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Communication

Antifouling Metabolites from the Mangrove Plant *Ceriops tagal*

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Abstract: The new diterpene methoxy-ent-8(14)-pimarenely-15-one (**1**) and three known metabolites: ent-8(14)-pimarene-15R,16-diol (**2**), stigmasterol (**3**) and β-sitosterol (**4**), were isolated from the roots of the mangrove plant *Ceriops tagal*. Their structures and relative stereochemistry were elucidated by means of extensive NMR, IR and MS analysis. Compounds **1**, **2**, **3** and **4** exhibited significant antifouling activities against cyprid larvae of the barnacle *Balanus albicostatus* Pilsbry, with EC_{50} values of 0.32 ± 0.01, 0.04 ± 0.00, 4.05 ± 0.15 and $18.47 \pm 0.40 \, \mu g/cm^2$, respectively, whereas their toxicities towards cyprids were very low, with LC_{50} values all above $10 \, \mu g/cm^2$.
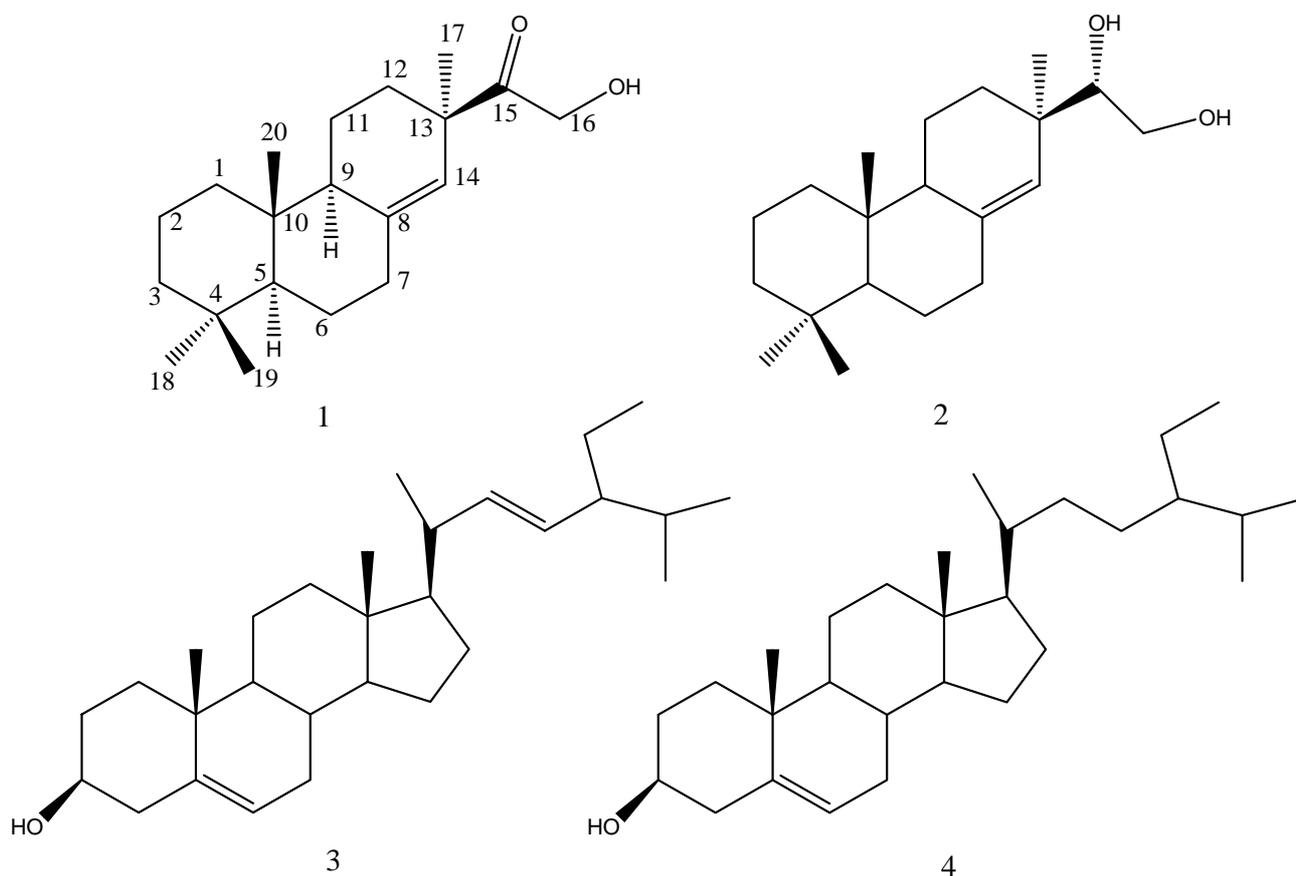
Introduction

