PHOTOBIOLOGY, PHOTOCHEMISTRY AND CHEMICAL ECOLOGY OF THIARUBRINES FROM THE ASTERACEAE

by

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ABSTRACT

This study investigated several aspects of the biology and chemistry of thiarubrines, a group of red, light-sensitive pigments found in the Asteraceae. Irradiation of thiarubrines with visible light resulted in the formation of 2,6-dithiabicyclo[3.1.0]hexene polyyne photointermediates ("photosulfides"), which were shown to be short-lived and unstable, yielding thiophenes and cyclooctasulfur upon standing at room temperature. The structures of six novel photosulfides, formed by photolysis of thiarubrines A, B and D, were elucidated using HPLC with UV-vis and mass spectral detection, and cold-temperature NMR spectroscopy. Thiarubrine A showed visible-light phototoxicity to *Candida albicans* suggesting that the photosulfides, or other molecules generated by the exposure of thiarubrines to light, have significant toxicity.

Investigation of the photoprotection of light-sensitive thiarubrines in the leaves of *Ambrosia chamissonis* revealed that anthocyanin pigments function as visible light screens that prevent thiarubrine photoconversion *in vivo*. Two anthocyanins, cyanidin 3-*O*-(6-*O*-malonyl-β-D-glucopyranoside) and cyanidin 3-*O*-(β-D-glucopyranoside), were isolated from *A. chamissonis*. Conversion of thiarubrines to thiophenes was shown to occur in *A. chamissonis* roots, which lack anthocyanins, but was prevented in leaves and stems, which contain high amounts of these light-screening compounds. Furthermore, solutions of cyanidin 3-*O*-(β-D-glucopyranoside) effectively prevented photoconversion of thiarubrine A *in vitro*.

The hypothesis that thiarubrine A is a biologically-active constituent of the leaves of *Wedelia* (formerly *Aspilia*) species swallowed whole by chimpanzees was tested. Leaf samples were analyzed using a quantitative HPLC assay and a bioassay for toxicity to

Candida albicans. Thiarubrines A, B, D and E and their corresponding thiophenes were not present in the leaves of four *Wedelia* species including *W. mossambicensis*, *W. rudis* and *W. pluriseta*, which are swallowed by chimpanzees at Mahale Mountains National Park and Gombe National Park, Tanzania. Candidicidal activity, indicative of the presence of thiarubrines, was also not detected in *Wedelia* leaf samples.

A novel 1,2-dithiin polyyne, thiarubrine M, was isolated from the roots of *Ambrosia chamissonis* and its structure determined by UV, IR, NMR and mass spectroscopy. Thiarubrine M, which contains a 1,2-dithiin group coupled to a terpenoid derived side-chain, is of mixed biosynthetic origin. Unlike other thiarubrines, thiarubrine M was found to lack significant antifungal activity.

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LIST OF ABBREVIATIONS

ANOVA = analysis of variance
APCI = atmospheric pressure chemical ionization
APT = attached proton test
AU = absorbance units
CC = column chromatography
CH_2Cl_2 = dichloromethane
$CHCl_3$ = chloroform
COSY = correlated spectroscopy
DMSO = dimethyl sulphoxide
DW = dry weight
EI = electron impact
FAB = fast atom bombardment
FW = fresh weight
HMBC = heteronuclear multiple bond correlation
HMQC = heteronuclear multiple quantum coherence
HPLC = high performance liquid chromatography
i.d. = internal diameter
LD = log dose
MeCN = acetonitrile
MeOH = methanol
MFW = methanol–formic acid–water
MIC = minimum inhibitory concentration
MS = mass spectrometry
ND = not detected
NMR = nuclear magnetic resonance
PDA = photodiode array
R_t = retention time
SDB = Sabouraud dextrose broth
TFA = trifluoroacetic acid
TLC = thin-layer chromatography
UV = ultraviolet
UV-vis = ultraviolet-visible
VLC = vacuum liquid chromatography

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Finally, I would like to thank my partner, Goya Ngan, and my brother, Nick Page, for their support during the last few years.

FOREWORD

The partial structures of photointermediates from thiarubrine A, which are described in Chapter 2 of this thesis, were previously published in a co-authored paper:

> Block, E., J. E. Page, J. Toscano, C.-X. Wang, X. Zhang, R. DeOrazio, C. Guo, M. S. Platz, R. S. Sheridan and G. H. N. Towers (1996). The photochemistry of thiarubrine A and other 1,2-dithiins: formation of 2,6-dithiabicyclo[3.1.0]hex-3-enes. Journal of the American Chemical Society 118: 4719-4720.

All experiments with plant-derived 1,2-dithiins (thiarubrines) presented in Block *et al.* (1996) were performed by J. E. Page in the laboratory of G. H. N. Towers.

The investigation of the chemical basis of chimpanzee leaf-swallowing presented in Chapter 4 of this thesis has been published as a co-authored paper:

> Page, J. E., M. A. Huffman, V. Smith and G. H. N. Towers (1997). Chemical basis for *Aspilia* (Asteraceae) leaf-swallowing by chimpanzees: a re-analysis. Journal of Chemical Ecology 23: 2211-2226.

The analyses described in Page *et al.* (1997) were conducted by J. E. Page in the laboratory of G. H. N. Towers while M. A. Huffman and V. Smith collected plant samples for analysis.

Professor G. H. N. Towers

CHAPTER 1

GENERAL INTRODUCTION

1.1. PLANT SECONDARY METABOLITES

In response to the biological limitations of living as sessile organisms, members of the kingdom Plantae have evolved a diverse array of organic molecules to mediate interactions between themselves and their environment, which includes other organisms. Such molecules, which for the most part are low molecular weight carbon-based compounds, have been termed secondary metabolites, a designation referring to the fact that they do not participate in primary metabolic processes. Unlike primary metabolites, which may be ubiquitous in all of the cells of a plant or animal, secondary metabolites are generally confined to a specialized cell type and restricted both in their taxonomic and ontogenetic distribution (Fraenkel, 1959; Berenbaum, 1995a).

Evolution has resulted in a staggering number of secondary metabolites; indeed, the molecular richness of the plant kingdom is one of the greatest expressions of biological diversity in Nature. Estimates of the total number of plant secondary metabolites are from 100,000 to 400,000 (Swain, 1977). Particularly diverse classes of plant chemicals are the alkaloids, of which ~10,000 have been isolated and characterized (Southon and Buckingham, 1989) and the terpenoids, with ~15,000 known compounds (Gershenzon and Croteau, 1991). There are perhaps 1000 polyynes (polyacetylenes), the class of metabolites which includes the title compounds, the thiarubrines (Lam and Hansen, 1990).

Although plant secondary metabolites have been studied, in the form of plant-derived drugs, for centuries, their ecological and physiological function in plants has only recently

begun to be understood. A landmark paper by Fraenkel (1959) reiterated the idea that the *raison d'être* of secondary metabolites is to defend plants from insect herbivores. This defensive function can be expanded to include protection from other groups of herbivores (i.e. mammals) as well as pathogenic fungi and microorganisms. In addition to their defensive role, plant chemicals also serve as visual and volatile signalling agents (e.g., Turlings *et al.*, 1990; Sembdner and Parthier, 1993), and as screens for UV light (Van de Staaj *et al.*, 1995).

A logical correlative of the defensive function of many plant secondary metabolites is that such chemicals possess biological activity, or the ability to effect a change in the biological processes of another organism. Biological activity usually involves the reaction of the plant chemical with a cellular target, such as a receptor, enzyme or membrane component, in the herbivore or pathogen which has consumed or infected the plant (Berenbaum, 1995a). This reaction results in a chemical change in the structure of the target molecule leading to alterations in the metabolism of the herbivore or pathogen. In the context of plant chemical defense, biological activity may be expressed as overt toxicity (i.e. causing death), feeding deterrence, inhibition of growth and development or a variety of other negative effects.

Humans exploit the biological activity of plant secondary metabolites in many aspects of our lives, in particular when we use them as medicines. The recent emergence of the diterpene taxol (paclitaxel) from *Taxus brevifolia* Nutt. (Taxaceae) as an effective chemotherapy drug and the antimalarial artemisin, a sesquiterpene from *Artemisia annua* L. (Asteraceae), highlight the importance of plant-derived drugs and underscore the rationale for continued investigations of biologically-active plant chemicals. In addition to their use as

medicines, the impressive array of chemical defenses produced by plants impacts humans in other ways. As animals that rely on vegetable matter (and on other animals that consume it) for our nutritional requirements, humans benefit from the success of wild and cultivated plants that grow and thrive by virtue of their chemical defenses. The failure of plant defenses against pathogens, and its effect on humans, is tragically illustrated by the Irish potato famine, in which the fungus *Phytophthora infestans* decimated the Irish potato crop in 1845–50 (Gregory, 1983). Humans also utilize plant chemicals as illicit drugs—principally alkaloids derived from the coca plant (*Erythroxylum coca* Lam., Erythroxylaceae) and the opium poppy (*Papaver somniferum* L., Papaveraceae), and preparations of *Cannabis* spp. (Cannabaceae) containing cannabinoids. International trade in such substances now contributes an estimated $560 Billion (CAN) annually to the global economy (United Nations Drug Control Programme, 1997).

1.2. PHOTOTOXIC PLANT SECONDARY METABOLITES

Sunlight has a major influence on the biological activity of plant secondary metabolites, and the activation and potentiation of toxicity by light is an important and successful defensive strategy in plants (Berenbaum, 1987; Arnason *et al.*, 1992). Such light-mediated biological activity has been termed phototoxicity, and the compounds responsible are known as phototoxins or photosensitizers. Compounds displaying phototoxicity are widespread in the plant kingdom and are known from at least 38 families (Berenbaum, 1995b). Of these, the Asteraceae and Apiaceae, which contain the phototoxic polyynes (including thiophenes) and furocoumarins, respectively, have perhaps received the most attention from chemists and biologists. Other phototoxic plant secondary metabolites include perylene quinones such as

hypericin from *Hypericum perforatum* L. (Guttiferae); alkaloids such as sanguinarine found in the Papaveraceae; furochromones; porphyrins and a range of other compounds such as the cinnamate derivatives, e.g., methyl *p*-methoxycinnamate, and terpenoids (Towers *et al.*, in press).

The biologically important portions of solar radiation striking the earth include the UV-B (290–320 nm) and UV-A (320–400 nm) wavebands, the former potentially damaging to living organisms, and visible light (400–760 nm), which drives the light reactions of photosynthesis. Longer wavelength infrared light (760–4000 nm) is important owing to its thermal effects on organisms.

Most phototoxicity is the result of the excitation of a photosensitizer by UV or visible light producing an excited molecule that can undergo two types of reactions: in type I reactions, the photosensitizer abstracts electrons or hydrogen atoms from, or donates electrons or hydrogen atoms to, other molecules yielding radicals or radical ions; in a competing process (type II reaction), the photosensitizer transfers energy to molecular oxygen by intersystem crossing giving an activated oxygen species, singlet oxygen ($^1O_2$) (Foote, 1987; Spikes, 1989). Singlet oxygen may cause oxidative damage to a variety of biological molecules such as unsaturated lipids, proteins and nucleic acids (Spikes and Straight, 1987) and many of the important classes of plant phototoxins, including polyynes, thiophenes and perylene quinones, function primarily as photooxidants (Arnason *et al.*, 1981; Scaiano *et al.*, 1987; Lenard *et al.*, 1993). Type I reactions involving the formation of reactive radicals are in competition with type II reactions, and plant phototoxins may also damage cells through the former process (Kagan *et al.*, 1993). In addition to oxidative or radical processes, photosensitizers may also react directly with molecules in affected cells.

For example, furocoumarins intercalate RNA and DNA in a dark reaction, and form cyclobutane adducts with bases in a photochemical reaction when irradiated with UV light (Song and Tapley, 1979).

1.3. THIARUBRINES

The research presented in this thesis focuses on the thiarubrines, a group of phototoxic sulfur-containing pigments found in the Asteraceae. These unusual molecules are derived from polyynes by the addition of two atoms of sulfur to yield a 1,2-dithiin (1,2-dithiacyclohexa-3,5-diene) ring coupled at carbons 3 and 6 to an alkyne or polyyne side-chain (Figure 1.1). Thiarubrines, the trivial name applied to the plant-derived 1,2-dithiins, recognizes the presence of sulfur in the molecules (Greek *theion* 'sulfur') and their distinct wine-red colour (Latin *rubr-* 'red'). Due to the unique characteristics of the 1,2 dithiin ring, the thiarubrines possess properties such as light sensitivity and potent phototoxicity and light-independent (dark) toxicity that set them apart both biologically and chemically from the polyyne and thiophene secondary metabolites found more commonly in the Asteraceae.

1.3.1. Botany and Distribution of Thiarubrines in the Asteraceae

The first reported occurrence of a 1,2-dithiin polyyne in nature was the isolation of 3-(3-buten-1-ynyl)-6-(1,3-pentadiynyl)-1,2-dithiin, thiarubrine B (**2**) from *Eriophyllum caespitosum* Dougl. (Asteraceae) by Mortensen *et al.* (1964). Since that time, phytochemical investigations have resulted in the isolation and characterization of eleven thiarubrines, the structures of which are shown in Figure 1.1.
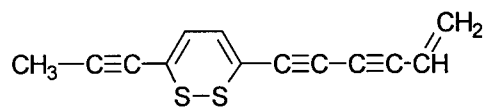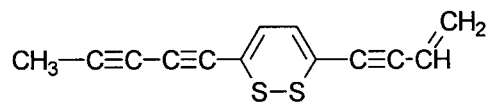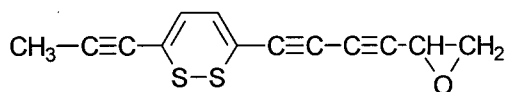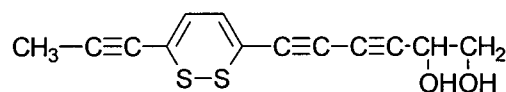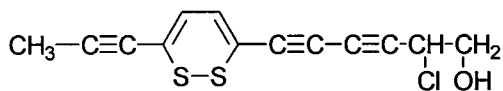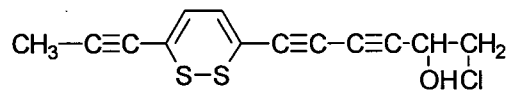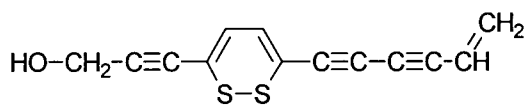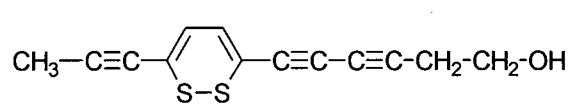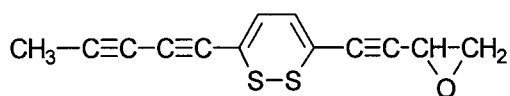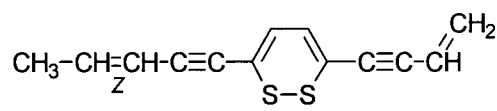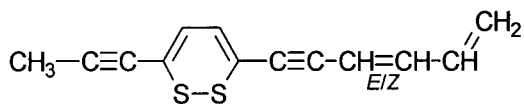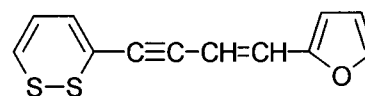
Figure 1.1. Structures of the thiarubrines (1–11) and a 1,2-dithiin-furan (12).

The distribution of the thiarubrines is limited to the Asteraceae where they are known to occur in 50 species representing 21 genera (Table 1.1). Thiarubrines are mainly restricted to the tribes Heliantheae and Helenieae, although there are a few reports from the Eupatorieae, Inuleae and Anthemideae. The most commonly occurring thiarubrines are 3-(5-hexen-1,3-diynyl)-6-(1-propynyl)-1,2-dithiin, thiarubrine A (**1**) and its isomer, thiarubrine B (**2**), which occur in 36 of the 50 species.

The genus *Ambrosia* L. has proven to be the richest source of thiarubrines thus far investigated (Balza *et al.*, 1989; Ellis *et al.*, 1995). Eight thiarubrines have been characterized from *Ambrosia chamissonis*, a tap-rooted herb distributed along the Pacific coasts of North and South America. In addition to thiarubrines A (**1**) and B (**2**), 1,2-dithiin polyynes present in *A. chamissonis* include an epoxide, 3-(5,6-epoxy-hex-1,3-diynyl)-6-(1-propynyl)-1,2-dithiin, thiarubrine D (**3**) (Balza *et al.*, 1989); a diol, 3-(5,6-dihydroxy-hex-1,3-diynyl)-6-(1-propynyl)-1,2-dithiin, thiarubrine E (**4**) (Balza *et al.*, 1989); chlorohydrins, 3-(5-chloro-6-hydroxy-hex-1,3-diynyl)-6-(1-propynyl)-1,2-dithiin (**5**) and 3-(6-chloro-5-hydroxy-hex-1,3-diynyl)-6-(1-propynyl)-1,2-dithiin (**6**) (Balza and Towers, 1990); alcohols, 3-(5-hexen-1,3-diynyl-6-(3-hydroxyprop-1-ynyl)-1,2-dithiin (**7**) (Balza and Towers, 1990) and 3-(6-hydroxyhex-1,3-diynyl)-6-(1-propynyl)-1,2-dithiin (**8**) (Ellis *et al.*, 1993). Lu *et al.* (1993) reported an epoxide derivative of thiarubrine B (**2**), 3-(3,4-epoxy-but-1-ynyl)-6-(1,3-pentadiynyl)-1,2-dithiin (**9**) from *Ambrosia trifida*. Due to the diversity and relatively high amounts of thiarubrines (*ca.* 0.2% of dry weight (Ellis, 1993)) present in *A. chamissonis*, and its availability in coastal British Columbia, all of the thiarubrines studied during the course of this thesis were isolated from this plant.

Table 1.1. Distribution of thiarubrines in the Asteraceae.

Taxon	Compound(s)	Reference
Anthemideae: Achilleinae		
Santolina chamaecyparissus L.	**12**	Lam _et al._ (1989)
Eupatorieae: Mikaniinae		
Mikania scandens (L.) Willd.	**2**	Bohlmann _et al._ (1973)
Inuleae		
Geigeria asperia Harv.	**11**	Bohlmann _et al._ (1982a)
Geigeria burkei Harv.	**11**	Bohlmann _et al._ (1982a)
Pegolettia senegalensis Cass.	**1, 2**	Bohlmann _et al._ (1983a)
Heliantheae: Ambrosiinae		
Ambrosia artemisiifolia L.	**1, 2**	Bohlmann and Kleine (1965); Gomez-Barrios _et al._ (1992)
Ambrosia chamissonis (Less.) Greene	**1, 2, 3, 4, 5, 6, 7, 8**	Rodriguez (1988); Balza _et al._ (1989); Lopez _et al._ (1989); Balza and Towers (1990); Ellis _et al._ (1993)
Ambrosia confertiflora DC.	**1**	Lopez _et al._ (1989)
Ambrosia cumanensis Kunth	**1**	Bohlmann _et al._ (1977)
Ambrosia eliator L.	**1**	Bohlmann and Kleine (1965)
Ambrosia psilostachya DC.	**1, 2**	Rodriguez (1988); Lopez _et al._ (1989)
Ambrosia trifida L.	**1, 2, 9**	Bohlmann and Kleine (1965); Lu _et al._ (1993)
Ambrosia trifoliata L.	**1**	Bohlmann and Kleine (1965)
Iva xanthifolia Nutt.	**1**	Bohlmann and Kleine (1965)

Heliantheae: Ecliptinae

Oyedaea lanceolata Rusby	2	Bohlmann and Zdero (1979)
Steiractinia sodiroi (Hieron.) S.F. Blake	2	Bohlmann and Zdero (1985)
Verbesina alata L.	2	Bohlmann et al. (1973)
Verbesina boliviana Klatt	2	Bohlmann et al. (1980a)
Verbesina cinerea Rusby	2	Bohlmann et al. (1980a)
Verbesina latisquamata S.F. Blake	1	Bohlmann and Lonitz (1978a)
Verbesina occidentalis (L.) Walter.	1	Bohlmann and Lonitz (1978b)
Wedelia hookeriana Gardner	1	Bohlmann et al. (1982b)
Wedelia (Aspilia) laevissima Baker	11	Ganzer et al. (1992)
Wedelia (Aspilia) mossambicensis (Oliv.) Wild	1, 2	Rodriguez et al. (1985); Lopez et al. (1989); Rodriguez (1988); Page et al. (1992); Norton et al. (1993); Huffman et al. (1996)
Wedelia (Aspilia) pluriseta Schweinf.	1	Rodriguez et al. (1985)
Wedelia Jacq. sp.	1	Bohlmann et al. (1983b)
Zexmenia hispida (Kunth) A. Gray	2	Bohlmann and Lonitz (1978c)

Heliantheae: Melampodiinae

Melampodium divaricatum (Rich. Ex Pres.) DC.	11	Bohlmann and Kleine (1965; Bohlmann and Van (1977)
Melampodium longifolium Cav.	11	Bohlmann and Kleine (1965)
Milleria quinqueflora L.	2	Bohlmann et al. (1973)

Heliantheae: Rudbeckiinae

Rudbeckia bicolor Nutt.	11	Bohlmann and Kleine (1965)
Rudbeckia hirta L.	1, 2, 11	Bohlmann and Kleine (1965); Constabel et al. (1988); Rodriguez (1988); Lopez et al. (1989); Guillet et al. (in press)
Rudbeckia neumanni Steud.	11	Bohlmann et al. (1973)

Species		Reference
Rudbeckia speciosa Wenderoth	11	Bohlmann and Kleine (1965)
Rudbeckia sullivantii Boyton & Beadle	11	Bohlmann et al. (1973)
Helenieae: Baeriinae		
Eriophyllum caespitosum Douglas ex Lindl.	2	Mortensen et al. (1964); Bohlmann and Kleine (1965)
Eriophyllum lanatum (Pursh) J. Forbes	1, 2	Bohlmann et al. (1981); Norton et al. (1985)
Eriophyllum staechadifolium Lag.	2	Bohlmann et al. (1981)
Lasthenia chrysostoma (Fisch. & C. A Mey.) Greene	10	Bohlmann and Zdero (1978)
Lasthenia coronaria (Nutt.) Ornduff	10	Bohlmann and Zdero (1978)
Helenieae: Chaenactidinae		
Bahia (Picradeniopsis) woodhousei (A.Gray) Rydb.	10	Bohlmann et al. (1976)
Chaenactis douglasii (Hook.) Hook. & Arn.	1, 2	Norton et al. (1985); Towers et al. (1985); Cosio et al. (1986); Hudson et al. (1986a); Hudson et al. (1986b); Constabel and Towers (1988); Constabel and Towers (1989)
Palafoxia hookeriana Torr. et A. Gray	1	Bohlmann et al. (1973)
Palafoxia texana DC.	1	Bohlmann et al. (1973)
Schkuhria advena Thell.	1	Bohlmann and Kleine (1965)
Schkuhria abrotanoides Roth	1	Bohlmann and Zdero (1985)
Schkuhria multiflora Hook. & Arn.	1	Bohlmann et al. (1980b)
Schkuhria pinnata (Lam.) Kuntze	1	Bohlmann and Kleine (1965); Bohlmann and Zdero (1977)
Schkuhria senecioides Nees	1	Bohlmann and Kleine (1965); Bohlmann and Zdero (1977)
Helenieae: Hymenopappinae		
Villanova titicaensis (Meyen et Walp.) Walp.	10	Bohlmann et al. (1984)

Other thiarubrines of scattered distribution in the Asteraceae include (Z)-3-(3-buten-1-ynyl)-6-(3-penten-1-ynyl)-1,2-dithiin (10) isolated from *Bahia woodhousei*, *Lasthenia chrysostoma* and *L. coronaria* and the *E/Z* isomers (Z)-3-(3,5-hexadiene-1-ynyl)-6-(1-propynyl)-1,2-dithiin and (E)-3-(3,5-hexadiene-1-ynyl)-6-(1-propynyl)-1,2-dithiin which together are termed thiarubrine C (11) (Bohlmann *et al.*, 1976; Bohlmann and Zdero, 1978). Lam *et al.* (1989) reported an interesting 1,2-dithiin-furan polyyne (12) from *Santolina chamaecyparissus*, although the limited amount of the compound that was available prevented its complete characterization. Thiarubrines have proven useful as chemotaxonomic characters as demonstrated by the lack of support for the inclusion of the genus *Eriophyllum* in the tribe Senecioneae based on the presence of these compounds in members of this genus (Bohlmann *et al.*, 1981).

Anatomical studies have shown thiarubrines to be localized in specialized resin canals (channels) which are visible as thin red veins in many thiarubrine-containing plant taxa. Resin canals commonly occur in the Asteraceae, Apiaceae and Araliaceae where they function to accumulate secondary metabolites such as polyynes (Sorensen, 1968). In *Ambrosia chamissonis*, thiarubrine-containing resin canals are found in the outer cortex and the immature periderm of the root where they run parallel to the vascular tissue (Ellis *et al.*, 1995). *Chaenactis douglasii* roots show a similar distribution of thiarubrine canals (Cosio *et al.*, 1986). Thiarubrine canals are also associated with the vascular tissue in *A. chamissonis* stems and leaves.

1.3.2. Thiarubrine Biosynthesis

Thiarubrines are biogenetically derived from polyynes, which are themselves formed from

acetate via the fatty acid biosynthetic pathway. The fatty acid precursor of polyynes in both higher plants and microorganisms is the 18-carbon molecule, oleic acid (Bohlmann *et al.*, 1973). In the early steps of thiarubrine biosynthesis, Bohlmann *et al.* (1973) proposed that oleic acid undergoes dehydrogenation to form crepenynic acid which, through further dehydrogenation and chain-shortening reactions, yields the 13-carbon tridecapentaynene. Using labelled [1-^{13}C], [2-^{13}C] and [1,2-^{13}C$_2$] acetates to probe thiarubrine biosynthesis in hairy root cultures of *Ambrosia artemisiifolia*, Gomez-Barrios *et al.* (1992) found that the ^{13}C-NMR spectra of labelled thiarubrine A (**1**) showed patterns of enrichment consistent with this proposed biosynthetic pathway. It is not clear whether the more abundant 1,2-dithiin polyynes such as thiarubrines A (**1**) and B (**2**) function as precursors to the minor thiarubrine derivatives (i.e. **3–9**) or whether the functional groups present on the latter are introduced before the 1,2-dithiin ring is formed.

Constabel and Towers (1989a) showed that hairy root cultures of *Chaenactis douglasii* fed labelled sulfur (^{35}SO$_4^{-2}$) incorporated ^{35}S into thiarubrine A (**1**) and B (**2**), as well as their corresponding thiophenes.

1.3.3. Chemistry of Thiarubrines

Because of the presence of the 1,2-dithiin ring, the chemical properties of the thiarubrines have attracted considerable attention. A remarkable property is their photosensitivity, with exposure of thiarubrines to UV or visible light resulting in the conversion of these red pigments to colourless thiophene photoproducts (Figure 1.2a). This conversion may also be effected by heat (Bohlmann and Kleine, 1965). As may be expected for such labile chemicals, thiarubrines in plant tissues are invariably accompanied by the corresponding

a.

hν (UV, Visible)
heat
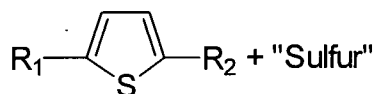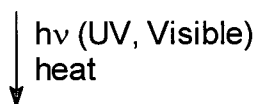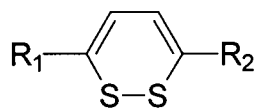
R_1: alkyne, polyyne
R_2: alkyne, polyyne
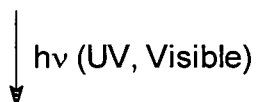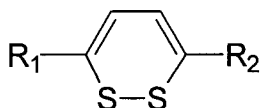
R_1——⟨S⟩——R_2 + "Sulfur"

b.

Biological Activity

R_1——⟨S–S⟩——R_2

antibacterial, antifungal (dark)

hν (UV, Visible)

"Activated Intermediates"

enhanced antibiotic (visible)

R_1——⟨S⟩——R_2 + "Sulfur"
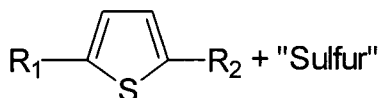
inactive (dark & visible)
antibacterial, antifungal, antiviral (UV)

Figure 1.2. Conversion of thiarubrines to thiophenes. **(a.)** Light- and heat-induced conversion of thiarubrines and other 1,2-dithiins to their corresponding thiophenes. **(b.)** Effect of UV and visible light on the biological activity of the thiarubrines.

thiophenes. Thiophenes present in thiarubrine-containing taxa may arise by the aforementioned photoconversion but may also be formed in a separate biosynthetic pathway (Constabel and Towers, 1989a). The corresponding thiophenes (**13–22**) of ten thiarubrines have been isolated and characterized (Figure 1.3).

Thiarubrines are of theoretical interest because of their eight π-electron antiaromatic ring system, a feature that makes them the only known antiaromatic molecules made by living organisms (Aihara, 1990). Relating to their antiaromatic character, early papers suggested that the 1,2-dithiin ring may be in equilibrium with its open-chain dithione tautomer (Bohlmann and Kleine, 1965; Bohlmann *et al.*, 1973; Bohlmann and Zdero, 1985). However, theoretical (Fabian and Birner, 1988; Cimiraglia *et al.*, 1991; Mann and Fabian, 1995) and spectroscopic (Radeglia *et al.*, 1988) studies have shown that the cyclic structure is energetically favoured. Synthesis of simple 1,2-dithiins was first achieved by Schroth *et al.* in 1967 (which resulted in the "discovery" of the "first" 1,2-dithiin three years after the isolation of thiarubrine B (**2**) from a plant!). Thiarubrine B (**2**) (Block *et al.*, 1994) and A (**1**) (Koreeda and Yang, 1994) have only recently been synthesized.

1.3.4. Biological Activity of Thiarubrines

Thiarubrines exhibit a diverse range of biological activities; without question, their extraordinary antibiosis places them among the most toxic of plant secondary metabolites. Potent antibacterial (Towers *et al.*, 1985; Constabel and Towers, 1989b; Ellis, 1993), antifungal (Towers *et al.*, 1985; Constabel and Towers, 1989b; Ellis, 1993; Towers *et al.*, 1993), cytotoxic (Rodriguez *et al.*, 1985; Hudson *et al.*, 1986), antitumour (only **11**, Freeman *et al.*, 1993), nematocidal (Rodriguez, 1988), insect antifeedant (Guillet, 1994) and
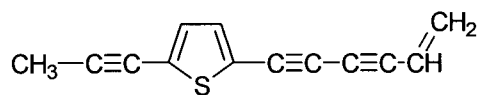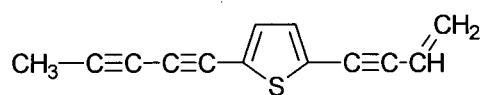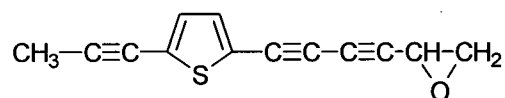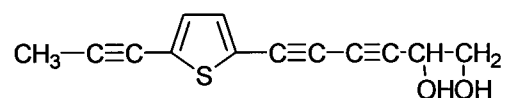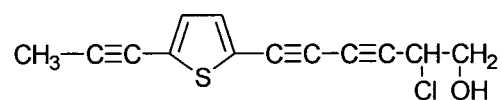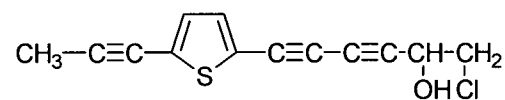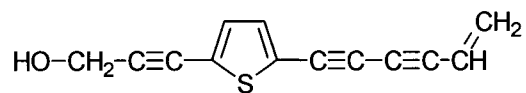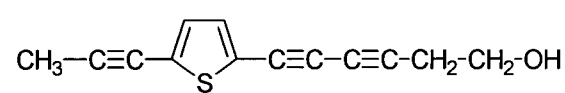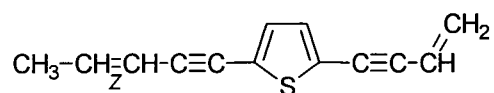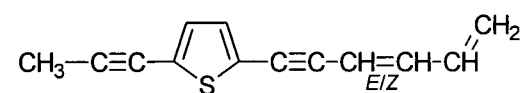
Figure 1.3. Thiophene photoproducts of thiarubrines.

insecticidal (Guillet *et al.*, in press) activities have been demonstrated in bioassays performed in the absence of light. Thiarubrine A (**1**) was found to have significant dermal and systemic (LD$_{50}$ 0.6 mg/kg) toxicity in mice (Truong *et al.*, 1995) and the use of the thiarubrines as antimicrobial drugs may be precluded by their toxic effects in mammals.

In most cases, assay of thiarubrine toxicity has been limited to testing the two most commonly occurring thiarubrines, A (**1**) and B (**2**). In a comparative study of antifungal activity, thiarubrines with polar side chains were found to possess higher activity than the unsubstituted thiarubrines A (**1**) and B (**2**) (Ellis, 1993). The same study showed that, in order of potency against *Candida albicans*, **4** > **8** > **7** > **5** > **6** > **3** > **1** > **2**.

Thiarubrines are also potently phototoxic and most of the biological activities demonstrated for the thiarubrines in the dark (i.e. antibacterial, antifungal, cytotoxic and insecticidal) have also been shown to occur with UV light irradiation (Rodriguez *et al.*, 1985; Towers *et al.*, 1985; Constabel and Towers, 1989b; Ellis, 1993; Guillet *et al.*, in press). Moreover, while thiarubrines lack antiviral activity in the dark, in combination with UV light they have been shown to be excellent antiviral agents against human immunodeficiency virus (Hudson *et al.*, 1993) and other membrane containing viruses (Hudson *et al.*, 1986a; Hudson *et al.*, 1986b). The thiophene photoproducts of the thiarubrines require UV-A light to manifest their antibacterial, antifungal and insecticidal activity and are wholly inactive in the dark (Towers *et al.*, 1985; Constabel and Towers, 1989b). Thiarubrine-derived thiophenes appear to exert phototoxicity through competing type I and II processes (Constabel and Towers, 1989b). Thiarubrines A (**1**) and C (**11**) have been claimed to undergo "redox cycling" and generate superoxide radicals (Freeman *et al.*, 1993) although no experimental evidence is available to support this assertion.

Interpretation of the light-mediated biological activities of the thiarubrines is complicated by their photolability (Figure 1.2b). Thus, UV light irradiation of thiarubrines results in the conversion of the 1,2-dithiin ring to a thiophene and the photosensitization of the thiophene photoproduct. In this way, many of the UV-mediated phototoxic effects of thiarubrines can be ascribed to the activity of thiophenes. For instance, leakage of glucose from monolamellar lipid vesicles has been observed with UV-A treatment of thiarubrines (Abramowski, personal communication), an effect that is most likely due to membrane damage caused by photosensitization of thiophenes. Surprisingly, irradiation of thiarubrines with visible light leads to enhanced antimicrobial (Constabel and Towers, 1989b) and antiviral (Hudson et al., 1993) activity compared to tests done in the dark, an effect that is not due to the thiophene photosensitization. Constabel and Towers (1989b) speculated that visible light yields "activated intermediates" that were responsible for this increase in activity, a hypothesis that is investigated in detail in Chapter 2.

The mechanism by which the thiarubrines exert their toxic effects has not been extensively investigated. Constabel and Towers (1989b) concluded that, in the dark, the cellular target of thiarubrines is not cell membranes (as is the case for thiophenes). With regards to light-independent (dark) toxicity, Bierer et al. (1995) reported a structure-activity study aimed at the synthesis of antifungal 1,2-dithiin compounds which lacked the overall toxicity of the thiarubrines. Their results suggest that the 1,2-dithiin ring is the active pharmacophoric element and that the polyyne moiety of the natural products could be removed without a decrease in activity. Furthermore, molecular modelling indicated that a single active-site nucleophile, such as a sulfhydryl moiety in a cysteine protease, is the biological target of 1,2-dithiin antifungals. Other mechanisms may need to be invoked to

explain the toxicity of the thiarubrines to organisms ranging from viruses to mammals; however, the involvement of the 1,2-dithiin ring seems likely.

1.3.5. Chemical Ecology of Thiarubrines

While little research has been directed at elucidating the ecological function of the thiarubrines, the demonstrated range of *in vitro* biological activities suggests that they have a defensive function. Their insecticidal and insect antifeedant properties, in particular, point to a role in protecting plants that contain these compounds from insect herbivory. This function is supported by the observation that thiarubrine canals in roots are arranged so that herbivores will contact them while eating. Further to a defensive role against fungal pathogens, Ellis (1993) showed that elicitation of *Ambrosia chamissonis* root cultures with cell wall preparations of the phytopathogenic fungus, *Phytopthora megasperma* f. sp. *glycine* led to an increase in the levels of the most potent antifungal thiarubrine, thiarubrine E (4).

An investigation of the consumption of *Ambrosia chamissonis* by larvae of the apparently specialist insect *Lascionycta wyatti* (Lepidoptera: Noctuidae) is currently underway in the Department of Botany, University of British Columbia. Preliminary results suggest that this insect is able to avoid thiarubrine toxicity, feeding on thiarubrine-containing diets with impunity, while generalist insect herbivores suffer toxic effects (Dojillo-Mooney, personal communication).

One aspect of the chemical ecology of the thiarubrines that has attracted a great deal of attention is their apparent role as mediators of the "medicinal" consumption of several species of the weedy shrub *Wedelia* (formerly classified as *Aspilia*) by chimpanzees (*Pan troglodytes*) in East Africa. *Wedelia* leaves purportedly contain high amounts of thiarubrine

A (1) (Rodriguez *et al.*, 1985). Considering the demonstrated antibiosis of the thiarubrines (including nematocidal activity), and the anomalous manner in which *Wedelia* leaves are consumed by chimpanzees, self-medication has provided a plausible, albeit controversial, explanation for the phenomenon of *Wedelia* leaf-swallowing.

1.4. OBJECTIVES

This thesis describes the results of an investigation into the chemical and biological properties of thiarubrines from the Asteraceae. The primary motive of this investigation was to gain a detailed understanding of the interactions, both chemical and biological, of the thiarubrines with light. As well, one aspect of thiarubrine chemical ecology, the *Wedelia–chimpanzee* interaction, was targeted for a rigorous analytical investigation. The objectives of this research were to:

1) determine the effect of visible light on the chemistry and toxicity of the thiarubrines;

2) elucidate the mechanism by which thiarubrines are protected from photoconversion *in vivo*;

3) examine the role of thiarubrines in mediating *Wedelia* leaf-swallowing behaviour by chimpanzees; and

4) isolate and assay the antifungal activity of a novel thiarubrine from *Ambrosia chamissonis*.

1.5. THESIS OVERVIEW

In the following chapters I present research aimed at addressing these objectives and, I hope,

resolving several outstanding questions concerning the thiarubrines. Chapter 2 describes the photochemistry of the thiarubrines, including the mechanism of desulfurization, the light-induced formation of novel photosulfide intermediates and the deposition of the photochemically-generated sulfur. The contribution of visible light to the toxicity of the thiarubrines to *Candida albicans* is also discussed.

Despite their photosensitivity, high amounts of the thiarubrines are present in the leaves and stems of *Ambrosia chamissonis*; the question of how they are protected from exposure to light *in vivo* is addressed in Chapter 3. I advance the hypothesis that anthocyanins function to screen thiarubrines from exposure to light, and present anatomical observations and experimental data to support this idea. An anthocyanin present in *Ambrosia chamissonis* leaves is shown to protect thiarubrines from photoconversion *in vitro*.

Chapter 4 reviews botanical and behavioural aspects of leaf-swallowing by chimpanzees, including the importance of thiarubrines and other biologically-active chemicals as mediators of this putative medicinal plant use. Chromatographic and biological assay of *Wedelia* species that are swallowed by chimpanzees conclusively shows that such leaves do not contain thiarubrines as previously reported. I discuss alternative explanations for this unusual behaviour in primates.

The isolation and characterization of a novel 28-carbon thiarubrine biogenetically derived from a 13-carbon polyyne coupled to a farnesyl chain of isoprenoid origin is described in Chapter 5. The lack of significant antifungal activity compared with other thiarubrines is discussed in relation to other structure-activity studies of the thiarubrines.

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CHAPTER 2

VISIBLE-LIGHT PHOTOCHEMISTRY AND PHOTOTOXICITY OF THIARUBRINES

2.1. INTRODUCTION

The thiarubrines are unique among plant pigments in exhibiting photolability, a property that is dramatically demonstrated by the rapid bleaching of a red thiarubrine solution upon exposure to UV or visible radiation. The characteristic colour of thiarubrines is due to the presence of a 1,2-dithiin chromophore that imparts a blue-light absorption band ($\lambda_{visible}$ 480–490 nm) in the UV-vis spectrum. Light-induced disruption of this chromophore, and its rapid conversion to a colourless thiophene photoproduct through desulfurization, are believed to be the primary photochemical events leading to the bleaching of both thiarubrines and synthetic 1,2-dithiins.

While the light sensitivity of thiarubrines has been recognized since their isolation in 1964 (Mortensen *et al.*, 1964), this property has received only cursory attention in the intervening years. In fact, the only experimental investigations of the interaction of thiarubrines with light have focussed on UV- and visible-light mediated phototoxicity. Several researchers have proposed that the process of light-induced desulfurization proceeds through an open-ring dithione (Bohlmann and Kleine, 1965; Freeman *et al.*, 1993; Schroth *et al.*, 1994) and Schroth *et al.* (1995) theorized that the thiarubrine–thiophene photochemical pathway may include bicyclic episulfide photointermediates. Two studies have demonstrated that the light-induced conversion of thiarubrines to their thiophenes results in greater toxicity than that attributable to either class of compound alone. Hudson *et al.* (1993) observed that UV-A irradiation of thiarubrine A (**1**), resulting in both the formation and photosensitization

of thiophene A (**13**), showed much higher antiviral potency than did UV-A treatment of thiophene A (**13**). Perhaps more suggestive was the finding that visible light irradiation of thiarubrine A (**1**), which leads to photoconversion without thiophene photosensitization, results in enhanced antibacterial (Constabel and Towers, 1989) and antiviral (Hudson *et al.*, 1993) activity compared to assay in the absence of light.

Constabel and Towers (1989) hypothesized that the aforementioned enhancement of biological activity by visible light is due to the formation of "activated intermediates". Support for such a hypothesis came from a serendipitous observation made during the course of my studies of thiarubrine chemistry: a red solution of thiarubrine A (**1**), once made colourless by exposure to sunlight, regains some of its red colour upon standing. Since the formation of 1,2-dithiins from thiophenes is thermodynamically prohibited, the reversible photolysis of thiarubrine A (**1**) led me to speculate on the presence of an intermediate chemical species between thiarubrines and thiophenes.

The objective of this study was to determine the effect of visible light on the chemistry and antifungal activity of the thiarubrines. Specifically, I sought to test the hypothesis that visible light exposure resulted in the conversion of thiarubrines to hitherto unknown intermediates. Here I report the characterization of a series of novel bicyclic photointermediates formed from visible light exposure of thiarubrines A (**1**), B (**2**) and D (**3**) isolated from *Ambrosia chamissonis*. In addition, I show that visible light results in phototoxicity to the pathogenic yeast, *Candida albicans*, suggesting the aforementioned photointermediates, or an activated form of sulfur, have significant biological activity.

2.2. MATERIALS AND METHODS

General experimental procedures

The HPLC-PDA system consisted of a Waters 600 pump controller, Waters 996 photodiode array detector and Waters 717 plus autosampler controlled by Millenium 2.1 software. The HPLC-MS system consisted of a Hewlett Packard 1090 Series II liquid chromatograph with autosampler interfaced with a Fisons VG Quattro mass spectrometer. A 3.9 mm X 150 mm (4 μm) C_{18} column (Waters NovaPak) equipped with NovaPak precolumn was used for analytical HPLC-PDA and HPLC-MS. Preparative HPLC was performed on a Waters 600 pump controller with a Waters U6K manual injector (3 ml sample loop) and Waters 994 photodiode array detector. UV-vis spectra were recorded on a Pye-Unicam 8720 scanning spectrophotometer in MeCN. NMR spectra were recorded in acetone-d_6 at 500 MHz (proton) and 125.7 MHz (carbon) on a Bruker AMX-500 spectrometer with the residual acetone (δ 2.08 ppm) signal as a reference. Low-resolution electron-impact mass spectrometry was performed on a Kratos MS50 instrument. A 300 W halogen lamp (Sylvania ELH) with less than 0.2 % of the spectral energy distribution in the UV region was used as a photochemical light source (Appendix 1) and light irradiance was measured using an International Light Inc. radiometer (model IL 1400A). Solvents were HPLC-grade (Fisher) except for MeOH, petroleum ether and $CHCl_3$ (reagent-grade, Fisher) used for thiarubrine isolation. Water was Milli-Q plus (Millipore). All procedures were performed in dim light to minimize photoconversion of thiarubrines.

Isolation of thiarubrines and thiophenes

Ambrosia chamissonis (Less.) Greene roots were collected near Centennial Beach,

Tsawwassen, Canada. A voucher specimen is deposited in the Herbarium of the University of British Columbia. Fresh roots (1.75 kg) were extracted with 5 l MeOH in a Waring blender and filtered through Whatman #1 filter paper. The resulting methanolic extract, after concentration to *ca.* 500 ml *in vacuo*, was partitioned with petroleum ether (4 X 200 ml) and $CHCl_3$ (3 X 200 ml). Evaporation of the petroleum ether fraction *in vacuo* gave a dark red residue that was pre-adsorbed on silica gel and subjected to vacuum liquid chromatography (VLC) (Coll and Bowden, 1986). The sample was applied to the top of a 350 ml sintered glass funnel (C) packed with Keiselgel G to a height of 4.5 cm and eluted with petroleum ether to give a red lipophilic fraction. After evaporation to dryness, the residue was resuspended in MeCN and subjected to column chromatography (CC) over "home-made" reversed-phase silica gel (Kuhler and Lindsten, 1983). Elution with MeCN–water (50:50) afforded three distinct red bands, the second of which was shown by analytical HPLC to be mainly composed of thiarubrines A (**1**) and B (**2**) and thiophenes A (**13**) and B (**14**). Separation of this mixture was achieved by repeated preparative HPLC on a 2.5 X 10 cm (5 µm) C_{18} radial compression column (Waters NovaPak) equipped with NovaPak precolumn. The mobile phase was composed of MeCN–water (74:26) at a flow rate of 27 ml/minute with detection at 340 nm. Injection volume was 100–500 µl. The resulting aqueous MeCN fractions were subjected to rotary evaporation and the aqueous solutions thus obtained extracted with CH_2Cl_2. Removal of the solvent *in vacuo* afforded thiarubrines A (**1**) and B (**2**) as red oils. Thiophenes A (**13**) and B (**14**) were obtained by isolation from the petroleum ether extract of *A. chamissonis* roots and by photoconversion of **1** and **2**, followed by purification by preparative HPLC (conditions as above).

Evaporation of the $CHCl_3$ fraction (above) *in vacuo* gave a dark red residue that was

shown by analytical HPLC to contain thiarubrine D (**3**) and thiophene D (**15**). Impure thiarubrine D (**3**) was obtained by separation of this residue by preparative reversed-phase HPLC (conditions as above). Final purification was achieved by preparative normal-phase HPLC on a 19 X 300 mm (10 μm) silica column (Waters μPorasil) equipped with a silica (Waters Resolve) precolumn eluted with a gradient with the following conditions: 0 minutes (100 %; hexane), 5 minutes (50:50; hexane–ethyl acetate), 10 minutes (50:50; hexane–ethyl acetate). The flow rate was 20 ml/minute, detection was at 340 nm and injection volume was 500 μl. Removal of the solvent *in vacuo* afforded thiarubrine D (**3**) as a red oil. Exposure of a solution of **3** to light followed by purification by reversed-phase and normal-phase preparative HPLC (conditions as above) afforded thiophene D (**15**).

Thiarubrines A (**1**), B (**2**) and D (**3**) and thiophenes A (**13**), B (**14**) and D (**15**) were >99% pure as determined by HPLC. All compounds were identified by comparison of their UV-vis and ^{1}H-NMR spectra with literature values (Gomez-Barrios *et al.*, 1992; Balza and Towers, 1993). The ^{13}C-NMR spectra of **1** and **2** were in agreement with published values (Gomez-Barrios *et al.*, 1992). The concentration of thiarubrine (ε = 3000 M^{-1} cm^{-1} at 490 nm) and thiophene (ε = 31,500 M^{-1} cm^{-1} at 340 nm) solutions was determined spectrophotometrically.

Thiarubrine A (**1**): UV-vis see Table 2.1; ^{1}H-NMR δ 7.03 (1 H, d, J = 6.7 Hz, H-8), 6.81 (1 H, d, J = 6.7 Hz, H-9), 6.16 (1 H, dd, J = 11.5, 17.5 Hz, H-2), 6.00 (1 H, dd, J = 1.9, 17.5 Hz (H-1$_a$), 5.90 (1 H, dd, J = 2.0, 11.4 Hz, H-1$_b$), 2.09 (3 H, s, Me-13) ppm; ^{13}C-NMR δ 138.1 (C-8), 134.0 (C-9), 133.8 (C-1), 118.6 (C-10), 116.1 (C-2), 110.7 (C-7), 99.9 (C-12), 86.1 (C-3), 81.1 (C-5), 77.3 (C-6), 77.0 (C-11), 73.6 (C-4), 4.5 (C-13) ppm. (See Appendix 2).

Thiarubrine B (**2**): UV-vis see Table 2.1; ^{1}H-NMR δ 7.00 (1 H, d, J = 6.7 Hz, H-7),

6.89 (1 H, d , J = 6.9 Hz, H-6), 6.20 (1 H, dd, J = 11.6, 17.7 Hz, H-2), 5.83 (1 H, dd, J

= 1.8, 17.6 Hz, H-1$_a$), 5.77 (1 H, dd, J = 1.7, 11.4 Hz, H-1$_b$), 2.11 (3 H, s, Me-13)

ppm; ^{13}C-NMR δ 137.4 (C-6), 134.9 (C-7), 130.7 (C-1), 116.8 (C-2), 115.8 (C-5),

112.9 (C-8), 98.5 (C-3), 88.6 (C-4), 86.5 (C-10), 83.3 (C-12), 69.3 (C-9), 63.7 (C-11),

4.3 (C-13) ppm. (See Appendix 2).

Thiarubrine D (**3**): UV-vis see Table 2.1; ^{1}H-NMR δ 7.05 (1 H, d, J = 6.9 Hz, H-8),

6.80 (1 H, d, J = 6.9 Hz, H-9), 3.77 (1 H, dd, J = 2.6, 4.1 Hz, H-2), 3.07 (1 H, dd, J =

4.2, 5.9 Hz, H-1$_b$), 3.03 (1 H, dd, J = 2.5, 5.9 Hz, H-1$_a$), 2.11 (3 H, s, Me-13) ppm.

(See Appendix 2).

Thiophene A (**13**): UV-vis see Table 2.1; ^{1}H-NMR δ 7.47 (1 H, d, J = 4.0 Hz, H-8),

7.22 (1 H, d, J = 4.0 Hz, H-9), 6.13 (1 H, dd, J = 11.5, 17.6 Hz, H-2), 5.96 (1 H, dd, J

= 1.9, 17.6 Hz, H-1$_a$), 5.87 (1 H, dd, J = 1.9, 11.4 Hz, H-1$_b$), 2.09 (3 H, s, Me-13)

ppm. (See Appendix 2).

Thiophene B (**14**): UV-vis see Table 2.1; ^{1}H-NMR δ 7.47 (1 H, d, J = 4.0 Hz, H-8),

7.22 (1 H, d, J = 4.0 Hz, H-9), 6.13 (1 H, dd, J = 11.7, 17.6 Hz, H-2), 5.96 (1 H, dd, J

= 1.9, 17.6 Hz, H-1$_a$), 5.87 (1 H, dd, J =1.9, 11.4 Hz, H-1$_b$), 2.05 (3 H, s, Me-13)

ppm. (See Appendix 2).

Thiophene D (**15**): UV-vis see Table 2.1; ^{1}H-NMR δ 7.34 (1 H, d, J = 3.9 Hz, H-8),

7.08 (1 H, d, J = 3.9 Hz, H-9), 3.63 (1 H, dd, J = 2.5, 4.1 Hz, H-2), 3.01 (1 H, dd, J =

4.1, 6.0 Hz, H-1$_a$), 2.95 (1 H, dd, J = 2.5, 6.0 Hz, H-1$_b$), 2.07 (3 H, s, Me-13) ppm.

(See Appendix 2).

Photolysis of thiarubrine A (1): spectrophotometry

Photolysis of thiarubrine A (**1**) was performed at room temperature in a small volume quartz

cuvette (1 X 0.4 X 3.5 cm i.d.). A solution of **1** in MeCN (1 ml, 0.24 mM) was exposed to

broad spectrum visible light (irradiance of *ca.* 950 mW/cm^2) from a halogen lamp for 15 s

after which the cuvette was sealed and placed in the UV-vis spectrophotometer. Absorbance

at 490 nm was determined immediately after photolysis and at 1, 2, 3, 4, 5, 10, 20, 30 and 60

minutes thereafter. Three separate experiments were performed and the results averaged.

Photolysis of thiarubrines: HPLC-PDA

Photolyses of thiarubrine A (**1**), B (**2**) and D (**3**) were performed at room temperature in

sealed 1 ml glass autosampler vials. MeCN solutions of **1**, **2** and **3** (0.8 ml, 0.15 mM) were

exposed to light from a halogen bulb (as above) for 30 s and analyzed immediately after

photolysis by HPLC. Twenty-μl aliquots were injected and eluted with MeCN–water (74:26)

at 1 ml/minute with detection over the wavelength range 200–600 nm. To examine the effect

of temperature on photointermediate stability, a solution of **1** (0.8 ml, 0.5 mM) was

photolysed and incubated at room temperature or at 0 °C in an ice bath. The resulting

solutions were analyzed by HPLC (conditions as above) immediately after photolysis and at

20, 40, 60 and 120 minutes thereafter. Three separate experiments were performed and the

results averaged.

Photolysis of thiarubrine A (1): HPLC-MS

Solutions of thiarubrine A (**1**) (2.14 mM) and thiophene A (**13**) (0.57 mM) were analyzed by HPLC with atmospheric pressure chemical ionization (APCI) mass spectrometric detection. The mobile phase consisted of MeCN–water containing 5 mM ammonium acetate buffer (74:26) at a flow rate of 1 ml/minute with no predetection splitting of the solvent stream. Injection volume was 20 μl. A solution of **1** was analyzed before and immediately after irradiation for 15 s with a halogen lamp.

Photolysis of thiarubrines: NMR

Samples (*ca.* 8–10 mg) of thiarubrine A (**1**), B (**2**) and D (**3**) were dried *in vacuo*, and taken up in acetone-d_6. The resulting solutions were transferred to a standard NMR tube and deaerated by three freeze-pump-thaw cycles (-195 °C, liquid N_2). After cooling with dry ice/acetone in a Dewar flask (-78 °C), the thiarubrine samples were irradiated with a halogen lamp until maximum bleaching was observed (4–5 minutes). ^{1}H-, ^{13}C- and heteronuclear multiple quantum coherence (HMQC)/heteronuclear multiple bond correlation (HMBC) NMR spectra were obtained at -60 °C.

