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HALOGENATED MONOTERPENES OF THE RED ALGA *MICROCLADIA*

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Key Word Index—*Microcladia borealis*; *M. californica*; *M. coulteri*; Rhodophyceae; monoterpenes; halogenation; toxicity; chemotaxonomy.

Abstract—The red marine algae *Microcladia borealis*, *M. californica* and *M. coulteri* produce several unusual halogenated monoterpenes including violacene, plocamene-B, plocamene-C, and plocamane-D. The isolation of these terpenes along with a study of their variation in each *Microcladia* at different locations are described.

INTRODUCTION

Marine algae from the division Rhodophyta have long been recognized as a good organo-halogen source [1,2]. Not unexpectedly, recent research upon certain algae of this division has shown that they contain a variety of halogenated organic components [3–5]. For example, an intriguing array of halo sesqui- and diterpenes have been isolated from almost every species of *Laurencia* [Rhodomelaceae, Ceramiales] so far examined [4–8]. Recent work has shown that a few other algae besides *Laurencia* can synthesize haloterpenes: polyhalogenated monoterpenes have been observed by us and others as constituents of the genus *Plocamium* [Plocamiacae, Gigartinales] [9–12] and the genus *Chondrococcus* [Rhizophyllidaceae, Cryptonemiales] [13, 14].

Aside from their uniqueness of structure and biosynthesis, algal haloterpenes are of interest because of their potential toxicity to other organisms. Crude alcohol extracts from *Laurencia pacifica* are toxic to test fish [15]. This toxicity may well be related to high concentrations of halogenated sesquiterpenes including prepacifenol [16], pacifenol [17], and johnstonal [18] recently observed from *L. pacifica*. We recently discovered that non-polar extracts from the red alga *P. cartilagineum* and *P. violaceum* are toxic to both fish and insects [9, 10]. Complex mixtures of halogenated monoterpenes actually constitute the major non-polar component of these extracts, and part of this toxic activity has been related [10] to specific monoterpenes such as plocamene-B (**1**) and violacene (**2**).

When wet, *Microcladia coulteri* Harvey and *M. californica* Farlow are strikingly similar in appearance to *Plocamium cartilagineum*; and similarities can be noted between *M. borealis* Ruprecht and *P. violaceum* [19]. These respective pairings of algae can also be found in exactly the same intertidal zones. Believing that the toxic terpenoids from *Plocamium* might serve as chemical defensive agents, it seemed intriguing to hypothesize that *Microcladia* [Ceramiaceae, Ceramiales] might also be a source of toxic haloterpenes.

Microcladia is related phyletically to several red algal species which are recognized as organo-halogen producers. *Microcladia* is in the same order as *Laurencia*, which elaborates halogenated sesquiterpenes [5], and it is in the same family as *Ceramium* and *Antithamnion*, both of which synthesize bromophenols. [3,4]. A chemical study of *Microcladia* appeared warranted since halogenated compounds are of current interest as potential taxonomic markers [4, 20].

The above arguments stimulated our belief that *Microcladia* should be capable of rich halo organic chemistry. We therefore examined all three species of *Microcladia* which are abundant in the Santa Cruz region. Described below are our observations which show that these algae are sources of polyhalogenated monoterpenes.

RESULTS

Microcladia coulteri freshly collected from Pebble Beach (Jan., 1975, subtidal) was extracted with $CHCl_3$. The resultant semi-mobile crude oil was dissolved in hexane and rapidly filtered through silica gel. Analysis by GC–MS (Fig. 1) showed a finger print which was similar to that which we previously observed from *P. violaceum* [10]. On the basis of GLC retention times and MS, four polyhalogenated monoterpenes could be identified which were common to both *Microcladia* and *Plocamium*. Two of these compounds have been described from our previous work on *Plocamium* and they include violacene (**2**) [or plocamene-A] and plocmane-B (**1**) [10]. The other two components have recently been characterized as plocamene-C† (**3**) [21] and plocame-D (**4**) [22]. Direct isolation of these components from this extract proved to be difficult because of their low levels relative to the non-halogenated lipids. The presence of these monoterpenes, albeit in low levels, in the *M. coulteri* oil was none the less quite significant. Therefore, the material from this same collection was subjected to more exhaustive work-up, involving continuous extraction with ethanol. Extraction of the resultant crude concentrate with

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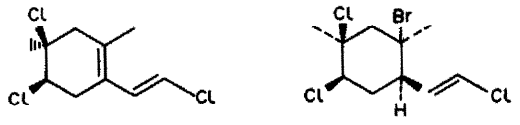
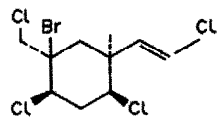
† An epimer of plocamene-C has been reported [23].

hexane yielded 0.33 g of oil which was purified by open column chromatography (silica gel) and hplc (porasil-A). A small amount of plocamene-C (3) was afforded by this procedure, and it was confirmed by comparison of its NMR spectrum (Fig. 2a) to that of an authentic sample (Fig. 2b) obtained from our studies on *P. violaceum* [21]. Based upon these results it is clear that polyhalomonoterpenes are isolable from *Microcladia* species.

