Laurencia rigida: Chemical Investigations of Its Antifouling Dichloromethane Extract[1]

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From the CH_2Cl_2 extract of the temperate marine red alga, *Laurencia rigida*, which has antifouling properties, eight sesquiterpenes (**1**−**8**) were isolated. Of these, four (3-acetoxy-*E*-γ-bisabolene (**1**), (−)-10α-bromo-9β-hydroxy-α-chamigrene (**2**), rigidol (**3**), and (+)-(10*S*)-10-bromo-β-chamigrene (**4**)), were shown to be new natural products. For the known compound deschloroelatol (**5**), reassignment of the ^{1}H- and ^{13}C-NMR data was found to be necessary on the basis of extensive NMR measurements. For elatol (**6**), complete ^{1}H- and ^{13}C-NMR data are also reported. The antimicrobial and antialgal activities of all isolates were assessed.

Laurencia rigida J. Agardh (Rhodophyceae, Ceramiales, Rhodomelaceae) is a commonly encountered marine red alga around the eastern and southern shores of Australia, which grows in shallow sub-littoral zones during spring and summer months. Although many neighboring marine plants and animals become heavily fouled over this period, *L. rigida* remains relatively free of fouling organisms. These observations and the results of screening the CH_2Cl_2 extracts in antifouling bioassays[2] indicated a chemical deterrence of common marine fouling organisms by the alga. In order to examine this effect further a detailed investigation of the natural product chemistry of the CH_2Cl_2 extract of this alga was undertaken. The results of this investigation are the basis of the current report.

Results and Discussion

Structural Chemistry. Eight compounds (**1**−**8**) were purified and characterized from the CH_2Cl_2 solubles extracted from the marine red alga *Laurencia rigida*.

Compound **1** was an unstable oil, with the molecular formula $C_{17}H_{26}O_2$ by mass spectrometry and NMR spectroscopy. Of the five degrees of unsaturation implied by the molecular formula of **1**, four were occupied by sp^2 hybridized carbon atoms. All remaining carbon atoms were sp^3 hybridized, indicating **1** to be a monocyclic molecule containing three carbon−carbon double bonds and one carbon−oxygen double bond; this latter was part of an acetoxyl function, which accounted for all of the oxygen atoms in the molecule. The results obtained from recording ^{1}H−^{1}H and ^{1}H−^{13}C (J = 150 Hz) 2D NMR COSY spectra of **1** enabled two main molecular fragments to be established. The first of these could be traced from the two olefinic methyl groups, H_3-12 and H_3-13, to C-1 and C-2, via an allylic coupling between these methyl groups and H-2. The fragment was further extended through the observed ^{1}H−^{1}H coupling between H-2 and H-3, and between H-3 and H_2-4. The second fragment was composed of the two adjacent methylene groups CH_2-10 and CH_2-11, whose protons inter-coupled; the olefinic methyl group H_3-15, which coupled to both H_2-10 and H-8, H_2-7, which long-range coupled to H_2-11; and the allylic methyl group H_3-14, which showed a long-range coupling to H_2-7. With this information and the results of an HMBC measurement [^{1}H−^{13}C (J = 8.3 Hz)], it was evident that the two fragments were connected via a carbon−carbon bond between C-4 and C-5, that C-7 and C-11 were both bonded to C-6, thus generating a cyclohexene ring, and that the acetoxyl group was located at C-3. The problems left to resolve were, then, the configuration at C-3 and the geometry of Δ.[5,6] The instability of **1** precluded any derivatizations that may have led to the absolute configuration at C-3 being resolved.[3] The geometry of $\Delta^{5,6}$ was clearly *E* on the basis of a NOE cross peak in the NOESY spectrum of **1** observed between H_3-14 and H_2-7. Clearly **1** is very closely related to the known compounds *E*-γ-bisabolene[4] and