Isolation and characterization of a sulfur precipitate

A dark precipitate present in a acetone-d_6 solution of thiarubrine A (**1**) that had undergone photolysis, after drying with a stream of N_2, was taken up in hexane and preadsorbed on silica gel (Keiselgel G). Separation by VLC on a 30 ml sintered glass funnel packed with 2.5 cm Keiselgel G eluted with hexane gave 4 fractions. Thin-layer chromatographic (TLC) analysis on Keiselgel 60 F_{254} aluminum backed plates (0.2 mm) developed with hexane

(detection with sodium azide spray reagent (Davies and Thuraisingham, 1968)) showed fraction 1 contained sulfur. After drying, fraction 1 was subjected to mass spectrometry.

Cyclooctasulfur (24): EI-MS *m/z* [M]$^+$ (intensity) 256 (33), 243 (9), 192 (24), 160 (27), 128 (31), 111 (34), 97 (46), 85 (49), 71 (68), 57 (100). (See Appendix 4).

Visible light phototoxicity of thiarubrine A (1)

Phototoxic antifungal activity was determined using a protocol modified from Towers *et al.* (1985). A 24-hour culture of *Candida albicans* (UBC #54), grown at 37 °C in Sabouraud dextrose broth, was centrifuged at 1600 rpm for 5 minutes. After decanting the supernatant, the pelleted cells were washed twice in saline (0.85% NaCl), and resuspended to Abs$_{520}$ = 0.1 in saline. Aliquots (2 ml) of the resulting cell suspension were transferred to small sterile Petri plates (35 X 10 mm) and 20 µl of test solutions of thiarubrine A (1) and thiophene A (13) (10 µg/ml in MeOH), or of MeOH (control) were added. The final concentration of 1 and 13 in the cell suspension was 0.1 µg/ml. After incubation of the plates for 30 minutes at 37 °C on a rotary shaker, 100-µl samples were removed and the plates were incubated for 10 minutes under broad spectrum visible light from a halogen bulb (7.4 mW/cm^2) or in darkness. After completion of light or dark treatment, 100-µl samples were again removed. Samples withdrawn at 0 and 10 minutes were serially diluted in saline (10^{-1}–10^{-4}), and 100-µl aliquots of these dilutions plated on potato dextrose agar. The number of viable cells was counted after incubation of the plates at 37 °C in the dark for 24 hours. Three experiments were performed and the results averaged. The toxicity (percent cell mortality) of 1, 13 and

the control in the presence and absence of visible light was compared by single factor analysis of variance (ANOVA).

2.3. RESULTS

2.3.1. Observation of Photointermediate (Photosulfide) Formation

The first physicochemical evidence that the photoconversion of thiarubrines to thiophenes proceeds through an intermediate chemical species (i.e. photointermediates) was obtained from spectrophotometric analysis of thiarubrine bleaching. Irradiation of a solution of thiarubrine A (1) with visible light resulted in a 94.5% decrease in the concentration of the compound as determined by spectrophotometry (Abs_{490}). When the resulting bleached solution was allowed to stand at room temperature, 9.6% of the prephotolysis concentration of thiarubrine A (1) was observed to reform, an effect that is illustrated in Figure 2.1. After initially proceeding rapidly, the reformation of 1 levelled off and reached a maximum after *ca.* 60 minutes. Liquid chromatographic analysis of an irradiated thiarubrine A (1) solution confirmed the presence of photointermediates, and showed that the reversibility of the bleaching was due to the reformation of the irradiated thiarubrine. Figure 2.2 illustrates the results of HPLC analysis of a solution of thiarubrine A (1) before and after photolysis. Prior to photolysis, thiarubrine A (1) was detectable as a single peak (R_t 7.6 minutes) that was replaced by two new peaks upon exposure of the solution to visible radiation. The less polar of these was identifiable as thiophene A (13) on the basis of its retention time and UV-vis spectrum, while the more polar peak was attributable to the newly formed photointermediate. In photolysis experiments conducted with thiarubrine B (2) and thiarubrine D (3), a similar
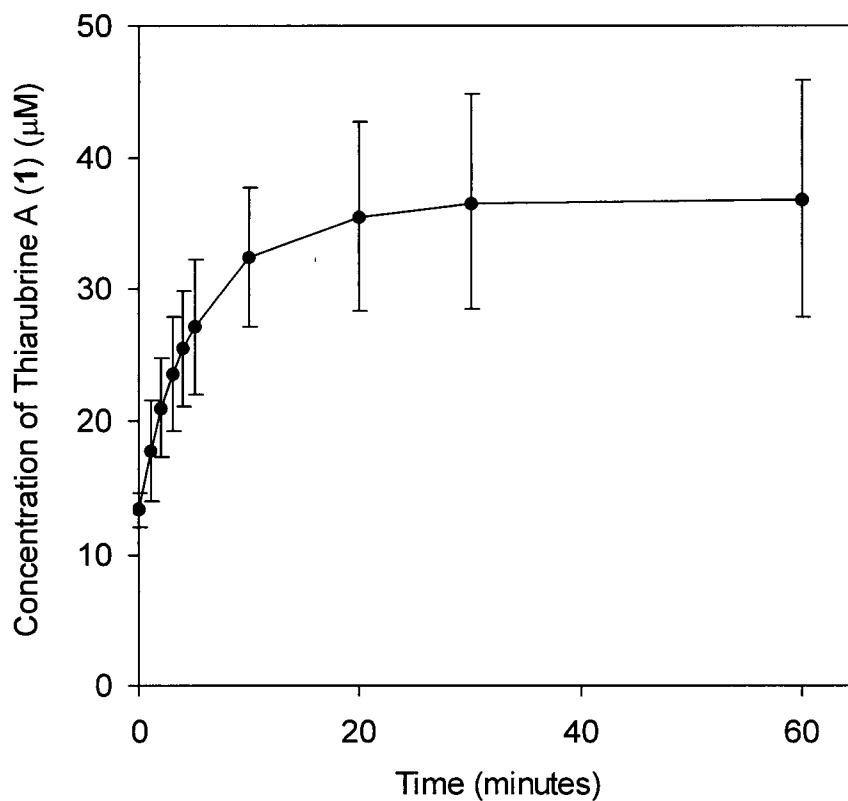
Figure 2.1. Kinetics of reformation of thiarubrine A (**1**) after photolysis. A cuvette containing a solution of **1** was bleached by visible-light exposure, and the amount of **1** present determined by spectrophotometry (Abs_{490}) at 0, 1, 2, 3, 4, 5, 10, 20, 30 and 60 minutes thereafter. Each point indicates the mean of three independent experiments. Error bars represent standard deviation.

a.

b.

c.

Thiarubrine A
Thiophene A
Photointermediate Peak
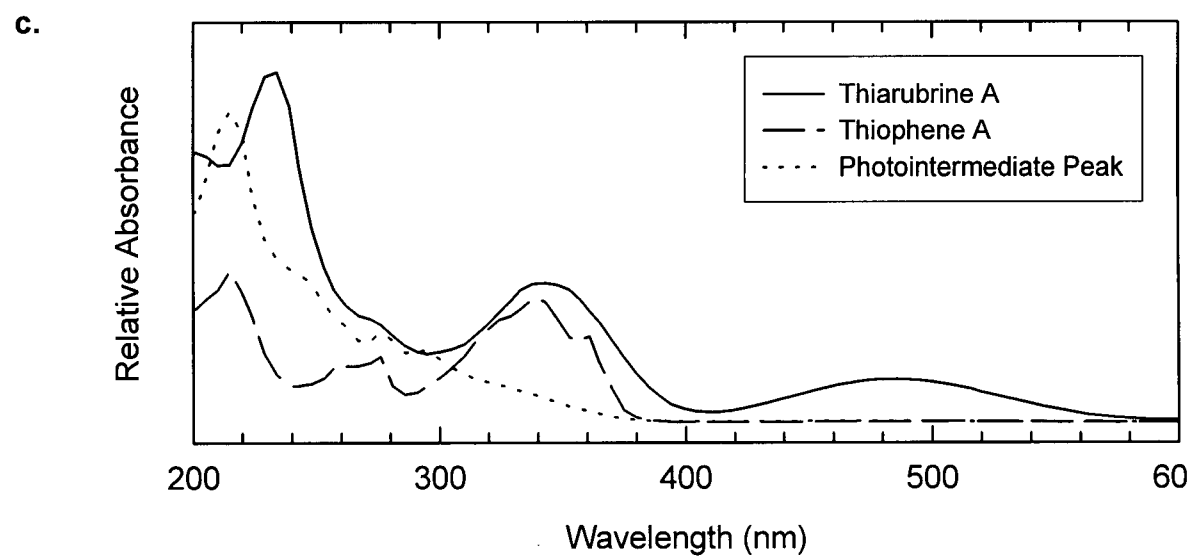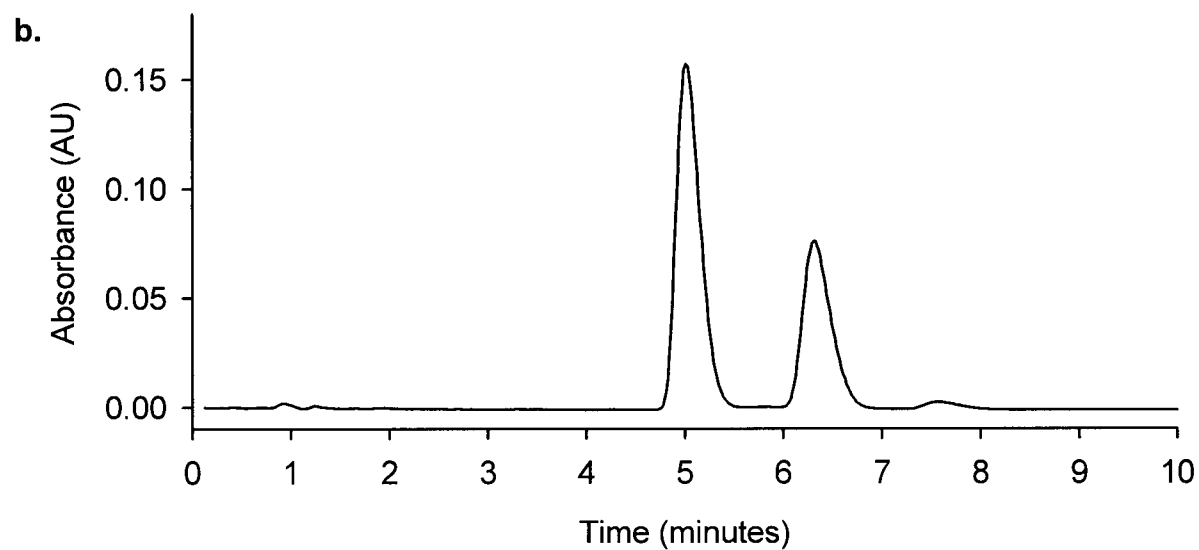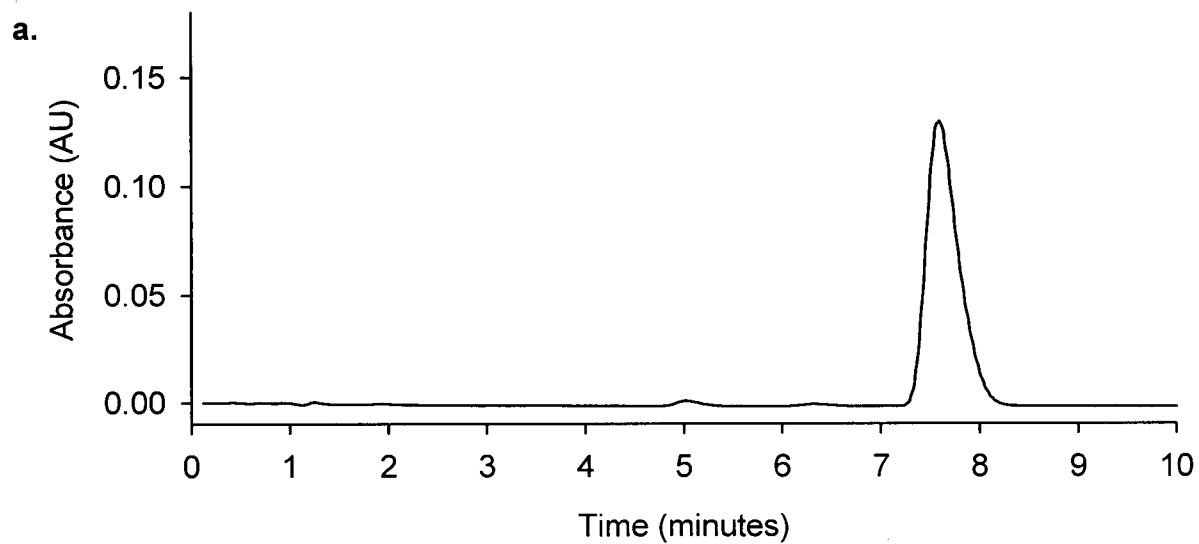
Figure 2.2. Photolysis of thiarubrine A (**1**) monitored by HPLC with photodiode array detection. Chromatograms were derived at 215 nm. (**a.**) Chromatogram obtained from injection of solution of **1** (R_t 7.6 min) prior to irradiation. (**b.**) Analysis of a solution of **1** immediately after 30 s irradiation with visible light. Peaks attributable to thiophene A (**13**) and to photointermediate(s) are visible at 6.1 min and 4.9 min, respectively. (**c.**) UV-vis spectra of thiarubrine A (**1**), thiophene A (**13**) and photointermediate(s) as determined by photodiode array detection.

chromatographic pattern was observed (Table 2.1). In all three cases, photointermediate peaks were found to elute before the corresponding thiophenes.

Thiarubrine photointermediates are short-lived and unstable; injection of photolysed solutions of thiarubrine A (**1**) at 20, 40, 60 120, 180, 240 and 300 minutes after photolysis showed that photointermediate peaks rapidly disappear with a concomitant increase in the amount of thiophenes and, to a lesser extent, thiarubrines (Figure 2.3). Cooling a photolysed solution of thiarubrine A (**1**) from room temperature to 0 °C led to quadrupling of the lifetime of photointermediates from *ca.* 60 minutes to *ca.* 240 minutes.

2.3.2. Characterization of Photosulfides

HPLC coupled to UV-vis spectroscopy and mass spectrometry proved useful in elucidating the structures of thiarubrine photointermediates. The UV-vis absorption spectra of thiarubrine A (**1**), thiophene A (**13**) and the photointermediate peak, obtained by on-line photodiode array (PDA) detection, are overlaid in Figure 2.2c. Table 2.1 summarizes the UV-vis spectral data for thiarubrines A, (**1**), B (**2**) and D (**3**), as well as their thiophenes and photointermediates. The photointermediates of thiarubrines **1–3** all show minimal absorption in the UV-A region (320–400 nm), where both thiarubrines and thiophenes strongly absorb, and exhibit none of the visible absorption of thiarubrines, indicating that the photointermediates lack the conjugated chromophore of either class of compound.

HPLC coupled to atmospheric-pressure chemical ionization (APCI) mass spectrometry allowed accurate measurement of the mass of the photointermediate peak of thiarubrine A (**1**). In the upper panel of Figure 2.4, the total ion chromatogram obtained after

Table 2.1. HPLC retention times and UV-vis spectra of thiarubrines A (**1**), B (**2**) and D (**3**) and their photolysis products.

	Thiarubrine Parent	Thiophene	Photointermediates
Thiarubrine A (**1**)	7.6[a] (239, 348, 491)[b]	6.1 (215, 276, 338, 361s[c])	4.9 (215, 276s)[d]
Thiarubrine B (**2**)	7.1 (229, 353, 491)	5.9 (220, 253, 334, 353s)	4.7 (268, 310s)[d]
Thiarubrine D (**3**)	4.1 (229, 348, 486)	3.5 (210, 253, 329, 343s)	3.0 (238, 300s)[d]

[a] retention time (minutes).
[b] spectral maxima (nm).
[c] s = shoulder.
[d] see Appendix 3 for UV-vis spectra of photointermediates.
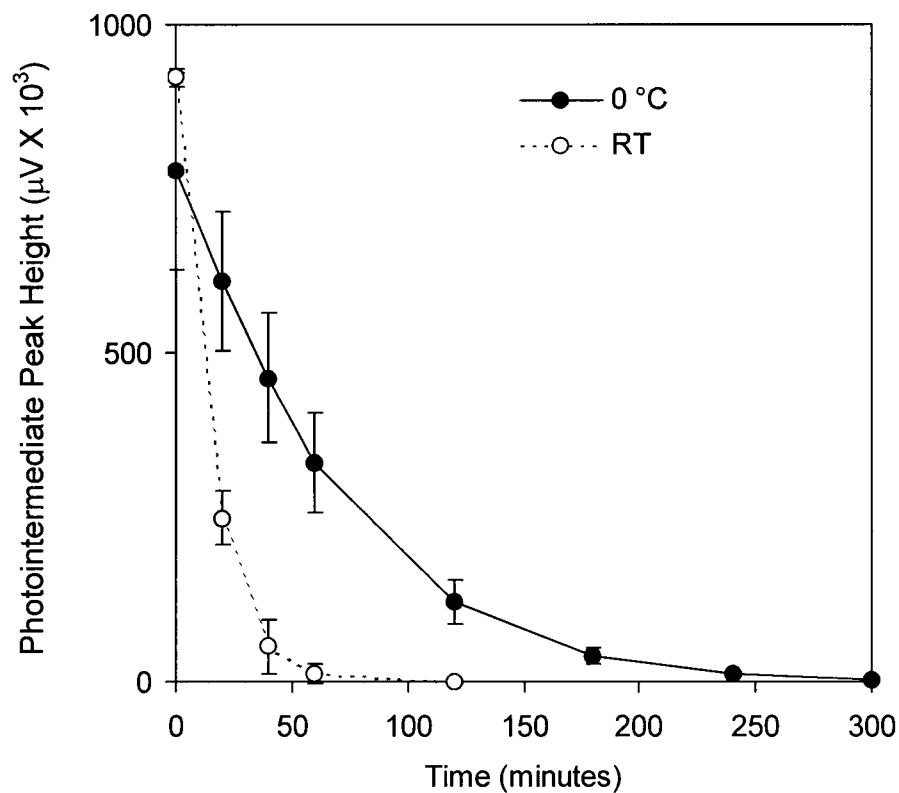
Figure 2.3. Effect of temperature on photointermediate stability as determined by HPLC analysis of irradiated solutions of thiarubrine A (**1**). Solutions maintained at room temperature or at 0 °C in an ice bath were irradiated for 30 s with visible light and the amount of photointermediate present determined chromatographically on the basis of peak height at 0, 20, 40, 60, 120, 240 and 300 minutes thereafter. Data points indicate means of three experiments. Error bars represent standard deviation.
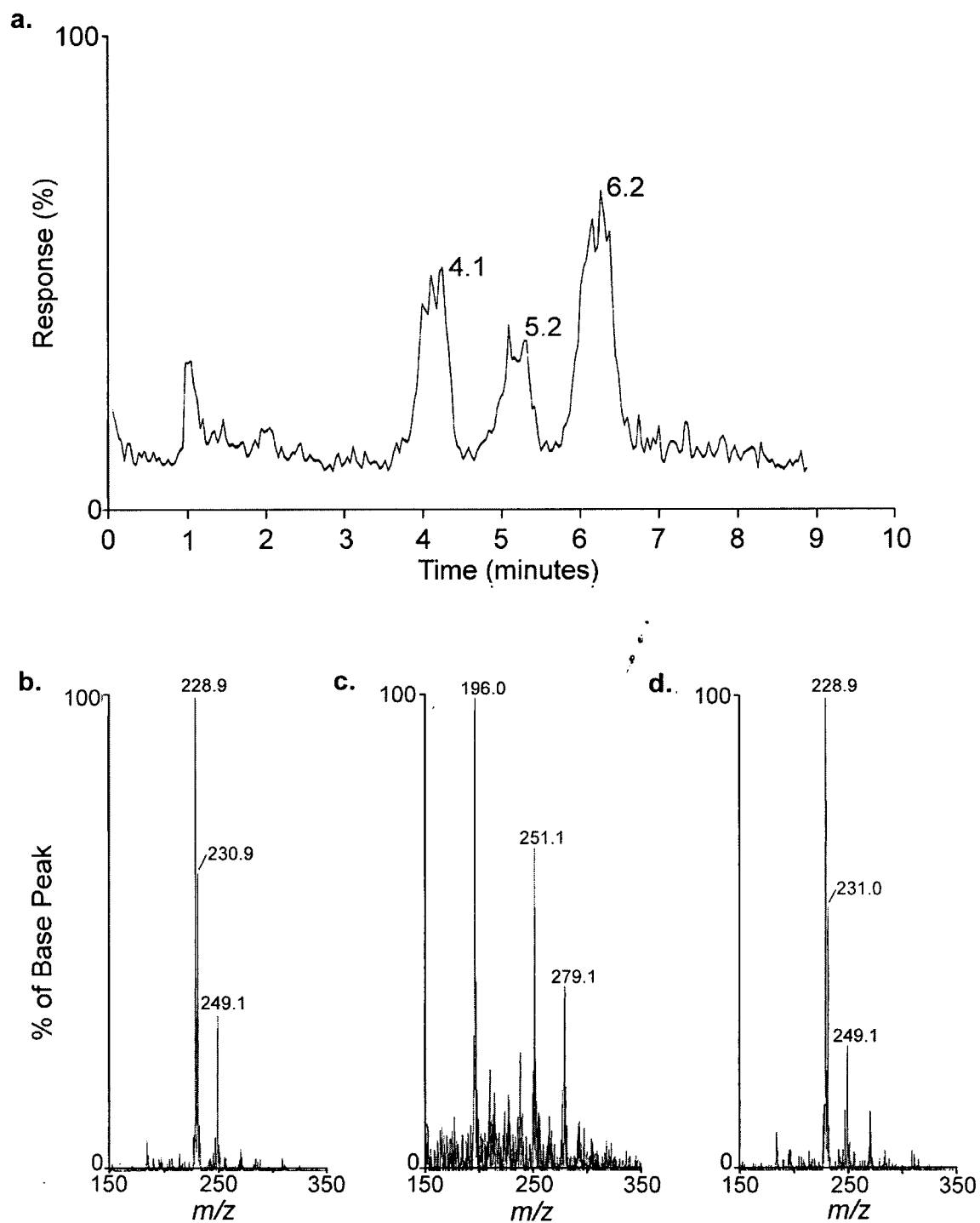
Figure 2.4. HPLC-MS analysis of an irradiated solution of thiarubrine A (**1**). (**a.**) Total ion chromatogram obtained from injection of a solution of **1** immediately after irradiation with visible light for 30 s. Peaks corresponding to **1**, **13** and photointermediate(s) are visible at 6.2, 5.2 and 4.1 minutes, respectively. (**b.**) Mass spectrum of **1** with molecular ion peak [M]⁺ at m/z 228.9 indicated. (**c.**) Mass spectrum of **13**. (**c.**) Mass spectrum of photointermediate(s) showing molecular ion peak [M]⁺ at m/z 228.9.

injection of an irradiated solution of thiarubrine A (1) shows three peaks: photointermediate (R_t 4.1 minutes), thiophene A (13) (R_t 5.2 minutes) and 1 (R_t 6.2 minutes). The mass spectrum of each peak is depicted in Figure 2.4b–d. Comparison of the mass spectrum of the photointermediate with that of its thiarubrine parent compound, 1, shows that the molecular ion peaks $[M]^+$ of both compounds have identical masses (m/z 228.9). Thus, on the basis of the results of HPLC-PDA and HPLC-MS analysis of irradiated thiarubrine A (1) solutions, I was able to conclude that although the photointermediates clearly lack a 1,2-dithiin (or thiophene) ring system, they have not yet lost an atom of sulfur.

Due to the instability of thiarubrine photointermediates, application of conventional NMR techniques to their spectroscopic characterization was impractical. This problem was overcome through the use of low-temperature NMR, which allowed spectroscopic experiments of up to 12 hours duration to be performed without substantial decreases in photointermediate concentration. In a simple procedure, de-aerated samples of thiarubrines in NMR tubes were submerged in acetone/dry ice in a Dewar flask and exposed to high intensity light with the mirrored parabolic bottom of the flask serving to focus the light on the sample. This bleaching technique, and the use of low-temperature NMR for studies of 1,2-dithiin photolysis, was developed in the laboratory of Professor E. Block, Chemistry Department, The State University of New York at Albany.

Figure 2.5 illustrates the ^{1}H-NMR spectra of thiarubrine A (1), photolysed 1 and thiophene A (13). The ring protons (H-9 and H-9) of a 1,2-dithiin system show a characteristic AB pattern of resonances in the δ 6.5–7.2 ppm region while those of thiophenes resonate further downfield (δ 7.0–7.5 ppm) (Balza and Towers, 1993). Photolysis of thiarubrine A (1) results in a mixture of three compounds (Figure 2.5b): a small amount of
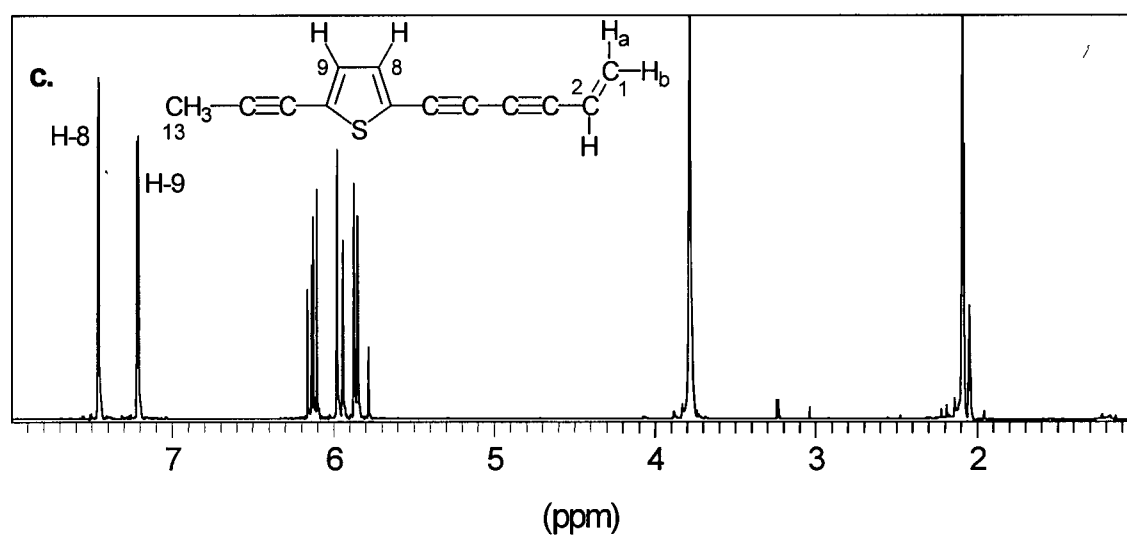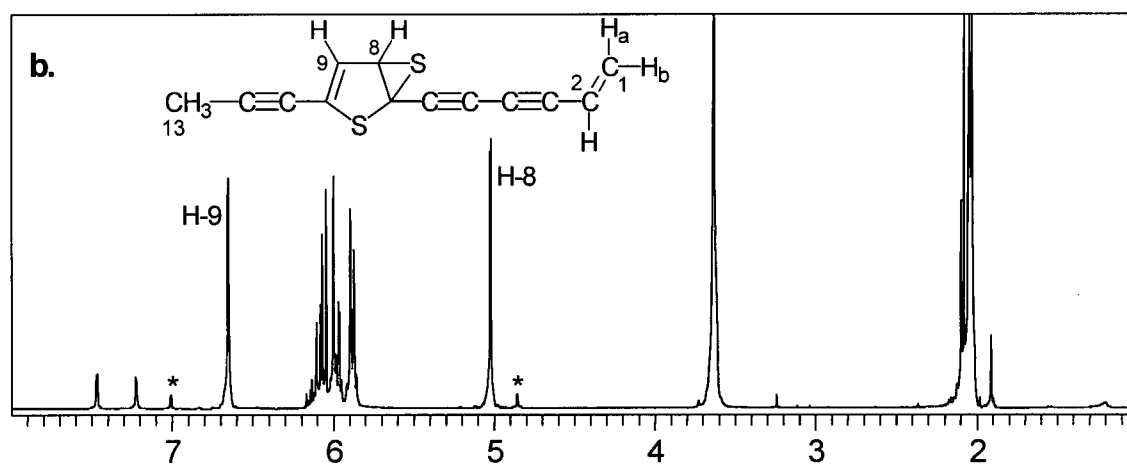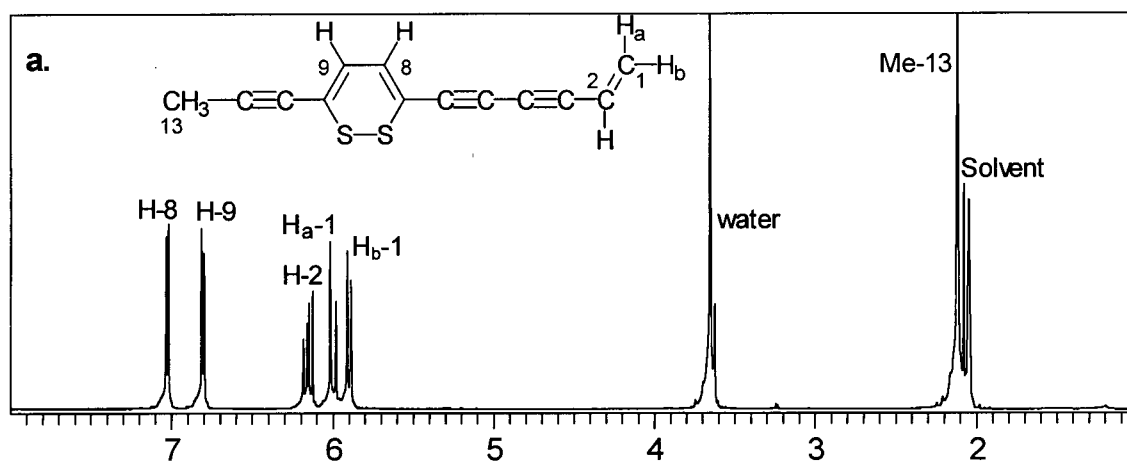
Figure 2.5. ^{1}H-NMR analysis of the photolysis of thiarubrine A (**1**). (**a.**) ^{1}H-NMR spectrum of **1** prior to irradiation. (**b.**) ^{1}H-NMR spectrum obtained after irradiation of **1** with visible light for 4–5 minutes. Asterisks indicate ring proton signals of minor photointermediate (**1b**). Sample was cooled to -60 °C during irradiation and acquisition of spectral data. (**c.**) ^{1}H-NMR spectrum of thiophene A (**13**). A water peak is visible at δ 3.6–3.8 ppm in all three spectra.

thiophene A (**13**) (integrating to 10.4% of the total area), identifiable on the basis of its ring proton signals at δ 7.22 ppm (H-9) and δ 7.47 ppm (H-8), and major and minor photointermediates representing 86.2% and 3.4% of the total area, respectively.

Both the major (**1a**) and minor (**1b**) photointermediates of thiarubrine A (**1**) displayed ^{1}H- and ^{13}C-NMR spectra that were similar to their thiarubrine parent except for radical upfield-shifts in resonances of one proton and two carbons that were formerly part of the 1,2-dithiin system. In the proton spectrum, these signals were observed at δ 5.03 ppm in **1a** and at δ 4.86 ppm in **1b** (Figure 2.5b). Due to the more limited sensitivity of ^{13}C-NMR, data for the mixture generated from photolysis of thiarubrine A (**1**) showed only the major photointermediate (**1a**) and a few of the more intense signals of thiophene A (**13**) (Figure 2.6). Of the thirteen major carbon signals observed in the carbon spectrum, only the upfield-shifted signals at δ 58.67 ppm and δ 53.89 ppm differed substantially from thiarubrine A (**1**). Analysis of the heteronuclear HMQC spectrum showed the former to be bonded to the upfield-shifted ring proton while the latter (δ 53.89 ppm) carbon resonance, by virtue of its low intensity and lack of attached protons, was concluded to be a quaternary ring carbon. While this work was in progress, 1,2-dithiin and other simple synthetic 1,2-dithiins were shown to yield novel 2,6-dithiabicyclo[3.1.0]hexenes possessing an episulfide (thiirane) ring as photointermediates (Block, personal communication, 1996; Block *et al.*, 1996). Comparison of the ^{1}H- and ^{13}C-NMR spectra of the photolysis products of thiarubrine A (**1**) with NMR spectral data obtained for 2,6-dithiabicyclo[3.1.0]hexenes showed that the upfield-shifted proton and carbon signals in **1a** and **1b** resulted from the formation of an episulfide ring (Figures 2.5b, 2.6b).
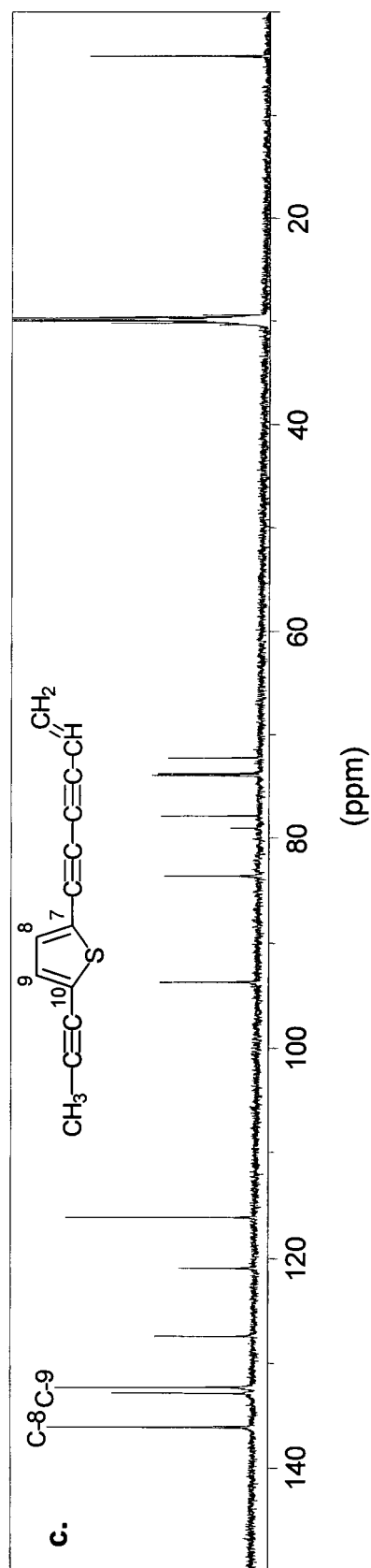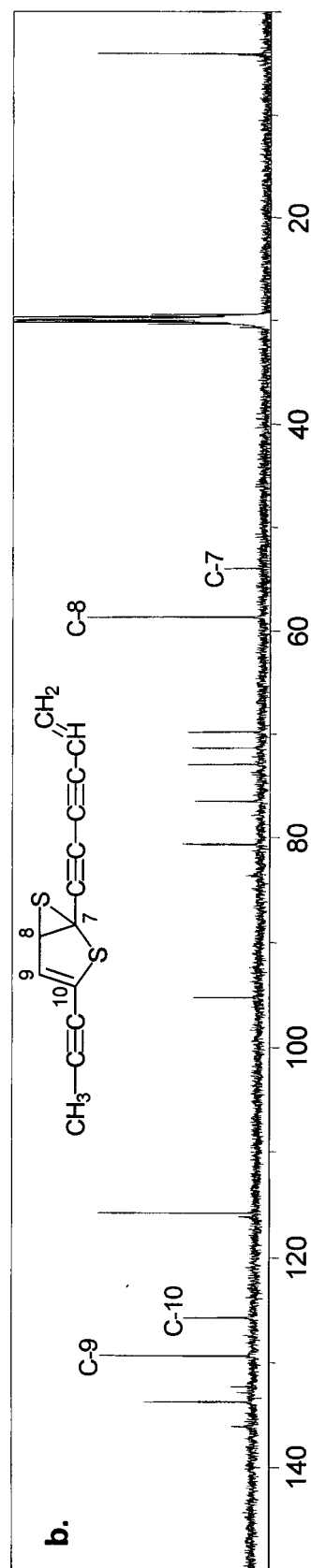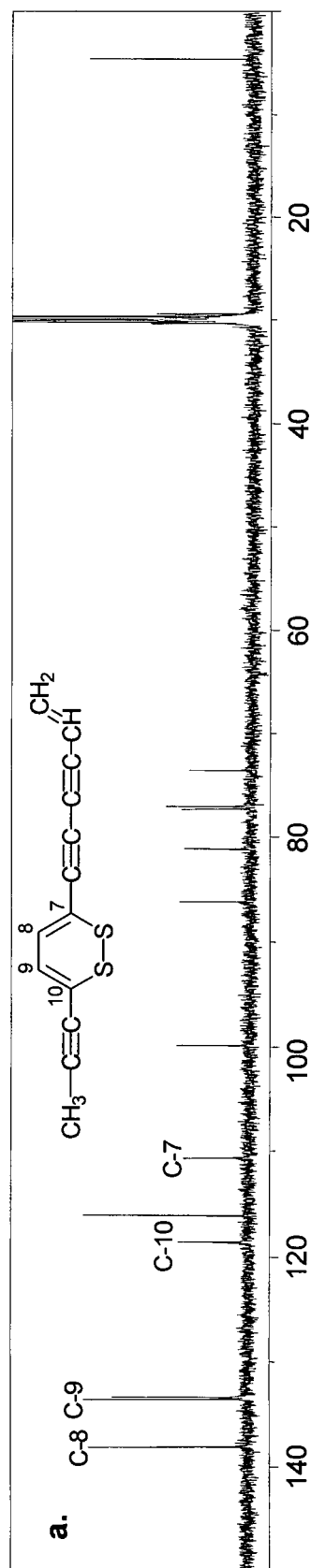
Figure 2.6. ^{13}C-NMR analysis of the photolysis of thiarubrine A (**1**). (**a.**) ^{13}C-NMR spectrum of **1** prior to irradiation. (**b.**) ^{13}C-NMR spectrum acquired after irradiation of **1** with visible light for ~4–5 minutes. Sample was cooled to -60 °C during irradiation and acquisition of spectral data. (**c.**) ^{13}C-NMR spectrum of thiophene A (**13**).

Due to the unsymmetrical side-chains present in thiarubrine A (1), the episulfide ring may be positioned either proximal or distal to the methyl-bearing side-chain. A heteronuclear HMBC spectrum, which is summarized graphically in Figure 2.7, allowed for the determination of the position of the episulfide ring in 1a and 1b relative to the polyyne side-chains. A correlation was established between the ring proton doublet at δ 6.66 ppm (H-9) of 1a and the acetylenic carbon at δ 71.36 ppm (C-11), a carbon signal that was also correlated to the protons of terminal methyl signal at δ 2.1 ppm (H-13). Hence, the position of the episulfide ring was proximal to the diyne-ene side-chain in the major isomer 1a and, by extension, distal to it in the minor isomer, 1b.

Thus, irradiation of thiarubrine A (1) yields the novel bicyclic photointermediates 1-(5-penten-1,3-diynyl)-5-(1-propynyl)-2,6-dithiabicyclo[3.1.0]hex-4-ene (1a) and 1-(1-propynyl)-3-(5-penten-1,3-diynyl)-2,6-dithiabicyclo[3.1.0]hex-3-ene (1b) (Figure 2.8). Recognizing their formation by light (Greek *photos* 'light') and the presence of the episulfide moiety in the bicylic photointermediates of thiarubrines, I have assigned the trivial name "photosulfides" to these previously unknown molecules (i.e. isomeric photosulfides 1a and 1b from thiarubrine A (1)). Based on the analysis of 2D NMR spectra (HMQC and HMBC), the complete ^{1}H- and ^{13}C-NMR assignments for photosulfide 1a and the incompletely assigned spectrum of photosulfide 1b are presented in Table 2.2.

Application of cold-temperature NMR to the photolysis of thiarubrines B (2) and D (3) showed that each yields isomeric bicyclic photointermediates in a similar fashion to thiarubrine A (1). Table 2.2 presents the complete ^{1}H- and ^{13}C-NMR assignments of photosulfide 2a, 1-(1,3-pentadiynyl)-3-(3-buten-1-ynyl)-2,6-dithiabicyclo[3.1.0]hex-3-ene, and photosulfide 3a, 1-(5,6-epoxy-hex-1,3-diynyl)-5-(1-propynyl)-2,6-dithiabicyclo[3.1.0]
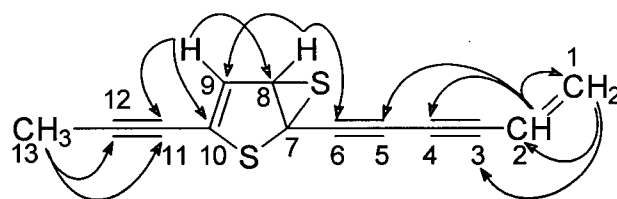
Figure 2.7. Selected HMBC correlations of photosulfide **1a**.
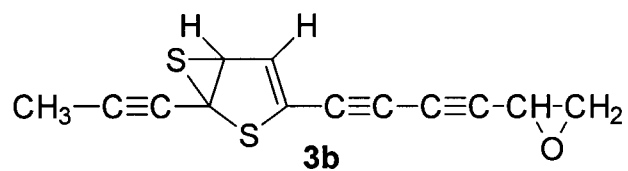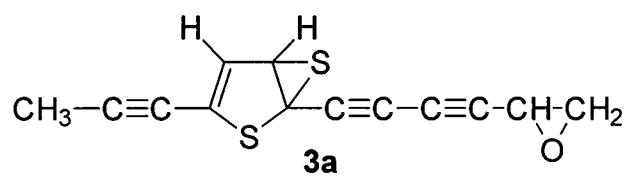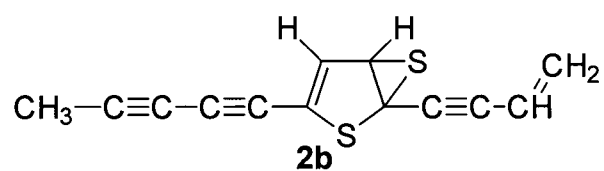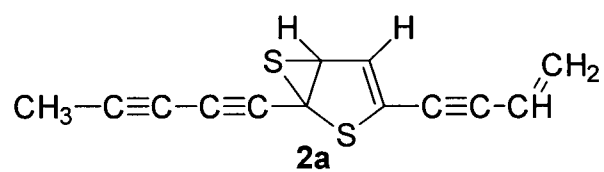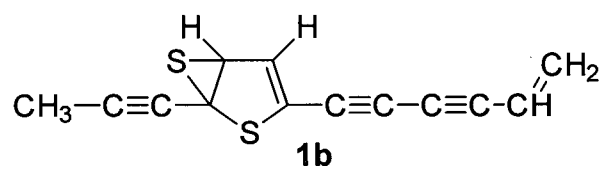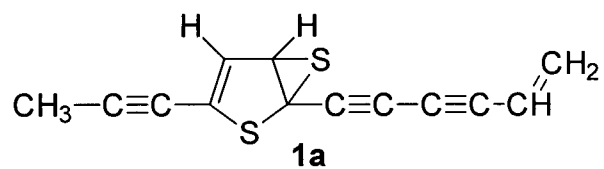
Figure 2.8. Structures of photosulfide intermediates.

Table 2.2a. ^{1}H- and ^{13}C-NMR assignments including HMBC (H–C) correlations of thiarubrine A (**1**) photointermediates.

Position	Photosulfide **1a** (major isomer)			Photosulfide **1b** (minor isomer)
	$\delta_H{}^a$ (mult, J)^b	$\delta_C{}^a$	HMBC (H to C)	δ_H (mult, J)
1a	5.99 (dd, 2.3, 17.6)	133.73	C-2, C-3	
1b	5.89 (dd, 2.1, 11.1)			
2	6.08 (dd, 11.1, 17.5)	115.84	C-1, C-4, C-5	
3		80.61		
4		72.92		
5		69.83		
6		76.51		
7		53.89		
8	5.03 (d, 2.7)	58.67	C-6, C-9, C-10	7.01 (d, 2.9)
9	6.66 (d, 2.5)	129.41	C-7, C-8, C-10, C-11	4.86 (d, 2.7)
10		125.80		
11		71.36		
12		95.21		
13	2.03 (s)	3.95	C-11, C-12	

^{a} δ in ppm
^{b} s, d = singlet, doublet; J in Hz

Table 2.2b. ¹H- and ¹³C-NMR assignments including HMBC (H–C) correlations of thiarubrine B (**2**) photointermediates.

Position	Photosulfide **2b** (major isomer)			Photosulfide **2a** (minor isomer)	
	δ_H [a] (mult, J) [b]	δ_C [a]	HMBC (H to C)	δ_H (mult, J)	δ_C
1a	5.82 (dd, 17.7, 1.9)	130.9	C-2, C-3	5.77 (dd, 17.5, 1.9)	130.6
1b	5.75 (dd, 11.5, 1.8)			5.70 (dd, 11.4, 1.9)	
2	6.11 (dd, 17.6, 11.4)	116.4	C-4	6.00 (dd, 17.6, 11.4)	116.3
3		94.7			
4		81.1			
5		124.7			
6	6.78 (d, 2.8)	131.4	C-4, C-5, C-7, C-8	4.92 (d, 2.8)	57.6
7	4.98 (d, 2.6)	58.3	C-5, C-6, C-9	6.94 (d, 2.6)	134.6
8		54.3			
9		68.7			
10		71.2			
11		63.0			
12		82.3			
13	2.02 (s)	3.8	C-10, C-11, C-12	2.07 (s)	4.1

[a] δ in ppm
[b] s, d = singlet, doublet; J in Hz

Table 2.2c. ^{1}H- and ^{13}C-NMR assignments (ppm) including HMBC (H–C) correlations of thiarubrine D (**3**) photointermediates.

Position	Photosulfide **3a** (major isomer)			Photosulfide **3b** (minor isomer)
	$\delta_H{}^a$ (mult, J)^b	$\delta_C{}^a$	HMBC (H to C)	δ_H (mult, J)
1a	3.03 (d, 4.9^c)	48.9	C-2, C-3	
1b	3.06 (d, 4.4^c)			
2	3.70 (s^c)	39.5	C-3, C-4	
3		80.6		
4		65.9		
5		70.2		
6		72.8		
7		53.4		
8	5.02 (d, 1.3)	58.7	C-6, C-9, C-10	7.01 (d, 2.9)
9	6.63 (s^c)	129.4	C-7, C-8, C-9, C-10	4.86 (d, 2.7)
10		125.8		
11		71.3		
12		95.3		
13	2.02 (s)	34.0	C-10, C-11, C-12	

^{a} δ in ppm
^{b} s, d = singlet, doublet; J in Hz
^{c} poorly resolved spin coupling pattern

hex-4-ene, the major photointermediates of thiarubrine B (**2**) and thiarubrine D (**3**), respectively. Limited ^{1}H-NMR spectral data are also presented for the less abundant photosulfide **2b**, 1-(3-buten-1-ynyl)-5-(1,3-pentadiynyl)-2,6-dithiabicyclo[3.1.0]hex-4-ene, and photosulfide **3b**, 1-(1-propynyl)-3-(5,6-epoxy-hex-1,3-diynyl)-2,6-dithiabicyclo[3.1.0] hex-3-ene. The structures of photosulfides **1a**, **1b**, **2a**, **2b**, **3a** and **3b** are shown in Figure 2.8.

Thiarubrines A (**1**), B (**2**) and D (**3**) gave different ratios of photosulfide isomers as determined by integration of ring proton peak area. The major photosulfide, **1a**, accounted for 96.2% of the photointermediates formed upon irradiation of thiarubrine A (**1**). Remarkably, photolysis of thiarubrine B (**2**) resulted in 81.4% of the opposite photosulfide isomer, **2a**, in which the episulfide ring is proximal to the methyl-bearing side-chain. The photointermediates of thiarubrine D (**3**) exhibited a similar isomeric ratio to those of thiarubrine A (**1**) with 98.7% of the photosulfides present existing as photosulfide **3a**.

2.3.3. Characterization of Cyclooctasulfur

Upon standing at room temperature, a dark precipitate was observed to form in a sample of thiarubrine A (**1**) that had undergone photolysis and cold-temperature NMR. Chromatographic separation of the precipitate followed by TLC showed that it was composed primarily of sulfur as determined by cochromatography with authentic elemental sulfur and reaction (white spot upon heating) with a sodium azide spray reagent specific for sulfur. Comparison of the low-resolution EI-mass spectrum of the sulfur-containing fraction with literature values (Cooper *et al.*, 1996) allowed for its identification as cyclooctasulfur (S_8) (**24**) (Figure 2.9) .

Cyclooctasulfur (24)
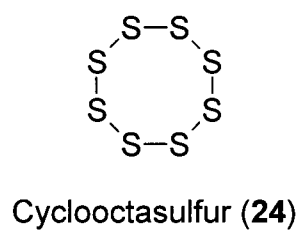
Figure 2.9. Structure of cyclooctasulfur (24) formed by photolysis of thiarubrine A (1).

2.3.4. Visible-Light Phototoxicity of Thiarubrine A

To obtain insight into the toxicity of the photoconversion process, and the molecules involved in it, I compared the toxicity of thiarubrine A (**1**) and thiophene A (**13**) to *Candida albicans* in the presence or absence of visible light. Cell suspensions were treated with **1, 13** or a solvent (MeOH) control, briefly stirred in the dark to allow the compounds to diffuse and incubated in the darkness or under visible light from a halogen bulb for 10 minutes. The number of viable *C. albicans* cells in the suspension was determined before and after the 10 minute incubation period. Table 2.3 presents the results of the phototoxicity bioassay. Thiarubrine A (**1**) exhibited pronounced visible-light phototoxicity to *C. albicans* with a 99.3% decrease in the number of viable cells resulting from 10 minutes of irradiation. This effect was highly significant ($P = 0.00005$, single factor ANOVA) compared to incubation of thiarubrine treated cells in darkness. In contrast, thiophene A (**13**) ($P = 0.558$) and the solvent control ($P = 0.646$) showed no phototoxicity with visible light exposure. The effect of visible light on the toxicity of thiarubrine A (**1**) and thiophene A (**13**) is photographically illustrated in Figure 2.10.

2.4. DISCUSSION

Although the existence of photointermediates between thiarubrines and thiophenes has been hypothesized on the basis of microbiological assays and theoretical considerations (see Introduction), none have been previously characterized. I demonstrate for the first time that irradiation of thiarubrines results in the formation of bicylic photosulfide intermediates that yield thiophenes via desulfurization. The bicyclic structure of the photosulfides has not previously been found in nature and makes them among the first members of this new class

Table 2.3. Effect of visible light on the toxicity of thiarubrine A (**1**) and thiophene A (**13**) to *Candida albicans*.

Compound	Light Regime	Number of Viable Cells[a] (per ml, X 10^4)		% Mortality	P value[b]
		0 minutes	10 minutes		
Thiarubrine A (**1**)	Light	13.1 (2.0)	0.1 (0.1)	99.3	0.00005
	Dark	14.0 (4.6)	14.0 (5.2)	3.1	
Thiophene A (**13**)	Light	72.4 (24.8)	78.5 (48.4)	0.0	0.558
	Dark	47.8 (18.1)	35.9 (16.4)	26.4	
MeOH Control	Light	80.4 (17.3)	93.2 (29.7)	0.0	0.646
	Dark	75.6 (35)	69.7 (5.8)	0.0	

[a] mean (standard error of the mean) (n = 3)
[b] single-factor ANOVA comparison of toxicity in the presence or absence of visible light
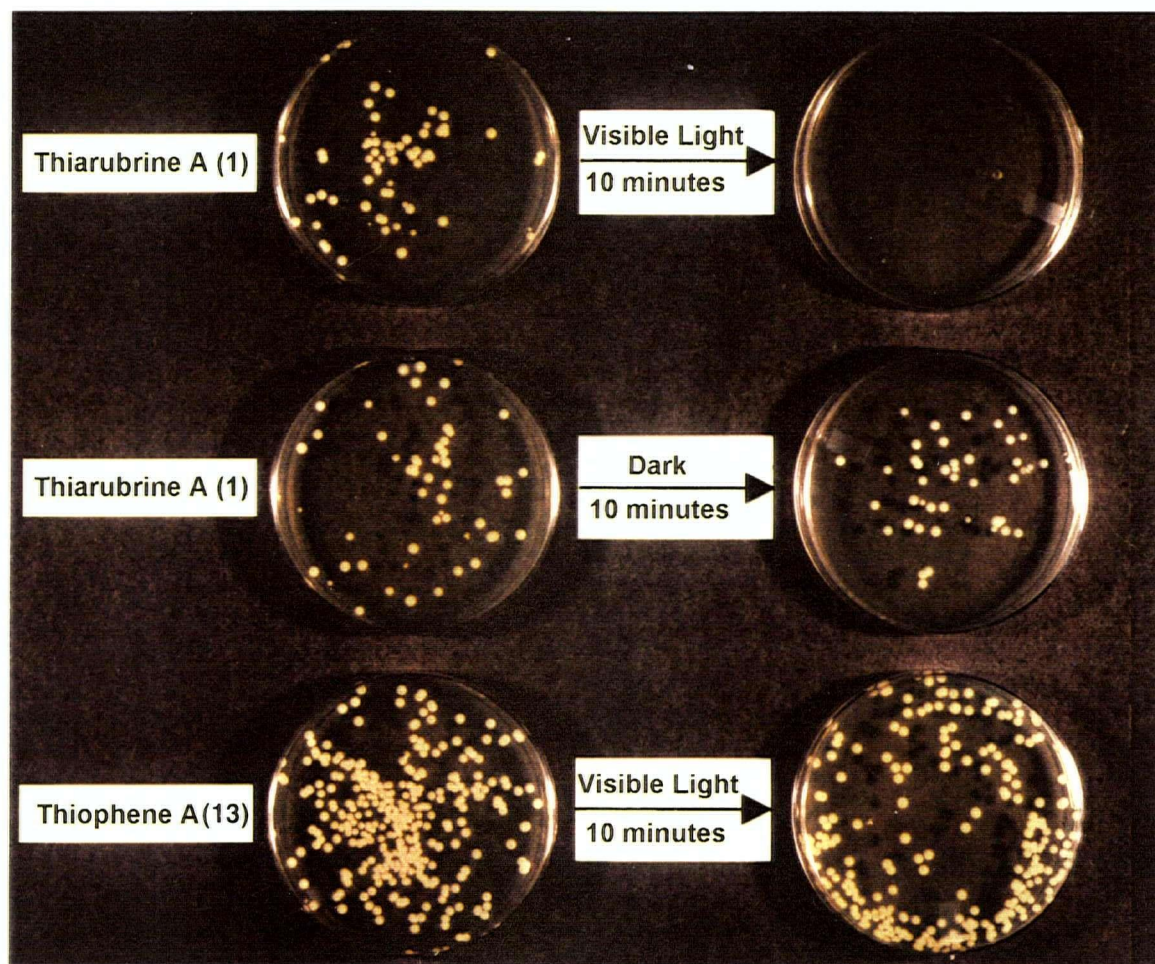
Figure 2.10. Visible-light phototoxicity of thiarubrine A (**1**) and thiophene A (**13**) to *Candida albicans*. Petri plates show number of viable cells (X 10^{-4} dilution) before and after irradiation of a drug treated cell suspension with visible light for 10 minutes (top and lower plates), or incubation in the dark for 10 minutes (middle plates).

of compounds[1]. Six novel photosulfides, photosulfides **1a** and **1b** from thiarubrine A (**1**), photosulfides **2a** and **2b** from thiarubrine B (**2**), and photosulfides **3a** and **3b** from thiarubrine D (**3**) were characterized using data obtained from HPLC-PDA, HPLC-MS and extensive cold-temperature NMR spectroscopy.

