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Biosynthesis of Haloterpenoids in Red Algae via Microbial-like Type I Terpene Synthases

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T.S.S. and I.B. contributed equally to this work. T.S.S., I.B., T.d.R., and B.S.M. designed research; T.S.S., I.B., M.L.M., and H.K.B. performed research; K.B., V.M.B., J.G., L.H., S.R., and J.T. performed genome sequencing (except *P. hornemannii*, which was done by T.S.S., M.L.M., and T.P.M.). All authors analyzed data. T.S.S., I.B., and B.S.M. wrote the paper with input from all authors.

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acschembio.3c00627.
General methods and materials of sequencing; biochemical validation; and structure elucidation experiments; as well as supporting figures and GC-MS analysis (PDF)

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Abstract

Red algae or seaweeds produce highly distinctive halogenated terpenoid compounds, including the pentabromochlorinated monoterpene halomon that was once heralded as a promising anticancer agent. The first dedicated step in the biosynthesis of these natural product molecules is expected to be catalyzed by terpene synthase (TS) enzymes. Recent work has demonstrated an emerging class of type I TSs in red algal terpene biosynthesis. However, only one such enzyme from a notoriously haloterpenoid-producing red alga (*Laurencia pacifica*) has been functionally characterized and the product structure is not related to halogenated terpenoids. Herein, we report 10 new type I TSs from the red algae *Portieria hornemannii*, *Plocamium pacificum*, *L. pacifica*, and *Laurencia subopposita* that produce a diversity of halogenated mono- and sesquiterpenes. We used a combination of genome sequencing, terpenoid metabolomics, *in vitro* biochemistry, and bioinformatics to establish red algal TSs in all four species, including those associated with the selective production of key halogenated terpene precursors myrcene, *trans*-β-ocimene, and germacrene D-4-ol. These results expand on a small but growing number of characterized red algal TSs and offer insight into the biosynthesis of iconic halogenated algal compounds that are not without precedence elsewhere in biology.

Graphical Abstract

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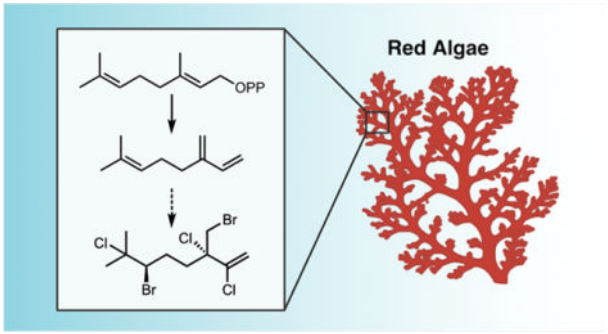

Terpenoids are the largest class of natural products and are ubiquitous across all kingdoms of life.[1] The pervasiveness of terpenoids can be attributed to their broad structural and biological versatility as signaling, defensive, or regulatory compounds.[2,3] Terpenoids have also found central applications as medicinal agents, where molecules like taxol and artemisinin are staples in the treatment of cancer[4] and malaria,[5] respectively. While both of these compounds were isolated from terrestrial plants, terpenoids can also be found in the marine environment exhibiting distinct structural, biological, and pharmacological properties.[6]

Red algae are a prolific source of distinctive halogenated mono- and sesquiterpenoids that are without precedence elsewhere in biology, except for the animals that graze upon them.[7] Although there is large structural diversity within red algal halogenated terpenoids, certain scaffolds are commonly encountered. Linear and carbocyclic monoterpenoids are extracted primarily from *Plocamium*[8] and *Portieria*[9,10] species, while chamigrane- and laurane-type sesquiterpenoids are isolated from *Laurencia* algae (Figure 1A).[11] These four structural groups make up approximately 40% of all known algal terpenoids that number about a thousand molecules.[1] Assessment of the pharmaceutical potential of haloterpenoids has brought forth some promising candidates like halomon (1), which showed highly selective activity against several cancer cell lines with low general cytotoxicity over 30 years ago.[12] However, the re-extraction of 1 at the time proved difficult as different regional populations of *Portieria hornemannii* produced distinct blends of carbocyclic and linear monoterpenoids, preventing reliable larger-scale access to 1.[9] No biotechnological solution exists today largely because of the dearth of coupled high-quality genomic and metabolomic information for red macroalgae.

