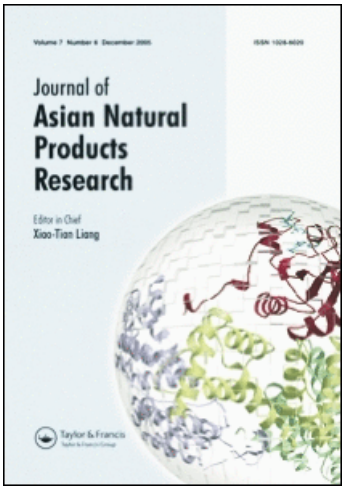

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Isolation and characterisation of some antifouling agents from the brown alga *Sargassum confusum*

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Isolation and characterisation of some antifouling agents from the brown alga *Sargassum confusum*

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Fats and phthalic acid derivatives were isolated and characterised based on their spectral analysis from the antifouling activity guided fractions of n-hexane and methanol extract of the brown alga *Sargassum confusum.* The fractions, as well as the isolated compounds, demonstrated significant antifouling activity against spores of a major fouling alga *Ulva pertusa* with 50–75% decrease of spore attachment on agar-coated slides.

Keywords: Antifouling activity; *Saragassum confusum*; Brown alga; Fats; Phthalic acid derivatives; Agar diffusion method

1. Introduction

Activity and reproduction of marine algae and other marine organisms are responsible for damage to ships, ship hulls, nets, etc. in various maritime occupations due to roughness and erosion caused by these fouling organisms. Some antifouling (AF) techniques have been attempted, mainly based on tributyl-tin (TBT) and other heavy metal-based derivatives being added to marine paints in order to control the fouling of target and non-target marine organisms [1]. However, these inorganic-based compounds cause toxicity and adversely affect the marine environment, which has led to restriction of these substances [2]. Marine natural product chemistry has become one of the promising alternatives since AF active constituents like tannins, bromophenol derivatives, diterpenes and halogenated furanones were isolated from various marine organisms [3–6]. The fats and fatty acids from marine organisms play an important role in pharmacological and biochemical centres due to the wide diversity of their biological characteristics and their oxidative enzymes leading to the formation of many other bioactive secondary metabolites [7]. The brown alga *Saragassum* genii are rich and most common on the Korean coast and the lower littoral and sublittoral

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belts [8]. The *Sargassaceae* family is known to contain chromenols, quinones and bioactive metabolites [9–13]. Several other species of *Sargassum* have a variety of farensylacetone derivatives, geranylphenyl derivatives, fuhalols and phloroethols, fats and their derivatives [14–17]. The isolation and characterisation of nine antifouling compounds, hexadecane (**1**), octadecane (**2**), eicosane (**3**),1-eicosanol (**4**), 1-pentadecanol (**5**), dibutyl phthalate (**6**), dioctyl phthalate (**7**), diisononyl phthalate (**8**) and dicyclohexyl phthalate (**9**), from n-hexane and methanol extracts of the marine brown alga *Sargassum confusum* and AF activity against fouling alga *Ulva pertusa* is described herein.

2. Results and discussion

The dried material of *Sargassum confusum* was successively extracted with n-hexane and methanol at room temperature for 72 h. Bioassay-guided fractions of these extracts led to the isolation of AF compounds **1**–**9** after thorough purification by repeated chromatography over silica gel, HPLC and prep.TLC (figure 1).

Compound **1** yielded a colourless liquid. The ^{1}H NMR spectrum signal appearing at δ 0.85 was assigned to a terminal methyl group and a broad singlet at δ 1.05–1.23 corresponded to methylene protons. The GC-MS spectrum gave its molecular ion at *m/z* 226 and afforded its identity as hexadecane, similar to the reported data in the literature [18–20].

Compound **2,** similarly obtained, yielded a low-melting solid. Its NMR and GC-MS spectral data revealed its identity as octadecane. Using similar procedures the compounds **3**, **4** and **5** were identified as eicosane, 1-eicosanol and 1-pentadecanol, respectively [18–20].