Schroth *et al.* (1995) proposed a mechanism for the light-induced conversion of 1,2-dithiins to thiophenes (Figure 2.11) that is supported by the results of this investigation. In the proposed photochemical pathway, irradiation of thiarubrines (e.g., **1**) leads to the electrocyclic ring opening of the 1,2-dithiin ring to give an open-chain dithione tautomer (**1c**). Rearrangement of the twisted form **1c'** via intramolecular cycloaddition yields the episulfide-containing photosulfide intermediates (**1a** and **1b**) from which the thiophene **13** forms via loss of sulfur. In this study there was no chromatographic or spectroscopic evidence for the presence of the dithione. However, matrix isolation spectroscopy and flash photolysis experiments performed on the parent compound, 1,2-dithiin, showed dithione formation with visible light irradiation at 25 K (-248 °C) (Block *et al.*, 1996). The reversibility of the bleaching process (i.e. 9.6% of the prephotolysis concentration of thiarubrine A (**1**) reforms after 60 minutes) presumably occurs because of the reformation of the 1,2-dithiin ring from the dithione. It is unclear if photosulfides contribute to this reversibility by reconverting to their immediate precursors, the dithiones.

The nature of the sulfur lost during the conversion of photosulfides to thiophenes has been clarified somewhat by the finding that cyclooctasulfur (S_8) (**24**) is formed by irradiating thiarubrine A (**1**). This result is not surprising since at room temperature elemental sulfur

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[1] 2,6-Dithiabicyclo[3.1.0]hex-3-ene photointermediates from synthetic 1,2-dithiins were characterized in the laboratory of Professor E. Block, Department of Chemistry, The State University of New York at Albany several weeks before the elucidation of thiarubrine photointermediates.
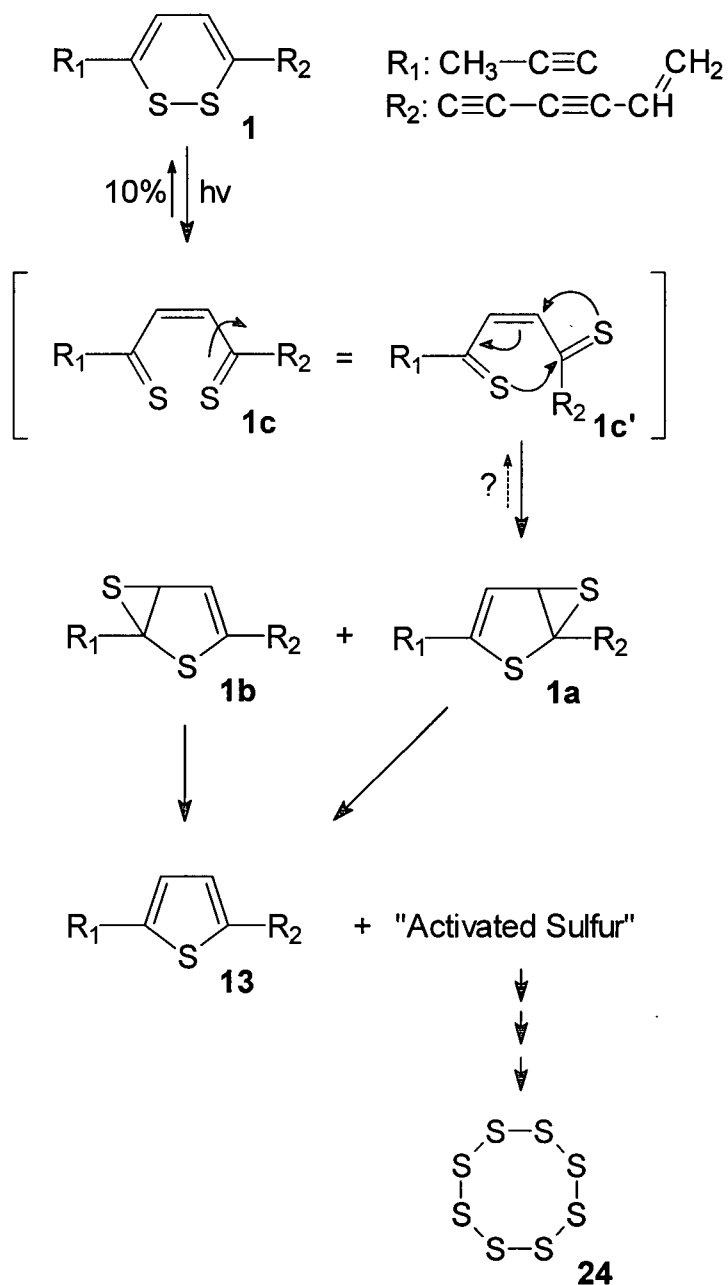
Figure 2.11. Photochemical pathway for conversion of thiarubrines to thiophenes.

exists predominantly as S_8 (Tebbe *et al.*, 1982). The immediate fate of the sulfur lost through desulfurization, however, is unclear. The extrusion of atomic sulfur from 1,2-dithiins has been suggested to be thermodynamically disfavoured (Block *et al.*, (1996) based on data of Mann and Fabian, (1995)) and it appears that a likely route for desulfurization is through a bimolecular process. It is known that thermolysis of ethylene episulfide leads to the loss of S_2 in a bimolecular process (Lown *et al.*, 1968). The photostable 1,2-dithiin, diborneno-1,2-dithiin forms diborneno-1,2,3-trithiepine through the insertion of a single atom of sulfur when incubated with photolabile 1,2-dithiins in light (Schroth *et al.*, 1995), raising the possibility that such a molecule may be useful in elucidating the nature of the sulfur released by functioning as a "sulfur trap".

An unexpected result of this study was the surprising regioselectivity of photosulfide formation that results in the irradiation of thiarubrines A (**1**) and B (**2**) yielding the opposite ratio of photosulfide isomers. That is, the episulfide ring is proximal to the diyne-ene containing side-chain in photosulfide **1a** (the major photosulfide from **1**) but occupies the opposite face of the 2,3-dihydrothiophene ring in the major photointermediate of thiarubrine B (**2**), photosulfide **2a**. As well, the amount of photosulfide **1a** exceeds the minor isomer **1b** by a ratio of 25:1 while the amount of **2a** is greater than that of **2b** by a ratio of only 5:1. These ratios are the opposite of what one might predict if the preservation of the conjugation of the diyne side-chain is the important factor determining the regioselectivity of the cyclization of dithiones to photosulfides. At this time no satisfactory explanation for this phenomenon can be put forward.

Thiarubrines possess significant visible absorption while their corresponding thiophenes do not. Consequently, the differences in the absorption spectra of thiarubrine A

(1) and thiophene A (13) allowed me to use visible light to dissect the thiarubrine–thiophene photochemical pathway and, in an indirect fashion, determine the toxicity of the photosulfides and other photointermediates. Visible-light irradiation of thiarubrine A (1) resulted in statistically significant phototoxicity to *Candida albicans* at a concentration of 0.1 μg/ml with less than 1% of the pretreatment number of viable cells remaining after light exposure. As expected, thiophene A (13), which requires UV light to manifest its toxicity, did not show phototoxicity under the halogen lamp used to irradiate the treated *C. albicans* cell cultures. Since it is probable the small amount of thiarubrine A (1) present in the cell suspension would, under constant irradiance, be converted wholly to the open-chain dithione, photosulfides 1a and 1b and finally sulfur, I suggest that observed phototoxicity is the result of one, and perhaps all three, of these molecular species.

Examination of the chemical properties of dithiones, episulfides and elemental sulfur reveals all have potential to exert toxic effects. The pronounced reactivity of the dithione intermediate, evidenced by the electrocyclic attack of the thione groups on the quaternary carbons of the short-lived dithione intermediates, may lead to toxicity through interaction (i.e. covalent bonding) with biological target molecules such as membrane components or proteins. The second group of light-generated molecules, the photosulfides, could also play a role in the phototoxicity since some episulfides are highly reactive (Brasen *et al.*, 1965; Huisgen, 1989) and episulfide intermediates have been implicated in the nephrotoxicity of halogenated alkenes (Commandeur *et al.*, 1996; Shim and Richard, 1997). A third, possibly toxic event in the thiarubrine–thiophene pathway is the extrusion of the episulfide sulfur from photosulfides, perhaps giving a reactive form of atomic sulfur or S_2 which may damage cells in a similar fashion to the excited states of oxygen. The toxicity of the extruded sulfur is

supported by evidence, albeit circumstantial, showing that the release of a reactive form of atomic sulfur mediates the toxicity of the insecticide parathion (Neal and Halpert, 1982). As well, photochemically-generated excited state sulfur atoms have been shown to react with alkenes and acetylenes (Strausz, 1967). The sulfur liberated by thiarubrine photolysis eventually forms cyclooctasulfur (S_8) (**24**), a compound that has moderate antibiotic (Izac *et al.*, 1982) and antifungal (Cooper *et al.*, 1996) activities. An important role for cyclooctasulfur in the observed phototoxicity is not supported by the short duration of the bioassay (10 minutes), which may not allow S_8 to both form and exert toxic effects, and the fact that thiarubrine A (**1**) was tested at concentrations ten-fold lower than those at which cyclooctasulfur exerts antifungal activity. Further research is required to determine which biologically-active molecule is responsible for the visible light mediated killing of *C. albicans* cells by thiarubrines.

Although this study did not seek to determine if thiarubrines function as photooxidants as suggested by Freeman *et al.* (1993), electrochemical studies of thiarubrine A (**1**) at the University of Arizona have shown that phototoxic superoxide generation by thiarubrines is not likely to occur (Block, personal communication, 1995).

Many plants use light to activate and potentiate the toxicity of chemical defenses (Arnason *et al.*, 1992; Towers *et al.*, in press). Most commonly, plant phototoxicity is mediated through the generation of singlet oxygen (i.e. thiophenes, polyynes, and perylene quinones) or through covalent interactions with DNA in the case of the photogenotoxic furocoumarins (Song and Tapley, 1979). Based on the results of this study, the thiarubrines appear to possess a unique mechanism of phototoxicity involving the light-induced formation of reactive intermediates including the novel photosulfides identified in this study, and the

uncharacterized open-chain dithiones and activated sulfur which are part of the thiarubrine–thiophene pathway.

The findings of this study, together with those of previous investigations of the biological activity of thiarubrines, demonstrate that the toxicity of the thiarubrines is the result of at least three distinct mechanisms. These consist of: 1) the toxicity of thiarubrines in the absence of light, an effect that is the result of the reactivity of the 1,2-dithiin ring with what has been hypothesized to be a single enzyme target (Bierer *et al.*, 1995); 2) visible-light phototoxicity caused by reactive intermediates as shown by this study; and, 3) photooxidative damage caused by the UV-induced formation of singlet oxygen by their ultimate photoproducts, the thiophenes. The utility of this three-pronged attack as a defensive strategy seems clear—each class of molecule may, through its own unique mechanism and specific cellular target, inflict damage to a pathogen or herbivore that infects or consumes a thiarubrine-containing plant.

The visible-light phototoxicity highlighted in this study suggests that thiarubrines may deserve a "second look" as antifungal drugs. Although the treatment of candidiasis and fungal diseases in humans with systemically administered thiarubrines is prevented by their toxicity, the potentiation of antifungal activity by visible light may allow for the use of low doses of thiarubrines for dermatomycoses.

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CHAPTER 3

PHOTOPROTECTION OF LIGHT-SENSITIVE THIARUBRINES IN *AMBROSIA CHAMISSONIS*

3.1. INTRODUCTION

Ambrosia chamissonis grows as a sprawling, perennial herb in high-irradiance environments along the Pacific coasts of North and South America. Despite the light-sensitivity of thiarubrines to UV and visible radiation, the leaves and stems of *A. chamissonis* contain significant amounts of these chemicals (Ellis, 1993; Chapter 4 "Results and Discussion"). The presence of thiarubrines in above-ground, photosynthetic tissues represents an apparent contradiction since the same visible wavelengths that drive the light reactions of photosynthesis also photoconvert thiarubrines to thiophenes. The consequences of photoconversion include the partial inactivation of thiarubrines as defensive chemicals and the loss of a substantial biosynthetic commitment in these complex sulfur compounds. Moreover, the results presented in Chapter 2 indicate that photoconversion *in vivo* may lead to autotoxicity in thiarubrine-containing plant tissues.

Although the problems of storing light-sensitive thiarubrine phototoxins may be unique to a small number of asteraceous taxa, many plant phototoxins are exposed to sunlight *in vivo*. Phototoxic members of the Asteraceae, Hypericaceae and Apiaceae prefer "well-lit" habitats (Arnason *et al.*, 1992) and potent photooxidants such as the polyynes (Arnason *et al.*, 1980) and perylene quinones (Fields *et al.*, 1990), and the photogenotoxic furocoumarins (Berenbaum, 1992) are localized in leaf tissues exposed to sunlight. However, most plant phototoxins do not absorb visible light (hypericin and other perylene quinones are

exceptions), and few exhibit the pronounced photolability of the thiarubrines. The photoprotection of plant phototoxins has received little experimental attention although Lam and Hansen (1990) suggested that the UV-induced decomposition of unstable polyynes is prevented by their interaction with conjugated molecules, a process that could prevent the formation of excited states.

In addition to the excitation and decomposition of endogenous phototoxins, the damaging effects of sunlight, which are primarily due to UV-B induced formation of photochemical lesions in nucleic acids and photoinhibition caused by excess visible radiation, are experienced by many terrestrial plants. Plants are protected from both types of light stress by the accumulation of screening pigments. In the case of UV-B, such molecules are primarily colourless UV-absorbing phenolic compounds such as flavonoids (Li et al., 1993; Kootstra, 1994; Van de Staaij et al., 1995) and sinapate esters (Sheahan, 1996) although anthocyanin pigments also have UV-screening properties (Takahashi et al., 1991; Klaper et al., 1996). Screening compounds accumulate in sub-epidermal cells of leaves (Day et al., 1993; Schitzler et al., 1996) and prevent UV-B light from reaching the leaf mesophyll. Photoinhibition, the decrease in the photosynthetic capacity of the plant through damage to the photosynthetic apparatus, occurs in high-irradiance visible light. Plants avoid photoinhibition by screening of visible wavelengths with anthocyanin pigments (Gould et al., 1995 but see Burger and Edwards, 1996) and the multifunctional involvement of carotenoids (Demmig-Adams, 1990; Sarry et al., 1994).

The persistence of thiarubrines in *Ambrosia chamissonis* leaves indicates that an as yet undescribed mechanism for their photoprotection exists. Since plants utilize UV and visible light absorbing pigments as light filters, and preliminary observations showed thiarubrines

were associated with anthocyanins in *A. chamissonis*, I hypothesized that a similar strategy may serve to photoprotect thiarubrines in the photosynthetic tissues of *A. chamissonis*. In this study, I examined the role of anthocyanin pigments in the photoprotection of thiarubrines.

3.2. MATERIALS AND METHODS

General experimental procedures

The analytical HPLC system consisted of a Waters 600 pump controller, Waters 996 photodiode array detector and Waters 717 plus autosampler controlled by Millenium 2.1 software. A 3.9 mm X 150 mm (4 μm) C_{18} column (Waters NovaPak) equipped with NovaPak precolumn was used for analytical HPLC-PDA. Preparative HPLC was performed on a Waters 600 pump controller with a Waters U6K manual injector (3 ml sample loop) and Waters 994 photodiode array detector. A 2.5 X 10 cm (5 μm) C_{18} radial compression column (Waters NovaPak) equipped with NovaPak pre-column was used for preparative-scale isolations. UV-vis spectra were recorded on a Pye-Unicam 8720 scanning spectrophotometer in MeCN (thiarubrines and thiophenes) and in 0.01% HCl–MeOH (anthocyanins). The concentrations of pigment solutions were quantified spectrophotometrically at 490 nm for thiarubrines (ε = 3000) and 520 nm for anthocyanins (ε = 32,000). ^{1}H-NMR spectra were recorded in DMSO-d_6–TFA (9:1) at 400 MHz on a Bruker WH-400 400 MHz spectrometer with the residual DMSO (δ 2.59 ppm) signal as a reference. Fast atom bombardment (FAB) mass spectra were obtained on a Kratos Concept II HQ mass spectrometer in positive mode with a thioglycerol matrix. A Carl Zeiss light microscope was used to examine and photograph *A. chamissonis* seedlings and light irradiance was measured with an International Light Inc. radiometer (model IL 1400A). Solvents were HPLC-grade (Fisher) except for the

reagent-grade petroleum ether, CH_2Cl_2 and ethyl acetate used for anthocyanin isolation. Water was Milli-Q plus (Millipore). All manipulations of thiarubrines were performed under dim light or under a dark room safe-light.

Cultivation of Ambrosia chamissonis seedlings

Seeds of *A. chamissonis* were collected from beneath mature plants growing at the interface of beach and sand dunes in Centennial Beach Regional Park, Tsawwassen, British Columbia. The bur-like seeds were separated from other debris and soaked overnight in water. After removal of excess water, the seeds were mixed with damp beach sand and the seed/sand mixture spread evenly on the surface of plastic pots (10 X 10 cm) containing beach sand. Pots were covered with plastic bags and placed in a plant-growth chamber under a 12 hour day (25 °C)–12 hour night (20 °C) regime with illumination provided by Vitalite fluorescent tubes at 45 μmol s^{-1} m^{-2}. Relative humidity was 75–90% and seedlings received liberal amounts of water.

Isolation of anthocyanins

Leaves and stems (0.96 kg fresh weight) of *A. chamissonis* were collected in Centennial Beach Regional Park, Tsawwassen, British Columbia. The plant material was homogenized in a Waring blender in 4 l MeOH–formic acid–water (70:2:28) ("MFW"), the extract filtered and the fibrous marc re-extracted with 2 l of MFW. The combined extracts, after concentration to 750 ml *in vacuo*, were extracted with 2 X 500 ml petroleum ether, 2 X 500 ml CH_2Cl_2 and 2 X 500 ml ethyl acetate to remove nonpolar constituents. The aqueous MeOH layer was evaporated to dryness to give a purple anthocyanin-containing residue (46.5

g). Alkaline hydrolysis was performed by the treatment of a small amount of this residue as a MFW solution with 2 drops of 1 M NaOH. A crude separation of anthocyanins **25** and **26** was achieved on a 35 ml C_{18} reversed-phase cartridge (Waters Sep-Pak 10 g) equilibrated with water and eluted with a gradient of MeOH–formic acid–water (0:8:92) to MeOH–formic acid–water (60:8:32). Preparative HPLC on a 2.5 X 20 cm (5 μm) C_{18} radial compression column (Waters NovaPak) equipped with NovaPak precolumn led to the final purification of the pigments. Compounds were eluted using a gradient system with the following conditions: 0 minutes (20:4:76; MeOH–formic acid–water), 10 minutes (30:4:66), 20 minutes (35:4:61); or isocratically using 8% MeCN–92% (trifluoroacetic acid–acetic acid–water (1:6:93)). Flow rate was 27 ml/minute with detection at 520 nm. Injection volume was 100–500 μl. The resulting fractions were dried *in vacuo*, resuspended in trifluoroacetic acid and precipitated with excess diethyl ether to yield trifluoroacetic acid salts of anthocyanins **25** and **26**.

Cyanidin 3-*O*-(6-*O*-malonyl-β-D-glucopyranoside) (**25**): UV-vis λ_{max} (0.01% HCl–MeOH) nm, 530 (+ 48 with $AlCl_3$), 283, 202, E_{440}/E_{530} = 22%; FAB-MS *m/z* 536 [M = $C_{24}H_{23}O_{14}]^+$, 288 [M - glucose - malonate]$^+$. ^{1}H-NMR see Table 3.1. (See Appendix 5).

Cyanidin 3-*O*-(β-D-glucopyranoside) (**26**): UV-vis λ_{max} (0.01% HCl–MeOH) nm, 529 (+ 45 with $AlCl_3$), 283, 205, E_{440}/E_{530} = 23%; FAB-MS *m/z* 449 [M = $C_{21}H_{21}O_{11}]^+$. ^{1}H-NMR see Table 3.1. (See Appendix 5).

Analysis of anthocyanins in plant samples

A. chamissonis seedlings (14–17 days post-germination) were rinsed free of sand and patted dry. Ten seedlings were selected at random, severed at the junction of stem and root and the combined root sample and combined leaf/stem sample accurately weighed. The samples, after grinding with 5 ml MFW in a mortar and pestle, were allowed to extract for 45 minutes. The extracts were decanted, the mortar rinsed with 5 ml MFW and the combined extracts filtered through cotton wool. Rotary evaporation gave an anthocyanin-containing residue that was resuspended in 200 µl MFW and analyzed by HPLC. *A. chamissonis* leaves collected from mature plants growing at the Tsawwassen site were extracted and analyzed in an identical manner to the leaves/stems of *A. chamissonis* seedlings.

Analysis of anthocyanins was performed using a gradient elution system adapted from Gao and Mazza (1994) with the following conditions: 0 minutes (10:90; MeOH–5% formic acid in water), 10 minutes (15:85), 20 minutes (20:80), 30 minutes (30:70), 40 minutes (35:65), 50 minutes (37:63), 60 minutes (90:10). Flow rate was 1.2 ml/minute with detection over the wavelength range 200–700 nm and specific detection at 520 nm. Injection volume was 20 µl. Quantification of anthocyanins was performed at 520 nm using peak area by comparison to a standard curve derived from injections of solutions of **26** ranging in concentration from 349 µg/ml to 1 µg/ml. The correlation coefficient value (r^2) for the standard curve was 0.998.

Thiarubrine photoconversion in A. chamissonis seedlings

Eighteen to 20 *A. chamissonis* seedlings (*ca.* 4 cm in height; 14–17 days post-germination) were removed from the sand in which they were grown, rinsed with water and placed in a

400 ml beaker containing water. To determine the thiarubrine A (**1**) and thiophene A (**13**) content of the seedlings before exposure to the experimental light regime, three seedlings were selected at random, patted dry and severed at the junction of root and stem. The combined root sample and combined leaf/stem sample were weighed, the samples transferred to a mortar and pestle and homogenized with either 1 ml MeCN for leaf/stem samples or 0.5 ml for root samples. Samples were allowed to extract briefly and transferred to a 1.5 ml Eppendorf tube. The extract was centrifuged at 10 000 rpm for 5 minutes and an aliquot of the supernatant analyzed by HPLC. To compare the photoconversion of thiarubrines in roots and leaves/stems, the remaining seedlings were irradiated with visible light. A water-filled beaker (400 ml) containing the seedlings was placed on a rotary shaker (100 rpm) in front of four fluorescent tubes (Sylvania cool-white, 20 W) providing an irradiance of 2.3 mW/cm^2 broad spectrum visible light incident on the submerged seedlings. Three seedlings were removed at 10, 30, 60, 120 and 240 minutes and prepared for analysis as described above.

The HPLC methodology for quantification of thiarubrines and thiophenes is described in Chapter 4 "Materials and Methods".

In vitro photoprotection of thiarubrines

To test the ability of anthocyanins to protect thiarubrines from photoconversion, an *in vitro* assay to measure the effect of anthocyanin screens of varying concentration on the photolysis of thiarubrine A (**1**) was developed. The "three-cuvette" apparatus used in this experiment is shown in Figure 3.1. Briefly, broad spectrum visible light from a fibre optic light source (Dolan-Jenner Industries Inc. Fiber-Lite High Intensity Illuminator Series 180) equipped with a 150 W halogen bulb (Sylvania EKE) was passed through a quartz cuvette (#1) containing a
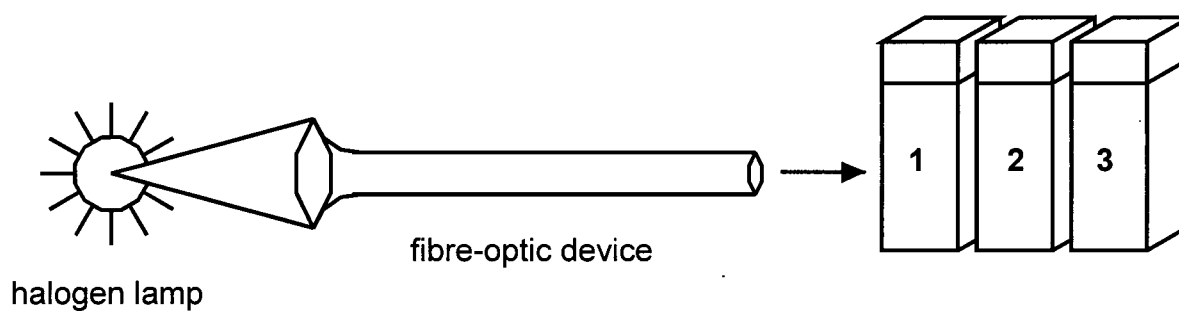
Figure 3.1. Schematic of "three-cuvette" apparatus used to measure photoprotectant effect of anthocyanin solutions. A thiarubrine A (**1**) solution in cuvette #3 was screened from visible radiation by cyanidin 3-*O*-(β-D-glucopyranoside) (**26**) test solutions contained in cuvette #2. A 10% $CuSO_4$ solution in cuvette #1 absorbed infrared light generated by the fibre-optic light source.

10% $CuSO_4$ solution to remove excess infrared radiation. The irradiance of the light incident on cuvette #1 was 105 mW/cm^2. The filtered light was directed through a quartz cuvette (#2) containing light-filtering solutions of **26** in 0.01% HCl–MeOH before irradiating a solution of a thiarubrine A (**1**) (0.8 ml; 0.5 mM) contained in a third quartz cuvette (#3). An opaque shutter allowed the duration of light exposure to be controlled. Two minutes of irradiation were shown to reduce the concentration of thiarubrine A (**1**) by 85% as determined by spectrophotometry. In triplicate experiments, solutions of **26** ranging in concentration from 3.2 mM to 0.5 mM were transferred to the middle (#2) cuvette and the "three-cuvette" system irradiated for 2 minutes. The concentration of the thiarubrine A (**1**) solution contained in the third cuvette was determined immediately after the 2 minute light exposure. New thiarubrine A (**1**) solutions were used for each replicate while anthocyanin solutions appeared stable under the light intensities used and were not changed between replicates.

3.3. RESULTS

3.3.1. Anatomy of Thiarubrine Photoprotection

Microscopic examination of seedlings and mature leaves of *Ambrosia chamissonis* allowed me to investigate the anatomical adaptations which may function to protect thiarubrines from light. Thiarubrines present in *A. chamissonis* leaves and stems are localized in canals that are visible as dark-red pigmented veins running on the underside of the leaf petiole and parallel to leaf vasculature (Figure 3.2B). Observation of longitudinal sections (1–3 cells in thickness) of *A. chamissonis* stems revealed that pigment veins are, in fact, composed of two cell types. In such veins, small diameter thiarubrine canals are surrounded by a sheath of purple-red cells that contain anthocyanin pigments, an arrangement that is shown in Figure

3.2C. Thiarubrine canals can be differentiated from anthocyanin cell layers by the bleaching of thiarubrines upon exposure to high intensity light. Thus, observation of a tissue preparation after exposure to *ca.* 2 minutes of visible light on the microscope stage, as illustrated in Figure 3.2E&F, clearly shows the discolouration of the thiarubrine canal while the surrounding purple-red anthocyanin-containing cells are unaffected. In contrast, the roots of *A. chamissonis* seedlings contain thiarubrine canals that are not surrounded by anthocyanin sheath cells (Figure 3.2D).

3.3.2. Isolation and Identification of Anthocyanins

Extraction of leaves and stems of mature *Ambrosia chamissonis* with MeOH–formic acid–water (70:2:28), an acidic solvent combination optimized for extraction of acylated anthocyanins (Gao and Mazza, 1994), gave a purple extract that was composed of four major (>10% of the total peak area each) and three minor anthocyanins as determined by HPLC (Figure 3.3). Alkaline hydrolysis of the extract indicated that six of the anthocyanins were acylated since only the most polar anthocyanin peak (R_t 25.0 minutes; **26**) was detectable after hydrolysis. Separation of the anthocyanin-containing extract by solvent-solvent partition, solid-phase extraction and reversed-phase preparative HPLC afforded the major anthocyanin pigment present in *A. chamissonis*, **25**, and the deacylated anthocyanin **26** as TFA salts. The addition of $AlCl_3$ to HCl–MeOH solutions of both compounds resulted in bathochromic shifts of the visible band (530 nm, 529 nm) indicating that adjacent free hydroxyl groups were present. A E_{440}/E_{vis} ratio of 22% and 23% for **25** and **26**, respectively, suggested that both compounds were 3-glycosides of cyanidin (Strack and Wray, 1989). The FAB-MS spectrum of **25** showed a molecular ion at *m/z* 536 corresponding to $C_{24}H_{23}O_{14}^+$
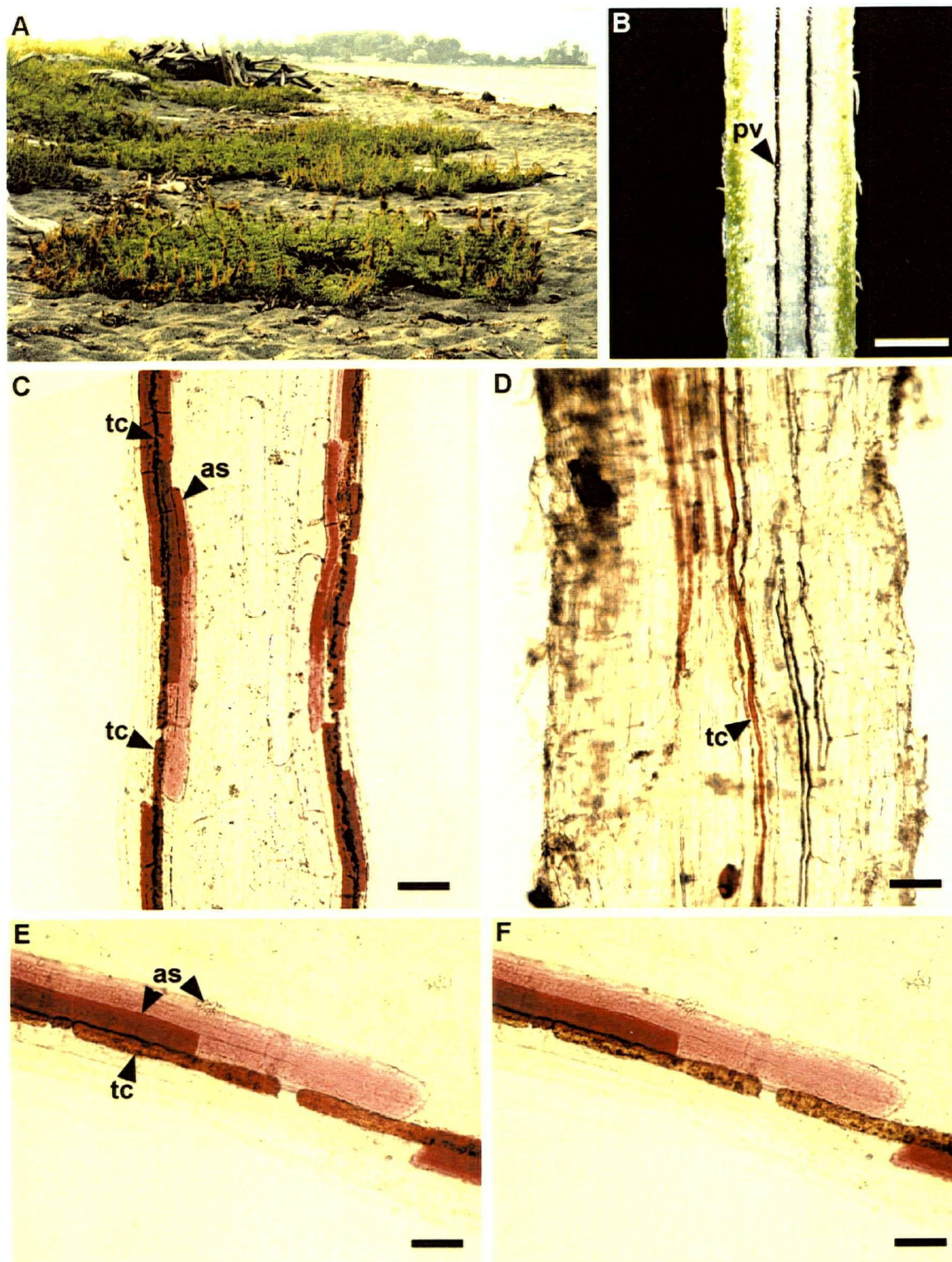
Figure 3.2. Anatomy of thiarubrine photoprotection in *Ambrosia chamissonis*. (**A**) Mature *A. chamissonis* plants growing at the interface of beach and maritime dunes, Centennial Beach regional Park, Tsawwassen, British Columbia. (**B**) Longitudinal section of mature *A. chamissonis* petiole with dark pigment veins (pv) indicated (bar = 1 mm). (**C**) Longitudinal view of thin tissue strip isolated from the stem of a 2-week old cultivated *A. chamissonis* seedling showing thiarubrine canals (tc) surrounded by anthocyanin sheath (as) cells. (bar = 200 μm). (**D**) Longitudinal section of the root of a 2-week old cultivated *A. chamissonis* seedling showing the absence of anthocyanin sheath cells surrounding thiarubrine canals (tc) (bar = 200 μm). Thiarubrine canals (tc) and anthocyanin sheath (as) cells before (**E**) and after (**F**) two-minutes irradiation with incandescent light. The discolouration and granular appearance of the thiarubrine canal after light exposure is visible (bars = 100 μm).
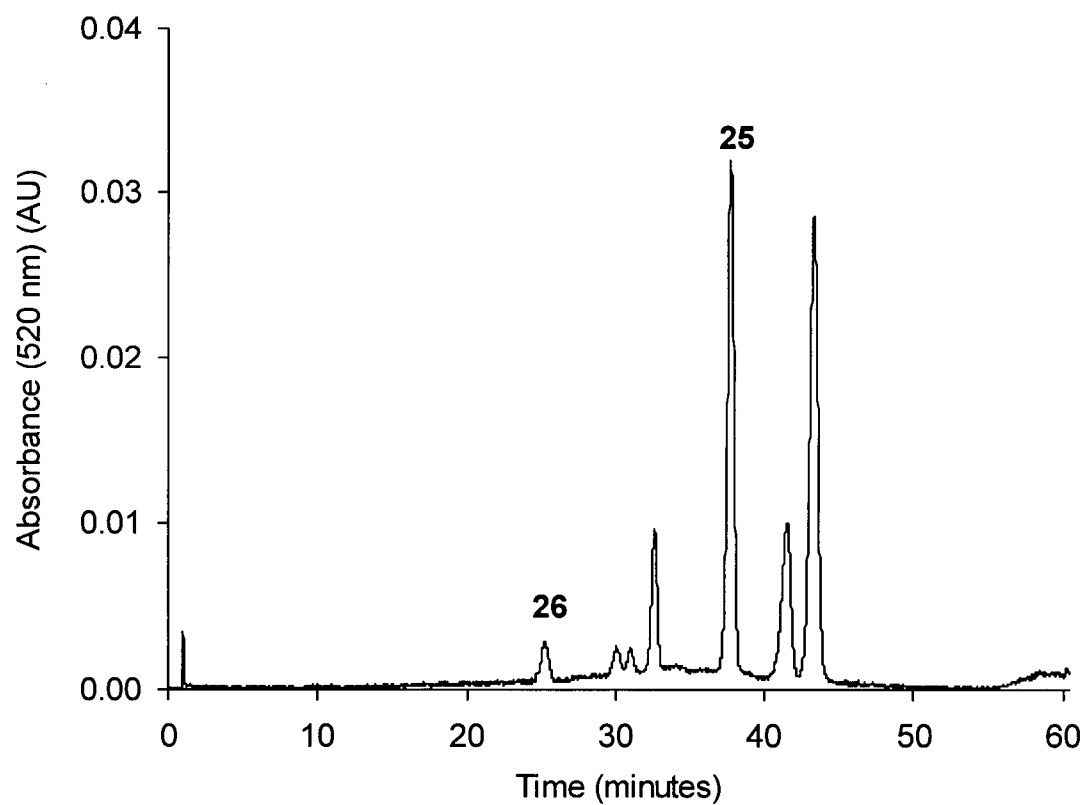
Figure 3.3. HPLC analysis of anthocyanins present in the leaves and stems of 15-day old *Ambrosia chamissonis* seedlings. Peaks corresponding to cyanidin 3-*O*-(6-*O*-malonyl-β-D-glucopyranoside) (**25**) and cyanidin 3-*O*-(β-D-glucopyranoside) (**26**) are indicated.

with the presence of a malonylglucose residue indicated by the fragmentation ion of m/z 288 [M - glucose - malonate]$^+$. As well, a resonance attributable to the malonyl $-CH_2-$ protons was observed at δ 3.36 ppm in the ^{1}H-NMR spectrum of anthocyanin **25**. Comparison of the ^{1}H-NMR spectrum of **25** (Table 3.1) with literature values (Bridle *et al.*, 1984; Saito *et al.*, 1988; Cheminat *et al.*, 1989) allowed for its identification as cyanidin 3-*O*-(6-*O*-malonyl-β-D-glucopyranoside). Compound **26** showed a molecular ion of m/z 449 corresponding to $C_{21}H_{21}O_{11}{}^+$ suggesting that it was the demalonyl derivative of **25**. This was borne out by comparison of its ^{1}H-NMR spectrum with previously reported data for cyanidin 3-*O*-(β-D-glucopyranoside) (Cheminat *et al.*, 1989). The assignment of the sugar residue as β-D-glucopyranosyl in both **25** and **26** was supported by the large coupling constant ($J = 7.7$ Hz) observed for the anomeric proton (glucose H-1) in each. The structures of the anthocyanins **25** and **26** isolated from *A. chamissonis* are shown in Figure 3.4.

3.3.3. Quantification of Anthocyanins

To quantify the amount of anthocyanins present in cultivated *A. chamissonis* seedlings and in mature leaves collected from plants in the wild, analytical methodology was developed using reversed-phase HPLC with photodiode array detection at 520 nm. All anthocyanins present in *A. chamissonis* were well resolved by the gradient elution method. Quantification was by comparison of the area of anthocyanin peaks to a standard curve derived from injection of a dilution series of solutions of **26**. The results of the HPLC analysis are presented in Table 3.2. Seedling leaves and stems, which were analyzed together, contained 10.5 μg/g FW of anthocyanins while roots did not contain detectable levels of these compounds. Mature

Table 3.1. ^{1}H-NMR spectral data of cyanidin 3-O-(6-O-malonyl-β-D-glucopyranoside) (**25**) and cyanidin 3-O-(β-D-glucopyranoside) (**26**).

Position	25 $\delta_H{}^a$ (mult, J)^b	26 δ_H (mult, J)
Aglycone		
4	8.81 (s)	8.89 (s)
6	6.78 (s)	6.69 (d, 1.9)
8	6.93 (s)	6.40 (br s)
2'	7.99 (d, 2.0)	8.00 (d, 2.4)
5'	7.03 (d, 8.7)	7.02 (d, 8.8)
6'	8.21 (dd, 2.2, 8.7)	8.23 (dd, 2.3, 8.8)
Glucose		
1	5.38 (d, 7.7)	5.33 (d, 7.7)
2	3.52 (dd, 9.2, 17.2)	3.49 (m)
3	3.39 (dd, 8.7, 17.9)	3.36 (m)
4	3.23 (m)	3.22 (m)
5	3.81 (m)	3.49 (m)
6a	4.10 (dd, 7.6, 11.8)	3.49 (m)
6b	4.43 (d, 10.9)	3.79 (d, 10.0)
Malonyl		
-CH$_2$-	3.36 (d, 2.7)	-

^{a} δ in ppm
^{b} s, d, m, br s = singlet, doublet, multiplet, broad singlet, respectively; J in Hz
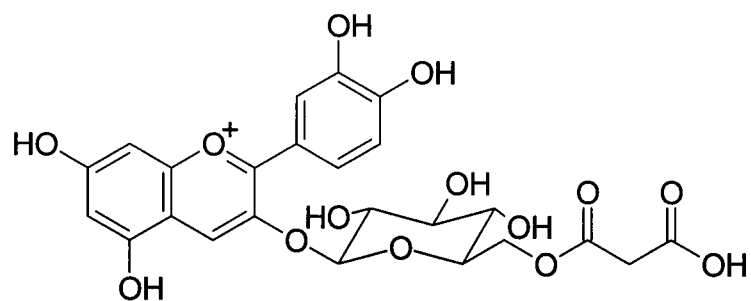
Cyanidin 3-O-(6-O-malonyl-β-D-glucopyranoside) (25)
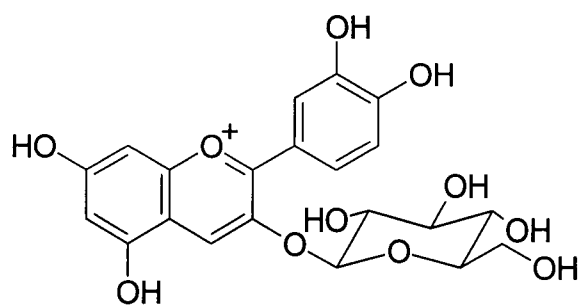
Cyanidin 3-O-(β-D-glucopyranoside) (26)

Figure 3.4. Structures of anthocyanins isolated from *Ambrosia chamissonis*.

Table 3.2. Anthocyanin Content of *Ambrosia chamissonis*.

Sample	Total Anthocyanins[a] (µg/g FW)
Seedling Leaves and Stems (n=5)	10.5 ± 6.8
Seedling Roots (n=5)	ND[b]
Mature Leaves (n=3)	2.9 ± 1.0

[a] mean $\pm$ standard deviation; expressed as cyanidin 3-*O*-(β-D-glucopyranoside) (**26**) equivalents.
[b] ND, not detected

leaves contained lower levels of anthocyanins (2.9 µg/g FW) than the leaves and stems of seedlings.

3.3.4. *In vivo* Thiarubrine Photoconversion in *A. chamissonis* Seedlings

To compare the photoconversion of thiarubrines in photosynthetic (above-ground) and nonphotosynthetic (below-ground) plant tissues, the effect of 4 hours of visible light exposure on the thiarubrine A (1) and thiophene A (13) content of whole *A. chamissonis* seedlings was measured. The results of the analysis are illustrated in Figure 3.5. Thiarubrine A (1) levels were greater than 900 µg/g FW in *A. chamissonis* roots prior to exposure to light but decreased by 94% after 30 minutes of irradiation; no thiarubrine A (1) was detectable in roots after 240 minutes light exposure. As expected, the photoconversion of thiarubrines in root tissues resulted in a concomitant increase in thiophene A (13) levels. Light exposure had little effect on the amount of thiarubrine A (1) or thiophene A (13) present in the leaves and stems of *A. chamissonis* seedlings. Although there was some variation in thiarubrine A (1) levels during the experiment (i.e. a 40% decrease was observed in the first 30 minutes of light exposure), the thiarubrine A (1) content of seedlings was nominally higher after 240 minutes compared to pre-irradiation levels.

3.3.5. *In vitro* Photoprotectant Effect of Cyanidin 3-O-(β-D-glucopyranoside) (26)

In order to determine if anthocyanins function as visible light screens that are able to prevent the photoconversion of thiarubrines to thiophenes, the photoprotectant effect of cyanidin 3-*O*-(β-D-glucopyranoside) (26) solutions of varying concentration was tested. Exposure to 2 minutes of visible irradiation with no anthocyanin screen (i.e. solvent control) led to an 85%

a.

b.
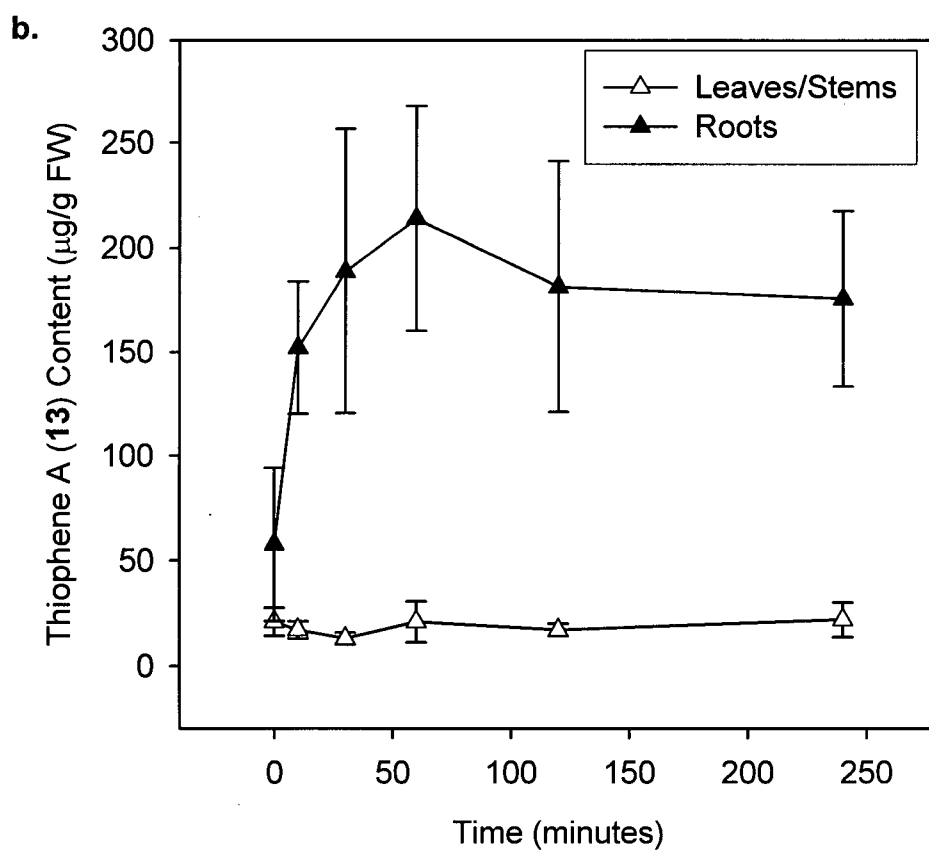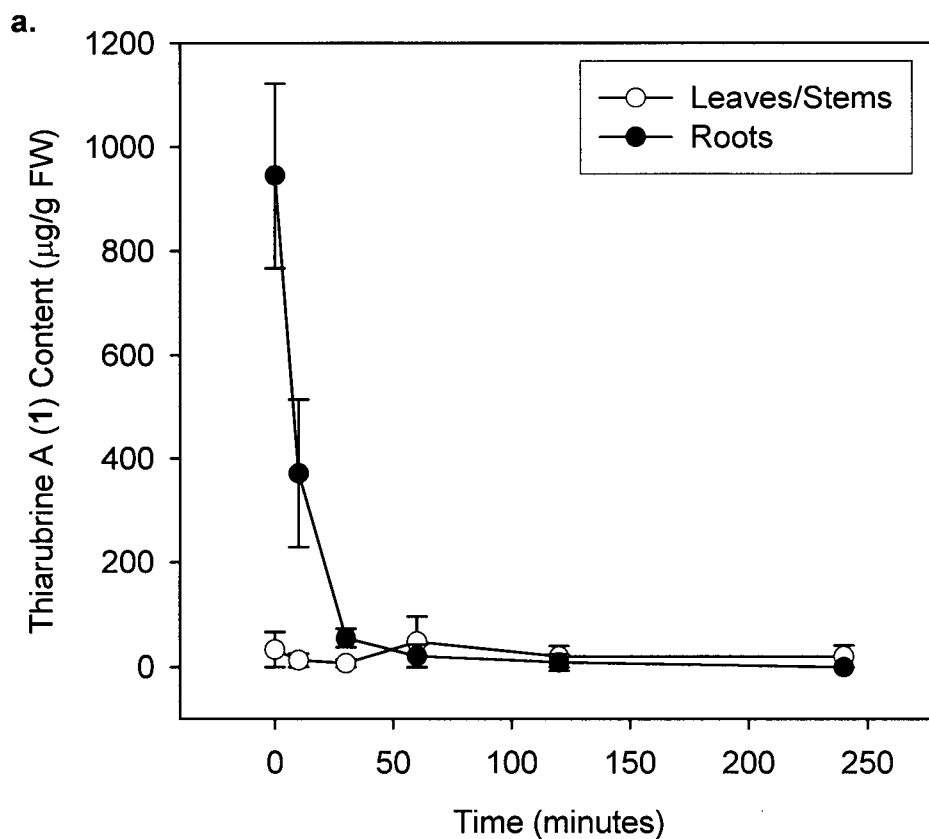
Figure 3.5. Comparison of the photoconversion of thiarubrine A (**1**) in the leaves/stems and roots of *Ambrosia chamissonis* seedlings. Seedlings (15–17 days) were removed from growth medium and exposed to visible light for 4 hours *in vitro*. Thiarubrine A (**1**) content (**a.**) and thiophene A (**13**) content (**b.**) of leaves/stems and roots was analyzed by HPLC before irradiation, and after 10, 30, 60, 120 and 240 minutes. Four separate experiments were performed and the results averaged. Error bars represent standard deviations.

(n = 3) decrease in the concentration of a thiarubrine A (**1**) solution (1.5 mM). As shown in Figure 3.6, anthocyanin solutions were able to prevent photoconversion with the amount of thiarubrine photoprotection increasing as a function of anthocyanin concentration. The data showed a sigmoidal distribution in the semi-log plot. Maximal photoprotection (< 5% decrease in thiarubrine A (**1**) concentration) occurred at anthocyanin concentrations greater than 0.1 mM.

3.4. DISCUSSION

Without a mechanism for photoprotection, sunlight would rapidly convert thiarubrines present in *Ambrosia chamissonis* leaves and stems to thiophenes. The observation that bleaching of thiarubrines occurs when thin longitudinal sections of *A. chamissonis* stems are exposed to visible radiation on the microscope stage indicates that photoconversion could occur *in vivo*. As well, it clearly shows that thiarubrine photoprotection is due to a mechanism, such as light screening, that functions outside of the canal structures containing these light-sensitive pigments.

In the present study, the role of visible-light absorbing anthocyanin pigments in thiarubrine photoprotection was investigated. The internal architecture of *Ambrosia chamissonis* stems and leaves contains a unique arrangement of anthocyanin-containing cells that appears to function in thiarubrine photoprotection. In many plants, anthocyanins are located in epidermal or sub-epidermal cells (Strack and Wray, 1989) although in a specialized subset of plants, primarily tropical understory herbs and sun-exposed aquatics, anthocyanins are found in mesophyll layers of leaf undersurfaces (Lee, 1983; Gould *et al.*, 1995). In *A. chamissonis*, anthocyanins are found in cells layers that sheath and surround
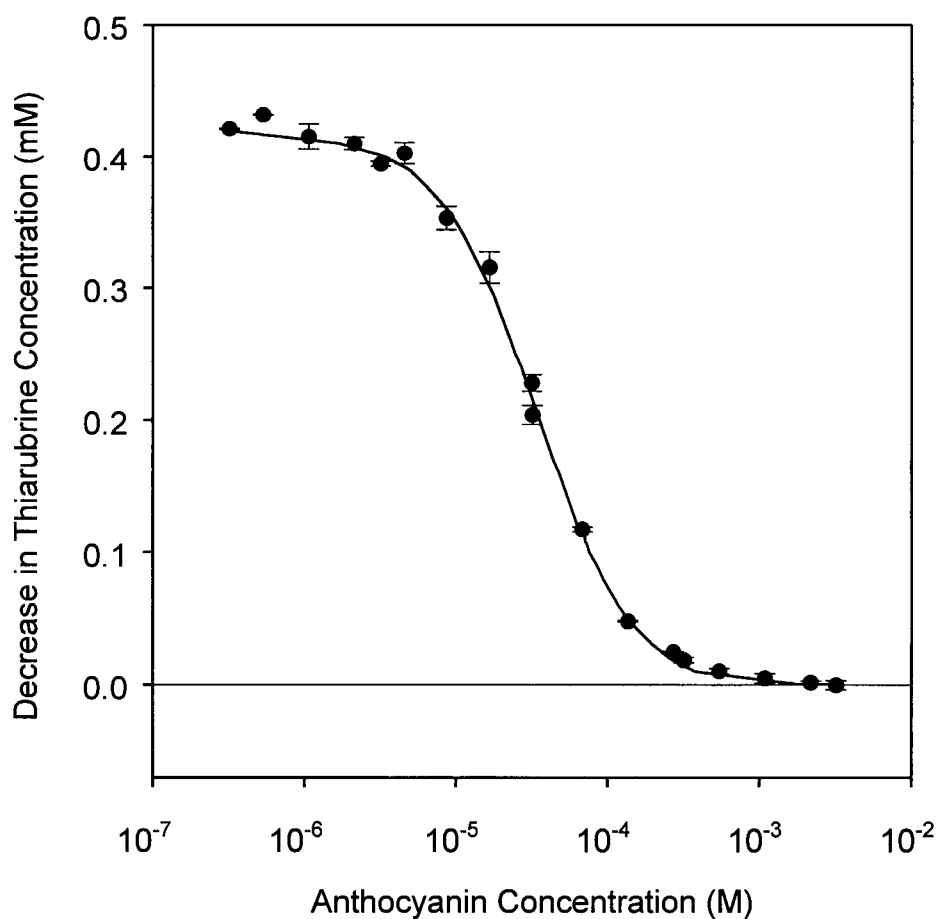
Figure 3.6. Photoprotection of thiarubrine A (**1**) by cyanidin 3-*O*-(β-D-glucopyranoside) (**26**). A 1.5 mM solution of thiarubrine A (**1**) was screened from visible radiation by solutions of cyanidin 3-*O*-(β-D-glucopyranoside) (**26**) ranging in concentration from 3.2 mM to 0.5 mM. After a 2 minute light exposure, the decrease in thiarubrine concentration was determined spectrophotometrically (Abs_{490}). Data points indicate the mean of three replicates with the error bars representing standard deviations.

thiarubrine canals, an anatomical adaptation that I suggest functions to prevent visible radiation from reaching thiarubrines. The protectant role of anthocyanins is supported by observations that thiarubrine-producing root cultures of *A. chamissonis* and *Chaenactis douglasii* accumulate anthocyanins when exposed to light (Ellis, 1993; Towers, personal communication).

No anthocyanins have been previously reported from *Ambrosia chamissonis* (NAPRALERT database, 1996)[1] and the isolation and characterization of cyanidin 3-O-(6-O-malonyl-β-D-glucopyranoside) (25) and cyanidin 3-O-(β-D-glucopyranoside) (26) represent new reports from this species. Cyanidin-3-glucosides commonly occur in the Asteraceae (Harborne, 1977) and acylation with malonic acid is frequently observed in this family (Takeda *et al.*, 1986). Comparison of the UV-vis spectra of thiarubrine A (1) and one of the anthocyanins present in *A. chamissonis*, cyanidin 3-O-(β-D-glucopyranoside) (26) (Figure 3.7), shows that the visible absorption band of cyanidin-based anthocyanins corresponds closely to that of thiarubrines. As well, anthocyanins absorb visible light more efficiently than thiarubrines, with the former possessing molar absorptivities an order-of-magnitude greater than do the thiarubrines (anthocyanins ε_{520} = ~30,000 M^{-1} cm^{-1}; thiarubrines ε_{490} = 3,000 M^{-1} cm^{-1}). Thus, anthocyanins have spectral characteristics that would allow them to function as visible light screens.

