THE QUINONOID PIGMENTS OF THE LICHENS *NEPHROMA LAEVIGATUM* AND *HETERODERMIA OBSCURATA*

by

PETER ALTMAN COHEN

B.G.S., The University of Maryland (College Park), 1984
M.Sc., The University of Washington (Seattle), 1989

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE STUDIES

(Department of Botany)

We accept this thesis as conforming
to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

June 1995

In presenting this thesis in partial fulfilment of the requirements for an advanced degree at the University of British Columbia, I agree that the Library shall make it freely available for reference and study. I further agree that permission for extensive copying of this thesis for scholarly purposes may be granted by the head of my department or by his or her representatives. It is understood that copying or publication of this thesis for financial gain shall not be allowed without my written permission.

(Signature)

Department of BOTANY

The University of British Columbia
Vancouver, Canada

Date August 25th 1995

DE-6 (2/88)

ABSTRACT

Field samples of the foliose lichens *Nephroma laevigatum* Ach. and *Heterodermia obscurata* (Nyl.) Trevis. were analyzed for anthraquinone and anthraquinone-like pigments. Both lichens were found to contain emodin, 7-chloroemodin and 7,7'-dichlorohypericin. In addition, the *N. laevigatum* specimen contained 7-chloro-1-*O*-methylemodin, 7-chloro-1-*O*-methyl-ω-hydroxyemodin (7-chlorocarviolin) and 2,2', 7,7'-tetrachlorohypericin, while the *H. obscurata* sample contained 5,7-dichloroemodin, flavoobscurin A and flavoobscurin B. Laboratory incubation studies with *N. laevigatum* (in the presence of sodium [1-^{13}C]acetate) also revealed the formation of 5-chloroemodin, 5-chloro-1-*O*-methylemodin, 5-chloro-ω-hydroxyemodin and 5-chloro-1-*O*-methyl-ω-hydroxyemodin (5-chlorocarviolin). These compounds had not been identified in any previous examination of field-collected lichen, and 5-chloro-1-*O*-methylemodin, although known from a fungus, has not been reported to occur in any lichen. The structures of the compounds were determined by a combination of UV, MS, ^{1}H NMR and ^{13}C NMR spectral methods.

Feeding experiments with sodium [2-^{14}C]acetate and sodium [1-^{13}C]acetate demonstrated that anthraquinones are biosynthesized in *Nephroma laevigatum* through the polyketide pathway, analogous to the pathways operating in fungi and higher plants. The lichen was also capable of chlorinating endogenous anthraquinones during incubation with sodium ^{36}chloride.

Biohalogenation experiments with a partially purified lichen chloroperoxidase and a commercially available fungal chloroperoxidase demonstrated enzyme-substrate specificity with respect to the production of different chlorinated anthraquinones. 5,7-Dichloroemodin was produced, in excellent yield, from 7-chloroemodin by the commercial enzyme; the lichen chloroperoxidase, however, yielded only 7-chloroemodin from emodin and did not further chlorinate 7-chloroemodin. 5-Chloroemodin was a product from both the control and commercial enzyme reactions.

Twelve lichen anthraquinones, bianthrones, hypericin derivatives, and synthetic hypericin were tested for their virucidal activity in end-point CPE (viral cytopathic effects) and plaque assays with herpes simplex virus type 1 (HSV-1). Emodin, 7-chloroemodin, 7-chloro-1-*O*-methylemodin, 5,7-dichloroemodin, hypericin and 7,7'-dichlorohypericin exhibited fair to good antiviral activity in the presence of light. In the plaque assay, 5,7-dichloroemodin, hypericin and 7,7'-dichlorohypericin completely inhibited the virus at a concentration of 1.0 μg/ml. Only hypericin was active at 0.01 μg/ml. The other anthraquinones and bianthrones were inactive at a concentration of 5.0 μg/ml.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ACKNOWLEDGEMENTS

The author extends sincere thanks to Professor Neil Towers for his kindness, invaluable guidance, advice and criticism in all matters pertaining to the research performed, and scientific results published, under his direction, as well as to Dr. James Hudson for his kindness and scientific support in doing all the antiviral work and assisting in its interpretation. The author also thanks Dr. Michael Adam, Professor Raymond Andersen, Professor Peter Hochachka, and Professor Alan Lewis for their patience, kindness, advice and scientific support during the course of the author's stay in the Department of Botany, and in the production of this thesis. Finally, I thank the external and university examiners for their critical evaluations of this thesis.

Financial assistance rendered during the course of this work by the Natural Sciences and Engineering Research Council of Canada, and by the Department of Botany, UBC is gratefully acknowledged.

The author wishes to record his appreciation to the Chemistry Department of UBC and the Laboratory of Chemistry, NIDDK, National Institutes of Health (Bethesda, Maryland, USA) for their assistance in recording NMR and mass spectra.

Finally, the author extends his deepest appreciation to his parents for their love, constant and generous support, and invaluable guidance, as well as to Xiaoqiu Tang for her strong spirit, selflessness, constant encouragement and love.

FOREWORD

The objectives of the research performed over the last three years, as described in this thesis, were to: 1) contribute to the extant knowledge of the chemistry of lichens, in particular, pigment-containing lichens such as *Nephroma laevigatum* and *Heterodermia obscurata*; 2) establish the ubiquity of the polyketide pathway as the primary source of anthraquinones in all major classes of organisms, including lichens; 3) examine the role of chlorination in *Nephroma laevigatum*, by the isolation and purification of a chlorinating enzyme (chloroperoxidase), and the study of the comparative properties of the lichen enzyme and a fungal chloroperoxidase, in a series of in vitro chlorination experiments with putative precursors of chlorinated anthraquinones in *N. laevigatum*; and 4) examine the virucidal properties of several structurally diverse lichen anthraquinonoid natural products in two assays, using HSV-1 (herpes simplex virus type 1).

CHAPTER 1: OVERVIEW

1.1 LICHEN CHEMISTRY AND CHEMOTAXONOMY

Lichens consist of symbiotic associations between algae and fungi, with approximately 20,000 species distributed throughout the world, in all types of ecosystems (Ahmadjian and Hale, 1973) . Traditionally, the study of lichens has relied on morphological and chemical characters as tools in their taxonomic classification; in fact, lichenology can claim one of the earliest uses of chemistry as a taxonomic tool in any of the botanical and mycological subfields (Elix, 1992). More recent developments in cell biology and molecular biology, which have been established in mycology for some time, are beginning to make themselves felt in lichenology, augmenting traditional taxonomic and physiological approaches.

The use of chemical methods in lichen taxonomy is not without controversy. While there are few detractors with respect to the inclusion of chemical spot tests (and thin-layer chromatography or TLC) in the set of criteria used to classify lichens, there are differing opinions regarding the relative importance of chemical criteria and how they should be interpreted (Brodo, 1986). The use of chemical criteria in classifying lichens began with W. Nylander in 1866, who investigated the reaction of lichen compounds with a variety of chemical reagents, producing characteristic colour changes (Nylander, 1866a, 1866b, 1866c). In 1907, W. Zopf published "Die Flechtenstoffe", describing his extensive chemical investigations of lichens (Zopf, 1907). Asahina (1937) proposed two principles for the application of chem-

ical methods to lichen classification: 1) two morphologically indistinguishable lichens, which are found to contain different metabolites under the same environmental conditions, should be regarded as different species; 2) the concentration ratio of two or more secondary metabolites may vary depending on the lichen habitat, and thus cannot be regarded as a reliable criterion in lichen taxonomic classification. In the intervening years, microchemical analyses of lichens have progressed to the modern field of lichen chemistry.

The debate dealing with the usefulness of chemistry in lichen classification has been thoroughly reviewed by S. Shibata (1964, 1973), M. E. Hale (1966, 1983), C.F. and W.L. Culberson (1969, 1970), D.L. Hawksworth (1976), I. Brodo (1986), R.S. Egan (1986), R. Rogers (1989), J. Poelt (1991), and J. Elix (1992). The most comprehensive treatment was that of Culberson and Culberson (1970), who systematically analyzed the distribution of 209 secondary metabolites in 2,315 species of lichens in order to discern chemical patterns that might be indicative of phylogenetic characteristics. The researchers reached several conclusions: 1) most lichen genera and families that are morphologically well-defined exhibit uniformity in their chemistry; 2) genera, which have atypical chemical patterns for the families in which they are members, exhibit affinities with other genera or families based on morphological data; 3) the highest degree of chemical variation occurs in the order Lecanorales, particularly with the systematically useful depside and depsidone classes of compounds; and 4) comparative phytochemistry of lichens is the most reliable method available to aug-

ment traditional morphological data in evaluating the "naturalness" of present systems of lichen classification.

Hale (1966), in one study, found a high degree of association between lichen secondary metabolites and particular morphological traits. He also suggests that there may exist a correlation between the degree of *O*-methylation of lichen depsides and depsidones and the structural complexity of the lichen genera containing them. According to this approach, he concluded that *Cladonia* represented the most advanced genus as it had the highest percentage of *O*-methylation; indeed, *Cladonia* is acknowledged by most lichenologists to be one of the most advanced lichen genera. Similarly, he cited the Sphaerophoraceae as an advanced family, and *Umbilicaria* as a "primitive" genus (the latter conclusion is also consistent with the current phylogenetic view). Large genera, such as *Parmelia*, contain sections that are chemically diverse (and may be undergoing speciation) and chemically uniform groups that may represent older stabilized lichen populations. Hale does recognize the limitations inherent in using a single chemical trait in assessing the taxonomic rank of lichens, and points out that such data should be used in conjunction with other chemical and morphological information

Brodo describes three fundamental problems in the application of chemistry to lichen taxonomy: practical problems, biological problems and philosophical problems. He emphasizes the importance of basic chemical techniques in systematics (spot tests and thin-layer chromatography), as well as the increasing recognition of the im-

portance of more rigorous methods, such as high performance liquid chromatography (HPLC), mass spectrometry (MS) and nuclear mag netic resonance spectroscopy (NMR). While the accessibility of even the "advanced" chemical instrumental methods (such as NMR spectroscopy and X-ray crystallography) has increased, the biological problems associated with the use of chemical criteria still remain. Brodo points out, for example, that some lichen compounds may be compartmentalized within the lichen, or may be produced during different stages of growth (Brodo, 1986). Culberson and Culberson (1958), in examining the genus *Lasallia*, found no variation in chemical composition during different stages of growth. The anthraquinones of *Heterodermia* are known to be restricted to the lower ecorticate surface of the lichens, while *Nephroma* anthraquinones are strictly medullary pigments. Obviously, chemical examinations that do not take into account the whole gamut of lichen macro and microstructures, as well as the varying growth stages, are incomplete. Furthermore, as Poelt (1991) has pointed out, "it is very difficult, and may even be impossible, to discriminate between homologies and analogies." Poelt and Leuckert (1993), in discussing the pitfalls of taxonomic classifications based on chemical patterns, provide numerous examples of compound substitution and supplementation in a wide range of lichen genera.

For example, some subtropical and tropical *Caloplaca* species contain usnic acid, rather than the usual assembly of anthraquinones: *Caloplaca cuyabensis* (Malme) Zahlbr. and *C. stenospora* (Malme) Zahlbr. are two such lichens. *Caloplaca*

variabilis (Pers.) Müll. Arg. appears not to contain simple anthraquinones at all, but rather greyish-violet amorphous pigments which may bear a biosynthetic relationship to anthraquinones. Krog (1970) reported the occurrence of a sample of *Solorina crocea* (L.) Ach., in Finland, whose lower surface was completely devoid of the expected solorinic acid and related anthraquinones. How does one deal with *Acroscyphus sphaerophoroides* Lév., in which no less than five major structural groups of compounds were reported (Shibata et al., 1968) ? *A. sphaerophoroides* is described as having morphological similarities to another member of the Caliciales, *Thelomma*, which has a very different chemistry (Tibell, 1984). Yet, *Acroscyphus* contains chemical products of all the known lichen secondary metabolic pathways. Does this complexity of assembly lines represent a primitive stage of lichen evolution, from which more advanced genera evolved by selectively discarding unnecessary metabolic machinery, or does such complex chemistry signify the highest possible state of lichen morphological and physiological development ?

A clue leading to a partial resolution of this dilemma may be found in the increasing attention being given to the study of lichen genes (DePriest and Gargas, 1994; Gargas et al., 1995). The available evidence (from other biological systems) suggests that there are close relationships between gene structure and biochemical pathways, and alteration, nonexpression or elimination of genes will influence the nature and quantity of chemical products (Rogers, 1989). Culberson, Culberson and Johnson (1988) have examined the relationships between gene expression, lichen

development and chemical production in the *Cladonia chlorophaea* complex. They found that, in the Appalachian Mountains, two distinct chemotypes belonging to a single interbreeding population were reproductively isolated from another chemotype from the Coastal Plain of North Carolina. The chemistry of the offspring appeared to be reflective of the ability of any one particular chemotype to cross with another. The authors conclude that "The probable biosynthetic relationships of the diagnostic compounds give no clue to the limits of the interbreeding populations," and further, "The old morphology/ chemistry argument in lichen taxonomy becomes irrelevant in a problem now analyzable by experimentation." Culberson thus proposed that chemical variants in lichens constitute sibling species, and single differences in chemical compositions warrant the attribution of distinct species status. Rogers (1989), in a review on chemical variation in lichens, counters that some chemical variation may be "genetically trivial" or environmentally induced. While acknowledging the role genes play in chemotypic variation, he does not believe that small changes in the structures of lichen compounds (i.e., functional group modifications) are necessarily indicative of genetic isolation or population discontinuities. Rogers thus comes down on the side of "selection of strains by the habitat, not of habitats by strains." An additional point emphasized by Rogers is the notion that biochemical pathways, rather than end products per se, should be considered when making taxonomic assessments; it was sug-

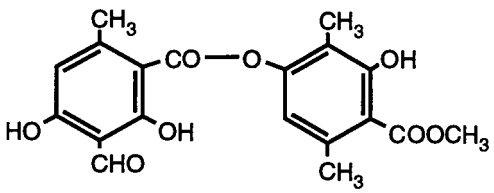
gested that the identification of a unique biosynthetic pathway (and its associated enzymes) should determine the suitability of assigning a unique taxonomic status to a particular lichen, along with proven genetic, morphological or physiological differences.

Egan (1986), in a detailed study of chemical variation and lichen morphology and geography, described three categories based on the literature data: 1) distinct correlations between lichen chemistry and lichen morphology or geography; 2) weak correlations between lichen chemistry and morphology or geography; 3) chemical strains in lichen species, where there does not appear to be any correlation between chemistry and morphology or geography. In his own study of *Xanthoparmelia* from Texas, Egan (1982) found chemosyndromic variation within different barbatic acid-producing species. Thus, each species of *Xanthoparmelia* produced a characteristic set of biogenetically related depsides in the medulla. In each case, there was at least one major product and at least one minor product, but the primary constituent in any given species occurred as a minor component in all the others. In analyzing the various viewpoints regarding chemical applications to lichenology, it would seem there is near unanimity among lichenologists regarding the inclusion, at some level, of chemical variation in lichens in any evaluation of taxonomic status, even if there is still debate about the manner of interpretation of the data.

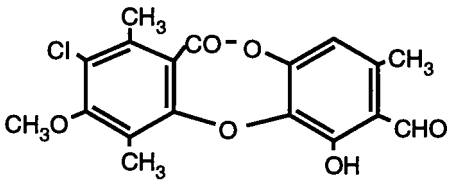
As early as the 1860s, when Nylander was analyzing lichens for chemical substances, the uniqueness of lichen compounds was recognized. To date, approxi-

mately 5,000 lichen species have been analyzed for lichen substances, constituting about one third of all known species (Elix, 1992). Lichen compounds are structurally unique and are rarely encountered in other organisms. The depsides and depsidones (Figure 1), the largest group of lichen compounds, are almost restricted to lichens, with a few exceptions known from lower fungi. Dibenzofurans (Figure 1) are also unique to lichens. Xanthones are known from lower fungi, lichens and higher plants; chlorinated xanthones (Figure 1), however, are ubiquitous in lichens and unknown in higher plants. Similarly, anthraquinones are widely distributed among all classes of organisms, yet chlorinated anthraquinones (Figure 1) are practically restricted to lichens (a few examples are known from lower fungi, basidiomycetes and marine echinoderms). Mevalonate-derived terpenes and steroids (Figure 1), prevalent in both lower fungi and higher plants, are fairly uncommon in lichens. Pulvic acid derivatives (Figure 1) are restricted to lichens and fungi. Finally, lichens are the only known class of organism totally devoid of alkaloids (although a few amino acid and peptide derivatives are known) (Figure 1).

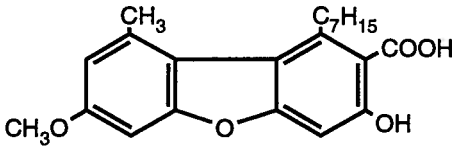
One rather obvious, but hardly trivial, observation is that lichen chemistry must somehow be a consequence of the symbiotic partnership between the mycobiont (fungus) and phycobiont (alga). While it is generally thought that the mycobiont is the source of a majority, if not all, of the lichen metabolites, experiments with cultured lichens and individual mycobionts have provided only a few clues as to the physiological bases for metabolite production (Renner and Gerstner, 1978b, 1980). In a few



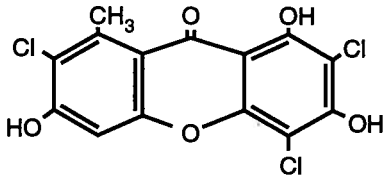
Atranorin (depside)



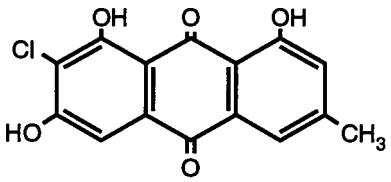
Pannarin (depsidone)



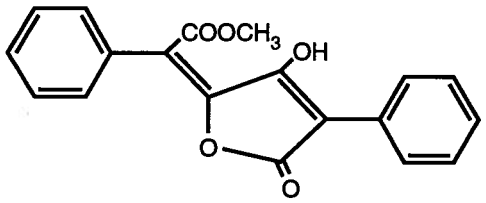
Melacarpic acid (dibenzofuran)



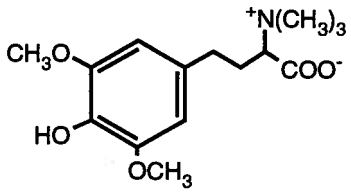
Arthothelin (xanthone)



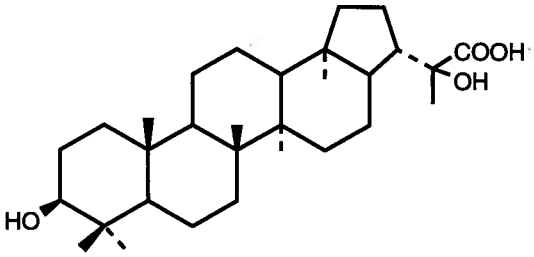
7-Chloroemodin (anthraquinone)



Vulpinic Acid (pulvic acid)



Solorinine (amino acid)



Pyxinic Acid (triterpene)

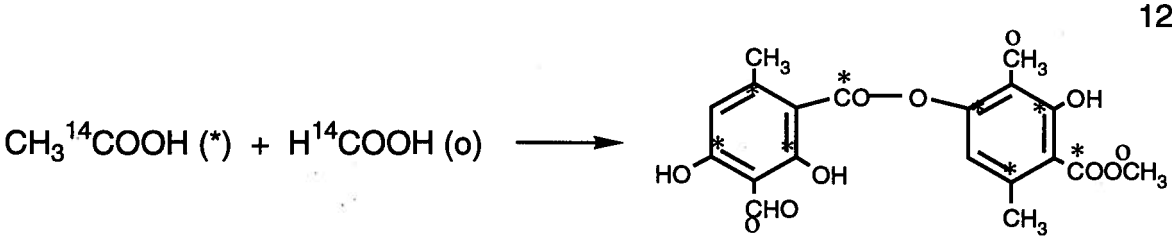
Figure 1. Lichen secondary metabolites.

reported instances, individually cultured mycobionts produce compounds not normally seen in the lichen association (Miyagawa et al., 1994). The reason for this difference is not yet clear, although it may involve either physiological or genetic adaptations to the culture conditions. Miyagawa et al. (1994), in their work with cultured lichen mycobionts, have suggested that the production of anomalous metabolites might be related to the osmotic stress induced during culturing of the mycobiont. In most cases, though, the mycobiont-derived metabolites were of a structural class of compound known from the lichen association (Hamada and Ueno, 1990). In one particular study, Mathey et al. (1980) cultivated the mycobiont from the tropical lichen, *Trypethelium eluteriae* Sprengel. They isolated several 1,2-napthoquinone pigments; these compounds were lacking in the intact lichen and were unexpected, representing as they do a structural type previously known only from a few higher plant genera, such as *Streptocarpus* (Gesneriaceae).

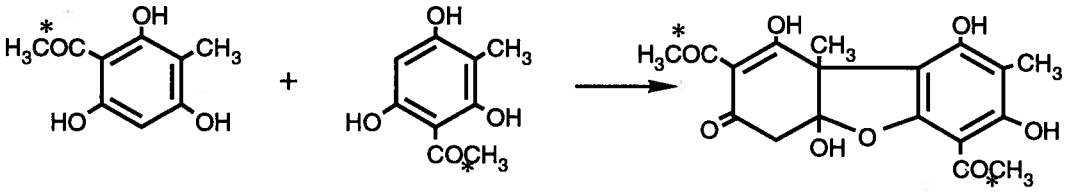
Culberson and Armaleo (1992) reported on the formation of a lichen-specific secondary metabolic pathway in the cultured mycobiont of *Cladonia grayi* G.K. Merr. ex Sandst. The cultured mycobiont produced the same set of depsides and depsidones found in the natural lichen. The researchers also noted that the sequence of biogenesis of the metabolites was consistent with contemporary theories on depside transformations to depsidones, and metabolite productivity was comparable to that seen in some nonlichen fungi. They ultimately concluded that the phycobiont (alga) is unnecessary for the production of depsides and depsidones in lichens.

Where parallels do exist between lichen and other plant or fungus-derived natural products, they probably reflect the presence of ubiquitous biosynthetic pathways. Surprisingly, there is very little literature discussion of the comparative phytochemistries of lichens and other organisms; this lack may simply reflect the scarcity of published data on the biogenesis of lichen compounds (Mosbach, 1973). Of the major types of lichen compounds, only the depsides, depsidones, pulvic acid derivatives, usnic acid, and tyrosine derivatives have been studied biogenetically (Figure 2).

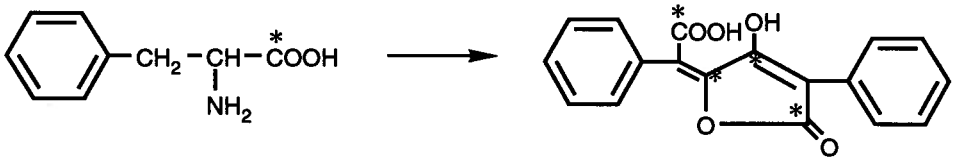
The earliest detailed reports on the biogenesis of lichen compounds were Mosbach's studies of gyrophoric acid (depside) and vulpinic acid (pulvic acid derivative) (Mosbach, 1964a, 1964b, 1967). Mosbach firmly established the origin of gyrophoric acid, in *Lasallia papulosa* (Ach.) Llano, from malonyl-SCoA, using [1,3-^{14}C] diethyl malonate. He also elucidated the origin of vulpinic acid, in *Letharia vulpina* (L.) Hue, from phenylalanine, using [1-^{14}C]-DL-3-phenylalanine. Subsequent research by Yamazaki and Shibata (1965, 1966) established the origin of the depsides lecanoric acid (from [1-^{14}C] acetate) and atranorin (from [1-^{14}C] acetate and [^{14}C] formate), using the lichen *Parmotrema tinctorum* (Nyl.) Hale. Taguchi et al. (1966) and Pentillä and Fales (1966) concurrently demonstrated the formation of usnic acid from acetylmethylphloroglucinol. In a series of experiments, Bloomer et al. (1968, 1969 and 1970) studied the biosynthesis of (+)-protolichesterinic acid in *Cetraria islandica* (L.) Ach.; the origin of this acetogenin from ^{14}C-labelled



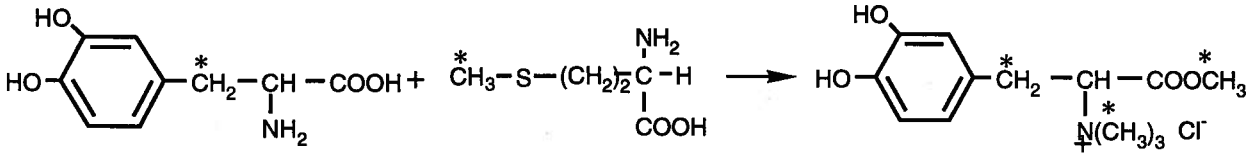
Biosynthesis of atranorin (depside) from labelled acetate and formate in *Parmotrema tinctorum* .



Biosynthesis of usnic acid (dibenzofuran) from [$^{14}CH_3$-CO]-acetylmethyl-phloroglucinol in *Usnea* and *Cladonia* spp.



Biosynthesis of pulvic acid from [1-^{14}C]-DL-phenylalanine in *Pseudo-cyphellaria crocata*.



Biosynthesis of sticticin (tyrosine derivative) from 3,4-DL-dihydroxyphenyl-[3-^{14}C]alanine and L-[methyl-^{14}C]methionine in *Lobaria laetevirens*.

Figure 2. The biosynthesis of lichen compounds.

acetate and succinate was firmly established. The bitter substance portentol, an unusual lactone from *Roccella* species, was found to be derived from both acetyl-SCoA and malonyl-SCoA (Aberhart et al., 1969, 1970). The biogenesis of the pulvic acid derivatives, calycin, vulpinic acid, pulvinic acid dilactone, pulvinamide, epanorin, pinastric acid, leprapinic acid and rhizocarpic acid were established using [1-^{14}C] phenylalanine incorporated into *Pseudocyphellaria crocata* (L.) Vainio (Maass et al., 1964; Maass and Neish, 1967). In the intervening years, Blanco et al. (1984) demonstrated that [^{14}C]urea was efficiently incorporated into evernic acid, atranorin and chloroatranorin in *Evernia prunastri* (L.) Ach., and Bernard and Goas (1981) studied the formation of amines from [2-^{14}C]glycine in Stictaceae and later, the biosynthesis of tyrosine derivatives in *Lobaria laetevirens* (Lightf.) Zahlbr. from L-[U-^{14}C]tyrosine and DL-[3-^{14}C]DOPA (Bernard et al., 1981).

Considering the number of identified lichen compounds (about 550), the state of our knowledge regarding their biogenesis is still in its infancy. When compared to the data available for the biosyntheses of fungal, plant, microbial, insect and animal natural products, the limited information about the origins of lichen substances is clearly inadequate. What may be even more revealing is the fact that since lichen biosynthetic studies began to ebb around 1970 (a quarter century ago), there have been only six additional papers in this area of research. In 1969, Mosbach explained the justification for investigating the biogenesis of lichen compounds as consisting of three factors: the uniqueness

of lichen compounds and their scarcity in other organisms; the uniqueness of lichens as symbiotic forms of life, and thus, the unique interactions the individual symbionts must adopt in order to produce lichen substances; and the contributions that elucidation of biosynthetic pathways would make towards a more formative chemotaxonomy of lichens.

Although the utility of lichen compounds for systematic purposes is generally firmly established, the roles they may play in the lichen itself are poorly understood. Lawrey (1986) recognized two categories of functions for lichen substances: 1) antimicrobial, antiherbivore and allelopathic protective roles; and 2) light screening, photobiont regulation and detoxification roles. While the evidence for the first category is fairly good, as a consequence of detailed ecological, physiological and pharmacological data, the experimental evidence for the second category is mixed and inconclusive.

In one study, Lawrey (1989) found a correlation between the antimicrobial properties of several lichen compounds and the ability of some lichens to deter potential herbivores. He compared two lichens known to be consumed by the slug, *Pallifera varia*, with two species avoided by the slug. Acetone extracts of the preferred lichens exhibited lower antimicrobial activity than extracts of the avoided lichens, for all the bacteria tested. In addition, the lichen compounds vulpinic acid, evernic acid and usnic acid were all found to have effective antimicrobial activity against the same bacteria. Lawrey thus concluded that lichen compounds can

protect the lichen from attack by either herbivores or microorganisms, but not in any specific manner. Gerson and Seaward (1977) demonstrated that snails (*Helix hortensis*) avoided certain lichens, but if the depsides had been removed by washing the lichens with a dilute soda solution, the snails consumed the same lichens. Experiments with insects (Slansky, 1979; Emmerich et al., 1993) have shown that certain lichen compounds are repellent to insects, while others do not deter feeding, or may alter normal insect growth and development after ingestion. Studies with vertebrates are few, and tend to be anecdotal. While it is well known that reindeer (*Rangifer tarandus*) eat a variety of lichens, the specific lichen compounds that may act as attractants or deterrents to grazing are not known (Richardson and Young, 1977). While human beings are generally not considered major consumers of lichens, several lichen species are known for their use as food or in herbal preparations: *Umbilicaria esculenta* (Miyoshi) Minks (Richardson and Young, 1977), *Bryoria fremontii* (Tuck.) Brodo and Hawks. (Turner, 1977), *Parmotrema tinctorum* (Nyl.) Hale and *Cetraria islandica* (L.) Ach. (Richardson, 1975).

Lichens and lichen compounds have been shown to be inhibitory towards the growth of plants, fungi, bacteria and other lichens (Huneck and Schreiber, 1972; Vartia, 1973; Rundel, 1978; Gardner and Mueller, 1981; Whiton and Lawrey, 1982, 1984; Ingólfsdóttir et al., 1985, 1994; Higuchi et al., 1993; Lawrey et al., 1994). In particular, lichens and their constituents have been shown to have strong enzyme inhibitory, antimicrobial and antiviral acitivities. Higuchi et al. (1993) tested forty-six

species of cultured lichen tissues for their inhibitory effect on the enzyme tyrosinase (an enzyme involved in the production of the skin pigment melanin). Three cultured lichens exhibited strong inhibitory activity; however, extracts of the natural lichen showed only weak inhibition. Interestingly, none of the tested lichens known to have anthraquinones showed major inhibitory activity. Work by Ingólfsdóttir et al. (1985) on the antimicrobial activity of seventeen lichen species from Iceland corroborated the results of Higuchi et al. (1993) that the primary compounds responsible for the antimicrobial activity were simple phenolics (e.g., methyl —orsellinate), depsides (e.g., atranorin and chloroatranorin) and pulvic acid derivatives (e.g., vulpinic acid). Usnic acid from several *Cladonia* species has long been used as an antibiotic in the treatment of dermatitis, eczema and other skin problems (Richardson, 1975). Lichens have also attained some importance, particularly in Japan, for the reputed antiviral activity of the constituent polysaccharides (Shibata et al., 1968; Nishikawa et al., 1970; Takahashi et al., 1974; Nishikawa et al., 1979; Gorin and Iacomini, 1984). In particular, polysaccharides from the genera *Umbilicaria*, *Lasallia*, *Lobaria*, *Sticta* and *Usnea* were found to be active. Although there has been only one report on the biological activities of anthraquinones isolated from lichens (Cohen et al., 1995), known lichen anthraquinones that are also produced by fungi and higher plants have been examined for their enzyme inhibitory and antimicrobial activities. Anke et al. (1980a, 1980b) reported on the antimicrobial activities of emodin, parietin, parietin anthrone, catenarin, and several other anthraquinones from the fungus, *Aspergillus*

glaucus. The first four anthraquinones are known from several lichen genera, including *Nephroma*, *Asahinea*, *Xanthoria* and *Caloplaca*. Nikaido et al. (1984) tested several higher-plant and synthetic anthraquinones, several of which were also known from lichens. The researchers found that emodin, 7-chloroemodin, citreorosein and chrysophanol exhibited strong inhibitory activity against cAMP phosphodiesterase. These anthraquinones are known from the lichen genera *Nephroma*, *Caloplaca*, *Lasallia*, *Heterodermia* and *Acroscyphus*. Several other anthraquinones, also known from lichens, exhibited little or no activity. These included skyrin (*Pyxine*, *Phaeophyscia*, *Acroscyphus*), parietin (*Nephroma*, *Xanthoria*) and emodin anthrone (*Heterodermia*).