Compound **6**, a colourless liquid, was analysed as $C_{16}H_{24}O_4$. The GC-MS spectrum showed a molecular ion at *m/z* 278; the other fragments at *m/z* 166, 149 (base peak) indicated the presence of phthalic anhydride moiety in the parent molecule. The ^{1}H NMR spectrum showed a multiplet at δ 7.74 and 7.52 (2H, each) indicating the presence of a disubstituted benzene ring. The protons at δ 4.30 as complex multiplet was assigned to methylene protons adjacent to oxygen function and a triplet at δ 0.96 was assigned to methyl function. In the ^{13}C NMR spectrum the signal at δ 169.5 revealed the presence of a carbonyl carbon of ester group and aromatic carbons resonated in the region δ 122–136. The remaining methylenes appeared in the corresponding region with the expected values (see section 3). The above data were identical to those reported for dibutyl phthalate in the literature [21,22].

Similarly the compounds **7**, **8** and **9** were identified as dioctyl phthalate, diisononyl phthalate, and dicyclohexyl phthalate by comparing their NMR spectral data with the literature data [21,23].

As a part of AF activity determination, the fractions derived from column chromatography of the n-hexane and methanol extracts were subjected to motility spore examination against the fouling alga *Ulva pertusa* at 125, 250, 500, 1000, 2000 and 4000 μg ml^{-1} concentration [24] (see table 1). Two fractions (F4 and F7) were found to be active. The active fractions yielded the above nine pure compounds that were subjected to the spore attachment test [25], and the results are tabulated in table 2.

The results indicated that the maximum number of spore attachments found to be in the control was 118.75 ± 10.33 mm^2 in 10 h. The nine AF candidates tested at three concentration levels (100, 10 and 1 μg ml^{-1}) showed a significant AF effect on spore

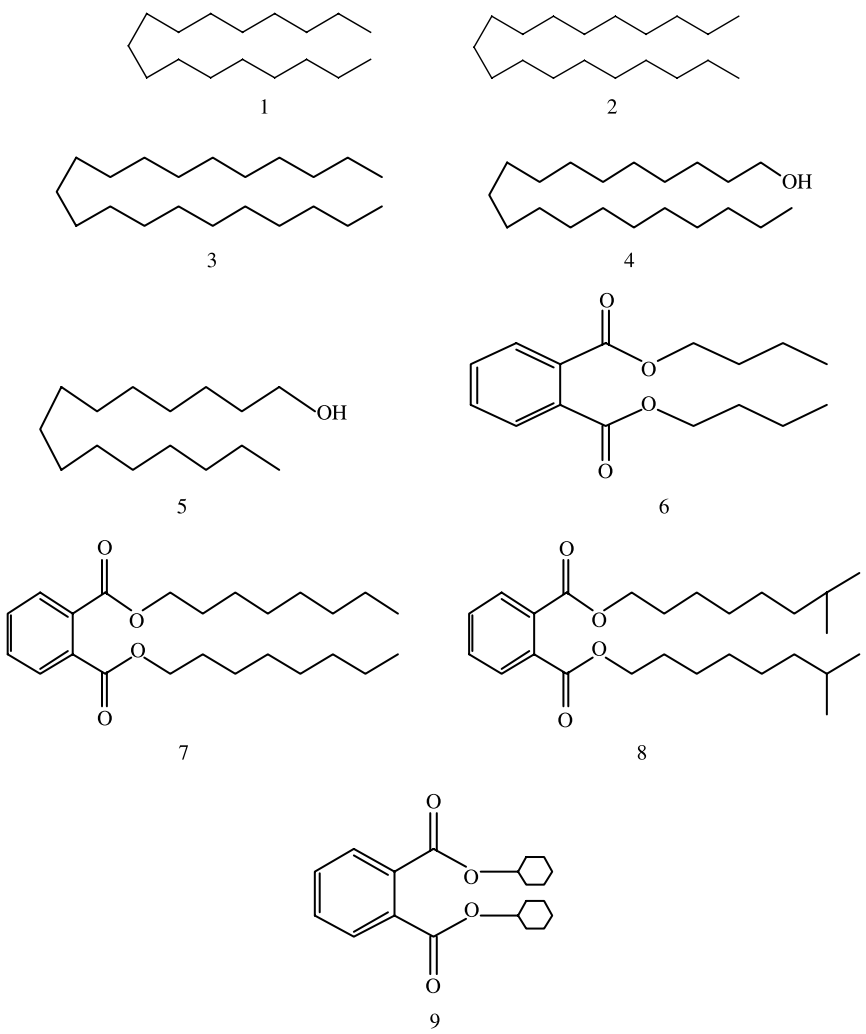

Figure 1. The structures of compounds **1**–**9**.

