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Biosynthesis and Function of Polyacetylenes and Allied Natural Products

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Abstract

Polyacetylenic natural products are a substantial class of often unstable compounds containing a unique carbon-carbon triple bond functionality, that are intriguing for their wide variety of biochemical and ecological functions, economic potential, and surprising mode of biosynthesis. Isotopic tracer experiments between 1960 and 1990 demonstrated that the majority of these compounds are derived from fatty acid and polyketide precursors. During the past decade, research into the metabolism of polyacetylenes has swiftly advanced, driven by the cloning of the first genes responsible for polyacetylene biosynthesis in plants, moss, fungi, and actinomycetes, and the initial characterization of the gene products.

The current state of knowledge of the biochemistry and molecular genetics of polyacetylenic secondary metabolic pathways will be presented together with an up-to-date survey of new terrestrial and marine natural products, their known biological activities, and a discussion of their likely metabolic origins.

Keywords

Acetylenes; Polyacetylenes; Secondary Metabolism; Desaturases; Lipids; Polyketides

1. Introduction to Acetylenic Natural Products

Acetylenic natural products include all compounds with a carbon-carbon triple bond or alkynyl functional group. While not always technically accurate, the term “polyacetylenes” is often used interchangeably to describe this class of natural products, although they are not polymers and many precursors and metabolites contain only a single acetylenic bond. Acetylenic natural products are widely distributed, occurring in plants, moss and lichens, fungi, marine algae, sponges, tunicates, insects, frogs and, in traces quantities, humans. The compounds themselves tend to be unstable, succumbing either to oxidative, photolytic, or pH-dependent decomposition, which originally provided substantial challenges for their isolation and characterization.

The earliest isolated alkyne-bearing natural product was dehydromatricaria ester (**1A**), which was isolated, but not fully characterized, in 1826 (Fig. 1). No compound was characterized as

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being acetylenic until 1892 (tariric acid, **5T**) [1,2], after which only a handful of compounds were isolated before 1952 (Fig. 1). A lecture by N. A. Sørensen to the Royal Chemical Society in Glasgow describes the early history of polyacetylenic natural product chemistry [3]. From the 1960s to the late 1980s, natural products began to yield to the increasing powers of separation and analysis, bolstered by the ability of organic chemists to synthesize highly unsaturated natural products and putative biosynthetic intermediates and cope with their fragility. It was during this 25-year growth spurt that the exploration into the synthesis, isolation, and biogenesis of naturally occurring acetylenes was dominated by the groups of Sir Ewart H. R. Jones (1911–2002) and Prof. Ferdinand Bohlmann (1921–1991) [4,5]. While Jones was at Manchester carrying out a research program that included the use of acetylenic chemistry toward the synthesis of polyenes and vitamin A, John D. Bu'lock became an assistant lecturer in 1951 at the same institution and began studying polyacetylenic compounds from fungi [6]. The first secondary metabolite that they reported was fatty acid-derived agrocybin (**1B**) [7] (n.b. an antifungal peptide from *Agrocybe cylindracea* has been confusingly given the same name [8]) and together they communicated the first biosynthetic studies of polyacetylenes [9]. Jones departed to chair the chemistry department at Oxford in 1955, but not before Bu'lock's enthusiasm for exploring fungal secondary metabolism drew Jones into a programmatic study of polyacetylenes that blossomed over 30 years. From the early days at Oxford, Viktor Thaller (with Jones) was involved in developing syntheses of isotopically labeled polyacetylene intermediates, a research area he maintained until 1989. Independently, Bohlmann, starting in 1953 at Braunschweig and then the Technical University of Berlin in 1959 with his assistant C. Zdero, investigated primarily plant-derived polyacetylenes from Asteraceae (Compositae) [10]. Card files from their extensive studies have been made freely available in a searchable online database that currently covers 6258 plants, mostly from Asteraceae, and 20,000 compounds of which 1223 are acetylenic [11]. Working independently at common purposes, the two groups' extensive and methodical natural product isolations and tracer studies expanded the compendium of known acetylenic natural products to *ca.* 1000 compounds and provided the underpinnings of polyacetylene biosynthetic pathways. As analytical methods have improved, many initial reports of the prevalence of acetylenic metabolites in plant families have been revised consistently upward.

During the past 25 years, substantial changes, both in growth and direction, have been occurring in the area of acetylenic natural products biosynthesis. The expansive screening effort spearheaded by the National Cancer Institute reinvigorated the discovery of new compounds. This has had the effect of disproportionately increasing the number of new polyacetylenes from marine organisms. More than 2000 polyacetylenes are known, with more than 1100 in the plant family Asteraceae. The molecular biology revolution has allowed the biochemical processes involved in polyacetylene biosynthesis to be probed with significant progress being made in the cloning of acetylene biosynthetic genes and the characterization of their protein products. As such, the goals of this review are to provide the state of knowledge in polyacetylene biosynthesis framed around progress made during the past 10 years and to review the new natural products isolated from 1997 to date. In doing this, an effort was made to highlight the biosynthetic parallels between terrestrial and marine pathways that, to our knowledge, have not been reviewed previously. Coverage of selected areas that have not seen recent progress are included to provide a fair overview of the expanse of acetylenic natural products chemistry.

There are a number of reviews that the reader should consider that examine earlier efforts in the area. A book by Bohlmann provides a golden resource, covering the synthesis, isolation, and chemotaxonomy of acetylenic natural products through 1972 [12]. Reviews focusing on the systematic relationships of polyacetylenes within specific plant families and tribes have been published [13–18]. Unusual fatty acids in seed oils were cataloged in 1981 by Badami and Patil, whose concentration on acetylenic acids updated portions of the Bohlmann monograph [19]. New structures of fatty acids and derivatives, their synthesis and

biotechnological aspects in the period preceding the current article have been reviewed [20, 21]. A recent and extensive review of the anticancer activity of natural and synthetic acetylenic lipids was written by Dembitsky [22]. A review of advances in the synthesis of acetylenic natural products has also recently appeared [23].

2. Biochemistry and Molecular Genetics

2.1 Biosynthesis of Fatty-Acid Derived Metabolites

As initial points of departure from primary metabolism, three fatty acids comprise the roots of most acetylenic natural products: crepenynic acid (**2E**), stearolic acid (**5A**) and tariric acid (**5T**) (Fig. 1). In this section, we will provide a brief overview of their biosynthetic pathways, the major types of metabolic transformations modifying the basic acids and, finally, consider the latest developments in the genetics and biochemistry of plant and fungal acetylenic natural product biosynthesis. Standard lipid nomenclature is used periodically to denote chain lengths (l) and the number of sites of desaturation, (d) as l:d, with the locations of acetylenic (a) bonds and the stereochemistry of alkenes (c, cis or *Z*- and t, trans or *E*-) indicated in the superscripts.

2.1.1 Models for the Biosynthesis of an Acetylenic Bond—During the early 1960s, two models put forward for the biosynthesis of polyacetylenes, the oxidative dehydrogenation (desaturation) mechanism and a decarboxylative enol elimination mechanism, used divergent approaches to the formation of the second π -bond (Fig. 2). The desaturation model, supported by the landmark work of Bloch on unsaturated fatty acid formation [24], was first tested by Bu'Lock in 1967 [25,26]. The enol elimination hypothesis was based upon the premise of Wakil [27] that acylmalonate derivatives were involved in poly(acetate) biosynthesis and was advanced prior to the entrenchment of Bloch's work. While at this time it seems improbable that enol eliminations play a large role in plant acetylenic secondary metabolism, the concept provided a facile means of introducing acetylenic bonds during the elongation of an acyl chain. As a proof-of-concept, Fleming tested the hypothesis and showed that phosphates and sulfates of 3-hydroxy-2-alkene carboxylates eliminated to acetylenes under mild conditions [28,29]. Further examples have been recently reported [30].

2.1.2 Primary Substrates for Acetylenic Fatty Acid Biosynthesis—Saturated fatty acids are synthesized *de novo* by multifunctional fatty acid synthase complexes, which append in a head-to-tail fashion malonyl units to a growing acyl chain. In most eukaryotes, this chemistry occurs in the cytosol; the major exception is green plants, where saturated fatty acids originate from the stroma of plastids (Fig. 3). The well established series of four reactions begin from acetyl-CoA: Claisen-like condensation of a malonyl group that elongates the acyl chain by two carbons to a β -ketoacyl-ACP, reduction to β -hydroxyacyl-ACP, dehydration leading to the α,β -unsaturated acyl-ACP and, finally, reduction to the C_2 -homologated acyl chain. A complex of soluble enzyme subunits catalyzes these reactions. In plants, the condensing activities involved are dependent upon the length of the nascent chain: β -ketoacyl synthase III (KASIII) carries out the first condensation to butyryl-ACP, KASI elongates the lipid chain to C_{16} , and KASII completes the formation of stearyl-ACP. In fungi, animals, and certain bacteria, multifunctional polypeptides termed Type I fatty acid synthases carry out analogous reactions in the cytosol. In plants, the soluble stearyl-ACP desaturase (FAB2) desaturates the majority (commonly >75%) of the C_{16} and C_{18} chains regiospecifically at C-9. In lower eukaryotes, acyl-CoA desaturases perform the analogous chemistry, whereas in bacteria both anaerobic desaturation and oxygen-dependent desaturation with acyl-lipid substrates are operative [31]. Aerobic desaturation, prevalent in eukaryotes, is catalyzed by non-heme-diiron desaturases that use molecular oxygen and an electron transport system to excise two vicinal C-H bonds on the alkyl chain of a fatty acid derivative regiochemically generating the π -bonds of an alkene. As a result of its relative stability and ease of overexpression, X-ray

crystallography, supplemented with other spectroscopic techniques, has provided a high-resolution picture of stearoyl-ACP desaturase [32,33]. Additional efforts to dissect structural attributes controlling the regio- and chemoselectivity for the soluble desaturases are yielding new insight into the enzymology of this key enzyme [34–36]. The fate of oleate produced by FAB2 is ruled by compartmentalization in plants, with a portion released to the cytosolic compartment as 18:1-CoA for the synthesis of eukaryotic glycerolipids (PC, PE, PI) and the remainder incorporated into chloroplast membranes. Solid reviews of fatty acid biosynthesis in plants and yeast may be found elsewhere [37,38]. At this point, it appears that the chief reservoir of fatty acids involved in acetylene biosynthesis is PC in the ER membranes.

Desaturation at the ER occurs on a nominally full-length C₁₆–C₁₈ acyl chain, which can also undergo chain elongation and oxidative cleavage processes. Elongation pathways, which synthesize acyl chains C₂₀ and longer, are reviewed elsewhere [39]. Desaturation occurring in plastids and on the endoplasmic reticulum leads to distinct lipid products in part because the lipid conjugates (e.g., acyl-CoA, acyl-ACP, and PC derivatives) act both as specific substrates and temporary repositories. As will be discussed in more detail later, numerous gene sequences for “diverged” desaturases have been published beginning in 1995 that are related to the microsomal oleate Δ^{12} -desaturase (named *FAD2* in plants) but that have an atypical chemical function (e.g., hydroxylation rather than alkane dehydrogenation) [40]. While it is not without ambiguity, trends are becoming apparent among diverged desaturases with peculiar regio- or chemoselectivity allowing the discernment of function based upon primary sequence.

2.1.3 The Crepenynate Pathway—The crepenynate pathway for acetylenic natural product biosynthesis has been examined repeatedly in plants and fungi over the past 50 years (Fig. 4). The pathway is fed with acetate-derived acyl lipids provided from primary metabolism and diverges with the conversion of linoleic acid (**2C**) to crepenynic acid (**2E**) with the installation of the initial acetylenic bond between C-12 and C-13. Desaturation of **2E** to (14Z)-dehydrocrepenynic acid (**2G**) has been demonstrated in fungi but, with a couple of exceptions, **2G** does not accumulate in plants [41,42]. Three H₂ equivalents are excised from **2G** by a series of uncharacterized transformations leading to the triynoic acid **2M**. At each dehydrogenation level in the crepenynate pathway, fatty acids can be diverted and chain-shortened resulting in a metabolic web with chain lengths between C₈ and C₁₈. Certain diversions from the main dehydrogenation series appear to lead to large groups of polyacetylenes, such as the multistep shunt to (ene)-diyne-diene **2O**, which is a precursor of the non-alkamide-related C₁₄ metabolites in Heliantheae [14]. Major chemical distinctions have been drawn between plant families. A significant example of taxonomic variation is the contrast between natural products in the Asteraceae family, in which the fatty acid carboxyl group is metabolized to a vinyl terminus, and the umbels (Apiaceae), where the same atoms are reduced to an alkane. Widespread transformations and specific enzymes that lead to many of the acetylenic metabolites are described in Section 2.3 and representative new plant metabolites are outlined in Section 3. (Place Fig. 4)

Crepenynic acid (**2E**) rarely accumulates in plant tissue but has been found in several seed oils, including those from *Crepis* spp. [43], *Saussurea candicans* [44], and *Afzelia cuanzensis* [45]. The earliest *in vitro* study of the biosynthesis of crepenynate-derived polyacetylenes examined a *Chrysanthemum flosculosum* (Asteraceae) cell-free preparation that incorporated low levels of [1-¹⁴C]oleate (**2A**) into **3A** (Fig. 5) [46]. Haigh carried out seminal biosynthetic studies of **2E**, where time courses of crepenynic acid production showed that accumulation begins several days after flowering and reaches its maximum 14–28 days later [47]. Radiochemical tracer studies supported **2A** as the precursor and were consistent with a model in which acetylenic groups were formed by the modification of fatty acids with prevailing chain lengths of C₁₆ and C₁₈ [47]. Subsequently, Bohlmann *et al.* showed that long-chain fatty acids

were necessary for the initial stages of polyacetylene biosynthesis; 14:1^{5c} and 16:1^{7c} were poorly incorporated into three plant polyacetylenes **3A**, **3B**, and **3C**) [48].

Falcarinol (**4B**) is distributed through many Apiaceae and Araliaceae species (Fig. 6A) [49]. It was shown by Barley and coworkers that [9-¹⁴C] and [10-³H]crepenynate (**2E**) were incorporated at 0.08–0.1% levels into **4B** in carrot tissue culture [50]. The identification of certain *Dendropax trifidus* metabolites, like C₁₈ acid **4A**, has been used as support for the formation of (–)**4B** through decarboxylation. Aldehyde **4D**, known from the Apiaceae species *Pastinaca sativa*, has been proposed as a precursor to falcarinone (**4E**) [51]. It is probable that a single pathway, likely initiated by 2-hydroxylation of **4A**, links the conversions resulting in **4B–4E**. In this hypothesis, decarboxylation of 2-hydroxyacid **4A** leads to the C₁₇ aldehyde homolog of **4D** that is sequentially reduced through an alcohol to an alkane. Metabolites with a primary hydroxy group that would result from alcohol dehydrogenase activities (e.g., **4F**, **8C–D**) are infrequently observed, implying that the subsequent elimination/reduction conversions are efficient.

A number of polyacetylenic natural products are known that occur with their dihydro derivatives suggesting a biogenetic relationship. Reduction of the 8,9- π bond in a dienyl system has been used to rationalize several metabolite pairs, including **4G** and **4H** from *Oenanthe crocata* (Fig. 6B). Two diene-forming pathways may be involved in the conversion of a methylene-skipped ene-diyne, such as **2I** or a derivative, to the conjugated diene **4G**. First, a functionally diverged desaturase can accommodate the expansion of the C-9 alkene to the 8,10-polyene. Conjugases are now known that provide examples of this activity by elaborating upon Δ^9 -unsaturation to produce 1,3-dienyl fatty acids (e.g., parinaric, α -eleostearic, and calendic acids) [52,53]. Second, lipoxygenase/peroxidase activities may hydroxylate an acetylenic intermediate providing the putative substrate for a 1,4-dehydrative elimination to **4G** (see Sections 2.3.1.2 and 2.3.1.3). If either case is operative, a Δ^8 -alkene reductase activity yielding **4H** should be detectable.

β -Hydroxyoleic acid (**4I**) has been shown to incorporate into allylic acetate **4J** in *Oenanthe pimpinelloides* and dehydrofalcarinone **4K** in *Artemisia atrata* and has been proposed as a general crepenynate pathway precursor to the C₁₇ Araliaceae polyacetylenes, whereby expulsion of CO₂ leads to a terminal alkene that may be further reduced (Fig. 6C) [54]. Alternatively, the general fatty acid catabolic enzymes could dehydrate and reduce **4I** to a saturated fatty acid and α -oxidation, as proposed in Section 2.3.1.2, would effect the chain-shortening. Introduction of the terminal acetoxy group, while likely an enzymatic process, is a facile acid-catalyzed reaction known to occur chemically with falcarinol and falcarinone endgroups [12].

In macrofungi, the incorporation of oleic, linoleic, crepenynic, and dehydrocrepenynate into polyacetylenes is well established. Circa 1960, *Polyporus anthracophilus* feeding experiments with [1-¹⁴C]acetate showed labeling of the odd-numbered carbons in matricaria ester **3D** (Fig. 5) [55]. A related experiment demonstrated the alternating [1-¹⁴C]acetate labeling of nemotinic and odyssic acids (**3F**, **3G**) from basidiomycete B. 841 (*Poria* sp.) [56], compounds related to the potent, but unstable, tuberculosis treatment mycomycin **3H** [57]. [2-¹⁴C]Malonate labels the even-numbered carbons in dehydromatricariol **3I**, while C-9 and C-10 specifically originated from acetate [9]. [10-¹⁴C]Oleate was transformed to linoleate, crepenynate, dehydrocrepenynate and **3I** by the basidiomycete *Melanoleuca (Tricoloma) grammopodicum* [26]. In contrast to plants, it is relatively common to observe the accumulation of acetylenic fatty acids in fungi.

M. grammopodium, *Poria sinuosa*, *Serpula (Merulius) lacrymans*, *Coprinus quadrididus*, and Basidiomycete A67 generally incorporated [10-³H, 9-¹⁴C]crepenynate into fungal acetylenes

with variable levels of tritium loss [58]. *Fistulina hepatica* and *Fistulina pallida* appear to also have non-crepenynate polyacetylene biosynthesis pathways available, as an incompletely characterized triynenediol was labeled at 200X that of tetrayne **3K** by [¹⁴C]**2E**. This tetrayne and diol **3J** retained only 0.01% of the ¹⁴C-label of crepenynate but showed 10-fold higher incorporations of acetate consistent with their formation through an alternate route [58]. Incorporation levels into polyacetylenic metabolites, such as **3L**, when the preferred (14*Z*)-isomer of dehydrocrepenynate was fed were 30–300X that of the (14*E*)-isomer in the fungi *M. grammopodium*, *Lepista diemii*, *C. quadrifidus*, and *S. lacrymans* [59]. In *Marasmius ramealis*, incorporation of esters **1A** and **3M** into (–)-marasin **3N** (0.8 and 2.0%, respectively) occurred without scrambling of the carboxyl groups [60]. In their analysis of this study, Davies et al. clearly stated that the order of four modifications – oxidation of the methyl group of **1A** to the carboxyl group and 10-decarboxylation, reduction of the 1-carboxyl group to an alcohol, and hydrogenation – was unresolved, as the substrate specificities are not known for any of the likely activities. For *S. lacrymans*, it remains unclear whether **1A** is a direct metabolic precursor to **3L**, the free acid of **3M**. Two studies presenting [¹⁴C]**1A** to *S. lacrymans* by the same research group showed very disparate labeling of **3L**, 11% and 0.45% [61]. It is quite conceivable that the *S. lacrymans* secondary metabolic grid contains multiple, distinct sequences of functional group interconversions beginning with distinct building blocks leading to individual metabolites (e.g., **3L**). The flux of intermediates through this grid may be linked to culture conditions and metered through a myriad of regulatory factors, which determine the final polyacetylene distributions visible in most plants and fungi.

2.1.3 Stearic and Tariric Acid Related Pathways—The production of the stearic acid series of acetylenic fatty acids (**5A–5G**), where the initial alkynyl group is installed at C-9, requires a distinct set of acetylenase activities versus the crepenynate pathway (Fig. 7A). The monoacetylene **5A** had been isolated from a single *Pyricularia* species (Santalaceae) during a survey of the genus [62], as well as *Exocarpos cupressiformis* [63], and *Stericulia foetida* [64]. Recent reports have shown it to be more widely distributed at low levels in members of the families Santalaceae (*Jodina rhombifolia*) [65] and Olacaceae (*Ochanostachys amentacea*) [66]. A preponderance of trans-alkenes is peculiar to compounds derived from **5A**, including **5B–K**. Leaves of *Nanodea muscosa*, a Patagonian herb used in folk medicine, contained the atypical acetylenic fatty acids **5L** and (13*E*)-octadecen-11-yneic acid (**5M**), with known ene-diyneic acid **5D** [67]. El-Jaber and coworkers proposed that **5M** forms from *cis*-vaccenic acid (18:1^{11c}) by a Δ¹¹-acetylenase followed by a cationic allylic isomerization (Fig. 7B). Compounds resulting from analogous rearrangements are widespread and their formation may be catalyzed by a lipoyxygenase-like species. Soybean lipoyxygenase contains a high oxidation potential pentacoordinate ferric hydroxide that initiates a proton-coupled electron transfer prior to oxygen binding [68]. It is plausible that a variant enzyme, deficient in peroxy radical formation, may be capable of a proton rebound reaction catalyzing the formation of enynes such as **5M**. This is shown figuratively in Fig. 7B, although other hydrogen atom donor/acceptor pairs may be involved including tyrosyl radicals or iron-oxo species. In each of three more recent studies, a variety of more highly unsaturated metabolites presumably derived from stearolate were quantified (e.g., **5J** and **5K** from *J. rhombifolia* and **5H**, **5I**, and **5M** from *Heisteria sylvanii*) [65,69,70].

Sterculic acid (**5O**), the 9,10-cyclopropenyl derivative of stearic acid, accumulates to 55–78% of the total seed lipids in *S. foetida* (Fig. 7C). The C₁₇ homolog malvalic acid (**5Q**) occurs at approximately 10% of the level of **5O**. Both compounds have been isolated with and chemically synthesized from stearic acid (**5A**) [71], but it has been known for some time that **5O** and **5Q** biochemically arise from oleic acid rather than **5A**. Because of the recent isolation of genes involved in **5O** and the co-existence of **5O** and **5A**, sterculic acid formation will be described here. Yano showed that [¹⁴CH₃]methionine, [1-¹⁴C]oleate and [1-¹⁴C]dihydrostericulate were converted to [¹⁴C]stericulate in a number of *Malva* species [72]. These experiments suggested

that formation of the double bond after the generation of the three-membered ring in dihydrostericulate (**5N**) completed the biosynthesis of the cyclopropenyl fatty acids. The timing of chain shortening, presumably by α -oxidation, was not determined but free fatty acids were the proposed substrate. Bao and coworkers isolated the cyclopropyl fatty acid synthase (*Sf*CPA-FAS) from *S. foetida* using an EST screening approach, where 0.4% of the sequenced plasmids from a library of developing *S. foetida* seed DNA contained a gene related to bacterial cyclopropyl fatty acid synthase [64,73]. The carboxy-terminal portion of the 864-aa *Sf*CPA-FAS showed 32% identity (122 of 376 residues) with the bacterial synthase. The 438-aa N-terminal domain of *Sf*CPA-FAS contained a hypothetical flavin-binding site (GXGXGXXA) and was most similar to *Arabidopsis* tryptophan 2-monooxygenase; the function of this domain has not been determined. In *Mycobacterium tuberculosis* and *Agrobacterium tumefaciens*, related flavin-binding proteins appear to be encoded 200 and 802-nt, respectively, upstream of the cyclopropyl fatty acid synthase genes [73]. The *Sf*CPA-FAS activity was localized to the *S. foetida* seed microsomal pellet and, with this membrane fraction, *S*-[¹⁴C]methionine and [1-¹⁴C]oleic acid were shown to be substrates [64,73]. The product **5N** was almost exclusively found in the PC fraction of microsomal assays, and in a subsequent investigation, it was shown the oleoyl groups in the *sn*-1 position of PC are likely the direct substrate for cyclopropane formation, a position unprecedented for ER-localized lipid modification reactions in plants [73]. Expression of *Sf*CPA-FAS in tobacco cells did not result in the formation of C₁₇ cyclopropanes or any cyclopropenyl fatty acids. The enzymes responsible for desaturation of **5N** to a cyclopropene and the chain-length-modifying activity remain to be discovered.

Given the formation of the acetylenic and cyclopropenyl fatty acids in *S. foetida*, it would be interesting to know if the compounds are related by a common diverged desaturase that can carry out both dehydrogenations. In any case, whether the desaturation occurs at the *sn*-1 or *sn*-2 position of PC, or whether it is linked to a different acyl carrier is not yet clear. While 91% of **5N** was found at the *sn*-1 position of PC in developing *S. foetida* seeds, **5O** was equally distributed between the *sn*-1 and *sn*-2 positions. As a result of these acyl preferences and differences observed in the location of the cyclopropanated fatty acids between mono- and di-CFA-containing TAG, the authors noted that multiple acyl transfers appear to occur during the journey to TAG [73]. The apparently anomalous features of this pathway suggest that substrates or chain specificities rare in normal fatty acid metabolism might be central to the formation of unusual fatty acids, including the acetylenic fatty acids.

In plants, the soluble Δ^9 -acyl-ACP desaturase, AAD1, normally introduces an initial 9:10-double bond into saturated acyl-ACP in the plastid and variants of AAD1 catalyze front-end dehydrogenations (dehydrogenation occurring between C-9 and the carboxyl group). Petroselenic acid **5R**, the Δ^6 -isomer of oleic acid, is widely distributed and occurs notably in members of the Apiaceae, Araliaceae, and Simarubiaceae families (Fig. 7D). A Δ^6 -palmitoyl-ACP variant desaturase from the endosperm of *Thunbergia alata* is known [74]. Details of the Δ^4 -desaturation of palmitoyl-ACP by a coriander enzyme related to AAD1 and elongation of the product by a 16:1^{4c}-specific KASA condensing enzyme to **5R-ACP** have been reported [75–77]. Structure-function studies have shown that within a series of Δ^9 -18:0, Δ^6 -16:0, and Δ^9 -16:0 desaturases, 2–5 residue substitutions can shift the regiochemistry or substrate preference to that of a different isozyme [34]. Additionally, two genes from burning bush *Bassia scoparia* (Amaranthaceae) were reported with 79.8 and 85.5% similarity to castor 18:0-ACP desaturase, the first of which is believed to be a variant with Δ^5 regiospecificity [78]. The identification of a novel acetylenase that accepts a monounsaturated fatty acid substrate, such as **5R-ACP**, as the direct precursor to tariric acid (**5T**) would extend the current paradigm for acetylenic fatty acid biosynthesis. Plant genera *Alvaradoa* and *Picramnia* belong to the newly formed family Picramniaceae and may provide a source of such a diverged ACP-desaturase, since petroselenic acid **5R** and the unusual fatty acids **5T** (58%), **5S** (1.4%), **5U** (trace), and

5V (15%) have been reported from *Alvaradoa amorphoides* [79,80]. The formation of **5U** likely occurs by the action of a Δ^6 -acetylenase on eicosenoic acid **5S**. A Δ^6 -acyl-ACP desaturase for *Picramnia pentandra* has been reported [81], although the action of an elongase and a Δ^4 -18:1 desaturase paired with either a Δ^4 - or Δ^6 -acetylenase remains conceivable.

Distinct from the higher plants, moss, ferns and liverwort produce arachidonic acid and acetylenic fatty acids by front-end desaturation (Fig. 8). *Ricca* spp. contain 18:2^{6a,9c}, **6B**, and **6C** in their lipid fractions [82]. The most studied organism, *Ceratodon purpureus*, stores polyacetylenic triglycerides in protonemata. [¹⁴C]18:3^{9c,12c,15c} (**2P**) and [U-¹⁴C] 18:4^{6c,9c,12c,15c} (**6D**) were converted to 18:4^{6a,9c,12c,15c} (**6C**) and EPA (**6E**) showing that the acetylenic bond is formed in a discrete step [83]. The sequence of desaturation events in moss was explored by Kohn [84]. When *C. purpureus* protonemata were incubated with radiolabeled precursors, it was demonstrated that γ -linolenate (**6A**) was converted to acetylenic fatty acid **6C** and dehydrogenation of **6D** led to **6C** and tetraenoic acid **6E**. The presence of Δ^6 -acetylenic fatty acids among the bryophytes has been put forth as support for the evolution of the moss genus *Fontinalis* and certain liverworts in the orders Monocleales and Marchantiales from a common ancestor linked through a Δ^6 -acetylenase progenitor [85].

Acetylenic groups were distributed primarily among TAG, DGDG in *Ricca duplex*, and a small amount of 18:1^{6a,9c} was present in MGDG [85]. Triple-bond containing acyl groups were completely excluded from the phospholipids [85]. Although acetylenic acid **6C** is probably accumulated in TAG as an energy reserve for the *C. purpureus*, it is not a precursor for polyalkenyl fatty acids as fed [¹⁴C]**6C** is disassembled and reincorporated as acetate into common fatty acids [83]. The effect of environmental factors upon lipid metabolism has been explored in the moss *Dicranum scoparium* [86]. Growth in light increased the production of **6C** in TAG and the acetylenic TAG reserves were preferentially diminished when culture conditions were switched to darkness. Pb(II) and, to a lesser degree, Cu(II) reduced the accumulation of acetylenic lipids. The results were consistent with increased desaturation/acetylenation when reducing equivalents are provided through photosynthesis and the potential inhibition of desaturases by heavy metals. Finally, the authors noted that unlike *C. purpureus*, no **6B** was accumulated and hypothesized that 18:3^{6c,9c,12c} was the substrate for the acetylenase in *D. scoparium* and certain other mosses.

While research has focused on Δ^6 -acetylenic lipids in moss, several monoacetylenic regioisomers 18:1^{6a}, 18:1^{9a}, and 18:1^{12a} are now known, as are a range of acetylenation sites in polyenoic acids (18:2^{6a,9c}, 18:2^{9a,12c}, 18:2^{9c,12a}, 18:3^{6a,9c,12c}, 20:3^{8a,11c,14c}, 20:4^{5a,8c,11c,14c}) [87]. The regiochemistry of desaturation and acetylenation in aquatic liverworts *Calliergon cordifolium*, *Drepanocladus lycopodioides*, *Fontinalis antipyretica* and aquatic mosses *Riccia fluitans* and *Pellia neesiana* appears to follow a x+3 pattern.

2.1.4 Other Desaturation Pathways—Certain lepidopteran moths produce acetylene-containing sex pheromones (Fig. 9) [88]. For example, a vineyard pest, the grape leafroller (*Desmia funeralis*), produces a mixture of 11-hexadecynal (**7C**), (Z,Z)-11,13-hexadecadienal (**7D**), and (Z)-11-hexadecenal (**7E**) and only the three-component blend is effective as a female sex pheromone [89]. The same acetylenic aldehyde has also been found as a pheromone component for *Heterocampa guttivitta* [90]. In the most studied genus, *Thaumetopoea*, the ability to form the acetylenic compounds **7A** and **7B** is not universal. The pheromone pathway is regulated by the Pheromone Biosynthesis Activating Neuropeptide (PBAN), whose presence allows the conversion of the enynic acid to the acetylated pheromone [91]. While labeling experiments have shown that *Thaumetopoea pityocampa* Δ^{11} -desaturates 16:0, installs the acetylenic bond, and then dehydrogenates C-13:14 in strict sequence prior to the modifications at the carboxylate group, *Thaumetopoea processionea* was found to produce only the corresponding 11,13-dienyl esters [92,93]. Formation of the dienyl acetate in *T.*

processionea also required hormonal activation by PBAN. The key differences between the pathways were interpreted to be the absence of a Δ^{11} -acetylenase in *T. processionea* and the presence of an enynic acid-specific reductase in *T. pityocampa*. It remains to be explored whether distinct proteins catalyze the Δ^{11} -desaturase and acetylenase steps or if a bifunctional enzyme is involved. In either case, the gain/loss of acetylenase activity is a critical factor in the species-selective pheromone signaling for both of these forest pests.

The wealth of diversity found among the microsomal diverged Δ^{12} - or FAD2 desaturases has placed them in the spotlight over the past decade. Yet, it is quite possible that front-end acyl-CoA desaturase or acyl-ACP desaturase variants are required enzymes in a number of unsaturated natural product biosyntheses. A sizeable number of acyl-CoA desaturases *sans* the *Cytb5* fusion are known from insects with Δ^{11} -regiospecificity (albeit yielding both (*E*)- and (*Z*)-FA isomers) [94,95]. Expression of one of the 13 *Cytb5*-desaturases from microalgae *Thalassiosira pseudonana*, TpDESN, in *Saccharomyces cerevisiae* showed a strict substrate specificity for the conversion of 16:0 to 16:1^{11c} [96]. Consequently, the microalgal desaturase allows one to wonder how many other organisms and enzymatic families beyond the known diverged AAD1, FAD2, and Δ^n ($n \leq 9$) desaturases might carry out atypical oxidative modifications to fatty acid derivatives. Given the number of novel fatty acid substrates encountered in secondary metabolism and continued detection of new desaturase isozymes, the possibilities for new regiochemical variations on desaturation-like reactions and functional diversity are still vast.

In passing, it is notable that there is a (single) example of an acetylenic fatty acid detected in a mammal. The urine of healthy adults contains 5-decynedioic acid and traces of the 4-isomer [97]. There have been no experiments reported that address the source of these compounds, but it is presumed that they have dietary origins.

2.2 Genetics of Acetylenic Bond Formation in Terrestrial Plants

Several major advances have been made in the cloning and characterization of acetylene biosynthesis genes. Because of the wealth of knowledge concerning lipid metabolism in higher plants, these organisms have been the initial access to the biochemistry of acetylenic natural products. It had been long noted that desaturases were the most probable general enzymatic system for alkyne bond formation. Initially, the application of T-DNA tagging to *A. thaliana* allowed the cloning of *FAD2*, the endoplasmic reticulum (ER)-localized oleoylphosphatidylcholine Δ^{12} -desaturase [98]. The Stymne group made the decisive leap by employing a cloning strategy that targeted Δ^{12} -desaturase-like genes from developing seeds of the flower *Crepis alpina*, which accumulates in excess of 60% crepenynic acid as triacylglycerol. In 1998, they isolated a gene encoding a Δ^{12} -acetylenase and, in doing so, confirmed the desaturase hypothesis [99]. Further design of cloning experiments has regularly used the existing knowledge of lipid metabolism, aerobic desaturation and conserved amino acid sequence data for the plant microsomal desaturases to screen for gene candidates.

2.2.1 Cloning and Expression of the Lipid-Desaturating Components of Polyacetylene Biosynthesis—The Asteraceae species *C. alpina* and *Crepis palaestina* contain approximately 70% crepenynic acid (**2E**) and 60% vernoleic acid (**2Q**), respectively. Microsomal preparations of seeds from the *Crepis* sp. were capable of the NADP(H)-dependent formation of the corresponding [¹⁴C] unusual fatty acids from [¹⁴C]oleate [99].

Acetylene- and epoxide-forming reactions were expected to involve similar chemistry to desaturation. The Stymne lab used polymerase-chain reaction to clone gene fragments from *C. alpina* and *C. palaestina* seeds that were similar to other plant membrane-bound desaturases [99]. The full-length variant desaturase gene from *C. alpina* encoded a 375-amino acid protein, dubbed Crep1, with 56% identity to *A. thaliana* microsomal oleate desaturase FAD2. Crep1

contained at least four hydrophobic domains, which in the model of FAD2 enzymes function as transmembrane segments and three histidine (His) box motifs (GHECGHHAFS, SHRXHH, THVXHHLF). It had been previously shown by mutagenesis that the His box domains were absolutely required for the function of stearoyl-CoA desaturase [100]. When expressed in *S. cerevisiae* with exogenous linoleic acid, Crep1 produced crepenynate that was detected at $\leq 0.3\%$ of TFA. Even at this low abundance, the identity of the crepenynate was detectable as the diethylamide derivative. In yeast, which are only capable of providing saturated and monounsaturated glycerolipids as substrate, an octadecadienoic acid was produced demonstrating the bifunctional desaturase/acetylenase activity of Crep1. The fact that the 18:2 in the transgenic yeast was not a substrate for Crep1 acetylenase activity was mysterious; it was possible that cellular localization impeded access to the linoleate, the 18:2 may have been present in an acyl derivative that was unsatisfactory as an acetylenase substrate, or the level of 18:2 was simply insufficient to produce detectable amounts of **2E**.

Transient *de novo* biosynthesis of unsaturated fatty acids at extramicrosomal sites, such as the plastid, may be relevant during fungal infection and is apparently linked to polyacetylene biosynthesis. In *Petroselinum crispum*, the fungal elicitor peptide Pep25 has been shown to stimulate rapid 18:3^{9c,12c,15c} (α -linolenate, **2P**) production and, concomitantly, decrease 18:2 levels over a 2-hour period in cultured cells, protoplasts, and plant leaves [101]. α -Linolenate is a necessary lipid for the biosynthesis of the octadecanoid jasmonate in plants, a signaling component involved in defensive response and development, while polyunsaturated fatty acids generally provide an oxidative sink to consume reactive oxygen species [102]. The induction of ω -3 desaturation upon fungal infection may correlate with the production of increasingly desaturated metabolites, particularly late in the polyacetylene pathways, and serve as a responsive mechanism that integrates polyacetylene biosynthesis throughout a plant.