The role of lichen compounds as light-screening agents was originally suggested by Ertl (1951) and supported by Barkman (1958), who cited evidence for the presence of higher concentrations of cortical substances in exposed lichens than in lichens growing in the shade. Several other studies have attempted to corroborate this conclusion (Scott, 1964; Hill and Woolhouse, 1966; Richardson, 1967; Rundel, 1969). Most of these studies dealt with the anthraquinone parietin. For example, Richardson (1967) transplanted *Xanthoria* thalli from a variety of different habitats (but all containing parietin) to new locations. The surviving lichen thalli showed morphological changes and varying parietin content, probably as a response to the new habitat conditions. While there does seem to be a correlation between light intensity in a given lichen habitat and the concentrations of cortical

or medullary substances, additional studies are needed in order to corroborate the earlier findings (Lawrey, 1986).

Finally, the potential role of lichen secondary metabolites as phycobiont regulators has been examined by Follmann et al. (1960, 1963, 1965, 1966), who demonstrated that lichen compounds can alter the permeability of cell membranes, and thus cause the excretion of nutrients from the phycobiont (alga) to the mycobiont (fungus). Green (1970), however, attributed this effect to the acidity of the culture medium, and suggested that a buffered medium would considerably reduce any observed permeability changes resulting from lichen secondary metabolites. Regulation of enzyme activity was also indicated as another possible regulatory role for the lichen substances. Studies by Brown et al. (1982) showed that urease activity in the blue-green-alga containing *Peltigera canina* (L.) Willd. declined in response to the addition of urea. Vicente et al. (1976, 1978, 1979) found that several endogenous lichen phenolic substances inhibited urease activity, and suggested that chelation of Mn^{+2} (a cofactor for photosynthetic enzymes) in algal cells, by depsides like chloroatranorin, might represent a photosynthesis-regulating function for secondary metabolites. Clearly, much more research needs to be done before the whole range of biological, chemical and physical properties of lichen substances can be fully appreciated.

1.2 PHYTOCHEMISTRY OF ANTHRAQUINONES

Anthraquinones are pigments widely distributed among all classes of organisms, including lichens (Thomson, 1971, 1987). So far, only the bryophytes have been found to be consistently lacking in anthraquinones. The anthraquinones represent the largest and best studied of the quinones. At present, about 40 different anthraquinones have been characterized from lichens (Elix et al., 1984; Huneck, 1984; Thomson, 1987; Huneck, 1991), with only eight new anthraquinones (and two new napthoquinones) having been identified in the last ten years (Huneck et al., 1991, 1994; Himmelreich et al., 1994; Cohen and Towers, 1995a, 1995c). In contrast, around 80 new anthraquinones from lower fungi and basidiomycetes have been discovered in the last decade (Gill, 1994, 1995). Despite the slow progress in the discovery of new lichen anthraquinones, many lichen genera still remain to be systematically analyzed for new compounds.

While anthraquinones are known from a taxonomically and morphologically diverse range of lichens, they are more prominent in some families and genera than others. Thus, anthraquinones are particularly prevalent in the lichen families Teloschistaceae (*Caloplaca*, *Xanthoria*, *Teloschistes*, *Fulgensia*), Psoraceae (*Psora*, *Protoblastenia*), Physciaceae (*Heterodermia*, *Pyxine*), Umbilicariaceae (*Lasallia*), Parmeliaceae (*Asahinea*), Haematommataceae (*Haematomma*), Nephromataceae (*Nephroma*), Solorinaceae (*Solorina*). There are a few families that have not been thoroughly examined, or for which there are occasional reports of anthra-

quinones: Parmeliaceae (*Cetraria, Parmelia, Nephromopsis, Esslingeriana, Xanthoparmelia*), Usneaceae (*Usnea, Oropogon*), Coccocarpiaceae (*Coccocarpia*), Physciaceae (*Phaeophyscia*), Caliciaceae (*Acroscyphus*), Laureraceae (*Laurera*), Sphaerophoraceae (*Sphaerophorus*) and Cladoniaceae (*Cladonia*) (Table 1).

There are several important aspects to the distribution of anthraquinones, and natural products in general, in lichens. First, there does not appear to be an obvious association between the presence of a particular anthraquinone (or other metabolite) and conspicuous morphological characters for a given lichen. Despite the generally accepted use of depsides and depsidones as chemical markers, their broad phytochemical distributions, and the lack of knowledge regarding why they are made, make it difficult to arrive at conclusions about supposed chemical affinities in lichens.

In their study of chemical evolution in the cetrarioid lichens, Kärnefelt and Thell (1993) found chemical representatives of all the major lichen metabolic pathways in the cetrarioid genera. They ultimately concluded that the only discernible chemical affinity was the presence of higher aliphatic compounds among a majority of the cetrarioid genera. Pulvic acid derivatives (shikimate pathway) show some chemical affinities in *Pseudocyphellaria* (Lobariaceae), Candalariaceae, *Thelomma*, *Cyphelium* and *Acroscyphus* (Caliciaceae). Triterpenes (mevalonate pathway) show chemical affinities in *Lobaria* and *Pseudocyphellaria* (Lobariaceae), *Nephroma* (Nephromataceae) and *Peltigera* (Peltigeraceae). In the case of anthraquinones, however, any purported chemical affinities with known lichen taxa are even less obvious.

Table 1. Lichen taxa containing anthraquinones.[4]

Family	Genus
Coccocarpiaceae	*Coccocarpia*
Solorinaceae	*Solorina*
Nephromataceae	*Nephroma*
Lecideaceae	*Lecidea*
Lopadiaceae	*Lopadium*
Mycoblastaceae	*Mycoblastus*[2]
Psoraceae	*Protoblastenia*
	Psora
Umbilicariaceae	*Lasallia*
Haematommataceae	*Haematomma*
Ophioparmaceae	*Ophioparma*[1]
Squamarinaceae	*Squamarina*[1]
Placolecidaceae	*Placolecis*
Parmeliaceae	*Asahinea*
	Cetraria[2]
	Esslingeriana
	Nephromopsis
	Parmelia
	Xanthoparmelia
Usneaceae	*Oropogon*
	Usnea[2]
Physciaceae	*Heterodermia*
	Phaeophyscia
	Pyxine[2]
Teloschistaceae	*Caloplaca*
	Fulgenisa
	Teloschistes
	Xanthoria
Caliciaceae	*Acroscyphus*
Sphaerophoraceae	*Sphaerophorus*
Arthoniaceae	*Arthonia*
Pyrenulaceae	*Pyrenula*
Acarosporaceae	*Biatorella*
Laureraceae	*Laurera*
Chiodectonaceae	*Chiodecton*[1]
Trypetheliaceae	*Trypethelium*[3]
Cladoniaceae	*Cladonia*[2]

[1]Only napthoquinones reported.
[2]Anthraquinones and napthoquinones reported.
[3]Napthoquinone reported from cultured mycobiont; anthraquinones in native lichen.
[4]Data from Culberson (1969), Mathey et al. (1980), Huneck et al. (1994) and Himmelreich et al. (1994).

Lichen species have rarely been assigned a taxonomic status on the basis of their anthraquinone constituents alone. There are, however, a few instances where the presence (or absence) of anthraquinones, along with distinguishing morphological traits, have been sufficient to warrant segregation of individual species into new genera. For example, Rogers and Hafellner (1988) segregated the species *Haematomma ventosum* (L.) Massal. into the genus *Ophioparma* Norman (in a new family Ophioparmaceae Rogers and Hafellner) based on its non-lecanoroid ascus structure, arctic-boreal distribution, saxicolous substratum preferences and the presence of the acetone-soluble red napthoquinone, haemoventosin, in the apothecia. In contrast, *Haematomma puniceum* (Ach.) Massal. and *H. ochroleucum* (Necker) Laundon were maintained taxonomically, in part, on the basis of having anthraquinone apothecial pigments (e.g., haematommone). The genus *Lasallia* consists of about 15 species, of which seven are known to contain anthraquinones (Posner et al., 1990, 1991). Llano (1950), in his definitive study of the Umbilicariaceae, segregated *Lasallia* from *Umbilicaria* based on the pustulate thallus, spore number in the ascus and mode of reproduction. Even though the chemical structures of the *Lasallia* pigments were not fully characterized until 1969-1972 (Bohman, 1969b; Fox et al., 1969; Briggs et al., 1972), the pigmented thalli of several *Lasallia* species were recognized by Llano (1950), Culberson and Culberson (1958) and others. *Asahinea* was segregated from *Cetraria* by Culberson and Culberson (1965) on the bases of the absence of rhizines or marginal ciliae, imperforate apothecial discs, geographical

distribution, absence of aliphatic compounds and the presence of purple pigments, later characterized as anthraquinones (Mischenko et al., 1980). The genera *Pyxine* and *Heterodermia* (Physciaceae) contain species having anthraquinones. Poelt (1965) originally proposed a new classification of Physciaceae, in which *Heterodermia* was segregated from *Anaptychia* primarily on the basis of morphological characters. Subsequently, Culberson (1966) maintained that *Heterodermia* should be given separate status on the basis of chemistry as well. We now know that approximately 16 of the 81 known *Heterodermia* species contain anthraquinones, while they are completely absent from *Anaptychia* (Yosioka et al., 1968c; Kurokawa, 1973; Trass, 1992). In the case of *Pyxine*, opinions differ as to the relative merits of quinonoid pigment production as a taxonomic criterion. This appreciably reflects the lack of chemical information about the exact chemical structures of the compounds, as well as a lack of diagnostic uniformity in the assigning of taxonomic status. Rogers (1986), in his study of *Pyxine* in Australia, felt that cortical chemistry was an important taxonomic criterion, but medullary (pigment) chemistry was useful only as a confirmatory character. Swinscow and Krog (1975), in their examination of East African *Pyxine*, placed a greater emphasis on the presence or absence of medullary pigments in the species they examined, but ruled out pigmentation of the internal stipe as a valid criterion. Imshaug (1957) in his systematic study of *Pyxine* in the New World, was the most enthusiastic of all about using the medullary and stipe pigments as valid markers in taxonomic classification. At present, the chemical structures of many pigments in

Pyxine species (Huneck, 1976) and *Heterodermia* species (Kurokawa, 1973) remain to be elucidated. This void would seem to support then, in principle, the advocacy of using caution in relying too heavily on any purported taxonomic implications of secondary chemistry in lichens, unless that chemistry was very well understood.

The biogenesis of anthraquinones in lower fungi (Gatenbeck, 1958, 1960, 1962), higher fungi (Steglich et al., 1972; Gill and Giménez, 1990a, 1990b, 1992) and higher plants (Leistner, 1971, 1973; Yagi et al., 1978) has been well documented. The majority of anthraquinones are derived from acetate (as acetyl CoA) or malonate through the polyketide pathway (Weiss and Edwards, 1980). A few anthraquinones from higher plants are derived, instead, from shikimic acid (Leistner, 1973); but all known fungal and lichen anthraquinones are believed to be derived solely from acetate or malonate (Culberson, 1969; Mosbach, 1969; Gill and Steglich, 1987; Gill, 1994). The anthraquinone emodin (Figure 3) was shown to be formed from acetate in *Penicillium islandicum* (Gatenbeck, 1958, 1960, 1962), in the higher plant genera *Rhamnus*, *Rheum* and *Polygonum* (Leistner, 1971), and in the basidiomycete genera *Cortinarius* and *Dermocybe* (Gill and Steglich, 1987; Gill, 1994, 1995). Feeding experiments in *Dermocybe* have established the polyketide origin of emodin, and its transformation into a variety of structurally modified anthraquinones (Steglich et al., 1972). In addition, several anthrone precursors, leading to a host of anthraquinone end products in *Cortinarius* and *Dermocybe,* have been isolated, and their transformations into anthraquinones studied in vivo using ^{13}C-labelled acetate (Gill and Giménez, 1990a, 1990b, 1991, 1992).

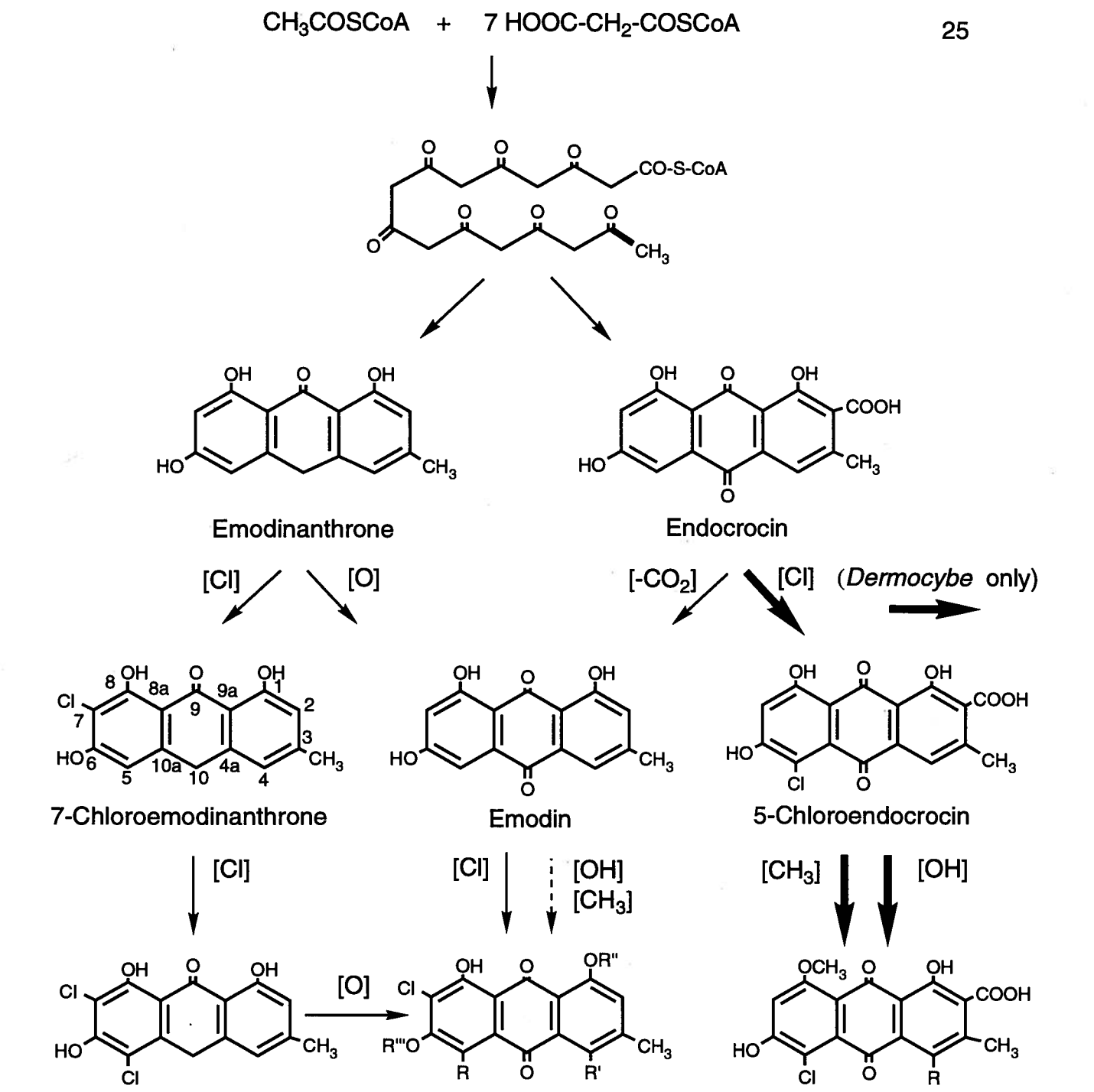


Figure 3. The biosynthesis of anthraquinones in lichens and *Dermocybe* (basidiomycete).

The few biosynthetic studies on lichen compounds have dealt primarily with depsides and pulvic acid derivatives (Maass et al., 1964, 1967; Yamazaki et al., 1965, 1966; Maass, 1970; Bloomer et al., 1970). So far, there have been no studies on the biogenesis of anthraquinones in lichens; the assumption has been made that they originate from acetate and malonate through the polyketide pathway (Shibata, 1964; Culberson, 1969; Mosbach, 1969; Santesson, 1970a).

Santesson (1970a) proposed a scheme describing the possible biogenetic relationships between the anthraquinones found in the genus *Caloplaca.* He suggested that anthrones might be precursors to corresponding anthraquinones, based on their co-occurrence in some lichens. In addition, Santesson (1970a) was the first to propose that chlorinated anthraquinones could be formed, in lichens, by direct halogenation of emodin and related compounds (Figure 3). Steglich et al. (1969) had suggested a similar mechanism for the formation of chlorinated anthraquinones in the basidiomycete genus *Dermocybe* (Figure 3). Although such proposals have yet to be tested experimentally for lichens or basidiomycetes, a mechanism for the chlorination of anthraquinones in the lower fungus, *Aspergillus fumigatus*, has been examined (Yamamoto et al., 1968). The authors grew cultures of the fungus with varying concentrations of chloride ion in the medium. They were able to establish an optimum concentration of chloride ion which resulted in the highest yield of chlorinated anthrones and anthraquinones in the fungus. When bromide ion was substituted for chloride ion in the culture medium, the corresponding brominated anthrone was produced by the fungus.

1.3 THE CHEMISTRY OF *NEPHROMA* AND *HETERODERMIA*

The genus *Nephroma* was reviewed extensively by Gyelnick (1931, 1932), later followed by Wetmore (1960) who revised the North American taxa. In the intervening years, Renner et al. (1982) categorized the South American species of *Nephroma* into nine chemical groups. Finally, James and White (1987, 1988) systematically studied the European, Macronesian, northern temperate and southern temperate species. The researchers analyzed the chemistry, by TLC, of a total of 28 species, in an attempt to formulate phytochemical groupings within the genus.

The earliest studies of the chemistry of *Nephroma* were those of Bachmann (1887). Despite some confusion surrounding the identity of the lichen substances, Bachmann was able to recognize the presence of a yellow emodin-like compound in the medulla of *Nephroma laevigatum* Ach. (as *N. lusitanicum* (Ach.) Nyl.) which turned purple with KOH. The presence of emodin in higher plants (*Frangula alnus*) had already been established. Hesse (1898a) also confirmed the presence of an hydroxyanthraquinone (nephromin) in *N. laevigatum*, but regarded the substance as unique to lichens. Zopf (1907) later compared nephromin to the yellow substance studied by Bachmann, and found they were identical. Bendz et al. (1967) separated nephromin into five compounds, and characterized each by mass spectrometry. Bohman (1968), continuing this work, fully established the identity of the five anthraquinones, utilizing mass spectrometry, IR, UV and ^{1}H NMR

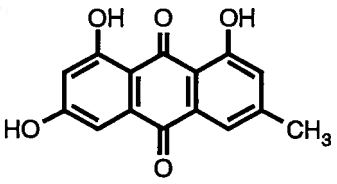
spectroscopy. In addition, Bohman isolated a triterpene (possibly nephrin) and a fatty acid. The *Nephroma* compounds are shown in Figure 4. Several other lichen substances have been isolated from *Nephroma* species, including nephroarctin (depsidone from *N. arcticum* (L.) Torss.; Nuno et al., 1969), phenarctin (depside from *N. arcticum*; Brüun, 1971), gyrophoric acid and tenuiorin (depsides from *N. pseudoparile* Räs.; Renner et al., 1982), perlatolic and glomelliferic acids (depsides from *N. cellulosum* (Sm.) Ach.; Renner and Gerstner, 1978a), ergochromes (dimeric xanthones from *N. analogicum* Nyl.; Renner et al., 1982), and a series of hopane triterpenoids present in almost all *Nephroma* species (James and White, 1987; White and James, 1988).

In their phytochemical analyses of 15 South American species of *Nephroma*, Renner et al. (1982) recognized nine chemical groupings. One species, *N. analogicum*, was found to contain yellow ergochrome pigments (secalonic acids A and C), which had been known previously only from the genera *Parmelia* (Yosioka et al., 1968d) and *Nephromopsis* (Yosioka et al., 1972; Kärnefelt and Thell, 1993). There was no evidence, however, for the presence of anthraquinones in any of the southern species.

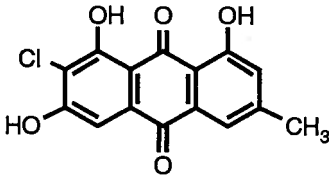
James and White (1987) reexamined the European and Macronesian *Nephroma*, recognizing seven distinct chemical groupings (Table 2). Anthraquinones were identified in three species, *N. laevigatum*, *N. tangeriense* (Maheu and A. Gillet) Zahlbr. and *N. venosum* Degel. The authors report that up to 16 anthra-

quinones may be present in some varieties of *N. laevigatum,* according to TLC, but also indicate that nonpigmented or pigment-deficient morphotypes have been found in Portugal and the Canary Islands. *N. tangeriense* was said to bear a similarity to *N. laevigatum* chemically, although it is restricted to southern Europe and North Africa and generally lacks apothecia. *N. venosum* is endemic to the Azores and morphologically distinct from *N. laevigatum* or *N. tangeriense.* Finally, in their study of the southern temperate *Nephroma*, White and James (1988) expanded on the earlier work of Renner et al. (1982). Their chemical results are consistent with the phytochemical data obtained by Renner et al. (1982), and again confirm the absence of anthraquinones in the southern temperate species (Table 2).

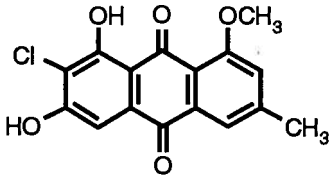
The chemical study of *Heterodermia* was initiated in 1898 with Hesse's identification of the hydroxyanthraquinone pigment blastenin in *Heterodermia obscurata* (Nyl.) Trevis. (as *Anaptychia heterochroa* Vain.) (Hesse, 1898b, 1901). Asahina and Yosioka (1940) isolated the triterpene zeorin and depside atranorin from *Heterodermia speciosa* (Wulf.) Trevis. (as *Anaptychia speciosa* (Wulf.) Mass.), *H. hypoleuca* (Ach.) Trevis. (as *A. hypoleuca* (Ach.) Mass.), and *H. obscurata* (as *A. heterochroa*). They also recognized the presence of a yellow-orange undersurface in *H. obscurata*, and attributed this colour to an hydroxyanthraquinone (identical with blastenin) which they then isolated. Hale (1956) analyzed several North American lichens, including *Heterodermia obscurata* (as *Anaptychia heterochroa*) and observed the presence of a yellow anthraquinone in the lower surface of this lichen. The first systematic study



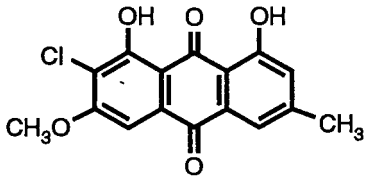
Emodin



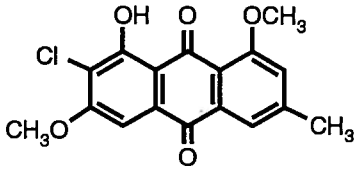
7-Chloroemodin



7-Chloro-1-O-methylemodin



7-Chloro-6-O-methylemodin



7-Chloro-1,6-di-O-methylemodin

Figure 4. The anthraquinones of *Nephroma laevigatum*.

of *Heterodermia* and *Anaptychia* was that of Kurokawa (1962). In his monograph, the author revised all species classified in the genus *Anaptychia*. Using chemical and morphological data, Kurokawa proposed a new infrageneric classification of *Anaptychia*, but did not feel the chemical data warranted the need for segregation of taxa into new genera. Poelt (1965), on the other hand, proposed a new classification of the Physciaceae, in which *Anaptychia* would be segregated into *Anaptychia* and *Heterodermia*.

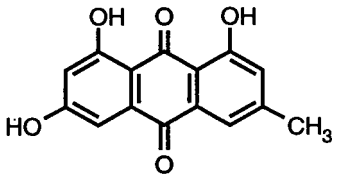
According to Poelt's classification, *Anaptychia* is primarily characterized by the presence of thin-walled spores and the absence of atranorin, while *Heterodermia* has thick-walled spores and contains atranorin. It was later shown by Culberson (1966) and Kurokawa (1973) that, in fact, atranorin is present in some *Anaptychia* species. This was followed by a major study by Culberson (1966). The author examined 14 species of *Anaptychia* from North and South Carolina. Following the principles set forth by Poelt (1965), Culberson separated *Heterodermia* from *Anaptychia* on the basis of spore morphology as well as chemistry. The author demonstrated that all species of *Heterodermia* contained, in addition to atranorin, other lichen substances (e.g., zeorin, salazinic acid, norstictic acid and anthraquinones) completely absent from *Anaptychia*.

Yosioka et al. (1968a, 1968b) examined, in some detail, the chemistry of *Heterodermia obscurata*. Several anthraquinones and bianthrones were isolated from the lichen growing in Japan (Figure 5). In a survey of several other pigmented

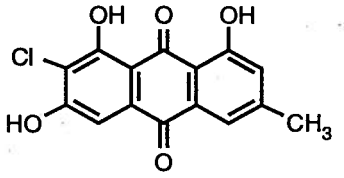
Table 2. Major lichen compounds in *Nephroma* species.[1,8]

Nephroma species	Phenarctin (P) Nephoarctin (N)	Gyrophoric acid (G)	Usnic acid (U)	Stictic acid (S)	Hyposalazinic acid (HZ)	Anthraquinones	Hopane triterpenoids
N. analogicum Nyl.						EC AA & AB[6]	
N. antarcticum (Jacq.) Nyl.	P		U	S/C/HC/HS[5]	[HZ]		T3
N. arcticum (L.) Torss.	N		U				T3
N. areolatum James & White							T3-T6
N. australe A. Rich Race 1			U	HS	HZ		T3
N. australe A. Rich Race 2			U				T3
N. bellum (Sprengel) Tuck. Race 1							T1-T6
N. bellum (Sprengel) Tuck. Race 2							T2/T3/T5
N. cellulosum (Ach.) Ach.				PR/SP/GL[4]			T3
N. chubutense Lamb			U				T6
N. expallidum (Nyl.) Nyl.							T2/T3/T5
N. foliolatum James & White							T3
N. helveticum Ach.							[T1]/T4
N. hensseniae James & White							T1/T3/T4/T6
N. isidiosum (Nyl.) Gyelnik							[T1]/T4
N. kuehnemannii Lamb	P		[U]				
N. laevigatum Ach. Race 1							T6
N. laevigatum Ach. Race 2							T3
N. microphyllum Henssen	P		[U]			AQ	
N. occultum Wetmore	[P]/N		U				T3
N. papillosum White & James			U				T3
N. parile (Ach.) Ach. Race 1 & 2[7]		[G]					T2/T3/T5
N. parile (Ach.) Ach. Race 3							T1/T3/T4/T6
N. plumbeum (Mont.) Mont.							
N. pseudoparile (Räsänen) Zahlbr.	T[2]	G/MG/MGA[3]	U				T3
N. resupinatum (L.) Ach.							
N. rufum (Church. Bab.) James							[T2]/T5
N. silvae-veteris Goward & Goffinet			U				
N. skottsbergii White & James			U				T3
N. sulcatum James & White							T3/T4/T5/T6
N. tangeriense Maheu & A. Gillet						AQ	[T2]/T3/[T4]/T6
N. venosum Degel.						AQ	T3

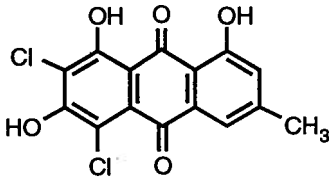
[1] Symbol key: compounds detected by TLC are indicated by "U" (present) or "[U]" (present in some samples).
[2] Contains tenuiorin (T).
[3] Contains methyl gyrophorate (MG) and 4-*O*-methylgyrophoric acid (MGA).
[4] Contains perlatolic acid (PR), stenosporic acid (SP) and glomelliferic acid (GL).
[5] Contains constictic acid (C), hypoconstictic acid (HC) and hypostictic acid (HS).
[6] Contains ergochromes AA and AB (EC AA & AB).
[7] Unknown xanthone detected in Race 2.
[8] Data from Renner et al. (1982), James and White (1987) and White and James (1988).



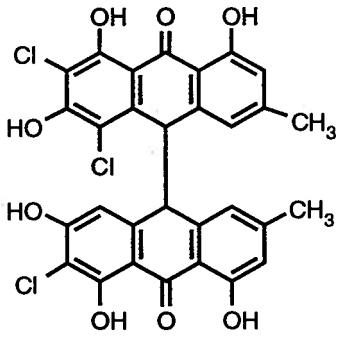
Emodin



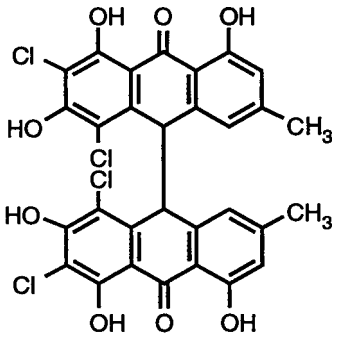
7-Chloroemodin



5,7-Dichloroemodin



Flavoobscurin A



Flavoobscurin B

Figure 5. The anthraquinones of *Heterodermia obscurata*.

Heterodermia (Yosioka et al., 1968c), anthraquinones and anthrones were detected, by TLC, in eight tropical and semi tropical *Heterodermia* (Table 3). In addition, several yellow non quinonoid pigments (possibly pulvic acid derivatives) were detected in two species.

The last comprehensive study of *Heterodermia* (as *Anaptychia*) was that of Kurokawa (1973), who reviewed all known species, performed TLC analyses, discussed phytochemical variations within the genus, provided geographical distributions, and reclassified *Anaptychia* into two subgenera: subgenus *Anaptychia* and subgenus *Heterodermia*. *Anaptychia* was defined as having subascending or erect lobes, and an upper cortex with varying thickness; *Heterodermia*, in contrast, has adnate lobes and a rather uniform upper cortex. As in his earlier work (Kurokawa, 1962), chemical variation was not considered as a factor in taxonomic reassessment.

Finally, Trass (1992) compiled the known information for the 81 accepted species of *Heterodermia* in tabular form, providing morphological and geographical data, and indicating the presence or absence of pigments in each species.

Table 3. Anthraquinone and anthrone pigments in *Heterodermia* species as determined by TLC.[1,3]

Heterodermia Species	Range	7-Chloroemodin	7-Chloroemodin anthrone	5,7-Dichloro-emodin	Flavoob-scurin A	Flavoob-scurin B
H. cyathiformis (Kurok.)	S. Africa	++		++	+	+
H. dendritica (Pers.) Poelt	E. , S. E. Asia	++	++	++	+	++
H. fauriei (Kurok.)[2]	Hawaii, Thailand					
H. firmula (Nyl.) Trevis.	E. , S. E. Asia	++	++	+	++	++
H. flabelatta (Fée) Awasthi	S. Am., Asia, E. Afr.	++	++	++	++	++
H. hypocaesia (Yasuda) Awasthi	Aus., Asia, S. Africa	++/-				
H. hypochraea (Vain.) Swinscow & Krog	S. Am., E. Afr., Asia	++	++	+		++/-
H. loriformis (Kurok.) Swinscow & Krog	E. Africa		++	++		
H. lutescens (Kurok.) Follm.	S. Am., Africa, Asia	+/-				
H. obesa (Pers.) Trass[2]	Hawaii					
H. obscurata (Nyl.) Trevis.	Aus., N.Z., USA, Eur. S.Am., Asia, E. Afr.	++	++	++	++	++
H. pacifica (Kurok.)	Japan, Taiwan	++				
H. pandurata (Kurok.) Trass	Japan, Taiwan, Thai.	++	++	++/-		
H. propagulifera (Vain.) Day	S. Am., Asia, USA (?)	++	++	++	++	++
H. rugulosa (Kurok.) Trass	Mexico, S. W. USA	++	++	++	++	++
H. subascendens (Asah.) Trass	Japan, Taiwan, China	++	++			
H. vulgaris (Vain.) Follm. & Rédon[2]	S. Am., E. & S. Africa					

[1]Symbol key: compounds demonstrated by TLC are marked with ++ (significant) or + (trace). Compounds demonstrated by TLC in a portion of the samples are marked ++ (+) or - (absent).