Table 1. Effect of *Sargassum confusum* extract-fractions on spore motility of *Ulva pertusa.*

	Activities							
Concentration ($\mu g\ ml^{-1}$)	*F1*	*F2*	*F3*	*F4*	*F5*	*F6*	*F7*	*F8*
4000	++	+++	++	++++	++	++	++++	++
2000	++	++	++	++++	++	++	++++	++
1000	++	++	++	++++	++	++	++++	++
500	++	+	+	++++	+	+	++++	+
250	+	–	–	++++	–	–	++++	+
125	–	–	–	+++	–	–	+++	–

++++, strong: 100%;+++, moderate: 95–75%;++, active: 75–50%; +, weak, <50%; –, no response.

Table 2. Isolated compounds **1–9** against spore attachment of *Ulva pertusa.*

	Concentration ($\mu g\ ml^{-1}$)			
Compound	*100*	*10*	*1*	*Control*
1	$21.50 \pm 2.50^{****}$	$29.25 \pm 1.48^{***}$	$45.75 \pm 7.50^{**}$	118.75 ± 10.33
2	$22.50 \pm 3.30^{****}$	$30.75 \pm 1.20^{***}$	$45.75 \pm 5.89^{**}$	118.75 ± 10.33
3	$26.50 \pm 2.96^{****}$	$57.50 \pm 3.84^{**}$	$72.00 \pm 6.96^{*}$	118.75 ± 10.33
4	$27.25 \pm 2.77^{****}$	$35.00 \pm 3.32^{***}$	$47.25 \pm 4.21^{**}$	118.75 ± 10.33
5	$21.50 \pm 2.29^{****}$	$29.50 \pm 2.69^{***}$	$41.00 \pm 4.36^{**}$	118.75 ± 10.33
6	$21.75 \pm 1.93^{****}$	$39.00 \pm 4.95^{***}$	$52.00 \pm 3.16^{**}$	118.75 ± 10.33
7	$20.25 \pm 1.92^{****}$	$37.50 \pm 6.10^{**}$	$53.25 \pm 3.70^{**}$	118.75 ± 10.33
8	$28.75 \pm 1.92^{****}$	$40.25 \pm 3.77^{***}$	$46.50 \pm 4.72^{**}$	118.75 ± 10.33
9	$21.00 \pm 1.87^{****}$	$30.00 \pm 1.58^{***}$	$44.75 \pm 3.96^{**}$	118.75 ± 10.33

All the values are expressed in mean ± S.D. $^{*}P < 0.01$; $^{**}P < 0.005$; $^{***}P < 0.0025$; $^{****}P < 0.001$, significantly different from control.

attachment. The spore attachment rate was found to be 25–45% at the tested concentrations, except that compound **3** showed a moderately high spore attachment rate at 1 μg ml^{-1} level. All these AF candidates showed a relative 50–75% reduction of spore attachment against the fouling alga *Ulva pertusa*. The phthalic acid esters from the marine alga *Sargassum wightii* showed antibacterial properties [22]; that these phthalic acid esters **6–9** displayed AF activity confirms that no impurity was taken up by the alga from the environment.

The existing metal-based compounds such as TBT displayed 40–50% reduction at the lower concentration of 0.25–1 ppm, and copper sulphate affected the spore attachment by 60–80% at the 1–5 ppm level against the fouling alga *Ulva conglobata* [26]. Non-toxic compounds like epiagelasine C was reported to inhibit about 85–90% spore attachment against *Ulva conglobata* [27] and the enzymes like pronase and α-amylase showed significant spore inhibition of *Enteromorpha intestinalis* [28]. The present investigation reveals that simple fatty compounds and aromatic carboxylic acid derivatives inhibited spore attachment at moderate concentrations. This is the first report on the presence of AF active compounds of *Sargassum confusum*.