Marine fouling organisms often cause technical and economic problems by settling on artificial surfaces submerged in seawaters [1]. Although organotin compounds such as tributyltin (TBT) and

tributyltin oxide (TBTO) have been widely used for controlling these fouling organisms, they were also found to be toxic to many non-target marine organisms [2]. The Marine Environment Protection Committee of the International Maritime Organization has proposed a ban on the application of TBT-based antifouling paints from January 1, 2003 and the ban of the presence of such paints on the surface of vessels from January 1, 2008 [3]. Currently, some booster biocides are used for antifouling paints, but they may also pollute the aquatic environments [4]. Therefore, effective and environmentally friendly antifoulants are urgently needed. An important source of such effective and environmentally friendly antifoulants is the antifouling active substances isolated from marine organisms. To date, a variety of natural products with antifouling activities have been isolated from lots of different marine organisms, including marine bacteria, algae, seagrass, sponge, coral, bryozoa, ascidian, etc. [5,6]. However, there is scant information on isolation of antifouling compounds from mangrove species, which is one kind of marine higher plants [7].

Figure 1. Structure of the compounds.

Marine organisms use the diverse mechanisms to protect their own surfaces from fouling [8]. Within the marine ecosystem, evolution has allowed for the development of certain antifouling properties. Marine organisms have both physical and chemical methods to protect themselves from the harmful process of biofouling [8,9]. The key chemical antifouling mechanism of marine organisms occurs via the production of secondary metabolites (also known as natural products) which deter foulers [1]. During our search for structurally interesting and antifouling natural products from the

mangrove plants, we found that compounds from the roots of *Ceriops tagal* inhibited significantly larval settlement of the barnacle *Balanus albicostatus*, which is one important fouling organism in East Asian coastal waters. These antifouling active compounds included one new diterpene, methoxy-ent-8(14)-pimarenely-15-one (1) and three known metabolites, designated as ent-8(14)-pimarene-15R,16-diol (2), stigmasterol (3) and β-sitosterol (4) (Figure 1). The isolation, structural elucidation and antifouling activity of these compounds are described in this paper.

Results and Discussion

One new diterpene, methoxy-ent-8(14)-pimarenely-15-one (**1**), and three known metabolites ent-8(14)-pimarene-15R,16-diol (**2**), stigmasterol (**3**) and β-sitosterol (**4**) were isolated from the roots of *C. tagal*. These compounds showed potent non-toxic antifouling activities against larval settlements of *B. albicostatus*.
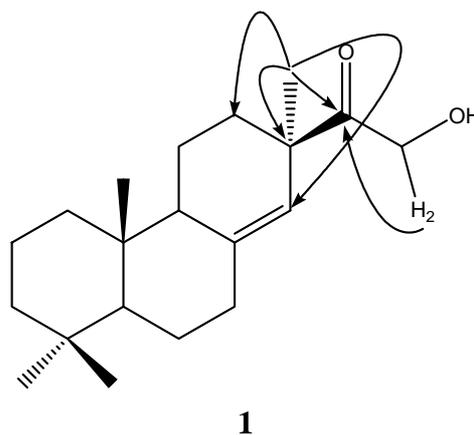
Methoxy-ent-8(14)-pimarenely-15-one (**1**) was obtained as amorphous powder, $[\alpha]_D^{25}$-2.37° (*c* -0.002, CHCl$_3$). Its molecular composition of $C_{20}H_{32}O_2$ was established by HRESIMS at *m/z* 305.2487 [M + 1]$^+$ (calcd. 305.2481 for $C_{20}H_{33}O_2$). The ESIMS of **1** showed quasimolecular ion peaks at *m/z* 305.4 [M + 1]$^+$. The IR absorptions at 3433, 1735 cm^{-1} showed the presence of hydroxyl and carbonyl groups. Analysis of the ^{1}H-NMR, ^{13}C-NMR, DEPT and HMQC spectra revealed not only the presence of four methyls, eight methylenes (including one oxygenated methylene at δ_C 65.9, t), three methines and five quaternary carbon (including one carbonyl group at δ_C 214.9, s), but also the presence of one trisubstituted double bond (Table 1). Four singlet methyls (δ_C 22.4, q; 22.0, q; 33.6, q; 14.5, q) and one trisubstituted double bond (δ_C 143.3, s; 122.8, d), in addition to the optical rotation data of **1**, suggested that a pimarane diterpenoid structure might be presented [10-14].

