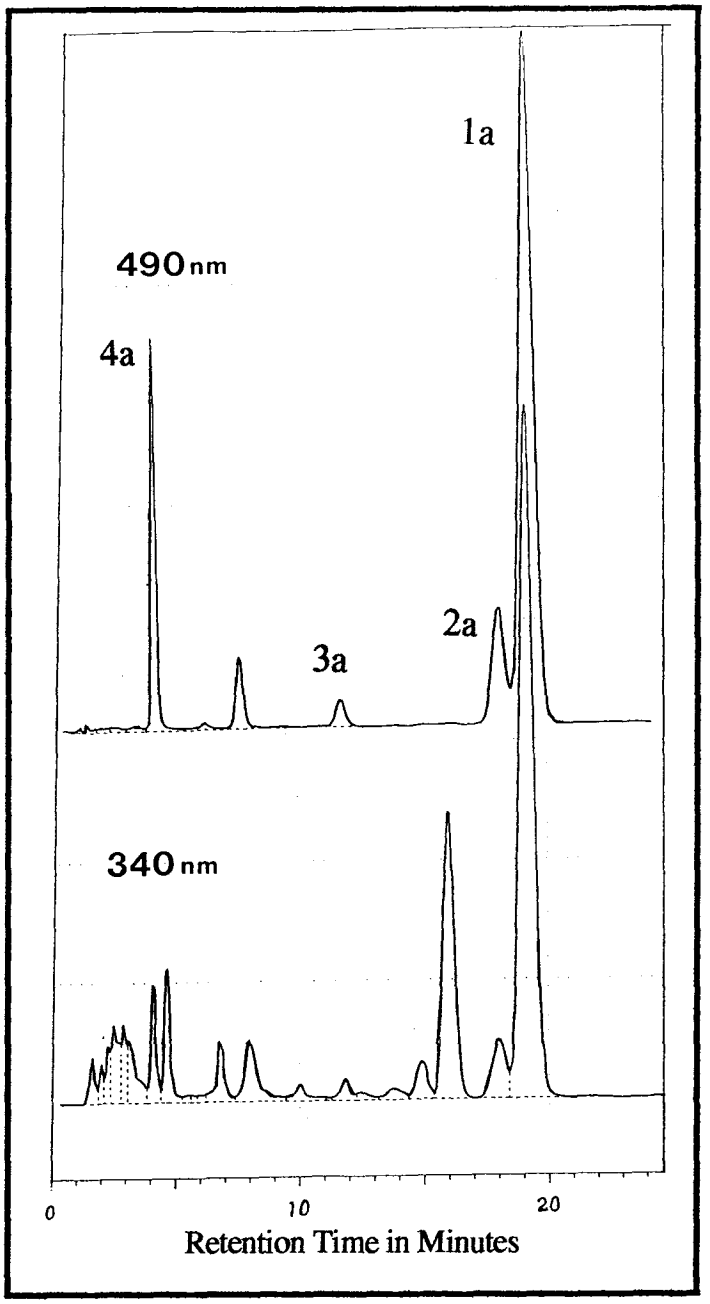

Figure 2.6 HPLC Trace of Crude Root extract at 340 nm and 490 nm at a Flow Rate of 1 ml/min H_2O:CH_3CN (28:72).

Table 2.1 Rf Values of the Thiophenes on Analytical Thin Layer Chromatography on Silica Gel F_{254}.

Compound	Hexane:Diethyl Ether (9:1)	Dichloromethane:Acetone (30:1)
1b	0.50	0.80
2b	0.50	0.80
3b	0.30	0.72
4b	0.00	0.10
5b	-	0.51
6b	-	0.55
7b	-	0.40

*Solvent Mixture at 3ml/min..

Extraction procedures using petroleum ether furnished only the less polar thiarubrines **1a**, **2a**, and **3a**, as well as very low concentrations of their corresponding thiophenes. The compounds are more stable in petroleum ether than methanol as indicated by lower concentrations to the thiophenes. Methanol, however, readily extracted the more polar thiarubrines (**4a**, **5a**, **6a**, **7a**) as well as residual non-polar thiarubrines and thiophenes. A nonpolar compound was detected at Rt = 27.33 (CH_3CN:H_2O, 72:28, flow rate 1 ml/min). This represents the pentaynene according to the spectrum determined by Bohlmann *et al.*, 1973 (UV λ_{max} nm 412, 382, 353, 330, 285, 266).

Preparative TLC was used to isolate thiophenes on a small scale. The band at Rf = 0.1 was scraped off and extracted with CH_3CN:CH3OH (7:1). White crystals precipitated. These were subjected to GC-MS. It had a similar retention time to other sesquiterpene lactones which had been identified (Felipe Balza, personal communication), but the fragmentation pattern was different from any sesquiterpene lactone known from *Ambrosia chamissonis* (Geissman *et al.*, 1973) (see Appendix V for mass spectrum)

Column chromatography with dichloromethane was employed for initial isolation of all compounds. HPLC analysis of the fractions before and after irradiation confirmed the retention times of the thiarubrines and their corresponding thiophenes. UV-Vis spectra of thiophenes

furnished by the HPLC photodiode array detector are shown in Appendix II. The thiarubrines tend to degrade and therefore gave less defined spots. Thiarubrine **1a** and **2a** came off together as the first red band followed by thiarubrine **3a.** Two red bands eluted next. The first one was the already identified primary alcohol **7a** as monitored by HPLC co-injected with a standard. HPLC of crude samples revealed that there was another polyyne which eluted at the same retention time causing additional peaks in the UV-Visible spectrum. This was removed with column chromatography. The chlorohydrins were not visualized but were detected by HPLC. They appeared in fractions between the primary alcohol **7a** and the epoxide **3a.** A band closely followed **7a,** whose identification will be described in Chapter 3. Thiarubrine **4a** eluted only after acetone was added to eluting solvent. The thiophenes co-chromatographed with their corresponding thiarubrines.

Preparative HPLC conditions were varied depending on the polarity of the thiarubrines and thiophenes to be separated. The retention times and solvent systems are presented in Table 1.3. for the thiarubrines and thiophenes. The purification of thiarubrines **1a** and **2a** and the thiophenes **1b** and **2b** was difficult because they could not be separated on the column and thus could only be separated if lower concentrations were injected at a time on HPLC (~2 mg), whereas the others can be loaded 8-10 mg at a time. Mass spectral data of a mixture of **1a** and **2a** is presented in Figure 2.7. When a crude or pure thiarubrine solution is irradiated it turns yellow. When the resulting thiophene is isolated by HPLC the pure compound is colorless.

Table 2.2 Analytical HPLC Retention Times (in Minutes) of the Thiarubrines and Thiophenes With Different Solvent Systems (H_2O:CH_3CN).

Compound	28:72	50:50	55:45
1a	18.62	-	-
1b	15.66	-	-
2a	17.52	-	-
2b	14.63	-	-
3a	11.72	69.52	-
3b	9.99	52.09	-
4a	4.88	12.13	17.86
4b	4.37	9.46	13.33
5a	(8.39)	39.50	71.32
5b	(7.50)	30.10	52.08
6a	(7.26)	34.74	62.58
6b	(6.36)	26.07	45.42
7a	(8.05)	35.35	58.00
7b	(6.91)	25.15	41.59
8a	(8.05)	30.31	51.00
8b	(6.91)	23.02	37.50
9	-	27.73	43.02
"Polyyne #1"	13.40	-	-

values in brackets indicate that at this solvent system the peaks are not ressolved

Table 2.3 Preparative HPLC Retention Times (in Minutes) for the Purification of Thiarubrines and Thiophenes with Different Solvent Systems.

Compound	H_2O:CH_3CN	R_t(min)
1a	35/65	41.82
1b	35/65	33.96
2a	35/65	39.02
2b	35/65	31.46
3a	35/65	22.59
3b	35/65	18.72
4a	60/40	42.86
4b	60/40	29.07
5a	50/50	42.77
5b	50/50	31.82
6a	50/50	38.94
6b	50/50	26.94
7a	50/50	38.46
7b	50/50	29.40

*Solvent Mixture at 3ml/min., Rt = Retention time

Although relative retention times and UV spectra are known for some of the thiophenes mass spectral data substantiated the identities of these compounds. The hydroxylated thiophenes such as the chlorohydrin thiophenes (**5b** and **6b**) in Figure 2.8, the primary alcohol thiophene (**7b**) in Figure 2.8 (ii) and the diol (**4b**) in Figure 2.9 (i) were identified using GC-MS of the TMS derivatives. The chlorohydrins have very distinctive fragmentation patterns which distinguish one from the other (Figure 2.10). Being chlorinated compounds they have very distinct spectra due to the relative abundances of the chlorine isotopes. Solid probe was used for the thiarubrines. Thiarubrine **4a** for instance, crystallized in concentrated solution from preparative HPLC. The crystals were subjected to solid probe MS revealing the characteristic mass spectrum (Figure 2.10 ii).

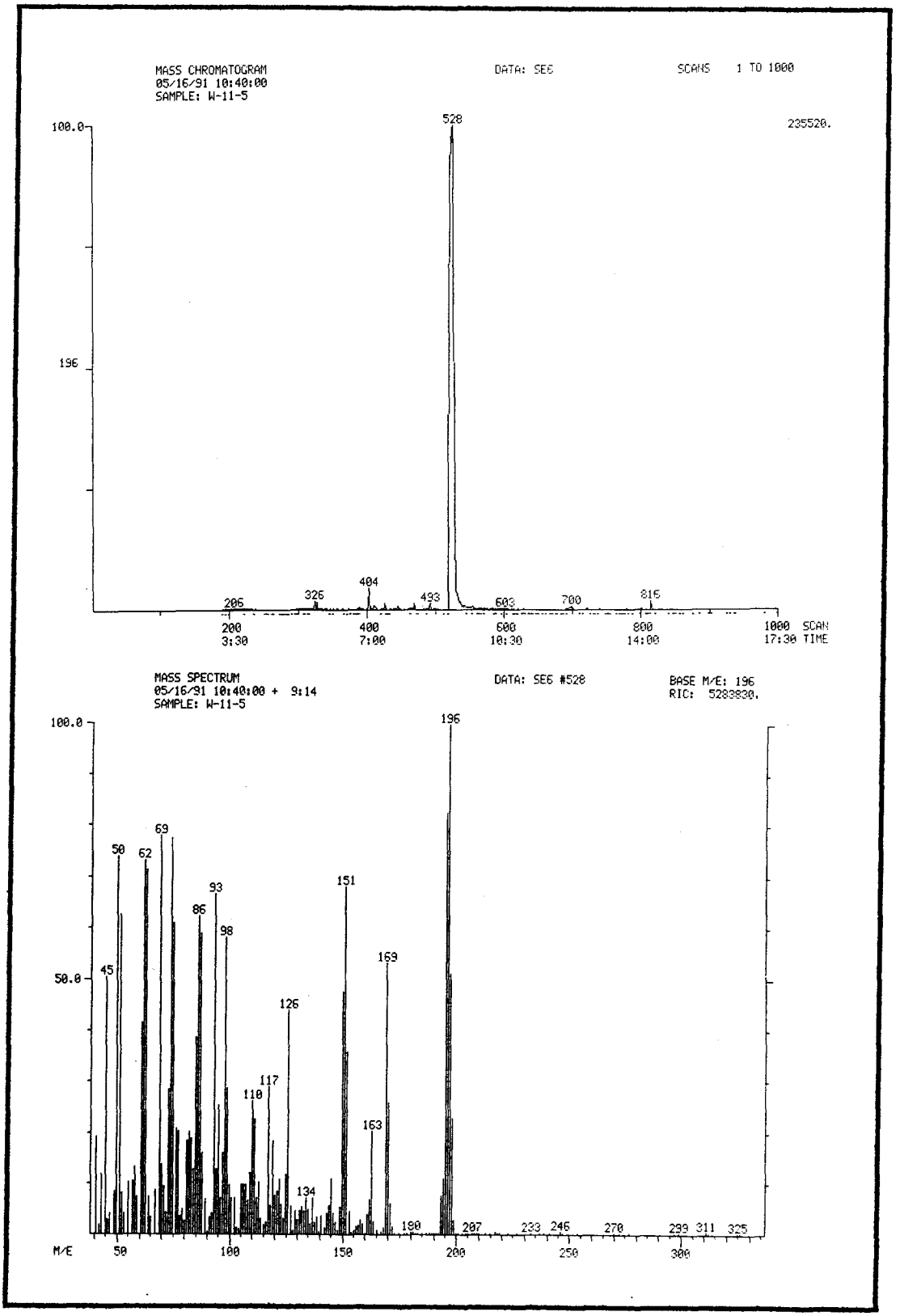

Figure 2.7 Mass Chromatogram and Spectrum of a Mixture of Thiarubrines **1a** and **2a** Isolated by Column Chromatography.

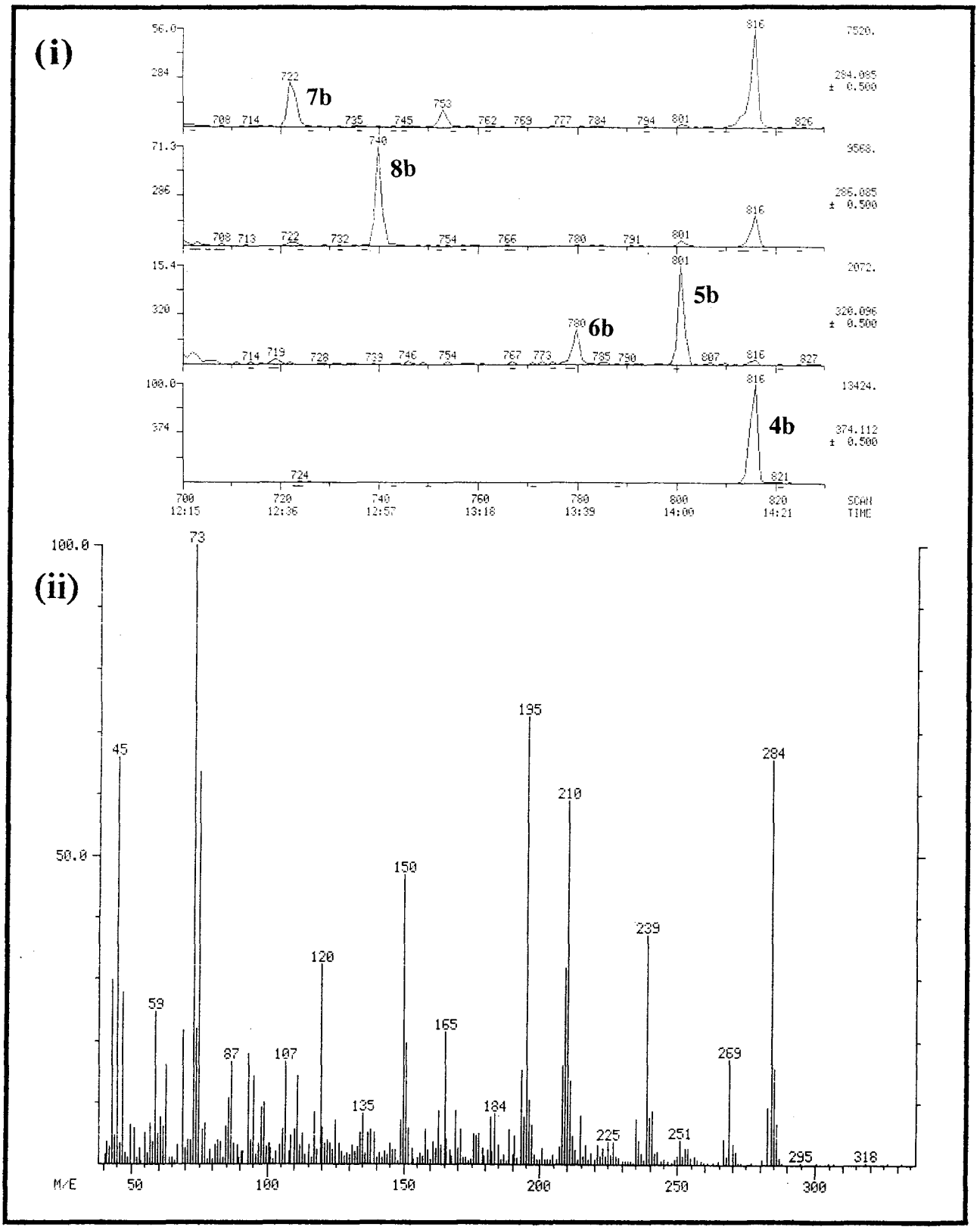

Figure 2.8 (i) GC-Mass Chromatogram of the TMS-Derivatives of Thiophenes **4b**, **5b**, **6b**, **7b**, **8b**. (ii) Mass Spectrum of the TMS-Derivative of Thiophene **7b**.

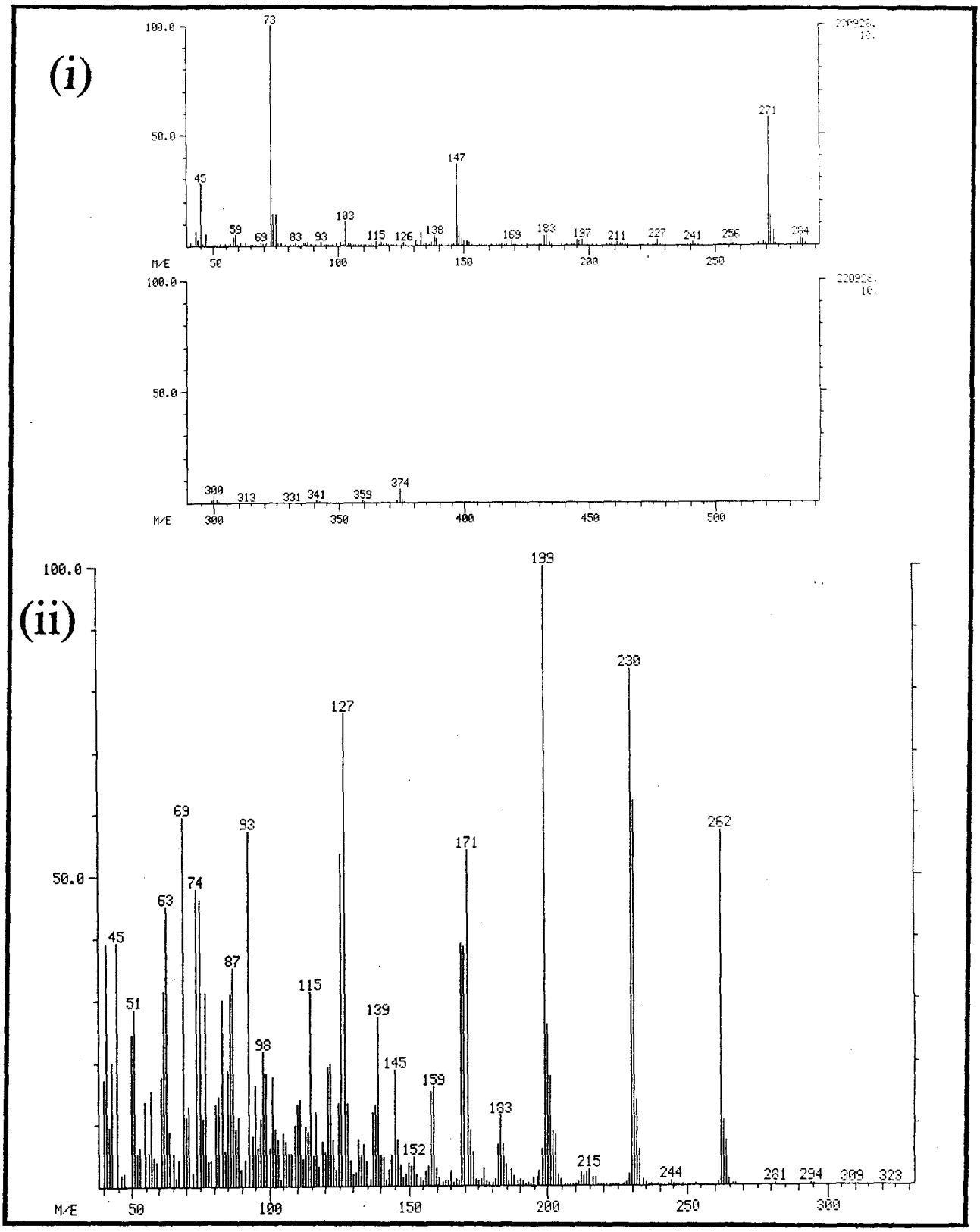

Figure 2.9 (i) GC-Mass Spectrum of the TMS-Derivative of Thiophene **4b** and (ii) Solid Probe Mass Spectrum of Thiarubrine **4a** Crystallized from Preparative HPLC.

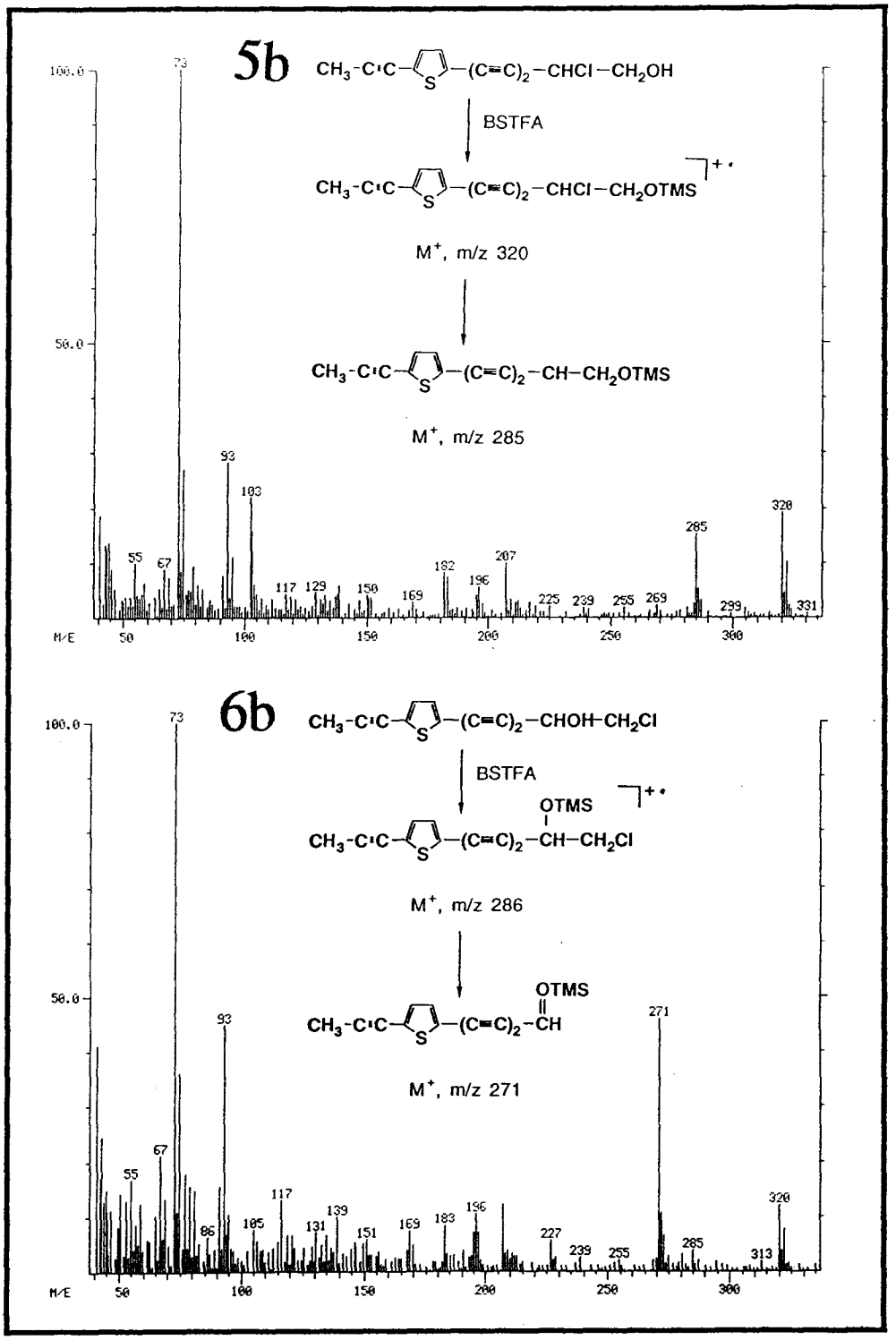

Figure 2.10 GC-Mass Spectra of the TMS-Derivatives of Chlorinated Thiophenes **5b** and **6b**.

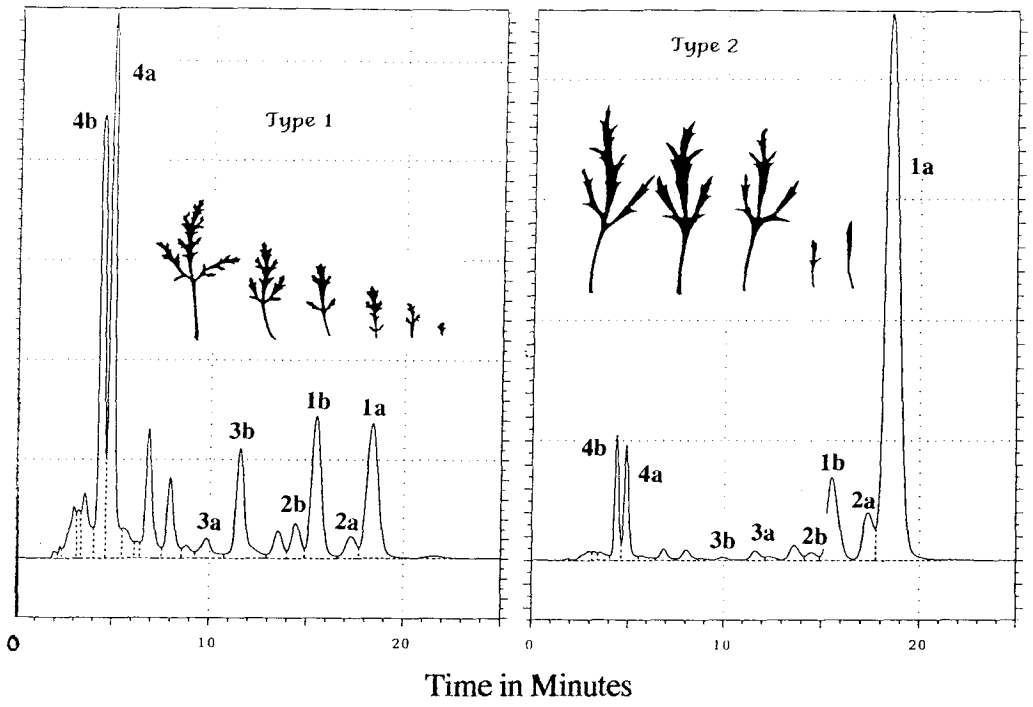

Figure 2.11 HPLC Traces of Roots Collected from Plants with Two Types of Leaf Morphology

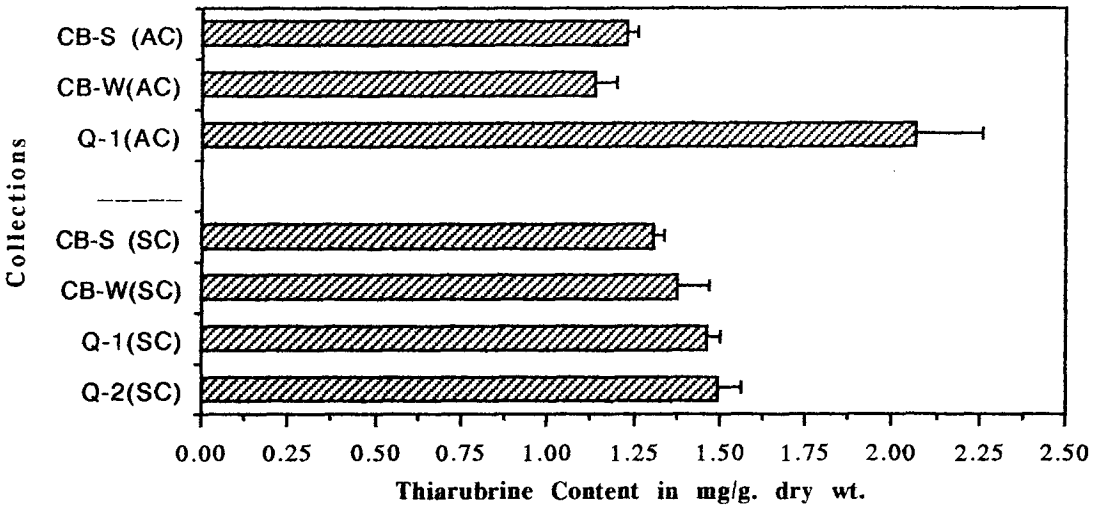

Figure 2.12 Thiarubrine Content of Collections from Centennial Beach (CB) (S=Succulent, W=Woody) and the Queen Charlotte Islands (Q) in the Spring (SC) and Autumn (AC). Variation is presented as standard deviation (n=3).

The HPLC traces of the roots from the collections of *Ambrosia chamissonis* from the Queen Charlotte Islands demonstrated different thiarubrine profiles (Figure 2.11). The chemical profile of Type 2 were very similar to those from Centennial Beach which were similar in leaf morphology to Type 1. Type 1 exhibited the highest yield of thiarubrines on a dry weight basis (Figure 2.12).

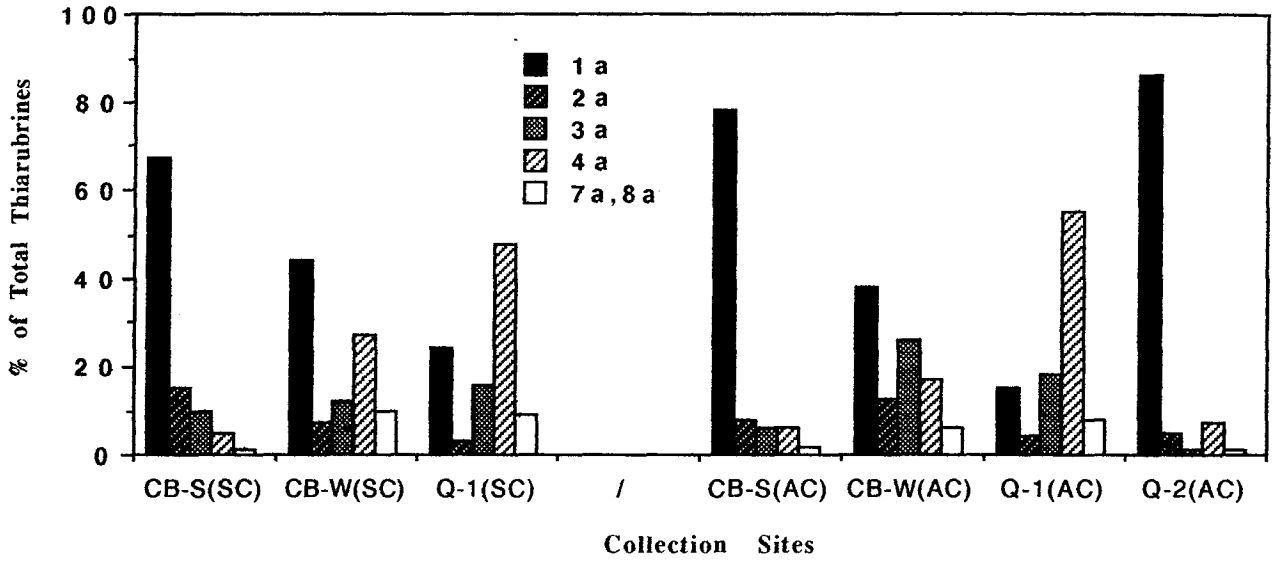

Figure 2.13 Thiarubrine Profiles of Collections from Centennial Beach (CB) (S=Succulent, W=Woody) and the Queen Charlotte Islands (Q-1 and Q-2, representing Type-1 and Type-2 leaf morphologies) in the Spring (SC) and Autumn (AC).

The chemical profile of the succulent roots did not vary with season in the Tsawassen samples, whereas the woody roots did. The latter had much higher levels of thiarubrine **3a** in the autumn than in the spring. The converse was true for thiarubrine **4a**.

The roots of the QCI 1 and QCI 2 collections did not differ in appearance. They both had very little in the way of succulent or lateral branching, and were generally much smaller than those from Centennial Beach. The roots of QCI-1 had very high levels of thiarubrine **4a**, whereas QCI-2 had a very similar profile to CB-S. Examination of HPLC traces of the minor thiarubrines and thiophenes revealed that the primary alcohols **7a**, **7b**, **8a**, and **8b** were present in appreciable

concentrations (~4% of the total thiarubrine/thiophene content). The chlorohydrins were detectable by "in flight" UV-Vis spectra in some cases, but often they were not integrated by the HPLC.