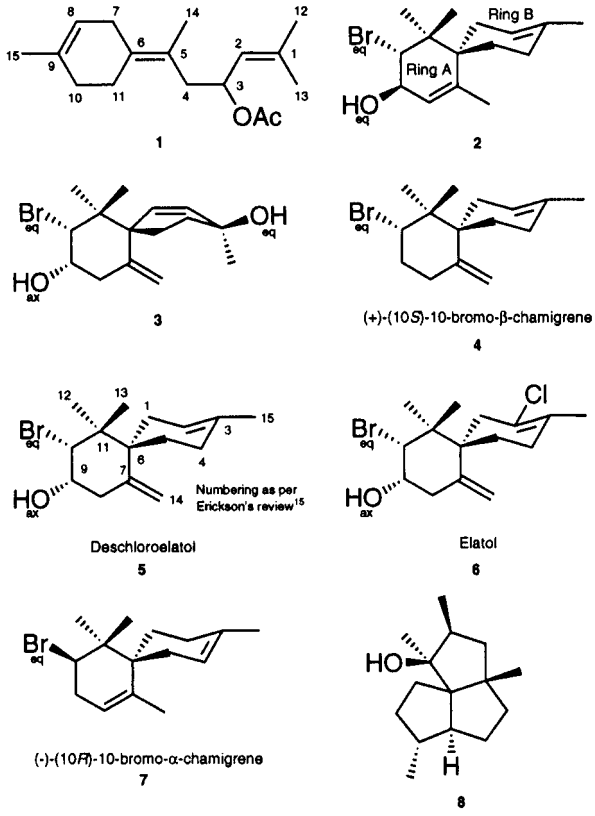

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Table 1. ^{13}C-NMR (75.5 MHz, $CDCl_3$) Data for Compounds **1**−**6**

carbon	**1**	**2**	**3**	**4**	**5**	**6**
1	136.6 s	29.6 t	136.4 d	30.3 t[a]	30.1 t	38.6 t
2	123.8 d	121.7 d	131.0 d	119.7 d	119.4 d	128.0 s
3	70.8 d	134.3 s	78.5 s	132.7 s	132.5 s	124.1 s
4	39.3 t	28.6 t	28.6 t	27.5 t	27.6 t	29.3 t
5	131.6 s	31.0 t	22.7 t	25.6 t	25.8 t	25.6 t
6	121.4 s	44.3 s	51.4 s	47.0 s	47.0 s	49.1 s
7	29.8 t	144.4 s	143.7 s	145.6 s	141.1 s	140.7 s
8	120.4 d	122.8 d	37.9 t	33.0 t	37.9 t	38.0 t
9	134.1 s	73.6 d	72.0 d	35.7 t	70.3 d	72.1 d
10	31.5 t	73.3 d	70.5 d	66.1 d	71.8 d	70.8 d
11	26.9 t	44.3 s	42.6 s	42.7 s	43.1 s	43.1 s
12	18.3 q	17.8 q	21.5 q	17.5 q	20.7 q	20.7 q
13	25.8 q	25.3 q	26.6 q	23.9 q	24.2 q	24.2 q
14	19.2 q	23.0 q	116.5 t	112.6 t	115.7 t	115.8 t
15	23.3 q	23.3 q	24.5 q	23.1 q	23.0 q	19.4 q
OAc	170.3 s					
	21.4 q					

[a] Multiplicity by DEPT, s = C, d = CH, t = CH_2, q = CH_3.

12-hydroxy-*E*-γ-bisabolene,[5] and hence the trivial name 3-acetoxy-*E*-γ-bisabolene is proposed.

Compound **2** analyzed for $C_{15}H_{23}OBr$ by MS and NMR spectroscopy. Comparison of its MS, IR, and ^{1}H- and ^{13}C-NMR data with those for **4** indicated the two molecules to be very similar. In contrast to **4**, compound **2** had no NMR resonances associated with an *exo* carbon−carbon double bond, but it did have resonances for an allylically coupled methyl group (CH_3-14/H-8). It also had an IR absorption and ^{1}H- and ^{13}C-NMR resonances consistent with the presence of a secondary hydroxyl function. These data could be rationalized by the presence of an extra hydroxyl function at C-9 in **2**, and the migration of the $\Delta^{7,14}$ double bond in **4**, into ring A, Δ,[7,8] in **2**. Stereochemically, **2** and **4** were found to be identical, in a relative sense, on the basis of the comparable NOE interactions between H-10 and H_2-5 in both molecules. The fact that the coupling constant between H-9 and H-10 was 8.7 Hz clearly placed the OH function at C-9 as equatorial and β. Thus, **2** is best described as (−)-10α-bromo-9β-hydroxy-α-chamigrene.

