



## Ecological leads for natural product discovery: novel sesquiterpene hydroquinones from the red macroalga *Peyssonnelia* sp.

Amy L. Lane<sup>a,†</sup>, Laurlynn Mular<sup>b</sup>, Elizabeth J. Drenkard<sup>b</sup>, Tonya L. Shearer<sup>b</sup>, Sebastian Engel<sup>b</sup>, Suzanne Fredericq<sup>c</sup>, Craig R. Fairchild<sup>d</sup>, Jacques Prudhomme<sup>e</sup>, Karine Le Roch<sup>e</sup>, Mark E. Hay<sup>b</sup>, William Aalbersberg<sup>f</sup>, Julia Kubanek<sup>a,b,\*</sup>

<sup>a</sup>School of Chemistry and Biochemistry, Georgia Institute of Technology, Atlanta, GA 30332, USA

<sup>b</sup>School of Biology, Georgia Institute of Technology, Atlanta, GA 30332, USA

<sup>c</sup>Department of Biology, University of Louisiana at Lafayette, Lafayette, LA 70504, USA

<sup>d</sup>Bristol-Myers Squibb Pharmaceutical Research Institute, Princeton, NJ 08543, USA

<sup>e</sup>Department of Cell Biology and Neuroscience, University of California at Riverside, Riverside, CA 92521, USA

<sup>f</sup>Institute of Applied Sciences, University of the South Pacific, Suva, Fiji

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### ABSTRACT

Pharmacologically-motivated marine natural product investigations have yielded a large variety of structurally unique compounds with interesting biomedical properties, but the natural roles of these molecules often remain unknown. While secondary metabolites may function as antimicrobial chemical defenses, few studies have examined this hypothesis. In the present investigation, chromatographic fractions from 69 collections of Fijian red macroalgae representing at least 43 species were evaluated for growth inhibition of three microbial pathogens and saprophytes of marine macrophytes. At least one microbe was suppressed by fraction(s) of all evaluated algae, suggesting that antimicrobial defenses are common among tropical seaweeds. From these leads, peyssononic acids A–B (**1–2**), novel sesquiterpene hydroquinones, were isolated from the crustose red alga *Peyssonnelia* sp. At ecologically realistic concentrations, both compounds inhibited growth of *Pseudoalteromonas bacteriolytica*, a bacterial pathogen of marine algae, and *Lindra thalassiae*, a fungal pathogen of marine algae, and exhibited modest anti-neoplastic activity against ovarian cancer cells. The peyssononic acids included one novel carbon skeleton and illustrated the utility of ecological studies in natural product discovery.

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### 1. Introduction

Marine organisms including invertebrates, microbes, and seaweeds are widely recognized sources of structurally novel secondary metabolites.<sup>1,2</sup> These natural products have provided promising drug leads, offered targets for synthetic organic chemists, and afforded opportunities for elucidation of unusual biosynthetic pathways. Secondary metabolite pathways probably evolved as a result of complex interactions between organisms in their native habitats, but the role of natural products in mediating such interactions remains poorly understood in the majority of cases.

Secondary metabolites may play a particularly important role in mediating marine host–microbe interactions in the ocean.<sup>3,4</sup> While most microbes may be innocuous or beneficial to hosts, reports of

disease outbreaks in a variety of marine invertebrates and seaweeds suggest the negative impact of some microbes on coral reef health and thus the potential for selection to resist pathogenic microbes.<sup>5,6</sup> Among marine plants, coralline lethal orange disease devastated South Pacific coralline algal populations during the 1990s,<sup>7</sup> red spot disease has impacted commercially valuable kelp populations,<sup>8</sup> a slime mold wasting epidemic destroyed nearly all *Zostera marina* eelgrass in the North Atlantic during the 1930s,<sup>9</sup> and the pathogenic fungus *Lindra thalassiae* has been reported to cause raisin disease in the brown algae *Sargassum* spp. as well as disease in seagrasses.<sup>10,11</sup> Microbial pathogens not only affect susceptible populations, but can also disturb the structure and function of entire marine communities.<sup>5</sup>

Disease outbreaks in marine plants appear sporadic and pathogens may target specific hosts.<sup>5</sup> One possible explanation for this limited disease prevalence is that secondary metabolites defend some species against microbial attack,<sup>4</sup> but only a handful of previous studies investigated this possibility and even fewer identified specific defensive metabolites from marine plants. Two previous

\* Corresponding author. Tel.: +1 404 894 8424; fax: +1 404 385 4440.

E-mail address: [julia.kubanek@biology.gatech.edu](mailto:julia.kubanek@biology.gatech.edu) (J. Kubanek).

† Present address: Scripps Institution of Oceanography, University of California at San Diego, 9500 Gilman Dr., La Jolla, CA 92093, USA.

surveys provided evidence for antimicrobial chemical defenses among these organisms.<sup>12,13</sup> Known antimicrobial defenses among marine plants include six secondary metabolite classes: (1) halogenated furanones from the red alga *Delisea pulchra* impede colonization by a variety of genera of marine bacteria,<sup>14</sup> (2) a polybrominated 2-heptanone from the red alga *Bonnemaisonia hamifera* inhibits growth of co-occurring marine bacterial strains,<sup>15</sup> (3) a macrocyclic polyketide from the brown alga *Lobophora variegata* inhibits growth of a pathogenic fungus,<sup>16</sup> (4) a flavone glycoside from the seagrass *Thalassia testudinum* is growth inhibitory toward a zoosporic fungus,<sup>17</sup> (5) sulfated triterpenes from *Penicillus capitatus* and *Tydemania expeditionis* are effective antifungal defenses at ecologically realistic concentrations,<sup>18,19</sup> and (6) diterpene-shikimate natural products from the red alga *Callophycus serratus* found at heterogeneous sites on algal surfaces defend this alga against a pathogenic marine fungus.<sup>20</sup>