One functionally uncharacterized gene from *P. crispum*, *ELI12*, is upregulated by Pep25 [103]. The predicted translation of the gene sequence revealed that *ELI12* contained the hallmarks of a FAD2 desaturase: two pairs of transmembrane domains separated by two histidine box motifs with a third histidine box in the final carboxy terminal segment. However, unlike most oleate desaturases *ELI12* displayed no function during yeast expression. Cahoon and coworkers expressed *ELI12* in developing soybean seed embryos, which accumulated crepenynate and (14Z)-dehydrocrepenynate and thus uncovering a role for *ELI12* in the formation of acetylenic fatty acids from endogenous linoleate [104]. The source of the apparent Δ^{14} -desaturase activity was not defined, but recent studies from our lab indicate that an endogenous FAD2 activity may be involved (R.E. Minto, unpublished results).

One of the major advances during the past decade has been the exponential expansion of DNA sequence data through cost-reductions and improved technologies. With this, there has been change in mind-set that valuable information can be efficiently gleaned from judiciously selected EST and large-scale sequencing endeavors. Within the study functionally assigning *ELI12* as an acetylenase, the comparison of two EST sequences and five genomic PCR products with published nucleotide sequences made evident three evolutionarily distinct groups of diverged desaturases: the desaturase/hydroxylases, the Asteraceae epoxygenases and acetylenases, and the acetylenases from Apiaceae and Araliaceae [104]. The schism between the acetylenases is clear; at the protein level, Apiaceae/Araliaceae sequences showed <60% identity with their Asteraceae counterparts while maintaining 88–94% identity within each group. The regulation of acetylenase expression appears to correlate with fungal defensive response through a range of species. In addition to *ELI12*, a functionally characterized acetylenase from *Sclerotinia sclerotiorum*-challenged *Helianthus annuus* (sunflower) flower heads was cloned and other instances of viral-induced diverged FAD2s are known from Solanaceae species [104,105]. Other pathogen-responsive *P. crispum* genes likely to be acetylenases have a novel cis-regulatory element and are not upregulated in response to

wounding, jasmonate, ultraviolet light, or ethylene [106]. How acetylenic secondary metabolism is integrated into pathogen response and understanding the selectivity of the defensive mechanism still poses many interesting questions.

The factors that affect the levels of unusual fatty acids accumulated by the expression of acetylenases and epoxygenases have been examined. When real-time RT-PCR was used to quantitate transcription of several *FAD2* isoforms, *FAD3*, and acetylenases in *C. alpina* and *Helianthus annuus*, it was observed that while the *Crep1* gene is expressed in flowers, the level of gene expression is 10^4 -fold lower than in developing seeds and that competition with **2P**-producing *FAD3* for 18:2 substrate may limit the biosynthetic activity of the acetylenase in the flower (refer to Fig. 4) [107]. A higher demand for **2D** as the substrate for any diverged *FAD2* desaturase would, on first view, be expected to result in the coordinate depletion of linoleate levels. In a separate study, modest seed-specific expression of the *C. palaestina* Δ^{12} -epoxygenase (*Cpal2*) gene in *Arabidopsis* seeds led to the accumulation of **2Q** (4.2–7% of TFA), greatly reduced amounts of 18:2 and surprisingly higher levels of 18:1 versus wild-type seeds [108]. Two potential factors considered for the increase in oleate, the substrate for *FAD2*, were homology-dependent gene silencing of endogenous *Atfad2* and homology-based protein-protein interaction. However, no significant changes in *Atfad2* transcript levels were noted. In an attempt to raise **2Q** yields, co-expression of *C. palaestina* Δ^{12} -desaturase (*Cpdes*) with *Cpal2* in *A. thaliana* increased 18:2 levels above that of the wild-type line. In seeds homozygous for *Cpdes* and *Cpal2*, **2Q** levels rose to maximally 9–10% of TFA highlighting substrate availability as one factor controlling diverged desaturase activity [108, 109]. A second potential mechanism for the reduced levels of 18:2 in *Cpal2* seeds may be translational or post-translational interactions between the epoxygenase and *FAD2* desaturase. Limiting levels of 18:2 were concluded not to be the primary cause of the lower levels of accumulation of **2Q** in transgenic *Arabidopsis* versus levels observed in *C. palaestina*.

In both *C. alpina* and *H. annuus*, accumulation of acetylenic fatty acids was not observed in flower tissue even though gene expression was detected [107]. Specific breakdown of unusual fatty acids in vegetative tissue has been advanced as the reason for the absence of expected novel fatty acids in transgenic plants and may be relevant in limiting the presence of atypical fatty acids, including acetylenic species, particularly outside of developing seeds [110,111]. Phospholipid acyl hydrolases may direct acetylenic fatty acids to either degradation or productive natural product anabolism [112]. Apparently, four levels of control situationally affect detectable acetylenase activity: transcript numbers for acetylenases, unsaturated substrate availability, product degradation, and targeting by lipid speciation. Identifying the functions and control mechanisms of so-called “cryptic” acetylenase expression, those pathways that do not seem to produce any acetylenic or acetylene-derived products, is an essential, yet poorly explored, aspect of acetylenic secondary metabolism.

The chemoselectivity of the diverged desaturases is amazing; each member of the class produces either a methylene-skipped alkene, conjugated polyen(yne), alkyne, epoxide, or alcohol as its chief enzymatic product. Unfortunately, unlike the soluble Δ^9 -stearoyl-ACP desaturase [32,33,36], no crystallographic data has been reported for the membrane-bound desaturases. Consequently, models of the microsomal enzymes are based solely upon primary sequence data, mutagenesis studies, and immunochemical experiments. In the absence of crystallographic data, identifying specific determinants of the chemical path has been difficult. For *A. thaliana* *FAD2*, residues 148 and 324 were shown to modulate hydroxylase and desaturase activity [113]. While tempting, drawing analogies about the composition of the diiron active site, reaction intermediates, and mechanistic details of microsomal desaturase from the plastidal desaturases should be made with trepidation. Some fundamental differences exist between the soluble and microsomal enzymes. For example, the cryptoregiochemistry of the hydrogen abstraction in the soluble Δ^9 -desaturase family, which favors abstraction of the

pro-(*R*) hydrogen distal to the carbonyl group (e.g., H-10*R* in castor desaturases) independent of regiochemistry [114], is opposite to the membrane desaturases, which remove the pro-(*R*) hydrogen proximal to the carbonyl group. The model currently used for the active site is that of a compound Q-like oxidant based on the more developed enzymology of soluble methane monooxygenase [115]. Recommended overviews of desaturation systems and the current mechanistic models have been written by Shanklin & Cahoon [116] and Buist [117], respectively.

While it is a conversion that initially seems unlikely, the known enzymatic formation of acetylenic bonds occur by the direct net dehydrogenation of *cis*-alkenes (Fig. 2A). Desaturative acetylenic bond synthesis cleaves two strong 107 kcal/mol (448 kJ/mol) vinyl C-H bonds with the formation of a strikingly weaker 64 kcal/mol (268 kJ/mol) C-C π bond [118]. The 150 kcal/mol (628 kJ/mol) enthalpic deficit of alkyne formation is well offset by net 4 e⁻ reduction of oxygen to water, which includes the consumption of one equivalent of NADPH. The electrons provided by NADPH oxidation are routed through the auxiliary proteins NADPH-cytochrome *b*₅ oxidoreductase and cytochrome *b*₅.

The kinetic isotope effects (KIE) for the abstraction of H-12 and H-13 during the *C. alpina* acetylenase catalyzed desaturation of linoleate have been recently measured. A k_H/k_D ratio of 14.6 ± 3.0 was found at C-12 consistent with a primary KIE and a smaller secondary KIE of 1.25 ± 0.08 was measured at C-13 [119]. Several conclusions can be made from this data: alkyne formation involves asynchronous C-H bond cleavage and the bond breakage is initiated at the carboxyl-proximate carbon C-12. Following the initial abstraction, either cationic and radical species may be involved in the dehydrogenation but experimental evidence characterizing the intermediate is lacking. Cleavage of the C-13-H bond should be rapid, as it is weakened by the neighboring electron-deficient center. For a radical-like intermediate, the reduction of the C-H bond strength at C-13 has been calculated to be approximately 71 kcal/mol (297 kJ/mol) [118]. The large primary KIE exceeds the nominal values predicted by transition state theory (k_H/k_D 6.9–7.5) suggesting the involvement of quantum mechanical tunneling. This effect had been reported by Abad and coworkers for an $\Delta^{9,11}$ -acyl-CoA conjugase, but many microsomal desaturases exhibit tunneling-free values, so the significance and generality of this effect is not known [120].

Perhaps one of the most striking aspects of the acetylenic bond formation in crepenynate is the absence of epoxidation activity. Epoxidizing reagents commonly initiate reactions through the interaction of π -electrons with an electrophilic oxygen. The ring strain inherent in the conversion of an alkene to an epoxide is handily offset by the sacrifice of a weaker C=C π bond for stronger C-O bonds. Strict positioning of the alkene π and C-H bonds at the desaturation site may limit the approach trajectory of the active oxygen species to the exposed hydrogens, as is implied in Fig. 10. Alternatively, changes to the electronic states of the iron centers or the involvement of a distinct activated oxygen intermediate may cause a shift in the balance toward hydrogen abstraction from epoxidation. These general factors have been used to explain in the control of cytochrome P450 reaction outcomes [121].

The production of trans fatty acids has emerged as a common attribute of the acetylenases. When Crep1 was expressed in *S. cerevisiae* in the absence of exogenous 18:2, a diastereomeric mixture of (9*Z*,12*Z*)- and (9*Z*,12*E*)-18:2 isomers was produced (0.18 – 0.47% of TFA) [122]. Production of 18:2^{9c,12t} was observed when Crep1 was expressed under a seed-specific promoter in *A. thaliana* wild-type and *fad2* mutant lines (2.3 and 3.5% of TFA, respectively). A higher level of 18:1 in the *fad2* *A. thaliana* seeds expressing Crep1 correlated with the higher 18:2^{9c,12t} load suggested that Crep1 had *trans*-desaturase activity with 18:1. A fungal acetylenase recently expressed in our lab has exclusively Δ^{12} -desaturase activity when expressed in the absence of linoleate (B.J. Blacklock and R.E. Minto, unpublished data). In all

examined cases, lipid conformations allowing *syn*-abstraction of the two hydrogens by desaturases appear to be active (Fig. 10A) [123]. We propose that the stereochemical flexibility apparent through the *trans*-desaturase chemistry results from the changes to the binding pocket for an acetylenase necessary to accommodate the sp^2 to sp linearization as 18:2 is converted to crepenynate (Fig. 10B). In *Spodoptera littoralis*, a mixture of alkene stereoisomers is observed for Δ^{11} -desaturase and $\Delta^{10,12}$ -conjugase activities with myristate and (11*Z*)-tetradecenoate, but not with the corresponding C_{16} substrates [124]. The model proposed by Serra *et al.* involves two distinct binding pockets for the desaturase activity, where C_{14} substrates can bind in two modes resulting in the diastereomeric products and the longer substrate homologs can be accommodated only in the large pocket (Fig. 10C, D) [124]. The exposure of the reactive carboxyl-proximate hydrogen is expected to align the C-H σ orbital with the approaching oxygen electrophile. For Crep1, the methyl terminus of bound oleate may partition between two distinct binding modes, which rationalizes the production of both 18:2 isomers. As 18:2^{9c,12c} is converted to crepenynate, in the absence of a conformational twisting of the alkyl chain, a binding arc of nearly 60° is required to allow the repositioning of the ω -end of the acyl groups. In contrast to the desaturases and hydroxylases, a smaller glycine replaces alanine at the amino acid immediately preceding the first His box (His box Ia) in the known acetylenases. This residue may relieve steric hindrance in the ensuing acetylenase chemistry.

In 2000, Sperling and coworkers cloned, sequenced and heterologously expressed a bifunctional Δ^6 -desaturase/acetylenase and a Δ^6 -desaturase from *C. purpureus* [125]. Degenerate primers based upon Δ^5 - and Δ^6 -acyl lipid desaturases and Δ^8 -sphingolipid desaturases were used in PCR reactions to clone prospective gene fragments. Full length CPDes6 and CPAcet6 genes coded for 520 and 483-aa proteins, respectively. One of the most striking features of the acetylenase domain of CPAcet6 is its low similarity to any other moss, fungal, plant, worm or mammalian Δ^6 -acyl desaturases. Comparison with known front-end desaturases showed the presence of an amino-terminal cytochrome b_5 domain in both *C. purpureus* polypeptides, including the diagnostic HPGG heme-binding motif. The three histidine box regions in the desaturase domain contain seven conserved histidines and the last motif starts with an invariant glutamate, QXXHHLFP. A functional difference between CPAcet6 and the Δ^{12} -acetylenases is the ability of the moss acetylenase to produce its own Δ^{6c} -unsaturated substrate, rather than the non-viable *trans*-fatty acids that are produced by acetylenases from other organisms.

In summary, the model for the formation of crepenynate fits within the existing paradigm for alkene-producing desaturases. Substrate binding favors cisoid conformations, either through the stereochemistry of the (*Z*)-alkene or the arrangement of two methylene groups in an eclipsed conformation, and the chemistry proceeds via an asynchronous oxidative dehydrogenation. As a consequence of the growing family of fatty acids produced through pairings of desaturase and acetylenase reactions, it is reasonable to assume that additional acetylenase variants will be found.

2.2.2 Roles of Acyl Conjugation in the Secondary Metabolism of Acetylenes—

A conundrum emanating from the studies of the *Crepis* acetylenase, the related FAD2-like hydroxylase and MCFA-related fatty acid biosynthetic genes is the biological mechanism that restrictively targets unusual fatty acids to TAG while maintaining the integrity of membrane compositions (i.e., “normal” glycerolipids derived from 16:0, 18:0, 16:1, 18:1, 18:2, and 18:3). By what means do plants, fungi, and mosses (in particular, as these organisms can accumulate high concentrations of acetylenic lipids) channel atypical fatty acids and avoid catastrophic changes to the vital structures and functions of membranes and associated polypeptides? Within the glycerol-3-phosphate pathways, enzymatic specificities in the thioesterases [126], acyltransferases, cytidine diphosphocholine-diacylglycerol choline phosphotransferase (CDP-

CPT) and phospholipases (PLs) each have been shown to have potential for exerting control on acyl chain flux (Fig. 3). Mechanistic insight provided by pathways seemingly peripheral to alkyne biosynthesis, those at the heart of primary lipid metabolism, may foreshadow studies of secondary metabolic flux for the polyacetylenes. The following enzymes provide mechanisms for targeting and speciation of acetylenic lipids.

Lysophosphatidic Acid Acyltransferase (LPAAT): For an LPAAT in *Cuphea*, it has been observed that the *sn*-1 group on the phosphatidic acid acceptor strongly influences acyl selectivity at the *sn*-2 position, with the bias in this case favoring the incorporation from CoA derivatives with similar fatty acyl chain lengths. For example, isoforms of LPAAT selectively generate 10:0/10:0- and long-chain phosphatidic acids [127]. With the decanoyl lipids segregated, the action of a 10:0/10:0-specific DAGAT completes the channeling of these medium-chain lipids to tricaproin.

CDP-CPT: As the enzyme that controls the levels of DAG and PC via a reversible transfer of phosphocholine, CDP-CPT could mediate the conversion of PC containing unusual acyl groups, including acetylenic fatty acids, to DAG. This would, in turn, promote the channeling of these atypical fatty acids to TAG, particularly in plants, such as *Crepis* sp., and fungi, such as *Cantharellus* sp., that accumulate acetylenic TAGs. While CDP-CPT is an appealing branch point for acyl lipids, *in vitro* studies for VLCFA, MCFA, and hydroxy fatty acid-producing plants indicate that CDP-CPT is of minor importance as it has little acyl group selectivity [128].

Phospholipases: Phospholipase A₂ (PLA₂) activities in plants that produce unusual fatty acids are often high for the uncommon acid. In organisms that do not routinely produce oxygenated acyl groups, hydroxy fatty acids seem to be cleaved from PC by PLA₂ through a housekeeping mechanism associated with the elimination of unselectively oxidized and peroxidation-degraded acyl groups from membranes [129].

Transacylation: Transacylation, catalyzed by enzymes that are outside of the standard glycerol-3-phosphate pathway, have been shown to have potential for the direct transfer of unusual acyl groups to TAG. Phospholipid diacylglycerol acyltransferase (PDAT), mediates the transfer of acyl groups from PC to DAG with the net result of producing TAG and lysoPC. Selective transfer was shown for 18:1-OH (**2R**) over 18:1 from the *sn*-2 position of PC with PDAT isolated from *Ricinus communis* [130]. The elimination of the 18:1-OH from PC without returning it to DAG is an attractive mechanism for accomplishing the observed ricinoleate exclusion from membrane lipids in castor bean. A cloned and functionally characterized *Arabidopsis thaliana* PDAT (*AtPDAT*) was found to use both PE and PC, to prefer transferring groups from the *sn*-2 position over *sn*-1, and to favor shuttling short chain, oxygenated, and unsaturated acyl groups [131]. *AtPDAT* transferred acetylenic groups from crepenynoyl PC at approximately half the rate of 18:2 [131]. Balancing the metabolite flux between the cytoplasmic acyl-CoA pool and glycerolipids is crucial in very long chain (VLC)-PUFA biosynthesis in transgenic linseed, as Δ^6 -elongation occurs on CoA [132]. PDAT and diacylglycerol acyltransferase (DGAT) occlude substrates from the pool of desaturable lipids through TAG incorporation, while equilibration by lysophosphatidylcholine acyltransferase (LPCAT) and the action of LPAAT have the potential to increase the available polyunsaturated glycerophospholipid substrates [133]. The exact role of *AtPDAT* remains an open question; it may control the distribution of oxygenated acyl groups formed during membrane repair or it may gate the production of lysophospholipid in signaling pathways [131]. For secondary metabolism, it is quite probable that the enzymes that regulate the lipid pool composition (PC, CoA, free FA) will be found to strongly control the extent and/or regiochemistry of acetylenation/desaturation processes leading to polyacetylenes and be at the heart of issues faced in acetylenic lipid/natural product metabolism in transgenic plants.

Beyond these central glycerolipid metabolic enzymes, thioesterases, the segregation of pathway components, and metabolite catabolism may provide the control elements that direct the flow of unusual lipids. For early steps in linoleate-derived polyacetylene biosynthesis, it seems unlikely that thioesterases limit pathway flux, as microsomal desaturation and acetylenation occur on PC. However, the extent of chain-shortening for C₉–C₁₆ polyacetylenes (see below) may be partially coupled to the thioesterase releasing acetylenic β -oxidation intermediates.

For terminal products of the polyacetylene biosynthetic pathways, levels of polyacetylenes are often transient and tissue specific. For most instances, it remains to be determined if specialized vesicles act as repositories for the potentially toxic natural products, if polyacetylene precursors are locally retained and converted to mobile species upon appropriate stimuli, and if a passive, systemic transport of the molecules is tied to efficient degradation of the polyacetylenes after their usefulness has been exhausted. For example, futile cycling of ricinoleate and vernoleate has been proposed as a mechanism for disposing of acyl chains excluded from membranes, reducing the lipids to polyhydroxyalkanoates [134]. β -Oxidation may catabolize the unusual fatty acids impacting attempts to design of transgenic plants for renewable sources of new seed oils and polyacetylene secondary metabolism alike, when acetylenic fatty acids or thioesters are not efficiently targeted for storage in TAG or converted to more refractory metabolites (e.g., fatty alcohols or alkanes).

2.3 Elaboration of Acetylenic Natural Products

Distinctive polyacetylenes that result from the elaboration of the monoacetylenic compounds are found throughout the kingdoms, but are most prevalent in higher plants. Sorensen has reviewed the fatty-acid derived polyacetylenes of Asteraceae and highlighted several trends [135]. Composite flowers are nearly the exclusive source of cyclic polyacetylenes, both of the aromatic types – benzene, furan, thiophene, and coumarin ring systems – and the non-aromatic forms – pyrans and spiroketals. Certain Asteraceae species produce compounds that contain no acetylenic bonds, such as simple thiophenes, but almost certainly have the enzymatic machinery for acetylenic bond formation because the thiophenes have been established to originate from alkynes. With rare exceptions, all other families of plants and the Basidiomycete fungi produce linear polyacetylenes. Chemotaxonomic analysis has been used as confirmation of phylogenetic relationships and to construct models for the chemical genesis of metabolites; a series of papers by Christensen and coworkers cover the most recent systematic efforts in this regard [13–17,136]. For example, most of the screened Asteraceae in Heliantheae, Cynareae, Astereae and Anthemideae produce acetylenic natural products (9200 species and 524 genera are known; 15% and 41% of the species and assigned genera, respectively, have been investigated). The tribe Heliantheae produces enepentayne, dienetetrayne, and enetetrayne conjugation patterns that serve as precursors to the thienyl metabolites, but Anthemideae and Astereae make shorter conjugation patterns and no thiophenes. The thiophenes may correlate with acetylenase activities in Heliantheae that lead to longer extended π -systems. Additionally, enzymatic systems for sulfur addition may be limiting; Astereae has not been found to make thioether, sulfoxide or sulfone metabolites. In a second case, Anthemideae and Heliantheae are the only two tribes within the family that make alkamides, suggesting that one or more element of alkamide biosynthesis is absent in the remainder of Asteraceae. Molecular genetic studies can be expected to uncover the origins of the Composite polyacetylenes, a research area with good potential given the breadth of distribution and number of biosynthetic trends in this plant family.

It has been previously noted that many of the specialized reactions that occur during plant lipid secondary metabolism are subtle variations upon primary metabolic transformations, resulting in products that are capable of integration into the existent biochemical framework [137]. For

example, the ability to catalyze chemically similar transformations in taxonomically distant organisms, such as Δ^{12} -acetylenation in Asteraceae and Apiaceae (described above), suggests that evolutionary pressures have led organisms to acquire this trait independently. In the case of the acetylene biosynthesis, the enlistment of unsaturated glycerolipids in the crepenynate pathway together with a primary metabolic Δ^{12} -desaturase as the likely acetylenase progenitor comprise a possible example of biochemical symbiosis. In this Section, the intermingling, or potential for it, between fatty-acid derived polyacetylene biosynthesis and established biochemical transformations from other pathways will be examined. In sharp contrast, prokaryotes exchange, through horizontal transfer, gene clusters, cassettes with ready-made multi-gene biosynthetic pathways that often provide the recipient with a suite of unusual biogenetic capacities. Cases involving such genetic modules in the biosynthesis of acetylenic natural products will be explored in Section 2.4.

2.3.1 Chain Length Adjustment—For the most part, it appears that the desaturative chemistry to the acetylenic derivatives precedes the reduction of fatty acyl chain lengths since shorter homologs of oleic acid possessing fewer methylene groups between the alkene and carboxyl group were not incorporated into C₉–C₁₅ acetylenic natural products [48]. Chain length adjustment may proceed by α , β , and ω -oxidation.

2.3.1.1 β -Oxidation— β -Oxidation has been invoked for the chain-shortening of virtually all polyacetylenes with backbones C₁₆ and shorter. Methyltrienoic fatty acids are presumed to be shortened to C₁₄ and C₁₃ fatty acid and alcohols (**8A–8D**) via either two β -oxidation steps or two β -oxidations combined with an α -oxidation (Fig. 11A) [12]. However, in no case has the metabolic course of these two-carbon chain adjustments been experimentally shown for an acetylenic natural product. Peroxisomal β -oxidation is involved in indole-3-acetic acid and jasmonate plant hormone biosyntheses [138–140]. Bohlmann has proposed that 3-hydroxyoleic acid (**4I**), normally a β -oxidation intermediate, is connected to the falcarinol and dehydrofalcarinol pathway in Araliaceae via a β -elimination process (Fig. 6C) [54,141].

2.3.1.2 α -Oxidation—While α -oxidation has not been directly shown with acetylenic substrates, substantial progress has been made recently in plants and algae in identifying the enzymatic players. Likely scenarios include direct α -oxidation of fatty acids and deviations from normal β -oxidation reactions prior the retro-Claisen fragmentation (Fig. 11B). Acyl decarbonylation and reduction pathways from plant cuticular wax formation are included here as they are rational alternatives leading to C₁-shortened alkanes, fatty aldehydes and alcohols. The pathways outlined below are expected to encompass the C₁ loss mechanisms for many of the odd-chain acetylenic fatty acids.

In the first case, direct stereoselective α -oxidation of fatty acids leads to (*R*)-2-hydroperoxy acids that spontaneously collapse to aldehydes with the expulsion of CO₂. Originally shown with the diatom *Thalassiosira rotula*, the (*R*)-stereochemistry of the 14:0 to 16:0 2-hydroperoxides (≥ 99 %ee) have been shown *in vitro* for extracts of *Ulva pertusa*, *Ulva conglobata*, and several red and brown algal species [142,143]. Enzymes related to prostaglandin endoperoxide synthase-1 and -2 have been implicated in the α -oxidation of linolenic acid (**2P**) and (13*S*)-hydroxylinoleic acid [144]. These α -dioxygenases initially produce (2*R*)-hydroperoxy acids (**8E**), which can undergo non-enzymatically reductive fragmentation to a fatty aldehyde chain-shortened by one carbon (83%, **8I**) and (2*R*)-hydroxy fatty acid (15%, **8G**), respectively, as well as a small amount of the C₁₇ fatty acid homolog. In tobacco leaves, the products are 2-hydroxy C₁₇ fatty acid (90–95%), 8,11,14-heptadecatrienoic acid (5–10%), with the aldehyde being undetectable. In plants, several α -dioxygenases are present which have distinct expression patterns linked to either biotic or abiotic stress. For a concise survey of plant α -dioxygenases, a current review by Hamberg is available [145].

Pea α -dioxygenase has been demonstrated to be bifunctional, possessing peroxidase activity dependent upon the availability of a suitable reductant [146]. Additionally, both peroxiredoxins and peroxygenase have wide substrate specificities that are capable of reducing hydrogen peroxide, alkylhydroperoxides, and oxidized amino groups. In plants, four peroxiredoxin classes are dispersed among the nucleus, chloroplasts, and probably the peroxisomes [147]. While their normal physiological function may be to protect biological macromolecules and to protect cells from hydrogen peroxide that escapes the action of catalase, concurrent function of the α -dioxygenases and their reducing partners has the potential to produce the lower polyacetylenic homologs [148]. It is not clear at this time in what organelle(s) reduction occurs nor which molecular species are the preferred substrates.

Developments in the area of plant cuticular wax biosynthesis have been reviewed recently, and include good metabolic alternatives to the chemistry discussed above [149]. Fatty acyl-CoA reductases (FARs) enzymatically produce, without carbon loss, fatty alcohols and/or fatty aldehydes (**8M-N**), which are functional group modifications commonly seen in lipid-derived acetylenic natural products, from acyl-CoAs. *A. thaliana cer4* mutants lack the ability to produce C₂₄ to C₂₈ fatty alcohol, and the cloned FAR gene led to the production of C₂₄ and C₂₆ VLC alcohols when expressed in yeast [150]. From pea, 28-kDa and 58-kDa enzymes were isolated by size-exclusion and affinity chromatography that showed FAR activity capable of generating aldehydes and alcohols, respectively [151]. A potential congener of the pea enzyme, WAX2 from *A. thaliana*, has a sterol/fatty acid desaturase-like domain at its amino-terminus and bears similarity to the SDR family of dehydrogenases (NAD(P)H oxidoreductases) toward the carboxy end [152]. *wax2* mutants produce greatly minimized levels of hydrocarbons and aldehydes in the stem and leaf cuticular waxes. As one of its activities, WAX2 may produce intermediates for the synthesis of alkanes (**8J**). Finally, reduced methyl termini are common in many polyacetylenes, including the falcarinol derivatives (Fig. 6). It is not yet clear whether they form through decarboxylation followed by alkene reduction or directly. The FAR-produced aldehyde intermediates could be chain-shortened by a process distinct from α -oxidation. As an activity that could support a direct decarbonylative reaction, CER1 has been suggested to be an aldehyde decarbonylase in *Arabidopsis*, however, the assignment of this activity is far from clear [149,153]. Biochemical activities for aldehyde decarbonylation have been clearly observed in preparations from *P. sativa* leaves and the green alga *Botryococcus braunii* [154,155]. The plant enzyme appears to require Cu(II), while the algal form needs Co(II) and porphyrin or corrin. In both cases, provided [¹⁴C]octadecanal led to heptadecane and ¹⁴CO being released without the need of a nicotinamide cofactor.

The loss of C₁ units from fatty acid thioesters also occurs, but the scope of this chemistry outside of plant wax biosynthesis is not yet clear. In one instance, a methylketone synthase found in the wild tomato *Lycopersicon hirsutum* is responsible for the decarboxylation of β -ketoacyl-CoA species (**8L**) central to the ubiquitous acetyl-CoA liberating β -oxidation process to the carbonyl derivative **8H**. [156]. Decarboxylation of β -hydroxyacyl derivatives **8K**, confusingly referred to as α -oxidation in the literature [15,17], has been repeatedly suggested for C₁ loss from polyacetylenic intermediates presumably leading to alkenes (**8F**), yet no biochemical proof has been provided beyond experimental incorporation of **4I** into polyacetylenes [12].

2.3.1.3 Intra-chain oxidation—A second distinct pathway in macroalgae and plants is critical to the formation of fatty aldehydes. Intrachain oxidation yields hydroperoxide derivatives capable of cleavage to short chain aldehydes. Hydroperoxide lyase has also been solubilized from brown algae, which cleaves (9*Z*,11*E*,12*S*)-12-hydroperoxy-9,11-octadecadienoic acid to hexanal, in a manner analogous to higher plant wounding and pest responses [157,158]. Cell lysates from the green alga *U. conglobata* produce midchain (9*R*)-hydroperoxides from **2C** and **2P** [159], but cleave arachidonic acid via a (11*R*)-hydroperoxy

intermediate to (2E,4Z) and (2E,4E)-decadienal [160]. Control of the cleavage reactions is exerted by strict substrate specificity, as shown by the action of partially purified *Laminaria angustata* hydroperoxide lyase on (13S)-hydroperoxy and (15S)-hydroperoxides of linoleate and arachidonate, respectively [157]. Other activities, such as a heme-c containing protein, may be involved in the production of racemic 9- and 13-hydroperoxides in certain algae [161]. In silica-less diatoms, such as *Phaeodactylum tricornutum*, 9- and 12-lipoxygenases and a hydroperoxide lyase convert primary metabolic arachidonic acid, EPA and C₁₆ PUFAs in TAG to toxic aldehydes and alkenes in response to herbivores (examples described in [162]). Although speculative, it is through these processes that certain short-chain acetylenic fatty aldehydes may originate from TAG, independent of α - and β -oxidation.

Mid-chain cleavage of nascent polyacetylenes has been proposed to occur by a Bayer-Villiger like oxygen-insertion reaction (Fig. 12A). It has been attributed to Jones that chain-shortening to the C₉- and C₁₀-methyltrienes (e.g., **1A**) may be formed from ⁹-desaturated lipids by oxidation of a keto intermediate **9A** to **9B** [12]. Sequential 9-lipoxygenase, dehydrogenase, and Bayer-Villigerase activities may account for the formation of an optically active glycerol triester **9C** in Euphorbiaceae seed oils from the invasive Chinese tallow tree *Sapium sebiferum* [163], *Sebastiani ligustrina* [164], and recently *Sebastiania commersoniana* [165]. The allene, which is found in the *sn*-3 position, could be formed by the isomerization of a Δ^6 -acetylenic bond. While rare, other polyacetylenes have previously been detected in this plant family [166].

Rare vinyl ether metabolites maracin A (**9D**) and maracen A (**9E**) that exhibited activity against the non-pathogenic indicator species *Mycobacterium phlei* were isolated from *Sorangium cellulosum* strains So ce880 and So ce1128 (Fig. 12B) [167]. Labeling studies with [1,2-¹³C₂]acetate showed a contiguous series of nine intact acetate units terminated by a methyl-derived acetate carbon at C-1 of **9D**. A Hock rearrangement similar to that involved in divinyl ether synthesis [168], has been proposed that is initiated from a 6-hydroperoxide species and followed by an allene-acetylene isomerization cumulatively attaching the oxygen atom to the acetylenic ether and fracturing the unsaturated chain.

2.3.2 ω -Modification of acetylenic fatty acids—Compounds found ubiquitously in plant families are strong indicators of the interrelationships between species and have been used extensively for chemotaxonomy [12,169,170]. One trend, the presence of a 3-hydroxy or 3-oxohept-1-ene-4,6-diyne ω subunit, is characteristic for all compounds from Araliaceae (Fig. 13). The metabolic order of the reactions leading to these ω -modifications is not yet certain; even the mere existence of the enzymatic components remains obscure. The action of a terminal desaturase may directly provide acetylenic compounds such as **5J–K**, **10H–I**, and a number of marine natural products to be described later. A recently reported Δ^{12}/Δ^{15} -desaturase from *Acanthamoeba castellanii* produced a *n*-1 fatty acid 16:3^{9c,12c,15c} [171], which heightens the likelihood that falcarinol derivatives remain as glycerolipids (**10A–I**) until the chain-end desaturation occurs in final stages of biosynthesis (Fig. 13). With modern approaches like T-DNA insertions, gene knockdowns and metabolomics, the identification of the genetic loci and enzymatic activities that underlie taxonomic correlations has now become an attainable target.

ω -Oxidation of fatty acids by cytochrome-P₄₅₀ enzymes occurs widely and has major relevance in cutin and suberin biosynthesis [172,173], protection against postgenital organ fusion [174], and other aspects of plant development. Related CYP₄₅₀s with specific reductant requirements have been found in *Fusarium oxysporum* [175]. NADPH is the required reductant for a terminal hydroxylase from wheat microsomes, whereas an NADH-requiring CYP₄₅₀ that favors ω -1 to ω -3 hydroxylation of medium-chain fatty acids is known from etiolated wheat shoots and *Helianthus tuberosus* [176,177]. Organisms generally harbor many P₄₅₀ isozymes,

some promiscuous and others that appear to have restricted functions. For example, in *Petunia hybrida*, CYP76B9 expressed in flowers and leaves catalyzed ω -oxidation of capric and lauric acids [178]. From the same plant, CYP92B1 is transcribed in early flower development and converted lauric, linoleic and linolenic acids to primary alcohol-bearing fatty acids, which seem to be important to fertilization [179]. Specific CYP₄₅₀ homologs are expected to mediate the corresponding chemistry in acetylenic natural product metabolism. In contrast to pathways where allylic and propargylic alcohols may spontaneously eliminate water, elimination of either a primary or secondary alcohol would presumably necessitate a specific dehydratase to provide a thermodynamically less favored monosubstituted alkene. In a case potentially relevant to the formation of terminal acetylenic C₉-alkamides in Heliantheae, CYP94A5 from tobacco catalyzed the ω -hydroxylation of C₁₂–C₁₈ saturated and unsaturated fatty acids, with a preference for 9,10-epoxystearate, and the expressed enzyme was capable of oxidizing the terminal methyl group to a carboxylic acid in yeast microsomes [180]. α , β -Acetylenic carboxylic acids are well known to readily decarboxylate non-enzymatically leading to terminal acetylenes and this process is believed to occur in numerous instances (e.g., oxidative conversion of **3G** to **3F** in Fig. 5 and terminal acetylenic alkamides). Cytochrome P₄₅₀-dependent fatty acid hydroxylases from plants has been recently reviewed [181].

Other mechanisms for the introduction of oxidative functionalities in acetylenic fatty acids include C-H activation at allylic or propargylic positions catalyzed by enzymes other than lipoxygenases and diverged desaturases, singlet-oxygen-mediated ene reactions, alkene/allene epoxidations, and (reversible) alkene/alkyne hydration [12]. In the carotenoids, allene oxidase appears to be critical, although this conversion is not known outside of algae. The accessibility of fast, low-cost genome sequencing and gene-chip transcript analysis hold promise for fettering out these specialized activities, particularly as the arrays of C₁₆–C₁₈ dehydrogenative modifications become established.

2.3.3 Sulfur Addition—Sulfur, a heteroatom commonly introduced into polyacetylenes, is found in ecologically significant thiophenes and dithienes. Naturally occurring sulfur-containing heterocycles and thioethers, first identified in the 1940s, are produced by Asteraceae and fungi [3,12]. For the >150 reported thiophene derivatives, marigolds (*Tagetes* spp.) are one of the major producers. Structure variation lies in the number of rings (1–3) and the degree of unsaturation of the side chains (ene/yne) (Fig. 14A).

Marigolds were initially flagged for their invulnerability to root-knot nematodes and, as early as 1956, it was recognized that interplanting or rotation of marigolds with selected crops provides an environmentally sound protection against soil nematodes [182]. Field testing has shown that tobacco plants benefited from two years of protection in plots that had grown *Tagetes patula* (French marigold) or *Tagetes erecta* (African marigold) cultivars [183]. One of the most active antinematodal agents was identified as α -terthienyl (α -T, **11Ch**) [184]. A hydrogenated derivative of the thiophene BBT **11Ca**, 5-butyl-2,2'-bithienyl, was also found to control these nematodes. A survey of 175 Asteraceae species showing that 67 organisms completely suppress the lesion nematode *Pratylenchus penetrans*; the number of nematode-resistant organisms positively determined to contain sulfur heterocycles was 15, 15, and 12, when analyzed for thiophenes **11Ca** and **11Ch** and thiorubrine **11Dj**, respectively. Toxicity requires soil irradiation indicating that the heterocycles are protoxins. Additional naturally occurring and synthetic linear acetylenes tested for nematotoxicity have been described by Chitwood [185]. Compounds that tend to be most active are conjugated, often bearing photoactivating carbonyl and aryl groups.

