ABSTRACT

A NEW POLYACETYLENIC ALCOHOL IN *FISTULINA HEPATICA*: PROGRESS TOWARDS THE IDENTIFICATION OF ACETYLENASES IN BASIDIOMYCETES

by Errol A. Huffman, Sr.

Cultures of the basidiomycete *Fistulina hepatica* have been studied to determine the times of appearance of acetylenic natural products. A triacetylenic alcohol has been isolated from the ethereal extract of mycelial cultures of *F. hepatica*. On the basis of spectroscopic data, its structure was determined to be cis-3-tridecene-5,7,9-triyne-1,11-diol. The structure has also been determined for an unsaturated fatty acid, which accumulates when a parsley desaturase is expressed in soybeans. The compound, identified as (14Z)-dehydrocrepenynic acid by NMR and mass spectral data, is believed to be a widely used intermediate in the crepenynate pathway to polyacetylene production in plants and, presumably, fungi. A yeast expression system capable of overexpressing FAD2-type desaturases that may be involved in the biosynthesis of acetylenic natural products was optimized with respect to growth conditions and host cell strain. This system may be suitable for the expression of fungal acetylenases from *F. hepatica.*
A NEW POLYACETYLENIC ALCOHOL IN *FISTULINA HEPATICA*: PROGRESS TOWARDS THE IDENTIFICATION OF ACETYLENASES IN BASIDIOMYCETES

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Dedication

I dedicate this work to my wife, Diane; sons, Kyle Miller and Les Huffman; parents, Robert and Marjorie Huffman; brother, Larry Huffman; grandparents, Robert and Faye Young; and grandchildren.
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1. Introduction

Preface

Desaturases comprise a class of enzymes that catalyze dehydrogenation of fatty acids, which, in turn, play central roles in membrane structure, prostaglandin formation and the biosynthesis of interesting derivatives such as polyacetylenes [1-4]. Antiviral and antitumor activities have been attributed to many acetylenic metabolites making them targets of study for biosynthetic production in industrially important plants and micro-organisms [5].

The fatty acids from which polyacetylenes are derived contain unusual oxidative modifications such as acetylenic bonds and epoxy and hydroxyl groups, and could become industrially important as renewable resources if they could be produced efficiently in substantial quantities. However, a complete understanding of the regulation of plant lipid metabolism and the genes involved is essential to engineering genetic improvements in oil quality and quantity [3].

Gene cloning and sequence analysis of a predicted oxidative, desaturase-like enzyme can provide information useful in deducing the topology of the protein and the structure and location of the putative active site. Availability of the appropriate desaturase genes permits the manipulation of fatty acid composition through heterologous expression [2]. These approaches may lead to successful manipulation of plant lipids through the transfer of genes for fatty acid-modifying enzymes from wild species to common agricultural crops.

1.1 Biosynthesis of fatty acid derivatives in plants

Singer and Nicolson’s fluid mosaic model of membrane structure, common in all organisms, aptly describes the dynamic interaction between membrane-bound proteins floating in a sea of fluid lipid. The fluid nature of the membranes of cells and organelles derives from the activity of stearoyl-CoA and stearoyl-ACP desaturases that introduce a Δ9-double bond in stearic acid [6]. This and subsequent steps in the desaturation of fatty acids is crucial for regulating the membrane fluidity of all living cells. In addition to this
important aspect of primary metabolism, many organisms further modify the resulting unsaturated fatty acids through pathways of secondary metabolism which are less well understood [1,7,8]. The initial primary metabolism phase proceeds through a common pathway and uses the ubiquitous fatty acid biosynthesis machinery. Fatty acid biosynthesis generates major products of 16 or 18 carbon chains containing one to three methylene-interrupted double bonds in the cis configuration.

Acetyl-CoA is the basic building block for fatty acids. Synthesis begins with condensation of acetyl-CoA with malonyl-CoA followed by carbonyl reduction, dehydration, enoyl reduction and repetition of this cycle. During synthesis in plants, fatty acids are esterified to a water-soluble acyl-carrier protein (ACP) localized in the plastids (Figure 1). Subsequently, the fatty acyl-ACP esters are used for lipid synthesis within the plastids or hydrolyzed to free acids for transport through the plastid membranes during export to the endoplasmic reticulum (ER). During transport they are converted to acyl-CoA esters by an acyl-CoA synthetase associated with the plastid envelope [3].

Functional desaturation of membrane lipids in plants is catalyzed by membrane-bound desaturases of the ER and the soluble stearoyl-ACP desaturase in the plastids. The initial steps of lipid synthesis in the ER probably involve the transfer of 16:0 and 18:1 acyl-CoA esters to sn-glycerol-3-phosphate in the synthesis of phosphatidic acid (PA). PA is then converted to 1,2-diacyl-sn-glycerol (DAG) before modification with a polar alcohol at the sn-3 position in forming a variety of phospholipids crucial to the structure of all membranes except those of plastids [3]. The transformation essential to polyunsaturation in plants is catalyzed by the ER 18:1 desaturase using 18:1 esterified to phosphatidylcholine (PC) as substrate [6].

Subsequent desaturations and installation of various functional groups have resulted in a vast array of unusual fatty acid derivatives including several thousand polyacetylenic compounds which, in many cases, exhibit antitumor, antiviral and antibacterial activities. This secondary metabolism phase is not unique to a narrow class of organisms. Polyacetylenes derive from a broad range of phyla representing plants, fungi, bryophytes, amphibians and algae as well as marine- and micro-organisms.
Figure 1. Lipid synthesis in leaves of the model plant system *Arabidopsis thaliana*. Pathways are initiated by the synthesis of palmitic acid (16:0), esterified to acyl-carrier protein (ACP), within the plastid. While some 16:0 is esterified to the *sn*-2 position of lysophosphatidic acid (LPA) for local lipid synthesis, most is elongated to stearoyl-ACP (18:0-ACP) and then desaturated to oleoyl-ACP (18:1-ACP); a small portion of 16:0 is exported to the endoplasmic reticulum (ER). The 18:1-ACP is used in the synthesis of phosphatidic acid (PA). Most PA synthesis occurs in the ER. Approximately 50% of total acyl-ACPs are hydrolyzed to free fatty acids for transfer from the plastid to the ER where they are converted to acyl-CoA esters. PA made in the plastid is used for synthesizing phosphatidylglycerol (PG), diacylglycerol (DAG), monogalactosyldiacylglycerol (MGD), digalactosyldiacylglycerol (DGD) and sulfolipid (SL). PA made in the ER may be converted, through DAG using activated headgroups, to phospholipids such as phosphatidylcholine (PC) and phosphatidylethanolamine (PE). Alternatively, a nucleotide-activated form of DAG, cytidine diphosphate-DAG (CDP-DAG), may be used to form phospholipids such as phosphatidylinositol (PI). The ER 18:1 desaturase (FAD2) and 18:2 desaturase (FAD3) modify fatty acids at both the *sn*-1 and *sn*-2 position of PC. Triacylglycerol (TAG) synthesis proceeds mainly through DAG. A large part of the DAG produced in the ER is transferred to the plastid for synthesis of plastid lipids [3].
Examples of the biologically active compounds produced by plants include the violently toxic substance cicutoxin, which is found concentrated in the rhizomes of water hemlock (Figure 2). This highly unsaturated alcohol, first isolated and characterized in 1953, has caused many deaths of humans and animals over the centuries [9-11]. Another closely related unsaturated alcohol, falcarinol, is a product of Devil's Club, a North American shrub possessing medicinal properties [12]. It commonly occurs in Umbelliferae and is a well-known component of *Panax ginseng* and the common carrot. This polyyne, toxic in high concentrations, exhibits significant antibacterial, antimycobacterial and anti-*Candida* activity [12]. Minquartynoic acid, occurring in the twigs of a subcanopy hardwood tree of the Indomalaysia region, has been proven to effectively inhibit HIV-1 [13]. Cis-1,9,16-heptadecatriene-4,6,diyne-3,8-diol, extracted from the leaves of the Angelica Tree in Costa Rica, has shown cytotoxic activity against a variety of tumor cell lines [13,14]. The natural bioactivity of compounds such as these identify them as possible molecular targets that may one day be produced by industrially important plants and microorganisms. Although their potential individual values are obvious, the general biosynthesis of these compounds is not well understood.

1.2 Characteristics of fatty acid modification systems

An important element in designing the industrially important transgenic systems referred to above is a complete understanding of the metabolic pathways in both the original and the host species. Although knowledge of secondary pathways generating unusual fatty acids is incomplete, the enzymes responsible for the desaturation of polyunsaturated fatty acids during primary metabolism are well known and may provide clues crucial to elucidating details of the secondary metabolic mechanisms.

For example, linoleate (18:2<sup>Δ9,12</sup>) is the precursor of α-linolenate (18:3<sup>Δ9,12,15</sup>) and, with linolenate, serves as a crucial membrane structural component in plants as well as an essential fatty acid in human diets [2]. In addition to these roles, 18:2 is an important precursor for secondary metabolic products. Nearly all 18:2 is synthesized in the ER of plants by an integral membrane desaturase whose activity in *Arabidopsis* arises from the
Figure 2. Biologically active compounds in plants include minquartynoic acid, which effectively inhibited lymphoblastoid cell killing by HIV-1 in anti-HIV assays. Falcarinol exhibits antibiotic activity as the causative agent of allergic contact dermatitis [16]. It has also exhibited antifungal activity in carrots [17]. Cicutoxin is an extremely potent natural poison, which, if ingested, induces strong clonic convulsions in humans and animals [9]. The antitumor agent cis-1,9,16-heptadecatriene-4,6-diyne-3,8-diol exhibits cytotoxic activity against Hep-G2, A-431, H-4IIE and L-1210 tumor cell lines [14].
Minquartynoic Acid

*Ochanostachys amentacea*
(tree stems, Malaysia)

Antitumor Agent

(-)-Falcarinol

Devil's Club
(tree bark, western N. America)

Antibiotic

Cicutoxin

Water Hemlock
(rhizomes, water hemlock)

Neurotoxin

cis-1,9,16,-Heptadecatriene-4,6-diynediol-3,8-diol

Angelica Tree
(tree leaves, Costa Rica)

Antitumor Agent
fatty acid desaturation 2 (Fad2) locus [15]. FAD2 is an ER-localized oleoyl-CoA Δ^{12}-desaturase that introduces a cis-double bond between carbons 12 and 13 of oleoyl-CoA to form linoleoyl-CoA [18]. Only a small portion of 18:2 is synthesized by a plastidial 18:1 desaturase.

Sequencing of cDNA clones of Fad2 and other membrane-localized desaturases has revealed the presence of three motifs containing eight histidine residues [6,19]. Alignments of amino acid sequences produced the consensus sequence

\[ HX(3 \text{ or } 4)HX(20-50)HX(2 \text{ or } 3)HHX(100-200)HX(2 \text{ or } 3)HH \]

and site-directed mutagenesis of each of the eight His residues in the stearoyl-CoA Δ^{9}-desaturase from rat liver resulted in loss of desaturase activity. The histidine-boxes are believed to bind two iron ions required for catalysis [20]. All integral-membrane proteins known to contain the eight-histidine motif catalyze oxygen-dependent reactions with hydrocarbon substrates and represent at least 11 different activities: five desaturases (four fatty acid, one steroid), three hydroxylases (fatty acid, xylene, alkane), two oxidases (C-4 sterol methyl, carotene) and one decarbonylase (aldehyde) [20].

In addition to containing these conserved His-boxes, these enzymes have a catalytic dependence on molecular oxygen, nonheme iron, NADH and cytochrome b_{5} [21]. They also have similar responses to chemical inhibitors, maintain the same stereospecificity of the desaturase reaction and display the same kinetic isotope effects for C-H bond cleavage in all cases reported to date [19]. Structurally, the enzymes have similar molecular weights, possess local sequence homology near the His motifs and orient the His motifs on the cytoplasmic side of the membrane [21]. Recently, immunofluorescence microscopy analyses of differential permeabilization experiments confirmed that the N-termini of both FAD2 and FAD3 (the Arabidopsis linoleoyl CoA Δ^{15}-desaturase) are exposed on the cytosolic side of the ER membrane [18].

1.3 Fad2-type enzymes

Sequence analysis of Fad2 and related desaturases, in addition to revealing the His-boxes conserved in membrane-bound desaturases, has also provided information necessary for predicting the structure and location of a putative active site [2,21].
Hydropathy analysis of the sequences for yeast and rat liver \( \Delta^9 \)-desaturases reveal two positionally conserved hydrophobic domains (50 residues each) that could potentially form two transmembrane-spanning loops each [22]. With the N-terminus exposed to the cytosol, as verified for FAD2 and FAD3, insertion of these hydrophobic regions into the ER membrane would orient the remainder of the protein in the cytosol where the conserved catalytic His-boxes presumably interact with the electron carrier protein cytochrome \( b_5 \). Functional verification of the proposed topology of the two enzymes was provided when a yeast-rat \( \Delta^9 \)-desaturase fusion gene was able to functionally replace the native \( \Delta^9 \)-desaturase in \( S. \) cerevisiae [22]. Hydropathy analyses of six additional membrane protein sequences (five desaturases and one hydroxylase) were consistent with this structural model [19].