DISCUSSION

The roots of *Ambrosia chamissonis* produce the widest spectrum of thiarubrines and thiophenes known from any other species in the Asteraceae. They are all based on the structure of thiarubrine **1a** with the exception of thiarubrine **2a**. Figure 2.14 demonstrates the proposed biosynthetic relationship between some of the thiarubrines. Extraction and purification procedures have been established to obtain the compounds with their differing polarities. It may be that other species examined have a wider array of chemicals which have not been detected due to the extraction procedures used. Column chromatography was useful for the isolation of the minor thiarubirnes and thiophenes, which could be analyzed by GC-MS. The low production of these compounds implies that their defensive function in the plant may be minimal. The presence of chlorohydrins may reflect the abundance of chlorine ions in the habitat of this species. Chlorohydrins can be artifactually derived from epoxides, but the relative abundances of the primary and secondary chlorohydrins suggest that this is not occurring. If they were artifacts the primary chlorohydrin would be three times the abundance of the secondary. HPLC traces indicate that the converse is true of extracts from roots and root cultures of *Ambrosia chamissonis.*

Differences in plant morphology were observed in and between two populations. The observations of Geissman *et al.*(1973) that correlation cannot be made between leaf morphology and chemistry hold true for those samples examined from the Queen Charlotte Islands and Tsawassen. The plants which had similar leaf morphology had very different chemical profiles, whereas plants that were different (CB-S and QCI-2) had very similar profiles. The morphological types as well are not steadfast; some plants observed had varying leaf morphology within one individual. Although there is variability in both chemistry and morphology the chemicals present

are unique to this group. These compounds may be chemical markers in the identification of species in these groups of plants which are troublesome taxonomically.

Nothing is known about how the plant responds to the sulphur that is extruded from the thiarubrine molecule. The yellow coloration of the thiarubrine extract after irradiation is probably due to the sulphur since the thiophenes are colorless. Other sulphur forms such as sulphite and sulphur dioxide are normally converted into sulphate which can be stored in the cell vacuole (Rennenberg, 1984). It is this form which is reduced and subsequently utilized by the plant. In the case of thiarubrines, which are stored in canals, the canals may be a storage place for the sulphur as well. The canals do not only contain thiarubrines, thiophenes, and their byproducts; Throughout the many extractions and attempts at isolation there is an oil in which these substances are dissolved. Nothing is known about its chemical nature.

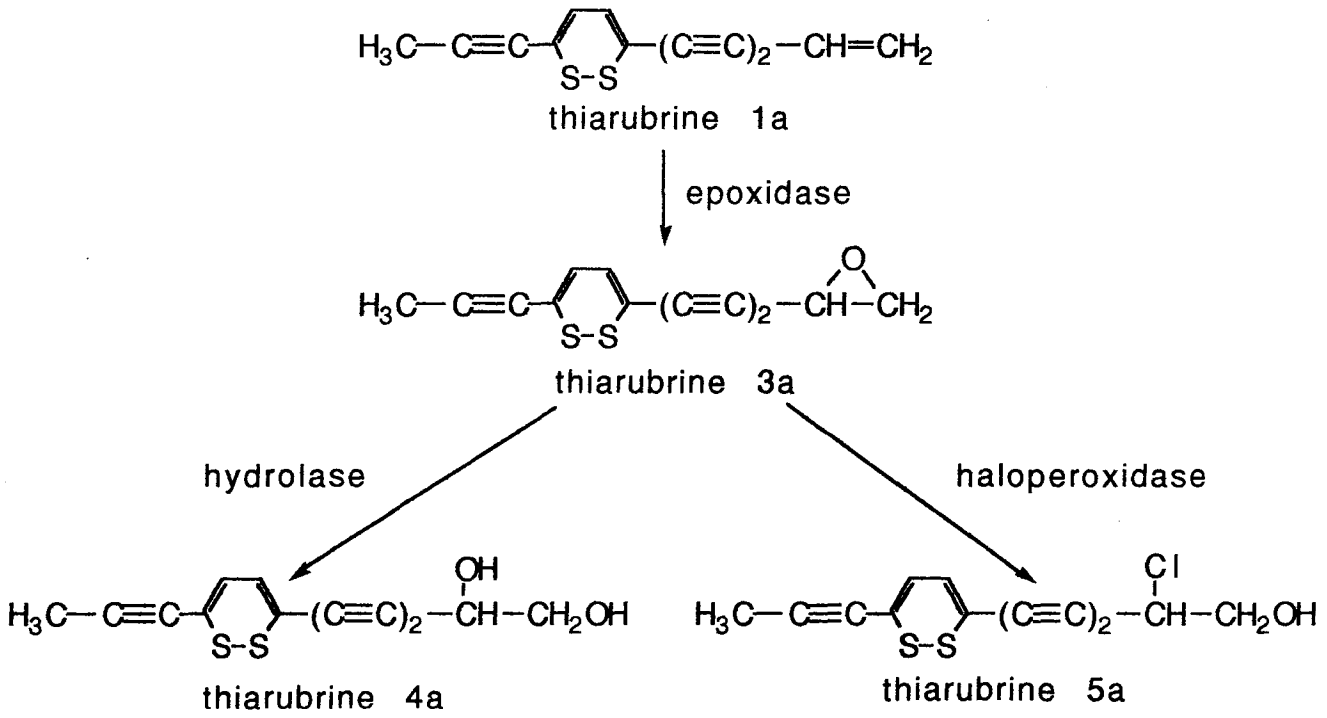

Figure 2.14 Proposed Biosynthetic Relationship Between some of the Thiarubrines.

CHAPTER THREE

COMPARISON OF THIARUBRINE AND THIOPHENE PRODUCTION BETWEEN ROOTS AND ABOVE GROUND PORTIONS OF *AMBROSIA CHAMISSONIS*

INTRODUCTION

Species in the Asteraceae, such as *Chaenactis douglasii* (Cosio *et al.*, 1985) and *Verbesina* ssp. (Bohlmann *et al.*, 1980), contain no thiarubrines or thiophenes in their above ground portions. Roots are generally considered the major sources of these classes of compounds. The thiarubrines are unstable in light and both thiarubrines and thiophenes are bioactive upon irradiation. Theoretically organs exposed to sunlight seem to be suitable for storage because of possible photo-auto-toxicity. There are plants, however, which do store photo-unstable polyynes, such as phenylheptatryine, in their leaves.

The leaves of the plants collected at Centennial beach in Tsawwasen, B.C. exhibited little variation in morphology as indicated in Chapter Two. In this chapter the distribution of thiarubrines and thiophenes will be examined in the entire plant. Quantitative HPLC data was adjusted to take into account the differences in absorptivity by the two classes of compounds. Thus the peaks on the HPLC reflect the amounts of compounds based on their absorptivity at the particular wavelength. At 340 nm the molar absorptivity of thiarubinre **1 a** is 10,000, whereas that of thiophene **1 b** is 31,000.

The location of thiophenes has not been determined accurately. X-ray emmission techniques have been employed to determine the sulphur distribution in *Tagetes patula* seedling roots (Makjanic *et al.*, 1988). The lowest concentration was found to be in the epidermis whereas the highest was found to be in the endodermis. The secondary roots of *A. chamissonis* have a well-developed bark. This means that the endodermis is lost, since the pericycle, the layer internal

to the endoderm, develops into the periderm The localization of these compounds has not been examined in other parts of the plant. Unlike the colourless thiophenes, the red thiarubrines are easy to visualize.

MATERIAL AND METHODS

Plant Material

Collections of *Ambrosia chamissonis* were obtained from Centennial Beach, Tsawwassen, British Columbia October 8, 1989. Three plants with thick succulent stems were selected (ie. shoots without periderm). The entire plants were placed in dark plastic garbage bags to minimize the exposure to light..

Histological Examination

Sectioning using resin embedding techiniques (JB-4) was not succuessful due to pigment degradation under the conditions of fixing and embedding. Hand-sectioning in dim light gave much better results. Roots, stems and leaves of each sample were hand-sectioned to determine the presence and localization of the red compounds.

Extraction

Leaves (7-10 cm long), green stems, woody stems, and roots were separated and samples of approximately 20 grams of each were weighed out. All samples were frozen at -70°C and freeze-dried. Five grams of each sample were weighed and ground and extracted overnight in methanol:acetonitrile (7:1). The solution was filtered through Whatmann No. 1. The thiarubrine concentration of each extract was determined using the Beer-Lambert equation for the roots and woody stems. The thiarubrine content of the green stems and leaf extract could not be determined at this stage because of other pigments which absorb at the wavelengths used for calculations of concentration.

Evaluation of Extracts with HPLC

The extract was brought down to a small volume and then partitioned between chloroform and water. The chloroform layer was partitioned repeatedly with water until the aqueous layer was clear. The organic layer was dried with anhydrous $MgSO_4$, concentrated *in vacuo,* resuspended in HPLC grade acetonitrile, and filtered for HPLC. Analysis by HPLC was done using a Waters HPLC with a photodiode array detector. Examination of all extracts was performed by HPLC using a Waters System with a MCH-10 column (4 mm x 300 mm) at a flow rate of 1 ml/min eluting isocratically with CH_3CN:H_2O (72:28). All runs were performed at 340 nm except for some of the leaf extracts which were also analyzed at 484 nm. All samples were irradiated and then analyzed by HPLC to verify that the peaks were thiophenes and thiarubrines. All other procedures were carried out in dim light to prevent the photo-conversion of thiarubrines to thiophenes. Calculations used peak area percentages from HPLC traces. The thiophene values were adjusted by dividing the percent by 3.1 and then overall thiarubrine and thiophene levels were calculated as a percent fom the total.

Column Chromatography

Half of each of the three extracts of the green stem and the leaf were combined. The extracts were separated by column (5cm x 45cm) with a cold water jacket packed with silica gel (230-400 mesh) and hexane:diethyl ether (9:1). The stem and leaf extracts were loaded onto the column and chromatographed with the same solvent mixture. A red band (thiarubrines) was collected and the overall thiarubrine content was calculated. The fraction was analyzed by HPLC to ensure that all of the thiarubrines wer present in the fraction.

RESULTS

In the spring young shoots have conspicuous red stripe which upon extraction reveal that they are thiarubrines and anthocyanins. The thiarubrines can be seen in canals running longitudinally in the upper stem and around the hollow stem of the lower shoot (Figure 3.1). Mature plants were also examined chemically.

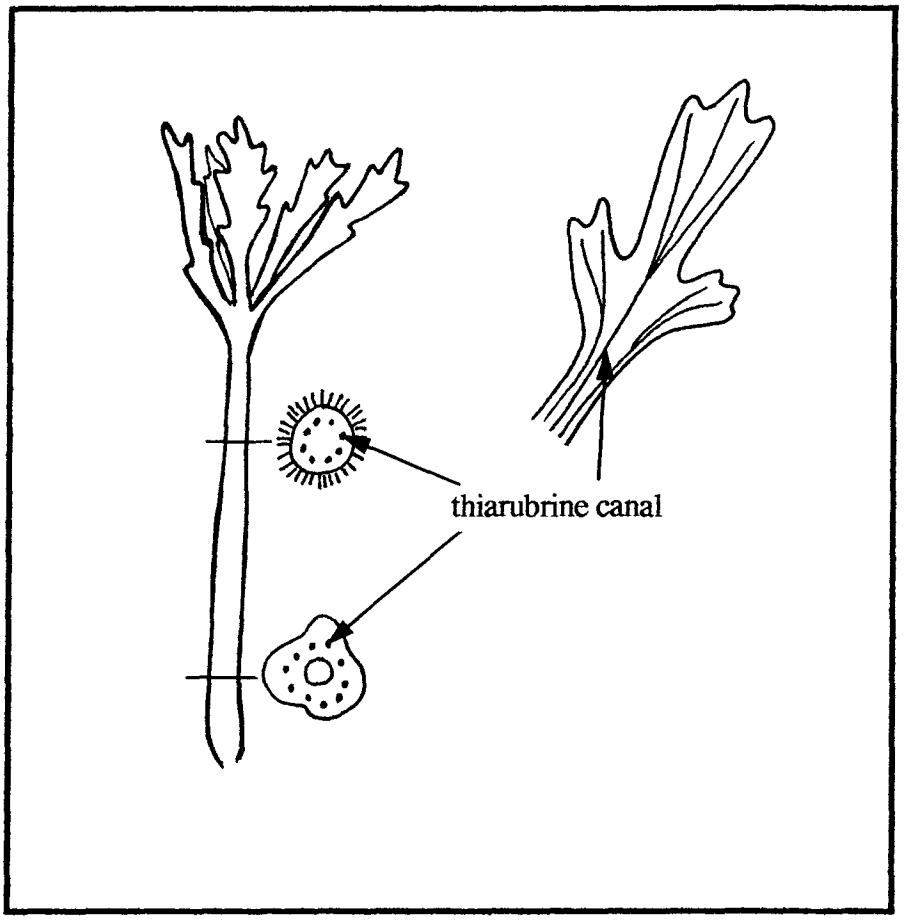

Figure 3.1 Thiarubrine Canals in the Young Stem (i) and Leaf (ii) of *Ambrosia chamissonis.*

In general leaves and stems of young tissues, contain more thiarubrines than older, larger leaves. Woody stems also contain thiarubrines, but are impossible to discern histologically using

the present method because of dark brown pigmentation and the brittleness of this material. Extraction revealed the presence of thiarubrines and thiophenes.

Table 3.1 Overall Thiarubrine Content of the Different Organs of *Ambrosia chamissonis*

Plant Organ	Overall Thiarubrine Content (mg/g dry wt.)
Roots	2.1 ± 0.021
Woody Stems	0.234 ± 0.040
Green Stems	0.102
Leaves	0.166

Error is expressed as Standard Deviation (n=3), no error was given for leaves and green stems because these samples were combined

Preliminary HPLC analysis indicated that the thiarubrine in highest abundance was **1a**. Methanolic extracts were partitioned with petroleum ether to separate the more polar components. Column chromatography successfully separated the thiarubrines from the other pigments found in the leaves. An orange band was conspicuous which separated into a fast yellow band and a slower red band. The yellow band was collected and the UV-Vis spectrum was examined (λmax 343 nm, 452 nm, 477 nm) which is characteristic of carotenoids. The red band split into a yellow and a red one. These fractions were not used for calculations of content because much of the thiophene is lost during the process. The UV-Vis spectrum of the former indicated this fraction contained thiophenes, whereas the latter contained a mixture of thiarubrines and thiophenes. Increasing the proportion of hexane in the eluting solvent eluted a green band, which gave a UV-Vis spectrum typical for chlorophyll (λmax 664, 437).

The root profile is of course similar to the extracts examined in Chapter Two. There was variation in the thiarubrine **3a** content as is reflected by the large error bars of thiarbrines **3a** and **1a** (Figure 3.2). There is a very low thiophene content. The chemical profile of the woody stems (Figure 3.3) mimicked that of the roots except that there was a higher ratio of thiophenes to

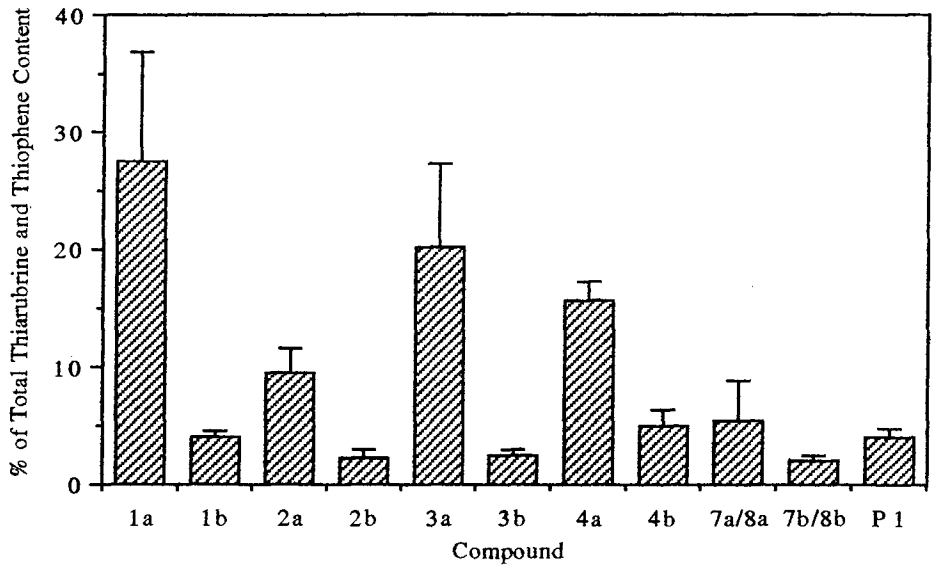

Figure 3.2 Percent of Thiarubrines (**1a, 2a, 3a, 4a, 7a, 8a**) and Thiophenes (**1b, 2b, 3b, 4b, 7b, 8b**) from the Total Polyyne Content of the Root Extracts. Standard Deviation is Expressed in Error Bars (n=3). (P 1=Unknown Polyyne #1)

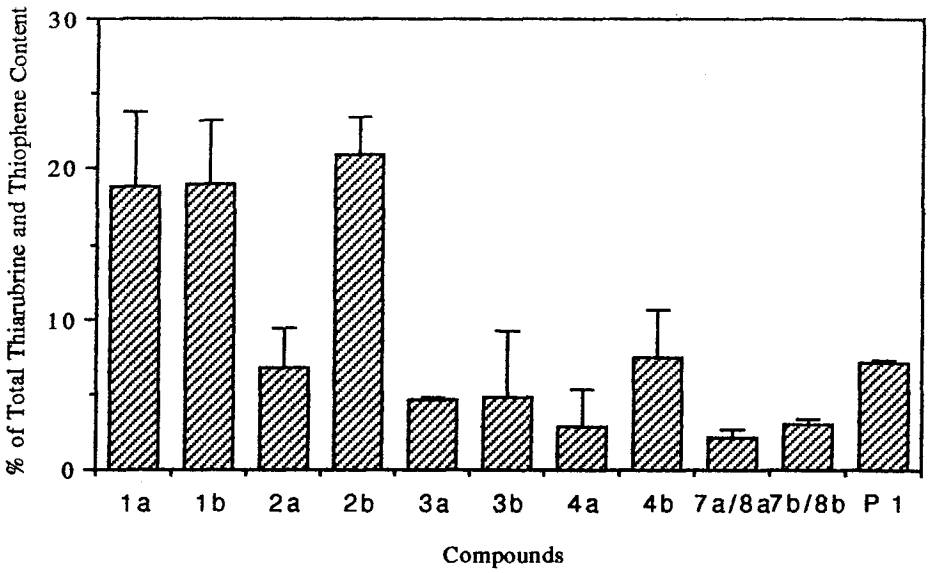

Figure 3.3 Percent of Thiarubrines (**1a, 2a, 3a, 4a, 7a, 8a**) and Thiophenes (**1b, 2b, 3b, 4b, 7b, 8b**) from the Total Polyyne Content of the Woody Stem Extracts. Standard Deviation is Expressed in Error Bars (n=3). (P 1=Unknown Polyyne #1)

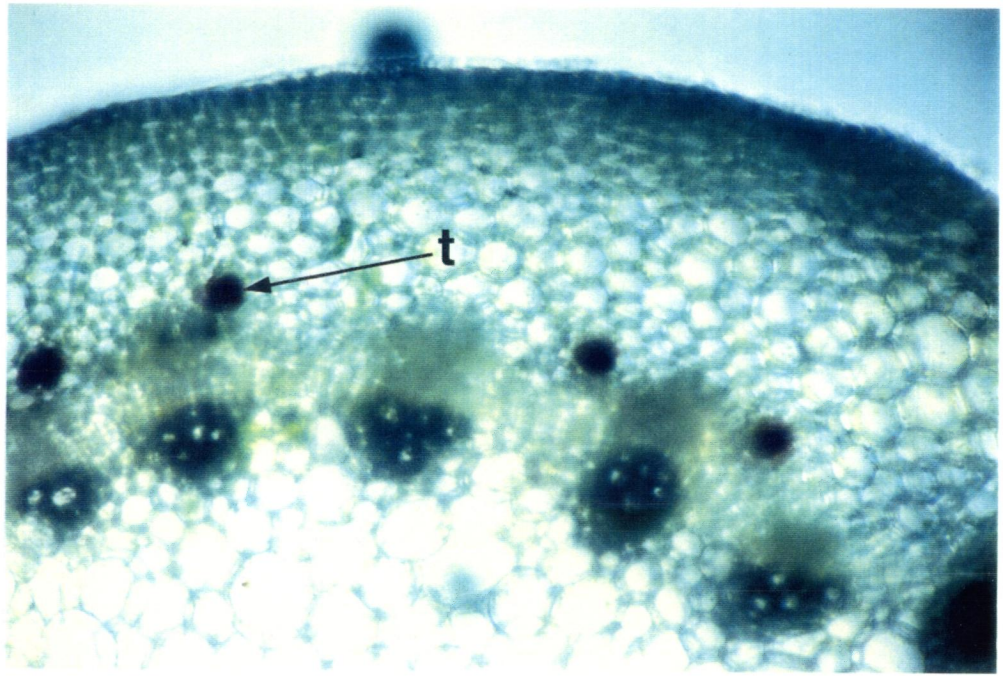

Figure 3.4 Cross-section Through Green Stem with Thiarubrine Canals of *Ambrosia chamissonis*. (t = thiarubrine canal)

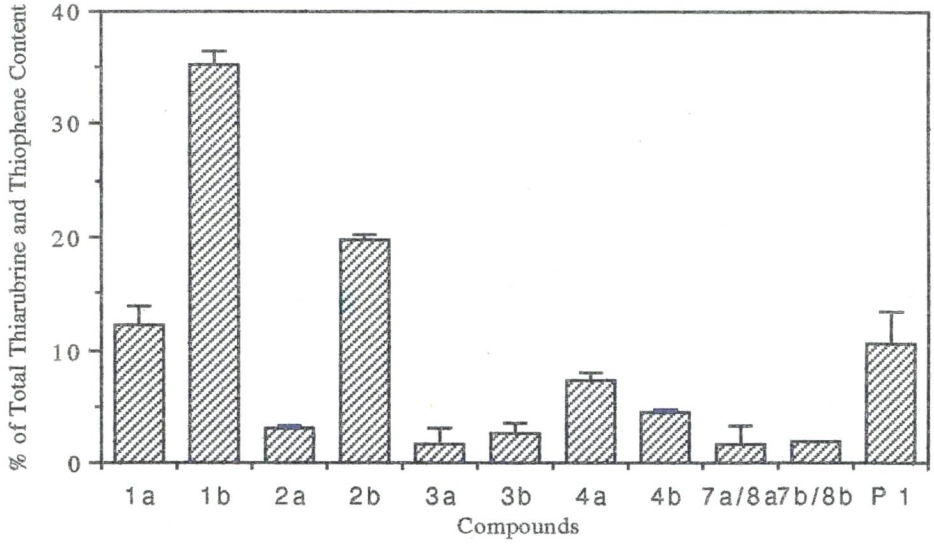

Figure 3.4 Percent of Thiarubrines (**1a, 2a, 3a, 4a, 7a, 8a**) and Thiophenes (**1b, 2b, 3b, 4b, 7b, 8b**) from the Total Polyyne Content of the Crude Green Stem Extracts. Standard Deviation is Expressed in Error Bars (n=3). (P 1=Unknown Polyyne #1)

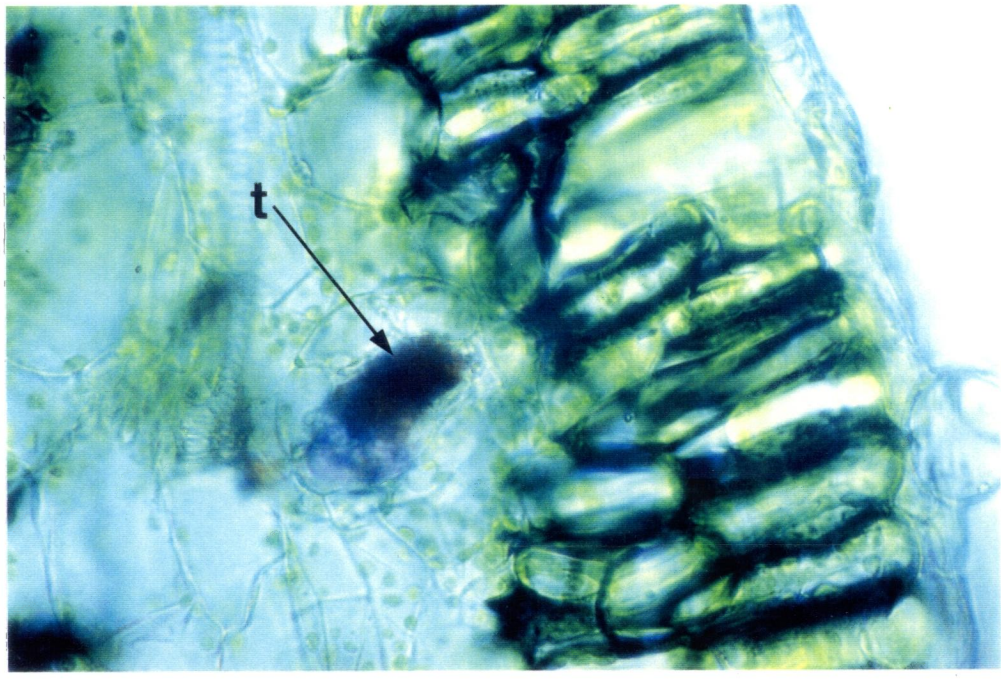

Figure 3.6 Cross-Section Through the Leaf, with Thiarubrine Canal, of *Ambrosia chamissonis.* (t = thiarubrine canal)

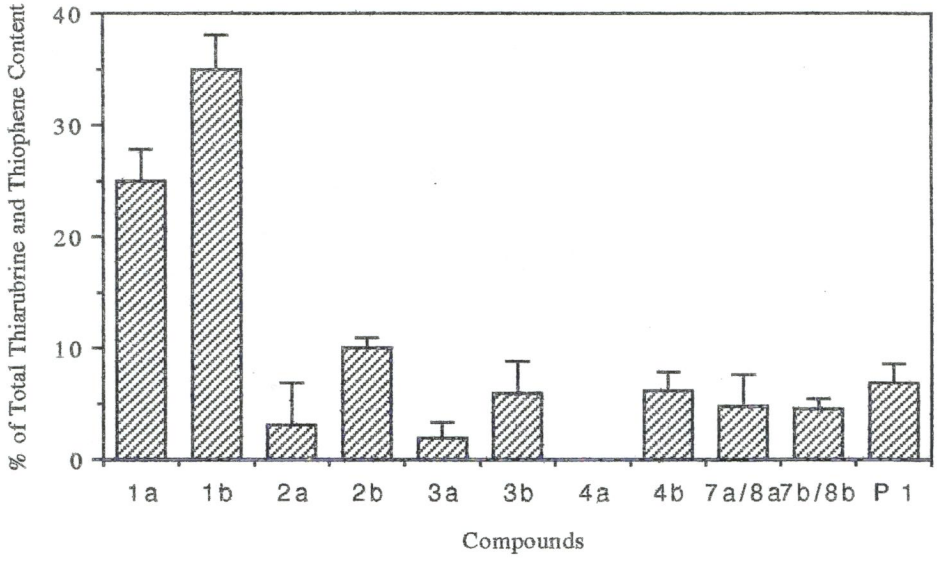

Figure 3.7 Percent of Thiarubrines (**1a, 2a, 3a, 4a, 7a, 8a**) and Thiophenes (**1b, 2b, 3b, 4b, 7b, 8b**) from the Total Polyyne Content of the Crude Leaf Extracts. Standard Deviation is Expressed in Error Bars (n=3). (P 1=Unknown Polyyne #1)

thiarubrines (approximately 1:1 as estimated). There was also a very high concentration of **2b** exceeding that of the other thiophenes including thiophene **1a**. Column chromatography was not required for the visualization of the polyynes in woody stems because there were no other pigments absorbing at 490 nm.

The green stems and leaves have a very high thiophene to thiarubrine level (Figures 3.5 and 3.7), but as the TLC and visual inspection indicate thiarubrines are present (Figures 3.4 and 3.6). The former have a higher content of thiarubrine **4a** than the latter in which the level is negligible.

In all samples the minor thiarubrines and thiophenes discussed in Chapter Two were detected. The chlorohydrins were in negligible amounts whereas the primary alcohols **7a**, **7b**, **8a**, and **8b** could be quantified. They were in small quantities, therefore their values were combined. The thiophene content relative to the thiarubrines was 1:3 in the aerial parts and 3:1 in root extracts.

A root extract was column chromatographed under the same conditions as the leaf and green stem extracts. Five percent of the yield was lost in this procedure, and the chemical profile demonstrated a lower thiophene content.

DISCUSSION

Thiophenes have been shown to have an independent biosynthesis to the thiarubrines (Constabel and Towers, 1989b). In the case of the leaves where there is a high concentration of thiophene relative to thiarubrine presumably much of the former is derived through the degradation of the latter in the shoots and leaves. Thiophene **2b** is in much higher concentrations in the woody stems than would be predicted for normal degradation when compared with the other thiophenes. This may be the result of differential stability of this molecule or it may be due to elevated synthesis of thiophene **2b** independent of the thiarubirne **2a**.

The thiarubrines, although considered root chemicals, are also found in other portions of the plant, albeit in lower concentrations. These chemicals are powerful phototoxins when irradiated yet surprisingly they are present in the leaves and stems. The canals are very important

for the maintenance of the compounds and the protection of the plant from the chemicals it is synthesizing. They are especially noticeable in young tissues and presumably function as defensive sinks. How the plant maintains them is not known. Defensively it is a good strategy; exposure by the invading organism to light after consumption leads to its instantaneous destruction. Why should a phototoxic chemical occur in the roots if predation is in the dark? One possible reason is that the predator may surface after consumption and thus be effected at a later time. An important feature of the thiaubrines is their light and dark reactions. There is some evidence also that thiophenes, such as α-terthienyl, can be electronically excited, in the absence of light, as a result of enzymatic reactions which produce singlet oxygen or superoxide anions. Peroxidase activity in *Tagetes* roots increased with plants infected with nematodes (Gommer *et al.*, 1988). Thus we have the same reaction that would occur photochemically.

CHAPTER FOUR

IDENTIFICATION OF A NOVEL THIARUBRINE AND THIOPHENE

INTRODUCTION

As indicated in Chapter Two, reverse phase HPLC revealed a novel thiarubrine and thiophene in *A. chamissonis* roots. Detection by UV-Vis spectrophotometry is important because most of the polyacetylenes exhibit characteristic spectra. To identify these compounds a number of chromatographic techniques such as preparative thin layer chromatography, high performance liquid chromatography are employed. Mass spectrometry is an important tool for the elucidation of chemical structures. Not only does it supply the molecular formula, but the fragmentation patterns also give an indication of structure. The fragmentation patterns of the chlorohydrins **5b** and **6b**, for example, are clearly distinguishable (Balza *et al.*, 1990, Chapter 1). Gas chromatography-mass spectroscopy (GC-MS), of the TMS derivatives, can be employed when examining mixtures of alcohols and acid derivatives of thiophenes, but thiarubrines are not stable enough at the high temperatures required for this procedure. Information about the thiarubrines can be acquired using solid probe electron impact mass spectrometry.

As already mentioned ^{13}C-NMR spectroscopy has not been used very much for structural information of these compounds, and by far the most useful technique for the elucidation of the chemical structures of both a thiarubrine and its corresponding thiophene is ^{1}H-NMR spectroscopy which allows for characterization of the protons in a compound. The characteristic shifts and couplings of thiophene and thiarubrine protons make interpretation of data rather simple. Proton NMR data for several thiophenes was presented by Bohlmann and Zdero (1985). They indicated that the acetylenic methyl groups is always at lower fields at around 2.05 ppm. The long-range deshielding effect of the ring protons are also characteristic.

MATERIALS AND METHODS

Plant Material.

Ambrosia chamissonis (Less.) Greene was collected in British Columbia at Centennial Beach (CB), Tsawwassen, British Columbia in May and September, 1991. Voucher specimens are deposited at the herbarium of the Department of Botany, University of British Columbia.

Extraction.

Freeze-dried ground roots were extracted with CH_3OH:CH_3CN (7:1) for 24 hours. The sample was filtered through Whatman 1 filter paper and evaporated *in vacuo* to almost dryness then resuspended in CH_3CN. The volume was reduced and partitioned at least twice between $CHCl_3$ and H_2O. The organic layer yielded the thiarubrines. Preliminary examination of all extracts and fractions were performed by HPLC using a Waters System with a MCH-10 column (4 mm x 300 mm) at a flow rate of 1 ml/min eluting isocratically with CH_3CN:H_2O (72:28). All procedures were carried out in dim light to prevent the photo-conversion of thiarubrines to thiophenes.

Isolation and Identification.

Preliminary separation of **8a** was carried out using a Si gel 60 column (230-400 mesh) eluted with CH_2Cl_2 and monitored by analytical HPLC (see Chapter Two). Compound **8a** was further purified by preparative HPLC using a Varian 5000 with a MCH-10 (8 mm x 300 mm) reverse phase column, eluting isocratically with H_2O:CH_3CN (1:1) at 3 ml/min. High and low resolution electron impact mass spectral analysis was carried out at the University of British Columbia Mass Spectrometry Facility.

For the identification of the thiophenes a small amount of crude red oil obtained from the column and determined by HPLC to contain thiarubrine **8a** was irradiated to produce the corresponding thiophenes. Preparative TLC (CH_2Cl_2:CH_3COCH_3, 30:1) yielded a mixture of thiophenes **7b** and **8b** (Rf 0.28). These were converted to the trimethylsilyl (TMS) ethers by

drying the sample and then adding 10 ml of dry pyridine and 10 ml of N,O-bis-trimethylsilyltrifluoroacetamide (BSTFA) at room temperature. GC-MS analysis was carried out on the TMS-derivatives using an automated Finnigan 1020 GC-MS equipped with a fused-silica capillary column (30 mm x 0.25 mm) of SE-54 and operated in the EI mode (70 eV) with an ion-source temperature of 95°C. GC-MS was carried out on a mixture of the hydroxylated thiophenes to determine their relative retention times. **8a** and **8b** were purified by preparative HPLC and submitted for ^{1}H NMR spectroscopic analysis.