Thiophene derivatives are found in all tissues of marigolds. The complexities of investigating the biosynthetic spectrum of marigold thiophenes in plant tissue culture have been commented upon in several instances. The differences between differentiated tissue cultures and callus

were investigated in *T. patula* [186]. The major source of bithenyls **11Ca**, **11Ce**, **11Cg**, and **11Ch** is root tissue, irrespective of whether the root is cultured or part of an intact plant. The total thiophene production is substantial in root culture and intact plant roots, however only traces of **11Ca** and **11Ce** are found in callus. Throughout the plants, the proportions of thiophenes differ widely. In achenes, no thiophenes are found and in the shoots, the major compounds are **11Cc** and **11Ce** with only traces of **11Ca** present. α -T, which is present in the root but not detectable in shoots, accumulates in flowers. Despite many experiments, it remains to be shown whether the thiophene metabolites originate exclusively in the roots and specific species are preferentially accumulated among different plant parts, or whether enzymatic components of the thiophene pathway are expressed in a tissue-specific manner.

Reaction stoichiometry suggests that an equivalent of H₂S is incorporated through addition reactions to produce the thiophenes. Cysteine may be the proximate sulfur carrier. In marigold, sulfide is not directly integrated into **11Ch**, however sulfur derived from both Na₂³⁵SO₄ and [³⁵S]methionine are added [187]. The slow response of root cultures when the sulfate level is raised in low sulfur medium has been used as support for the molecular rather than metabolic regulation of thiophene production [188].

Labeled oleate and acetate sources result in low isotope incorporation in marigold. To circumvent these difficulties, the biosynthesis of 5-(3-buten-1-ynyl)-2,2'-bithiophene **11Ca** was examined through [U-¹³C]- and [1-¹³C]glucose feeding experiments with root cultures of *T. patula*. High glucose incorporation allowed retrobiosynthetic analysis, a method that accurately tracks label distributions within primary metabolites. These experiments confirmed the involvement of acetate or a related 2-carbon homologating unit culminating in the biosynthetic scheme shown in Fig. 14B [189]. The demethylation of bithienyl derivatives has been explored by feeding [³⁵S]-labeled **11F**, **11Ca**, **11Cb**, and **11Cl** and it was found that methyl cleavage occurs prior to the formation of the second thiophene ring (Fig. 14C) [190]. Additionally, **11F** was not incorporated into α -T (**11Ch**), bringing into question the order of sulfur additions leading to this important metabolite.

2.3.4 Alkamides—Alkamides are fatty acid amides that are usually unsaturated and frequently contain acetylenic bonds. The nearly 200 reported alkamides result from the apparent combinatorial ligation of alkyl amines apparently derived from amino acids with short- and medium-chain fatty acids [191]. The acetylenic fatty acyl groups vary in length from C₉ to C₁₈. Extracts of the purple coneflower (*Echinacea purpurea*) have insecticidal, immune-system-stimulating, and anti-inflammatory properties that are attributed to the acylamides [192]. Alkamides have been shown to affect root growth and morphology in *Arabidopsis* indicating a potential role in development [193].

Very limited studies exist on the biosynthesis of alkamide metabolites but, in the case of *E. purpurea*, it was shown by Bohlmann *et al.* that crepenynate pathway metabolites [10-¹⁴C] **2A** and [16-¹⁴C]**2I** could be incorporated into **12B** at 0.05% efficiency and that anacyclin (**12A**) is a likely precursor to the **12B** (0.1% incorporation of [12-¹⁴C]**12A**) (Fig. 15) [194]. *In vitro* germinated seedlings metabolized [¹⁵N]valine as detected by enrichment of alkamides in *E. purpurea* [195].

2.4 Acetylenic Polyketide and Non-Ribosomal Peptide Biosynthesis

A large number of microbial and marine polyacetylenes are polyketides or peptidic in origin. Over the past 15 years, there has been an explosion of studies examining the molecular biochemistry of PK biosynthesis; a recent splendidly written review was penned by Hill [196]. The modification of PKs through module shuffling and chimeragenesis has the potential for the production of a wide array of bioactive PKs. While marine-derived polyacetylenes are distributed through a range of organisms, most are concentrated in certain red and green algae

and the marine sponges. It appears that a number of the isolated polyacetylenes are actually produced by symbionts or are consumed in diet. The genetic and biochemical organization of alkyne-yielding PK and NRPS pathways will be delineated below.

2.4.1 Eneidyne Antibiotics

2.4.1.1 Structural Subtypes: The enediynes natural products are a class of PKs which can thermally cyclize to biradical intermediates when they are actively triggered or uninhibited. These reactive intermediates, when appropriately intercalated in the minor groove of ds-DNA through a glycoaromatic recognition element, aggressively cleave DNA with sequence specificity by hydrogen atom abstraction and subsequent reaction of the DNA-derived radicals with dissolved oxygen [197]. Although the lack of tissue specificity and high toxicities of the enediynes natural products has precluded their direct use as pharmaceuticals, the development of calicheamicin-monoclonal antibody conjugates and polymer-bound neocarzinostatin derivatives has led to marketed antitumor agents [198,199].

A variety of cyclization triggers are present within the enediynes antibiotics, varying from strained epoxy and aziridino groups to labile trisulfide linkages. When an appropriate triggering reaction occurs, access to a cyclizable intermediate is allowed. For example, nucleophilic attack upon labile substituents of certain C₉ cycloenediynes generates a reactive allene-ene-yne functionality that can be cyclized via a Myers–Saito cyclization to a σ , π -toluene diradical species (Fig. 16A). Stabilizing apoproteins that non-covalently bind the chromophores prevent premature cyclization and have been found with each of the nine-membered cycloenediynes [200]. For neocarzinostatin (**13A**) and related structures, conversion of the natural product to the enyne allene intermediate increases the ring strain for the triggered species to 15 kcal/mol above the σ , π -diradical energy [201]. Cycloaromatization can occur to a 2,6- or 2,7-diradical; the latter regioselectivity is believed to be favored for systems with a small, fused cyclopentane ring [202]. In the case of the C₁₀ enediynes, cleavage of the trisulfide linkage with a reductant, such as glutathione, causes a Michael addition to a constraining cyclohexenone ring (Fig. 17A) [203]. The bridged system transduces ring strain to the enediynes via the σ -bonding framework, often decreasing the distance between the acetylenic termini. While disagreement remains as to the exact structural change that initiates the Bergman cycloaromatization that forms the *para*-benzyne 1,4-diradical (Fig. 17A), calculations generally support ground-state destabilization as the controlling factor [204].

Five discrete enediynes 9-membered cores (chromophores) (**13A–E**) and an equal number of 10-membered cores (**14A–G**) have been identified, each of which are diversified through complex polysaccharide and aromatic targeting domains (Figs. 16 and 17). Both terrestrial microbial and marine invertebrate sources have been identified for enediynes with identical chromophores. Namenamicin **14C** [205] and shishijimicins A–C (**14D–F**) [206], isolated from the ascidians *Polysyncraton lithostrotum* and *Didemnum proliferum*, respectively, and calicheamicin **14A**, procured from the actinomycete *Micromonospora echinospora* [207], differ only in the level of glycosylation and the presence of the aromatic orsellinic thioester (R₃, Fig. 17B).

The biosyntheses of neocarzinostatin (**13A**), dynemicin A (**14G**), and esperamicin A₁ (**14B**) were probed between 1989 and 1993 (Fig. 18A) [208–210]. The head-to-tail connectivity provided grounding for either PK or fatty acid biosynthetic hypotheses. Cultures of *Streptomyces carzinostaticus* grown with [¹³C]-labeled acetates and radiolabeled bicarbonate, [³H₃]methionine and acetate provided the incorporation patterns for **13A** [208]. Of the three labeling patterns considered, the acetate labeling supported only connectivity that results from the cleavage of two acetate units with the loss of a methyl and carboxyl groups. Through the feeding of [¹³C]-labeled acetates, [¹⁵N,2-¹³C]glycine and [¹³CH₃]methionine to *Micromonospora chersina* cultures, **14G** was shown to potentially arise from the coupling of

two heptaketides – the anthraquinone and the enediyne-bearing moieties [209]. The *O*-Me group was labeled by both methionine and glycine. As a result of the isotopic incorporation pattern, it was hypothesized that two heptaketides were connected between C-8 and C-9 via a Friedel-Crafts-like acylation [209]. A common progenitor hypothesis to **13A**, **14B** and **14G** was initially suggested where scission of a C₂ unit from an octaketide would occur (or C₄ from **2E**) (Fig. 18B) [209]. For **13A** and **14G**, atypical desaturation between C-8 and C-13 of “crepenynate” would be required to gain the contiguous enediyne carbons. The effects of cerulenin inhibition on the production of **13A** were not ameliorated by added oleate as might be expected for a **2E**-derived enediyne. Finally, the carbons of the diyne units in **14G** were found to be derived from two acetates, whereas in **13A** they each arose from an intact acetate pointing toward two distinct intermediates leading to the **14** enediynes and a separate precursor to **13A**. Recent experiments seem to have resolved this incongruity through the identification of PK enediyne-producing gene clusters.

In contrast to the lack of evidence for plant polyacetylene biosynthetic gene organization, secondary metabolic pathways are typically clustered in microorganisms. Consequently, gene walking that begins at a confirmed gene locus has been used to clone a number of enediyne biosynthetic gene megaclusters. The means of identifying gene clusters has evolved as the knowledge of the biosynthetic origins of enediynes has increased. Initially, without a clearly defined target FAS or PKS for the enediyne antibiotics, transformations central to the production of the unusual deoxyhexoses found in these metabolites were used to gain entry to the gene clusters. Primers targeting conserved regions within the dNDP-glucose 4,6-dehydratases, enzymes shown by Bechthold to be readily cloned by virtue of their high primary sequence homology [211], have been used for PCR amplification of enediyne antibiotic clusters. Locating the nearly 100-kb, 74 ORF calicheamicin gene cluster (*cal*) was one of the successes of this approach [212].

Type I PKSs, those proteins where the enzymatic functions occurring during one round of chain elongation are carried by a single polypeptide, have two distinct architectures – iterative and modular. Iterative PKS enzymes found in actinomycetes are responsible for the 9- and 10-membered scaffolding rings that form the chromophoric cores of the enediyne antibiotics [213], and combinations of modular PKS domains with NRPS modules are responsible for compounds described in Sections 2.4.2 and 2.4.3.

The genetics and biochemistry of the nine-membered ring enediyne C-1027 (**13E**), a characteristic example of this group of natural products, will be outlined here. For **13E**, as with all known enediynes, the initially formed enediyne cores have been hypothesized to form by the homologation of an acetyl-CoA starter unit to seven malonyl-CoA precursors. The C-1027 gene cluster from *Streptomyces globisporus* possesses 67 ORFs extending over 85 kb and includes a single iterative PKS (SgcE) comprised of four domains (KS, AT, KR, DH) (Fig. 18C) [214]. Disruption and complementation experiments show necessity of SgcE for the formation of **13E** [214]. Enediyne PKSs do not possess enoyl reductase (ER) activities within their modules, which is consistent with the synthesis of a polyunsaturated PK. While the ACP domain is less clearly defined than in other PKSs and several functionally unassigned domains lie within the polypeptide, general structural conservation is found within the enediyne PKSs. The final PKS domain in SgcE is a 4'-phosphopantetheinyl transferase (PPTE) that may specifically activate the secondary metabolic ACP domain. To complete **13E**, the benzoxazolate, β-amino acid, and deoxyamino sugar are proposed to be synthesized via their respective biosynthetic precursors anthranilate, tyrosine, and glucose-1-phosphate and convergently assembled to the acetylenic core [214]. The functional need for several other genes at the *sgcAB* locus have been verified (*sgcA*, *sgcC1*, *sgcD6*) and *sgcC* has been shown to be an aryl hydroxylase, as its disruption results in the accumulation of deshydroxy metabolite **13F**.

For the calicheamicin biosynthetic cluster, 16 ORFs are associated with orsellinic and enediyne PKSs, 18 are involved in carbohydrate chemistry, 9 are regulatory, and 22 have functions that have not yet been discerned [212]. CalE8, the 1919-aa enediyne PKS involved in **14A** biosynthesis, directly aligns with SgcE and the two gene translations are 56% identical and 67% homologous [212]. By comparing the known enediyne PKS genes, two genetically distinct groups were found based upon ring size, but on the whole, the conservation at both the sequence and gene structure levels point to divergent evolution of all the PKSs from one ancient gene. This analysis was developed into a predictive model that was applied to the analysis of 12 unknown PKS sequences found in the genomes of cultured actinomycetes that were not known to produce enediynes. Seven of the organisms contained sequences that bore homology to the C₁₀-chromophore-producing PKSs and the remaining five possessed sequences that were related to the C₉-enediyne-yielding PKSs [215]. At this point, it appears that unassigned coding sequences in the gene cluster are responsible for the acetylenic bond formation in the enediynes.

CalE8 is also remarkably similar to the PUFA PKSs. The prokaryotic marine bacterium *Shewanella* produces eicosapentaenoic (EPA, 20:5^{5,8,11,14,17}) and docosahexenoic (22:6^{4,7,10,13,16,19}) acids and the eukaryotic microflora *Schizochytrium* accumulates EPA and docosapentenoic acid (22:5^{2,5,8,11,14}), both by anaerobic PK pathways. Four *Shewanella* open-reading frames contain KS, KS α , KS β , AT(A), AT, ER, multiple ACPs, and DH domains; similar domains are organized into three ORFs in *Schizochytrium* [216]. The “standard” methylene-skipped polyene arrangement in these FAs results from the action of 2,2- and 2,3-isomerase activities associated with the DH segments. This unexpected commonality between PUFA biosynthesis in these disparate prokaryotic and eukaryotic organisms could be attributed to horizontal gene transfer. Whether PKS-driven polyacetylene formation in marine bacteria and invertebrates originates from analogous genetic processes should be explored. The scope of the chemistry and the interrelationships among organisms merits further study, as will be clear from the examples in Section 3.1.4.

2.4.2 Sporolides—During the past 600 million years, metazoans have formed a symbiotic relationship with marine bacteria and many sponges are sources of polyacetylenic natural products. Straight-chain polyacetylenes were characterized by HPLC in primary cultures of the sponge *Xestospongia muta* that had structurally differentiated in a manner consistent with the native organism [217]. The polyacetylenes were ascribed to the presence of heterotrophic bacteria. Through the analysis of the KS domains of 19 sponge species, Fiesler and coworkers identified PKSs found in culture-independent sponge metagenomes that were attributed to symbiotic sponge bacteria [218]. In their study, 88% of the PKS sequences identified formed a class of sponge-related genes distinct from all previously reported PKS and FAS genes. To date, no similarity has been found between sponge metagenome PKSs and the calicheamicin iterative type I PKS [218].

Genomics, coupled with progress in culture of marine organisms, has unveiled the large diversity of secondary metabolic pathways in marine actinomycetes. The biosynthesis of the PK sporolides is a case in point. *Salinispora tropica* CNB-440 was found by Udvary, Moore, and co-workers to devote 9.9% of its 5.18-Mbp circular genome to major PKS, mixed PKS/NRPS, NRPS and siderophore-producing natural product gene clusters [219]. Most of the clusters are localized within a single quadrant of the genome, which also contains a high proportion of mobile genetic elements. Both 9- and 10-membered-ring-producing enediyne PKS gene clusters (*spo* and *pksI*, respectively) were identified in the *S. tropica* sequence that were not found in the prolific antibiotic soil actinomycete, *Streptomyces coelicolor* A3(2). The assignment of the genus *Salinispora* to the family Micromonosporaceae, from whose species **14A**, **13A**, and other enediyne natural products originate, is worth noting. The 50-kb *spo* cluster contains 39 ORFs hypothetically responsible for the synthesis of sporolides A and B (**15B–C**), aromatic hybrid natural products with origins in the PK and shikimate pathways (Fig. 19A).

The function of the *pksI* cluster remains to be determined, although the absence of genes required for deoxyhexose biosynthesis and attachment to the chromophore points is consistent with a yet-to-be determined cyclized enediyne metabolite. The similarity of the *spo* PKS (Stro2697) to maduropeptin PKS is consistent with a cyclization pathway involving prosporolide (**15A**). It has been shown that halides can attack 1,4-dehydrobenzene leading to haloaromatic products **15B** and **15C** [220] and it was earlier shown that cyanosporasides A and B (**15D–E**) from *Salinispora pacifica* CNS103 can originate via a similar halo enediyne cyclization (Fig. 19B) [221]. Consequently, it is proposed that following the synthesis of **15A**, the enediyne is non-enzymatically cyclized to form a *p*-benzyne intermediate that is trapped sequentially by Cl^- and a proton. The observation that the chemistry of the 9-membered enediynes can be subdivided into metabolites that undergo Bergman cyclizations (maduropeptin **13C** and kedarcidin **13D**) and those that proceed by the allene-ene closure is interesting. The Myers-Saito closure has long been recognized to have substantial polar character, whereby the mode of reactivity is solvent-dependent [201,222]. As a result, it is anticipated that polycyclic natural products from actinomycetes will be found that are formed by ionic Myers-Saito cyclizations of PKSs.

2.4.3 Jamaicamides—Jamaicamides A–C (**16A–C**, Fig. 20A) natural products of mixed PK-NRPS origin, were isolated from *Lyngbya majuscula* strain JHB in a brine shrimp toxicity screen [223]. These compounds had LC_{50} 's of 15 μM for cytotoxicity against human lung and mouse neuroblastoma cell lines, as well as possessing sodium channel-blocking activities. Gerwick and coworkers contributed structure elucidations, isotopic feeding studies, and the sequencing of the *Jam* gene cluster in 2004. The structures of these natural products contain a series of unusual features: a pyrrolinone, a vinyl chloride, and, in **16A**, a 1-bromoalkyne. $[1-^{13}\text{C}]$, $[2-^{13}\text{C}]$, and $[1,2-^{13}\text{C}_2]$ acetate, *S*- $[^{13}\text{CH}_3]$ methionine, L- $[1-^{13}\text{C}]$ and L- $[3-^{13}\text{C}]$ alanine, and $[^{13}\text{C}_3, ^{15}\text{N}]$ β -alanine were provided to the cyanobacteria in a feeding study to build a biogenetic framework and yielded the labeling pattern shown in Fig. 20B. The 58-kb gene cluster is comprised of 17 ORFs and is flanked by several likely transposase coding sequences (Fig. 20C). Each of the ORFs is transcribed colinearly, except *jamQ*. Based upon preliminary annotation, the cluster contains 6 distinct PKS modules; *jamE*, *jamJ*, *jamK*, part of *jamL*, *jamM* with *jamN*, and *jamP*; and 2 NRPS modules; part of *jamL* and *jamO* (Fig. 20C). ATP-PPi exchange rates for purified His₆-tagged acyl-ACP synthetase *JamA* were assayed in the presence of a series of C₄–C₈ and aromatic fatty acids. It was found that 5-hexenoic (75% r.a.) and 5-hexynoic acid (100% r.a.) exchanged most efficiently supporting desaturation and/or acetylation prior the PKS/NRPS reactions.

Potentially impacting the biosynthesis of the alkynoic fatty acid segment, *JamB* encodes a 321-aa polypeptide with 48% amino acid similarity to the *Saccharomyces cerevisiae* Ole1p Δ^9 -desaturase, but there are three surprising aspects to its function. Related Δ^5 -desaturases are known from plants, fungi, and humans, however the primary structure of *JamB* is distinct from each of these. The third His box domain in *JamB* aligns with the Δ^5 -/ Δ^6 -desaturases, where the consensus sequence is QXXHH [224]. The functional significance of variations in this signature domain is not known. There is no evidence for a Cyt-*b*₅ domain in *JamB*, implying that *JamB* relies upon “free” Cyt-*b*₅ for the influx of the 2 electrons. This is distinct from Ole1p, as free cytochrome-*b*₅ was not sufficient to restore function to a yeast expressing an amino-terminal truncation of Ole1p [225]. While there is no direct evidence, *JamB* appears to be a terminal desaturase. Finally, the apparent use of a free fatty acid or ligation of a C₆ starter to ACP prior to dehydrogenation is unexpected as desaturases most frequently accept CoA or glycerolipid substrates. Irregardless of the lipid speciation, clear examples of terminal desaturation are sparse. A recent study by Napier identified a terminal desaturation product [171], in addition to an earlier report of a terminal monoacetylenic acid **16E** that is presumed to form by dehydrogenation of co-occurring 10-undecenoic acid **16D** [226].

The potential terminal desaturase/acetylenase activity encoded by *jamB* may have direct relevance to the biosynthesis of the cyclodepsipeptides and many of the *Pellosia*-like long-chain acetylenic alcohols. Each of these compounds appear to be formed from a short-chain terminal acetylenic acid or could be assembled by a PKS using an acetylenic starter unit. JamB is the first example of a likely desaturase intertwined with a microbial PKS pathway. Probing for the colocalization of desaturase and PKS genes may lead to the identification of new acetylenic natural product gene clusters. An unexpected observation, given the numerous examples of self-resistance and transport genes associated with highly toxic PKS products, was that no gene(s) appeared to encode protection for *L. majuscula* against the effects of the jamaicamides. It has been suggested that jamaicamides may act specifically upon eukaryotic cellular functions of predators or competitors and the prokaryotic cyanobacterium is resistant through the absence of the jamaicamides' molecular target. An offshoot to this observation is that screening for the presence of self-resistance genes may not be generally useful in locating marine prokaryotic gene clusters.

Although gene disruption/complementation studies are not yet possible in *L. majuscula*, the sequential biological activities in the gene cluster could be assembled with the feeding data and two biochemical studies resulting in a compelling sequence of chemical steps. In the current proposal for the biosynthesis of **16A**, either 5-hexenoic or 5-hexynoic acid is activated with ATP by acyl-ACP synthetase homolog JamA, modified by JamB, and transferred to the acyl carrier protein JamC. By homology, JamD is weakly related to a cyanobacterial flavoprotein (21% identity over 250 of 683 residues); its activity remains to be determined. *jamE*, *jamF*, *jamG*, *jamH*, *jamI*, and possibly a portion of *jamJ* form a β -keto modifying gene cassette, which is responsible for the vinyl chloride side-chain. Claisen condensation of acetate or malonate with the growing PK chain produces a 3-hydroxythioester similar to mevalonate formation. Hydrolysis and decarboxylation coupled with dehydration, and potentially alkene isomerization, leads to the alkylidene side chain. A series of three consecutive acyl carrier protein domains may provide a conduit of way stations through which side-chain modification intermediates are shuttled. This ACP cluster is an arrangement previously observed in gene clusters for *pksX*, mupirocin, and leinamycin [227–229]. The timing and mechanism of both the alkene chlorination and alkyne bromination steps have not yet been defined. A good candidate for the chlorinating agent is JamE, whose sequence homology to the phytanoyl-CoA dioxygenase family aligns it with related enzymes involved in marine halogenated NRPS and PK-NRPS products (barbamide and syringomycin) [230,231]. Extension of the linear precursor continues during modules 2–8 with *C*- and *O*-methyltransferases located in modules 2 and 6, respectively. The high selectivity of the adenylation subunit in JamO for L-alanine, as predicted from the labeling study and by its similarity to an analogous adenylation subdomain in bleomycin [232], was demonstrated by an ATP-PPi exchange assay. A thioesterase in PKS-like module 8, which is most related to a NRPS TE domains from *Bacillus subtilis*, may offload the acid product to be acted upon by JamQ during the final cyclization to the pyrrolinone. Alternatively, JamQ (or JamP and JamQ in concert) acts to close the heterocycle and release the finished natural product.

2.5 Biosynthesis of Acetylenic Terpenoids

Green algae from the single-genus Caulerpaceae family are causing ecological damage by displacing other plants and animals over large swaths of the Mediterranean Ocean and along the southern California coast. Their incursion is, in part, due to the ichthyotoxic effects of acetylenic terpene dienolesters, such as caulerpenyne **17B**. In addition to triacetoxo **17B**, *Caulerpa prolifera* from Greece has yielded a broad assortment of fatty esters **17C** comprised of the C₁₄–C₂₀ saturated acyl groups and unsaturated acyl chains to 5,8,11,14,17-eicosapentenoate (Fig. 21A). In **17C**, the alkene has isomerized between the 2- and 3(3')-positions of the C-1 dihydro derivative of **17B**.

The biosynthesis of **17B** has been investigated in intact macroalgae [233]. Unialgal *Caulerpa taxifolia* isolates grown in artificial seawater cultures were presented sodium [1-¹³C]acetate and ¹³CO₂ while segregated in plastic tubes. While acetate was installed exclusively into the ester appendages, the ¹³CO₂ was uniformly incorporated the terpene backbone consistent with carbon dioxide fixation. This data corroborates isoprenoid biosynthesis via the mevalonate-independent MEP (**17A**) pathway (Fig. 21A) and the role of cytosolic acetate in the late-pathway tailoring reactions. There is now substantial evidence that all isoprenoids, both cytosolic (e.g., sterols) and plastidal (e.g., phytols) origins, found in members of the Chlorophyta class of green algae are formed exclusively by the MEP route [234]. The cytosolic mevalonate-mediated pathway appears to have been lost in Chlorophyta, while retained by ancestral flagellate green algae that are more closely allied to terrestrial higher plants. No experimental evidence exists for the formal dehydrogenative conversion of the farnesyl chain to the acetylene. Natural products with an alkene at the site corresponding to the acetylenic carbons in **17B** are known, suggesting a subsequent 2e⁻ oxidation [235].

Molluscs benefit by modifying dietary secondary metabolites to defensive allomones; analysis of certain lipids found in *Oxynoe olivacea* have shown them to be pure oxytoxin-2 (**17E**). The action of two lipases, LIP-1 and LIP-2, from *O. olivacea* on caulerpenyne **17B** have been investigated *in vitro* (Fig. 21B) [236]. LIP-1 concertedly hydrolyzes the enol ester and eliminates acetate and LIP-2 hydrolyzes the isolated enol acetate. The order of action is believed to be LIP-2 followed by LIP-1 *in vivo* leading to **17E**, although preoxytoxin-2 **17D** can be generated in cell-free extracts. Volvatellin (**17F**), found in the mantle and white mucus of the mollusc *Volvatella* sp. has been used to corroborate the metabolic processing of secondary metabolites by gastropods [237]. A pair of lactones **17G–H** that resemble *Caulerpa* metabolites were purified from a Caribbean sacoglossan during an ecological study [238].

The gelliosterols A-D (**18A–B**, **18D–E**), C₂₃ acetylenic cholesterol derivatives with variable oxidation states at C-7 and C-25, were isolated by Scheuer's lab (Fig. 22A) [239]. Earlier Steiner and coworkers had isolated a series of acetylenic (**18C**, **18F**) and C₂₃ alkenyl sterols from the sponge *Calyx nacaensis* [240]. The cyclopropene calysterol (**18G**) that may be formed through an alternate dehydrogenation channel was found with the isopropyl-bearing acetylenic sterols. A mechanism for acetylenic sterol biosynthesis has been proposed that invokes a formal retro-carbene addition reaction modeled on biosynthetic studies by Djerassi (Fig. 22B) [241]. The precursor 24H-isocalysterol provides the backbone for the formation of both methyl and isopropylacetylenic derivatives. Acetylenic sterols have also been reported from the terrestrial plant *Gynostemma pentaphyllum* (Curcubitaceae) [242]. It was proposed that dehydrogenation at C-22 leads to these plant sterols which possess longer side-chains.

A striking group of acetylenic quinones and hydroquinones **19A–O** bearing isopentenyl-derived side-chains are distributed between ascomycetes, hymenomycetes, and both marine and terrestrial Basidiomycetes (Fig. 23). Functional group diversity is scant, consisting of alkoxy and C₁ modifications to the aryl ring and, rarely, modification of the sesquiterpene unit. Representative compounds include eutypine (**19A**), eulatinol (**19E**), culpin (**19H**) from the ascomycete *Preussia* sp., and *Stereum hirsutum* metabolites **19G–K** [243–245]. The grapevine-attacking ascomycete *Eutypa lata*, the causative pathogen for “dying-arm disease”, produces a strain-dependant mixture of acetylenic phenols, chromenes and benzofurans [246, 247]. Aldehyde **19A**, originally purported to be the major virulence factor for the disease, is carried though the plant sap from fungally infected trunks and has been found to uncouple mitochondrial oxidative phosphorylation and causes proton leakage act as a cyclic protonophore [248,249]. Eutypine reductase rapidly detoxifies the acetylenic aldehyde leading to the alcohol eutypinol (**19B**) [250]. *Vitis vinifera* leaves from *Eutypa*-resistant vines were more proficient at reducing **19A**. From these leaves, eulatachromene (**19L**) has emerged as a

new, potentially more phytotoxic metabolite [247]. Also, through the examination of product mixtures and grape leaf chlorophyll loss assays, it is mixtures of **19A**, **19B**, **19E**, **19L**, and 2-isopropenyl-5-formylbenzofuran (**19M**) that seem to most contribute to pathogenicity. Siccayne (**19D**), the only antibiotic isolated thus far from a marine basidiomycete *Halocyphina villosa*, is also found in *E. lata* and the hymenomycete *Helminthosporium siccans* [251]. It is structurally similar to the bacteriostatic compound frustulosin (**19F**) from the terrestrial basidiomycete *Stereum frustulosum* [252–254]. Siccayne has cytotoxic activity which appears to derive from the blockage of nucleoside precursor uptake and it inhibits mitochondrial respiration in yeast [252,255]. Further information on the biological activities and synthesis of *Eutypa* metabolites can be found in a review by Jiménez-Teja [256].

The origins of aromatic eutypine skeleton, its derivatives and the chromene and benzofuran natural products have not been directly investigated in *E. lata*; however studies that may have bearing on the biosynthesis have been reported from other organisms. [257–259]. Feeding of [¹⁴C]- and L-[2-¹⁴C]phenylalanine and the use of phenylalanine ammonia lyase inhibitors with the flowering plant *Ageratum houstonianum* demonstrates that benzofuran **19M** likely originates from cinnamic acid via the shikimate pathway [260].

2.6 Biosynthesis of Acetylenes Originating from the Shikimate Pathway

Acetylenic natural products derived exclusively from the shikimate pathway are rare (Fig. 24). Diaryl metabolites **20A–C** are produced by *Asparagus officinalis* (Liliaceae) [261,262]. Analysis of “No Nuclear Overhauser Effect” (NNE) ¹³C NMR and mass spectra for **20A** and **20B** derived from [*d*₅-Ar], [1-¹³C], and [3-¹³C]phenylalanine metabolism in suspension-cultured cells led to the illustrated labeling pattern. Collectively, labeling studies support an intermediate structurally consistent with **20D** derived from a single phenylalanine unit as the source of the C₃–O–C₆(B-ring) portion. Phenylalanine decarboxylation provides the acetylenic C₆–C₂ unit. There have been no studies investigating whether a specific short-chain aryl acetylenase, or a series of specialized oxidative dehydrogenative processes are involved. Optically active acetylenic norlignan virgatyne (**22A**, Fig. 26), a potential shikimate-derived natural product, was identified with tannins and flavonoid sulfonates from *Phyllanthus virgatus* [263].

2.7 Acetylenic Alkaloids

While uncommon, acetylenic compounds are found among the myriad of azaspiro[5.5]undecane and decahydroquinolines alkaloids that stand as a formidable chemical defense in certain dendrobatid and mantelline amphibians [264]. A dietary hypothesis for the origin of poison-dart frog toxins was strengthened during the past decade by identifying the link between myrmicine ants and the accumulation of decahydroquinoline alkaloids in the amphibian's skin [265]. These heterocycles are related to frog-accumulated histrionicotoxin (**21A**) and gephyrotoxin (**21B**), which are high-affinity noncompetitive inhibitors of nicotinic acetylcholine receptors (Fig. 25A). The metabolic origins of **21A** and **21B** have been difficult to pinpoint [266]. Compounds **21D** and **21E** were found in both dendrobatid frogs and virgin queens of *Solenopsis azteca* collected from a Puerto Rican hillside, while captive frogs are known not to accumulate the alkaloid toxins. The sequestration of groups of alkaloids in skin glands that mirror ant secondary metabolites suggests that frog alkaloids may not be chemically modified by the frogs [267]; an observation which has found few exceptions [268]. The formation of the bicyclic 2,5-disubstituted decahydroquinoline (e.g. **21G** and **21H**) and histrionicotoxin (THX) ring systems is presumed to occur from enamine **21F** via electrophilic catalysis (Fig. 25B). C₁₉ *trans*-pyrrolidines, commonly found in the venom of myrmicine ants within the Western hemisphere, are considered the precursors to the acetylenic alkaloid lehmizidine **21I** via dihydro metabolite **21J** but to date, no lehmizidines or acetylenic pyrrolidines have been detected in ants (Fig. 25B) [269]. Even though the C₁₉ precursors to

the bicyclic metabolites appear to be related, *Dendrobates histrionicus* and *Dendrobates lehmanni* only produce either THX or lehmizidines, respectively. This is puzzling given that each species is distinct and lives at non-overlapping elevations so, if a single ant provides the alkaloid precursors, the consumed organism would need a much broader range. Whether acetylenic bond formation occurs in the amphibians or arthropods is unknown. The recent observation of defensive alkaloids from *Mantella* sp. poison frogs and *Tetramorium electrum* ants from southeastern Madagascar, including acetylenic 5,8-disubstituted indolizidine **21C**, has been used to support convergent evolution of defensive alkaloid acquisition and/or biosynthetic pathways between Malagasy ant species and Neotropical ants [268]. Given the local distributions of the frog/ant ecosystems, it is tempting to consider that the acetylenic compound may be produced by an ant symbiont or local flora and acquired by the ants for their benefit.

2.8 Use of Higher Plant Cell Cultures to Study Polyacetylene Biosynthesis

Agrobacterium rhizogenes-transformed hairy root plant cultures have been investigated on a number of occasions for the production of acetylenic natural products. While comprehensive coverage is not provided here, these systems have promise for the enhanced production of acetylenic metabolites useful in biosynthetic studies. Several examples briefly show the benefits of and experimental variables for hairy root culture.

Hairy root cultures of goldenrod (*Solidago altissima* L.) produced 45–93 µg/g fresh weight *cis*-dehydromatricaria ester (**1A**, Fig. 1) under dark conditions, which was 2–3 times the production from untransformed roots [270]. While the greenish roots cultured under illumination grew more rapidly, which was presumed to be due to hemiautotrophy, the production of the polyacetylenes was consistently lower. Light regulation can have either a positive or negative effect on polyacetylene production; hairy roots for *Acmella oppositifolia*, have enhanced growth and polyacetylene production when cultured with light [271]. Naturally grown *S. altissima* roots produced 303 µg/g fresh weight of **1A**; however, *in vitro* plants produced only 0.2–1.2 µg/g fresh weight **1A**, whether grown from axenic seed or regenerated hairy roots. It follows that other stimuli, as experienced by the wild-grown roots, may be required for maximal polyacetylene production. It had previously been observed that *Bidens sulphureus*, an Asteraceae species, mycelial extracts of *Pythium aphanidermatum* stimulated polyacetylene production [272].

A number of Campanulaceae metabolites have been produced using *A. rhizogenes*-transformed tissue (Fig. 26) [273–276]. *Platycodon grandiflorum* hairy root was found to produce *ca.* 19 times greater levels of lobetyol (**22B**) and 39-fold increase yields of lobetyolin (**22C**) with lobetyolinin (**22D**) when compared to the roots of intact plants [277]. Callus and hairy root cultures of *Pratia nummularia* produced the rutinoid pratialin-A (**22E**) and glycoside pratialin-B (**22F**), respectively [278].

For hairy root cultures of *Ambrosia maritima*, the production of thiarubrine A (**11Dj**) and two derivatives was increased by methyl jasmonate [279]. In the presence of methyl jasmonate, cell suspension cultures only produced the biosynthetic precursor pentaynene **11E** and at a level 9.6-fold lower than hairy roots.

3. New Natural Products

3.1 Acetate-Derived Structural Subtypes

3.1.1 Plant Fatty Acids—From higher plants, the isolation and characterization of new acetylenic compounds regularly appear in print. As mentioned in the Introduction, hard work from many labs during the extended period from 1890 to 1985 provided an extensive library of acetylenic plant metabolites. Below, an attempt has been made to give comprehensive

coverage of the novel and important structures reported since 1997. Compounds are organized by structure type or host plant taxonomy.

A diacetylenic 1,6-dioxaspiro[4.5]decane gymnasterkoreayne G (**23A**) was isolated from the aerial parts of *Matricaria aurea* (Asteraceae) and, along with four known gymnasterkoreaynes, was active in a transcription factor inhibitory screen of *Gymnaster koraiensis* leaf extract [280,281]. Elevated levels of the NFAT transcription factor has been linked to autoimmune responses and inflammation. While **23B** showed lower NFAT inhibition than the *threo*-diol-containing gymnasterkoreayne E (**23C**), the differential activities of **23B**, **23C**, and the epoxydiyne gymnasterkoreayne B (**23D**) illuminate the importance of the stereochemical arrangement of the oxygen functionalities in maximizing this inhibitory effect. Several of the gymnasterkoreaynes A-F exhibit anticancer activity (Fig. 27) [282]. The gymnasterkoreaynes are found with polyacetylenes **23F** and **23E**, the latter being their likely direct precursor (Fig. 27). Three new diacetylenic spiroketals (**23G–I**) were isolated from *Plagius flosculosus* and examined for cytotoxicity [283]. They were found to be less active against Jurat T and HL-60 leukemia cells than known compounds that contained two unsaturated rings. Reduced sensitivity of Bcl-2-overexpressing cells to these natural products suggested a mechanism of action involving the mitochondrial apoptotic pathway.