Due to the UV-A absorption of thiarubrines, UV radiation also results in conversion of thiarubrines to thiophenes. Although anthocyanins have been suggested to have a role in screening of UV-B light (Takahashi *et al.*, 1991; Klaper *et al.*, 1996), it is unclear whether

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[1] One ethnobotanical use of *Ambrosia chamissonis* seems to be related to the presence of anthocyanins (and perhaps thiarubrines) in this plant. The Clayoquot Sound Scientific Panel (1995) reported that: "Children at Hesquiat [Clayoquot Sound, British Columbia] played with the juice of this plant [*A. chamissonis*], which turns red when first exposed to air, pretending it is blood".
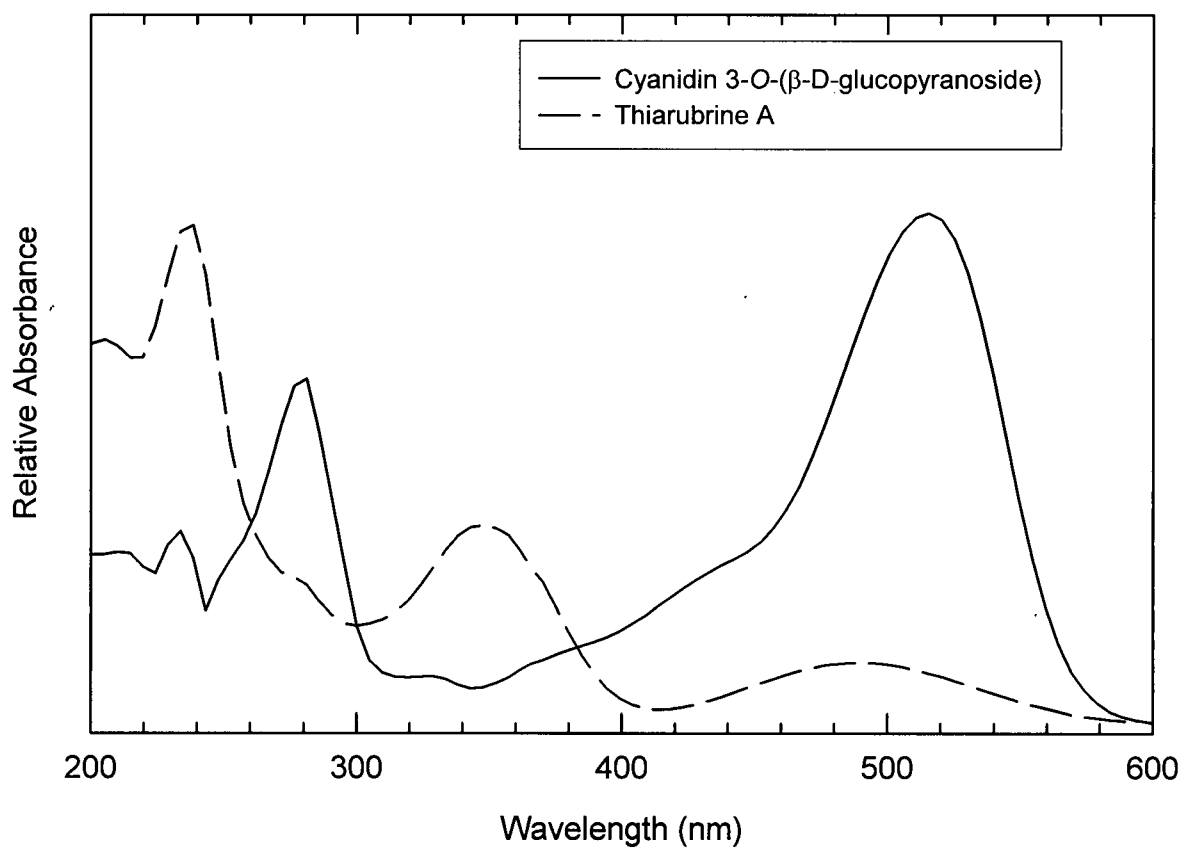
Figure 3.7. Comparison of the UV-vis spectrum of cyanidin 3-*O*-(β-D-glucopyranoside) (**26**) and thiarubrine A (**1**) showing overlap of the visible absorption band (400–600 nm) of both pigment types. Spectra were obtained from photodiode array detection.

they absorb enough light in the critical 340–350 nm range to photoprotect thiarubrines at these wavelengths. HPLC analysis of the formic acid–MeOH extract of *A. chamissonis* leaves showed high amounts of UV-A absorbing phenolics present (data not shown) which may participate in UV screening of thiarubrines.

Ambrosia chamissonis seedlings provided an ideal model system with which to examine the photoprotectant role of anthocyanins *in vivo*. As determined by HPLC, exposure of whole seedlings to visible light resulted in the rapid and irreversible conversion of thiarubrine A (**1**) and other thiarubrines present in roots to their corresponding thiophenes. In leaves and stems, however, thiarubrine levels were unaffected. Since both light microscopy (Figure 3.2) and HPLC analysis (Table 3.2) showed that the roots of *A. chamissonis* seedlings lack anthocyanins, a possible explanation for these results is that thiarubrines undergo photoconversion to thiophenes in the absence of photoprotective pigments. An alternative explanation for this pattern is that the rate of *de novo* synthesis of thiarubrines is higher in leaves/stems compared with roots; thus, thiarubrines lost through photoconversion would quickly be replaced in above-ground plant tissues. Two lines of evidence argue against an explanation of differential metabolism. Thiophene A (**13**) levels in leaves/stems did not increase during the 4 hour light treatment, as one would expect if thiarubrines were being synthesized at a rapid pace and quickly converted to thiophenes in above-ground parts. As well, thiophene A (**13**) levels in roots levelled off and decreased after the initial 60 minutes of light-exposure, supporting the assertion that thiarubrine synthesis is not occurring during the 4 hour experiment. Furthermore, Constabel and Towers (1989) found no evidence of rapid turnover of thiarubrines in hairy root cultures of *Chaenactis douglasii*.

By using a "three-cuvette" apparatus, I tested the *in vitro* photoprotectant effect of acidic solutions of cyanidin 3-*O*-(β-D-glucopyranoside) (**26**), the deacylation product of the seven anthocyanins present in *A. chamissonis*. Such solutions proved efficacious as light screens and, at concentrations above 0.1 mM, led to near complete photostability of thiarubrine A (**1**) during a 2 minute period of high-irradiance visible light. The sigmoidal curve that results from plotting the amount of thiarubrine bleaching versus anthocyanin concentration (Figure 3.6) requires some explanation. A possible reason for the high-concentration component of this trend is that anthocyanins in their cationic form (i.e. in acidic solution) deviate from the Beer-Lambert Law due to self-association, an effect that results in marked increases in light absorbance at high concentrations (Timberlake, 1980). Thus, self-association may lead to non-linear increases in light interception, and hence photoprotection of thiarubrines, at high anthocyanin concentrations.

Other screening mechanisms may also be contributing to thiarubrine photoprotection in *Ambrosia chamissonis*. The leaves of this plant have a silvery appearance due to a covering of epidermal hairs. Leaf pubescence has been shown to modify the optical properties of leaves, resulting in light reflection and scattering (Robberecht *et al.*, 1980; Karabourniotis *et al.*, 1992), and such structures may serve to attenuate the amount of light entering leaf tissues. The localization of thiarubrine canals (and their associated anthocyanin layers) on the undersides of mature *A. chamissonis* leaves and leaf petioles may shield these tissues. Finally, other classes of plant pigments including carotenoids and chlorophylls also absorb visible radiation: the blue band (400–500 nm) maxima of β-carotene and chlorophyll-*b* are 466 and 497, and 448 nm, respectively. The contribution of such pigments to thiarubrine photoprotection is probably small. Significant amounts of carotenes were not

detected during work-up of *A. chamissonis* extracts nor were these orange-red pigments visible during microscopic examination of *A. chamissonis* seedlings. As well, an important screening role for chlorophyll is not supported by the finding that thiarubrines in *A. chamissonis* stems are protected from light despite few cells containing chloroplasts in these tissues.

In conclusion, the results of this study provide evidence that thiarubrines in *Ambrosia chamissonis* leaves and stems are protected from light by the visible-light screening properties of anthocyanin pigments. Anthocyanins have been suggested to play a role in attenuation of UV-B damage (Takahashi *et al.*, 1991; Klaper *et al.*, 1996) and photoinhibition (Gould *et al.*, 1995). This study is the first to report that these widespread pigments are utilized to screen phototoxic, light-sensitive secondary metabolites. As well, the localization of anthocyanins in cell layers surrounding thiarubrine canals represents a unique and previously unreported anatomical adaptation for photoprotection.

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CHAPTER 4

CHEMICAL ECOLOGY OF *WEDELIA* LEAF-SWALLOWING BY CHIMPANZEES:

THE ROLE OF THIARUBRINES

4.1. INTRODUCTION

Animals may deliberately ingest plants containing secondary metabolites that have beneficial effects on health (Janzen, 1978), a behavioural process that has been termed zoopharmacognosy (Rodriguez and Wrangham, 1993). Examples of plant consumption for the purpose of self-medication have been suggested to be the ingestion of *Vernonia amygdalina* Del. (Asteraceae) pith (Huffman and Seifu, 1989; Ohigashi *et al.*, 1994) and the swallowing of whole leaves by chimpanzees (*Pan troglodytes*) (Wrangham and Nishida, 1983; Rodriguez *et al.*, 1985; Wrangham and Goodall, 1989). In the latter behaviour, chimpanzees carefully select, fold and swallow whole leaves of three *Wedelia*[1] (Asteraceae) species and over fifteen other plant taxa (Figure 4.1). The nutritional contribution of such leaves, which are found intact and undigested in chimpanzee feces following bouts of leaf-swallowing, appears to be minimal.

The peculiar method of ingesting *Wedelia* leaves, and the possibility that whole leaves contain pharmacologically-active substances, prompted investigation of the phytochemistry of *Wedelia* species. Rodriguez *et al.* (1985) reported that thiarubrine A (**1**) was present in high concentrations in *W. mossambicensis* Oliv. and *W. pluriseta* O. Hoffm. leaves. In light

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[1] *Wedelia mossambicensis*, *W. pluriseta*, *W. rudis* and *W. africana* were formerly classified as members of the genus *Aspilia* Thouars. *Aspilia* has been reduced to synonymy with *Wedelia* Jacq. (Robinson, 1992; Turner, 1992), a change that appears to have gained acceptance in the botanical community (Mabberley, 1997).

Figure 4.1. Chimpanzee selecting and swallowing leaves of *Wedelia mossambicensis* in Mahale Mountains National Park, Tanzania. (Photograph © M. A. Huffman. Used with permission).

of the biological activity of thiarubrine A (**1**), which includes nematocidal activity, and the incidence of parasite infection in chimpanzees (Myers and Kunst, 1972), *Wedelia* leaf-swallowing was hypothesized to have an anthelmintic function (Rodriguez *et al.*, 1985). Other possible functions of this anomalous plant use have been suggested to be fertility regulation, based on the presence of uterostimulatory kaurene diterpenes in *W. mossambicensis* (Page *et al.*, 1992), reduction of intestinal pain (Wrangham, 1995) and intestinal nematode expulsion (Huffman *et al.*, 1996).

Despite the isolation of thiarubrine A (**1**) from one collection of *Wedelia* leaves, other researchers have been unable to replicate this finding. High performance liquid chromatographic (HPLC) analysis of *Wedelia mossambicensis* leaves collected in Mahale in 1991 and 1993–1994 from plants utilized by chimpanzees, as well as from greenhouse-grown specimens, failed to detect thiarubrine A (**1**) (Page *et al.*, 1992; Huffman *et al.*, 1996). As a result of the uncertainty surrounding the presence of this compound in *Wedelia* leaves, the impact of thiarubrine A (**1**) on chimpanzee health and the role of plant chemicals in mediating leaf-swallowing is unclear.

In this study I attempt to determine if thiarubrine A (**1**) is present in *Wedelia* leaves using a sensitive HPLC assay for the detection and quantification of thiarubrines A (**1**), B (**2**), D (**3**) and E (**4**) and their thiophenes (**13–16**). In addition, I employ an antifungal bioassay for toxicity to *Candida albicans*, a human pathogenic yeast that is extremely sensitive to thiarubrines. I describe the application of both assays to extracts of fresh and dried samples of *Wedelia* species from four locations in East Africa as well as other asteraceous taxa known to contain thiarubrines.

4.2. MATERIALS AND METHODS

General experimental procedures

Plant samples were collected with the appropriate permission from each host country. Samples were collected in Canada by J. E. Page and G. H. N. Towers, in Tanzania by M. A. Huffman, in Uganda by G. H. N. Towers and in Kenya by V. Smith and M. A. Huffman. Samples received minimal exposure to light after collection and all procedures were performed in dim light to minimize the photoconversion of thiarubrines. All solvents were HPLC-grade (Fisher) except for reagent-grade MeOH or EtOH used in field extractions. Water was Milli-Q plus (Millipore). The HPLC system consisted of a Waters 600 pump controller, Waters 996 photodiode array detector, Waters 717 plus autosampler controlled by Millenium 2.1 software. A 3.9 mm X 150 mm (4 μm) C_{18} column (Waters NovaPak) equipped with NovaPak precolumn was used for HPLC analyses. UV-vis spectra were obtained on a Pye-Unicam 8720 scanning spectrophotometer in MeCN. The ^{1}H- and ^{13}C-NMR spectra of thiarubrine E (**4**) were recorded in acetone-d_6 on a Bruker AMX-500 spectrometer. The ^{1}H-NMR spectrum of thiophene E (**16**) was recorded in CDCl$_3$ on a Bruker AC-200E spectrometer. The residual acetone (δ 2.08 ppm) or CHCl$_3$ (δ 7.24 ppm) signal were used as references.

Field-extracted plant material

Samples of *W. mossambicensis* were collected in Mahale Mountains National Park, Tanzania in December 1993 through February 1994. Nine leaf samples were collected from *W. mossambicensis* plants concurrently with direct observations of leaf-swallowing and five from plants that showed indirect evidence of leaf-swallowing, including discarded stalks with

missing leaves. The age and size of the leaves sampled, as well as their location on the plant, were closely matched to leaves consumed by the chimpanzees. Leaves of an unidentified *Wedelia* sp. (#1) were collected in Gombe Stream National Park, Tanzania from a plant which the male chimpanzee Freud was observed to swallow leaves. Plant identifications were verified by S. Mathenge (Dept. of Botany, University of Nairobi, Kenya) and E. Knox (East African Herbarium, National Museums of Kenya). Voucher specimens are deposited in the Herbarium of the University of Nairobi and the East African Herbarium, National Museums of Kenya. No voucher specimen was available for the Gombe *Wedelia* sp. (#1) which was preserved in EtOH.

Additional samples were collected during November 1995 through February 1996. Samples of *W. mossambicensis, W. rudis* and an unidentified *Wedelia* sp. (#2) were obtained at Gombe; *W. mossambicensis* was collected in Nanyuki, Kenya; and one sample of *W. mossambicensis* leaves was collected at Mahale from a plant from which a chimpanzee was observed to swallow leaves. Voucher specimens are deposited in the East African Herbarium, National Museums of Kenya (Mahale and Gombe specimens) and in the Herbarium of the University of Nairobi and the East African Herbarium, National Museums of Kenya (Nanyuki specimens). Samples of the plant material of interest were collected, accurately weighed (3.0 g) and placed in amber vials containing 20 ml MeOH. Smaller samples (2.0 g or 2.6 g) were collected if plant material was limited.

Dried plant material

A bulk collection of *W. mossambicensis* leaves was made at Mahale in January 1996 from plants known to be frequented by chimpanzees. Leaves and roots of *W. mossambicensis* and

W. africana Pers. C.D. Adams were collected in the Kampala District of Uganda in November 1995 and identification confirmed by T. Katenge (Makerere University Herbarium, Uganda). Plants of *W. mossambicensis* were grown in the greenhouse of the Botany Dept., U.B.C. from seeds collected at Mahale. *Ambrosia chamissonis* (Less.) Greene samples were collected at Iona Island and Comox, British Columbia, Canada in June 1995 and May 1996, respectively. *Eriophyllum lanatum* (Pursh) Forbes was collected on Hornby Island, B.C. in May 1996 and *Chaenactis douglasii* (Hook.) H. & A. was collected near Princeton, B.C. in August 1996. Voucher specimens of the greenhouse-grown *W. mossambicensis* and the North American taxa are deposited in the U.B.C. Herbarium. Material for extraction was ground to a fine powder and 1.0 g samples accurately weighed. If plant material was limited, <1.0 g samples were prepared.

Sample preparation

The MeOH extract of field-extracted samples was decanted and the plant material washed with 3 ml of MeOH. The extracts were combined, passed through a C_{18} cartridge (Waters) and the volume adjusted to 25.0 ml. A 15.0 ml aliquot was dried *in vacuo* and the residue resuspended in MeCN to yield a solution of 1 g fresh weight (FW)/ml concentration. This solution was filtered through a 0.45 μm filter (Gelman) and analyzed by HPLC.

Dry plant samples were extracted with 3 X 25 ml CH_2Cl_2. Three rounds of extraction effectively removed >99 % of the thiarubrines as determined by triplicate extraction of an *Ambrosia chamissonis* root sample monitored spectrophotometrically at 490 nm. The combined extracts were evaporated to dryness and the residue resuspended in 2.0 ml MeCN

to yield a solution of 0.5 g dry weight (DW)/ml. The solution was filtered through a 0.45 μm filter (Gelman) and analyzed by HPLC.

For assessment of antifungal activity, a 500-μl aliquot of the MeCN solutions was dried by a stream of N$_2$ and reconstituted in 500 μl MeOH.

Standards

Thiarubrines A (**1**), B (**2**) and D (**3**) were isolated from MeOH extracts of *Ambrosia chamissonis* roots collected near Centennial Beach, Tsawwassen, Canada as described in Chapter 2. Thiophenes A (**13**), B (**14**) and D (**15**) were isolated from the same source and by photoconversion of the corresponding thiarubrines. Thiarubrine E (**4**) was isolated from a CHCl$_3$ fraction obtained from a methanolic extract of *A. chamissonis* roots (Chapter 2 "Materials and Methods") by solvent-solvent partition. The CHCl$_3$ fraction was concentrated *in vacuo* to yield a dark red residue that was separated by repeated preparative HPLC on a 2.5 X 10 cm (5 μm) C$_{18}$ radial compression column (Waters NovaPak) equipped with NovaPak precolumn. The mobile phase was composed of MeCN–water (74:26) at a flow rate of 27 ml/minute with detection at 340 nm. Injection volume was 100–500 μl. Final purification was achieved by preparative normal-phase HPLC on a 19 X 300 mm (10 μm) silica column (Waters μPorasil) equipped with a silica (Waters Resolve) precolumn eluted with a gradient with the following conditions: 0 minutes (100%; hexane), 5 minutes (50:50; hexane–ethyl acetate). The flow rate was 20 ml/minute, detection was at 340 nm and injection volume was 500 μl. Removal of the solvent *in vacuo* afforded thiarubrine E (**4**) as a red oil. Exposure of a solution of **4** to light followed by purification by reversed-phase and normal-phase preparative HPLC (conditions as above) afforded **16**.

The purity of all standards was >99% by HPLC. Thiarubrine E (**4**) and thiophene E (**16**) were identified spectroscopically (UV-vis and ^{1}H-NMR) by comparison with literature values (Balza and Towers, 1993). The concentration of thiarubrine ($\varepsilon = 3000$ M^{-1} cm^{-1} at visible maximum 483–490 nm) and thiophene ($\varepsilon = 31,500$ M^{-1} cm^{-1} at UV-A maximum 324–340 nm) solutions was determined spectrophotometrically.

Thiarubrine E (**4**): UV-vis see Table 2.1; ^{1}H-NMR δ 6.89 (1 H, d, $J = 6.8$ Hz, H-8), 6.67 (1 H, d, $J = 6.8$ Hz, H-9), 4.51 (1 H, t, $J = 5.7$ Hz, H-2), 3.64 (1 H, br s, H-1$_b$), 3.63 (1 H, br s, H-1$_a$), 2.08 (3 H, s, Me-13) ppm. (See Appendix 2).

Thiophene E (**16**): UV-vis see Table 2.1; ^{1}H-NMR δ 7.01 (1 H, d, $J = 14.9$ Hz, H-8), 6.86 (1 H, d, $J = 14.4$ Hz, H-9), 5.28 (1 H, m, H-2), 5.03 (2 H, m, H-1$_a$, H-1$_b$), 2.16 (3 H, s, Me-13) ppm. (See Appendix 2).

Chromatographic conditions and standard curves

Analysis was performed using a step gradient elution system with the following conditions: pre-injection (55:45; water–MeCN), 0 minutes (30:70), 10 minutes (10:90), 15 minutes (0:100), 32 minutes (55:45). Flow rate was 1 ml/minute with detection over the wavelength range 200–600 nm and specific detection at 340 nm. Injection volume was 20 μl. Thiarubrine and thiophene peaks in samples were identified on the basis of retention time and comparison of UV-vis spectra with those of authentic standards as determined by photodiode array detection. Quantification of thiarubrines and thiophenes was performed at 340 nm using peak area by comparison to standard curves derived from replicate injections of solutions ranging

in concentration from ~1000 µg/ml to ~0.1 µg/ml for thiarubrines and ~125 µg/ml to ~0.1

µg/ml for thiophenes (Table 4.1). The correlation coefficient values (r^2) for all compounds

exceeded 0.99 (Table 4.1). Six consecutive injections of a solution of thiarubrine A (**1**) (100

µg/ml) gave a deviation in area of 0.8%. For thiarubrine A (**1**), the coefficient of variation

(CV) for 5 independent analyses of a sample of *Ambrosia chamissonis* roots was 3.5% where

$$CV = \frac{\text{standard deviation}}{\text{mean}} * 100\%.$$

Detection limits

Detection limits for the HPLC assay were less than 50 ng/ml for thiarubrine and thiophene

standards. To determine the practical detection limit of thiarubrine A (**1**) in plant extracts

where interfering peaks are present, dried *W. mossambicensis* leaves (5.0 g) which previous

analysis had shown were devoid of thiarubrine A (**1**), were finely ground and extracted with

CH_2Cl_2. The extract was dried *in vacuo* and taken up in 10 ml MeCN. After "spiking" with

200 µg/g (based on 5.0 g extracted weight) of thiarubrine A (**1**), the solution was serially

diluted to yield solutions ranging in concentration from 200 µg/g DW to 0.78 µg/g DW.

Aliquots (500 µl) were dried with N_2 and resuspended in 500 µl of MeOH for

determination of the detection limit of the antifungal bioassay.

Antifungal assay

Antifungal activity was determined using the broth dilution method modified from Towers *et*

al. (1985). A 48-hour culture of *C. albicans* (UBC #54) grown at 37 °C in Sabouraud dext-

Table 4.1. Retention time and calibration values of thiarubrine and thiophene standards.

Compound	Retention Time[a] (minutes)	Range (μg/ml)	r^2
Thiarubrine A (**1**)	16.1 ± 0.04	0.1 – 999.4	0.999
Thiarubrine B (**2**)	15.5 ± 0.08	0.1 – 992.6	0.999
Thiarubrine D (**3**)	10.9 ± 0.06	0.1 – 1011.0	0.999
Thiarubrine E (**4**)	5.3 ± 0.02	0.1 – 1021.8	0.999
Thiophene A (**13**)	14.3 ± 0.04	0.02 – 125.4	0.998
Thiophene B (**14**)	13.6 ± 0.20	0.03 – 123.2	0.999
Thiophene D (**15**)	9.8 ± 0.03	0.03 – 129.2	0.999
Thiophene E (**16**)	4.1 ± 0.03	0.06 – 114.6	0.999

[a] mean ± standard deviation (n = 14)

rose broth (SDB) was diluted to ~1.0 X 10^4 cells/ml. MeOH solutions of plant extracts were diluted 100-fold and a 200-μl aliquot transferred to a well in row 2 of a 96-well microtitre plate (8 lanes X 12 rows) (Costar). MeOH was used as a solvent (negative) control. A MeOH solution of thiarubrine A (1) (114 μg/ml) was tested as a reference compound. Solutions were diluted in a series of nine two-fold dilutions while row 12 served as an untreated control and received 100 μl of untreated SDB. A 100-μl aliquot of diluted *C. albicans* culture was transferred to each well of rows 2 to 12. Row 1 received growth medium only (200-μl SDB). Plates were incubated in the dark at 37 °C for 24 hours and the optical density of each well read at 600 nm using an ELISA reader (Titertek Multiskan) zeroed on culture medium (row 1).

Solutions were screened for activity in single experiments in which one lane was used for each solution. Solutions which showed activity (>30% inhibition of growth in the first well) were retested in two experiments of three replicate lanes each. Minimum inhibitory concentration (MIC) values were determined visually as the lowest dilution which resulted in a clear well and the values from each experiment averaged.

4.3. RESULTS AND DISCUSSION

4.3.1. HPLC Assay

The HPLC assay allowed for the detection and quantification of thiarubrine A (1) and three related 1,2-dithiin polyynes, thiarubrine B (2), thiarubrine D (3) and thiarubrine E (4), known to occur in the Asteraceae. Exposure of thiarubrines to visible light results in the formation of photosulfide intermediates which convert to the corresponding thiophenes (Chapter 2). Due to the potential for photoconversion during sample collection and transit, the HPLC assay

included the detection and quantification of thiophenes A (**13**), B (**14**), D (**15**) and E (**16**) which are the photoproducts of the thiarubrines of interest. All 1,2-dithiin or thiophene polyynes included in the analysis exhibit distinct UV-vis spectra, and their unambiguous detection in extracts was on the basis of retention time and UV-vis spectroscopy by photodiode array detection. A chromatogram of a standard mixture of compounds (**1–4**, **13–16**) is illustrated in Figure 4.2a.

Previous studies of thiarubrine chemistry have relied on isocratic HPLC methods which have been unable to separate polar thiarubrines with oxygenated side-chains (i.e. thiarubrines D (**3**) and E (**4**)) and those with diyne-ene side-chains (i.e. thiarubrines A (**1**) and B (**2**)) in a single chromatographic run (Balza and Towers, 1993; Norton *et al.*, 1993; Huffman *et al.*, 1996). The use of gradient elution resulted in resolution of all major thiarubrines and thiophenes. Extraction efficiency for dried plant material was >99% for thiarubrines determined by spectrophotometry. Although field extraction probably resulted in somewhat less efficient extraction of thiarubrines, the MeOH extracts which resulted contained most of the soluble pigments and subsequent re-extraction was deemed unnecessary.

4.3.2. Antifungal Assay

Thiarubrines are toxic to many microorganisms and exhibit particularly potent activity against the pathogenic yeast *Candida albicans* (Towers *et al.*, 1985; Bierer *et al.*, 1995; Ellis *et al.*, 1995; Chapter 5). Based on the sensitivity of *C. albicans* to thiarubrines, an antifungal bioassay was developed for use as a complementary method for detection of thiarubrines in crude plant extracts. Although compounds other than thiarubrines may be expected to be

Time (minutes)
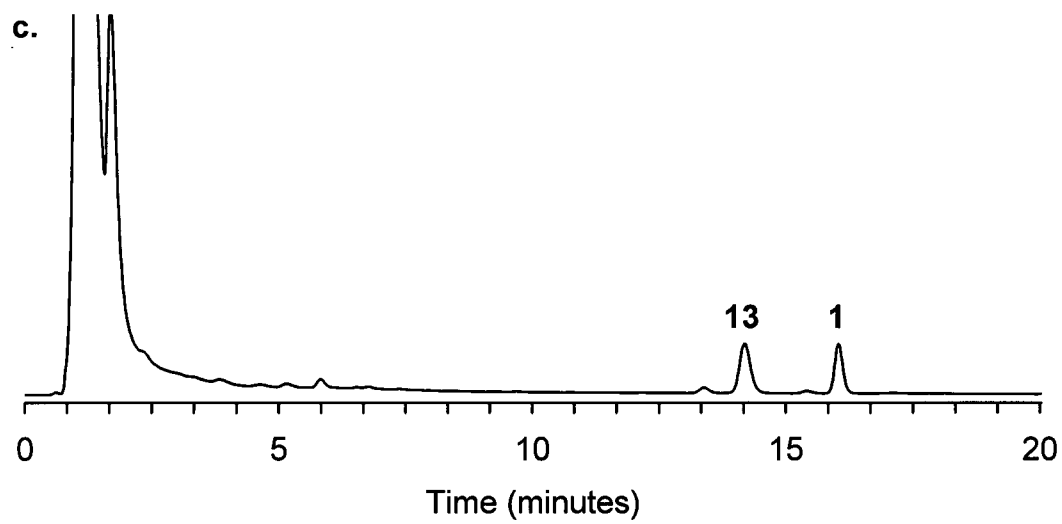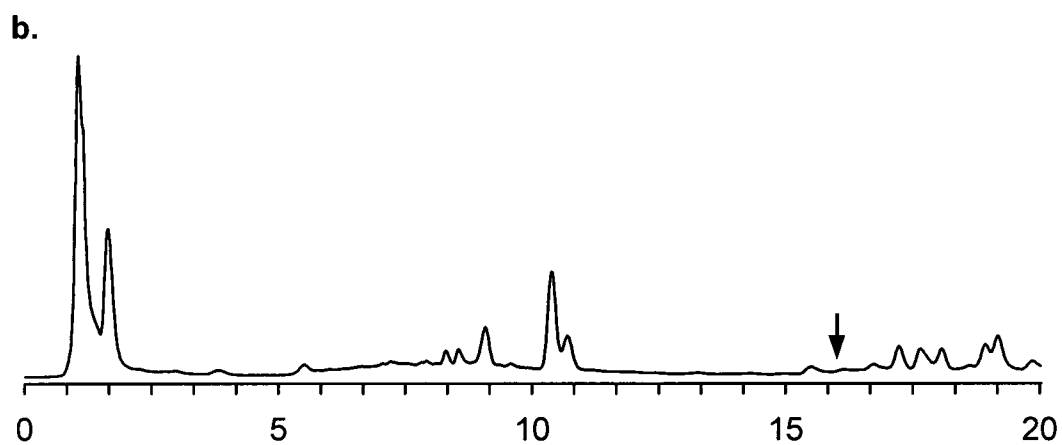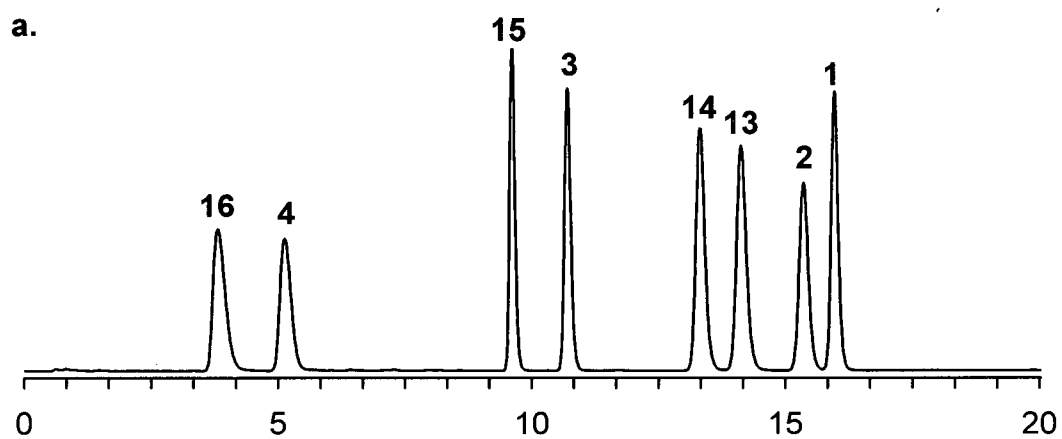
Figure 4.2. HPLC analysis of thiarubrines and thiophenes in *Wedelia mossambicensis*. (**a.**) Chromatogram of injection of standard mixture of thiarubrines (**1–4**) and thiophenes (**13–16**). (**b.**) Chromatogram obtained from analysis of field-extracted *W. mossambicensis* leaves collected at Mahale from plant which the chimpanzee Iwan was observed to swallow nine distal leaves on February 7, 1994. Peaks at ~10.8 minutes and ~15.5 minutes were shown not to be thiarubrines by UV-vis spectroscopy. Arrow indicates the retention time of thiarubrine A (**1**). (**c.**) Chromatogram of field-extracted *W. mossambicensis* roots collected at Gombe on February 2, 1996. Detection: 340 nm. A.U. full-scale: 0.25.

toxic to *C. albicans*, the bioassay detected antifungal activity only in plant samples which were shown to contain thiarubrines by HPLC. The bioassay was not used to quantify thiarubrines nor was it able to detect thiophenes which lack activity against *C. albicans* in the absence of UV light (Towers *et al.*, 1985). Solvent controls (MeOH) did not inhibit *C. albicans* growth at concentrations used in the assay. Bioassay of a solution of pure thiarubrine A (**1**) as a reference compound showed it had an MIC of 0.12 μg/ml.

4.3.3. Detection Limits

The sensitivity of the HPLC assay and antifungal bioassay were assessed using an extract of dried *W. mossambicensis* leaves from Mahale that was shown by prior analysis to lack thiarubrines or antifungal activity. The extract was "spiked" with a known amount of thiarubrine A (**1**) and serially diluted with additional extract. Analysis of the resulting solutions showed the detection limit for the HPLC assay was 6 μg/g DW. Bioassay of the "spiked" solutions showed that a concentration of 25 μg/g DW of thiarubrine A (**1**) resulted in >90% inhibition of *C. albicans* growth in the well containing the highest concentration of test solution.

Rodriguez *et al.* (1985) estimated that *Wedelia* leaves contained 5 mg of thiarubrine A (**1**) per leaf based on the analysis of a chloroform extract of dried leaves. This amount was later revised to 100 μg per leaf (Rodriguez and Wrangham, 1993). Calculation using an average dry leaf weight of 0.092 g (n = 28) for young leaves of cultivated *W. mossambicensis* plants reveals that the HPLC assay and the antifungal bioassay should be capable of detecting levels <3 μg per leaf. For confirmatory purposes, I "spiked" fresh *W. mossambicensis* leaves from plants in cultivation with 100 μg thiarubrine A (**1**) per leaf. The leaves were lyophilized,
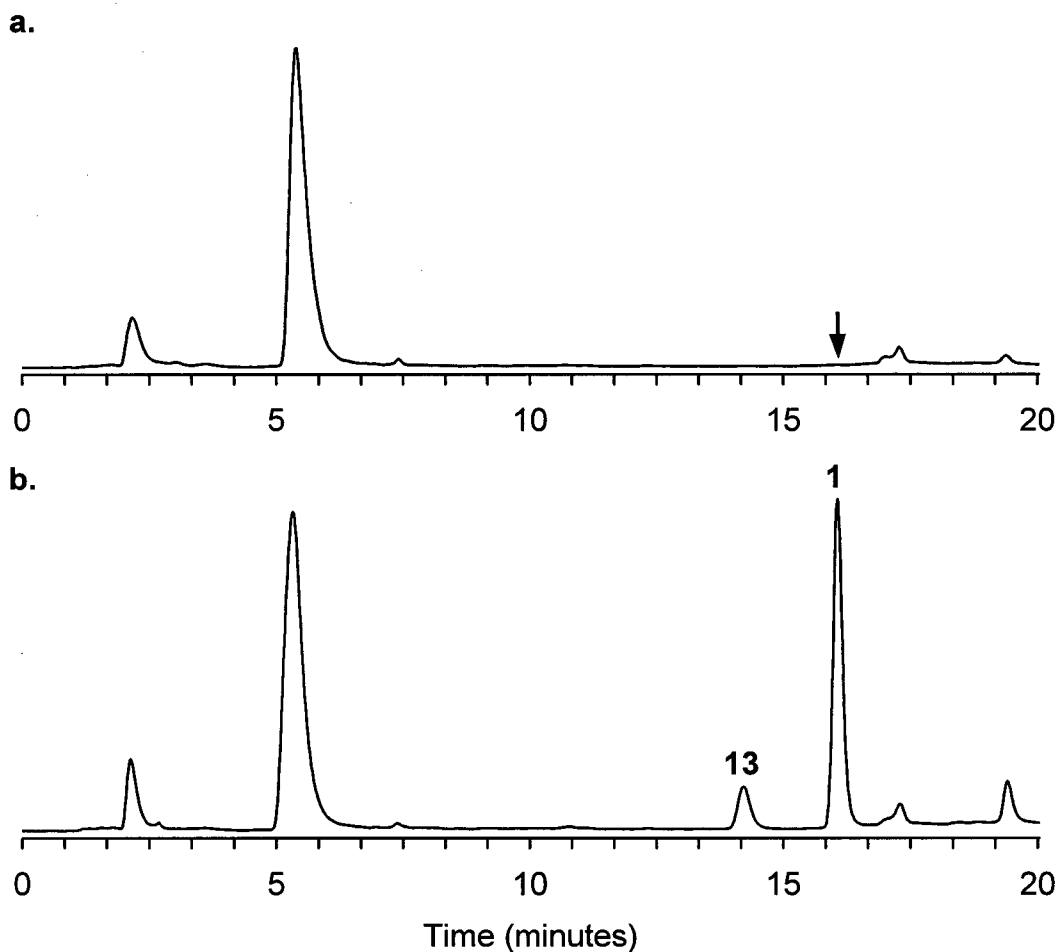
Figure 4.3. HPLC analysis of thiarubrine A (**1**) "spiked" *Wedelia mossambicensis* leaf samples. Chromatograms obtained from analysis of (**a.**) an extract of *W. mossambicensis* leaves from greenhouse-grown specimens and (**b.**) an extract of *W. mossambicensis* leaves from the same source "spiked" with 100 µg thiarubrine A (**1**) per leaf. Arrow indicates the retention time of thiarubrine A (**1**). The peak at ~5.5 minutes was shown not be a thiarubrine (i.e. **4**) by UV-vis spectroscopy. Detection: 340 nm. A.U. full-scale: 0.25.

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processed using the methodology developed for dry plant material and analyzed in the HPLC

and *C. albicans* assays. A comparison of chromatograms obtained from analysis of "spiked"

and "unspiked" leaves (Figure 4.3) clearly shows that 100 µg thiarubrine A (**1**) per leaf was

well within the detection limit of the HPLC assay. As well, the "spiked" sample had

significant activity in the antifungal assay (Table 4.3).

4.3.4. Analyses

Table 4.2 summarizes the results of the HPLC and bioassay analysis of field-extracted

Wedelia leaf, stem and root samples. The results of the analysis of dried samples of *Wedelia*

species and three North American asteraceous taxa, *Ambrosia chamissonis*, *Eriophyllum*

lanatum and *Chaenactis douglasii* are shown in Table 4.3. Thiarubrines and thiophenes have

been isolated from the latter three species and their inclusion in the study served both to

validate the methods of extraction and analysis, and to compare the levels of thiarubrines in

Wedelia species with those of other plants.

A total of 27 samples (19 fresh and 8 dried) of *Wedelia* leaves representing

collections of *W. mossambicensis*, *W. rudis* and *W. africana*, from four sites in East Africa

and from plants in cultivation in North America, contained no detectable thiarubrine A (**1**),

thiophene A (**13**) or any related thiarubrines (**2–4**) or thiophenes (**14–16**). The absence of

these compounds is illustrated by Figure 4.2b, which shows a chromatogram obtained by

analysis of a field-extracted sample of *W. mossambicensis* leaves collected at Mahale from a

plant from which chimpanzees swallowed leaves. Furthermore, *Wedelia* leaf samples showed

no detectable antifungal activity.

Table 4.2. Thiarubrine and thiophene content and antifungal activity of field-extracted *Wedelia* species.

Sample	Location (Date)	Total Thiarubrines[a] (μg/g FW)	Total Thiophenes[a] (μg/g FW)	MIC[b] (mg FW/ml)
W. mossambicensis leaves[c] (n=13)	Mahale (1993-94)	ND[d]	ND	inactive[e]
stems (n=2)		ND	ND	inactive
roots (n=2)		1.0 ± 0.6 (**1**[f])	1.1 ± 0.01 (**13, 14**)	inactive - 6.3
W. mossambicensis leaves[c] (n=1)	Mahale (1995-96)	ND	ND	inactive
W. mossambicensis leaves (n=1)	Gombe (1995-96)	ND	ND	inactive
roots (n=1)		9.1 (**1, 2**)	2.7 (**13, 14**)	0.6
W. mossambicensis leaves (n=3)	Nanyuki (1995-96)	ND	ND	inactive
W. rudis leaves (n=1)	Gombe (1995-96)	ND	ND	inactive
Wedelia sp. (#1) leaves[c] (n=1)	Gombe (1993-94)	ND	ND	inactive
Wedelia sp. (#2) leaves (n=3)	Gombe (1995-96)	ND	ND	inactive
roots (n=1)		ND	ND	inactive

[a] mean ± standard deviation

[b] mean (range)

[c] leaf samples collected after direct or indirect observations of leaf-swallowing behaviour

[d] ND, not detected

[e] inactive, no inhibition of *Candida albicans* growth at highest concentration (5 mg FW/ml) tested

[f] numbers in brackets refer to compounds present in sample

Table 4.3. Thiarubrine and thiophene content and antifungal activity of *Wedelia* spp. and other asteraceaeous taxa.

Sample	Location (Date)	Total Thiarubrines[a] (µg/g DW)	Total Thiophenes[a] (µg/g DW)	MIC[b] (µg DW/ml)
W. mossambicensis leaves (n=2)	Mahale (1995-96)	ND[c]	ND	inactive[d]
W. mossambicensis leaves (n=2)	Uganda (1995)	ND	ND	inactive
roots (n=1)		ND	ND	inactive
W. mossambicensis leaves (n=2)	U.B.C. (1996)	ND	ND	inactive
spiked leaves (n=1)		125.1 (**1**)	5.2 (**13**)	468.8
roots (n=2)		164.4 ± 85.7 (**1, 2, 3, 4**[e])	10.0 ± 0.8 (**13, 14, 15, 16**)	58.6 (39.1 – 78.1)
W. africana leaves (n=2)	Uganda (1995)	ND	ND	inactive
roots (n=1)		ND	12.0 (**13, 14**)	inactive
Chaenactis douglasii aerial parts (n=2)	Princeton (1996)	ND	ND	inactive
roots (n=1)		1396.4 (**1, 2**)	104.6 (**13, 14**)	19.5
Eriophyllum lanatum aerial parts (n=2)	Hornby Is. (1996)	ND	ND	inactive
roots (n=2)		214.1 ± 7.0 (**1, 2**)	6.8 ± 0.09 (**13, 14**)	97.7 (78.1 – 117.2)

Ambrosia chamissonis B.C. (1996)

leaves (n=3)	1442.9 ± 1107.4 (**1, 2**)	388.1 ± 228.4 (**13, 14**)	74.9 (29.3 – 156.3)
stems (n=1)	3694.3 (**1, 2, 3, 4**)	538.5 (**13, 14, 15, 16**)	4.9
roots (n=3)	491.7 ± 87.1 (**1, 2, 3, 4**)	48.5 ± 5.2 (**13, 14, 15, 16**)	14.6 (4.9 – 19.5)
Thiarubrine A (**1**)	-	-	0.12
MeOH	-	-	inactive

[a] mean ± standard deviation
[b] mean (range)
[c] ND, not detected
[d] inactive, no inhibition of *Candida albicans* growth at highest concentration (2500 μg DW/ml) tested
[e] numbers in brackets refer to compounds present in sample

Ten of the *W. mossambicensis* leaf samples from Mahale and one sample of leaves of an unidentified *Wedelia* sp. (#1) from Gombe were collected from plants from which chimpanzees were observed to swallow leaves, immediately after observations of leaf-swallowing were made. The complete absence of thiarubrines and antifungal activity in these samples underscores the finding that chimpanzees do not acquire significant levels of thiarubrines when they ingest *Wedelia* leaves.

Two samples of *W. mossambicensis* stem and one sample of *W. pluriseta* stem included in the analysis were found to be devoid of thiarubrines or antifungal activity.

Several of the field-extracted root samples contained low but detectable levels of thiarubrines and their thiophenes. Two samples of *W. mossambicensis* roots from Mahale contained 0.6 and 1.4 µg/g FW of thiarubrine A (**1**), respectively as well as small amounts of thiophenes A (**13**) and B (**14**). Antifungal activity was found only in the sample containing the higher concentration of thiarubrine A (**1**). Moderate levels of thiarubrines A (**1**) and B (**2**) (total 9.1 µg/g FW), and thiophenes A (**13**) and B (**14**) (total 2.7 µg/g FW) were found in *W. mossambicensis* roots from Gombe (Figure 4.2c) which also showed activity in the bioassay (MIC 0.6 mg FW/ml). Neither thiarubrines nor thiophenes were found in a single root sample of *Wedelia* sp. (#1) from Gombe.

Of the dry root samples analyzed, *W. mossambicensis* roots from Uganda did not contain thiarubrines or thiophenes. Thiophenes A (**13**) and B (**14**) (total 12.0 µg/g DW) were present in a Ugandan sample of *W. africana*, a species which has not previously been shown to contain thiarubrines or thiophenes (NAPRALERT, 1996). The roots of both *Wedelia* species from Uganda lacked antifungal activity.

Root samples from greenhouse-grown *W. mossambicensis* plants contained moderately high concentrations of thiarubrines A (**1**), B (**2**), D (**3**) and E (**4**) as well as their corresponding thiophenes, and showed antifungal activity. Thiarubrine D (**3**) and thiarubrine E (**4**) and their corresponding thiophenes (**7** and **8**) have not previously been reported from *Wedelia* species. Total mean thiarubrine and thiophene levels were 164.4 µg/g DW and 10.0 µg/g DW, respectively. These quantities are substantially lower than the 2.03 mg/g DW of thiarubrines A (**1**) and B (**2**) reported in *W. mossambicensis* roots by Lopez *et al.* (1989) but similar to the 230 µg/g DW found by Norton *et al.* (1993).

It is not clear why Rodriguez *et al.* (1985) found high levels of thiarubrine A (**1**) in *Wedelia* leaves when all subsequent analyses of this plant have been unable to detect even small amounts of this chemical in samples collected from Mahale or other sites. One possible explanation is that the *W. mossambicensis* "leaf" sample originally obtained from Mahale, or the *W. pluriseta* sample from Kenya with which it was combined, were contaminated with root material. The trace amounts of thiarubrines present in *Wedelia* roots may have been enriched through the chromatographic purification process to the point where sufficient amounts could be isolated for spectroscopic characterization.

Sulfur-containing polyynes have been previously reported to be present in several *Ambrosia* species (Ellis *et al.*, 1995) and in *C. douglasii* and *E. lanatum* (Norton *et al.*, 1985). The pattern of thiarubrine distribution observed in *Wedelia* samples (i.e. thiarubrines present in the roots but absent from leaves and stems) was found for two of the North American taxa sampled. The leaves and stems of both *C. douglasii* and *E. lanatum* lacked thiarubrines or thiophenes, and above-ground plant material of both species was inactive in the bioassay. Cosio *et al.* (1986) were also unable to detect thiarubrines or thiophenes in the leaves and

stems of *C. douglasii*. However, high levels of thiarubrines and potent antifungal activity were found in *C. douglasii* roots with total amounts of thiarubrines A (**1**) and B (**2**) of 1396.4 μg/g DW and thiophenes A (**13**) and B (**14**) of 104.6 μg/g DW. *E. lanatum* roots contained five-fold lower levels of **1** and **2** (total 214.1 μg/g DW) and **5** and **6** (total 6.8 μg/g DW) compared with *C. douglasii*.

Analysis of *Ambrosia chamissonis* found high concentrations of thiarubrines and thiophenes in leaves, stems and roots (Table 4.3). It is worth noting that Ellis (1993) found the opposite distribution of thiarubrines and thiophenes, with higher levels in roots compared to leaves, from the distribution shown by this study. *Ambrosia chamissonis* extracts showed high activity in the antifungal assay with mean MIC values of 74.9, 4.9 and 14.6 μg DW/ml for leaves, stems and roots, respectively.

The findings of this study are somewhat anomalous in that total thiarubrine content and antifungal activity are not well correlated. For instance, although the roots and leaves of *A. chamissonis* were found to contain 491.7 and 1442.9 μg/g FW total thiarubrines, respectively, the roots were five-fold more active. An explanation for this pattern is discussed in Chapter 5.

4.3.5. Conclusions

These results demonstrate that thiarubrine A (**1**) is not present in detectable concentrations in the leaves of *Wedelia* species swallowed by chimpanzees. This finding does not support the hypothesis that the basis for leaf-swallowing is the ingestion of pharmacologically-active (i.e. nematocidal (Rodriguez *et al.*, 1985)) plant chemicals. Recently, Messner and Wrangham (1996) reached a similar conclusion when they reported that *in vitro* tests on extracts of *Rubia*

cordifolia L. (Rubiaceae) leaves, which are swallowed by chimpanzees at Kibale National Park, Uganda, showed a lack of antiparasitic activity against *Strongyloides* nematode larvae. Moreover, no sign of nematocidal activity has been noted in detailed observations of nematode (*Oesophagostomum stephanostomum*) expulsion directly associated with leaf-swallowing at Mahale using *W. mossambicensis* or any other species (Huffman *et al.*, 1996). Such adult nematodes were always motile at the time of expulsion, and even when left *in situ* with leaf and dung, remained alive and motile for up to four days thereafter. It is worth noting that chemicals in swallowed leaves may function to repel or reduce the motility of parasites, rather than directly acting as nematocides.

The results of this investigation showing the absence of thiarubrines in *Wedelia* spp. leaves indicate that factors other than this class of chemicals are important determinants of leaf-swallowing behaviour. Moreover, the utility of biologically-active plant chemicals as an explanation for leaf-swallowing by African apes has been called into question by the growing list of plant species from diverse taxa (24 species representing 16 families; Huffman, personal communication) that are swallowed whole in the manner of *Wedelia* leaves. It seems unlikely, given the fact that most secondary metabolites have restricted taxonomic distribution, that a single chemical (or chemicals), or type of biological activity, is present in all swallowed plant species. Remarkably, a characteristic shared by all leaves swallowed by chimpanzees and other apes is not chemical but physical; the presence of trichomes and a rough leaf surface (Huffman *et al.*, 1993; Huffman and Wrangham, 1994). As an alternative explanation for leaf-swallowing, Huffman *et al.* (1996) have hypothesized that such physical characteristics may function to trap and expel parasites.

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CHAPTER 5

THIARUBRINE M: A NOVEL THIARUBRINE OF MIXED BIOSYNTHETIC ORIGIN FROM *AMBROSIA CHAMISSONIS* ROOTS

5.1. INTRODUCTION

Previous investigations of the sulfur-containing polyynes of *Ambrosia chamissonis* have resulted in the isolation of eight thiarubrines (compounds **1–8**) (Rodriguez, 1988; Balza *et al.*, 1989; Lopez *et al.*, 1989; Balza and Towers, 1990; Ellis, 1993). Of these compounds, thiarubrine E (**4**) is the most polar, eluting first on a reversed-phase chromatographic column, while thiarubrine A (**1**) is the least polar. HPLC analysis of a crude methanolic extract of *A. chamissonis* roots revealed the presence of a peak that displayed the characteristic UV-vis spectrum of a 1,2-dithiin polyyne yet had a retention far exceeding that of thiarubrine A (**1**) (Figure 5.1). Here I report the isolation, structural elucidation and biological assay of this novel 1,2-dithiin polyyne (**27**), which has been assigned the trivial name thiarubrine M.

5.2. MATERIALS AND METHODS

General experimental procedures

The HPLC-PDA system consisted of a Waters 600 pump controller, Waters 996 photodiode array detector and Waters 717 plus autosampler controlled by Millenium 2.1 software. A 3.9 mm X 150 mm (4 μm) C_{18} column (Waters NovaPak) equipped with NovaPak precolumn was used for analytical-scale separations. A Waters 600 pump controller with a Waters U6K manual injector (3 ml sample loop) and Waters 994 photodiode array detector were used for preparative HPLC. UV-vis spectra were recorded on a Pye-Unicam 8720 scanning
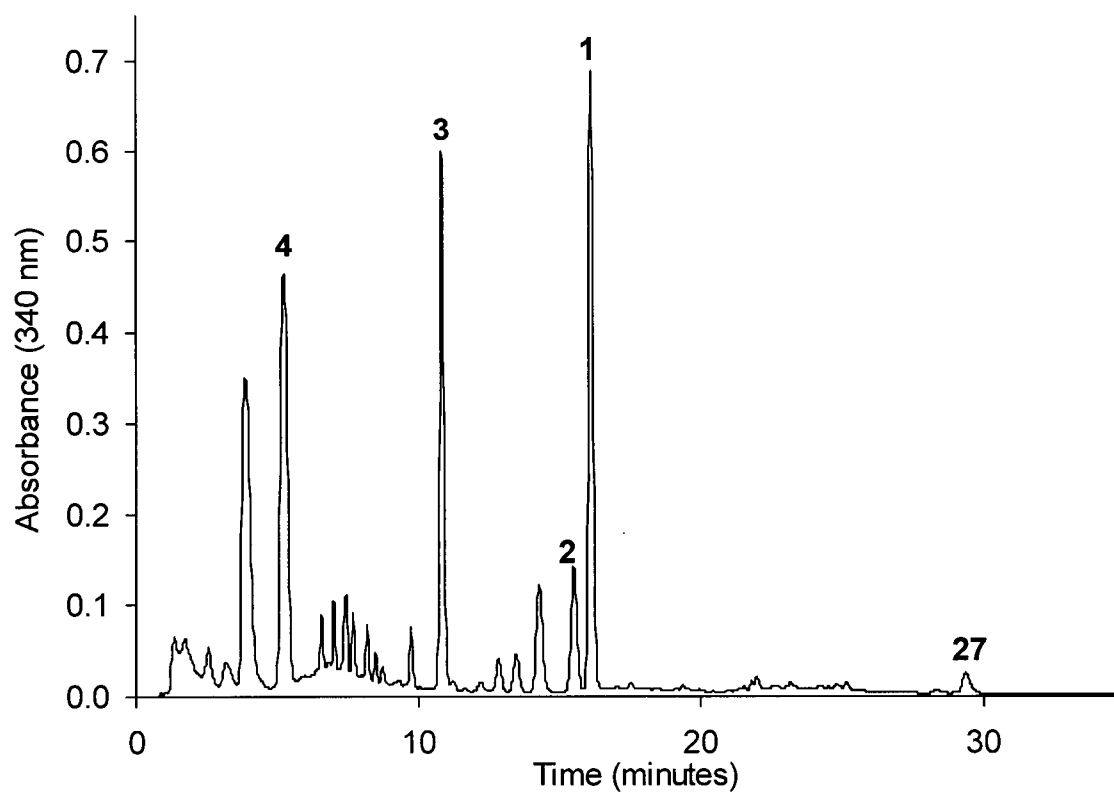
Figure 5.1. Chromatogram obtained from HPLC analysis of *Ambrosia chamissonis* root. Thiarubrine M (**27**) is visible as a peak at 29.5 minutes.

spectrophotometer in MeCN. ^{1}H-, ^{13}C- and HMQC/HMBC NMR spectra were recorded in acetone-d_6 at 500 MHz (proton) and 125.7 MHz (carbon) on a Bruker AMX-500 spectrometer, the ^{1}H–^{1}H-COSY spectrum was obtained in CDCl$_3$ at 400 MHz on a Bruker WH-400 spectrometer and the Attached Proton Test (APT) ^{13}C-NMR spectrum was determined at 75.4 MHz in CDCl$_3$ on a Varian XL-300 spectrometer. The residual acetone (δ 2.08 ppm) or CHCl$_3$ (δ 7.24 ppm) signals were used as references. Low- and high-resolution electron-impact mass spectra were obtained on a Kratos MS50 instrument. The infrared spectrum was measured using a Bomem-Michaelson-100 FT/IR and the specific rotation was determined on a Perkin Elmer 241MC polarimeter. Solvents were HPLC-grade (Fisher) except for petroleum ether (reagent-grade, Fisher) used for thiarubrine isolation. Water was Milli-Q plus (Millipore). All procedures were performed in dim light to minimize photoconversion of thiarubrines.