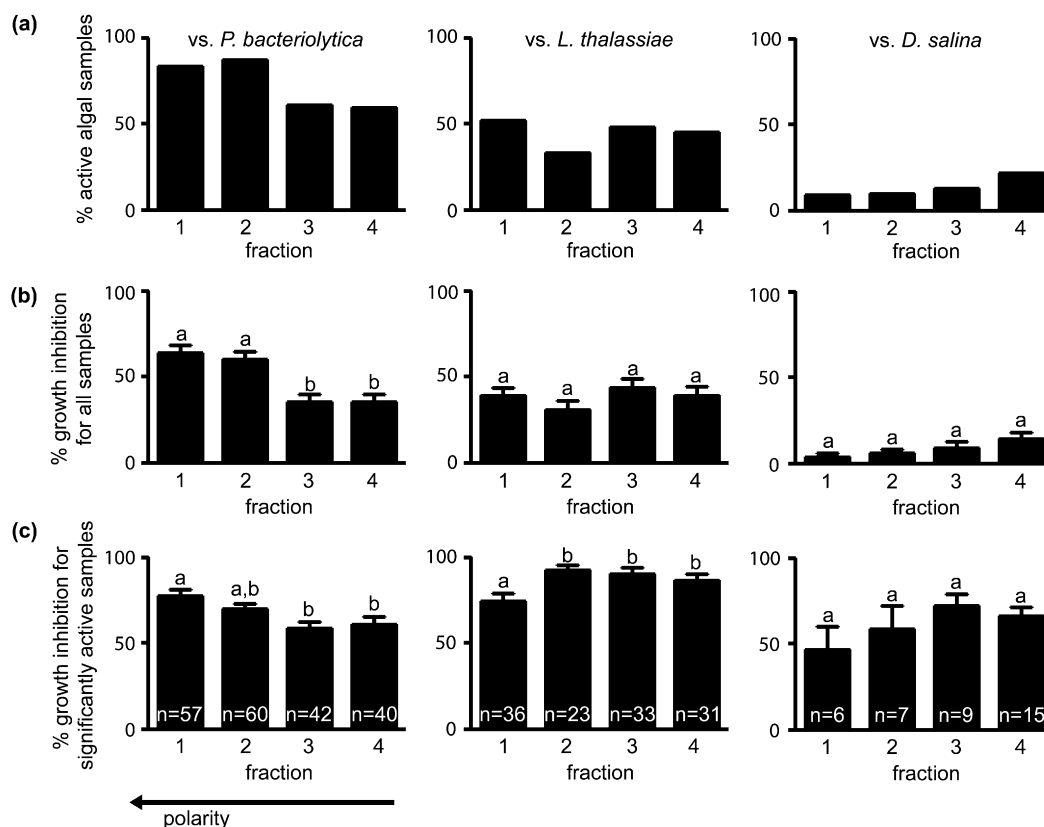
Herein, we evaluate antimicrobial chemical defenses for 69 collections of Fijian red macroalgae, providing evidence that chemical defenses span a wide range of polarities and suggesting these defenses are not broad spectrum but instead active against specific microbes. Although not commonly investigated as sources of novel natural products, members of the algal genus *Peyssonnelia* exhibited particularly strong antimicrobial activities in ecological assays. Bioassay-guided fractionation of extracts from *Peyssonnelia* sp. resulted in the discovery of peyssonic acids A–B (**1–2**), growth inhibitors of both a bacterial and fungal pathogen of marine algae. The peyssonic acids include a novel carbon connectivity pattern and illustrate the potential of ecologically-motivated studies in the discovery of novel chemistry.

## 2. Results and discussion

### 2.1. Survey of 69 Fijian red macroalgae reveals antimicrobial chemical defenses are common

To determine the frequency of antimicrobial chemical defenses among tropical red macroalgae as well as better understand properties of these defenses, Fijian seaweeds were extracted and these extracts separated into four chromatographic fractions using reversed-phase liquid chromatography. Among 69 collections of red macroalgae, including at least 43 distinct species (Supplementary data), antimicrobial chemical defenses were prevalent against the bacterial pathogen *Pseudoalteromonas bacteriolytica*, known to cause red spot disease in kelp,<sup>8</sup> and the fungal pathogen *L. thalassiae*, reported to infect phylogenetically distant hosts including *Sargassum* spp. brown algae and seagrasses.<sup>10</sup> Both of these marine pathogens were inhibited by chromatographic fractions ranging from polar to lipophilic (Fig. 1a), suggesting that a variety of classes of secondary metabolites are employed as antimicrobial chemical defenses.