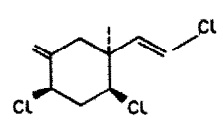


Plocamene-B (1)

Plocamene-C (3)



Violacene (2)



Plocamene-D (4)

A unique observation that the relative compositions of polyhalomonoterpenes vary markedly at different collection sites has been characteristic in our work upon *Plocamium* [9, 10]. We were motivated to determine whether a similar phenomenon existed for *Microcladia* monoterpenes. Hence, our studies were expanded to three locations north of Santa Cruz from which we had thoroughly studied *Plocamium*. In addition to *M. coulteri* both *M. borealis* and *M. californica* could easily be obtained from this area.

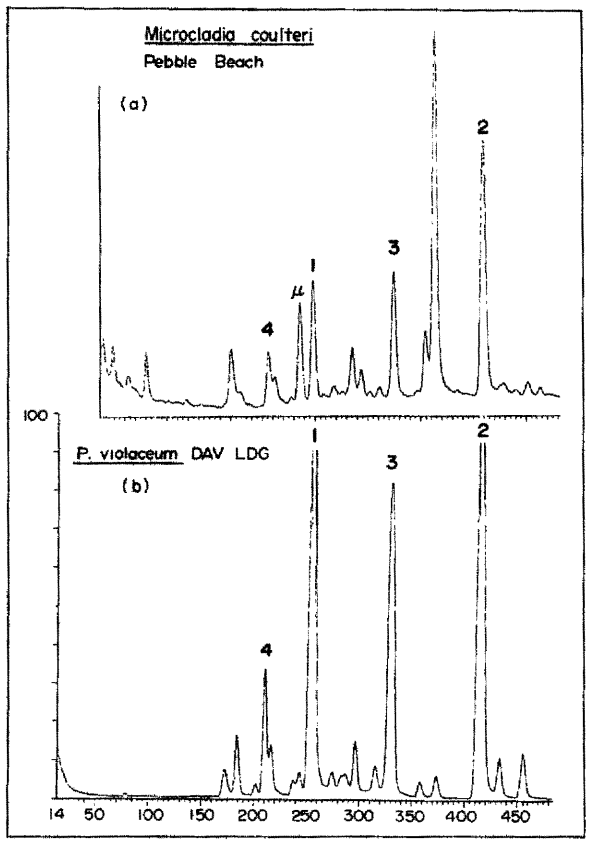


Fig. 1. GLC analysis of extracts containing plocamene-B (1), violacene (2), plocamene-C (3), and plocamene-D (4) from (a) *Microcladia coulteri* and (b) *Plocamium violaceum*.

M. borealis from four northern Santa Cruz locations (Nov., 1974) was pooled and extracted twice with CH_2Cl_2. The resultant dark oil (2.2 g) was chromatographed (silica gel) and yielded a hexane fraction (47 mg) which contained violacene (2) on the basis of a comparison of its NMR spectrum (Fig. 2c) to that of an authentic sample (Fig. 2d) isolated from *Plocamium* [10]. Re-extraction of this same algal material with ethanol yielded after purification additional violacene (50 mg) for a total yield of 0.08% (vs dry plant wt).

We next examined *M. californica* collected from Davenport Landing (March, 1975). It, along with its host, *Egregia*, were separately extracted with $CHCl_3$ followed by ethanol. Examination of each of these crude extracts by TLC showed, for the *Microcladia* sample, spots at the R_f (0.4–0.5 in hexanebenzene, 80:20) characteristic of monoterpene hydrocarbons **1–4**. This R_f region, however, was blank for the *Egregia* extract. Further extraction of the *Microcladia* material with ethanol gave a dark crude oil (10 g). Purification of the combined alcohol extracts by column chromatography and hplc gave a fraction (22 mg) having a TLC $R_f = 0.43$ indicative of haloterpene hydrocarbons. Further examination of this fraction by pulse NMR gave a spectrum (Fig. 2e) which showed many identical features to a spectrum of authentic plocamene-B (Fig. 2f) [10]. These observations indicated *M. californica* to contain plocamene-B plus other lipid impurities in *ca* 0.005% yield (vs plant dry wt).