Mass spectral analysis of **3**, the final new metabolite isolated in this study, indicated its molecular formula to be $C_{15}H_{23}O_2Br$. From its IR and NMR data it was evident that the functionality within **3** clearly consisted of a secondary bromo-function [70.5 (d) ppm], a secondary [3385 cm^{-1}, 72.0 (d) ppm] and a tertiary hydroxyl group [3385 cm^{-1}, 78.5 (s) ppm], and two carbon−carbon double bonds, one *exo* [143.7 (s), 116.5 (t) ppm] and one *endo* [131.0 (d), 136.4 (d) ppm]; the molecule is thus bicyclic. From the ^{1}H- and ^{13}C-NMR data of **3**, **5**, and **6**, the A ring of **3** could be clearly identified as the same as in **5** and **6**. The remaining part of the molecule had three main fragments; the *endo* carbon−carbon double bond, two methylene groups whose protons intercoupled, and a tertiary carbon bearing OH and CH_3. The two olefinic protons (δ 6.00, 5.73) strongly coupled with each other (J = 10.3 Hz). The proton with resonance at δ 6.00 coupled with one of the H_2-5 protons, while the other (δ 5.73) coupled with one of the H_2-4 protons. Hence, C-3 and C-6 must separate the carbon−carbon double bond at both ends from the $-CH_2-CH_2-$ unit, giving rise to the second ring within **3**. With the basic structure of **3** deduced only the relative configuration at C-3 and C-6 required resolution. From the NOESY spectrum of **3**, cross peaks between H-10 and H_2-5 enabled the relative configurations at both C-10 and C-6 to be proposed as shown in **3**. Cross peaks between H_3-15 and one of the protons at C-14 indicated CH_3-15 to be pseudo-axial and α, suggesting the B ring to have a twist-boat conformation. The trivial name of rigidol is suggested for **3**.

Compound **4** was recently described by us as (+)-(10*S*)-10-bromo-β-chamigrene in a paper relating specifically to the use of pulsed field gradients (PFGS) in NMR spectroscopy of natural products.[6]

Compounds **5** and **6** were characterized as deschloroelatol[7,8] and elatol,[7−9] respectively. For both of these isolates it was necessary to repeat all NMR measurements and assignment work, as the original data,[8]

Table 2. ^{1}H-NMR (300 MHz, $CDCl_3$) Data for Compounds **1**−**3**, **5**, and **6**[a]

proton	**1**	**2**	**3**	**5**	**6**
1		1.87 (m), 2.25 (m)	6.00 (dd, J = 1.7, 10.3 Hz)	2.08 (dm, J = 17.7 Hz)	2.08 (br d, J = 17.5 Hz)
				2.19 (dm, J = 17.7 Hz)	2.19 (br d, J = 17.5 Hz)
2	5.13 (dm, J = 9.1 Hz)	5.42 (m)	5.73 (dd, J = 1.7, 10.3 Hz)	5.27 (m)	
3	5.58 (ddd, J = 6.9, 7.1, 9.1 Hz)				
4	2.21 (dd, J = 6.9, 13.3 Hz)	1.93 (m)	1.40 (ddd, J = 2.5, 13.6, 13.8 Hz)	~1.6 (m), ~1.8 (m)	1.82 (m), 1.96 (m)
	2.52 (dd, J = 7.1, 13.3 Hz)		1.99 (m)		
5		1.84 (ddd, J = 6.1, 11.0, 11.1 Hz)	1.68 (dm, J = 13.0 Hz)	~1.6 (m), ~1.8 (m)	1.62 (m), 1.80 (m)
		1.65 (m)	2.03 (ddd, J = 2.9, 13.0, 13.6 Hz)		
6					
7	2.69 (br m)				
8	5.34 (m)	5.40 (m)	2.57 (dd, J = 2.3, 15.0 Hz)	2.45 (dd, J = 2.6, 14.4 Hz)	2.49 (dd, J = 2.8, 14.4 Hz)
			2.71 (br d, J = 15.0 Hz)	2.67 (dm, J = 14.4 Hz)	2.19 (dm, J = 14.4 Hz)
9		4.30 (br d, J = 8.7 Hz)	4.15 (m)	4.13 (br ddd, J = 2.6, 3.3, 6.1 Hz)	4.14 (m)
10	2.00 (m)	4.56 (d, J = 8.7 Hz)	4.62 (d, J = 2.9 Hz)	4.66 (d, J = 3.3 Hz)	4.61 (d, J = 2.9 Hz)
11	2.34 (m)				
12	1.66 (br s)	0.97 (s)	1.03 (s)	1.02 (s)	1.06 (s)
13	1.71 (br s)	1.10 (s)	1.21 (s)	1.02 (s)	1.07 (s)
14	1.58 (s)	1.72 (br s)	4.79 (br s), 5.07 (br s)	4.78 (br s), 5.06 (t, J = 1.8 Hz)	4.79 (br s), 5.12 (br s)
15	1.66 (br s)	1.65 (br s)	1.28 (s)	1.56 (br s)	1.70 (br s)
	1.99 (s, OAc, C*H*$_3$)	2.30 (br s, O*H*)		2.12 (br s, O*H*)	2.19 (br s, O*H*)

[a] All assignments are based on extensive 1D and 2D NMR experiments, including COSY90, HMQC, HMBC, HETCOR, NOE difference, and NOESY.