[2]Uncharacterized anthraquinones and anthrones.

[3]Data from Yosioka et al. (1968) and Kurokawa (1973).

CHAPTER 2: CHEMICAL PROPERTIES OF LICHEN QUINONOID PIGMENTS

2.1 ISOLATION OF *NEPHROMA LAEVIGATUM* NATURAL PRODUCTS

2.1.1 INTRODUCTION

The lichen *Nephroma laevigatum* is widely distributed throughout western Europe and the Mediterranean, where its range extends east to Israel (James & White, 1987). In North America, it can be found on both the Atlantic and Pacific coasts. In the east, the lichen is known from Massachusetts to Labrador, while in the west it occurs from northern California to British Columbia (Wetmore, 1960). This particular species is the most "oceanic" of the North American *Nephroma*, restricted to coastal areas near the Atlantic and Pacific Oceans (Figure 6; Appendix 1).

In British Columbia, *Nephroma laevigatum* is abundant on sub-basic shoreline rocks on islands dotting the mainland coast north to Alaska. It can also occasionally be found growing on the trunks and branches of Big-Leaf Maple (*Acer macrophyllum*) near the coast.

2.1.2 MATERIALS AND METHODS

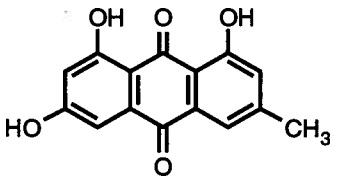
Several collections of the lichen were made from different islands in southwestern British Columbia: Bowen Island, Gabriola Island and Galiano Island. Attempts were made to locate the lichen on the adjacent mainland, but without success. *Nephroma laevigatum*, in British Columbia, would appear to be highly localized in humid relic coastal forests on the numerous islands adjacent to the

Figure 6. *Nephroma laevigatum* from Gabriola Island, British Columbia.

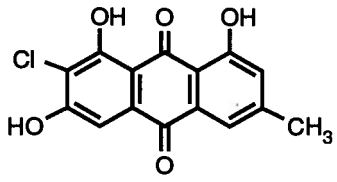
mainland.

The different collections were cleaned of soil, moss and other debris and air dried. Two reference samples were deposited in the UBC Botany Department Herbarium. Small quantities of lichen were extracted with ice-cold diethyl ether for 10 minutes, and the extracts examined by TLC. The patterns of chemical products were identical for all the samples examined; this suggests that there is little (or no) chemotypic variation within the lichen communities sampled. This uniformity does not exclude, however, the possibility that *N. laevigatum* chemotypes exist in other localities within British Columbia. In fact, James & White (1987) reported on the existence of two distinct chemical races of *N. laevigatum*, differing only by the substitution of one hopane triterpenoid with another. The rarer Race 2 (Table 2) was found in only two collections from the Azores Islands. In addition, rare pigment deficient varieties have been found on the Canary Islands, Madeira and in Portugal (James & White, 1987). These chemotypes are morphologically and chemically identical to the common *N. laevigatum*, the only difference being the complete or partial absence of anthraquinones in the medulla.

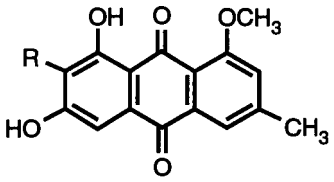
The dried lichen material was combined (0.4 Kg) and extracted, in succession, with cold diethyl ether, acetone and methanol. The extracts were concentrated to small volumes and examined by TLC. The diethyl ether extract showed 12 coloured spots, the primary constituent being 7-chloroemodin (2) (R_f 0.6; 9:1 chloroform:methanol). The other prominent compounds present in the extract



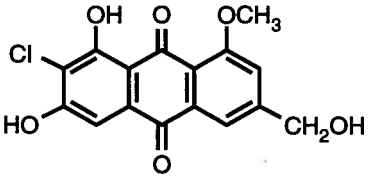
Emodin (1)



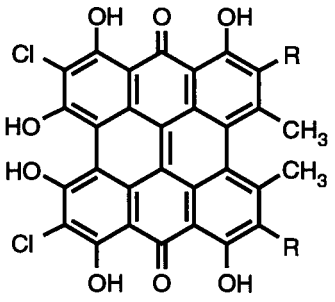
7-Chloroemodin (2)



R = Cl; 7-Chloro-1-*O*-methylemodin (3)
R = H; 1-*O*-methylemodin (7)



7-Chlorocarviolin (4)



R = H; 7,7'-Dichlorohypericin (5)
R = Cl; 2,2',7,7'-Tetrachlorohypericin (6)

Figure 7. The anthraquinones of *Nephroma laevigatum*.

were: emodin (1) (R_f 0.8); 7-chloro-1-*O*-methylemodin (3) (R_f 0.7); 7-chloro-1-*O*-methyl-ω-hydroxyemodin (4) (R_f 0.3); 7,7-dichlorohypericin (5) (R_f 0.2); and 2,2',7,7'-tetrachlorohypericin (6) (R_f 0.2). These compounds are shown in Figure 7. Minor coloured constituents, evident from the TLC plate, were presumed to be other anthraquinones or anthrones based on their UV fluorescence, but were not present in sufficient quantities to permit conclusive identification. Two additional non coloured spots that fluoresced under UV light were probably phenolic constituents, such as depsides.

Following TLC examination, the orange-red extract was concentrated to a brown-red solid. The solid (10 g) was divided into three equal portions. One portion (3.3 g) was purified by column chromatography on 100 g of Sephadex LH-20, using a gradient of chloroform:methanol (9:1) to methanol. Fractions (20 ml) were collected and analyzed by TLC. Fractions 2-4 contained emodin (1) and 7-chloro-1-*O*-methylemodin (3). Fractions 5-7 contained 7-chloro-1-*O*-methylemodin (3). Fractions 6-8 contained 7-chloro-1-*O*-methylemodin (3) and 7-chloroemodin (2). Fractions 9-12 contained 7-chloroemodin (2). 7-Chloro-1-*O*-methyl-ω-hydroxyemodin (4) came from fractions 13-18. Small amounts of 7,7'-dichlorohypericin (5) and 2,2',7,7'-tetrachlorohypericin (6) were found in fractions 19-22. In order to obtain the bulk of compounds 5 and 6, the stationary purple layer was extruded from the column and then extracted with pyridine for 16 hours. The purple extract

was concentrated to give a mixture of compounds 5 and 6. Separation of the mixture was accomplished by column chromatography on Sephadex LH-20, using methanol as the eluant. Prior to spectroscopic analysis, all recrystallized compounds were checked for purity by TLC and reversed-phase HPLC.

2.1.3 RESULTS AND DISCUSSION

Spectral analysis of compound 2 supported the structure as 7-chloroemodin. The EIMS (Electron Ionization Mass Spectrometry) shows peaks at 306 and 304 and the CIMS (Chemical Ionization Mass Spectrometry) reveals peaks at 307 and 305. The 2D COSY (Two-Dimensional Correlated Spectroscopy) ^{1}H NMR spectral data are shown in Table 4. The assignment of the chlorine to position 7 is based on the chemical shifts of H-5, H-4 and H-2, which are consistent with those previously reported (Bohman, 1968; Yamamoto et al., 1968; Yosioka et al., 1968a). Several NOE (Nuclear Overhauser Effect) experiments demonstrated an enhancement of the signals of H-2 and H-4 upon irradiation of the methyl protons, as well as an enhancement of H-5 upon irradiation of the hydroxyl proton at C-6. These results are consistent with structure 2. ^{13}C NMR assignments listed in Table 5 are based on APT (Attached Proton Test) and HETCOR (Heteronuclear Chemical Shift Correlation) experiments, and calculations of carbon chemical shifts using the methods described by Ewing (1979) and Silverstein et al. (1991).

Compound 3 proved to be the 1-*O*-methyl derivative of 2 by analysis of the

Table 4. [1]H NMR data for emodin (1), 7-chloroemodin (2), 7-chloro-1-*O*-methylemodin (3) and 1-*O*-methylemodin (7).[1]

Proton	1[2]	2[3]	3[3]	7[2]
2	7.15, s	7.14, s	7.45, s	7.45, s
4	7.58, s	7.50, s	7.62, s	7.67, s
5	7.25, d (2.5)	7.26, s	7.22, s	7.20, d (2.5)
7	6.65, d (2.5)			6.67, d (2.5)
1-OH	12.05, s	11.92, s		
6-OH				
8-OH	12.15, s	12.78, s		
1-OMe			3.98, s	4.05, s
3-Me	2.47, s	2.48, s	2.52, s	2.50, s

[1]Chemical shifts (δ) are reported in ppm from TMS internal standard. The coupling constants are given in Hz.
[2]The spectra were recorded in Me_2CO-d_6 at 300 MHz.
[3]The spectra were recorded in DMSO-d_6 at 500 MHz.

Table 5. ^{13}C NMR data for emodin (1), 7-chloroemodin (2) and 7-chloro-1-*O*-methylemodin (3).[1]

Carbon	1	1[2]	2	2[2]	3	3[2]
1	161.3	158.7	161.4	158.7	160.9	163.2
2	124.0	120.1	124.0	120.1	120.8	118.5
3	148.1	142.4	148.5	142.4	148.3	142.0
4	120.3	125.2	120.4	125.2	120.5	124.9
5	108.7	111.8	108.6	113.2	106.9	113.2
6	165.5	162.8	163.1	163.2	162.1	163.2
7	107.8	106.7	121.3	112.9	118.5	112.9
8	164.4	160.2	162.8	160.6	161.1	160.6
9	189.5	189.3	191.0	187.4	187.5	187.1
10	181.1	173.9	181.7	173.9	182.6	173.5
4a[4]	132.6	131.5	133.2	131.5	134.5	131.1
8a[3]	108.8	110.0	110.1	111.3	111.4	111.0
9a[3]	113.1	107.1	114.2	107.1	114.2	107.1
10a[4]	134.9	132.8	133.0	130.9	132.3	129.3
1-OMe					56.4	
3-Me	21.5		21.6		21.7	

[1]Chemical shifts (δ) are reported in ppm from TMS internal standard. The spectra were recorded in DMSO-d_6 at 125 MHz.
[2]Values calculated according to the methods described in Ewing (1979) and Silverstein et al. (1991).
[3]Values for C-8a and C-9a can be interchanged.
[4]Values for C-10a and C-4a can be interchanged.

2D COSY ^{1}H NMR (Table 4) and EIMS spectra. The EIMS of 3 shows peaks at 320 and 318, while the CIMS shows peaks at 321 and 319. The UV and 2D COSY ^{1}H NMR spectral assignments are consistent with previously reported data (Bohman, 1968; Ayer and Trifonov, 1994). A series of NOE experiments confirmed the identity of 3. Upon irradiation of the methyl protons, there was a corresponding increase in the signal intensities of H-2 and H-4. Irradiation of the methoxy protons resulted in an enhancement of the H-2 signal. Finally, irradiation of the hydroxyl proton at C-6 produced an enhanced signal for H-5. The ^{13}C NMR data are shown in Table 5. Assignments were made on the basis of APT and HETCOR experiments, as well as carbon chemical shifts calculated according to the methods described by Ewing (1979) and Silverstein et al. (1991). Additional structural proof was afforded by reduction of 3 with Raney nickel to give the dechlorinated product (Figure 7). The 2D COSY ^{1}H NMR shifts (Table 4) of this product are consistent with those previously reported for 1-*O*-methylemodin (7) (Ayer and Trifonov, 1994). A comparison of TLC R_f values for compound 7 and published data for 1-*O*-methylemodin also helped in confirming the identity of compound 3 (Ayer and Trifonov, 1994; Cameron and Crossley, 1977).

Emodin (1) was characterized by UV, EIMS, CIMS and 2D COSY ^{1}H NMR spectra (Table 4). Several NOE experiments confirmed that compound 1 was emodin. Irradiation of the methyl protons produced a corresponding increase in the signal intensities of H-2 and H-4. Irradiation of the hydroxyl proton at C-8 also produced an increase in the signal intensity of H-7. Table 5 gives the ^{13}C NMR data as well. The

shift assignments were made on the basis of APT and HETCOR experiments, as well as carbon chemical shifts calculated according to the methods described by Ewing (1979) and Silverstein et al. (1991). The data are in agreement with those previously reported (Bohman, 1968; Gill et al., 1988).

Compound 4 was quite different from the other anthraquinones. It was apparent from the 2D COSY ^{1}H NMR spectral data that the signal corresponding to the methyl group of emodin had been replaced by a new one at δ 4.73 ppm. This suggested the presence of an hydroxymethyl group at C-3 (Table 6). Further confirmation was obtained from the CIMS and UV spectra, which demonstrated the compound to be 7-chloro-1-*O*-methyl-ω-hydroxyemodin (7-chloro-1-*O*-methyl citreorosein or 7-chlorocarviolin) (4). An NOE experiment was performed in which irradiation of the hydroxymethyl protons resulted in increases in the signal intensities of H-2 and H-4. 7-Chlorocitreorosein had been isolated from *Aspergillus fumigatus* (Yamamoto et al., 1968), and 1-*O*-methylcitreorosein (carviolin) has been obtained from a culture of *Penicillium roseo-purpureum* (Hind, 1940). Compound 4 thus represents a new natural anthraquinone.

Compounds 5 and 6 are related to the well-known natural product hypericin, found in *Hypericum* species (Brockmann and Sanne, 1957; Falk and Schmitzberger, 1992) and in a basidiomycete, *Dermocybe austroveneta* (Gill et al., 1988). Hypericin is the subject of intensive medical scrutiny because of its antiviral activity

(Lopez-Bazzocchi et al., 1991). Meruelo and coworkers, in 1988, showed that hypericin inhibited the spread of the Friend and radiation leukemia viruses in vitro and in vivo (Meruelo et al., 1988). The same group also reported that hypericin can inactivate human immunodeficiency virus (HIV), when measured by reverse transcriptase (RT) activity; it would appear, however, that the purified enzyme is not the main target of hypericin activity (Lavie et al., 1989). Thus, the mode of action of hypericin still remains a topic of debate (Kraus et al., 1990).

The only known natural halogenated hypericin-like compounds are the gymnochromes, brominated phenanthroperylenequinones in the crinoid, *Gymnocrinus richeri* (De Riccardis et al., 1991). The identities of compounds 5 and 6 became apparent on an examination of the UV spectra, which were similar to that of hypericin; they were also in very close agreement with the UV spectra of the gymnochromes (De Riccardis et al., 1991), and synthetic brominated derivatives of hypericin (Falk and Schmitzberger, 1992).

The negative-ion LSIMS (Liquid Secondary Ion Mass Spectrometry) spectrum of 5 shows three peaks at 575, 573 and 571 (relative intensity 1:4.2:6). This is indicative of two chlorine atoms in the molecule. The combination of 2D COSY ^{1}H NMR (Table 6) and LSIMS data indicate that 5 is a symmetrical dimer. A series of NOE experiments confirmed that 5 has the structure shown. Irradiation of the methyl protons produced an enhancement of protons H-2 and H-2'. Irradiation of the HO-8 (HO-8') hydroxyl proton did not, however, produce an NOE enhancement

Table 6. [1]H NMR data for 7-chloro-1-*O*-methyl-ω-hydroxyemodin (4), 7,7'-dichlorohypericin (5) and 2,2',7,7'-tetrachlorohypericin (6).[1]

Proton	4[2]	5[3]	5[4]	6[3]
2,2'	6.78, s	7.42, s	7.20, s	
4,4'	7.78, s			
5,5'	7.43, s			
7,7'				
1,1'-OH		13.79, s		13.95, s
6,6'-OH		18.28, s		
8,8'-OH		15.55, s		15.65, s
3-OMe	3.95, s			
3-CH_2OH	4.63, d (5.0)[5]			
3-Me, Me'		2.65, s	2.70, s	2.80, s

[1]Chemical shifts (δ) are reported in ppm from TMS internal standard. The coupling constants in parentheses are given in Hz.
[2]The spectrum was recorded in DMF-d_6 at 300 MHz.
[3]The spectra were recorded in DMSO-d_6 at 400 MHz.
[4]The spectrum was recorded in MeOH-d_4 at 400 MHz.
[5]The hydroxymethyl protons were coupled to a broad hydroxyl proton. When the hydroxyl proton was exchanged by addition of a small amount of MeOH-d_4, this signal became a singlet.

at positions C-7 and C-7'.

The UV spectrum of 6 is very similar to that of 5. The LSIMS (negative ion) spectrum shows four peaks at 645, 643, 641 and 639 (relative intensity 1:3:5:3.5). This is indicative of four chlorine atoms in the molecule. The 2D COSY ^{1}H NMR spectral data are shown in Table 6. Compound 6, like 5, must also be a symmetrical dimer based on the spectroscopic evidence.

The number of free hydroxyl groups in each assigned structure was confirmed by peracetylation with acetic anhydride in pyridine. Mass spectra were determined by direct injection of the acetylation mixtures. Observation of the progressive loss of ketene was especially helpful in confirming the dimeric nature of 5 and 6.

The biogenesis of the anthraquinones and the perylenequinones presumably takes place through the polyketide pathway (Figure 3). Detailed studies on the biosynthesis of emodin and related compounds in fungi and higher plants have been carried out (Gatenbeck, 1962; Zenk and Leistner, 1968; Leistner, 1971; Steglich et al, 1972; Leistner, 1973; Shibata, 1974; Casey et al., 1978; Weiss and Edwards, 1980; Steyn et al., 1981; Turner and Aldridge, 1983; Gill and Steglich, 1987; Gill, 1994). Hypericin is believed to be formed by the linkage of two emodinanthrone units, with subsequent oxidation leading to the perylenequinone structure (Brockmann and Sanne, 1953). The mechanism and stage of formation of the chlorinated anthraquinones and hypericins is, however, uncertain at present (Figure 3).

2.1.4 SYNTHESES OF 1-*O*-METHYLEMODIN (7), 7,7'-DICHLOROHYPERICIN (5) AND 2,2',7,7'-TETRACHLOROHYPERICIN (6)

7-Chloro-1-*O*-methylemodin (3) (1.5 mg, 0.005 mmol) was dissolved in 5 ml of 0.015 M NaOH. Raney nickel (3.0 mg) was added, in one portion, to the stirred solution. The reaction mixture was heated at reflux for 15 minutes, cooled to room temperature and filtered. The red filtrate was acidified to pH 7 with 6 N HCl. The yellow solution was extracted three times with 50 ml portions of diethyl ether. The combined organic layers were dried and concentrated to a yellow solid. Recrystallization from 3:1 toluene:chloroform afforded 1.0 mg of 1-*O*-methylemodin (7) (0.004 mmol, 75 % yield). The R_f of the product was 0.3 (5:1 benzene:ethyl acetate), 0.15 (3:3:1 toluene:chloroform:ethyl acetate) and 0.6 (70:20:8:2 chloroform: petroleum ether:ethyl acetate:methanol). The product was characterized by CIMS and 2D COSY ^{1}H NMR spectra (Table 4).

Hypericin (7 mg, 0.014 mmol) was dissolved, with stirring, in 10 ml of dry *N*,*N*-dimethylformamide. To the stirred, deep purple solution was added *N*-chlorosuccinimide (4 mg, 0.03 mmol). After four hours at ambient temperature, solvent was removed under reduced pressure, and the residual purple solid was dried, in vacuo, for 24 hours. This material was chromatographed on a column of Sephadex LH-20 with methanol:pyridine (9:1) as the eluant. Compound 6 (1.8 mg from acetic acid, 20 % yield) was eluted first and was characterized by UV, LSIMS and 2D COSY ^{1}H NMR spectra. The compound proved to be identical with the natural product in all respects. Compound 5 (4 mg from acetic acid, 50

% yield) was characterized by UV, LSIMS, 2D COSY ^{1}H NMR and ^{13}C NMR spectra. The ^{13}C NMR shifts for compound 5 (Table 7) were determined using APT and HETCOR techniques. Several NOE experiments confirmed the presence of protons at C-2 and C-2'. Irradiation of the methyl protons resulted in an increase in each proton signal at C-2 and C-2'. Thus, the location of the chlorine atoms was at C-7 and C-7'. The compound proved to be identical with the natural product in all respects.

2.1.5 PHYSICAL AND CHEMICAL DATA FOR NATURAL PRODUCTS

Emodin (1) (3 mg, 0.002 % yield, based on dry tissue weight) was obtained as orange crystals from ethyl acetate: MP 256-257° C; UV (ethanol) λ max (log ε) 253 (4.31), 265 (4.29), 289 (4.36), 438 (4.18); CIMS *m/z* $[MH]^{+}$ 271 (94), 197 (100); CIMS *m/z* $[MH]^{-}$ 270 (100). For ^{1}H and ^{13}C NMR data respectively, see Tables 4 and 5.

7-Chloroemodin (2) (78 mg, 0.06 % yield) was obtained as orange crystals from ethyl acetate: MP 281-283° C; UV (ethanol) λ max (log ε) 257 (4.24), 315 (4.22), 325 (4.08), 437 (3.88), 504 (3.70); CIMS *m/z* $[MH]^{+}$ 307 (26), 305 (100); EIMS (70 eV) *m/z* $[M]^{+}$ 306 (35), 304 (100). For ^{1}H and ^{13}C NMR data respectively, see Tables 4 and 5.

7-Chloro-1-*O*-methylemodin (3) (82 mg, 0.06 % yield) was obtained as orange crystals from ethyl acetate: MP 289-291° C; UV (ethanol) λ max (log ε) 256 (4.24), 286 (4.23), 423 (3.78); CIMS *m/z* $[MH]^{+}$ 321 (30), 319 (100); EIMS (70 eV) *m/z* $[M]^{+}$ 320 (35), 318 (100). For ^{1}H and ^{13}C NMR data respectively, see Tables 4 and 5.

7-Chloro-1-*O*-methyl-ω-hydroxyemodin (4) (1 mg, 0.008 % yield) was obtained as pink crystals from ethanol: MP > 290° C; UV (ethanol) λ max (log ε) 222 (4.50), 250 (4.20), 300 (4.23), 434 (3.95), 452 (3.90), 490 (3.78), 525 (3.60); CIMS *m/z* $[MH]^+$ 337 (3), 335 (12); (4) triacetate: CIMS *m/z* $[MH]^+$ 480 (49), 478 (100), 463 (35), 461 (79). For ^1H NMR data, see Table 6.

7,7'-Dichlorohypericin (5) (1.4 mg, 0.001 % yield) was obtained as purple crystals from acetic acid: MP > 350° C; UV (dimethylsulfoxide) λ max (log ε) 251 (4.70), 294 (4.60), 332 (4.50), 388 (3.93), 485 (4.08), 553 (4.29), 597 (4.58); LSIMS *m/z* $[M-H]^-$ 575 (9), 573 (38), 571 (54). For ^1H NMR data, see Table 6.

2,2',7,7'-Tetrachlorohypericin (6) (0.8 mg, 0.0006 % yield) was obtained as purple crystals from acetic acid: MP > 350° C; UV (dimethylsulfoxide) λ max (log ε) 251 (4.70), 295 (4.60), 332 (4.50), 390 (3.90), 484 (4.06), 554 (4.27), 598 (4.56); LSIMS *m/z* $[M-H]^-$ 645 (2), 643 (6), 641 (10), 639 (7). For ^1H NMR data, see Table 6.

Table 7. ^{13}C NMR data for 7,7'-dichlorohypericin (5) and hypericin.[1]

Carbon	5[2]	Hypericin[2]	Hypericin[3]
1,1'	162.8	161.4	161.2
2,2'	117.2	118.9	118.6
3,3'	144.3	143.4	143.5
6,6'	175.2	174.2	174.7
7,7'	107.7	105.5	105.4
8,8'	167.5	168.1	166.0
9,10	184.3	183.6	183.3
1a,9a	101.6	102.0	101.9
1b,9b[4]	120.3	120.6	119.2
3a,3b[4]	120.4	120.7	120.7
6a,6b[5]	125.2	127.0	126.6
8a,10a	109.6	108.3	108.3
8b,10b[5]	124.3	126.0	126.0
9c,10c	120.8	121.2	121.2
3-Me, 3'-Me	23.8	23.6	23.6

[1]Chemical shifts (δ) are reported in ppm from TMS internal standard.
[2]The spectra were recorded in DMSO-d_6 at 125 MHz.
[3]The spectrum was recorded in DMSO-d_6 at 90 MHz (Falk and Schmitzberger, 1992).
[4]Values for C-1b/C-9b and C-3a/C-3b can be interchanged.
[5]Values for C-6a/C-6b and C-8b/C-10b can be interchanged.

CHAPTER 2: CHEMICAL PROPERTIES OF LICHEN QUINONOID PIGMENTS

2.2 ISOLATION OF *HETERODERMIA OBSCURATA* NATURAL PRODUCTS

2.2.1 INTRODUCTION

The lichen *Heterodermia obscurata* can be found in temperate and tropical areas of the world. It has been reported from East Asia, Southeast Asia, India, Nepal, Australia, New Zealand, Polynesia, Hawaii, Central and South America, Western Europe, Russia, East Africa and North America (Kurokawa, 1962; Culberson, 1966; Kurokawa, 1973; Swinscow and Krog, 1976, Hale, 1979; Swinscow and Krog, 1988; Trass, 1992). Of the 16 *Heterodermia* species known to contain anthraquinones, *Heterodermia obscurata* is the only one to be found in temperate regions (Table 3).

Heterodermia obscurata is abundant in deciduous forests of the eastern United States (Figure 8; Appendix 2). It can be found from Florida to Pennsylvania and west to Texas and Wisconsin (Kurokawa, 1962; Hale, 1979). Isolated populations of the lichen have also been reported from Arizona (Weber, 1963), southern Ontario (Brodo, 1988), New Brunswick (Gowan and Brodo, 1988), the Black Hills of South Dakota (Wetmore, 1968) and Hawaii (Kurokawa, 1962).

In Maryland, the lichen is fairly common in forested areas; in particular, it can be found growing on the trunks of Black Oak (*Quercus velutina*), Ash (*Fraxinus* spp.), Black Walnut (*Juglans nigra*), Sweet Gum (*Liquidambar styraciflua*), Hickory (*Carya* spp.) and occasionally on acidic rocks in hilly areas.

Figure 8. *Heterodermia obscurata* from Cedarville State Forest, Maryland.

2.2.2 MATERIALS AND METHODS

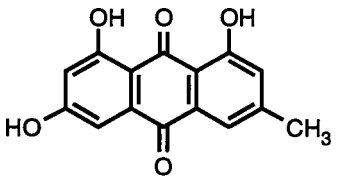
Several collections of *Heterodermia obscurata* were made from different areas within Cedarville State Forest in southern Maryland. Two reference samples were deposited in the UBC Botany Department Herbarium. The lichen was cleaned of debris and air-dried.

Immersion of the dried lichen in ice-cold diethyl ether for ten minutes produced a yellow extract which was examined by TLC (9:1 chloroform:methanol). The TLC patterns for all the individual collections were indistinguishable from one another, suggesting a chemical uniformity in *H. obscurata* populations within the park. In fact, the TLC pattern was practically identical to the one reported by Yosioka et al. (1968c) for *H. obscurata* collected in central Japan.

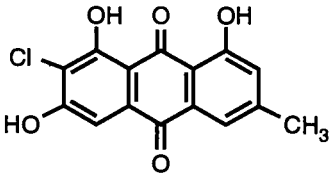
The lichen collections were combined and extracted, in succession, with ice-cold diethyl ether, acetone and methanol. All the extracts were concentrated to minimal volumes and examined by TLC. 7-Chloroemodin (2) was the main constituent of the three extracts. The ether extract indicated the presence of several yellow pigments, believed to be anthrones. This was supported by their UV fluorescence colors (dark red) and similarities in R_f values with anthrones reported earlier in *H. obscurata* (Yosioka et al., 1968c). The structural identities of the anthrones, however, could not be confirmed, as they were present in the extract in only minute amounts. Six compounds were ultimately identified in the three extracts (TLC: 8:2 chloroform:methanol): emodin (1) (R_f 0.88); 7-chloroemodin (2)

(R_f 0.72); 5,7-dichloroemodin (3) (R_f 0.32); flavoobscurin A (4) (R_f 0.42); flavoobscurin B (5) (R_f 0.20); 7,7'-dichlorohypericin (6) (R_f 0.30); and atranorin (R_f 0.55). Compounds 1-5 were reported to be present in *H. obscurata* from Japan (Yosioka et al., 1968a, 1968b) (Figure 5); 7,7'-dichlorohypericin (6), also isolated from *Nephroma laevigatum*, is a novel derivative of hypericin (Cohen and Towers, 1995a). The structures of the lichen compounds are shown in Figure 9.

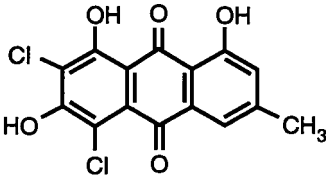
Following TLC examination, the yellow-orange extract from 10 g of lichen was concentrated to 25 ml, and filtered. The material remaining on the filter proved to be atranorin (180 mg, 1.8 % yield) based on a TLC comparison with commercial atranorin (R_f 0.85 for both substances in 12:12:1 chloroform:petroleum ether:methanol) and mixed melting point (198-200°C; no depression). The mixture of pigments (40 mg) was chromatographed on a column of Sephadex LH-20 (100 g). A gradient of chloroform:methanol (8:2) to methanol was used for elution of the lichen compounds. The order of elution was: 1) emodin; 2) 7-chloroemodin; 3) 5,7-dichloroemodin; 4) flavoobscurin A; 5) flavoobscurin B; and, finally 6) 7,7'-dichlorohypericin. 7-Chloroemodin was purified by recrystallization from hot ethyl acetate; the other compounds were purified by preparative TLC (8:2 chloroform:methanol). The immobile purple layer remaining on the column was extruded and extracted with 100 ml of pyridine for 16 hours. The extract was concentrated, and dried under vacuum, to give pure 7,7'-dichlorohypericin.



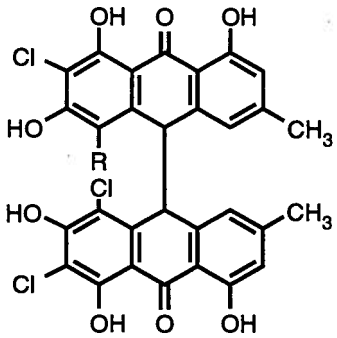
Emodin (1)



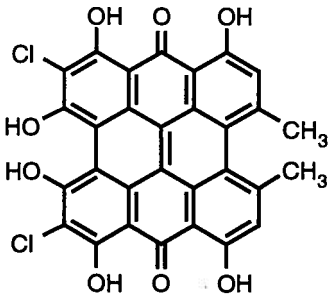
7-Chloroemodin (2)



5,7-Dichloroemodin (3)



R = H; Flavoobscurin A (4)
R = Cl; Flavoobscurin B (5)



7,7'-Dichlorohypericin (6)

Figure 9. The anthraquinones of *Heterodermia obscurata*.

2.2.3 RESULTS AND DISCUSSION

The identity of compound 2 was confirmed by analysis of the CIMS and 2D COSY ^{1}H NMR spectra. The positive-ion CIMS spectrum shows peaks at 307 and 305; the negative-ion CIMS spectrum shows peaks at 306 and 304. The 2D COSY ^{1}H NMR spectral data are shown in Table 8. The CIMS and 2D COSY ^{1}H NMR data are consistent with those previously reported (Bohman, 1968; Yosioka et al., 1968a; Yamamoto et al., 1968). A series of NOE experiments confirmed the identity of 2. Irradiation of the methyl protons resulted in the enhancement of the H-2 and H-4 proton signals. Irradiation of the hydroxyl proton at C-6 resulted in a signal intensity enhancement of H-5. ^{13}C NMR assignments were obtained from APT and HETCOR experiments, and are shown in Table 9.

Compound 3 proved to be 5,7-dichloroemodin on the basis of the MS and 2D COSY ^{1}H NMR spectra. The LSIMS spectrum shows peaks at 341, 339 and 337 (relative intensity 1:2:3). This is indicative of two chlorine atoms in the molecule. The 2D COSY ^{1}H NMR spectral data are shown in Table 8. The data agree well with earlier results (Yosioka et al., 1968a; Lam et al., 1972). An NOE experiment confirmed the identity of 3. Irradiation of the methyl protons produced a corresponding increase in the proton signal intensity of H-2 and H-4.