Having established that halogenated monoterpenes are isolable from all three species of *Microcladia*, we next sought to compare the relative compositions of components **1–4** among each species from different locations. Analyses by GC–MS proved to be the method of choice especially in view of the relatively low concentrations of the haloterpenes observed above. To minimize variation due to seasonal effects each of the three *Microcladia* species was recollected at three northern Santa Cruz intertidal locations during June, 1975. In addition, to rule out artifacts due to inhomogeneous collections, representative single plant samples were analyzed.

Examination of the crude $CHCl_3$ extract of *M. borealis* (Fig. 3b) showed marked variations in the composition of the four terpenes **1–4** with the former two predominating at the three collection locations: Four Mile beach, Davenport Landing and Pigeon Point (Table 1). Similarly, two components, **1** and **2**, could be consistently observed from *M. californica* (Fig. 3c) at each of the three collection sites. Surprisingly, *M. coulteri* displayed very low amounts of these halogenated monoterpenes and only **2** could be barely observed in the oil from Pigeon Point. This last result stands in sharp contrast to the composition observed for the *M. coulteri* oil from Pebble Beach. Finally, analyses of the extract of individual plant specimens of *M. borealis* and *M. californica* (Fig. 3d) from Pigeon Point clearly showed the presence of violacene (**2**) in the former and violacene (**2**) and plocamene-B (**1**) in approximately equal amounts in the latter.

DISCUSSION

The monoterpenes **1–4** have structural features which suggest their close biogenetic relatedness. In addition their halogenation pattern contrasts to that observed from terpenes and halocarbons previously isolated from

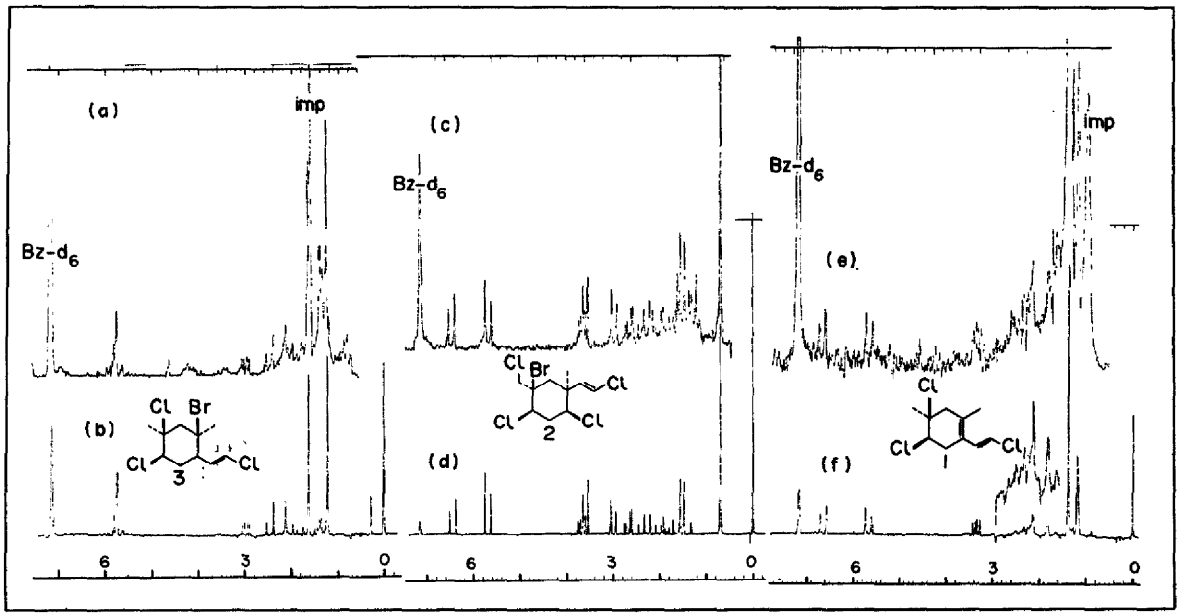
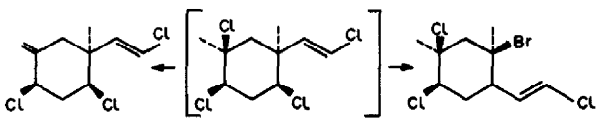


Fig. 2. NMR spectra in benzene-d_6 at 100 MHz of plocamene-C (3) from (a) *M. coulteri* at Pebble Beach and (b) *P. violaceum*; violacene (2) from (c) *M. borealis* at four locations north of Santa Cruz, and (d) *P. violaceum*; plocamene-B (1) from (e) *M. californica* at Davenport Landing and (f) *P. violaceum*.