At least one chromatographic fraction from all 69 seaweed samples significantly inhibited growth of one or more evaluated microbes, suggesting that red algae have been selected to deter deleterious microbes via chemical defenses. This high prevalence of defense corresponds with findings reported by Engel et al.<sup>12</sup> and Puglisi et al.<sup>13</sup> in surveys of antimicrobial defenses among tropical red, green, and brown algae. In the present study, for 99% of the 69 algal collections, at least one chromatographic fraction at natural whole tissue concentration was significantly inhibitory toward



**Figure 1.** Antimicrobial activities of four chromatographic fractions prepared from extracts of 69 collections of Fijian red macroalgae against pathogenic bacterium *Pseudoalteromonas bacteriolytica*, pathogenic fungus *Lindra thalassiae*, and saprophytic fungus *Dendryphiella salina*. (a) Frequency of significant antimicrobial activity for fractions at natural whole tissue concentrations ( $n=69$  algal collections). (b) Comparison of inhibitory potency among all fractions evaluated at natural whole tissue concentrations. Different letters indicate fractions differing significantly in antimicrobial activity ( $p<0.05$ ,  $n=69$  algal collections; one-way ANOVA with Bonferroni's multiple comparisons test; bars denote one standard error). (c) Comparison of inhibitory potency among significantly active algal collection fractions evaluated at natural whole tissue concentrations. Different letters indicate fractions differing significantly in antimicrobial activity ( $p<0.05$ ; one-way ANOVA with Bonferroni's multiple comparisons test; bars denote one standard error);  $n$  represents the number of significantly active algal sample fractions compared.

growth of pathogenic *P. bacteriolytica*; for 74% of collections, at least one fraction was active against pathogenic *L. thalassiae*. *Dendryphiella salina*, a saprophytic marine fungus, was more resistant to algal chemical defenses, with only 30% of collections exhibiting at least one fraction with significant activity against this saprophyte.

The most polar fractions were significantly more inhibitory toward *P. bacteriolytica* than less polar fractions ( $p < 0.05$ ,  $n = 69$ ; one-way ANOVA with Bonferroni's multiple comparison test; Fig. 1b), when both active and inactive fractions were considered. Given this high frequency of antibacterial activity among polar chromatographic fractions, these samples should be the focus of future studies. As most known natural products from red macroalgae are lipophilic, it is likely these fractions contain novel antibacterial metabolites. In contrast, polar and non-polar fractions did not differ significantly in their ability to suppress growth of the fungi *L. thalassiae* or *D. salina* ( $p > 0.05$ ,  $n = 69$ ; one-way ANOVA with Bonferroni's multiple comparison test; Fig. 1b). However, when only significantly inhibitory fractions were evaluated, less polar fractions were significantly more potent than more polar fractions against *L. thalassiae* ( $p < 0.05$ , one-way ANOVA with Bonferroni's multiple comparison test; Fig. 1c). The reverse was true for fractions inhibitory toward *P. bacteriolytica* (Fig. 1c), suggesting that different polar classes of compounds defend against this bacterium than against the fungus *L. thalassiae*. Further, no correlations were observed between antimicrobial potencies of individual fractions against *L. thalassiae* versus *D. salina*, *L. thalassiae* versus *P. bacteriolytica*, or *D. salina* versus *P. bacteriolytica* (data not shown). This overall lack of broad-spectrum antibiotic activity suggests that algal chemical defense molecules are not multifunctional against a wide variety of microbial genera, but instead are more targeted in their effects.

The high prevalence of antimicrobial activities observed among chromatographic fractions from several orders of red macroalgae (Supplementary data) suggests the potential of ecology-driven studies in the discovery of novel chemistry. A search of the MarinLit database revealed secondary metabolites have been previously reported for only 53% of these evaluated genera ( $n = 29$  evaluated genera; Supplementary data). Further, antimicrobial chemical defense compounds have been isolated and identified from only one of these genera,<sup>20</sup> indicating a wealth of ecologically active natural products remain to be discovered.

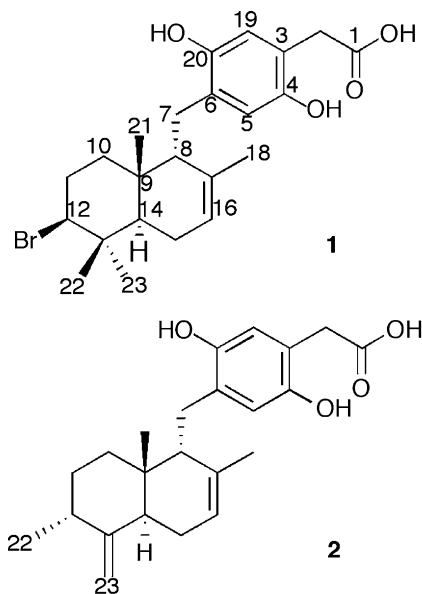
In addition to the abundance of chemical defense observed across different species, substantial variation in antimicrobial defense was observed among multiple collections identified as the same algal species by morphological- and/or molecular-based analyses (Supplementary data). Despite the indication from 18S rRNA analyses that samples G-0011 and G-0163 were both *Neogoniolithon frutescens*, fractions from G-0011 were significantly more inhibitory toward both *P. bacteriolytica* and *L. thalassiae* than were fractions from G-0163. Analogously, among two collections identified as *Corynocystis prostrata*, collection G-0008 exhibited significantly greater antifungal activity than collection G-0026. Together, these results as well as additional comparisons of chemical defenses among members of the same species (Supplementary data) suggest intraspecific variation in antimicrobial chemical defenses is common. The importance of intraspecific variation in secondary metabolite production has previously been recognized among macroalgal antiherbivore defenses,<sup>21,22</sup> and such intraspecific variation may result from a variety of biotic and abiotic factors.<sup>23</sup>

In contrast to variability observed for antimicrobial chemical defenses within several species and genera of red algae, some genera were consistently well defended against microbial pathogens. For example, extracts from all nine collections of *Peyssonnelia* spp. were strongly inhibitory toward both *P. bacteriolytica* and *L. thalassiae*, suggesting *Peyssonnelia* represents a particularly well-defended algal genus. This prevalence of chemical defense, together with the paucity of known secondary metabolites from

*Peyssonnelia* spp.,<sup>24,25</sup> implicated these algae as a promising source of novel chemistry and insights into ecological function.