Emodin was characterized by UV, CIMS and 2D COSY ^{1}H NMR spectra (Table 8). Several NOE experiments confirmed that 1 was emodin. Irradiation of the methyl protons resulted in increases in the proton signal intensities of H-2 and H-4. Irradia-

Table 8. [1]H NMR data for emodin (1), 7-chloroemodin (2) and 5,7-dichloroemodin (3).[1,2]

Proton	1	2	3
2	7.19, s	7.18, s	6.95, s
4	7.57, s	7.61, s	7.43, s
5	7.26, d (2.5)	7.43, s	
7	6.69, d (2.5)		
1-OH		11.97, s	11.72, s
6-OH			13.66, s
8-OH		12.20, s	12.80, s
3-Me	2.48, s	2.48, s	2.40, s

[1]Chemical shifts (δ) are reported in ppm from TMS internal standard. The coupling constants are given in Hz.
[2]The spectra were recorded in Me_2CO-d_6 at 400 MHz.

Table 9. ^{13}C NMR data for emodin (1), 7-chloroemodin (2), 5,7-dichloroemodin (3) and flavoobscurin B (5).[1]

Carbon	1	1[2]	2	2[2]	3	3[2]	5
1	161.9	158.7	161.4	158.7	160.0	158.7	160.4
1'							160.4
2	124.8	120.2	124.2	120.1	124.6	120.1	122.1
2'							122.1
3	149.3	142.4	148.3	142.4	145.4	142.4	146.0
3'							146.0
4	121.2	125.3	120.4	125.3	119.4	125.3	117.2
4'							117.2
5	109.7	111.9	108.5	113.2	119.3	119.4	115.4
5'							115.4
6	167.0	160.4	163.1	160.8	168.5	161.2	166.6
6'							166.6
7	108.6	106.7	121.0	112.9	122.7	114.2	117.3
7'							117.3
8	165.9	160.2	162.8	160.6	160.4	158.7	161.6
8'							161.6
9	191.9	189.3	189.0	187.4	210.9	185.5	188.8
10	182.2	161.7	180.8	161.7	182.4	163.0	188.8
1a							116.0
1b							139.5[5]
4a (9b)	134.0[4]	131.5	132.8[4]	131.5	127.0[4]	129.6	139.5[5]
8a	110.0[3]	110.0	110.8[3]	111.3	111.0[3]	112.6	112.9
8b							139.6[5]
9a	114.4[3]	107.1	113.4[3]	107.1	113.6[3]	107.1	116.0
9c							30.8
10a	136.6[4]	132.8	132.6[4]	130.9	133.6[4]	131.3	112.9
10b							139.6[5]
10c							30.8
3-Me	21.8		21.6		21.5		21.6
3-Me'							21.6

[1]Chemical shifts (δ) are reported in ppm from TMS internal standard. The spectra were recorded in DMSO-d_6 at 125 MHz.
[2]Values calculated according to the methods described in Silverstein et al. (1991).
[3]Values for C-8a and C-9a can be interchanged.
[4]Values for C-10a and C-4a can be interchanged.
[5]Values for C-1b/9b and C-8b/10b can be interchanged.

Table 10. [1]H NMR data for flavoobscurin A (4), flavoobscurin B (5) and 7,7'-dichlorohypericin (6).[1]

Proton	4[2]	5[2]	6[3]
2,2'	6.54, s	6.68, s	7.42, s
4,4'	5.11, s	5.74, s	
5,5'	6.96, s		
7,7'			
10,10'	4.66, d (3.0)[4]	4.95, s[4]	
10,10'	4.72, d (3.0)[4]		
1,1'-OH			
6,6'-OH			
8,8'-OH			
3-Me, 3'-Me	2.32, s	2.32, s	2.65,s

[1]Chemical shifts (δ) are reported in ppm from TMS internal standard. The coupling constants are given in Hz.
[2]The spectra were recorded in Me_2CO-d_6 at 400 MHz.
[3]The spectrum was recorded in DMSO-d_6 at 400 MHz.
[4]No attempt has been made to assign stereochemistry.

tion of the C-8 hydroxyl proton also produced a proton signal enhancement of H-7.

Compounds 4 and 5 are chlorinated bianthrones, originally isolated by Yosioka et al. (1968b) (Figure 9). Nonhalogenated bianthrones have been found in a crinoid, *Lamprometra palmata* (Rideout and Sutherland, 1985) and in the fungi *Aspergillus chevalieri* (Bachmann et al., 1979) and *A. wentii* (Assante et al., 1980). The structures of 4 and 5 were proven by LSIMS, CIMS and 2D COSY ^{1}H NMR spectra. The two compounds produce characteristic fragmentation patterns in their mass spectra. The negative-ion LSIMS spectrum of 4 shows peaks corresponding to the parent bianthrone structure, as well as the two nonsymmetrical monomers. The negative-ion LSIMS spectrum of 5 also shows the parent peak, along with a single set of monomeric fragments. Only the monomeric fragments can be seen in the CIMS spectra of the two compounds; they do display, however, the same overall patterns seen in the two LSIMS spectra. The 2D COSY ^{1}H NMR chemical shifts for 4 and 5 are shown in Table 10. Table 9 lists the ^{13}C NMR shifts for flavoobscurin B, which were not reported by Yosioka et al. (1968b).

7,7'-Dichlorohypericin (Figure 9) gave a negative-ion LSIMS spectrum with parent peaks at 575, 573 and 571 (relative intensity 1:2:3). This is indicative of two chlorine atoms in the compound. This conclusion was supported by the 2D COSY ^{1}H NMR data (Table 10). An NOE experiment also confirmed the identity of compound 6. Irradiation of the methyl protons produced an increase in the proton signal intensity of H-2 (H-2').

Many lichen genera produce anthraquinones, and anthrones have been detected in several species by TLC (Bohman, 1968; Yosioka et al., 1968c; Kurokawa, 1973; Steiner et al., 1974). Bianthrones are probably formed by oxidative coupling of anthrones. A TLC, performed a few hours after sample collection, clearly showed the presence of bianthrones and 7,7'-dichlorohypericin. Thus, it seems unlikely that these natural products are exclusively artifacts generated during extraction and sample manipulation.

2.2.4 SYNTHESES OF 7-CHLOROEMODIN (2) AND 5,7-DICHLOROEMODIN (3)

Emodin (40 mg, 0.15 mmol) was added to 50 ml of dry *N,N*-dimethylformamide. The red solution was allowed to stir at ambient temperature until all the emodin had dissolved (40 minutes). *N*-Chlorosuccinimide (40 mg, 0.30 mmol) was added, in one portion, to the stirred solution. The reaction mixture was maintained at room temperature for 24 hours. The solvent was removed, under reduced pressure, to give an orange solid. The solid was dried, in vacuo, for 24 hours. Column chromatography of the crude mixture on Sephadex LH-20 (8:2 chloroform:methanol) afforded (in order of elution) emodin (1) (8 mg from ethyl acetate, 20 % recovery), 7-chloroemodin (2) (22 mg from ethyl acetate, 48 % yield) and 5,7-dichloroemodin (3) (12 mg from methanol, 24 % yield). All compounds were characterized by UV, CIMS, EIMS, ^{1}H NMR and ^{13}C NMR spectra. They were found to be identical with the natural products in all respects.

2.2.5 PHYSICAL AND CHEMICAL DATA FOR NATURAL PRODUCTS

Emodin (1) (3 mg, 0.03 % yield, based on dry tissue weight) was obtained as orange crystals from ethyl acetate: MP 255-256°C; UV (ethanol) λ max (log ε) 262 (4.30), 288 (4.34), 434 (4.20); CIMS *m/z* $[MH]^-$ 270 (100). For ^1H and ^{13}C NMR data respectively, see Tables 8 and 9.

7-Chloroemodin (2) (12 mg, 0.12 % yield) was obtained as orange crystals from ethyl acetate: MP 280-282°C; UV (ethanol) λ max (log ε) 257 (4.24), 315 (4.22), 325 (4.08), 437 (3.88), 504 (3.70); CIMS *m/z* $[MH]^+$ 307 (38), 305 (100); CIMS *m/z* $[MH]^-$ 306 (33), 304 (100). For ^1H and ^{13}C NMR data respectively, see Tables 8 and 9.

5,7-Dichloroemodin (3) (1 mg, 0.01 % yield) was obtained as red crystals from methanol: MP 268-270°C; UV (ethanol) λ max (log ε) 262 (4.43), 320 (4.20), 457 (4.00), 524 (3.90); LSIMS *m/z* $[M-H]^-$ 341 (12), 339 (25), 337 (35); CIMS *m/z* $[MH]^+$ 343 (7), 341 (38), 339 (55); CIMS *m/z* $[MH]^-$ 342 (12), 340 (68), 338 (100). For ^1H and ^{13}C NMR data respectively, see Tables 8 and 9.

Flavoobscurin A (4) (2 mg, 0.02 % yield) was obtained as lemon-yellow crystals from acetic acid: MP > 350°C; UV (ethanol) λ max (log ε) 273 (4.25), 410 (4.30); LSIMS *m/z* $[M-H]^-$ 615 (2), 613 (4), 611 (5), 326 (19), 324 (75), 322 (100), 290 (9), 288 (18); CIMS *m/z* $[MH]^+$ 329 (17), 327 (80), 325 (100), 293 (47), 291 (86); CIMS *m/z* $[MH]^-$ 327 (7), 325 (27), 323 (33), 290 (40), 288 (66). For ^1H NMR data, see Table 10.

Flavoobscurin B (5) (3 mg, 0.03 % yield) was obtained as lemon-yellow crystals from acetic acid: MP > 350°C; UV (ethanol) λ max (log ε) 276 (4.20), 403 (4.26); LSIMS *m/z* $[M-H]^-$ 651 (2), 649 (6), 647 (12), 645 (9), 327 (7), 325 (26), 323 (32); CIMS *m/z* $[MH]^+$ 329 (11), 327 (54), 325 (79); CIMS *m/z* $[MH]^-$ 327 (8), 325 (31), 323 (40). For ^1H and ^{13}C NMR data respectively, see Tables 10 and 9.

7,7'-Dichlorohypericin (6) (0.8 mg, 0.008 % yield) was obtained as purple crystals from acetic acid: MP > 350°C; UV (ethanol) λ max (log ε) 259 (4.70), 292 (4.60), 332 (4.51), 485 (4.08), 552 (4.26), 594 (4.56); LSIMS *m/z* $[M-H]^-$ 575 (19), 573 (39), 571 (50). For ^1H NMR data, see Table 10.

CHAPTER 3: BIOSYNTHETIC STUDIES OF QUINONOID PIGMENTS IN *N. LAEVIGATUM*

3.1 INTRODUCTION

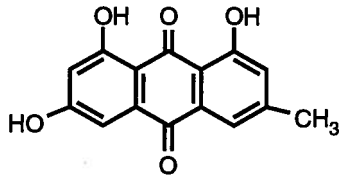
In order to determine if the lichen anthraquinones are derived from acetate through the polyketide pathway, sodium [2-^{14}C]acetate and sodium [1-^{13}C]acetate were administered to *Nephroma laevigatum*, maintained in aqueous culture, in two separate experiments. In a third experiment, sodium ^{36}chloride was fed to the lichen, in the hope of observing in situ chlorination of endogenous anthraquinones, or their precursors.

3.2 MATERIALS AND METHODS

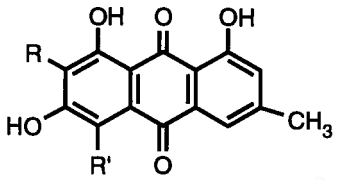
Lichen thalli of *Nephroma laevigatum* were collected from shoreline rocks on Gabriola Island, British Columbia in October, 1994. The lichen (100 g) was carefully cleaned of moss, soil, infected or damaged lichen thalli and other debris, and washed several times with sterile distilled water. The lichen (20 g) was placed in each of three plastic dishes (245 x 245 x 20 cm). In three different experiments, the lichen was incubated with aqueous solutions of sodium [2-^{14}C]acetate (50 microcuries, 99.9 atom % ^{14}C), sodium [1-^{13}C]acetate (0.12 M solution, 99 atom % ^{13}C) and sodium ^{36}chloride (25 microcuries, 99 atom % ^{36}Cl). Each isotope was dissolved in a sufficient amount of sterile distilled water to thoroughly moisten the lichen, but not submerge it. The total liquid (100 ml) was absorbed within a few minutes. The incubation experiments were maintained in an indoor greenhouse at 27°C under constant conditions of light/dark cycles (16 hours light/8 hours dark)

and temperature. After five days, the lichen was harvested, dried and weighed.

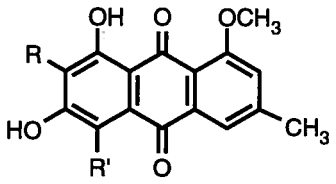
The isolation and characterization of the labelled anthraquinones follow the same procedures utilized in the initial identification of compounds in *Nephroma laevigatum* (Chapter 2). The primary constituent in the mixture of pigments obtained from the sodium [2-^{14}C]acetate incorporation experiment was 7-chloroemodin (2) (TLC; R_f 0.5; 9:1 chloroform:methanol). The TLC pattern revealed the presence of two additional compounds, which were subsequently isolated and characterized as: emodin (1) (R_f 0.8) and 7-chloro-1-*O*-methylemodin (3) (R_f 0.7). Five products were identified in the pigment mixture obtained from the sodium [1-^{13}C]acetate incorporation experiment: 7-chloro-1-*O*-methylemodin (3) (R_f 0.7), 5-chloro-1-*O*-methylemodin (5) (R_f 0.65), 5-chloroemodin (6) (R_f 0.55), 5-chloro-1-*O*-methyl-ω-hydroxyemodin (7) (R_f 0.3) and 5-chloro-ω-hydroxyemodin (8) (R_f 0.25). Finally, the pigment mixture from the sodium ^{36}chloride incorporation experiment contained 7-chloroemodin (2) (R_f 0.5), 7-chloro-1-*O*-methylemodin (3) (R_f 0.7) and 7-chloro-1-*O*-methyl-ω-hydroxyemodin (4) (R_f 0.35). The structures of these compounds are shown in Figure 10. The lichen was extracted successively with acetone and methanol. After TLC examination, the combined extracts were concentrated to a brown-red solid. The solid was purified by column chromatography on Sephadex LH-20, using a gradient of chloroform:methanol (9:1) to methanol. Fractions (20 ml) were collected and analyzed by TLC. All compounds were further purified by preparative TLC (8:2



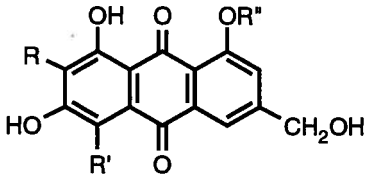
Emodin (1)



R = Cl, R' = H; 7-Chloroemodin (2)
R = H, R' = Cl; 5-Chloroemodin (6)
R = Cl, R' = Cl; 5,7-Dichloroemodin (9)



R = Cl, R' = H; 7-Chloro-1-*O*-methylemodin (3)
R = H, R' = Cl; 5-Chloro-1-*O*-methylemodin (5)



R = Cl, R' = H, R" = CH_3; 7-Chloro-1-*O*-methyl-ω-hydroxyemodin (4)
R = H, R' = Cl, R" = CH_3; 5-Chloro-1-*O*-methyl-ω-hydroxyemodin (7)
R = H, R' = Cl, R" = H; 5-Chloro-ω-hydroxyemodin (8)

Figure 10. Anthraquinones from isotope labelling experiments with *N. laevigatum*.

Table 11. [1]H NMR data for emodin (1), 7-chloroemodin (2), 7-chloro-1-*O*-methylemodin (3), 7-chloro-1-*O*-methyl-ω-hydroxyemodin (4), 5-chloro-1-*O*-methylemodin (5), 5-chloroemodin (6), 5-chloro-1-*O*-methyl-ω-hydroxyemodin (7), and 5-chloro-ω-hydroxyemodin (8) from isotope labelling experiments.[1]

Proton	1(^{14}C)[2]	2(^{14}C)[2]	2(^{36}Cl)[3]	3(^{14}C)[2]	3(^{13}C)[4]	3(^{36}Cl)[3]	4(^{36}Cl)[2]	5(^{13}C)[4]	6(^{13}C)[4]	7(^{13}C)[4]	8(^{13}C)[4]
2	7.21, s	7.09, s	7.20, s	7.50, s	7.48, s	7.45, s	6.90, s	7.38, s	7.08, s	7.46, s	7.12, s
4	7.57, s	7.49, s	7.62, s	7.68, s	7.62, s	7.68, s	7.80, s	7.58, s	7.38, s	7.73, s	7.54, s
5	7.24, d (2.5)	7.11, s	7.48, s	7.29, s	7.25, s	7.35, s	7.50, s				
7	6.65, d (2.5)							6.78, s	6.68, s	6.78, s	6.63, s
1-OH											
6-OH											
8-OH											
1-OMe				4.05, s	4.00, s	4.03, s	4.00, s	3.92, s		3.92, s	
3-CH_2OH							4.73, d (5.0)[5]			4.63, d (5.0)[5]	4.54, d (5.0)[5]
3-Me	2.48, s	2.48, s	2.50, s	2.50, s	2.50, s	2.50, s		2.47, s	2.40, s		

[1]Chemical shifts (δ) are reported in ppm from TMS internal standard. The coupling constants are given in Hz. The radioactive/stable isotope label or isotope used in the feeding study is shown in parentheses.

[2]The spectra were recorded in DMF-d_6 at 400 MHz.

[3]The spectra were recorded in Me_2CO-d_6 at 400 MHz.

[4]The spectra were recorded in DMSO-d_6 at 400 MHz.

[5]The hydroxymethyl protons are coupled to a broad hydroxyl proton. If the hydroxyl proton is exchanged by addition of a small amount of MeOH-d_4, this signal becomes a singlet.

Table 12. Properties of radiolabelled anthraquinones isolated from *Nephroma laevigatum*.

Compound	Isotope	Yield (%)[1]	DPM[2]	DPM/mg	μC_i[3]	mC_i/mM[4]	% Incorp.[5]
1	^{14}C	2 mg (0.02)	8,188	4094	0.004	0.00054	0.01
2	^{14}C	30 mg (0.23)	112,716	3757	0.05	0.00051	0.1
2	^{36}Cl	5 mg (0.06)	18,200	3638	0.01	0.00061	0.04
3	^{14}C	22 mg (0.17)	23,804	1082	0.01	0.00014	0.02
3	^{36}Cl	5 mg (0.06)	2,010	402	0.001	0.00006	0.004

[1]Yields are based on lichen dry weight.
[2]Disintegrations per minute.
[3]Total radioactivity (microcuries).
[4]Specific activity.
[5]Percentage (absolute) incorporation ([DPM of isolated product/DPM of isotope fed to lichen] % 100).

chloroform:methanol), and repeatedly recrystallized from a suitable solvent until purity (> 99 %) could be established on the basis of reversed-phase HPLC. Radiolabelled compounds were recrystallized to constant specific activity, and purity checked by reversed-phase HPLC. All compounds were characterized by UV, MS, ^{1}H NMR and ^{13}C NMR spectra.

Autoradiographs of the radiolabelled compounds were made by exposing 2D silica gel TLC plates of anthraquinones and isotope to X-ray film, after solvent development in chloroform:methanol (8:2) and acetone:acetic acid (8:2). After two months exposure, the X-ray film was developed and the radioactive "halos", corresponding to either ^{14}C or ^{36}Cl-labelled anthraquinones, marked (Figure 11). Radioactive "halos" representing sodium ^{36}chloride or sodium [2-^{14}C]acetate were clearly distinguishable from the radiolabelled anthraquinones. Development of the 2D TLC plate in either solvent system did not result in the migration of isotopically labelled reagent from the original point of application. Furthermore, there was no evidence for the presence of either isotope at the locations where the samples of radiolabelled anthraquinones were applied to the TLC plate. It was therefore concluded that the purified radiolabelled anthraquinones were free from any contamination by the isotopes used in the lichen tracer studies.

3.3 RESULTS AND DISCUSSION

The identity of 2 was established by ^{1}H NMR (Table 11) and mass spectra. The spectral data are consistent with our previous results (Cohen and Towers,

1995a, 1995b). Table 12 provides the specific activities and percentage incorporations for ^{14}C and ^{36}Cl-labelled 7-chloroemodin (2).

Emodin (1) was characterized by ^{1}H NMR (Table 11) and mass spectra. The spectral data are indistinguishable from those of authentic material (Cohen and Towers, 1995a, 1995b). The specific activity and incorporation levels for ^{14}C-labelled emodin (1) are shown in Table 12. The amount of emodin produced in the sodium [2-^{14}C]acetate incubation experiment was insufficient for a ^{13}C NMR spectrum to be taken.

7-Chloro-1-*O*-methylemodin (3) was identified by ^{1}H NMR (Table 11), ^{13}C NMR (Table 13) and mass spectra. The results of an NOE experiment performed on compound 3 were consistent with the assigned structure. Irradiation of the H-2 proton resulted in an enhancement of the methoxy protons at C-1. The percentage incorporation of ^{14}C label into compound 3 was 20 % of the level for 7-chloroemodin, and the specific activity of ^{14}C-labelled 7-chloro-1-*O*-methylemodin was also 20 % of the values reported for 7-chloroemodin and emodin (Table 12).

The ^{13}C NMR spectrum of 7-chloro-1-*O*-methylemodin isolated from the feeding experiment shows specific incorporation at carbon atoms 1, 3, 4a, 6, 8, 9 and 10a (Table 13). This is entirely consistent with its formation from an octaketide precursor, itself derived from acetate. The ^{13}C isotopic enrichments were measured by comparing the peak intensities in both the natural abundance and enriched spectra after normalization (Casey et al.,1978). The enrichments values are consistent with

Table 13. ^{13}C NMR data and isotope enrichments for 7-chloro-1-*O*-methylemodin (3).[1]

Carbon	Chemical Shift (δ)	% Enrichment[2]
1	160.0	3.1
2	120.5	0.7
3	147.8	3.7
4	120.0	0.7
4a	131.8	5.2
5	118.0	0.6
6	161.0	4.9
7	106.4	0.0
8	160.2	3.1
8a	112.4	0.0
9	186.2	6.0
9a	113.5	0.0
10	182.0	1.9
10a	135.6	3.8
OMe	56.5	0.0
Me	21.8	-0.5

[1]The lichen was fed 0.12 M sodium [1-^{13}C] acetate. The spectrum was recorded in DMSO-d_6 at 125 MHz. Chemical shifts (δ) are reported in ppm from TMS internal standard.

[2]Percentage ^{13}C enrichments (greater than 1.1 % natural abundance) were calculated from the ratio in peak height obtained from the ^{13}C NMR spectra of labelled and unlabelled products, respectively.

Table 14. ^{13}C NMR data for 7-chloroemodin (2), 7-chloro-1-*O*-methylemodin (3) and 5-chloro-1-*O*-methylemodin (5).[1]

Carbon	2(^{14}C)	3(^{14}C)	3(^{13}C)	5(^{13}C)
1	161.4	160.0	160.0	160.2
2	124.0	120.3	120.5	120.0
3	148.5	148.1	147.8	145.8
4	120.4	120.1	120.0	119.8
5	108.6	106.6	106.4	118.6
6	163.1	160.7	161.0	161.0
7	121.3	119.8	118.0	112.6
8	162.8	160.5	160.2	160.2
9	191.0	186.4	186.2	191.8
10	181.7	182.0	182.0	183.6
4a[2]	133.2	131.1	131.8	130.6
8a[3]	110.1	109.8	112.4	112.6
9a[3]	114.2	113.1	113.5	113.4
10a[2]	133.0	134.4	135.6	134.8
OMe		56.4	56.5	56.5
Me	21.6	21.7	21.8	21.8

[1]Chemical shifts (δ) are reported in ppm from TMS internal standard. The spectra were recorded in DMSO-d_6 at 125 MHz. The isotope label is shown in parentheses.

[2]Values for 4a and 10a can be interchanged

[3]Values for 8a and 9a can be interchanged.

levels obtained from biosynthetic studies of anthraquinones in *Penicillium islandicum* (Casey et al., 1978), perylenequinones in *Pyrenochaeta terrestris* (Kurobane et al., 1981) and anthraquinones in *Dermocybe* (Gill and Giménez, 1990a, 1990b).

5-Chloro-1-*O*-methylemodin (5) had a mass spectrum similar to that of the 7-chloro isomer; the ^{1}H NMR spectrum was quite different however. The location of the chlorine at C-5 was based on the chemical shift for H-7 (6.78 ppm) which is characteristic for an aromatic proton situated between two aromatic hydroxyl groups (Table 11). The location of the methoxy group was determined by an NOE experiment; irradiation of the methoxy protons at C-1 resulted in an increase in the signal intensity for the H-2 proton.

5-Chloro-1-*O*-methylemodin had previously been isolated from the fungus *Phialophora alba* (Ayer and Trifonov, 1994). Although two other 5-chloro-substituted anthraquinones are known from the fungal genus *Dermocybe* (Steglich et al., 1969), they have not previously been reported from lichens collected in the field.

7-Chloro-1-*O*-methyl-ω-hydroxyemodin (4), a new metabolite previously isolated by us (Cohen and Towers, 1995a), was also obtained from our lichen feeding experiments. We could not, however, isolate sufficient quantities for accurate measurements of isotope enrichments. The structure of 4 was proven by ^{1}H NMR, NOE experiments and mass spectra. The location of the methoxy protons at C-1 was confirmed by their irradiation, which produced an increase in the ^{1}H NMR signal intensity of the H-2 proton.

5-Chloroemodin (6) was confirmed by ^{1}H NMR and mass spectra. The CIMS mass spectra of 6 and 6 triacetate indicated that it was a monochloroemodin. The ^{1}H NMR spectrum showed an aromatic signal consistent with an H-7 proton (6.70 ppm) (Table 11).

5-Chloro-1-*O*-methyl-ω-hydroxyemodin (7) was confirmed by ^{1}H NMR, NOE experiments and mass spectra. The CIMS mass spectrum was similar to that of 4; however, the presence of an H-7 proton shift (6.78 ppm) indicated that the chlorine must be at C-5 (Table 11). The position of the methoxy protons was shown by an NOE experiment; irradiation of the methoxy protons resulted in an increase in the ^{1}H NMR signal intensity of H-2.

5-Chloro-ω-hydroxyemodin (8) was confirmed by ^{1}H NMR and mass spectra. The EIMS mass spectrum gave a parent molecular ion corresponding to the molecular mass of 8. The presence of an H-7 proton shift (6.63 ppm) was readily apparent from the ^{1}H NMR spectrum, and indicated that the chlorine must be at C-5 (Table 11).

3.4 PHYSICAL AND CHEMICAL DATA FOR NATURAL PRODUCTS

3.4.1 PRODUCTS FROM SODIUM [2-^{14}C]ACETATE INCORPORATION

Emodin (1) (2 mg, 0.02 % yield, based on dry tissue weight) was obtained as orange crystals from ethyl acetate: MP 256-257°C; UV (ethanol) λ max (log ε) 253 (4.31), 265 (4.29), 289 (4.36), 438 (4.18); CIMS *m/z* $[MH]^+$ 271 (100), 242 (77). For ^{1}H NMR data, see Table 11.

7-Chloroemodin (2) (30 mg, 0.23 % yield) was obtained as orange crystals from ethyl acetate: MP 281-283°C; UV (ethanol) λ max (log ε) 257 (4.24), 315 (4.22), 325 (4.08), 437 (3.88), 504 (3.70); CIMS *m/z* $[MH]^+$ 307 (26), 305 (100); EIMS (70 eV) *m/z* $[M]^+$ 306 (35), 304 (100). For ^1H and ^{13}C NMR data respectively, see Tables 11 and 14.

7-Chloro-1-*O*-methylemodin (3) (22 mg, 0.17 % yield) was obtained as orange crystals from ethyl acetate: MP 289-291°C; UV (ethanol) λ max (log ε) 256 (4.24), 286 (4.23), 423 (3.78); CIMS *m/z* $[MH]^+$ 321 (30), 319 (100); EIMS (70 eV) *m/z* $[M]^+$ 320 (35), 318 (100). For ^1H and ^{13}C NMR data respectively, see Tables 11 and 14.

3.4.2 PRODUCTS FROM SODIUM [1-^{13}C]ACETATE INCORPORATION

7-Chloro-1-*O*-methylemodin (3) (3 mg, 0.02 % yield) was obtained as orange crystals from ethyl acetate: MP 288-290°C; UV (ethanol) λ max (log ε) 256 (4.24), 286 (4.23), 423 (3.82); CIMS *m/z* $[MH]^+$ 321 (33), 319 (100). For ^1H and ^{13}C NMR data respectively, see Tables 11, 13 and 14.

5-Chloro-1-*O*-methylemodin (5) (3 mg, 0.02 % yield) was obtained as orange crystals from ethyl acetate: MP 252-253° C; UV (ethanol) λ max (log ε) 228 (4.30), 259 (4.33), 314 (4.25), 440 (4.02); CIMS *m/z* $[MH]^+$ 321 (33), 319 (100). For ^1H and ^{13}C NMR data respectively, see Tables 11 and 14.

5-Chloroemodin (6) (2 mg, 0.01 % yield) was obtained as orange crystals from ethyl acetate: CIMS *m/z* $[MH]^+$ 307 (33), 305 (100); EIMS *m/z* $[M]^+$ 306 (22), 304 (100); 5-chloroemodin triacetate: EIMS *m/z* $[M]^+$ 390 (9), 388 (27), 348 (9), 346 (25), 306 (51), 304 (100). For ^1H NMR data, see Table 11.

5-Chloro-1-*O*-methyl-ω-hydroxyemodin (7) (2 mg, 0.01 % yield) was obtained as salmon crystals from methanol: UV (ethanol) λ max (log ε) 218 (4.50), 257 (4.22), 322 (4.08), 496 (3.56); CIMS *m/z* $[MH]^+$ 337 (33), 335 (100); 5-chloro-1-*O*-methyl-ω-hydroxyemodin triacetate: CIMS *m/z* $[MH]^+$ 463 (33), 461 (100); EIMS *m/z* $[M]^+$ 420 (20), 418 (46), 378 (18), 376 (55), 336 (2), 334 (7), 318 (42), 316 (100), 306 (20), 304 (51). For ^{1}H NMR data, see Table 11.

5-Chloro-ω-hydroxyemodin (8) (2 mg, 0.01 % yield) was obtained as orange crystals from methanol: EIMS *m/z* $[M]^+$ 322 (33), 320 (100). For ^{1}H NMR data, see Table 11.

3.4.3 PRODUCTS FROM SODIUM ^{36}CHLORIDE INCORPORATION

7-Chloroemodin (2) (5 mg, 0.06 % yield) was obtained as orange crystals from ethyl acetate: MP 281-282°C; UV (ethanol) λ max (log ε) 257 (4.24), 315 (4.22), 325 (4.10), 437 (3.88), 504 (3.80); CIMS *m/z* $[MH]^+$ 307 (30), 305 (100). For ^{1}H NMR data, see Table 11.

7-Chloro-1-*O*-methylemodin (3) (5 mg, 0.06 % yield) was obtained as orange crystals from ethyl acetate: MP 289-291°C; UV (ethanol) λ max (log ε) 256 (4.24), 286 (4.22), 423 (3.80); CIMS *m/z* $[MH]^+$ 321 (33), 319 (100). For ^{1}H NMR data, see Table 11.

7-Chloro-1-*O*-methyl-ω-hydroxyemodin (4) (1 mg, 0.01 % yield) was obtained as pink crystals from ethanol: MP > 290°C; UV (ethanol) λ max (log ε)

222 (4.50), 250 (4.20), 300 (4.23), 434 (3.95), 452 (3.90), 490 (3.78), 525 (3.60). CIMS *m/z* $[MH]^+$ 337 (3), 335 (12); 7-chloro-1-*O*-methyl-ω-hydroxyemodin tri-acetate: CIMS *m/z* $[MH]^+$ 480 (49), 478 (100), 463 (35), 461 (79). For ^1H NMR data, see Table 11.