The biosynthesis of algal haloterpenoids has remained an enigma for decades. Solving this mystery would shed light on new halogenation reactions in biology and provide opportunities for providing a reliable and sustainable supply of bioactive compounds like 1, ochtodene (2), pacifenol (3), and laurinterol (4). However, to date, little is known about the algal enzymes involved in the biosynthesis of halogenated mono- and sesquiterpenoids. Generally, all terpenoids are constructed from two simple five-carbon building blocks, namely, dimethylallyl diphosphate (DMAPP) and isopentenyl diphosphate (IPP). DMAPP and IPP derive from two possible biosynthetic pathways, namely, the mevalonate pathway or the methylerythritol 4-phosphate pathway.[13,14] The production of most specialized, nonsteroidal terpene scaffolds in terrestrial plants,[15] fungi,[16] and bacteria[17] is catalyzed by

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class I terpene synthases (TS).[18] TSs utilize oligoprenyl diphosphate substrates to generate an enormous array of carbon scaffolds, with their products covering the chemical space from linear diphosphate-elimination products to complex, polycyclic structures harboring multiple stereocenters. These hydrocarbons are then subject to downstream tailoring reactions resulting in great structural diversity.[18] Terpenoid biochemistry in marine organisms is much less investigated, but recently, class 1 TSs could also be identified in the genomes of corals,[19] sponges,[20] and red algae.[21]

For red algal terpene biosynthesis specifically, there are two studies showing the occurrence of class I TSs. In the first study, transcriptomes of the known haloterpenoid-producing species *Laurencia pacifica* and *Laurencia dendroidea* were each found to contain genes for three different class I TSs, one of which was biochemically characterized to selectively produce the sesquiterpene prespatane (5, Figure 1B).[22] In a second study, the red algae *Porphyridium purpureum* and *Erythrolobus australicus* were each found to have one and two TSs, respectively; however, these species are not known to produce haloterpenoids.[21] The products of the sole *P. purpureum* TS as well as one of the *E. australicus* TSs produced a mix of sesquiterpenes containing aristolene (6) and β-copaene (7), while the second *E. australicus* TS converts farnesyl diphosphate (FPP) to nerolidol (8, Figure 1B).[21] In a phylogenetic analysis, the TSs from all four organisms form a monophyletic clade within otherwise bacterial TS sequences, therefore representing microbial-like terpene synthases (MTSs) rather than plant-like terpene synthases.[21] The characterized enzymatic terpene products from the red algal clade show no resemblance to the major groups of haloterpenoids, and the enzymes seemingly occur in haloterpenoid producers as well as other red algae. Further, the general distribution of these genes within red algae seems to be sparse, as only sequencing data for four out of 41 investigated species showed their presence.[21] Since only *L. pacifica* and *L. dendroidea* transcriptomic data sets are available, we sought to determine if this family of terpene synthases is involved in the production of haloterpenoids by a more comprehensive study of haloterpenoid-producing red algal species.

Here, we report the discovery of 10 new red algal MTSs from *Plocamium pacificum*, *Portieria hornemannii*, and *Laurencia subopposita* facilitated by metagenome sequencing. We also biochemically characterized the remaining MTSs from *L. pacifica*. Phylogenetic analysis and inspection of TS-containing contigs demonstrated a clear algal origin of the genes. We show through *in vitro* reconstitution and biochemical characterization of algal TS sequences the selective production of both mono- and sesquiterpene scaffolds, including key halogenated terpene precursors. This work expands the red algal terpene synthase family and provides insight into the biosynthetic pathways of algal halogenated natural products.

RESULTS AND DISCUSSION

Seaweed Collection and Chemotyping.

We collected *L. pacifica*, *L. subopposita*, and *P. pacificum* from La Jolla, California, and *P. hornemannii* from Oahu, HI, USA. All samples were collected in coastal tidepools. *P. hornemannii* and *P. pacificum* are reported to exclusively form halogenated monoterpenoids, while *L. pacifica* and *L. subopposita* are described as sesquiterpenoid producers. Because of the widely reported variability of terpene production by different collections of

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identical-looking algae,[23–25] we started by chemotyping all collected specimens. Gas chromatography–mass spectrometry (GCMS) analysis of hexane extracts generated from all samples revealed putative halogenated terpenoids as constituents.

Halomon (1) was confirmed as a major constituent in our *P. hornemannii* sample by comparison to a synthetic standard[26] (Figure 2) in addition to other halogenated monoterpenoids of lower mass (Figure S1). The structure of 1 shows a halogenation pattern corresponding to the double-bond configuration of myrcene (9), making it a likely biosynthetic precursor (Figures 2 and S2).

P. pacificum showed a complex mixture of halogenated monoterpenoids. Because of the lack of available standards, we carried out a preparative extraction, resulting in the isolation and structural elucidation of the hexabromochlorinated 10 (Figure 2) and three other, structurally related compounds by NMR spectroscopy (Figure S2). All identified compounds showed halogenation patterns that correspond to the double-bond configuration of ocimene (11) (Figures 2 and S2). The identified compounds corresponded perfectly to the constituents reported from this local population of *P. pacificum* almost 50 years ago.[27,28]

