



## Isolation of elatol from *Laurencia microcladia* and its palatability to the sea urchin *Echinometra lucunter*

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### ABSTRACT

Elatol was isolated as the major compound from the red alga *Laurencia microcladia* Kütz. collected in Southern Brazil. This is the first report of elatol in this species. We also investigated the herbivore behaviour of the black sea urchin *Echinometra lucunter* (Linnaeus, 1758) towards *L. microcladia*, *Ulva fasciata* Delile and *Gracilaria domingensis* (Kütz.) Sond. ex Dickie through live algal multiple-choice feeding assay, as well as artificial feeding assay. The sea urchins ate the crude algae *L. microcladia* and pellets containing the powdered algae, extract and all tested concentrations of elatol, suggesting that this seaweed and its main compound are palatable for *E. lucunter*.

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### 1. Introduction

The *Laurencia* complex Lamouroux (Rhodophyceae) comprises three genera (Nam, 2006) that are some of the most prolific producers of secondary metabolites in the marine environment (Wright et al., 2003; Lyakhova et al., 2004). Secondary metabolites from these algae are predominantly sesquiterpenes like majapolene B from *Laurencia majuscula* (Blunt et al., 2008); diterpenes like neorogiolol B from *Laurencia obtusa* (Blunt et al., 2005) and C<sub>15</sub>-acetogenins like laurenyne from *Laurencia yonaguniensis* (Blunt et al., 2004; Wright et al., 1991; Bittner et al., 1985; Howard and Fenical, 1976) that usually are characterised by the presence of halogen atoms in their structures (König and Wright, 1997a).

Previous phytochemical investigations on *Laurencia microcladia* from the Mediterranean Sea lead to the isolation of calenzanol (Guella et al., 2001) and 6,8-cycloeuodesmane sesquiterpenes (Guella et al., 2002). Recently several cytotoxic cuparene sesquiterpenes (Kladi et al., 2005), besides (E)-2-tridecyl-2-heptadecenal, bromolaurenisol and laurinterol were isolated from the extracts of samples collected at Chios Island, in the North Aegean Sea (Kladi et al., 2006). From the same collection, other aromatic and cyclolaurane sesquiterpenes with cytotoxic activity, as well as (+)- $\alpha$ -isobromocuparene and (-)- $\alpha$ -bromocuparene were reported (Kladi et al., 2007).

During our ongoing investigation of marine organisms from the coast of Santa Catarina, Southern Brazil we performed thin layer chromatography (TLC) analysis of several crude extracts from these organisms, including the black sea urchin (*Echinometra lucunter*). A remarkable observation in this preliminary analysis was the detection of a compound with the same

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chromatographic behaviour in the extracts of *Laurencia microcladia* and *Echinometra lucunter*, suggesting the hypothesis of herbivory.

To investigate this hypothesis we isolated the main compound from *Laurencia microcladia* collected in the coast of Santa Catarina, Brazil, and conducted herbivory experiments with the black sea urchin toward *L. microcladia*, *Gracilaria domingensis* and *Ulva fasciata*.

## 2. Materials and methods

### 2.1. Collection of organisms

The samples of *Laurencia microcladia* and *Echinometra lucunter* used for the chemical investigation were collected by hand in March 2006 at the lower intertidal zone of Praia da Sepultura (27°07'54"S and 48°31'40"W), Santa Catarina, Southern Brazilian coast.

For the herbivory experiments samples of the seaweeds *L. microcladia*, *G. domingensis* and *U. fasciata*, and of the black sea urchin were collected from the same place as above in December 2007 and March 2008. Voucher samples of these seaweeds are kept at the Herbarium of the Department of Botany, Federal University of Santa Catarina (FLOR 14516–14520).

### 2.2. Chemical experiments

#### 2.2.1. Isolation of *Laurencia microcladia* major metabolite

Fresh material of *Laurencia microcladia* (2.1 kg) was exhaustively extracted with ethanol at room temperature for 3 days (three times). The extract after evaporation of the solvent afforded a green oily residue (9.5 g) that was partitioned with ethyl acetate and water, affording 4.5 g of aqueous fraction and 5 g of ethyl acetate fraction. This late fraction was further fractionated over silica gel (Merck 60), using n-hexane with increasing amounts of ethyl acetate as mobile phase. Fractions 2 (10% ethyl acetate in n-hexane) and 3 (20% ethyl acetate in n-hexane) were combined (1.6 g) and further purified by column chromatography on silica gel using chloroform with increasing amounts (5%) of ethyl acetate as mobile phase, to yield the pure compound **1**. The structure of compound **1** was established through <sup>1</sup>H, <sup>13</sup>C NMR and correlation spectra (Bruker Avance 2), IR (Shimadzu -IR Prestige-21) and MS (Shimadzu QP-5000) analysis and comparison with literature data.