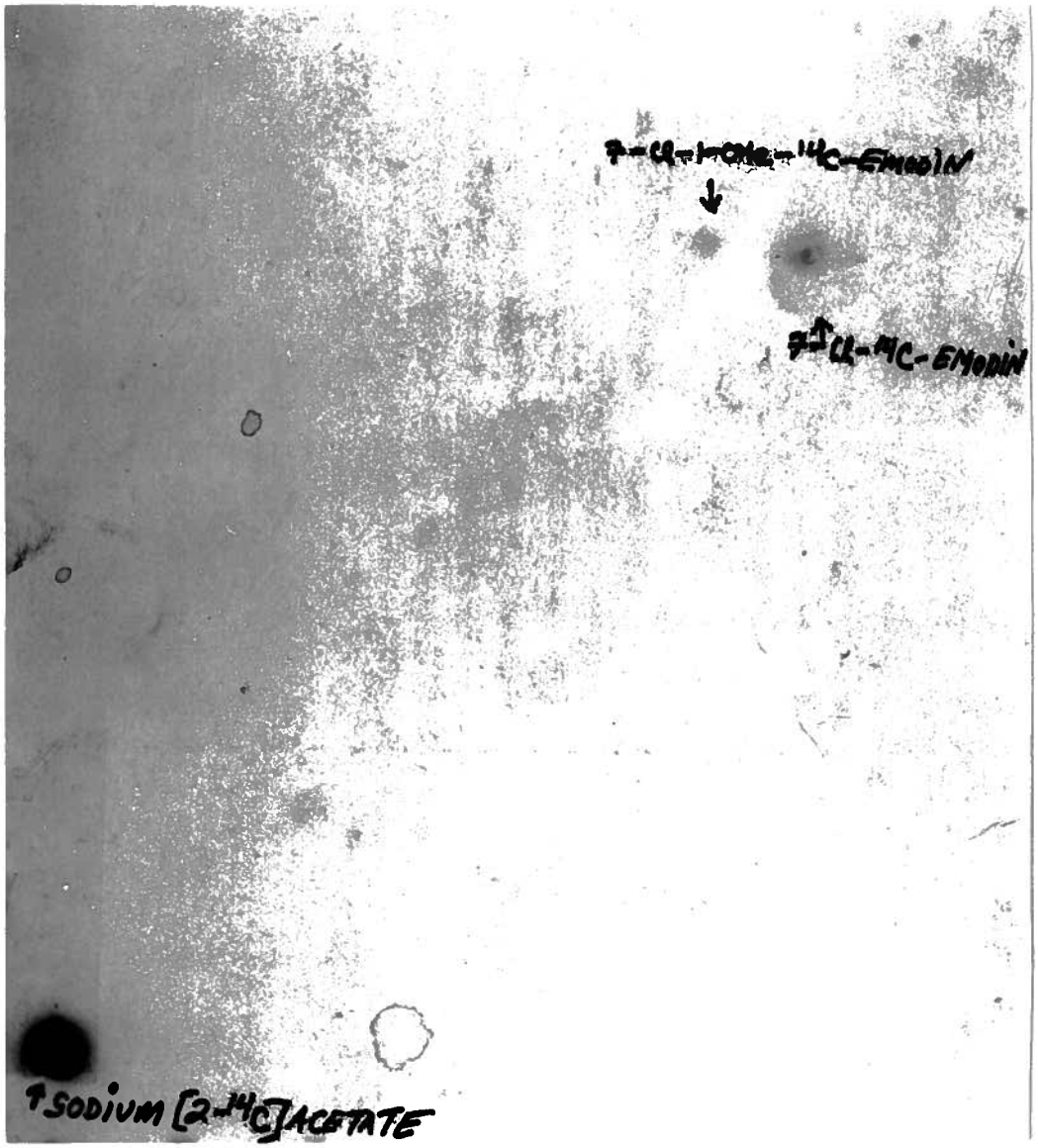


Figure 11. Autoradiographs of 2D TLC of radiolabelled compounds.

CHAPTER 4: BIOHALOGENATION STUDIES OF QUINONOID PIGMENTS

4.1 INTRODUCTION

Since the discovery of the first halogenating enzymes in the early 1950s, our knowledge about the physiological roles, molecular structures, and mechanisms of action of haloperoxidases has greatly expanded (Neidleman and Geigert, 1986; van Pée, 1990; Franssen and van der Plas, 1992; Butler and Walker, 1993). Three classes of haloperoxidases are now known: chloroperoxidases (which can chlorinate, brominate, and iodinate various substrates); bromoperoxidases (bromination and iodination), and iodoperoxidases (iodination only). The first systematic investigation of a haloperoxidase began with the isolation and mechanistic study of a chloroperoxidase from the fungus *Caldariomyces fumago* (Morris and Hager, 1966; Hager et al., 1966). In the intervening years, additional chloroperoxidases have been isolated from bacteria and fungi; bromoperoxidases from bacteria, marine algae, marine worms and sea urchins; and iodoperoxidases from mammals, birds, higher plants and brown algae (Neidleman and Geigert, 1986; Franssen and van der Plas, 1992). Fungi appear to contain only chloroperoxidases, although basidiomycetes have yet to be examined in any detail. The prevalence of chlorinated natural products in fungi (Gribble, 1992) is consistent with the presence of chloroperoxidases; although the same enzymes can also utilize higher halogens, the scarcity of brominated or iodinated compounds in fungi probably reflects the scarcity of these halogens in terrestrial substrates. The single report of a lichen haloperoxidase is that of a bromoperoxidase isolated from *Xanthoria parietina* (Plat

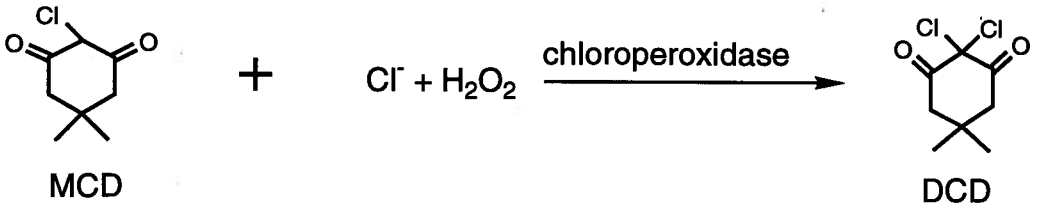
et al., 1987). The investigators found that the enzyme contained vanadium, essential for catalytic activity, and was remarkably thermostable, maintaining full enzymatic activity at 50°C. In addition, the bromoperoxidase had a high affinity for bromide ion and was only active at low concentrations. The enzyme was also inhibited by chloride and fluoride ions, and exhibited a pH optimum at pH 5.5. Considering the absence of brominated compounds in lichens, and the ubiquity of chlorinated substances, it is surprising that the lichen would produce a bromoperoxidase, rather than a chloroperoxidase. Interestingly , a bromoperoxidase isolated from the green alga *Penicillus capitatus* was later shown to become a chloroperoxidase at a lower pH (Manthey and Hager, 1989; Franssen and van der Plas, 1992). Had the vanadium-containing bromoperoxidase from *Xanthoria parietina* been studied at lower pH, it might also have shown chloroperoxidase activity (Soedjak and Butler, 1990).

4.2 MATERIALS AND METHODS

Since *Nephroma laevigatum* produced several chlorinated anthraquinones, and there was evidence from the sodium ^{36}chloride incorporation experiment for in situ chlorination, it seemed worthwhile to try to identify a chloroperoxidase in the lichen.

Ultimately, a semi-purified chloroperoxidase preparation was obtained from the lichen in the following manner. The lichen (400 g) was homogenized in a blender with 2 liters of 0.1 M potassium phosphate buffer, pH 5.8. The homo-

Dimedon Assay



MCD DCD

Reaction mixture: (2 ml) 0.2 M potassium phosphate buffer, pH 3.0
(0.1 ml) 50 mM potassium chloride
(0.1 ml) 2 mM MCD
(0.1 ml) 10 mM hydrogen peroxide
(10 µl) chloroperoxidase

Procedure:

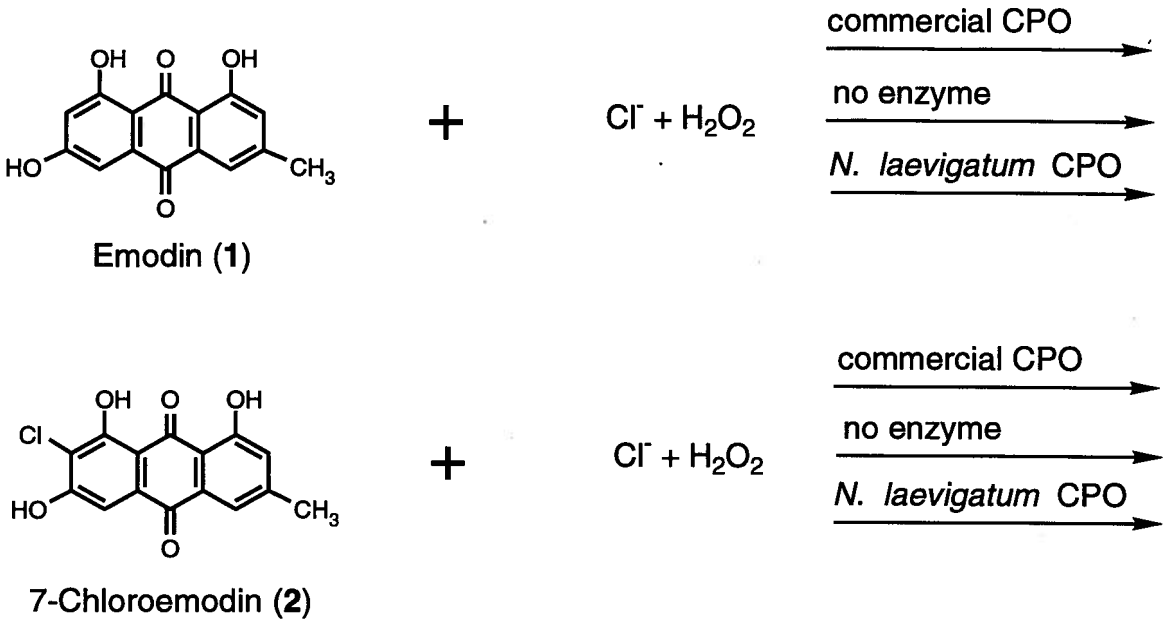
1. Mix all the ingredients, except hydrogen peroxide, in a vial.
2. Initiate chlorination reaction by the addition of peroxide.
3. Assay mixture continuously in a quartz UV cuvette. Monitor the decrease in absorbance (292 nm) corresponding to the consumption of MCD.
4. The MCD reaction is the standard assay used to assign the activity of a haloperoxidase enzyme.

Bradford Assay

Procedure:

1. Dissolve 100 mg Coomassie Blue G-250 in 50 ml 95% ethanol. To this solution add 100 ml 85% (w/v) phosphoric acid. Dilute the resulting solution to a final volume of 1 liter.
2. Prepare protein solutions (bovine serum albumin) by dissolving an appropriate amount of BSA in 0.15 M NaCl. Prepare standard solutions containing 10 to 100 µg protein per 0.1 ml.
3. Pipet 0.1 ml of each protein solution into test tubes and adjust volume, if necessary, to 0.1 ml with 0.15 M NaCl.
4. Add 5 ml of Coomassie Blue Reagent, mix contents well and measure absorbance (595 nm) after 2 minutes and 45 minutes in a quartz cuvette (against reagent blank containing dye and buffer)
5. Plot weight of protein against corresponding absorbances. Determine the protein content in unknown samples from the standard curve.

Figure 12. Dimedon and Bradford assays.



Emodin (**1**)

7-Chloroemodin (**2**)

Experimental protocols:

1. Control reaction: 3 mg (10 μmol) of 1 or 2 and 100 mg (1.4 mmol) KCl was placed in 34 ml of 6:4 0.2 M potassium phosphate-DMF buffer, pH 3.4. To the stirred solution was added 17 μl (280 μmol) of 50 % aq. H_2O_2. The reaction mixture was maintained at ambient temperature, checked periodically by TLC and stopped after 68 hours by extraction of dried products with ethyl acetate. The dried extract was fractionated by column chromatography and compounds purified by preparative TLC (8:2 chloroform:methanol).
2. Commercial (fungal) chloroperoxidase (CPO) reaction: 3 mg (10 μmol) of 1 or 2, 100 mg (1.4 mmol) KCl, 6 units CPO and 17 μl (280 μmol) of 50 % aq. H_2O_2 in 34 ml of 6:4 0.2 M potassium phosphate-DMF buffer, pH 3.4. Commercial chloroperoxidase obtained from Sigma Chemical Co.
3. *Nephroma laevigatum* chloroperoxidase (CPO) reaction: 3 mg (10 μmol) of 1 or 2, 100 mg (1.4 mM) KCl, 6 units lichen CPO and 17 μl (280 μmol) of 50 % aq. H_2O_2 in 34 ml of 6:4 0.2 M potassium phosphate-DMF buffer, pH 3.4.
4. All products were characterized by MS and ^1H NMR spectra.

Figure 13. Biohalogenation experiments.

Table 15. Reaction products from chlorination experiments with emodin (1) and 7-chloroemodin (2).[1]

Reaction	Emodin (1)	7-Chloroemodin (2)	5-Chloroemodin (3)	5,7-Dichloroemodin (4)
Control (1)[2]	+	+ (40 hours)	+ (40 hours)	+ (68 hours)
Control (2)[2]		+		-
Com. CPO (1)[3]	+	+ (20 hours)	+ (20 hours)	+ (68 hours)
Com. CPO (2)[3]		+		+ (40 hours)
N. l. CPO (1)[4]	+	+ (20 hours)	-	-
N. l. CPO (2)[4]		+		-

[1]Symbol key: + (present) or - (absent). The time when the product first appeared (by TLC) is shown in parentheses. All reaction products characterized by UV, EIMS and ^{1}H NMR spectra.

[2]No enzyme.

[3]Commercial fungal chloroperoxidase (*Caldariomyces fumago*); Sigma Chemical Co.

[4]*Nephroma laevigatum* semi-purified chloroperoxidase.

genate was filtered through cheese cloth, and centrifuged at 10,000 rpm for 1 hour. The extraction process was repeated three times, and the filtered extracts centrifuged. After centrifugation, the supernatants were combined, and adjusted to 40 % saturation with ammonium sulphate. The extract was left at 0° for 24 hours. After centrifugation at 10,000 rpm (1 hour), the supernatant was brought to 60 % saturation with ammonium sulphate, and left standing at 0° for several hours. The pellet was suspended in 0.1 M potassium phosphate buffer (pH 5.8), and then dialyzed against 10 mM phosphate (pH 5.8) for 24 hours (fraction 1). The 60 % ammonium sulphate solution was centrifuged at 10,000 rpm for 40 minutes, and the pellet collected. The pellet was suspended in 0.1 M phosphate buffer (pH 5.8), and dialyzed against 10 mM phosphate (pH 5.8) for 24 hours (fraction 2). Fractions 1 and 2 were assayed by the dimedon method (Neidleman and Geigert, 1986). Fraction 1 displayed little activity; fraction 2 was purified on a DEAE-Sephadex A 50 column by gradient elution with 0.1 M to 0.2 M potassium phosphate buffer, pH 6.0. The column fractions were assayed, and the active portions combined, and dialyzed against 10 mM phosphate buffer (pH 5.0) for 16 hours. The semi-purified enzyme preparation was lyophilized, and the crude chloroperoxidase (in 25 ml of 0.1 M potassium phosphate, pH 5.0) assayed once again. Two isolations and purifications of lichen chloroperoxidase were undertaken. The yield of crude chloroperoxidase per run was 6 mg, with a specific activity of 0.67 units/mg of protein for each purification (for a combined total of 12 mg chloroperoxidase). The protein content and specific

activity were calculated according to the Bradford dye-binding method (Bradford, 1976) and dimedon assay (Neidleman and Geigert, 1986), respectively. The semi-purified chloroperoxidase was used in the experimental protocols described in Figure 13.

The UV-visible absorption spectrum of the chloroperoxidase shown in Appendix 6 shows the characteristic absorption band (280 nm) for protein, but the absence of absorption bands for a heme or flavin prosthetic group suggests that the lichen enzyme lacks such a prosthetic group. Several nonheme chloroperoxidases are known from fungi and bacteria (Franssen and van der Plas, 1992).

4.3 RESULTS AND DISCUSSION

In order to examine whether *N. laevigatum* chloroperoxidase was capable of chlorinating anthraquinones in vitro, emodin (1) and 7-chloroemodin (2) were incubated with the chloroperoxidase preparation from the lichen. For comparison, commercial chloroperoxidase (from the fungus *Caldariomyces fumago*) was also tested for its ability to chlorinate the anthraquinones.

The results of the incubation experiments can be seen in Table 15. Incubation of emodin (1) with *N. laevigatum* chloroperoxidase (at pH 3.4) produced 7-chloroemodin (2), but not 5,7-dichloroemodin (3); similarly, incubation of 7-chloroemodin with the lichen enzyme failed to give 5,7-dichloroemodin. In contrast, commercial fungal chloroperoxidase catalyzed the conversion of emodin to 7-chloroemodin and 5,7-dichloroemodin; 7-chloroemodin was also converted to 5,7-dichloroemodin. The latter reaction took place with extraordinary efficiency; after 40 hours

at ambient temperature, the 7-chloroemodin had been completely converted to 5,7-dichloroemodin. The control reaction (no enzyme) also converted emodin to 7-chloroemodin and 5-chloroemodin, as well as a small quantity of 5,7-dichloroemodin; surprisingly, 7-chloroemodin was not further chlorinated in the control reaction (Table 15).

The time course of the reactions suggests that chlorination in the lichen and fungal enzyme reactions are, indeed, catalyzed by the chloroperoxidase, and are not simply a consequence of hydrogen peroxide-mediated chlorination. 5-Chloroemodin and 7-chloroemodin were detected, by TLC, at least 20 hours earlier than in the control reaction (Table 15). This indicates that both enzymes increased the reaction rate for the formation of the product. 5,7-Dichloroemodin was formed, from emodin, at the same rate for both the fungal enzyme and control reactions, but was synthesized from 7-chloroemodin only in the fungal chloroperoxidase reaction.

The results of the incubation experiments are consistent with the known chemistry of *Nephroma laevigatum*. The chlorination of 7-chloroemodin to 5,7-dichloroemodin by the lichen chloroperoxidase would not be expected, since neither 5- nor 5,7-dichloroemodin is found in the lichen (Cohen and Towers, 1995a). While 5,7-dichloroemodin is known from the lichen *Heterodermia obscurata* (Yosioka et al., 1968b; Cohen and Towers, 1995b), until now only a few 5-chloroanthraquinones have been isolated from fungi (Steglich et al., 1969; Ayer and Trifonov, 1994). Steglich and coworkers (1969) proposed a biogenetic scheme in which late chlorination of an anthraquinone in ring position 5 might take place in the genus *Der-*

mocybe. However, no isotope incorporation experiments in *Dermocybe* with ^{36}Cl have been performed, nor has a chloroperoxidase been isolated from a basidiomycete.

It is thus presently unclear why *Nephroma laevigatum* would produce the 5-chloro-substituted emodins in the ^{13}C feeding experiment, while they were not evident, by TLC, in either the sample selected for tracer studies, the other two incubation studies, or in all the previous work on the lichen. It is further unclear why the chloroperoxidase, once isolated from the lichen, appears incapable of effecting 5-chlorination. A typical electrophilic chlorination (Cl^+) reaction should give both the 5 and 7-chloro-substituted products. This expectation was observed with the hydrogen peroxide-mediated chlorination of emodin (control reaction) and in the reaction catalyzed by the fungal enzyme. The ^{13}C incubation mixture (which produced the 5-chloro isomers) was not supplemented with exogenous chloride ion, but the lichen chloroperoxidase medium contained 40 mmol KCl. Perhaps excess chloride ion inhibits the ability of this enzyme to effect 5-chlorination. It is also conceivable that 5-chlorination was effected by another enzyme, which was not removed from lichen tissue or was lost during purification. Finally, it is conceivable that the 5-chloro isomers are present in the wild lichen, but were not previously detected due to their low concentrations and R_f values so close to their 7-chloro counterparts.

4.4 PHYSICAL AND CHEMICAL DATA FOR REACTION PRODUCTS

4.4.1 PRODUCTS FROM CONTROL (NO ENZYME) REACTION

7-Chloroemodin (2) was obtained as orange crystals from ethyl acetate: EIMS *m/z* $[M]^+$ 306 (33), 304 (100). TLC (8:2 chloroform:methanol): R_f 0.50.

5,7-Dichloroemodin (3) was obtained as red crystals from methanol: EIMS *m/z* $[M]^+$ 384 (2), 382 (14), 380 (22), 342 (12), 340 (66), 338 (100). TLC: R_f 0.20.

5-Chloroemodin (4) was obtained as orange crystals from ethyl acetate: EIMS *m/z* $[M]^+$ 306 (37), 304 (100); ^{1}H NMR (DMSO-d_6, 400 MHz) δ 7.38 (s, 1H, H-4), 7.03 (s, 1H, H-2), 6.60 (s, 1H, H-7), 2.48 (s, 3H, Me). TLC: R_f 0.60.

4.4.2 PRODUCTS FROM COMMERCIAL CHLOROPEROXIDASE REACTION

7-Chloroemodin (2) was obtained as orange crystals from ethyl acetate: EIMS *m/z* $[M]^+$ 306 (33), 304 (100). TLC: R_f 0.50

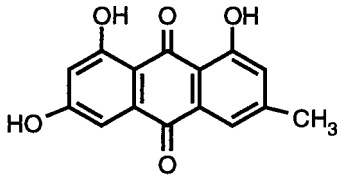
5,7-Dichloroemodin (3) (from emodin) was obtained as red crystals from methanol: EIMS *m/z* $[M]^+$ 342 (12), 340 (68), 338 (100). TLC: R_f 0.20

5,7-Dichloroemodin (3) (from 7-chloroemodin) was obtained as red crystals from methanol: EIMS *m/z* $[M]^+$ 342 (13), 340 (68), 338 (100); ^{1}H NMR (DMSO-d_6, 400 MHz) δ 7.40 (s, 1H, H-4), 7.05 (s, 1H, H-2), 2.40 (s, 3H, Me). TLC: R_f 0.20.

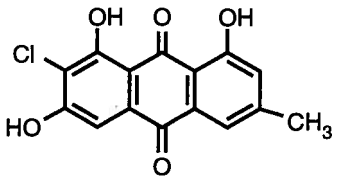
5-Chloroemodin (4) was obtained as orange crystals from ethyl acetate: EIMS *m/z* 306 (37), 304 (100); ^{1}H NMR (DMSO-d_6, 400 MHz) δ 7.35 (s, 1H, H-4), 7.05 (s, 1H, H-2), 6.60 (s, 1H, H-7), 2.48 (s, 3H, Me). TLC: R_f 0.60.

4.4.3 PRODUCTS FROM *N. LAEVIGATUM* CHLOROPEROXIDASE REACTION

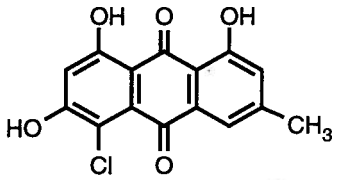
7-Chloroemodin (2) was obtained as orange crystals from ethyl acetate: EIMS *m/z* $[M]^+$ 306 (33), 304 (100). TLC: R_f 0.50.



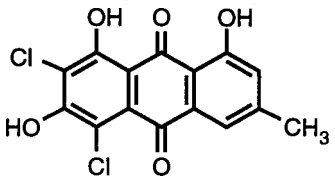
Emodin (1)



7-Chloroemodin (2)



5-Chloroemodin (3)



5,7-Dichloroemodin (4)

Figure 14. Substrates and products from chlorination experiments.

CHAPTER 5: ANTIVIRAL ACTIVITIES OF LICHEN QUINONOID PIGMENTS

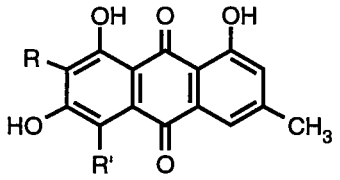
5.1 INTRODUCTION

Hypericin, and a number of structurally related anthraquinones and bianthrones, have been shown to posses antiviral activities against several animal viruses with membranes, including HIV-1 (Meruelo et al., 1988; Schinazi et al., 1990; Tang et al., 1990; Kraus et al., 1990; Anderson et al., 1991; Sydiskis et al., 1991; Barnard et al., 1992). None of these earlier studies, however, examined the role of light in mediating virucidal activity. Hypericin is a known photosensitizer, and its virucidal activity has been shown to be dependent on light (Hudson et al., 1991; Carpenter and Kraus, 1991; Hudson et al., 1993; Lenard et al., 1993). This may also be true for anthraquinones and bianthrones. In addition, the antiviral activity of hypericin has been shown to be affected by a variety of assay parameters (Anderson et al., 1991; Hudson et al., 1993). Thus, relative activities must be compared under uniform and optimal conditions.

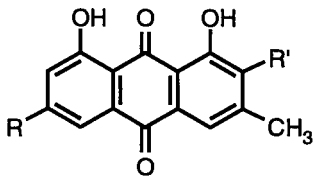
The compounds used in this study (Figure 15) were isolated either from the lichens *Nephroma laevigatum* and *Heterodermia obscurata*, or were natural products obtained previously. All of the compounds, with the exception of hypericin, are known constituents of lichens.

5.2 MATERIALS AND METHODS

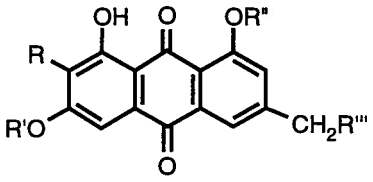
The anti-HSV assays follow the protocols described previously (Marles et al.,



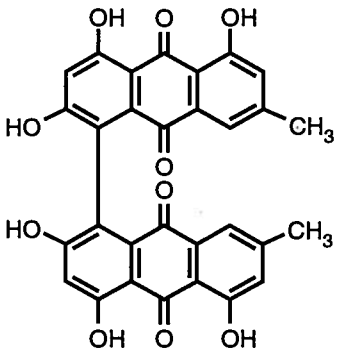
R = R' = H; Emodin (1)
R = Cl, R' = H; 7-Chloroemodin (2)
R = R' = Cl; 5,7-Dichloroemodin (5)



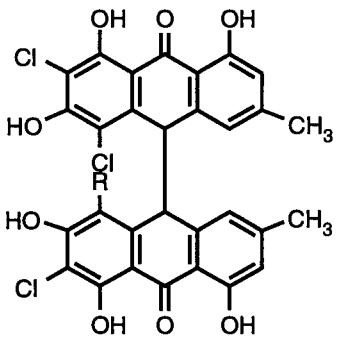
R = OH, R' =COOH; Endocrocin (7)
R = R' = H; Chrysophanol (8)



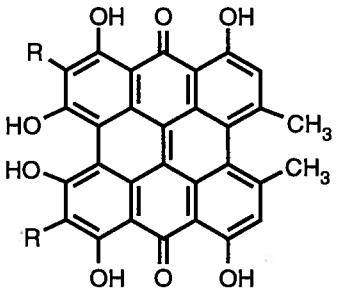
R = R'' = R''' = H, R' = CH_3; 6-O-Methylemodin (2)
R = Cl, R' = R''' = H, R'' = CH_3; 7-Chloro-1-O-methylemodin (4)
R = Cl, R' = H, R'' = CH_3, R''' = OH; 7-Chloro-1-O-methyl-ω-hydroxyemodin (13)



Skyrin (6)



R = H; Flavoobscurin A (9)
R = Cl; Flavoobscurin B (10)



R = H; Hypericin (11)
R = Cl; 7,7'-Dichlorohypericin (12)

Figure 15. Structures of lichen compounds used in antiviral assays.

Table 16. Minimum inhibitory concentrations of lichen compounds against HSV-1 virus.[1]

Compound	Complete Inactivation	Partial Inactivation
Emodin (1)	2	1
6-*O*-Methylemodin (2)	inactive	inactive
7-Chloroemodin (3)	2	1
7-Chloro-1-*O*-methylemodin (4)	2	0.5
5,7-Dichloroemodin (5)	0.25	0.125
Skyrin (6)	inactive	inactive
Endocrocin (7)	inactive	inactive
Chrysophanol (8)	inactive	inactive
Flavoobscurin A (9)	inactive	inactive
Flavoobscurin B (10)	inactive	inactive
Hypericin (11)	< 0.06	<< 0.06
7,7'-Dichlorohypericin (12)	< 0.06	<< 0.06
7-Chloro-1-*O*-methyl-ω-hydroxyemodin (13)	inactive	inactive

[1]μg/ml.

1992). Briefly, the standard reaction mixtures were prepared by adding a known amount of HSV-1 (100 pfu or plaque-forming units) to serial two-fold dilutions of the test compound made up in Dulbecco MEM medium plus 0.1 % serum, previously shown to be the optimum conditions for the antiviral activity of hypericin (Hudson et al., 1994). The range of concentrations of the compounds was 2.0 µg/ml down to 0.06 µg/ml. The reaction mixtures were irradiated at 9 Kjoules, supplied by fluorescent lamps, for 30 minutes. The irradiation was provided by several General Electric F20 T12/cw lamps which gave a measured incident energy of 1.5 mW/cm^2 (the emission was between 380 and 700 nm). Controls included reactions kept dark by covering the assay plates with aluminum foil, and virus without compound. All reactions were carried out in duplicate, and every compound was tested at least twice.

The cultures were inspected daily microscopically for residual HSV-1 infectivity, as characteristic CPE (cytopathic effects), and for comparison with virus only and cells only controls. In the case of the untreated HSV-1, CPE involved the entire culture by day 3-4. At this time, cultures that still displayed no viral CPE were considered free of infectious virus (complete viral inactivation in the reactions).

Compounds that displayed good anti-HSV activity in the end-point tests were evaluated in more detail by use of plaque assays. Reaction mixtures were prepared with several concentrations of the compounds and HSV-1 (10^5 pfu/ml). Following irradiation with light as before, and with appropriate controls, the mixtures were se-

Table 17. Inhibition of HSV-1 virus in plaque assay.

Compound[1]	# of Plaques[2]	% of Plaques[3]
None (control)	936	100
Emodin (1)	784	84
7-Chloroemodin (3)	904	97
7-Chloro-1-*O*-methylemodin (4)	1062	113
5,7-Dichloroemodin (5)	0	< 0.1
Hypericin (11)	0	< 0.1
7,7'-Dichlorohypericin (12)	1	< 0.1

[1]1.0 μg/ml.
[2]pfu/ml x 10^{-2} dilution factor.
[3]Relative to control.

Table 18. Effect of light on HSV-1 inhibition at different substrate concentrations.[1]

Compound	Dark/Light	1.0 µg/ml	0.1 µg/ml	0.01 µg/ml
5,7-Dichloroemodin (5)	Dark	100	-	-
	Light	< 0.01	52	100
Hypericin (11)	Dark	34	100	100
	Light	< 0.01	0.33	24
7,7'-Dichlorohypericin (12)	Dark	43	100	100
	Light	0.17	50	100

[1]Values reported as % pfu (plaque-forming units) remaining.

rially diluted 10^{-1} to 10^{-4}. The 10^{-2} and 10^{-4} dilutions were each inoculated in duplicate onto monolayers of Vero cells in culture dishes (60 mm diameter) to permit adsorption of remaining infectious virus to the cells (60 minutes at 37°C). The inocula were then removed by aspiration and replaced by molten agarose overlays (at 42°C), which consisted of 0.5 % final concentration of agarose, Dulbecco MEM and 5 % serum. When the overlays had set, the cultures were returned to the incubator until plaques could be visualized (4 days). In order to facilitate the enumeration of plaques, the cell monolayers were fixed in 10 % formalin in phosphate-buffered saline, and stained with 1 % crystal violet in water. Plaques appeared as discrete round "holes" in the uniform blue monolayer of intact cells. The number of plaques at each dilution was calculated as pfu/ml, and compared to the corresponding number in the untreated virus control.