We also examined extracts from *L. pacifica* that showed a complex mixture of halogenated signals in GCMS analyses, but the later retention times of the chromatogram showed signs of thermally degraded materials (Figure S4). Again, a preparative extraction was carried out, identifying prepacifenol (12) as a major compound. In total, three compounds related to 12 were isolated and their structures determined by NMR, while a fourth was tentatively identified by its MS fragmentation pattern (Figure S4). This series of compounds is consistent with the hypothesis that halogenated chamigrane terpenoids originate from γ-bisabolene (13, Figure S2). In fact, 13 was identified in the *L. pacifica* extract by its mass spectrum (Figure S4). All isolated chamigrane sesquiterpenoids degraded thermally upon GCMS analysis. Pacifenol (3), formerly reported as a main constituent in *L. pacifica* was not isolated, but an NMR sample of prepacifenol (12) rearranged quantitatively and selectively to 3 in $CDCl_3$ over a time of 14 days, highlighting the ease of this nonenzymatic conversion.[29] As a member of the laurane sesquiterpenoids, laurinterol (4) was also identified by extraction and NMR. All identified compounds have been previously described from *Laurencia* species.[11]

The final red alga that we examined in this study was *L. subopposita*, which showed fewer, mostly low-abundance halogenated signals than those from the other investigated algae. We tentatively assigned one compound as oppositol (14) that was previously identified from an *L. subopposita* sample collected in La Jolla, CA (Figure S5).[30] Two major constituents identified by MS spectra were nonhalogenated laurene-type sesquiterpenoids (Figure S5), also previously reported from *L. subopposita*[31] as well as germacradien-4-ol (15), which was identified by its mass spectrum. Germacradien-4-ol (15) is a likely precursor for the brominated oppositol (14) (Figures 2 and Figure S2).

Sequencing and Genome Mining.

Following validation of haloterpenoid production, we sequenced draft metagenomes and transcriptomes of each alga using a combination of short- and long-read technologies (Table

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S1). Macroalgae prove to be challenging materials for nucleotide extraction due to the complex mixture of polysaccharides and polyphenolics present in their cell walls, as well as endophytic or tightly attached eukaryotes that may contaminate and confound genome assembly. Algal genome completeness was assessed with the eukaryota benchmarking universal single-copy orthologs (BUSCO v4.0.5) database.[32] Metagenome assemblies of all four samples contain at least 70% of complete eukaryota BUSCOs, with publicly available algal data sets containing on average 65.2% of complete eukaryota BUSCOs (Figure S6). In both the *P. pacificum* and *L. pacifica* assemblies, there was a significant proportion of duplicated BUSCOs, which may indicate erroneous assembly of haplotypes or the presence of a contaminating eukaryotic sequence. Of the four samples, the *P. hornemannii* sample produced the highest-quality genome, containing 73.7% of complete, single-copy eukaryotic gene orthologs. This improvement may be attributed to an updated high-molecular-weight DNA extraction protocol for algal tissue.[33] To complement metagenome sequencing, we performed RNA-seq experiments using total RNA extracted from the combined algal tissues of each sample. Notably, the *P. hornemannii* genome had a 95.44% overall alignment rate of raw RNA-seq reads. All other samples, on average, mapped 70% of raw RNA-seq reads.

To identify red algal haloterpenoid biosynthesis genes, we queried the algal sequencing data sets using a custom Hidden Markov model (HMM) generated from a larger diversity of characterized TS sequences from octocorals, bacteria, and fungi.[19] To complement HMM results, we performed tBLASTn searches with known red algal microbial-type class I TS sequences as genetic hooks.[22] Querying the genome and transcriptome of each respective organism yielded a total of 10 previously unknown well-scoring hits, all with canonical class I TS sequence motifs (Figure S7).[14] Our analysis of the *L. pacifica* sequencing data found only the three previously identified MTSs. Six new MTSs were identified from *L. subopposita*, three new MTS sequences from *P. hornemannii*, and notably, only a single MTS was identified from *P. pacificum*. Phylogenetic analysis revealed a well-supported (bootstrap > 99%) monophyletic clade composed exclusively of red algal MTSs (Figure 3A). We maintained the nomenclature of red algal TS sequences from the literature for continuity[18,20] (Figure 3B).

The expanded red algal clade nests within the broader microbial-type terpene synthase clade alongside bacterial, fungal, and nonseed plant TS sequences. This contrasts with recent work exploring sponge and coral TSs that form distinct monophyletic clades while still maintaining the overall shared structure and conserved key active site residues with bacterial sequences, suggesting an ancient horizontal gene transfer (HGT) event.[19,20] Similarly, our findings and previous studies[21] do not support red algal type I TS evolution via a recent gene duplication and neofunctionalization of a red algal isoprenyl diphosphate synthase (IDS) like in insect TS evolution (Figure 3A).[34] Red algal IDS sequences from both publicly available genomes (Table S2) and sequences from this study are more closely related to other IDSs than to newly identified TS sequences. Overall, the sparse taxonomic distribution, nested monophyly within the broader microbial clade, and conserved active site residues support a recent HGT event as the mode of evolution for red algal MTSs.

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Characterization of Red Algal Type I Terpene Synthases.