Acetylenic thiophenes are of interest for their insecticidal activities. Three new thienylacetylenes xanthopappins A–C (**24A**, **24C**, **24F**, Fig. 28) were purified from *Xanthopappus subacaulis* (Asteraceae) that have LC₅₀ values of 0.53–0.95 µg/ml when UV-activated against 4th-instar larvae of the Asian tiger mosquito [284]. Known thiophenes **24B** and **24D–E** were found in the same species.

Feeding experiments have indicated that C₁₃ acid **8A**, produced through several α - and β -oxidation chain cleavages, is the precursor to acetylenic coumarins in Asteraceae [12]. A series of carbanionic cyclization steps reminiscent of PK chemistry close the aromatic ring. Coumarins **24G–L** have been isolated from *Artemisia dracunculus* and *Chamaemelum mixtum* [285].

A number of new acetylenic glucosides have been identified and it appears that such complexly modified fatty alcohols are appearing in the literature at higher frequency, possibly as a result of advances in compound isolation and structure elucidation (Fig. 28). Carthamosides A₁ (**24M**) and A₂ (**24N**) were found with **24O** at low abundances in dried *Carthamus tinctorius* petals [286]. The simple glucosides bidenosides C and D (**24O**, **24P**) were purified from the aerial parts of *Bidens bipinnata* [287]. Both *Atractylodes lancea* (*so-jutsu*) and *Atractylodes ovata* (*byaku-jutsu*) are important medicinal plants from Japan, China, and Korea and are often prescribed without distinction. The 1-*O*- β -D-glucopyranoside of (2*E*,8*E*)-2,8-decadiene-4,6-diyne-1,10-diol (**24Q**) was extracted from rhizomes of *A. lancea* [288]. In contrast, *A. ovata* produces an unusual β -D-apiofuranosyl-(1 6)- β -D-glucopyranoside linked at the alternate 8-hydroxyl site **24R**, which together with the identified terpene natural products and phylogenetic analysis supports the assignment of *A. ovata* as a unique species. Additionally, a number of esterified diacetylenic alcohols have been isolated from a related species. Atractylayne (**24S**) and its 1-(3-methylbutanoyl) isomer **24T** have been extracted in copious quantities from *Atractylodes chinensis*, a traditional digestive aid and diuretic [289]. The (*R,R*)-enantiomer of dehydrofalcariol (**24U**) was reported for the first time, isolated from *Artemisia monosperma*, with the related C₁₀ triol in free and β -glucopyranosyl forms (**24V–W**) [290, 291].

New compounds continue to be identified from Araliaceae species, particularly the ginsengs (*Panax* sp.) (Fig. 29). From the dried roots *Panax quinquefolium*, PQ-1 (**25A**) and 3-oxo-PQ-1 (**25B**), which possess uniformly *R* stereocenters, were uncovered [292]. Through the analyses

of **25A**, **25B**, PQ-3 (**25C**), PQ-8 (**25D**), panaxytriol, panaxydol, acetylpanaxydol, and panaxydiol and synthetic stereoisomers, the growth inhibitory effectiveness of these metabolites was linked to the presence of a (3*S*)-chiral center. This unnatural configuration at C-3 decreased IC₅₀ values by 10-fold. An indole-3-acetyl ester of (3*R*,8*S*)-faltarindiol **25E** was purified with trienediyne **25F** from flower bud extracts of *Hedera rhombea* [293]. Ester **25E** inhibited the growth of dicotyledonous plants with effective concentrations (EC₅₀) values in the range of 3×10^{-5} to 9×10^{-8} M; faltarindiol and **25F** were ineffective. A dextrorotatory triol (**25G**) from *Cussonia barteri* related to antimycotic **25H** was active against Gram-positive bacteria [294]. Three new polyols with immunosuppressive activity were located in fractions from *Hydrocotyle leucocephala* (Apiaceae) [295]. The saponin polyacetyleneginsenoside-Ro (**25J**) inhibited the replication of HIV-1 cells [296]. Faltarindiol (**4C**) was the most active constituent assayed in *Angelica pubescens*, a Chinese traditional treatment for arthritis, inhibiting 5-lipoxygenase with an IC₅₀ of 0.4 μM [297]. The (3*S*,8*S*) and (3*R*,8*S*)-isomers of **4C** were shown to have antimycobacterial activity and to be active against multi-drug resistant strains of *S. aureus* [298,299]. Acetoxy modifications found in the known Apiaceae acetate **25I** and oplopandiol acetate (**25H**) from *Oplonanax horridus* increase selectivity towards *Mycobacterium* spp.. Faltarindiol ethers with furanocoumarins, named japoangelols A–D (**25J–M**), are novel structures with the potential for enhanced contact toxicity [300]; both polyacetylenes and furanocoumarins in Apiaceae species are known to cause dermatitis. Among these metabolites, faltarindiol had greater *in vitro* inhibitory activity against human gastric adenocarcinoma cells than any of the ethers **25J–M** [301].

Among the higher plants, an increasing number of novel structures have been reported from families other than Asteraceae, Apiaceae and Araliaceae. Two unusual long-chain tetraynoic acids (**26A–B**) with a pattern of unsaturation reminiscent of the stearolic acid pathway were found in the fruits and stems of *Mkilua fragrans* (Annonaceae) (Fig. 30) [302]. From the same family, *Mitrephora celebica* produces **26D** and oropheic acid (**26C**), which are more saturated C₁₈ acids that seem to be biosynthetically related to **26A–B** [303]. Acid **26C** has been isolated once previously [304]. The furan-containing very long-chain acids, hydrocarbons and esters **26E–H** were separated from air-dried roots of *Polyalthia evicata* [305]. The methyl esters of **26E** and **26F** showed cytotoxicity against *Plasmodium falciparum* and *M. tuberculosis*, respectively.

Pentagonia gigantifolia roots contained **5T** (Fig. 7D) and 9-nonadecyenoic acid [306]. Both compounds were effective in antifungal assays surveying fluconazole-susceptible and resistant *Candida albicans* strains (IC₅₀ 0.25–0.90 μg/ml; amphotericin B 0.23 μg/ml). Dihydrospingosine out-competed the inhibitory effects of the acetylenic acids in a sphingolipid reversal assay, suggesting that interference with sphingolipid biosynthesis may be the mode of action of these simple acetylenic acids.

Minquartynoic acid (**26I**), of interest for its toxicity to the protozoans *P. falciparum* and *Leishmania major* [307], anti-HIV [308], and antitumor properties, was purified from *O. amentacea* with two related C₁₈ acids (**26J–K**) (Fig. 30) [309]. Acid **26I** has also been identified in two related Lauraceae species [310,311]. Eneidyenoic acids with C-8,10 acetylenic bonds, and a triynoic acid with the additional triple bond at C-12 (**26M–O**) and 6-acetylenic acid (**26L**) were found in *Heisteria acuminata* bark cyclooxygenase bioassay-guided purification [312]. These compounds may be related through α- and β-oxidation chain-shortening, although **26L** appears to be derived from a C₁₇ precursor. *Scurrula atropurpurea*, a plant used in folk medicine as a cancer treatment, provided the known triynoic acid **26M** with the more saturated C-10 analogs **26N–O** [313].

There have been several reports of Lauraceae metabolites possessing terminal acetylenic groups. Ketone **26P** was found with acids **26Q–R** in the bark of *Litsea rotundifolia* (Lauraceae)

[314]. Unripe avocado (*Persea americana*) fruit were found to contain the antimycotic 1,2,4-trihydroxyheptadec-16-yne (**26S**) with the corresponding saturated and 16-alkenyl triols [315]. Acetylene **26S** has an (*R,R*)-configuration deduced from an acetone derivative and showed activity as mosquitocide in excess of rotenone. Litsealactone B (**26T**) was extracted from leaves of the evergreen *Litsea japonica* [316].

3.1.2 Acetylenic Fatty Acids of the Bryophytes—The first brominated fatty acids from a terrestrial organism were reported in 1999 (Fig. 31A) [317]. The lichen *Acorospora gobiensis* grows in salt-laden soil surrounding brackish Lake Issyk-Kul in the Tian Shan mountains produces two compounds, 18-Br-18:2^{5t,15a,17t} (**27A**) and 18-Br-18:0^{5a,7a,17a} (**27C**). A further six unusual brominated acids (**27B**, **27D–H**) were later identified from a mixture of lichens at the same locality [318]. Haloperoxidases, such as those operative in the lichen *Xanthoria* sp., may mediate these brominations. Electrophilic bromination and trapping by hydroxide (or water) is a reasonable, but untested, proposition that has been made to account for two bromoallenes **27I–J** that occur in *A. gobiensis* (Fig. 31B) [319]. This idea may be extended as a route for halogen introduction into the previously reported set of terminal bromoacetylenic compounds and may be important for the production of many brominated marine polyacetylenes (see Section 3.1.4).

3.1.3 Fungal Fatty Acids and Polyketides—Few fatty acid derived acetylenes are known from Ascomycota, but in recent years they have been the source of several unique pharmacophores (Fig. 32). Phomallenic acids A–C (**28A–C**) were identified from a *Phoma* sp. (Ascomycota) by a novel whole-cell differential screening assay with *Staphylococcus aureus* for cell-permeable FabF/H fatty acid synthase inhibitors [320]. Of these promising allenic antibiotics, C₁₈ **28C** was the most active compound with 20-fold higher potency than thiolactomycin or cerulenin [321]. While the biosynthetic origins of **28A–C** have not been investigated, stereospecific formation of the (*R*)-allene is expected to be enzymatic.

The 3,4,5,6-tetrahydro-6-hydroxy derivative of mycomycin, antibiotic 07F275 (**28D**) was purified from submerged cultures of a filamentous fungus originating on a Panamanian tree bark [322]. As had been observed with nemotinic acid and mycomycin, this antibacterial and antifungal compound was too unstable to isolate in a pure form.

Three of the yellow pigment components **28E–G** from the basidiomycete *Xerula melanotricha* cultures have been investigated as 3-hydroxy-3-methylglutarylCoA (HMGCoA) synthase inhibitors. Xerulin (**28E**) and dihydroxerulin (**28F**) synergistically inhibit the synthase *in vitro* and reduce cholesterol biosynthesis in human cervical cancer cells HeLa S3 [323]. A definite mechanism of action is not known; other γ -alkylidenebutenolide natural products with long hydrocarbon chains have been observed to have similar activities [324].

An unusual C₁₀ 2,3-dihydroxyacid acetylenic acid (**28H**) was purified from *Laetiporus sulphureus* var. *miniatus* (Basidiomycota) [325]. Media broth for *Clitocybe catinus* yielded three new acetylenic alcohols **28I–K** including a very-short chain C₆ acetylenic diol [326].

3.1.4 Marine Fatty Acids and Polyketides—Sponges, in the phylum Porifera have yielded a diverse array of oxygenated and halogenated lipids with likely PK or fatty acid origins. Potently bioactive acetylenic acids with high molecular weights have been found in many members of the genera *Petrosia* and *Xestospongia*. Characteristic of these molecules are chain lengths ranging from C₄₄ to C₄₇ that are decorated with a large number of hydroxy, keto, acetylenic and non-conjugated *cis*-/*trans*-alkenes. Most *Petrosia* polyacetylenes are highly cytotoxic against a range of tumor cell lines and have been observed to possess RNA-cleaving ability and to inhibit PLA₂, Na⁺/K⁺-ATPase, reverse transcriptase and DNA replication.

Interference with DNA synthesis has been mentioned to be a likely cause of their cytotoxicity [327].

Atlantic and Mediterranean *Petrosia* sp. grow in illuminated water with the symbiotic alga *Aphanocapsa feldmannii* and solitary in dark caves where they are preyed upon by the nudibranch *Peltodoris atromaculata* [328]. Regardless of the presence of the alga, similar types of polyacetylenes are detected, the majority of which are very-long chain C₄₆ unbranched polyols, although the distributions of specific metabolites are variable. Petroformynes 1–8 and isopetroformynes (**29A–T**) have been isolated from geographically distant colonies of *Petrosia* sponges (Fig. 33) [328–333]. In individuals isolated from the North Atlantic Ocean, all hydroxy-bearing stereocenters examined possessed the (*S*) configuration (not shown on structures). When subjected to a brine shrimp assay, these compounds had intense toxicity with LD₅₀ values of 0.002–0.12 µg/ml [330]. C₃₁ Petroformynic acid (**29U**) was isolated from white *Petrosia ficiformis* [333] and was similar to a compound previously reported with an additional six methylene groups and two double bonds in the central undefined segment [328]. The petrocortynes A–F (**30A–C**, **30I**, **30K–L**, Fig. 34) from the Korean South Sea are distinguished from the petroformynes by the presence of a 1,4-dialkyn-3-ol functionality in mid-chain and, with the exception of **30B**, bear (*R*)-configurations at all assigned asymmetric centers [334, 335]. The identification of the dideoxypetrosynols, petrotetrynols (**30H**, **30R–U**, **30W**), petrotriyndiol (**30V**), and the homologous and epimeric petrocortynes (**30D–G**, **30J**, **30M**, **30O**, **30Q**) greatly expanded the number of observed alcohol configurations, chain lengths, and levels of desaturation (Fig. 34) [327, 336–338], suggesting that this class of metabolites may be assembled from available unsaturated fatty acid or PK precursors and subjected to environmentally controlled or subspecies-dependent hydroxylation. It was proposed by Shin that hydroxide attack on the C-21 or C-27 alkenes followed by hydride abstraction was the origin of petrocortynes E–H (**30I**, **30L**, **30N**, **30P**) [327]. A chemically more facile hypothesis is that a lipoxygenase or heme-based oxygenase activity, analogous to that described earlier from algae (Section 2.3.1.3), is involved. Corticatic acids A–C (**31A–C**) were reported in 1994 from *Petrosia corticata* (Fig. 35) [339]. **31A** and the related acids D and E (**31D–E**) have potential as selective antifungal agents as they were found to inhibit yeast geranylgeranyltransferase type 1, which has only 30% sequence homology to the human enzyme [340]. Acids **31A–C** bear great resemblance to the petrocortynes when compared at the C1-ends, but differ in the locations of the internal unsaturation (C-17 to C-20). The 1,4-diene is a potential site of reactivity in **31E**, which is a hypothetical substrate for acetylenase and allylic hydroxylation reactions leading to the other corticatic acids. Aztèquynols A and B (**31F–G**) were found to possess an 18-methyl branch and a fully saturated central region [341]. Callyspongynic acid (**31H**), a specific inhibitor of α -glycosidase (IC₅₀ 0.25 µg/ml), contained the α , β -acetylenic acid and a 4-en-1-yn-3-ol termini as was observed in **31A–B** and **31D–E**. However, the stereochemistry of the alcohol was inverted [342].

A series of C₄₅ to C₄₇ compounds were purified from a Korean *Petrosia* sp. [327, 343]. With its C₄₇ linear chain, nepheliosyne A (**31I**), isolated from the marine sponge *Xestospongia* sp., is one of the longest marine acetylenic acids to date (Fig. 35). It had much weaker *in vitro* cytotoxicity against L-1210 lymphoma and human epidermal carcinoma KB cells (IC₅₀ >20 µg/mL) than other *Petrosia* polyacetylenes [344]. Structurally related, highly oxygenated compounds haliclonyne **31J** extracted from a *Haliclona* sp. sponge [345] and osirisynes A–F (**31K–P**) from *Haliclona osiris* have been reported [346]. The α -acetylenic carboxylic acid functionality, only seen previously in the corticatic acids [339], indicates a distinctive biogenetic route.

Reniera fulva, a related species to the *Petrosia* sp. based on ribosomal RNA analysis [347], produces the C₂ symmetric metabolite fulvinol (**31Q**, Fig. 35). This structure is strongly suggestive of a dimerization of two identical lipids that have the same half-chain length as the

unoxygenated segments of petrocortynes A and B. Petrosynol (**31R**), which also a symmetric metabolite, and petrosolic acid (**31S**) have been isolated from a Red Sea *Petrosia* sp. [348]. Petrosolic acid is notable because C₄₄ carboxylic acids are relatively rare and it inhibited DNA polymerase activity of HIV reverse transcriptase. The relatively short-chain polyol **31R** is an inhibitor of sea urchin cell division and does not contain the dominant 1,4-diyne-3-ol core but, like **31Q**, is intriguingly symmetric.

The biogenesis of the petrosynes and related polyacetylenic polyols has not been deduced, but portions of the mycolic acid biosynthetic pathway may provide hints toward their genesis (Fig. 36). Mycolic acids are very long-chain fatty acids produced by specialized elongation and condensation reactions of common long-chain lipids, formed by a Type I FAS, in mycobacteria. Unfortunately, experiments to date have given contradictory results impeding the development of a consensus model for their biosynthesis [349]. Early studies showed the inhibition of corynomycolic acid production by avidin in cell-free extracts of *Corynebacterium diphtheriae*, which the authors suggested supported the involvement of a biotin-dependent carboxylation of palmitate [350]. For dehydrocorynomycolate (**32B**), a Claisen condensation of the α -anion of the activated C₁₆ thioester with a second palmitate moiety by a 3-ketoacyl synthetase creates the (2*R*)-C₃₂ ketone **32A** (Fig. 36A). Enzymatic reduction to the (3*R*)-alcohol **32B** subsequently occurs *in vivo*, although *C. diphtheriae* extract was incapable of this transformation. Later studies of *Bacterionema matruchotii* that did not show avidin inhibition cast doubt on the universality of the carboxylation hypothesis [351]. Deuterium labeling studies with 2,2-dideuteropalmitate resulted in the isolation of [2,4,4-*d*₃]corynomycolate that is unlikely to be formed by a carboxylative process unless deuterium can be returned to the alkyl chain at C-2. Most recently, gene deletion studies in *Corynebacterium glutamicum* showed that gene clusters *accD2* and *accD3* are involved in 26:0 carboxylation [352]. Two homologous proteins *AccD4* and *AccD5* from *M. tuberculosis* involved in α -mycolic acid formation are known to mediate the carboxylation of 26:0-CoA, which occurs in advance of β -carboxylated acyl transfer to the PPB domain of the specialized polyketide synthase *pks13* [353]. While it lies to others to resolve the differences between the individual organisms, it is undeniable that carboxylative activation of acyl chains leads to fatty acid coupling in certain species.

In the case of the *Petrosia* polyacetylenes and related marine PKs, a ketoacid analogous to those proposed in the *C. diphtheriae* study would be expected to be in equilibrium with the enol tautomer **32C** (Fig. 36B). Conversion of the enol intermediate to a species with a good leaving group, perhaps through phosphorylation (e.g., **32D**), provides a mechanistically viable path to an acetylenic bond. Species analogous to **32D** has been shown through model compounds by Fleming and Harley-Mason to eliminate to an acetylenic acid (see also Section 2.2.1) [28,354]. We propose that an extension of the Fleming hypothesis uses condensing units produced by standard intermediary metabolism and allows the installation of acetylenic bonds within the very long-chain metabolites.

Triangulynes A–H (**33A–H**) and triangulynic acid **33I** were characterized from the marine sponge *Pellina triangulata* (Fig. 37) [355]. A differential cytotoxicity profile for the triangulynes was similar to the C₄₃ bis-enynol vasculyne from *Cribrorhynchus vasculum*, but not the C₂₀–C₂₃ enynols from an earlier collection of the same organism. Lembehynone A **33J**, derived from an Indonesian *Haliclona* sp. sponge, has a structure suggestive of PK origins. This linear C₃₆ diacetylenic alcohol induces neuritogenesis in Neuro 2A neuroblastoma cells resulting in bipolar neuritis [356]. A photoaffinity probe based on **33J**, [¹²⁵I]-azido-LB-18, specifically labeled *in vivo* a 30-kDa protein in Neuro 2A cells that is anticipated to be the target of **33J** [357]. The presence of a (3*R*)-propargylic alcohol on a minimally C₁₈ alkyl chain were necessary for strong neuritogenic activity [358].

Shorter chain metabolites that have been subsequently uncovered may provide insight into the biosynthetic course. Durissimols A and B (**34A–B**, Fig. 38) were isolated from *Strongylophora durissima* (a genus recommended for amalgamation with *Petrosia* [359]), the second compound showing cytotoxicity against human gastric tumor cells at concentrations of 10 μ M [360]. Hydroxylation at the 6-position in a compound related to **34A** may yield the strongylodiols A–J (**34C–L**), which were isolated from an Okinawan *Petrosia* (*Strongylophora*) sp. as enantiomeric (6*R*:6*S*) mixtures ranging from 97:3 to 69:31 for **34L** and **34G**, respectively [361,362]. It is uncommon to isolate natural products as enantiomeric mixtures; in this case, it is not yet known whether the mixture is an isolation artifact or reflective of the enzymatic activities. The flux of strongylodiol metabolites may be regulated by diastereomeric interactions of epimeric compounds with subsequent enzymes. Strongylodiol E (**34G**) is likely desaturated to related diols **34I** (60 %ee) and **34H** (92 %ee), the latter appearing to be selectively formed by oxidation of the (*R*)-epimer. The timing of methyl branching is not obvious.

Peroxyacarnolic acids A and B (**34M–N**) were isolated from the Red Sea sponge *Acarnus* cf. *bergquistae* found in Eritrea (Fig. 38) [363]. Based on the direction of the specific rotation, an absolute configuration of (3*S*,6*R*) has been assigned to **34M–N**. The unusual 1,2-dioxane rings of these chiral structures may be formed by a cyclooxygenase-like reaction, where closure of the peroxide occurs at a ketone. Examples of COX-1 and COX-2 have been cloned and heterologously expressed from the corals *Gersemia fruticosa* and *Plexaura homomalla* [364, 365]. Amazingly, a pair of *R*- and *S*-stereospecific *P. homomalla* COX-2 isozymes showed 97% identity at the amino acid level and had seven highly conserved residues when compared to known cyclooxygenases; a single substitution of Ile-349 for Val in the substrate binding channel switched the stereochemistry from 15*R* to 15*S* for 70% of the prostaglandin produced [366].

Halogenated C₉–C₁₈ fatty acids have been isolated from geographically isolated *Xestospongia* species found in Australia and the Red Sea (Fig. 39). The C₁₈-bromodiacetylenic acid **35A** was characterized in isolates of *Xestospongia testudinaria* from Australia and Mayotte (Comoros Is.) and a *Xestospongia* sp. from the Red Sea [367–369]. Additionally, *X. testudinaria* yielded four additional C₁₆–C₁₈ bromofatty acids **35B–E** and C₉ fatty acid **35F**, which is a possible degradation product of **35C**. The diacetylene-containing fatty acid **35G** has been co-isolated with the structurally related trans-enyne **35A** [368]. A later study found a series of brominated fatty acids with acetylenic groups at the 5, 7, 13, 15, and 17 positions (**35D**, **35H–K**, **35N–O**) [370]. In contrast to plant lipids, these brominated natural products contained exclusively trans-alkenes. While the close associations of marine bacteria and animals often leave the origins of marine secondary metabolites undefined, the localization of the internally brominated C₂₄–C₂₆ fatty acid esters **35P–R** in sponge cells has been ascertained in the sponge *Amphimedon terpenensis* by Ficoll density gradient fractionation [371,372]. These compounds may, nevertheless, originate from bacteria ingested by the animals since their hydrophobic nature would make bacterial excretion and reabsorption by the sponge unlikely. In either case, these unusual brominated $\Delta^{5,9}$ -diunsaturated fatty acid derivatives may be formed through alkene bromination or alkyne hydrobromination reactions. It can be envisioned that elongation of C₂₀ and C₂₁ fatty acids may be followed by chain-length specific Δ^5 -desaturation to provide the precursors. *Oceanapia*, a genus closely related to *Xestospongia*, has yielded a C₁₄ fatty acid containing an ene-yne-ene-yne conjugated system [373]. A sponge isolated in the Philippines *Diplastrella* sp. produced a collection of brominated acetylenic diols that are similar in many regards to both the *Xestospongia* bromoacids and *Callispongia* sp. polyacetylenic diols and triols (*vide infra*). Diplynes A–E (**35W–X**, **35Z**, **35AB**, **35AD**) were located by an HIV-1 integrase inhibition assay and purified with diplyne A 1-sulfate (**35Y**), diplyne C 1-sulfate (**35AA**), and deoxydiplyne D sulfate (**35AC**), although the non-sulfated compounds were later found to be inactive [374].

A distinct group of chlorinated dienetriynes alcohols and esters **36A–J** have been isolated from the Pacific sponge *Haliclona lunisimilis* (Fig. 40) [375]. These compounds appear to be fatty acid derivatives that result from β -oxidation of *cis* Δ^9 -alkene-bearing C₁₈ unsaturated fatty acids. The variations in chlorodiene stereochemistry are consistent with long-lived resonance-stabilized cations present during electrophilic chlorination at the terminal position. Acetylenes **36A–B**, **36D–F**, and **36I–J** together with ketones **36K** and **36L** had been earlier found in a nudibranch *Diaulula sandiegensis*, collected in the same vicinity, that feeds on the sponge. The consumption and likely oxidation of the secondary alcohols to ketones, which are absent in the sponge, fits with the oxidation of foraged metabolites by the nudibranch to yield more potent defensive excretions.

A series of C₁₂–C₁₇ diacetylenes, named montiporic acids and esters (**37A–O**), have been procured from the methanolic extract of scleractinian coral with the montiporynes A–F (**37P–U**) [376,377]. The network of 2,4-diyne from the *Montipora* sp. coelenterate is suggestive of allied biogenetic origins involving α -oxidation and ω chain-oxidation outlined in Fig. 41A. During reproduction, these hermaphroditic corals release convoluted bundles of sperm and eggs, the latter release polyacetylenic alcohols that are chemoattractants for sperm.

The acetylenic 3-alkylpyridines hachijodines F and G (**38A**, **38C**), xestamine A (**38B**), niphatyne A (**38D**), and bear long alkyl chains terminated by *N*-methoxy-*N*-methylamino or *N*-methylhydroxylamino substituents and are found in the *Xestospongia* and *Amphimedon* sponges (Fig. 42A) [378–380]; niphatesine H **38E** was subsequently isolated from *Niphates* sp. [381]. The 3-alkylpyridines contain isolated acetylenic and *cis*-enyne groups distributed along the alkyl chain and have been found with the corresponding oximes. While not directly examined in sponges, the hydroxylamines are expected to be precursors to the corresponding oximes, based upon analogies to cyanogenic glucoside, glycosinolate, and camalexin biosyntheses in plants [382–384]. Cytochrome P-450 isozymes CYP71, CYP79, CYP83 oxidatively decarboxylate amino acids to oximes, as exemplified by linamarin and lotaustralin in cassava [385]. The hachijodines were found to have substantial cytotoxicity against P388 murine leukemia cells (1.0 $\mu\text{g}/\text{mL}$). Recently, three acetylenic 3-alkylpyridine glycosides and an alkene-containing variation, coined the amphimedosides (**38F–I**), were extracted from *Amphimedon* sp. [386]. β -Glycosylation has little effect on the cytotoxicity of the **38F–I** when compared to their aglycons. A biogenetic proposal exists for pyridine derivatives in sponges from the order Haplosclerida, in which the 3-alkylpyridine is formed by the condensation of an α , γ -alkanedial, acrolein, and ammonia [387]. We feel that an alternate cyclization proposal, shown in Fig. 42B, involving the addition of a formaldehyde-derived iminium ion with the tethered enol equally accounts for the formation of the 3-alkylpyridines. The involved intermediate could originate either as a PK or by the oxidation of an unsaturated fatty acid. A mixture of the unusual diamine alkaloids clathculins A–B (**38J–K**) was isolated from the brittle pink Indo-Pacific sponge *Clathrina* aff. *reticulum* [388].

Apparently formed by α -oxidation, (2*S*)-methoxy fatty acids (Fig. 43A) have previously been isolated from the sponge *Higginsia tethyoides* [389]. Members of the genus *Stelletta* have produced interesting methyl-branched acetylenic monoglycerides (**39A**), lysophosphatidylcholines (**39B**), and free fatty acids (**39C–F**) [390,391]. Since acetylenic fatty acids have not been observed to accumulate in membrane lipids in plants, it is perhaps not surprising that **39A–B** were very minor sponge metabolites.

Distinct from the acylglycerolipids, acetylenic plasmenyglycerolipids are occasionally major lipid components distributed through several orders of marine animals (Fig. 43B). In the New Zealand marine sponge *Petrosia hebes*, diacetylenic 1-alkenylglycerols **39G–H** comprise up to 7% of the membrane lipids [392]. These compounds, which unlike the C₃₀–C₅₅ acetylenic alcohols from other *Petrosia* sp. (order Haplosclerida) are inactive against leukemia cells, are

related to the 1,5-dien-3-yne ether lipid raspailynes from *Raspailia pumila* and *Raspailia ramosa* (order Axinellida) [393]. In *Raspailia* spp., (1Z,5Z)-, (1E,5Z)-, and (1Z,5E)-dienynes are readily degraded by triplet oxygen to 1-formylglycerol and the 4-en-2-ynyl aldehydes (Fig. 43C) [394], which are reminiscent of the toxic, reactive *Caulerpa* terpene dialdehydes (Fig. 21). A subsequent report expands the substitution patterns for *Petrosia* sp. to include linear and methyl-branched (Z,Z)-1,5-dien-3-yne (**39I–K**) and (Z)-1-en-3-yne (**39L–N**) [395]. Biosynthetic details for these intriguing compounds are scarce. In mammals, a mixed function Δ^1 -desaturase has been reported which uses NADPH, Mg^{2+} , ATP, and free cytochrome-b₅ to oxidize 2-acyl-1-alkylglyceroethanolamine to acylplasmeneylethanolamine (Fig. 43C) [396]. Whether a divergent set of front-end desaturase and acetylenase enzymes that act specifically on *O*-linked glycerolipids are operative or an alkyldihydroxyacetone phosphate synthase incorporates acetylenic fatty alcohols into **39O** during the synthesis of acetylenic ether lipids **39G–N** remains to be determined.

Other sponges from the order Haplosclerida, exemplified by the Japanese sponge *Callyspongia truncata*, are rich sources of polyacetylenic alcohols that have core structures distinct from the 1,4-pentadiyn-3-ols (and their derivatives) found in *Petrosia* spp. (Fig. 44). Callyspongynes A and B (**40A–B**), as major (C₃₃) and minor (C₃₅) components, were purified from Australian sponges and found to have an absolute configuration of (3*R*) [397]. Callypentayne (**40C**), callyberyne B (**40D**), and callytetrayne (**40E**) are simple hydrocarbons, which is unusual for marine polyacetylenes [398,399]. The hypothesis has been put forward that these *Callyspongia* sp. C₂₁ enynes are produced by the decarboxylation of C₂₂ carboxylic acids [398]. Callydiyne (**40F**), a symmetric C₁₆-bis(enyne), had been reported earlier [400]. The first acetylenic lipids found in the phylum Chordata (**40G–J**) are structurally related to **35V** [401]. Tsukamoto *et al.* identified five pentaacetylenic triols (**40K–O**) which both disrupted settlement of barnacle larvae and induced metamorphosis of larvae for the ascidian *Halocynthia roretzi* [399]. Such secondary metabolites with antifouling characteristics may provide an alternative to the persistent organotin toxins. A series of C₂₂ alcohols, callyspongynols A–C (**40P–R**) were identified by a P388 cytotoxicity screen [402]. Sulfated acetylenic alcohols (**35Y**, **35AB**, **35AC**) have been isolated both from *Diplastrella* spp. and *C. truncata* (Fig. 39) [403]. Callyspongins A and B (**35U–V**) specifically interfered with oocyte fertilization membrane formation at concentrations of 6.3 and 50 μ M (MIC), respectively, without affecting embryo and egg development [403]. The stereochemistry of **35U** matched that of the known non-sulfonated metabolite siphonodiol **35T** [404]. The more highly saturated C₂₄ enyne callyspongynol sulfate A (**40S**) was found to be a membrane type 1 matrix metalloproteinase inhibitor (Fig. 44) [405]. A second α -hydroxylated carboxylic acid, aikupikanyne F (**40T**), alcohols (**40U–V**) and hydrocarbons (**40W–Z**) were isolated from a Red Sea *Callyspongia* sp [406].

A range of linear and branched C₁₆ to C₃₃ metabolites containing a 2,4-dien-1-ol moiety that suggests common biogenetic origins have been isolated from a range of sponges (Fig. 45). Durissimol B (**41A**) was detected in a screen for cytotoxicity to human gastric tumor cells [360]. The small branched metabolite durissimol A (**41C**) occurred in the same organism, *S. durissima* with the earlier reported compounds reneirin-2 (**41D**) and 18-hydroxyreneirin-2 (**41E**) [360,407]. Pellynol F (**41B**) was isolated with pellynols G and H (**41F–G**) [408]. Ketone **41G** is formed by the ready oxidation of petrocortynes F–H and **41F** [335,408].

Alkamides, while widespread in plants, are relatively uncommon from oceanic species. Callyspongamide A (**41H**) was found in the MeOH/CH₂Cl₂ extracts of *Callyspongia fistularis* (Fig. 45) [409]. The C₁₇ enediynoic segment of **41H** is surprisingly consistent with other *Callyspongia* metabolites. In the cases of the malyngamides A–D [410,411] and I [412] and hermitamides A–B [413], a group of 7(*S*)-methoxytetradec-4(*E*)-enoic acid and 7-methoxy-9-methylhexadec-4(*E*)-enoic acid amides, it has been proposed that the compounds

originate from the consumption of *L. majuscula*, or epiphytic cyanobacteria associated with algae, by specialist sea hares. It would be interesting to determine whether *C. fistularis* has a NRPS or amide synthetase activity that produces the amide *de novo* or whether the sponge sequesters metabolites produced by a cyanobacterial partner. The simplicity of the phenethylamide in **41H** is quite different from the generally non-aromatic and halogenated amine moieties in marine cyanobacterial amides [414]. Taurospongins A (**41I**) is, to our knowledge, a unique acetylenic lipid containing a taurine amide, a methyl-branched trihydroxydecanoic acid, and C₂₅ dimethylene-interrupted acetylenic acid. Isolated from a *Hippospongia* sp. sponge, **41I** was found to be a potent inhibitor of DNA polymerase β and HIV reverse transcriptase [415].

Trichloromethyl groups are found in a number of cyanobacterial natural products, where they are the apparent result of free-radical chlorination of the pro-(*S*) methyl group of leucine and its derivatives. Four simple perchlorinated acetylenic acetamides (**42A–D**) isolated from *Microcoleus lyngbyaceus* are remarkable due to their lack of branching (Fig. 46) [416]. It remains to be determined whether these compounds are produced using isopropyl halogenation chemistry common to barbamide [230] and herbacic acid [417] followed by demethylation or, alternatively, through repetitive chlorination of methyl termini on linear alkanes. A putative precursor to these compounds, 6-acetamidotridecane, was purified in the same study. Unsaturated acetamides with chlorovinyl and dichlorovinyl groups known as the taveuniamides A–K (**42E–O**) were isolated from a mixture of blue-green algae including *Schizothrix* spp. and *L. majuscula* collected in Fiji [418]. Given the two similar groups of compounds, haloalkenyl termini for **42E–O** and the jamaicamides are hypothesized to form by the dehydrohalogenation of the 1,1,1-trichloroethyl groups.

Acetylenic glycosides are not exclusively plant-derived; several glycosylated marine PKs have been reported (Fig. 47). Powerfully ichthyotoxic and hemolytic toxins prymnesin 1 and 2 (**43A–B**) were isolated from the red-tide algae *Prymnesium parvum* [419,420]. These exceedingly complex PKs contained one to three glycosylation sites. Even though **43A–B** are presumably enzymatically cyclized, very recent experiments addressed a long-standing problem concerning the biosynthesis of such polyether ladders [421]. The solvent water was found to be critical in guiding ring size selectivity for the tandem epoxide ring openings initiated from a single tetrahydropyran template. Callipeltosides A–C (**43C–E**) are chlorocyclopropanated 14-membered macrolides appended to highly oxygenated sugars and aminosugars through α -*O*-glycosidic bonds [422,423]. Methyl-branched sugars are often microbial in origin; *O*-methylevalose, the sugar portion of **43D** is similar to sugar from *Micromonospora carbonaceae*. **43C–E** may be secondary algal or bacterial products accumulated by the host sponge. The 13-membered peptidomacrolide spongidepsin (**43F**) was characterized from the Vanuatu sponge *Spongia* sp. that has 0.42–0.66 μ M IC₅₀ values in a cancer cell cytotoxicity assay [424].

Aromatic PKs are not common from cyanobacteria (Fig 47). Nostocyclone A (**43G**), an aromatic cyclophane was procured from an algal growth on the floor of an Israeli greenhouse [425]. The suggested folding pattern for this likely PK is shown (**43H**). The acetogenins from *Laurencia* spp. and related red algae contain an abundance of acetylenic and other unsaturated groups. While not covered here, Erickson has described research in this area, including biosynthetic aspects [426], and updates on recent isolates have been made in a series of reviews on marine natural products [427–433].

3.2 Acetylenic Lipopeptides and Cyclodepsipeptides

L. majuscula, a marine blue-green alga, is the most prodigious cyanobacterial source of bioactive natural products currently known. This ubiquitous organism is found in a variety of habitats and morphologies, and the distribution of natural products can vary markedly

depending upon its source. A recent report noted that 75% of the 110 known fatty acid, alkaloid and pyrrole metabolites from this organism have a detectable biochemical activity [434].