Table 3. Biological Activities of Compounds **1**−**8**

	inhibition zone (cm)						
	Fungi				Bacteria		Alga
compound (μg)	*Ustilago violacea*	*Mycotypha microspora*	*Eurotium repens*	*Fusarium oxysporum*	*Bacillus megaterium*	*Escherichia coli*	*Chlorella fusca*
1 (34)	0	0	0	0	0	0	0.3
2 (33)	0	0.3	0	0	0	0	0.3
3 (45)	0.5	0.4	0	0	0	0	0
5 (25)	0	2.0	1.8	0	0.1	0	0.2
6 (135)	0.3	2.5	2.5	0.7	0.2	0	0
8 (40)	0	0	0	0	0	0	0.2
controls (μg)							
benzylpenicillin potassium salt (85)					2.0	0	
streptomycin sulfate (65)					0.5	0.3	
miconazole nitrate (50)	Total[a]	0.7	1.8	0.1			
cyclohexamide (60)	Total	0.4	0.5	0.3			

[a] Total, indicates the control-caused complete inhibition of fungal growth on the test plate. Compounds **4** and **7** were inactive at the 50-μg level in all assay systems.

which were recorded in acetone-d_6, contained a number of incomplete and incorrect assignments. It also appears that these authors had made a minor error in their structural representations of deschloroelatol and elatol, since the configuration at C-6 is inverted. In Tables 1 and 2 complete assigned ^{1}H- and ^{13}C-NMR data ($CDCl_3$) are reported for **5** and **6**.

The remaining two isolates, **7** and **8**, were found to be identical with compounds previously reported by Howard and Fenical[10] and Coll and Wright,[11] respectively.

Biological Activity. All isolates were tested for their antimicrobial and antialgal activities employing agar diffusion assays as previously described.[12] The results of these bioassays are presented in Table 3. From this table it is evident that deschloroelatol (**5**) and elatol (**6**) have the most potent and widest spectrum of activity in the applied test systems, **5** being the more active. Comparable inhibition zones were only obtained for **5** and **6** when the latter was applied in the test systems in approximately a fivefold higher concentration to that of **5** (see Table 3). In general, our bioassays tend to suggest that compounds **5** and **6**, and to a lesser extent **2** and **3** have moderate antifungal properties. It might also be reasonably concluded that compounds **1**, **2**, **5**, and **8** have moderate antialgal activity, and that none of the tested compounds have any antibacterial properties of note.

Bioassays investigating the antifouling properties were originally performed with the CH_2Cl_2 extract of the algae and then with the pure compounds deschloroelatol (**5**) and elatol (**6**).[2,13] From the results of the bioassays conducted by de Nys *et al.*,[2,13] it seems likely that elatol (**6**) and deschloroelatol (**5**) are the components responsible for the observed antifouling activity of the CH_2Cl_2 extract of the algae. Elatol (**6**) at a concentration of 100 ng/cm^2, completely inhibited the settlement of *Balanus amphitrite* larvae, and deschloroelatol inhibited it to 90%.[13] Both compounds also deterred settlement of the bryozoan *Bugula neritina* at low concentrations.[13] These metabolites, however, act by being toxic, severely affecting the survival rate of nauplii larvae of *B. amphitrite*. Thus, the commercial development of **5** and **6** as antifouling agents is not likely.[13]

Experimental Section

General Experimental Procedures. The experimental procedures were as previously reported.[14]

Plant Material. The algal material was obtained in May 1993, at Cape Banks, Sydney, Australia. Plants growing at depths between 1 and 3 m were collected, deep frozen, and, on return to the laboratory, freeze dried. A voucher specimen is deposited at the Herbarium of the Royal Botanical Gardens, Sydney, NSW, Australia (voucher number NSW A 011283).