To determine the biosynthetic product of each terpene synthase, codon-optimized forms of each gene were synthesized, expressed heterologously in *Escherichia coli*, and screened *in vitro* for activity with common terpene diphosphate precursors (Figure S8). We analyzed the resulting reaction products by GCMS (Figures S9–S20). All recombinant proteins, except LsTS-6, showed TS activity (Figures 4A and S20). This is unsurprising as LsTS-6 was not present in the transcriptome of *L. subopposita* and appears to be C-terminally truncated in the genome of *L. subopposita* (Figure S7).

We first examined *P. pacificum* and *P. hornemannii* as these two algae produce a similar yet distinct mixture of mixed halogenated monoterpenes. Two of the enzymes, PpTS-C2 and PhTS-1, acted as selective monoterpene synthases, converting geranyl diphosphate (GPP) to acyclic terpene products (Figures S9 and S10). The single TS identified from *P. pacificum* PpTS-C2 selectively produced *trans*-β-ocimene (11), the proposed terpene precursor to all linear halogenated monoterpenoids isolated from *P. pacificum* like compound 10. The monoterpene-producing enzyme was obtained from *P. hornemannii*, PhTS-1, instead selectively produced the isomer β-myrcene (9), which is the hypothesized biosynthetic precursor of halomon (1) and other known haloterpenoids from *P. hornemannii*. Two additional TSs identified in both the genome and transcriptome of *P. hornemannii*, PhTS-2 and PhTS-3, showed the selective formation of germacrene D (22, Figure S11) and valencene (21, Figure S12), respectively, despite no sesquiterpenoids being reported from this organism in the literature. After identification in our enzymatic assays, revisiting our GCMS analysis of *P. hornemannii* revealed that valencene (21) is also found in the crude extract of this species (Figure S1). Notably, both monoterpene synthase genes did not show an N-terminal signal sequence and therefore are unlikely to be localized in a plastid, which is typical in plant monoterpene synthases.[35] This would point to cytosolic monoterpene production in both species.

We next turned our attention to the two *Laurencia* strains that produce halogenated sesquiterpenes. Of the five new functional sequences from *L. subopposita* and two uncharacterized *L. pacifica* sequences, all acted as selective sesquiterpene synthases, converting FPP to terpene products (Figure 4B). LphTPS-B showed production of a mixture containing bicyclogermacrene (17, Figure S13), while LphTPS-C selectively produced a sesquiterpene alcohol that was not identifiable by its mass spectrum (Figure S14). Isolation from a preparative assay and structure elucidation by NMR revealed structure 19. A compound with the same NMR data has been reported from *L. nipponica* with different relative configuration,[36] but was revised to the structure shown here. Three of the *L. subopposita* synthases showed sequence identity of more than 40% with one respective *L. pacifica* TS (Figure 4A). LsTS-5 produced prespatane (5), like its homologue LphTPS-A. LsTC-4 selectively converted FPP to ledene (16), which can be formed by a very similar cyclization mechanism as bicyclogermacrene (17), produced by its *L. pacifica* homologue. However, LsTC-3 produced germacrene B (18), a compound that is only remotely similar to the LphTPS-C product 19. The two remaining functional *L. subopposita* TSs did not have a clear homologue in the other *Laurencia* species and selectively produced (*Z*,*E*)-α-farnesene (20, LsTS-1) and germacrene D-4-ol (15, LsTS-2), one of the major metabolites of *L.*

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subopposita identified by GCMS. Oxidized terpenes showing the same carbon scaffold as 15 and 16 have previously been reported from *L. subopposita*.[31] To determine the absolute configuration of 15, it was isolated from a preparative assay and subjected to polarimetry, establishing the configuration of the alcohol to be the same as reported for 14,[30] verifying 15 as a reasonable precursor. Notably, none of the TSs found in *L. pacifica* make a bisabolane-type scaffold even though 13 is detected in the crude extract. Also, no TS in both *Laurencia* species generates a carbon scaffold that can be connected to laurane-type sesquiterpenoids, which are present in *L. pacifica* as well as *L. subopposita* extracts (Figures S4 and S5). This suggests that the missing terpene precursors are produced by either a microbial symbiont or a different enzyme class.

Genomic Distribution of Algal Terpene Synthases.

We next examined the genomic context of each terpene synthase to validate their origin as algal and to explore the physical colocalization, or clustering, of biosynthetic genes. Eukaryotic biosynthetic gene clusters (BGCs) are typically spaced over several kilobases, have large intergenic regions, and are often flanked, or contain, viral retrotransposable elements.[37] For example, in octocorals, TS genes were found to be physically colocalized with genes encoding putative terpenoid tailoring enzymes.[19,38] In red algae, there are a handful of examples of gene clustering; however, algal BGCs known to date either contain vanadium-dependent haloperoxidase (VHPO)[39] or MTS encoding genes.[40,41] Previously identified MTS-containing gene clusters are associated with kainoid-producing red macroalgae, where the MTS catalyzes an *N*-prenylation reaction.