#### 2.2.2. Chromatographic analysis

The internal organs from fresh individuals of the sea urchin *Echinometra lucunter* were separated from the skeleton and macerated in ethanol during 3 days. The fresh material of *Laurencia microcladia* was extracted in the same conditions. The ethanolic crude extracts were analysed by TLC (silica gel 60 F254, aluminium support plates, Macherey–Nagel) in chloroform:ethyl acetate (8:2) using compound **1** as reference and the plate was sprayed with anisaldehyde/H<sub>2</sub>SO<sub>4</sub> reagent.

#### 2.2.3. Feeding assays

Multiple-choice feeding assays were conducted according to Erickson et al. (2006) with live algae commonly found in the rocky shore habitat of Praia da Sepultura: *Laurencia microcladia*, *Gracilaria domingensis* and *Ulva fasciata*. One sea urchin and about 700 mg of each alga were placed into each aquarium (10 × 34 × 25 cm, 3 L) containing sea water under constant aeration for 7 days. A control aquarium without sea urchins was used to measure the biomass variation during the experiment. Water parameters as pH (8.0–8.5), temperature (22–25 °C) and salinity (35–36) were checked every day during the experiment as well and algae weight measurements were performed each 48 h. The ANOVA one way and post hoc Tukey's honest significant difference (HSD) test were used to identify significant differences in the consumption ( $P < 0.05$ ).

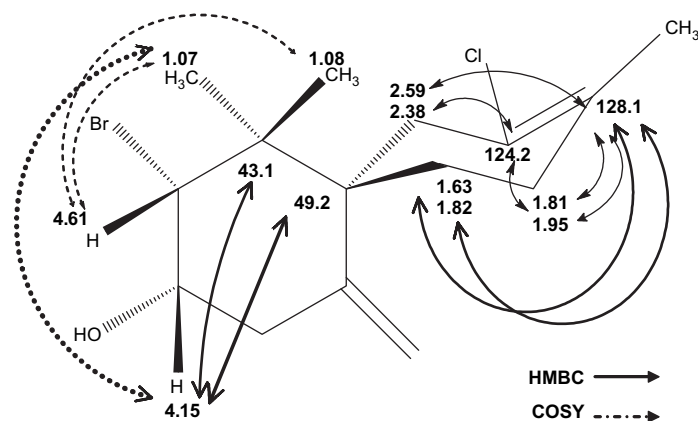
The artificial feeding assay was performed with the methodology proposed by Hay et al. (1994). Different types of agar pellets were prepared in the same size (3.5 × 8.5 × 1.5 cm) containing elatol (0.05, 0.1 or 0.2 mg pro pellet); powdered algae (2 g *L. microcladia* or *U. fasciata* pro pellet) or ethanol (solvent control, 2 mL pro pellet), and one further control was prepared containing just pure agar. Five sea urchins and one of each type of pellets were placed into each of the six tanks (300 L) containing sea water under constant aeration and water parameters as pH (8.0–8.1), temperature (21–23 °C) and salinity (35–37) were verified every day. Pellets weight measurements were performed each 24 h for 7 days to obtain the daily weight variation and non-parametric Wilcoxon signed-ranks test was used to evaluate the consumption percentage rates of pellets. Differences were considered significant when  $P < 0.05$ . After the experiment, all sea urchins were sacrificed, their internal organs were extracted and the extracts were analyzed by TLC in the same ways as described before (item 2.2.2).

## 3. Results

### 3.1. Chemical experiments

By TLC analysis of extracts, a major metabolite (compound **1**) was visualised after spraying with sulphuric anisaldehyde as a blue spot at R<sub>f</sub> 0.82 in the lanes corresponding to *Laurencia microcladia* and *Echinometra lucunter* extracts.