5.3 RESULTS AND DISCUSSION

Table 16 presents the results of the survey of 12 lichen compounds and hypericin for their inhibitory activity against HSV-1 virus. Emodin (1), 7-chloroemodin (3) and 7-chloro-1-*O*-methylemodin (4) completely inactivated HSV-1 at a concentration of 2 μg/ml. Partial inactivation was also seen for compounds 1 (1 μg/ml), 3 (1 μg/ml) and 4 (0.5 μg/ml). The most active anthraquinone was 5,7-dichloroemodin (5). Complete inactivation of the virus was attained at a concentration of 0.25 μg/ml, and 0.125 μg/ml gave partial inactivation. The remaining

five anthraquinones (2, 6, 7, 8, 13) were completely inactive, even at 5 μg/ml. The bianthrones flavoobscurin A (9) and flavoobscurin B (10) were also inactive at 5 μg/ml. Finally, hypericin (11) and 7,7'-dichlorohypericin (12) showed comparable inhibitory activity against HSV-1; complete inactivation took place at less than 0.06 μg/ml.

Table 17 compares the antiviral activities of the six active compounds in a plaque assay. As was the case with the initial end-point CPE method, this assay was conducted following irradiation with light. Of the six compounds tested, 5,7-dichloroemodin (5), hypericin (11) and 7,7'-dichlorohypericin (12) completely inhibited HSV-1 at 1.0 μg/ml. Thus, of the 11 anthraquinones and bianthrones tested, only 5,7-dichloroemodin exhibited light-mediated anti-HSV activity comparable to that of hypericin and 7,7'-dichlorohypericin.

This result is illustrated in Table 18, where the effects of light and dark activities of the three compounds are compared. At a higher concentration (1.0 μg/ml), both hypericin and 5,7-dichloroemodin were appreciably more active than 7,7'-dichlorohypericin in the light. In the dark, however, 5,7-dichloroemodin was totally inactive; the two hypericins exhibited comparable moderate anti-HSV activity. At an intermediate concentration (0.1 μg/ml), the activities of 5,7-dichloroemodin and 7,7'-dichlorohypericin in the light started to decrease. At this concentration, all three compounds were inactive in the dark. Finally, at the lowest concentration (0.01 μg/ml), only hypericin showed any anti-HSV activity in the light.

There are several significant findings to be gleaned from this study. The first observation is the higher inhibitory activity of 5,7-dichloroemodin, when compared with 7-chloroemodin and emodin. There also appears to be no difference in activity between the monochlorinated anthraquinones and the nonchlorinated parents. This finding suggests that, in the emodin series at least, anti-HSV activity occurs when both the C-5 and C-7 ring positions are substituted; the possibility that 5-chloroemodin might also be active cannot be excluded, however. Replacement of the 1-OH in emodin by a 1-OMe group (compound 4) resulted in an initial partial inactivation, but the plaque assay results suggest that the viral CPE were merely delayed, and that eventually the CPE reached 100 %. Thus, for 1,3 and 4, lower concentrations of the compound gave little or no decrease in the progress of CPE, while higher concentrations gave complete inactivation of the virus.

Modifications of the basic emodin structure (Figure 15) by placing an OMe group at C-6 (compound 2), a COOH group at C-2 (compound 7), removal of the OH group at C-6 (compound 8), and substitution of a CH_2OH for a methyl group (compound 13) all gave inactive compounds. Of the dimeric structures, only the hypericins (compounds 11 and 12) showed anti-HSV properties; skyrin (6) (bianthraquinone) and flavoobscurins A (9) and B (10) (bianthrones) were completely inactive.

In general, the results suggest that the phenanthroperylenequinone structure of hypericin and 7,7'-dichlorohypericin is more active than either the less condensed

bianthrone structure, or the monomeric/dimeric anthraquinones (Table 16). The presence of several chlorine atoms in flavoobscurin A and B did not appear to positively influence their activities against HSV; in fact, the structural conformations of the two compounds may account for their lack of activity against the virus. In contrast, placing chlorines in ring positions 5 and 7 of emodin resulted in greatly enhanced virucidal activity for 5,7-dichloroemodin. In the case of 7,7'-dichlorohypericin, substitution of two chlorine atoms in the 7 and 7' ring positions of hypericin slightly reduced the antiviral activity of the halogenated analogue in the light. Meruelo et al. (1988) had shown that pseudohypericin (with a CH_2OH group in place of a CH_3) was significantly less active than hypericin. Compound 13, which also has a CH_2OH group in place of a methyl group, was completely inactive.

In conclusion, this study provides evidence for the anti-HSV activities of several lichen anthraquinones and a chlorinated hypericin derivative. In particular, hypericin, 7,7'-dichlorohypericin and 5,7-dichloroemodin demonstrated significant virucidal properties in light at a concentration of 1.0 μg/ml. Hypericin and 7,7'-dichlorohypericin also displayed moderate anti-HSV activities in the dark at 1.0 μg/ml. Hypericin is known to be a photodynamic compound that mediates biological activities via singlet oxygen (Thomas and Pardini, 1992; Thomas et al., 1992; De Witte et al., 1993; Hudson et al., 1994). It is not known, however, if the dark reaction follows the same mechanistic course as the light-mediated process. In fact, there may be several different light and dark processes operating concurrently, or subject to

varying reaction conditions. This would appear to be the case with 5,7-dichloro-emodin, which showed the same light-mediated virucidal activity as hypericin, but was totally inactive in the dark at a concentration of 1.0 μg/ml.

CHAPTER 6: CONCLUSIONS

This thesis addresses several different aspects of the chemistry, biochemistry and medicinal properties of quinonoid constituents of the lichens *Nephroma laevigatum* and *Heterodermia obscurata*. I have undertaken the isolation and characterization of twelve anthraquinone, bianthrone and phenanthroperylenequinone pigments from the two lichens, examined in vitro and in vivo the biogenesis of several anthraquinones, and tested thirteen structurally diverse lichen quinonoid compounds for their potential antiviral activities.

The first study dealt with the isolation and identification of pigments in *N. laevigatum* and *H. obscurata*. Four emodin and two hypericin derivatives were isolated from *N. laevigatum* collected in British Columbia. This lichen was found growing only in the littoral zones of islands between the mainland and Vancouver Island. The preferential lichen substratum was granitic rock along the shoreline, but occasionally the lichen could be found on the trunks and branches of Big-Leaf Maple (*Acer macrophyllum*). TLC surveys of several samples collected from different geographical locations and substrata showed a uniformity in chemical composition. Although minor constituents, such as 7,7'-dichlorohypericin, 2,2', 7,7'-tetrachlorohypericin and 7-chloro-1-*O*-methyl-ω-hydroxyemodin gave only faint TLC spots, they were present in all lichen extracts examined. There did not appear to be, therefore, any chemical variation within the regions and different lichen substrata examined. Two chemical races of *N. laevigatum* are known

(Table 2), but they differ only in their triterpene compositions. Occasionally, non pigmented forms of the lichen have been collected in Madeira, Canary Islands and in Portugal (James and White, 1987).

The only previous chemical studies of *Nephroma laevigatum* were those of Bendz et al. (1967) and Bohman (1968). Bohman (1968) identified five anthraquinones in the lichen growing in Sweden. Of the five compounds characterized by Bohman, I was able to isolate three, which are apparently common to both the British Columbian and Swedish samples (emodin, 7-chloroemodin and 7-chloro-1-*O*-methylemodin). I did not, however, find either 7-chloro-6-*O*-methylemodin or 7-chloro-1,6-di-*O*-methylemodin in any of my samples. Furthermore, Bohman did not indicate in her paper how much lichen material was used in the study. Thus, her failure to find 7-chloro-1-*O*-methyl-ω-hydroxyemodin, 7,7'-dichlorohypericin and 2,2',7,7'-tetrachlorohypericin may reflect her having worked with insufficient material, or this may truly represent chemical variation between two different samples of the same species (Cohen and Towers, 1995a).

Three anthraquinones, two bianthrones and 7,7'-dichlorohypericin were isolated from *Heterodermia obscurata* collected in Maryland. As with *N. laevigatum*, *H. obscurata* displayed chemical uniformity, according to TLC, for samples collected from different locations in Cedarville State Forest. In addition, with the exception of 7,7'-dichlorohypericin, the compounds obtained from the lichen growing in Maryland were identical to those found in a Japanese sample studied by Yosioka et al.

(1968a, 1968b) (Cohen and Towers, 1995b). Again, 7,7'-dichlorohypericin may not have been identified by the Japanese group as a result of a lack of sufficient lichen material, or it may be restricted to particular varieties of *H. obscurata.* Thus, a systematic chemical study of *N. laevigatum* and *H. obscurata* from a variety of different geographical and environmental conditions is necessary before generalizations about the chemistry of these two lichens can be made.

The presence of emodin anthrones in both *N. laevigatum* and *H. obscurata* was suggested by the TLC of lichen extracts. Several pale yellow spots were evident in the thin-layer chromatographs of all extracts of both lichens. Although not definitive, the R_fs of the spots correspond closely to purported emodin anthrones in *N. laevigatum* (Bohman, 1968) and *H. obscurata* (Yosioka et al., 1968c; Kurokawa, 1973).

My second objective was to examine the biogenesis of emodin and chlorinated emodin derivatives in *N. laevigatum* using radioactive and stable isotopes. Lichen anthraquinones are believed to be formed from acetate, in the same manner as fungal anthraquinones. The polyketide pathways leading to fungal anthraquinones have been well established (Franck, 1984; Gill, 1994, 1995). I have demonstrated, using sodium [2-^{14}C]acetate and sodium [1-^{13}C]acetate, the polyketide origins of emodin, 7-chloroemodin and 7-chloro-1-*O*-methylemodin in *N. laevigatum.* Figure 16 shows the percentage incorporations for the ^{14}C-labelled emodins, and the percentage ^{13}C enrichments (over and above the 1.1 % natural abundance)

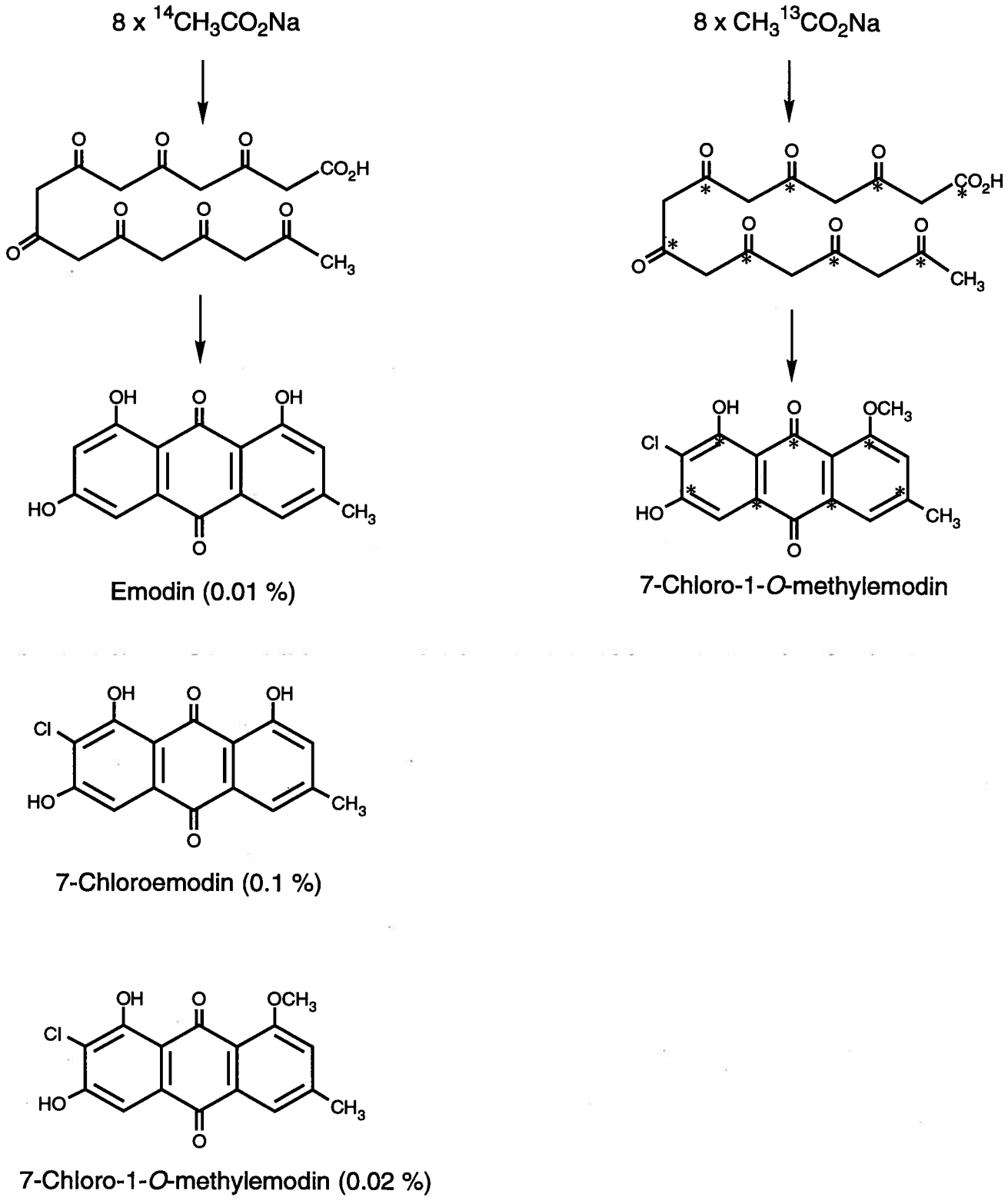


Figure 16. The lichen polyketide pathway in *Nephroma laevigatum*.

at each alternately-labelled carbon in 7-chloro-1-*O*-methylemodin. Absolute incorporation rates of 0.1 % or higher for polyketide biosynthesis in fungi were considered significant by Franck (1984); similar values for incorporations of [1-^{14}C]acetate and [2-^{14}C]acetate into *Rumex alpinus* and *Rhamnus frangula* anthraquinones were also regarded as significant by Leistner (1971, 1973). In their study of the biosynthesis of the aliphatic acid, (+)-protolichesterinic acid, Bloomer et al. (1968, 1969) obtained incorporation rates of 0.01-0.08 % of [1-^{14}C]acetate into the lichen *Cetraria islandica*. Finally, Gill and Giménez obtained, in their study of the biosyntheses of anthraquinones in *Dermocybe sanguinea*, ^{13}C atom % enrichment levels ranging from 0.3 to 1.3 % from sodium [1-^{13}C]acetate (1990b).

The degree of labelling is highly subject to the experimental conditions and target organism of the feeding study: higher plants, bacteria, marine algae, and some lower fungi tend to have higher metabolic turnover rates than most basidiomycetes and lichens. Not surprisingly, therefore, there have been few feeding studies with mushrooms and lichens. In general, based on previous studies with plants and fungi, the degree of labelling of any isolated product will depend on the amount of isotope supplied, the metabolic or reproductive stage of the organism at the time of precursor application, the number of potential biochemical pathways to which the labelled precursor may contribute, cellular compartmentalization of metabolites and the purity of the final labelled product (Luck-

ner, 1990). Several of the problems associated with whole-organism feeding studies can be reduced by using cell cultures or enzyme preparations, which bypass complications associated with metabolite compartmentalization. In addition, use of purified enzymes and structurally-pertinent labelled precursors reduces the likelihood of unwanted biochemical transformations, yet can result in the demonstration of direct precursor-product relationships.

This has been shown for several transformations of anthraquinones: ^{14}C-labelled emodin converted to radioactive geodin and dihydrogeodin in *Aspergillus terreus* (Fujimoto et al., 1975), conversion of ^{3}H-emodin to radioactive parietin in a cell-free preparation from *Aspergillus parasiticus* (Anderson, 1986b), deoxygenation of emodin to chrysophanol by emodin deoxygenase from *Pyrenochaeta terrestris* (Ichinose et al., 1993), and oxidation of emodin and chrysophanol anthrones to the corresponding anthraquinones by emodinanthrone oxygenase from *Aspergillus terreus* (Chen et al., 1995).

In particular, Sankawa and coworkers have established the enzymatic processes leading to the oxidation of emodin anthrone to emodin, and the *O*-methylation of emodin to 8-*O*-methylemodin, in *Aspergillus terreus* (Fujii et al., 1982, 1991; Chen et al., 1992, 1995). Two enzymes have been isolated from the fungal culture: emodin anthrone oxygenase and emodin *O*-methyltransferase. In a survey of ten anthraquinone-producing microorganisms, Fujii et al. (1991) found anthrone oxygenase activity in all of them; this confirms the ubiquity of the biosyn-

thetic process of anthrone oxidation to anthraquinone in microorganisms. Emodin *O*-methyltransferase exhibited a broad substrate specificity with respect to methylation, when tested on sixteen anthraquinones and anthrones (Fujii et al., 1982; Chen et al., 1992). Emodin showed the highest relative activity (100 %), followed by 4-hydroxyemodin (80 %), ω-hydroxyemodin (22 %) and 7-chloroemodin (18 %). The remaining substrates showed little or no activity. The implications of these results for the formation of 7-chloro-1-*O*-methylemodin and 7-chloro-1-*O*-methyl-ω-hydroxyemodin in *Nephroma laevigatum* are: 1) it is reasonable to assume that anthraquinone-producing lichens contain analogous enzymes catalyzing the formation of anthraquinones from anthrones; and 2) *O*-methylation of emodin occurs preferentially, but not exclusively, before chlorination of the anthraquinone ring.

Taking into account the slow growth and metabolism of most lichens, I anticipated that the in vivo absolute incorporation rates and enrichments of ^{14}C and ^{13}C-labelled acetate, respectively, into *N. laevigatum* would be lower than might be expected for analogous feeding studies with plants or fungi. Therefore, future experiments designed to ascertain the biosynthetic pathways leading to anthraquinones in lichens should include the use of cell-free preparations or purified enzymes, ^{14}C-labelled anthrones and anthraquinones as precursors, [1,2-^{13}C]acetate (which would provide unequivocal evidence for the transformations of octaketide precursors into anthraquinones), and in vivo time-course studies in order to examine the patterns and progressions of precursor metabolism in the "cultured" lichen.

Since there is tentative evidence for the presence of anthrones in lichens, and anthrones have been established as anthraquinone precursors in some fungi (Sankawa et al., 1973; Franck, 1984; Gill and Giménez, 1991; Gill et al., 1992; Gill, 1994, 1995) and higher plants (Labadie et al., 1972; Yagi et al., 1978; Vederas and Nakashima, 1980; Grün and Franz, 1980, 1981; Sigler and Rauwald, 1994; Chen et al., 1995), biosynthetic transformations of anthrones to anthraquinones in lichens should be studied using labelled precursors in both in vivo and in vitro systems.

Finally, the origin(s) of the 5-chloro-substituted emodins, obtained from the [1-^{13}C]acetate feeding experiment, should be examined in greater detail. Systematic chemical surveys of natural populations of *Nephroma laevigatum* (and other lichens) should be undertaken in order to determine if 5-chloroanthraquinones are ubiquitous or occasional constituents of lichens; or if they represent anomalous natural products produced by *N. laevigatum* only under laboratory conditions. The production of the unexpected compounds may be a result of having perturbed the metabolism of the lichen by using a relatively large amount (1 gram) of 0.12 M sodium [1-^{13}C]acetate, or a consequence of changes in the ionic strength or pH of the lichen's environment during the course of the experiment (Holker et al., 1974).

The third area of research involved the study of the chlorination of anthraquinonoid pigments in *N. laevigatum*. Incorporation of sodium ^{36}chloride into the lichen established that exogenous chloride could be used by the lichen to make chlorinated anthraquinones, although this experiment by itself cannot rule out

the possibility of nonenzymatic chlorination, as lichens may contain sufficient amounts of hydrogen peroxide and endogenous chloride to carry out chlorinations in situ.

An attempt was made, therefore, to isolate a chloroperoxidase (chlorinating enzyme) from *N. laevigatum*. Ultimately, a semi-purified enzyme fraction was obtained from the lichen by a series of purification steps. Using the Dimedon and Bradford assays, I determined the specific activity and protein content of the crude chloroperoxidase, respectively.

Chlorination experiments with the semi-purified lichen chloroperoxidase, and a commercially available fungal chloroperoxidase, demonstrated substrate specificity for both enzymes. The lichen chloroperoxidase failed to catalyze the formation of 5,7-dichloroemodin from either emodin or 7-chloroemodin as anticipated, while 5,7-dichloroemodin was synthesized in excellent yield from 7-chloroemodin by the commercial enzyme, and from emodin in the control (no enzyme) reaction. In addition, 5-chloroemodin was produced from emodin in both the commercial and control reactions. Interestingly, although 5-chloroemodin was found to be produced by the lichen during the [1-^{13}C]acetate feeding experiment, it has not been previously identified in *N. laevigatum*, nor was it produced in the lichen chloroperoxidase reaction. This supports the hypothesis that the lichen is not normally capable of biosynthesizing 5-chloroemodin from emodin, and the presence of the 5-chloroemodin derivatives in the "cultured" lichen is a probable consequence of having perturbed the lichen's normal metabolism during the course of the [1-^{13}C] acetate incorporation study.

Chloroperoxidases represent a fairly large class of halogenating enzymes, known primarily from fungi and bacteria (Neidleman and Geigert, 1986; Asplund, 1992; Franssen and van der Plas, 1992). A majority of the chloroperoxidases contain heme prosthetic groups, but several nonheme chloroperoxidases have been obtained from these sources (Neidleman and Geigert, 1986; Franssen and van der Plas, 1992). In particular, a nonheme chloroperoxidase isolated from *Pseudomonas pyrrocinia* was shown to catalyze the chlorination of indole to 7-chloroindole, although the product was verified only by comparison of the HPLC retention time with authentic samples of chloroindoles (Wiesner et al., 1986). This enzyme would thus represent the first haloperoxidase for which a regioselective halogenation has been demonstrated, as all previous studies of haloperoxidase-catalyzed halogenation reactions have shown a complete absence of any regio-selectivity or stereoselectivity (Morrison and Bayse, 1973; Ramakrishnan et al., 1983; Neidleman and Geigert, 1986; Franssen and van der Plas, 1992).

The results of the biohalogenation reactions suggest that the *N. laevigatum* chloroperoxidase exhibits regioselectivity with respect to the substrate emodin. The enzyme catalyzed the chlorination of emodin to give only 7-chloroemodin. Although the exact mechanism for any chloroperoxidase-mediated chlorination has yet to be fully established, the fact that the lichen haloperoxidase catalyzed the formation of only one of the two expected products of electrophilic chlorination of emodin suggests that the enzymatic chlorination mechanism in the lichen may be

somewhat different than the mechanisms proposed for fungal and algal haloperoxidases.

The final topic of my thesis dealt with the antiviral properties of lichen compounds. Several lichen anthraquinones, bianthrones and hypericin derivatives were examined in end-point CPE (viral cytopathic effects) and plaque assays with HSV-1 (herpes simplex virus type 1). Emodin, 7-chloroemodin, 7-chloro-1-*O*-methylemodin, 5,7-dichloroemodin, hypericin and 7,7'-dichlorohypericin showed fair to good virucidal activity in the presence of light. In the plaque assay, hypericin, 7,7'-dichlorohypericin and 5,7-dichloroemodin completely inhibited the virus at 1.0 μg/ml concentrations; only hypericin was active, however, at 0.01 μg/ml. The remaining compounds were found to be inactive at a concentration of 5.0 μg/ml.

The results of the HSV-1 assays demonstrate that substitution of two chlorine atoms on the emodin structure enhances the virucidal activity substantially. On the other hand, 7-chloroemodin showed the same activity as emodin in the assay. Substitution of chlorine atoms in the hypericin structure did not enhance its activity, and the two chlorinated bianthrones (flavoobscurin A and B) were completely inactive. In addition, *O*-methylation at position 1 of 7-chloroemodin did not effect the antiviral activity. An unexpected result was that 5,7-dichloroemodin exhibited virucidal activity (1.0 μg/ml) only under the influence of light.

Anthraquinones, and a number of structurally related compounds, have been shown to possess antiviral and associated activities (Brown et al., 1980; Konoshima et al., 1989; Kraus et al., 1990; Schinazi et al., 1990; Andersen et al., 1991; Sydiskis et al., 1991; Barnard et al., 1992; Tagahara et al., 1992; Cohen et al., 1995). In addition, hypericin and related perylenequinones have demonstrated antiviral activities against a variety of viruses (Meruelo et al., 1988; Lavie et al., 1989; Kraus et al., 1990; Schinazi et al., 1990; Tang et al., 1990; Andersen et al., 1991; Carpenter and Kraus, 1991; Hudson et al., 1991; Lopez-Bazzocchi et al., 1991; Barnard et al., 1992; Hudson et al., 1993; Lenard et al., 1993; Hudson et al., 1994; Cohen et al., 1995). The role of light in mediating the virucidal activity of hypericin has been shown by Hudson et al. (1991, 1993), Carpenter et al. (1991) and Lenard et al. (1993). Until now, however, light dependence had not been demonstrated for anthraquinones or anthrones. Thus, 5,7-dichloroemodin represents the first anthraquinone requiring light for its antiviral activity (Cohen et al., 1995). While most of the reports involve the testing of plant, fungal or synthetic anthraquinones and hypericin derivatives against viruses, the results presented here represent the first reports of antiviral naturally occurring chlorinated anthraquinones and hypericin derivatives.

Finally, experiments planned for the future should include the testing of 5-chlorinated anthraquinones, as well as of other halogenated and nonhalogenated structural analogues of emodin and hypericin, in order to ascertain the struc-

ture-activity relationships that determine the natural product's activities against different viruses under the influence of a host of reaction parameters.

BIBLIOGRAPHY

Aberhart, D. J., K. H. Overton, and S. Huneck. 1969. Portentol: a novel polypropionate from the lichen *Rocella portentosa*. Chem. Commun. 162-163.

Aberhart, D. J., A. Corbella, and K. H. Overton. 1970. The biosynthesis of portentol: assembly of a linear pentapropionate from acetate and methionine. Chem. Commun. 664-665.

Ahmadjian, V. and M. E. Hale. 1973. The Lichens. Academic Press, New York.

Andersen, D. O., N. D. Weber, S. G. Wood, B. G. Hughes, B. K. Murray, and J. A. North. 1991. In vitro virucidal activity of selected anthraquinones and anthraquinone derivatives. Antiviral Res. 16: 185-196.

Anderson, J. A. 1986a. Conversion of emodin to chrysophanol in a cell-free system from *Pyrenochaeta terrestris*. Phytochemistry 25: 103-106.

Anderson, J. A. 1986b. Conversion of emodin to physcion by a cell-free preparation of *Aspergillus parasiticus*. Phytochemistry 25: 1115-1117.

Anke, H., I. Kolthoum, H. Zähner, and H. Laatsch. 1980a. Metabolic products of microorganisms. 185. The anthraquinones of the *Aspergillus glaucus* group. I. Occurrence, isolation, identification and antimicrobial activity. Arch. Microbiol. 126: 223-230.

Anke, H., I. Kolthoum, and H. Laatsch. 1980b. Metabolic products of microorganisms. 192. The anthraquinones of the *Aspergillus glaucus* group. II. Biological activity. Arch. Microbiol. 126: 231-236.

Asahina, Y. 1937. Über den taxanomischen Wert der Flechtenstoffe. Bot. Mag. 51: 759-764.

Asahina, Y. and I. Yosioka. 1940. Untersuchungen über Flechtenstoffe, XCV. Mitteil.: Über die Zeorin-Gruppe (II). Chem. Ber. 73: 742-747.

Asplund, G. 1992. On the Origin of Organohalogens Found in the Environment. Linköping Studies in Art and Science, Linköping.

Assante, G., L. Camarda, and G. Nasini. 1980. Secondary mould metabolites. IX. Structure of a new bianthrone and of three new *seco*anthraquinones from *Aspergillus wentii* Wehmer. Gazz. Chim. Ital. 110: 629-631.

Ayer, W. A. and L. S. Trifonov. 1994. Anthraquinones and a 10-hydroxyanthrone from *Phialophora alba*. J. Nat. Prod. 57: 317-319.

Bachmann, E. 1887. Emodin in *Nephroma lusitanica*. Ber. Deut. Bot. Ges. 5: 192-194.

Bachmann, E. 1890. Über nichtkristallisierte Flechtenfarbstoffe. Ein Beitrag zur Chemie und Anatomie der Flechten. Jb. Wiss. Bot. 21: 1-61.

Bachmann, E., J. Lüthy, and C. Schlatter. 1979. Toxicity and mutagenicity of molds of the *Aspergillus glaucus* group. Identification of physcion and three related anthraquinones as main toxic constituents from *Aspergillus chevalieri*. J. Agric. Food Chem. 27: 1342-1347.

Barkman, J. J. 1958. Phytosociology and Ecology of Cryptogamic Epiphytes. Van Gorcum, Assen.

Barnard, D. L., J. H. Huffman, J. L. B. Morris, S. G. Wood, B. G. Hughes, and R. W. Sidwell. 1992. Evaluation of the antiviral activity of anthraquinones, anthrones, and anthraquinone derivatives against human cytomegalovirus. Antiviral Res. 17: 63-77.

Bendz, G., G. Bohman, and J. Santesson. 1967. Chemical studies on lichens 9. Chlorinated anthraquinones from *Nephroma laevigatum*. Acta Chem. Scand. 21: 2889-2990.

Bernard, T. and G. Goas. 1981. Biosynthèse de la sticticine chez le lichen *Lobaria laetevirens*. Physiol. Plant. 53: 71-75.

Bernard, T., G. Goas, J. Hamelin, and M. Joucla. 1981. Characterization of DOPA betaine, tyrosine betaine and *N*-dimethyltyrosine from *Lobaria laetevirens*. Phytochemistry 20: 2325-2326.

Blanco, M. J., C. Suárez, and C. Vicente. 1984. The use of urea by *Evernia prunastri* thalli. Planta 162: 305-310.

Bloomer, J. L., W. R. Eder, and W. F. Hoffman. 1968. The biosynthesis of (+)-protolichesterinic acid. Chem. Commun. 354-355.

Bloomer, J. L. and W. F. Hoffman. 1969. On the origin of the C_3-unit in (+)-protolichesterinic acid. Tetrahedron Lett. 4339-4340.

Bloomer, J. L., W. R. Eder, and W. F. Hoffman. 1970. Biosynthesis of (+)-protolichesterinic acid in *Cetraria islandica*. J. Chem. Soc. (C). 1848-1850.

Bohman, G. 1968. Chemical studies on lichens 11. Anthraquinones from *Nephroma laevigatum*. Arkiv Kemi. 30: 217-223.

Bohman, G. 1969a. Anthraquinones from the genus *Caloplaca*. Phytochemistry 8: 1829-1830.

Bohman, G. 1969b. Chemical studies on lichens 22. Anthraquinones from the lichen *Lasallia papulosa* var. *rubiginosa* and the fungus *Valsaria rubricosa*. Acta Chem. Scand. 23: 2241-2244.

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72: 248-254.

Briggs, L. H., D. R. Castaing, A. N. Denyer, E. F. Orgias, and C. W. Small. 1972. Chemistry of fungi. Part VIII. Constituents of *Valsaria rubricosa* and the identification of papulosin with valsarin. J. Chem. Soc. Perkin I. 1464-1466.

Brockmann, H. and W. Sanne. 1953. Zur Biosynthese des Hypericins. Naturwiss. 40: 509-510.

Brockmann, H. 1957. Photodynamically active plant pigments. Proc. Chem. Soc. 304-312.

Brodo, I. M. 1986. Interpreting chemical variation in lichens for systematic purposes. Bryologist 89: 132-138.

Brodo, I. M. 1988. Lichens of the Ottawa Region. Ottawa Field-Nat. Club Spec. Pub. No. 3.

Brown, D. H., C. Vicente, and E. Legaz. 1982. Urease activity in *Peltigera canina* (L.) Willd. Cryptogamie, Bryologie et Lichénologie 3: 33-38.

Brown, J. P. 1980. A review of the genetic effects of naturally occurring flavonoids, anthraquinones and related compounds. Mutation Res. 75: 243-277.

Brüun, T. 1971. Phenarctin, a fully substituted depside from *Nephroma arcticum*. Acta Chem. Scand. 25: 2831-2836.