The planar structure of **1** was elucidated by the analysis of 2D-NMR data and by comparison with the NMR data of **2** (Table 1). The NMR spectra of **1** were similar to those of **2**, except for the fact that the chemical shift of C-15 (δ_C 214.9, s) were shifted more upfield than that of **2** (δ_C 78.7, d), indicating that the oxygenated methine of **2** was replaced by carbonyl group. Furthermore, The HMBC correlations from Me-17 (δ_H 1.12, s) to C-15 (δ_C 214.9, s), C-14 (δ_C 122.8, d), C-13 (δ_C 46.8, s) and C-12 (δ_C 32.8, t), and from H-16 (δ_H 4.36, s) to C-15 (δ_C 214.9, s) indicated the connectivity between C-13/C-15 and C-15/C-16 (Figure 2). Thus, the gross structure of **1** was assigned as a new pimarane diterpenoid.

In addition, the comparison of its NMR data with that of compound **2** and the NOE cross-signals confirmed the relative stereochemistry of compound **1**. Comparison of the NMR data of **1** with **2** showed that the Me-18 (33.6, q), Me-17 (22.4, q) was in an α-orientation, while Me-19 (22.0, q) was in a β-orientation. Then, the 3-D structure was obtained using CHEM 3D ULTRA V 9.0, with MM2 force-file calculations for energy minimization in the computer (Figure 3). NOE correlations between Me-19 and Me-20 (2.013 Å) confirmed that Me-20 was in a β-orientation. Then, the cross-peaks of NOE correlations between Me-18 and H-5 (2.306 Å) and between H-5 and H-9 (2.286 Å) indicated that the H-5 and H-9 were on the β-side of **1**.

Table 1. ^{1}H-NMR Data (500 MHz) and ^{13}C- NMR Data (125 MHz) for Compound **1** (CDCl$_3$).

No.	δ_C	δ_H (J = Hz)
1	38.8 t	1.01 (m), 1.57 (m)
2	18.8 t	1.43 (m)
3	42.0 t	1.20 (m), 1.39 (m)
4	33.3 s	
5	54.6 d	1.07 (m)
6	22.4 t	1.36 (dd, 4.5, 13), 1.64 (m)
7	35.8 t	2.11 (ddd, 5, 13.5, 18.5), 2.36 (m)
8	143.3 s	
9	51.0 d	1.75 (m)
10	38.5 s	
11	20.1 t	1.61 (m)
12	32.8 t	1.13 (m), 2.31 (m)
13	46.8 s	
14	122.8 d	5.37 (s)
15	214.9 s	
16	65.9 t	4.36 (s)
17	22.4 q	1.12 (s)
18	33.6 q	0.88 (s)
19	22.0 q	0.83 (s)
20	14.5 q	0.64 (s)

Figure 2. Key HMBC correlations of Compound **1**.

1

The known compounds ent-8(14)-pimarene-15*R*,16-diol (**2**), stigmasterol (**3**) and β-sitosterol (**4**) were identified by comparison of their physical and spectral data with those in the literature [14-16].

The antifouling activities of four compounds are shown in Figure 4. Compounds **1**, **2**, **3** and **4** exhibited significant antifouling activities against cyprid larvae of the barnacle *B. albicostatus* Pilsbry, with EC$_{50}$ values of 0.32 ± 0.01, 0.04 ± 0.00, 4.05 ± 0.15 and 18.47 ± 0.40 µg/cm^2, respectively. On the other hand, the LC$_{50}$ values of four compounds were all above 10 µg/cm^2 (Figure 4), indicating that their toxicities to cyprids were quite low and they all inhibited cyprid settlement in a non-toxic way.