Compound **1** (Fig. 1) was obtained after successive chromatographic procedures as colourless oil (1.0 g), corresponding to a relative yield of ca. 10% (w/w) to the ethanolic extract of *L. microcladia*. MS and NMR data and comparison with literature



**Fig. 1.** Structure of elatol showing the main assignments according to the complete NMR data and some correlations in the HMBC and COSY experiments (key correlations in bold).

data confirmed the structure of the halogenated chamigrene known as elatol (Sims et al., 1974; König and Wright, 1997b; Vairappan et al., 2001). This is the first time elatol is reported for *Laurencia microcladia*. Although  $^1\text{H}$ -NMR and  $^{13}\text{C}$ -NMR spectral data were reported before (Sims et al., 1974; Kennedy et al., 1988; König and Wright, 1997b; Vairappan et al., 2001), we performed unambiguous assignments of all C–H correlations by combined DEPT, COSY, HSQC, HMBC and ROESY spectra (Table 1). Despite of differences in  $^1\text{H}$  NMR data among different authors (Table 2), the  $^{13}\text{C}$  NMR chemical shifts are quite similar, but there are divergences in the attributions of signals for quaternary C-atoms (Table 3).

### 3.2. Herbivory experiments

The experiment with live algae (Fig. 2) showed herbivore behaviour of *Echinometra lucunter* to the three species of algae, but the sea urchins ate more *Laurencia microcladia* than *Gracilaria domingensis* and *Ulva fasciata* with statistical significant differences.

The results of the artificial feeding assay are presented in Fig. 3. All types of pellets were bitten by sea urchins. The pellets containing the powdered seaweeds *L. microcladia* and *U. fasciata* had the highest consumption rate, but with no significant differences to the other pellets (due to the high standard deviation). Pellets containing 0.1 mg elatol were more consumed than pellets containing 0.2 or 0.05 mg elatol or containing just agar, with statistically significant differences. The control pellets containing ethanol were the less consumed.

**Table 1**

$^1\text{H}$  and  $^{13}\text{C}$  NMR data in  $\text{CDCl}_3$  (500 and 125 MHz, respectively) for elatol (compound 1).

C-atom	$^{13}\text{C}$ ( $\delta$ )	DEPT 145	HSQC – $^1\text{H}$ ( $\delta$ )	HMBC	ROESY
1	38.6	$\text{CH}_2$	2.59 ( <i>d</i> , $J = 17.0$ Hz) 2.38 ( <i>d</i> , $J = 17.0$ Hz)	C140.8/C124.2 C25.6/C49.2/C124.2/C128.2/C140.8 C19.5/C38.6	
2	124.2	C		C19.5/C38.6/C25.6/C29.4	
3	128.1	C		C128.1/C124.2/C 49.2 C19.5/C49.2/C38.6/C128.1/C140.8	2.59/2.63
4	29.4	$\text{CH}_2$	1.95 ( <i>m</i> ) 1.81 ( <i>m</i> )	C29.4/C49.2/C38.6/C128.1/C140.8 C38.6/C29.4/C49.2/C128.1/C140.8	2.50/2.59/2.63
5	25.6	$\text{CH}_2$	1.82 ( <i>m</i> ) 1.63 ( <i>m</i> )	C20.8/C24.3 C25.6/C38.0/C38.6/C72.2/C115.9 C115.9/C140.8	
6	49.2	C		C70.9/C49.2/C72.2/C140.8/C115.9	
7	140.8	C		C38.0/C43.1/C70.9/C140.8	2.50/2.63
8	38.0	$\text{CH}_2$	2.63 ( <i>d</i> , $J = 14.6$ Hz) 2.50 ( <i>dd</i> , $J = 14.6/2.5$ Hz)	C20.8/C24.1/C43.1/C115.9	1.07/1.08/1.63/1.70/ 1.81/1.82/2.63/4.79
9	72.2	CH	4.15 ( <i>br d</i> , $J = 3.4$ Hz)	C38.6/C38.0/C29.4/C25.6/C24.3 C24.3/C43.1/C49.2/C70.9/C72.2	1.63/2.38/2.50/2.59
10	70.9	CH	4.61 ( <i>d</i> , $J = 2.6$ Hz)	C20.8/C43.1/C49.2/C70.9/C72.2 C38.0/C49.2	1.07/2.50/4.79
11	43.1	C		C38.0/C49.2/C140.8	1.07/1.70/2.38/2.59/ 4.61/5.12
12	20.8	$\text{CH}_3$	1.07 ( <i>s</i> )		
13	24.3	$\text{CH}_3$	1.08 ( <i>s</i> )		
14	115.9	$\text{CH}_2$	5.12 ( <i>s</i> ) 4.79 ( <i>s</i> )		
15	19.5	$\text{CH}_3$	1.70 ( <i>s</i> )	C29.4/C124.2/C128.1	

**Table 2**Comparison with previously published <sup>1</sup>H NMR data of elatol (numbering scheme according to Sims et al., 1974).