Butler, A. and J. V. Walker. 1993. Marine haloperoxidases. Chem. Rev. 93: 1937-1944.

Cameron, D. W. and M. J. Crossley. 1977. Synthesis of emodin methyl ethers. Aust. J. Chem. 30: 1161-1165.

Carpenter, S. and G. A. Kraus. 1991. Photosensitization is required for inactivation of equine infectious anemia virus by hypericin. Photochem. Photobiol. 53: 169-174.

Casey, M. L., R. C. Paulick, and H. W. Whitlock. 1978. Carbon-13 nuclear magnetic resonance study of the biosynthesis of daunomycin and islandicin. J. Org. Chem. 43: 1627-1634.

Chen, Z. G., I. Fujii, Y. Ebizuka, and U. Sankawa. 1992. Emodin *O*-methyltransferase from *Aspergillus terreus*. Arch. Microbiol. 158: 29-34.

Chen, Z. G., I. Fujii, Y. Ebizuka, and U. Sankawa. 1995. Purification and characterization of emodinanthrone oxygenase from *Aspergillus terreus*. Phytochemistry 38: 299-305.

Cohen, P. A. and G. H. N. Towers. 1995a. The anthraquinones and phenanthroperylenequinones of *Nephroma laevigatum*. J. Nat. Prod. 58: 520-526.

Cohen, P. A. and G. H. N. Towers. 1995b. The anthraquinones of *Heterodermia obscurata*. Phytochemistry. In press.

Cohen, P. A. and G. H. N. Towers. 1995c. Biosynthetic studies of chlorinated anthraquinones in the lichen *Nephroma laevigatum*. Phytochemistry. Submitted for publication.

Cohen, P. A., J. B. Hudson, and G. H. N. Towers. 1995. Antiviral activities of anthraquinones, bianthrones and hypericin derivatives from lichens. Experientia. In press.

Culberson, C. F. and W. L. Culberson. 1958. Age and chemical constituents of individuals of the lichen *Lasallia papulosa*. Lloydia 21: 189-192.

Culberson, C. F. 1969. Chemical and Botanical Guide to Lichen Products. Univ. North Carolina Press, Chapel Hill.

Culberson, C. F., W. L. Culberson, and A. Johnson. 1988. Gene flow in lichens. Amer. J. Bot. 75: 1135-1139

Culberson, C. F. and D. Armaleo. 1992. Induction of a complete secondary-product pathway in a cultured lichen fungus. Exper. Mycol. 16: 52-63.

Culberson, W. L. and C. F. Culberson. 1965. *Asahinea*, a new genus in the Parmeliaceae. Brittonia 17: 182-190.

Culberson, W. L. and M. E. Hale, Jr. 1965. *Pyxine caesiopruinosa* in the United States. Bryologist 68: 113-116.

Culberson, W. L. 1966. Chemistry and taxonomy of the lichen genera *Heterodermia* and *Anaptychia* in the Carolinas. Bryologist 69: 472-487

Culberson, W. L. and C. F. Culberson. 1970. A phylogenetic view of chemical evolution in the lichens. Bryologist 73: 1-31.

DePriest, P. T. and A. Gargas. 1994. Lichens as models for the study of molecular evolution: structure and variation of rRNA predicted by optional group I introns. Fifth International Mycological Congress, Abstracts, Vancouver, August.

De Riccardis, F., M. Iorizzi, L. Minale, R. Riccio, B. R. de Forges, and C. Debitus. 1991. The gymnochromes: novel marine brominated phenanthroperylene-quinone pigments from the stalked crinoid *Gymnocrinus richeri*. J. Org. Chem. 56: 6781-6787.

De Witte, P., P. Agostinis, J. Van Lint, W. Merlevede, and J. R. Vandenheede. 1993. Inhibition of epidermal growth factor receptor tyrosine kinase activity by hypericin. Biochem. Pharmacol. 46: 1929-1936.

Egan, R. E. 1982. *Xanthoparmelia barbatica* (Elix) comb. nov. new to North America. Bryologist 85: 129-130.

Egan, R. E. 1986. Correlations and non-correlations of chemical variation patterns with lichen morphology and geography. Bryologist 89: 99-110.

Elix, J. A., A. A. Whitton, and M. V. Sargent. 1984. Recent progess in the chemistry of lichen substances. Prog. Chem. Org. Nat. Prod. 45: 103-234.

Elix, J. A. 1992. History of lichen chemistry and development of analytical techniques. Flora Australia 54: 23-29.

Emmerich, R., I. Giez, O. L. Lange, and P. Proksch. 1993. Toxicity and antifeedant activity of lichen compounds against the polyphagous herbivorous insect *Spodoptera littoralis*. Phytochemistry 32: 1389-1394.

Ertl, L. 1951. Über die lichtverhältnisse in Laubflechten. Planta 39: 245-270.

Ewing, D. F. 1979. ^{13}C Substituent effects in monosubstituted benzenes. Org. Magn. Res. 12: 499-524.

Falk, H. and W. Schmitzberger. 1992. On the nature of "soluble" hypericin in *Hypericum* species. Monatsh. Chem. 123: 731-739.

Falk, H. and G. Schoppel. 1992. On the synthesis of hypericin by oxidative trimethylemodin anthrone and emodin anthrone dimerization: isohypericin. Monatsh. Chem. 123: 931-938.

Falk, H. and W. Schmitzberger. 1993. On the bromination of hypericin: the gymnochrome chromophores. Monatsh. Chem. 124: 77-83.

Follmann, G. 1960. Die Durchlässigkeitseigenschaften der Protoplasten von Phycobionten aus *Caldonia furcata* (Huds.) Schrad. Naturwiss. 47: 405-406.

Follmann, G. and M. Nakagawa. 1963. Keimhemmung von Angiospermensamen durch Flechtenstoffe. Naturwiss. 50: 696-697.

Follmann, G. and V. Villagrán. 1965. Flechtenstoffe und Zellpermeabilität. Z. Naturforsch. 20b: 723.

Follmann, G. and R. Peters. 1966. Flechtenstoffe und Bodenbildung. Z. Naturforsch. 21c: 386-387.

Fox, C. H., W. S. G. Maass, and T. P. Forrest. 1969. Papulosin, a novel chlorinated anthraquinone from *Lasallia papulosa* (Ach.) Llano. Tetrahedron Lett. 919-922.

Franck, B. 1984. Mycotoxine aus Schimmelpilzen-Waffen ungebetener Tischgenossen von Mensch und Tier: Wirkungen, Biosynthese und Schutzmöglichkeiten. Angew. Chem. 96: 462-474.

Franssen, M. C. R. and H. C. van der Plas. 1992. Haloperoxidases: their properties and their use in organic synthesis. Adv. Appl. Microbiol. 37: 41-99.

Fredenhagen, A., P. Hug, H. Sauter, and H. H. Peter. 1995. Paeciloquinones A, B, C, D, E and F: new potent inhibitors of protein tyrosine kinase produced by *Paecilomyces carneus*. II. Characterization and structure determination. J. Antibiotics 48: 199-204.

Fujii, I., Y. Ebizuka, and U. Sankawa. 1982. Partial purification and some properties of emodin-*O*-methyltransferase from (+)-geodin producing strain of *Aspergillus terreus*. Chem. Pharm. Bull. 30: 2283-2286.

Fujii, I., Z. G. Chen, Y. Ebizuka, and U. Sankawa. 1991. Identification of emodinanthrone oxygenase in fungus *Aspergillus terreus*. Biochem. Int. 25: 1043-1049.

Fujimoto, H., H. Flasch, and B. Franck. 1975. Biosynthese der Seco-anthrachinon Geodin und Dihydrogeodin aus Emodin. Chem. Ber. 108: 1224-1228.

Gardner, C. R. and D. M. J. Mueller. 1981. Factors affecting the toxicity of several lichen acids: effect of pH and lichen acid concentration. Amer. J. Bot. 68: 87-95.

Gargas, A., P. T. DePriest, M. Grube, and A. Tehler. 1995. Multiple origins of lichen symbioses in fungi suggested by SSU rDNA phylogeny. Science 268: 1492-1495.

Gatenbeck, S. 1958. Incorporation of labelled acetate in emodin in *Penicillium islandicum*. Acta Chem. Scand. 12: 1211-1214.

Gatenbeck, S. 1960. On the biosynthesis of the pigments of *Penicillium islandicum*. II. Acta Chem. Scand. 14: 296-302.

Gatenbeck, S. 1962. The mechanism of the biological formation of anthraquinones. Acta Chem. Scand. 16: 1053-1054.

Gerson, U. and M. R. D. Seaward. 1977. Lichen-invertebrate associations. *In* Lichen Ecology. M. R. D. Seaward (editor), Academic Press, London, pp. 69-119.

Gill, M. and W. Steglich. 1987. Pigments of fungi (macromycetes). Prog. Chem. Org. Nat. Prod. 51: 1-317.

Gill, M., A. Giménez, and R. W. McKenzie. 1988. Pigments of fungi, part 8. Bianthraquinones from *Dermocybe austroveneta*. J. Nat. Prod. 51: 1251-1256.

Gill, M. and A. Giménez. 1990a. Pigments of fungi. Part 11. (+)-Austrocorticin, austrocorticinic acid, austrocorticone, and related pigments; the first naturally occurring anthraquinone derived from a propionate-triggered octaketide. J. Chem. Soc. Perkin Trans. I. 1159-1167.

Gill, M. and A. Giménez. 1990b. Pigments of fungi. Part 17. (*S*)-(+)-Dermochrysone, (+)-dermolactone, dermoquinone, and related pigments; new nonaketides from the fungus *Dermocybe sanguinea* (sensu Cleland). J. Chem. Soc. Perkin Trans. I. 2585-2591.

Gill, M. and A. Giménez. 1991. Austrovenetin, the principal pigment of the toadstool *Dermocybe austroveneta*. Phytochemistry 30: 951-955.

Gill, M., A. Giménez, and R. Watling. 1992. Pigments of fungi, part 26. Incorporation of sodium [1,2-$^{13}C_2$]acetate into torosachrysone by mushrooms of the genus *Dermocybe*. J. Nat. Prod. 55: 372-375.

Gill, M. 1994. Pigments of fungi (macromycetes). Nat. Prod. Rep. 67-90.

Gill, M. 1995. Pigments of Australasian *Dermocybe* toadstools. Aust. J. Chem. 48: 1-26.

Gorin, P. A. J. and M. Iacomini. 1984. Polysaccharides of the lichens *Cetraria islandica* and *Ramalina usnea*. Carbohydr. Res. 128: 119-132.

Gowan, S. P. and I. M. Brodo. 1988. The lichens of Fundy National Park, New Brunswick, Canada. Bryologist 91: 255-325.

Green, T. G. A. 1970. The biology of lichen symbionts. Ph.D. dissertation, University of Oxford, England.

Gribble, G. W. 1992. Naturally occurring organohalogen compounds. J. Nat. Prod. 55: 1353-1395.

Grün, M. and G. Franz. 1980. Studies on the biosynthesis of aloin in *Aloe arborescens*. Planta Med. 39: 288.

Grün, M. and G. Franz. 1981. In vitro biosynthesis of the C-glycosidic bond in Aloin. Planta 152: 562-564.

Gyelnik, V. 1931. Nephromae novae et criticae. Ann. Cryptog. Exot. 4: 121-149.

Gyelnik, V. 1932. *Nephroma*-Studien. Hedwigia 72: 1-30.

Hafellner, J. 1988. Principles of classification and main taxonomic groups. *In* Handbook of Lichenology. M. Galun (editor), CRC Press, Boca Raton, Vol. 3, pp. 41-52 .

Hager, L. P., D. R. Morris, F. S. Brown, and H. Eberwein. 1966. Chloroperoxidase II. Utilization of halogen anions. J. Biol. Chem. 241: 1769-1777.

Hale, Jr., M. E. 1956. Studies on the chemistry and distribution of North American lichens. Bryologist 59: 114-117.

Hale, Jr., M. E. 1966. Chemistry and evolution in lichens. Israel J. Bot. 15: 150-157.

Hale, Jr., M. E. 1979. How to Know the Lichens. Wm. C. Brown Co., Dubuque.

Hale, Jr., M. E. 1983. The Biology of Lichens. Edward Arnold, London.

Hamada, N. and T. Ueno. 1990. Lecanoric acid from the mycobiont of the lichen *Stereocaulon curtatum*. Phytochemistry 29: 678-679.

Hawksworth, D. L. 1976. Lichen Chemotaxonomy. *In* Lichenology: Progress and Problems. D. H. Brown, D. L. Hawksworth, and R. H. Bailey (editors), Academic Press, London, pp. 139-184.

Hesse, O. 1898a. Beitrag zur Kenntniss der Flechten und ihrer charakteristischen Bestandtheile; Zweite Mittheilung. J. Prakt. Chem. 57: 442-445.

Hesse, O. 1898b. Beitrag zur Kenntniss der Flechten und ihrer charakteristischen Bestandtheile; Zweite Mittheilung. J. Prakt. Chem. 58: 485-487.

Hesse, O. 1901. Beitrag zur Kenntniss der Flechten und ihrer charakteristischen Bestandtheile; Zweite Mittheilung. J. Prakt. Chem. 63: 549-550.

Higuchi, M., Y. Miura, J. Boohene, Y. Kinoshita, Y. Yamamoto, I. Yoshimura, and Y. Yamada. 1993. Inhibition of tyrosinase activity by cultured lichen tissues and bionts. Planta Med. 59: 253-255.

Hill, D. J. and H. W. Woolhouse. 1966. Aspects of the autecology of *Xanthoria parietina* agg. Lichenologist 3: 207-214.

Himmelreich, U., S. Huneck, G. B. Feige, and H. T. Lumbsch. 1994. Squamaron, ein Napthochinon aus der Flechte *Squamarina cartilaginea*. Z. Naturforsch. 49b: 1289-1291.

Hind, H. G. 1940. The constitution of carviolin: a colouring matter of *Penicillium carmino-violaceum* Biourge. Biochem. J. 34: 577-579.

Holker, J. S. E., R. D. Lapper, and T. J. Simpson. 1974. The biosynthesis of fungal metabolites. Part IV. Tajixanthone: ^{13}C nuclear magnetic resonance spectrum and feedings with [1-^{13}C]- and [2-^{13}C]-acetate. J. Chem. Soc. Perkin I. 2135-2140.

Hudson, J. B., I. Lopez-Bazzocchi, and G. H. N. Towers. 1991. Antiviral activities of hypericin. Antiviral Res. 15: 101-112.

Hudson, J. B., L. Harris, and G. H. N. Towers. 1993. The importance of light in the anti-HIV effect of hypericin. Antiviral Res. 20: 173-178.

Hudson, J. B., E. A. Graham, and G. H. N. Towers. 1994. Antiviral assays on phytochemicals: the influence of reaction parameters. Planta Med. 60: 329-332.

Huneck, S. and K. Schreiber. 1972. Wachstumsregulatorische Eigenschaften von Flechten und Moos-Inhaltsstoffen. Phytochemistry 11: 2429-2434.

Huneck, S. 1973. Nature of lichen substances. *In* The Lichens. V. Ahmadjian and M. E. Hale (editors), Academic Press, New York, pp. 495-522.

Huneck, S. 1977. Inhaltsstoffe von *Pyxine coccifera*. Phytochemistry 15: 799-801.

Huneck, S., W. Steglich, and G. Höfle. 1977. Canarion, ein neues Naptho-chinon aus *Usnea canariensis*. Phytochemistry 16: 121-123.

Huneck, S. 1984. Fortschritte der Chemie von Flechtenstoffen. *In* Beiträge zur Lichenologie: Festschrift J. Poelt: Beiheft 79 zur Nova Hedwigia. H. Hertel and F. Oberwinkler (editors), J. Cramer, Vaduz, pp. 793-838.

Huneck, S. 1991. New results in the chemistry of lichens. Symbiosis 11: 225-248.

Huneck, S., C. F. Culberson, W. L. Culberson, and J. A. Elix. 1991. Haema-tommone, a red pigment from apothecia of *Haematomma puniceum*. Phytochemistry 30: 706-707.

Huneck, S., U. Himmelreich, J. Schmidt, V. John, and U. Zeybek. 1994. Zur Chemie von Flechten aus der Türkei. Struktur von Nemetzon, dem Apo-thecienpigment von *Haematomma nemetzii*. Z. Naturforsch. 49b: 1561-1565.

Ichinose, K., J. Kiyono, Y. Ebizuka, and U. Sankawa. 1993. Post-aromatic deoxygenation in polyketide biosynthesis: reduction of aromatic rings in the biosyntheses of fungal melanin and anthraquinone. Chem. Pharm. Bull. 41: 2015-2021.

Imshaug, H. A. 1956. The lichen genus *Pyxine* in North and Middle America. Contr. Herb. Mich. State Univ. 56: 246-269.

Ingólfsdóttir, K., S. F. Bloomfield, and P. J. Hylands. 1985. In vitro evaluation of the anti-microbial activity of lichen metabolites as potential preservatives. Antimicrobial Agents and Chemotherapy 28: 289-292.

Ingólfsdóttir, K., K. Jurcic, B. Fischer, and H. Wagner. 1994. Immunologically active polysaccharide from *Cetraria islandica*. Planta Med. 60: 527-531.

Inoue, K., Y. Shiobara, H. Nayeshiro, H. Inouye, G. Wilson, and M. H. Zenk. 1984. Biosynthesis of anthraquinones and related compounds in *Galium mollugo* cell suspension cultures. Phytochemistry 23: 307-311.

James, P. W. and F. J. White. 1987. Studies on the genus *Nephroma* I. The European and Macronesian species. Lichenologist 19: 215-268.

Jong, S. C. and R. Donovick. 1989. Antitumor and antiviral substances from fungi. *In* Advances in Applied Microbiology, Academic Press, New York, Vol. 34, pp. 183-262.

Kärnefelt, I. and A. Thell. 1993. Chemical evolution in Cetrarioid lichens. *In* Phytochemistry and Chemotaxonomy of Lichenized Ascomycetes-A Festschrift in Honour of Siegfried Huneck: Bibliotheca Lichenologica. G. B. Feige and H. T. Lumbsch (editors), J. Cramer, Berlin, Vol. 53, pp. 115-127.

Konoshima, T., M. Kozuka, J. Koyama, T. Okatani, K. Tagahara, and H. Tokuda. 1989. Studies on inhibitors of skin tumor promotion, VI. Inhibitory effects of quinones on Epstein-Barr virus activation. J. Nat. Prod. 52: 987-995.

Kraus, G. A., D. Pratt, J. Tossberg, and S. Carpenter. 1990. Antiretroviral activity of synthetic hypericin and related analogs. Biochem. Biophys. Res. Commun. 172: 149-153.

Krog, H. 1970. Forekomst av en upigmentert *Solorina crocea* i Finnmark. Blyttia 28: 181-182.

Kurobane, I., L. C. Vining, A. G. McInnes, D. G. Smith, and J. A. Walter. 1981. Biosynthesis of elsinochromes C and D. Pattern of acetate incorporation determined by ^{13}C and ^{2}H nmr. Can. J. Chem. 59: 422-430.

Kurokawa, S. 1962. A Monograph of the Genus *Anaptychia*. J. Cramer, Weinheim.

Kurokawa, S. 1973. Supplementary notes on the genus *Anaptychia*. J. Hattori Bot. Lab. 37: 563-607.

Labadie, R. P., J. Otten, and A. B. Svendsen. 1972. An investigation on the anthracene derivatives in *Rumex hydrolapathum* Huds. (II). Pharm. Weekblad 107: 541-547.

Lam, J. K. K., M. V. Sargent, J. A. Elix, and D. O'N. Smith. 1972. Synthesis of valsarin and 5,7-dichloroemodin. J. Chem. Soc. Perkin I. 1466-1470.

Lavie, G., F. Valentine, B. Levin, Y. Mazur, G. Gallo, D. Lavie, D. Weiner, and D. Meruelo. 1989. Studies of the mechanisms of action of the antiretroviral agents hypericin and pseudohypericin. Proc. Nat. Acad. Sci. US 86: 5963-5967.

Lawrey, J. D. 1986. Biological role of lichen substances. Bryologist 89: 111-122.

Lawrey, J. D. 1989. Lichen secondary compounds: evidence for a correspondence between antiherbivore and antimicrobial function. Bryologist 92: 326-328.

Lawrey, J. D., A. Y. Rossman, and R. Lowen. 1994. Inhibition of selected hypocrealean fungi by lichen secondary metabolites. Mycologia 86: 502-506.

Leistner, E. 1971. A second pathway leading to anthraquinones in higher plants. Phytochemistry 10: 3015-3020.

Leistner, E. 1973. Biosynthesis of morindone and alizarin in intact plants and cell suspension cultures of *Morinda citrifolia*. Phytochemistry 12: 1669-1674.

Lenard, J., A. Rabson, and R. Vanderoef. 1993. Photodynamic inactivation of infectivity of human immunodeficiency virus and other enveloped viruses using hypericin and rose bengal. Proc. Natl. Acad. Sci. US 90: 158-162.

Llano, G. A. 1950. A monograph of the lichen family Umbilicariaceae in the western hemisphere. Office of Naval Research, Washington, D. C.

Lopez-Bazzocchi, I., J. B. Hudson, and G. H. N. Towers. 1991. Antiviral activity of the photoactive plant pigment hypericin. Photochem. Photobiol. 54: 95-98.

Luckner, M. 1990. Secondary metabolism in microorganisms, plants and animals. Springer-Verlag, Berlin, pp. 62-68.

Maass, W. S. G., G. H. N. Towers, and A. C. Neish. 1964. Flechtenstoffe. I. Untersuchungen zur Biogenese des Pulvinsäureanhydrid. Ber. Deut. Bot. Ges. 77: 157-161.

Maass, W. S. G. and A. C. Neish. 1967. Lichen substances II. Biosynthesis of calycin and pulvinic dilactone by the lichen, *Pseudocyphellaria crocata*. Can. J. Bot. 45: 59-72.

Maass, W. S. G. 1970. Lichen substances. IV. Incorporation of pulvinic-^{14}C acids into calycin by the lichen *Pseudocyphellaria crocata*. Can. J. Biochem. 48: 1241-1248.

Manthey, J. A. and L. P. Hager. 1989. Characterization of the catalytical properties of bromoperoxidase. Biochemistry 28: 3052-3057.

Marles, R. J., J. B. Hudson, E. A. Graham, C. Soucy-Breau, P. Morand, R. L. Compadre, C. M. Compadre, G. H. N. Towers, and J. T. Arnason. 1992. Structure-activity studies of photoactivated antiviral and cytotoxic tricyclic thiophenes. Photochem. Photobiol. 56: 479-487.

Mathey, A., B. Steffan, and W. Steglich. 1980. 1,2-Napthochinon-Derivate aus Kulturen des Mycosymbionten der Flechte *Trypethelium eluteriae* (Trypetheliaceae). Liebigs Ann. Chem. 5: 779-785.

Mayrhofer, H. 1982. Ascosporen und Evolution der Flechtenfamilie Physciaceae. J. Hattori Bot. Lab. 52: 313-321.

Meruelo, D., G. Lavie, and D. Lavie. 1988. Therapeutic agents with dramatic antiretroviral activity and little toxicity at effective doses: aromatic polycyclic diones hypericin and pseudohypericin. Proc. Nat. Acad. Sci. US 85: 5230-5234.

Mishchenko, N. P., L. S. Stepanenko, O. E. Krivoshchekova, and O. B. Maksimov. 1980. Anthraquinones of the lichen *Asahinea chrysantha*. Khim. Prir. Soedin. 2: 160-165.

Miyagawa, H., N. Hamada, M. Sato, and T. Ueno. 1993. Hypostrepsilic acid, a new dibenzofuran from the cultured lichen mycobiont of *Evernia esorediosa*. Phytochemistry 34: 589-591.

Miyagawa, H., N. Hamada, M. Sato, and T. Ueno. 1994. Pigments from the cultured lichen mycobionts of *Graphis scripta* and *G. desquamescens*. Phytochemistry 36: 1319-1322.

Morris, D. R. and L. P. Hager. 1966. Chloroperoxidase I. Isolation and properties of the crystalline glycoprotein. J. Biol. Chem. 241: 1763-1768.

Morrison, M. and G. Bayse. 1973. Stereospecificity in peroxidase-catalyzed reactions. *In* Oxidases and Related Redox Systems. T. E. King, H. S. Mason, and M. Morrison (editors), University Park Press, Baltimore, Vol. 1, pp. 375-388.

Mosbach, K. 1964a. On the biosynthesis of lichen substances. Part II. The pulvic acid derivative vulpinic acid. Biochem. Biophys. Res. Commun. 17: 363-367.

Mosbach, K. 1964b. On the biosynthesis of lichen substances. Part I. The depside gyrophoric acid. Acta Chem. Scand. 18: 329-334.

Mosbach, K. 1967. On the biosynthesis of lichen substances. Part 4. The formation of pulvic acid derivatives by isolated lichen fungi. Acta Chem. Scand. 21: 2331-2334.

Mosbach, K. 1969. Biosynthesis of lichen substances, products of a symbiotic association. Angew. Chem. Internat. Edit. 8: 240-250.

Mosbach, K. 1973. Biosynthesis of lichen substances. *In* The Lichens. V. Ahmadjian and M. E. Hale (editors), Academic Press, New York, pp. 523-546.

Neidleman, S. L. and J. Geigert. 1986. Biohalogenation: principles, basic roles and applications, Ellis Horwood, Chichester.

Nikaido, T., T. Ohmoto, U. Sankawa, S. Kitanaka, and M. Takido. 1984. Inhibitors of adenosine 3',5'-cyclic monophosphate phosphodiesterase in *Cassia* seed. Chem. Pharm. Bull. 32: 3075-3078.

Nishikawa, Y., M. Tanaka, S. Shibata, and F. Fukuoka. 1970. Polysaccharides of lichens and fungi. IV. Antitumor active *O*-acetylated pustulan-type glucans from the lichens of *Umbilicaria* species. Chem. Pharm. Bull. 18: 1431-1434.

Nishikawa, Y., K. Yoshimoto, R. Horiuchi, K. Michishita, M. Okabe, and F. Fukuoka. 1979. Studies on the water-soluble constituents of lichens. III. Changes in antitumor effect caused by modifications of pustulan- and lichenan-type glucans. Chem. Pharm. Bull. 27: 2065-2072.

Nuno, M., Y. Kuwada, and K. Kamiya. 1969. The structure of nephroarctin. Chem. Commun. 78.

Nylander, W. 1866a. Circa novum in studio lichenum criterium chemicum. Flora 49: 198-201.

Nylander, W. 1866b. Quaedam addenda ad nova criteria chemica in studio lichenum. Flora 49: 233-234.

Nylander, W. 1866c. Hypochlorite of lime and hydrate of potash, two new criteria in the study of lichens. J. Linn. Soc. Bot. 9: 358-365.

Oxford, A. E., H. Raistrick, and P. Simonart. 1939. Studies in the biochemistry of microorganisms. LX Griesofulvin, $C_{17}H_{17}O_6Cl$, a metabolic product of *Penicillium griseo-fulvum* Dierckx. Biochem. J. 33: 240-248.

Pentillä, A. and H. M. Fales. 1966. On the biosynthesis in vitro of usnic acid. Chem. Commun. 656-657.

Petersen, F., A. Fredenhagen, H. Mett, N. B. Lydon, R. Delmendo, H. B. Jenny, and H. H. Peter. 1995. Paeciloquinones A, B, C, D, E and F: new potent inhibitors of protein tyrosine kinases produced by *Paecilomyces carneus*. I. Taxonomy, fermentation, isolation and biological activity. J. Antibiotics 48: 191-198.

Plat, H., B. E. Krenn, and R. Wever. 1987. The bromoperoxidase from the lichen *Xanthoria parietina* is a novel vanadium enzyme. Biochem. J. 248: 277-279.

Poelt, J. 1965. Zur Systematik der Flechtenfamilie Physciaceae. Nova Hedwigia 9: 21-32.

Poelt, J. 1973a. Systematic evaluation of morphological characters. *In* The Lichens. V. Ahmadjian and M. E. Hale (editors), Academic Press, New York, pp. 91-115.

Poelt, J. 1973b. Classification. *In* The Lichens. V. Ahmadjian and M. E. Hale (editors), Academic Press, New York, pp. 599-632.

Poelt, J. 1986. Morphologie der Flechten. Fortschritte und Probleme. Ber. Deut. Bot. Ges. 99: 3-29.

Poelt, J. 1991. Homologies and analogies in the evolution of lichens. *In* Frontiers in Mycology. D. L. Hawksworth (editor), Surrey, pp. 85-97.

Poelt, J. and C. Leuckert. 1993. Substitution and supplementary addition of secondary products in the evolution of lichenized ascomycotina. *In* Phytochemistry and Chemotaxonomy of Lichenized Ascomycetes-A Festschrift in Honour of Siegfried Huneck. G. B. Feige and H. T. Lumbsch (editors), J. Cramer, Berlin, Vol. 53, pp. 201-215.

Posner, B., G. B. Feige, and S. Huneck. 1990. Phytochemische Untersuchungen an west-europäischen *Lasallia*-Arten. Z. Naturforsch. 45c: 161-165.

Posner, B., G. B. Feige, and C. Leuckert. 1991. Beiträge zur Chemie der Flechtengattung *Lasallia* Mérat. Z. Naturforsch. 46c: 19-27.

Ramakrishnan, K., M. E. Oppenhuizen, S. Saunders, and J. Fisher. 1983. Stereoselectivity of chloroperoxidase-dependent halogenation. Biochemistry 22: 3271-3277.

Renner, B. and E. Gerstner. 1978a. Dünnschichtchromatographische Isolierung von Flechtenstoffen und deren Identifizierung. Z. Naturforsch. 33c: 340-345.

Renner, B. and E. Gerstner. 1978b. Anthrachinone aus der Mycobiontenkultur und dem Thallus von *Caloplaca ferruginea*. Naturwiss. 65: 439-440.

Renner, B. and E. Gerstner. 1980. Mediumabhängige Anthrachinon-Bildung in Mycobiontenkulturen von *Caloplaca ferruginea*. Naturwiss. 67: 352-353.

Renner, B., A. Henssen, and E. Gerstner. 1982. Zur Phytochemie südamerikanischer *Nephroma*-Arten. Z. Naturforsch. 37c: 739-747.

Richardson, D. H. S. 1967. The transplantation of lichen thalli to solve some taxonomic problems in *Xanthoria parietina* (L.) Th. Fr. Lichenologist 3: 386-391.

Richardson, D. H. S. 1975. The Vanishing Lichens. David and Charles, North Pomfret, pp. 95-97.

Richardson, D. H. S. and C. M. Young. 1977. Lichens and vertebrates. *In* Lichen Ecology. M. R. D. Seaward (editor), Academic Press, London, pp. 121-144.

Richardson, D. H. S. 1988. Medicinal and other economic aspects of lichens. *In* Handbook of Lichenology. M. Galun (editor), CRC Press, Boca Raton, Vol. 3, pp. 93-108.

Rideout, J. A. and M. D. Sutherland. 1985. Pigments of marine animals. XV. Bianthrones and related polyketides from *Lamprometra palmata gyges* and other species of crinoids. Aust. J. Chem. 38: 793-808.

Rogers, R. W. 1986. The genus *Pyxine* (Physciaceae, lichenized ascomycetes) in Australia. Aust. J. Bot. 34: 131-154.

Rogers, R. W. and J. Hafellner. 1988. *Haematomma* and *Ophioparma*: two superficially similar genera of lichenized fungi. Lichenologist 20: 167-174.

Rogers, R. W. 1989. Chemical variation and the species concept in lichenized ascomycetes. Bot. J. Linn. Soc. 101: 229-239.

Rundel, P. W. 1969. Clinal variation in the production of usnic acid in *Cladonia subtenuis*. Bryologist 72: 40-44.

Rundel, P. W. 1978. The ecological role of secondary lichen substances. Biochem. Syst. Ecol. 6: 157-170.

Sankawa, U., Y. Ebizuka, and S. Shibata. 1973. Biosynthetic incorporation of emodin and emodinanthrone into the anthraquinonoids of *Penicillium brunneum* and *P. islandicum*. Tetrahedron Lett. 2125-2128.