RESULTS

Analytical reversed-phase HPLC analyses with CH_3CN:H_2O (1:1), revealed that **8a** (Rt=30.3 min) has an intermediate polarity between **7a** (Rt=35.4 min) and its corresponding thiophene (Rt=25.2 min), whereas the thiophene **8b** (Rt=23.0 min) has a slightly lower polarity than the thiophene **7b** (Rt=25.2 min). Figure 4.1 shows the UV-Vis spectra of thiophenes **8b** and **7b**. The UV-Vis spectrum of thiarubrine **8a** is presented in Figure 4.2. Preparative HPLC was useful in the purification of thiarubrine **8a** and thiophene **8b**. The optimum conditions were a flow rate of 3 ml/min with CH_3CN:H_2O (1:1). Thiarubrine **8a** had a retention time of 35.17 min whereas thiophene **8b** was eluted after 26.60 min.

Compound **8a** was determined to have a molecular formula of $C_{13}H_{10}S_2O$ ($M^+\cdot$; m/z 246.0174) by high resolution EI mass spectrometry (Figure 4.3). Other major fragments included m/z 214, suggesting the loss of a sulphur atom and $[M\text{-}CH_2OH]^+$ (m/z 215) which is characteristic of a primary alcohol. The UV-Vis spectrum of **8a** is very similar to those of the other thiarubrines, whereas the absorption spectrum of the corresponding thiophene (**8b**) is distinctive, resembling that of **4b** and **5b** in having an additional UV maximum which is indicative of an alcohol functionality. The conversion of thiarubrines to thiophenes was carried out by irradiation of the red fractions, which turned yellow. Upon purification by HPLC the thiophene **8b** was obtained as a colorless solution.

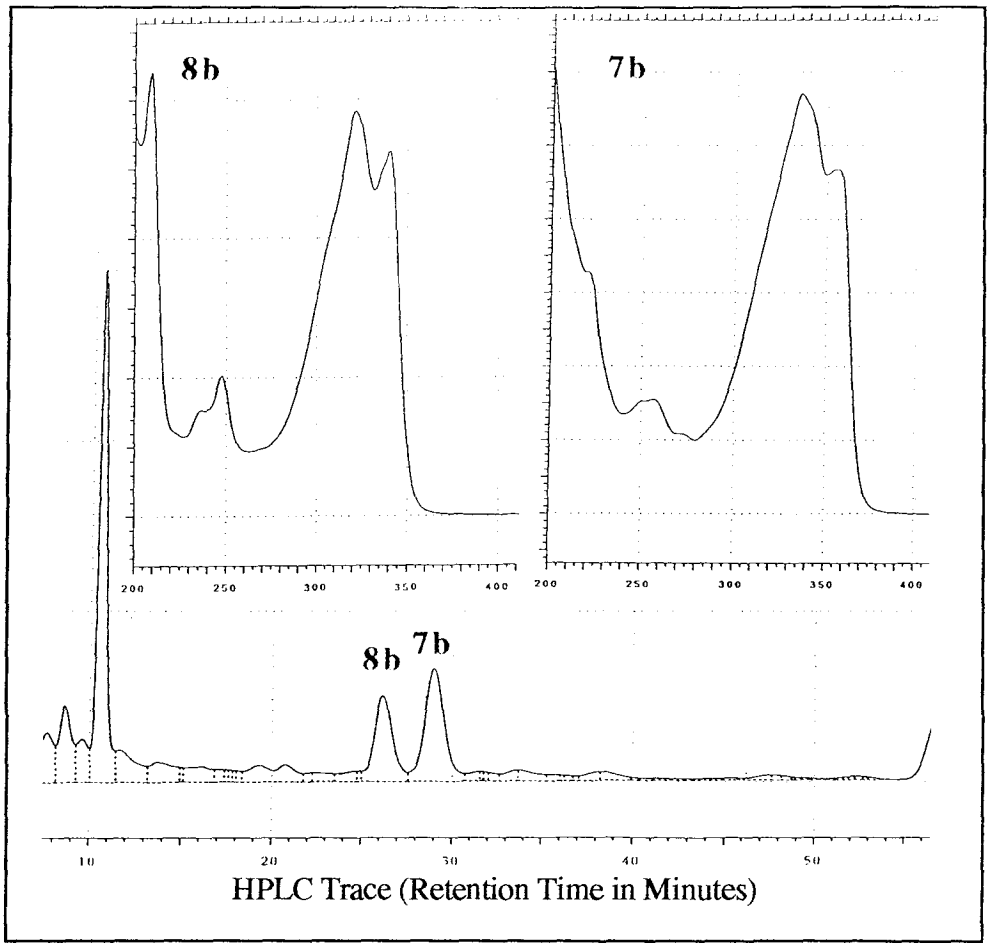

Figure 4.1 "In Flight" UV-Visible Spectra of Thiophene **7b** and **8b** from HPLC.

The GC-MS analysis of the trimethylsilyl ether derivatives of the sample isolated from TLC furnished two peaks corresponding to a molecular ion at m/z 286 (thiophene **8b**) and a molecular ion at m/z 284 (thiophene **7b**) (Figure 4.4). The mass spectrum of the former (Figure 4.5), and subsequent analysis of samples purified by HPLC, revealed major peaks at $[M\text{-}CH_2OTMS]^+$ (m/z 183) and $[CH_2OTMS]^+$ (m/z 103) indicating the presence of a primary hydroxyl group. The GC-MS analysis of a mixture of the hydroxylated thiophenes from *Ambrosia chamissonis* (**4b**, **5b**, **6b**, **7b**, and **8b**) demonstrated their relative retention times (Figure 4.6).

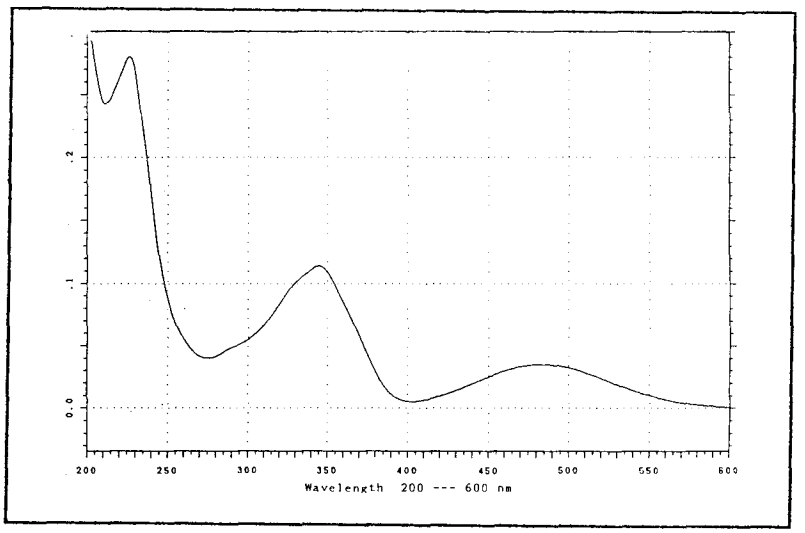

Figure 4.2 UV-Visible Spectrum of Thiarubrine **8a**.

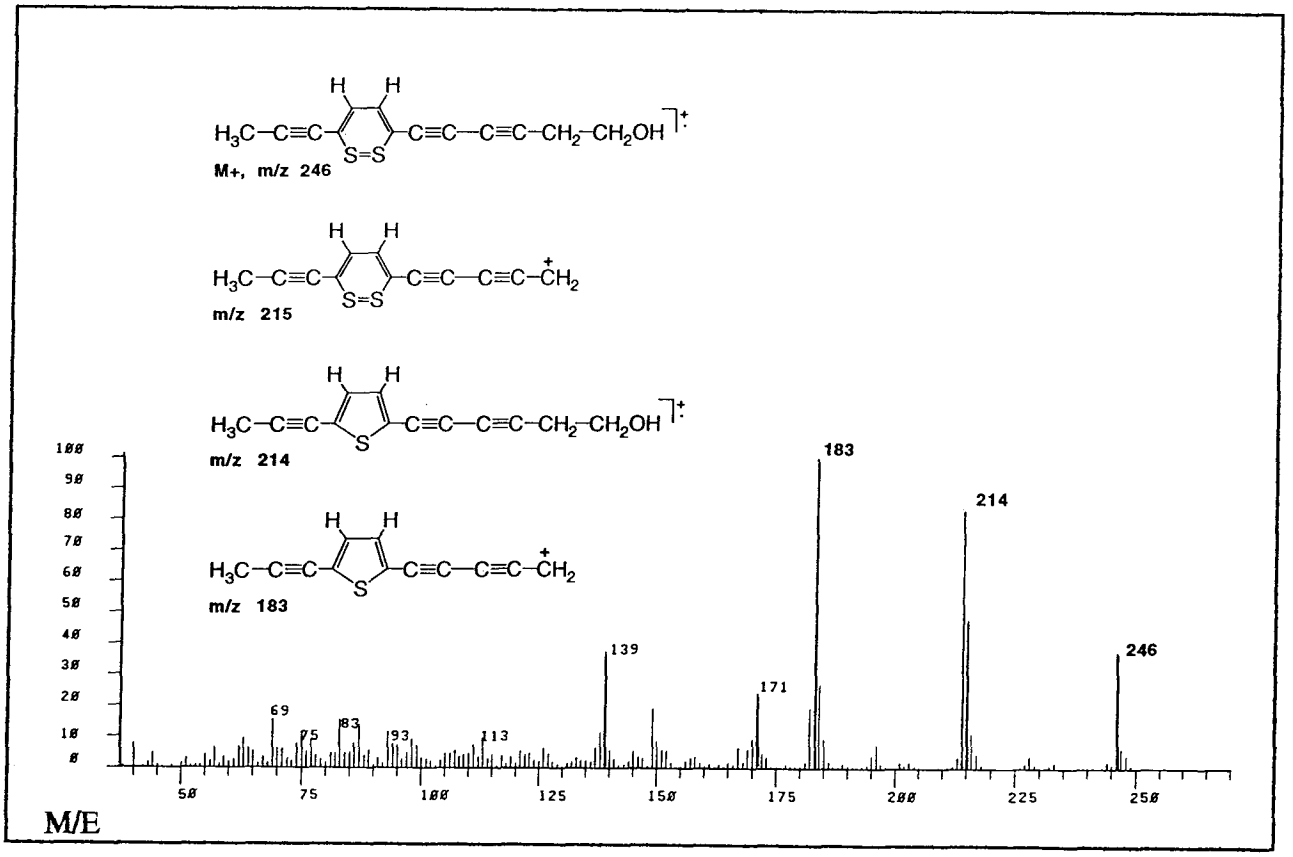

Figure 4.3 High Resolution Mass Spectrum of Thiarubrine **8a**.

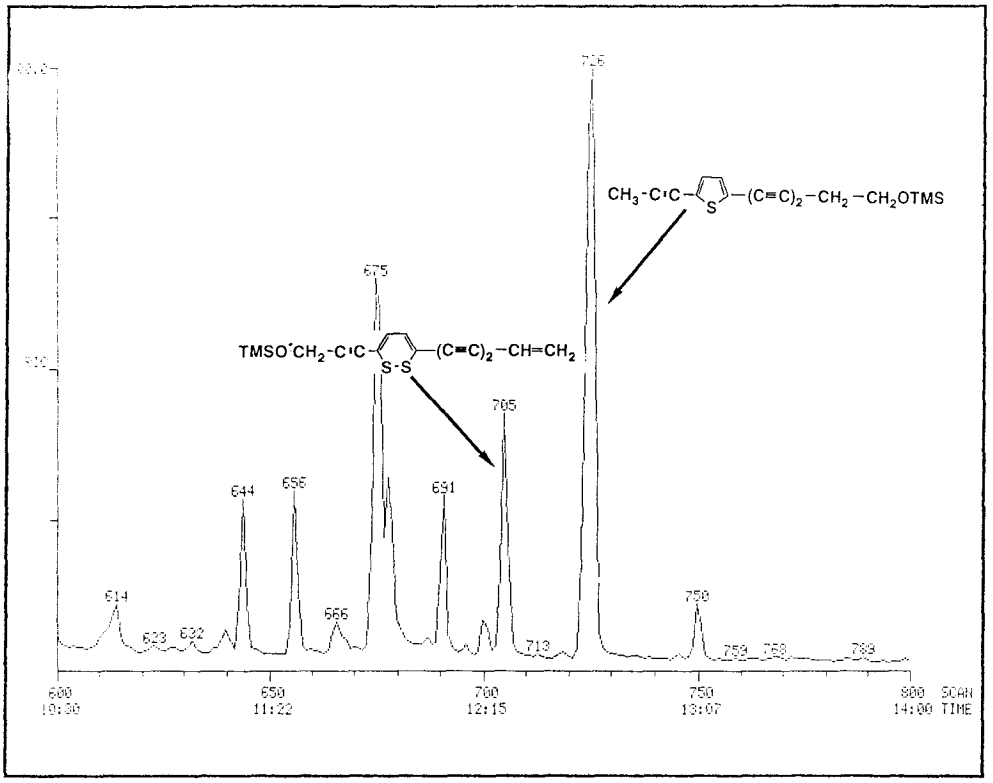

Figure 4.4 GC-Mass Chromatogram Of Sample Isolated by TLC.

The 300 MHz ^{1}H NMR spectrum of the thiophene **8b** (Figure 4.7.) showed a signal corresponding to an acetylenic methyl singlet at 2.07 ppm and an AB doublet of doublets at 7.10 ppm (H-8) and 6.93 ppm (H-9). Resolution enhancement showed a long-range coupling (^{6}J=0.58) between the latter (H-9) and the methyl group, which aided in the identification of the ring proton of the thiophene moiety. An oxygen-bearing methylene at 3.86 ppm (t, J=6.22, 6.30), coupled to a methylene at 2.66 ppm (t, 2H, J=6.22, 6.30), typical of protons of a carbon atom bonded to a carbinol and acetylenic groups, was also observed. Irradiation of the signal at 3.86 ppm collapsed that at 2.65 ppm to a singlet, inferring a primary alcohol function in the molecule. The ^{1}H NMR (300 MHz) spectrum of **8a** revealed similar resonances to that of the thiophene. The acetylenic methyl signal was found at 2.06 ppm (s, 3H), and the ring proton resonances at 6.64 ppm (H-8) and 6.49 ppm (H-9). As above, a long-range coupling (^{6}J=0.79) was also observed

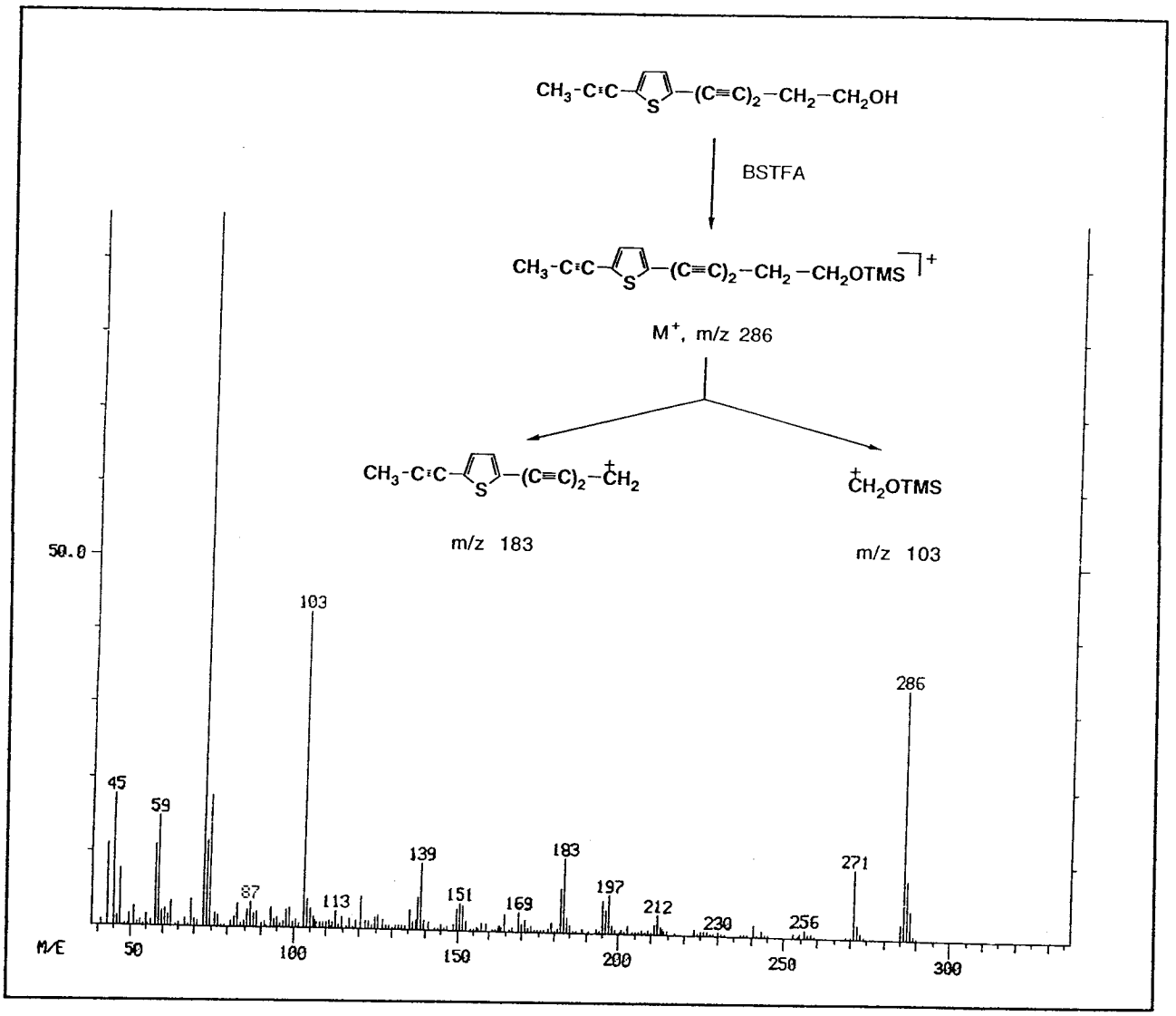

Figure 4.5 GC Mass Spectrum of Thiophene **8b** and Fragmentation Pattern of the Molecule.

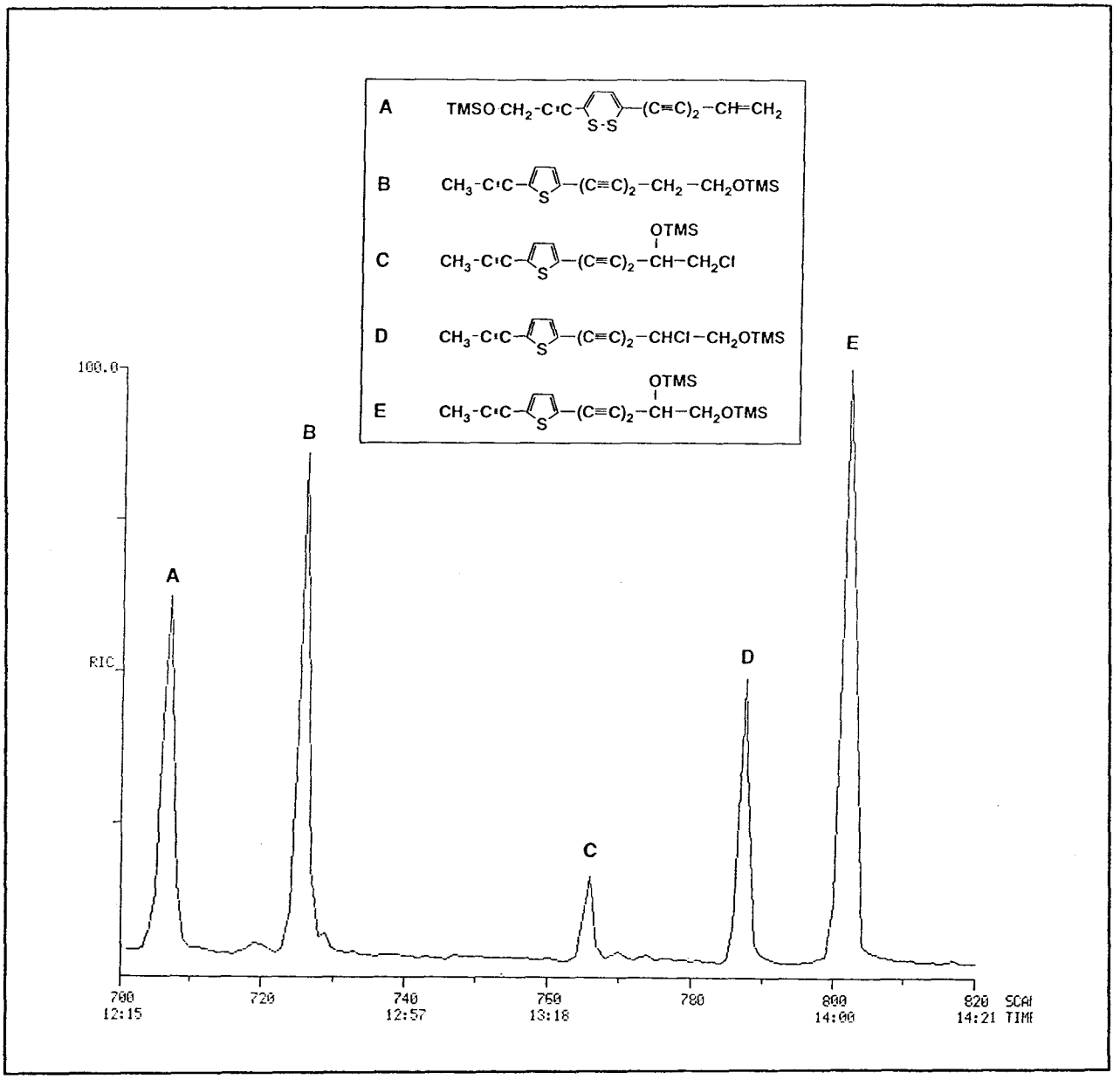

Figure 4.6 GC Mass Chromatogram of the TMS Derivatives of the Hydroxylated Thiophenes.
(A = TMS derivative of thiophene **7 b**, B = TMS derivative of thiophene **8 b**, C = TMS derivative of thiophene **6 b**, D = TMS derivative of thiophene **5 b**, E = TMS derivative of thiophene **3 b**)

between the acetylenic methyl group and the H-9 proton, which afforded ready distinction between the six-membered ring protons signals. Thiarubrine **8a** was thus characterized as 3-(1-propynyl)-6-(6-hydroxyhex-3-yn-1ynyl)-1,2-dithiacyclohexadiene and the thiophene **8b** as 2-(1-propynyl)-6-(6-hydroxyhex-3-yn-1ynyl)-thiophene.

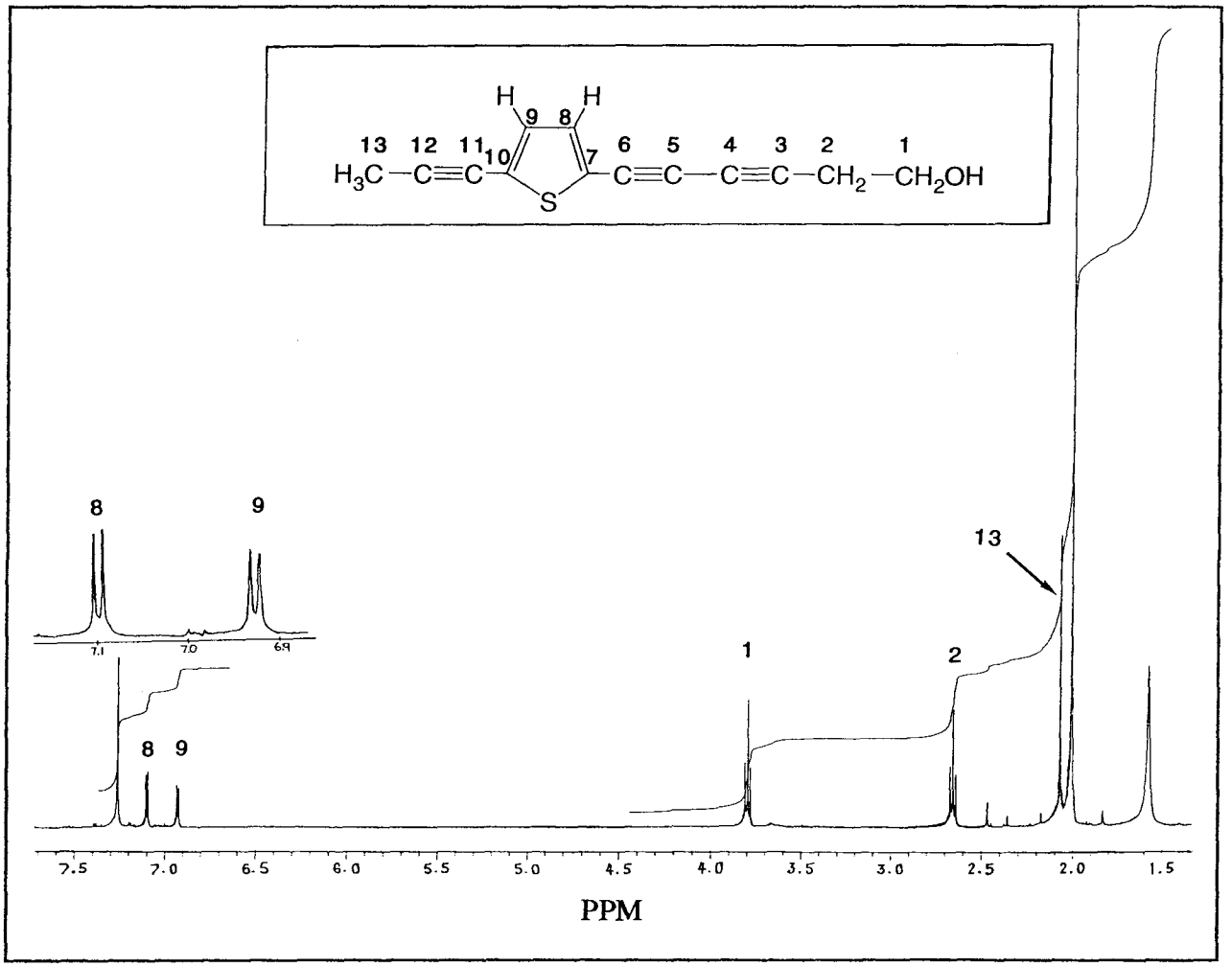

Figure 4.7 300 mHz ^{1}H NMR of Thiophene **8b**.

DISCUSSION

These results bring to date the presence of eight thiarubrines, and their corresponding thiophenes, in *Ambrosia chamissonis* roots. UV-Vis spectra are very useful in the diagnosis of the

thiophenes, although the UV-Vis spectrum of the new secondary alcohol was somewhat misleading. Based on similar molecules, the extra absorbance peak (341 nm) suggested, that the hydroxyl group was probably adjacent to an acetylene group, implying that the compound is a secondary alcohol (Felipe Balza, dir. comm.). This was ressolved by derivatizing the hydroxyl group followed by GC-MS. This fragmentation pattern furnished the position of the hydroxyl function. The thiarubrine does not lend itself well to GC-MS analysis due to the high temperatures required. Low and high resolution solid probe electron impact mass spectrometry of the pure thiarubrine however, supplied the required information. By far the most powerful technique for the elucidation of the structure of the thiophene and thiarubrine was ^{1}H NMR spectroscopy. It readily allows for the distinction between positional isomers; thiarubrines based on the **1a** skeleton exhibit coupling between the ring proton (H-9) and the acetylenic methyl group, whereas those based on the **2a** skeleton do not exhibit coupling at all between these two moieties.

Thiarubrine **8a** is presumably biosynthesized through thiarubrine **3a** via hydrogenation to produce the secondary alcohol. There is no evidence of the presence of the related primary alcohol.

CHAPTER FIVE

ESTABLISHMENT OF ROOT CULTURES

INTRODUCTION

Tissue culture is potentially a good alternative source of valuable phytochemicals. The problems confronting this technology include: low yields of chemicals, problems with establishment of continuous cultures, and the fact that many desired compounds are rarely exuded into the medium. The production of the naphthoquinone pigment shikonin, an important compound in the cosmetic industry for coloring lipstick and in the medical field for the treatment of burns and hemorrhoids, is one of the few examples of industrial utilization of plant tissue culture for the gain of chemicals (Fujita *et al.*, 1981). A two stage system using suspension culture of *Lithospermum erythrorhizon* is utilized to produce shikonin. In the first stage cells proliferate in a defined medium whereas the second stage medium is modified for chemical production.

Goleniowski *et al.*(1990) examined the sequiterpene lactone production in callus culture of *Ambrosia tenuifolia.* They claimed that levels of these compounds were higher than in the whole plant growing under natural conditions. Preliminary studies reveal, however, that thiarubrines are not produced in callus cultures of *Ambrosia chamissonis.* There are many examples which show that for production of certain secondary compounds some degree of organization is required (Cosio *et al.*, 1986). Roots of *Ambrosia chamissonis* are the best source of thiarubrines and thiophenes, as has been demonstrated in previous chapters, thus root cultures were examined. Roots are easily induced through modification of growth regulators in the medium, such as the use of a higher ratio of auxin to cytokinin which characteristically favours the formation of roots.

Another way to propagate roots in culture is to employ *Agrobacterium rhizogenes.* An excellent review article is presented by Zambryski *et al.* (1989). This gram-negative plant pathogen causes hairy root disease so-called because it induces the proliferation of roots at the site

of infection. The mode of activity is much the same as for *A. tumefaciens*. *Agrobacterium* species have plasmids called root-inducing (Ri) plasmids in the case of *A. rhizogenes* and the tumour-inducing (Ti) plasmids in *A. tumefaciens*. The bacterium introduces a piece of DNA from the Ri-plasmid, known as transfer DNA (T-DNA), into a wounded plant cell where it becomes stably incorporated into the nuclear genome (Figure 5.1). *Vir* genes, on the plasmid, facilitate this transfer. Factors released from the plant cell upon wounding enhance the expression of these genes; for example, acetosyringone from tobacco, has been identified as a virulence inducer.

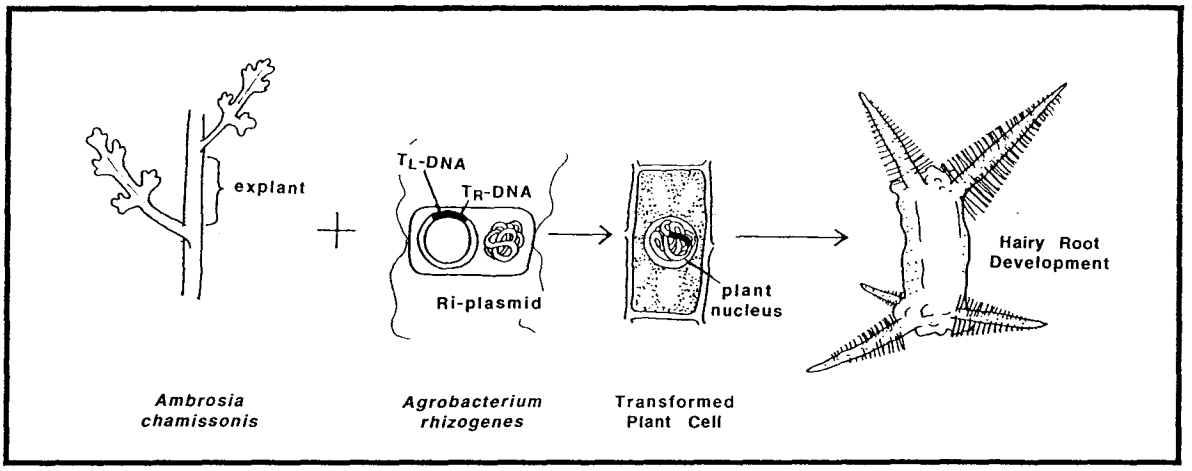

Figure 5.1 Transformation of *Ambrosia chamissonis* with *Agrobacteriu rhizogenes.*

Among other things, the T-DNA encodes for the synthesis of opines. The Ri-plasmid contains genes, which not only encode for the replication and transportation of the T-DNA, but also encode for the metabolism of opines. The plant cell is thus reprogrammed not only for the proliferation of tissue, but also for the production of a carbon and nitrogen source for the bacteria which is not metabolizable by the plant. There are two main types of *A. rhizogenes* strains based on the types of opines encoded for. The most common is the agropine-type characterized by having two T-DNAs. Put simply, one of them (T_R-DNA) encodes for the production of auxin. The T_L-DNA sensitizes the plant tissue to endogenous auxin by virtue of root loci (*rol*) genes. The

mannopine-type has one T-DNA which has high homology with the T_L-DNA. Transformed roots are therefore the result of increased sensitivity to endogenous auxin levels.