H-atoms	Sims et al. (1974), 100 MHz, CDCl <sub>3</sub>	Kennedy et al. (1988), 250 MHz, Me <sub>2</sub> CO-d <sub>6</sub>	König and Wright (1997b), 300 MHz, CDCl <sub>3</sub>	Our data, 500 MHz, CDCl <sub>3</sub>
<b>1</b>	*	<b>2.59 m</b> <b>2.30 br d</b> (12.5)	<b>2.19 br d</b> (17.5) <b>2.08 br d</b> (17.5)	<b>2.59 br d</b> (17.0) <b>2.38 br d</b> (17.0)
<b>4</b>	*	2.08 <i>dd br</i> (15, 12) 2.00 <i>dd br</i> (15, 5)	1.96 <i>m</i> 1.82 <i>m</i>	1.95 <i>m</i> 1.81 <i>m</i>
<b>5</b>	*	1.78 <i>d br</i> (17) 1.59 <i>ddd</i> (17, 12, 5)	1.80 <i>m</i> 1.62 <i>m</i>	1.82 <i>m</i> 1.63 <i>m</i>
<b>8</b>	*	<b>2.72 dddd</b> (6.5, 3, 15, 1.5 × 2) <b>2.38 dd</b> (15, 3)	<b>2.49 dd</b> (2.8, 14.4) <b>2.19 dm</b> (14.4)	<b>2.63 d</b> (17.0) <b>2.50 dd</b> (14.6, 2.5)
<b>9</b>	4.16 <i>m</i>	4.13 <i>m</i> (6.5, 6, 3)	4.14 <i>m</i>	4.14 <i>br d</i> (3.4)
<b>10</b>	4.60 <i>d</i> (3)	4.71 <i>d</i> (3)	4.61 <i>d</i> (2.9)	4.61 <i>d</i> (2.6)
<b>12</b>	1.10 <i>s</i>	1.06 <i>s</i>	1.06 <i>s</i>	1.07 <i>s</i>
<b>13</b>	1.10 <i>s</i>	1.05 <i>s</i>	1.07 <i>s</i>	1.08 <i>s</i>
<b>14</b>	5.12 <i>s</i>	5.01 <i>t br</i>	5.12 <i>br s</i>	5.12 <i>br s</i>
	4.80 <i>s</i>	4.85 <i>s br</i>	4.79 <i>br s</i>	4.79 <i>br s</i>
<b>15</b>	1.70 <i>s</i>	1.66 <i>d br</i> (1.2)	1.70 <i>br s</i>	1.70 <i>br s</i>
<b>OH</b>		<b>3.58 d</b> (6)	2.19 <i>br s</i>	2.19 <i>br s</i>

Chemical shifts ( $\delta$ ) in ppm and coupling constants (Hz) are described as in the original works. Controversies among authors are represented in bold. Some values (\*) are not reported by the authors.

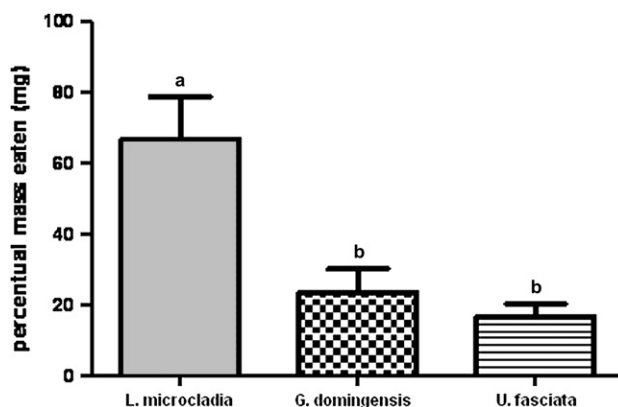
**Table 3**Comparison of published <sup>13</sup>C NMR data ( $\delta$  ppm) from elatol (numbering scheme according to Sims et al., 1974; just controversial chemical shifts are represented).

