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SECONDARY METABOLITE CHEMISTRY OF THE AUSTRALIAN BROWN ALGA *ENCYOTHALIA CLIFTONII*: EVIDENCE FOR HERBIVORE CHEMICAL DEFENCE

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IN HONOUR OF PROFESSOR JEFFREY HARBORNE'S SIXTY-FIFTH BIRTHDAY

Key Word Index—*Encyothalia cliftonii*; Sporochnales; marine algae; bis-prenylphenols; feeding deterrents; chemical defence.

Abstract—Two bis-prenylated phenols have been isolated from the Western Australian brown alga *Encyothalia cliftonii* Harvey. The structures of these compounds were determined by spectral and chemical methods. The major metabolite, 2,4-bis(3-methylbut-2-enyl)phenol, previously isolated as a minor metabolite from the related alga *Perithalia caudata*, showed significant feeding deterrence toward the herbivorous sea urchin *Tripneustes esculentus* (Leske). The minor metabolite is a new compound in which hydration of one of the isopentenyl olefinic bonds has taken place.

INTRODUCTION

In recent years, it has become clear that selected families of marine algae have evolved effective chemical defences against marine herbivores. Chemical deterrents have been isolated from all major phyla of marine plants, and in many cases their effects against a variety of herbivores have been demonstrated in both field and laboratory experiments [1–4]. As part of our continuing program to explore the various families of chemically-defended tropical marine algae, we recently encountered a large population of the brown alga *Encyothalia cliftonii* Harvey near Exmouth along the Northwest Cape of Australia, an area of intense herbivore activity. Because algae of the order Sporochnales are generally rare, little data are available on their relative palatability to herbivores. Recently, we described the structures of the sporochnols, monoterpenoid phenols from the related Caribbean brown alga *Sporochnus bolleanus* [5]. These compounds were found to exhibit significant feeding deterrence properties toward herbivorous Caribbean fishes. In this paper, we report the chemical composition of *E. cliftonii* and the defensive properties, against an herbivorous sea urchin, of two phenolic metabolites, **1** and **2**, isolated from the alga.

RESULTS AND DISCUSSION

Encyothalia cliftonii was collected near Exmouth, Western Australia in December 1989, and stored and transported frozen. The chloroform extract of the freshly defrosted algae was fractionated by silica flash column chromatography, and further purified by silica HPLC to yield phenol **1** as the major component (isolated yield 13% extract) and phenol **2** (isolated yield 3.7% extract). The structures of these alkylated phenols were determined by spectral analyses and by chemical conversion to suitable derivatives.

Phenol **1** was obtained as a viscous oil which analysed for $C_{16}H_{22}O$ by high resolution mass spectrometry. The UV spectrum for this compound (λ_{max} 280 nm) suggested the presence of a phenol. This was also indicated by a significant bathochromic shift (to 297 nm) observed upon the addition of base. Confirmation was obtained by acetylation of **1** under standard conditions to yield the corresponding phenol acetate **3**. Unfortunately, NMR data showed that phenol **1** was not completely pure. Repeated HPLC purifications failed to cleanly separate **1** from contaminants which were identified as mixtures of the corresponding rearranged olefin isomers [double bonds at the 3′-5′(4′) and/or 3-5(4) positions]. However, since the contaminants composed less than 10% of the mixture, the major NMR bands (Tables 1 and 2) associated with **1** could easily be discerned. Characteristic aromatic proton resonances for a 1,2,4-trisubstituted benzenoid moiety were observed, as well as methyl resonances at δ1.70, 1.73, 1.75 and 1.77, two benzylic methylenes at δ3.23 and 3.33, and two olefin protons at δ5.31, which indicated the presence of two isopentenyl substituents. The ^{13}C NMR data for **1** (Table 2) showed the expected substituted phenol carbons as well as those of two isopentenyl substituents. To clarify the spectra and

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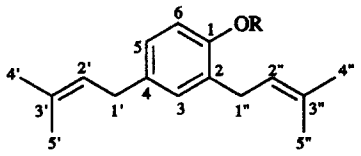