A. tumefaciens, in particular, has been used as a "natural" genetic engineer, making it possible to incorporate foreign DNA into plant cells which can then be regenerated into entire plants. Regenerated plants from hairy roots have characteristic morphologies due to the *rol* genes. These features include wrinkled leaves, stunted growth and decrease in seed output. The application of *A. rhizogenes* ito tissue culture is slightly different than that with *A. tumefaciens.* The latter has been extensively used in genetic manipulation and engineering, whereas the former is mainly used as a source of phytochemicals.

Tissue cultures are established by introducing *A. rhizogenes* to wounded plant tissue, the transformed cells developing into roots. These roots can be maintained on media devoid of growth regulators, therefore modification of the medium is not required. The morphology of transgenic roots is different from that of normal root cultures. They grow ageotropically ie. they grow upward, away from gravity and are thus easily recognized. Proof of transformation is established in a number of ways. High voltage paper electrophoresis is commonly used as a "simple" separation technique for the detection of the opines. Over time some cultures may lose their ability to produce these compounds and a negative result may be obtained. A number of researchers have used DNA probes to detect the incorporated T-DNA.

The suspension culture system already described for shikonin production, has not been without its problems. Due to the genetic instability of non-differentiated tissue, Shimomura *et al.* (1991a) examined the production of shikonin in hairy-root cultures. They were able to extract this compound from the medium without changing the culture conditions. The production was enhanced by using an adsorbent (XAD-2 column) and 5.2 mg per day could be obtained from a 2-liter batch.

Constabel and Towers (1988) developed hairy root cultures of *Chaenactis douglasii* and examined the production of thiarubrines. Through selection of high-yielding lines they were able to obtain cultures which produced twice as much thiarubrine as non-transformed root cultures.

Shimomura *et al.* (1990b) suggest that since hairy root cultures of *Hyoscyamus*, generated from two different strains, exhibited different chemical profiles; it is the insertion of different "plasmids" which is responsible. They did not specify how many lines they examined, however, so it may be a matter of selection of a higher yielding line. Mano *et al.* (1986) indicate that lines from the same strain show variations in chemical profile.

Mukundan and Hjortso (1990) studied the production of thiophenes in root cultures of *Tagetes erecta.* Like many researchers they found that the overall production of thiophenes in normal root cultures versus hairy root culture was less. This was calculated on the basis of the yield per flask rather than the amount of thiophene per gram of root material. The chemical profile of the transformed roots matched that of the intact roots. This was also observed by Parodi *et al.* (1988) in a study of the bithiophenes and benzofurans of hairy root cultures of *Tagetes patula.*

Efforts have been made to up-scale production of desired chemicals through the use of fermenters eg. shikonin production in suspension cultures of *Lithospermum.* Hilton and Rhodes (1990) found problems with growing root cultures using the same style of fermenter. The impellers of the culture vessel, which keep the culture and medium circulating, ensuring adequate aeration, caused damage to the roots. This problem was solved through modification of the vessel by segregating the impeller with a cage. Another solution is to use an air-lift system, whereby the medium is sparged with air from the bottom of the vessel (Toivonen *et al.*, 1990). Toivonen *et al.* demonstrated that there is no difference in the chemical profiles between shaker flasks and the fermenter.

Various culture systems, including fermenters, were examined. The chemistry of tissue cultured roots often reflects that of the roots. Examination of the roots of *Ambrosia chamissonis* (Chapter Two) indicated that the chemistry of the root varies depending on which part of the root is extracted. In this chapter the chemistry of normal root cultures was compared with that of hairy root cultures.

Material and Methods

Plant Material

Most collections of *Ambrosia chamissonis* were obtained from Centennial Beach, Tsawwassen, British Columbia. Samples were also taken from North Beach, Queen Charlotte Islands, British Columbia.

Bacterial Cultures

Strains A4, 15834, and TR7 of *Agrobacterium rhizogenes* were obtained from Dr. L. Moore, Department of Plant Pathology, Oregon State University, Corvalis, Oregon. Bacterial cultures were grown up on Potato Dextrose Agar (Difco), stored at 25°C and then transferred to Potato Dextrose Broth (Difco) and placed on an orbital shaker at 100 rpm incubated at 28°C. Bacterial liquid cultures were used to prepare stocks which were stored in 50% glycerol at -70°C in Eppendorf tubes.

Explant Preparations

Stems leaves and petioles were examined for their tissue culture potential. Roots were excluded because they are difficult to sterilize.

Green stems which had widely spaced leaves were selected. They were defoliated, washed, and cut into segments 6-7 cm long. Under sterile conditions the segments were immersed, for 15 minutes, in a 30% bleach (Javex) solution with 2% Tween-20 (BDH) (Figure 5.2). They were rinsed several times with sterile distilled water, washed in 70% ethanol for 2 minutes and blotted dry on sterilized filter paper. The bleach-damaged ends were excised and discarded. The rest of the segment was cut into pieces 3-5 mm in length. Both normal and transgenic root cultures were generated from these segments.

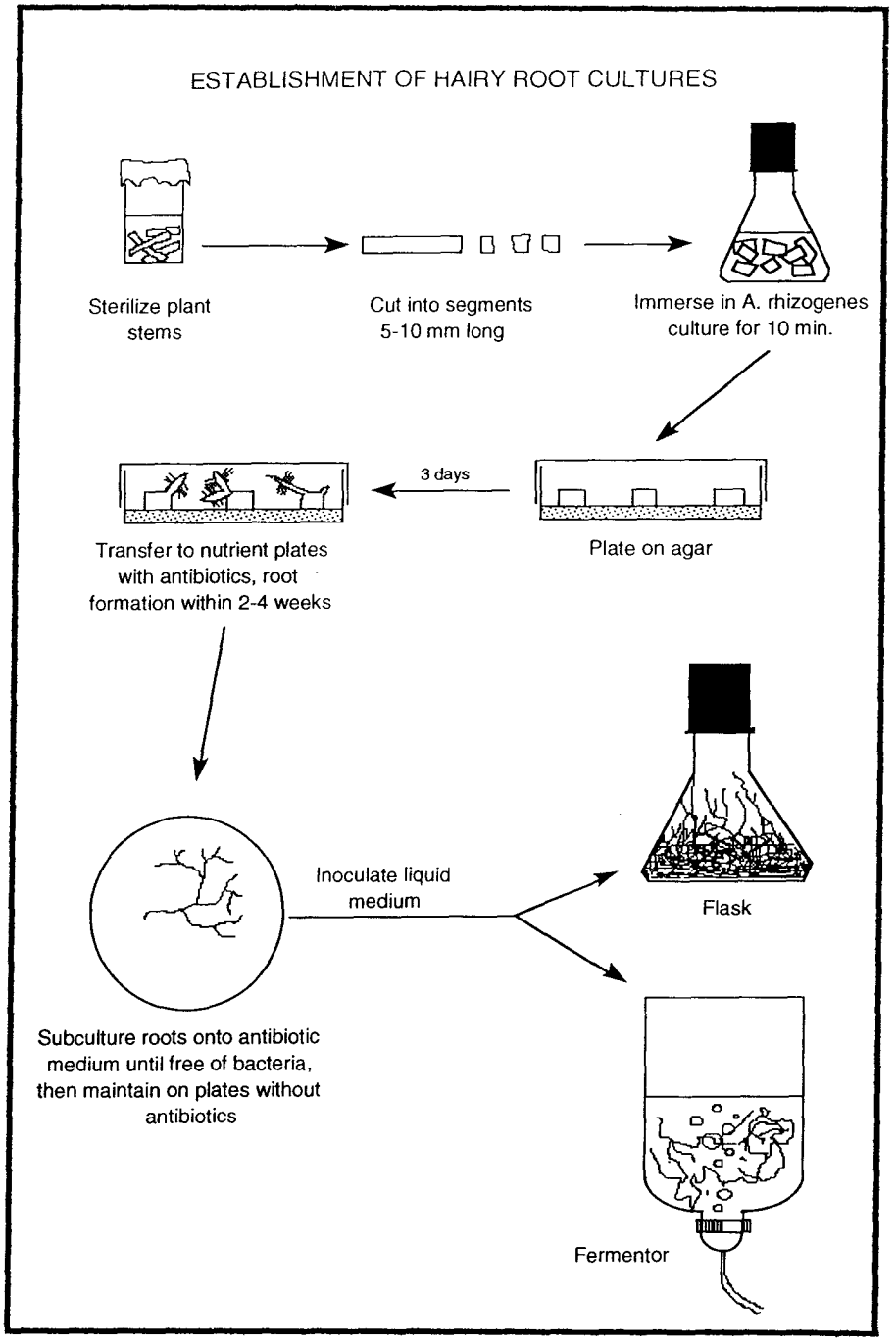

Figure 5.2 Transformation of *Ambrosia chamissonis* Stem Segments with *Agrobacterium rhizogenes.*

Normal Root Cultures

The explants were placed on solid Schenck and Hildebrandt medium (Schenck and Hildebrand 1972) and Murishige and Skoog medium (Murishige and Skoog) supplemented with

various concentrations of the auxins NAA (naphthalene acetic acid) and IBA (indole butyric acid) as well as the cytokinin BAP (benzylaminopurine)

Transgenic Root Cultures

The explants once cut were immediately immersed into a 18 hour bacterial culture for 10 minutes (Figure 5.2). They were blotted dry on sterilized filter paper and placed on plates of water/agar. Control segments were immersed in PD broth. After incubation for three days they were transferred onto antibiotic SH plates. Carbenicillin (300 mg/L) and vancomycin (250 mg/L) were filter-sterilized through 22 µm millipore filters into medium that had cooled to less than 30°C.

Preparation of Acetosyringone

Acetosyringone (7.84 mg) was dissolved in 100 µl DMSO and brought to 200 ml with buffer (0.9% NaCl, MES pH 5.5) to make a 200 µM solution. Various concentrations (0-200 µM) of acetosyringone were filter sterilized into the bacterial culture prior to treatment to see if transformation was enhanced.

Verification of Transformation

To verify that the hairy root cultures were transformed paper electrophoresis was employed to detect the opines (Petit *et al.*, 1983). Fresh root samples were ground in 1% HCl (1 mg/g tissue) for 10 minutes in a boiling water bath. The homogenate was centrifuged at 2000 g for 10 minutes. The supernatant was evaporated under reduced pressure at 40°C and dissolved in water (0.2 ml/g tissue). Ten microlitres of the extract was spotted on Whatman 3 MM paper and electrophoresced at 90 V/cm (900 or 1500 V) for 8-11 min on a Desaga apparatus with a BioRad powerpack. The buffer used was formic acid:acetic acid:water (30:60:910). The electrophorogram was dried and dipped in silver nitrate solution prepared as outlined by Trevelyan *et al.*, (1950). The papers were dried and then developed, at room temperature, in a solution of 20 g NaOH in 100 ml H_2O made to 1L with 95% ethanol (Ellis *et al.*, 1984), immersed in fixer and washed for 3

hours with running water. Standards of agropine, agropinic acid, mannopinic acid and mannopine were kindly supplied by Dr. Petit (Groupe de Recherche sur les Interactions entre Plantes et Microorganisms, Universite de Paris-Sud, Institut de Microbiologie, Orsay, France). Results were recorded as a positive or negative for the presence or absence of standard opines.

A Southern blot was prepared with DNA from transformed roots with different strains of *A. rhizogenes* and non-transformed roots, but the probe of T-DNA from *A. tumefaciens* used was not successful.

Thiarubrine Localization

Hand sections of normal and hairy roots were made. Inititially JB-4 embedding techniques were employed, but due to the loss of thiarubrines and the success of hand sections they were not incorporated into this study. All sectioning was done in dim light to minimize the conversion of thiarubrine to thiophene prior to viewing.

Growth Curve of Transformed and Normal Root Cultures.

Forty milliliters of SH medium were placed in 125 ml *Erlenmeyer* flasks and inoculated with 5 root tips approximately 1 cm long from normal root cultures or hairy root cultures generated from *Agrobacterium rhizogenes* strain A4. The medium for the normal root cultures was supplemented with 2 mg/l of NAA. There was a total of 36 flasks set up for each line. The cultures were maintained on a rotary shaker at 80 r.p.m.s in the dark at 24°C. Four flasks of each were weighed and analyzed every four days. Thiarubrine content was determined by UV-Vis spectrometry. The four samples were combined and prepared for HPLC analysis.

Chemical Analysis of all Cultures

The root cultures were blotted dry and then rinsed three times with distilled water and frozen to -70°C. They were freeze-dried on a Virtis Lyophilizer. The dried tissue was weighed, ground and extracted with methanol:acetonitrile (7:1) for 24 hours. The sample was filtered

through Whatman 1 filter paper. Overall thiarubrine content of the filtrate was calculated at this point using a molar absorptivity of 3000. The solution was evaporated under reduced pressure at 28°C and resuspended in HPLC grade CH_3CN. Filtering through glass wool was required to remove precipitates of more polar components. The filtrate was reduced *in vacuo* and resuspended in HPLC grade acetonitrile. This was repeated until no residue was left on the side of the flask. The sample was passed through a Sep-Pak and injected into a Waters HPLC equipped with a photodiode array detector. The column used was a Varian Micro Pak MCH-10 reverse-phase column at 1 ml/min. The first runs were isocratic with $CH_3CN:H_2O$ (72:28). Samples were then injected and run isocratically with $CH_3CN:H_2O$ (50:50).

Fermenter

Eight liter air-lift fermenters (manufactured by Dr. M. Hjortso, Dept. of Chemical Engineering, Louisiana State University , U. S. A.) contained 4 liters of SH medium. The medium for the normal roots was supplemented with 2 mg/L NAA (naphthalene acetic acid) whereas the transgenic roots (strain A4) were maintained in hormone-free medium. Each fermenter was innoculated with two grams of roots. Air was supplied from the bottom and the fermenters were kept in the dark. After the cultures had reached stationary phase the media as well as the roots were extracted and analysed for thiarubrines and thiophenes. The medium was partitioned with chloroform, dried with $MgSO_4$, concentrated *in vacuo*, and resuspended in HPLC grade acetonitrile. Thiarubrine concentration was calculated. Two fermenters were set up for each line.

Sulphate

A low yielding transgenic root line was chosen for this experiment to see if supplementation of sulphate would enhance the production. The control flask contained the normal SH medium that had been used for growth of the cultures. In treatment 1 the flasks were enhanced with 0.8 g/L of $MgSO_4$, in treatment 2 they were enhanced with 3.2 g/L Four flasks were set up in

each treatment and cultured in the dark at 24°C for 28 days. The cultures were extracted as previously described and the thiarubrine content was calculated per flask. Each sample was analysed by HPLC.

Malic Acid

SH medium was prepared with different concentrations of malic acid (0.1, 5.0, 10.0, 50.0, 100.0 mg/L) and the pH adjusted to 5.6 with KOH. Four 250 ml Erlenmeyer flasks were set up for each treatment. Each flask contained forty milliliters of the medium and were innoculated with four, approximately 1 cm long, tips of hairy roots transformed with *A. rhizogenes* 15834. They were incubated in the dark on a rotary shaker at 100 r.p.m.s at 24°C. The cultures were harvested after 28 days, blotted dry, freeze-dried and prepared for HPLC as already described.

Cultures Initiated From Plants Collected From the Queen Charlotte Islands

Plants were collected from North Beach, Queen Charlotte Islands (see Chapter Two). The two types were prepared for hairy root culture as already outlined. Only stem segments from type 2 initiated roots. All of Type 1 stem segments were contaminated with fungi and could not be retrieved.

RESULTS

Normal Root Cultures

Cultures were found to grow best on SH medium with 4 mg/L NAA and the explant of choice was found to be young green stems. MS medium enhanced the production of anthocyanin pigments. These were detected by extracting with acidified methanol and examining the UV-Visible spectrum. The acetylenes were not stable in this extraction solvent. Although the thiarubrines were produced the cultures did not grow as rapidly as on SH, therefore SH medium was used for the rest of the studies. Over time the requirement for growth regulators changed and

the medium was subsequently altered with NAA and BAP to improve growth. Root cultures which did not exhibit callus growth were extracted.

Hairy Roots Cultures and Opine Detection

All strains of *Agrobacterium rhizogenes* employed, induced roots on the explants within two weeks of inoculation. Detection of opines using paper electrophoresis verified that the cultures were transformed. Agropinic acid was detected in all hairy root cultures. Agropine was detected from hairy roots derived from A4 and 15834, whereas TR7 strains produced mannopinic acid and mannopine. In one case a line from TR7 opines were not detected. No opines were detected in normal root cultures.

Transformation was apparent because the roots exhibited ageotropic growth characteristic of "hairy roots". The roots transformed with A4 and 15834 exhibited more extensive branching than those transformed with TR7. Transformation was efficient (3-8 roots initiated per stem segment), therefore acetosyringone was not required.

Chemistry of Root Cultures

The thiarubrine profile of the hairy root cultures did not differ from that of most normal root cultures. Some of the latter possessed callus, therefore, the overall thiarubrines produced was less than cultures comprised solely of roots. The data presented in Table 5.1 and Figure 5.3 were obtained with normal cultures which did not exhibit callus formation. The problem with the normal cell cultures was to keep the growth medium at the optimum hormone levels otherwise growth rate decreased and callus content increased.

Cultures on medium supplemeted with BAP generated leafy shoots which ultimately generated roots. Interestingly canals were evident in the leaves prior to formation of the roots.

Table 5.1 Overall Thiarubrine Content of the Root Cultures.

Root Culture	Overall Thiarubrine Content (mg/g dry wt)
Normal Root Culture	1.02 (± 0.40, n=7)
Hairy Root-A4	1.24 (± 0.15, n=6)
Hairy Root-15834	1.03 (± 0.50, n=9)
Hairy Root-TR7	1.07 (± 0.24, n=7)

Error is presented as standard deviation (n varies)

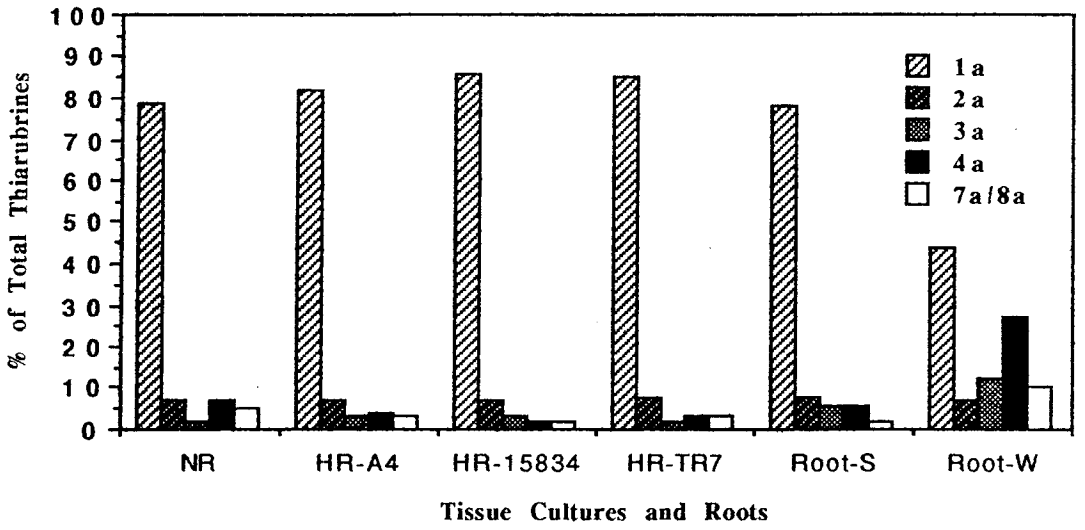

Figure 5.3 Comparison of Thiarubrine Composition in Roots and Root Cultures.
"normal"=root cultures generated with growth regulators; HR=hairy root cultures (different strains of *Agrobacterium* are identified); Root-S=Succulent Root; Root-W=Woody Root.

Localization

Examination of cross-sections of root cultures revealed that both transformed and non-transformed roots possessed easily discernible canals between the double endodermal layers. These red canals run longitudinally throughout the tissues. Root cultures exposed to light for an hour exhibited purple coloration. Upon sectioning the purple color was found within the endodermal cells themselves. These are presumably anthocyanins which may absorb light and prevent autotoxicity.

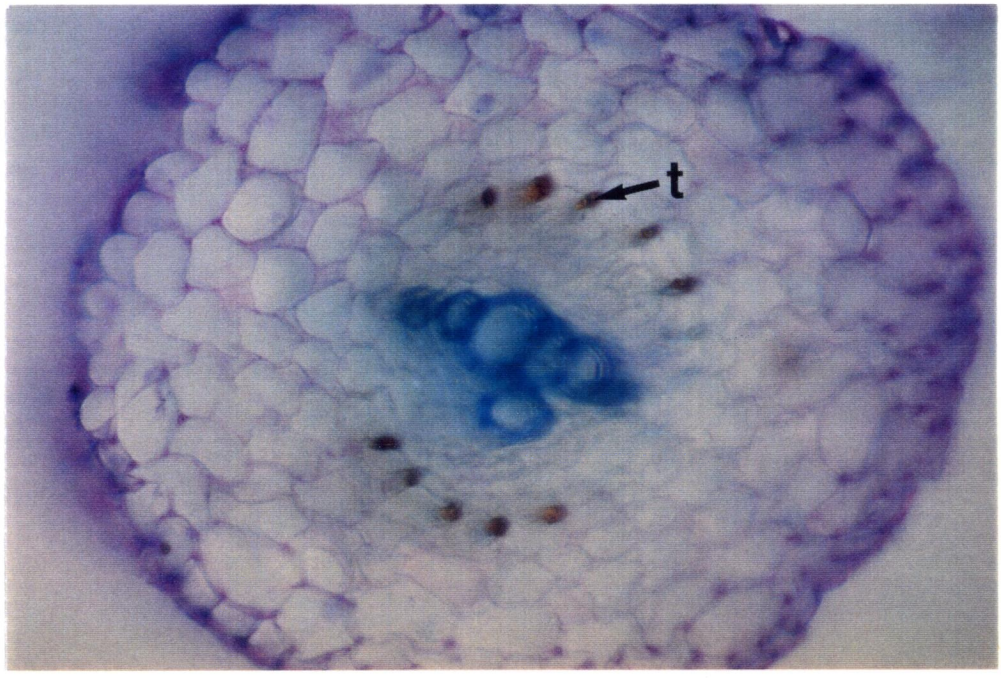

Figure 5.4 Cross-Section, Stained with Toluidine Blue, of a Transgenic Root. (t = thiarubrine canal)

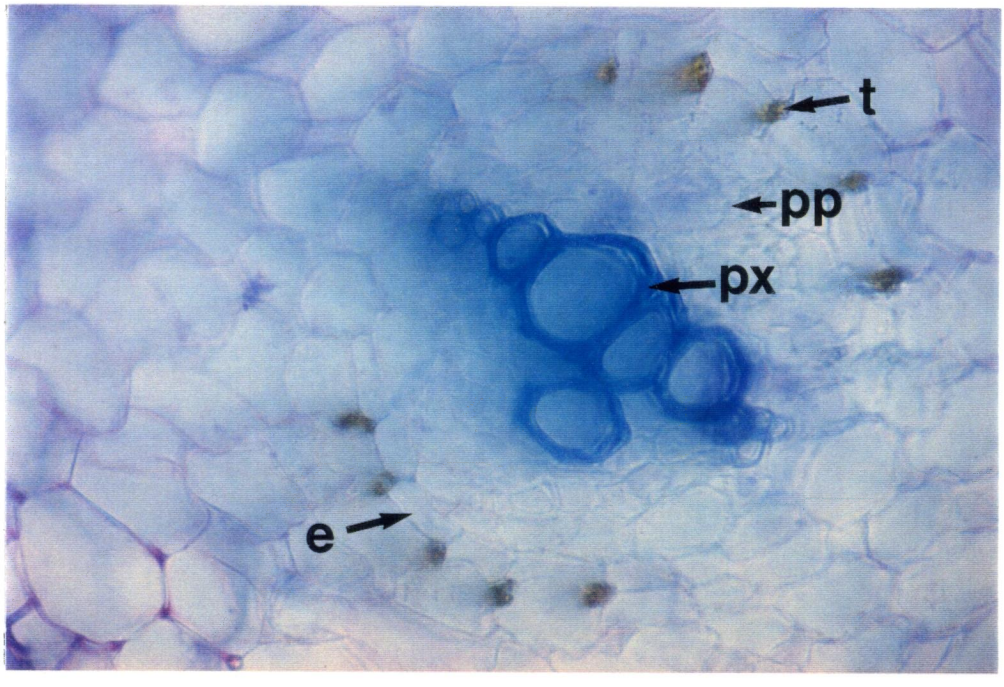

Figure 5.5 Cross-Section, Stained with Toluidine Blue, of a Transgenic Root. (t = thiarubrine canal, e = endoderm, pp = primary phloem, px = primary xylem)

Growth curve

The growth curve of the root cultures was similar to that of other root cultures reported. There is a characteristic lag phase followed by a growth phase (logarithmic phase) and terminating

in a stationary phase. Although this pattern was observed in the transgenic and non-transgenic cultures stationary phase was reached much sooner in transgenic cultures (Figure 5.6).

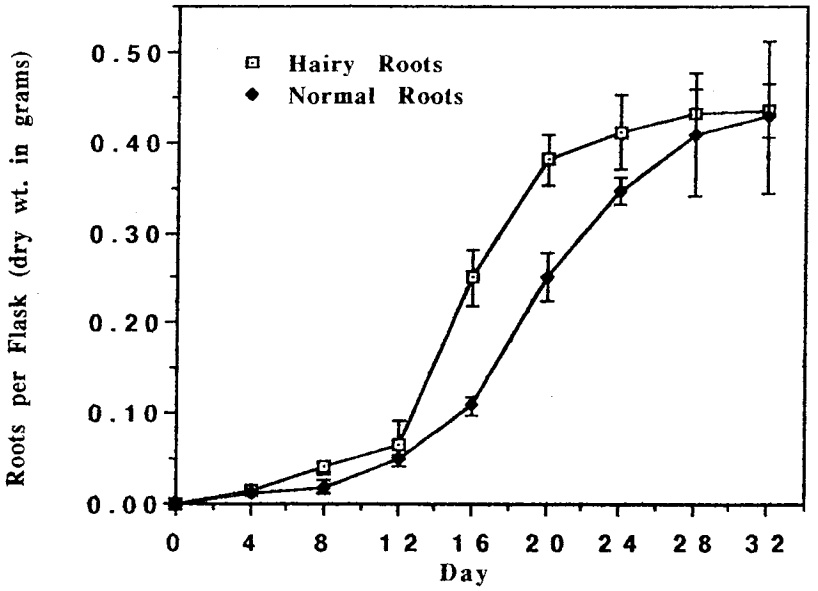

Figure 5.6 Growth Curves of Transgenic Root Cultures and Normal Root Cultures.

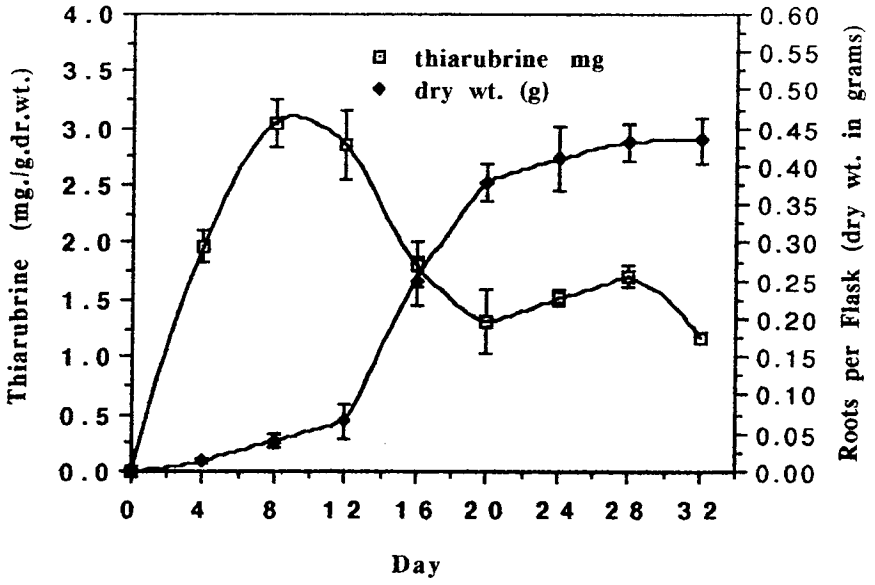

Figure 5.7 Growth Curve of Transgenic Root Cultures and Thiarubrine Production (in mg/ g dry wt.).

The chemistry of the hairy root cultures changed over time. During the lag phase (to day 12) a thiarubrine was observed at 52 minutes with H_20:CH_3CN (50:50) in higher quantities than the alcohol thiarubrines (Figure 5.8). This was intermediate between the thiarubrine **3a** and thiophene **3b**. However, the small amounts precluded analysis at this time.

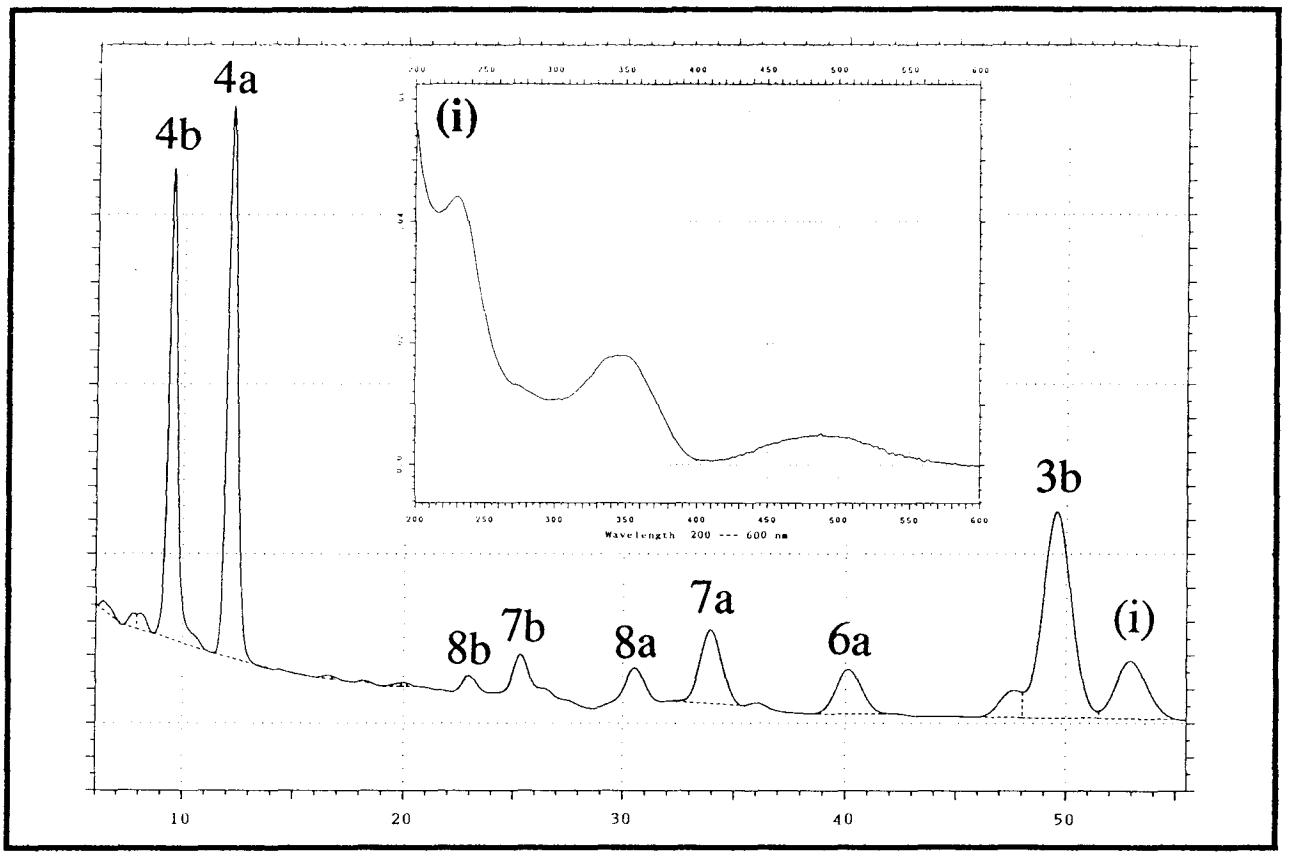

Figure 5.8 HPLC Trace of 8 Day Old Hairy Root Culture Extract at a Flow Rate of 1 ml/min at 50:50 (H_2O:CH_3CN) with the UV-Vis Spectrum of an Unknown Thiarubrine (i).

Fermenter

The growth rate of the transgenic roots was more rapid than that of the non-transgenic roots. The hairy root cultures were ready to harvest at four weeks whereas the normal roots required six weeks. In both cases the roots grew as a dense mat without callus formation. The more polar thiarubrines were detected in the medium (Table 5.2). This is due to the branch roots which originate from the pericycle of the root inside the endodermis and rupture the cells of the

parent root releasing the contents of the canals. The chemical profiles of the roots (Figure 5.9) did not differ from the shaker flask cultures. The increase in biomass was substantially enhanced in the fermenter. In four weeks the transformed cultures grew from 1.0 g to 550.0 g. The weight was not accurate because of the residual medium.

Table 5.2 Thiarubrine Yield From Freeze-Dried Cultures from Fermenter.