All MTS genes identified in this work are placed on contigs that are unambiguously red algal as determined by their structure and closest homologues of their neighboring genes. To identify possible haloterpenoid BGCs in red algae, we used a targeted genome mining approach to screen the draft genome assemblies of *L. pacifica*, *L. subopposita*, *P. hornemannii*, and *P. pacificum* with the Pfams for terpene synthases (PF19086), VHPOs (PF01569), and polyprenyl synthetases (PF00348). We complemented this data set with the previously generated results from screening each genome with a custom HMM targeting MTSs.[19] Most of the MTS genes did not show colocalization with putative tailoring enzymes, even after checking extended up- and downstream regions across the contigs they were placed on. Exceptions were two sets of gene pairs in *L. pacifica* and *L. subopposita* (Figure 5A,B). In *L. pacifica*, two of the biochemically characterized red algal TSs were found to colocalize 152kb apart on a single contiguous sequence 484kb in length (Figure 5A). In *L. subopposita*, one of the biochemically characterized red algal TSs, LsTS-2, was found to cluster with a VHPO and the fragmented sequence of LsTS-6 (Figure 5B). This cluster is roughly 25kb upstream of a coding sequence annotated as a glycosyltransferase using a Conserved Domain Database search; closest BLASTp hits support this sequence as algal in origin (Table S3). Both gene pairs are interspersed with retrotransposable (RT) elements and other mobile genetic elements common in eukaryotic genomes.[42]

Overall, the distribution of algal TS genes appears to not be widespread, with only a few sparsely distributed species of the classes Porphyridiophyceae and Florideophyceae known to harbor MTSs or 7 out of 49 investigated species to date (Table S4). This raises

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intriguing questions about the origin of the red algal MTS sequences. On the one hand, the sequences are monophyletic, but the whole clade is nested within fungal and bacterial TSs (Figure 3). On the other hand, most red algal species do not harbor these genes and the ones that do belong to the orders Gigartinales (*Portieria hornemannii*), Plocamiales (*Plocamium pacificum*), Ceramiales (*Laurencia* spp.), and Phorphyridiales (*Porphyridium purpureum* and *Erythrolobus australicus*) (Table S4), which are not closely related to each other (Figure S21). These observations collectively suggest distinct, more recent horizontal gene transfer events rather than an ancestral event spanning both classes, Florideophyceae and Porphyridiophyceae, followed by the loss of this gene in almost all red algae. Because of the similarity of all red algal MTS sequences, the vector acting in the putative HGT events was likely related, although its identity is yet unknown.

CONCLUSIONS

In summary, we report the discovery of the first terpene synthases from the haloterpenoid-producing red macroalgae *L. subopposita*, *P. pacificum, and P. hornemannii*, and characterize previously uncharacterized sesquiterpene synthases from *L. pacifica* by the combination of transcriptomics, genomics, and *in vitro* biochemical characterization. We showed that red macroalgae can produce both mono- and sesquiterpenes via class I microbial-type terpene synthases and that these terpene scaffolds are likely on the pathway toward the unusual bouquet of haloterpenoids unique to red macroalgae.

We suspect that specialized halogenases function as (mixed) dihalogenases to introduce bromine and chlorine atoms across the double bonds of terpene substrates. The draft genomes of the four haloterpenoid-rich red algae reported here are each replete with putative VHPOs, which are well known from red algae to catalyze monobromination reactions.[43] In the case of *L. subopposita*, we identified an MTS-VHPO gene pair that we suspect may be involved in the biosynthesis of oppositol (14) from the biochemically validated germacrene D-4-ol (15) synthase LsTS-2. Further sequencing and expression work is actively underway to establish whether there are functional links between these algal enzymes in the production of halomon and other polyhalogenated terpenes distinctive to red macroalgae.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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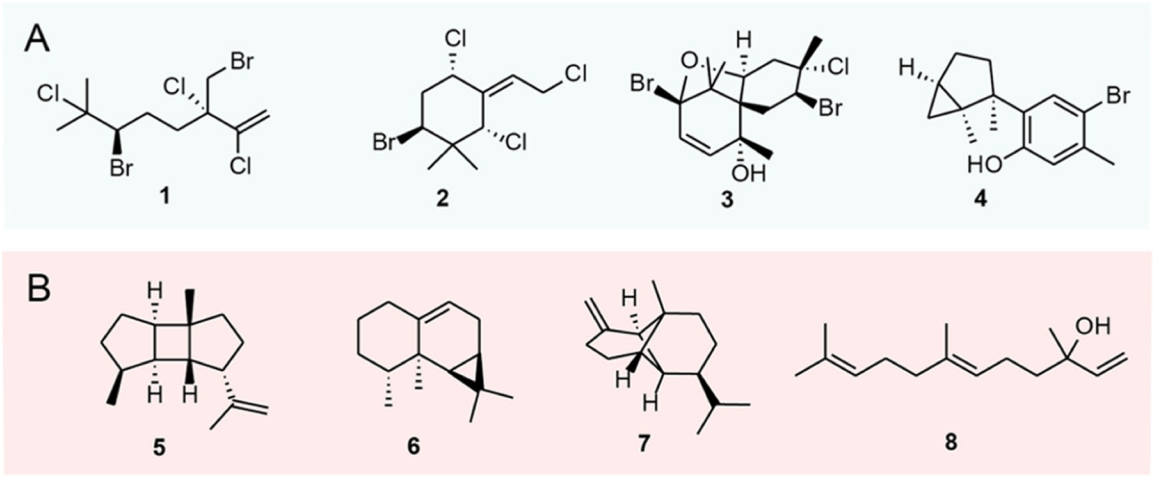

Figure 1.