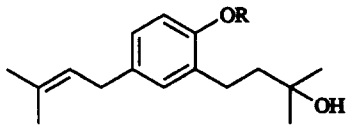

	R
1	H
3	Ac
5	H, $\Delta^{2'3'}$, $^{2''3''}$tetrahydro

	R
2	H
4	H, $\Delta^{2'3'}$ dihydro
6	Me, $\Delta^{2'3'}$ dihydro

Table 1. ^{1}H NMR data for compounds **1–6**

C	1	3	5
3	6.91 *br s*	7.06 *br s*	6.91 *br s*
5	6.88 *dd* (J=8.8, 2.0 Hz)	7.03 *dd* (J=8.0, 1.9 Hz)	6.86 *dd* (J=8.0, 2.0 Hz)
6	6.70 *d* (J=7.0 Hz)	6.94 *d* (J=8.0 Hz)	6.65 *d* (J=8.0 Hz)
1′	3.23 *d* (J=7.4 Hz)	3.25 *d* (J=7.0 Hz)	2.54 *m*
2′	5.31 *m*	5.27 *t* (J=7.0 Hz)	1.47 *m*
3′			1.59 *m*
4′	1.77* *s*	1.79* *s*	0.95 *d* (J=6.5 Hz)
5′	1.75* *s*	1.75* *s*	0.95 *d* (J=6.5 Hz)
1″	3.32 *d* (J=7.0 Hz)	3.34 *d* (J=7.3 Hz)	2.54 *m*
2″	5.31 *m*	5.36 *t* (J=7.3 Hz)	1.47 *m*
3″			1.59 *m*
4″	1.73* *s*	1.79* *s*	0.92 *d* (J=6.5 Hz)
5″	1.70*	1.75* *s*	0.92 *d* (J=6.5 Hz)
-Ac		2.29 *s*	
	2	**4**	**6**
3	6.87 *br s*	6.88 *br s*	6.96 *br s*
5	6.86 *dd* (J=7.7, 2.4 Hz)	6.86 *dd* (J=8.6, 2.1 Hz)	7.09 *m*
6	6.73 *d* (J=7.7 Hz)	6.72 *d* (J=8.7 Hz)	6.75 *d* (J=8.9 Hz)
1′	2.67 *m*	2.48 *m*	2.52 *m*
2′	1.77 *m*	1.44 *m*	1.59 *m*
3′		1.55 *m*	1.50 *m*
4′	1.27 *s*	0.90 *d* (J=6.5 Hz)	0.92 *d* (J=6.5 Hz)
5′	1.27 *s*	0.90 *d* (J=6.5 Hz)	0.92 *d* (J=6.5 Hz)
1″	3.22 *d* (J=7.3 Hz)	2.67 *m*	2.67 *m*
2″	5.28 *t* (J=7.3 Hz)	1.77 *m*	1.74 *m*
3″			
4″	1.72 *s*	1.27 *s*	1.28 *s*
5″	1.69 *s*	1.27 *s*	1.28 *s*
-OMe			3.79 *s*

Spectra were recorded in $CDCl_3$ solutions at 300 and 360 MHz.
*These resonances could not be assigned with confidence.

prove that the contaminants were double bond isomers, phenol **1** was hydrogenated with Pt/C in ethanol to yield the tetrahydro derivative **5** in the expected (quantitative) yield. The NMR data for **5** were completely consistent with the structure assigned. These data indicated that phenol **1** could be assigned as 2,4-bis(3-methylbut-2-enyl)phenol, a structure identical to a metabolite recently isolated by Blackman *et al.* from the related Australian alga *Perithalia caudata* [6]. Also, phenol **1** had been previously synthesized [7, 8]. Indeed, comparison of our data with published information showed the molecules were identical.