A similar model containing six transmembrane segments has been presented for the \( Pseudomonas \) oleovorans alkane hydroxylase AlkB, which is an integral cytoplasmic membrane protein whose primary sequence contains eight potential membrane-spanning sections [23]. Chimeric enzymes were used to test this model. Alkaline phosphatase (PhoA), which lacks an export signal, exhibits high activity when fused to periplasmic domains but is relatively inactive when fused to cytoplasmic domains of membrane proteins. On the other hand, \( \beta \)-galactosidase (LacZ) is active when fused to cytoplasmic domains but shows reduced activity when fused to periplasmic domains of membrane proteins. Protein fusions linking different amino-terminal fragments of AlkB with PhoA and LacZ, at specific sites in the primary sequence of AlkB, were used to provide complementary catalytic information for determining whether these sites were located near the cytoplasmic or periplasmic membrane face [23]. The observed activity indicated that AlkB has three domains that span the membrane twice each with the remainder of the protein oriented in the cytoplasm.

FAD2-type enzymes catalyze a variety of reactions in plants, animals, fungi and bacteria [21]. In plants, \( \Delta^{12} \)-desaturase-like enzymes catalyze at least four types of reactions including those involving desaturase, acetylenase, epoxidase and hydroxylase activities (Scheme 1) [21,24]. Sequencing of \( Fad2 \) has also made possible the design of probes used in cloning a set of structurally related desaturases in \( Arabidopsis \) [1]. Lipid
Scheme 1. Examples of four desaturase-like reactions in plants are shown (reviewed by Shanklin and Cahoon) [21]. The reactions are thought to occur on substrates esterified to phospholipids. In *Arabidopsis*, FAD2 converts oleic acid to linoleic acid. The 12-hydroxylase of *Ricinus communis* L. catalyzes the production of ricinoleic acid from oleic acid. CREP1 and CPAL2 of *Crepis* convert linoleic acid to crepenynic acid and vernolic acid, respectively.
12-hydroxylase

\[ \text{oleic acid} \xrightarrow{\Delta^{12}-\text{desaturase}} \text{linoleic acid} \xrightarrow{\Delta^{12}-\text{epoxygenase}} \text{vernolic acid} \]

\[ \text{ricinoleic acid} \xrightarrow{\Delta^{12}-\text{acylase}} \text{crepeninic acid} \]
analysis of these *Arabidopsis* mutants, each deficient in one of eight desaturases studied, revealed a pattern of changes in relative lipid levels that, when viewed together, have contributed to a greater understanding of the regulation and mechanisms of these enzymes [3].

**1.4 Identification and cloning of the acetylenase gene Crep1**

The *Crepis alpina* acetylenase gene *Crep1* has been cloned and sequenced by Stymne [24]. The enzyme, in a desaturation reaction on PC substrate, catalyzes dehydrogenation of the double bond at the $\Delta^{12}$-position of linoleate to form crepenylic acid (Scheme 2) [25]. This unusual fatty acid, first discovered in the seed oil of *C. foetida*, is an important precursor in the biosynthesis of acetylenic natural compounds [4,26]. A cDNA library from *C. alpina* was screened for the $\Delta^{12}$-desaturase-like sequences resulting from PCR experiments that used degenerate primers based on endoplasmic reticular desaturase sequences. Examination of deduced amino acid sequences of CREP1 predicts a microsomal enzyme of 375 amino acid residues with structural features consistent with the desaturase model described above. The protein is anchored by four putative transmembrane segments, with the histidine motifs oriented near the cytoplasmic face of the membrane (Figure 3). Alignment of amino acid sequences indicates that CREP1 has 59% identity with the castor bean 12-hydroxylase and 56% identity with *Arabidopsis* Fad2 [24].

The *Crep1* gene was expressed in *Saccharomyces cerevisiae* (YN94-1 strain) and crepenylic acid (18:1A) was detected when linoleic acid (18:2) was added to the media [24]. It has been reported that yeast cultures grown without exogenous 18:2 produced 18:2 at levels of 0.4% w/w of total fatty acids, indicating that the enzyme also acts as a $\Delta^{12}$-desaturase. However, subsequent conversion to 18:1A was not observed. 18:2 produced by CREP1 appears not to serve as a substrate for the enzyme.

The epoxygenase CPAL2, from *C. palaestina*, was also cloned by Stymne. CPAL2 uses 18:2 as a substrate to convert the double bond at the $\Delta^{12}$-position to an epoxy group yielding vernoleate. Both CREP1 and CPAL2 share high homology with $\Delta^{12}$-desaturases [24]. In addition, CREP1 and CPAL2 exhibit biochemical characteristics
Scheme 2. The general biosynthesis of crepenylic acid is represented. In feeding experiments with plants and fungi, stearic acid is uniformly labeled at alternate carbon atoms of the chain by incorporation of [1-14C]acetate [4]. [2-14C]malonate is incorporated in a complementary pattern excluding the terminal ethyl group, which is derived from acetate. [14C]linoleate is converted to [14C]crepenynate by microsomes from developing seeds of *C. alpina* [25].
acetyl-CoA + malonyl-CoA

Fatty acyl synthase

\[ \text{CH}_3(\text{CH}_2\text{CH}_3)_8\text{COOH} \]

stearic acid

\[ \text{CH}_3(\text{CH}_2)_{12}\text{COOH} \]

oleic acid

\[ \text{CH}_3(\text{CH}_2)_{14}\text{COOH} \]

linoleic acid

\[ \text{CH}_3(\text{CH}_2)_{16}\text{COOH} \]

crepenynic acid
Figure 3. Proposed membrane topology of CREP1. Examination of the CREP1 sequence indicates a likely membrane-bound orientation of the protein with the putative catalytic histidine motif located adjacent to the cytoplasmic face of the membrane. Strong $\alpha$-helix breakers such as glycine and proline, as well as charged residues, occur between locations 65-76 and 250-254. Bold bars denote transmembrane domains and lightly filled ovals mark the iron-binding histidine-rich motifs.
consistent with the microsomal desaturases; they require NADH and are inhibited by cyanide (but not carbon monoxide) and cytochrome bs antibodies, presumably indirectly.

Crepenynate occurs with vernoleate in the seeds of the Crepis species C. aurea, C. biensis, C. intermedia and C. occidentalis; therefore, these species may contain both an acetylenase and epoxygenase [27]. However, as illustrated by the Δ^{12}-hydroxylase and Δ^{6}-acetylenase examples below, these Crepis species may, instead, contain one dual-function enzyme.

1.5 A bifunctional Δ^{6}-acetylenase/desaturase from Ceratodon purpureus

A second acetylenase gene encoding a bifunctional enzyme was recently cloned and expressed in S. cerevisiae. Transgenic yeast expressing a Δ^{6}-acetylenase/desaturase from the moss Ceratodon purpureus introduced a Δ^{6}-cis-double bond in linoleic acid (18:2Δ^{9,12}) and subsequently converted the product, γ-linolenic acid (18:3Δ^{6,9,12}), to the main fatty acid found in C. pupureus, 9,12-(Z,Z)-octadecadien-6-ynoic acid (18:2AΔ^{6A,9,12}) [28]. Importantly, γ-linolenic acid, and not α-linolenic acid (18:3Δ^{9,12,15}), serves as substrate for Δ^{6}-acylenation, providing support for the suggested biosynthesis of 9,12,15-(Z,Z,Z)-octadecatrien-6-ynoic acid (18:3AΔ^{6A,9,12,15}), the dominant fatty acid of the triglyceride fraction in C. purpureus [7]. Incorporation of labeled precursors indicated a preferred pathway where linoleic acid is desaturated twice in the Δ^{6}-position to yield 18:2Δ^{6A,9,12}, which undergoes Δ^{15}-desaturation forming 18:3AΔ^{6A,9,12,15}.

1.6 Enzymes related to the Crepis acetylenase

Included in the family of nonheme iron-containing integral-membrane enzymes is the bacterial alkane ω-hydroxylase AlkB from Pseudomonas oleovorans, discussed above [20]. Mssbauer studies have provided evidence that AlkB contains a dinuclear iron cluster with spectroscopic properties similar to clusters found in the soluble stearoyl-ACP Δ^{9}-desaturase and other enzymes that catalyze oxygen-dependent hydrocarbon oxidations [20,29]. In the presence of air and a substrate octane, the dinuclear center
was oxidized and 1-octanol was formed indicating that the diiron center of this enzyme is located in the active site. Because of its specific occurrence in this group of proteins, the conserved consensus-binding motif may imply a common catalytic core [20].

Ricinoleic acid, (R)-12-hydroxy-(9Z)-octadecenoic acid, is an industrially important hydroxylated fatty acid produced agriculturally by the castorbean \textit{R. communis}. In vitro synthesis by microsomal membranes is biochemically similar to the microsomal \(\Delta^{12}\)-desaturases; it requires oxygen, NADH and cytochrome \(b_5\) [30]. The reaction also displays the same substrate and regiospecificity as microsomal desaturases. Furthermore, catalysis is inhibited by cyanide (but not carbon monoxide) indicating a requirement for iron but distinguishing this enzyme from cytochrome P-450. Based on these similarities, the gene encoding a \(\Delta^{12}\)-hydroxylase, capable of producing ricinoleic acid in transgenic tobacco and \textit{Arabidopsis}, was cloned from a \textit{R. communis} cDNA library [30]. Alignment of deduced amino acid sequences from the \textit{Ricinus} 12-hydroxylase and Fad2 from \textit{Arabidopsis} indicates the two enzymes share 67% identity. The similarity of sequence, and the biochemical correspondence of the \textit{Ricinus} 12-hydroxylase with desaturases in general, probably result from a closely related reaction mechanism.

Species of the genus \textit{Lesquerella} also accumulate hydroxy fatty acids, including ricinoleic acid [31]. Based on nucleotide sequence similarity with the oleate 12-hydroxylase of \textit{R. communis}, an oleate 12-hydroxylase (LFAH12) was isolated from \textit{L. fendleri}. Interestingly, LFAH12, which is homologous to the \textit{Ricinus} 12-hydroxylase as well as various \(\Delta^{12}\)-desaturases, is capable of both desaturase and hydroxylase activities. Overexpression of the gene in yeast results in accumulation of small amounts of both linoleic and ricinoleic acid. Sequence comparisons of castor and \textit{L. fendleri} \(\Delta^{12}\)-hydroxylase cDNA sequences with several \(\Delta^{12}\)-desaturases showed seven residues conserved within desaturases but absent from the hydroxylases [32]. Site-directed mutagenesis of the hydroxylase positions for the desaturase residues in the \textit{Lesquerella} clone resulted in an increase greater than 3:1 in linoleic:ricinoleic acid levels compared to wild type controls[32]. The dual functionality of the LFAH12 and the sequence similarity of hydroxylases and desaturases provide additional evidence for a reaction mechanism common to both types of enzyme.
The variety of FAD2-related reactions was extended with the discovery that conjugated double bonds can arise from an existing cis-double bond [33]. A pulse-chase experiment using 14C-labeled precursors with developing seeds of *Momordica charantia* demonstrated that linoleic acid (18:2Δ9c,12c) is a precursor of α-eleostearic acid (18:3Δ9c,11t,13t). Furthermore, expression in *S. cerevisiae*, transformed with cDNA encoding a putative desaturase from *Impatiens balsamina*, resulted in conversion of linoleic acid to α-eleostearic acid or conversion of α-linolenic acid (18:3Δ9c,12c,15c) to α-parinaric acid (18:4Δ9c,11t,13t,15c) [34].

The conjugated trans-Δ11- and trans-Δ13-double bonds of α-eleostearic acid in seeds of *M. charantia* and α-parinaric acid in seeds of *I. balsamina* are synthesized by enzymes analogous to microsomal FAD2s [33]. The deduced polypeptides share 53% amino acid sequence identity between themselves and from 50-60% identity with *Arabidopsis* FAD2, the castor oleic acid hydroxylase and the *Crepis* acetylenase and epoxygenase. They also contain histidine motifs found in membrane-bound diiron-oxo proteins. However, in contrast to FAD2 and related desaturases previously discovered, the conjugases do not install a cis-Δ12-double bond in oleate but, instead, modify an existing cis-Δ12-double bond to conjugated trans-Δ11- and trans-Δ12-double bonds.

Even broader functional variety has been realized among FAD2-related enzymes with the discovery of a class that modifies a Δ9-double bond to produce the conjugated 8E, 10E-double bonds of calendic acid (18:3Δ8t,10t,12c) [35]. In substrate requirement, the process is similar to α-eleostearate production. Two closely related cDNAs from *Calendula officinalis*, CoFADX-1 and CoFADX-2, converted linoleic acid and α-linolenic acid to calendic acid when expressed in *S. cerevisiae* [35]. The deduced amino acid sequences of these enzymes share 40-50% homology with other FAD2 and FAD2-type enzymes. Furthermore, alignment of amino acid sequences using the ClustalX program show that the *Calendula* enzymes exhibit divergence similar to CREP1 and CPAL2 when compared with other FAD2-type enzymes.

FAD2-type enzymes thus exhibit a broad range of substrate specificity, yet studies to date indicate substrate preference within small groups (as reviewed in reference [21]). Δ12-desaturases and 12-hydroxylases function on oleic acid whereas Δ12-epoxygenases
and \( \Delta^{12} \)-acetylenases require linoleic acid as substrate. Additionally, the *Impatiens* \( \Delta^{12} \)-conjugase is capable of functioning on at least two substrates as demonstrated when expression in *S. cerevisiae* of cDNA for the *Impatiens* conjugase resulted in accumulation of \( \alpha \)-eleostearic acid from linoleic acid substrate and \( \alpha \)-parinaric acid from \( \alpha \)-linolenic acid.

The sequence and biochemical similarities of CREP1 with nonheme diiron desaturase-like enzymes support the evolution of a common reaction mechanism within this group of proteins. Complete elucidation of this mechanism is necessary for understanding the biosynthesis of acetylenic compounds, in plants as well as fungi, and will provide information useful in designing enzyme systems for oilseed crops.