Terpenoid structures derived from red algae. (A) Representatives of the largest structural families of terpenoids in red algae. Halomon (1) and ochtodene (2) are linear and carbocyclic halogenated monoterpene, respectively (>140 and >60 structures known, respectively); pacifenol (3) is a halogenated chamigrane sesquiterpenoid (>140 structures); and laurinterol (4) is representative of aromatic sesquiterpenoids based on the closely related laurane, cyclolaurane, and cuparane scaffolds (>90 structures). (B) Products previously characterized from red algal terpene synthases.

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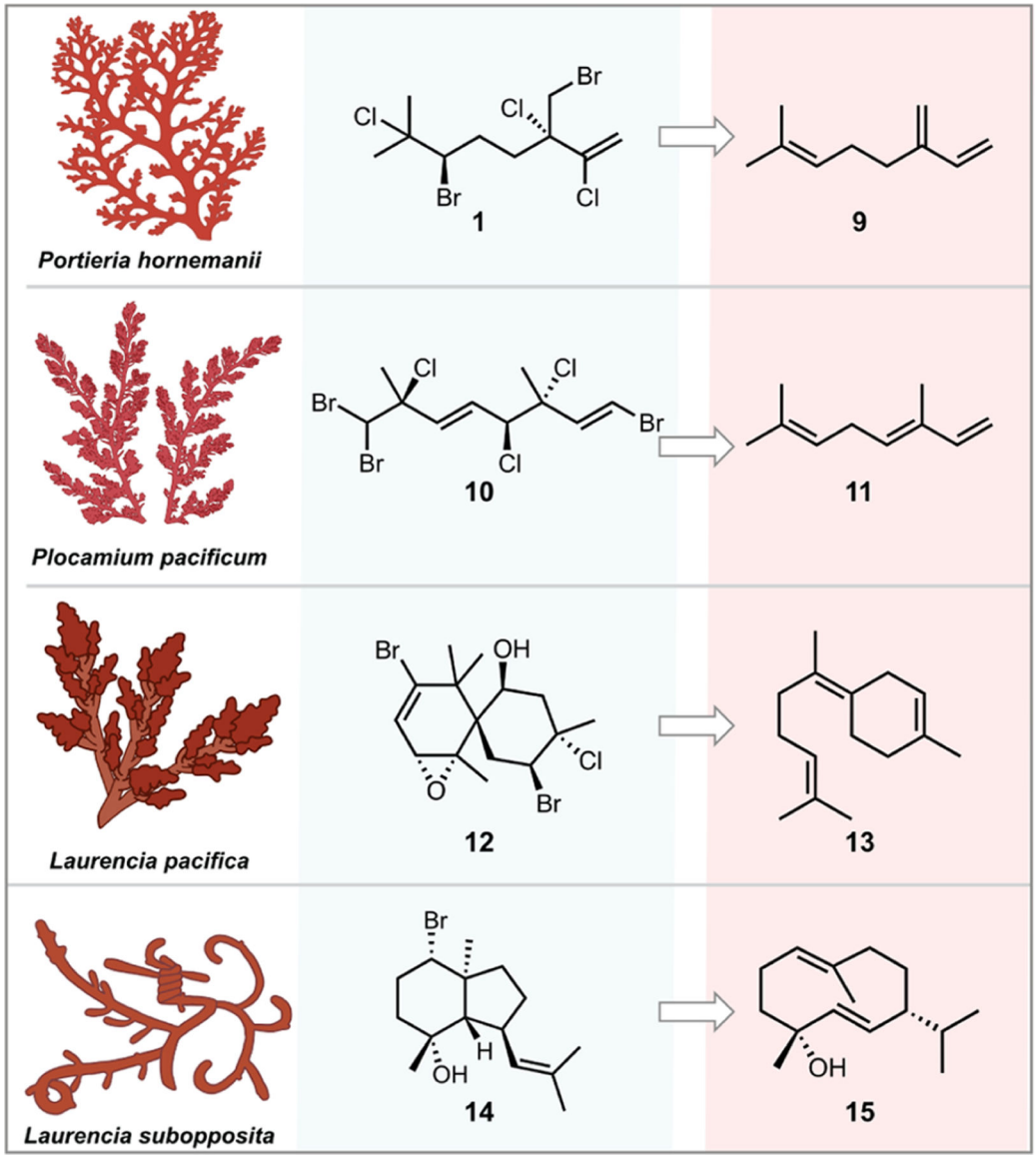

Figure 2.
Identified representative haloterpenoids from collected red agal samples and their putative biosynthetic terpene precursors.