Isolation of thiarubrines

Ambrosia chamissonis roots were collected near Centennial Beach, Tsawwassen, Canada. Fresh roots (3.35 kg) were homogenized with 6 l MeOH in a Waring blender and filtered through Whatman #1 filter paper. The resulting methanolic extract, after concentration to *ca.* 500 ml *in vacuo*, was extracted with 3 X 300 ml hexanes. Evaporation of the hexane layer *in vacuo* gave a dark-red residue that was analyzed by HPLC using the step-gradient methodology described in Chapter 4 "Materials and Methods". Detection of the unknown peak (R$_t$ = 29.5 minutes) in this and subsequent fractions guided the isolation of compound **27**. After pre-adsorption on silica gel, the hexane residue was separated by VLC. The sample was applied to the top of a 350 ml sintered glass funnel (C) packed with Keiselgel G to a

height of 4.5 cm and eluted with petroleum ether to give a red lipophilic fraction. After

evaporation to dryness, the residue was resuspended in MeCN and subjected to reversed-

phase CC over "home-made" reversed-phase silica gel. Elution with MeCN–water (50:50)

afforded three distinct red bands, of which the latest eluting was shown to be mainly

composed of compound 27 and traces of other minor thiarubrines and thiophenes. Separation

of this mixture was achieved by extensive reversed- and normal-phase preparative HPLC.

Reversed-phase preparative HPLC was performed on a 2.5 X 10 cm (5 μm) C_{18} radial

compression column (Waters NovaPak) equipped with NovaPak precolumn. The column was

eluted with MeCN at a flow rate of 27 ml/minute with detection at 340 nm. Injection volume

was 100–500 μl. A 19 X 300 mm (10 μm) silica column (Waters μPorasil) equipped with a

silica (Waters Resolve) precolumn was used for normal-phase preparative HPLC. The mobile

phase was hexane at a flow rate of 10 ml/minute, detection was at 340 nm and injection

volume was 500 μl. After the final chromatographic separation, the purified fraction was

dried *in vacuo* to yield thiarubrine M (27) as a red oil. Crystallization of 27 in MeCN gave

small ruby needles.

Thiarubrine M (27): $[\alpha]^{23}_D$ -490° (*c* 0.72, CHCl$_3$); UV (MeCN) λ_{max} 228.3, 345.2,

482.4 nm; IR (CHCl$_3$) ν_{max} 3013-2830 (C–H, aliphatic), 2255 (C≡C), 2218 (C≡C),

1603, 1448, 1373, 1242, 1033, 918, 818 cm^{-1}; HR EI-MS *m/z* 432.1924 (calcd for

$C_{28}H_{32}S_2$: 432.1946); LR EI-MS *m/z* [M]$^+$ 432 (13), 400 (36), 196 (100), 69 (96); ^{1}H-

NMR and ^{13}C-NMR see Table 5.1. (See Appendix 6).

The isolation of thiarubrines A (**1**), B (**2**) and D (**3**), and of thiarubrine E (**4**) is described in Chapter 2 and Chapter 4, respectively.

Antifungal bioassay

Antifungal activity was determined using the broth dilution method modified from Towers *et al.* (1985). For determination of MIC values, thiarubrines A (**1**), B (**2**), D (**3**) and E (**4**) were taken up in MeOH and the concentrations of the resulting standard solutions adjusted to 0.5 mM. Due to its limited solubility in MeOH and low activity in preliminary experiments, thiarubrine M (**27**) was solubilized at a concentration of 50 mM in DMSO. MeOH and DMSO were used as solvent controls; a 5 mg/ml of nystatin in DMSO was used as a positive control. A 48-hour culture of *C. albicans* (UBC #54) grown at 37 °C in Sabouraud dextrose broth (SDB) was diluted to ~1.0 X 10^4 cells/ml. Test solutions of thiarubrines were diluted 100-fold and a 200-µl aliquot transferred to a well in row 2 of a 96-well microtitre plate (8 lanes X 12 rows) (Costar). Solutions were diluted in a series of nine two-fold dilutions while row 12 served as an untreated control and received 100 µl SDB. A 100 µl aliquot of diluted *C. albicans* culture was transferred to each well of rows 2 to 12. Row 1 received growth medium only (200 µl SDB). Plates were incubated in the dark at 37 °C for 24 hours and the optical density of each well read at 600 nm using an ELISA reader (Titertek Multiskan) zeroed on culture medium (row 1).

Thiarubrines were tested in three experiments with three replicate lanes in each experiment. Minimum inhibitory concentration (MIC) values were determined as the concentration which resulted in a well having an optical density <15% of the untreated control. This determination was confirmed by visual inspection of plates.

Regression analysis of antifungal activity versus thiarubrine content

Data on the antifungal activity (MIC value) and thiarubrine content of *Aspilia mossambicensis* roots, *Chaenactis douglasii* roots, *Eriophyllum lanatum* roots and *Ambrosia chamissonis* roots, stems and leaves were obtained from the analyses presented in Chapter 4 "Results and Discussion". Linear regression analysis was performed using the least squares method with Microsoft Excel software.

5.3. RESULTS AND DISCUSSION

5.3.1. Elucidation of the Structure of Thiarubrine M

The MeOH extract of *A. chamissonis* roots was partitioned with hexane to give an organic fraction containing nonpolar thiarubrines. Chromatographic separations using VLC on silica gel and CC on reversed-phase silica gel resulted in the partial purification of compound **27**. Final purification by extensive reversed- and normal-phase preparative HPLC afforded compound **27** as a red oil.

The high-resolution EI mass spectrum of compound **27** exhibited a molecular ion at *m/z* 432.1924 corresponding to the molecular formula $C_{28}H_{32}S_2$. Although not particularly diagnostic, infrared absorptions at 2255 and 2218 cm^{-1} indicated acetylenic groups, and an unsymmetrical conjugated diene was suggested by the absorption at 1603 cm^{-1}. Absorption bands at 482.4 and 345.2 nm in the UV-vis spectrum indicated that the molecule contained a 1,2-dithiin chromophore. This conclusion was supported by a fragment at *m/z* 400 [M - 32]$^{+}$ (intensity 36) in the low-resolution EI mass spectrum indicating a loss of sulfur and the presumed formation of a thiophene.

Analysis of the low resolution mass spectrum showed a fragment at *m/z* 196 [M -

$236]^+$ (intensity 100) that was found to have a molecular formula of $C_{13}H_8S$ by high resolution mass spectrometry. This molecular formula is identical to that of both thiophene A (**13**) and thiophene B (**14**), suggesting that the 1,2-dithiin group in compound **27** was part of a thiarubrine A (**1**) or thiarubrine B (**2**) subunit. This supposition was borne out by comparison of the ^{1}H- and ^{13}C-NMR spectra of **27** with those of thiarubrine A (**1**). Proton resonances at δ 2.06, 6.46 and 6.59 ppm corresponded to the terminal methyl, and ring proton signals of thiarubrine A (**1**), respectively, although the latter resonance showed some upfield shifting. As well, acetylenic carbon resonances (C-2, C-3, C-8, C-9) exhibited chemical shifts that were in good agreement with the corresponding signals in thiarubrine A (**1**). The presence of a thiarubrine A-like subunit was verified by analysis of the HMBC spectrum (Figure 5.2; Table 5.1) which showed correlations between the terminal methyl protons and acetylenic C-3, and between ring proton H-5 and this carbon. Remarkably, long-range coupling was observed between the terminal methyl and ring proton H-5. The resonances of acetylenic carbons C-10 and C-11 of compound **27** were shifted somewhat from the equivalent carbons of thiarubrine A (**1**) but it seemed a reasonable assumption that they were the second pair of such carbons in a diyne chain. Both were shown to have connectivity to the H-12 proton resonance at δ 2.69 ppm. In this manner C-1 to C-12 of **27** were assigned.

The remaining part of the molecule of **27** contained 16 carbons which the APT spectrum showed consisted of three methyls (δ 16.0, 17.7, 25.7 ppm); seven methylenes (δ 26.2, 26.7, 27.0, 28.3, 30.9, 37.6, 39.7 ppm); three methines (δ 118.3, 124.0, 124.4 ppm) and three quaternary carbons (δ 131.3, 135.2, 137.0 ppm). The protons attached to each carbon were characterized on the basis of the HMQC spectrum. Calculation of the degree of unsaturation of compound **27** (total = 13) indicated that the 16 carbon portion, in addition
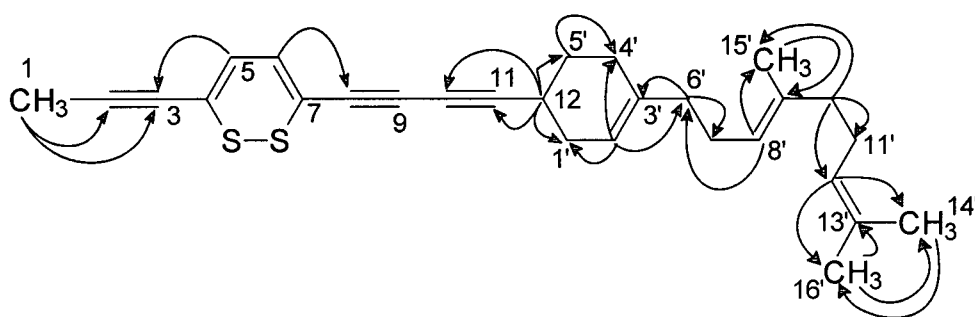
Figure 5.2. Selected HMBC correlations for thiarubrine M (**27**).

Table 5.1. ^{1}H- and ^{13}C-NMR assignments including HMBC (H–C) correlations of thiarubrine M (**27**).

Position	$\delta_H{}^a$ (mult, J)^b	$\delta_C{}^a$	HMBC (H to C)
Me-1	2.06 (s)	5.0	C-2, C-3
2	-	97.4	
3	-	77.1	
4	-	117.6	
5	6.46 (dd, 0.4, 6.7)	132.4	C-3, C-7
6	6.59 (d, 6.8)	135.5	C-4, C-8
7	-	112.7	
8	-	71.5	
9	-	82.6	
10	-	64.7	
11	-	93.9	
12	2.69 (m)	26.6	C-10, C-11, C-1', C-5'
1'a	2.29 (d, 17.6)	30.9	
1'b	2.12 (d, 7.4)		
2'	5.32 (br s)	118.3	C-1', C-4', C-6'
3'	-	137.0	
4'a	1.90–2.05 (m)	~26	
4'b	1.90–2.05 (m)		
5'a	1.90 (m)	28.3	C-1', C-4'
5'b	1.71 (m)		
6'	1.96 (t, 7.1)	37.6	C-2', C-3', C-7'
7'	~2.02 (m)	~26	
8'	5.07 (t, 6.4)	124.0	C-6', C-7', Me-15'
9'	-	135.2	
10'	1.96 (t, 7.1)	39.7	C-9', C-11', C-12', Me-15'
11'	~2.02 (m)	~26	
12'	5.07 (t, 6.4)	124.4	C-10', C-11', Me-14', Me-16'
13'	-	131.3	
Me-14'	1.66 (s)	25.7	C-12', C-13', Me-16'
Me-15'	1.57 (s)	16.0	C-8', C-9', C-10',
Me-16'	1.56 (s)	17.7	C-12', C-13', Me-14'

^{a} δ in ppm
^{b} s, d, t, m, br s = singlet, doublet, triplet, multiplet, broad singlet, respectively; J in Hz

to the presence of three double bonds, also contained a cyclic structural feature.

Using the H-12 proton multiplet and the protons of the three methyl groups as starting points, HMBC correlations allowed the deduction of several partial structures. The H-12 signal exhibited correlations with two methylene carbons at δ 28.3 ppm (C-5') and δ 30.9 ppm (C-1'). The latter carbon were adjacent to the olefinic methine at δ 118.3 ppm (C-2'), as determined by analysis of the ^{1}H–^{1}H-COSY spectrum showing correlations between H-1'a,b and H-2'. The H-2' resonance showed connectivity to the methylene carbon at δ 37.6 ppm (C-6'), a signal which was found to be bonded both to the quaternary C-3' (δ 137.0 ppm). The cyclohexene ring was established by correlations of the H-5' (H-5'a and H-5'b) and H-2' protons with carbon C-4' (δ ~26 ppm).

A second partial structure was deduced using correlations of the methyl signal at δ 1.57 ppm (Me-15'). This resonance was correlated to carbon signals at δ 135.2 (C-9', quaternary), 124.0 (C-8', methine) and 39.7 ppm (C-10', methylene). Linkage of this methyl-bearing fragment to the C-12–C-7' partial structure was established by the ^{1}H–^{1}H COSY (Table 5.2) and HMBC spectra. Since the HMQC spectrum indicated that the single proton attached to the C-8' methine was one proton of an overlapping two proton triplet at δ 5.07 ppm, it was deduced to be adjacent to a –CH$_2$– group. This conclusion was supported by ^{1}H–^{1}H COSY correlations between H-8' and a methylene signal at ~2.02 ppm. Because of the poor resolution in this region of the proton spectrum, and the substantial overlap in the ~26 ppm region of the carbon spectrum, it proved impossible to accurately assign this and several of the methylene proton and carbon signals. However, since the HMBC spectrum showed the H-8' proton to be correlated with C-6' <u>and</u> a methylene carbon at δ ~26 (i.e. C-7'), the connectivity C-6' through to C-8' was apparent. A third partial structure was suggested by

Table 5.2. ^{1}H–^{1}H COSY correlations for thiarubrine M (**27**).

Position	Position
Me-1	H-5
H-5	H-6
H-12	H-1', H-5'
H-1'a,b	H-2'
H-7'	H-8'
H-11'	H-12'
Me-14'	H-12'
Me-15'	H-8'
Me-16'	H-12'

the methyl signals at δ 17.7 (Me-16') and 25.7 (Me-14') ppm: the HMBC spectrum showed that both methyls were substituents of the quaternary carbon at δ 131.3 ppm (C-13') and both were correlated with C-12' (δ 124.4 ppm, methine). Thus, this dimethyl group appeared to be one of the termini of compound **27**. The proton of C-12' was found to be part of the overlapping triplet at δ 5.07 ppm. In a similar fashion to H-8', the H-12' resonance showed cross peaks in the HMBC spectrum with the C-10' methylene and one of the poorly resolved methylene carbons at ~26 ppm (i.e. C-11'). Thus, the connectivity from Me-15' to the terminal dimethyl group was established, completing the structure of compound **27**.

While spectroscopic determination of the stereochemistry at the C-8' double bond was not possible with the NMR experiments described above, on the basis of biosynthetic considerations it seems likely that this bond exists in a *E* configuration.

Recrystallization of compound **27** from MeCN gave a single crystal which was suitable for limited X-ray diffraction studies. The results of the crystallographic analysis

spectroscopy. Thermal motion in the C_{15} side-chain prevented determination of the stereochemistry at C-12 and C-8'. Thus, compound **27** was shown to be either 3-(4-(4-((3*Z*)-4,8-dimethyl-3,7-nonadienyl)-3-cyclohexenyl)-1,3-butadiynyl)-6-(1-propynyl)-1,2-dithiin or 3-(4-(4-((3*E*)-4,8-dimethyl-3,7-nonadienyl)-3-cyclohexenyl)-1,3-butadiynyl)-6-(1-propynyl)-1,2-dithiin. The two possible structures of this molecule, (*E*)- and (*Z*)-**27**, which has been assigned the trivial name thiarubrine M[1], are shown in Figure 5.4. The complete [1]H- and [13]C-

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[1] The use of letters to designate thiarubrine derivatives has led to some confusion in the literature. Ellis *et al.* (1995) assigned letters to 11 thiarubrines and a sulfoxide of thiarubrine A (**1**), resulting in the designation of thiarubrines A to L. Since the sulfoxides lack the chemical and biological properties of the thiarubrines, and may in fact be artifacts of isolation, the designation of a sulfoxide as "thiarubrine J" seems inappropriate. Nevertheless, the name thiarubrine L has priority in the literature and I have used the next available letter, M, for the trivial name of compound **27**.
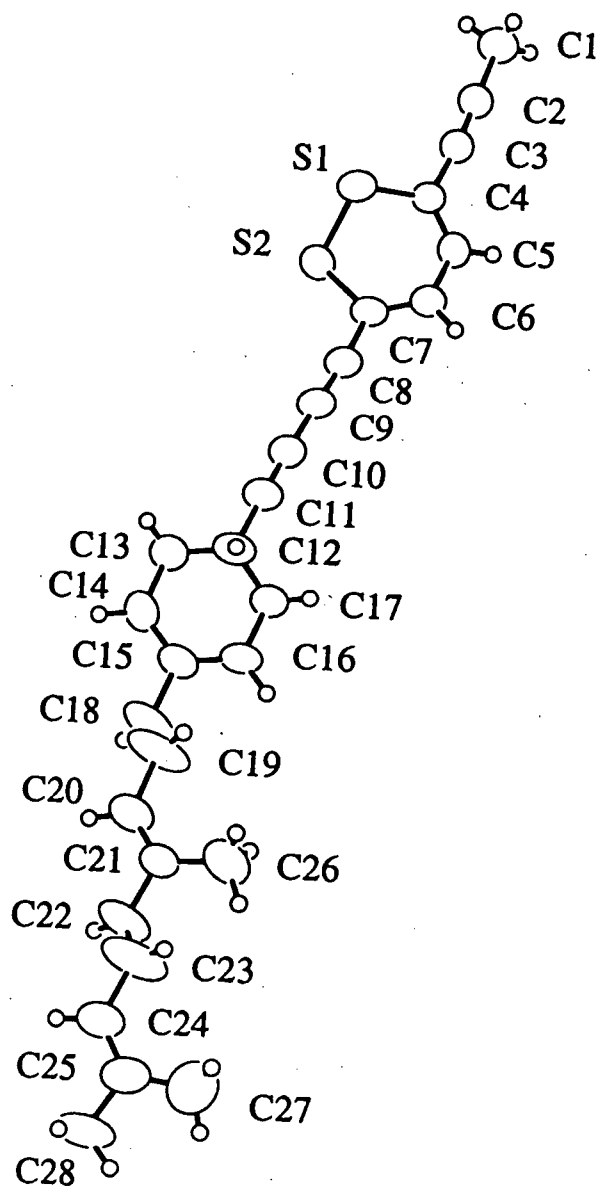
Figure 5.3. Molecular projection of the gross structure of thiarubrine M (**27**) based on x-ray diffraction analysis.

(E)-thiarubrine M (27)
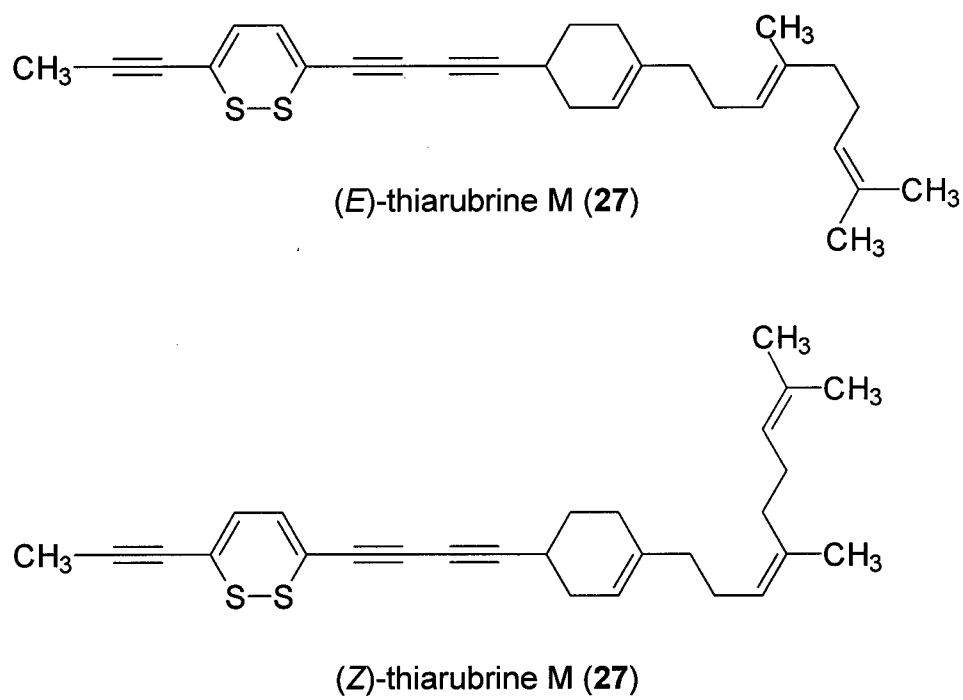
(Z)-thiarubrine M (27)

Figure 5.4. Possible structures of thiarubrine M (27).

NMR assignments for compound **27** are presented in Table 5.1. Thiarubrine M (**27**) is a novel molecule that is unique in two respects: it is the first thiarubrine isolated that is larger than 13 carbons, and it is of mixed biosynthetic origin. Based on the principles of biosynthesis, thiarubrine M (**27**) is formed by the condensation of a C_{15} farnesyl group of isoprenoid origin with a fatty-acid derived thiarubrine A (**1**) molecule. Biogenetically this appears to be an unusual situation and there are no other reports in the literature of plant secondary metabolites composed of both polyyne- and isoprenoid- derived subunits.

5.3.2. Antifungal Activity

The antifungal activity of thiarubrine M (**27**) and of thiarubrines A (**1**), B (**2**), D (**3**) and E (**4**) toward the pathogenic yeast *Candida albicans* was tested using a broth dilution method. The results of the bioassay are presented in Table 5.3. Thiarubrines **1–4** were shown to have potent candidicidal activity, a finding which confirms earlier studies of these compounds (Towers *et al.*, 1985; Ellis, 1993). It is worth noting that this study found somewhat lower MIC values for the thiarubrines than those reported by Ellis (1993) and Bierer *et al.* (1995). For example Ellis (1993) and Bierer *et al.* (1995) reported MIC's of 1.6 μM and 0.7 μM, respectively, for thiarubrine A (**1**) compared to 0.5 μM determined in this study.

Surprisingly, thiarubrine M (**27**) did not display antifungal activity even when assayed at a concentration two orders-of-magnitude higher (250 μM) than that at which thiarubrine A (**1**) exerts toxicity. Although this lack of activity may be due to the limited solubility of thiarubrine M (**27**) in the aqueous culture medium, the use of DMSO as carrier solvent and the apparently uniform red colour of the test wells, suggests that thiarubrine M (**27**) was well-dispersed in the test medium and able to act on *Candida* cells.

A more likely explanation for the inactivity of thiarubrine M (27) is that the isoprenoid side-chain impairs the ability of this compound to reach or interact with the cellular target of thiarubrines and other 1,2-dithiins. Two possible ways in which the C_{15} side-chain may lead to this diminished activity are by affecting either the lipophilicity or the steric properties of thiarubrine M (27). Based on the trend apparent from Table 5.3, lipophilicity may be a primary determinant of the biological activity of thiarubrines. Thiarubrines A (1) and B (2), which have similar retention times by HPLC, and hence similar lipophilicities, show reasonably close MIC values (Table 5.4). The slightly more polar thiarubrine D (3), which contains an epoxide-bearing side-chain, has four-fold higher antifungal activity and thiarubrine E (4), the most polar of the compounds tested here, also exhibits the lowest MIC value. Thiarubrine M (27) is much more lipophilic than thiarubrines A (1) and B (2), a property that may not allow it to behave in the same manner as the other thiarubrines with regards to its ability to cross cell membranes. In their structure-activity study of synthetic 1,2-dithiins, Bierer *et al.* (1995) found that the size of substituents on a series of diester derivatives greatly affected their candidicidal activity: the presence of bulky groups led to pronounced decreases in antifungal activity. Since the cellular target of thiarubrines is unknown at this time, it is perhaps specious to suggest that the bulky farnesyl side-chain of thiarubrine M (27) prevents its interaction with such a site. Nevertheless, steric hindrance of the putative pharmacophore, the 1,2-dithiin moiety, by the C_{15} side-chain is a possible reason for the lack of toxicity of thiarubrine M (27) towards *Candida albicans*.

In Chapter 4, the analysis of thiarubrines in plant samples by HPLC and biological assay led to an unresolved question: why were the levels of thiarubrines determined by HPLC so poorly correlated with the antifungal activity of plant samples? The relationship

Table 5.4. Antifungal activity of thiarubrines isolated from *Ambrosia chamissonis* roots.

Compound	MIC (μM)[a]
Thiarubrine A (**1**)	0.52 ± 0.18
Thiarubrine B (**2**)	0.63 ± 0.00
Thiarubrine D (**3**)	0.12 ± 0.07
Thiarubrine E (**4**)	0.02 ± 0.02
Thiarubrine M (**27**)	inactive (> 250)
MeOH	inactive
DMSO	inactive
Nystatin	0.42 pM

[a] minimum inhibitory concentration (mean $\pm$ standard deviation)

between total thiarubrine content (i.e. the sum of compounds 1–4) of plant extracts and antifungal activity (MIC) of the extract is shown in Figure 5.5a. The regression coefficient (R^2 = 0.416, P < 0.023) determined by linear regression analysis indicates that these two factors are not well correlated. The measurement of the MIC values for thiarubrines A (1), B (2), D (3) and E (4), which are the major thiarubrines present in *A. chamissonis*, allowed for the chromatographically-determined thiarubrine content to be adjusted to account for the antifungal potency of the quantified compounds. Basing the potency scale on thiarubrine A (1), which has a MIC of 0.52 µM, thiarubrine B (2) (MIC = 0.63 µM) is 0.83 times less active, thiarubrine D (3) (MIC = 0.12 µM) is 4.33 times more active, and thiarubrine E (4) (MIC = 0.02) is 26.0 times more active. Thus, multiplication of the concentration of each thiarubrine in the plant samples by its "potency factor" allowed for the calculation of adjusted total thiarubrine content. Figure 5.5b shows the relationship between adjusted total thiarubrine content and antifungal activity (MIC). The regression was highly significant (R^2 = 0.783, P < 0.0001) indicating that, as well as the total amount of thiarubrines present, the potency of the individual components is an important determinant of the antifungal activity of thiarubrine-containing plant samples.
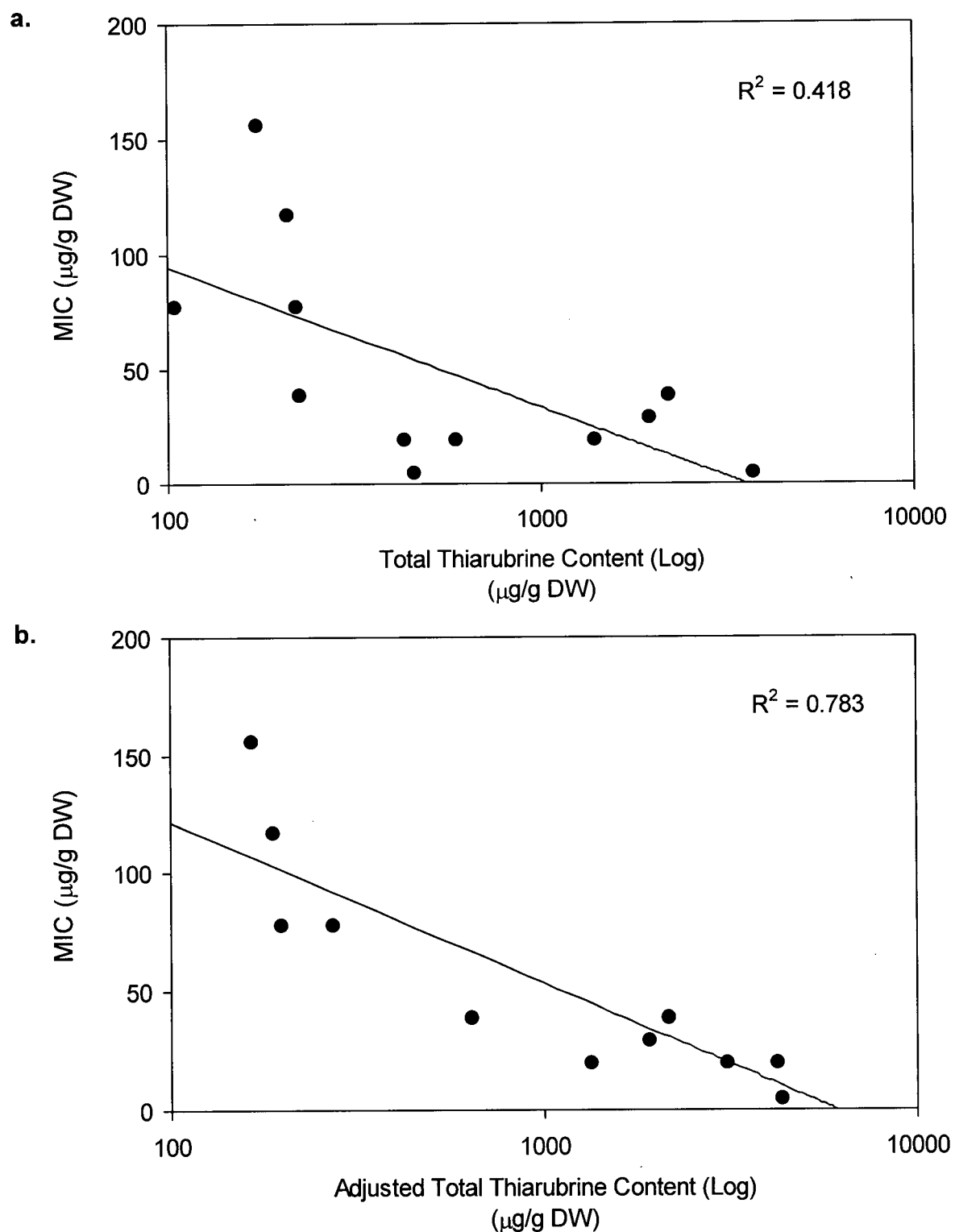
Figure 5.5. Regression analysis of antifungal activity versus thiarubrine content of asteraceous plant samples. (**a.**) Antifungal activity versus total thiarubrine content. (**b.**) Antifungal activity versus thiarubrine content adjusted to take into account the antifungal potency of the thiarubrines present.

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CHAPTER 6

GENERAL DISCUSSION

6.1. RESEARCH HIGHLIGHTS

The work described in the previous four chapters examined several different aspects of the photochemistry, photobiology, chemical ecology and toxicity of thiarubrine secondary metabolites isolated from members of the Asteraceae. These studies have contributed significant new information about the thiarubrines, and have been highlighted by four key findings:

1. the discovery of photosulfide intermediates;

2. the elucidation of the role of anthocyanins in thiarubrine photoprotection;

3. the conclusive refutation of the long-standing hypothesis that thiarubrines are the pharmacological basis for *Wedelia* leaf-swallowing by chimpanzees;

4. the discovery of a novel thiarubrine in *Ambrosia chamissonis* roots.

The first study, presented in Chapter 1, addressed the broad objective of determining how visible light affects the chemistry and toxicity of the thiarubrines. Experiments with thiarubrines A (**1**), B (**2**) and D (**3**) isolated from *Ambrosia chamissonis* roots showed that exposure of thiarubrines to visible radiation results in their conversion to transient photointermediates that further decompose to thiophenes. Application of HPLC coupled with UV-vis spectroscopy and mass spectrometry, as well as low-temperature NMR spectroscopy allowed for the characterization of these "photosulfide" photointermediates as 2,6-dithiabicyclo[3.1.0]hexene polyynes. The structures of six photosulfides (**1a, 1b, 2a, 2b, 3a,**

3b), all of them novel compounds, were elucidated. By virtue of their bicyclic ring structure, the photosulfides represent an entirely new class of organic compound, a finding that underscores one of the basic rationales for studying plant biochemistry—that plants contain unusual, interesting and potentially useful molecules.

The thiarubrines exhibit potent light-independent (dark) toxicity that has been suggested to be due to the reactivity of the 1,2-dithiin moiety present in these molecules (Bierer *et al.*, 1995). Whether as an accidental by-product of the instability of the 1,2-dithiin ring or through evolutionary design (see below), the thiarubrines are also phototoxic. As part of my investigation of the photoconversion of thiarubrines, the visible-light generated photosulfides and other intermediates of the thiarubrine–thiophene pathway (i.e. the short-lived dithiones, "activated" sulfur) were shown to possess significant *in vitro* phototoxicity to the pathogenic yeast *Candida albicans*. The majority of phototoxic plant secondary metabolites exert their toxic effects through UV or visible light mediated oxidative damage (type I or II processes) or by the UV-induced formation of cyclobutane adducts with DNA bases (Arnason *et al.*, 1992). While the mechanism by which thiarubrine phototoxicity is manifested (and which reactive molecular species is responsible) still remains to be elucidated, it is clear from the results of this study that the photochemical mode of action is unlike that of any other plant phototoxin.

The use of the inherently reactive and unstable 1,2-dithiin ring for chemical defense has not occurred without its costs, however, and the formation of thiarubrines in above-ground plant tissues requires that a mechanism exist to protect them from conversion by sunlight. Chapter 3 examined the photoprotection of thiarubrines in *Ambrosia chamissonis*, a plant that contains thiarubrines in its leaves and stems. While both UV and visible light are

capable of converting thiarubrines to their corresponding thiophenes, the blue-light absorption band of thiarubrines is more problematic because penetration of light of blue wavelengths (i.e. 400–500 nm) into plant tissues is important for photosynthesis. The results of this study show that *A. chamissonis* has overcome the problem of storing thiarubrines in leaves and stems in a novel fashion: thiarubrine canals are surrounded by a sheath of cells containing visible-light absorbing anthocyanin pigments. *A. chamissonis* contains seven anthocyanins of which two were isolated and identified as cyanidin 3-*O*-(6-*O*-malonyl-β-D-glucopyranoside) (**25**) and cyanidin 3-*O*-(β-D-glucopyranoside) (**26**). The visible absorption band of cyanidin-based anthocyanins shows considerable overlap with that of the thiarubrines, hence the red colour of both, and *in vitro* experiments with anthocyanin **25** demonstrated that anthocyanins are effective photoprotectant screens for thiarubrines. This is the first report of the photoprotection of light-sensitive phototoxins in plants, and represents an example of the use of one class of secondary metabolite, the phenolic-derived anthocyanins, to protect the products of a second independent branch of secondary metabolism, the fatty-acid derived thiarubrines.

In Chapter 4, the roots of four asteraceous taxa (*Wedelia mossambicensis, Eriophyllum lanatum, Chaenactis douglasii* and *Ambrosia chamissonis*) were found to contain thiarubrines. Of these plants, only *A. chamissonis* contained thiarubrines in its leaves and stems, an attribute that may be related to the mechanism of anthocyanin photoprotection described above. In addition to *A. chamissonis*, thiarubrines have been found in seven other *Ambrosia* species (Table 1.1). Most phytochemical investigations of *Ambrosia* species have focussed on roots as sources of these compounds and it is unclear from the literature if the

majority of species also contain thiarubrines in their aerial parts. Lu *et al.* (1993) reported an epoxide derivative of thiarubrine B, compound **9**, from the stems of *A. trifida*.

A third objective of this thesis was to determine what role, if any, thiarubrines play in the swallowing of whole *Wedelia* leaves by chimpanzees. The characteristic UV-vis spectra of the thiarubrines makes them ideal molecules for chromatographic analysis, and Chapter 4 presents a rigorous HPLC methodology that is capable of resolving the major asteraceous thiarubrines and thiophenes, and detecting thiarubrine levels as low as 6 μg/g DW in plant material. As well, the potent antifungal activity of these compounds makes them amenable to microbiological assay as a complementary detection method. Thiarubrines were not found in the leaves of any of the *Wedelia* species analyzed, including those sampled directly after leaf-swallowing behaviour was observed in the field.

In the twelve years since thiarubrine A (**1**) was first reported to be present in *Wedelia mossambicensis* and *W. pluriseta* leaves (Rodriguez *et al.*, 1985), this chemical has been touted in the both the scientific (Wrangham and Goodall, 1989; Rodriguez and Wrangham, 1993; Berry *et al.*, 1995) and popular (Cowen, 1990; Rodriguez, 1995) literature as evidence that primates engage in self-medication— the "antibiotic discovered by chimps" (Ghitelman, 1986). This view is not supported by the findings presented in this thesis. However, plant chemicals have important roles in determining patterns of primate plant use and other examples of the consumption of medicinal or antiparasitic plants (e.g., the chewing of the bitter pith of *Vernonia amygdalina* (Huffman *et al.*, 1994)) are corroborated by the presence of biologically-active chemicals in these plants. It is my hope that the elimination of thiarubrines as an explanation for *Wedelia* leaf-swallowing will spur rigorous, clear-headed research in this area.

The final subject of my thesis was the isolation and identification of a novel 1,2-dithiin polyyne, thiarubrine M (27). This compound had eluded detection and isolation during previous investigations of the chemistry of *Ambrosia chamissonis* roots (Balza *et al.*, 1989; Balza and Towers, 1990; Ellis *et al.*, 1993), perhaps because of its lipophilic properties and low concentration in crude extracts. Thiarubrine M (27) was purified by a combination of normal-phase and reversed-phase column chromatography and preparative HPLC, and the structure elucidated by the application of mass spectrometry, and UV-vis, infrared and NMR spectroscopy. This 28-carbon compound differs from the other eleven known thiarubrines, which are 13-carbon molecules, because of the presence of a C_{15} isoprenoid subunit that appears to have condensed with the vinyl group of the diyne-ene side-chain of thiarubrine A (1) giving rise to a cyclohexene ring.

A surprising property of thiarubrine M (27) is that it wholly lacks antifungal activity even when tested at concentrations several orders-of-magnitude higher than those at which thiarubrines A (1), B (2), D (3) and E (4) show toxicity.

6.2. EVOLUTION OF THIARUBRINES

The light-mediated and light-independent toxicity of the thiarubrines has important implications concerning the evolution of the biosynthetic pathway leading to these unusual sulfur derivatives. Recent discussions by phytochemists and chemical ecologists about the adaptive significance of the diversity of plant secondary metabolites have focussed attention on the evolutionary processes by which this diversity is initially generated and how it is maintained (see Romeo *et al.*, 1996). While my thesis did not explicitly attempt to investigate the evolution of secondary metabolites, trends in the biological activity of thiophene and 1,2-

dithiin polyynes may be relevant to discussions of the evolution of chemical diversity in plants as a whole.

Many members of the Asteraceae contain straight-chain polyynes, while thiophene polyynes have a more limited distribution and 1,2-dithiin polyynes (thiarubrines) occur infrequently. Since it has been determined from tracer studies that thiophenes and thiarubrines are formed from polyyne precursors (Gomez-Barrios *et al.*, 1992), the ability to synthesize sulfur-containing polyynes clearly represents a derived state. I would further speculate that while it is apparent from the sulfur-labeling experiments of Constabel and Towers (1989) that thiophenes are not the precursors of thiarubrines, the ability of a plant to make thiarubrines probably results from some heritable change in the enzymes or biosynthetic processes that synthesize thiophenes. This supposition is not new; Bohlmann *et al.* (1973) proposed that thiophenes may arise by the addition of sulfur (e.g. H_2S) across two adjacent triple bonds in a polyyne precursor while the double addition of sulfur and the subsequent bonding of the adjacent free sulfur atoms could yield 1,2-dithiins.

Based on a straight-chain polyyne–thiophene polyyne–1,2-dithiin polyyne sequence, I would argue that evolution and diversification of polyyne secondary metabolites in plants is driven by successive increases in biological activity (Table 6.1). In general, straight-chain polyynes show limited light-independent (dark) toxicity and exhibit UV-mediated phototoxicity through photooxidative and nonoxidative mechanisms (McLachlan *et al.*, 1984). It is worth noting that the designation of a compound as "inactive" or "nontoxic" is fraught with exceptions, and compounds such as the polyyne neurotoxin, cicutoxin, (Wittstock *et al.*, 1995) and pentaynene (Guillet *et al.*, in press) possess significant light-independent toxicity. Several studies have shown that thiophene derivatives of polyynes are

Table 6.1. Evolution of polynes and their derivatives.

Evolutionary Sequence	Example	Biological Activity		
		Dark	UV	Visible
Straight-chain polyyne		$-(+)^{a,b}$	$+$	$-$
Thiophene		$-$	$++$	$-$
Thiarubrine		$++$	$(++)^c$	$++$

[a] − indicates lack of biological activity under the light regime while + indicates presence of biological activity. The number of + characters denotes the relative potency of the biological activity.
[b] Straight-chain polyynes show limited dark toxicity.
[c] UV-mediated biological activity due to UV-induced conversion of thiarubrines to thiophenes.

more phototoxic against microorganisms than are straight-chain polyynes (MacLachlan *et al.*, 1986) and the addition of a thiophene heterocycle generally leads to increased phototoxicity (Hudson and Towers, 1991). Therefore, the increase in the biological activity, and hence the defensive properties of a polyyne, brought about by the addition of one atom of sulfur (forming a thiophene ring) may be a key step in the evolution of polyyne secondary metabolites. The addition of two atoms of sulfur, yielding a 1,2-dithiin ring, leads to further increases in biological activity. The result of this extra biosynthetic step (or steps) is the formation of a class of molecules, the thiarubrines, that show more potent toxicity than either straight-chain or thiophene polyynes <u>and</u> that also exhibit light-independent (dark) toxicity. In addition, the light-sensitivity of 1,2-dithiin polyynes allows the plant to make use of the toxicity generated by their UV and visible-light induced photoconversion and the UV-photosensitized phototoxicity of the resulting thiophene photoproducts. Furthermore, the "three-pronged attack" strategy discussed in Chapter 2 not only leads to increases in toxicity but also a diversification in the cellular targets of the active compounds. While polyynes and thiophenes act primarily on cell membranes, thiarubrines appear to have other target sites (e.g. enzymes). Guillet *et al.* (in press) have recently argued that the evolution of polyyne diversity in *Rudbeckia hirta*, which produces polyynes, thiophenes and thiarubrines, may be due to different modes of action of the various compound types present. Thus, increases in biological activity may be invoked as the evolutionary force driving the diversification of the polyyne pathway from simple, straight-chain polyynes to thiophene polyynes, and finally, to 1,2-dithiin polyynes (thiarubrines).

6.3. FUTURE RESEARCH DIRECTIONS

The results presented in this thesis raise many questions concerning the thiarubrines. What is the nature of the sulfur extruded by the photosulfides? What determines the ratio of photosulfide isomers? How does the conversion of thiarubrines to thiophenes result in increased toxicity? Do other *Ambrosia* species (or members of other thiarubrine-containing genera) photoprotect thiarubrines with anthocyanin light filters?

6.3.1. Basic Research

The most obvious gaps in basic knowledge about the thiarubrines are the mechanism by which they exert their toxicity (both light-mediated and light independent) and how they are formed in plants. With regards to the former, considering that the thiarubrines show extremely potent antifungal activity towards an organism, *Saccharomyces cerevisiae*, whose physiology and genetics are becoming so well understood, determining the cellular target of these compounds seems a feasible goal. With this goal in mind, a study of the cellular basis of thiarubrine toxicity is currently underway in the Towers laboratory, Department of Botany, The University of British Columbia.

The latter area, thiarubrine biosynthesis, may be much more difficult problem to tackle. However, in light of the fact that the Asteraceae is one of the most diverse plant families in Nature, and the polyynes (including their sulfur derivatives, the thiophenes and thiarubrines) have such ecological importance, a study of polyyne biosynthesis, perhaps using a molecular genetic approach, may be a rewarding undertaking.

6.3.2. Applications

Thiarubrines have yet to live up to their early promise as antifungal drugs. However, the extraordinary potency of their toxicity to fungal pathogens including *Candida albicans* and the findings of this study demonstrating that this toxicity is significantly enhanced by visible light treatment suggests that the thiarubrines may be useful in phototherapy of dermatomycoses or infections of the oral cavity. Such an approach may allow the topical application of small amounts of thiarubrines to infected areas followed by brief irradiation with visible light.

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Romeo, J. T., J. A. Saunders and P. Barbosa (1996). Recent Advances in Phytochemistry, Volume 30, Phytochemical Diversity and Redundancy in Ecological Interactions, Plenum Press, New York.
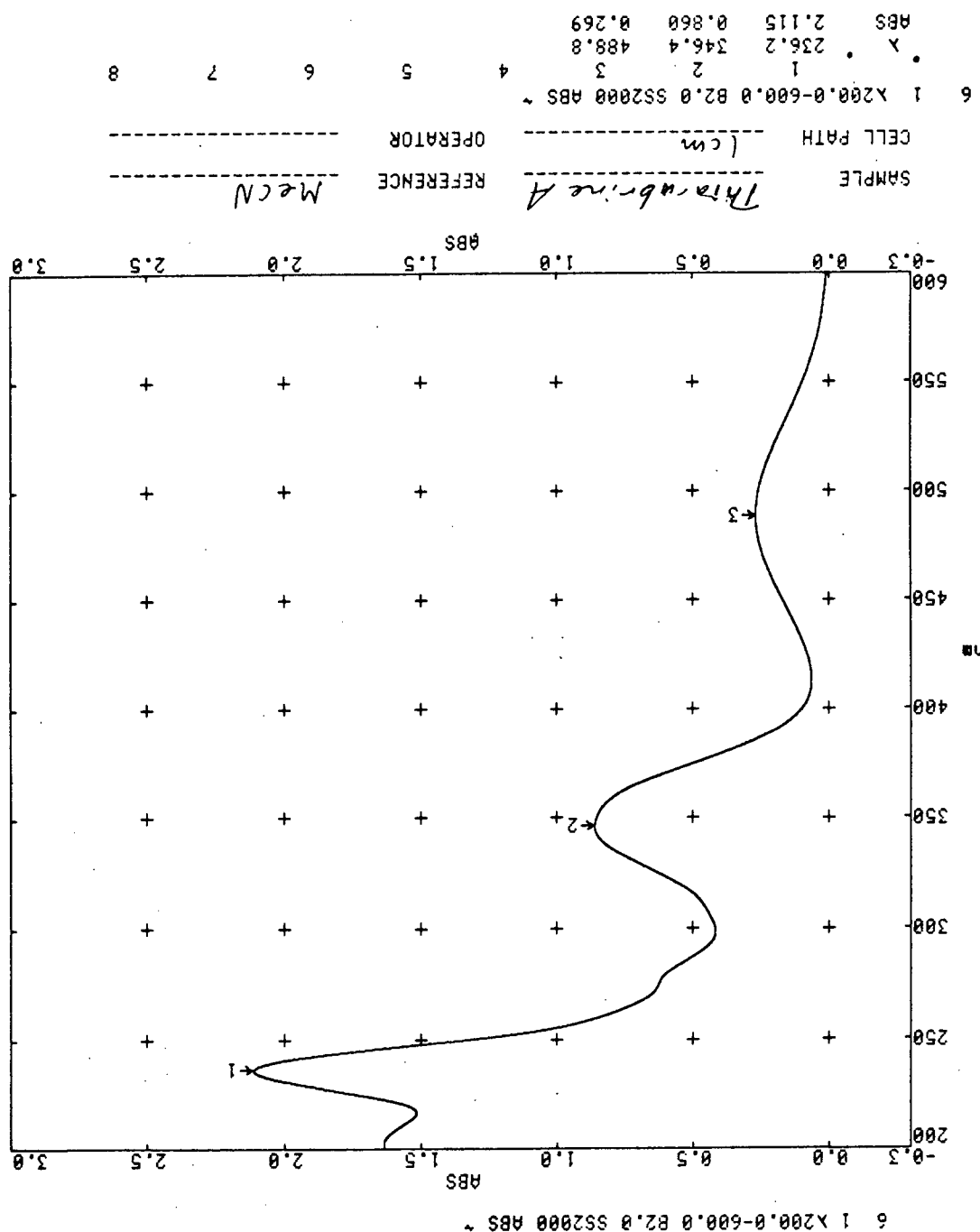
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APPENDIX 1: SPECTRAL ENERGY DISTRIBUTION FOR HALOGEN LAMP
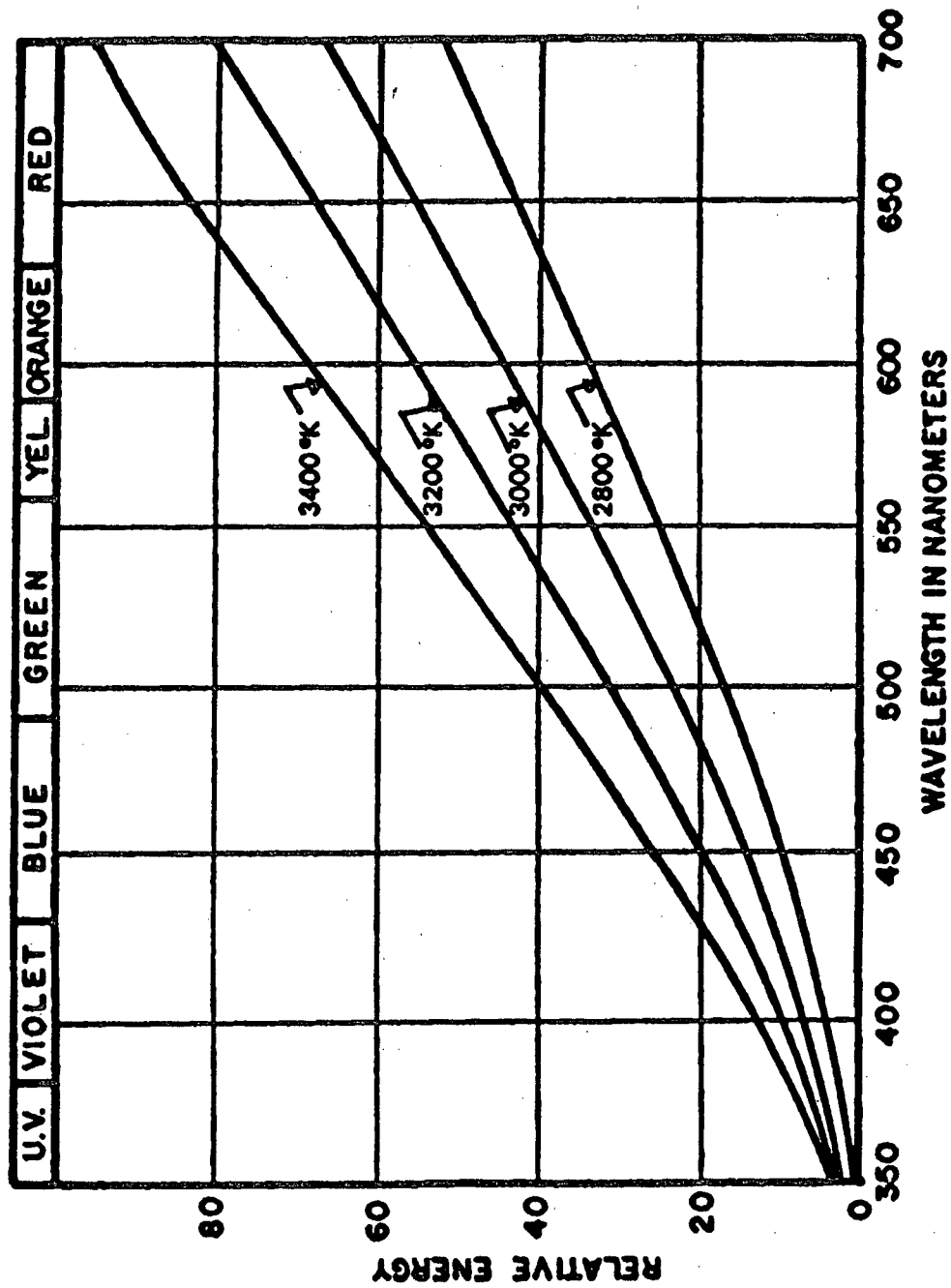
APPENDIX 2A: UV-VIS SPECTRUM OF THIARUBRINE A (1)

	1	2	3
λ	236.2	346.4	488.8
ABS	2.115	0.860	0.269

6 1 x200.0-600.0 82.0 SS2000 ABS

SAMPLE _Thiarubrine A_

REFERENCE _MeCN_

CELL PATH (cm)