### 1.7 Unusual fatty acids as seed oil crops

In addition to the relatively few types of fatty acids serving important structural roles in membranes, over 300 naturally occurring fatty acids have been discovered in seed oils [36]. Chain length varies from 8 to 24 carbons and they possess novel functional groups on their acyl chains. These less-common fatty acids may occur in only a few species but, frequently, can account for the majority of fatty acid content in the seed oil of a species.

Unusual fatty acids offer interesting and valuable possibilities for commercial development as raw materials used in producing lubricants, paints, polymers and pharmaceuticals. However, their origins are generally not suited to industrial-scale production [3,36,37]. Unlike common agricultural crops, the plants producing unique oils may not incorporate them into seed oils, may not produce profitable yields or may not be amenable to segregation from crops in adjacent fields. Therefore, transgenic varieties of commercial seed crops are sought as a practical alternative to the original source through biogenetic engineering [37]. Genes encoding enzymes capable of forming unusual fatty acids can be transferred into appropriate commercial crops such as soybean, canola, and rapeseed. A transgenic crop encoding the genes necessary for synthesis, transport and storage of a unique fatty acid is envisioned that would function as a chemical factory turning out a unique oil on an industrial scale.
However, challenges inherent in heterologous expression systems must first be met. Wild plants channel unusual fatty acids from PC to TAG by acyl- and stereo-specific phospholipase enzymes (sn-1 and sn-2) and phospholipid/diacylglycerol acyltransferase (PDAT) enzymes (sn-2) which catalyze the hydrolysis and transacylation of acyl groups from PC [38-40]. TAGs in plant oil seeds are also formed by the diacylglycerol acyltransferase (DAGAT) enzyme through acetylation of DAG with acyl-CoA [39].

Plants and yeast that do not accumulate unusual fatty acids are also known to express phospholipases or PDATs that are specific for certain types of these acids. For example, *A. thaliana* likely produces seed-localized phospholipases or PDATs compatible with ricinoleic acid (18:1OH) because the ratio of hydroxylated fatty acids between TAG and PC was observed to increase from 6:1 in developing seeds to 17:1 in mature seeds [39]. However, *A. thaliana* appears to lack a similar transfer mechanism for crepenynic acid (18:1A), which remained at a constant level of 1.5% during seed development and increased to only 2% in mature seeds. The ratio of 18:1A between TAG and PC decreased from 1:1 to 0.6 over the same period compared with 335:1 in *C. alpina*, a natural source of 18:1A.

Transfer activity similar to that for 18:1OH in *A. thaliana* also occurs in transgenic rape where the ratio of laurate between TAG and PC increased from 1.5:1 to 21:1 [41]. To a lesser extent, sunflower was shown to transfer both 18:1OH and vernolic acid from PC to neutral lipids; and the budding yeast *S. cerevisiae*, capable of accumulating large reserves of TAG during stationary growth, has exhibited microsomal PDAT activity specific for sn-2-ricinoleoyl-PC as well as vernoloyl-DAG [40]. Acyltransferase activities such as these are involved in the selective removal and breakdown or remodeling of unusual fatty acids.

In spite of these challenges, two transgenic plant oils, each commercially produced by altering a single gene, provide novel mixtures of fatty acids. High-lauric acid canola, developed by Calgene finds useful applications in detergents as well as specialty foods [42]. DuPont’s transgenic soybean, with >85% oleic and low saturated fatty acids, likewise offers healthy alternatives when included in human diets (38). The high stability of oils such as these, relative to polyunsaturated fatty acids, also makes them useful as biodegradable lubricants.
In addition to these commercial products, numerous cDNA clones, encoding enzymes capable of biosynthesizing unusual fatty acids such as ricinoleic, linoleic, vernolic and crepenynic acids, currently exist and could be transformed into cultivated plants for the purpose of optimizing phenotypes for commercial seed crop production [43]. Biochemical characterization and results of structure/function studies of FAD2-type enzymes responsible for novel fatty acids will provide information useful in designing transgenic plants or microorganisms capable of efficiently producing acetylenic oils and, possibly, antitumor and antibiotic agents. Because of its high homology with FAD2-related enzymes, CREP1 is being used as a model FAD2 desaturase in our current yeast expression studies.

2. Functional expression of Crep1

2.1 Background

Reproducible functional expression of Crep1 is being developed to provide a reliable system capable of efficiently overexpressing related enzymes isolated from the basidiomycete fungi. These investigations will be essential for development of enzymatic assays as well as structure/function studies utilizing site-directed mutagenesis. Overexpression of Crep1 should be performed under optimal conditions in order to maximize protein production and activity. Expression of Crep1 in Arabidopsis has produced total fatty acids, from developing seeds of transgenic plants, containing up to 2.3% (w/w) crepenynic acid [39]. However, extraction of oils from the tiny Arabidopsis seeds, for the purpose of structure/function studies, would be inefficient.

An alternate expression system would be S. cerevisiae, which is known to produce only saturated and monoenoic fatty acids, thereby simplifying chromatographic analysis [28,44]. The growth media must be supplemented with linoleic acid (18:2) to ensure the presence of substrate for crepenynate production. However, incorporation of exogenous 18:2 does not adversely affect culture growth [44]. The cell doubling time for Saccharomyces (strain NCYC 1383) during exponential division in both unsupplemented and linoleate-supplemented (1 mM) cultures was approximately 2.4
hours. Nonetheless, low solubility of exogenous 18:2 in the culture media may limit incorporation by host cells, resulting in insufficient substrate for high levels of crepenynate production. The addition of Tergitol NP-40 (up to 1% wt/vol) to the growth media of *S. cerevisiae* has shown no discernible effect on cell division when compared with control cultures [44].

**Expression of diverged Δ12-desaturase**

Eukaryotic microbial hosts, such as *S. cerevisiae*, may provide a suitable membrane environment (ER) and electron donor (Cyt b₅) required for observable activity from a heterologous microsomal fatty acyl desaturase. As discussed in the introduction, the rat Δ⁹-desaturase, which shares only 36% identity with the *S. cerevisiae* Δ⁹-desaturase (OLE1), was expressed in a *S. cerevisiae* mutant (strain L8-14C), deficient in OLE1 activity, allowing complementation of the *ole1* mutation [22]. When expressed on a high copy number plasmid, the rat Δ⁹-desaturase gene resulted in accumulation of about 33% linoleic acid compared with 30% by cells transformed with *Ole1*. Cells containing the rat Δ⁹-desaturase gene on a single copy plasmid experienced growth rates reduced approximately 65% relative to wild type but still produced about 25% linoleic acid (18:2) compared with 37% in yeast transformed with *Ole1*.

The complementation of L8-14C was later instrumental in assessing the functional significance of the conserved His residues in FAD-type enzymes. A fusion between the promoter/5′ region of the yeast *Ole1* gene and the rat Δ⁹-desaturase open reading frame, with and without mutations of the His→Ala residues, was transformed into the *ole1* mutant strain L8-14C [19]. Cells carrying the wild-type rat gene were viable when plated on media lacking unsaturated fatty acids while cells expressing the enzyme with mutated His residues failed to grow. Immunoblots of total yeast cellular extracts were performed using anti-rat Δ⁹-desaturase antibodies as a probe and compared with a Western blot analysis of total protein content. The comparison showed that the mutant fusion protein in L8-14C was successfully expressed with no decrease in mRNA or protein stability.
Expression of the *Lesquerella* hydroxylase (LFAH12) and the *Arabidopsis* desaturase (FAD2) in yeast, under the control of the *GAL1* promoter, has also been used successfully to produce heterologous fatty acids at levels sufficient for functional characterization. Expression of FAD2 resulted in accumulation of about 3.5% combined 18:2 fatty acids and expression of LFAH12, a dual function enzyme, caused accumulation of 0.75% each of the total species of 18:2 and 16:2 acids and 1.5% ricinoleic acid [45]. This level of production was useful for mutagenesis studies of the two proteins and it was subsequently determined that as few as six amino acid substitutions are sufficient to convert LFAH12 to a desaturase.

Additionally, successful functional expression of *Fad2* behind the *GAL1* promoter of pSE936 in *S. cerevisiae* (strain MKP-o) resulted in increased accumulation of dienoic acids with lower culture temperatures [8]. Linoleic acid was observed to accumulate to 9.2, 6.4 and 0.6% (w/w) at 15, 22 and 28 °C, respectively, while the levels of total fatty acids remained close to those in control cultures. Temperature dependence has also been observed in *S. cerevisiae* (strain INVSc1) cultures expressing the Δ⁹-conjugase CoFADX-1 behind the *GAL1* promoter of pESC-URA [35]. Calendic acid accumulated to 4.5% (w/w) in cells grown at 16 °C while almost none was detected in cultures grown at 30 °C. In another example, the *S. cerevisiae* cell strain MKP-o, described above and transformed with *Fad2* in pSE936 (*GAL1* promoter), displayed growth rates and total fatty acid levels comparable to untransformed cells [8]. However, polypeptide profiles of the two cell strains, determined by polyacrylamide electrophoresis, appeared similar, indicating that a low expression level of *Fad2* was sufficient to increase 18:2 levels from less than 1% of total fatty acids to 9.2%.

*Expression of Crep1*

When grown at 28 °C with 1 mM exogenous linoleic acid and 1% Tween 40 detergent, *S. cerevisiae* (YN-94-1 strain), transformed with *Crep1* in pVT100U [constitutive alcohol dehydrogenase (*ADH1*) promoter], produced total fatty acids containing approximately 0.3% crepenynic acid [24]. A constitutive promoter such as *ADH1* will, in theory, initiate transcription of RNA leading to the synthesis of active
protein in fixed amounts irrespective of the carbon source [46]. Transcription of mRNA under the control of \( ADH1 \) has been reported to be efficient in the presence of glucose although \( ADH1 \) protein synthesis declined 10-fold during cell growth into the stationary phase. While 0.3% crepenynate accumulation demonstrates the viability of the expression system, it is low compared with the examples above and illustrates the potential for increased accumulation of product by optimizing the culture conditions, expression system and enzyme activity.

To address the influence of these variables on crepenynic acid accumulation in \( S.\ cerevisiae \), the \( Crep1 \) expression experiments performed by Stymne were modified in this study to investigate the effects of temperature, promoter, cell strain, detergent and culture size on crepenynate production.

### 2.2 Materials

\( Taq \) DNA polymerase was acquired from Fisher and \( Pfu \) polymerase was obtained from New England Biolabs. Complete media without uracil was prepared with yeast nitrogen base without amino acids and ammonium sulfate (Difco), ammonium sulfate and dextrose or galactose (Fisher Scientific). Dropout powder without uracil was prepared by a published method with adenine (hemisulfate salt), arginine hydrochloride, aspartic acid, serine, threonine, tryptophan, tyrosine and valine purchased from Sigma [47]. Glutamic acid (monosodium salt), histidine, leucine, lysine hydrochloride and methionine were acquired from Fisher Biotech. Linoleic acid and tergitol NP-40 were obtained from Sigma and DMSO was supplied by Aldrich. Ethanol (100%) and hexanes were acquired from Pharmco (Brookfield, CT). The capillary column (DB-23, 30 m x 0.25 mm) used for gas liquid chromatography separation of FAMEs was purchased from J. and W. Scientific.
2.3 Methods

2.3.1 Expression in *Saccharomyces cerevisiae*

The *Crep1* gene was expressed in *S. cerevisiae* strain YN94-1 with the plasmid pVT-CREP1 and in strain INVSc1 with the plasmids pCREP1, pHisCREP1, pCREP1His and pMEG203. Transformed YN94-1 cells and construct were a gift from Sten Stymne, Department of Plant Breeding Research, Swedish University of Agricultural Science, Sval v, Sweden [24]. The plasmid pMEG203 was constructed by cloning the *NotI/XhoI*-digested ORF of *Crep1* from the Bluescript clone pCREP1 into the corresponding restriction sites behind the galactose-inducible promoter *GAL1* of the yeast expression vector pYES2. The pADH-based plasmids containing histidine tags were constructed as follows. The ORF of *Crep1*, originally cloned in pMEG203, was PCR amplified in two 30-µl volumes each containing 1 µg pMEG203 DNA; 5 pmol each of the forward oligonucleotide primer Agrocac-5: 5'-ACCTCGAGATG(CAT)6 GGATCCATG-3 with the reverse primers Agrosac1-3: 5'-TGCTCTAGAGCTCAGAATTTATTGTAC-3 (HisCREP1, CREP1) or Agrosac2-3: 5'-TGCTCTAGAGCTCA(ATG)6GAATTTATTGTAC-3 (HisCREP1, CREP1) or Agrosac2-3: 5'-TGCTCTAGAGCTCA(ATG)6GAATTTATTGTAC-3 (CREP1His); 3 µl of 10x PCR buffer (0.5 M Tris HCl, pH 9.0; 2.5 mg/ml BSA; 20% sucrose; 1 mM cresol red; 20 mM MgCl2); 50 µmol dNTP; 0.5 unit of *Taq* DNA polymerase and 0.2 units *Pfu*. Thirty cycles of denaturation (94 °C, 30 s), annealing (72 °C, 30 s), and extension (72 °C, 90 s) were performed followed by twenty-five additional cycles of denaturation (94 °C, 30 s), annealing (55 °C, 30 s), and extension (72 °C, 90 s) using a model PTC-200 Peltier thermocycler. The resulting 1.3 kb products were digested with *XhoI* and *XbaI* (HisCREP1); *BamHI* and *XbaI* (CREP1); *BamHI* and XbaI (CREP1His). The digestes were ligated into the corresponding sites of pADH (pYES2 containing an *ADH* promoter in place of *GAL1*, provided by Brenda Blacklock, Department of Chemistry and Biochemistry, Miami University, Oxford, OH) to give the plasmids pHisCREP1, pCREP1His and pCREP1. The plasmids pHisCREP1, pCREP1His, pCREP1 and pMEG203, and empty vectors pYES2 and pADH, were transformed into *S. cerevisiae* strain INVSc1 using a published method [48]. The sequences of the inserts of
pHisCREP1, pCREP1His and pCREP1 were confirmed to be identical to that reported by Stymne and in the sense orientation relative to the ADH promoter using the PRISM BigDye™ Terminator Cycle Sequencing System (Perkin-Elmer/Applied Biosystems) and a model 310 genetic analyzer (Applied Biosystems).