Tissue Extracted	Overall Thiarubrine Production
Hairy Roots	0.92 mg/g. dry wt.
Normal Cultured Roots	1.12 mg/g. dry wt.
Medium from Hairy Root Culture	0.050 mg/L
Medium from Normal Root Culture	0.067 mg/L

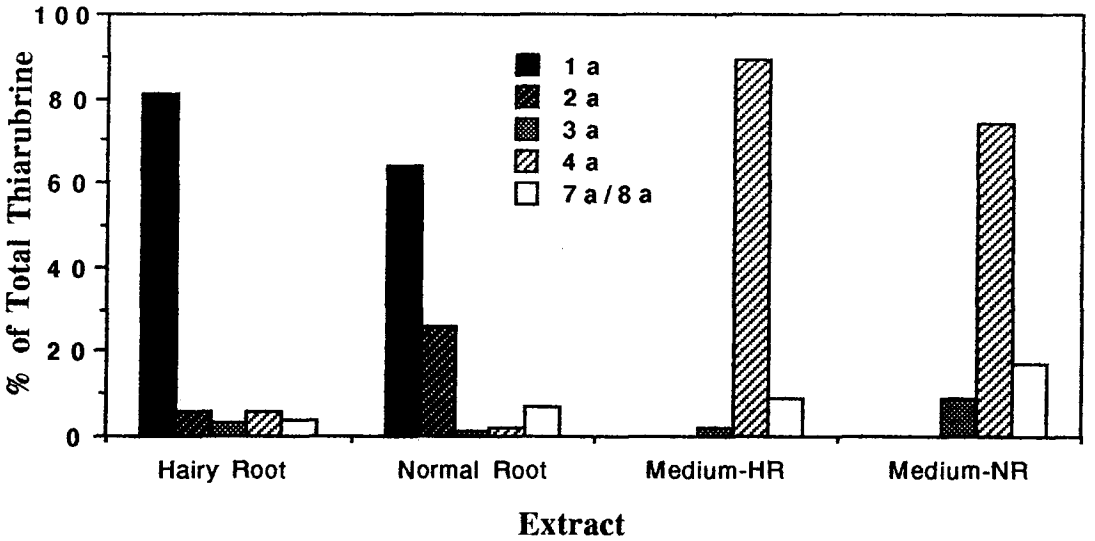

Figure 5.9 Thiarubrine Content of the Cultured Roots (HR=Hairy roots transformed with Agrobacterium rhizogenes A4, NR= Normal Root cultures) and the Culture Medium.

HPLC analysis of the medium revealed minute quantities of an unknown polyyne, suggested from its UV-Vis spectrum

Sulphate

There was no significant difference between the dry weight of the samples or the thiarubrine content between treatments (Table 5.3).

Table 5.3 Thiarubrine Production in Hairy Root Cultures (A4) Treated with Sulphate.

Treatment (mg/L $MgSO_4$)	Dry wt. of roots in grams	Thiarubrine Content in mg/g dry wt.
0 (SH)	0.3977 (± 0.0212)	0.4689 (±0.1655)
1 (SH + 0.8 g/L)	0.4135 (± 0.0480)	0.4720 (± 0.1510)
2 (SH + 3.2 g/L)	0.3513 (± 0.0760)	0.3966 (± 0.0482)

Malic Acid

The cultures which were treated with malic acid were darker brown with increasing concentration. Upon microscopic inspection one could see that the cortex of the roots was proliferating while the endodermis, with its canals, remained intact. The concentration of thiaurbrines decreased with increased concentration of malic acid (Table 5.4). HPLC profiles indicated that there was no difference chemically between the treatments except that there was only trace amounts of the thiarubrines and thiophenes of the primary alcohols, and the amount of thiosulphinate **9** was enhanced (Figure 5.10) All of the cultures including the controls exhibited peaks which overlapped the peaks of the thiarubrines and thiophenes of the primary alcohols (Figure 5.11).

Table 5.4 Thiarubrine Production in Transgenic Cultures Treated with Various concentrations of Malic Acid.

Malic Acid Treatment (mg/L)	Thiarubrine Concentration (mg/g dry wt)
0.0	0.60 ± 0.0340
0.1	0.57 ± 0.0141
5.0	0.53 ± 0.0621
10.0	0.49 ± 0.0083
50.0	0.38 ± 0.0484
100.0	0.38 ± 0.0205

Error is given as standard deviation, n=4, except in treatment 10, where n=3

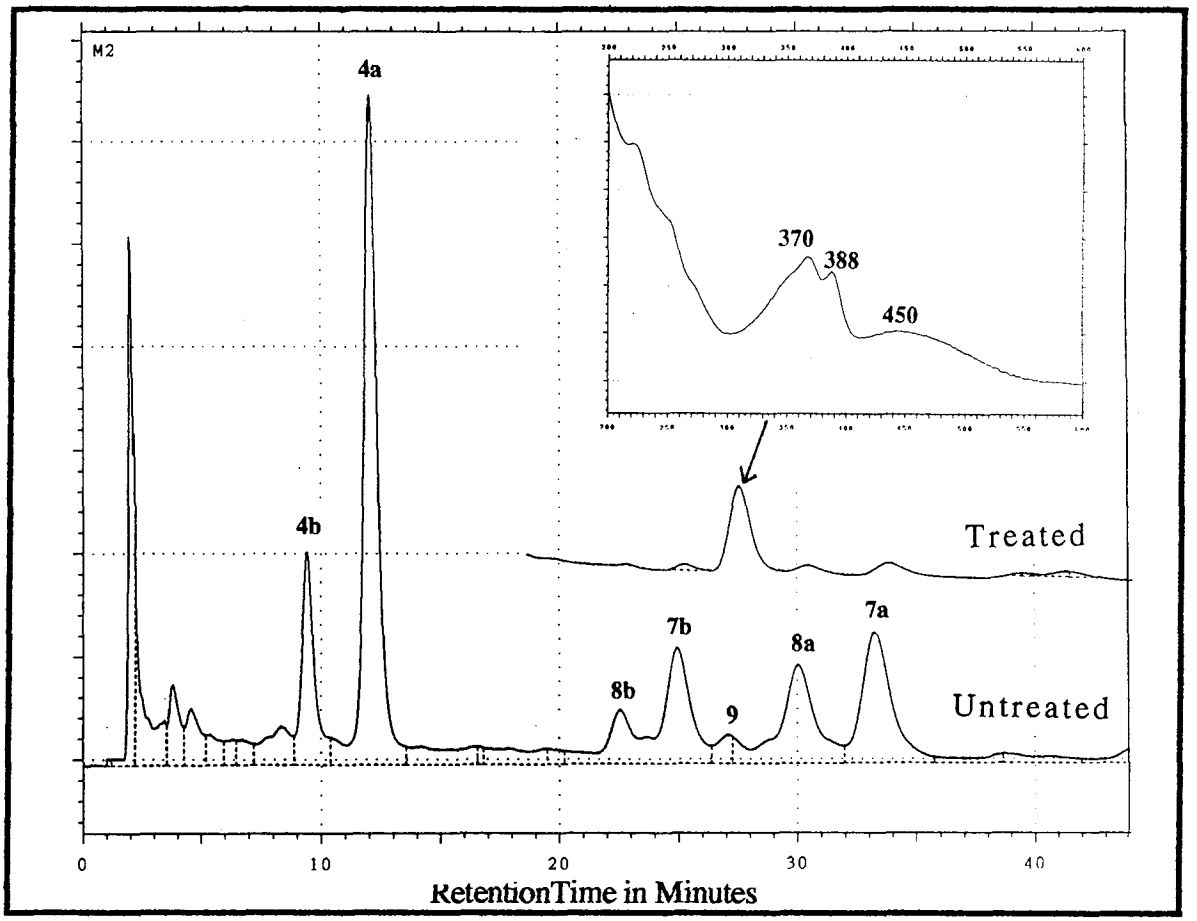

Figure 5.10 HPLC Traces of Untreated Versus Cultures Treated with Malic Acid and the UV-Vis Spectrum of Thiosulphinate **9**. Wavelengths are presented in nanometres.

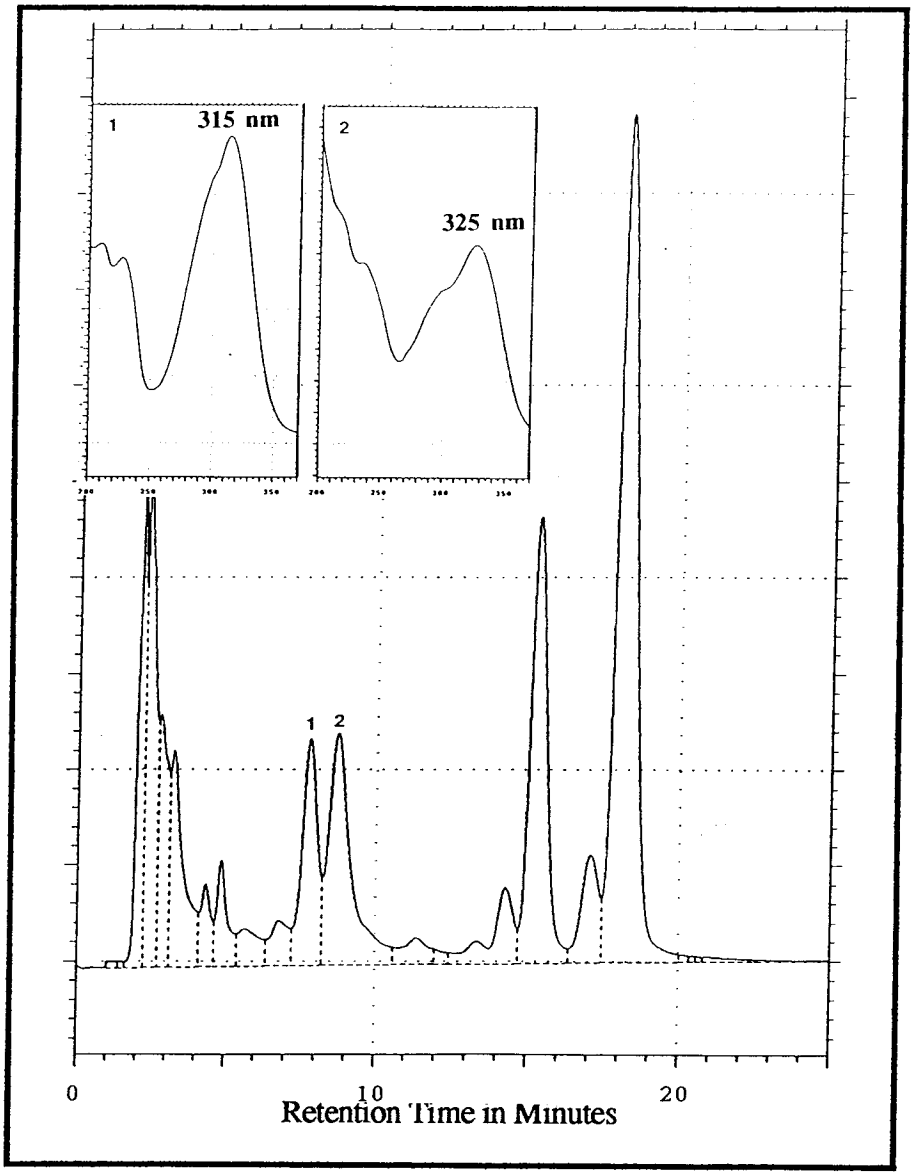

Figure 5.11 HPLC Trace of Extract from Malic Acid Experiment Representing Treated and Untreated Cultures with the "In flight" UV-Vis Spectra of Unknown Components.

Transgenic cultures generated with *Agrobacterium rhizogenes* (strain A4) from Type 2 Queen Charlotte Island plants exhibited the same chemical profile as the other cultured roots, except that the concentration of thiarubrine **2a** was higher than in the other cultures (30%). Subsequently thiarubrine **1a** was lower in contration (64%), but the rest were approximately the same: thiarubrine **3a** (1%), thiarubirne **4a** (3%), and thiarubirnes **7a** and **8a** (2%). The overall yield of thiarubrines was found to be 1.15 (±0.25) mg/g dry wt.

DISCUSSION

Tissue cultured roots, although a source of thiarubrines, did not yield large amounts. Enriching the growth medium with potential precursors, such as sulphate or malic acid, did not prove to be effective. This may be due to inaccessibility of sulphate or because the thiarubrines are being produced at their optimum concentration of this tissue system. Roots with secondary growth accumulated these chemicals in canals in the developing periderm. In the root cultures the canals were limited to primary tissues which may have reached their physiological capacity. Although the *Chaenactis* cultures in cross-section looked very similar to the *Ambrosia* root cultures the thiarubrine content of the former was twice that of the latter. Identification of the other constituents of the canal may reveal the reason(s) for this.

Since the TR7 strain of *Agrobacterium* contains only one piece of T-DNA it was not the absence of the genes encoding for the production of the opines which gave a nefative result in the opine detection procedure. Over time cultures may lose their ability to synthesize these compounds. The morphological characteristics of the culture and the fact that they grow on media devoid of growth regulators suggest that they are most probably transformed.

Croes *et al.* (1989) examined the role of auxin in root culture. Lateral branching of the roots is thought to be induced by high levels of endogenous auxin. Not only did the addition of high concentrations of IAA cause the production of root primordia, but they indicated that the agropine-type strains of *Agrobacterium* cause more branching in transformed tissue. This is not surprising since these strains introduce genes encoding for the production of IAA. The mannopine-type tested had much less in the way of secondary branching. The A4 and 15834 strains both exhibited more extensive branching than TR7, however, there was no difference in the overall production of thiarubrines. Croes *et al.* (1989) found that thiophenes accumulated in the root tips which had been severed indicating that auxin is important for the synthesis of thiophenes because this is where it accumulated. Theoretically roots which have lots of lateral branching should produce more of these compounds. Croes *et al.* (1988) indicated that the polyyne content

was highest in the roots tips. This does not appear to be true in light of the fact that the thiarubrine content of hairy roots transformed with TR7, a strain which does not cause the production of auxins and consequently does not have as extensive lateral branching, does not exhibit lower thiarubrine content that those transformed with strains which do encode for auxin synthesis.

Gomez-Barrios *et. al.* (1991) examined the thiarubrine production in the hairy root cultures of *Ambrosia artemisifolia.* Not only did they use the cultures to gain the thiarubrines completely assign ^{13}C NMR data they also verified the biosynthetic pathway suggested by Bohlmann (1973), which stated that C_{13} dithiacyclohexadienes are derived from C_{14} precursors.

Ambrosia chamissonis produces a wider array of the thiarubrines and therefore would be an interesting system to examine the biosynthesis of these compounds as well as to examine the inter-relationships between them.

One area of phytochemistry that is now being examined is the biotransformation of chemicals into more useful compounds through the use of tissue cultures. Many classes of chemical reactions are involved in the biotransformation of exogenous substrates by plant cell culture. These include: hydroxylation, oxido-reduction of ketones and aldehydes, reduction of the carbon-carbon double bond, glycosylation and hydrolysis. These processes are outlined in a review article by Suga and Hirata (1990). Suspension cultures have been traditionally the culture system of choice. These immobilized cultures have many advantages. Not only is sampling the culture over time easy, but addition of precursor and contact with virtually all cells in the system is accomplished. Suspension cultures of *Coffea arabica, Datura innoxia, Eucalyptus perriniana, and Nicotiana tabacum* fed (RS)-tropic acid, produced a number of glucosides and glucose-esters (Ushiyama and Furuya, 1988). Root cultures of *Panax ginseng,* using 2-phenolpropionic acid, produce glucose esters with high efficiency. Half of the product is excreted into the medium making this an ideal system for collection. Kawaguchi *et al.* (1990) demonstrated this by feeding *Panax ginseng* hairy root cultures digitoxigenin. Three esters and two glycosides were produced. In many cases glycosylation is desirable because it increases solubility of the product. Data are not yet available to compare the chemical output between suspension or callus culture systems and root

culture systems. It has become apparent that both systems have their merits. It may be that the systems will modify chemicals in different ways. In short, plant cultured cells have a high glycosylation activity for compounds with a small Mr and a small number of substituents. The use of tissue culture may allow one to produce difficult to synthesize chemicals.

Kennedy *et al.* (1993) compared the chemistry of roots with transgenic root cultures of *Artemisia absinthium.* This is not a reasonable comparison to make since the cultured roots are young and do not have secondary growth. A comparison should have been made between transgenic cultures and non-transformed root cultures. An interesting point which they did not address directly is the potential of root cultures as a system for determining where the components come from. In the plant resin canals often run through the roots and into the stem. The oil components may have different sites of synthesis. Culturing shoots and shoots separately may give some indication where the chemicals originate. Cultured roots are immature compared with the roots from normal plants. Esau (1977) points out that, in some members of the Asteraceae, cells of the endodermis may divide tangentially resulting in a double endodermis and that secretory canals can develop between the two-layers. This appears to be the case with *Ambrosia* cultured roots. However, more extensive investigation still needs to be done to clarify this.

CHAPTER SIX

ELICITATION WITH FUNGAL CELL WALL PREPARATIONS IN HAIRY ROOT CULTURES OF *AMBROSIA CHAMISSONIS*

INTRODUCTION

Secondary compounds are generally considered defensive in nature; the stresses of the environment can elicit their production. These stresses may induce the biosynthesis of novel chemicals (phytoalexins) or enhance the production of secondary metabolites already present in plant tissue. The phenomenon is known as elicitation and there are two major types of elicitor treatments: with biotic elicitors, which include preparations from pathogenic organisms such as bacteria and fungi, and abiotic treatments, which include the use of salts of heavy metals.

Tissue culture is a good system for studying elicitation of secondary metabolite production. Suspension cultures have been studied the most extensively. *Lithospermum erythrorhizon* suspension cultures, treated with a *Penicillium* preparation isolated from a contaminated culture, exhibited higher than normal levels of shikonin. The elicitor was prepared by grinding the *Penicillium* , autoclaving it, and then adding the product to the suspension culture. Elicited cultures which were extracted *in situ* had the highest levels of shikonin (Kim and Chang, 1990). Van der Heijen *et al.* (1988) examined *Tabernaemontana* species, which produce the indole alkaloid, apparcine, in suspension culture. Upon elicitation a number of new compounds were produced which exhibited antimicrobial activity, and thus, presumably function as defensive chemicals in the plant. Apparicine, the original compound of interest, was produced in low concentrations. This demonstrates the unpredictability of the natural products which will be produced upon elicitation.

The first consideration is the selection of a suitable elicitor preparation. In earlier work natural pathogens were used to elicite production of chemicals. It appears that effective candidates

are not necessarily restricted to these species. Conversely, not all elicitors will work on all systems. Schumaker *et al.* (1987), examining alkaloid production in *Escholtzia*, found the cell cultures would respond to elicitors derived from *Penicillium* and *Saccharomyces*, but not from *Phytophthora megasperma*. Munkandan and Hjortso (1990), on the other hand, examined thiophene production of *Tagetes patula* hairy root cultures treated with various fungal elicitors and found all species, including those not pathogenic in nature, enhanced the production of secondary metabolites. The second important consideration is the mode of application of the micro-organism to the culture system. Coculture has been employed, but since in most cultures, there is little if any physical defense against invading factors, the pathogenic species tend to overgrow the explant. A popular application utilizes complex preparations from the organism. The active components of many are unknown. Cell wall preparations are commonly performed by liberating the cell wall components through autoclaving, thus ruling out the role of enzymes. Whether the active components are naturally active or whether they are activated through heat is also not known. Treatment with cellulase and pectinase (derived from fungi) have also been shown to be active elicitors (van der Heijden *et al.*, 1988).

Elicitation of differentiated tissue, such as root and hairy root cultures, have only recently being examined. Fungal elicitors prepared from *Phytophthora* and *Pythium* have been shown to enhance the production of polyynes such as trideca-1,3,11-ene-5,7,9-yne in hairy root cultures of *Bidens sulphureus* (Flores *et al.*, 1988). Other species such as *Carthamus tinctorius*, *Bidens pilosus* and *Tagetes* also exhibited enhanced levels of polyynes after application of elicitator. Mukandan and Hjortso (1990) examined hairy root cultures of *Tagetes patula* and elicited thiophenes with preparations of *Fusarium conglutinans*. Thiophene formation varied although all were enhanced at elicitor treatments between 0.1 and 0.2 mg of glucose equivalents per ml of medium, especially bithienylbutinene (BBT). The most effective treatment was incubation with the elicitor for 48 hours.

Abiotic systems have also proved fruitful. Robbins *et al.* (1987) examined the production of the isoflavans, vestitol and sativan in HR cultures of *Lotus corniculatus* elicited with

glutathione. The activity of phenylalanine ammonia lyase (PAL) was enhanced at least three-fold over the elicitation period. Vestitol production was enhanced whereas, sativan was produced at low levels. The isoflavans accumulated in the medium as well as in the tissues. Robbins *et al.*, (1987) examined the response of tissue, of different ages, to elicitation and found that responses differed depending on age and on dosage of elicitor.

Recently Davies *et al.*, (1993) showed that a glycoprotein, isolated from a crude *Verticillium dahliae* preparation, elicited two different responses. In cotton and soybean cell suspension cultures, the protein component induced the production of phytoalexins, whereas the carbohydrate component caused the production of peroxide. This is one example of components of elicitor preparations exhibiting different chemical responses by the same plant tissue. How these responses relate to the plant as a whole is not known. It may be that responses to the different compounds are organ-specific.

Although the potential for the use of elicitors has been demonstrated, in some cases they do not enhance the production of the major characteristic secondary metabolites of a plant species or the compounds desired. For example, abiotic elicitors (copper and cadmium salts) increase the production of sesquiterpenoids rather than tropane alkaloids in *Datura stramonium* hairy root cultures (Furze *et al.*, 1991). This aspect of the elicitation phenomenon has been insufficiently explored and may actually lead to the development of "new" desirable compounds.

In this study, the elicitation of hairy root cultures of *Ambrosia chamissonis* was examined. Their superior growth to normal root cultures make them better candidates for the examination of the production of thiarubrine compounds. *Phytophthora megasperma*, a soybean pathogen, has been used successfully for the elicitation of thiophenes in various culture systems. Transgenic and non-transgenic root cultures, although producing thiarubrines and thiophenes, do not yield high concentrations of these potentially useful chemicals (Chapter Five). Elicitation offers a system to enhance these chemicals by presenting a "perceived" pathogen.

MATERIALS AND METHODS

Hairy Root cultures

Hairy root cultures were started from stem tissue inoculated with *Agrobacterium rhizogenes* strain A4 and established on solid SH medium (Chapter 4). Four to five root tips approximately 1 cm long were used to inoculate 40 ml of SH medium in each flask. Thirty-five 125 ml flasks were set up for each trial. The cultures were maintained in the dark at 23°C on a rotary shaker (100 rpm) for 26 days.

Elicitation with *Fusarium oxysporum* (FO) Elicitor

A preparation of elicitor from *Fusarium* was received as a gift from Dr. Carl Douglas. Four 25 day cultures were inoculated with 3ml of FO elicitor. The controls were given sterile distilled water. The cultures were incubated for 24 hours and then frozen, freeze-dried, extracted, and quantified as already outlined in Chapter Five.

Fungal Cultures

Phytophthora megasperma. f. sp. *glycinea.* cultures were obtained from Dr. Carl Douglas at the University of British Columbia. The oomycete was grown on solid V8 medium composed of 200 ml clarified vegetable juice, 3 g $CaCO_3$, 20 g agar, and 800 ml dH_2O. Once established, 500 ml of liquid medium in 2 L culture flasks, were inoculated with pieces of agar. The liquid medium was composed of 1.5% sucrose, 2 g/L asparagine, 0.2 g/L $MgSO_4 \times 7H_2O$, 10 g/L $CaCl_2 \times 2H_2O$, 1.04 g/L K_2HPO_4, 20 mg/L β-sitosterol, 3 g/L $CaCO_3$, 0.1 mg/L $FeSO_4 \times 7H_2O$, 0.1 mg/L thiamine-HCl, 0.1 mg/L $ZnSO_4 \times 7H_2O$, 0.02 mg/L $CuSO_4 \times 5H_2O$, 0.02 mg/L $NaMoO_4 \times 2H_2O$, 0.02 mg/L $MnCl_2 \times H_2O$ and the pH adjusted to 5.7 or 9.0. They were incubated at 23°C and harvested after 4 months of growth.

Elicitor Preparation

One hundred grams fresh weight of mycelia were harvested and washed in a Buchner funnel on 37 µm nylon mesh filter, with distilled H_2O until clear. The mycelium was rinsed with 1 L of 0.5 M NaCl, resuspended in 200 ml and homogenized in two portions in a Sorvall Omnimixer then filtered through 37 µm nylon mesh. The residue was resuspended in 500 ml Tris/EDTA (20 µM Tris/25 µM Na_2EDTA; 12.11 g Tris/46.52 g Na_2EDTA in 5 liters of dH_2O), homogenized, and then filtered. The filtrant was resuspended in 500 ml Tris/EDTA and stirred at 4°C for 18 hours. The slurry was then filtered and washed several times filtering under suction on 37 µm nylon mesh. The washing agents were: 1 L Tris/EDTA, 1 L dH_2O, 1 L $CHCl_3$:CH_3OH (1:1), and 1 L acetone. The residue was air-dried for 18 hours. This process yielded 8.8 g of mycelium preparation which was suspended in 880 ml dH_2O and autoclaved for 4 hours. After cooling the suspension was filtered through 37 µm nylon mesh and the filtrate was concentrated to approximately 1% volume under reduced pressure at 40°C. The concentrate was dialyzed against 4 L of dH_2O for 24 hours then was filtered through Whatman GF/A filter paper and centrifuged at 10,000 x g for 20 minutes. The supernatant was filtered through 0.45 µm membrane filters and freeze-dried. The elicitor was quantified in glucose equivalents determined by anthrone-glucose assay (Dische, 1962). This is a conventional way of expressing the amount of elicitor; it is not intended to imply that the active ingredients are carbohydrate in nature.

Elicitation

The elicitor was filter-sterilized and different concentrations (0.0, 2.5, 12.5, 25.0, 125, 500.0 and 1000.0 mg glucose equivalents) were added to the 26 day old cultures. There were four flasks set up for each treatment. The controls received sterile distilled water of the same volume (2 ml). The roots were harvested after 48 hours, rinsed with sterile distilled water, blotted dry and frozen at -70°C.

Extraction

The roots were freeze-dried, ground and extracted with CH_3OH:CHCN (7:1) overnight. The samples were filtered and total thiarubrine content for each was determined spectrophotometrically (refer to Chapter Two). The extracts for each treatment were combined and prepared for HPLC (refer to Chapter Two).

RESULTS

The cultures elicited with *Fusarium* exhibited no overall enhancement of thiarubrine production over the elicitor concentrations tested. In fact at 1000 mg glucose equivalents per flask the thiarubrine level was lower. Further studies using this elicitor were not pursued.

TABLE 6.1 Overall Thiarubrine Production of the Hairy Root Cultures Elicited with Pmg Elicitor.

TREATMENT (mg *glu E/flask)	OVERALL THIARUBRINE CONTENT (mg/g dry wt)	
	Trial 1	Trial 2
0.0	1.30 ± 0.07	0.92 ± 0.07
2.5	1.28 ± 0.06	0.90 ± 0.08
12.5	1.23 ± 0.20	0.93 ± 0.08
25.0	1.25 ± 0.05	1.01 ± 0.13
125.0	1.22 ± 0.14	0.78 ± 0.42
250.0	1.26 ± 0.08	0.88 ± 0.15
500.0	1.22 ± 0.16	0.87 ± 0.25

*glu E= glucose equivalents as determined by anthrone glucose assay (Dische, 1962).
Each trial was an average of 4 flasks, except for Trial 1 treatment with 1000 mg glu E which was three flasks. Error is expressed as Standard Deviation.

The overall thiarubrine yields did not vary substantially with the treatments of *Phytophthora megasperma* (Table 6.1). They did however show marked change in the chemical profile of the root cultures over the various treatments (Figures 6.1 and 6.2). Synthesis of thiarubrine **4a** was enhanced at what appeared to be the the expense of thiarubrine **1a**. The values for the thiarubrines with intermediate polarity were not expressed because their values were obscured due to peak overlap of another compound(s), as exhibited by the UV-Visible spectra.

The concentration of thiarubrine **1a** decreased as the concentration of elicitor increased. Thiarubrine **4a** on the other hand increased. In trial 2 at 250 mg Glu E/flask the concentration of **4a** fell. Upon inspection of the HPLC traces it was evident that there was a high thiophene **4b** concentration, implying that the lower values may have been due to degradation.

In all treatments including the control, two compounds previously undetected, were observed by HPLC, with CH_3CN:H_2O (72:28) at 1 ml/min, at 8.63 and 11.34 minutes. Their UV-Vis spectra are similar to the unknown polyyne mentioned in Chapter 1. The spectra are very simlar to those polyynes which have three triple bonds and one double bond according to Bohlmann (1973) (Appendix III). It probably has a hydroxyl function because it has the same polarity as the thiarubrine with the primary alcohol function. The production of the thiosulphinate is slightly elevated, whereas the concentration of the primary alcohols are lower with increasing concentration of elicitor. The minor thiarubrines and thiophenes are in such low concentrations that although they were detectable, but not quantifiable.

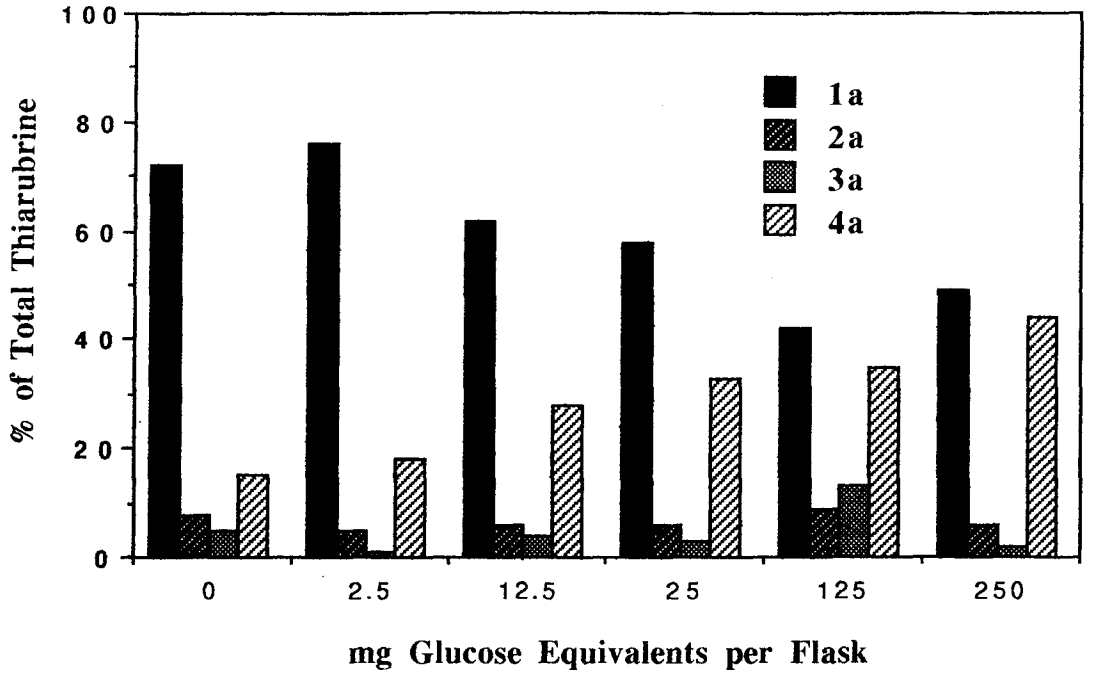

Figure 6.1 The Effect on Thiarubrine Profile of Hairy Root Cultures Elicited with Varying Concentrations of Pmg Elicitor-Trial 1.