3. Experimental

3.1 General experimental procedures

^{1}H NMR (200 MHz) and ^{13}C NMR (50 MHz) were recorded on a Bruker-200 instrument using $CDCl_3$ as solvent and TMS as internal standard. HPLC and prep.HPLC experiments were carried out on a Shimadzu LC-6A instrument, using a C18 MS column (5 μm, 6 × 250 mm; 5 μm, 25 × 250 mm) with an isocratic solvent containing MeOH/H_2O (8:2). GC-MS were recorded on a Shimadzu GC-10-AT QP5050A system equipped with a flame ionisation detector, HP-5 column (30 m × 0.25 mm, 0.25 μm), Oven temperature at120°C, column temperature initially held at 50°C for 2 min, raised to 150°C at 4°C min^{-1} and then to 250°C at 7°C min^{-1}. Argon was used as carrier gas at a flow rate of 1.0 ml min^{-1} with a split ratio 1:50. The MS was recorded at 70 eV under EI mode. Silica Ar/Merck 7GF pre-coated plates were used for TLC. Merck silica gel (100–200 mesh) was used for column chromatography.

3.2 Plant material

The brown alga *Sargassum confusum* was collected from Pohang, South Korea and kept at −20°C until extraction. The material was shade-dried and powdered.

3.3 Extraction and isolation

The powdered material (2 kg) was subjected to cold extraction with n-hexane and methanol successively for 72 h. The n-hexane extract (1.8 g) was subjected to column chromatography over silica gel, using solvents of increasing polarity from n-hexane through EtOAc. In all, 105 fractions (250 ml) were collected, and the fractions showing similar spots on TLC were combined to yield five major fractions (F1–F5). In a similar manner; the methanol extract (1.5 g) gave three fractions (F6–F8). These fractions were subjected initially for spore motility test against the fouling alga *Ulva pertusa* [24]. Two fractions (F4 and F7) showed positive for motility of spores. The bioassay-guided fractions (F4 and F7) and the residues therein were subjected to purification by repeated chromatography over silica gel, HPLC and prep.TLC, yielding compounds **1**–**9**. These compounds were individually tested for *Ulva pertusa* spore attachment by using the agar diffusion method [25].

The residue from the column fractions 74–89 (n-hexane/EtOAc, 7.5:2.5) of the n-hexane extract furnished hexadecane (**1**), octadecane (**2**), eicosane (**3**), dibutyl phthalate (**6**) and dioctyl phthalate (**7**), the residue from the column fractions 27–41 (n-hexane/EtOAc, 8.5:1.5) of the methanol extract yielded 1-eicosanol (**4**), 1-pentadecanol (**5**), diisononyl phthalate (**8**) and dicyclohexyl phthalate (**9**), respectively.

Hexadecane (**1**): Colorless liquid (12 mg), $C_{16}H_{34}$; ^{1}H NMR δ 0.85 (CH_3), 1.05–1.23 (CH_2); EI MS *m/z* (%int) 226(8), 197(11), 141(15), 127(15), 85(28), 71(55), 57(100), 43(91).

Octadecane (**2**): Low melting solid (10 mg), $C_{18}H_{38}$; ^{1}H NMR δ 0.88 (CH_3), 1.15–1.28 (CH_2); EI MS *m/z* (%int) 254(5), 225(5), 169(14), 85(32), 57(100), 43(85).

Eicosane (**3**): Low melting solid (10 mg), $C_{20}H_{42}$; ^{1}H NMR δ 0.88 (CH_3), 1.25–1.32 (CH_2); EI MS *m/z* (%int) 282(4), 253(7), 197(14), 127(15), 85(36), 57(100), 43(82).

1-Eicosanol (**4**): Colorless liquid (8 mg), $C_{20}H_{41}OH$; ^{1}H NMR δ 0.88 (CH_3), 1.15–1.28 (CH_2), 3.55 (OH); EI MS *m/z* (%int) 280(21), 251(14), 153(8), 139(12), 97(54), 55(100), 41(85), 31(23), 29(51).