Transformed S. cerevisiae was maintained on solid complete medium (CM-ura), prepared by a published method, containing dextrose (20 g), ammonium sulfate (5 g), YNB without amino acids and ammonium sulfate (1.7 g), dropout powder(-uracil) (1.3 g), agar (20 g) per liter [47]. For experimental purposes, transformed cells containing pVT-100U and pADH-based plasmids were cultivated in liquid CM-ura medium of the same composition as the solid CM-ura medium without agar. Transformed cells containing pYES2-based plasmids were cultivated at 30 °C in CM-ura medium (50 ml) supplemented with galactose as the carbon source and Tween-20. Culture volumes of 3 ml were grown in 16 x 150 mm culture tubes and culture volumes of 100 ml were grown in 500 ml-Erlenmeyer flasks.

YN94-1 cells transformed with pVT-based plasmids and INVSc1 cells transformed with pADH-based plasmids without histidine tags were grown in 3 ml and 100 ml culture volumes at 18 °C and 30 °C for 4 days with orbital shaking at 225 rpm. Cultures were supplemented with exogenous 18:2 (1mM) and NP-40 (0.1% w/v) as indicated. Ammonium linoleate was prepared by dissolving linoleic acid in 100% ethanol and adding 2 molar equivalents of NH₄OH (1 M) dropwise while vortexing. The ethanol was evaporated under vacuum and the ammonium linoleate was dissolved in DMSO.

YN94-1 cells transformed with pVT-based plasmids were grown at 30 °C and 15 °C in 100 ml culture volumes without NP-40 and 18:2. Two duplicate sets were supplemented with NP-40 (0.1% w/v) and/or 18:2 (1mM). INVSc1 cells transformed with pADH-based plasmids with histidine tags were grown at 30 °C in 100 ml culture volumes supplemented with 18:2 (1mM) and NP-40 (0.1% w/v).

All experimental cultures were innoculated to an optical density of 0.05 at 600 nm by using material from 48-hour starter cultures.
2.3.2 GC-MS analysis of fatty acids

Fatty acid methyl esters (FAMEs) were prepared from transformed yeast and authentic linoleic acid and crepenynic acid by heating at 80 °C for 60 min in 2% (v/v) methanolic H$_2$SO$_4$. The resulting FAMEs were extracted with hexanes (2 x 2 ml), the solvent was evaporated under a stream of nitrogen and the residue was dissolved in 150 µl hexanes. Samples were analyzed by GC-MS with a Hewlett-Packard 5890 GC coupled to a 5972A mass selective detector (MSD) using a DB23 column and oven temperature programming consisting of: 100 °C (3 min hold), 100 °C -250 °C at 20 °C/min, 250 °C (3 min hold), and 20 °C/min to 100 °C, (1 min hold). The MSD inlet temperature was 300 °C and the detector temperature was 280 °C. The column head pressure was 50 kPa of helium and the ionizing potential of the MSD was 70 eV.

2.4 Results and discussion

Culture conditions for Crep1 expression were examined to determine the effect of volume, cell strain, temperature and detergent on crepenynic acid production. Culture sizes of 3 ml and 100 ml were inoculated with S. cerevisiae strain YN94-1 transformed with pVT-CREP1 and empty vector pVT100U. The growth medium was supplemented with 18:2, which is the substrate for Δ$^{12}$-acetylenation, and Tergitol NP-40 detergent (0.1% w/v) to solubilize the exogenous 18:2. GC-MS analysis was used to confirm the presence of 18:1A in the fatty acids produced by the transgenic yeast. The methyl ester of a fatty acid from the transgenic yeast expressing Crep1 was identical with respect to the retention time (11.41 min) and was consistent with the EI-MS fragmentation pattern of synthetic 18:1A (Figure 4). Accumulation of 18:1A was not present in cells transformed with the empty vector (Figure 5B).

YN94-1 cultures were expected to produce at least 0.3% 18:1A [24]. Analysis of fatty acids recovered from YN94-1, transformed with pVT-CREP1 and grown at 30 °C in 100 ml cultures supplemented with exogenous 18:2 and NP-40, showed accumulation of 18:1A averaging 0.32% of endogenous total fatty acids (Table 1A, Figure 5A). GC analyses of fatty acids produced in culture volumes of 3 ml yielded an average of 0.36%.
Figure 4. Mass spectra of the methyl ester of a fatty acid extracted from YN94-1 yeast transformed with Crep1 and grown at 30 °C in 100 ml cultures with exogenous 18:2 and NP-40 (top) and methyl crepenynate (bottom). The parent ion is \( m/z = 292 \). Additional diagnostic ions include \( m/z = 236 \) and \( m/z = 249 \). All are present in authentic crepenynic acid and YN94-1 cells transformed with CREP1 but absent in cells transformed with empty vector.
Average of 11.388 to 11.399 min.: 12.D (-)

Scan 755 (11.421 min): 15.D
Figure 5. Truncated fatty acid profile of YN94-1 expressing Crep1 (A) and pVT100U (B). Cells were grown at 30 °C in CM-ura supplemented with 18:2 and NP-40. FAMEs were prepared from crepenynic acid and whole cells and analyzed by GC-MS. A fatty acid present in CREP1 transformants and absent in pVT100U transformants was consistent with respect to the retention time (11.41 min) and EI mass spectrum pattern of synthetic 18:1A.
Table 1. Effect of culture volume at 30 °C on accumulation of 18:1A in the YN94-1 and INVSc1 yeast strains. Fatty acid levels as a fraction of total endogenous fatty acids are indicated for cells transformed with empty vector and Crep1 grown in CM-ura at the temperature and culture conditions shown. Values averaged from triplicate samples are shown.
### A

<table>
<thead>
<tr>
<th>Fatty acid, peak area %</th>
<th>YN94-1/pVT100</th>
<th>YN94-1/pVT-CREP1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3 ml</td>
<td>100 ml</td>
</tr>
<tr>
<td>16:0 Me</td>
<td>64.22 ± 3.13</td>
<td>67.15 ± 4.95</td>
</tr>
<tr>
<td>16:1 Me</td>
<td>11.11 ± 1.51</td>
<td>10.86 ± 1.78</td>
</tr>
<tr>
<td>18:0 Me</td>
<td>15.24 ± 0.48</td>
<td>13.44 ± 1.37</td>
</tr>
<tr>
<td>18:1 Me</td>
<td>9.43 ± 1.30</td>
<td>8.56 ± 1.82</td>
</tr>
<tr>
<td>18:2a Me</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Culture conditions: 30 °C, 18:2 (1mM) and NP-40 (0.1% w/v) added to media

### B

<table>
<thead>
<tr>
<th>Fatty acid, peak area %</th>
<th>INVSc1/pADH</th>
<th>INVSc1/pCREP1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3 ml</td>
<td>100 ml</td>
</tr>
<tr>
<td>16:0 Me</td>
<td>61.62 ± 2.43</td>
<td>62.80 ± 1.15</td>
</tr>
<tr>
<td>16:1 Me</td>
<td>12.05 ± 1.27</td>
<td>12.31 ± 0.95</td>
</tr>
<tr>
<td>18:0 Me</td>
<td>15.84 ± 0.41</td>
<td>14.59 ± 0.95</td>
</tr>
<tr>
<td>18:1 Me</td>
<td>10.49 ± 1.57</td>
<td>10.30 ± 1.15</td>
</tr>
<tr>
<td>18:2a Me</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Culture conditions: 30 °C, 18:2 (1mM) and NP-40 (0.1% w/v) added to media
However, the 18:1A chromatographic peaks from analyses of the 100 ml cultures were more well defined compared with those from 3 ml cultures. Furthermore, the mass spectra of the 100 ml cultures contained relatively fewer contaminating ions, due, perhaps, to improved aeration from the increased surface area of the 100 ml cultures, resulting in an altered distribution of interfering lipids. Therefore, 100 ml culture volumes were judged to be more reliable for analysis of 18:1A production.

Crep1 was also expressed in *S. cerevisiae* strain INVSc1 to determine its influence on 18:1A accumulation compared with strain YN94-1. INVSc1 transformed with pADH-CREP1 and grown in 30 °C in 100 ml cultures supplemented with exogenous 18:2 and NP-40 accumulated an average of 0.25% (Table 1B). Accumulation of 18:1A was not present in cells transformed with the empty vector. Culture volumes of 3 ml yielded an average of 0.24%.

INVSc1 was also transformed with Crep1 containing histidine tags for eventual purification of the overexpressed protein (Data not shown). INVSc1/pHisCREP1 (N-terminus His-tag) grown at 30 °C in 100 ml cultures supplemented with exogenous 18:2 and NP-40 accumulated an average of 0.22% 18:1A, which is similar to the results of INVSc1/pADH-CREP1. INVSc1/pCREP1His (C-terminus His-tag) accumulated an average of only 0.07% 18:1A. While the mass spectra of the FAMEs eluting at 11.40 min did not resemble that of authentic 18:1A, preliminary GC-MS analyses of dimethyloxazoline (DMOX) derivatives of the fatty acids indicated that the compound is 18:1A (Data not shown). The placement of the His-tag apparently plays a role in 18:1A accumulation but a possible mechanism is not clear. (Accumulation of 18:1A was undetectable in preliminary expression trials with INVSc1 transformed with pMEG203. Expression with this vector was not pursued further.)

 Cultures of YN94-1 and INVSc1 transformed with Crep1 were grown at 15 °C to determine the effect of temperature on CREP1 activity. Increased accumulation of product was anticipated from the cultures grown at 15 °C due to the favorable influence of reduced culture temperatures observed in 18:2 production by MKP-o and calendic acid production by INVSc1. However, 18:1A was not detected in our cultures grown at 15 °C (Table 2A and 2B). CREP1 is known to convert 18:1 to 18:2 in the absence of
Table 2. Effect of culture volume at 15 °C on accumulation of 18:1A in the YN94-1 (A) and INVSc1 (B) yeast strains. Fatty acid levels as a fraction of total fatty acids are indicated for cells transformed with empty vector and Crep1 grown in CM-ura at the temperature and culture conditions shown. Values averaged from triplicate samples are shown.
### A

<table>
<thead>
<tr>
<th>Fatty acid, peak area %</th>
<th>YN94-1/pVT100</th>
<th>YN94-1/pVT-CREP1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3 ml</td>
<td>100 ml</td>
</tr>
<tr>
<td>16:0 Me</td>
<td>51.91 ± 3.32</td>
<td>34.27 ± 10.50</td>
</tr>
<tr>
<td>16:1 Me</td>
<td>25.53 ± 4.23</td>
<td>36.95 ± 7.53</td>
</tr>
<tr>
<td>18:0 Me</td>
<td>6.34 ± 0.98</td>
<td>6.20 ± 1.41</td>
</tr>
<tr>
<td>18:1 Me</td>
<td>16.23 ± 2.81</td>
<td>22.58 ± 4.51</td>
</tr>
</tbody>
</table>

Culture conditions: 15 °C, 18:2 (1mM) and NP-40 (0.1% w/v) added to media

### B

<table>
<thead>
<tr>
<th>Fatty acid, peak area %</th>
<th>INVSc1/pADH</th>
<th>INVSc1/pCREP1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3 ml</td>
<td>100 ml</td>
</tr>
<tr>
<td>16:0 Me</td>
<td>39.73 ± 3.14</td>
<td>44.94 ± 4.7</td>
</tr>
<tr>
<td>16:1 Me</td>
<td>28.21 ± 1.96</td>
<td>31.27 ± 1.23</td>
</tr>
<tr>
<td>18:0 Me</td>
<td>5.90 ± 0.54</td>
<td>4.66 ± 0.57</td>
</tr>
<tr>
<td>18:1 Me</td>
<td>26.16 ± 1.61</td>
<td>19.13 ± 3.21</td>
</tr>
</tbody>
</table>

Culture conditions: 15 °C, 18:2 (1mM) and NP-40 (0.1% w/v) added to media
exogenous 18:2. Conversion of 18:1 to 18:2 was not observed in our cultures of YN94-1 transformed with Crep1 incubated at 30 °C or 15 °C.

Yeast cells growing at reduced temperatures might be expected to incorporate increased levels of bent fatty acids such as 18:2 and calendic acid into their membranes in order to maintain fluidity. On the other hand, 18:1A is linear from C₁₁ to C₁₄ and can be viewed as reducing membrane fluidity by increasing the tendency of acyl chains to pack together. To protect membrane integrity at reduced temperatures, the cells may regulate membrane lipid composition through selective removal of acyl chains like 18:1A.

In any event, 18:1A accumulation in Saccharomyces is apparently temperature dependent because none was detected during preliminary trials performed with YN94-1 grown at 37 °C. This result might be due to reduced enzyme stability. In this study, accumulation of 18:1A was most effective in Saccharomyces strain YN94-1 transformed with pVT-CREP1 and grown in 100 ml cultures at 30 °C.