Santesson, J. 1969. Chemical studies on lichens 24. Norsolorinic acid in *Lecidea piperis*. Acta Chem. Scand. 23: 3270.

Santesson, J. 1970a. Anthraquinones in *Caloplaca*. Phytochemistry 9: 2149-2166.

Santesson, J. 1970b. Chemical studies on lichens 30. Anthraquinonoid pigments of *Trypetheliopsis boninensis* and *Ocellularia domingensis*. Acta Chem. Scand. 24: 3331-3334.

Santesson, J. 1970c. Some occurrences of the anthraquinone parietin in lichens. Phytochemistry 9: 1565-1567.

Santesson, J. 1973. Identification and isolation of lichen substances. *In* The Lichens. V. Ahmadjian and M. E. Hale (editors), Academic Press, New York, pp. 633-652.

Sargent, M. V., D. O'N. Smith, and J. A. Elix. 1970. The minor anthraquinones of *Xanthoria parietina* (L.) Beltram, the chlorination of parietin, and the synthesis of fragilin and 7-chloroemodin ('AO-1'). J. Chem. Soc. (C). 307-311.

Schinazi, R. F., C. K. Chu, J. R. Babu, B. J. Oswald, V. Saalmann, D. L. Cannon, B. F. H. Eriksson, and M. Nasr. 1990. Anthraquinones as a new class of antiviral agents against human immunodeficiency virus. Antiviral Res. 13: 265-272.

Scott, G. D. 1964. The lichen symbiosis. Advancement of Science (London) 31: 244-248.

Shibata, S. and T. Ikekawa. 1963. Metabolic products of fungi. XX. Biosynthesis of rugulosin. Chem. Pharm. Bull. 11: 368-372.

Shibata, S. 1964. Biogenetical and chemotaxonomical aspects of lichen substances. *In* Beiträge zur Biochemie und Physiologie von Naturstoffen-Festschrift Kurt Mothes zum 65 Geburtstag, Gustav Fischer Verlag, Jena, pp. 451-465.

Shibata, S., O. Tanaka, U. Sankawa, Y. Ogihara, R. Takahashi, S. Seo, D. M. Yang, and Y. Iida. 1968a. The constituents of *Acroscyphus sphaerophoroides* Lév. J. Jap. Bot. 43: 335-342.

Shibata, S., Y. Nishikawa, M. Tanaka, F. Fukuoka, and M. Nakanishi. 1968b. Antitumor activities of lichen polysaccharides. Z. Krebsforsch. 71: 102-104.

Shibata, S. 1973. Some aspects of lichen chemotaxonomy. Nobel Symposium 25: Chemistry in Botanical Classification. G. Bendz and J. Santesson (editors), Academic Press, New York, pp. 241-249.

Shibata, S. 1974. Anthraquinonoid mycotoxins. Int. Congr. Pure Appl. Chem. 2: 107-115.

Shu, Y. Z., M. Arcuri, M. R. Kozlowski, R. R. Wang, K. S. Lam, L. P. Chang, D. M. Pirnik, and P. M. Rose. 1994. Haloemodins, a new class of endothelin-1 type B (ET_B) receptor binding inhibitors. J. Antibiotics 47: 1328-1332.

Sigler, A. and H. W. Rauwald. 1994. *Aloe* plants accumulate anthrone-type anthranoids in inflorescence and leaves, and tetrahydroanthracenes in roots. Z. Naturforsch. 49c: 286-292.

Silverstein, R. M., G. C. Bassler, and T. C. Morrill. 1991. Spectroscopic Identification of Organic Compounds, John Wiley and Sons, New York, p. 241.

Slansky, Jr., F. 1979. Effect of the lichen chemicals atranorin and vulpinic acid upon feeding and growth of larvae of the yellow-striped armyworm, *Spodoptera ornithogalli*. Environ. Entomol. 8: 865-868.

Søchting, U. 1983. Anatomical and cytological characteristics of unpigmented *Caloplaca verruculifera* from Denmark. Bot. Tidskr. 68: 152-156.

Soedjak, H. S. and A. Butler. 1990. Chlorination catalyzed by vanadium bromoperoxidase. Inorg. Chem. 29: 5015-5017.

Steglich, W., W. Lösel, and V. Austel. 1969. Anthrachinon-Pigmente aus *Dermocybe sanguinea* (Wulf. ex Fr.) Wünsche und *D. semisanguinea* (Fr.). Chem. Ber. 102: 4104-4118.

Steglich, W., R. Arnold, W. Lösel, and W. Reininger. 1972. Biosynthesis of anthraquinone pigments in *Dermocybe*. J. Chem. Soc. Chem. Commun. 102-103.

Steglich, W. and K. F. Jedtke. 1976. Neue Anthrachinonfarbstoffe aus *Solorina crocea*. Z. Naturforsch. 31c: 197-198.

Steiner, M., K. W. Glombitza, A. Wagner, and J. Poelt. 1974. Anthraquinones of *Astroplaca opaca*. Phytochemistry 13: 273-274.

Steyn, P. S., R. Vleggaar, and P. L. Wessels. 1981. A ^{13}C NMR study of the biosynthesis of norsolorinic acid. S. Afr. J. Chem. 34: 12-17.

Swinscow, T. D. V. and H. Krog. 1975. The genus *Pyxine* in East Africa. Norw. J. Bot. 22: 43-68.

Swinscow, T. D. V. and H. Krog. 1976. The genera *Anaptychia* and *Heterodermia* in East Africa. Lichenologist 8: 103-138.

Swinscow, T. D. V. and H. Krog. 1988. Macrolichens of East Africa. British Museum (Natural History), London.

Sydiskis, R. J., D. G. Owen, J. L. Lohr, K. H. Rosler, and R. N. Blomster. 1991. Inactivation of enveloped viruses by anthraquinones extracted from plants. Antimicrobial Agents and Chemotherapy 35: 2463-2466.

Tagahara, K., J. Koyama, T. Ogura, T. Konoshima, M. Kozuka, H. Tokuda, H. Nishino, and A. Iwashima. 1992. Electronic properties and inhibitory effects on Epstein-Barr virus activation of mono- and di-substituted anthraquinones. Chemistry Express 7: 557-560.

Taguchi, H., U. Sankawa, and S. Shibata. 1966. Biosynthesis of usnic acid in lichens. Tetrahedron Lett. 5211-5214.

Takahashi, I., S. Nakanishi, E. Kobayashi, H. Nakano, K. Suzuki, and T. Tamaoki. 1989. Hypericin and pseudohypericin specifically inhibit protein kinase C: possible relation to their antiretroviral activity. Biochem. Biophys. Res. Commun. 165: 1207-1212.

Takahashi, K., T. Takeda, S. Shibata, M. Inomata, and F. Fukuoka. 1974. Polysaccharides of lichens and fungi. VI. Antitumor active polysaccharides of lichens of Stictaceae. Chem. Pharm. Bull. 22: 404-408.

Tang, J., J. M. Colacino, S. H. Larsen, and W. Spitzer. 1990. Virucidal activity of hypericin against enveloped and non-enveloped DNA and RNA viruses. Antiviral Res. 13: 313-326.

Thomas, C., R. S. MacGill, G. C. Miller, and R. S. Pardini. 1992. Photoactivation of hypericin generates singlet oxygen in mitochondria and inhibits succinoxidase. Photochem. Photobiol. 55: 47-53.

Thomas, C. and R. S. Pardini. 1992. Oxygen dependence of hypericin-induced phototoxicity to EMT6 mouse mammary carcinoma cells. Photochem. Photobiol. 55: 831-837.

Thomson, R. H. 1971. Naturally Occurring Quinones. Academic Press, London.

Thomson, R. H. 1987. Naturally Occurring Quinones III: Recent Advances. Chapman and Hall, London.

Tibell, L. 1984. A reappraisal of the taxonomy of Caliciales. *In* Beiträge zur Lichenologie: Festschrift J. Poelt: Beiheft 79 zur Nova Hedwigia. H. Hertel and F. Oberwinkler (editors), J. Cramer, Vaduz, pp. 597-713.

Trass, H. 1992. Synopsis of the lichen genus *Heterodermia* (Ascomycotina, Physciaceae sive Pyxinaceae). Folia Cryptogamica Estonica 29: 1-24.

Turner, N. J. 1977. Economic importance of black tree lichen (*Bryoria fremontii*) to Indians of western North America. Econ. Bot. 31: 461-470.

Turner, W. B. and D. C. Aldridge. 1983. Fungal Metabolites II. Academic Press, London.

van Pée, K. H. 1990. Einführung von Halogen durch Haloperoxidasen aus Bakterien. Kontakte 1: 41-47.

Vartia, K. O. 1973. Antibiotics in lichens. *In* The Lichens. V. Ahmadjian and M. E. Hale (editors), Academic Press, New York, pp. 547-561.

Vederas, J. C. and T. T. Nakashima. 1980. Biosynthesis of averufin by *Aspergillus parasiticus*; detection of ^{18}O-label by ^{13}C-N.M.R. isotope shifts. J. Chem. Soc. Chem. Commun. 183-185.

Vicente, C. A. and M. P. Estévez. 1976. Inhibition of photolysis by chloroatranorin in isolated chloroplasts from *Evernia prunastri*'s phycobiont. Boletin Real Sociedad Española de Historia Natural, Sección Biológica 74: 17-23.

Vicente, C. A., M. Palasí, and M. P. Estévez. 1978. Urease regulation mechanisms in *Lobaria pulmonaria* (L.) Hoffm. Revue Bryologique et Lichénologique 44: 83-89.

Vicente, C. A. and L. Xavier Filho. 1979. Urease regulation in *Cladonia verticillaris* (Raddi) Fr. Phyton (Buenos Aires) 37: 137-144.

Walter, B. and K. Ballschmiter. 1991. Biohalogenation as a source of halogenated anisoles in air. Chemosphere 22: 557-567.

Wat, C. K. and G. H. N. Towers. 1971. Biosynthesis of the benzoquinone, shanorellin, in *Shanorella spirotricha*. Phytochemistry 10: 103-107.

Weber, W. A. 1963. Lichens of the Chiricahua Mountains, Arizona. Univ. Colorado Studies, Biol. Ser. 10: 1-27.

Weiss, U. and J. M. Edwards. 1980. The Biosynthesis of Aromatic Compounds. John Wiley and Sons, New York.

Wetmore, C. M. 1960. The lichen genus *Nephroma* in north and middle America. Mich. State Univ. Mus. Pub. (Biol. Ser.) 1: 373-452.

Wetmore, C. M. 1968. Lichen flora of the Black Hills. Mich. State Univ. Mus. Pub. (Biol. Ser.) 3: 211-464.

Wetmore, C. M. 1980. A new species of *Nephroma* from North America. Bryologist 83: 243-247.

Wetmore, C. M. 1994. The lichen genus *Caloplaca* in North and Central America with brown or black apothecia. Mycologia 86: 813-838.

White, F. J. and P. W. James. 1988. Studies on the genus *Nephroma* II. The southern temperate species. Lichenologist 20: 103-166.

Whiton, J. C. and J. D. Lawrey. 1982. Inhibition of *Cladonia cristatella* and *Sordaria fimicola* ascospore germination by lichen acids. Bryologist 85: 222-226.

Whiton, J. C. and J. D. Lawrey. 1984. Inhibition of crustose lichen spore germination by lichen acids. Bryologist 87: 42-43.

Wiesner, W., K. H. van Pée, and F. Lingens. 1986. Detection of a new chloroperoxidase in *Pseudomonas pyrrocinia*. FEBS Lett. 209: 321-324.

Wiesner, W., K. H. van Pée, and F. Lingens. 1988. Purification and characterization of a novel bacterial non-heme chloroperoxidase from *Pseudomonas pyrrocinia*. J. Biol. Chem. 263: 13725-13732.

Yagi, A., M. Yamanouchi, and I. Nishioka. 1978. Biosynthetic relationship between tetrahydroanthracene and anthraquinone in *Aloe saponaria*. Phytochemistry 17: 895-897.

Yamamoto, Y., N. Kiriyama, and S. Arahata. 1968. Studies on the metabolic products of *Aspergillus fumigatus* (J-4). Chemical structure of metabolic products. Chem. Pharm. Bull. 16: 304-310.

Yamazaki, M., M. Matsuo, and S. Shibata. 1965. Biosynthesis of lichen depsides, lecanoric acid and atranorin. Chem. Pharm. Bull. 13: 1015-1017.

Yamazaki, M. and S. Shibata. 1966. Biosynthesis of lichen substances. II. Participation of C_1-unit to the formation of β-orcinol type lichen depside. Chem. Pharm. Bull. 14: 96-97.

Yosioka, I., H. Yamauchi, K. Morimoto, and I. Kitagawa. 1968a. Two new chlorine containing anthraquinones from a lichen, *Anaptychia obscurata* (Nyl.) Vain. Tetrahedron Lett. 1149-1152.

Yosioka, I., H. Yamauchi, K. Morimoto, and I. Kitagawa. 1968b. Three new chlorine containing bisanthronyls from a lichen, *Anaptychia obscurata* Vain. Tetrahedron Lett. 3749-3752.

Yosioka, I., K. Yamauchi, K. Morimoto, and I. Kitagawa. 1968c. The pigment constituents of some *Anaptychia* species. J. Jap. Bot. 43: 343-348.

Yosioka, I., T. Nakanishi, S. Izumi, and I. Kitagawa. 1968d. Structure of a lichen pigment entothein and its identity with secalonic acid A, a major ergot pigment. Chem. Pharm. Bull. 16: 2090-2092.

Yosioka, I., K. Morimoto, K. Murata, H. Yamauchi, and I. Kitagawa. 1971. Skyrin from two lichen species. Chem. Pharm. Bull. 19: 2420-2422.

Yosioka, I., H. Yamauchi, K. Murata, and I. Kitagawa. 1972. Coloring substance of a lichen *Cetraria ornata*. Chem. Pharm. Bull. 20: 1082-1084.

Zembower, D. E., C. M. Kam, J. C. Powers, and L. H. Zalkow. 1992. Novel anthraquinone inhibitors of human leukocyte elastase and cathepsin G. J. Med. Chem. 35: 1597-1605.

Zenk, M. H. and E. Leistner. 1968. Biosynthesis of quinones. Lloydia 31: 275-292.

Zopf, W. 1907. Die Flechtenstoffe in chemischer, botanischer, pharmakologischer und technischer Beziehung, Jena.

APPENDIX 1

Figure 17. Approximate phytogeographical distribution of *Nephroma laevigatum* in North America.

APPENDIX 2

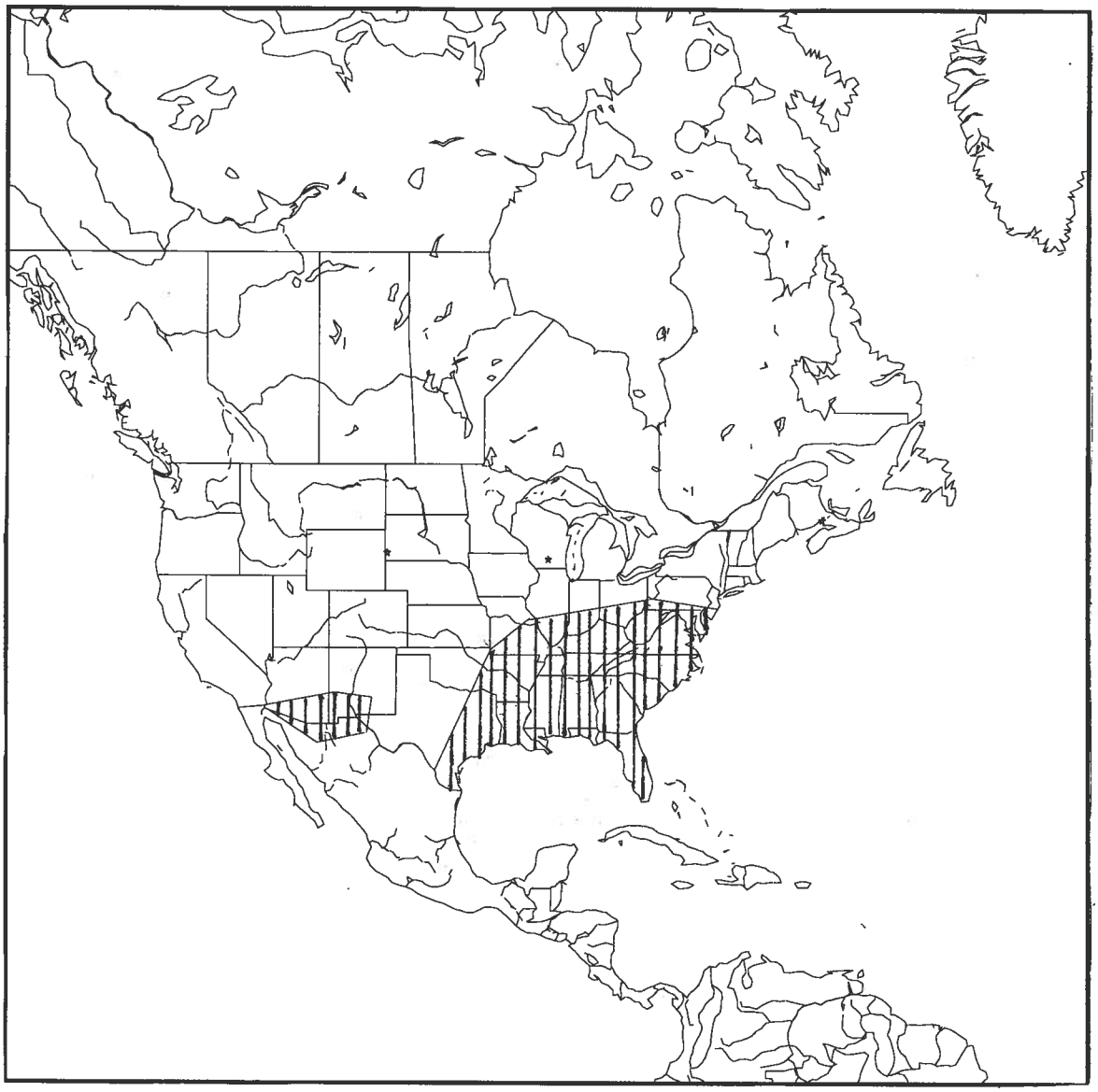
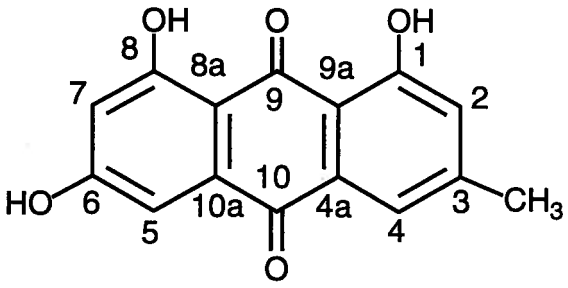
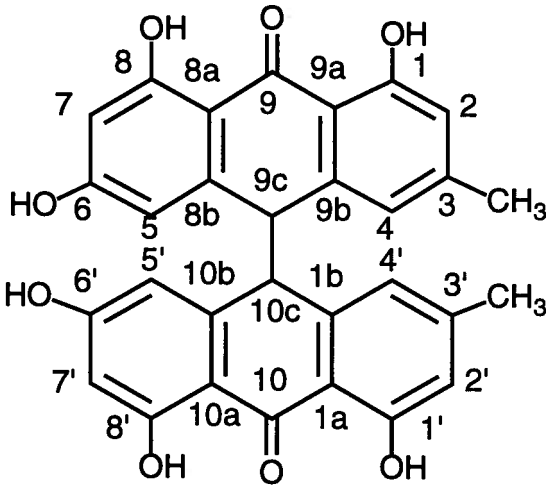


Figure 18. Approximate phytogeographical distribution of *Heterodermia obscurata* in North America.

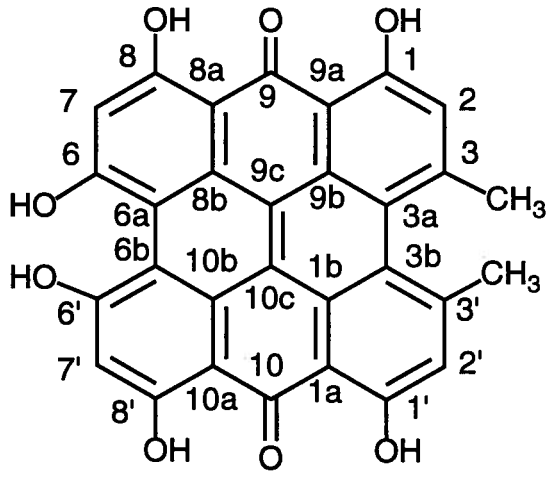
APPENDIX 3



Emodin (anthraquinone)



Emodin bianthrone (bianthrone)

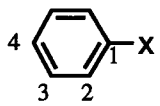


Hypericin (phenanthroperylenequinone)

Figure 19. Numbering systems in quinonoid natural products.

APPENDIX 4

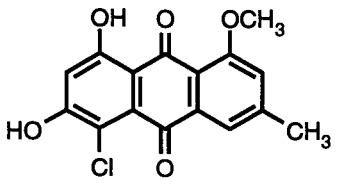
Sample Calculations of ^{13}C Carbon Chemical Shifts



$\sigma_C = 128.5 + z_i$ (all aromatic carbons)

$\sigma_C = 195.2 + z_i$ (C-9 and C-10)

Substituent x	z_1	z_2	z_3	z_4
-H	0.0	0.0	0.0	0.0
$-CH_3$	9.2	0.7	-0.1	-3.0
-Cl	6.3	0.4	1.4	-1.9
-OH	26.9	-12.8	1.4	-7.4
$-OCH_3$	31.4	-14.4	1.0	-7.7
-COphenyl	9.3	1.6	-0.3	3.7



5-Chloro-1-*O*-methylemodin

Carbon	Calculation	Value	Obs.
1	128.5 + 31.4(OCH_3) - 0.1 (CH_3) - 0.3 (C=O) + 3.7 (C=O)	163.2	160.2
2	128.5 - 14.4(OCH_3) - 0.3 (C=O) + 0.7 (CH_3)	114.5	120.0
3	128.5 + 9.2(CH_3) + 1.0 (OCH_3) + 3.7 (C=O)	142.4	145.8
4	128.5 + 0.7 (CH_3) - 7.7 (OCH_3) + 3.7 (C=O) - 0.3 (C=O)	124.9	119.8
5	128.5 - 0.3 (C=O) + 3.7 (C=O) - 7.4 (OH) - 12.8 (OH) + 6.3 (Cl)	118.0	118.6
6	128.5 + 26.9 (OH) + 3.7 (C=O) + 0.4 (Cl) + 1.4 (OH)	163.3	161.0
7	128.5 + 1.4 (Cl) - 12.8 (OH) - 12.8 (OH) + 3.7 (C=O)	108.0	112.6
8	128.5 + 26.9 (OH) + 1.4 (OH) - 1.9 (Cl) - 0.3 (C=O) + 3.7 (C=O)	159.1	160.2
9	195.2 + 1.0 (OCH_3) + 1.4 (OH) + 3.7 (C=O) - 1.9 (Cl)	199.4	191.8
10	195.2 - 3.0 (CH_3) - 7.7 (OCH_3) + 3.7 (C=O) - 7.4 (OH) - 7.4 (OH) + 1.4 (Cl)	175.2	183.6
4a	128.5 - 0.1 (CH_3) + 1.0 (OCH_3) + 1.6 (C=O) - 0.3 (C=O) - 1.9 (Cl)	128.8	130.6
8a	128.5 + 1.6 (C=O) - 0.3 (C=O) - 12.8 (OH) + 1.4 (Cl) - 7.4 (OH) - 7.7 (OCH_3)	103.5	112.6
9a	128.5 -14.4 (OCH_3) - 3.0 (CH_3) + 1.6 (C=O) - 7.4 (OH) - 0.3 (C=O)	105.0	113.4
10a	128.5 + 1.6 (C=O) - 0.3 (C=O) + 1.4 (OH) + 1.4 (OH) + 0.4 (Cl)	133.0	134.8

Figure 20. Calculations of ^{13}C carbon chemical shifts using the method of Ewing (1979).

APPENDIX 5

Calculations of Protein Content and Specific Activity:

1. A standard curve is prepared from standard solutions of BSA (Bovine Serum Albumin), containing 10 to 100 μg protein per 0.1 ml buffer, using the Bradford dye-binding assay. The weight of the protein is plotted against corresponding absorbances.

2. The protein content in a sample of the active semi-purified lichen chloroperoxidase fraction is determined from the standard curve.

3. Calculation:

a. 100 μl of semi-purified lichen chloroperoxidase gave absorbances of 0.184 and 0.200.

b. $A_{595\,nm}$ (0.184) is equivalent to 29.2 μg protein and $A_{595\,nm}$ (0.200) is equivalent to 31.6 μg protein.

c. Average weight of protein is (29.2 μg + 31.6 μg)/2 = 30.4 μg.

d. Protein concentration is 30.4 μg/100 μl or 0.3 μg/μl or 0.3 mg/ml.

e. Total protein content in semi-purified lichen chloroperoxidase:

   20 ml (total volume of fraction) x 0.3 mg/ml = 6 mg total protein.

f. 10 μl semi-purified lichen chloroperoxidase converted 2×10^{-7} moles MCD (monochlorodimedon) to DCD (dichlorodimedon) in 100 minutes (6000 seconds) in dimedon assay.

   2×10^{-7} moles DCD formed/6.0×10^{3} seconds = 3.33×10^{-11} moles sec^{-1}; for 20 ml total volume of fraction, this is 6.66×10^{-8} moles sec^{-1} or 0.066 μmoles sec^{-1}.

g. 1 Unit of enzyme catalyzes the formation of 1 μmole product per minute under defined conditions. Therefore, (0.066 μmoles sec^{-1}) x (60 seconds/minute) = 4 μmoles min^{-1} or 4 units of lichen chloroperoxidase

h. Specific Activity = 4 units/6 mg protein = 0.67 units/mg protein.

Figure 21. Calculations of protein content and specific activity of semi-purified chloroperoxidase from *Nephroma laevigatum*.

APPENDIX 6

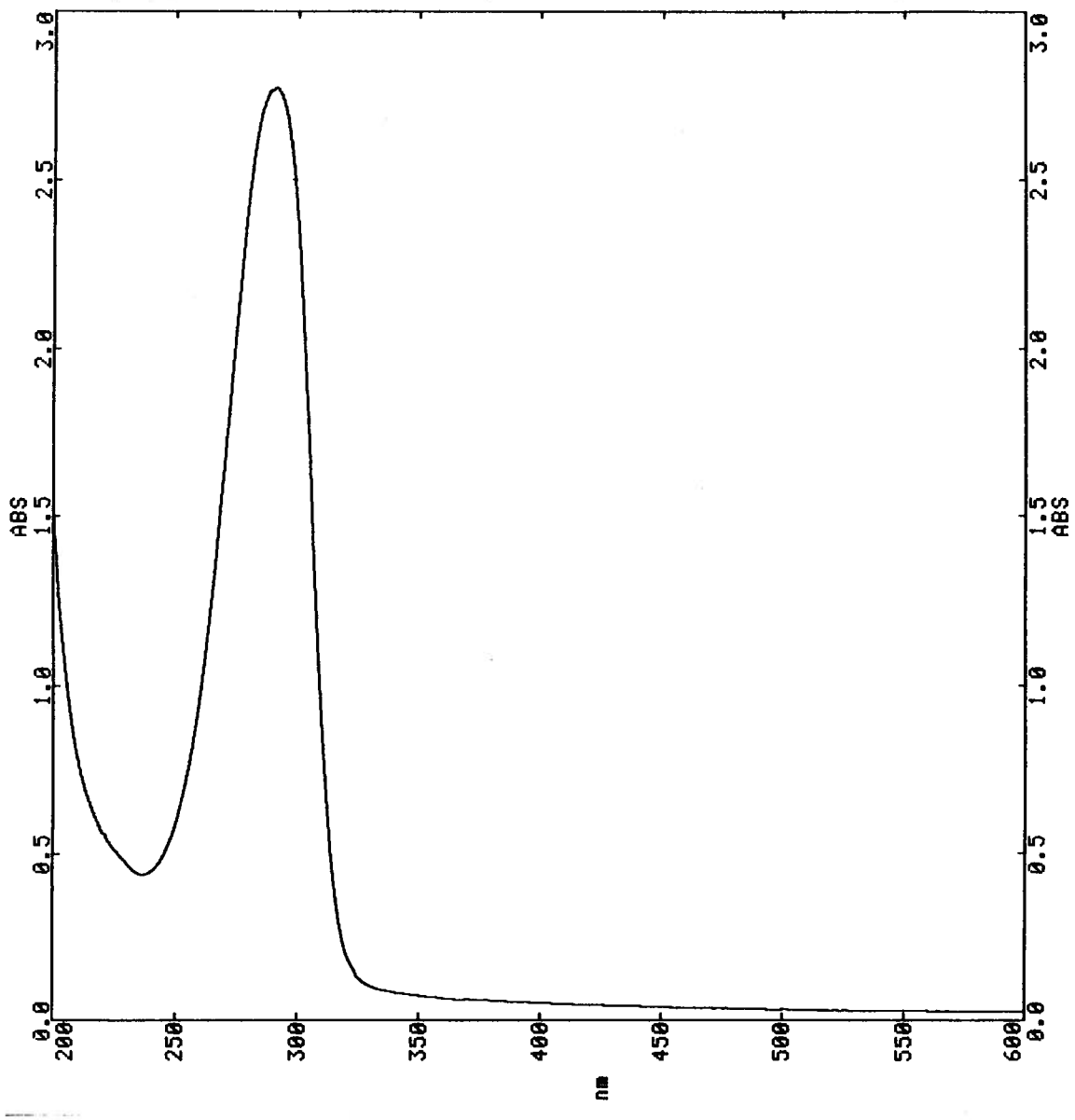


Figure 22. UV spectrum of semi-purified chloroperoxidase from *Nephroma laevigatum* (the spectrum was recorded in 0.1 M potassium phosphate buffer, pH 5.0, and a protein concentration of 0.3 mg/ml).

APPENDIX 7

Figure 23. Lichen feeding experiments with stable and radioisotopes.

APPENDIX 8

Table 19. HPLC data for lichen anthraquinones, bianthrones and hypericin derivatives.[1]

Compound	Source	Retention Time[2]	Solvent and Elution System
Emodin	*N. laevigatum*	4.58	1:1 MeOH-2 % AcOH in CH_3CN
7-Chloroemodin	*N. laevigatum*	4.20	1:1 MeOH-2 % AcOH in CH_3CN
7-Chloroemodin	*N. laevigatum*	4.23	1:1 MeOH-2 % AcOH in CH_3CN to 100 % MeOH (gradient; 15 min)
7-Chloroemodin	*H. obscurata*	4.15	60:40 MeOH-H_2O to 100 % MeOH (gradient; 15 min)
7-Chloro-1-*O*-methylemodin	*N. laevigatum*	4.24	1:1 MeOH-2 % AcOH in CH_3CN
7-Chloro-1-*O*-methyl-ω-hydroxyemodin	*N. laevigatum*	3.80	1:1 MeOH-2 % AcOH in CH_3CN
5,7-Dichloroemodin	*H. obscurata*	2.97	60:40 MeOH-H_2O to 100 % MeOH (gradient; 15 min)
Flavoobscurin A	*H. obscurata*	3.76	1:1 MeOH-2 % AcOH in CH_3CN
Flavoobscurin B	*H. obscurata*	3.53	1:1 MeOH-2 % AcOH in CH_3CN
Hypericin	Sigma Chem. Co.	13.44	60:40 MeOH-H_2O to 100 % MeOH (gradient; 10 min)
7,7'-Dichlorohypericin	*N. laevigatum*	13.85	60:40 MeOH-H_2O to 100 % MeOH (gradient; 10 min)
7,7'-Dichlorohypericin	Synthetic	13.93	60:40 MeOH-H_2O to 100 % MeOH (gradient; 10 min)
2,2',7,7'-Tetrachlorohypericin	*N. laevigatum*	14.18	60:40 MeOH-H_2O to 100 % MeOH (gradient; 10 min)

[1] All compounds were eluted from a reversed-phase Waters Bondapak C_{18} column (3.9 x 300 mm).
[2] Retention times are in minutes.