other algal sources. In terms of biosynthesis, violacene (2) and plocamene-D (4) along with plocamene-C (3) and plocamene-B (1) each form a set in which the components appear to be related by a dechlorobromination and a dehydrobromination respectively. A crossover from the isoprenoid set, 2 & 4, to the non-isoprenoid set, 1 and 3 can best be imagined via a *trans*-chloro vinyl migration. An as yet unencountered structure such as 5 could be a common intermediate to both families, and in the rearrangement of 5 to 3 the axial *trans*chloro



4 5 3

vinyl is set-up to migrate as the adjacent chlorine departs. It is noteworthy that the ratio of Cl to Br in 1–4 is very high. Of the halogens bromine appears to be most often incorporated into the synthesis of halo organics by red algae. From *Laurencia* bromine is without exception the dominant organic halogen because it is represented either alone or with chlorine in *all* the sesquiterpenes (and enyne ethers) reported to date [5–8, 16–18]. Bromide is also abundant in southern California *Plocamium* extracts [11, 12] and it is the dominant halogen observed both in the low molecular weight halo organics from *Bonnemaisonia* [24] and *Asparagopsis* [25, 26], and in the phenols from several red algae [4].

The results of this study provide some unique input related to a phyletic view of halomonoterpene synthesis by algae. Our observation that the *Microcladia* haloterpene components 1–4 vary in relative composition between different collection locations finds an unusual parallel to our similar observations for *Plocamium* [10, 21, 22]. Furthermore, a related situation can be noted in recent studies of *Chondrococcus hornemanni* [Cryptonemiales, Rhizophyllidaceae]. The major halomonoterpenoid components from this latter alga varied markedly between different collection sites including two Hawaiian locations studied by Moore [14] and one from Japan studied by Ichikawa [13]. One possible conclusion is that there well may be no unique pattern of monoterpene structure types to enable a differentiation between algae at the species level. A further relevant observation is that these unrelated algae, *Microcladia*, *Plocamium* and *Chondrococcus*, are able to synthesize either the same or similar monoterpene components. If it is accepted that the taxonomy of each of these genera is fully secure,* then there seems to be at present no obvious explanation of this phenomenon. These collective observations suggest to us that a correlation of polyhalogenated monoterpene structure type to algal systematics at the level of genus to order does not apparently exist. Therefore, the utility of these kind of structure types as a chemotaxonomic tool will be severely limited.

EXPERIMENTAL

NMR spectra were determined at 100 MHz at continuous wave or pulse/fourier-transform mode. Preparative high pressure liquid chromatographic (hplc) separations were carried out using an 8′ × 3/8″ column (Porisal-A) with an eluant of hexane–C_6H_6 (80:20). Analytical GC–MS was carried out with an all glass GLC system equipped with a 5′ × 1/8″ column (2% OV-17) operated with temperature programming from 150–250° at 2° per min. Column chromatography utilized Davison Chemical Co. silica gel Grade 62 (60–200 mesh). Hexane–C_6H_6 (80:20) was used for TLC work (Si gel). Compounds 1–4 could not be separated by this solvent system and gave an $R_f \approx 0.45$. Marine plant samples were identified according to Smith [19]. *M. californica* and *M. coulteri* are described as being similar in vegetative structure. In the Monterey Bay area, however, the former is epiphytic on *Egregia* and is much less abundant and smaller in size than the latter

* Discussions with Professor I. Abbott, Hopkins Marine Station and also ref. [19] support the proposed taxonomic assignments.

Table 1. Relative percentage compositions of the halogenated monoterpenes of northern Santa Cruz *Microcladia*

Location	Compound	*M. borealis* 1	2	3	4	*M. californica* 1	2	3	4	*M. coulteri* 1	2	3	4
Four Mile Beach		13	53	16	18	33	67	—	—	—	—	—	—
Davenport Landing		36	57	—	7	45	49	—	6	—	—	—	—
Pigeon Point		11	89	—	—	38	38	—	24	—	100	—	—