To determine the influence of detergent and exogenous 18:2 on fatty acid composition, 100 ml cultures of YN94-1 were grown at 30 °C with and without exogenous 18:2. A duplicate set of cultures received NP-40. In the absence of exogenous 18:2, the addition of NP-40 did not affect the fatty acid composition of YN94-1 cells transformed with pVT100 and pVT-CREP1 grown at 30 °C (Table 3A).

In Saccharomyces, 16:1 and 18:1, formed by the microsomal Δ⁹-desaturase OLE1, normally comprise >70% of total cellular fatty acids [49]. The OLE1 desaturase is strongly repressed by the addition of exogenous Δ⁹-unsaturated fatty acids, including 18:2 [50]. Wild type cells have been shown to preferentially incorporate exogenous 18:2 into membrane lipids, at levels >50% of total fatty acids, resulting in the loss of nearly all of the naturally occurring 16:1 and 75% of 18:1 due to repression of Ole1 mRNA levels.

In our study, YN94-1 transformed with pVT100 and pVT-CREP1 produced levels of 16:1 and 18:1 >85% of total fatty acids (Table 3A). However, in agreement with previous reports, the addition of 18:2 to the culture media suppressed the accumulation of endogenous 18:1 and 16:1. Addition of 18:2 to our YN94-1/pVT100 cultures, without NP-40, also resulted in a reduction of 16:1 and 18:1 levels (31% and 56%, respectively),
Table 3. Effect of Tergitol NP-40 and exogenous 18:2 on fatty acid distribution in the YN94-1 yeast strain. Fatty acid levels as a fraction of total fatty acids are shown for Transcriptional control by exogenous Δ⁹-unsaturated fatty acids in repressing *Ole1* mRNA levels represents only one mechanism for reducing endogenous unsaturation [48]. In addition to transcriptional control by Δ⁹-unsaturated fatty acids, fatty acids without Δ⁹-unsaturation are able to regulate desaturase activity post-transcriptionally [49]. pVT100U and pVT-CREP1 transformants grown in CM-ura at 30 °C. Values were averaged from triplicate samples.
### A

**Fatty acid, peak area %**

<table>
<thead>
<tr>
<th></th>
<th>YN94-1/pVT100</th>
<th></th>
<th>YN94-1/pVT-CREP1</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Without NP-40</td>
<td>With NP-40</td>
<td>Without NP-40</td>
<td>With NP-40</td>
</tr>
<tr>
<td>16:0 Me</td>
<td>10.96 ± 1.15</td>
<td>10.48 ± 0.43</td>
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<tr>
<td>16:1 Me</td>
<td>51.51 ± 1.61</td>
<td>52.36 ± 1.34</td>
<td>52.94 ± 1.33</td>
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<tr>
<td>18:0 Me</td>
<td>2.81 ± 0.20</td>
<td>2.75 ± 0.16</td>
<td>1.97 ± 0.48</td>
<td>2.33 ± 0.24</td>
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<tr>
<td>18:1 Me</td>
<td>34.64 ± 0.94</td>
<td>34.34 ± 0.76</td>
<td>36.29 ± 0.89</td>
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<tr>
<td>18:2 Me</td>
<td>0.08</td>
<td>0.01</td>
<td>0.06</td>
<td>0.04</td>
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</tbody>
</table>

Culture conditions: 100 ml without 18:2; 30 °C with and without NP-40 added to media

### B

**Fatty acid, peak area % excluding 18:2**

<table>
<thead>
<tr>
<th></th>
<th>YN94-1/pVT100</th>
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<th>YN94-1/pVT-CREP1</th>
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<tbody>
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<td>16:0 Me</td>
<td>42.28 ± 7.80</td>
<td>67.15 ± 4.95</td>
<td>39.89 ± 10.42</td>
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<td>16:1 Me</td>
<td>35.52 ± 4.90</td>
<td>10.86 ± 1.78</td>
<td>33.26 ± 5.08</td>
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<tr>
<td>18:0 Me</td>
<td>7.14 ± 1.05</td>
<td>13.44 ± 1.37</td>
<td>13.70 ± 3.05</td>
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<tr>
<td>18:1 Me</td>
<td>15.05 ± 2.64</td>
<td>8.56 ± 1.82</td>
<td>13.04 ± 4.15</td>
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<tr>
<td>18:2a Me</td>
<td>ND</td>
<td>ND</td>
<td>0.11</td>
<td>0.32</td>
</tr>
</tbody>
</table>

Culture conditions: 100 ml; 30 °C + 18:2 (1mM) with and without NP-40 (0.1% w/v) added to media
which was amplified in the presence of NP-40 (79% and 75%, respectively) (Table 3B). Cultures of YN94-1/pVT-CREP1 supplemented with 18:2 in the presence of NP-40 exhibited even more pronounced reductions, with 16:1 and 18:1 levels each reduced by 90%.

YN94-1/pVT-CREP1 readily incorporated 18:2 in the absence of detergent, but the presence of NP-40 in the culture media dramatically increased the repressive effect of exogenous 18:2 over Ole1. NP-40 also resulted in boosting 18:1A accumulation from 0.11% to 0.32%, which is equivalent to Stymne's results (Table 3B). Exogenous 17:1 Δ10 also strongly represses Ole1 mRNA levels and desaturase activity, even during continued transcription, through mechanisms that may control mRNA stability [49].

Furthermore, in wild type yeast cells, Ole1 transcription and mRNA levels are not affected by exogenous 18:1 Δ11, which is incorporated into membrane lipids and subsequently reduces endogenous 16:1 and 18:1 levels [49]. In view of the results from these previous studies and the reduced activity of the OLE1 Δ9-desaturase in our present work, exogenous 18:2 may activate controls over membrane fatty acid composition that also influence the activity of CREP1.

These controls may include selective removal and specific breakdown or remodeling of unusual fatty acids, such as 18:1A, through phospholipase, DAGAT or PDAT activity. Acyltransferases are crucial to the synthesis of both membrane lipids and TAG [36]. These enzymes are highly selective for acyl groups on both of the substrates involved in the transfer (reviewed by Millar, et al.) [36]. The acyltransferases of Saccharomyces may not efficiently transfer unusual fatty acids like 18:1A from PC to substrates in TAG synthesis. Alternatively, they may be very effective in transferring 18:1A away from the ER for remodeling or breakdown in order to maintain membrane integrity.

The reason for accumulation of 18:1A and 18:2 that is low compared with expression systems such as MKP-o is probably due to a combination of several factors. Improving the levels of 18:1A accumulation will require understanding its production, as well as the enzymes involved in its transfer to TAG, and those enzymes that may be active in maintaining the availability of 18:2 as an appropriately conjugated CREP1 substrate [51]. Understanding the complex regulatory circuit, including the roles of putative protein
sensors, that exerts control over native fatty acid desaturase activity is necessary to evaluate its influence over heterologous expression and activity.

**Conclusion**

The *Crep1* gene isolated from *Crepis alpina* encodes a fatty acid Δ\(^{12}\)-acetylenase that desaturates the double bond at the Δ\(^{12}\)-position of linoleic acid (18:2) to form crepenynic acid (18:1A). The conditions for reproducible functional expression of *Crep1* in *S. cerevisiae* strain YN94-1 were established. Cells incubated at 30 °C and supplemented with exogenous 18:2 and NP-40 accumulated 18:1A averaging 0.32% of total endogenous fatty acids. 18:1A accumulated to 0.22% and 0.07% respectively in strain INVSc1 transformed with pHis/CREP1 and pCREP1/His.

These levels of 18:1A accumulation are sufficient for functional identification of CREP1 and holds promise for application to related enzymes. Nonetheless, future developments in modifying endogenous controls over the fatty acid composition of *S. cerevisiae* will be required in order to achieve levels sufficient for protein purification. At that time, the His-tags will facilitate purification of active enzyme through affinity chromatography. The repression of *Ole1* activity by exogenous 18:2 indicates that exogenous fatty acids exert controls over membrane fatty acid composition that may repress CREP1 activity through the involvement of phospholipase, DAGAT or PDAT.

In addition to its acetylenase activity, CREP1 was previously reported to convert 18:1 to 18:2 in the absence of exogenous 18:2. The appearance of the fatty acid methyl ester of 18:2 at levels of 0.4% (compared with 18:1A levels of 0.3%) was reported to accumulate in YN94-1 transformed with pVT-CREP1 in those studies. However, using the same cell line and vector, accumulation of the methyl ester derivative of 18:2 was not observed in the current work (compared with 18:1A levels of 0.32%). The agreement between the two studies in results for 18:1A, but lack of observed 18:2 accumulation in the present work supports a conclusion that CREP1 is not bifunctional.
3. Structure elucidation of (14Z)-dehydrocrepenynic acid in soy beans transformed with ELI12 from Petroselinum crispum

3.1 Background

Pathogenic resistance in plants involves an array of mechanisms that have evolved to prevent penetration, growth and replication of invading organisms [52]. Plants must be able to both quickly perceive appropriate signals from the invading organism and metabolize defense-related compounds to effectively resist invasion. Investigation of the molecular mechanisms of these defenses is difficult because multiple infection events occur in a non-synchronized fashion in relatively few cells. Consequently, studies of intact non-host/pathogen systems are complemented with systems of plant cell cultures combined with fungal elicitors prepared from hyphal cell walls [52,53].

Petroselinum crispum (parsley) is a member of the Umbelliferae family and is known to produce the fungicidal polyacetylenes falcarinone, falcarinol and falcarindiol [4,54]. The non-host interaction of parsley with the Glycine max (soy) pathogenic fungus Phytophthora sojae has been imitated by suspension-cultured cells treated with a peptide elicitor from this fungus [53]. Treatment of parsley with elicitor also induces large increases in unsaturated fatty acids immediately after rapid and transient accumulation of mRNAs encoding a plastid-localized ω-3 FAD [53]. In addition to this desaturase, similar treatment rapidly induces expression of other Fad-like genes including ELI12 (elicitor-activated gene), a putative defense-related gene previously isolated during screening of a parsley cDNA library, and PcΔ12-FAD [53,55]. The deduced proteins share sequences similar to microsomal ω-6 desaturases from other plant species. PcΔ12-FAD encodes an enzyme that, when overexpressed in yeast, converts oleic (18:1) acid to linoleic acid (18:2) and, presented with 16:1 substrate, produces 16:2 fatty acid as well as 18:2 [53].

The accumulation of two products from one enzyme results from the regiospecificity of FAD2-related enzymes, which was demonstrated when the oleoyl-PC desaturase from Arachis hypogaea (peanut), expressed in yeast, consistently referenced an existing cis-double bond to install a methylene-interrupted cis-double bond on the distal end of the
substrate [56]. The cis-configuration is explained by the current model of desaturase binding geometry. Based on studies with the rat liver Δ⁹-desaturase, fatty acid substrates are bound in the eclipsed conformation preceding abstraction of the pro-(R)-hydrogens to yield a cis-double bond [57].

Yeast transformed with ELI12 failed to accumulate linoleic acid or hydroxylated fatty acids even though the deduced protein is 60% identical to various plant microsomal ω-6 FADs and castor bean 12-hydroxylase [30,53]. This sequence similarity, combined with rapid gene activation by elicitor, indicates that ELI12 may serve in a defense-related role, as do ω-3 FAD and ω-6 FAD. However, in spite of this similarity, ELI12 apparently requires a different substrate.

The unusual fatty acid derivative (14Z)-dehydrocrepenynic acid has been previously isolated from plants and fungi [58-60]. First identified in 1967 through feeding studies, dehydrocrepenynic acid was shown to accumulate in the fungus Tricholoma grammopodium [60]. The appearance of crepenynic acid and (14Z)-dehydrocrepenynic acid during polyacetylene biosynthesis by T. grammopodium confirmed the hypothesis that natural acetylenes are derived from common fatty acids. (14Z)-Dehydrocrepenynic acid is also known to exist in significant proportions as both the free fatty acid (25%) and as the triglyceride (78%) in Cantharellus cibarius (chanterelle mushrooms) [59].

Labeling studies have previously demonstrated that oleic, linoleic, crepenynic and dehydrocrepenynic acids are incorporated by both higher plants and micro-organisms and yield the corresponding diyne and triyne esters [4]. Later, feeding experiments with fungal cultures of Lepista diemii, Coprinus quadrifidus and Serpula lacrymans showed that only the (14Z)-isomer of dehydrocrepenynate was incorporated into the relevant polyacetylenes produced and the E-isomer was not [61]. This result confirmed that (14Z)-dehydrocrepenynate is a member of the crepenynate pathway, general for the biogenesis of polyacetylenes.

3.2 Materials

Soy bean embryos transformed with ELI12 in Petroselimum crispum were provided by Ed Cahoon (DuPont.Wilmington, DE). Cantharellus cibarius was purchased locally and
ground with a coffee mill (model K7450) obtained from Regal Ware. HPLC grade acetonitrile, hexanes, ethyl acetate, chloroform and methanol were obtained from Pharmco (Brookfield, CT). Phenacyl bromide and triethylamine were acquired from Aldrich. The capillary column (DB-23, 30 m x 0.25 mm) used for GC separation of FAMEs was purchased from J. and W. Scientific. The Econosil C18 column (250 x 10 mm, 10 µm particle size) used for HPLC separation was purchased from Alltech.