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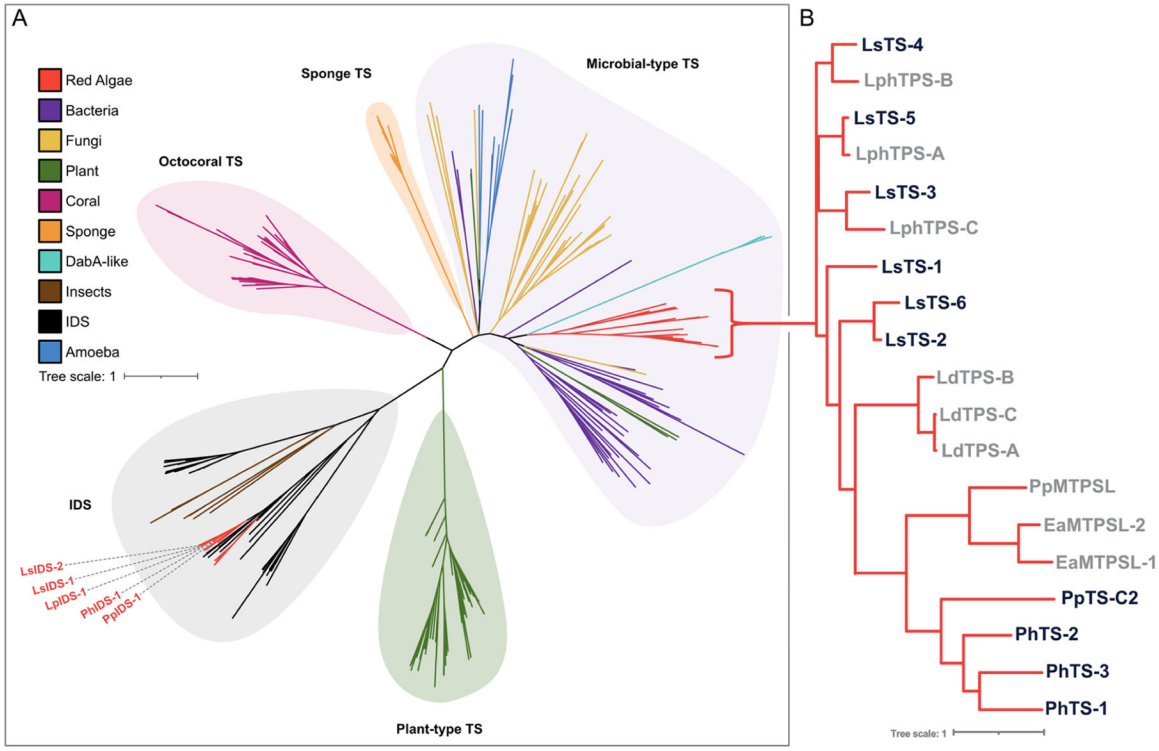

Figure 3.

Phylogenetic analysis of red algal TS sequences. (A) Phylogeny of TS sequences includes representative bacterial, fungal, amoeba, coral, sponge, and plant TS sequences. Putative red algal IDS sequences are highlighted in red, and those from this study are labeled. The scale measures evolutionary distances in substitutions per amino acid. (B) Subclade of red algal sequences, the first two or three letters of each TS name represent the taxonomy of the macroalgae from which it originated. Names of new sequences are in black, and previously identified sequences are in gray.