Phenol **2** was isolated from the more polar chromatography fractions as a viscous oil which analysed for $C_{16}H_{24}O_2$ by high resolution mass spectrometry and ^{13}C NMR methods. Phenol **2** retained many of the spectral characteristics of **1**, except that **2** appeared to possess an additional hydroxyl group. As with **1**, phenol **2** was also contaminated with a small amount (< 10%) of an inseparable second compound identified as the

Table 2. ^{13}C NMR data for compounds **1–6**

C	1	3	5	2	4	6
1	152.3	147.0 C	151.3	151.9 C	151.7	155.3
2	126.9	132.6 C	128.7	128.8 C	128.5	129.7
3	129.9	129.9 CH	129.9	129.8 CH	129.8	129.7
4	134.2*	139.5 C	135.0	133.7 C	134.8	135.2
5	127.0	126.7 CH	126.5	126.9 CH	126.9	126.5
6	115.7	122.0 CH	115.2	116.0 CH	115.9	110.6
1′	29.8	28.9 CH_2	27.9	33.5 CH_2	32.9	32.9
2′	122.2	121.5 CH	39.1	123.8 CH	41.1	41.1
3′	131.9	132.4 C	27.7	131.7 C	27.7	27.7
4′	25.6	25.6 Me	22.5	25.6 Me	22.5	22.5
5′	17.8	17.7 Me	22.5	17.7 Me	22.5	22.5
1″	33.6	33.8 CH_2	32.9	24.9 CH_2	24.8	25.2
2″	123.9	123.1 CH	41.1	43.5 CH_2	43.4	44.2
3″	134.0*	133.1 C	28.0	71.7 C	71.7	71.0
4″	25.6	25.6 Me	22.5	29.3 Me	29.3	29.2
5″	17.8	17.7 Me	22.5	29.3 Me	29.3	29.2
-OMe						55.5 Me
-OAc		169.3 C				
		20.7 Me				

Spectra were recorded at 50 MHz in $CDCl_3$ solutions. Numbers of attached protons for **2** and **3** were determined by DEPT sequence analyses. Assignments for **2**, **3** and **5** were made by XHCORR and COLOC experiments; assignments for the derivatives were by analogy. *These assignments could not be made with confidence.

$\Delta^{3'-5'(4')}$ double bond isomer. Hydrogenation of **2** yielded the dihydro phenol **4**, which was obtained in the expected quantitative yield. The overall NMR data for **2** and derivative **4** showed that the trisubstituted olefin in **1** had been hydrated in **2** to yield the corresponding tertiary alcohol. This complication introduced a regiochemical problem of substitution on the aromatic ring which was not present in phenol **1**. From the simple NMR data it was not possible to differentiate the relative positions of the hydroxyl, 3-hydroxy-3-methylbutyl and isopentenyl groups on the aromatic ring. Using COSY, COLOC and XHCORR experiments, a complete assignment of the ^{1}H NMR resonances for **2** was achieved (see Table 2). From these data the ^{1}H NMR confident assignments for derivative **4** were also feasible.

Treatment of derivative **4** with methyl iodide and potassium carbonate in acetone generated the expected phenol methyl ether **6** in 66% purified yield. The ^{1}H NMR features for **6** were, with the exception of the additional methoxy methyl resonance at δ3.79, virtually identical with **4**. In a NOE experiment, irradiation of the methoxy methyl group produced strong enhancements of the aromatic proton at C-6′ and the methylene protons at C-1″ of the 3-hydroxy-3-methylbutyl group. These data, along with COSY experiments, fixed the relative positions of substituents in **2** as drawn.

As part of our interest in the natural functions of algal metabolites, we assessed the effects of the crude chloroform extract of *E. cliftonii*, and the purified phenols, at natural concentrations, on the feeding response of the tropical sea urchin *Tripneustes esculentus*. The assays utilized were fashioned after an assay procedure originally designed to determine fish feeding responses *in situ* [9]. Crude extracts and purified metabolites were incorporated into a carageenan-based food prepared by homogenization of a suitable and preferred algal food, the brown alga *Sargassum* sp. Figure 1 illustrates the sizeable reduction in feeding observed with foods incorporating the crude extract at 1% of wet food mass, and with phenol **1** at 0.2% wet mass. At its natural concentration of 0.012% wet food mass, phenol **2** failed to reduce feeding. The greater deterrence observed from the crude extract, however, could be explained by the possible additive effects of both metabolites.