3.3 Methods

3.3.1 GC-MS

An authentic sample of dehydrocrepenynic acid was extracted from dried *Cantharellus cibarius* (chanterelle mushrooms), a known source of (14Z)-dehydrocrepenynate. The tissue (24 g) was finely pulverized in a coffee mill, transferred to a round-bottom flask and stirred in ethyl acetate (300 mL) for 5 h. The solvent was removed under vacuum at room temperature. The residue was dissolved in 2.0% (v/v) methanolic H2SO4 (2 mL) and heated at 80 °C for 60 minutes in screw-capped tubes. The transesterification reactions were diluted with H2O (2 mL) and were extracted twice with hexane (2 mL). Fatty acid methyl esters were recovered from the organic phase by evaporation under N2 to a film that was dissolved in hexane (150 µL) for GC-MS analysis. Transgenic *ELI12*-soybean embryos were treated similarly. Samples were analyzed by GC-MS with a Hewlett-Packard 5890 GC coupled to a 5972A MSD using a DB23 column and oven temperature programming of: 100 °C (3 min hold), 100 °C-250 °C at 20 °C/min, 250 °C (3 min hold), 20 °C/min to 100 °C, (1 min hold). The MSD inlet temperature was 300 °C and the detector temperature was 280 °C. The column head pressure was 50 kPa of helium and the ionizing potential of the MSD was 70 eV. Fatty acid methyl esters in the samples were identified by comparison with the retention times and fragmentation patterns of authentic samples.
3.3.2 Preparation of phenacyl derivatives

Finely ground soybean embryos were extracted using CHCl₃/MeOH (2:1, by volume). The solutions were stirred for 90 min and the solvent was removed using a rotary evaporator. The soy lipids and chanterelle-derived and synthetic (14Z)-dehydrocrepenynate were saponified with 0.5% (w/v) methanolic sodium hydroxide by heating in a water bath at 100 °C for 5 min, cooling to room temperature and repeating the heating. The solution was acidified with HCl and the fatty acids extracted twice with hexane. The solvent was evaporated in vacuo and the free fatty acids were dissolved in acetone (13 mL). Triethylamine (180 µL) was added. The solution was mixed prior to the addition of phenacyl bromide (156 mg). The solution was next placed in a 50 °C water bath for 2 h. Water (5 mL) was added and solution was extracted twice with hexane. Solvent removal by a rotary evaporator yielded the phenacyl derivatives. Phenacyl derivatives of commercially available fatty acids were prepared as chromatography standards.

3.3.3 Reverse phase HPLC

Phenacyl derivatives of fatty acids were separated on an Econosil C₁₈ column (250 x 10 mm). Elution of phenacyl derivatives was performed at a flow rate of 5 mL/min at 4 °C using a water-acetonitrile gradient. After injection of the sample, the ratio of solvent B to A increased with a linear gradient from 10:90 to 0:100 water:acetonitrile over 40 min. The column was then washed with 100% acetonitrile for 40 minutes. The elution was monitored at 210, 240, and 280 nm.

3.3.4 NMR analysis

¹H-NMR and ¹³C-NMR were performed on a 300 MHz Bruker Avance spectrometer. Spectra were recorded at 298 K in CDCl₃. Chemical shifts in ppm were solvent referenced and expressed downfield from tetramethylsilane.
3.4 Results and discussion

3.4.1 GC-MS

Methyl ester derivatives of authentic fatty acids were prepared to aid in analysis of the natural compounds. The GC-MS retention times were: methyl stearate (18:0), 9.92 min; methyl oleate (18:1<sup>9</sup>), 9.99 min; methyl linoleate (18:2<sup>9,12</sup>), 10.16 min; and methyl γ-linolenate (18:3<sup>6,9,12</sup>), 10.381 min. A component of transesterified soybean extract and the chanterelle-derived methyl dehydrocrepenynate eluted at 10.93 min. Co-injection of a mixture of the two esters demonstrated that the compounds resulting in the 10.93 min peak had identical elution properties. The fragmentation patterns for the mass spectrum of the soy-derived methyl ester were identical to methyl (14<sup>Z</sup>)-dehydrocrepenynate from chanterelle and synthetic material (Figure 6).

3.4.2 Reverse-Phase HPLC

A high degree of resolution for difficult-to-separate fatty acids can be achieved by HPLC analysis as their phenacyl esters [62]. The reaction in acetone produces 85-93% yield of esters in 1 h at room temperature and elution of derivatized compounds is conveniently monitored at 254 nm due to UV absorption by the phenacyl moiety [63]. HPLC retention times for the phenacyl derivatives of fatty acid standards were: stearate, 274.7 ml; oleate, 182.4 ml; linoleate, 128.4 ml; and myristate (14:0), 128.4 ml. The phenacyl derivatives of the biological samples from chanterelles and transgenic soybeans contained a major component that eluted at 68 ml with identical relative extinction coefficients at 210, 240, and 280 nm. The fractions eluting at 68 ml were collected separately and concentrated for NMR analysis.
Figure 6. The mass spectrum of methyl (14Z)-dehydrocrepenynate from *C. cibarius*, a known source of the compound, (A) and soybean embryos transformed with *ELI12* (B).
3.4.3 NMR

The synthetic material and natural product derivatives were identical by $^1$H- and $^{13}$C-NMR spectroscopy. Phenacyl dehydrocrepenynate isolated by HPLC was air-sensitive, particularly in concentrated solutions. During the course of data collection, the sample purity decreased to ca. 70%. Conclusive results were obtained from the methyl ester derivatives. A vinyl proton consistent by chemical shift (δ 5.80) and coupling constants ($dm, J=10.7, 7.3$ Hz) with a (Z)-enyne was observed from both biological sources and the synthetic material (Table 4). An additional diagnostic peak at δ 3.05, assigned to the methylene protons in the 1,4-enzyme system, was clearly visible in each sample.

Conclusion

The identity of the compound present in the FAMEs of soy bean embryos transformed with ELI12 from P. crispum was identified by comparison with the anticipated compound methyl (14Z)-dehydrocrepenynate from both a known natural source and synthetic material. With the observation of the simultaneous appearance of crepenynate, the dual-functionality of the enzyme encoded by ELI12 has been established as a $\Delta^{12}$-acetylenase/$\Delta^{14}$-desaturase. This example of $\Delta^{14}$-regioselectivity is unique in plants. The only example reported to date which may be related to ELI12 is the fatty acid hydroxylation by Peganum harmala yielding 9,14-dihydroxyoctadecanoic acid [27].

The primary metabolic path to polyunsaturation in plants follows the installation of methylene-interrupted double bonds, which predicts $\Delta^{15}$-desaturation of $\Delta^{12}$-substrates. However, (15Z)-dehydrocrepenynate has never been reported. Nonetheless, divergence from the usual pattern of methylene-skipped double bonds has been seen in the conversion of linoleic acid to $\alpha$-eleostearic and calendic acids, and the conversion of $\alpha$-linolenic acid to $\alpha$-parinaric acid [34,35].

The $\Delta^{14}$-regioselectivity of ELI12 has important consequences for polyacetylene biosynthesis in parsley, which produces falcarinone, falcarinol and falarindiol [4,54].
Table 4. $^1$H- and proton-decoupled $^{13}$C-NMR data of the methyl ester derivative of (14Z)-dehydrocrepenynate.

$^a$ Assignment not certain
<table>
<thead>
<tr>
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</table>
Falcarinol-related compounds are known to occur in the Umbelliferae family and have been hypothesized to derive from the crepenynic pathway to polyacetylene formation [4].

Like parsley, *Daucus carota* (carrot) also belongs to the Umbelliferae and produces falcarinol and falcarindiol [17]. These acetylenic compounds, in carrots, are involved in the defense mechanism against storage rot [17]. Most importantly, carrot tissue has been shown to incorporate labeled crepenynate and subsequently accumulate labeled falcarinol, indicating that crepenynate can function as an intermediate in the biogenesis of falcarinol [64].

Conversion of linoleic acid to dehydrocrepenynic acid by the *ELI12* gene product indicates that dehydrocrepenynic acid is probably a precursor to polyacetylene formation in parsley. *ELI12* gene function indicates that the biogenesis of falcarinone may proceed through a mechanism whereby *ELI12* desaturates the $\Delta^{12}$-double bond of linoleate, forming crepenylic acid, and subsequently desaturates the $\beta-\gamma$ positions to produce (14Z)-dehydrocrepenynate (Scheme 3). Further desaturation likely occurs while the substrate is bound to PC or other phospholipids. Following release of the substrate from PC, chain shortening, which may proceed through deformylation, and oxidation hypothetically yield falcarinone [64]. The eclipsed binding geometry hypothesis relevant to *ELI12* can be tested with a yeast expression system and evaluated by GC-MS analysis of (14Z)-dehydrocrepenynate formed from linoleate deuterated at the pro-(R)-positions of C-14 and C-15.

4. Isolation and preliminary structure determination of polyacetylenes from *Fistulina hepatica*

4.1 Background

4.1.1 Metabolites previously isolated from *F. hepatica*

Over 600 naturally occurring acetylenic compounds had been isolated by 1970, many from plants and basidiomycete fungi [4]. Approximately 70% of the polyacetylenes
Scheme 3. Proposed biosynthetic route for falcarinone from parsley. Labeling studies support the hypothesis that crepenynate participates as an intermediate and, therefore, desaturation of the distal half of the substrate occurs before chain shortening [64]. The 18 carbon keto-aldehyde preceding falcarinone has been observed in Pastinaca sativa, another member of the Umbelliferae, and lends additional weight to the scheme [64].
Linoleic acid

(14Z)-dehydrocrepenynic acid

Δ14-acetylénase

β-γ dehydrogenation

Hydrolysis, Reduction Oxidation

Deformylation

Falcarinone

R = phospholipid
produced by fungi are structurally 9 or 10 carbon chains. In earlier studies of \textit{F. hepatica} alkyne biosynthesis, Jones \textit{et al.} isolated five metabolites, typical of this structure, with unbranched 10 and 13 carbon chains (Scheme 4) \cite{65}. The metabolites were trideca-2,4,6,8-tetrayne \textbf{I}; trans-tridec-2-ene-4,6,8-triyne-1-ol \textbf{II}; \textit{cis}- and \textit{trans}-isomers of dehydromatricarianol \textbf{III}, \textbf{IV}; and 2\text{\textsubscript{D}}:3\text{\textsubscript{L}}:4\text{\textsubscript{L}}-trideca-5,7,9,11-tetrayne-1,2,3,4-tetraol \textbf{V}.

4.1.2 Biosynthesis of polyacetylenes and feeding studies

In both fungi and higher plants, feeding experiments with $^{14}$C-labeled acetate and malonate show that the biosynthesis of acetylenes is similar to that of fatty acids \cite{4}. Acetate acts as the starter unit and the unbranched carbon chain, common to nearly all natural acetylenes, is extended with malonate. Feeding studies with labeled oleic acid indicate that oleic acid is the precursor of natural acetylenic compounds \cite{4}.

The results of similar studies show incorporation of labeled linoleic, crepenynic and dehydrocrepenynic acids into acetylenic products (Scheme 5) \cite{4}. Taken together, these experiments support a hypothesis where a series of oxidative desaturations in basidiomycetes, catalyzed by CREP1 homologs and other FAD2-like enzymes, drives the conversion of linoleic acid into a variety of polyacetylenes, such as those isolated by Jones \cite{65}.

4.1.3 Biochemical and genetic considerations

Biochemical and genetic investigations of the dehydrogenation mechanisms of acetylenases and $\Delta^{12}$-desaturases in basidiomycetes requires the identification of putative substrates for each metabolic transformation. Consequently, the isolation and characterization of polyacetylenes produced by these fungi is essential to both developing a plausible metabolic hypothesis and an experimental design for enzyme characterization. Structure/function studies and enzyme assays described above will be performed in the future. For this purpose, poly (A)$^{\text{+}}$ RNA was purified from \textit{Cantharellus cibarius} (chanterelles) and used to construct a cDNA expression library.
Scheme 4. *F. hepatica* natural products isolated from shaken cultures by Jones and coworkers. The two tetra-acetylenic compounds represent the first hydrocarbon IV and the first tetraol V encountered in fungal metabolites. Tetrayne-tetraol V showed antibacterial activity comparable with cephalosporin C against *Staphylococcus aureus* (Oxford strain) and *Salmonella typhi* (strain Miss S.) (54). Compounds I and II are the *cis*- and *trans*- isomers of dehydromatricarianol.
<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure</th>
<th>mg/L of culture fluid</th>
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<tbody>
<tr>
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Scheme 5. Proposed pathway to polyynes via CREP1 homolog. Labeled linoleic, crepenynic and dehydrocrepenynic acids are incorporated by micro-organisms and higher plants yielding the corresponding diyne and triyne esters [4].
Acetylenase I (CREP1 homolog)

Desaturase/Acetylenase III

R=phospholipid
4.1.4 Culture conditions for the present study

In this study, culture conditions suitable for polyacetylenic secondary metabolite production in *F. hepatica* were determined. Nutrients, temperature and rotation rate of shaken cultures were selected to verify the similarity of acetylene production by *F. hepatica* ATCC 64428 with Jones' undisclosed *F. hepatica* strain [65]. The strains, if different, may accumulate different metabolites. However, reproducible culture conditions, regardless of strain identity, are necessary for reliable metabolite distribution and subsequent extraction of target compounds. Purified polyacetylene yields are low, 0.15 mg, or less, to 2 mg/L of culture [65]. Furthermore, maintaining reproducible conditions, free from competing fungal contamination, is crucial to the collection of mycelia for RNA isolation and cDNA synthesis required for cloning alkyne biosynthetic genes from the slow growing *F. hepatica*.