## 2.2. Bioassay-guided fractionation yields novel antimicrobial sesquiterpene hydroquinones from *Peyssonnelia* sp.

*Peyssonnelia* sp. collection G-0109, exhibiting particularly potent antimicrobial activity, was selected as a candidate for ecologically-guided natural product isolation and identification. Guided by growth inhibition of the pathogenic bacterium *P. bacteriolytica*, peyssonic acids A–B (1–2) were isolated by reversed-phase column chromatography and HPLC (see Experimental).



Structures of novel peyssonic acids A and B (1–2).

**Table 1**

<sup>13</sup>C and <sup>1</sup>H NMR spectral data for 1–2 (125 MHz for <sup>13</sup>C and 500 MHz for <sup>1</sup>H; in DMSO)

No.	1		2	
	$\delta^{13}\text{C}$	$\delta^1\text{H}$ ( $J_{\text{H,H}}$ )	$\delta^{13}\text{C}$	$\delta^1\text{H}$ ( $J_{\text{H,H}}$ )
1	175.3	—	175.3	—
2	44.5	3.09s	44.5	3.10s
3	122.6	—	122.5	—
4	150.1	—	150.1	—
5	117.8	6.35s	117.5	6.40s
6	126.6	—	126.7	—
7	30.3	2.13m, 2.77dd (6.2, 14.6)	29.4	2.21dd (2.4, 12.2), 2.90dd (7.2, 15.0)
8	53.7	1.78t (5.2)	50.9	1.97m
9	36.4	—	38.4	—
10	36.3	0.99m, 1.88m	29.5	0.68m, 2.06td (2.0, 10.9)
11	30.8	1.93m, 2.13m	28.4	1.28br d (12.7), 1.63m
12	70.9	4.20dd (3.0, 12.5)	38.5	2.53m
13	39.1	—	154.5	—
14	41.8	1.64m	32.6	2.42br t (7.9)
15	25.1	1.92m, 2.06m	25.8	1.90m
16	119.2	5.23s	119.2	5.29s
17	136.7	—	136.7	—
18	23.6	1.49s	23.4	1.60s
19	116.6	6.31s	116.6	6.33s
20	146.1	—	146.1	—
21	21.8	0.86s	19.0	0.65s
22	17.5	0.97s	19.6	1.09d (7.2)
23	29.9	1.00s	106.8	4.50s, 4.81s
OH	—	8.38br s	—	8.51br s
OH	—	13.39 br s	—	13.37 br s

Peyssononic acid A (**1**) displayed an  $[M-H]^-$  molecular ion with  $m/z$  449.1309 and a characteristic monobrominated isotopic pattern, supporting a molecular formula of  $C_{23}H_{31}O_4Br$ . The structure of **1** was determined through examination of 1D and 2D NMR spectral data (Table 1, Supplementary data). Assignments within the aromatic group of **1** were established by HMBC correlations from H-5 ( $\delta$  6.35) to C-3 ( $\delta$  122.6), C-4 ( $\delta$  150.1) and C-20 ( $\delta$  146.1), and from H-19 ( $\delta$  6.31) to aromatic C-4, C-6 ( $\delta$  126.6), and C-20. These assignments were confirmed and *para* dihydroxy substitution established by comparison of experimental  $^{13}C$  chemical shifts with empirical and literature values.<sup>25,26</sup> HMBC correlations from H-19 to C-2 ( $\delta$  44.5), along with correlations from H-2 ( $\delta$  3.09) to C-1 ( $\delta$  175.3), C-3, C-4, and C-19 ( $\delta$  116.6) then established an acetic acid substituent attached at C-3. The connection to the sesquiterpene group was assigned at C-6 on the basis of HMBC correlations from H-5 to C-7 ( $\delta$  30.3) and from both H-7a ( $\delta$  2.13) and H-7b ( $\delta$  2.77) to C-5 ( $\delta$  117.8), C-6, and C-20.

The bromine-substituted drimane-type sesquiterpene group of **1** was elucidated primarily through analysis of HMBC and COSY data. HMBC correlations from singlet Me-18 ( $\delta$  1.49) to C-8 ( $\delta$  53.7), C-16 ( $\delta$  119.2), and C-17 ( $\delta$  136.7) prompted attachment of this methyl group to C-17 and established C-8–C-17–C-16 connectivity. HMBC correlations from singlet Me-21 ( $\delta$  0.86) to C-8, C-9 ( $\delta$  36.4), and C-10 ( $\delta$  36.3) next established connectivity between these carbons. COSY correlations between H-10a ( $\delta$  0.99) and H-11a ( $\delta$  1.93), between H-10b ( $\delta$  1.88) and H-11b ( $\delta$  2.13), and between both H-11 protons and H-12 ( $\delta$  4.20) prompted linkage of C-10–C-11–C-12, with these assignments confirmed by an HMBC correlation from H-11b to C-10. A bromine substituent was assigned at C-12 on the basis of downfield  $^{13}C$  and  $^1H$  chemical shifts ( $\delta$  70.9 and  $\delta$  4.20, respectively). HMBC correlations from both Me-22 ( $\delta$  0.97) and Me-23 ( $\delta$  1.00) to C-12 and quaternary C-13 ( $\delta$  39.1) supported C-12–C-13 connectivity, while HMBC correlations from Me-21, Me-22, and Me-23 to C-14 ( $\delta$  41.8) established connectivity between C-9 and C-14, thus sealing this ring. COSY correlations between H-14 ( $\delta$  1.64) and both H-15 protons ( $\delta$  1.92,  $\delta$  2.06) as well as an HMBC correlation between H-16 ( $\delta$  5.23) and C-15 ( $\delta$  25.1) then sealed the second ring, completing the drimane skeleton.