OPERATOR

ABS

nm

6 1 x200.0-600.0 82.0 SS2000 ABS

APPENDIX 2A: ^{1}H-NMR SPECTRUM OF THIARUBRINE A (1)
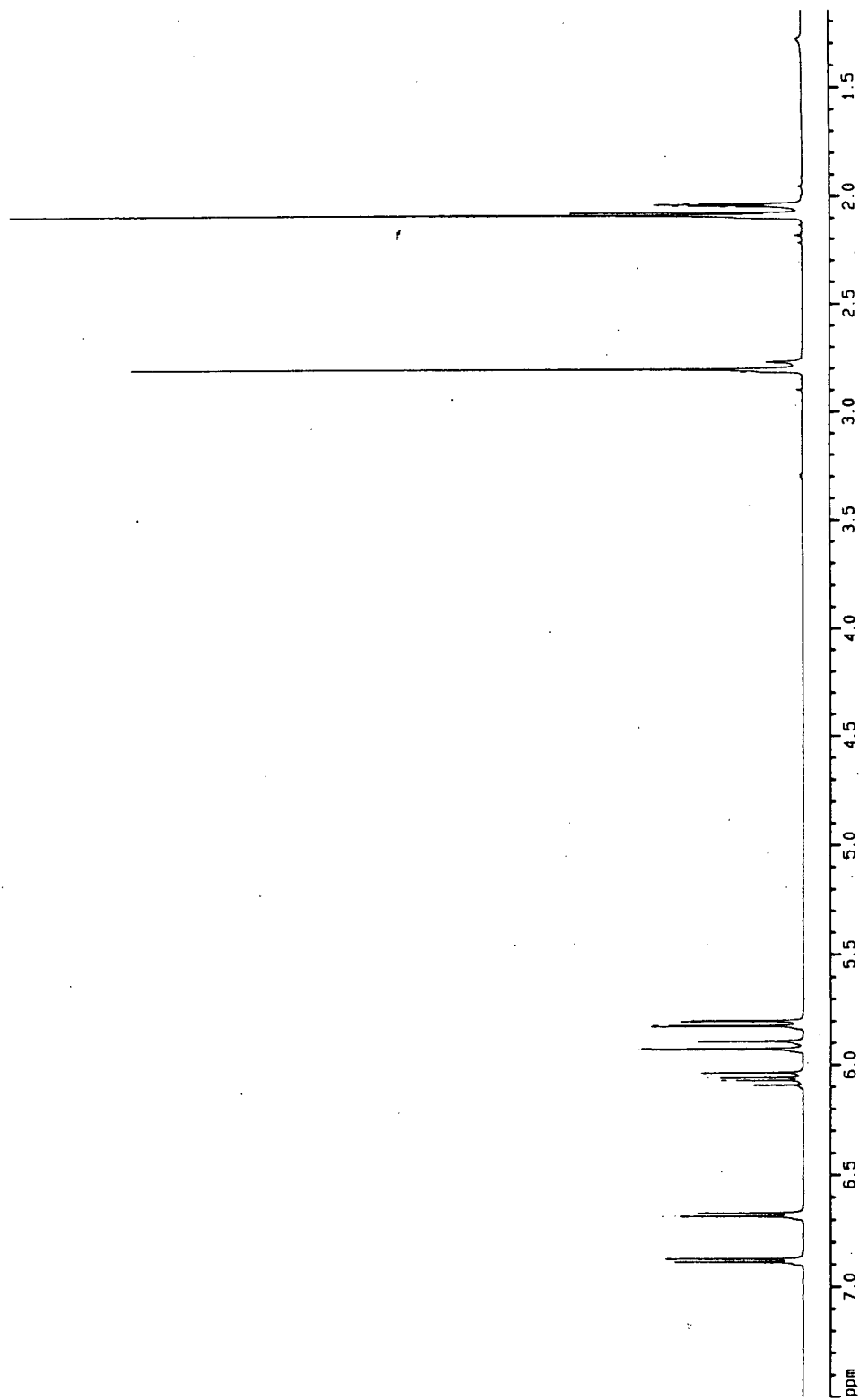
APPENDIX 2A: ^{13}C-NMR SPECTRUM OF THIARUBRINE A (1)
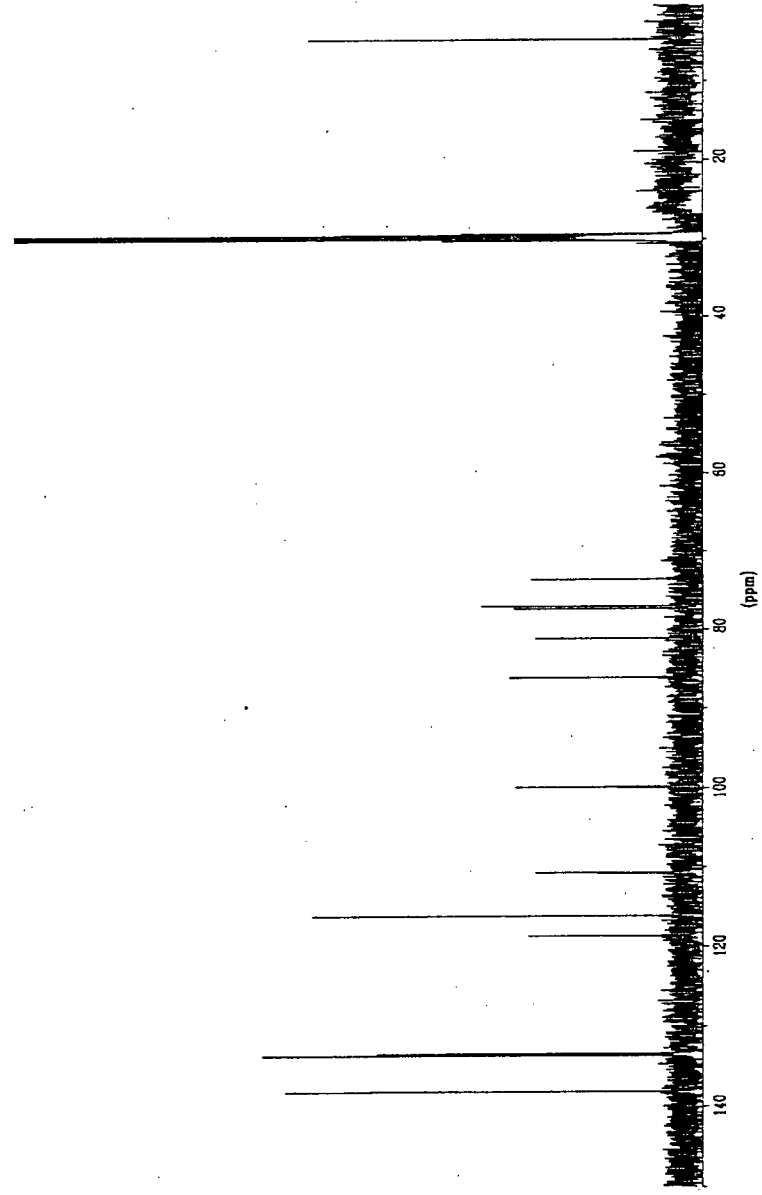
APPENDIX 2B: UV-VIS SPECTRUM OF THIARUBRINE B (2)

SAMPLE _Thiarubrine B_

REFERENCE _MeCN_

CELL PATH _l cm._

OPERATOR

4   1   X200.0-600.0 82.0 SS2000 ABS ˄

	X	ABS
1	354.7	1.097
2	492.9	0.345
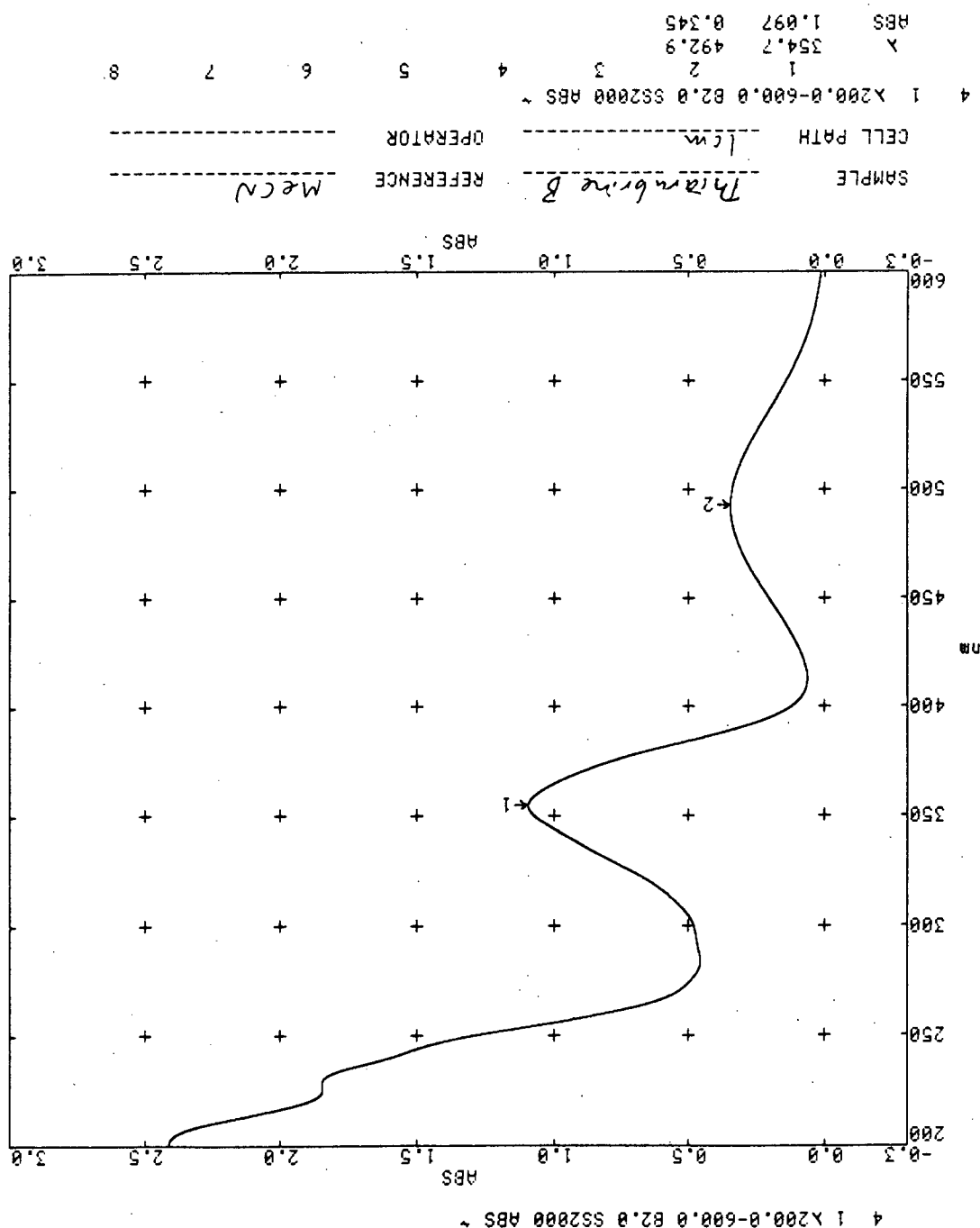
APPENDIX 2B: ^{1}H-NMR SPECTRUM OF THIARUBRINE B (2)
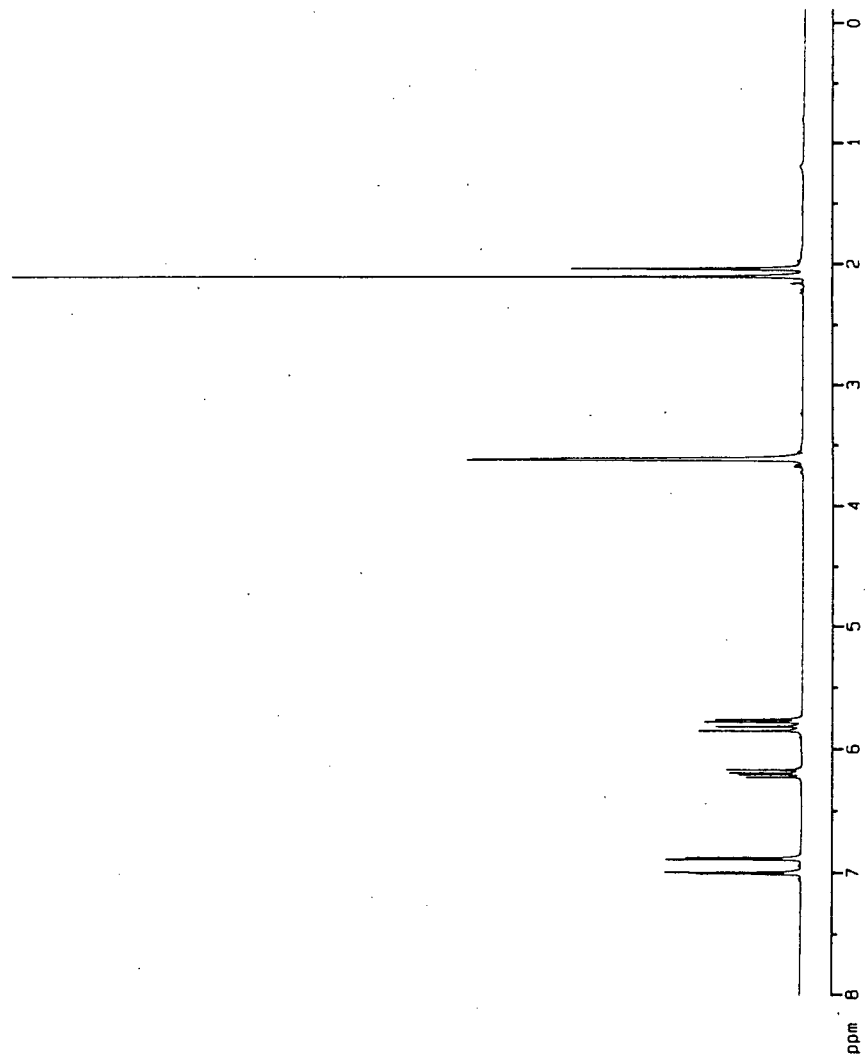
APPENDIX 2B: ^{13}C-NMR SPECTRUM OF THIARUBRINE B (2)
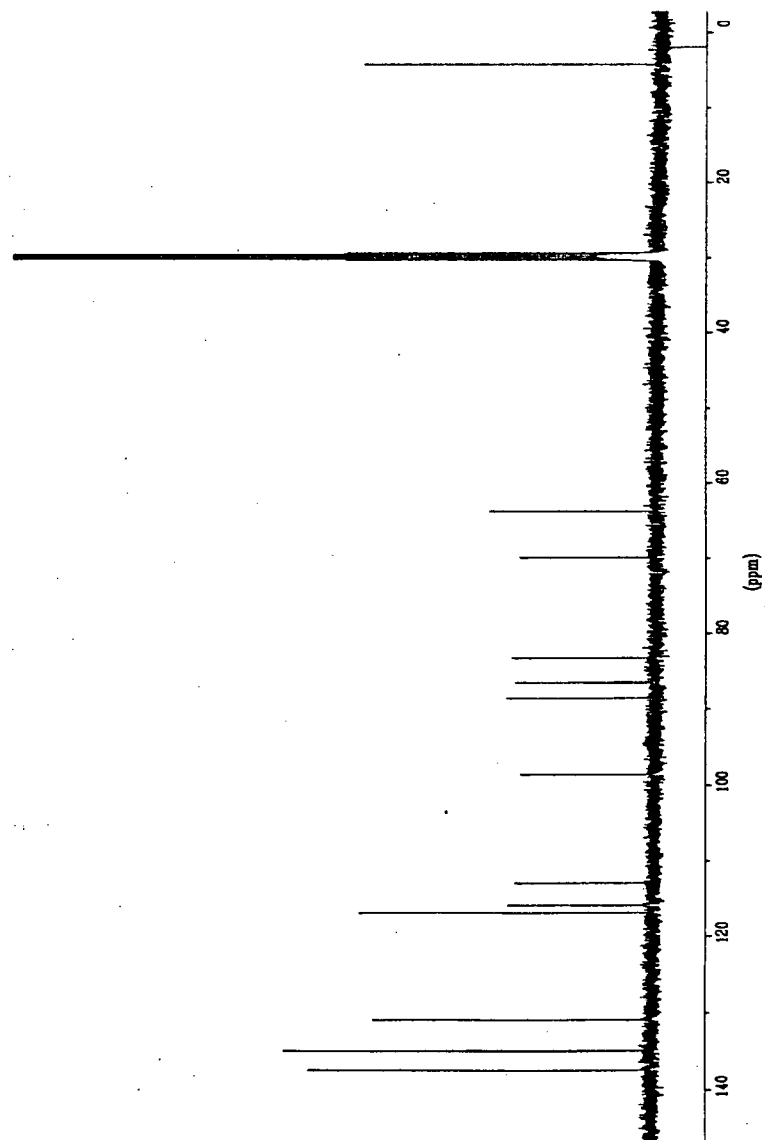
APPENDIX 2C: UV-VIS SPECTRUM OF THIARUBRINE D (3)

3 1 X200.0-600.0 B2.0 SS2000 ABS ^

SAMPLE	Thiarubrine D	CELL PATH	1cm
REFERENCE	MeCN	OPERATOR	------------

	1	2	3	4	5	6	7	8
X	228.8	304.8	346.4	484.9				
ABS	0.952	0.209	0.392	0.119				

3 1 X200.0-600.0 B2.0 SS2000 ABS ^
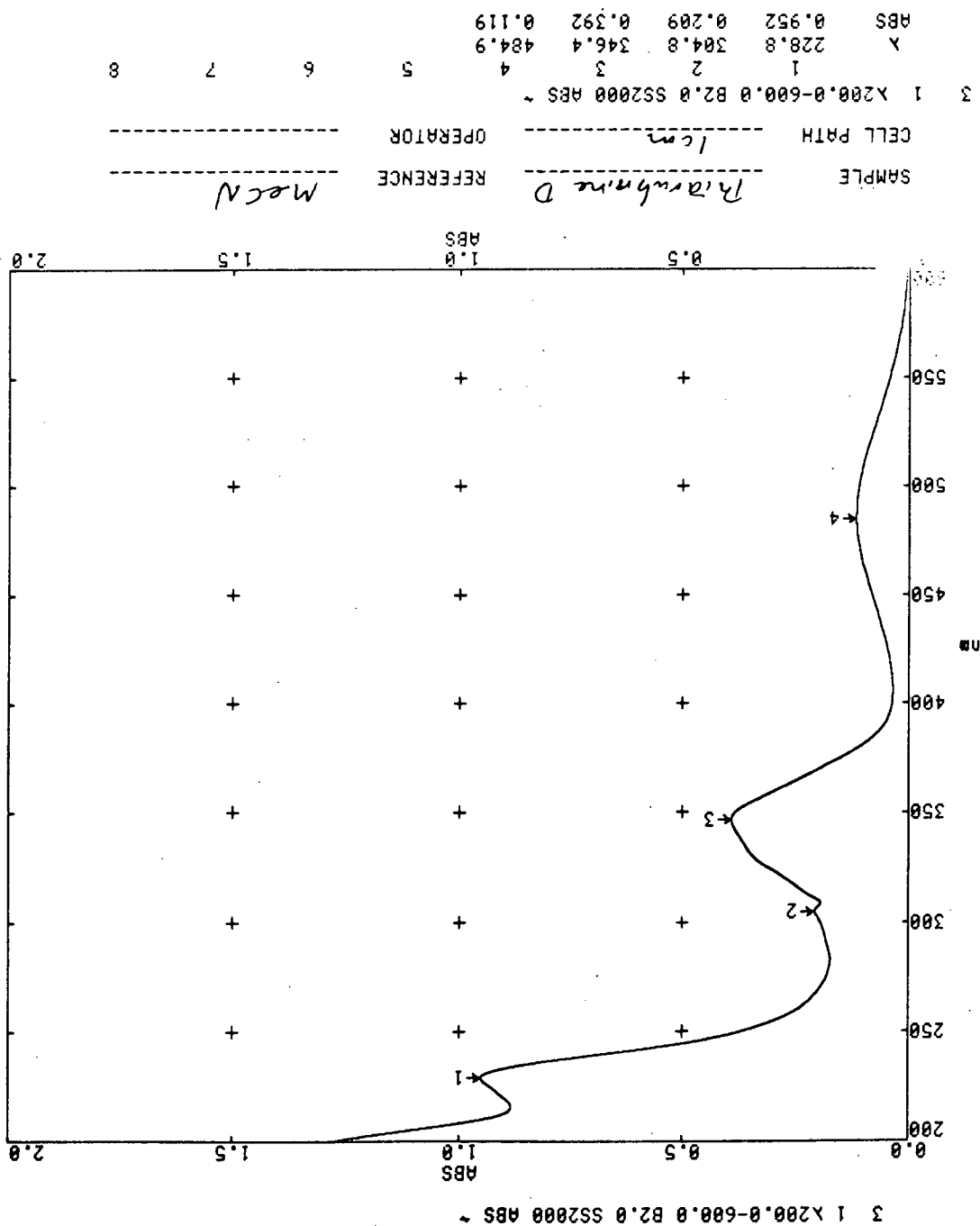
APPENDIX 2C: ¹H-NMR SPECTRUM OF THIARUBRINE D (3)
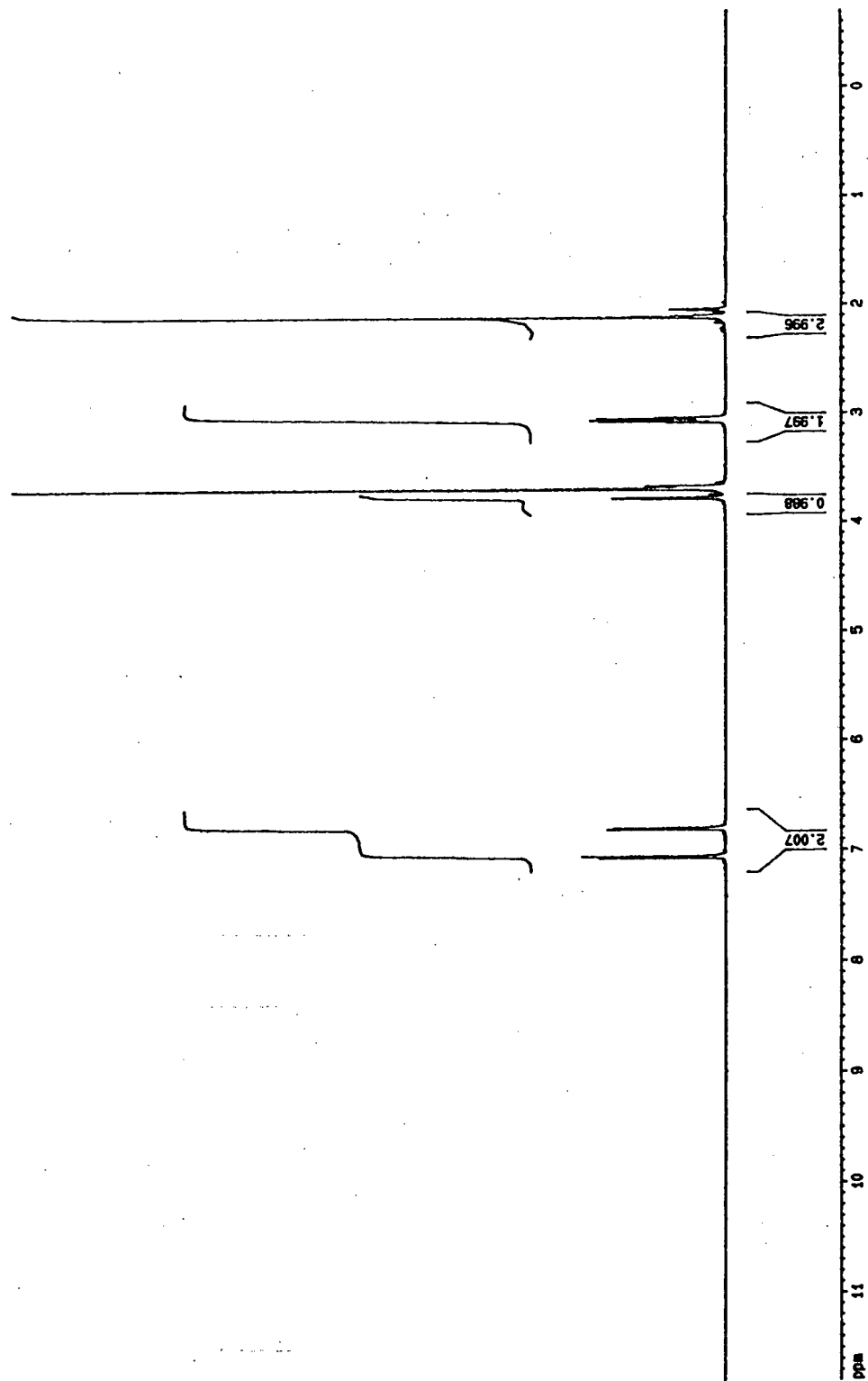
APPENDIX 2C: ^{13}C-NMR SPECTRUM OF THIARUBRINE D (3)
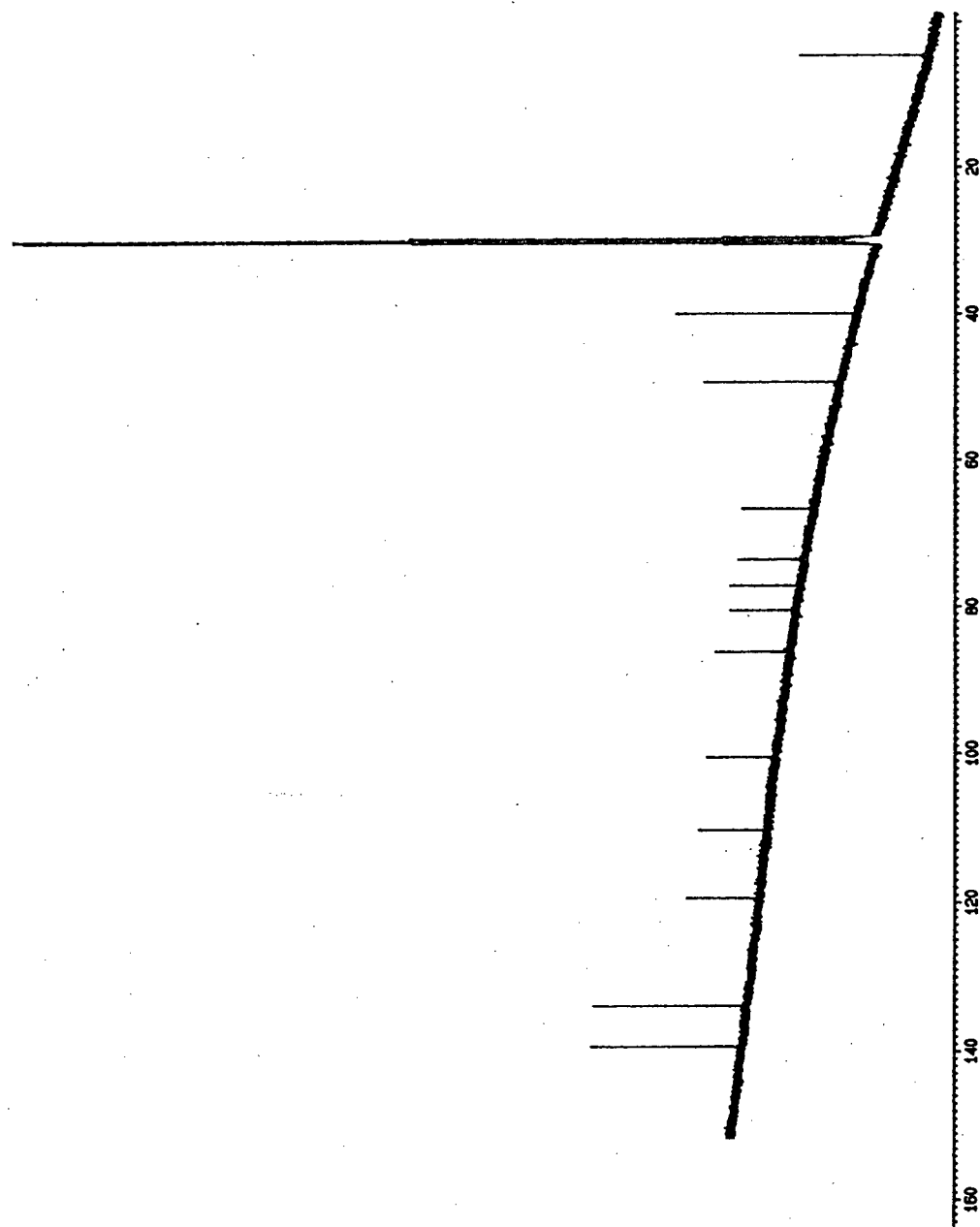
APPENDIX 2D: UV-VIS SPECTRUM OF THIARUBRINE E (4)

	1	2	3
λ	227.7	344.8	483.4
ABS	0.681	0.288	0.088

4 1 λ200.0-600.0 B2.0 SS2000 ABS ^

| 8 | 7 | 6 | 5 | 4 |

SAMPLE Thiarubrine E   CELL PATH 1 cm

REFERENCE MeCN   OPERATOR

ABS

nm

4 1 λ200.0-600.0 B2.0 SS2000 ABS ^
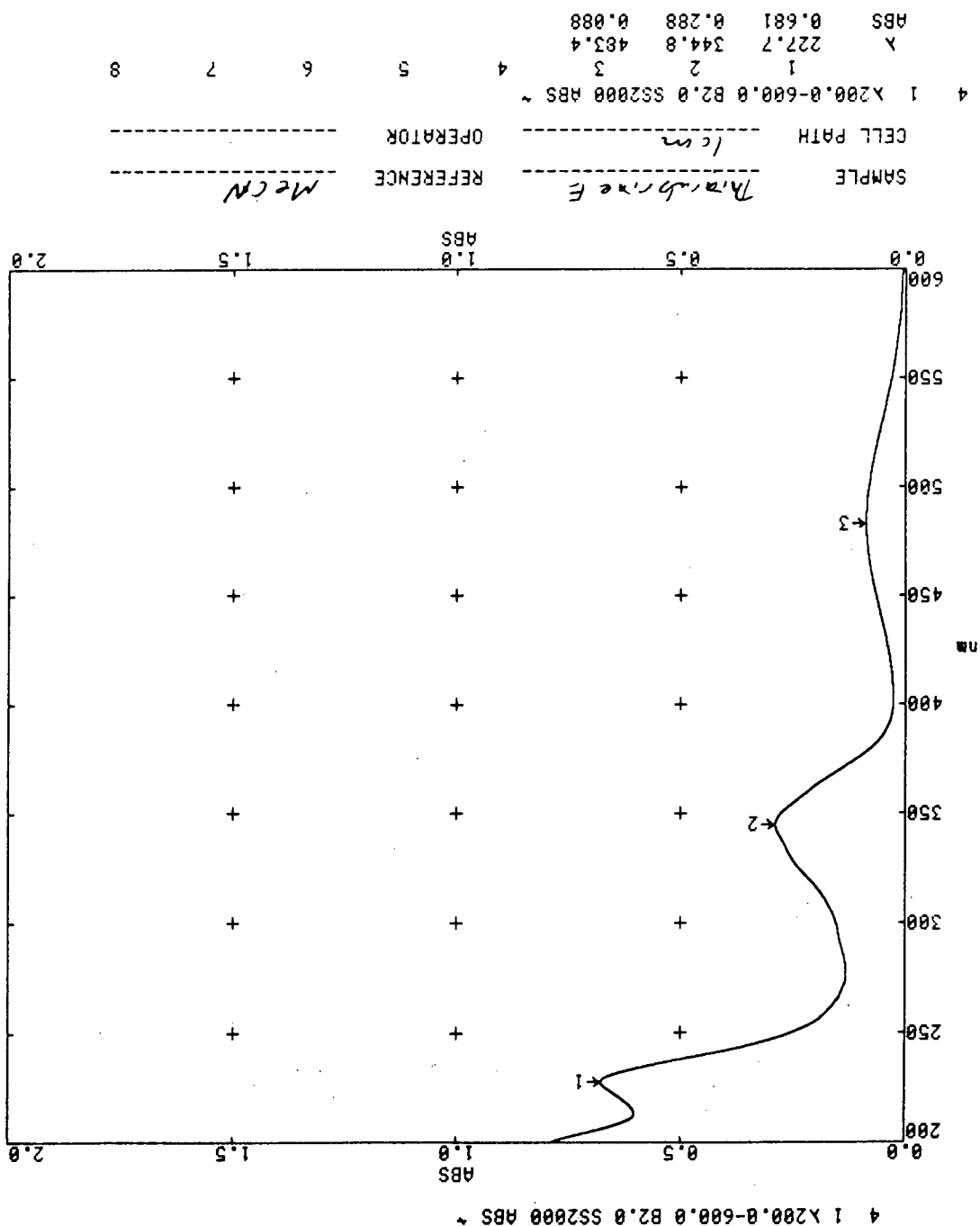
APPENDIX 2D: ^{1}H-NMR SPECTRUM OF THIARUBRINE E (4)
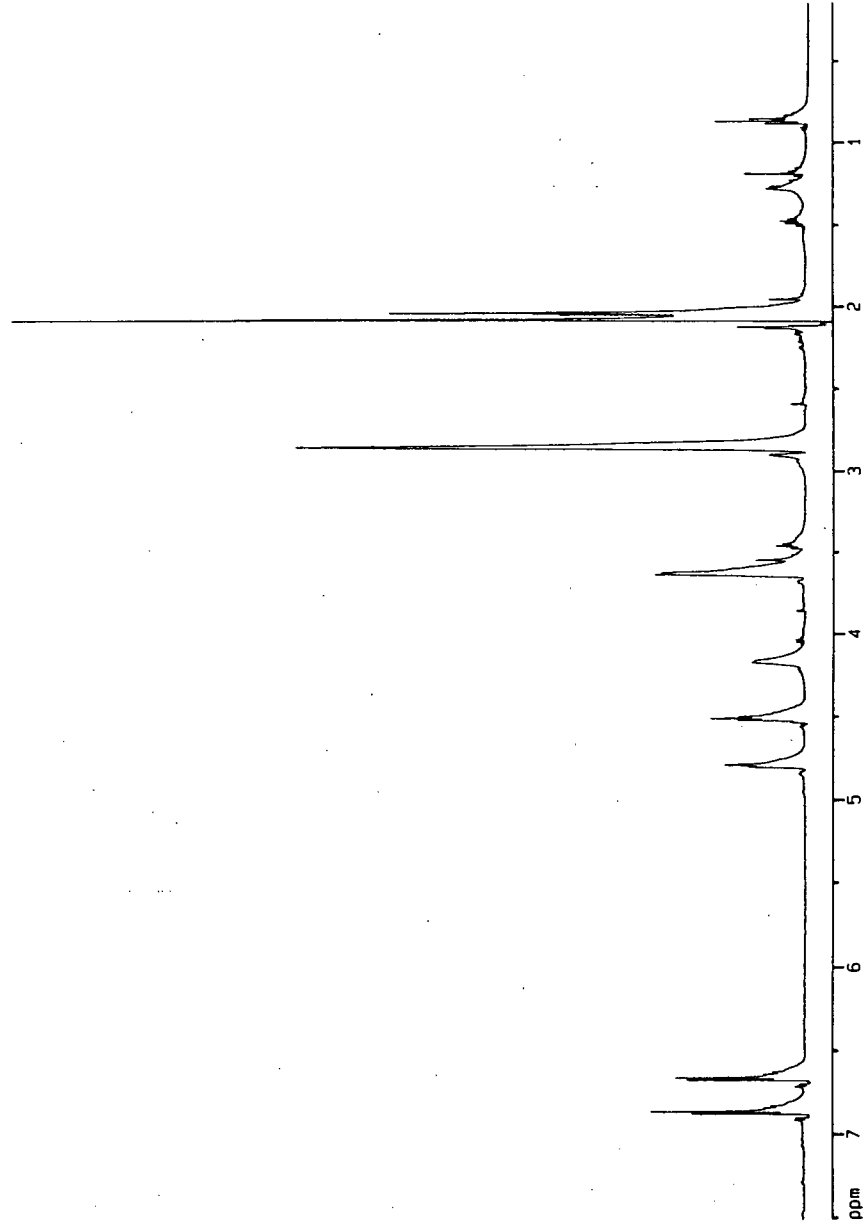
APPENDIX 2E: UV-VIS SPECTRUM OF THIOPHENE A (13)

	1	2	3	4	5	6	7	8
λ	214.5	268.3	274.4	339.4	360.7			
ABS	1.716	0.631	0.741	1.376	0.957			

8   1   X200.0-600.0 B2.0 SS2000 ABS ~
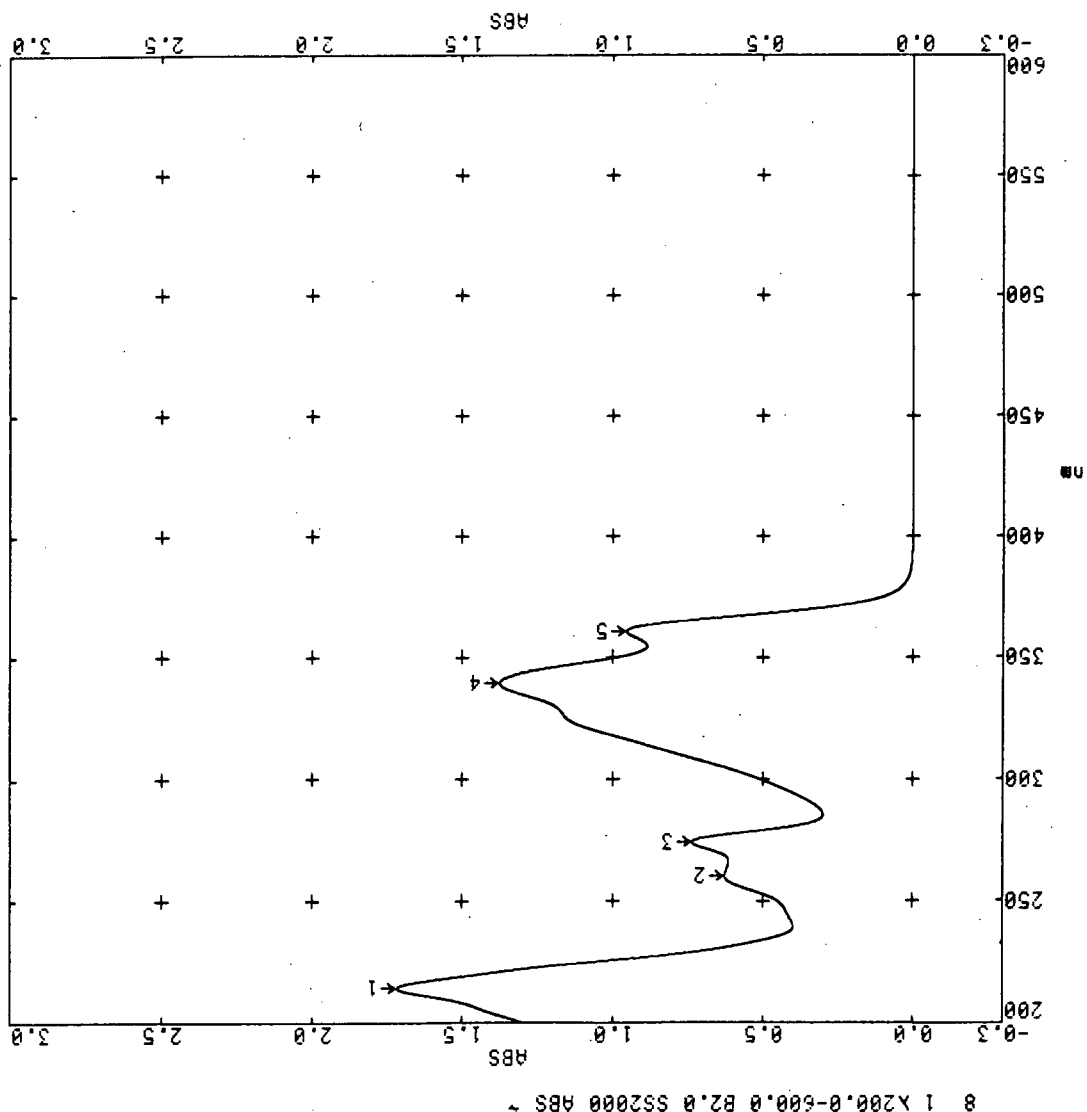
SAMPLE	_Thiophene A_	REFERENCE	_MeCN_
CELL PATH	_1 cm_	OPERATOR	----

8   1   X200.0-600.0 B2.0 SS2000 ABS ~

APPENDIX 2E: ^{1}H-NMR SPECTRUM OF THIOPHENE A (13)
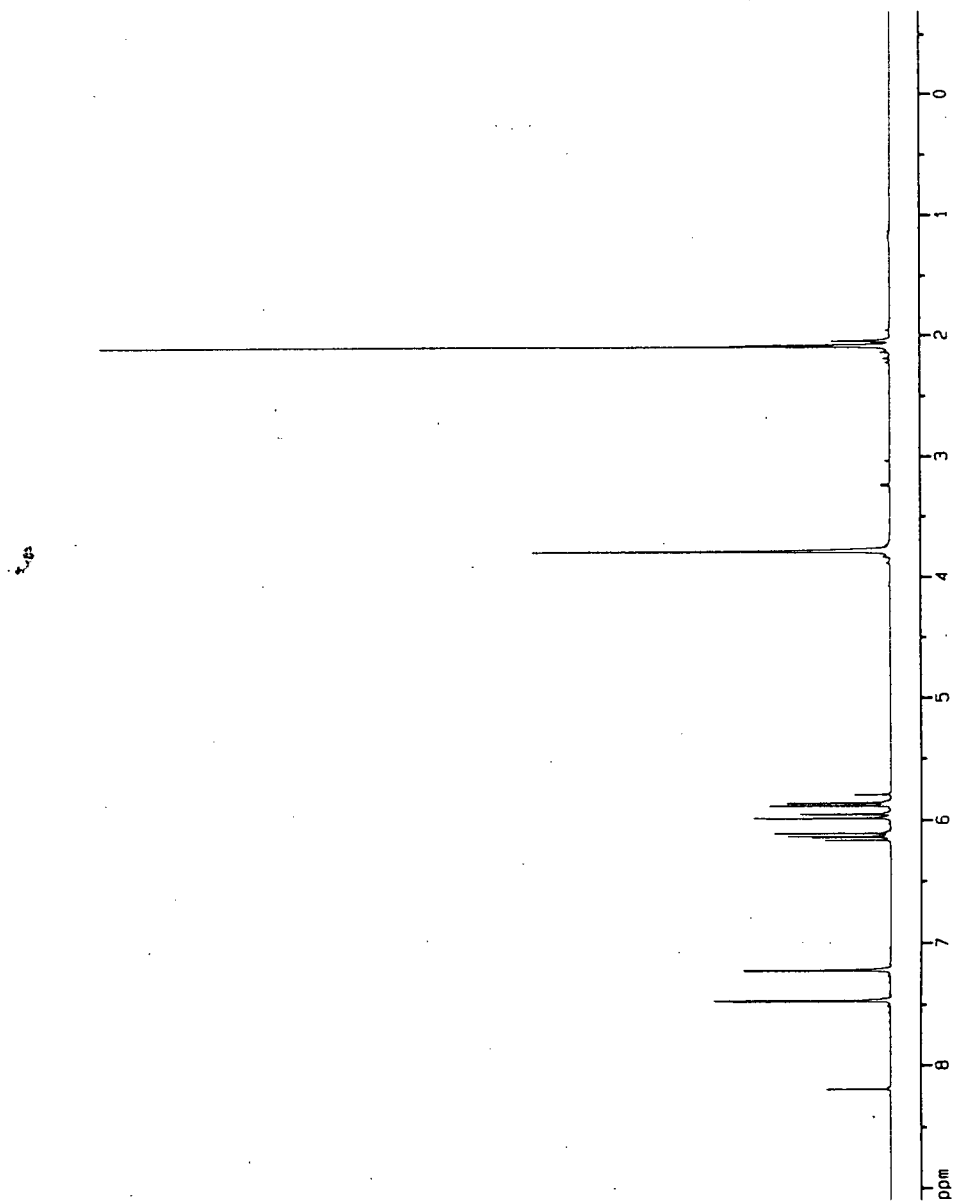
APPENDIX 2F: UV-VIS SPECTRUM OF THIOPHENE B (14)

	1	2	3
λ	251.2	335.5	355.3
ABS	0.398	1.654	1.481

4   5   6   7   8

11 1 X200.0-600.0 B2.0 SS2000 ABS ⌐

SAMPLE ___ Thiophene B ___   REFERENCE ___ MeCN ___

CELL PATH ___ 1.cm ___   OPERATOR ___

11 1 X200.0-600.0 B2.0 SS2000 ABS ⌐
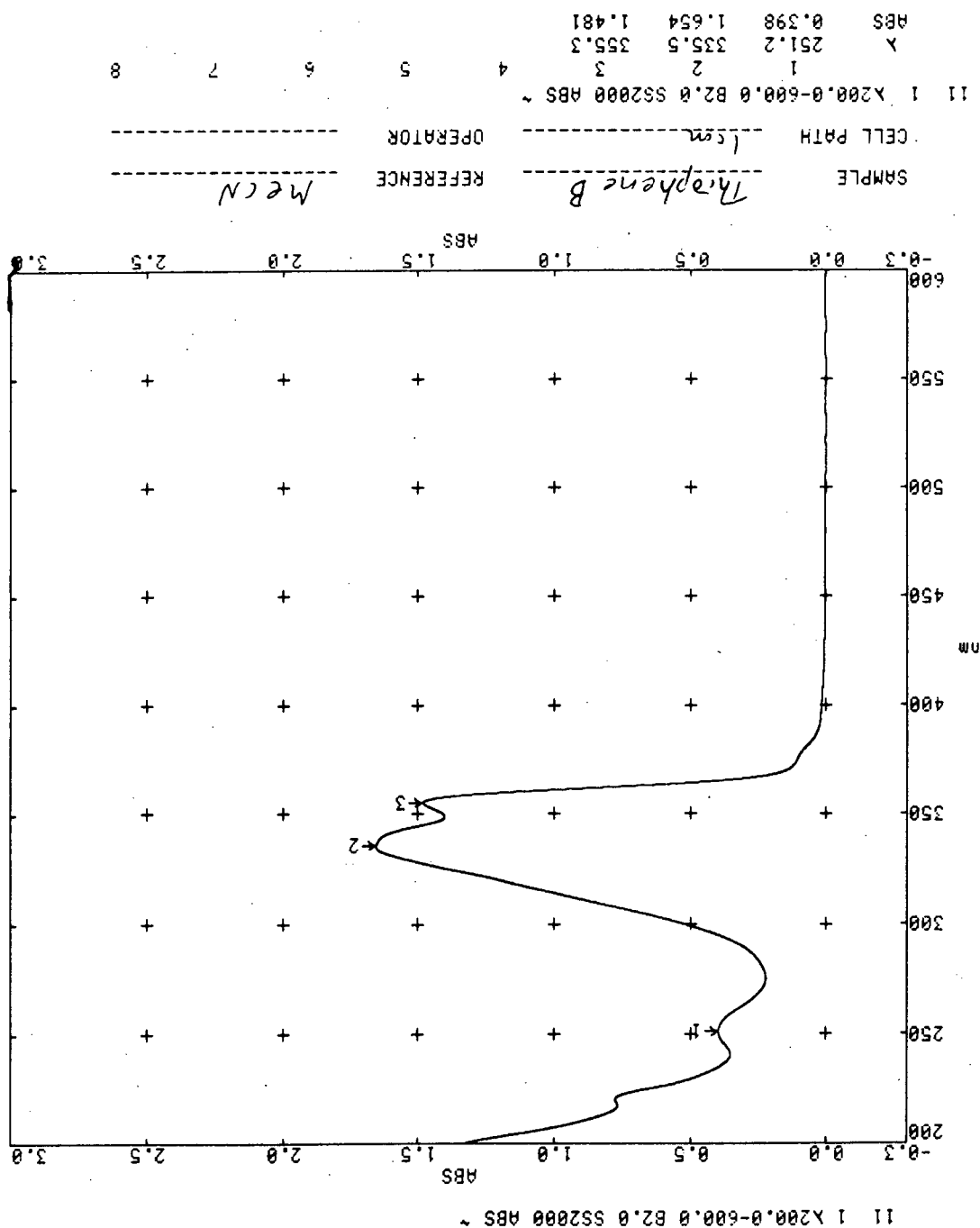
APPENDIX 2F: ^{1}H-NMR SPECTRUM OF THIOPHENE B (14)
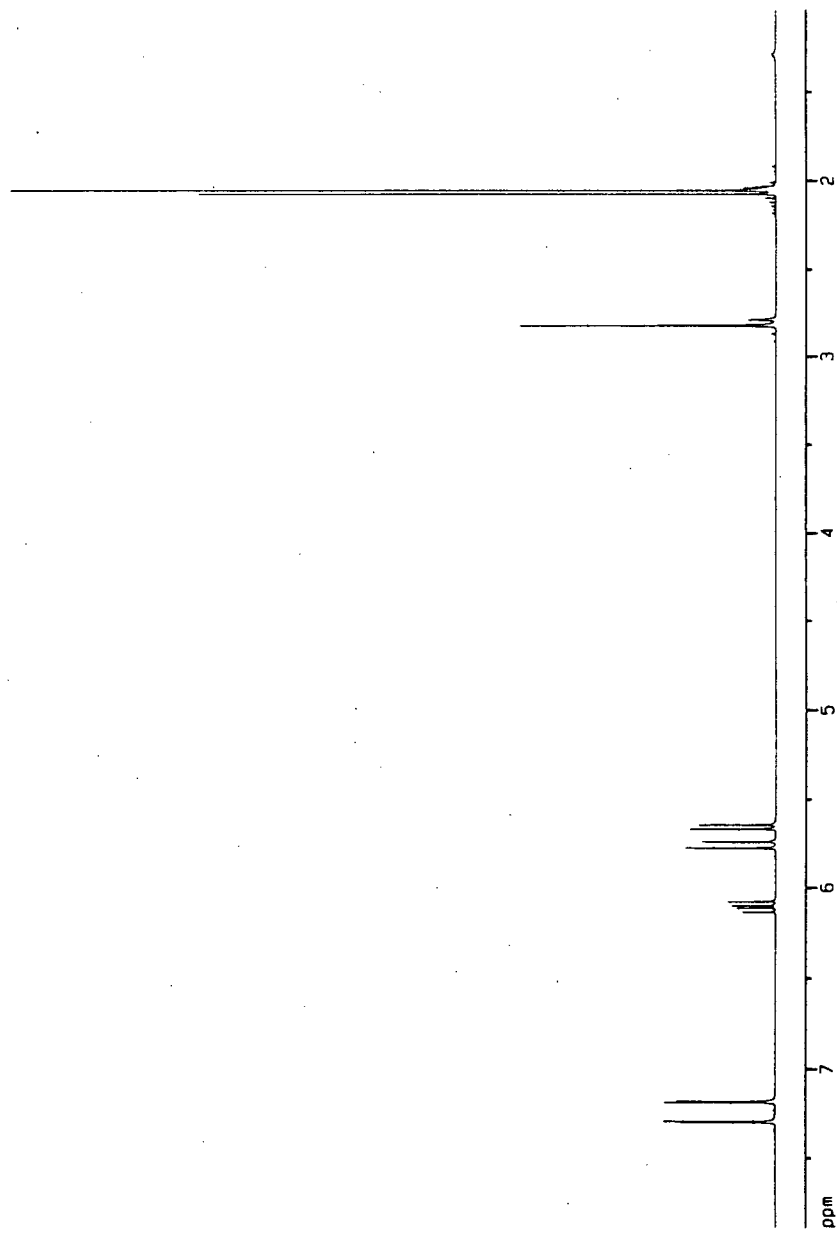
APPENDIX 2G: UV-VIS SPECTRUM OF THIOPHENE D (15)

SAMPLE __Thiophene D__   REFERENCE __MeCN__

CELL PATH __1 cm__   OPERATOR ____

1 1 X200.0-600.0 B2.0 SS2000 ABS ⌐

	1	2	3	4	5	6	7	8
X	211.3	252.9	327.3					
ABS	1.303	0.427	1.061					

1 1 X200.0-600.0 B2.0 SS2000 ABS ⌐
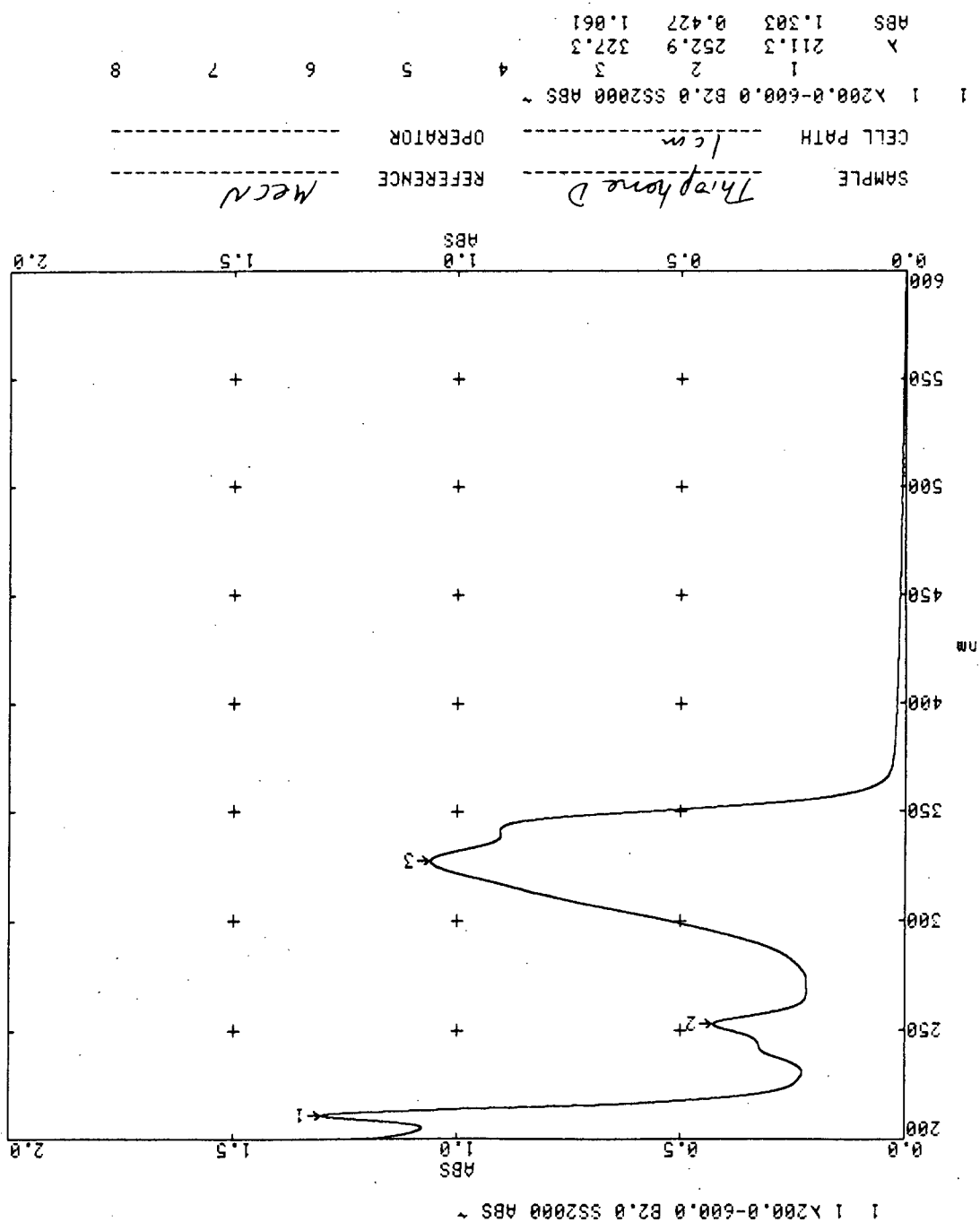
APPENDIX 2G: ¹H-NMR SPECTRUM OF THIOPHENE D (15)
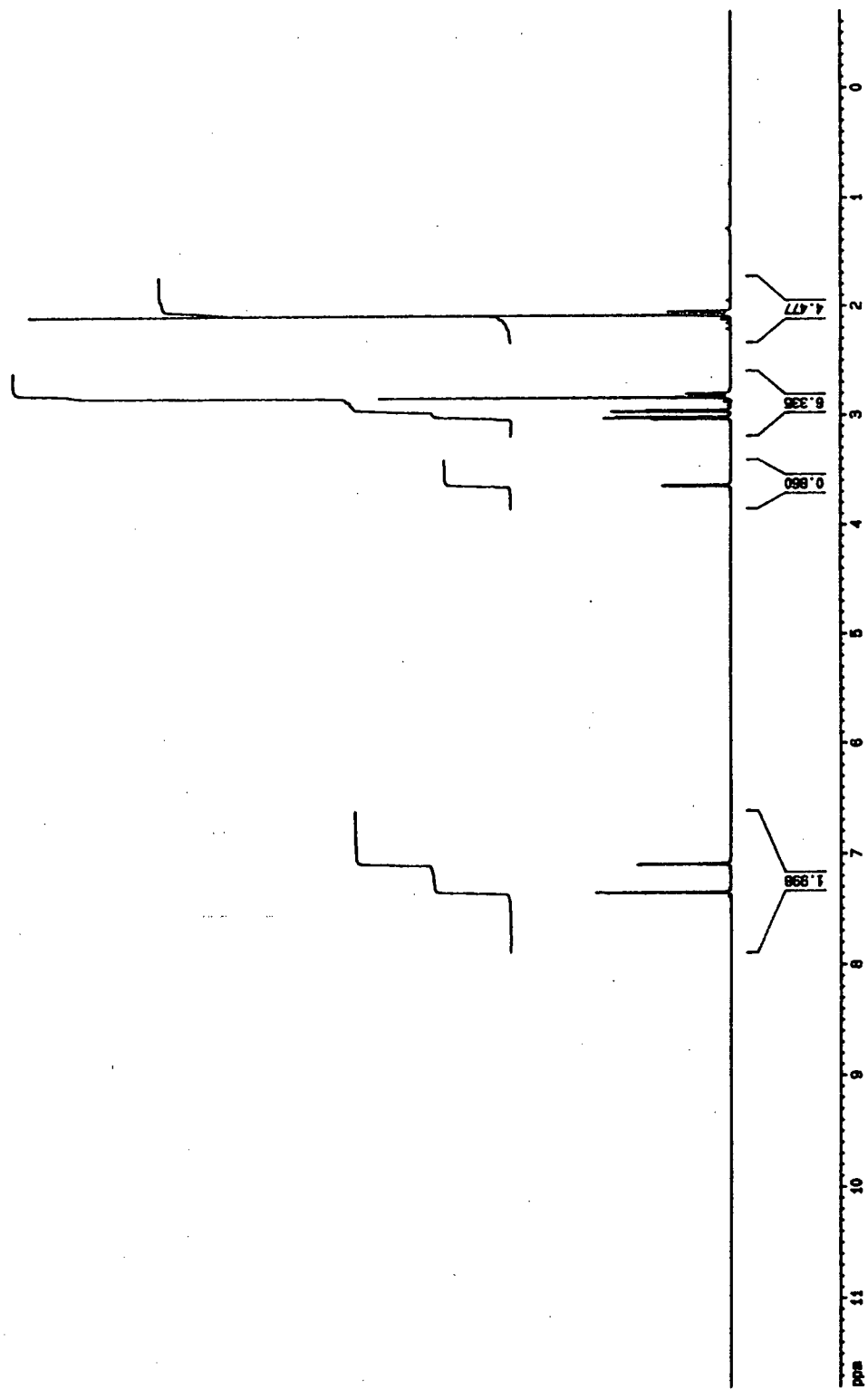
APPENDIX 2H: UV-VIS SPECTRUM OF THIOPHENE E (16)