4.1.5 Purification of acetylenes

Most acetylenes are thermally unstable and, therefore, are isolated most successfully from the aqueous media of fungal cultures by extraction with organic solvents near room temperature [4]. Fungal metabolites that accumulate in the media represent a wide range of polarities and can be recovered using appropriate solvents. Polyynes, including the relatively polar tetraol V, are most often isolated from mycelial culture fluids by continuous ether extraction [65,66].

Fractions of different polarity can be conveniently obtained through separation of the extracts by high-performance liquid chromatography (HPLC). This type of system is effective in separating geometric isomers of long-chain fatty acid methyl esters, which are similar in structure to acetylenic compounds previously isolated from basidiomycetes [67,68]. However, crude extracts contain a rich mixture of natural products further complicated by decomposition due to the inherent instability of polyacetylenes. Therefore, final purification of polyacetylenes co-eluting with other compounds can be necessary and may be accomplished by using column chromatography, thin layer chromatography (TLC) or repeated HPLC separation using modified solvent conditions.
4.1.6 UV screening of cultures

Polyeneynes produce highly characteristic ultraviolet (UV) spectra with sharp, vibrational fine structure. These spectra are characteristic of unique desaturation patterns important for preliminary identification of polyacetylenes [4]. Two sets of absorption bands can usually be seen in the spectrum: a short wavelength series of high intensity ($\varepsilon =10^6$ L mol$^{-1}$ cm$^{-1}$) and a longer wavelength series of lower intensity. Various arrangements of conjugated double and triple bonds yield distinct UV spectra unique to each conjugated system (Table 5).

The wavelength of observed maxima ($\lambda_{\text{max}}$), combined with their relative intensities, make UV spectroscopy a powerful tool for diagnosing desaturation patterns in conjugated systems. For example, comparison of the UV spectrum of the enetriynene, all \textit{trans}-trideca-3,11-diene-5,7,9-triyne-1,2-diol, with that of the ethyl ester of the dienetriyne, all \textit{trans}-tetradeca-4,6-diene-8,10,12-triyne-1-ol, illustrates the influence conjugated groupings of double and triple bonds exert on both the position and the relative intensity of $\lambda_{\text{max}}$ (Figure 7).

Furthermore, daily UV screening of shaken cultures, based on absorbance intensity of an appropriate $\lambda_{\text{max}}$, has proven effective in determining the period of maximum production of polyacetylenic compounds [65]. Presumably, this period also coincides with, or immediately follows, the maximum abundance of acetylenase mRNA, which is indispensable for molecular biology studies. However, a prediction of the amount of acetylenic compound present can not be based on the intensity of absorbance because the extinction coefficient $\varepsilon$ can only be determined after purification. Intense absorbtion may be due to a small amount of a strongly absorbing compound or it may, instead, be due to a large amount of a weakly absorbing substance.
Table 5. UV maxima of polyynes (Adapted from Bohlmann [4]). Major polyyne and polyynene parent chromophores are represented listing the wavelengths for vibrational maxima (nm) followed by the approximate extinction coefficient ($\times 10^3$ L mol$^{-1}$ cm$^{-1}$). The wavelength of the longest $\lambda_{\text{max}}$ for a given number of unsaturated bonds is determined by the number of conjugated triple bonds. For example, compounds containing five conjugated unsaturated bonds display the order: pentayne (392 nm); enetetryne (375 nm); dienetriyne (347 nm) and trienediyne (332 nm).
\[ R \rightarrow [\text{CH} \equiv \text{CH}]_n \rightarrow \text{[} \text{C} \equiv \text{C}]_{n'} \rightarrow [\text{CH} \equiv \text{CH}]_{n''} \rightarrow R \text{ in nm (} \varepsilon \times 10^3) \]

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Figure 7. The spectra of conjugated acetylenes are characterized by a series of low intensity bands ($\varepsilon$ 200-300) in the longer-wavelength series. A set of high intensity ($\varepsilon > 10^5$) bands appears in the accessible region of the UV spectrum when three or more acetylenic bonds are conjugated. A comparison of two spectra (enetriynene, upper; dienetriyne, lower) from the same system of conjugated chromophores, arranged differently, illustrates the diagnostic value of the positions and extinction coefficients of the $\lambda_{\text{max}}$. [From F. Bohlmann, *Naturally Occuring Acetylenes* (Copyright 1973 by Academic Press), Figures 10 and 11, p. 9. Reprinted by permission of Academic Press.]
4.1.7 Separation and analytical techniques

In addition to UV spectroscopy, improved separation and analytical technologies such as high-performance liquid chromatography (HPLC), liquid chromatography-mass spectrometry (LC-MS) and nuclear magnetic resonance (NMR) spectrometry should facilitate the characterization of additional, less stable compounds produced by *F. hepatica*. NMR and mass spectrometric data for the major acetylenic components in liquid mycelial cultures of this species will provide the necessary information for elucidating the structure of new compounds and deducing potential substrates for future enzyme studies.

4.2 Materials

Agar and liquid medium were prepared from Bacto yeast extract, malt extract, peptone, dextrose and agar obtained from Difco [69]. Benomyl was obtained as a gift from DuPont (Wilmington, DE). Tetracycline and dextrose were purchased from Fisher. Mycelia were ground in a model 52200R blender from Hamilton-Beach. HPLC grade acetonitrile and reagent grade diethyl ether, hexanes and methylene chloride were purchased from Pharmco (Brookfield, CT). UV analyses were conducted using a Carey 1E UV-visible spectrophotometer made by Varian (Humboldt, CT). HPLC columns were obtained from Alltech; analytical HPLC was performed on an Adsorbosil C\textsubscript{18} (250 x 4.6 mm; 5 µm particle size) HPLC column and preparative scale HPLC was performed using an Econosil C\textsubscript{18} (250 x 10 mm; 10 µm particle size) HPLC column. Analytical scale purifications were performed with a Beckman System Gold HPLC (Fullerton, CA). Preparative scale separations were conducted using an KTA™ Explorer 10S HPLC (Amersham Pharmacia Biotech, Piscataway, NJ). \textsuperscript{1}H-NMR and \textsuperscript{13}C-NMR were performed at 298 K in CDCl\textsubscript{3} on a 300 MHz Bruker Avance spectrometer. Deuterated solvents were acquired from Isotec. Chemical shifts in ppm were solvent referenced and expressed downfield from tetramethylsilane. Fresh *Cantharellus cibarius* was obtained from Earthly Delights (DeWitt, MI). Trizol reagent was purchased from GibcoBRL and
a poly(A)$^+$ RNA purification kit was purchased from Dynal. The cDNA library kit was obtained from Stratagene.

4.3 Methods

4.3.1 Growth and incubation of fungal cultures

_F. hepatica_ was maintained on solid YM medium, prepared by a published method, containing yeast extract (3 g), malt extract (3 g), peptone (5 g), dextrose (10 g), agar (20 g) per liter [47]. YM agar plates (100 mm) were inoculated from a slope culture of _F. hepatica_ and incubated at 26 °C. The agar and mycelia were ground in a blender. Two plates provided inoculum for each culture containing 2.5% malt extract media (1000 ml in a 2.8 L Fernbach flask). Cultures were incubated by shaking at 75 rpm and 26 °C.

After the third day of growth, samples (3 ml) were taken daily from the aqueous media of each flask and extracted with diethyl ether (3 ml). The extracts were examined spectroscopically between 200 and 400 nm. The polyyne content was assayed by measuring increases in the characteristic long wavelength absorption bands. The diagnostic peaks were plotted to determine the appearance of polyacetylenes in the cultures and the time of their maximum concentration.

One culture was used as an analytical time course study for unique acetylenes. Between the 9th and 21st days of growth, an aliquot (40 ml) was removed daily and extracted with diethyl ether (40 ml).

4.3.2 Extraction of acetylenic metabolites

The optimum extraction solvent was determined by extracting _F. hepatica_ media (2 ml) with various organic solvents (1 ml) and spectroscopically evaluating the intensities of the longest wavelength absorption bands in the ultraviolet region. For preparative scale HPLC purification, _F. hepatica_ cell broth (ca. 3.5 L) was isolated from mature 21-31 day submerged mycelial liquid cultures by passing it through Whatman 100 filter
paper. The mycelia were stored at -80 °C for molecular biology studies. The aqueous media was continuously extracted with diethyl ether (225 ml, 1:1 v/v) for 2 days. The organic phase of solvent extractions was concentrated on a rotary evaporator under vacuum, immediately redissolved in acetonitrile and passed through a 0.20 micron nylon filter.

### 4.3.3 Purification and analysis of polyacetylenes

Analytical-scale separation of metabolites by reverse-phase HPLC was optimized using an Adsorbosil C$_{18}$ HPLC column (250 x 4.6 mm) with an acetonitrile:water linear gradient (15 min, 50:50 to 60:40) and monitored by an on-line diode array detector.

Preparative-scale reverse-phase chromatography was performed by HPLC using an Econosil C$_{18}$ HPLC column (250 x 10 mm) and a two-step linear acetonitrile:water solvent gradient (9.6 min, 20:80 to 70:30; 11.5 min, 70:30 to 100:0). The elution was monitored by an on-line UV spectrophotometer at 288, 307 and 328 nm. Fractions that absorbed strongly were collected, concentrated and passed through the preparative column a second time using an acetonitrile:water linear gradient (15 min, 50:50 to 60:40). Intensely absorbing compounds were collected and concentrated on a rotary evaporator nearly to a film and dissolved in CDCl$_3$. The process of concentration was repeated before NMR analysis was performed.

### 4.3.4 Construction of cDNA library of *Cantharellus cibarius*

Total RNA was isolated from about 2 g frozen *C. cibarius* by grinding with a mortar and pestle chilled to -80 °C using Trizol reagent and following the manufacturer's instructions. Poly(A)$^{+}$ RNA was then purified using a system of poly(T) oligimers covalently bound to magnetic beads, following the manufacturer's protocol. cDNA, prepared from the poly(A)$^{+}$ RNA, was used to construct a cDNA expression library with EcoRI/XhoI-cut Uni-ZAP XR vector arms.
4.4 Results and discussion

4.4.1 UV screening of cultures

Daily screening of cultures indicated that polyacetylenes appeared in the media after 5 days growth, maintained a low level for 7-9 days, then peaked rapidly after approximately 12-14 days (Figure 8). Diagnostic peaks, used to monitor polyalkyne production, were selected from the maxima of a UV spectrum typical of crude extract from culture fluid (Figure 9). These maxima were located at $\lambda_{\text{max}}$, 240, 257, 270, 291, 306, 348 and 355 nm, which appear in the spectral signatures of enetriynes, dienetriynes and tetrynes and the compounds isolated by Jones [65].

Absorbances near 291 and 306 nm are caused in all five of the compounds isolated by Jones. Comparison of the relative intensities of these two peaks in compounds I, II and V with the time course of polyacetylene production indicates that these compounds are not the major contributors to the absorbance at these two maxima (no information for extinction coefficients have been reported for compounds III and IV). The extinction coefficients for I, II and V, at $\lambda_{\text{max}}$ 290 and 308, are approximately equal for the two maxima. In contrast, the time course indicates absorbance at 290 nm is over twice that at 306 nm. The *F. hepatica* strain used in the present study may be different from the strain examined by Jones and may produce some of the compounds isolated earlier either transiently or accumulate them during specific stages of growth or under unique environmental conditions. Unidentified fungal contaminates appeared in some of the cultures. A spectroscopic comparison of six of these with culture that were free of visible contaminates showed polyalkyne concentrations to be more than twice the level of those in the clean cultures. The spectra appeared otherwise similar, except for intensity, which may indicate the unknown fungus induced a defensive response from *Fistulina*, rather than producing the compounds itself.
Figure 8. Spectrophotometric time course for *F. hepatica* alkyne production. Polyacetylene content exponentially increased at 14 days after inoculation. The wavelengths monitored occur in the spectra of enetriynes, dienetriynes and tetranyes. The slow increase in polyacetylene concentration after 20 days is presumably due to evaporation of culture fluid.
Figure 9. Ultraviolet spectrum of a crude *F. hepatica* diethyl ether extract from a 16 day culture. Absorbance at 375nm clearly indicates the presence of enetetrayne chromophores.
4.4.2 Extraction of metabolites

Based on the intensity of the diagnostic peaks noted above, diethyl ether extracts produced a ten fold increase in nearly all monitored wavelengths compared with hexanes (data not shown). Ethyl acetate was as effective as ether but could not be monitored below 250 nm (Figure 10). Methylene chloride was least effective and could not be monitored below 240 nm. Additionally, the peaks at 348 nm, which appears in III, IV and dienetriynes, and 325 nm, appearing in dienetriynes, were greatly reduced in methylene chloride extracts compared with ether. Diethyl ether was selected for large-scale solvent extraction.

The unconcentrated ethereal extracts appeared to remain stable when stored in the dark at 4 °C. Spectra of crude extract stored this way for six months did not change appreciably from the original spectra of fresh extract. Nonetheless, individual compounds within the mixture may oxidize during storage, so, preparative-scale HPLC was performed only with fresh extract. However, analytical-scale HPLC of samples stored for two months at 4 °C revealed no significant change in retention times and desaturation patterns of intensely absorbing compounds when compared with the original data.