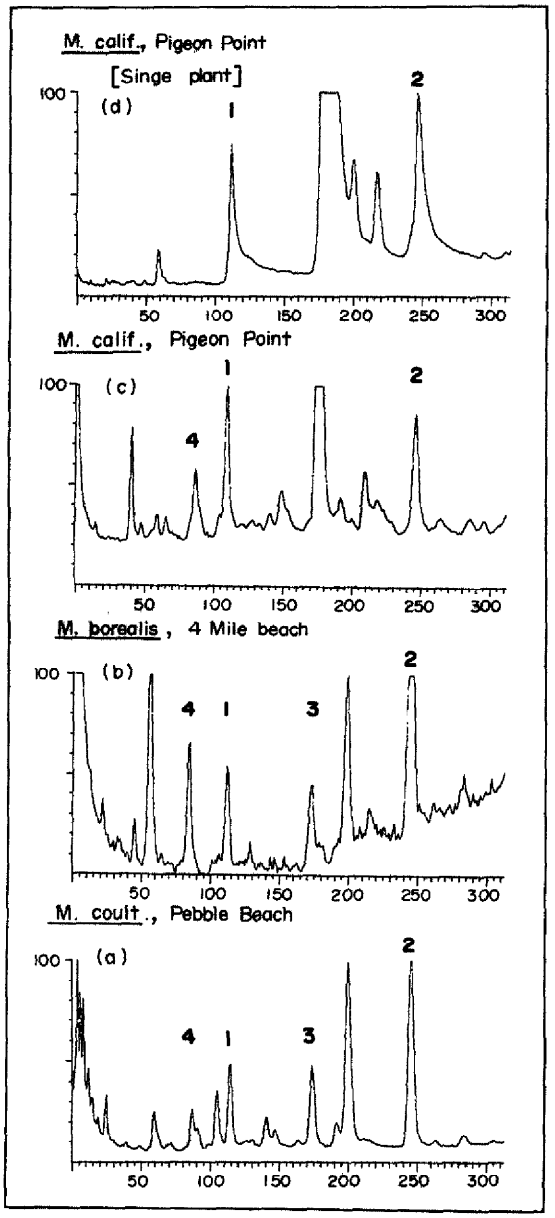


Fig. 3. GLC analysis of algal extracts containing plocamene-B (1), violacene (2), plocamene-C (3), and plocamene-D (4). Additional peaks in figs. b–d represent non-halogenated lipids.

which is epiphytic on *Gigartina* and *Prionitis*. These differences along with experience gained in noting differences of secondary branching and tapering at the ends between the two species, which were verified by inspection of sexually mature female plants of *M. californica* [#032976-6a–d], enabled us to distinguish unambiguously between the two plants. Voucher samples documenting the collections in this work are deposited in the herbarium of the Coastal Marine Lab at UCSC.

Extraction and isolations. Plocamene-C (3). *M. coulteri* (80 g dry wt) collected subtidally at Pebble Beach, Monterey County (V. #010875-1) was soaked without drying or grinding for several days in $CHCl_3$. The concentrate from this extraction (0.8 g) was dissolved in hexane and filtered through Si gel to give a yellow oil (152 mg). This sample was used for GC–MS analysis which revealed the presence of several halogenated monoterpenes and non-halogenated lipids. Multipulse NMR spectra showed resonance characteristic of haloterpenes but in very low levels relative to the non-halogenated lipids. A continuous extraction upon the same plant material with EtOH yielded a concentrate whose hexane solubles were chromatographed over Si gel. A resultant hexane fraction (0.33 g) was further fractionated by hplc to yield a fraction (~10 mg) which showed plocamene-C by NMR. Its MS (from GC–MS) showed expected major fragments at *m/e* 238, 240, 242; 239, 241, 243; 203, 205, 207; 167, 169; and 131.

Violacene (2). *M. borealis* (120 g, dry wt) from four collections: Four Mile Beach, Pigeon Point, Davenport Landing and Bonny Doon, (#111174 @ Dav. Landing) was pooled, extracted without drying or grinding and the resultant oil was purified as described. Violacene (2) was isolated and identified by comparison of its NMR spectra (Fig. 3) and MS [(from GC–MS) showing diagnostic peaks at *m/e* 352, 354, 356, 358; 317, 319, 321, 323; 301, 303, 305, 307, 309; 269, 271, 273] to that of authentic material.

Plocamene-B (1). *M. calfornica* (440 g, dry wt) from Davenport Landing (#032975-6) was extracted without drying or grinding and the resultant oil was purified as indicated. Semipure plocamene-B was isolated and identified by a comparison of its NMR spectrum (Fig. 4) and MS [(from GC–MS) diagnostic peaks at *m/e* 238, 240, 242; 203, 205, 207; 167, 169; 131] to that of authentic material. *Microcladia* samples were collected in June, 1975 for systematic GC–MS study. These samples were extracted immediately after collection with $CHCl_3$ which was worked-up by drying over $MgSO_4$ before evaporation. The conc oil was dissolved in hexane and rapidly filtered through Si gel to give a semi-depigmented oil for GC–MS examination.

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