	1	2	3	4	5	6	7	8
λ	210.1	249.3	323.5	341.6				
ABS	0.300	0.098	0.236	0.209				

1   1 X200.0-600.0 B2.0 SS2000 ABS ^

CELL PATH _l cm_ _ _ _ _ _ _   OPERATOR _ _ _ _ _ _ _ _ _ _ _

SAMPLE _Thiophene E_   REFERENCE _MeCN_

1   1 X200.0-600.0 B2.0 SS2000 ABS ^
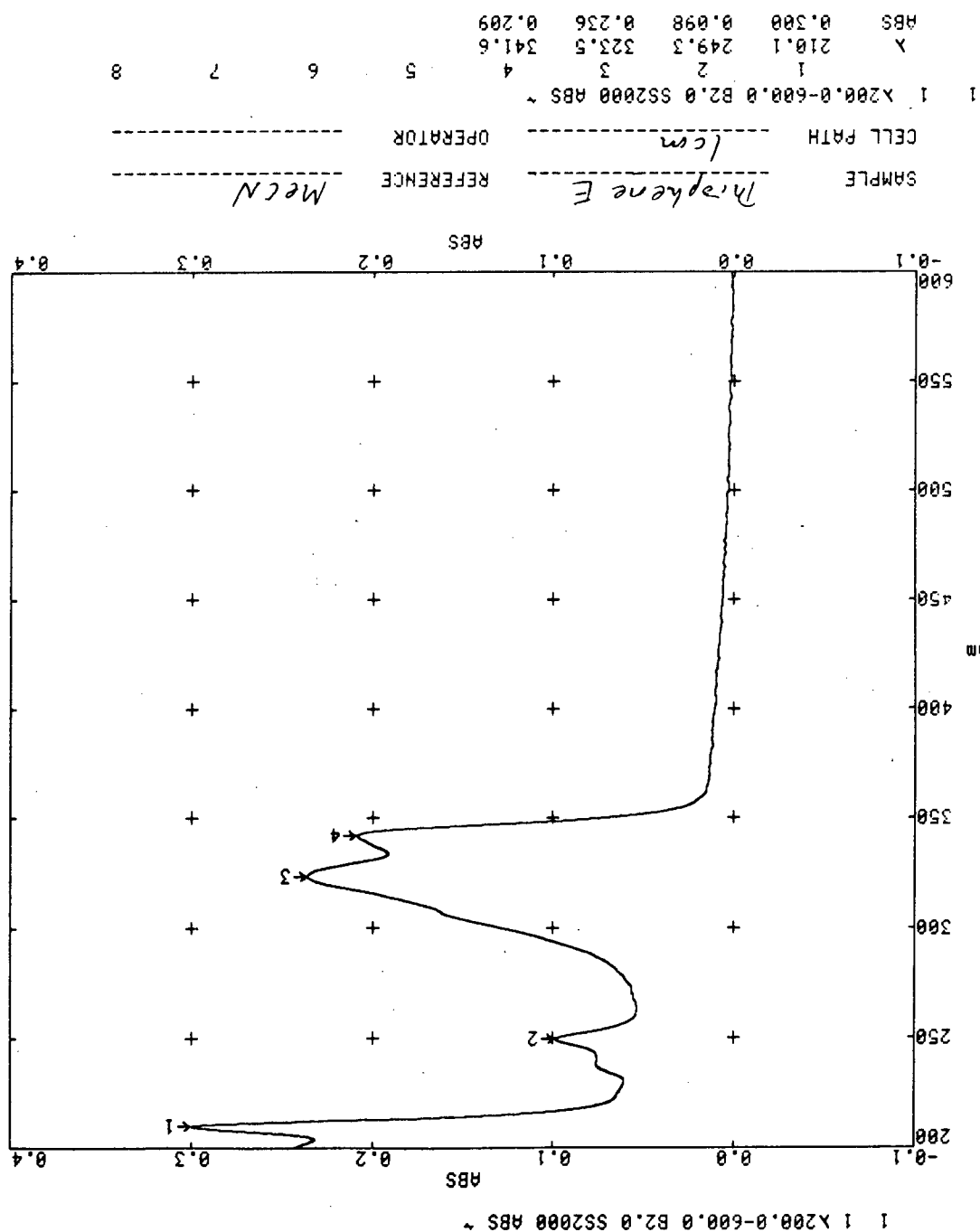
APPENDIX 2H: ¹H-NMR SPECTRUM OF THIOPHENE E (16)
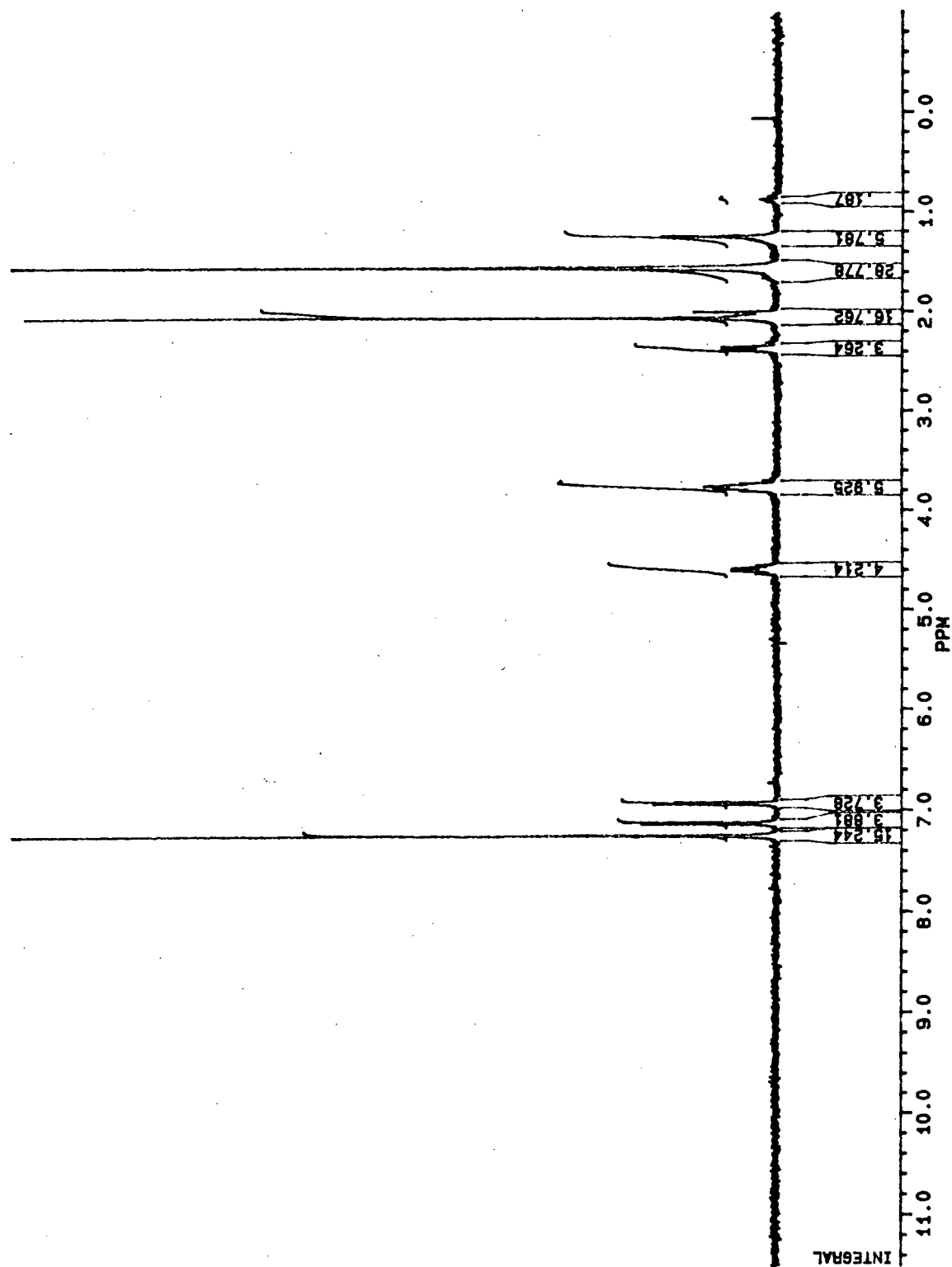
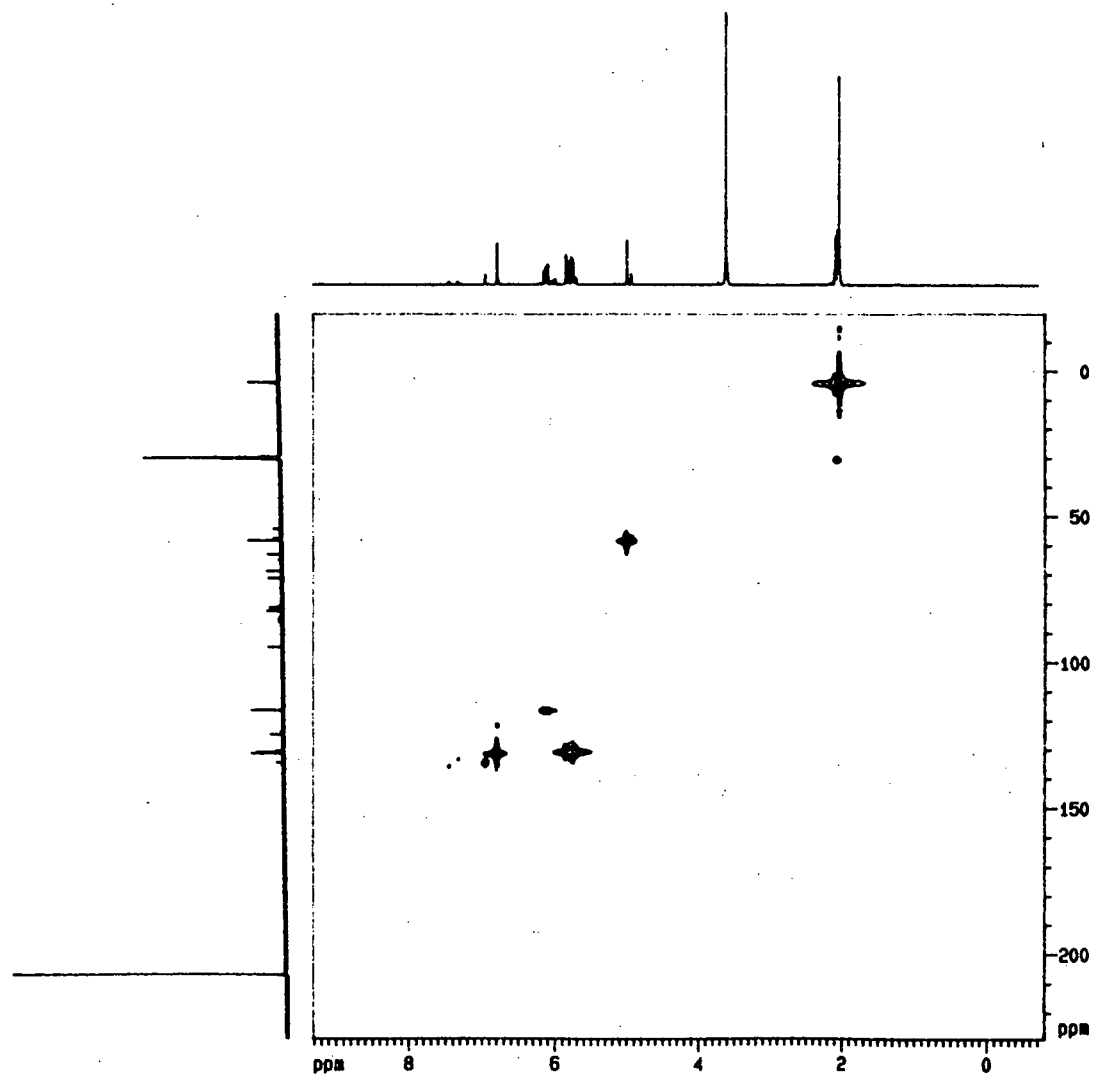**APPENDIX 3A: UV-VIS SPECTRUM OF PHOTOSULFIDES 1A AND 1B**
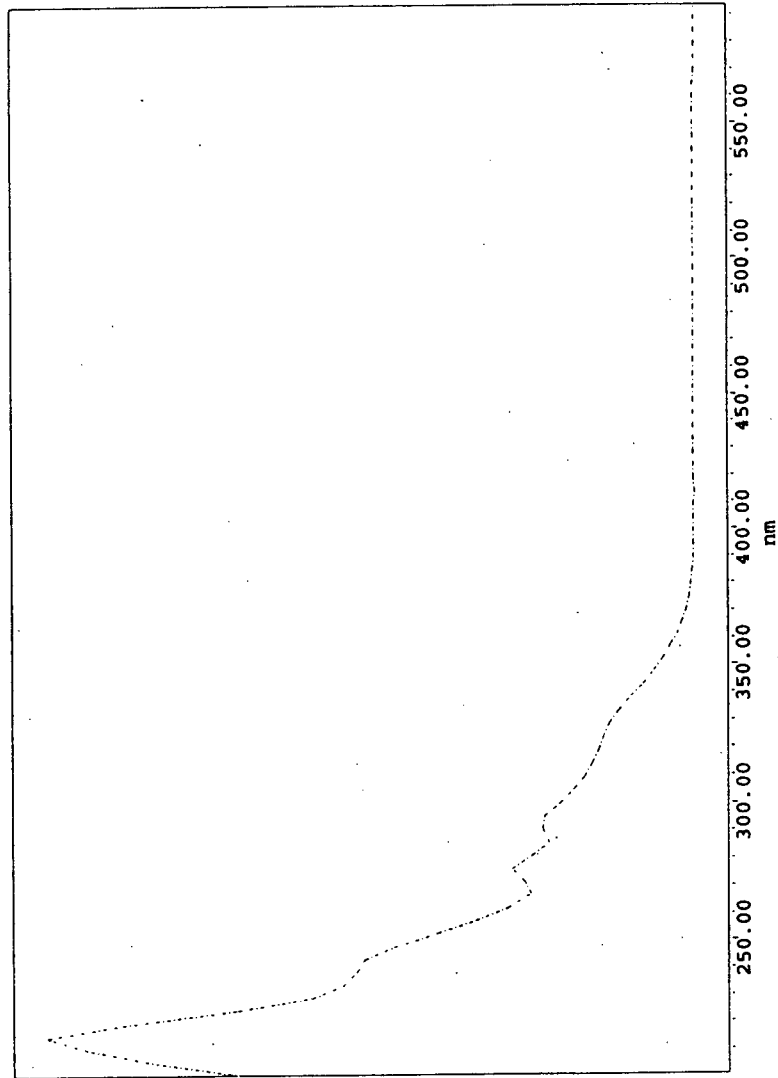
APPENDIX 3A: ¹H-NMR SPECTRUM OF PHOTOINTERMEDIATE MIXTURE OF THIARUBRINE A (1)
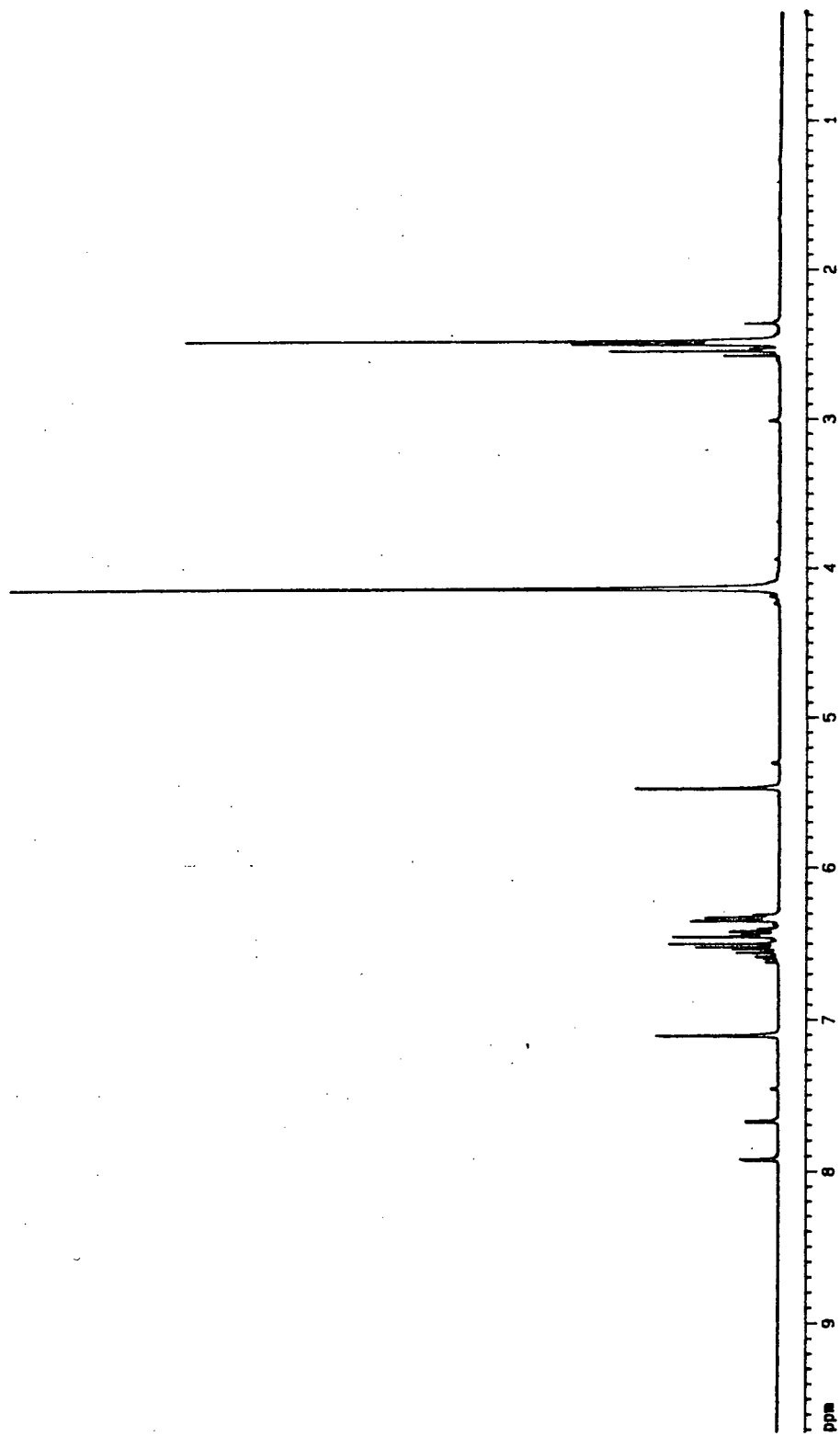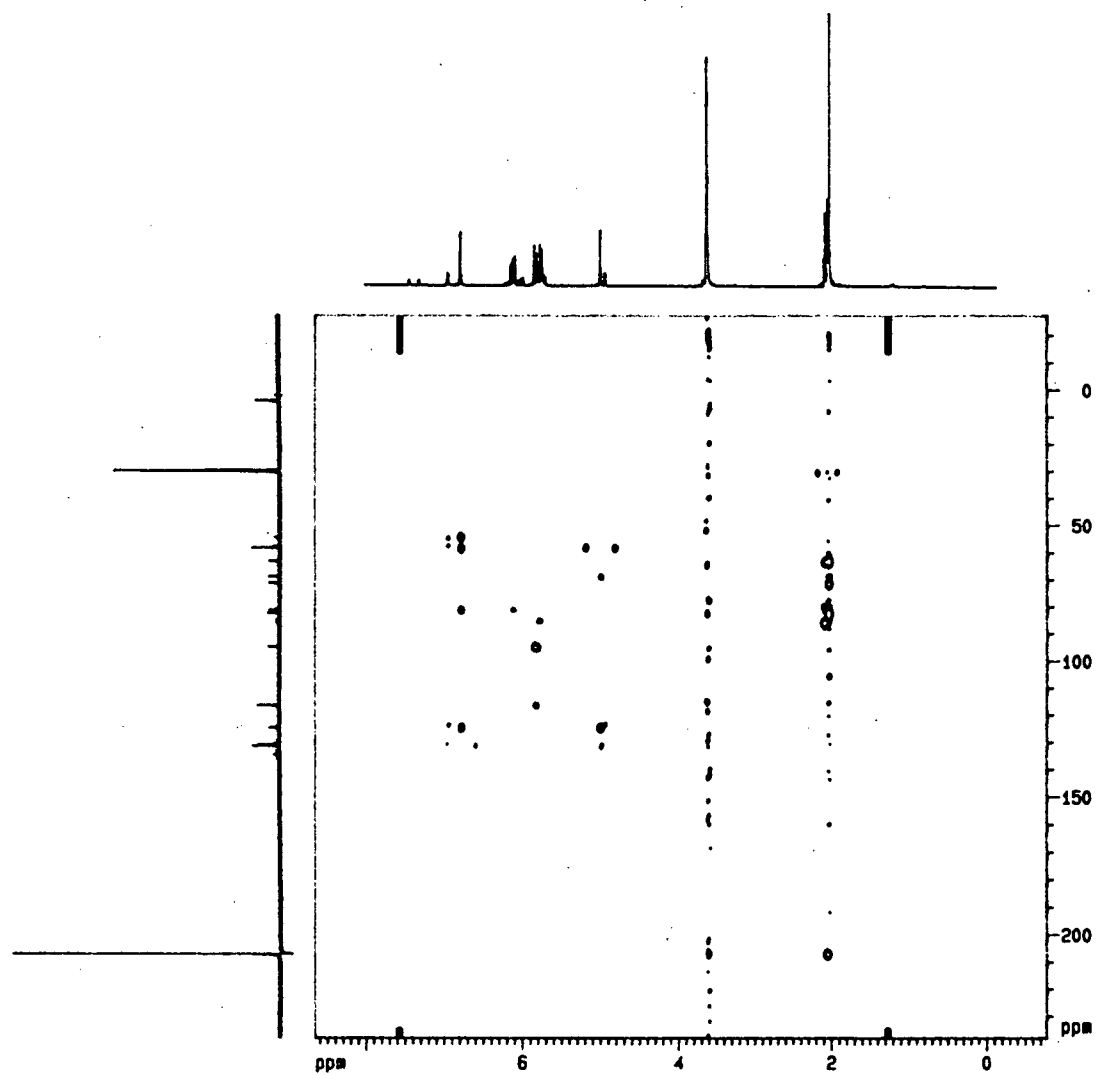
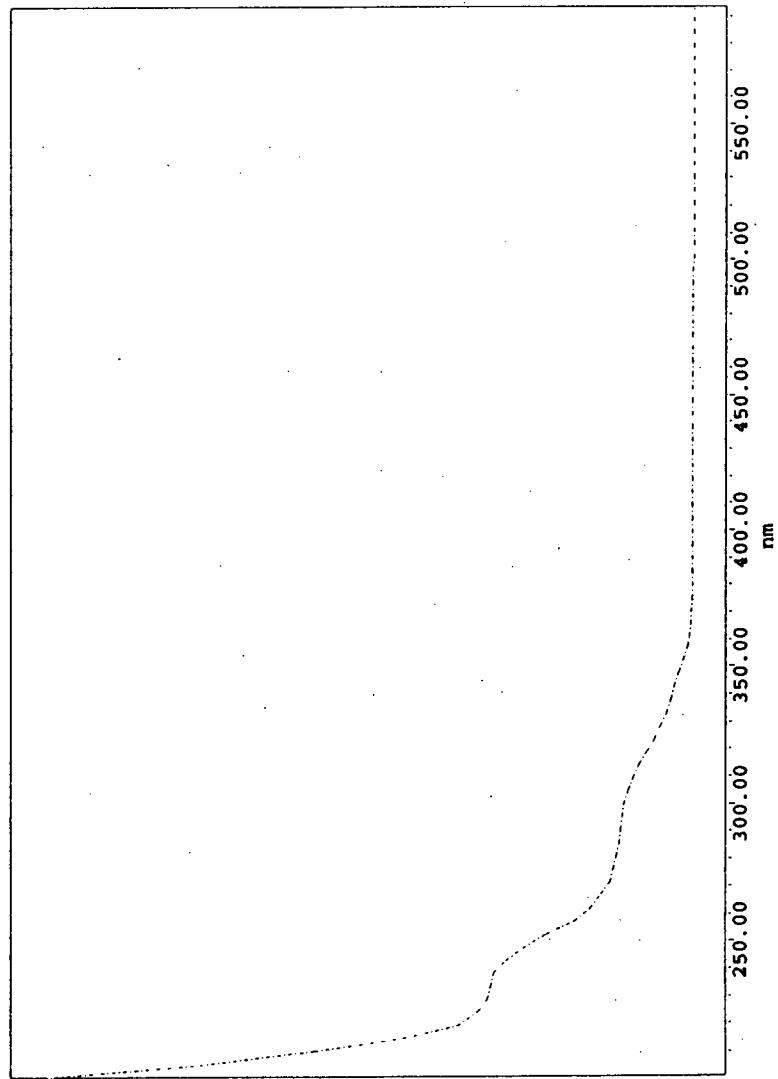APPENDIX 3A: ^{13}C-NMR SPECTRUM OF PHOTOINTERMEDIATE MIXTURE OF THIARUBRINE A (1)
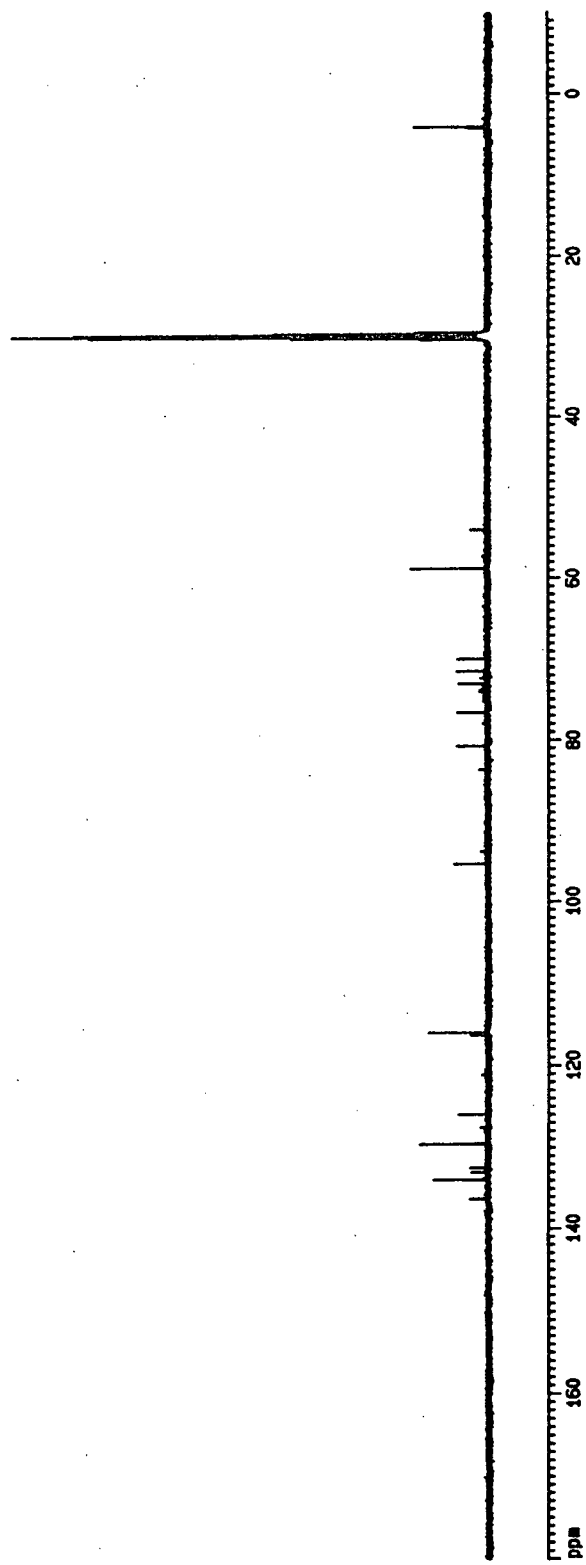
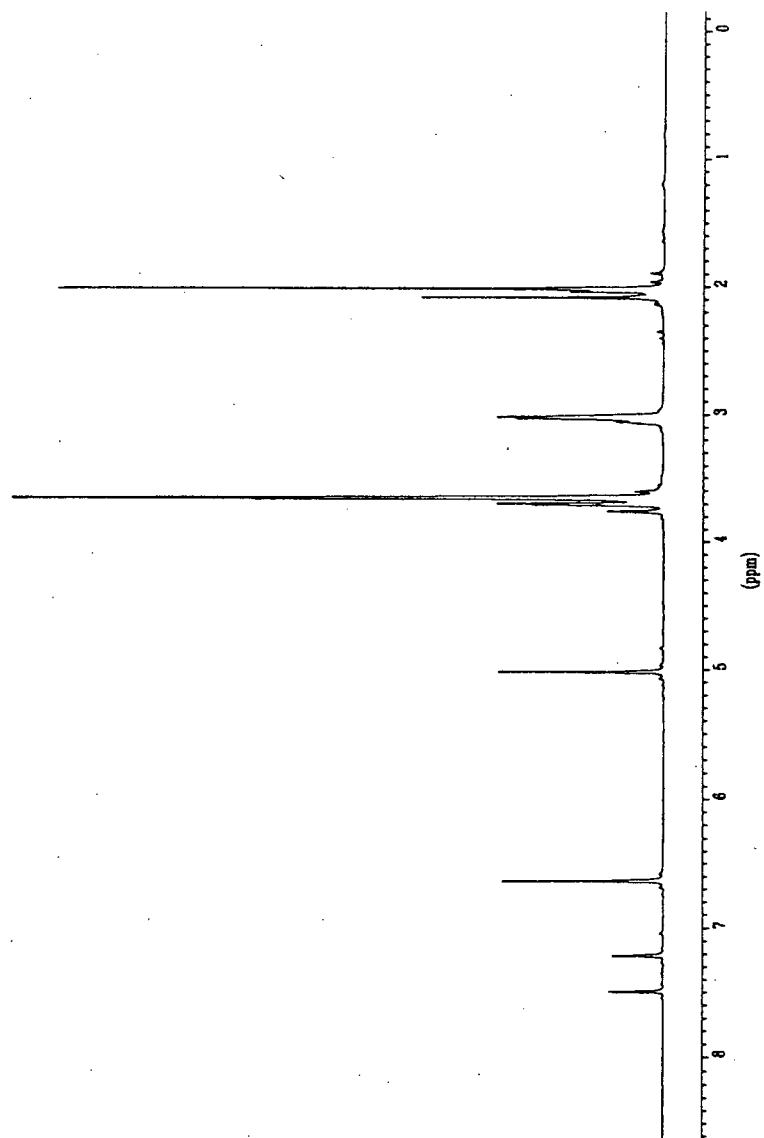# APPENDIX 3A: HMQC SPECTRUM OF PHOTOINTERMEDIATE MIXTURE OF THIARUBRINE A (1)
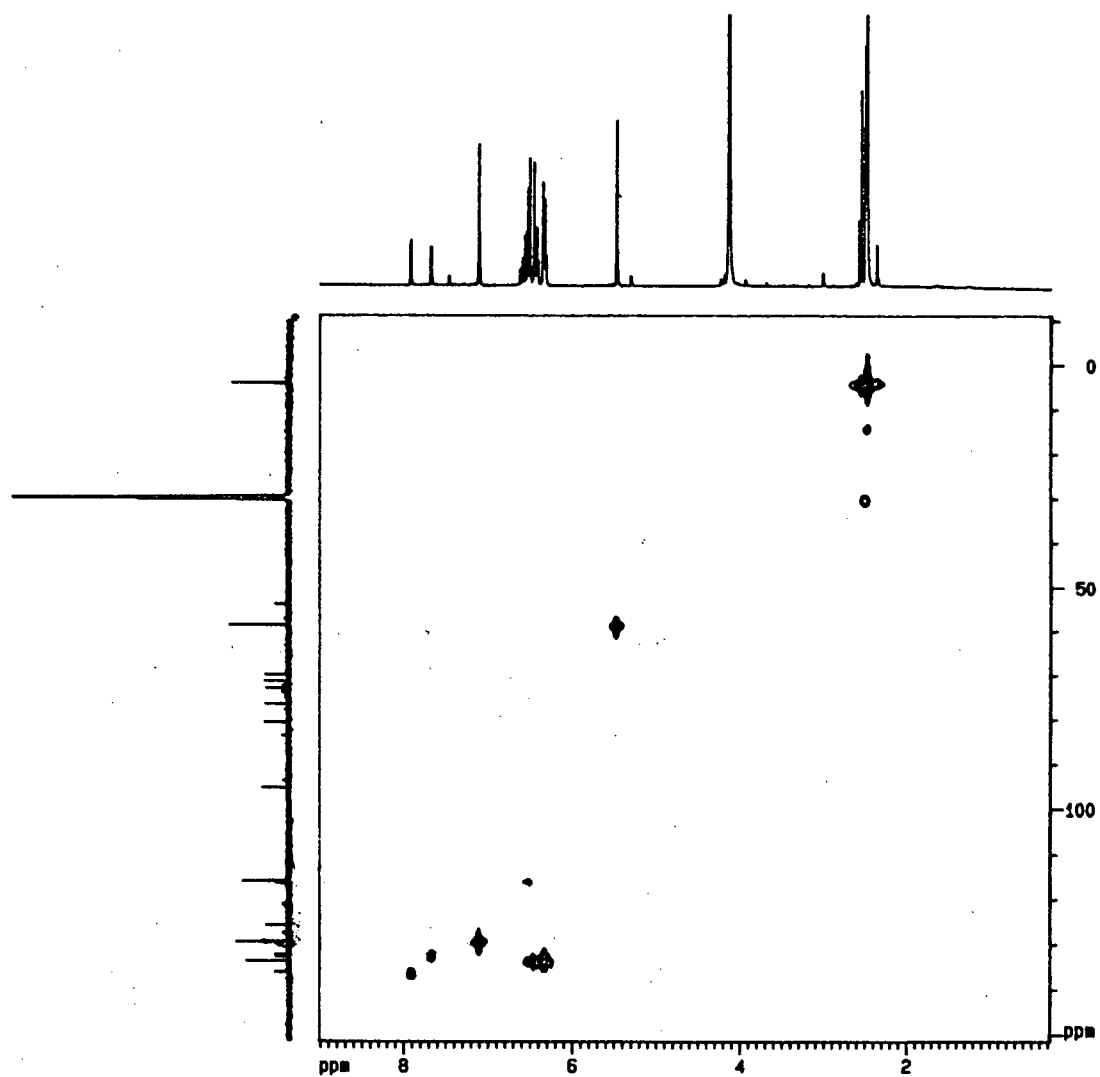
APPENDIX 3A: HMBC SPECTRUM OF PHOTOINTERMEDIATE MIXTURE OF THIARUBRINE A (1)
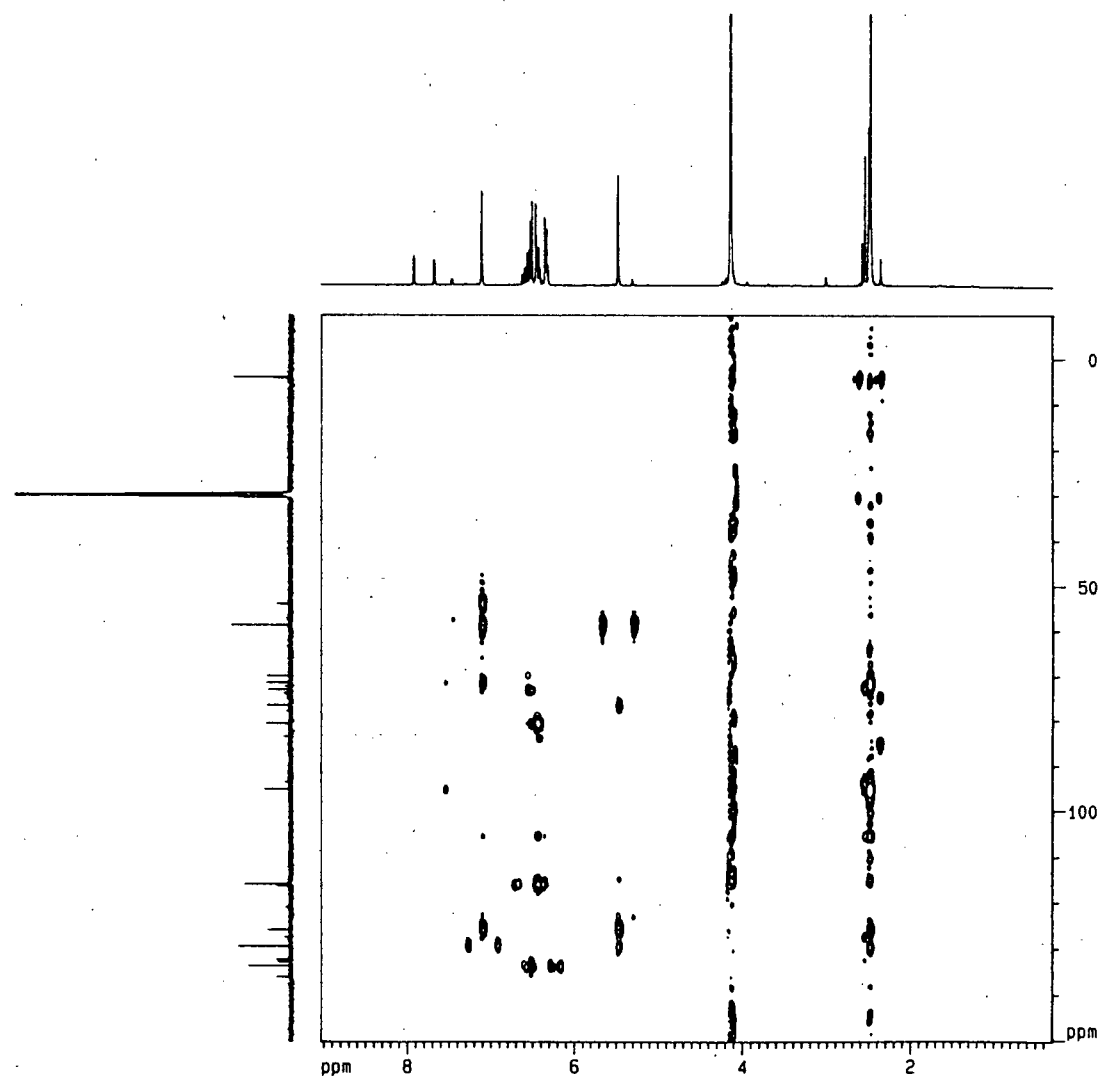
APPENDIX 3B: UV-VIS SPECTRUM OF PHOTOSULFIDES 2A AND 2B
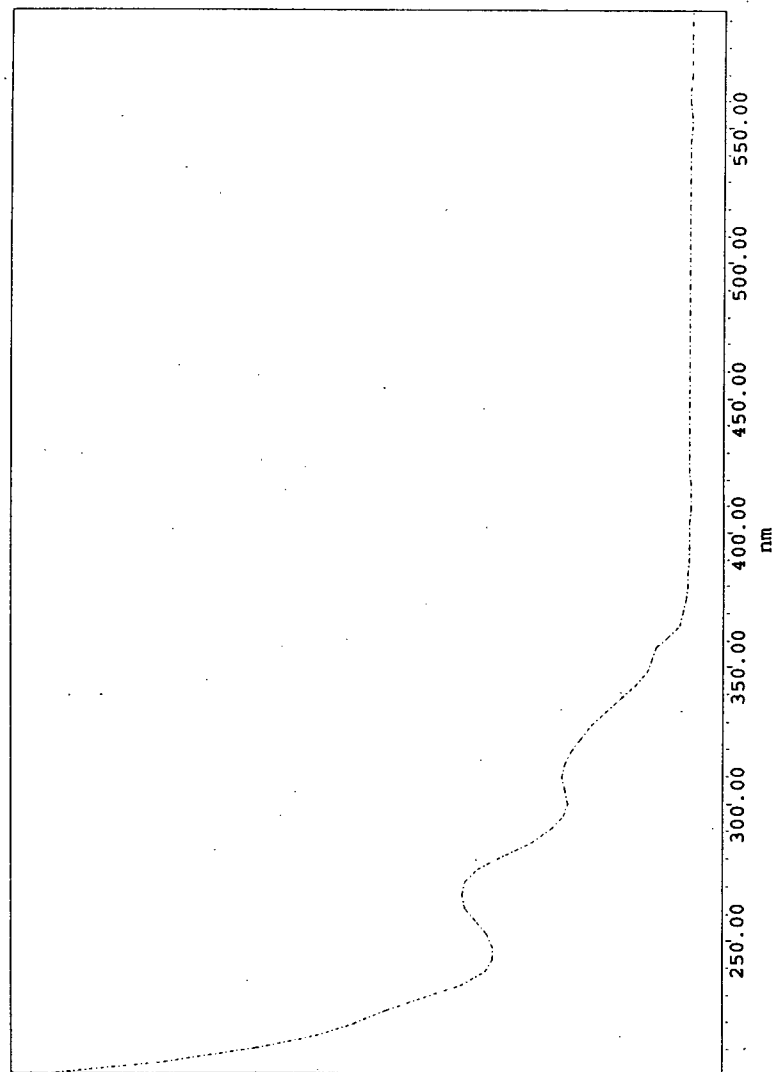
APPENDIX 3B: ^{1}H-NMR SPECTRUM OF PHOTOINTERMEDIATE MIXTURE OF THIARUBRINE B (2)
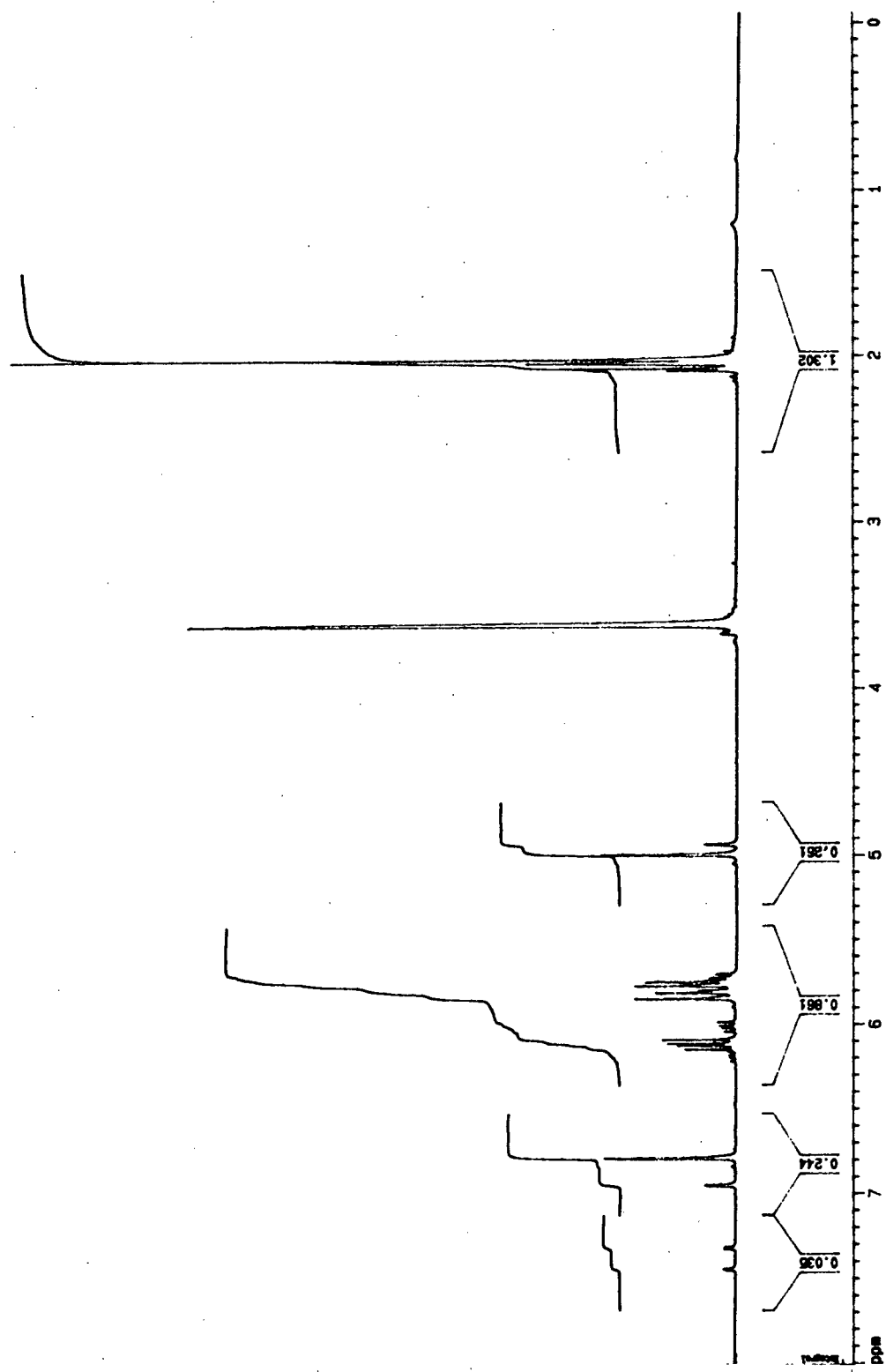
APPENDIX 3B: ^{13}C-NMR SPECTRUM OF PHOTOINTERMEDIATE MIXTURE OF THIARUBRINE B (2)
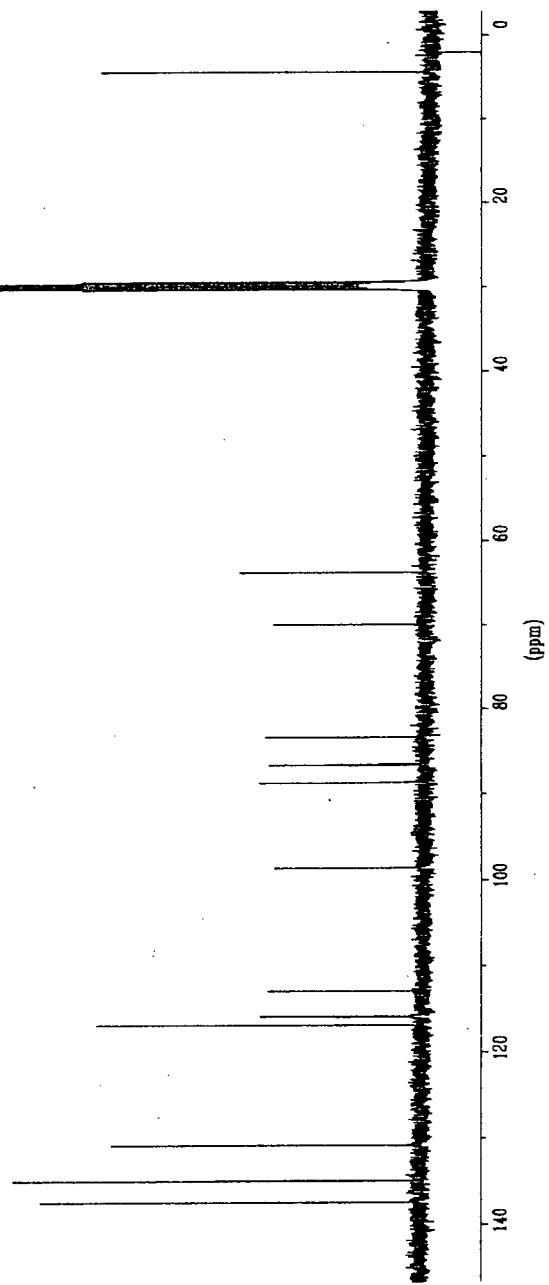
APPENDIX 3B: HMQC SPECTRUM OF PHOTOINTERMEDIATE MIXTURE OF THIARUBRINE B (2)

APPENDIX 3B: HMBC SPECTRUM OF PHOTOINTERMEDIATE MIXTURE OF THIARUBRINE B (2)
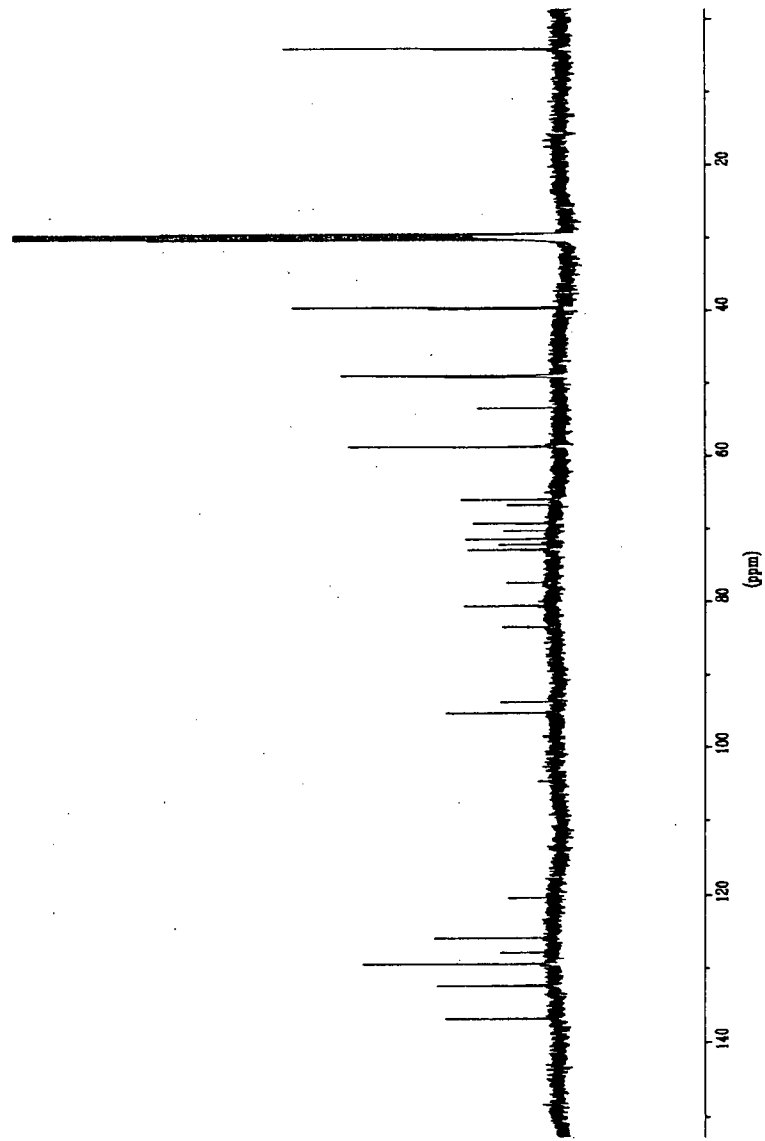
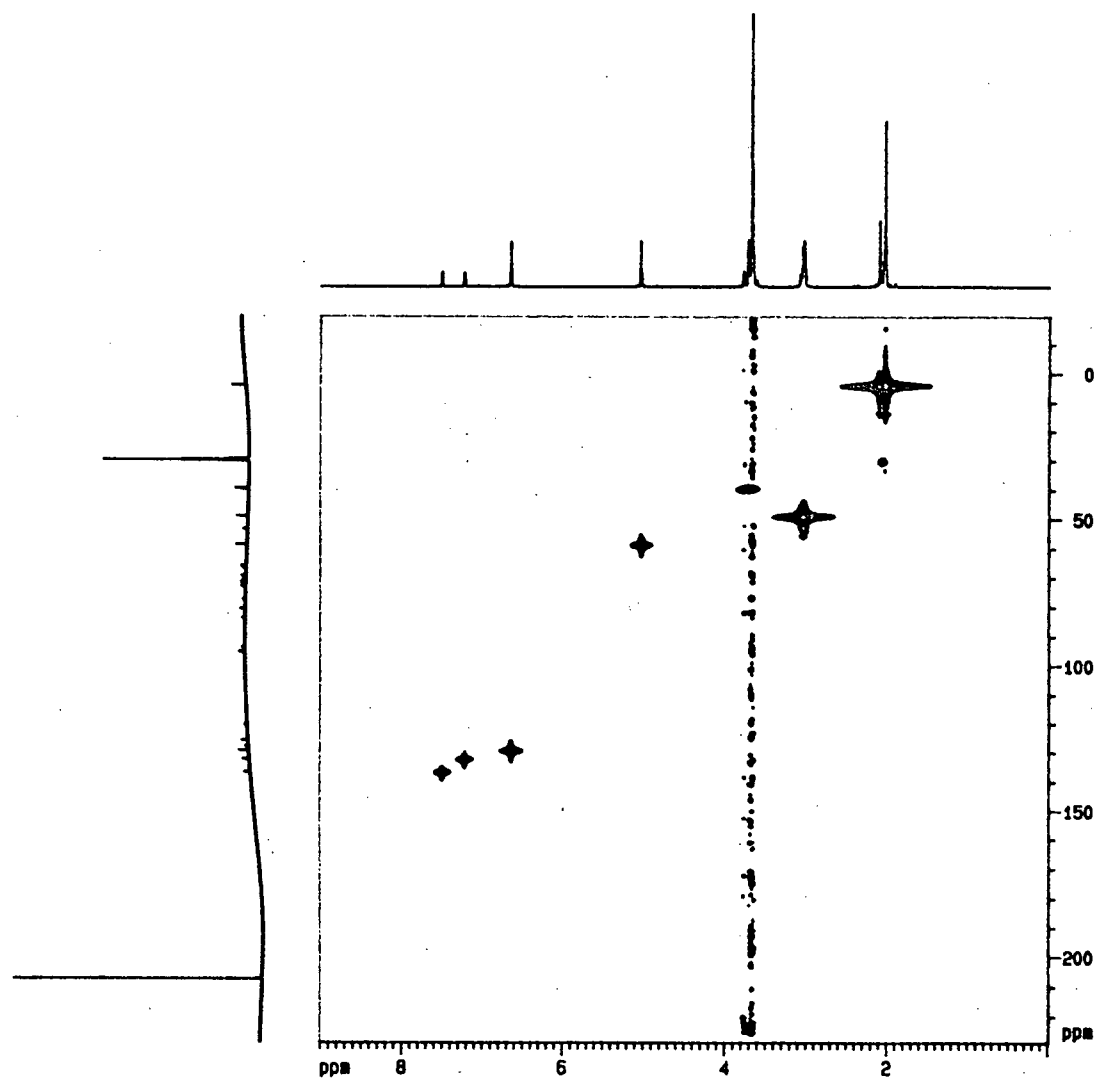## APPENDIX 3C: UV-VIS SPECTRUM OF PHOTOSULFIDES 3A AND 3B