Lipopeptides and cyclodepsipeptides have mixed PK/FA and NRPS origins. Apramides A–B (**44A–B**) and D–E contained variably methylated 7-octanoic acid termini and had negligible bioactivity [435]. The related planar metabolites dragomabin (**44C**) [436], carmabin (**44D**) [437], and dragonamides A–B (**44E–F**) [436,438] are also known; **44A–C** were isolated with 7-alkenoate and 42-keto lipid modifications, respectively. α -Methylation of the fatty amide group was (*S*) in each case (the original assignment for **44E** was corrected by total synthesis). Amides **44C–E** inhibited the W2 chloroquine-resistant strain of *P. falciparum* but **44F** was inactive, which suggested that an aromatic amino acid was required for antimalarial activity [436].

Of particular note, a family of cyclodepsipeptides has been identified from *L. majuscula* that contain residues derived from short-chain acetylenic fatty acids incorporated in a framework constructed with interesting *N*-methyl- and D- α -amino acids, β -hydroxy- and β -amino acids, and α -hydroxyacids. These compounds are produced by NRPS gene clusters and undergo an intramolecular cyclization controlled by the last functional domain, a thioesterase [439]. Palau' amide (**44G**) contains a complex 5,7-dihydroxy-2,6-dimethyldodec-2-en-11-ynoic acid residue, which is likely of PK origins [440]. This complex acid chemically appears to be derived from a 5-hexynoic acid starter unit that is elongated by three acetyl-CoAs (or an admixture with propionyl-CoA). The terminal acetylenic fatty acid is a potential biochemical link between palau' amide and ulongapeptin (**44H**) [441], which both originate from a Palau *Lyngba* strain, and other *L. majuscula* metabolites. The progression of fatty acid oxidation states from alkyne to alkane in the trungapeptins A–C (**44I–K**) [442] and antanapeptins A–D (**44L–O**) [443], as well as the alkene/alkyne metabolite pairing in pitepeptolides A–B (**44P–Q**) [444] suggest that *L. majuscula* produces hexanoic acid which is elongated by a PKS, desaturated and acetylenated, although the sequence of these steps is not clear.

Components, such as 2,2-dimethyl-3-hydroxy-7-octynoic (Dhoya) and 2,2-dimethyl-3-hydroxy-7-octenoic acid (Dhoya), are distinct and pervasive among marine natural products originating from cyanobacteria; consequently, these unusual acetylenic acids have been proposed as ecological markers [445]. Burja *et al.* authored a good review of cyanobacterial sources of bioactive metabolites and perspectives on biotechnology [414].

While the non-acetylenic cyclodepsipeptides often appear in marine sponges, the large C_{2-} symmetric cyclodecapeptide onchidin (**44R**), containing two 3-amino-2-methyloct-7-ynoic acid residues, was extracted from a pulmonate mollusk *Onchidium* sp. highlighting the pervasive qualities of compounds produced by cyanobacteria [446]. Georgiamide (**44S**) was found to contain the 2,2-dimethylated acid Dhoya [447], as do yanucamides A and B (**44T–U**) [444], kulolide-1 (**44V**) [448] and kulokainalide-1 (**44W**) [449]. The latter two compounds were sequestered by the bubble-shell mollusc *Philinopsis speciosa*, which consumes the sea hare *Stylocheilus longicauda* that, in turn, eats blue-green algae and presents a vivid molecular picture of a marine food chain. Sea hares collected in Papua New Guinea contained dolastatin-17 (**44X**), an acetylenic “dolayne” β -amino acid-possessing member of a pharmacologically important group of antineoplastic agents [450]. Guineamide C (**44Y**) contains the β -amino acid 2-methyl-3-aminooct-7-ynoic acid (Maoya) [451]. The unexpected presence of both L-*N,O*-dimethyltyrosine in **44Y** and its D-enantiomer in guineamide D is notable. The large 14-residue cyclic lipopeptide malevamide C (**44Z**), purified from *Symploca laete-viridis*, was constructed from 3-amino-2-methyl-7-octynoic acid [452]. *Lyngbya semiplena* was found to contain the acetylenic wewakpeptins A–D. One of the most promising of these, wewakpeptin A (**44AA**) was very active against human lung cancer cells (LC₅₀ 0.4 μ M) [453].

3.3 Acetylenic terpenoids

Acetylenic terpenoids are found both in terrestrial fungi and marine organisms. Freelingyne (**45A**) is an example of a small group of furanosesquiterpenes of plant origin containing a γ -alkylidenebutenolide moiety (e.g., xerulin, Fig. 32). The furan ring is likely produced by the reduction/dehydration of a lactone precursor. While two metabolites supporting this biosynthetic progression are known, the origin of the acetylenic bonds in this unique group of sesquiterpenes remains an open problem (Fig. 49) [454]. Hemiquinone **45B** was isolated from the culture broth of SDEF 678, an ectotrophic fungus associated with the roots of an Australian grass [455]. This hemiquinone had antifungal activity and incompletely characterized cyclohexene-1,4-diols presumed to be structurally related to **45B** that were extracted from the SDEF 678 cultures promoted the growth of barley seedlings. Generally, isopentenynyl quinoids have low antifungal activity. Weakly phytopathogenic serialynic acid (**19N**, Fig. 23) has been extracted from the culture agar of an *Antrodia serialis*, a terrestrial basidiomycete [456]. The trichloroquinone mycenon **19O** was purified from the culture broth of *Mycena* sp. TA 87202 and identified by X-ray crystallography (Fig. 23) [457]. This compound is a specific competitive inhibitor of isocitrate lyase. While uncommon in metabolites of Basidiomycete origin, **19O** contains a chlorovinylidene unit. Structurally, this haloalkene functionality is more prevalent in marine compounds (e.g., jamaicamide) [223]. Compound **19O** selectively inhibits isocitrate lyase in castor seeds (K_m 5.2–11.2 μ M).

A large number of marine carotenoids are known to be accumulated by both freshwater and oceanic shellfish [458]. Acetylenic carotenoids are broadly distributed among Bacillariophyceae, Cryptophyceae, Dinophyceae, Euglenophyceae, Haptophyceae, and Xanthophyceae species [459] and those with biological activity have been reviewed separately by Dembitsky [22].

4. Functional significance of polyacetylenes and related compounds

Acetylenic compounds are produced by many species that are deemed ethnopharmacologically important. Species such as *E. purpurea* have been used by Native Americans as treatments for a variety of maladies since the early 17th century [460]. For often more sinister purposes, water dropwort (*O. crocata*), which contains the polyacetylene oenanthotoxin that targets the central nervous system, has been recognized since the 12th century to be useful as a poison for fish and others [461]. Few studies have been published that precisely define the biological functions and ecological significance of the acetylenic natural products, individually or as a group. The majority of reports on polyacetylene bioactivities are derived from antibiotic assay data and anthropocentric screens for anticancer agents. In most cases, survival-based roles for the natural products, either as toxic or unpalatable antifeedants, allelochemicals, phytoalexins, or basally produced antibiotics seems likely. A review focusing on the biological activities of naturally occurring acetylenes as insect divertants, fungicides, allelopathic species, insecticides, and pharmaceuticals has appeared [462]. While evidence supports that acetylenic (and acetylene-derived) thiophenes function as sensitizers for the production of singlet oxygen, most polyacetylenes have light-independent functions. Studies on enzymatic targets relevant to the native biological function of the polyacetylenes are rare. Certain recent or well established studies are described below to frame the potential roles of acetylenic compounds.

The localization of plant polyacetylenes in specific tissues is consistent with the characteristics of secondary metabolites. In the case of plant roots, a number of recent studies have spectroscopically investigated the distribution of falcarinol (**4B**) and falcarindiol (**4C**) and found that polyacetylenes in both carrot and *P. quinquefolium* (American ginseng) are, in part, concentrated in oil-filled channels within the periderm/pericyclic parenchyma tissue running parallel to the length of the root [463–465]. In cultivated carrots, higher levels of polyacetylenes are found in high-carotene (HCM) varieties, with the higher concentrations of **4C** present in

the upper portion of the root and the periderm and phloem, while **4B** was more uniformly distributed [466]. Young secondary phloem tissue in HCM carrots contained **4B**, detected by Raman absorption, but the wild carrot *Daucus carota* ssp. *maritimus* had predominantly **4C**. Falcarindiol was distributed uniformly through the phloem [467,468]. HPLC analysis of *P. quinquefolium* root showed that the total content of panaxydol and falcarinol is inversely correlated with root diameter [469]. The localization of polyacetylenes in exterior layers of tissue is consistent with their role in providing an antifungal shield for young roots.

Epidemiological studies often tout the cancer-protective attributes of foods and vegetables; in this regard, many studies support the cytotoxic effects of **4B** and related polyacetylenes from carrot and ginseng [136,470,471]. The source-dependent chemoprotective behavior of carotene (*i.e.*, whether it was ingested in carrots or as a pure compound used as food additive) has been a paradox that may be resolved by the recognition of potentially chemoprotective polyacetylenes in the root crop. Recent investigations have focused on **4B** illuminating its potential as an anticancer agent. It was suggested that a delay in large tumor formation may be linked to the sensitization or induction of apoptosis by (-)-**4B**, presented as a pure compound or within carrots [472]. In these studies, the bioavailability of **4B** from carrot juice was shown by persistent plasma levels approaching $3 \text{ ng} \cdot \text{mL}^{-1}$. A recent study showed that the impact of **4B** is biphasic with respect to concentration in human colon carcinoma CaCo-2 cells [473]. When exposed to low concentrations of **4B**, a pro-proliferative effect was observed. At $>20 \mu\text{M}$ **4B**, cellular markers of apoptosis and cellular detachment increased while the intermediate concentration regime exhibited increased DNA single-strand breakage with minimal effect on cellular viability. The **4B** content of carrots was reduced by 70% when carrot pieces were boiled 12 minutes [474], however **4B**, **4C** and falcarindiol-2-acetate increased significantly over 4 months of storage at 1°C [475]. Consequently the nutritional effects of carrots or other Apiaceae species are dependent on food preparation. Chemoprotective behavior is not expected to be a general characteristic of polyacetylenes; nevertheless, these studies highlight the nutraceutical potential for certain polyacetylenes. A wide range of factors, including bioavailability, interactions between polyacetylenic species and other natural products, compound stability, concentration effects and context of dietary uptake need to be considered before the antiproliferative effects can become well-established. An experimentally established biological mechanism for the mammalian impact of **4B** as an anticancer agent remains unknown.

Falcarinol (**4B**) is a noted allergen [136,476]. One hypothesis for the sensitization by **4B** is that it undergoes an $\text{S}_{\text{N}}1$ -like substitution reaction via a resonance-stabilized carbocation allowing the formation of protein-**4B** antigenic conjugates through cysteine and amine-containing biomolecules. Other common acetylenic natural products, such as **4C**, have diminished lipophilicity that reduces their antigenicity.

Antifungal activity for **4C** has been proposed to stem from alteration of the plasma membrane or other membrane functions [477]. Chlamydospores of *Mycocentrospora acerina* incubated with **4C** accumulated the polyacetylene and underwent rapid electrolyte loss. Hyphal tips for *M. acerina* were seen to burst at $75 \mu\text{g/ml}$ **4C** and synthetic micelles at lower concentrations. Many polyacetylenes are formally oxylipins, but their potential roles in plant-fungal and fungal-fungal communication are uninvestigated. In a leading example, Bowers had previously recognized that a soil basidiomycete released the oxylipin 8-hydroxylinoleate, which symbiotically protects crop roots from the pathogenic fungus *Pythium ultimum* [478].

Slight variations in polyacetylene structure result in extreme variations in biological activities. Low toxicity cicutol (**46A**), **4B** and **4C** can be contrasted with lethal K^+ -current blocker cicutoxin from water hemlock (**46B**) [479]. The toxicity of **46B** was found to have three structural requirements: an allylic alcohol, a long-conjugated (*E*)-polyene, and a terminal

hydroxy group [480]. Derivatives with terminal carbonyl derivatives had lower lethal doses and were stronger inhibitors of GABA_A receptors in rat brains suggesting that the pharmacology of **46B** results from its conversion to an oxidized derivative.

Acetylenes, phenylpropanoids and isoprenoids are recognized to be phytoalexins, low molecular weight compounds produced by plants to respond to microbial attack, disease state, or abiotic stress (e.g., UV irradiation, metal salts, detergents) [481]. Polyacetylenes have been found in organisms, such as the solanaceous plants, that do not appear to produce basal levels of the compounds. The nonspecific elicitors RNase A and nigeran released **46C** (0.5 mg/L), **4C** (0.1 mg/L) and an incompletely described polyacetylene into the media of an eggplant (*Solanum melongena*) cell suspension culture [482]. Accumulation of suspected polyacetylenic phytoalexins, including **4B** and **46D**, was similarly observed in tomato fruits and leaves induced with *Cladosporium fulvum*, *Verticillium albo-atrum*, and *F. oxysporum* [483,484]. Sufficient aqueous solubility to allow metabolites to be exuded from healthy cells into necrotic tissue may be a precondition for bioactivity. Colonization of tomato by compatible strains of *C. fulvum* may result from cellular localization of **4C** in the tomato that renders **4C** ineffective and the rapid catabolism of **4C** by both tomato and the fungus that allows delayed but continual fungal growth [485]. Fungal stimulation of polyacetylene accumulation has been seen in the other plant families (**46E**, *C. tinctorius* Asteraceae [486–488]; **46F**, *Vicia faba* Leguminosae [489]; **4B–C**, *D. carota* Apiaceae [490]). Elicitor-stimulated upregulation of acetylenase genes in parsley and sunflower together with the above metabolite observations supports acetylenic natural products functioning as phytoalexins [104,491]. Still, many of the genes necessary for the formation of the putative antifungal polyacetylenes need to be discovered, their *in vivo* functions remain to be directly established, and their regulation deciphered.

Polyacetylenes have been shown in a number of cases to be allelochemicals, compounds that affect the metabolism and growth of plants subacutely. Thiophene **11Bk** (Fig. 14) is exuded from Russian knapweed (*Centaurea repens*) roots leading to soil levels of 4–5 ppm that are sufficient to prevent root elongation and establishment of competing species [492]. Through this action, *C. repens* is an invasive weed in North America. A recent EST study of the related invasive species *Centaurea maculosa* highlighted a series of acetylenase-like genes [493]. Other compounds are more acutely toxic; agrocybin (**1B**) strongly inhibits the growth of wheat, soybean and *Lemna minor* and may be the agent responsible for grass death by fairy rings [494]. Dehydrofalcarinol was identified as one of the seed germination inhibitory components from *Artemisia capillaris* [495]. The allelopathic activity of the common Asteraceae ester **1A** has been investigated many times but, in a recent study, the effectiveness of **1A** released from *S. altissima* was concluded to be small in most soils, as even light clay soil bound the metabolite tightly [496].

While several examples of plant-derived polyacetylenes involved in antiherbivory have been reported, the importance of this function seems under investigated. Insecticidal activities of structural subtypes found in *Rudbeckia hirta* were studied while varying the light flux [497]. Under dark conditions thiarubrine **11Di** was toxic to mosquito larvae (*Aedes atropalpus*, LC₅₀ 0.09 µg/ml) and tobacco hornworm *Manduca sexta* (larval mortality 62% at 50 µg/ml). Under near-UV irradiation, the toxicity of thiophene **11Bi** to the *A. atropalpus* larvae became apparent and polyacetylene **11E** was lethal under all conditions. The variable photoactivities are indicative of a range of mechanisms of actions. The level of **11E** in *R. hirta* increases with mineral stress consistent with stress activation of secondary metabolism [498]. The concentration of the new acetylenic alkamide **46G**, which was co-isolated with known alkamides (**46H–I**), in *Chrysanthemum morifolium* varieties tracked with the plant's resistance to the greenhouse pest western flower thrip (*Frankliniella occidentalis*) [499].

In a limited number of cases, acetylenic natural products from plants have been shown to act as allomones (*i.e.*, trans-specific chemical signals that induce a behavioral change in another species beneficial to the producer). The soldier beetle (*Chauliognathus* sp.) accumulates dihydromatricaria acid (**3E**) in its hemolymph, which it can exude from segmental glands [500]. This compound has been proposed to act as an antifeedent, and may be the root of the beetle's avoidance by jays, mice and ants. While the accumulation of lipophilic natural products may be expected for phytophagous insects, as they maintain high lipid/water ratios, have large guts, and consume many times their body weight of food, the mechanism of localization is not known. Charge transfer complexes between polyacetylenes and unidentified partners have been suggested as a sequestration mechanism [501]. Coupled with strong methoxyalkylpyrazine odorants and bitter 3-phenylpropanamide and 1-methyl-2-quinoline, 1,8-cineole and polyacetylenes are thought to provide a 3rd line of antifeedant defense for the beetle *Metriorrhynchus rhipidius* [502]. The interaction of plants with insects, whether by herbivores, pollinators, or co-inhabitants, should be considered reciprocally in terms of evolution. Plants in evolutionarily distant phylogenies may have chemical traits, such as acetylenic secondary metabolism, reinforced through colonization by insects [461].

5. Future Directions

In this review, we have taken a broad perspective and examined both the recently identified acetylenic metabolites and the current understanding of the reactions relevant to polyacetylene biosynthesis in terrestrial and marine organisms. This has pointed to a number of areas that promise to be fruitful in future investigations: 1) the symbiotic relationships between marine animals and microorganisms extending to polyacetylene biosynthesis, 2) the possibility that marine organisms may largely use the same strategies in polyacetylene biosynthesis as terrestrial organisms, and 3) the potential for engineering value-added crops using the plethora of new and as yet to be discovered genes involved in polyacetylene production.

We are intrigued by the growing recognition of the mutualistic relationships between marine animals and microorganisms. In terrestrial species, a relatively small number of prokaryotic species have been found to produce polyacetylenes, whereas marine bacteria appear to provide the alkynyl precursors to their hosts. The relative ease of collecting species that are in close association with invertebrates or that can be detected through their pathogenic traits has led to the bulk of recent marine fungal compounds being isolated from the ascomycetes and oomycetes. It is clear, however, that a more complete survey of marine basidiomycetes will require continuing improvements in screening and culturing techniques. Seemingly, the land-loving organisms (ca. 13000 land and 11 aquatic species), basidiomycete mushrooms, appear to have evolved through several land-water migrations. A phylogenetic tree of terrestrial, freshwater and marine homobasidiomycetes, including the acetylene-producing organisms wood-degrading *Fistulina hepatica* and marine-derived fungus *Halocyphina villosa*, demonstrates that these organisms are remarkably interrelated [503]. The collection of halotolerant *Physalacria maipoensis* from both upland and salt-water inundated mangroves highlights that these swamps are a type (and endangered) environment for habitat evolution, and the crossover of secondary metabolic pathways. It is expected that these fertile relationships will become increasingly apparent as natural product isolation and genomics provide a fuller vision of microbial and marine secondary metabolic pathways.

Recent studies of plant and marine acetylenic secondary metabolism have suggested that less distinction should be made between the biosynthetic potential of marine and terrestrial organisms as the involved reactions are, for the most part, quite similar. Other than the larger proportion of halogenated compounds, the marine compound diversity might be largely ascribed to the peculiar symbiotic relationships between bacteria, fungi, algae and sponges in

salt-water environments and the apparently large ecological advantage that metabolite accumulation has engendered.

From the preceding observations, a likely PK biogenesis of the highly oxygenated marine polyacetylenes can be proposed. The dominance of polycistronic operons in microbial PK biosynthesis should lead to the cloning of entire pathways for bacterially produced marine polyacetylenes and the heterologous production of pharmacologically useful compounds in pure bacterial cultures. This is a direction with shining prospects as marine organisms have been noted to be one of the most important sources of new drug targets, including the polyacetylenes, however the diversity of isolated strains, rarity of individual species, and intractability of the host organisms continue to present monumental challenges to their commercialization.

Ultimately, the production of acetylenic lipids in genetically-engineered crop plants may provide protection from disease or pests directly to the plant and allow for the synthesis of novel compounds. Heterologous expression of fungal polyacetylene biosynthetic genes and complete marine invertebrate secondary metabolic pathways face difficulties, as eukaryotic genes are not typically clustered. Identifying the specific attributes of the diverged desaturases that turn their chemoselectivity to acetylenation would be greatly accelerated by the structural characterization of the microsomal desaturases, a goal that would have a wide-ranging impact on lipid metabolic research.

Further knowledge of the intermediate structures in these natural product pathways, such as the acyl conjugation of highly unsaturated lipids and the effects of metabolic channeling by metabolite oxidation and chain cleavage upon the distributions of the polyacetylenes, are the initial challenges to exploring the biotechnological future of acetylenic natural products. Plants in the families Asteraceae, Apiaceae and Solanaceae, such as sunflower, carrot, and tomato, are likely to possess substantial portions of the metabolic framework that could lead to the enhanced production of native polyacetylenes that confer disease or pest resistance. Transfer of selected secondary metabolic genes into these organisms could cause large perturbations on the accumulated polyacetylenes and the augmentation of input traits, those beneficial to the crop producer [133]. Substantial accumulation of acetylenic lipids may provide material for the semi-synthetic production of economically valuable compounds. Genetically engineering actinomycetes and other microorganisms, particularly in instances where gene clusters bear self-resistance mechanisms, provide attractive starting points for the commercialization of bioactive compounds from novel organisms.

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List of Abbreviations

ACP	acyl carrier protein
AT	acyltransferase
CoA	Coenzyme A
CDP-CPT	cytidine diphosphocholine diacylglycerol choline phosphotransferase

CFA	cyclopropanyl fatty acid
COX	cyclooxygenase
CYP₄₅₀	cytochrome P ₄₅₀
DAG	diacylglycerol
DGAT, diacylglycerol	acyl-CoA acyltransferase
DGDG	digalactosyldiacylglycerol
DH	β -hydroxyacyl dehydratase
DHAP	dihydroxyacetone phosphate
Dhoea	2,2-dimethyl-3-hydroxy-7-octenoic acid
Dhoya	2,2-dimethyl-3-hydroxy-7-octynoic acid
dNDP	deoxynucleotide diphosphate
%ee	percent enantiomeric excess
EPA	eicosapentenoic acid
ER	endoplasmic reticulum
FAR	fatty acyl-CoA reductases
FAS	fatty acid synthase
Hmpa	2-hydroxy-3-methylpentanoic acid
LIP	lipase
LPAAT	lysophosphatidic acid acyltransferase
KASA	ketoacyl synthase A

KIE	kinetic isotope effect
KR	β -ketoacyl reductase
KS	β -ketoacyl synthase
MCFA	medium-chain fatty acid
Me	methyl
MEP	methylerythritol-4-phosphate
MGDG	monogalactosyldiacylglycerol
N-MeVal	<i>N</i> -methylvaline
NADH	nicotinamide adenine dinucleotide, reduced form
NADPH	nicotinamide adenine dinucleotide phosphate, reduced form
NRPS	non-ribosomal peptide synthase
nt	nucleotide
OPP	pyrophosphate
ORF	open-reading frame
PA	phosphatidic acid
PBAN	pheromone biosynthesis activating neuropeptide
PC	phosphatidylcholine
PCR	polymerase chain reaction
PE	phosphatidylethanolamine
PI	phosphatidylinositol

PKS	polyketide synthase
PK	polyketide
PL	phospholipase
PPTE	4'-phosphopantetheinyl thioesterase
r.a	relative activity
TAG	triacylglycerol
TFA	total fatty acids
VLCFA	very long-chain fatty acids

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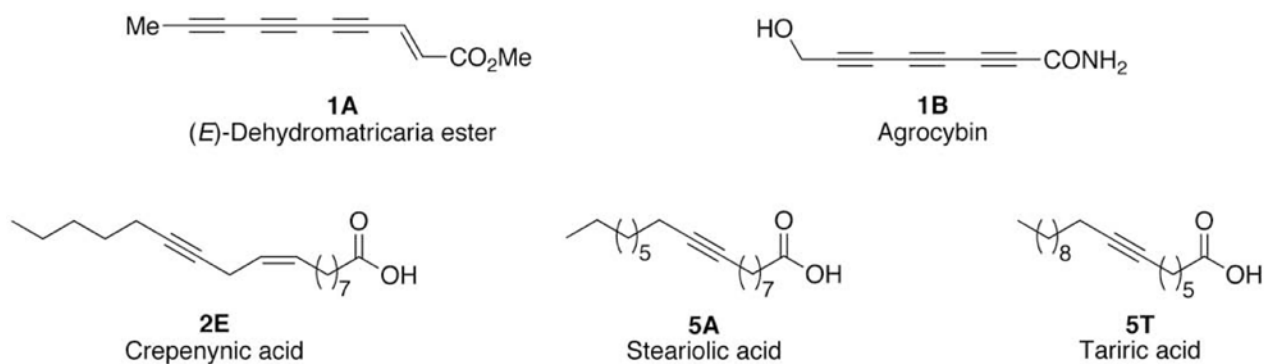
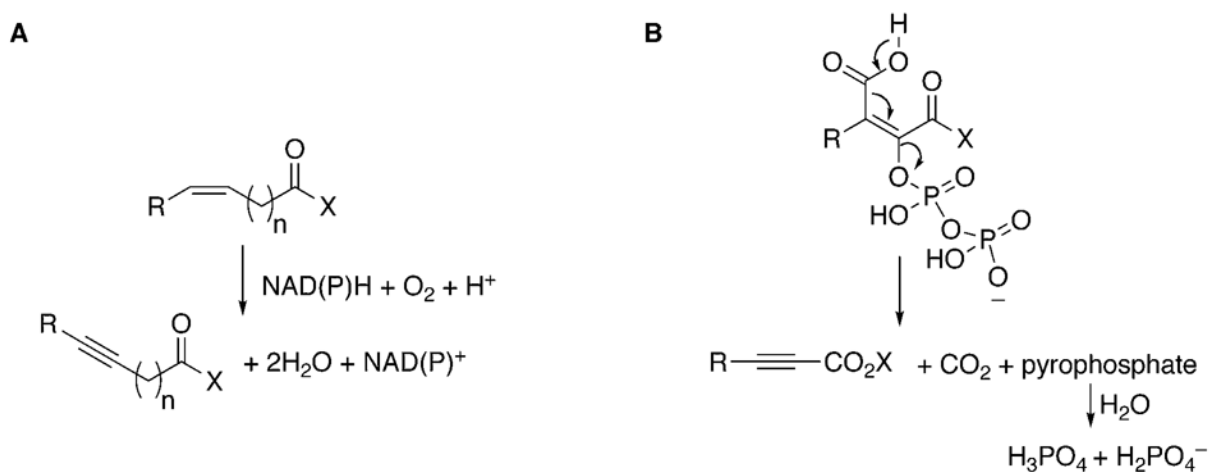
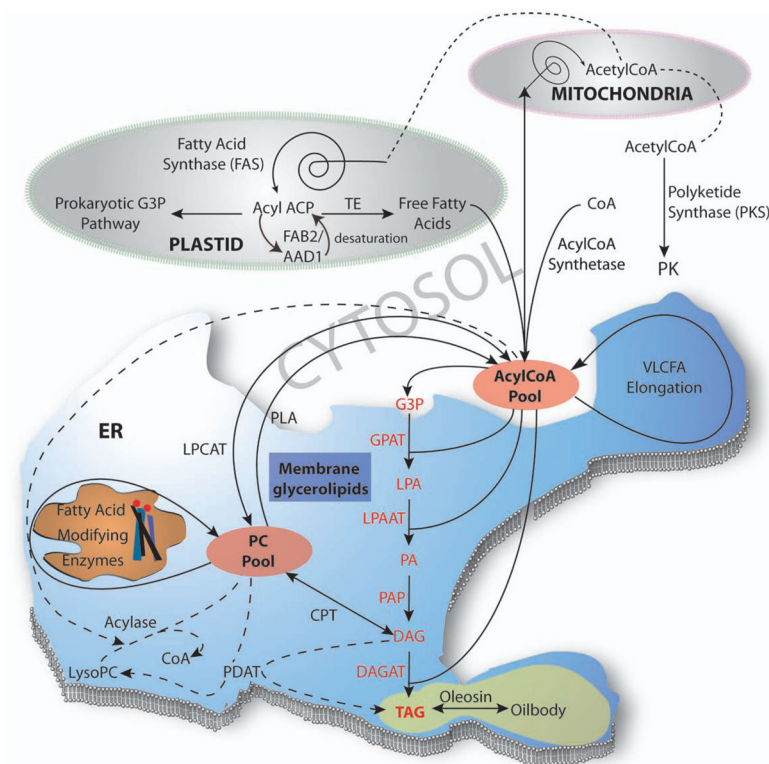


Fig. 1.
Historically and biosynthetically important acetylenic natural products.

**Fig. 2.**

Two distinct proposals for the biogenesis of acetylenic bonds. **A.** Desaturation of existing alkene functionality through an iron-catalyzed dehydrogenation with molecular oxygen [26]. Electrons are provided by either NADH or NADPH. **B.** The elimination of an activated enol carboxylate intermediate is thermodynamically driven by CO_2 formation, which may be coupled to the hydrolysis of pyrophosphate [28]. In the original hypotheses, path A would be operative with full-length acyl lipids and path B would install acetylenic groups during *de novo* fatty acid biosynthesis. Although current paradigm and all experiments dealing with fatty acid biosynthesis are consistent with the desaturase pathway, the elimination hypothesis remains valid for PK-derived acetylenic natural products.

**Fig. 3.**

An overview of the reactions modifying the lipidome and their cellular localization during lipid metabolism. The known processes that synthesize acetylenic bonds and many other oxidatively modified lipids occur at the endoplasmic reticulum, where substrate conjugation to PC appears to be required. In plants, unusual fatty acids are strongly biased to accumulate as TAG in oilbodies. While acyl redistribution is known to occur through the interplay of pathways shown, identification of the specific factors controlling unusual fatty acid accumulation is an area of active investigation. Each type of lipid conjugate can provide a specific entry point for the elaboration of C₈–C₁₈ acetylenic natural products. Both fatty acid and PK-derived secondary metabolism appear to be active in marine organisms.

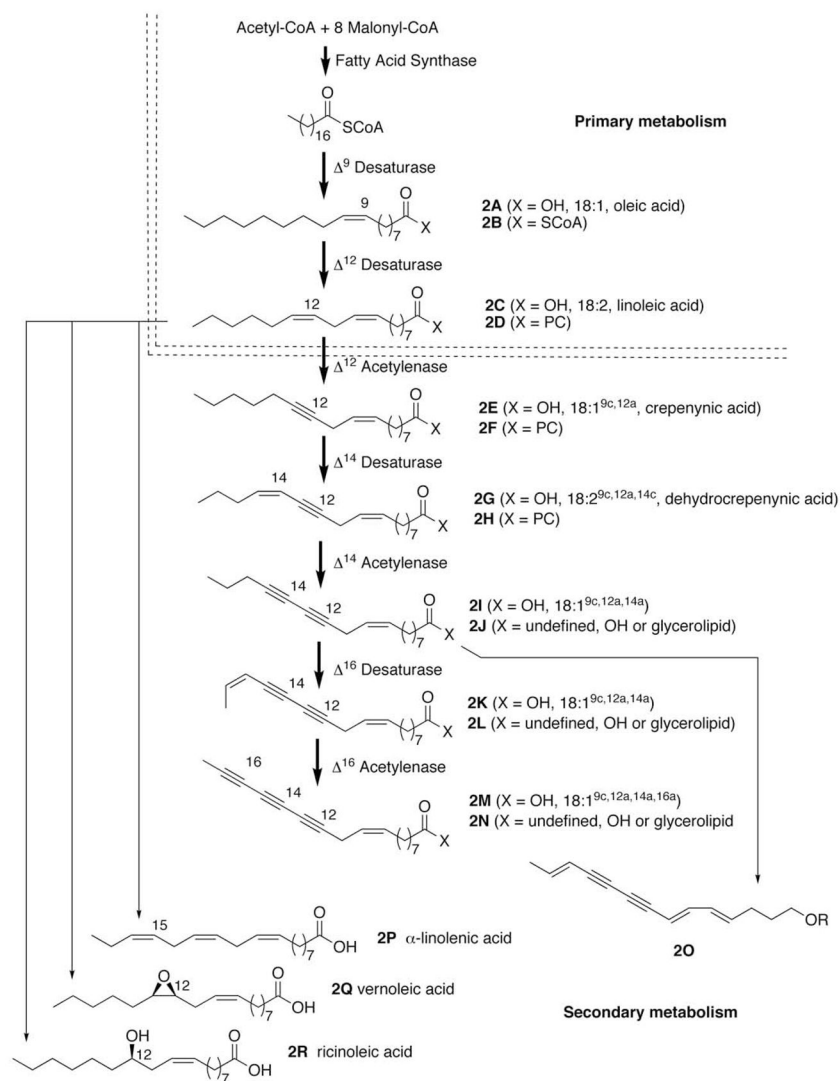


Fig. 4. Acetylenic fatty acids derived from the crepenynate pathway. Primary metabolism provides the pool of fatty acid precursors for **2E**. Fatty acids central to the crepenynate pathway and their most commonly employed acyl conjugates for enzymatic conversions to subsequent products are shown connected by heavy arrows. Pathways to products from other diverged desaturase activities and branches from the crepenynate pathway are shown with light arrows.

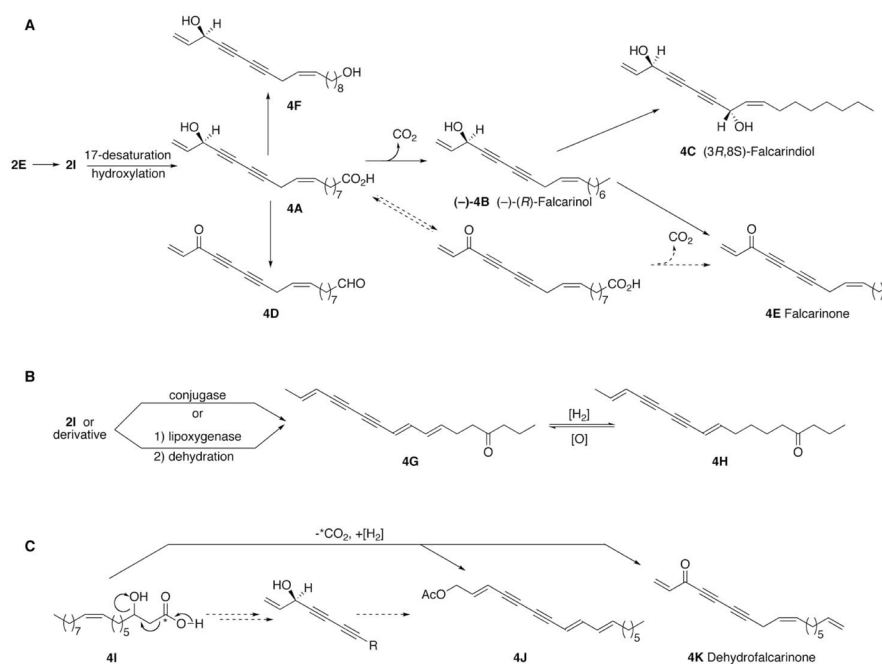
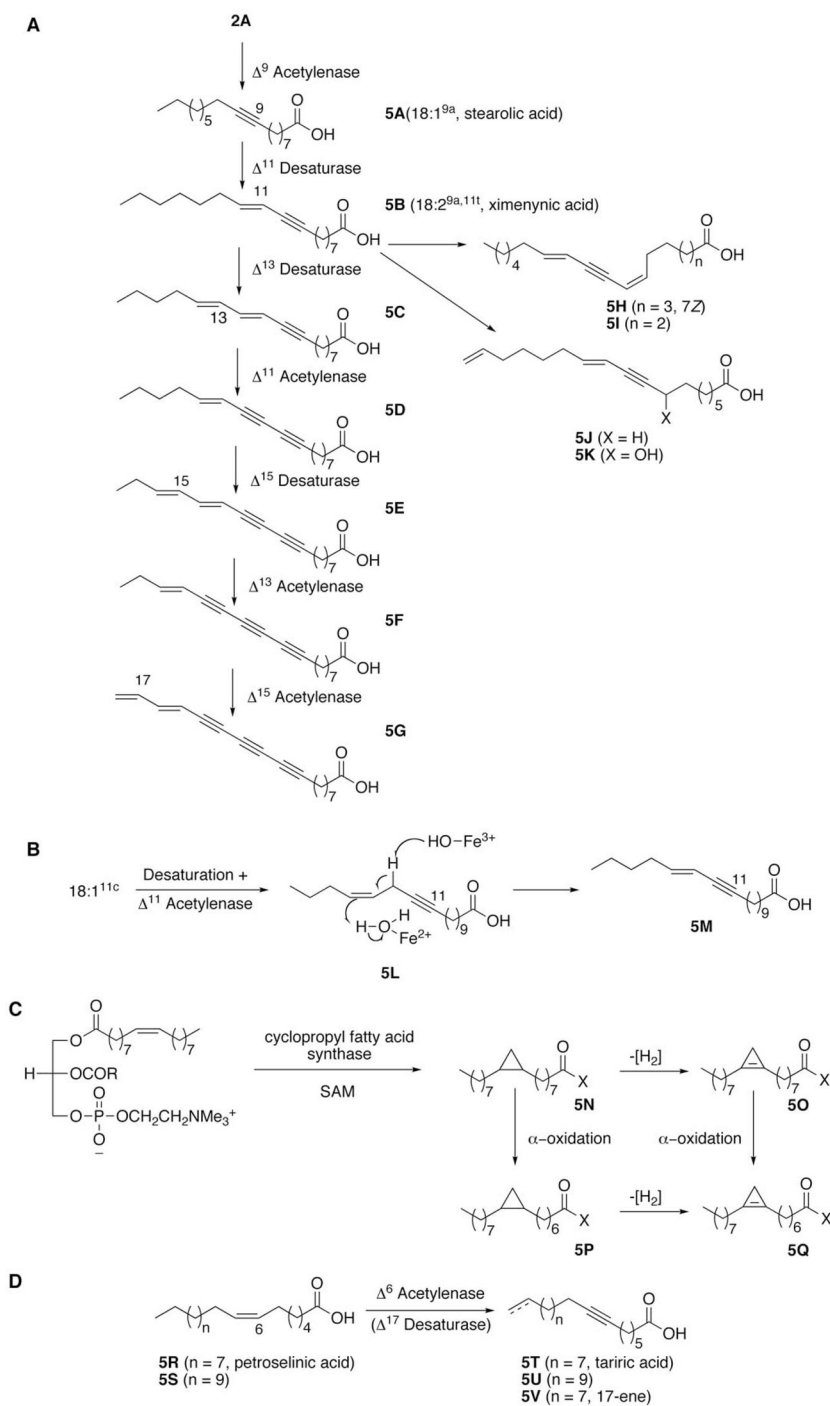


Fig. 6. Falcarinol and related metabolites are widespread amongst Apiaceae and Araliaceae species. **A.** The metabolic web to falcarindiol and falcarinone commences from crepenynic acid, where favored metabolic pathways are depicted with solid arrows. The ubiquitous enzymatic transformations that provide alternative routes to many of the natural products remain largely unexplored (one subset described in the text is marked with dashed lines). **B.** Accessing polyacetylenic metabolites with unsaturation patterns rarely encountered in other fatty acids occurs through combinations of novel desaturation and hydrogenation processes. Hydroxylation/dehydration processes remain viable hypotheses for many unsaturated metabolite pairs. **C.** Species from fatty acid catabolism, such as 3-hydroxyoleic acid, have been found to be metabolic precursors to falcarinol derivatives [54].