Relative stereochemical assignment for peyssononic acid A (**1**) commenced with assignment of H-12 ( $\delta$  4.20) in an axial position based on a large  $J$  coupling constant ( $J=12.5$  Hz) observed for this proton (as well as a smaller 3.0 Hz coupling), which supported an axial-axial relationship and  $180^\circ$  dihedral angle between H-12 and a proton at C-11.<sup>26</sup> Observation of a very intense COSY correlation between H-12 and H-11b ( $\delta$  2.13) prompted assignment of H-11b in an axial position on the opposite face of the ring from H-12. NOE correlations between H-12, H-14 ( $\delta$  1.64), and H-10b ( $\delta$  1.88) supported assignment of all of these atoms in axial positions on the same face of the drimane system. NOE correlations were not observed between any of these axial protons and Me-21 ( $\delta$  0.86) or H-11b. However, a strong correlation was noted between Me-21 and H-11b ( $\delta$  2.13), supporting assignment of Me-21 and H-11b in axial positions on the opposite face of the molecule. These assignments were reinforced by an NOE correlation observed between H-11b and Me-22 ( $\delta$  0.97), for which an axial position was supported by the upfield carbon chemical shift ( $\delta$  17.5) of this methyl relative to equatorial Me-23 ( $\delta$  29.9).<sup>27</sup> Thus, with axial H-11b, Me-21, and Me-22 assigned on one face of the drimane system and axial H-10b, H-12, and H-14 established on the opposite face, a *trans*-fused drimane ring configuration was proposed for **1**. The relative stereochemistry at C-8 was then assigned on the basis of NOE and  $J$  coupling constant arguments. A 6.2 Hz vicinal coupling observed for H-7b ( $\delta$  2.77) supported a dihedral angle of approximately  $30^\circ$  or  $130^\circ$  between H-7b and H-8 ( $\delta$  1.78). Further, the 5.2 Hz coupling observed for pseudotriplet H-8 suggested *gauche* relationships between this proton and both H-7a ( $\delta$  2.13) and H-7b. Observation

of NOE correlations between H-7b and axial H-10b and between H-8 and axial Me-21 then established relative stereochemistry at C-8.

High-resolution mass spectral data established the molecular formula of peyssononic acid B (**2**) as  $C_{23}H_{30}O_4$  ( $m/z$  369.2080  $[M-H]^-$ ). Comparison of  $^{13}C$  and  $^1H$  NMR spectral data as well as HMBC and COSY correlations between **1** and **2** indicated these molecules shared an acetic acid-substituted hydroquinone functionality and both possessed a sesquiterpene group attached at aromatic C-6 (Table 1, Supplementary data). Comparison of molecular formulae for **1** and **2** indicated the loss of a bromine group and gain of an alkene moiety within the sesquiterpene portion of **2**. An *exo*-methylene substituent was assigned at C-13 on the basis of HMBC correlations from singlets H-23a ( $\delta$  4.50) and H-23b ( $\delta$  4.81) to C-12 ( $\delta$  38.5), C-13 ( $\delta$  154.5), and C-14 ( $\delta$  32.6). HMBC correlations from doublet Me-22 ( $\delta$  1.09) to C-11 ( $\delta$  28.4), C-12, and C-13 then established attachment of Me-22 to methine C-12. HMBC and COSY correlations supported assignment of the remainder of this decalin-type system identical to that of **1**, and the sesquiterpene skeleton of **2** was verified by comparison with literature values.<sup>25</sup>

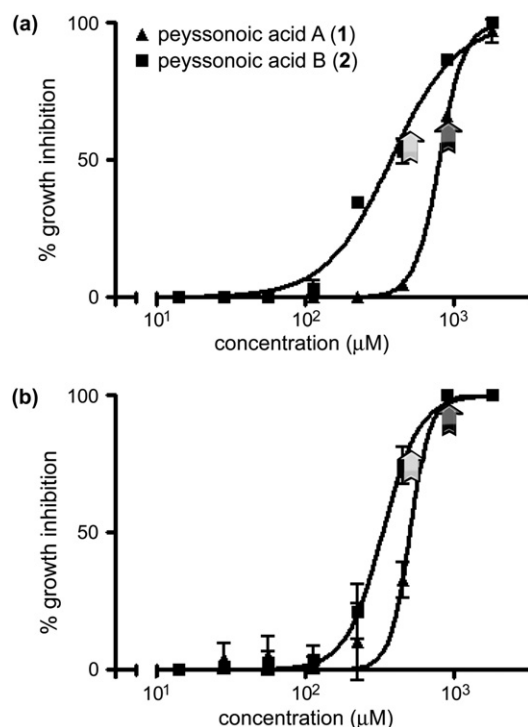
For peyssononic acid B (**2**), NOEs were observed between H-14 ( $\delta$  2.42) and Me-22 ( $\delta$  1.09), but not between H-12 ( $\delta$  2.53) and H-14, supporting assignment of H-14 and H-22 at axial positions on the same face of the drimane-type skeleton. NOEs between H-10b ( $\delta$  2.06) and both Me-22 and H-14 completed this series of 1,3-diaxial interactions. Me-21 was then assigned to an axial position on the opposite face based on NOEs observed between Me-21 ( $\delta$  0.65) and H-11b ( $\delta$  1.63), but not between Me-21 and H-14 or Me-22. This *trans* orientation of Me-21 and H-14 corresponded with the *trans*-fused bicyclic system proposed for **1**. Finally, assignment of the C-8 stereocenter was established analogously to **1**.