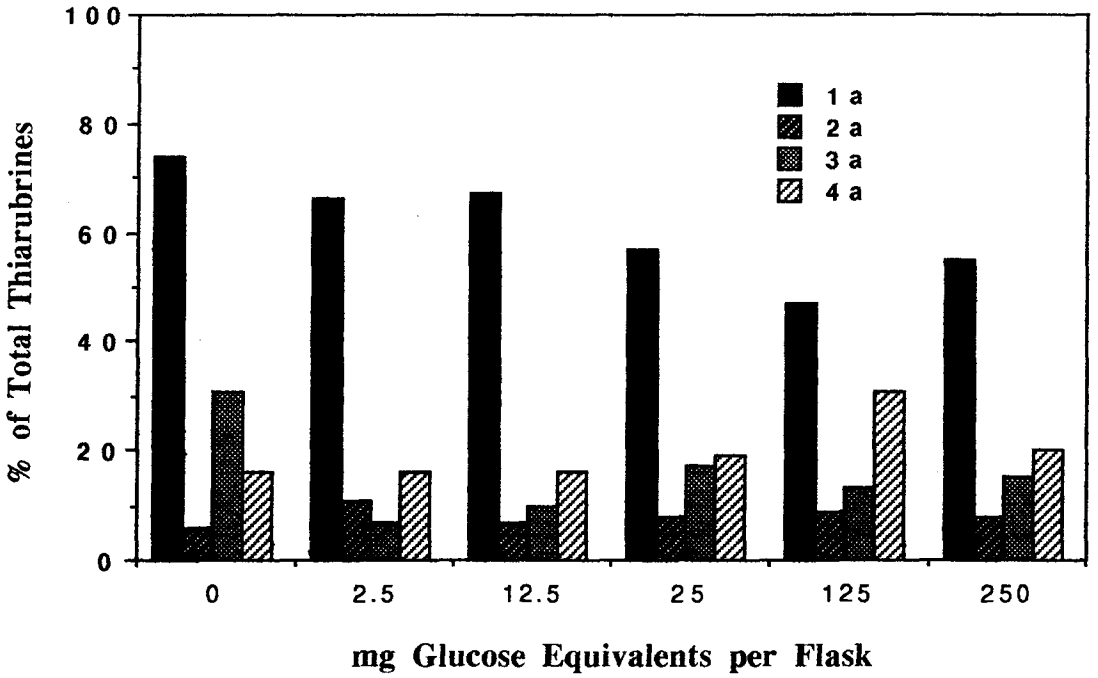

Figure 6.2 The Effect on Thiarubrine Profile of Hairy Root Cultures Elicited with Varying Concentrations of Pmg Elicitor-Trial 2

DISCUSSION

The thiarubrines appear to be biosynthetically related (Figure 2.13) eg. thiarubrine **4a** is synthesized through thiarubrine **3a** from **1a**. Elicitation results in the apparent conversion of biologically active thiarubrine **1a** into a related molecule (thiarubrine **4a**) which has a higher level of activity (Chapter 6). Thiarubrine **4a** is synthesized through thiarubrine **3a** from **1a**. This is a novel aspect of the elicitation phenomenon,

These results imply that the conversion process occurs in the thiarubrine canals themselves based on the fact that the amount of **1a**, already present in the canals, is reduced while **4a** is increased. Localization of the tissue response to an elicitor has not been examined. When applying a preparation there is little known about absorption by the cultured cells and subsequent transport to sites of chemical response. One benefit in using suspension cultures is that theoretically all cells are in contact with the medium and thus have the potential to respond. In root culture systems, however, the chemical production may be localized and therefore may rely on the translocation of the elicitor, or message, to the site. There has been very little investigation to compare suspension culture and root culture systems. Both systems have their merits and it may be that each produces a distinct array of compounds.

Elicitation not only shows potential for the enhancement of desired chemicals in tissue culture, but it also offers an experimental system to study the biosynthetic pathways and the molecular basis for the production of chemicals. As already stated suspension culture systems have been more extensively studied. *Phaseolus vulgaris* suspension cultures have been used to study the regulation of isoflavanoid biosynthesis (Dixon *et al.*, 1989). Dangl *et al.* (1987) developed protoplasts, which through elicitation with fungal cell wall preparations and UV light, excreted coumarins and flavonoid glycoside phytoalexins respectively, into the culture medium. They proposed this as a suitable system for analysing signal transduction and gene activation of the phenylpropanoid pathway from which these two classes of compounds biosynthetically arise. In this instance undifferentiated cultures have an advantage in their simplicity. For compounds, such

as the thiarubrines, which are particularly toxic to plant cells and must be sequestered into canals, differentiated tissues would be more suitable for studying biosynthesis.

The new polyynes had not been detected previously. It may be that the was probably induced by the use of a different incubator in this experiment. They may be precursors to the acetylenic compounds.

Although the potential of abiotic elicitors has not been explored in this study, they appear to be useful as a system for the enhancement of desired chemicals. They may also shed some light on the requirements of the plant in nature for synthesis of certain compounds. Corchete *et al.* (1991) demonstrated this when culturing *Digitalis thapsi* cells where he found that lithium enhanced digoxin production. The plant grows in acid soil with a high concentration of lithium and the results with cell cultures indicate that this metal plays an important role in the production of this compound. *Ambrosia chamissonis,* which grows in close proximity to the ocean, is exposed to many ions, whose concentrations vary over time. Such ions may be a key to the enhancement of thiarubrine production.

CHAPTER SEVEN

ANTIBIOTIC AND ANTIFUNGAL ACTIVITY OF THE THIARUBRINES

INTRODUCTION

Thiarubrine **1 a**, isolated from *Chaenactis douglasii,* displays antibacterial and antifungal activity, whereas thiophene **1 b** requires light for such activity (Towers *et al.* 1985). The thiarubrine exhibits enhanced toxicity in the presence of UV-A light against numerous bacteria via a complex mode of action (Constabel and Towers, 1989). It is not known whether it is the thiophene produced via photo-conversion or whether it is the process of conversion, which is responsible. Irradiation of the thiarubrine shows reduced toxicity to yeasts (Towers *et al.*, 1985). It was suggested that this is due to the light independent activity being greater than that of the phototoxicity of the corresponding thiophene. Towers *et al.* (1993) examination of the biological activities of other thiarubrines indicate that **3 a** and **4 a** are toxic at lower concentrations to the test organisms. Cytotoxicity tests, using mouse mastocytoma cells, show that the toxicity of these compounds on mammalian cells is less than that of thiarubrine **1 a** making these better candidates for medicinal agents.

There are different ways to assay for activity against micro-organisms. Disk-diffusion assays, also known as the Kirby-Bauer test, have been used extensively for the study of antibiotic and antifungal activities (Tortora *et al.*, 1989) and has been accepted by the Food and Drug Administation (FDA) as a method of testing for antibiotic activity. An extract is deposited on a paper disk which is placed on a thin lawn of the test organism. Activity is quantified by measuring the zone of growth inhibition around the disk. The problem with this method is that a large amount of the test compound is required and the results tend to be more qualitative than quantitative, since compounds may either diffuse differently or have different stabilities on the growth media.

Another method, the broth dilution test, requires less test compound. 96-Well Microtitek plates are used. Small amounts of compound can be applied and serially diluted. The test organism is applied and growth is detected by absorbance after a stated amount of time, allowing accurate measurements of activity to be made. This system has been applied to mammalian cytotoxicity testing where the test cells are usually cancer cells. This allows one to compare the dosage of the antibiotic required to kill a pathogenic organism with the toxicity to host cells. Mosmann (1983) established a technique using a tetrazolium salt to quantify colorimetrically the survival of mammalian cells and thus measure cytotoxicity. The cells are grown up and subjected to the tetrazolium salt which is cleaved by living cells. The formazan produced in the reaction precipitates, but can be solubilized with iso-propanol. Changes in pH dictate the color of the medium which interferes with absorbance readings of the blue MTT formazan measurement. This is remedied by converting the phenol red to yellow by acidification. Living cells will therefore cause the medium to turn yellow whereas the media in the wells with dead cells will be purple. The intensity of the yellow will reflect the number of living cells. Readings are taken on an Elisa plate reader using reference wavelength 600 nm.

The potency of a compound is quantified as the LC_{50}, which is defined as the lethal concentration of a chemical required to kill 50% of the test organism per specified unit of time. Quantification is done by plotting the concentration of the test compound along the x axis and the percentage mortality along the y axis on logarithmic probability paper, leaving space for, but not plotting the 0% and 100% mortality points (Litchfield and Wilcoxon, 1949). There should be at least two points above 50% mortality and two points below 50% mortality. A best-fit line is drawn through the points therefore representing the expected mortalities over the concentration range of the chemical. The value obtained at 50% mortality is called the LC_{50}.

Differences in the activities of thiarubrines may be due to the difference in stability. It has already been demonstrated that the diol is much more stable in aqueous solution and storage over

time (Towers *et al.*, 1993). Differences in pH may also dictate differences in stability which may be reflected in differing levels of biological activities.

The antifungal activity of thiarubrines is of particular interest because of the very low concentrations required to treat human pathogens, such as *Candida albicans* and *Aspergillus fumigatus* (Towers *et al.*, 1993, 1985). Thiarubrine **1a** is toxic, at very low concentration, to these organisms in the dark as opposed to bacteria for which light is required (Towers *et al.*, 1985) Much higher concentrations of compound was required for toxicity toward bacteria.

Comparisons of bioactivity of the thiarubrines against numerous organisms including gram-positive and gram-negative bacteria, a yeast and other fungi is presented. Most of these organisms are pathogenic to humans. The bacteria include *Pseudomonas aeruginosa*, an opportunistic pathogen causing infections which are difficult to treat in immuno-compromised patients. *Staphylococcus aureus* is of interest because it commonly causes infections particularly in the skin. *Escherichia coli*, long considered non-pathogenic, may cause serious infections. *Bacillus subtilis*, is considered non-pathogenic, but another species *Bacillus anthracis* is the causative agent of anthrax. The yeast *Candida albicans* causes diseases of the lungs, mouth, intestine and reproductive system. *Aspergillus fumigatus* causes a disease known as aspergillosis. This opportunistic fungus is a very serious problem to people with compromised immune defense systems. *Trichophyton mentagrophytes* and *Microsporum gypseum*, which are dermatophytes, cause ailments such as ringworm. Two imperfect fungi were also tested. These were *Fusarium trincticum.* and *Verticillium lateritium*, although considered plant pathogens, other related species have been implicated in mycotoxicoses. The activities of thiarubrines previously unpublished were compared ie. the chlorohydrins, the primary alcohols, and thiarubrine **2a.** Antibiotic assays are not presented for the thiophenes.

MATERIALS AND METHODS

Organisms for Antifungal, Antibacterial and Cytotoxicity Assays

Cultures of *Candida albicans* strain UBC #54 were obtained from the UBC culture collection. *Aspergillus fumigatus* (DAOM 150786) was received from the Canadian Collection of Fungus Cultures, Center for Land and Biological Resource Research, Agriculture Canada, Ottawa. *Pseudomonas aeruginosa* was received from Dr. Hancock, Microbiology Department, University of British Columbia. *Trichophyton mentagrophytes*, *Fusarium tricinctum*, *Verticillium lateritium*, and *Microsporum gypseum* were supplied by Dr. Robert Bandoni , Botany Department, University of British Columbia. *Staphylococcus aureus*, *Bacillus subtilis*, and *Escherichia coli* cultures were supplied by the Microbiology Department at the Univeristy of British Columbia.

Mouse mastocytoma P815 cells (ATCC TIB64) were maintained in fetal calf serum.

Chemical Preparation

All thiarubrines were purified by HPLC as outlined in Chapters Two and Four. They were quantified spectrophotometrically using the molar absorptivity for thiarubrine **1a** (ε=3000 at 490 nm) and resuspended in acetonitrile at a concentration of 0.1 mg/ml for antifungal and antibacterial tests and 1.0 mg/ml for mammalian cytotoxicity tests. Acetonitrile was determined to be non-toxic at the low levels attained in the bioasays All manipulations of the thiarubrines were done in dim light. α-Terthienyl was a gift from Zyta Abramowski (University of British Columbia), Gentamicin and Fungizone were purchased from Gibco, and Nystatin was supplied by Sigma.

Comparison of Antibiotic Activity of Thiarubrines using Paper Disk Assay

Cultures for Paper Disk Assays: Overnight liquid cultures of *Candida albicans*, *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, and *Pseudomonas aeruginosa* grown in Mueller Hinton (MH) medium were applied by swab to MH plates, except in the case of *C.*

albicans which was applied to Yeast Nitrogen Base (YNB) (Difco) and Sabouraud Dextrose (Difco) plates. Cultures of *Aspergillus fumigatus, Trichophyton mentagrophytes, Fusarium tricinctum, Verticillium lateritium* and *Microsporum gypseum* were established on YNB medium. Conidia were harvested and suspended in MH broth (~10^6 conidia/ml) and applied by swab to YNB plates.

Disk Assays: 1.0, 0.1, and 0.01 μg, of each test chemical and control antibiotics, were applied to sterilized paper disks (Schleicher and Schuell #740-E) and placed on the plates prepared with bacteria and fungal conidia. Control antibiotics were chosen depending upon test organism. Gentamicin was used in anti-bacterial studies, whereas nystatin was used for anti-fungal. α-Terthienyl was used for both. Six impregnated disks were placed on each plate. The bacterial plates and *C. albicans* were done in duplicate. After the cultures were allowed to equilibrate for 1 hour in a 37°C incubator one set of duplicate plates was placed under UV-A lights for 2 hours and then returned to the incubator. This permitted the compounds to diffuse prior to light treatment. The other fungal plates were not subjected to light, but grown at 23°C for varying incubation times. Measurements of all disk assays was done by measuring the zone of inhibition around the paper disk. All assays were done twice whereas for the *C. albicans* plates were done three times (except for the 1.0 μg, which were done once). Disk diffusion assays for activity against *C. albicans* on Sabouraud dextrose (SAB) and YNB media were compared. These assays were done three times in the dark and under UV-A light as already described.

Quantitative comparison of antibiotic activity of thiarubrines using microtiter plate assays.

Organism preparation: A 24 hour culture of *Candida albicans,* grown at 37°C in YNB medium was diluted to 2.5 x 10^4 cells/ml. *Aspergillus fumigatus* conidia were isolated from 48 hour cultures grown at 37°C and suspended in YNB and MH broth to 5 x 10^5 condia/ml. A 24 hour culture of *Pseudomonas aeruginosa,* grown at 37°C in MH broth, was diluted to 2.5 x10^4 cells/ml.

Broth dilution assay: Two hundred microlitres of MH medium were added to the first row (8 wells) of sterile microtiter plate (Falcon 3072 Microtest III™ Tissue Culture Plates) using a Titertek Multichannel Pipette. The second row was left empty, whereas 100µl were added to the rest of the wells. The test compounds were diluted in MH medium to 1 µg/ml for *C. albicans* and *A. fumigatus* assays and 2 µg/ml for *P. aeruginosa* assays in MH broth. Two hundred microlitres of test compound solution were added to each well of row 2. Two wells were set up for each compound. A series of 22 two-fold dilutions, starting with row two of the test samples, was accomplished over two plates. No test compound was added to the last row of either plate. The starting concentration was 500 ng/ml for testing against *C. albicans* and *A. fumigatus* whereas the starting concentration for *P. aeruginosa* was 1 µg/ml. One hundred microlitres of the conidia suspension were added to all wells except for the first row. Therefore the first row of each plate contained only growth medium (negative control), whereas the last row of each plate was devoid of test compound but reflected the normal germination of the conidia (positive control). The plates were incubated at 37°C in the dark.

C. albicans : Duplicate plates were set up. One with medium of pH 5.5 and the other with pH 7. After 24 hours the optical density (O. D.) was determined with the Titertek Multiskan at 620 nm. A cotton swab was used to take samples from the wells with the minimal inhibitory concentration for each compound and applied to YNB plates to determine if the compounds were fungistatic or fungicidal.

A. fumigatus : The O. D. at 620 nm of each solution in a test well was read 24 hours later to determine the germination of the spores and the development of the mycelia. After 48 hours the plates were examined and growth was recorded as a positive or negative for each concentration of test compound and the minimal inhibitory concentration was determined.

P. aeruginosa: Duplicate plates were set up. One set of plates was equilibrated in the incubator for 20 minutes and then irradiated for 25 minutes with UV-A light. Growth of the

bacteria was determined after 48 hours by examination of the plate and a rating of positive or negative for growth.

Cytotoxicity Tests

P815 mastocytoma cells were prepared at 10^6 cells/ml in fetal bovine serum. Fifty microlitres of fetal bovine serum were added to each well of a sterile microtiter plate (Falcon 3072 Microtest III™ Tissue Culture Plate) using a Titertek Multichannel Pipette except the first and second rows of wells. One hundred microlitres were added to the first row of wells. The test compounds were suspended in medium to 1µg /400 µl (preliminary trial) and 0.4 µg/400 µl (subsequent trials). One hundred microlitres were added to the second row of wells. The test samples, in 50 µl aliquots, were diluted in series from the second row onward except for the last row. Fifty microlitres of the cell suspension were added to each well from the second one onward including the final row. The initial concentration of the test sample was 1.25 µg/µl (preliminary trial) and 0.5 µg/ml (subsequent trials). The final row was inoculated with cells, but devoid of test compound (positive control). The plates were incubated for 22 hours at 37°C. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was dissolved in 5 mg /ml of PBS and filter sterilized. Ten microliters were added to each well and the plates were incubated at 37°C or 2 hours. One hundred microliters 0.04 N HCl in isopropanol was added to each well and mixed thoroughly. The plates were read on a Titertek Multiskan at 620 nm.

LC_{50} Determination

The absorbance gleaned from the Titertek Multiskan is used to calculate the % survival which can be extrapolated to get % mortality values. Quantification is done by plotting the concentration of the test compound along the x axis and percentage mortality along the y axis on logarithmic probability paper. A straight line was drawn through the points therefore representing

the expected mortalities over the concentration range of the chemical. The LC_{50} value is taken where the line intersects 50% mortality.

All bioassays were repeated at least twice. All manipulations of the test compounds were performed in dim light.

RESULTS

Paper Disk Assays

Pseudomonas aeruginosa was not susceptible to the compounds at the concentrations tested using the disk-diffusion assay, therefore the data are not presented. The susceptibility of the other bacteria and *C. albicans* is presented in Table 7.1. The bacteria tested exhibited susceptibility when irradiated with UV-A light, the gram-positive bacteria (*B. subtilis* and *S. aureus*) having the largest clearing zones. In the dark, *E. coli* was very resistant to all thiarubrines except thiarubrine **3a**. This activity was enhanced slightly by light. The other thiarubrines exerted activity only after treatment with UV-A light. Thiarubrines **1a** and **2a** were toxic at lower concentrations than the other thiarubrines to *B. subtilis*.

On YNB medium at lower concentrations (0.01 µg/disk) the less polar thiarubines **1a** and **2a** exhibited no activity against *Candida albicans* in the dark. The other thiarubrines demonstrated activity at this concentration having similar activity ie. clearing zones were approximately the same size. At higher concentrations (0.1 µg/disk and 1.0 µg/disk) all the thiarubrines were active. On YNB plates the activities, for all concentrations tested, of thiarubrines **1a** and **2a** were enhanced when irradiated, whereas the activities were reduced for the other thiarubrines. Thiarubrines **1a** and **2a** exhibited larger clearing zones. The initial results using YNB medium (Table 7.1) contradicted those of Towers *et al.* (1985) who observe less activity of thiarubrine **1a** when irradiated. Therefore Sabouraud (SAB) medium, which they used, was tested (Table 7.2). There was reduced activity of thiarubrines **1a** and **2a** after irradiation with UV-A.

Table.7.1 Disk-Diffusion Assay to Determine the Relative Toxicities of Thiarubrines to Microorganisms in Dark and 2 hr Exposure to UV-A Expressed as Zones of Growth Inhibition (mm).

Thiarubrine (μg/disk)		*B. subtilis*		*S. aureus*		*E. coli*		*C. albicans*	
		Dark	UV-A	Dark	UV-A	Dark	UV-A	Dark	UV-A
1a	1.0	+/+	25.0±0.0	+/+	19.0±4.0	-/-	11.5±0.5	32*	35*
	0.1	-/-	12.5±1.5	-/-	11.5±1.5	-/-	-/-	18.7±1.9	22.7±5.7
	0.01	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	-/-	16.3±5.6
2a	1.0	+/+	20.0±0.0	+/+	14.5±1.5	-/-	9.5±0.5	31*	34*
	0.1	-/-	10.0±0.0	-/-	9.5±1.5	-/-	-/-	17.3±2.5	23.7±3.3
	0.01	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	-/-	15.0±4.1
3a	1.0	+/+	8.5±1.5	8.0±0.0	9.5±0.5	8.5±0.5	9.0±0.0	29*	20*
	0.1	-/-	-/-	-/-	7.5±0.5	-/-	-/-	21.3±1.9	17.3±2.1
	0.01	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	15.7±0.9	12.0±0.8
4a	1.0	+/+	9.5±0.5	+/+	9.0±0.0	-/-	+/-	30*	15*
	0.1	-/-	-/-	-/-	+/+	-/-	-/-	22.0±1.0	12.7±1.2
	0.01	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	14.3±0.9	9.7±1.2
5a	1.0	+/+	10.0±0.0	+/+	10.0±2.0	-/-	+/+	n.t.	n.t.
	0.1	-/-	-/-	-/-	8.0*	-/-	-/-	16.0±3.7	12.3±0.9
	0.01	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	10.7±2.7	9.7±0.5
6a	1.0	+/+	10.0*	+*	12.0*	-/-	+*	26*	19*
	0.1	-/-	-/-	-/-	9.0*	-/-	-*	20.0*	15*
	0.01	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	13.0*	10*
7a	1.0	+/+	10.5±0.5	+/+	10.5±0.5	-/-	+/+	25*	17*
	0.1	-/-	-/-	-/-	8.0±0.0	-/-	-/-	18.0±1.6	13.3±1.7
	0.01	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	12.7±0.9	10.0±0.8
8a	1.0	+/+	9.5±0.5	+*	9.5±0.5	-/-	+/-	30*	15*
	0.1	-/-	-/-	-/-	+/+	-/-	-/-	21.0±1.0	15.0±0.0
	0.01	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	12.7±1.2	10.3±0.9
Gent	1.0	13.5±1.1	11.5±1.1	13.5±1.5	16.5±0.5	11.0±2.0	12.0±0.0	8* (N)	8* (N)
	0.1	-/-	-/-	-/-	+/-	-/-	-/-	-/- (N)	-/- (N)
	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/- (N)	-/- (N)
α-tert	1.0	-/-	29.5±0.5	-	24.5±0.5	-/-	-/-	-*	20*
	0.1	-/-	15.0±1.0	-	15.5±0.5	-/-	-/-	-/-	10±0.5
	0.01	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	-/-	9.0±1.0

Values were averages of two bioassays except *C. albicans*, which was tested four times, and those values with "*" which were tested only once. Standard deviations are presented.

"+" or "-" values represent activity which was detected but not measurable.

(N) indicates the antibiotic tested was nystatin instead of gentamicin

Gent=gentamicin, α-tert=α-terthienyl, n.t.=not tested

Table 7.2. Disk-Diffusion Assay to Determine Antimicrobial Activity of Thiarubrines Against *C. albicans* on on Different Media in Dark and 2 hr Exposure to UV-A, Expressed as Zones of Growth Inhibition (mm).

Thiarubrine (1µg/disk)	YNB		Sabouraud (Difco)		Sabouraud*	
	Dark	UV-A	Dark	UV-A	Dark	UV-A
1a	30.3±1.7	35.3±2.7	33.5±3.5	31.5±0.5	32.3±1.4	29.8±1.6
2a	27.5±1.8	33.3±2.4	33.5±1.5	31.5±0.5	31.8±2.1	29.3±2.4
3a	23.5±1.5	18.5±2.1	23.5±1.5	18.0±0.0	23.7±1.7	17.0±2.5
4a	25.0±0.0	14.7±0.9	24.0±0.0	14.0±0.0	22.7±0.5	13.0±1.0
5a	n.t.	n.t.	19.5±0.5	14.0±0.0	20.0±0.0	13.0±0.5
7a	n.t.	n.t.	20.0±0.0	14.5±0.5	20.0±0.0	13.5±0.5
α-Terthienyl	-/-/-	20.0±0.1	-/-/-	15.0±0.0	-/-/-	15.0±0.0

Variation is given as standard deviation, n=3.

Table 7.3 Disk-Diffusion Assay to Determine the Dark Toxicities of Thiarubrines to Fungi Expressed as Zones of Growth Inhibition (mm).

Thiarubrine (µg/disk)		*F. tricinctum*	*V. lateritium*	*M. gypseum*	*T. mentagrophytes*
1a	0.1	15.0±0.0	8.8±2.5	25.0±5.0	›30/›30
	0.01	-/-	-/-	-/-	14.0±1.0
2a	0.1	15.0±0.0	8.8±2.5	22.5±7.5	›30/›30
	0.01	-/-	-/-	-/-	12.0±3.0
3a	0.1	15.0±0.0	12.0±0.0	27.5±2.5	›30/›30
	0.01	-/-	+/-	-/-	23.5±1.5
4a	0.1	20.0±0.0	15.5±0.5	25.0±5.0	›30/›30
	0.01	-/-	+/-	-/-	11.5±0.5
5a	0.1	14.0±1.0	13.0±2.0	25.0±5.0	›30/›30
	0.01	-/-	+/-	+/-	15.5±0.5
7a	0.1	15.5±0.5	14.5±0.5	26.5±6.5	›30/›30
	0.01	-/-	+/-	+/-	16.5±1.5
8a	0.1	17.5±1.5	12.5±0.5	25.0±5.0	›30/›30
	0.01	-/-	+/-	-/-	13.5±1.5
Nystatin	0.1	15.0±0.0	12.0±0.0	28.0±2.0	›30/›30
	0.01	8.0±0.0	-/-	12.0±2.5	14.4±0.5

Incubation times are given in brackets. Variation is given as standard deviation, n=3.

In Table 7.2 the toxicity of the thiarubrines against *C. albicans* was compared on YNB and SAB media. The more polar **3a** and **4a** did not respond differently on the two media whereas **1a** and **2a** differed in their responses to light. On YNB, as indicated in Table 7.1, the activity of these thiarubrines was enhanced upon irradiation. On SAB there was a reduction in activity like the rest of the compounds tested. α-Terthienyl was active only in the light.

The chemicals were toxic toward all of the fungi tested (Table 7.3). They had similar activity to nystatin as indicated by the clearing zones. The compounds were especially active against *T. mentagrophytes*. The chemicals are tested on the same medium which means differences in activity toward the different organisms tested, can be attributed to differential toxicity toward these organisms.

The cytotoxicity test on the P815 mastocytoma cells were initially performed at high concentrations of test compounds to find out what the concentration range of the tests should be. Several trials were performed, but due to problems with cell growth, calculations of LC_{50} were presented for one run for which cell growth was good. These data are presented in Table 7.4. When plotting the LC_{50} curves the slope of the line for thiarubrine **1a** was shallow compared to the others. Thus, there is a bigger difference between the MIC and the LC_{50} for thiarubrine **1a** as compared with the others. Other thiarubrines, such as **4a**, **5a** and **6a** have steeper slopes and therefore the MIC and the LC_{50} are closer in value.

Preliminary studies were performed to establish the duration of incubation and the pH of the medium for broth dilution test on *C. albicans*. The LC_{50} was evaluated at 24 hours and at 48 hours for activity against *C. albicans* in YNB medium at pH 5.5 and pH 7.0. At 24 hours there was a much lower LC_{50} for the compounds in pH 7.0 than 5.5 probably due to a longer lag period in the growth at that pH. 48 hour incubation time was chosen as the optimum incubation time because it gave the best cell growth. These results are presented in Table 7.4. Two trials were carried out and data from both are presented. The samples from the wells which did not exhibit

Table 7.4 Comparison of the LC_{50} and Minimum Inhibitory Concentrations (MIC) For P815 and *Candida albicans*.

Thiarubrine	LC_{50} and MIC During 24 hr Exposure in ng/ml			
	P815 (24 hours)		*C. albicans* (48 hour)	
	LC_{50}	MIC	LC_{50} trial 1 / trial 2	MIC trial 1 / trial 2
1a	8.0	500.0	50.0 / 50.0	500.0 / 250.0
2a	63.0	500.0	35.0 / 90.0	500.0 / 500.0
3a	37.0	250.0	7.0 / 16.0	250.0 / 62.5
4a	9.0	31.3	1.3 / 3.5	7.8 / 7.8
5a	70.0	250.0	0.5 / 4.0	15.6 / 15.6
6a	16.0	62.5	3.5 / 7.0	31.3 / 31.3
7a	60.0	250.0	0.8 / 7.8	7.8 / 31.3
8a	21.0	125.0	0.3 / 6.0	7.8 / 15.6
Fungizone	-	>500.0	35.0 / 37.0	160.0 / 160.0

Table 7.5 Toxicity Indices of Thiarubrines for P815 and *C. albicans*.

Thiarubrine	$\frac{LC_{50}P815}{LC_{50}\ C.\ albicans}$	$\frac{MIC\ P815}{MIC\ C.\ albicans}$
1a	0.16	1.00
2a	1.00	1.00
3a	3.22	1.60
4a	3.75	4.00
5a	31.10	16.03
6a	3.04	2.00
7a	13.95	12.79
8a	6.67	10.68

growth (MIC) when spread on nutrient medium, did not resume growth, indicating that the test compounds are fungicidal rather than fungistatic. Toxicity indices calculated using the LC50 and MIC values are presented in Table 7.5. The values for P815 cells are divided by the *C. albicans* values.

Table 7.6 Broth Dilution Assay to Determine Minimal Inhibitory Concentration (MIC) for (A) *P. aeruginosa* in the Dark vs. 2 hr Exposure to UV-A Light and (B) *A. fumigatus* for two Different Media.

Thiarubrine	MIC During 48 hr Exposure in ng/ml			
	(A) *P. aeruginosa*		(B) *A. fumigatus*	
	dark	UV-A	YNB	M-H
1a	>500	>500	250	250
2a	>500	>500	500	250
3a	>500	>500	500	500
4a	>500	>500	125	250
5a	500	>500	125	250
6a	500	>500	125	500
7a	>500	>500	250	125/250*
8a	>500	>500	125	250
Gentamicin	>500	>500	250	500

All tests were done twice. Differing results are presented*. A value of >500 means that there was no activity at 1000 µg/µl, but there was at 500 µg.

P. aeruginosa and *A. fumigatus* were not susceptible to the test compounds except at very high concentrations (Figure 7.6). There was a slight enhancement of activity with light for *P. aeruginosa.* The *A. fumigatus* was tested on two different media. MH media is a basic medium for bacteria and was devloped for toxicity testing.

DISCUSSION

In the disk-diffusion assays the thiarubrines did not exhibit toxicity toward *P. aeruginosa* in either light or dark, whereas *E. coli* was susceptible only after exposure to UV-A light except in the case of thiarubrine **3a** which was also active in the dark. The gram-positive bacteria, *S. aureus* and *B. subtilis,* were susceptible in light, and to a lesser degree, in the dark. The differences between gram-negative and gram-positive bacteria are based on the presence in the former of a more complex cell wall. Gram-negative bacteria are generally more resistant than gram-positive to antibiotics, which is not surprising since most antibiotics act on the bacterial cell wall. Towers *et al.* (1985) found that thiarubrine **1a** was more toxic to the yeasts *S. cerevisiae* and *C. albicans* in the dark. The present study reveals that light activity is dependent on the medium used when testing. The larger clearing zones for thiarubrines **1a** and **2a** indicate that these compounds may be more soluble in the growth medium. At lower concentrations, however, thiarubrines **1a** and **2a** exhibited no activity toward *C. albicans*, whereas the other thiarubrines were active in both light and dark implying that the clearing zone is not a good indicator of potency.

It has already been indicated that the medium is very important when testing chemicals. Some media nullify the effects of antibiotics. Hoeprich and Huston (1976) tested numerous antifungal compounds, using four types of media. SAB and YNB were found to exhibit different MIC's depending on the compound tested. They found that the synthetic formulations (YNB and Synthetic amino acid medium) did not antagonize the activity of miconile or clotrimazole, whereas SAB and brain-heart infusion, which are undefined media, did. Hoepich and Finn (1972) suggested that the presence of pyrimidines and pyrimidine ribosides, in the medium, is at least partially responsible for this.

The results for *C. albicans*, in the broth dilution assays, were presented in two trials rather than averaging because some of the values varied for some chemicals tested. Nonetheless it is evident that the more polar compounds are active at lower concentrations than the more non-polar

1a and **2a**. It must also be kept in mind that the compounds are tested in serial dilution which would have a larger difference in the test concentrations at the higher concentrations, which accounts for the large differences in these values. To gain more accurate MIC values for thiarubrine **1a**, for example, one would have to set up tests for concentrations between 250 and 500 mg/ml. The purpose of this paper is to emphasize the potential of the more polar ones.

For a compound to be a potential drug the LC_{50} and the MIC values for a cytotoxicity test, such as the P815 assay, should be higher than those for the pathogen (*C. albicans*) This means that the low levels required to kill the yeast will have little effect on the host cells. Thiarubrines **5a**, **7a**, and **8a** are the best candidates form this point of view as demonstrated by the toxicity indices presented in Table 7.5. Thiarubrine **5a**, for instance, can be applied in a concentration 1/31 a dosage which is lethal to 50% of the mammalian cells, whereas the dosage of thiarubrine **1a** required to kill 50% of the *C. albicans* is lethal to the mammalian cells. Both the MIC and the LC_{50} calculations are important as is demonstrated for thiarubrine **1a**, where the LC_{50} was much lower than the MIC. There is toxicity over a wide range of concentration as opposed to a compound such as thiarubrine **5a**, in which the mortality curve, when calculating the LC_{50}, was very steep, indicating there is a critical concentration where the compound is toxic and therefore the MIC and LC_{50} are relatively close.

The mechanisms of action of the thiarubrines are not known, although it has been suggested that the disulphide ring is important to biological activity (Constabel and Towers, 1989c). They exhibit activity in the light and in the dark. The light requiring toxicity is probably partially due to the toxicity exerted by the thiophene molecule which is produced after exposure to light. Some of the toxicity may result from the conversion process itself. Antimicrobial compounds have numerous modes of action. Some drugs inhibit the synthesis of the cell wall (eg. vancomycin) whereas others cause injury to the plasma membrane (eg. nystatin). Protein and nucleic acid syntheses can be inhibited as well as enzyme activity.