**Fig. 7.**

Acetylenic fatty acids derived from the stearolic and tariric acid pathways. **A.** Stearolic acid metabolites relies on a Δ^9 -acetylenase activity and their structures are distinguished by the presences of uniformly *trans*-alkenes. **B.** The formation of the (*E*)-alkenes has been proposed by El-Jaber to pass through an allylic cationic rearrangement [67], although other dehydration or *trans*-desaturation routes are plausible. **C.** Cyclopropenyl fatty acids, originally postulated to rise from acetylenic fatty acids, are created stepwise by an *sn*-1-selective cyclopropane synthase followed by an uncharacterized desaturation activity (X = PC). The coexistence of

cyclopropenyl and acetylenic fatty acids may indicate a common mode of desaturation. **D.** A plant Δ^6 -acetylenase is believed to catalyze the formation of tariric acid and related compounds.

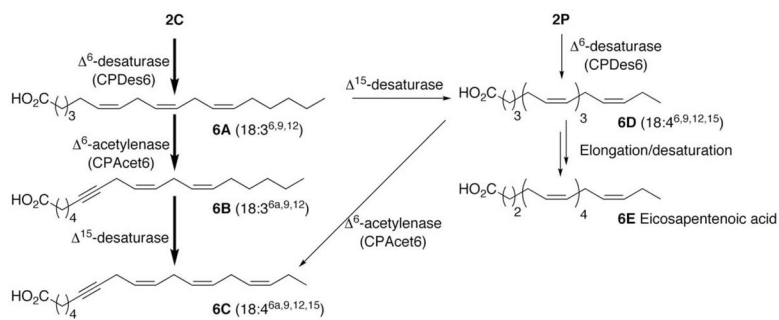


Fig. 8. Acetylenic fatty acid biosynthesis in moss. Conversions of common bryophyte precursors are shown and the favored pathway in *C. purpureus* is marked with heavy arrows.

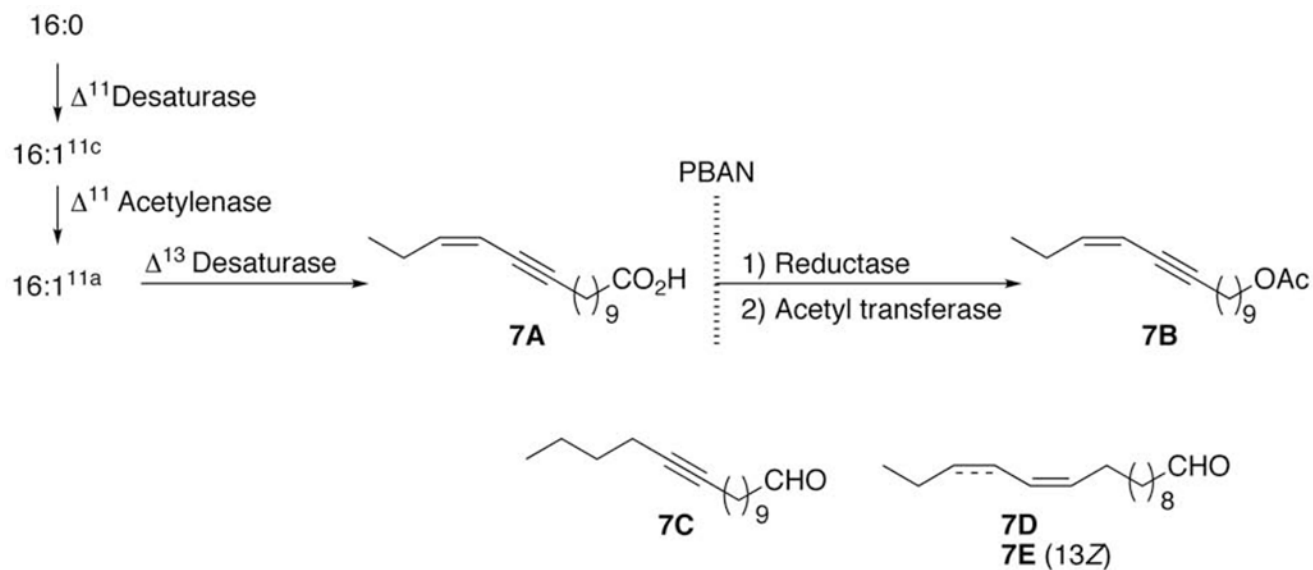
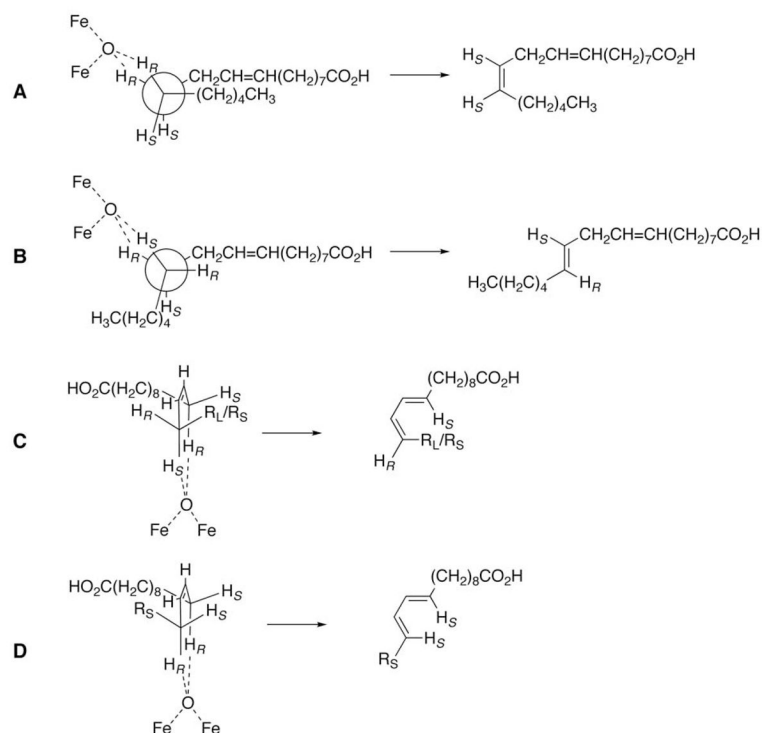


Fig. 9. Acetylenic pheromone biosynthesis in insects. In *T. pityocampa*, the transformation of the acetylenic fatty acid to the pheromone **7B** is blocked in the absence of the hormone PBAN [91].

**Fig. 10.**

Stereochemistry of acetylenase/desaturase activities. An acetylenase, such as Crep1, forms both (9Z,12E)- and (9Z,12Z)-isomers of linoleate. **A.** For the normal (12Z)-isomer, an alkyl chain eclipsed to the carboxylate-bearing chain allows the abstraction of the pro-(R) hydrogens from C-12 and C-13. **B.** During the formation of the (12E)-isomer, a diastereomeric conformation with eclipsing alkyl-hydrogen interactions is proposed to place the alkyl chain in a broadened, or second distinct, binding pocket. In this model, the binding pockets for acetylenases and trans-desaturases are similar and allow for the linearization of the nascent acetylenic lipid. For the bifunctional $\Delta^{11}/\Delta^{10,12}$ -desaturase from the insect *Spodoptera littoralis*, a two binding pocket model has been developed to accommodate the observed stereoselectivity [124]. R_S is a smaller ethyl segment in tetradecenoyl substrates and R_L is a longer butyl projection for hexadecenoyl substrates. **C.** For (11Z)-hexadecenoate, only the binding conformation leading to (10E,12Z)-hexadecadienoate is sterically allowed. **D.** In the case of (11Z)-tetradecenoate, both conformations shown in figure panels **C** and **D** are possible resulting in a mixture of (10E,12E)- and (10E,12Z)-tetradecadienoate. The stereochemical outcomes with myristate and palmitate are modeled by a similar sterically limiting binding pocket model where a large pocket produces the (11Z)-C₁₄ and C₁₆ isomers and a smaller pocket produces solely (11E)-tetradecenoate.

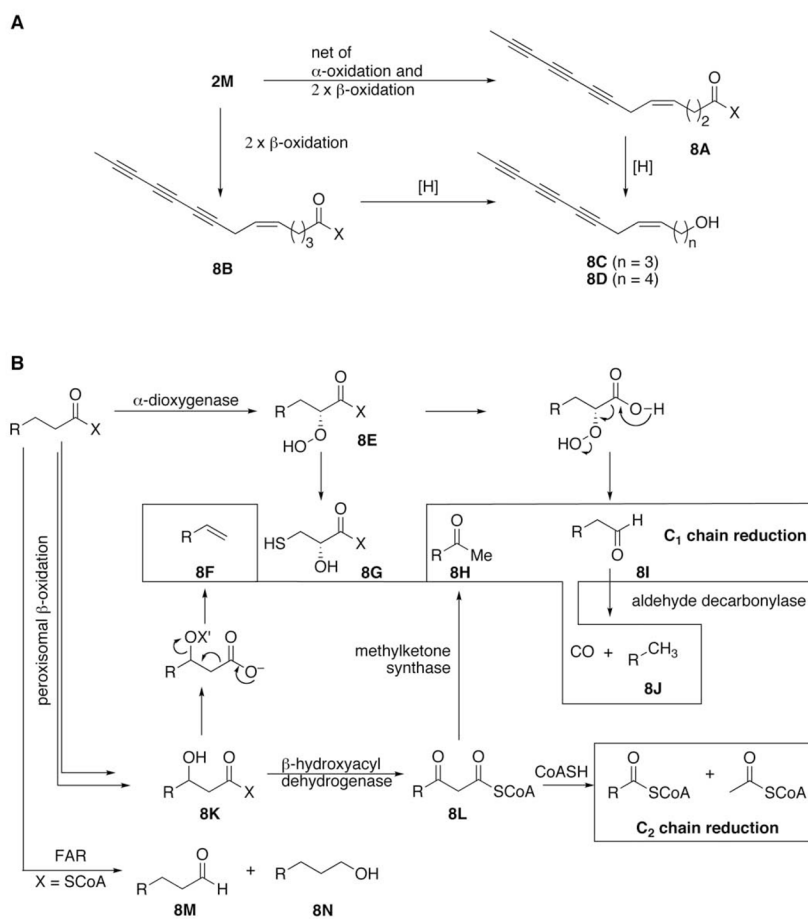


Fig. 11. Carboxy-terminal chain-shortening pathways for fatty acids. **A.** Important C_{13} and C_{14} fatty acids and alcohols result from apparent combinations of α - and β -oxidation pathways. **B.** Aldehyde, alkene, and methyl ketone chain termini are directly formed through α -oxidation chemistry, although the latter two are proposed to arise through fragmentation of β -oxidation intermediates **8K** and **8M**, respectively. X' is an undefined group, such as phosphate or pyrophosphate, that may assist in elimination.

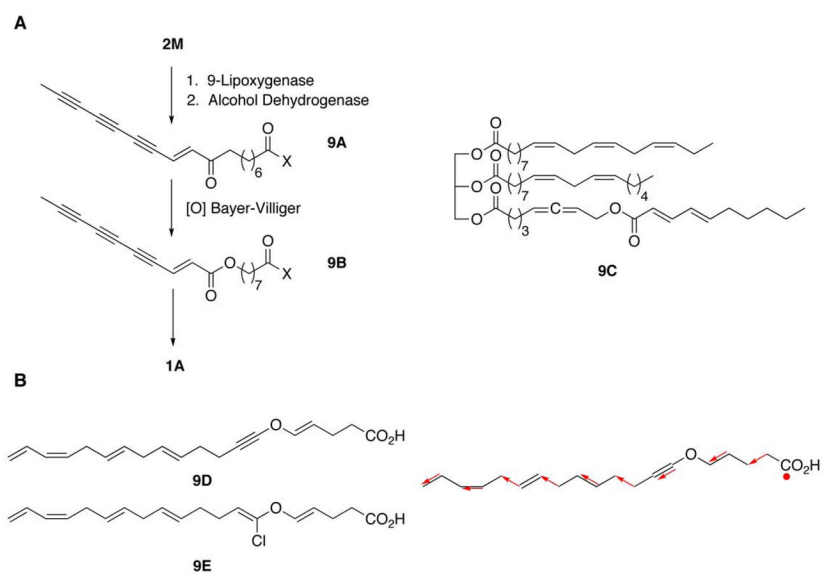


Fig. 12. Intra-chain cleavage pathways. Two processes may be relevant to the insertion of oxygen into an acetylenic fatty acid chain: **A**, Bayer-Villiger oxidation to C_{10} polyacetylenes [12] and **B**, Hock rearrangement of a bis-allylic hydroperoxide leading to maracin A (**9D**) and maracen A (**9E**) [167]. The labeling pattern for **9D** is shown in the lower right.

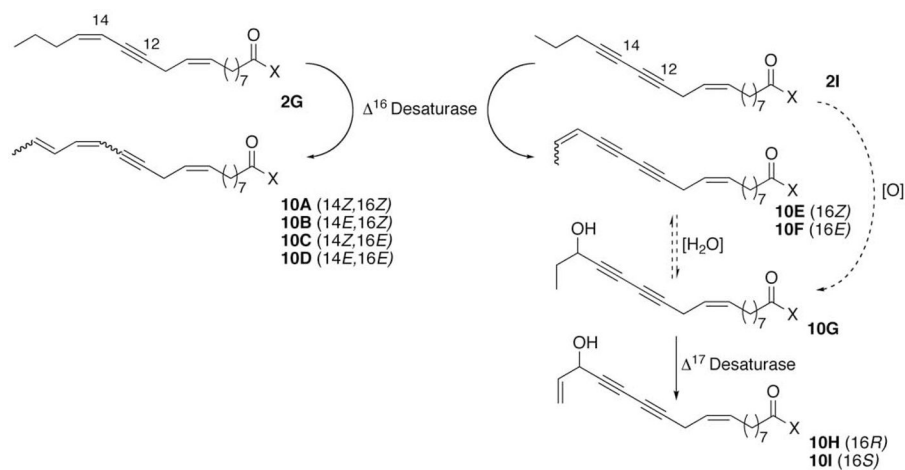


Fig. 13. Modifications to acetylenic fatty acids distal to the carboxyl group. A metabolic grid is presumed to generate a variety of ω -oxidized polyacetylenes, and the functionalization is necessary for their biological functions. For falcarinol, accumulated incorporation data and the recent identification of a terminal desaturation activity support the outlined proposal that many of the metabolic steps occur as acylglycerolipids; however, many details of these reactions remain to be ascertained.

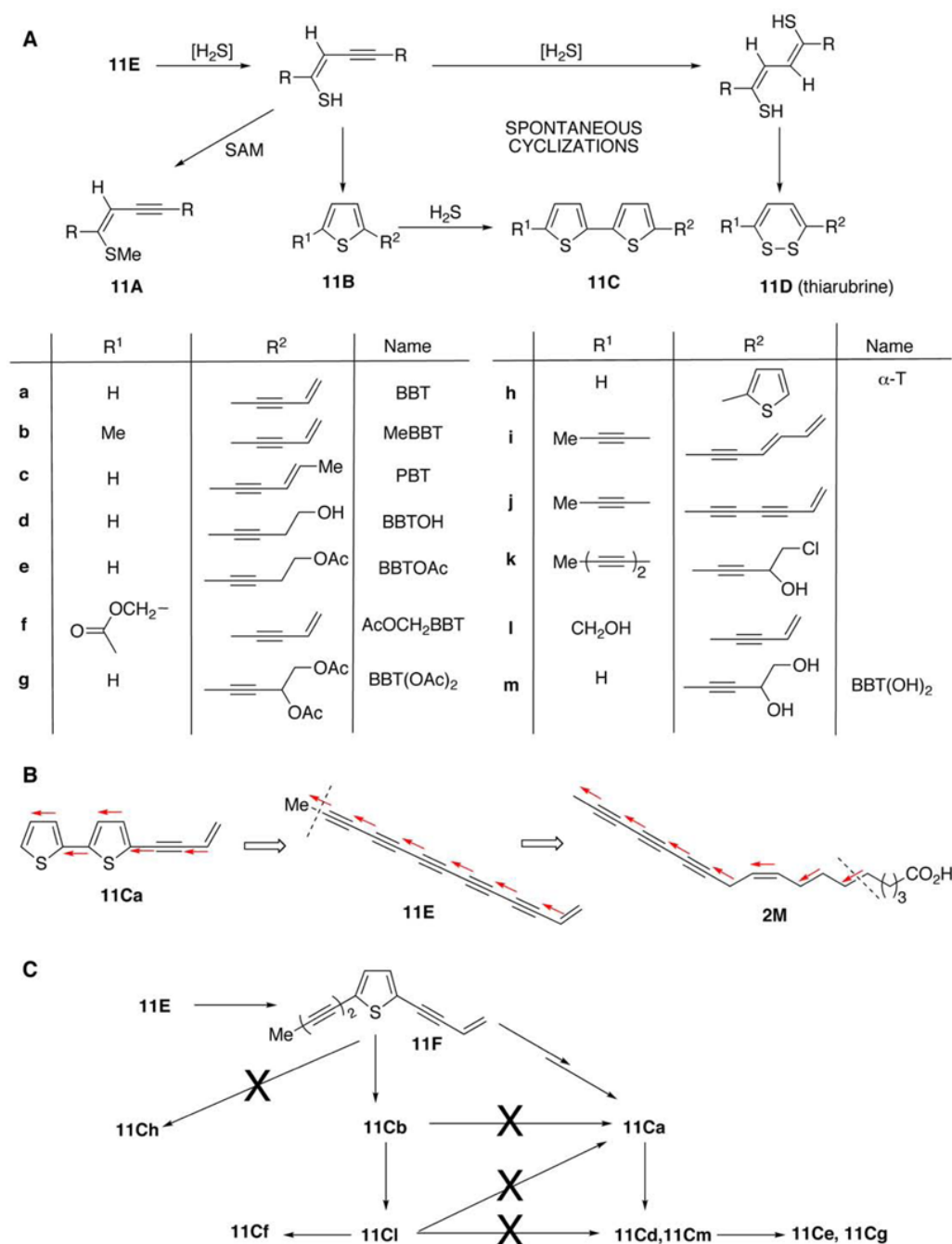


Fig. 14. Sulfur addition to polyacetylenes. **A.** Sulfur addition to a diene unit leads to a thiophene through a stepwise process. Formal addition of H₂S produces vinyl thiols that are intercepted in certain Asteraceae species to produce thioethers (**11A**). Ring closure results in thiophenes (**11B**) and bithienes (**11C**) and oxidative formation of disulfide linkages produces dithienes (**11D**). Combinations of substituent R¹ and R² described in the text are tabulated and designated by a suffix. **B.** Acetate incorporation patterns for dithienyl **11Ca** and its precursors tridecenepentayne (**11E**) and octadecenetriynoic acid (**2M**) determined through [¹³C]glucose feedings of *T. patula* [189]. **C.** The summarized incorporation experiments indicate that methyl

cleavage reactions occur prior to the formation of the second heterocycle creating two distinct reaction manifolds [190].

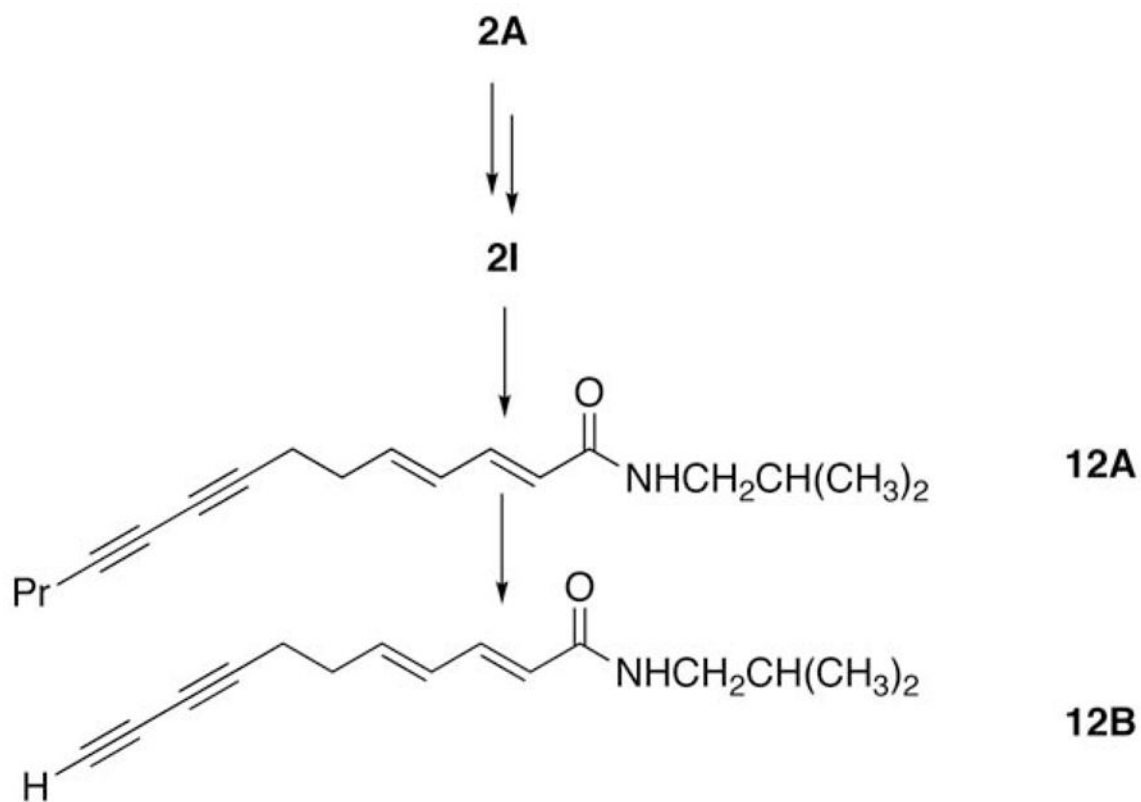


Fig. 15.

Proposed biosynthesis of alkamides. The ligation of an alkyl or aryl amine with a polyalkenyl or acetylenic fatty acid produces the alkamides. The progression from **2A** to **12B** has been verified in *Echinaceae pupurea* by radiotracer studies with $[10\text{-}^{14}\text{C}]$ methyl oleate, $[16\text{-}^{14}\text{C}]$ methyl enediyanoate, and $[12\text{-}^{14}\text{C}]$ anacyclin, however, the enzymatic participants have not been identified [194].

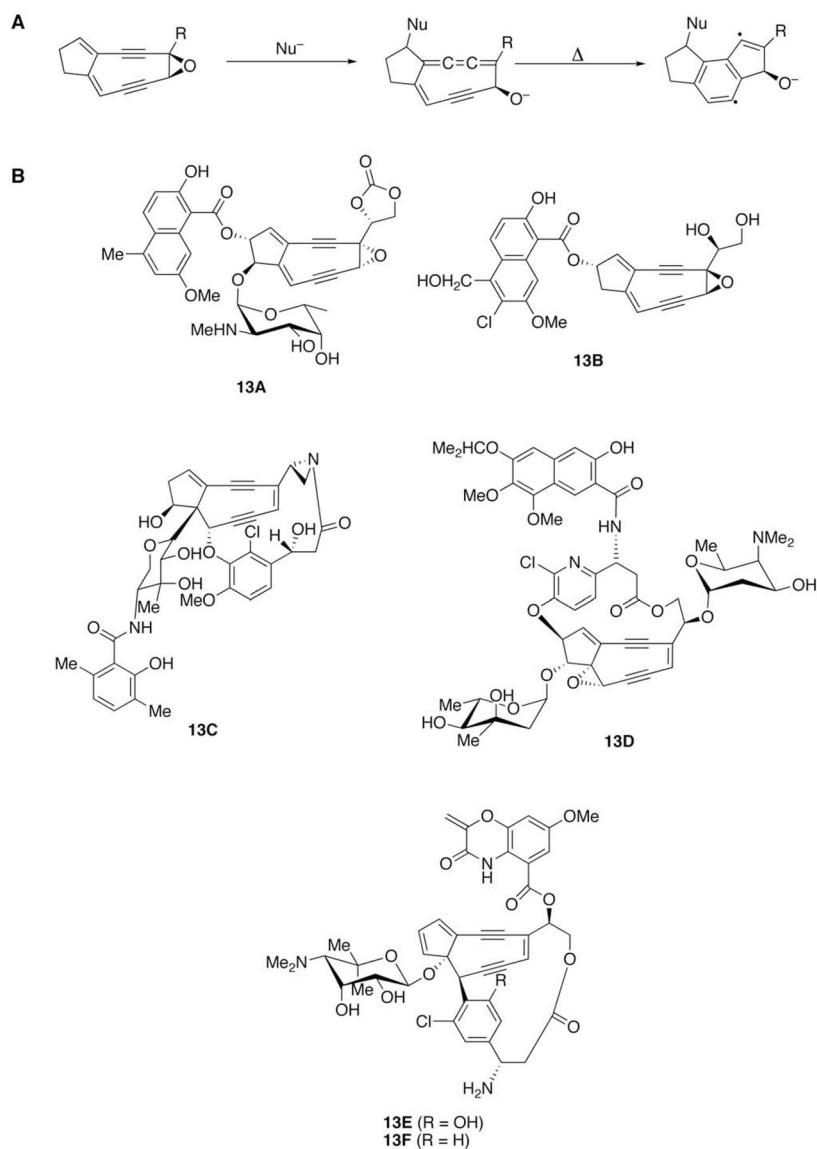


Fig. 16. Nine-membered ring cycloenediyne. **A.** Cyclization commonly occurs by nucleophilic attack on a diacetylenic chromophore yielding a strained cumulene-ene-yne (shown) or by perturbing a moiety stabilizing an enediyne. **B.** Structures of C_9 cycloenediyne natural products.

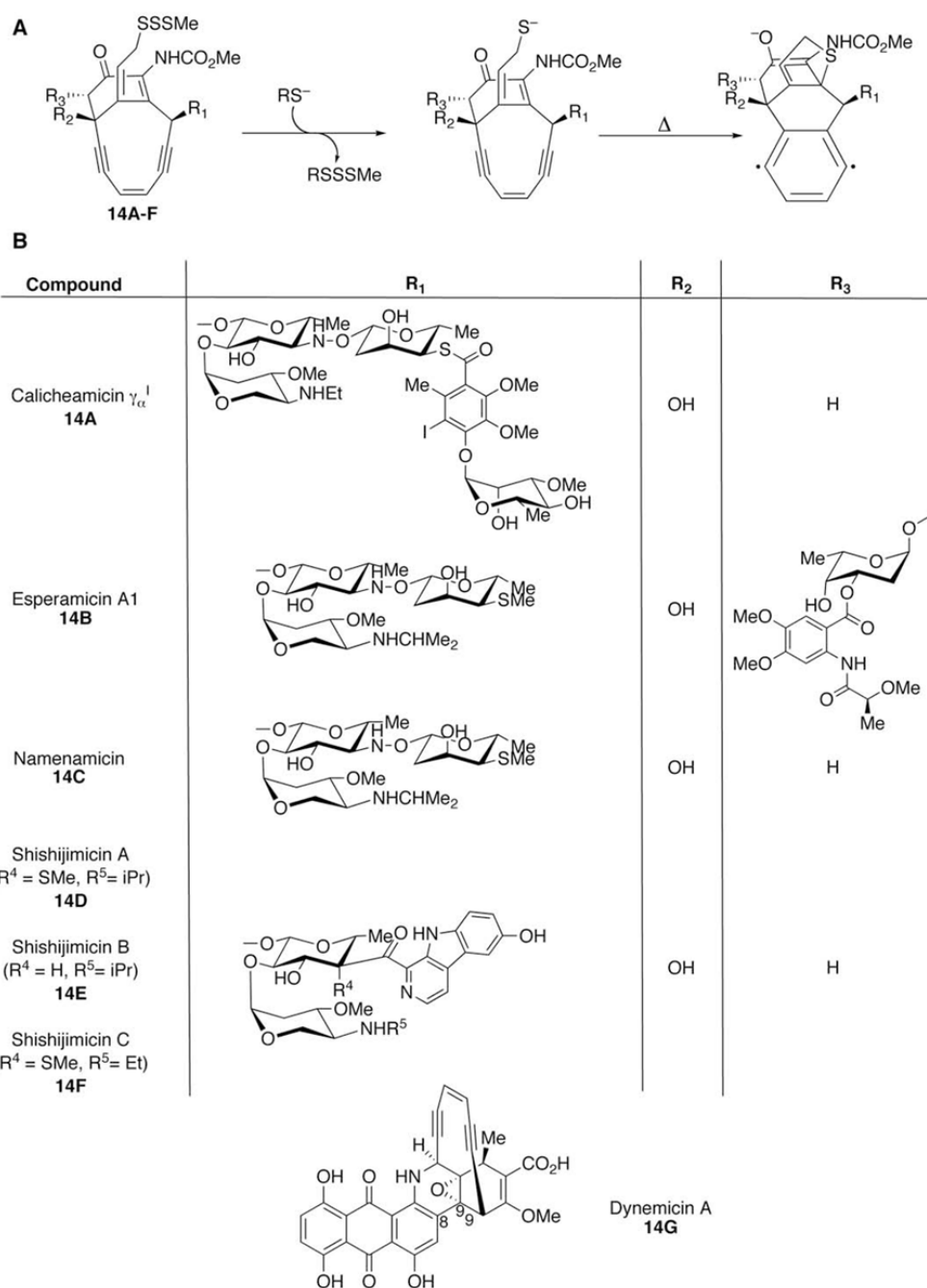
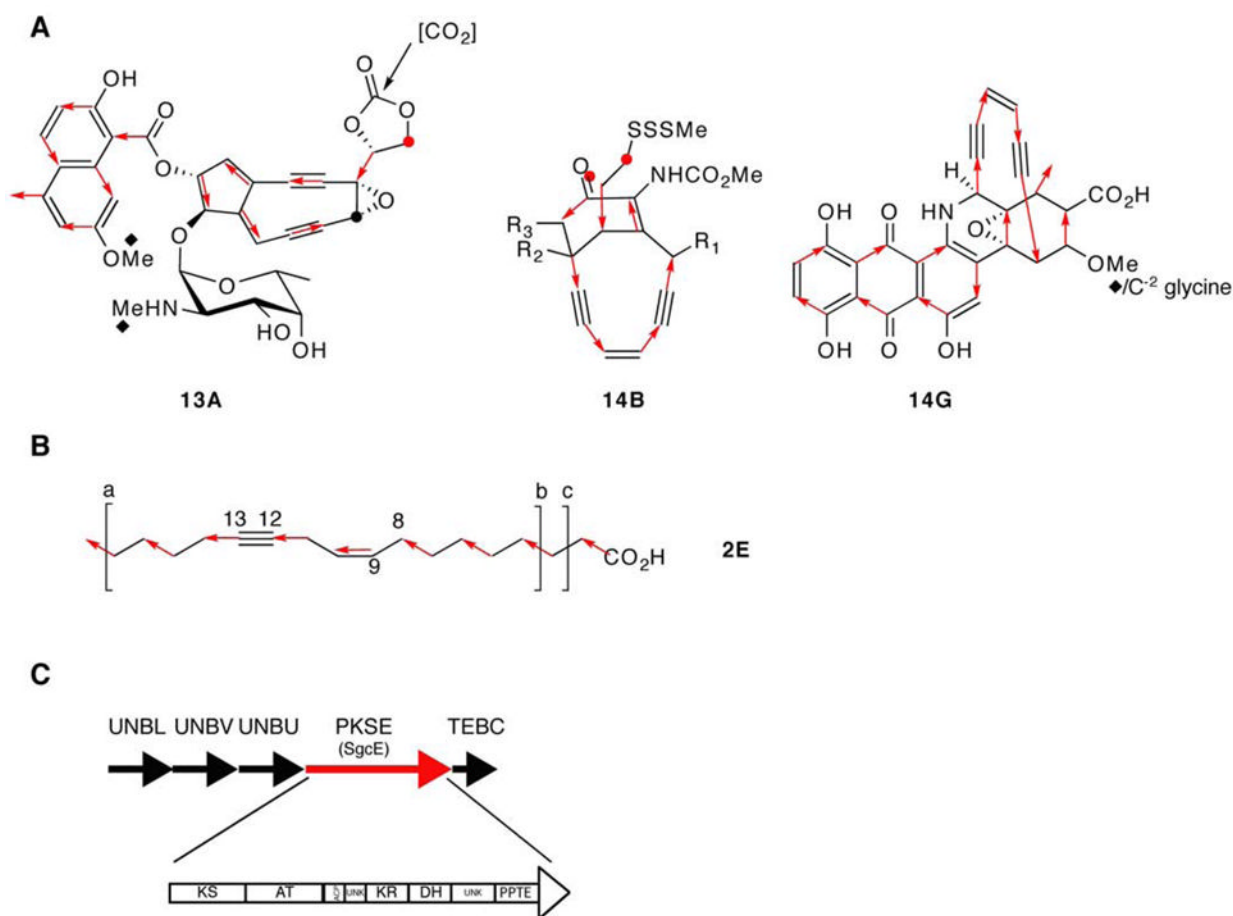


Fig. 17. Ten-membered ring cycloenediynes. **A.** The calicheamicin-like metabolites cyclize through a thiol-activated Michael addition activating the subsequent Bergman cyclization. **B.** Structures of the C₁₀ cycloenediyne natural products.

**Fig. 18.**

Biosynthesis of the enediynes. **A.** Incorporation patterns have been ascertained for three of the enediyne natural products [208–210]. **B.** Crepenynate was originally suggested as a progenitor, which was cleaved to a C₁₄ species. In this model, the ab fragment leads to **13A**; either ac or b fragments provide the labeling pattern for **14A–F** [209]. **C.** The organization of SgcE, the PKS for C-1027 biosynthesis, is consistent with an iterative PKS enzyme and includes an ORF for a specialized PPTE domain and several ORFs of unknown function [214]. PKS subdomains are: ACP, acyl carrier protein; AT, acyltransferase; DH, dehydratase; KR, β -ketoacyl reductase; KS, β -ketoacyl synthase; PPTE, phosphopantetheinyl thioesterase; and UNK, domain of unknown function.

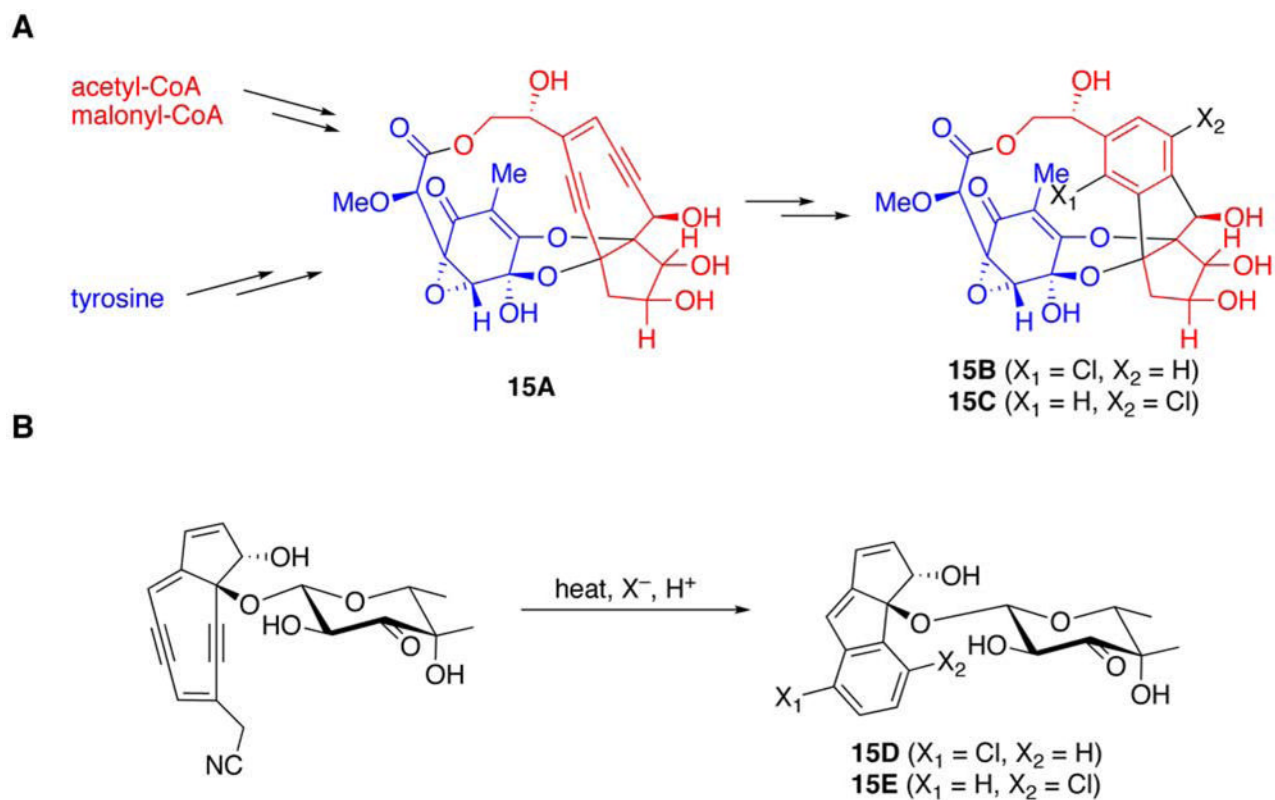


Fig. 19. Metabolites produced via haloenediyne cyclizations. **A.** **15B** and **15C** originate from the mixed PK enediyne presporolide, which undergoes a haloenediyne cyclization to produce the aromatic core [220]. **B.** Halide accelerations of enediyne cycloaromatization had been recently shown with for the cyanosporasides **15D–E** [221].