C-atoms	Vairappan et al. (2001), 100 MHz, CDCl <sub>3</sub>	Kennedy et al. (1988), 62.9 MHz, Me <sub>2</sub> CO-d <sub>6</sub>	König and Wright (1997b), 75.5 MHz, CDCl <sub>3</sub>	Our data, 125 MHz, CDCl <sub>3</sub>
<b>2</b>	124.86	128.61	128.0	124.2
<b>3</b>	128.76	124.81	124.1	128.1
<b>6</b>	43.85	49.98	49.1	49.2
<b>11</b>	49.88	44.01	43.1	43.1

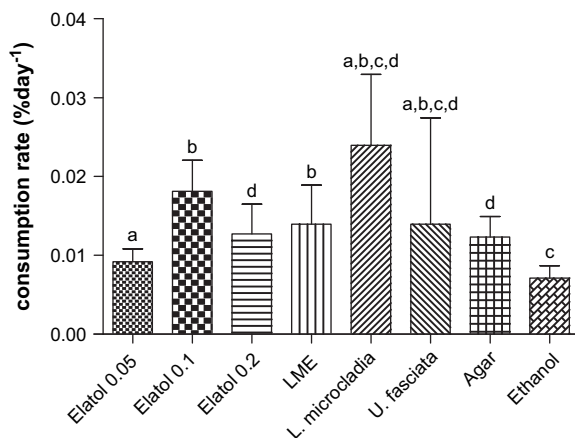
By TLC analysis and co-chromatography the presence of elatol was identified in all extracts from sea urchins exposed to *Laurencia microcladia*, but not in the extracts of sea urchins from the control aquarium in the live algae feeding assay.

#### 4. Discussion

Elatol was first isolated from *Laurencia elata* from Australia (Sims et al., 1974), and its occurrence in other species of *Laurencia* has been reported, frequently as the major metabolite from specimens collected between 33°S and 28°N (González et al., 1976; Brennan et al., 1987; Kennedy et al., 1988; König and Wright, 1997b; Juagdan et al., 1997; Vairappan et al., 2001). Despite of the many works on *L. microcladia* from the Mediterranean Sea (collected from latitudes 38 to 42°N), this is the first report of elatol in this species.



**Fig. 2.** Average percentage of total consumption of *L. microcladia*, *G. domingensis* and *U. fasciata* offered to the sea urchins *E. lucunter* in a live algal feeding assay. Different letters above bars denote significant statistical differences to  $P < 0.05$  (one way ANOVA followed by Tukey HSD test); vertical bars = SD;  $n = 9$ .



**Fig. 3.** Average consumption rate by sea urchins *E. lucunter* in artificial feeding assay. Elatol 0.05, 0.1 and 0.2: pellets containing elatol 0.05, 0.1 and 0.2 mg/pellet; LME: pellets with 4 mg crude algae extract of *L. microcladia*; *L. microcladia*: pellets with 2 g of powdered crude algae *L. microcladia*; *U. fasciata*: pellets with 2 g of powdered crude algae *U. fasciata*; agar: pellets with agar; ethanol: pellets with ethanol. Different letters above bars denote significant statistical differences to  $P < 0.05$  (Wilcoxon signed-ranks test); vertical bars = SD;  $n = 10$ .

Concerning the NMR data, some assignments are not in complete agreement with data published before. Based on correlations in the HMBC experiment (Table 1 and Fig. 1) C-3 was attributed to the C-atom at 128.1 ppm, since the H-atoms at C-5 (1.63 and 1.82 ppm) correlate with this C-atom and show no correlation with the C-atom at 124.2 ppm, so this late C-atom should be C-2. The other correlations observed are consistent with these attributions.

The high concentration of elatol in *Laurencia* species of tropical and subtropical areas suggests this compound to play an important role for these species. Previous analysis of anti-herbivory proprieties of metabolites from *Laurencia* species (including elatol) have been conducted (Granado and Caballero, 1995; Hay et al., 1987; Paul et al., 1998; Pereira et al., 2003), but no investigation was reported for *Laurencia microcladia*. We also found no report involving *Laurencia* species and the sea urchin *Echinometra lucunter*, whose feeding behaviour concerning other algae has been described (Erickson et al., 2006; Hiratsuka and Uehara, 2007).

The sea urchins used in our experiments were collected in a region with high density of *L. microcladia* having elatol as the major compound. In the live algae feeding assay just the extracts of sea urchins exposed to *Laurencia microcladia* presented in TLC a spot corresponding to elatol. This suggests the presence of elatol in the internal organs of *Echinometra lucunter* is a consequence of the ingestion of *L. microcladia*. Since *E. lucunter* ate the crude algae *L. microcladia*, as well as pellets containing powdered algae, *L. microcladia* extract and elatol, it seems that this sea urchin species can tolerate this compound at least in the tested concentrations.

Even if elatol does not deter *Echinometra lucunter*, its high abundance in *Laurencia microcladia* from Southern Brazil suggests this metabolite is somehow important for this species, possibly deterring other predators since this algae is very abundant in its habitat. In this aspect, further experiments with other local animal species are being performed.

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