The most structurally similar known relatives of peyssononic acids A and B (**1–2**) are peyssonols A–B, isolated from a Red Sea collection of *Peyssonnelia* sp. on the basis of activity against HIV-1 reverse transcriptase-associated RNA-dependent DNA polymerases.<sup>25</sup> Peyssonol A differs from peyssononic acid A (**1**) by substitution with a formyl group instead of an acetic acid group at C-3 on the hydroquinone ring and by a *trans*-fused drimane skeleton in **1** versus a *cis*-fused orientation in peyssonol A. Hence, peyssononic acid A (**1**) represents a novel carbon skeleton with one additional carbon relative to peyssonol A. Peyssononic acid B (**2**) shares a carbon skeleton with peyssonol B, and differs from this known metabolite in the presence of an acetic acid-substituted hydroquinone versus a methyl acetate group at the corresponding position in peyssonol B as well as in regioisomerization of one site of unsaturation in the drimane group. To our knowledge, **1** and **2** represent the first examples of terpene hydroquinone natural products bearing an acetic acid-substituted hydroquinone.

### 2.3. Ecological and pharmacological activities of peyssononic acids A–B (**1–2**)

Both peyssononic acids A–B (**1–2**) inhibited growth of the bacterial pathogen *P. bacteriolytica* and the fungal pathogen *L. thalassiae* (Fig. 2), suggesting their role as antimicrobial chemical defenses of *Peyssonnelia* sp. Against *P. bacteriolytica*, peyssononic acid A (**1**) exhibited an average  $IC_{50}$  value of 799  $\mu M$ , while peyssononic acid B (**2**,  $IC_{50}$  377  $\mu M$ ) was significantly more inhibitory toward this pathogen (Fig. 2;  $p < 0.0001$ ,  $F$ -test of log  $IC_{50}$  values). Against *L. thalassiae*, peyssononic acid B (**2**,  $IC_{50}$  331  $\mu M$ ) was also significantly more inhibitory than peyssononic acid A (**1**,  $IC_{50}$  506  $\mu M$ ; Fig. 2;  $p < 0.0001$ ,  $F$ -test of log  $IC_{50}$  values). For both compounds,  $IC_{50}$  values observed against each marine pathogen were lower than natural isolated concentrations of these natural products. This suggests that, despite their weak potencies when compared to typical biomedical activity data, these secondary metabolites are present within organism tissues at concentrations





**Figure 2.** Comparison of isolated concentrations (denoted by black arrow for **1** and gray arrow for **2**) of peyssononic acids A–B (**1–2**) and growth inhibition curves against ecologically-relevant pathogens (a) *P. bacteriolytica* and (b) *L. thalassiae* ( $n=3$  subsample assays at eight concentrations). In all cases, natural isolated concentrations were greater than experimental  $IC_{50}$  values, indicating these compounds represent effective antimicrobial chemical defenses of whole algal tissues. Further, peyssononic acid B (**2**) was significantly more inhibitory toward both marine pathogens than peyssononic acid A (**1**) ( $p<0.0001$ ;  $F$ -test of log  $IC_{50}$  values). Error bars denote one standard deviation.

sufficient for inhibition of these microbes in nature. Despite significant inhibition of *D. salina* observed with crude extracts, neither of these compounds inhibited growth of this marine saprophyte, suggesting *Peyssonnelia* sp. harbors other unidentified chemical defenses against this fungus.

While peyssononic acids A–B (**1–2**) were strongly inhibitory toward a marine pathogenic bacterium and fungus at ecologically realistic concentrations, they were only modestly inhibitory toward human pathogenic bacteria and fungi (Table 2). The most notable pharmacological activity for both compounds was against human ovarian cancer cell lines, with **2** exhibiting stronger anticancer activity ( $IC_{50}$  13.5 μM) than **1** ( $IC_{50}$  34.5 μM).

**Table 2**  
Pharmacological activities of **1–2**

Compound	Antibacterial $IC_{50}$ (μM)			Anticancer $IC_{50}$ (μM)			Antifungal MIC (μM)	
	MRSA <sup>a</sup>	VREF <sup>b</sup>	Antitubercular	Mean <sup>c</sup>	A2780/DDP-S <sup>d</sup>	Cell line selectivity ( $IC_{50}$ max/ $IC_{50}$ min)	WTCA <sup>e</sup>	ARCA <sup>f</sup>
1	>550	>550	>100	52.7	34.5	1.6	>550	>550
2	533	230	>100	28.0	13.5	4.6	>550	>550

<sup>a</sup> Methicillin-resistant *Staphylococcus aureus*.

<sup>b</sup> Vancomycin-resistant *Enterococcus faecalis*.

<sup>c</sup> Mean of 11 cancer cell lines (see Experimental section for details).

<sup>d</sup> Ovarian tumor cell line.

<sup>e</sup> Wild-type *Candida albicans*.

<sup>f</sup> Amphotericin B-resistant *C. albicans*.

## 2.4. Conclusions

In the present study, all 69 evaluated tropical red algal samples harbored significant antimicrobial chemical defenses against at

least one of three evaluated marine microbes. These chemical defenses spanned a full range of polarities, indicating a variety of chemical classes are involved in defending marine macroalgae against microbial attack. This abundance of defenses suggested the largely untapped potential of ecology-driven investigations in the discovery of novel chemistry. The potential of such approaches was illustrated by isolation and identification of peyssononic acids A–B (**1–2**), novel antimicrobial defenses from the red alga *Peyssonnelia* sp. These sesquiterpene hydroquinones mark the first report of terpenoid natural products bearing an acetic acid-substituted hydroquinone and include one novel carbon framework.