There is evidence that phototoxicity of thiophenes is due to the production of singlet oxygen and subsequent oxidation of lipid components of membranes (McRae *et al.*, 1985). There has been some work to determine the mode of action of these compounds. Two well-studied compounds of acetylenic origin α-terthienyl and phenylheptatriyne (PHT) have been examined for their activity. It was originally believed that the thiophene was oxygen dependent in its activity whereas PHT was active aerobically and anaerobically (Weir *et al.*, 1984). Kagan *et al.* (1984) found that upon irradiation, PHT was a good singlet oxygen producer. This activity was shown to require oxygen. It may be that the procedures employed by the former group did not accomplish an anaerobic environment. Gong *et al.* (1988) examined the effect of PHT on red blood cells irradiated with near UV light. They found that again there was a dependence on oxygen for toxicity. The membrane proteins were established as the targets.

Constabel and Towers (1989c) examined the structural characteristics, of the thiarubrines, which contribute to the biological activity by comparing the activity of thiarubrine **1 a** with that of thiarubrine **11 a**. The difference in these molecules is the triple bond of the former is replaced by a double bond in the latter. There was no difference in the light-independent activities. They concluded that the disulphide ring is the most important feature for activity of the molecules. The mode of action of the dark reactions as well as the light reactions is not known. Thiarubrine **1 a** has been shown to have light-requiring anti-viral activity (Hudson, *et al.*, 1987). Membrane viruses are more susceptible indicating that the target may be the membrane.

Useful antimicrobial drugs must possess a number of properties. The most important being selective toxicity. The compound should be toxic to the pathogen and exhibit little or no toxicity toward the host. Because bacteria and eucaryotic cells differ so much in composition bacteria are generally easier to treat selectively than eucaryotic cells. Fungi, on the other hand are very similar to higher organisms at the cellular level and therefore may be more difficult to control without negative effects to the host. The second criterion is that the drug must be soluble in body fluid. This problem has slowed the development of the drug taxol for the treatment of cancers.

Due to its low solubility a large quantity of carrier solution has been required for its application which is itself toxic. Thirdly, the drug must be stable in both the body and on the shelf. These represent only a few of the many important features.

It has been demonstrated that thiarubrines have great potential as antifungal and antibacterial agents. A fundamental problem with evaluating them, based on bioassay results, is that the conditions may not reflect the physiological environment of application. It is very important, when testing these compounds, to employ bioassays which are appropriate. When examining chemicals for medicinal activity the experimental conditions should reflect those in which the drug would be administered, as well if one is studying the activity of these compounds in nature it is best to mimic the environment of the chemicals.

Tested independently these compounds demonstrate a high level of toxicity to a number of organisms. It is important to keep in mind that in the natural system they co-occur not only with each other, but also with other compounds. There is little doubt that these compounds have a defensive role; the question is how do they operates in the plant. Toxicity in nature often infers that there is some potential against pathogens in other systems. There is an extensive amount of screening chemicals must go through before they will be accepted as medicinal agents. Initial screenings on crude samples are important. This is best accomplished by quick and easy tests such as disk-diffusion testing. Fractionating must be done to determine what the active components are.

CHAPTER EIGHT

GENERAL SUMMARY

It is important to emphasize the potential of the plant root as a chemical factory for the production of medicinal compounds. Roots, for the most part, have been under-utilized as a source of interesting and useful chemicals and often they are important sources of novel drugs, eg. ginseng. In these times of concern for the conservation of our forests and plants in general, tissue culture has potential as a source of plant-derived pharmaceuticals. Unexpectedly, a novel thiarubrine and thiophene were isolated from roots and root cultures, making it possible to explore spectroscopic techniques to identify them. *Ambrosia chamissonis* produces a family of these sulphur heterocyclic compounds with dramatic antifungal and antibacterial activities. There is little known about the biosynthesis of these thiarubrines and thiophenes. Data with elicited cultures suggest that at least the conversion from one thiarubrine to another (**1a** to **4a**) takes place in canals. It is possible the synthesis of the basic structure of thiarubrine **1a** also occurs here. Although there are many modifications to **1a**, thiarubrine **2a** is present without related derivatives. Many members of the Asteraceae produce only thiophenes such as *Echinops,* which produce an extremely wide array of thiophenes based on the structure of thiophene **2b**, without the presence of the thiophene **1a** type.

Canals containing the thiarubrines can be seen throughout the plant. Anatomical and chemical investigations showed the canals to contain an oil in which the active thiarubrine and thiophene occur. The oil appears to have a stabilizing effect on the these light-sensitive compounds, as indicated by their presence in parts of the plants exposed to sunlight eg. leaves.

Studies on the bioactivity against numerous pathogenic fungi and bacteria indicated that these compounds differ in their activities. Thiarubrine **1a** which was the first to be examined, in some cases was more potent against yeasts. It was demonstrated that because of their high toxicity to mammalian cells (presented as toxicity indices) it has little potential as a therapeutic agent,

whereas thiarubrines **5a** and **7a** are less toxic to mammalian cells at the doses required for treatment of the pathogen. Thiaubrine **3a** displayed antibacterial activities that the other thiarubrines did not exhibit. How these compounds interact with the test organisms remains unknown. Antimicrobial activity suggests that these chemicals have a defensive function in nature, but again this is an area which requires much more investigation.

Elicitation of root cultures with a fungal pathogen preparations did not result in the elevation of the thiarubrine level as was expected. Instead, thiarubrine **4a** was synthesized presumably from thiarubrine **1a**; the former being more fungicidal at lower concentrations than the latter. This study demonstrates that tissue culture may be a very useful source of antibiotic phytochemicals and indicates that through modification of growth conditions, improvements in chemical yields may be achieved. *Ambrosia chamissonis* with its spectrum of thiarubrines and thiophenes, is a good subject for biosynthetic studies of these chemicals about which so little is known.

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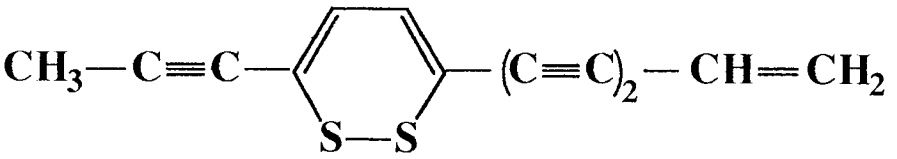
APPENDIX I: Thiarubrines and Their Corresponding Thiophenes

1a



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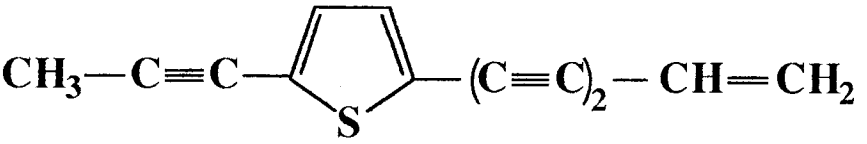
1b



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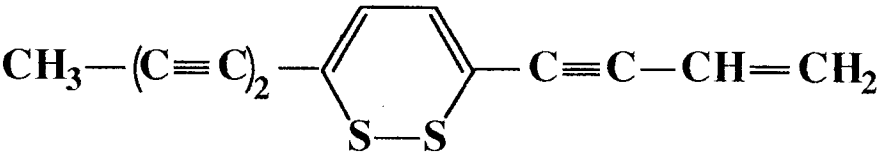
2a



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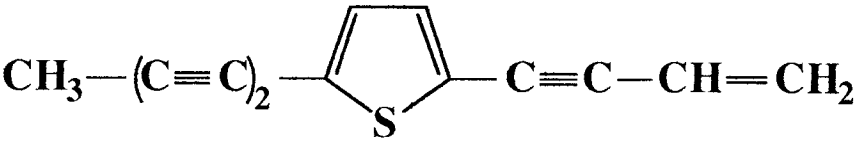
2b



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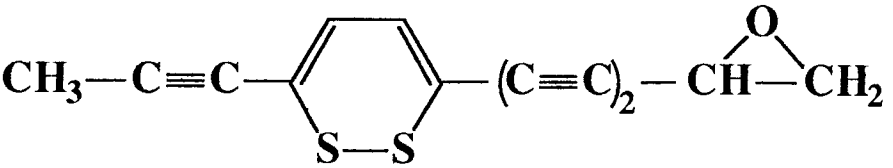
3a



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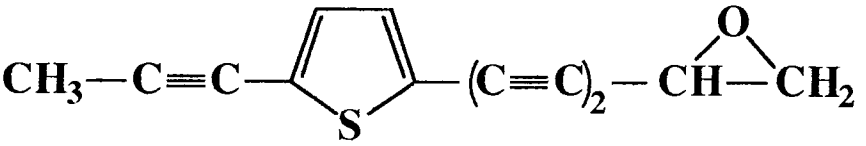

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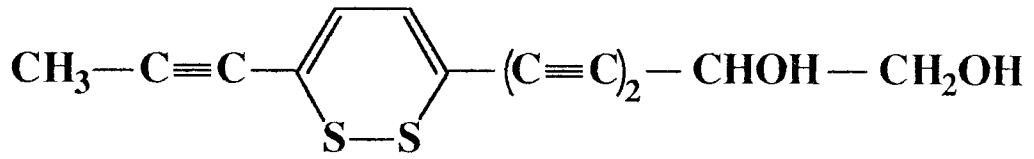

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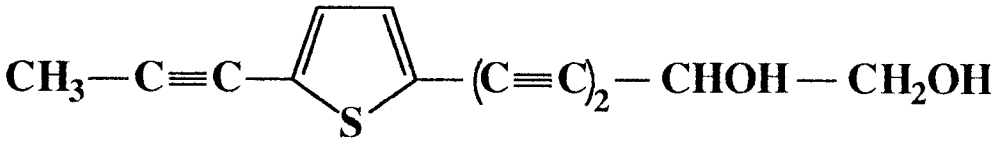

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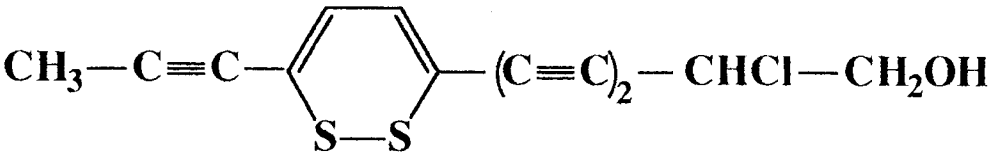

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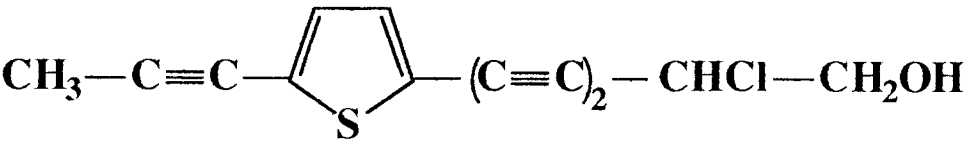
5b



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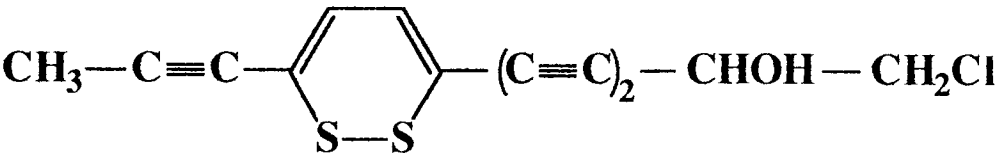

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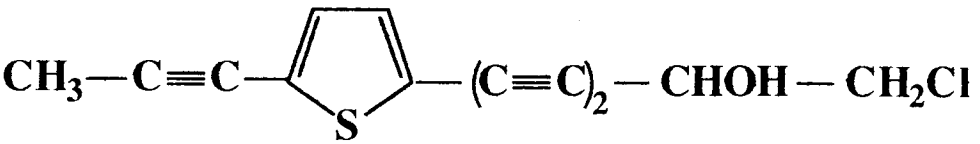

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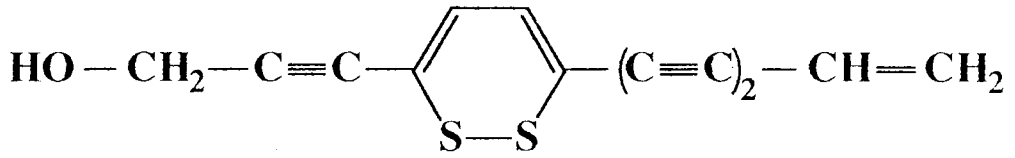

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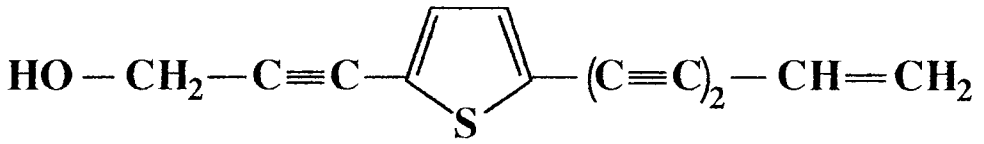
7b



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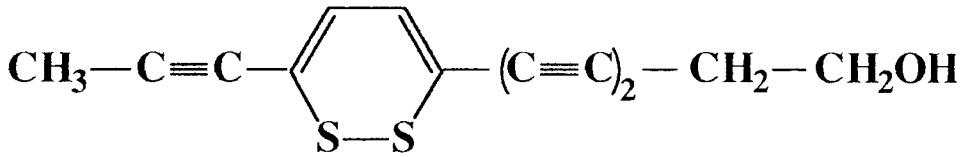

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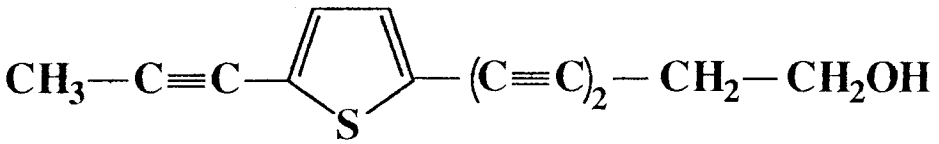

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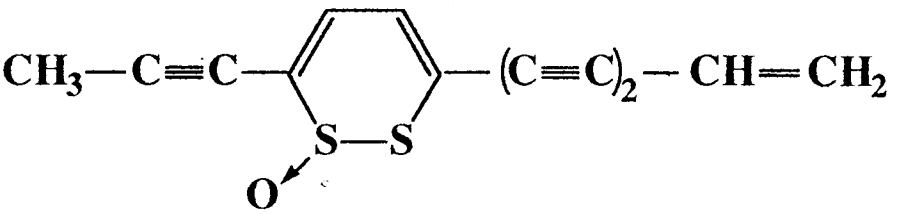
9



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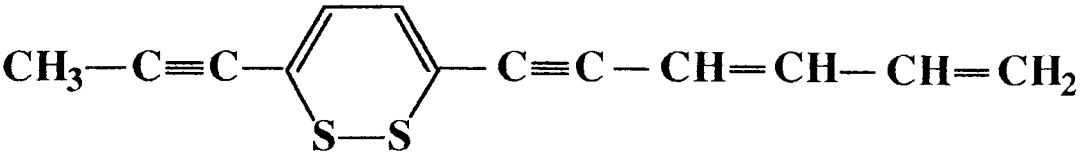
10a



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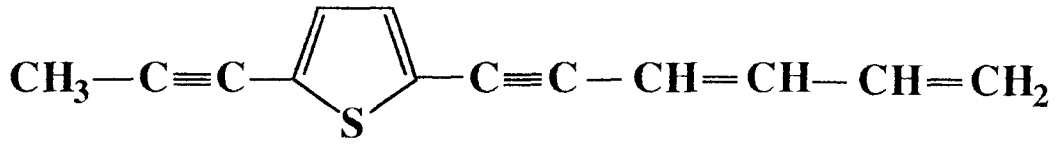
10b 

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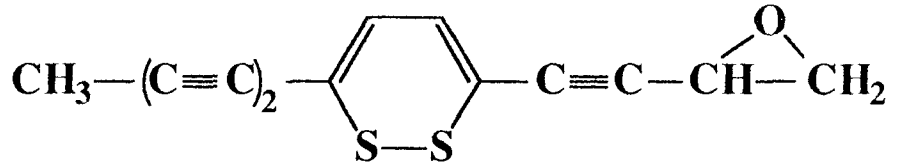
11a 

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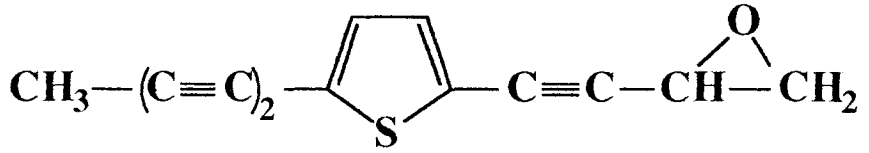
11b 

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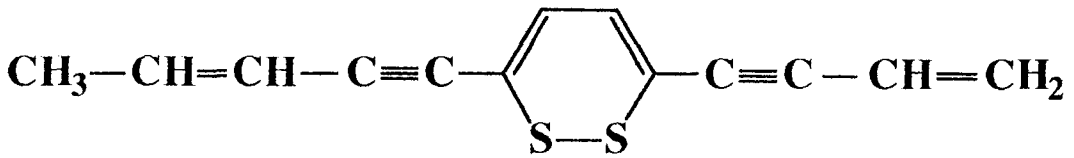
12a 

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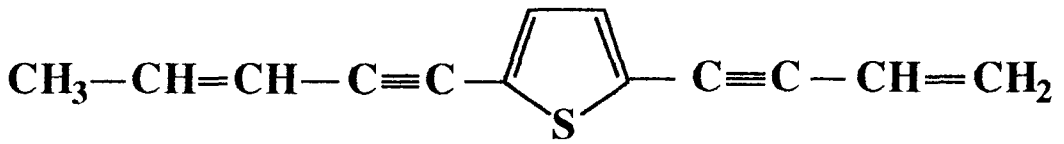
12b 

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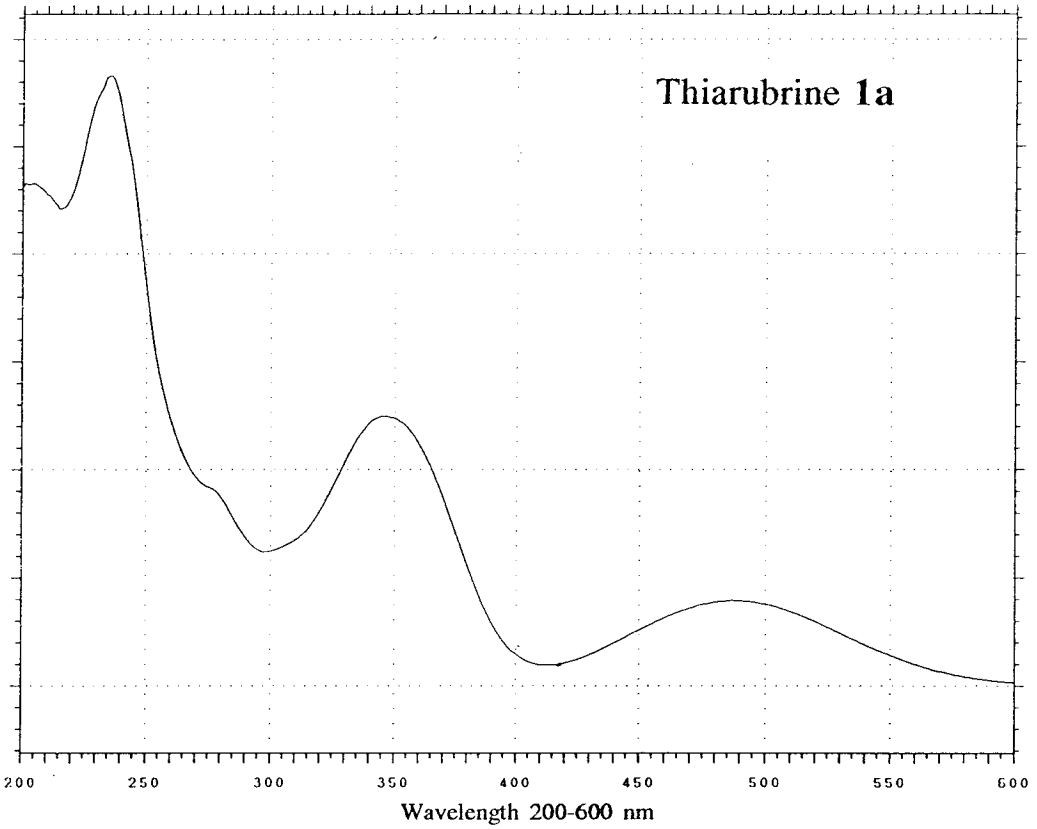
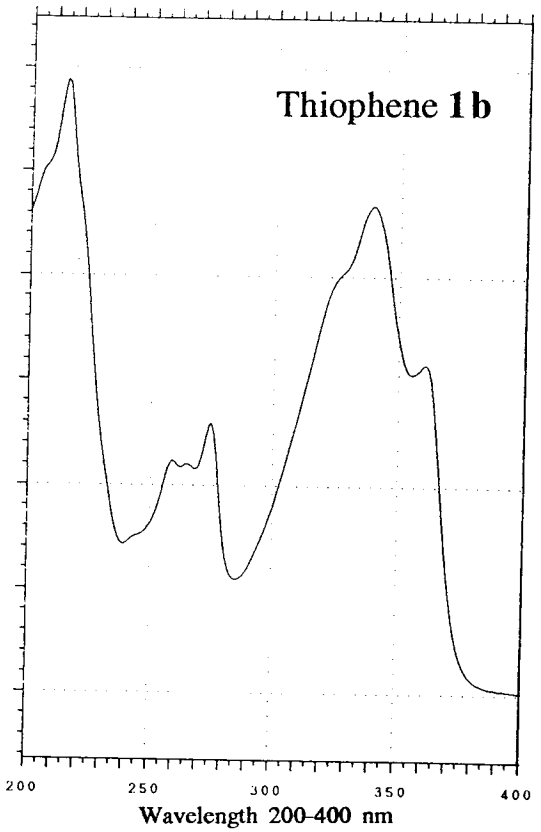
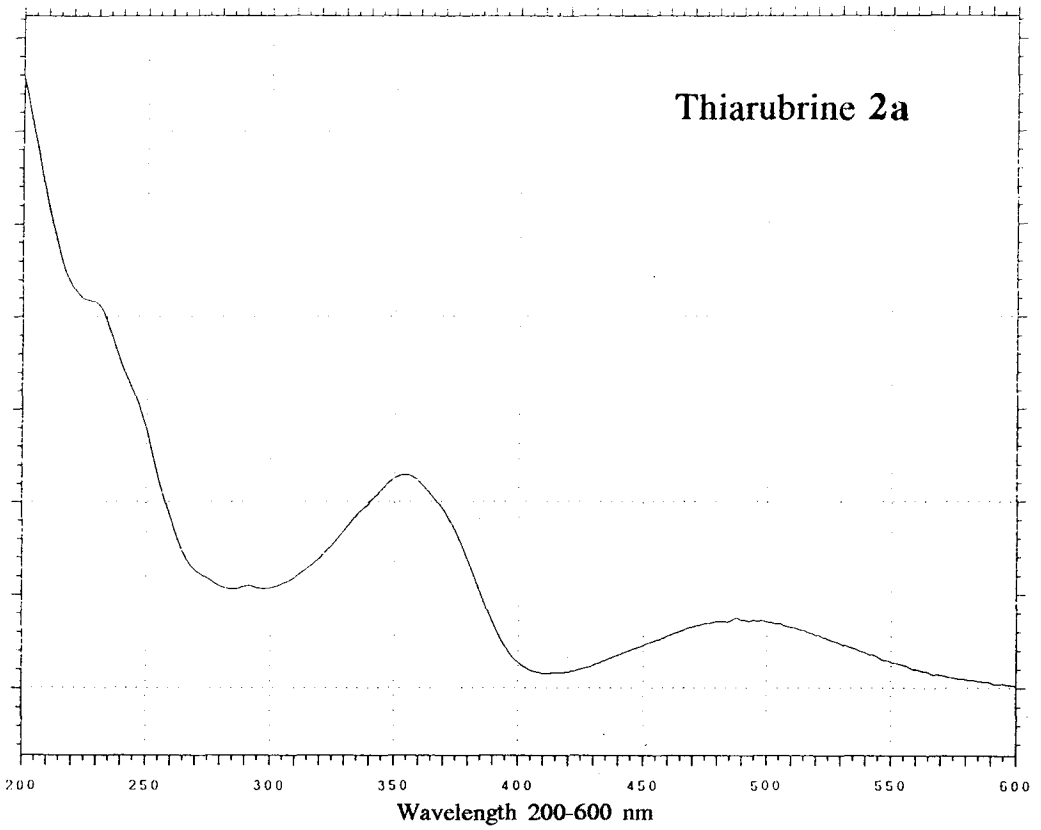
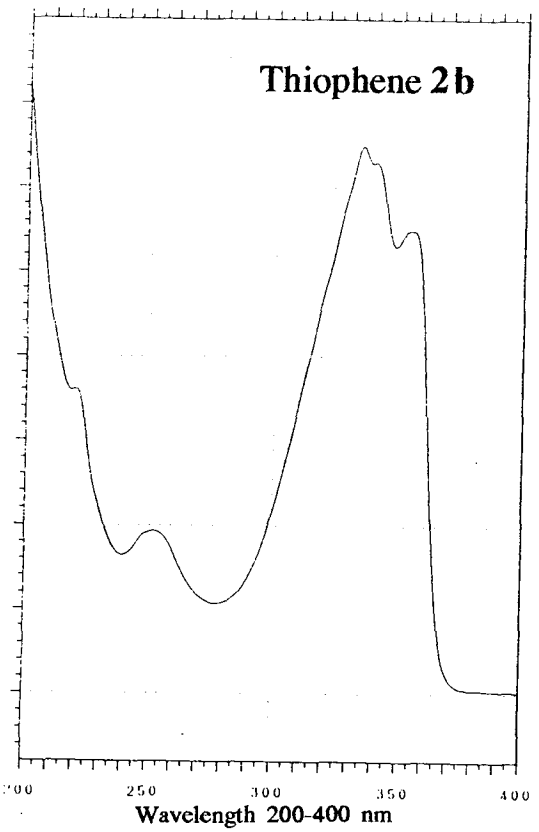
APPENDIX II(a): UV-Visible Spectra of Thiarubrine **1 a** and Thiophene **1 b**.





APPENDIX II(b): UV-Visible Spectra of Thiarubrine **2a** and Thiophene **2b**





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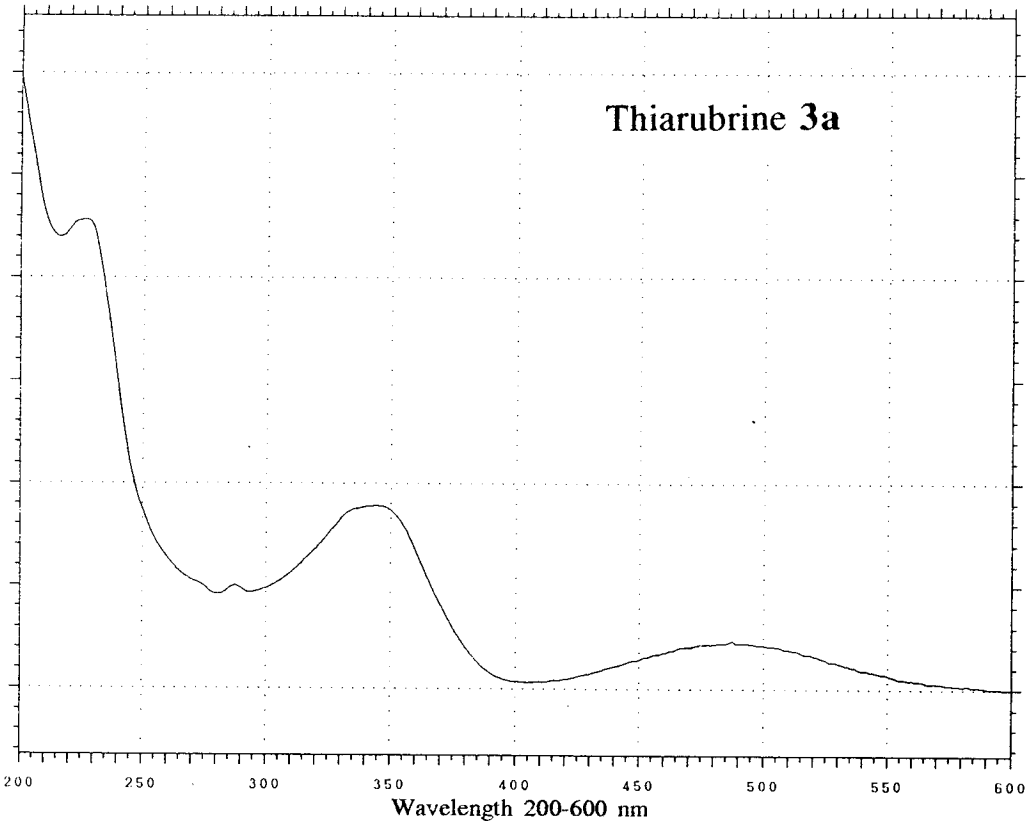
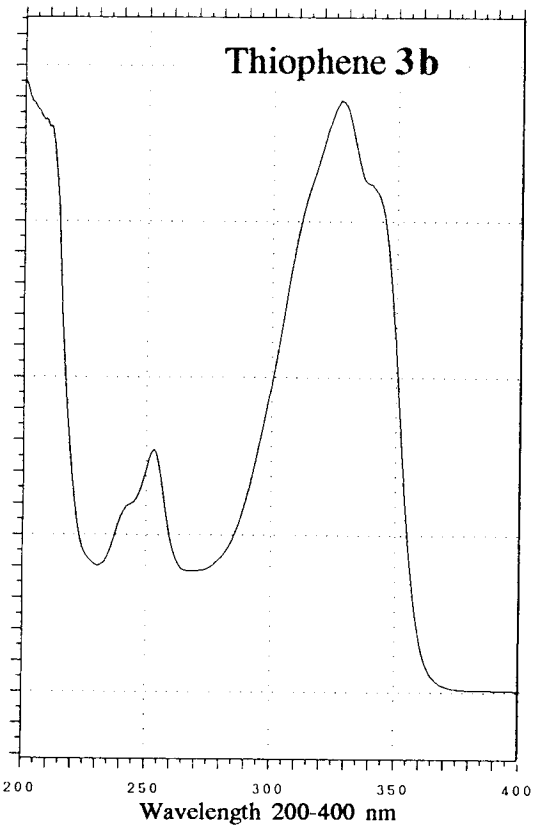
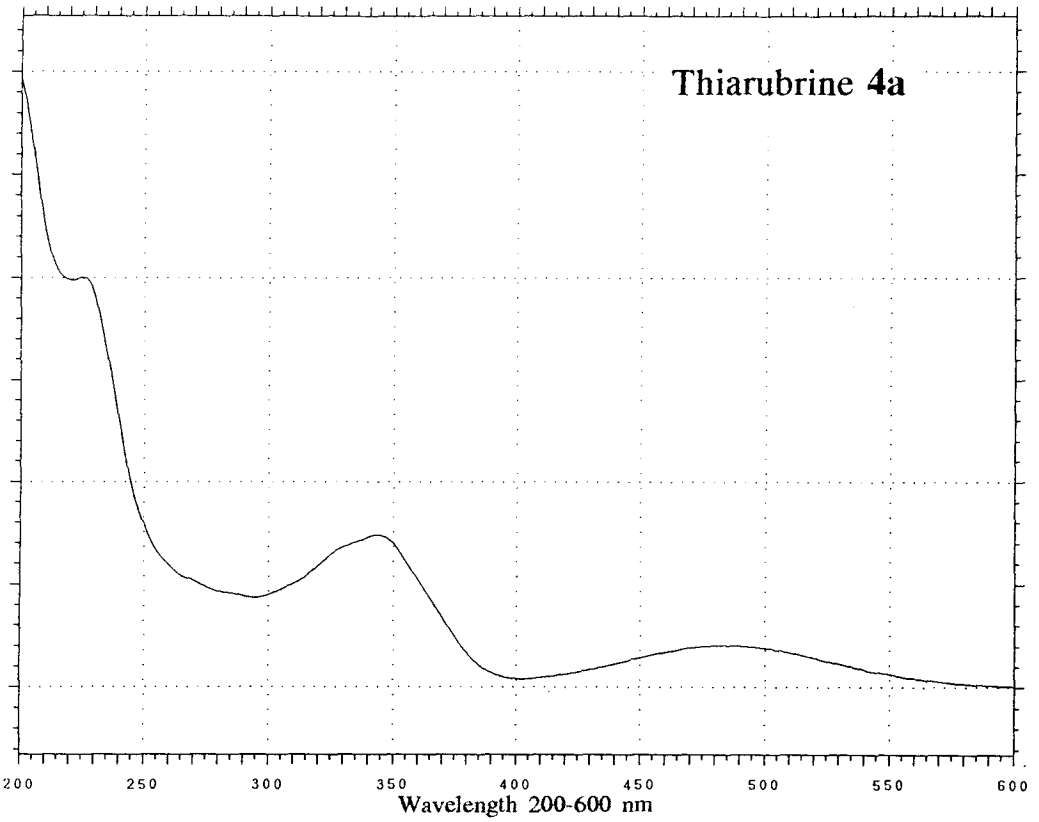
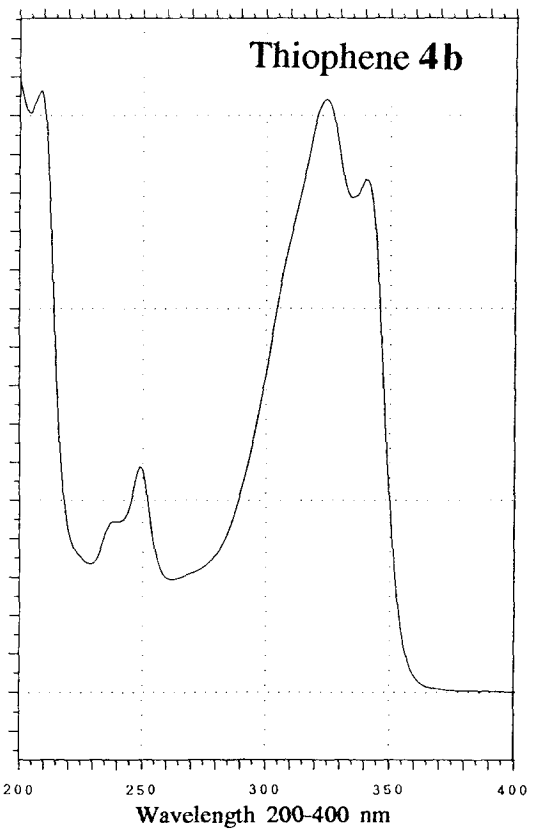