Figure 3. Key NOE correlations of compound **1** and corresponding interatomic distances (Å).
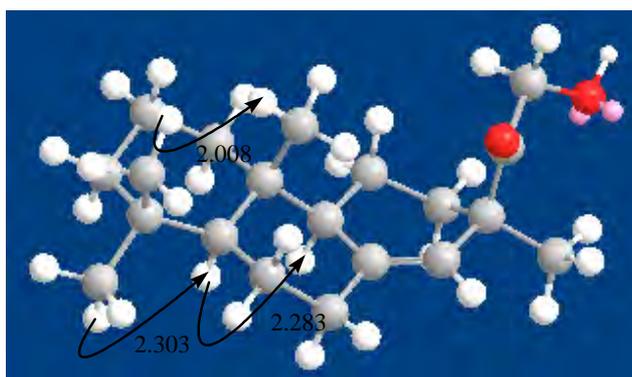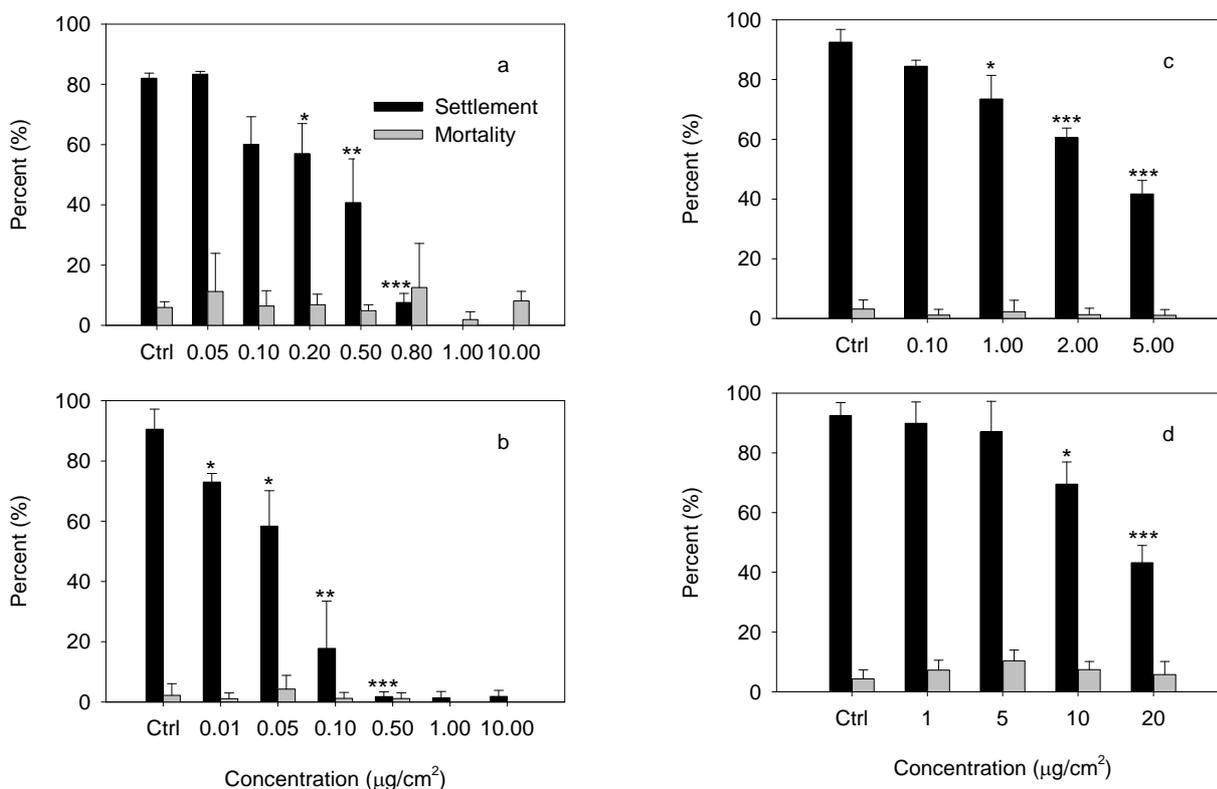

Figure 4. Effects of Compounds **1-4** on settlement and survival of cyprid larvae in *B. albicostatus*. a) Methoxy-ent-8(14)-pimarenely-15-one (**1**); b) Ent-8(14)-pimarene-15*R*,16-diol (**2**); c) Stigmasterol (**3**); d) β-sitosterol (**4**). Data were analyzed using one-way ANOVA, where $*P < 0.05$; $**P < 0.01$ and $***P < 0.001$ were significantly different from the control.

Pimarane diterpenoid had a stronger antifouling activity than phytosterin. Our structure-activity relationship analysis of pimarane diterpenoid suggested that the antifouling activity might be due to the number of free hydroxyl group in side chain of pimarane diterpenoid. Compound **2**, with two free hydroxyl groups had the higher antifouling activity than compound **1** with only one free hydroxyl

group. However, there was no great effect of the number of free hydroxyl group on the toxicity of pimarane diterpenoids. In addition, the structure-activity relationship analysis of phytosterin suggested that the presence of double bond in side chain of phytosterin might be important in expression of the antifouling activity in these compounds. Compound **3** had the stronger antifouling activity than compound **4** due to its double bond group. But, it seemed that the double bond in side chain of phytosterin did not influence the toxicity of phytosterins.