Extraction and Isolation. The dry algal tissue (463 g) was exhaustively extracted with 2 L of CH_2Cl_2 and then with 1.5 L of MeOH, to afford 20.9 g (4.5%) of CH_2Cl_2-soluble material. Vacuum liquid chromatography (VLC) of 5.8 g of this material over Si gel, using hexane containing increasing proportions of EtOAc as eluent, afforded nine fractions of 90 mL each. TLC and ^{1}H-NMR investigation of these fractions indicated fractions 2−5 to be of further interest.

HPLC separation of fraction 2 over normal-phase silica, with hexane containing 2% EtOAc as eluent, yielded compounds **1**, **4**, **7**, and **8**. HPLC separation of combined VLC fractions 3 and 4, over normal-phase silica employing hexane containing 9% EtOAc as eluent, afforded the three chamigrene derivatives, **2**, **5**, and **6**. HPLC separation of VLC fraction 5, over normal-phase silica employing hexane containing 20% acetone as eluent, yielded the chamigrene derivative **3**.

Compound 1 (3-acetoxy-*E*-γ-bisabolene): isolated as an unstable oil (5.6 mg, 0.0045%); $[\alpha]^{25}_D$ +1.0° (*c* 0.56, $CHCl_3$); IR ν_{max} (film) 3400, 2930, 1740; ^{1}H NMR, see Table 2; ^{13}C NMR, see Table 1; EIMS *m*/*z* $[M - H]^+$ 261 (<1), 260 (2), 218 (2), 202 (8), 149 (12), 135 (17); HRMS 261.1854 (calcd for $C_{17}H_{25}O_2$ 261.1830).

Compound 2 [(−)-10α-bromo-9β-hydroxy-α-chamigrene]: isolated as an oil (19.0 mg, 0.015%); $[\alpha]^{25}_D$ −49.4° (*c* 0.95, $CHCl_3$; IR ν_{max} (film) 2975, 1450; ^{1}H NMR, see Table 2; ^{13}C NMR, see Table 1; EIMS *m*/*z* $[M]^+$ 300 (<1), 298 (<1), 283 (1), 282 (1), 281 (1), 280 (1), 219 (23), 201 (47), 151 (85), 119 (100); HRMS 280.0781 (calcd for $C_{15}H_{21}{}^{79}Br$ $[M - H_2O]^+$ 280.0826) and 219.1735 (calcd for $C_{15}H_{23}O$ $[M - Br]^+$ 219.1750).

Compound 3 (rigidol): isolated as an oil (27.3 mg, 0.021%); $[\alpha]^{25}_D$ −6.5° (*c* 1.37, $CHCl_3$); IR ν_{max} (film) 3385,

2970, 1390; ^{1}H NMR, see Table 2; ^{13}C NMR, see Table 1; EIMS *m/z* $[M]^+$ 316, 314 (7, 8), 301 (11), 299 (32), 297 (23), 217 (100), 201 (55), 199 (45), 173 (35), 105 (85); HRMS 316.0861 (calcd for $C_{15}H_{23}O_2{}^{81}Br$ 316.0850).

Compound 4 [(+)-(10*S*)-10-bromo-*β*-chamigrene]: an oil (34.5 mg, 0.027%); ^{13}C NMR, see Table 1; all other physical and spectroscopic data as reported.[6]

Compound 5 (deschloroelatol): isolated as a clear oil (64.3 mg, 0.05%); ^{1}H NMR, see Table 2; ^{13}C NMR, see Table 1; other spectroscopic and physical data comparable to those reported previously.[7, 8]

Compound 6 (elatol): isolated as a clear oil (150.8 mg, 0.12%); ^{1}H NMR, see Table 2; ^{13}C NMR, see Table 1; remaining physical and spectroscopic data comparable to those reported.[7−9]

Compound 7 [(−)-(10*R*)-10-bromo-α-chamigrene]: isolated as an oil (6.4 mg, 0.005%) with comparable physical and spectroscopic data to those reported by Howard and Fenical.[10]

Compound 8: isolated as a clear oil (9.2 mg, 0.0072%) with comparable physical and spectroscopic data to those reported by Coll and Wright.[11]

Bioassays. The preliminary antifouling assays were performed as described by de Nys *et al*.[13] The antifungal, antibacterial, and antialgal bioassays reported in Table 3 were performed as described by Schulz and coworkers.[12]

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References and Notes

(1) Presented, in part, at the Thirty-Sixth Annual Meeting of the American Society of Pharmacognosy, 23−27 July, 1995, University of Mississippi, Mississippi, USA.
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