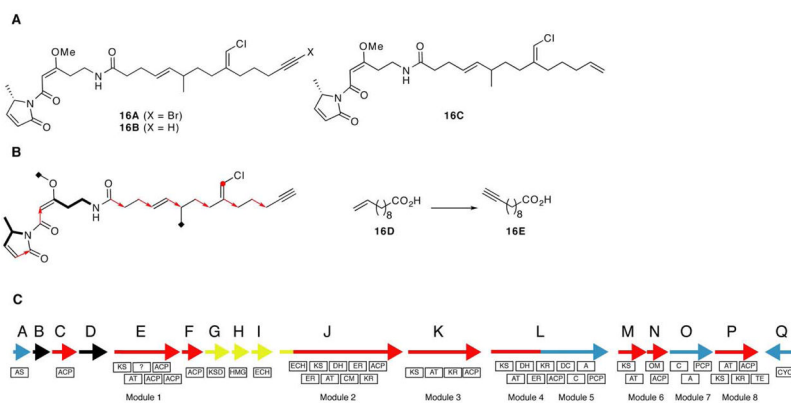


Fig. 20. Jamaicamides and their biosynthesis [223]. **A.** Structures of jamaicamides A-C (**16A–C**). **B.** Analysis of isotopic incorporation studies for jamaicamide B. ω Acetylenic fatty acids, which have been previously reported and may be formed through acetylenase action (e.g., **16D** to **16E**), are potential starter units for these PKS-NRPS metabolites. **C.** The jamaicamide biosynthetic gene cluster. JamB bears resemblance to a fatty acid desaturase. A portion of JamE is of unknown function, possessing weak similarity to the Fe^{2+} / α -ketoglutarate-dependent dioxygenases. Open-reading frames are shown as arrows. The color of an arrow indicates its assigned pathway: blue, NRPS; red, PKS; yellow, other assigned function; black, function not annotated. Boxes provide the order of the subdomains for the multifunctional polypeptides: A, adenylation domain; ACP, acyl carrier protein; AS, acyl-ACP synthetase; AT, acyltransferase; C, condensation domain; CM, C-methyltransferase; CYC, cyclase; DC, decarboxylase; DH, dehydratase; ECH, enoyl-CoA hydratase/isomerase; ER, enoyl reductase; HMG, 3-hydroxy-3-methylglutaryl-CoA synthase; KR, β -ketoacyl reductase; KS, β -ketoacyl synthase; KSD, β -ketoacyl synthase/dehydratase; OM, O-methyltransferase; PCP, peptidyl carrier protein; and TE thioesterase.

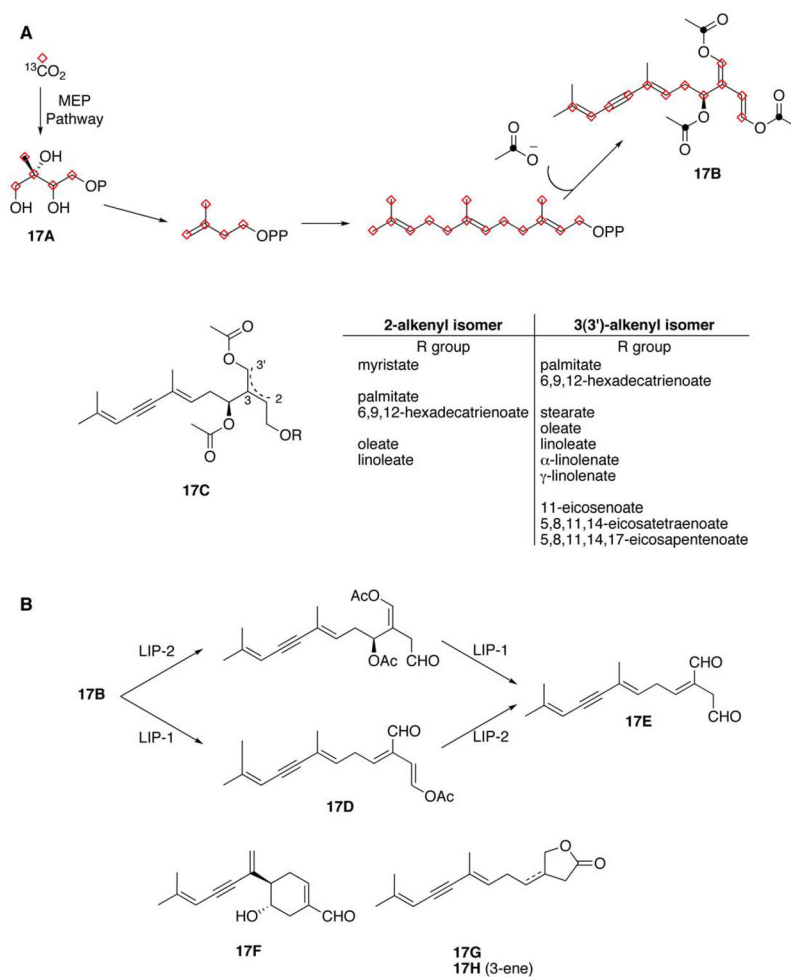


Fig. 21. Marine terpenoids. **A.** Incorporation studies of caulerpenyne have clearly shown this metabolite to arise from the MEP pathway [233]. A diverse set of acylated metabolites has been identified. **B.** The order of lipase reactions leading to the highly toxic secretion oxytoxin-2 are believed to follow the upper path *in vivo* [236]. Other cyclized species, such as **17F–H**, are known in this metabolic family.

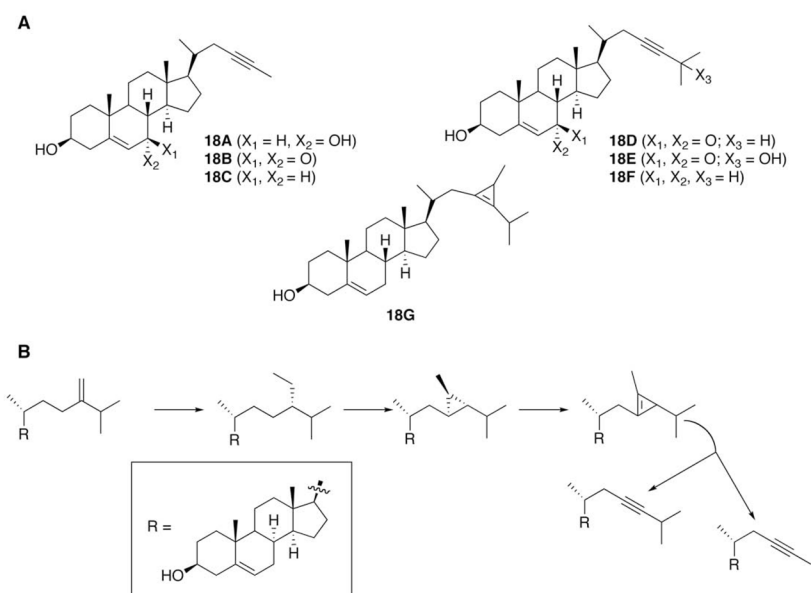
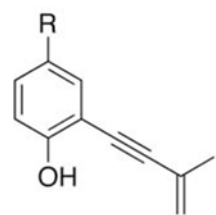
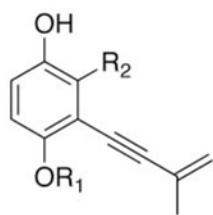


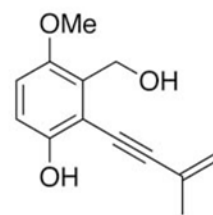
Fig. 22. **A.** Structures of acetylenic sterols from marine sources. **B.** Biosynthesis of C_{23} -alkynyl steroids from 24-methylenecholesterol [241].



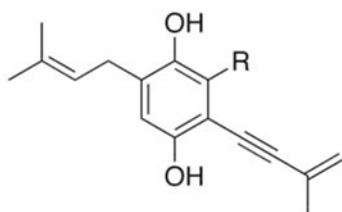
19A (R = CHO)
19B (R = CH₂OH)
19C (R = CO₂H)



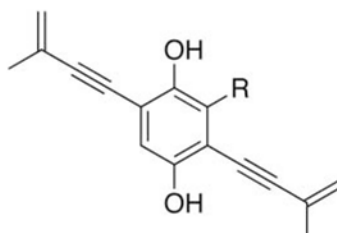
19D (R₁ = H, R₂ = H)
19E (R₁ = Me, R₂ = H)
19F (R₁ = H, R₂ = CH₂OH)



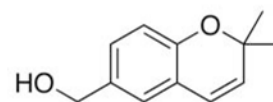
19G



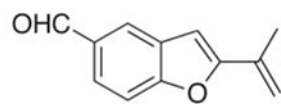
19H (R = H)
19I (R = CHO)



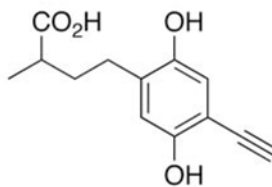
19J (R = CH₂OH)
19K (R = CHO)



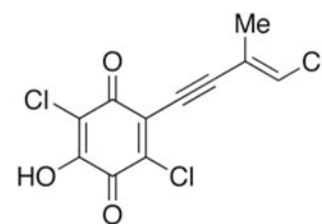
19L



19M



19N



19O

Fig. 23.
 Structures of quinoid natural products with acetylenic substituents and related aromatic heterocycles.

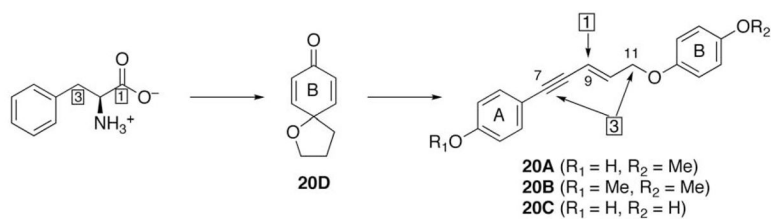


Fig. 24. Biosynthesis of polyacetylenes via the shikimate pathway. While it is rare that acetylenic metabolites result from only shikimate intermediates, the aryl ethers **20A–C** have been proposed to arise from two such fragments via the hypothetical cyclic ether **20D** [261,262].

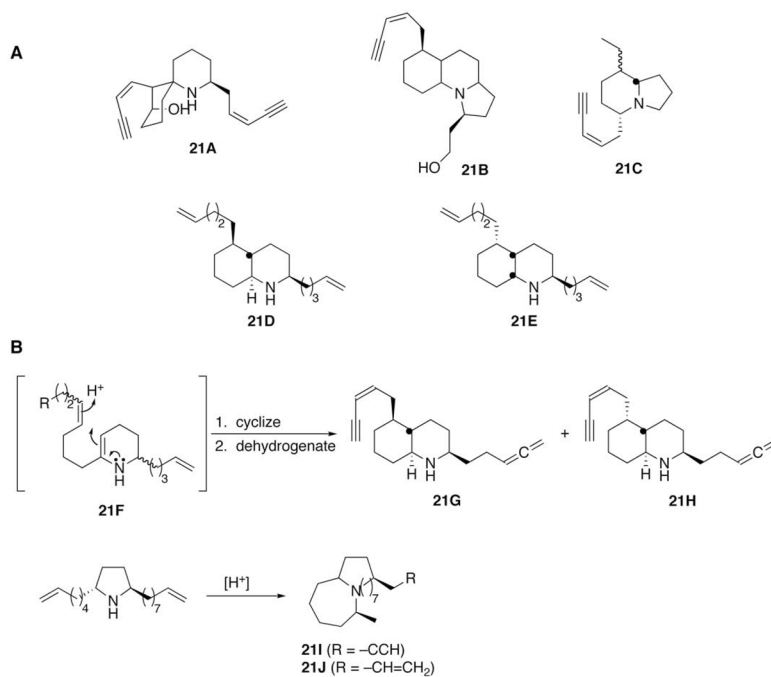


Fig. 25. Acetylenic alkaloids from amphibians. **A.** Structures of well known acetylenic polycyclic amines isolated from ants and/or frogs and their precursors. **B.** Enamine cyclizations are believed to expand the ring systems, and subsequent dehydrogenations occur to form acetylenic and allenic derivatives.

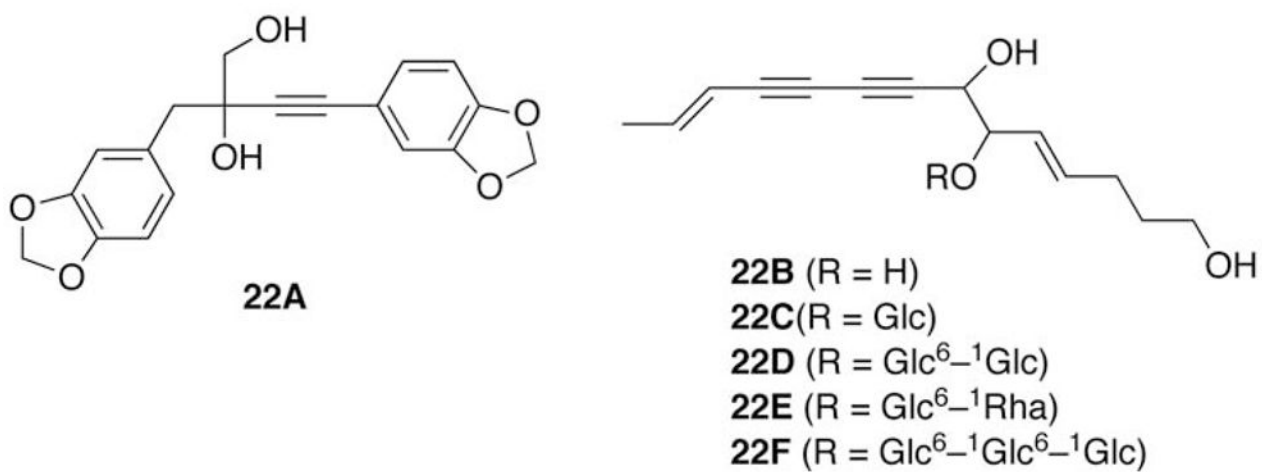


Fig. 26.
 Acetylenic secondary metabolites from the Campanulaceae family.

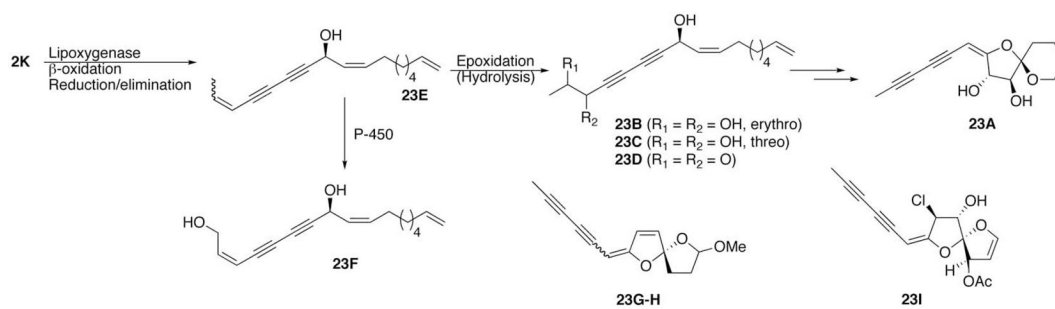


Fig. 27.
Proposed biosynthesis of acetylenic spiroketals found in Asteraceae species.

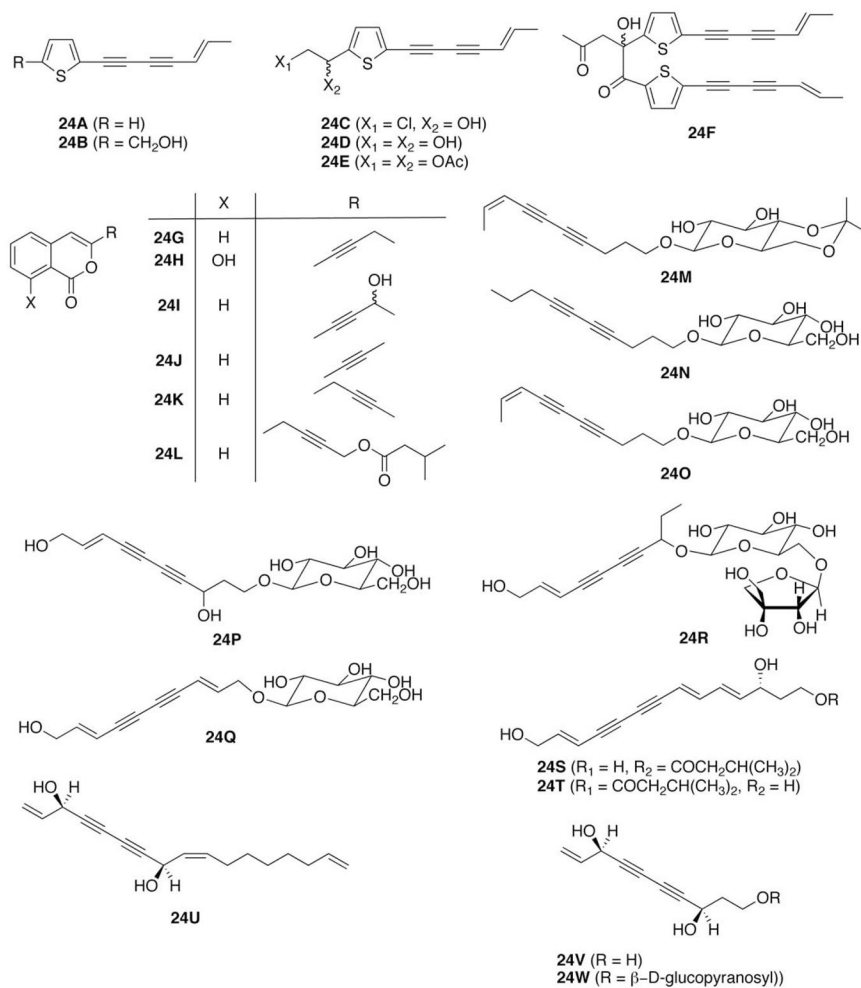


Fig. 28. Acetylenic natural products from the Asteraceae species include thiophenes, polyols, glycosides, and coumarins.

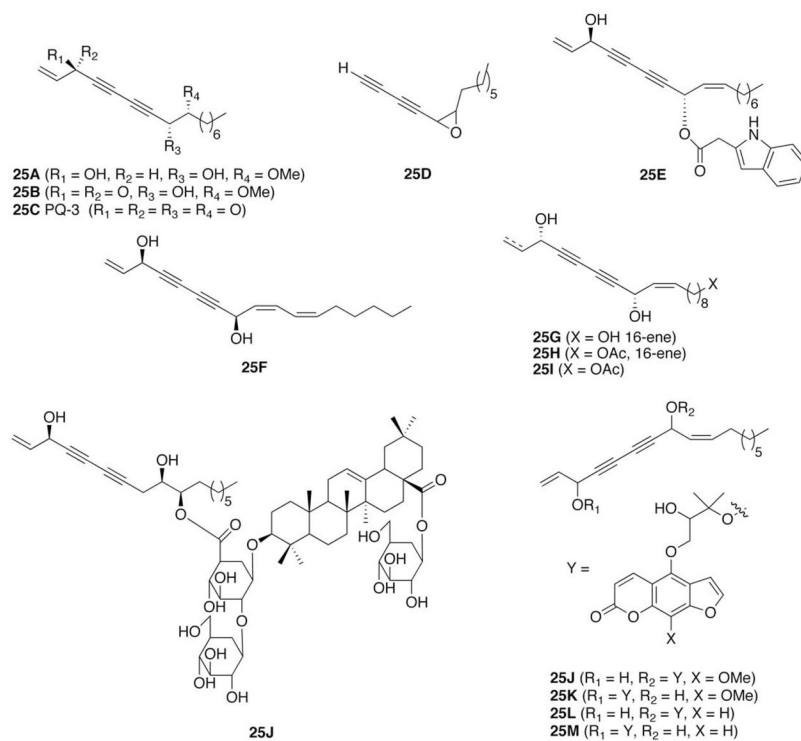


Fig. 29.
 Polyacetylenes from the plant families Apiaceae and Araliaceae.

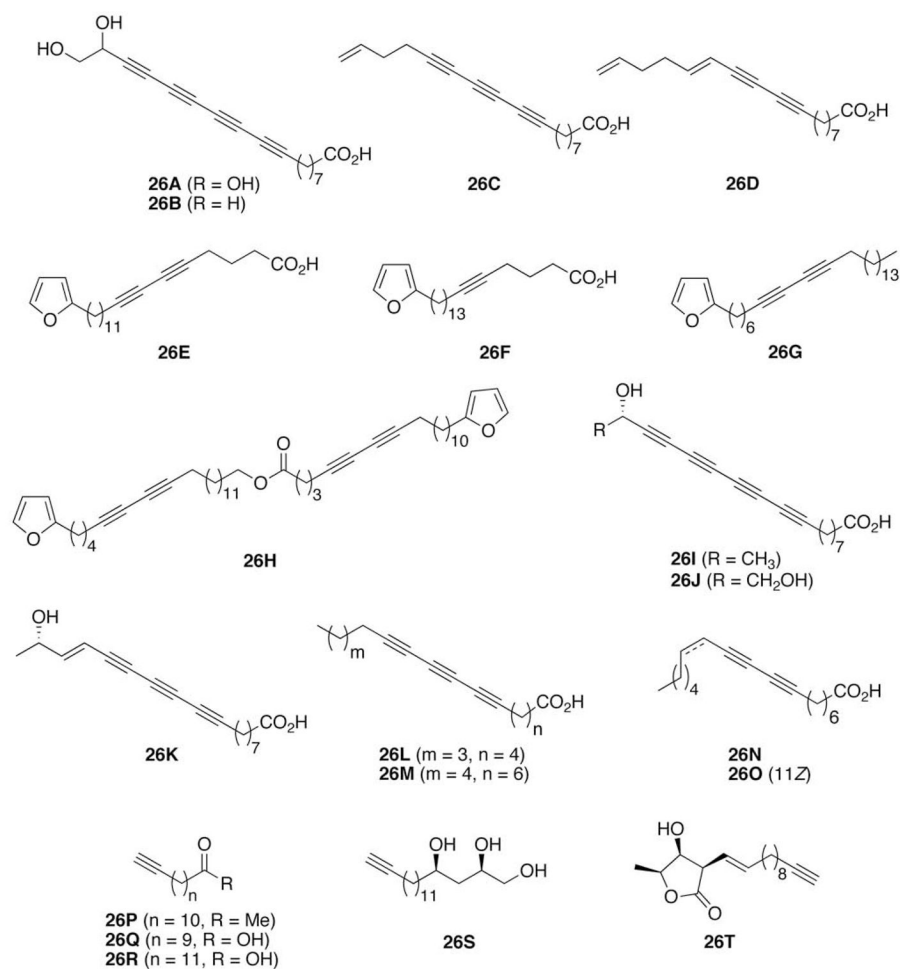


Fig. 30. Acetylenic secondary metabolites from the families Annonaceae and Lauraceae.

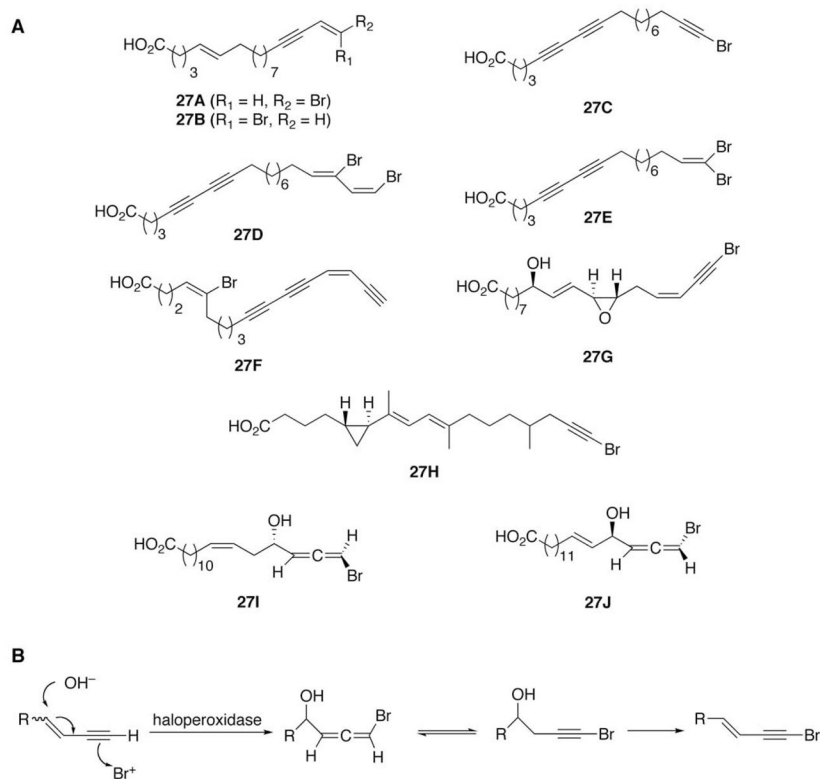


Fig. 31. **A.** Brominated acetylenic and allenic fatty acids from lichen. **B.** Hypothetical biosynthetic pathway to 1-haloacetylenic fatty acids in these organisms [319].

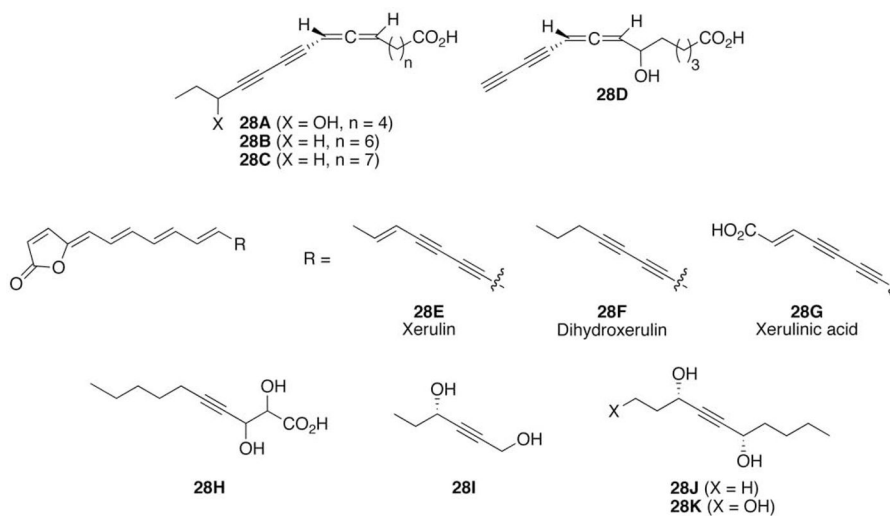


Fig. 32. Polyacetylenes including the phomallenic acids, 07F275 and the xerulins have been recently identified from fungi.

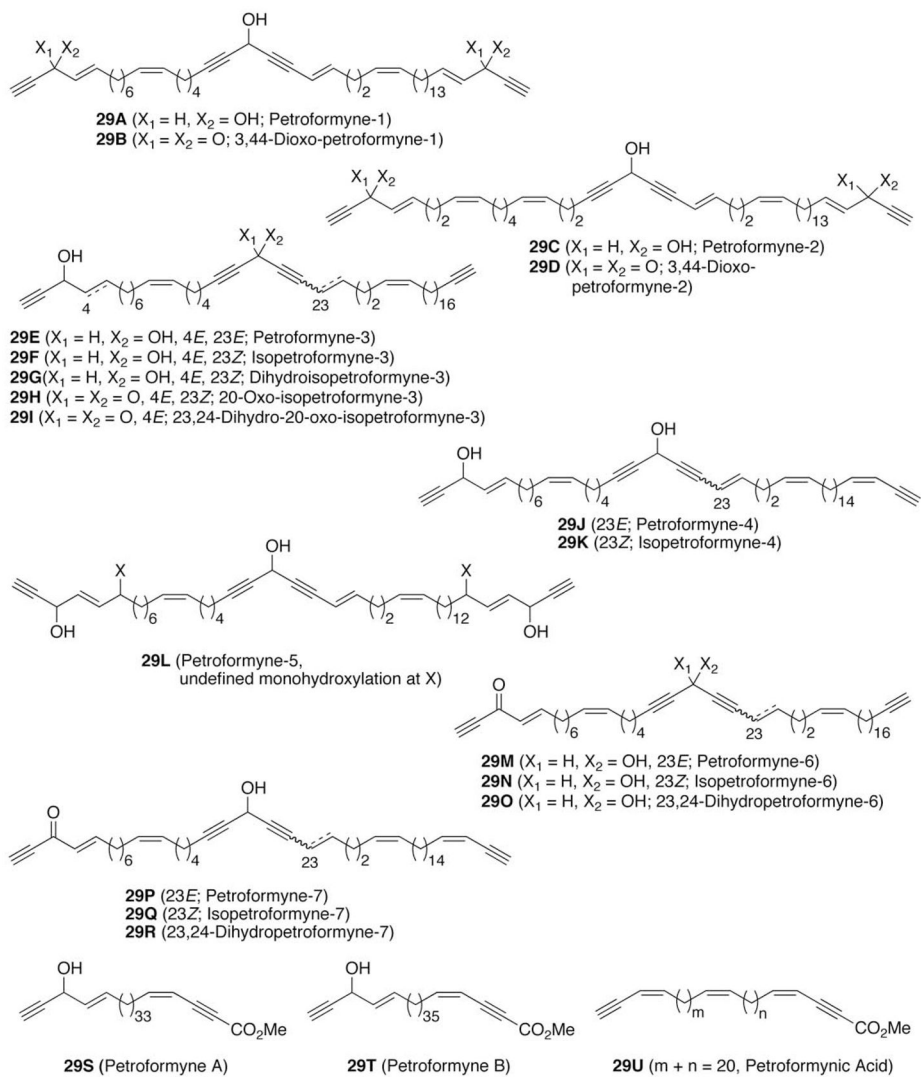


Fig. 33.
The petroformyne family of sponge secondary metabolites.

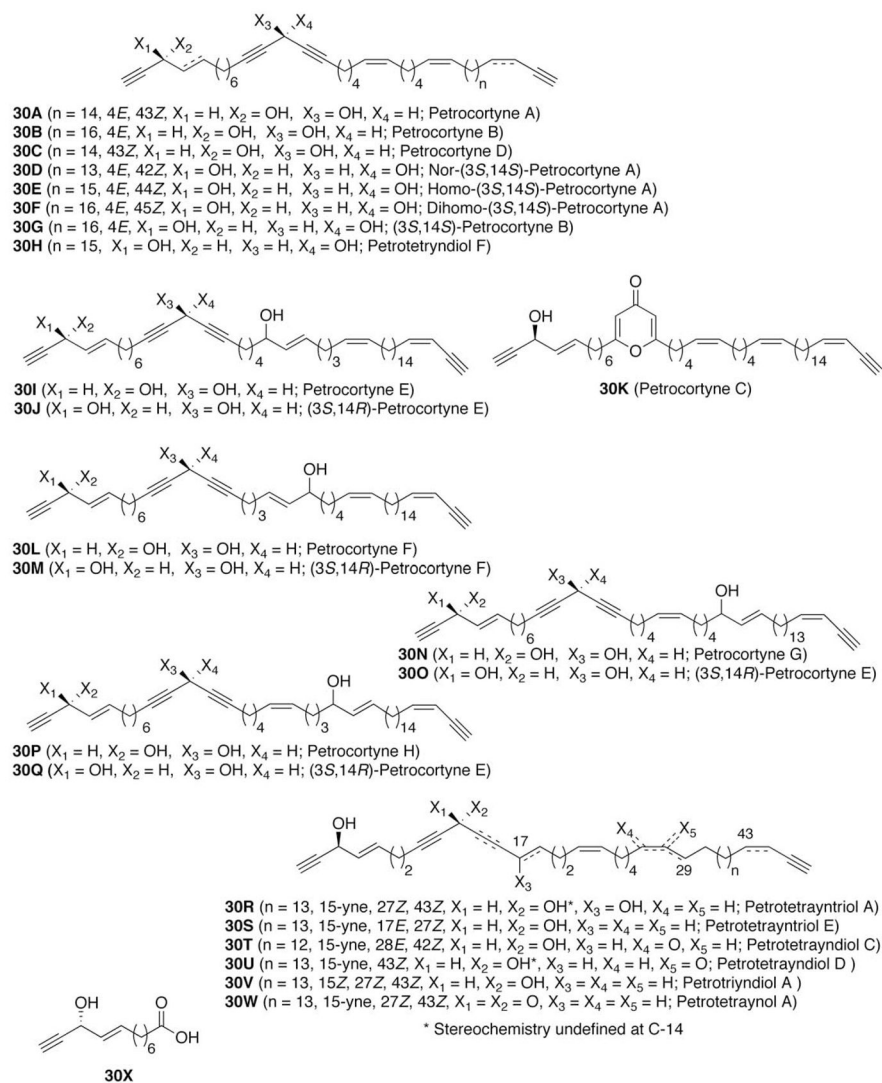


Fig. 34. The petrocortynes family of sponge natural products and other *Petrosia* ssp. polyacetylenic alcohols.

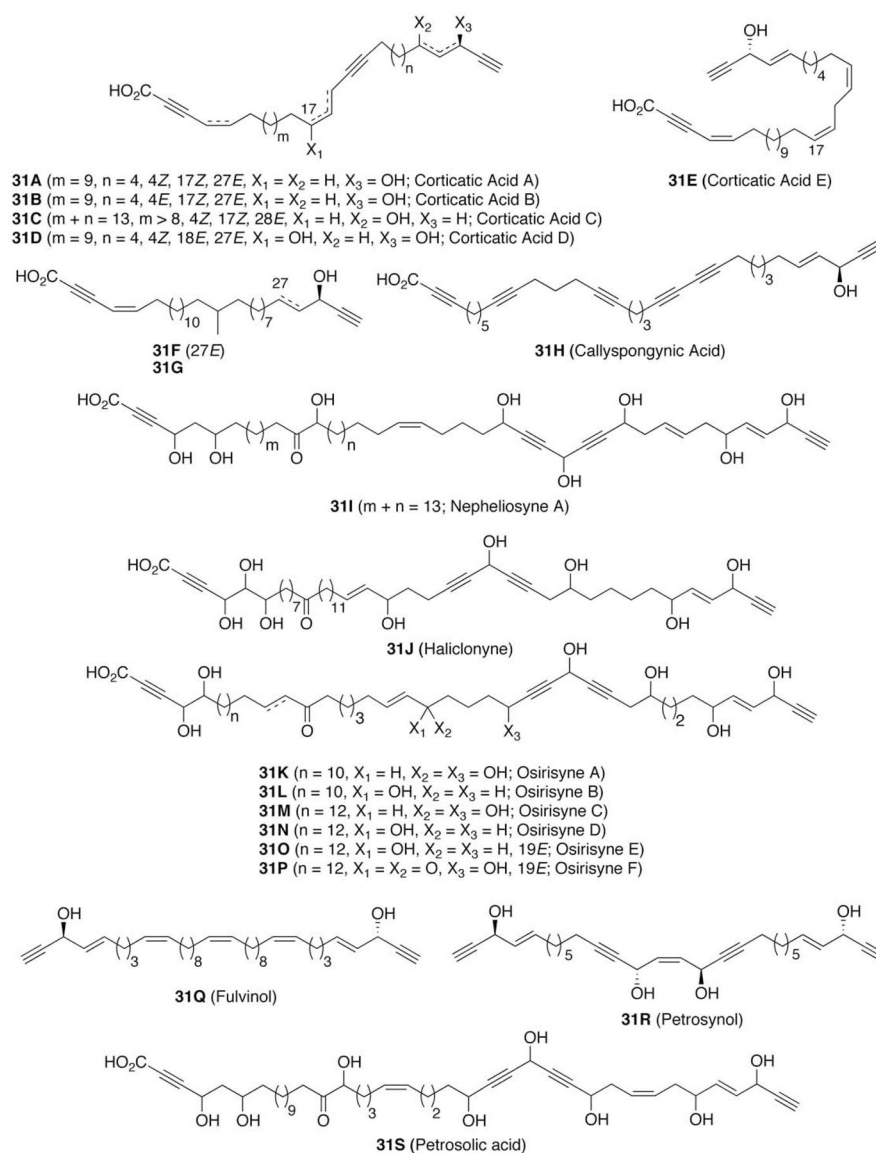


Fig. 35. Very long-chain acetylenic acids, including the corticatic acids (**31A–E**) and nepheliosyne A (**31I**), have structural similarities suggesting the involvement of common biochemical activities, including terminal oxidases. The size and symmetrical nature of di- and tetrahydroxyacetylenes **31Q** and **31R** support the involvement of hypothetical condensation reactions of long-chain fatty acid precursors.