## 3. Experimental

### 3.1. General

Semipreparative HPLC was performed with a Waters 1525 or 515 pump and a Waters 2996 diode-array UV detector or a Waters 2487 dual-wavelength absorbance detector. <sup>1</sup>H, <sup>13</sup>C, DEPT-135, HSQC, HMBC, COSY, NOESY, ROESY, and DPFGSE-NOE NMR experiments were conducted in DMSO with a Bruker DRX-500 instrument using a 5 mm broadband or inverse detection probe, and referenced to residual DMSO ( $\delta$  2.49 and  $\delta$  39.9 ppm for <sup>1</sup>H and <sup>13</sup>C, respectively). High-resolution mass spectra were acquired using electrospray ionization with an Applied Biosystems QSTAR-XL hybrid quadrupole-time-of-flight tandem mass spectrometer and Analyst QS software. UV spectra were recorded in methanol with a Spectronic 21D spectrophotometer, and optical rotations were measured with a Jasco P-1010 spectropolarimeter. All statistical analyses were completed with either SYSTAT version 9 (Systat Software, Inc., Chicago, IL) or GraphPad version 4 (GraphPad Software, Inc., La Jolla, CA). HPLC grade solvents were used in semi-preparative HPLC (Fisher Scientific Co.), and NMR solvents were obtained from Cambridge Isotope Laboratories.

### 3.2. Algal collection and identification

Algae were collected at depths of 2–20 m offshore from several islands in Fiji: Beqa (18°23'56" S, 177°57'59" E; 18°22'47" S, 177°59'37" E; 18°20'86" S, 178°2'1" E), Viti Levu (18°12'9" S, 177°39'65" E; 18°14'51" S, 177°47'27" E; 18°9'61" S, 178°25'57" E; 18°14'41" S, 177°46'85" E), Taveuni (16°48'97" S, 179°50'84" W; 16°52'32" S, 179°52'68" W; 16°50'44" S, 179°52'12" W; 16°52'47" S, 179°52'4" W; 16°52'33" S, 179°52'67" E), and Kadavu (18°42'71" S, 178°29'53" E; 18°42'49" S, 178°32'35" E). Peyssononic acids A–B (**1–2**) were isolated from *Peyssonnelia* sp. (collection ID# G-0109), collected at Kadavu (18°42'49" S, 178°32'35" E).

Algal specimens were identified by comparison with previously described morphological traits<sup>28</sup> and via 18S rRNA sequencing. Genomic DNA from ethanol-preserved red algal samples was extracted using the DNeasy Tissue Extraction Kit (Qiagen) and

purified using polyethylene glycol. A region of the nuclear small subunit ribosomal RNA (18S rRNA) gene was amplified via PCR using established primers and reaction conditions.<sup>29</sup> 18S rRNA fragments were sequenced in both directions. Sequences were manually edited in BioEdit version 7.0.5.3<sup>30</sup> and aligned using ClustalW.<sup>31</sup> Sequences were deposited in GenBank (accessions GQ227510–GQ227534). Phylogenetic relationships among sequences were determined in PAUP\* (Sinauer Associates, Inc.) using parsimony criteria and 1000 bootstrap replicates, including the poriferan, *Halichondria melanodocia*, as an outgroup (GenBank accession AY737639). Vouchers are housed at the University of the South Pacific in Suva, Fiji, and at the Georgia Institute of Technology.

### 3.3. Algal extraction

Fresh algal material was extracted successively in MeOH (2×) and MeOH/DCM (2:1, 1:1). Extracts were reduced in vacuo and subjected to reversed-phase fractionation with HP20ss resin (Supelco). Fractions 1 and 2 were eluted with MeOH/H<sub>2</sub>O (1:1 and 4:1, respectively), fraction 3 with MeOH, and fraction 4 with acetone.

### 3.4. Ecological antimicrobial assays

Chromatographic fractions were evaluated for inhibition of three ecologically-relevant marine microbes (see below). Assays were designed to approximate natural concentrations of metabolites experienced by microbes invading whole algal tissues. All fractions were tested at maximum concentrations volumetrically equivalent to those in the whole alga; fractions corresponding to a 1 mL wet volume of alga were incorporated into 1 mL of media, which was inoculated with an evaluated microbe.

**3.4.1. Antifungal assays.** Applying previously described methods,<sup>16</sup> antifungal assays were conducted with *L. thalassiae* (ATCC 56663), a fungal pathogen of a variety of marine macrophytes, and *D. salina*, a fungal saprophyte of marine plants. Chromatographic fractions, corresponding to 1.2 mL of wet tissue volume, were each solubilized in a minimal volume of methanol or acetone and incorporated into of 1.2 mL molten YPM agar (16 g/L granulated agar, 2 g/L yeast extract, 2 g/L peptone, 4 g/L D-mannitol, 250 mg/L of both streptomycin sulfate and penicillin G in 1 L of natural seawater). For each fraction, three 400  $\mu$ L subsamples of this mixture were dispensed into sterile 24-well microtiter plates, allowed to solidify, and an aliquot of *L. thalassiae* or *D. salina* suspension in sterile seawater added to each well. Control wells were prepared with YPM agar and solvent but no algal material. Plates were incubated at 28 °C for three days and digital photographs collected for each well. The percent of each well covered in fungal hyphae was determined using the area calculator feature of ImageJ software (NIH), and treatments and controls compared using one-way ANOVA with Dunnett's post-test. Antifungal assays with pure **1–2** were completed at 1:1 serially diluted concentrations ranging from 1812  $\mu$ M to 14  $\mu$ M, and percent growth inhibition at each concentration was calculated relative to solvent-only controls. Growth inhibition data were fit to a sigmoidal dose–response curve, and mean log IC<sub>50</sub> and standard error values computed. Reported IC<sub>50</sub> values were calculated as the antilog of mean log IC<sub>50</sub> values.<sup>32</sup> Antifungal activities of **1–2** were statistically compared with an *F*-test of the log IC<sub>50</sub> value for each compound.