APPENDIX 3C: ¹H-NMR SPECTRUM OF PHOTOINTERMEDIATE MIXTURE OF THIARUBRINE D (3)
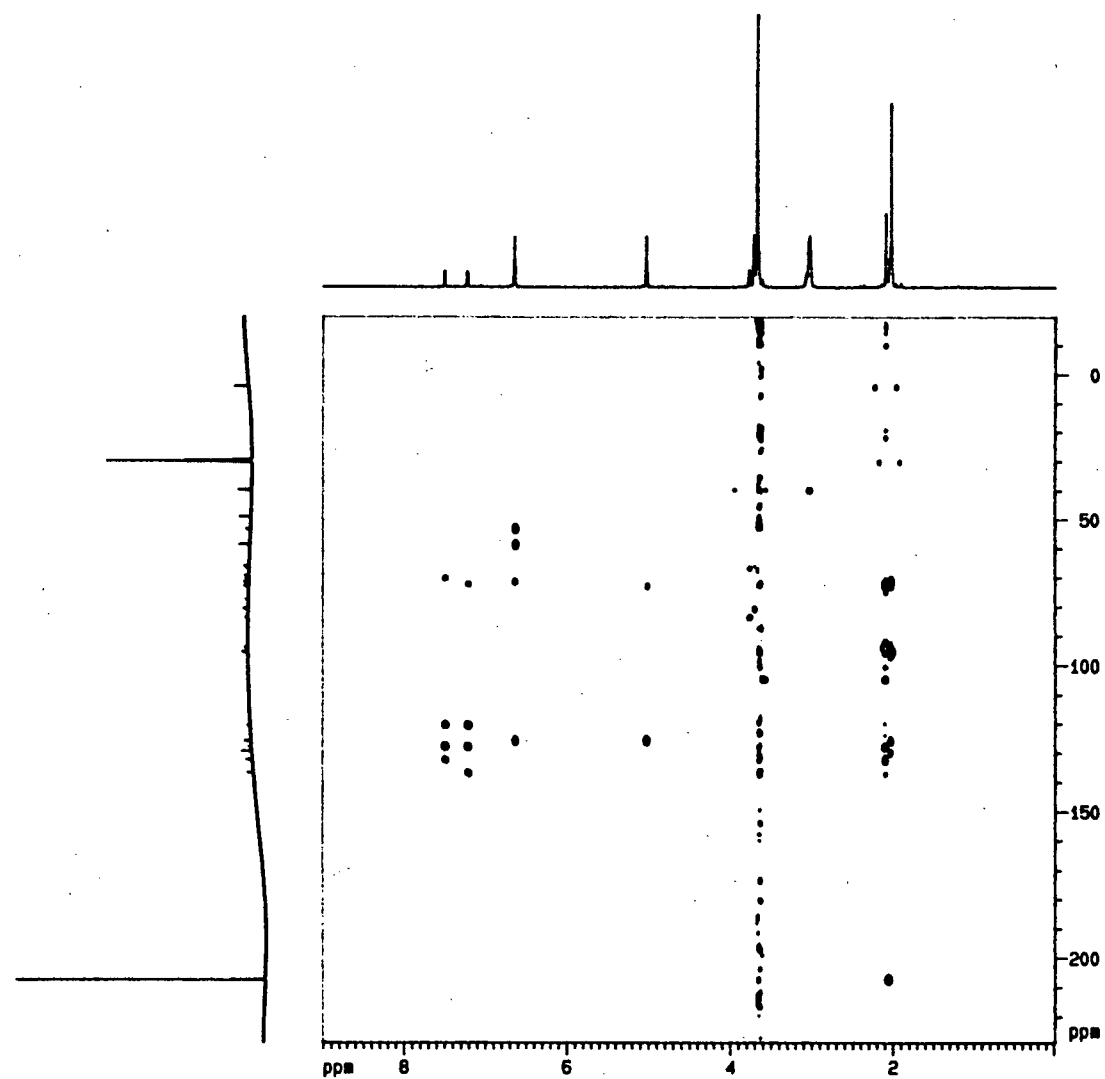
APPENDIX 3C: ^{13}C-NMR SPECTRUM OF PHOTOINTERMEDIATE MIXTURE OF THIARUBRINE D (3)
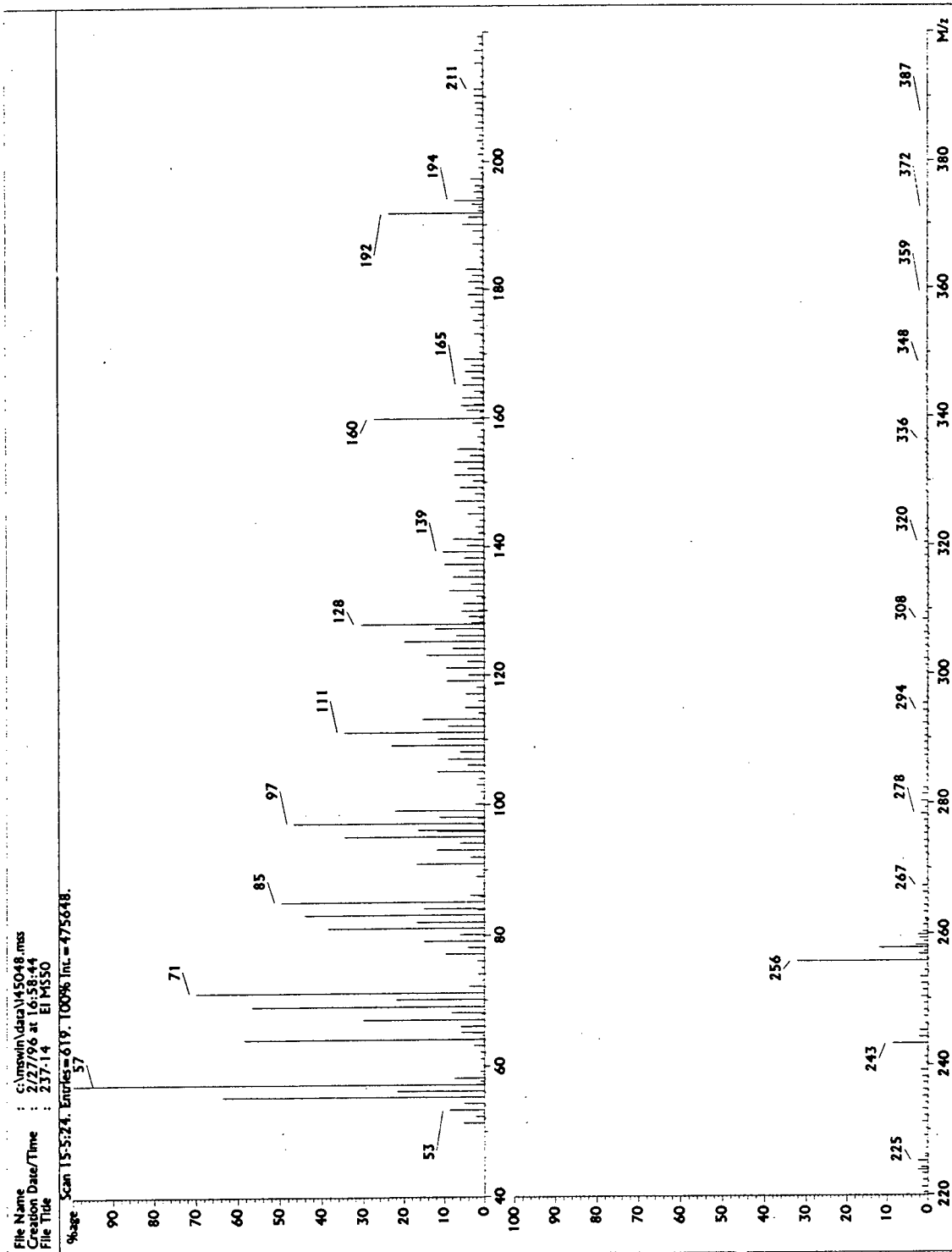
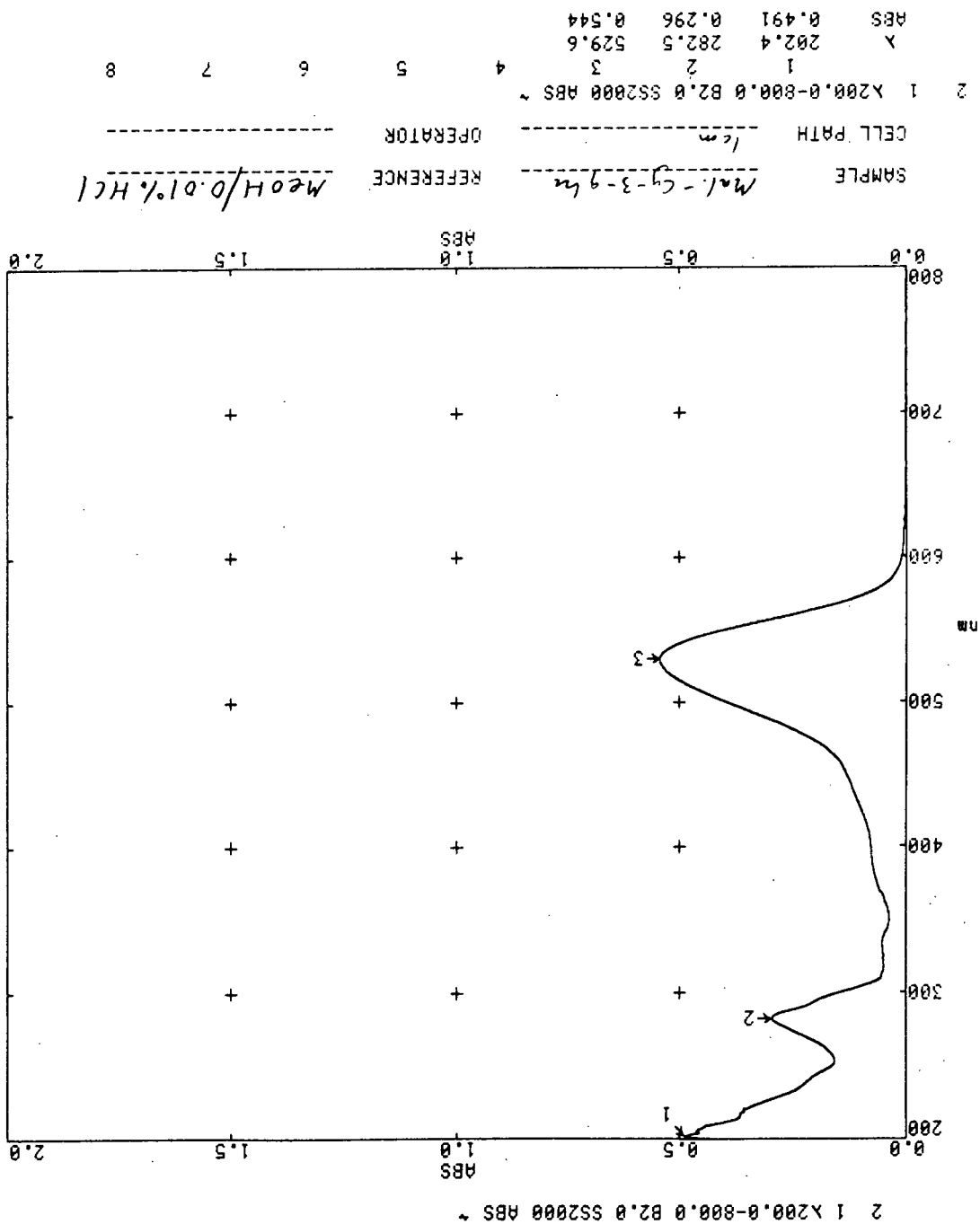# APPENDIX 3C: HMQC SPECTRUM OF PHOTOINTERMEDIATE MIXTURE OF THIARUBRINE D (3)

APPENDIX 3C: HMBC SPECTRUM OF PHOTOINTERMEDIATE MIXTURE OF THIARUBRINE B (3)

APPENDIX 4: LOW-RESOLUTION EI-MASS SPECTRUM OF CYCLOOCTASULFUR (24)

File Name : c:\mswin\data\V4SO48.mss
Creation Date/Time : 2/27/96 at 16:58:44
File Title : 237-14   EI MSSO

APPENDIX 5A: UV-VIS SPECTRUM OF CYANIDIN 3-O-(6-O-MALONYL-β-D-GLUCOPYRANOSIDE) (25)

SAMPLE   Mal-Cy-3-glu   REFERENCE   MeOH/0.01% HCl

CELL PATH   1cm   OPERATOR

2 1 X200.0-800.0 B2.0 SS2000 ABS

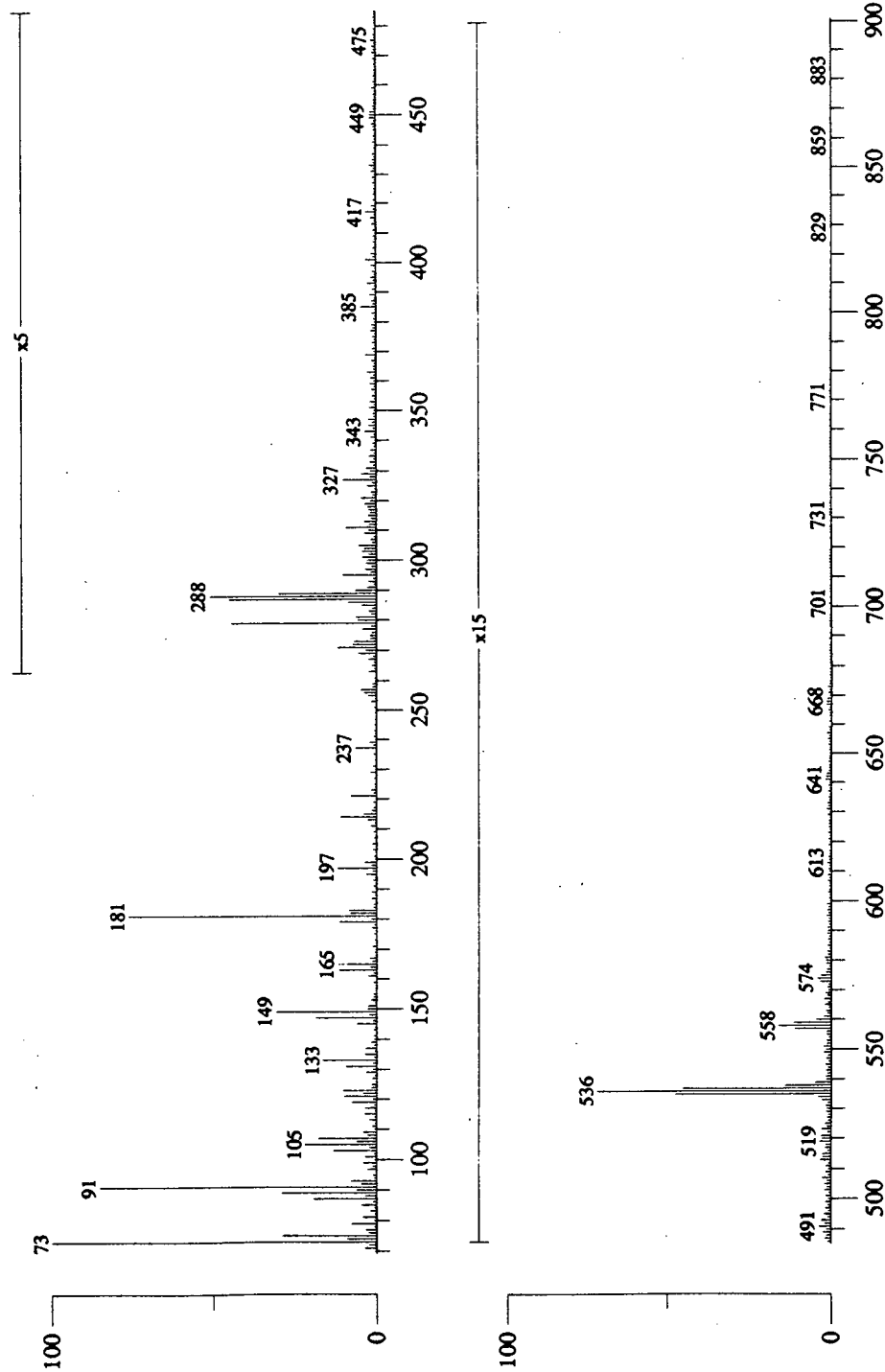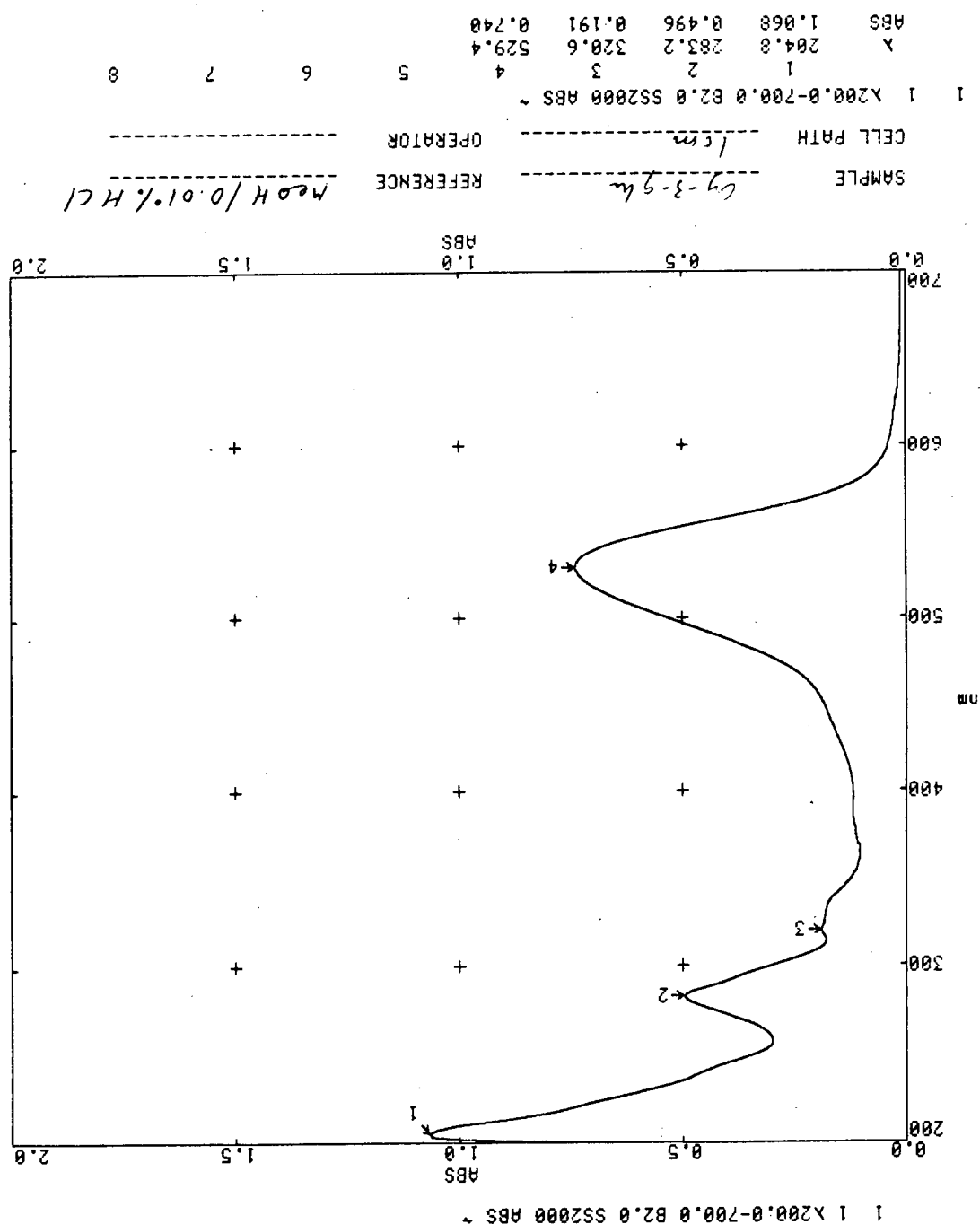|	λ	ABS
1	282.4	0.491
2	282.5	0.296
3	529.6	0.544

2 1 X200.0-800.0 B2.0 SS2000 ABS

APPENDIX 5A: ¹H-NMR SPECTRUM OF CYANIDIN 3-O-(6-O-MALONYL-β-D-GLUCOPYRANOSIDE) (25)

APPENDIX 5A: FAB MASS SPECTRUM OF CYANIDIN 3-*O*-(6-*O*-MALONYL-β-D-GLUCOPYRANOSIDE) (25)
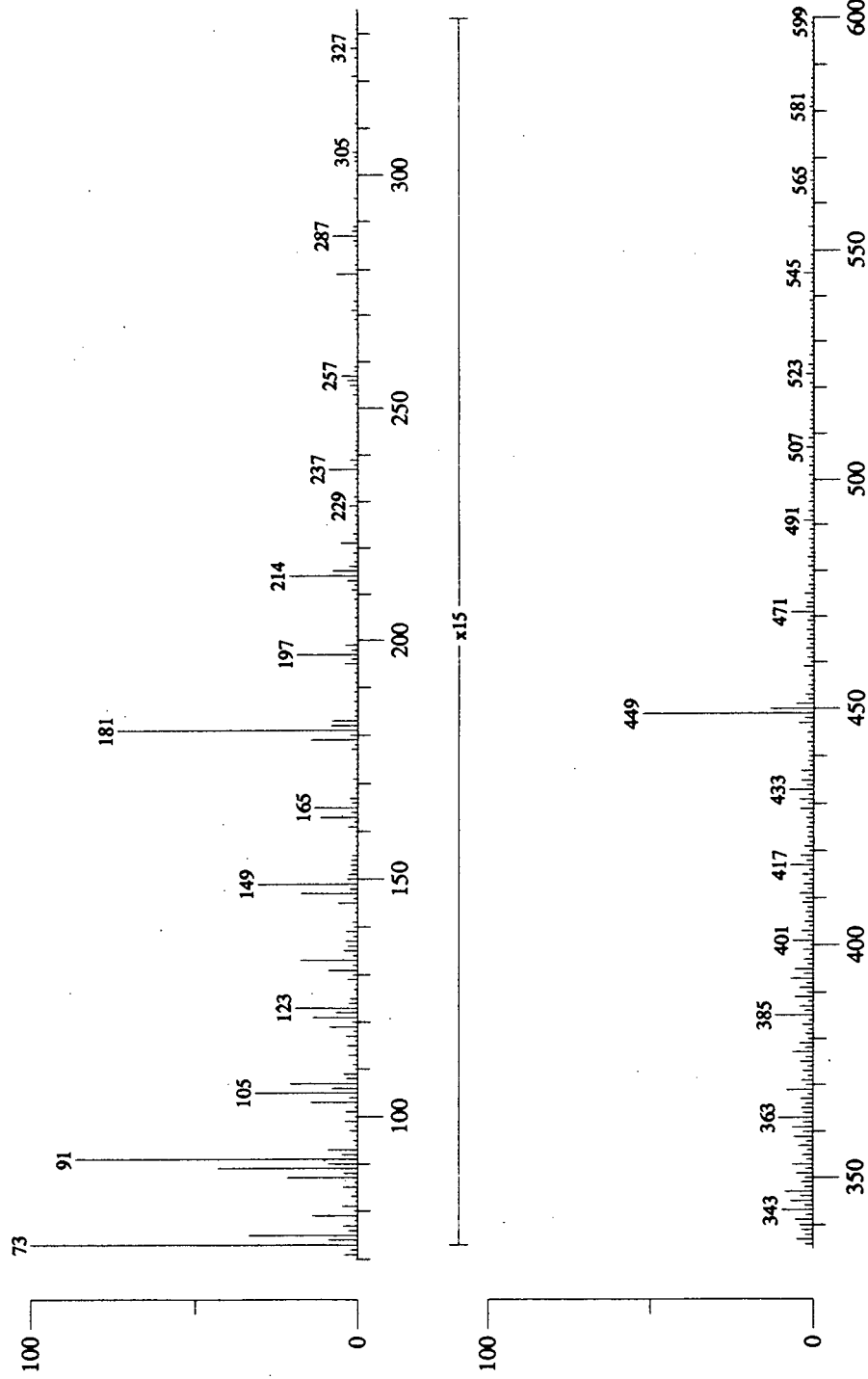
lsims3647 Scan 1   (Av 7-11 Acq)   100%=134255 mv 21 May 97 9:36
LRP +LSIMS SL 7778   * Matrix: Thioglycerol

APPENDIX 5B: UV-VIS SPECTRUM OF CYANIDIN 3-O-(β-D-GLUCOPYRANOSIDE) (26)

	1	2	3	4	5	6	7	8
λ	204.8	283.2	320.6	529.4				
ABS	1.068	0.496	0.191	0.740				

1 1 X200.0-700.0 B2.0 SS2000 ABS ^

SAMPLE   Cy-3-glu
CELL PATH   1 cm
REFERENCE   MeOH/0.01% HCl
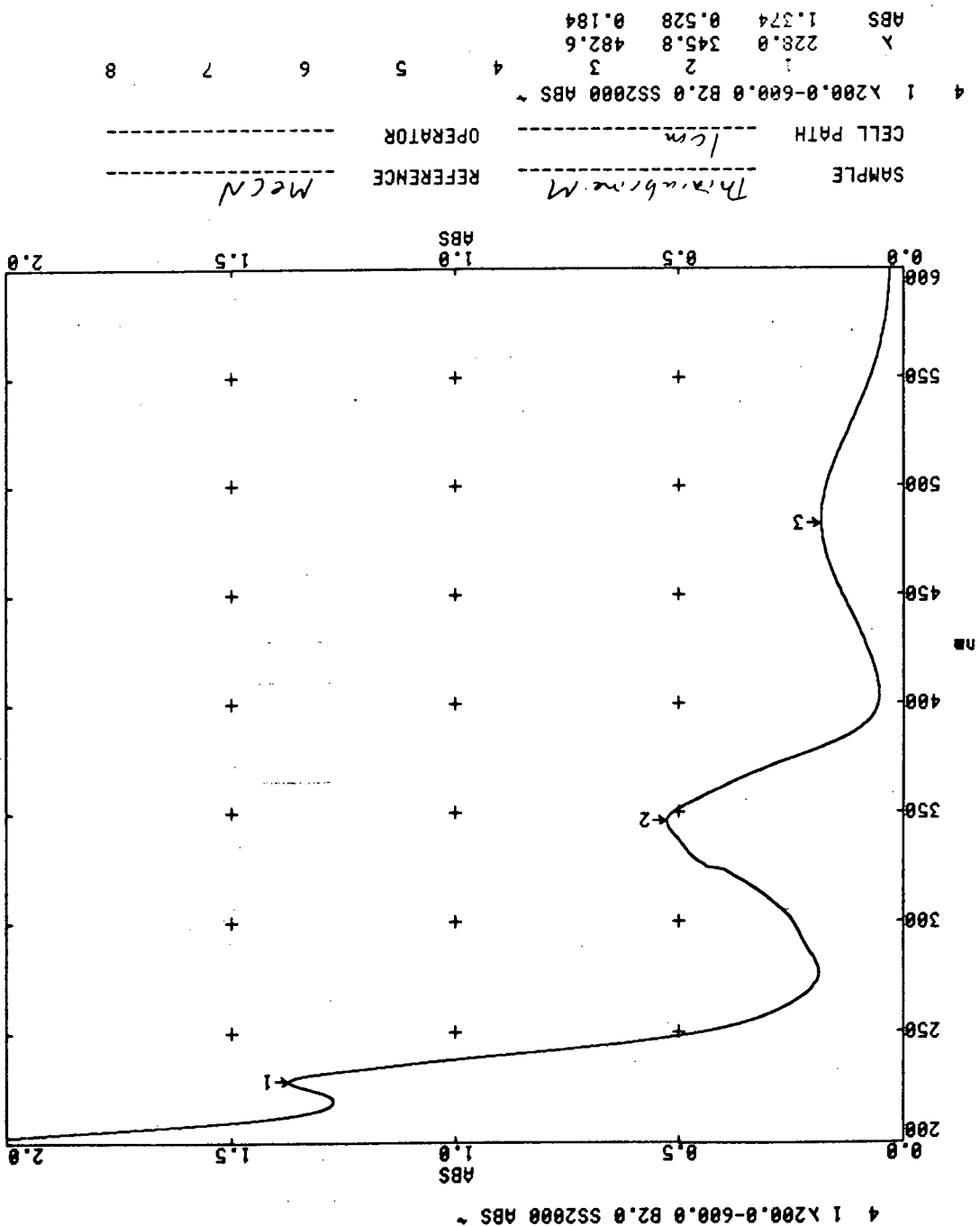OPERATOR   ------------------

APPENDIX 5B: ¹H-NMR SPECTRUM OF CYANIDIN 3-O-(β-D-GLUCOPYRANOSIDE) (26)

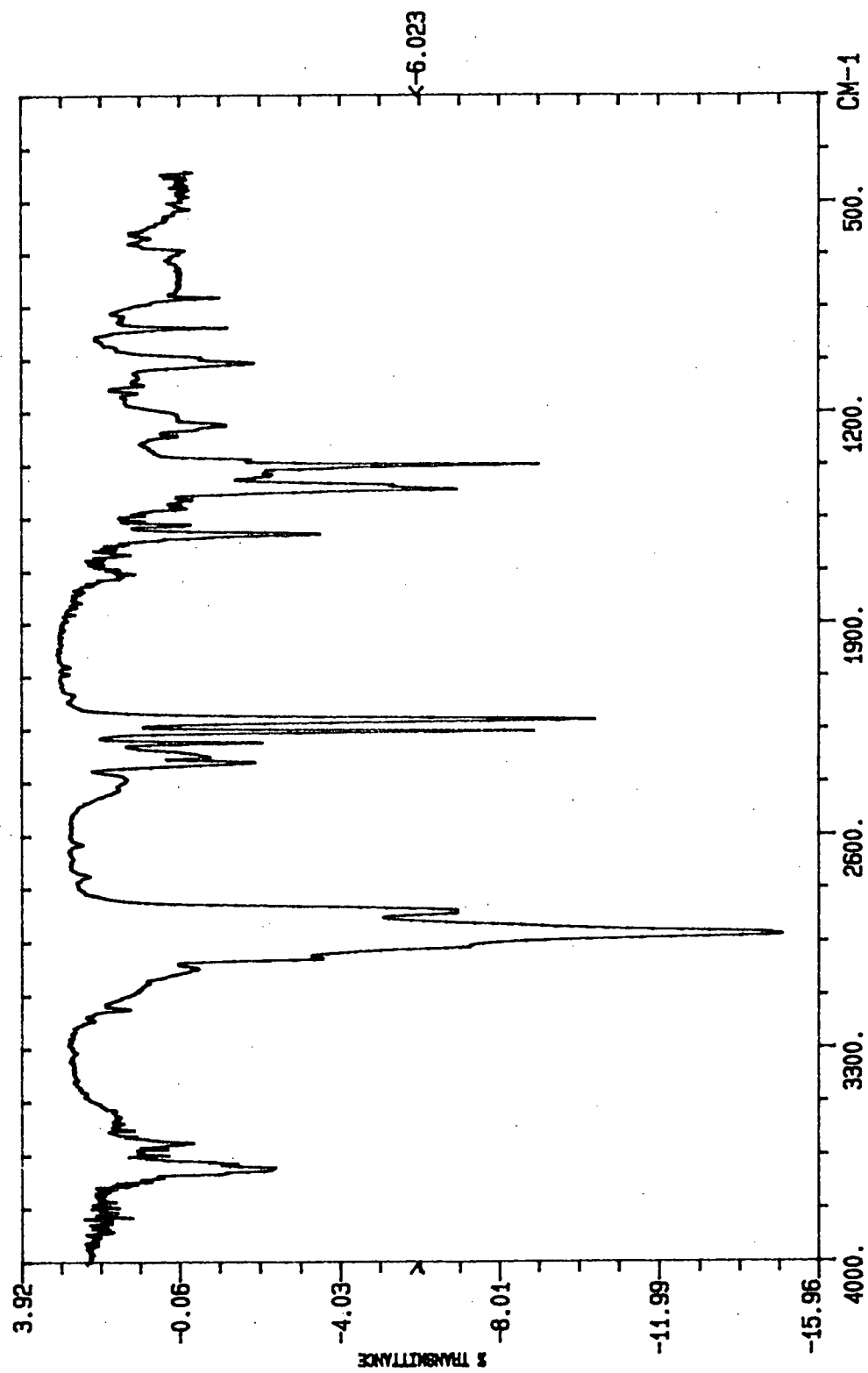# APPENDIX 5B: FAB MASS SPECTRUM OF CYANIDIN 3-O-(β-D-GLUCOPYRANOSIDE) (26)

lsims3897 Scan 1   (Av 10-20 Acq) 100%=70308 mv 5 Jun 97 10:53
LRP +LSIMS SL 7827   * Matrix: Thioglycerol
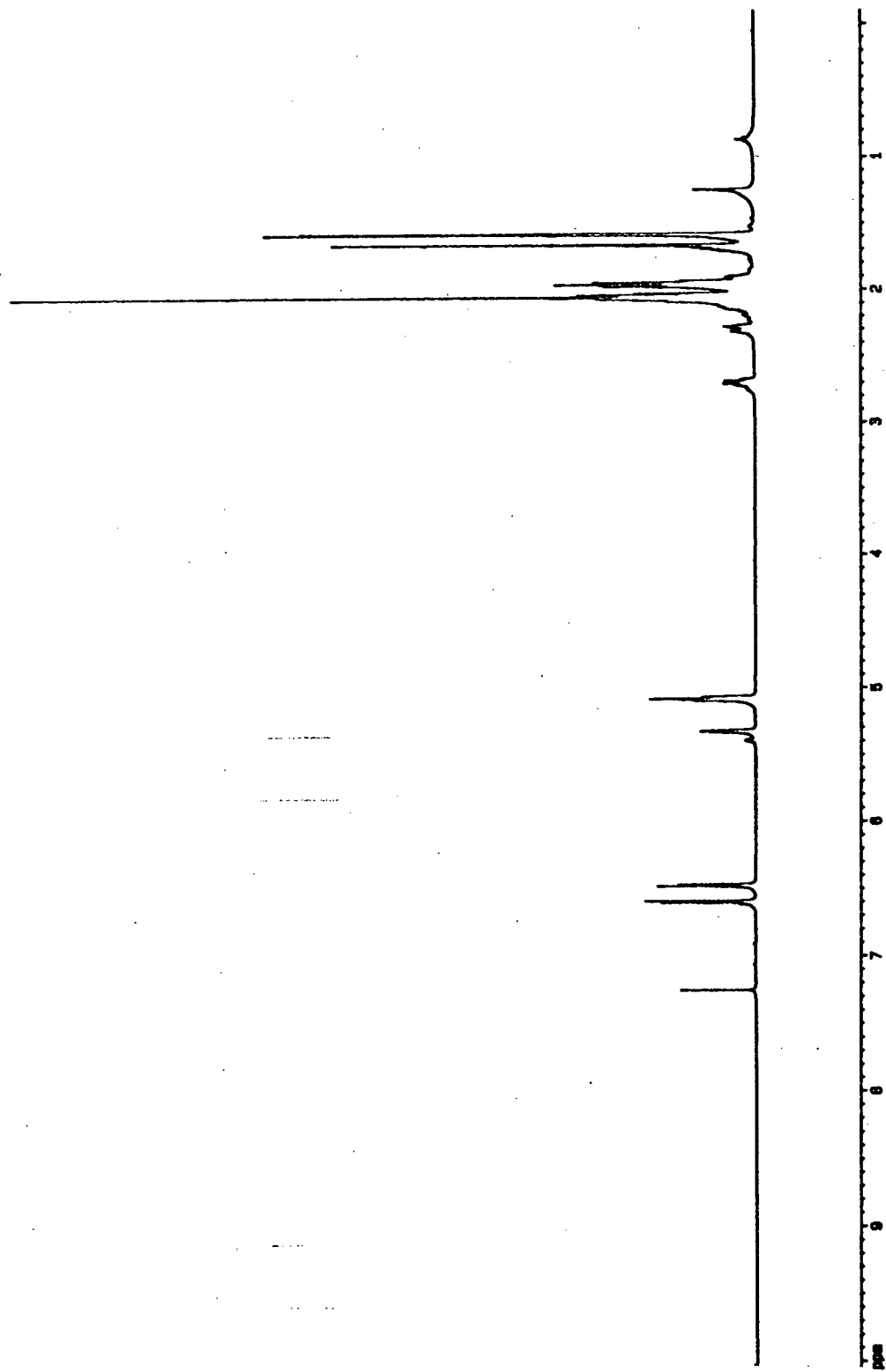
APPENDIX 6: UV-VIS SPECTRUM OF THIARUBRINE M (27)

	1	2	3	4	5	6	7	8
λ	228.0	345.8	482.6					
ABS	1.374	0.528	0.184					

4 1 X200.0-600.0 B2.0 SS2000 ABS

SAMPLE _Thiarubrine M_
REFERENCE _MeCN_
CELL PATH _1cm_
OPERATOR _____

4 1 X200.0-600.0 B2.0 SS2000 ABS
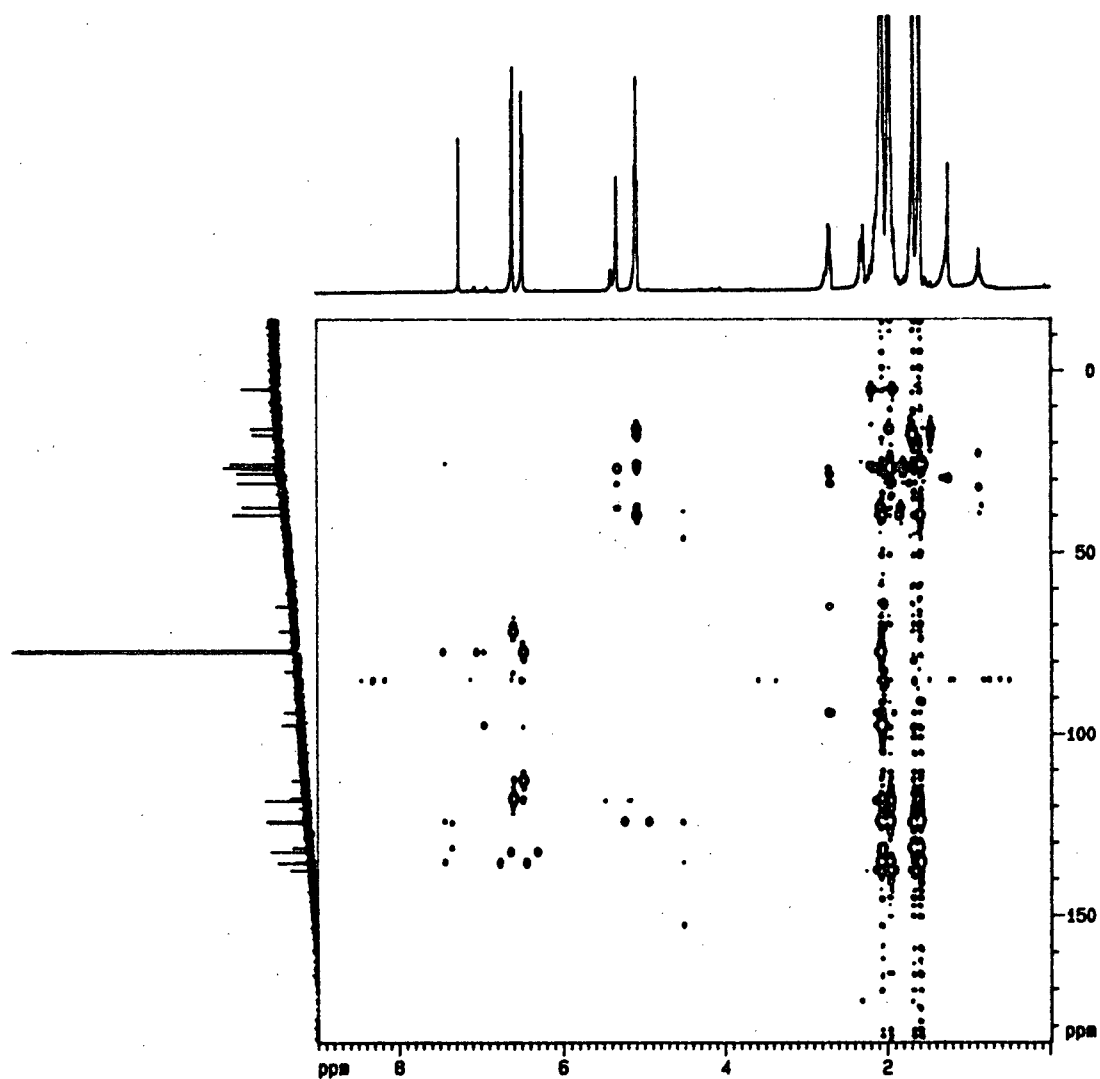
APPENDIX 6: IR SPECTRUM OF THIARUBRINE M (27)
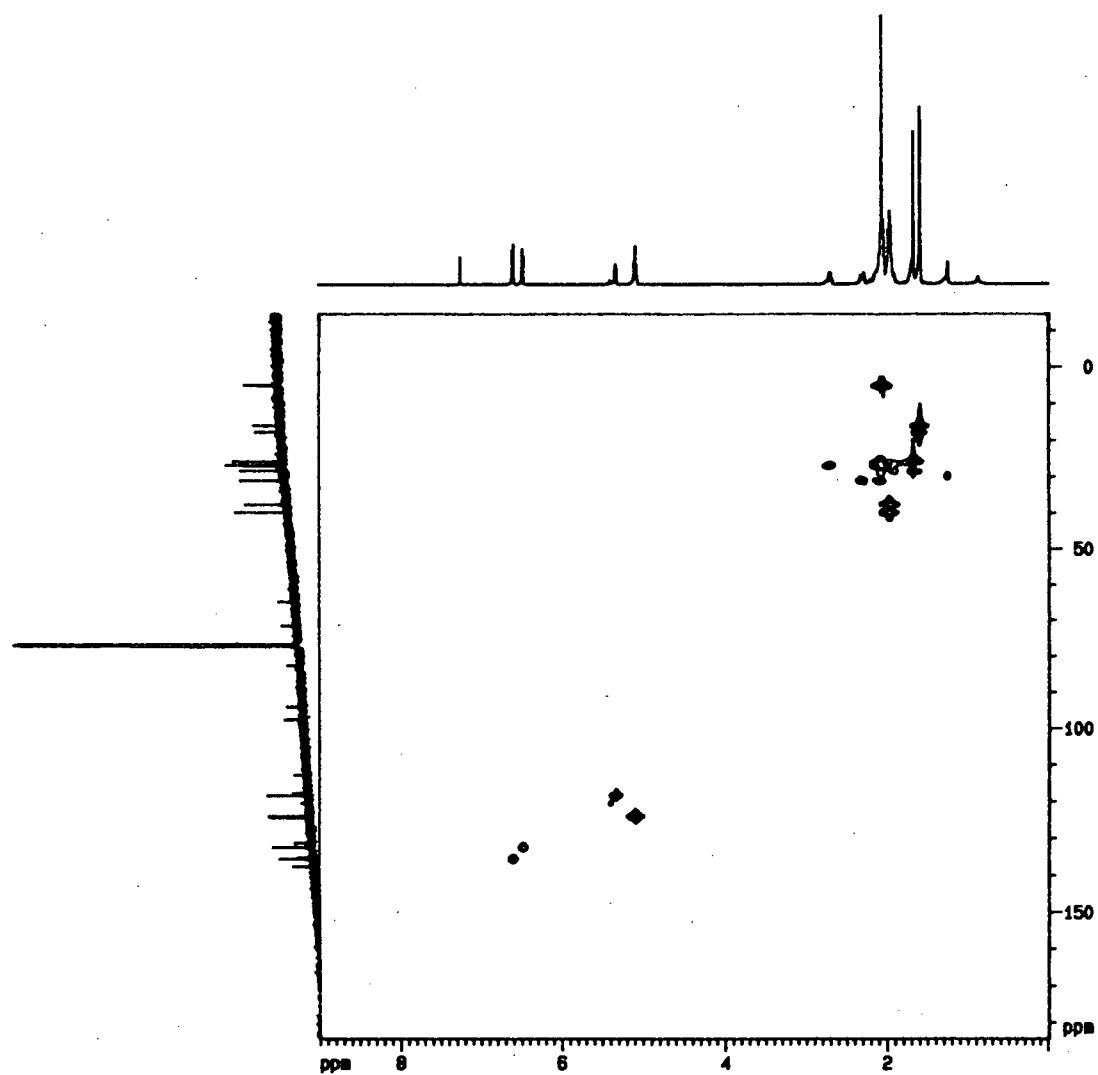
APPENDIX 6: ^{1}H-NMR SPECTRUM OF THIARUBRINE M (27)

APPENDIX 6: ^{13}C-NMR SPECTRUM OF THIARUBRINE M (27)

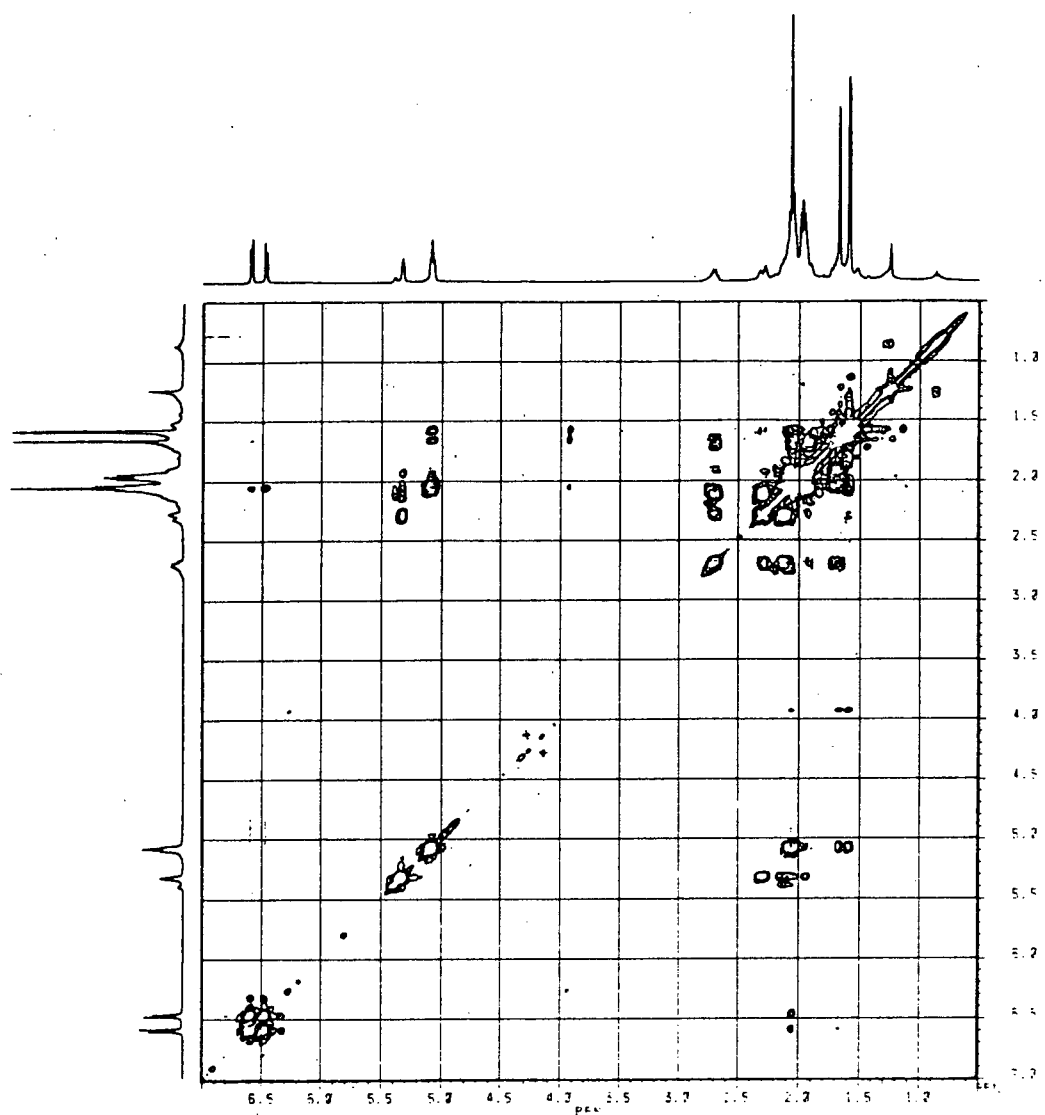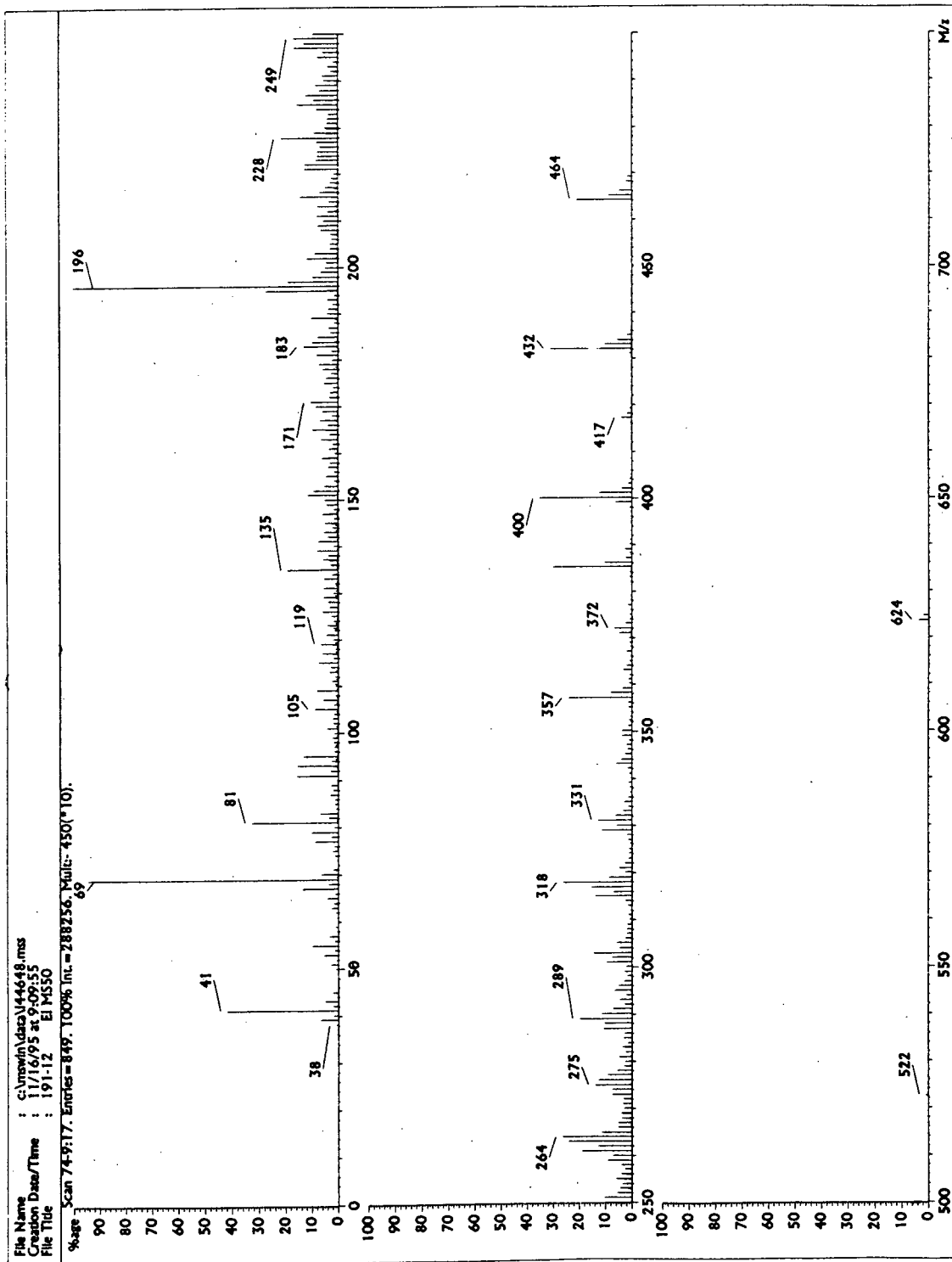# APPENDIX 6: HMQC SPECTRUM OF THIARUBRINE M (27)

APPENDIX 6: HMBC SPECTRUM OF THIARUBRINE M (27)

APPENDIX 6: ^{1}H-^{1}H COSY SPECTRUM OF THIARUBRINE M (27)

APPENDIX 6: LOW RESOLUTION EI MASS SPECTRUM OF THIARUBRINE M (27)

File Name : c:\mswin\data\V44648.mss
Creation Date/Time : 11/16/95 at 9:09:55
File Title : 191-12 EI MS50

Scan 74-9:17. Entries=849. 100% Int.=288256. Mult: 450(*10).

APPENDIX 6: HIGH RESOLUTION EI MASS SPECTRUM OF THIARUBRINE M
(27)

Mass	%age	Calculated	ppm	mmu	R/DB	Formula
					ATOMIC COMPOSITION REPORT	
433.19488	4.38	433.20236	17.3	7.5	14.5	$C_{28}H_{33}S_2$
432.19238	13.67	432.19455	5.0	2.2	15.0	$C_{28}H_{32}S_2$
		432.19791	12.8	5.5	11.0	$C_{25}H_{36}S_3$
		432.20129	20.6	8.9	7.0	$C_{22}H_{40}S_4$
431.18927	0.48	431.19009	1.9	0.8	11.5	$C_{25}H_{35}S_3$
		431.18671	-5.9	-2.6	15.5	$C_{28}H_{31}S_2$
		431.19345	9.7	4.2	7.5	$C_{22}H_{39}S_4$
420.17787	0.41	----- No match found -----				
418.18447	0.42	418.18564	2.8	1.2	7.0	$C_{21}H_{38}S_4$
		418.18228	-5.2	-2.2	11.0	$C_{24}H_{34}S_3$
		418.17889	-13.3	-5.6	15.0	$C_{27}H_{30}S_2$
417.18125	0.79	417.17783	-8.2	-3.4	7.5	$C_{21}H_{37}S_4$
		417.17444	-16.3	-6.8	11.5	$C_{24}H_{33}S_3$
		417.18881	18.1	7.6	14.5	$C_{27}H_{29}O_2S$
417.16725	1.29	417.17108	9.2	3.8	15.5	$C_{27}H_{29}S_2$
		417.17444	17.2	7.2	11.5	$C_{24}H_{33}S_3$
417.00146	0.51	417.00439	7.0	2.9	24.5	$C_{26}H_9O_2S_2$
		417.00778	15.2	6.3	20.5	$C_{23}H_{13}O_2S_3$
		417.01114	23.2	9.7	16.5	$C_{20}H_{17}O_2S_4$
414.15785	0.21	414.15433	-8.5	-3.5	9.0	$C_{21}H_{34}S_4$
		414.15097	-16.6	-6.9	13.0	$C_{24}H_{30}S_3$
		414.16534	18.1	7.5	16.0	$C_{27}H_{26}O_2S$