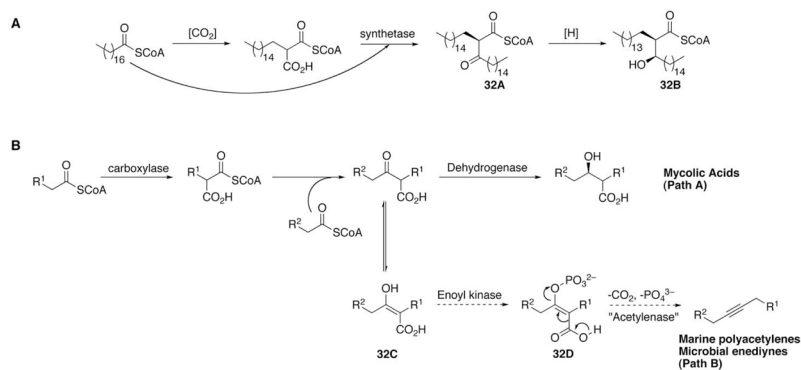


Fig. 36.

A. Mycolic acid biosynthesis involves a specialized condensing enzyme system initially furnishes a 2-alkyl-3-ketoacid [350,352,353]. **B.** Expanding upon the Fleming-Harley-Mason hypothesis, the carboxylation of a C₁₆-C₁₈ acetylenic fatty acid may be coupled with an acceptor acyl derivative to assemble the marine PKs.

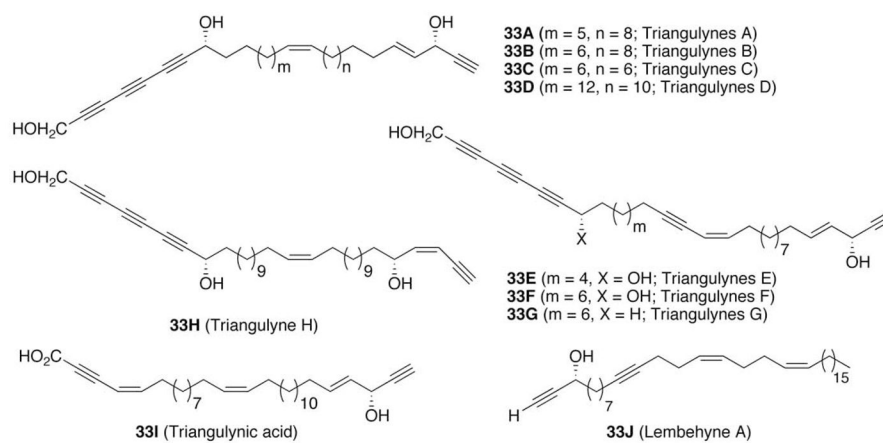


Fig. 37.
Other very-long chain marine polyacetylenic alcohols and acids.

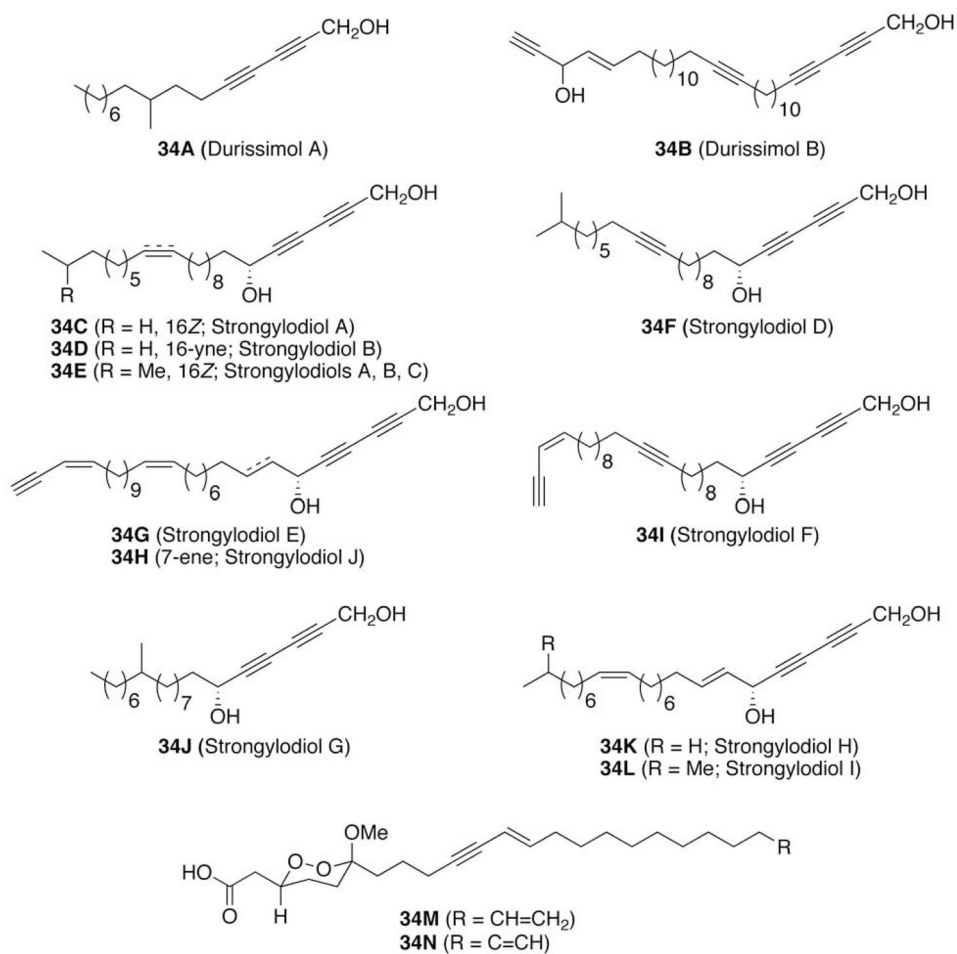


Fig. 38. Marine organisms occasionally accumulate relatively short-chain acetylenic metabolites. The strongylodiols are novel, as they are produced as epimeric mixtures at C-6, and the peroxyacarnic acids contain a distinctive 1,2-dioxane.

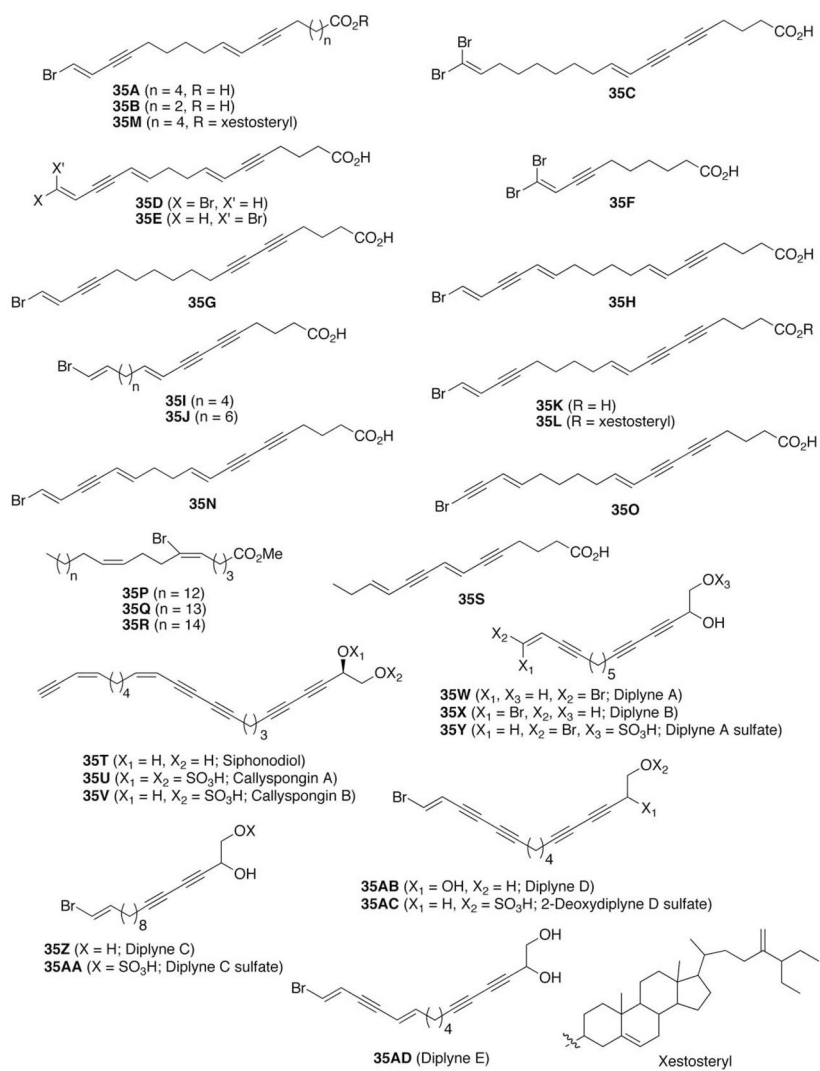


Fig. 39. A wide array of bromoacetylenic acids have been uncovered in marine sponges.

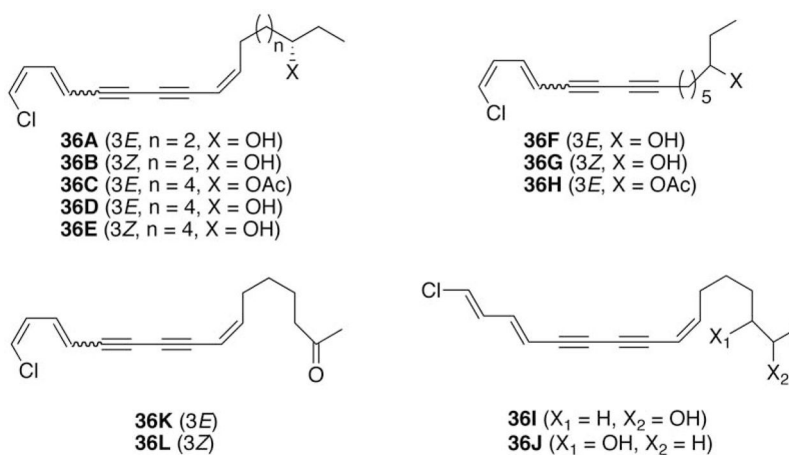
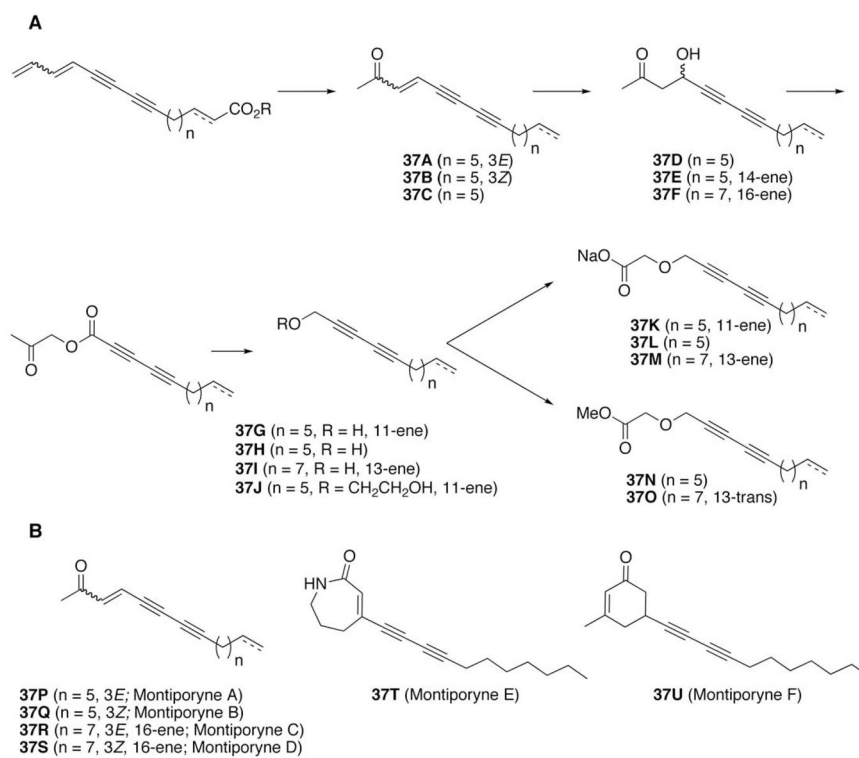


Fig. 40. Chlorinated polyacetylenic acids produced by *Haliclona lunisimilis* may be consumed and modified by the nudibranch *D. sandiegensis* to augment defensive secretions.

**Fig. 41.**

A. The diacetylenic montiporic acids appear to be formed by a series of α - and ω -oxidations analogous to terrestrial plants and fungi. The timing of the chain modifications is indeterminate.

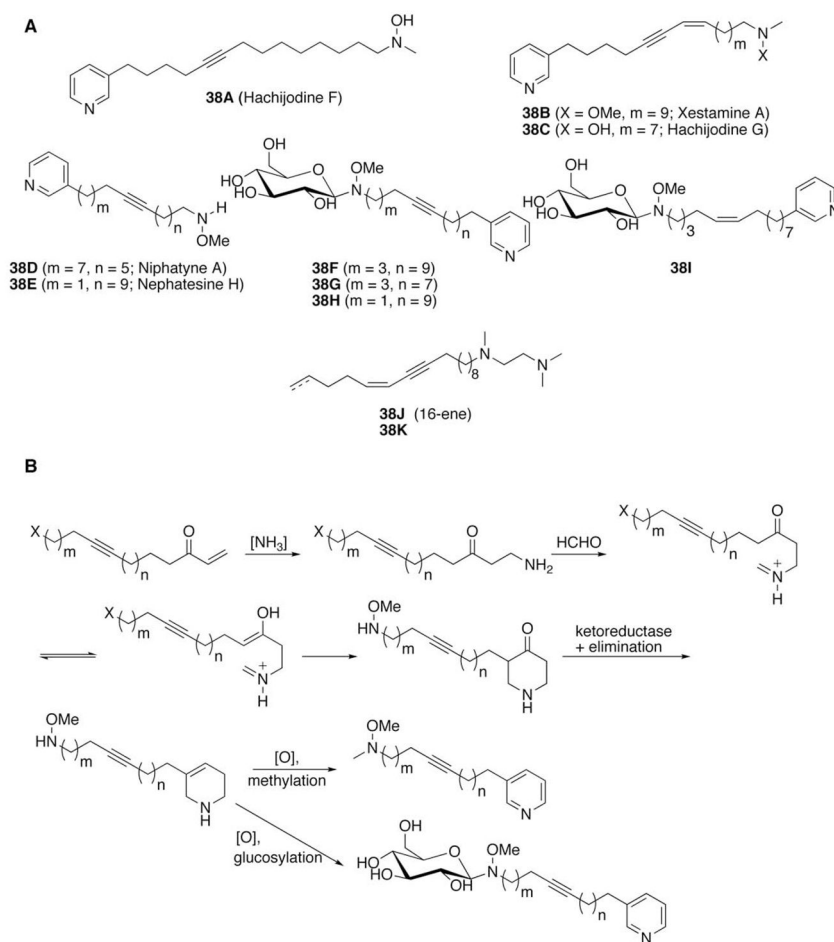
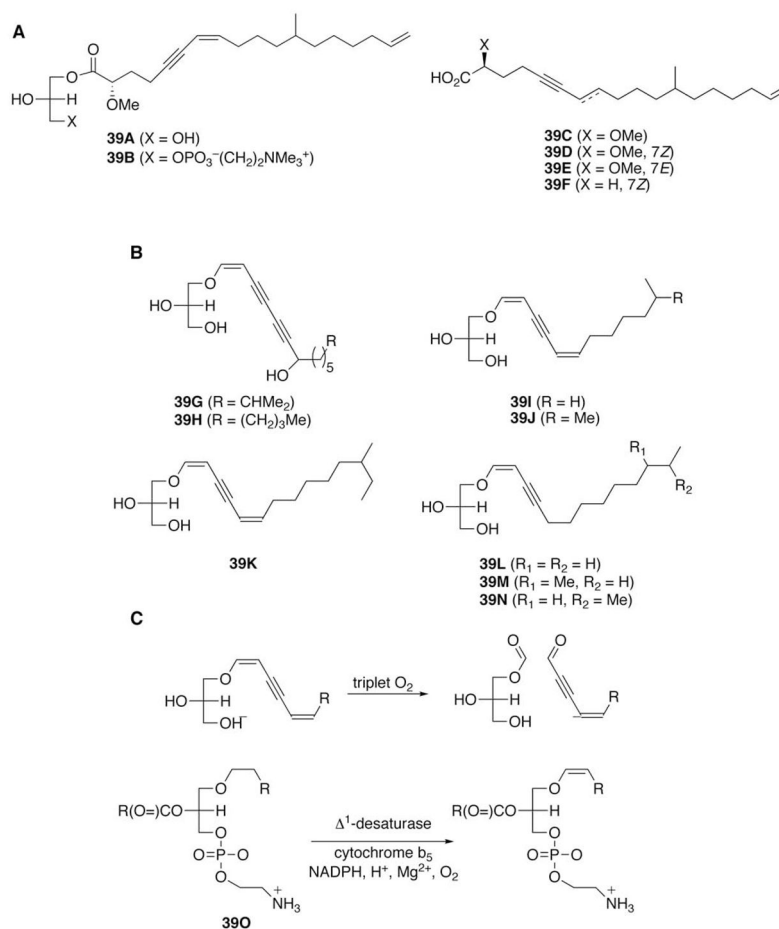


Fig. 42.

A. Alkaloids from the genera *Xestospongia*, *Amphimedon*, *Niphates*, and *Clathrina*. **B.** The biosynthesis of the acetylenic 3-alkylpyridines can be rationalized by a Mannich reaction involving a formaldehyde-derived iminium ion. The hydroxylamino termini are presumed to have origins similar to plant glucosinolates.

**Fig. 43.**

A. α -Methoxy fatty acids found in sponge species. **B.** Acetylenic 1-alkenylglycerols are likely furnished either by the exchange of acetylenic alcohols into 1-acyl DHAPs by alkyl dihydroxyacetonephosphate synthase or the direct desaturation of ether lipids. **C.** In terrestrial organisms, the desaturation to 2-acyl-1-alkylphosphatidylethanolamines involves the action of a Δ^1 -desaturase [396]. An analogous dehydrogenating system is expected in marine sponges. It has been demonstrated that 1-alkylglycerol derivatives can be cleaved by triplet oxygen producing toxic α , β -acetylenic aldehydes [394].

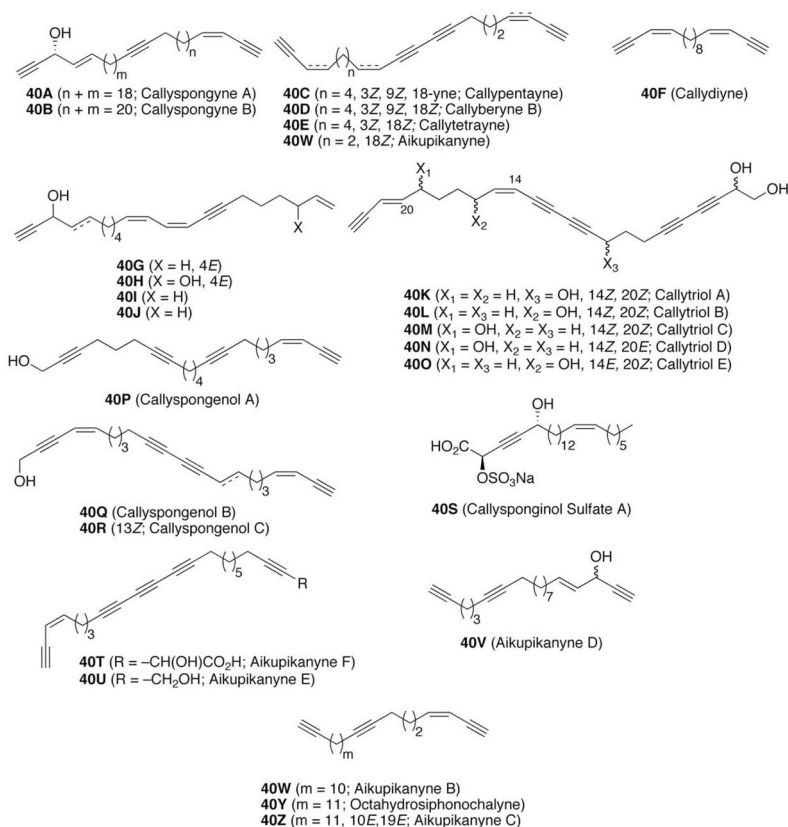


Fig. 44. *Callyspongia* and *Diplastrella* spp. produce acetylenic alcohols and sulfates absent the 1,4-pentadiyn-3-ol core of the petroformyne and petrocortyne metabolites.

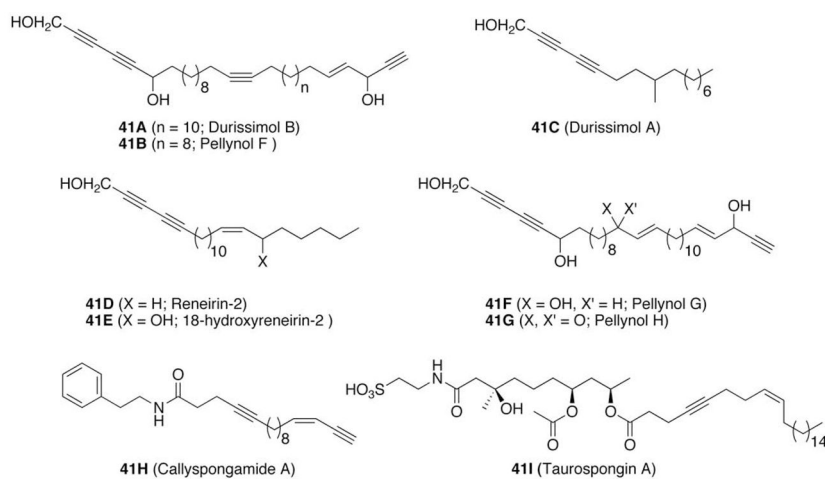
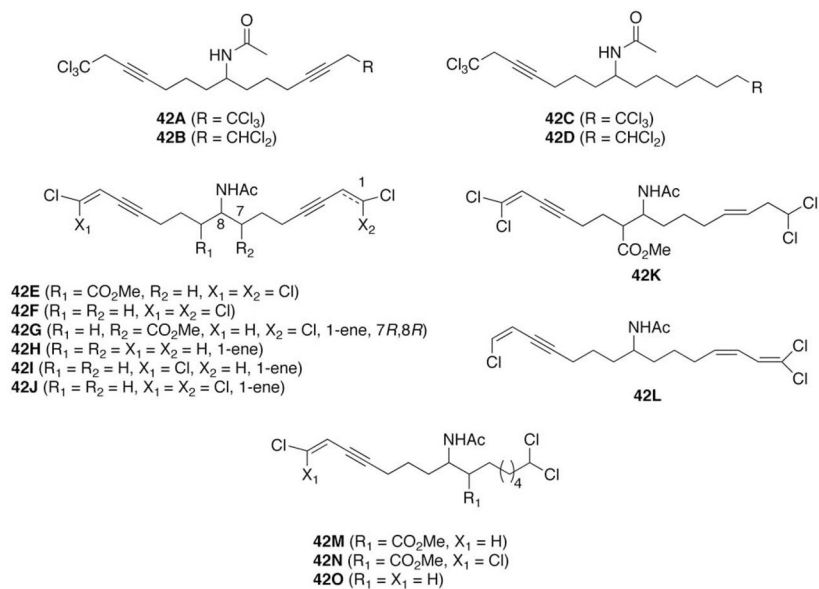


Fig. 45.
Marine diacetylenic propargyl alcohols and alkamides.

**Fig. 46.**

Certain chlorinated natural products result from the free-radical halogenation of methyl groups. The acetylenic acetamides are presumed to arise by a similar path followed by the elimination of HCl.

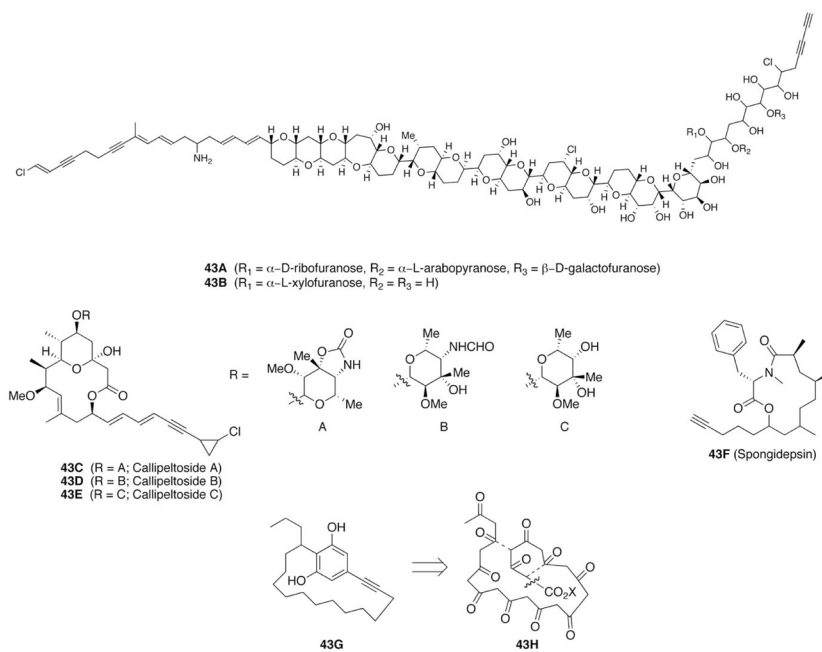
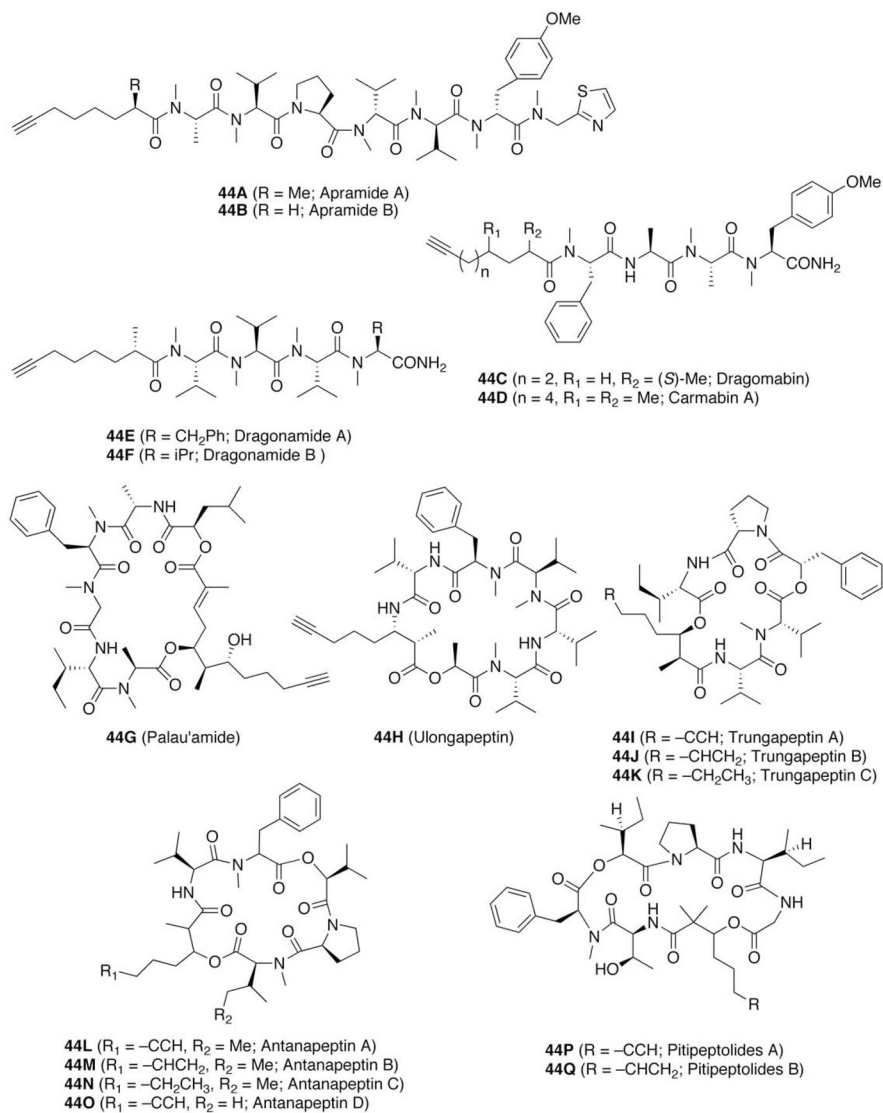
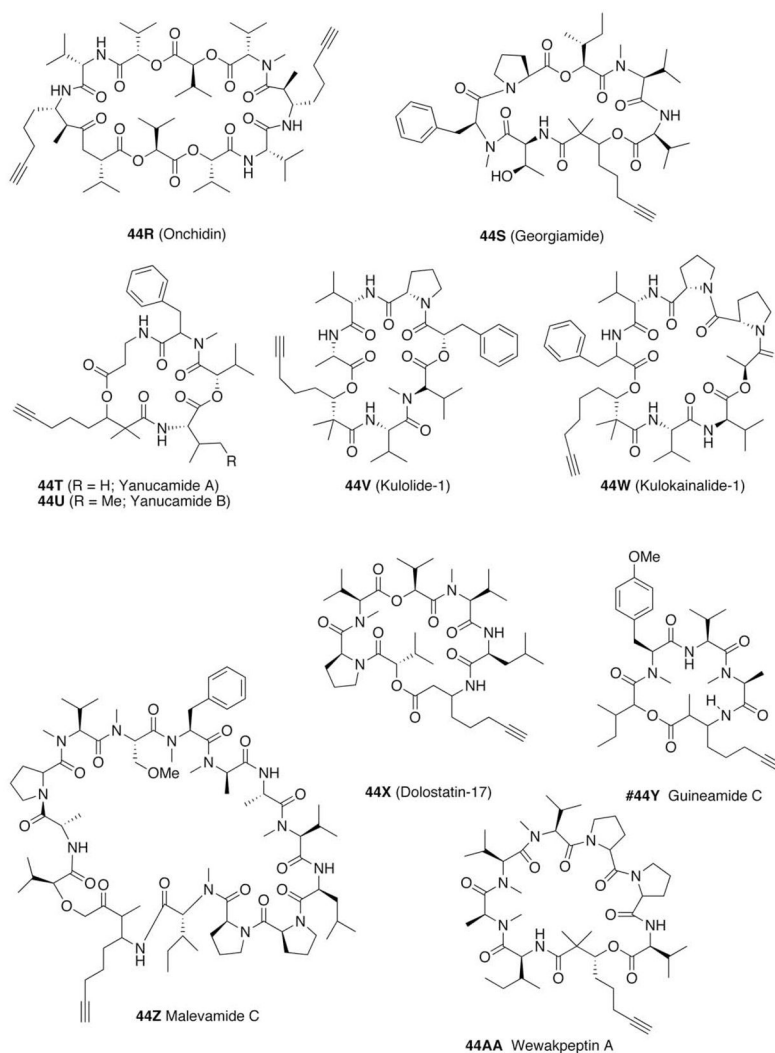


Fig. 47. Structurally novel marine PKs (**43A–E**) expand the distribution of glycosylated acetylenic metabolites from terrestrial to marine organisms. Other PK motifs, shown in **43F–G** appear to be rare [425].



**Fig. 48.**

A wide array of lipopeptides and cyclodepsipeptides containing terminal acetylenic branches are known. The acetylenic units and their more saturated congeners may arise from fatty acyl starter units that undergo stepwise desaturation. It remains to be determined which marine invertebrates accomplish aspects of acetylene biosynthesis and which compounds may be dietarily acquired from cyanobacteria.

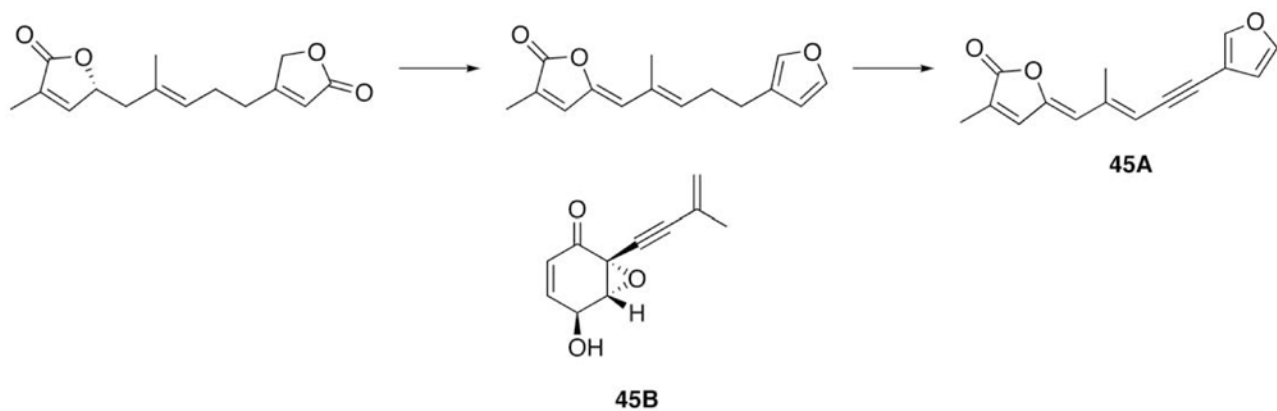


Fig. 49. Acetylenic modification of the terpenoids appears to occur on assembled metabolites. Acetylenases acting upon this class of substrates remain to be discovered.

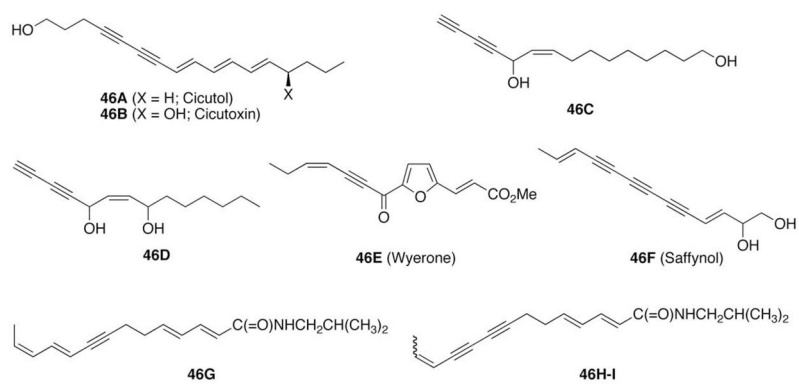


Fig. 50. Selected acetylenic compounds for which ecological and ethnopharmacological roles have been described.

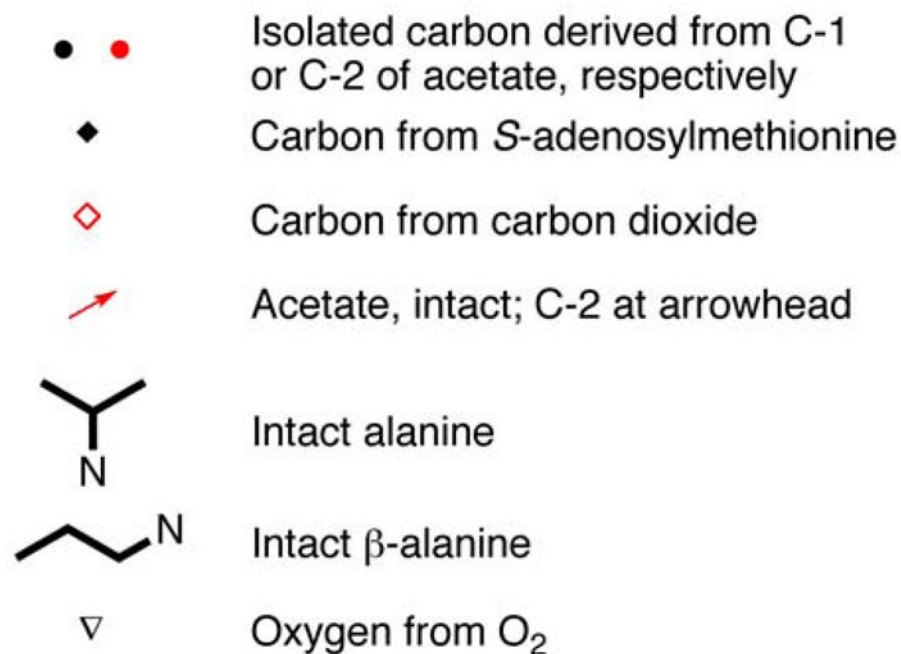


Fig. 51. Legend for Biosynthesis

Key to the experimentally determined isotopic labeling patterns that are superimposed on structures found in Figures (e.g., Fig. 5, 12, etc.). (Place unnumbered Figure, "Legend for Biosynthesis")