Several concepts seem to be emerging as a result of studies of the chemistry of marine algae of the order Sporochnales [5, 6, 10]. First, plants from this group are chemically unique in their production of phenols with either multiple isoprenoid or monoterpenoid substituents. Second, the observations of significant feeding deterrence activities from these metabolites against fish and invertebrate herbivores would seem to suggest that chemical defence is a well developed characteristic of the group.

EXPERIMENTAL

General. High resolution EI mass spectra were provided by the University of Minnesota Mass Spectrometry Service Laboratory. The ^{1}H NMR spectra were recorded on either Bruker 300 MHz or Nicolet 360 MHz instruments. ^{13}C NMR, DEPT sequence, COLOC and

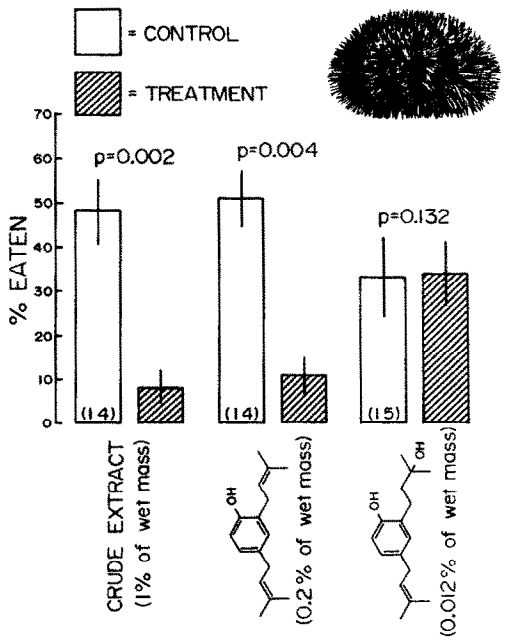

Fig. 1. The effects of the crude chloroform extract of *Encyothalia cliftonii*, at 1% of food mass, phenol **1**, at 0.2% of food mass, and phenol **2**, at 0.012% food mass, on feeding by the herbivorous sea urchin *Tripneustes esculentus*.

XHCORR spectra were recorded on a Bruker WP-200SY spectrometer. For TLC analyses, silica gel plates (Merck) were used and detection was by visualization under UV light and by spraying with 50% H_2SO_4 and heating.

Isolation of phenols **1** *and* **2**. The freshly defrosted alga was repeatedly extracted with chloroform. The combined extracts were dried, concd to a dark green tar under vacuum, and fractionated by flash CC on TLC-grade silica gel using various proportions of EtOAc in isooctane. Frs eluted with 40% EtOAc in isooctane contained phenol **1** [2,4-bis(3-methylbut-2-enyl)phenol] as 13% of the crude extract. Frs eluted with 80% EtOAc in isooctane yielded phenol **2** [2-(3-hydroxy-3-methylbutyl),4-(3-methylbut-2-enyl)phenol], as 3.7% of the extract.

2,4-*Bis*(3-*methylbut-2-enyl*)*phenol* (**1**). Phenol **1** showed IR ν_{max}^{neat}: 3429, 2965, 2923, 2854, 1712, 1610, 1505, 1437, 1376, 1198, 1096, 814 cm^{-1}; UV λ_{max}^{MeOH} nm: 280 (ε = 3330), shifts to 297 nm in base; HREIMS: $[M]^+$ *m/z* obsd 230.1658, calc. 230.1665 for $C_{16}H_{22}O$; LRMS *m/z* (rel. int.): 230 $[M]^+$ (53), 215 (18), 213 (16), 175 (34), 159 (37), 69 (100), 41 (44).