4.4.3 Purification of polyacetylenes

The components of the concentrated crude extract from mature culture filtrates were separated on an analytical scale by reverse-phase HPLC. At least 20 compounds with spectral signatures for highly conjugated eneyne natural products were observed. A time course analysis of these analytes revealed that unsaturation concentration increases in later culture periods (Figure 11). An enediynene accumulates before day 11 after inoculation and elutes at 5.71 min. Two compounds that appear to be enetriynes, eluting at 10.20 min and 10.96 min, begin to accumulate after day 11. A metabolite with the spectral characteristics of a dienetetrayne, which has been suggested to be the precursor
Figure 10. Ultraviolet spectra of *F. hepatica* metabolites extracted with diethyl ether, ethyl acetate and methylene chloride. Diethyl ether was most effective for the extraction of compounds absorbing at the $\lambda_{\text{max}}$ monitored during screening.
Figure 11. Time course analysis of polyacetylenic metabolites indicates that the level of unsaturation in the culture fluid increases in the later stages of *F. hepatica* growth.

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to tetraol V, also appears after day 14 (Figures 11 and 12). Examination of the peak shapes from the analytical UV spectra of these analytes indicates that, in many cases, resolution is not achieved during the first HPLC separation.

In mature cultures, the most intensely absorbing compounds, which are among the least polar metabolites, are enetetraynes by UV spectroscopy. These compounds eluted at 12.18 min, 17.66 min and 18.81 min (Figure 13). In contrast to earlier studies, where tetrayne concentration reached a maximum 9-14 days after inoculation, tetraalkyne products predominated at long culture times (Figure 14) [65]. Separation of components by reverse-phase preparative-scale HPLC was optimized by monitoring diagnostic wavelengths, but large-scale collection of fractions from the complex mixture was deemed impractical (Figure 15). Furthermore, in agreement with the analytical HPLC data, UV analysis indicated that the fractions containing the most intensely absorbing compounds were mixtures requiring additional separation. Therefore, fractions (20 ml) containing groups of compounds of similar polarity were collected from serial runs and combined (Figure 16). These samples were concentrated and passed through the preparative column a second time using a modified solvent gradient. Fractions containing intensely absorbing compounds were collected for NMR analysis.

Purified samples were concentrated to a film in glass scintillation vials by lyophilization or evaporation under a vacuum. The process produced, in many cases, intractable residues that remained, at least partially, insoluble in acetone, chloroform and methanol. The compounds prepared in this manner possibly reacted with the glass in a way that rendered them insoluble or polymerized through reactions with air. This negative reaction was avoided in subsequent preparations by concentrating the samples to very low volumes, adding deuterated solvent and repeating the process.
Figure 12. Putative dienetetrayne, a possible precursor to tetraol V, appears in the culture media after day 14 and increases in concentration through day 21; it is not visible in mature culture fluid (ca. 31 days).
Absorbance (mAu)

Wavelength (nm)

24.76 min
Figure 13. Spectra of putative enetetraines eluting at 12.18, 17.66 and 18.81 minutes. The $\lambda_{\text{max}}$, 239, 258, 272, 303, 324, 348 and 375 are clearly visible.

Green  12.18 min
Blue    17.66 min
Red     18.81 min
Figure 14. Contour plot of highly conjugated metabolites from a mature shaken culture of *F. hepatica*. The predominant desaturation pattern is enetetrayne, revealed by compounds eluting 12.18, 17.66 and 18.81 min. Intense absorption by a pair of enetriynes (10.20 and 10.96 min) is also prominent in the chromatogram.
Figure 15. Chromatogram of crude extract from 21-31 day *F. hepatica* cultures. Preparative-scale reverse-phase chromatography was performed by HPLC using an Econosil C<sub>18</sub> HPLC column (250 x 10 mm) and a two-step linear acetonitrile:water solvent gradient (9.6 min, 20:80 to 70:30; 11.5 min, 70:30 to 100:0).
Figure 16. Chromatogram of preliminary separation of crude extract from 21-31 day *F. hepatica* cultures (compare with Figure 14). The column was overloaded with sample and fractions (20 ml) containing intensely absorbing compounds were collected, concentrated and passed through the preparative column a second time using an acetonitrile:water linear gradient (15 min, 50:50 to 60:40). Intensely absorbing compounds were collected for NMR analysis.
4.4.4 Structure determination of cis-tridec-3-ene-5,7,9-triyne-1,11-diol

Analysis of the culture fluids of *F. hepatica* revealed the presence of a putative enetriynediol at 10.20 min. The structure of this metabolite was established by $^1$H NMR, electrospray ionization mass spectrometry and UV spectrometry (Table 6). Compound VI produced a UV spectrum typical of an enetriyne. The formula mass of 202.21 from MS analysis is in accord with a molecular formula of C$_{13}$H$_{14}$O$_2$ and degree of unsaturation consistent with an enetriyne. Vinyl protons consistent by chemical shift and coupling constants ($\delta$ 5.59, $d, J=10.4$ Hz and $\delta$ 6.26, $dt, J=11.2, 6.4$ Hz) with a (Z)-enetriyne conjugated system with an allylic methylene group were observed in the $^1$H NMR spectrum. Also present was a peak with a chemical shift typical of a proton geminal to a hydroxyl group adjacent to a conjugated triyne system ($\delta$ 4.41, $dd, J=1.5, 6.4$ Hz). A final diagnostic peak characteristic of the geminal protons of a primary alcohol coupled to a methylene group ($\delta$ 3.72, $t, J=6.0$ Hz) provided evidence for the proposed structure of VI as cis-tridec-3-ene-5,7,9-triyne-1,11-diol.

Initial attempts to purify VI produced a white film (1.0 mg) that represented a yield of 1.6 mg/L of culture filtrate. Unfortunately, the insolubility of the sample in organic solvents precluded NMR analysis. A subsequent sample was prepared as described in the methods section and the solubility problem was avoided. However, the method leaves no means for direct measurement of mass. Perhaps the inherent instability of polyacetylenes renders them subject to oxidation if not maintained in solution. The UV spectra of crude ethereal extracts stored at 4 °C for two months remained unchanged, even when concentrated to 10% of original volume, compared with the spectra of the same sample when first extracted. The pathway to polyyne production through crepenynic acid (Scheme 5) can be modified to accommodate the chain shortening and oxidative reactions that are required for the rich variety of polyacetylenes produce by plants and fungi. A route that apparently operates in the formation of methyl triyne compounds results in chain shortening of a dehydrocrepenynic acid derivative followed by desaturation of the conjugated double bonds to form a 14 carbon diyne derivative (Scheme 6) [4]. This route may also operate in the biosynthesis of compounds related to methyl triynes [4]. Additional oxidations and a sequence of common biochemical reactions involving...
Table 6. A new polyacetylenic alcohol VI in *F. hepatica* is *cis*-tridec-3-ene-5,7,9-triyne-1,11-diol. The structure was elucidated by $^1$H NMR, electrospray ionization mass spectrometry and UV spectrometry. The $^1$NMR spectrum revealed a doublet of multiplets and doublet of triplets due to protons at positions 3 ($\delta$ 6.28, $dt$, $J$=11.2, 6.4 Hz) and 4 ($\delta$ 5.59, $dm$, $J$=10.4 Hz) that flank a *cis* double bond. The proton at position 11 ($\delta$ 4.41, $dd$, $J$=1.5, 6.4 Hz) is located at a chiral center and shows separate coupling to the diastereotopic protons at position 12 ($\delta$ 1.75, $m$).
<table>
<thead>
<tr>
<th>Position</th>
<th>$\delta$ H</th>
<th>$J$, Hz</th>
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<tbody>
<tr>
<td>1</td>
<td>3.72, $t$</td>
<td>6.0</td>
</tr>
<tr>
<td>2</td>
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<td>6.6</td>
</tr>
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<tr>
<td>4</td>
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<td>10.4</td>
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<td>5</td>
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<td>6.4, 1.5</td>
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<tr>
<td>12</td>
<td>1.75, $m$</td>
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</tr>
<tr>
<td>13</td>
<td>1.01, $dd$</td>
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</table>
Scheme 6. Proposed pathway for the biosynthesis of VI. The route proceeds through crepenynate and includes biochemical transformations known to occur in plants and fungi. Dehydrocrepenynate undergoes Δ^{14}\text{-}desaturation and two rounds of β-oxidation followed by oxidation and dehydration to yield a 14 carbon dienediyne. Oxidation at the β-position of the carbonyl group and subsequent loss of CO₂ and H₂O result in a 13 carbon dienetriyne which can be oxidized to form the alcohol. Oxidation and isomerization yields VI. Further reduction and oxidation leads to II and, potentially, I. The strong, early and persistent appearance of VI in the culture fluids indicate an important role, perhaps associated with a branch point in polyacetylene biosynthesis in *F. hepatica*. Dehydration and reduction combined with a series of oxidations potentially leads to V and other tetraynes.
Crepenynic acid

Dehydrocrepenynic acid

\[ \text{R} = \text{phospholipid} \]
loss of H₂O and CO₂ yield a 13 carbon dienetriyne that can be further modified to form I, II, V and VI.

Conclusion

Five polyacetylenic metabolites have previously been isolated from *F. hepatica*, including a tetranye-tetraol V, which exhibited antibacterial activity. In the present study, a new alcohol VI was isolated and the preliminary structure was determined as cis-tridec-3-ene-5,7,9-triyne-1,11-diol. Culture conditions and a protocol for extraction and purification of VI and related metabolites was established.

A time course analysis was performed which shows that unsaturation concentration in culture fluids increases during culture growth. The timing of the appearance of unique metabolites is useful for predicting the course of desaturation events during polyacetylene biosynthesis in *F. hepatica*. Additionally, the change in metabolite concentrations over time may provide information useful in designing substrates for future structure/function studies of *F. hepatica* acetylenase genes and the eventual elucidation of the metabolic pathways of polyacetylenic metabolites.

In this regard, characterization of acetylenic metabolites in *F. hepatica* will be crucial in probing the sequence and mechanisms of the transformations between metabolites. Furthermore, the number of acetylenases involved in the synthesis of polyacetylenic linkages must be determined. *Fistulina* may utilize one or a few multi-functional enzymes of low specificity or several regio- and substrate-specific enzymes. Identification of putative substrates for each transformation will provide the probes necessary for investigating the mechanisms and the number of acetylenases involved in each transformation.

5. Summary

Polyalkynes, compounds containing conjugated carbon-carbon triple bonds, have previously been isolated from a broad range of organisms including plants, fungi, bryophytes, amphibians, algae and marine- and micro-organisms. These compounds may
be the result of desaturase-like reactions during secondary metabolism. Antiviral and antitumor activities have been attributed to many of the naturally occurring acetylenes making them targets of study for ultimate biosynthetic production in industrially important plants and micro-organisms.

The Crep1 gene of Crepis alpina encodes an enzyme which catalyzes the formation of an acetylenic bond from the Δ^{12}-double bond of linoleate to yield crepenyenic acid. Crep1 is related by its chemistry and predicted amino acid sequence to desaturase-like plant enzymes that modify fatty acids by installing unusual functional groups such as conjugated double bonds and epoxy and hydroxyl groups.

In previous studies, *S. cerevisiae* has proven to be a suitable host for overexpressing desaturase-like enzymes and was utilized successfully in the present work for the expression of Crep1. Optimal results were obtained with YN94-1 transformed with pVT-CREP1 and incubated at 30 °C in media volumes of 100 ml supplemented with NP-40. However, the low level (0.32% total endogenous fatty acids) of 18:1A accumulation and the observed repression of endogenous Olel indicates that mechanisms that control fatty acid composition in heterologous hosts like *S. cerevisiae* must be elucidated before increased activity of transgene products can be realized.

In light of the current success with expression of Crep1, the YN94-1/pVT-CREP1 system is anticipated to provide a reliable starting point for the future expression and characterization of fungal acetylenases from *F. hepatica*, *C. cibarius* and other basidiomycetes, which may use enzymes related to CREP1 to produce polyacetylenes. Construction of the *C. cibarius* λ-ZAP cDNA library represents the first step towards the eventual cloning and overexpression of these enzymes. Screening of transcribed genes homologous to Crep1 can next be performed using probes designed from the conserved regions of known desaturases.

To this end, genetic characterization of the parsley ELI12 Δ^{12}-acetylenase expands the body of information available for designing hybridazation probes. The characterization of the defense-related ELI12 as a bifunctional Δ^{12}-acetylenase/Δ^{14}-desaturase that catalyzes the accumulation of both crepenyenic acid and (14Z)-dehydrocrepenyenic acid implicitly demonstrates a role for CREP1-related enzymes in polyacetylenic biosynthesis in parsley, which produces the diyne falcarinol. Carrot, a relative of parsley, has been shown to
incorporate labeled crepenynate and subsequently accumulate falcarnol, which the plant utilizes in a defensive mechanism against storage rot. Defensive roles for polyacetylenes likely influence fungal, as well as plant, metabolism, as observed in the occurrence of the antimicrobial tetrane-tetraol V in *F. hepatica*.

The isolation and preliminary structure assignment of the enetriynol VI, combined with the time course analysis of unique metabolites, is an initial foray leading toward an informed hypothetical pathway of polyacetylene biosynthesis which includes I, II, III, IV and V in *F. hepatica*. The early and persistent appearance of VI in *F. hepatica* culture fluids hints at an important role for this metabolite, such as association with a branch point in polyalkyne biosynthesis.

Structural knowledge of VI and other acetylenic metabolites from *F. hepatica* is necessary for designing putative substrates for future enzyme structure/function studies. Identification of these substrates is an integral component of the elucidation of the sequence and mechanisms of the transformations between metabolites and the substrate-and regio-specificity of the acetylenases involved. Understanding these mechanisms and their regulatory elements is likewise required for the construction of efficient expression systems. The availability of acetylenase genes that are involved in the biosynthesis of natural bioactive compounds will permit the design of genetic systems that may be transferred into industrially important plants or microorganisms.
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