Experimental

General

^{1}H-NMR spectra were measured with Varian UNITY PLUS 500 spectrometer (USA). ^{1}H-, ^{13}C- and 2D-NMR spectra were measured with Varian INOVA 600 spectrometer (USA). High-resolution ESI mass spectra data were measured with a Waters Q-TOF Micro^{TM} spectrometer (USA) and were provided by Zhengzhou University, China. Low-resolution ESI mass spectra data were recorded on an AB 3200Q TRAP spectrometer (USA). IR spectra were measured using a Nicolet 380 FT-IR spectrophotometer (USA). The optical rotation data were obtained on a Rudolph Autopol IV polarimeter (USA).

Extraction and Isolation

The roots of *C. tagal* were collected from Hainan Province, China in July 2005. The air-dried and powdered material (4.1 kg) was extracted with 95% EtOH at room temperature. The extract was evaporated under vacuum to give a residue (715 g), and then the residue was partitioned between petroleum ether, ethyl acetate and H_2O. The petroleum ether extract (11.5 g) was chromatographed on a silica gel column, eluted with a petroleum ether – ethyl acetate gradient to obtain 13 fractions (A-M). Fraction H (1.1 g) was chromatographed over silica gel and Sephadex LH-20 repeatedly (elution with 24:1 hexane - acetone) to yield compound **1** (23 mg). Compound **2** (200 mg) was recrystallized with acetone –MeOH (4:1) from fraction M (1.562 g). Fraction L (438 mg) was chromatographed over Sephadex LH-20 column eluting with $CHCl_3$-MeOH (1:1) and the major fraction obtained was then purified by silica gel column chromatography eluting with petroleum ether- acetone (60:1) to yield compound **3** (11 mg). Fraction J (850 mg) was subjected to silica gel column chromatography eluting with petroleum ether- acetone (9:1) to yield compound **4** (646 mg).

Methoxy-ent-8(14)-pimarenely-15-one (**1**): $C_{20}H_{32}O_2$; amorphous powder; IR v_{max} (KBr): 3433, 1735 cm^{-1}; ESIMS, *m/z* 305.4 [M + 1]$^+$, HRESIMS at *m/z* 305.2487 [M + 1]$^+$ (calcd. 305.2481 for $C_{20}H_{33}O_2$); ^{1}H-NMR and ^{13}C-NMR data for **1** are listed in Table 1.

Antifouling Assay

Antifouling efficacies of the four compounds towards *C. tagal* were evaluated by the settlement inhibition assay with cyprid larvae of *B. albicostatus*. Adult *B. albicostatus* were obtained from

intertidal zone in Xiamen, China. In the laboratory, the nauplius I and II were released by subjecting the adult barnacles to aerial exposure for a period of 12 h, followed by immersion in filtered seawater (FSW 0.22 μm, salinity 30‰, and temperature 25°C). Naupilar larvae were collected using a pipette and placed into 8-liter glass beakers containing FSW. Naupilar larvae were reared at a density of 1 larvae mL^{-1} on a diet of the diatom *Chaetoceros muelleri* with a concentration of 2.0×10^5 cells·mL^{-1}. The seawater and diet were renewed everyday. After 5~6 days, the cultures were monitored for the presence of cyprids and the cyprids were harvested within 24 h after molting.

Tested compounds were dissolved in CH_2Cl_2. Aliquots of the solution were added to the glass dishes with diameter of 6 cm and air-dried. Then, 30 cyprids and 10 mL FSW were placed into each glass dish. Cyprids were exposed to different concentrations of the compounds determined as shown in Figure 4. There were three replicates for the FSW control and each concentration. After 48 h incubation at 25°C, the numbers of larvae which settled, died or did not settle in each replicate were enumerated with the aid of a stereomicroscope.

Statistical analysis

Differences in the settlement or mortality percentages of experimental and control treatments were tested for significance by one-way ANOVA. Significance was set at the 5% level. The EC_{50} (the concentration that reduces the settlement rate by 50% relative to the control) and LC_{50} (the concentration that results in 50% mortality relative to the control) both with a 95% confidence interval were estimated by using the Spearman-Karber method [17,18].

Acknowledgements

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Sample Availability: Samples of the compounds are available from authors.