2-(3-*Hydroxy*-3-*methylbutyl*), 4-(3-*methylbut*-2-*enyl*) *phenol* (**2**). Phenol **2** showed IR ν_{max}^{neat}: 3429, 2965, 2923, 2854, 1712, 1610, 1505, 1437, 1376, 1198, 1096 and 814 cm^{-1}; UV λ_{max}^{MeOH} nm: 280 (ε = 3330), shifts to 297 nm in base; HREIMS: $[M]^+$ *m/z* obsd 248.1771, calc. 248.1776 for $C_{16}H_{24}O_2$; LRMS *m/z* (rel. int.):248 $[M]^+$ (18), 230 (43), 203 (30), 175 (100), 159 (32), 69 (75), 59 (23), 41 (40).

Acetylation of phenol **1**. Phenol **1** (10 mg) was treated with Ac_2O (1 ml) and pyridine (1 ml) at 60° for 15 hr. The solvents were removed under high vacuum to yield acetate **3** as a viscous oil which by TLC and NMR analysis required no further purification (see Tables 1 and 2 for NMR data).

Hydrogenation of phenols **1** *and* **2**. In separate experiments, phenols **1** and **2** (15 mg each) were dissolved in 5 ml EtOH, 20 mg Pt/C was added, and the soln stirred under an atmosphere of H_2 at 65° for 20 hr. The soln was filtered through celite and the solvents were removed under vacuum to yield, from **1**, the tetrahydro derivative **5**, and from **2**, the dihydro derivative **4**. The yields of **4** and **5** were quantitative. Analysis of the reaction products by silica TLC and NMR showed that no further purifications were required. NMR data for **4** and **5** are found in Tables 1 and 2.

Methylation of dihydrophenol **4**. Phenol **4**, 23 mg in 15 ml dry Me_2CO, was combined with excess K_2CO_3 (*ca* 0.5 g) and MeI (*ca* 1–2 ml) and refluxed under N_2 for 20 hr. The soln was diluted with 50 ml cold H_2O and extracted with EtOAc. After drying with anhydrous $MgSO_4$, the solvents were removed under vacuum to yield the crude methyl ether **6**, as a viscous oil, which was purified by silica HPLC (20% EtOAc in isooctane) to yield the pure ether (16 mg) in 66% isolated yield (see Tables 1 and 2 for NMR spectra).

Herbivore bioassays. All bioassays were conducted onboard the research vessel *Columbus Iselin* in the Bahamas in June, 1989. The experimental animal was the common Caribbean sea urchin *Tripneustes esculentus*. All feeding experiments were conducted in one pint freezer containers with holes to facilitate water flow. In each container was placed one urchin of average size 3–5 cm, one piece of control food measuring 0.2 × 1 × 4 cm, and one treated food of identical size. Prior to initiation of the assays, urchins were conditioned to regularly feed on control food. The artificial food utilized in the assays consisted of a slurry of the readily consumed brown seaweed *Sargassum* sp. dispersed in a carageenan gel. To prepare the carageenan gels, 100 ml of freshly collected *Sargassum* was blended with 300 ml H_2O. The resulting slurry was filtered through coarse screen, and 30 ml aliquots were combined with 2 g carageenan and 20 ml H_2O and heated in a microwave oven for 75 sec at the highest setting. While still liquid, test extract and purified compounds in dichloromethane (1% volume), or dichloromethane solvent alone for the control, were vigorously stirred into the mixt. The liquified gel was poured into 0.2 × 1 × 4 cm molds and allowed to cool. When cool, the artificial foods were placed in the urchin containers, the containers were placed into a flowing seawater aquarium, and the experiment was allowed to run overnight. The food pieces remaining were removed and the per cent consumed from treated and controls were measured using a square millimeter grid. For the 3 experiments run, 14–15 replicate measurements were made. The crude extract was tested at 1% wet mass food, phenol **1** at 0.2% and phenol **2** at 0.012%. The data, shown in Fig. 1,

were validated by Wilcoxon's Paired-Samples Test analysis. *P* values for these experiments are shown in Fig.1.

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