1-Pentadecanol (**5**): Colorless liquid (8 mg), $C_{15}H_{31}OH$; ^{1}H NMR δ 0.92 (CH_3), 1.15-1.28 (CH_2), 3.72 (OH); EI MS *m/z* (%int) 210(16), 181(8), 153(5), 97(48), 69(45), 55(100), 41(82), 31(25), 29(56).

Dibutyl phthalate (**6**): Colorless liquid (15 mg), $C_{16}H_{22}O_4$; ^{1}H NMR δ 0.98 (CH_3), 4.23 (—OCH_2), 7.54 and 7.74 (ArH); ^{13}C NMR δ 168.5 (>C═O), 122–136 (aromatic), 58.8 (OCH_2), 31.2, 28.8, 14.9; EI MS *m/z* (%int) 278(18), 166(42), 149(100), 121(38), 77(48).

Dioctyl phthalate (**7**): Colorless liquid (10 mg), $C_{22}H_{38}O_4$; ^{1}H NMR δ 0.98 (CH_3), 1.27, 2.45, 4.23 (—OCH_2), 7.54and 7.74 (ArH); ^{13}C NMR δ 171.8 (>C═O), 128–134 (aromatic), 65.10 (OCH_2), 39.5, .28.8, 22.7, 14.6; EI MS *m/z* (%int) 390(18), 278 (15), 166(42), 149(100), 121(38), 77(48).

Diisononyl phthalate (**8**): Colorless liquid (7 mg), $C_{26}H_{42}O_4$; ^{1}H NMR δ 0.92 (CH_3), 1.08–1.25, 2.28, 4.23 (—OCH_2), 7.64 and 7.87 (ArH); ^{13}C NMR δ 171.8 (>C═O), 125-136 (aromatic), 59.5 (OCH_2), 39.5, 28.8, 22.7, 16.5.

Dicyclohexyl phthalate (**9**): Colorless liquid (9 mg), $C_{20}H_{26}O_4$; ^{1}H NMR δ 1.45–1.64 (CH_2), 1.84, 2.15, 5.15 (—OCH), 7.64 and 7.87 (ArH); ^{13}C NMR δ 168.8 (>C═O),

128–135 (aromatic), 73.2 (OCH), 31.50, 25.7, 22.9; EI MS *m/z* (%int) 330(18), 166(42), 149(100), 121(38), 77(48).

3.4 Spore motility bioassay

Ulva pertusa was thoroughly washed with sterilized sea water to remove the epiphytes. Then algal material was drenched in filtered sea water and dried for some time to facilitate the release of spores. After a few minutes algal material was taken out from the sea water and the remaining spore suspension was used for the spore motility bioassay. The extracted fractions each of about 5 mg were mixed with 10 μl DMSO and spore suspension and prepared at the different concentrations (125, 250, 500, 1000, 2000 and 4000 μg ml^{-1}). After 1 min, about 25–30 μl aliquot of *Ulva pertusa* spore suspension was taken out from the test concentration sample and the motility of spores was observed under the microscope. The results are given in table 1.

3.5 Spore attachment (agar diffusion method)

Thalli of the green fouling alga *Ulva pertusa* was thoroughly washed with 0.2 μM filtered sea water and placed in dry Petri dishes under fluorescent light at room temperature overnight. After drying, the *Ulva pertusa* material (500 g) was covered with filtered sea water (1000 ml) to release the motile cells as spore suspension with 30,000 spores ml^{-1}. Test compounds **1**–**9** (about 5 mg) were dissolved in 0.5 ml DMSO and 0.5 ml distilled water as stock solution and the desired concentrations 1, 10 and 100 μg ml^{-1} were prepared. 2% of Agar gel (2 g Bactoagar dissolved in 100 ml distilled water) with the above concentrations of test compounds was coated onto acid-clean glass slides about 2 cm × 2 cm in area. Agar-coated film without the test compounds served as control. The agar-coated slides were placed in *Ulva pertusa* spore suspension for 10 h in the dark. Later, the plates were thoroughly washed with distilled water and the number of spores attached per mm^2 counted, by microscope (Olympus BH2, 400 × magnification); the results are presented in table 2.

3.6 Statistical analysis

The data are expressed as mean ± S.D. for four replicates of each compound against control. Significant differences (P) between concentrations were measured using Students t-test.

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