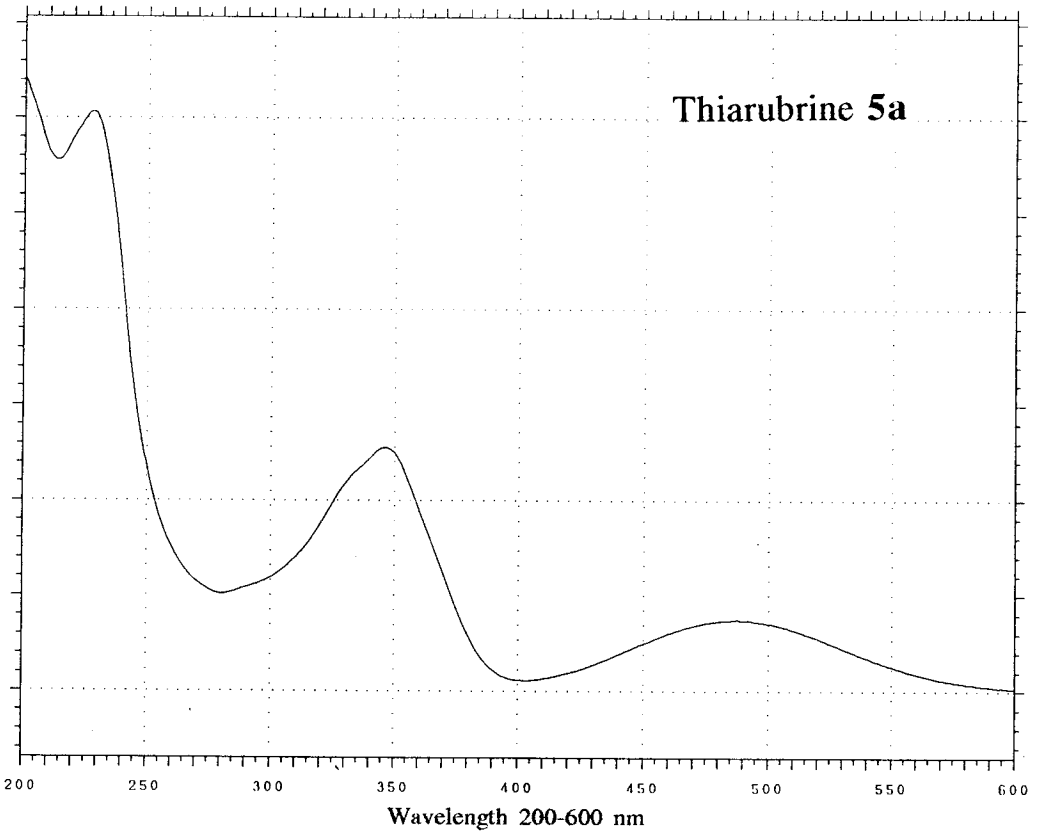


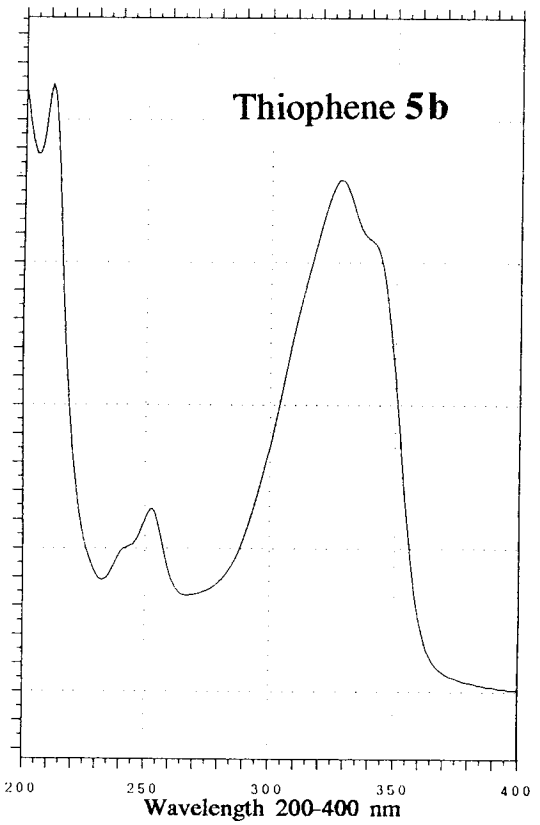
APPENDIX II(d): UV-Visible Spectra of Thiarubrine **4a** and Thiophene **4b**.



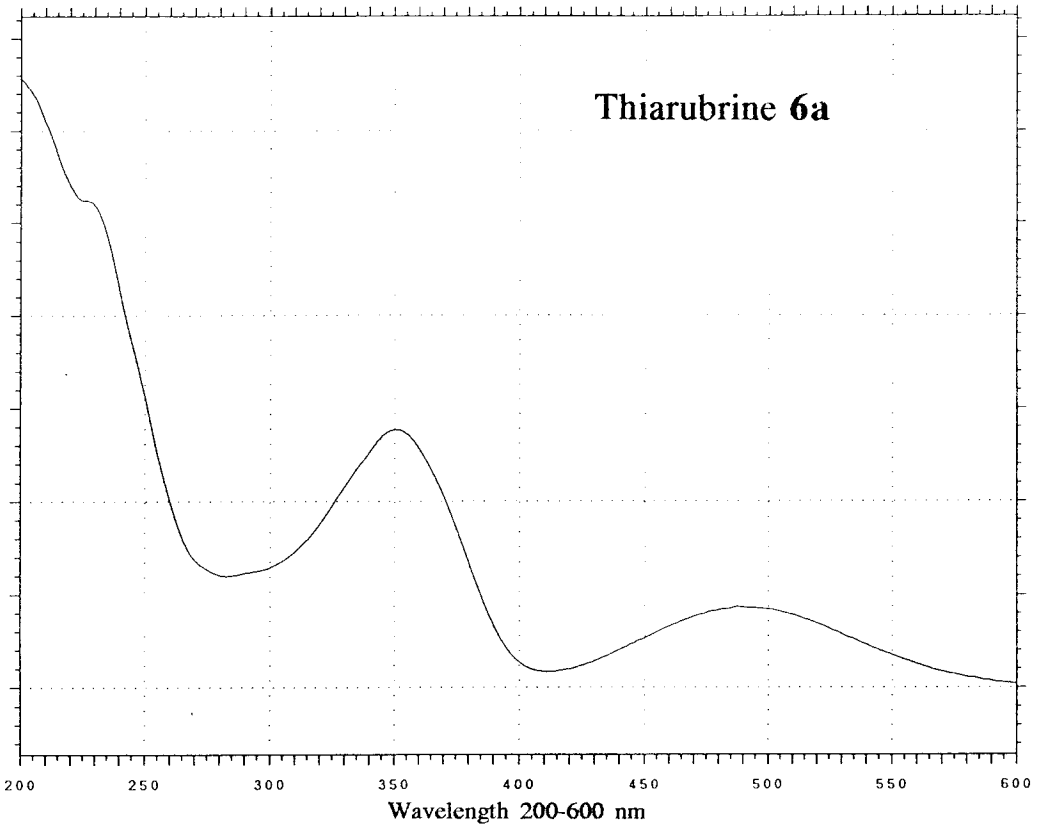


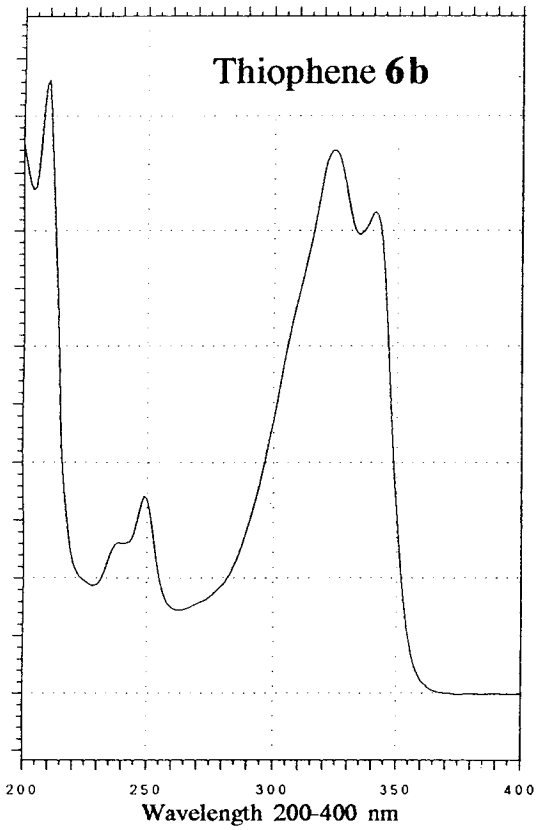
APPENDIX II(e): UV-Visible Spectra of Thiarubrine **5a** and Thiophene **5b**.



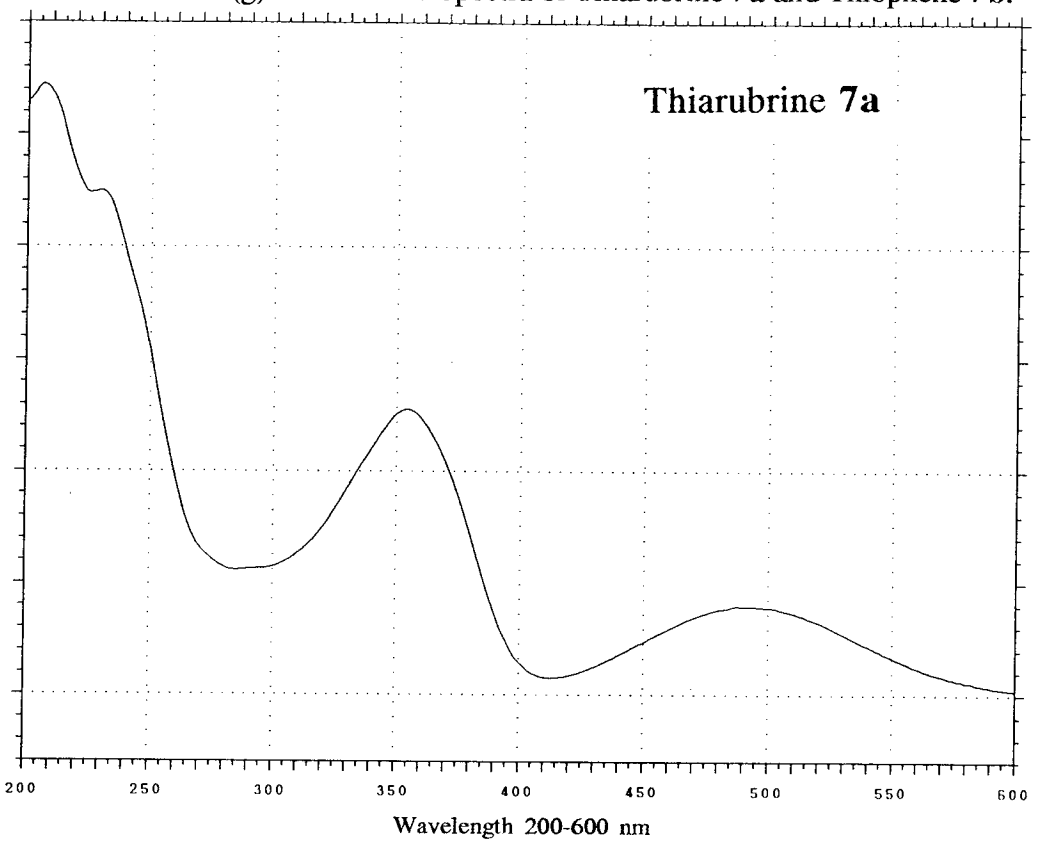


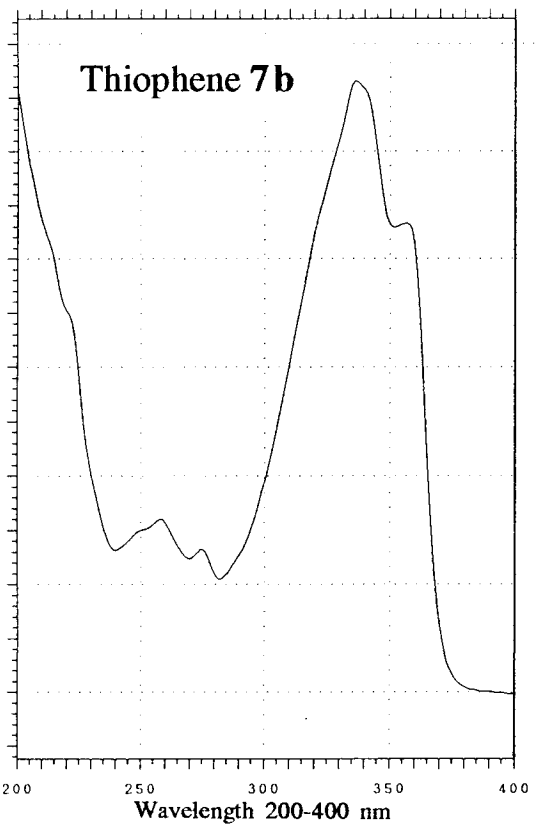
APPENDIX II(f): UV-Visible Spectra of Thiarubrine **6a** and Thiophene **6b**.



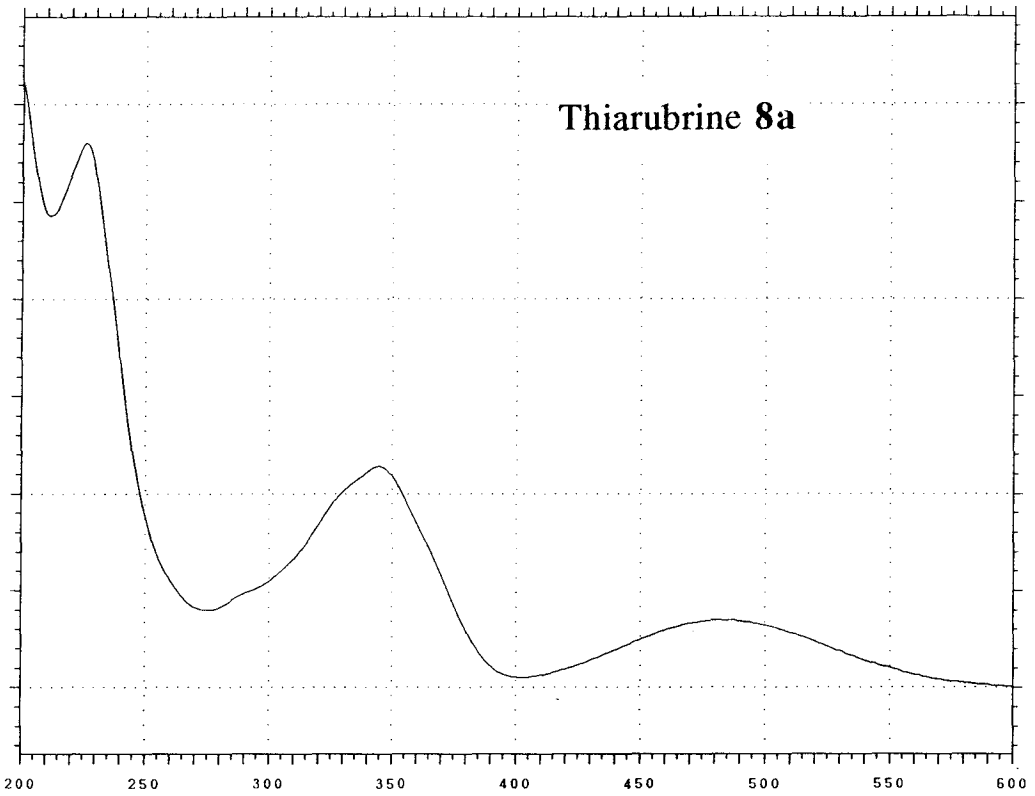


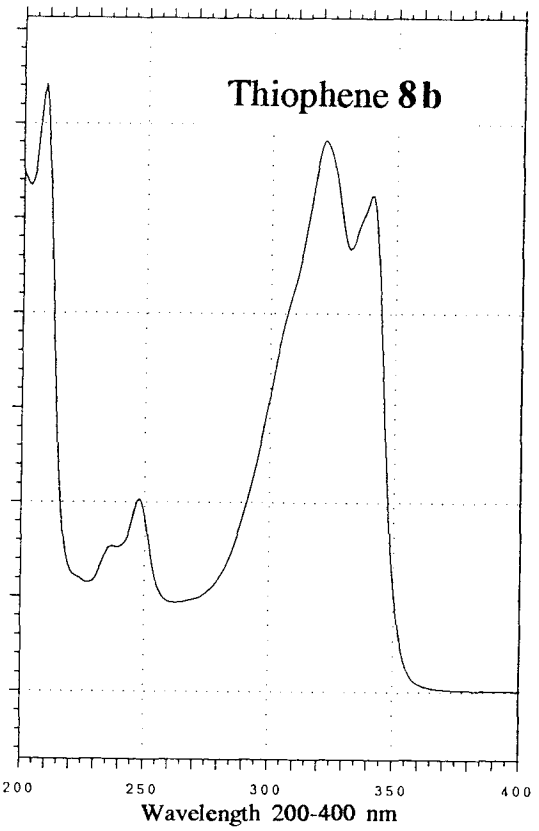
APPENDIX II(g): UV-Visible Spectra of Thiarubrine **7a** and Thiophene **7b**.



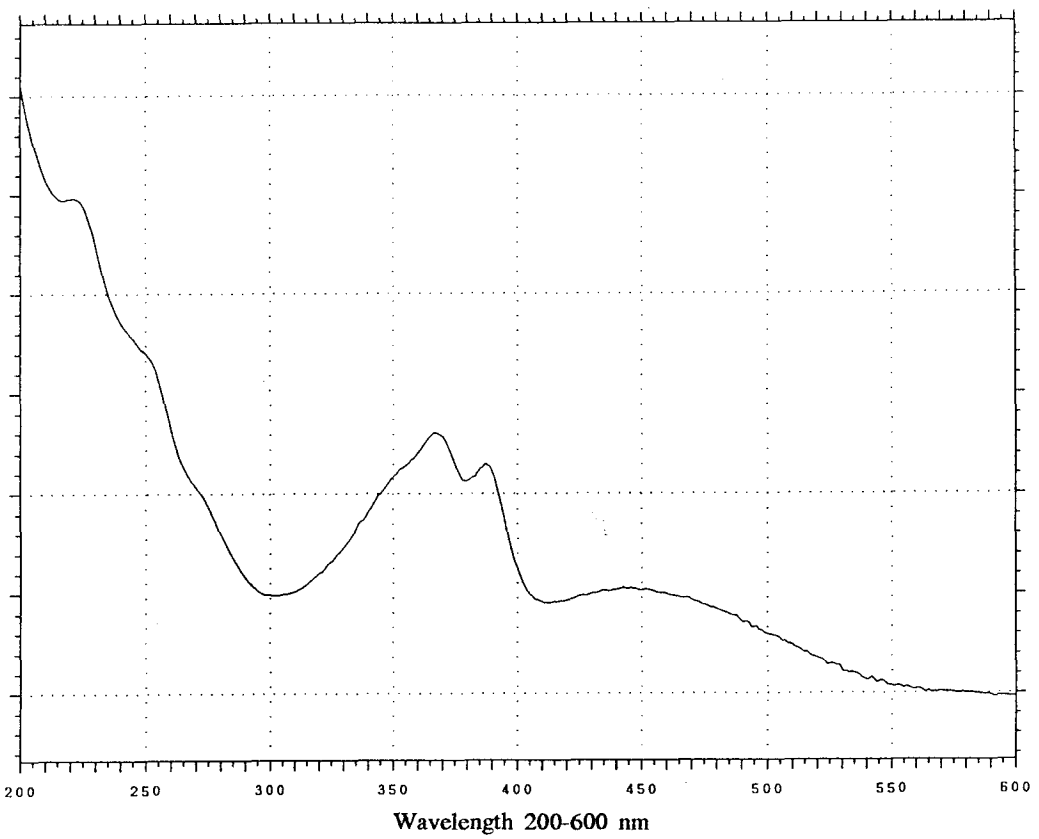


APPENDIX II(h): UV-Visible Spectra of Thiarubrine **8a** and Thiophene **8b**.





APPENDIX II(i): UV-Visible Spectrum of Thiosulphinate **9**.



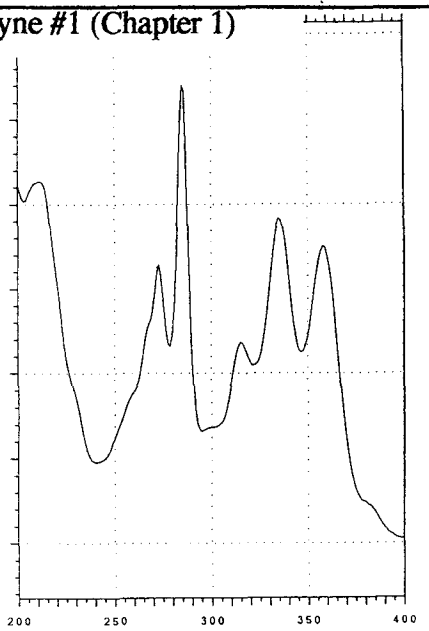
APPENDIX III: UV-Vis Spectral Data for Unknown Polyynes

UV-Vis Maxima

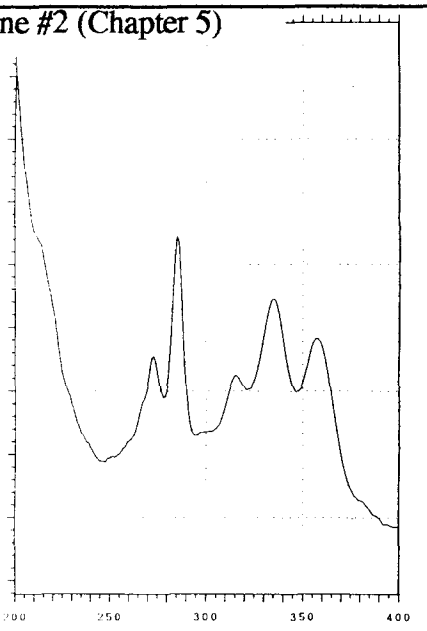
	Long wavelengths group			Lower wavelengths group		
Unknown Polyyne #1	358	336	316	286	272	
Unknown Polyyne #2	358	336	316	286	272	
Unknown Polyyne #3	350	328	308	292	270	260
Known Polyyne	356	333	312	283	271	

UV-Vis Spectra

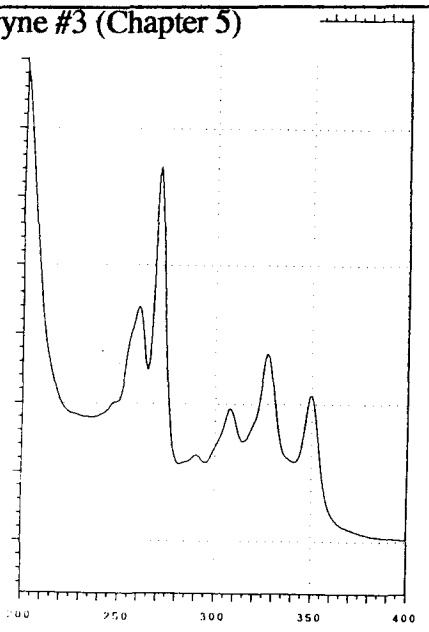
Polyyne #1 (Chapter 1)



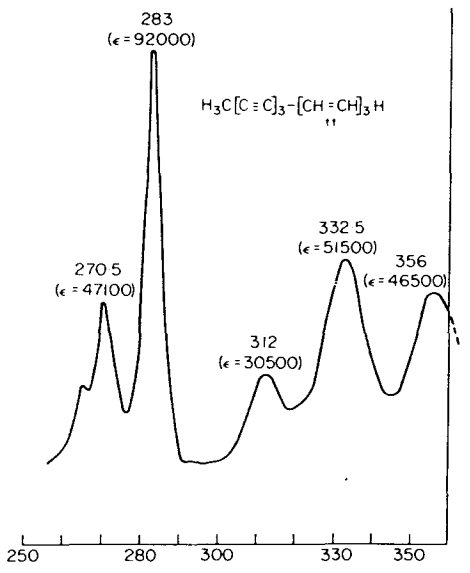
Polyyne #2 (Chapter 5)



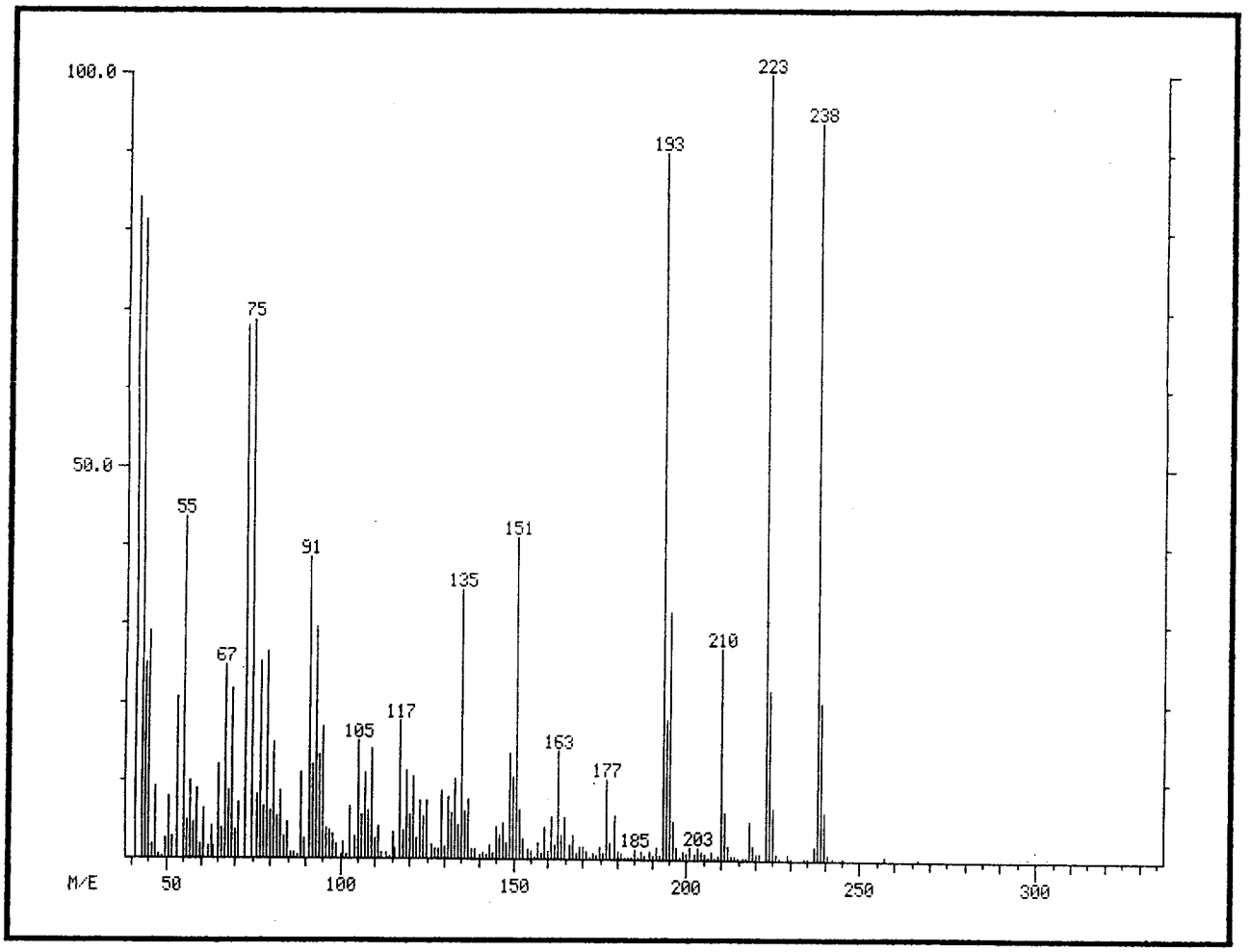
Polyyne #3 (Chapter 5)



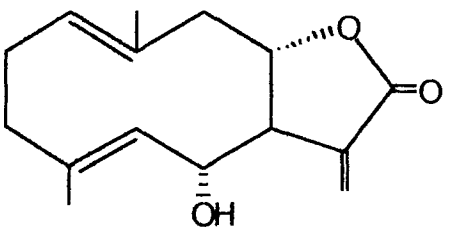
Known Polyyne (Bohlmann, 1973)



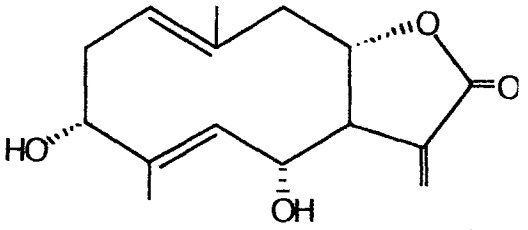
APPENDIX IV: Mass Spectrum of Isolated Crystals (Chapter 2)



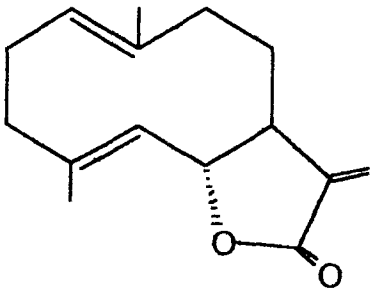
APPENDIX V: Sesquiterpene Lactones from Ambrosia chamisonis



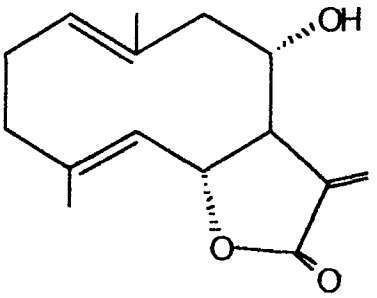
Chamissellin



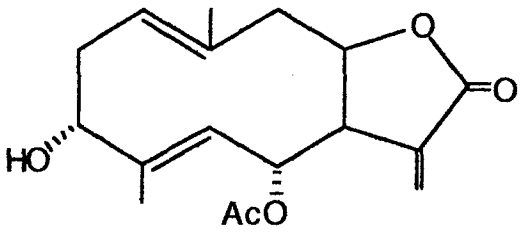
Chamissonin



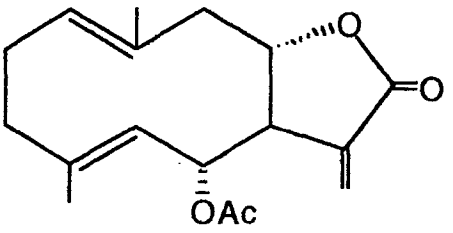
Costunolide



Chamissanthin



Chamissarin



Laurenobiolide

APPENDIX VI: Electron Impact High Resolution Mass Spectrometry of Thiarubrine **8a**

C	H	S	O	DEV	MEAS MASS	#PTS	%INT
13	10	2	1	0.1	246.0174	35	56.25
12	7	2	0	-0.4	214.9985	29	51.29
13	10	1	1	0.9	214.0461	29	26.90
12	7	1	0	-0.4	183.0264	29	41.53
11	7	1	0	-0.7	171.0262	35	58.55
11	7	0	0	-0.8	139.0539	29	32.72

APPENDIX VII: Spectral Data of Thiarubrine **8a** and Thiophenes **7b** and **8b**

7b: UV λ_{max} nm 357, 342(sh), 334, 249. MS (of TMS derivative) *m/z* (rel. int.): 284 $[M]^+\cdot$(58), 195 $[M\text{-}OTMS]^+$(62).

8a: UV λ_{max} nm 490, 345. MS (of TMS derivative) *m/z* (rel. int.):246 $[M]^+\cdot$(38), 214 $[M\text{-}S]^+\cdot$(83), 215 $[M\text{-}CH_2OH]^+$(83), 183 $[MS\text{-}S\text{-}CH_2OH]^+$(100).

8b: UV λ_{max} nm 341, 322, 246, 235. MS (of TMS derivative) *m/z* (rel. int.): 286 $[M]^+\cdot$(33), 183 $[M\text{-}CH_20TMS]^+$(10), 103 $[CH_20TMS]^+$(42).