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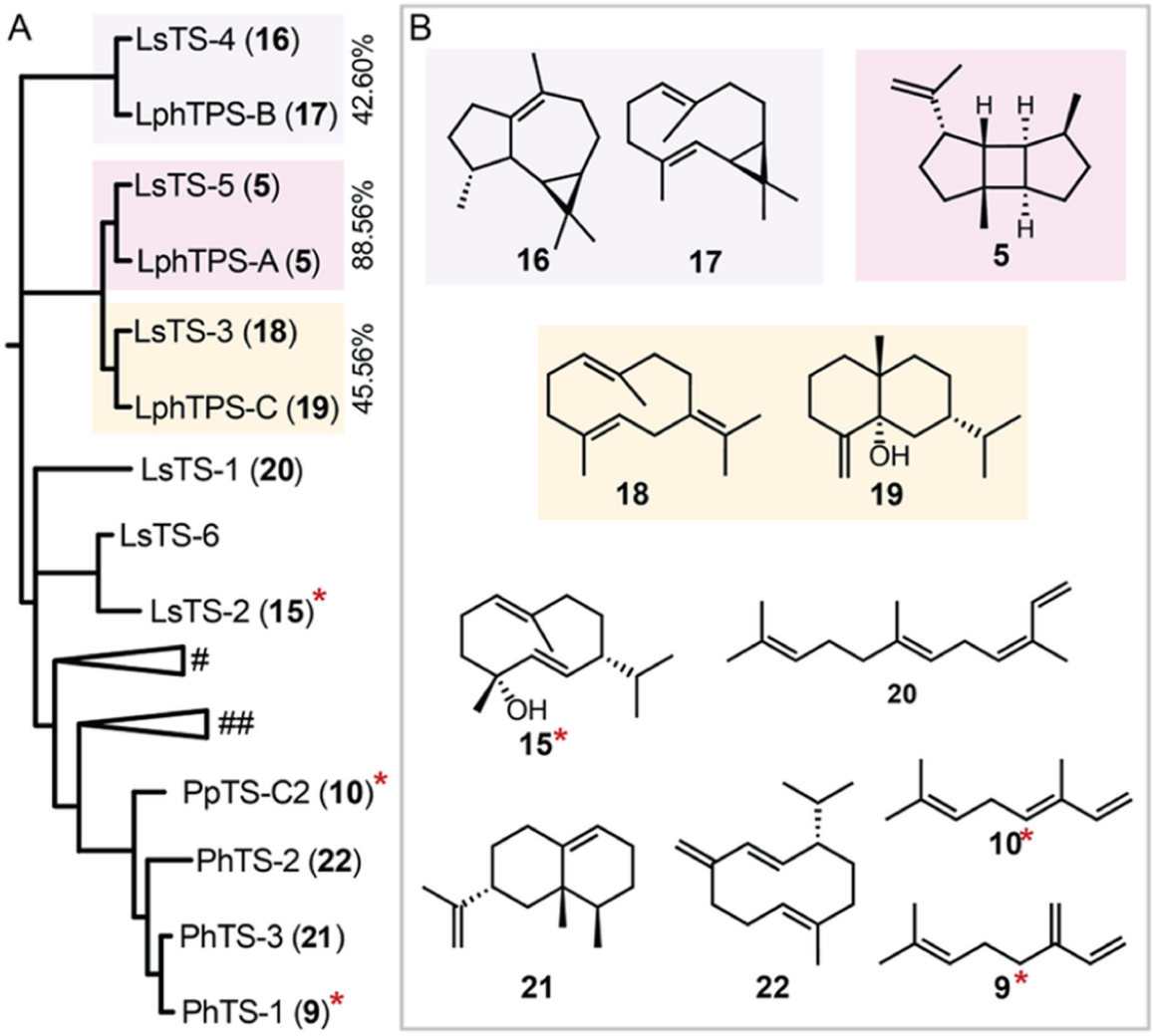

Figure 4.

Algal TS biochemical characterization. (A) Phylogeny of newly characterized red algal terpene synthases and (B) their respective products. Algal sequences not included in this study are collapsed. Sequences highlighted with a red asterisk produce products predicted to be on pathway toward algal haloterpenoid products. Percent similarity by amino acid sequence is shown next to each *Laurencia* TS pair. #: *E. australicus* and *P. pupureum* TSs; ##: *L. dendroidea* TSs.

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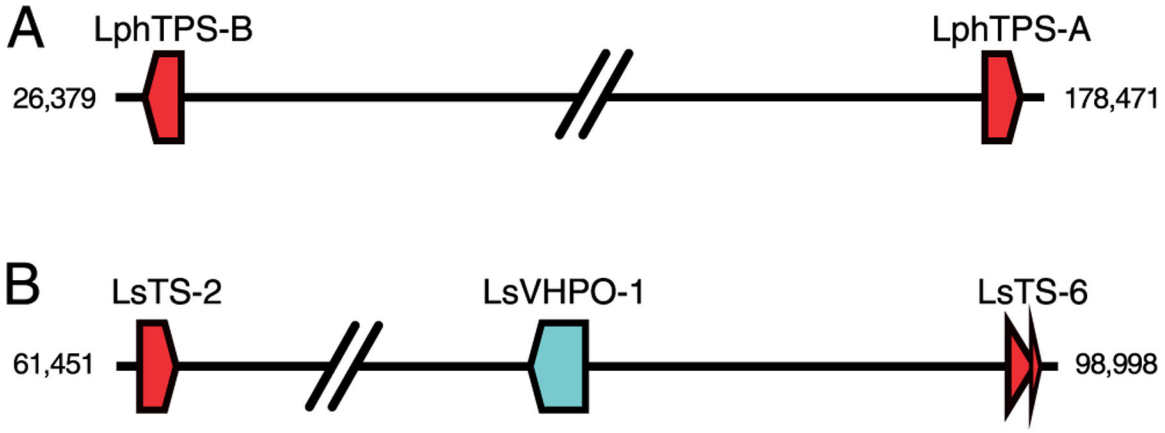

Figure 5.
Identified terpene synthase containing gene pairs from (A) *L. pacifica* and (B) *L. subopposita*.