**3.4.2. Antibacterial assays.** Assays using *P. bacteriolytica*, a known marine plant pathogen, were adapted from previous methods.<sup>16</sup> A 24 h shake culture of this bacterium was diluted 1:160 in Difco Marine Broth 2216 (BD Biosciences) and 195  $\mu$ L of this mixture

added to duplicate treatment and control wells of a 96-well plate. An equal amount of sterile broth was added to blank wells. Five microliters of 40× concentrated chromatographic fractions in DMSO were dispensed into treatment and blank wells, yielding a final concentration approximating that found in whole algal tissues; 5  $\mu$ L of DMSO were added to corresponding control wells. Plates were incubated for 20 h at 30 °C, and turbidity measured at 600 nm. We corrected for the natural absorbance of chromatographic fractions by subtracting algal-containing sterile blank turbidities from values obtained for treatments. Corrected treatment turbidity values were compared with controls using one-way ANOVA with Dunnett's post-test. For HPLC fractions and pure compounds from *Peyssonnelia* sp. (ID# G-0109), assays were completed at 1:1 serially diluted concentrations; log IC<sub>50</sub> values computed and statistically compared analogously to antifungal assays.

### 3.5. Pharmacological assays

Pure peyssononic acids A and B (**1–2**) were evaluated for activity against tumor cell lines BT-549, DU4475, MDA-MD-468, NCI-H446, PC-3, SHP-77, LNCaP-FGC, HCT116, MDA-MB-231, A2780/DDP-S, and Du145, representing breast, colon, lung, prostate and ovarian cancer cells. In vitro cytotoxicity was evaluated with the (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt) MTS dye conversion assay as previously described.<sup>33</sup> Antimalarial activity was determined with a previously reported SYBR Green based parasite proliferation assay.<sup>34,35</sup> Antibacterial assays were performed against methicillin-resistant *Staphylococcus aureus* (ATCC 33591) and vancomycin-resistant *Enterococcus faecium* (ATCC 700221), and antifungal assays against both wild-type and amphotericin B-resistant *Candida albicans* (ATCC 32354 and ATCC 90173, respectively), using previously reported methods.<sup>36</sup> Antitubercular activity was assessed against *Mycobacterium tuberculosis* strain H37Rv (ATCC 27294) using the previously described Alamar blue susceptibility test (MABA).<sup>37</sup>

### 3.6. Isolation of sesquiterpene hydroquinones A–B

Frozen *Peyssonnelia* sp. (ID# G-0109) was extracted exhaustively in methanol (2×) and methanol/dichloromethane (2:1 and 1:1) and fractionated with HP20ss resin (Supelco), following the same procedure applied in the algal survey (Experimental 3.3). Fraction 2, with the strongest activity against ecologically-relevant pathogen *P. bacteriolytica*, was subjected to multiple rounds of semipreparative reversed-phase HPLC with an Agilent Zorbax SB-C18 column (5  $\mu$ m, 9.4×250 mm) using methanol/water and acetonitrile/water gradient mobile phases. Antibacterial properties of HPLC fractions were measured with the *P. bacteriolytica* assay described above, and final purification of active compounds **1–2** achieved using a Phenomenex Develosil C<sub>30</sub> column (5  $\mu$ m, 4.6×250 mm) with a methanol/water gradient mobile phase.

**3.6.1. Peyssononic acid A (1).** Brown gum (8.2 mg; 0.13% plant dry mass);  $[\alpha]_D^{24}$  35.0 (c 0.343 g/100 mL, MeOH); UV (MeOH)  $\lambda_{max}$  (log  $\epsilon$ ) 229 nm (4.24), 270 nm (3.89); <sup>1</sup>H NMR (DMSO, 500 MHz) and <sup>13</sup>C/DEPT NMR (DMSO, 125 MHz) data, Table 1; NOE, COSY, HMBC NMR data, Supplementary data; HRESIMS [M–H]<sup>–</sup> *m/z* 449.1309 (calcd for C<sub>23</sub>H<sub>30</sub>O<sub>4</sub>Br, 449.1333).

**3.6.2. Peyssononic acid B (2).** Brown gum (3.1 mg; 0.05% plant dry mass);  $[\alpha]_D^{24}$  200.0 (c 0.0800 g/100 mL, MeOH); UV (MeOH)  $\lambda_{max}$  (log  $\epsilon$ ) 230 nm (4.35), 268 nm (3.81); <sup>1</sup>H NMR (DMSO, 500 MHz) and <sup>13</sup>C/DEPT NMR (DMSO, 125 MHz) data, Table 1; NOE, COSY,

HMBC NMR data, [Supplementary data](#); HRESIMS  $[M-H]^-$   $m/z$  369.2080 (calcd for  $C_{23}H_{29}O_4$ , 369.2071).

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### Supplementary data

Species identification, phylogenetic analysis, and antimicrobial chemical defense potencies for individual algal collections;  $^1H$  and  $^{13}C$  NMR spectra for **1–2**; COSY, HSQC, HMBC, and NOE NMR spectral data for **1–2**. Supplementary data associated with this article can be found in online version at doi:10.1016/j.tet.2009.11.042.

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